1. Cell Organization:

Techniques and a Preliminary View

Since the invention of the light microscope and our first vistas of cells, technological and conceptual developments have gone hand in hand. Hence a review of some of the methodology is useful, although in the chapters that follow, results and techniques are frequently considered together. Our review first examines the techniques more traditionally associated with cell biology: microscopy and the isolation of cell components. These sections are followed by the approaches of molecular biology, such as recombinant DNA technology and immunological techniques, which have revolutionized every aspect of biology and have obvious significance in studies concerning cell function and structure. More traditional biochemical techniques that have become part of biological studies are also included. A preliminary view of cell organization is then presented; other chapters will expand on the themes raised in this section.

I. TECHNIQUES USED IN THE STUDY OF CELLS

A. Microscopic Techniques

The application of video technology and computer-aided analysis of images has allowed studies of cell structure and function that were not possible before. Movie and video-tape recording of microscopic data is being superseded by *digital image acquisition*. This technique is convenient, fast and the duplication of the images does not lose information (see Shaw et al., 1995, 1997). Application of digital image technology has specific computer requirements, no longer difficult to obtain. Furthermore, a video camera must be interfaced to a computer through an analog to digital board. However, digital cameras are also available requiring a camera interface board. Digital to video conversion can be obtained in a variety of ways such as a scan converter. The practical requirements for this technique has been recently reviewed (Waterman-Storer et al., 1997).

Refinements of more conventional approaches, such as *cytochemistry* and *autoradiography* have also contributed valuable information. Cytochemistry allows the detection and quantitative estimation of cell components or enzyme reactions, whereas autoradiography detects photographically the presence of radioactive isotopes incorporated into cell components. Similarly, immunological techniques have allowed recognition of the distribution of specific proteins inside cells. In situ hybridization, which allows recognition of specific nucleotide sequences, identifies the location of nucleic acids.

Light microscopy

Fig. 1 illustrates the principles of two of the light microscopic methods currently used in cell biology: conventional light microscopy (Fig. 1a) and phase and interference microscopy (Fig. 1b).

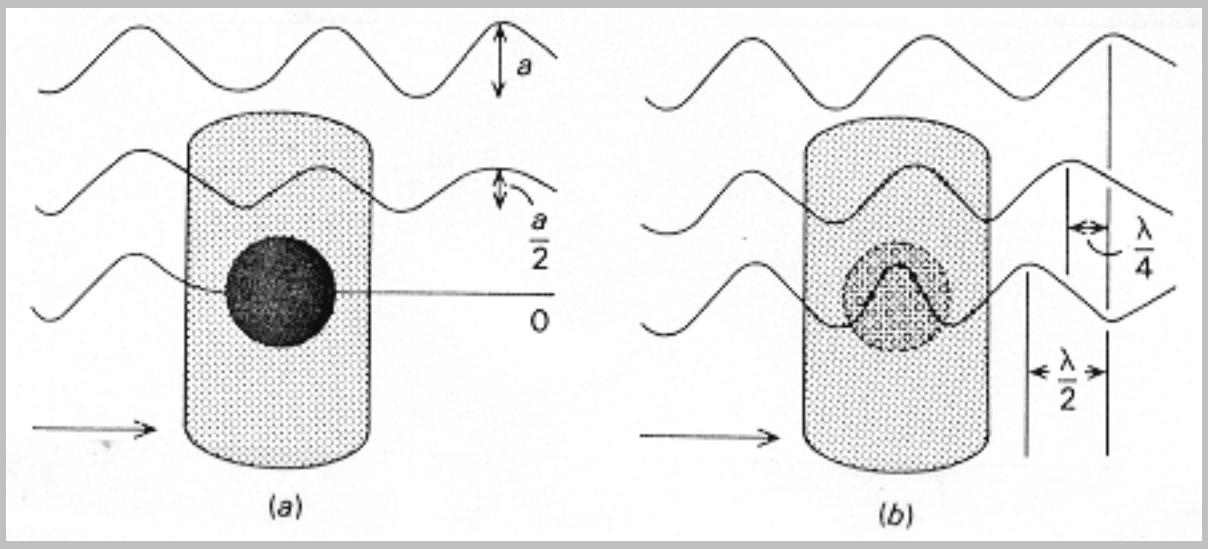


Fig. 1 Comparison of absorption and phase interference methods in light microscopy. Based on a diagram from Ross (1967). Reproduced by permission.

With visible light and an ordinary light microscope, the various cell components offer little contrast unless they have been stained or absorb light. Generally, before being examined with the light microscope, a specimen must be fixed, a procedure that immobilizes the macromolecular components either by denaturing or cross-linking them. Then embedding in either plastic or paraffin is needed, generally preceded by dehydration. The material is then sectioned and stained. The absorption or fluorescence of the dyes can be measured quantitatively with a variety of techniques. Fig. 1a illustrates the absorption of light through a stained cell in conventional microscopy.

In light microscopy, immunological techniques have been used by attaching an antibody to a label, a dye with characteristic light absorption or fluorescence (*immunocytochemistry*). Similarly, sites of enzyme activity can be identified by using substrates, sometimes analogs of the natural substrates, which the enzymes convert into insoluble compounds with characteristic absorption or compounds that can be readily precipitated (*enzyme cytochemistry*). (Refer to Use of Antibodies section)

The interference methods illustrated by the special case of phase-contrast microscopy in Fig. 1b do not require any of the preparatory procedures, so they are best suited for the study of living cells whose dynamics can be recorded on videotape. Many past contributions were made with cinematography. These methods take advantage of light interference phenomena. When two coherent beams of light are recombined, they can interfere with each other constructively or destructively to produce a contrast image. The beam passing through an object will be retarded in proportion to the thickness and density of the object. In *phase-contrast microscopy* the beam diffracted by the object is recombined with a reference

beam that has passed through the medium or the background material. The reference beam has been advanced or retarded a quarter-wavelength to maximize the interference. The difference in phase between the two beams will give positive or negative interference, the former showing the object as darker and the latter as lighter than the background. In *interference microscopy*, the phase of the reference beam can be varied in relation to the specimen beam, allowing measurement of the retardation of the specimen beam. The method can be used as a quantitative tool to estimate the dry weight of objects.

Nomarski *differential interference contrast microscopy* (DIC) is based on the interference between two closely separated points in the object. The beam passing through the specimen is split by a birefringent plate (a modified Wollaston prism). The image is a gradient of the phase difference between these two adjacent points. Mathematically, the contrast is the derivative of the path differences with respect to distance. The image has a directional contrast resembling a shadow-cast relief map of cellular details. This technique provides a resolution closer to the theoretical limit than any other light microscopic technique. Furthermore, objects above and below the plane of focus are excluded from the image, which provides essentially an optical section. Nomarski optics are ideally suited for observing objects with well-defined boundaries, such as fibers or condensed chromosomes.

Less powerful techniques, such as modulation contrast (Hoffman) microscopy (Hoffman and Gross, 1975) and other asymmetric illumination methods (Kachar, 1980), provide images that resemble DIC images. Hoffman microscopy has been used recently because of its economy and its advantages in common with DIC, such as viewing of optical sections, increased visibility, and contrast of unstained specimens. As in DIC, the images are shadowed and show no halos. However, the principles involved are very different.

Fig. 2 allows a comparison of images obtained with phase-contrast and Nomarski differential interference microscopy. With Nomarski microscopy (Fig. 2a) the details are sharp and each cell component is clearly defined. With phase contrast (shown in Fig. 2b with positive contrast) the details appear more diffuse and, where slightly out of focus, are surrounded by halos.

Generally, under optimal conditions, light microscopy can distinguish as separate images two points that are no less than 0.2 μm apart. This is considered the practical limit of resolution for these techniques. With shorter wavelengths, much closer points can be resolved; this principle has been exploited in electron microscopy, which uses electrons instead of visible light. The resolution with the electron microscope under optimal conditions approaches atomic dimensions.

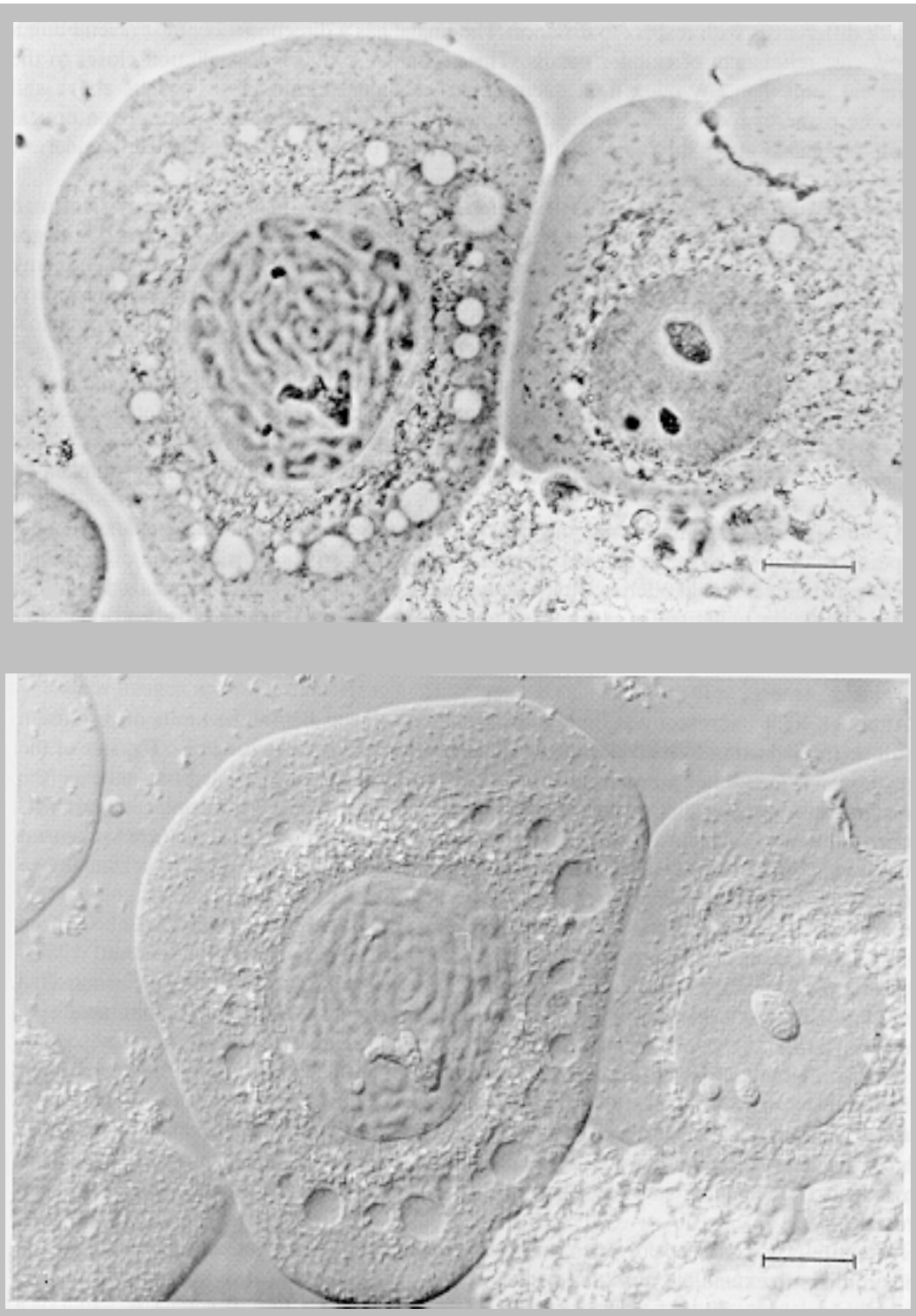


Fig. 2 Endosperm cells of *Hemanthus katherinae* shown with (a) Nomarski differential interference. Type some more Endosperm cells of *Hemanthus katherinae* shown with (b) positive phase contrast. The bar represents 20 µm. Photomicrographs by R. D. Allen, reproduced by permission.

Although light microscopy is limited in resolution, objects below the limits of resolution can still be studied using Nomarski optics and high-performance video cameras. The size of the image, however, does not correspond to that of the object. The video methods enhance the contrast of the objects by subtracting the background and amplifying the remaining signal. This approach allows viewing of single microtubules that are only 25 nm in diameter. Contrast improvements and light intensity measurements have also been provided by digital image processors that filter and average signals.

Confocal microscopes for imaging either fluorescence emission or reflected light have been developed and are now commercially available (White and Amos, 1987; White et al., 1987). Like DIC, they allow the study of optical sections. The instruments contain a computer-controlled system that scans the specimen rapidly in three dimensions with a single small laser illuminated spot (ideally 0.2 μm in diameter). Light from the specimen passes through an aperture and is focused on the plate of a video camera. Unlike conventional microscopes, they view a region coincident (i.e., confocal) with the illuminated spot. Only the region lying within a narrow depth of focus produces an image that corresponds to a precise location in space. Ideally, out-of-focus areas are not a problem since they are excluded by the pinhole aperture. The image can be digitized and stored on computer disks for analysis with conventional programs. Confocal microscopes are ideal for studying the three-dimensional organization of cells with fluorescent dyes.

Confocal microscopy is a powerful technique capable of providing a wealth of 3-D information on a living specimen. However, the entire specimen is exposed to the light producing bleaching and photodynamic damage mediated by the fluorophore or other absorbing molecules. Because only a fraction of the emitted light is used, the amount of light needed is substantial with subsequent increase in damage. In addition, with thick specimen the information is restricted to structures about 20 μm from the surface because of distortions caused by scatter. Most of these problems can be avoided by the use of *multiphoton optical absorption* to mediate excitation as done with *two photon-fluorescence microscopy* (Denk et al., 1990).

Molecules can absorb simultaneously two or more photons delivered by intense laser pulses (see Denk and Svaboda, 1997). The high intensity laser used has a short duration (below 1 ps) and at 100 MHz it boosts the frequency of two photon absorption. The exciting light, in the infrared range (twice the wavelength of the absorption peak from single photons), is not absorbed from single photon eliminating background fluorescence and background scattering. The fluorescence is generated virtually only in the vicinity of the focal point. In scanning, fluorescence excitation is limited to a focal "slice". In addition, all fluorescence photons are used. It has been found possible to make observations on very thick specimen (e.g. 200 μm)

Evanescent field fluorescence microscopy and the availability of novel fluorescent substances has opened new avenues in the study of events occurring in or close to plasma membranes. When a beam of light passes from a medium of high refractive index (e.g., glass) to that of a lower refractive index (e.g., water or the surface of a cell) the light undergoes *total internal reflection* if the angle of incidence is sufficiently high (see Steyer and Almers, 2001). This reflection produces a thin layer of light, the *evanescent field* (EF). Consequently molecules in this thin layer (about 100 nm) are illuminated, whereas molecules outside this zone remain in the dark. In addition to exciting fluorescent molecules in the thin illuminated layer, the technique provides information on the position of the fluorescent object which brightens as it approaches the interface and dims when it leaves it. Applications of this technique to the study of the interactions of myosin with an ATP analog and in the study of exocytosis are discussed below (Section IIB)

An entirely new concept of microscopy developed by M. Isaacson and A. Lewis (Pool, 1988) promises to extend the resolving power of the light microscope to as little as 50 nm. This microscope scans the illuminated specimen in 15-nm steps with a tiny detector consisting of a tube with an aperture as small as 50 nm in inner diameter.

3D reconstruction has allowed the mapping of gene expression patterns in development (see Davidson and Baldock, 2001) using light methods. A variety of techniques have been developed for 3D reconstruction of large objects such as embryos. The problems are somewhat different from conventional microscopy because of the large size of the specimen. Furthermore, these techniques do not require the high resolution needed for the study of cells. Confocal microscopy allows making observations in specimen as thick as 1 mm. *Optical coherence tomography* (OCT) can use specimen as thick as 2 to 3 mm (Boppart et al., 1996) by measuring backscattered infrared light and can be used with living specimen. The technique is similar to radar except that in this case backscattering or reflections of light rather than sound are detected at various specimen depths. *Optical projection tomography* (OPT) has been developed to produce 3D images of stained or fluorescent labelled prepared biological specimens with a thickness of up to 15 mm (Sharpe et al., 2002). With this technique, the specimen is rotated 360 degrees at angular steps of 0.9 degrees leading to a reconstruction of the image.

Electron Microscopy

Electron microscopy has brought the study of cell structure to the so-called *submicroscopic* or *ultrastructural* level, where even macromolecules can now be observed. The electron microscope (EM) depends on the fact that electron beams can be bent and focused by electric or magnetic fields, allowing them to form magnified images in much the same way as light in light microscopy. The electron beam originates from a filament and, after passing through an evacuated microscope column, is focused on a fluorescent screen or a photographic plate.

Transmission Electron Microscopy With the *transmission electron microscope* (TEM), the electron beam passes through the specimen. The denser areas in the specimen scatter more electrons and the corresponding regions in the image appear darker. Electron stains of high density are usually used to enhance the contrast. The specimen preparation generally used for TEM resembles in principle the fixation, embedding, sectioning, and staining used for the light microscope. Because of the increased magnification and spacial resolution offered by EM, specimen preparation is especially critical. Ideally, in order to obtain an accurate image of their structure, the components of cells and tissues must be maintained in a configuration corresponding to their original ultrastructure. Many fixatives such as OsO_4, also serve as stains. Others, such as glutaraldehyde, do not add to the contrast and additional treatment with electron stains is needed. Generally, the sections have to be much thinner than those used for light microscopy, which must be embedded in plastic. In part, the thinner sections must be used because of the limited penetrating power of conventional TEM; in part, they are needed to avoid the superposition of features in the image above or below the structures of interest, because through focus optical sectioning or confocal microscopy is not possible in EM.

High-voltage or intermediate-voltage electron microscopes are now in use. Because of the increased penetration of the electrons provided by the higher voltages, thicker specimen, sometimes even intact cells, can be used. Together with tilting of the EM stage, these techniques provide stereoscopic information and a wealth of detail. However, because of the overlap of the information in these images, they are difficult to interpret. Developments in computer analysis of such images are providing greater information about the three-dimensional organization of cells.

Isolated cell components, macromolecules, and macromolecular assemblies such as ribosomes and viruses have been studied with the TEM using *negative staining*. With this technique the particles are suspended, generally in a solution of an electron-dense compound (e.g., phosphotungstate or uranyl salts). The preparation is sprayed or spread in some manner on a grid and then dried. Structures that are not permeated by the salt appear light, whereas those containing the salt, for example, the previously hydrated areas-appear dark.

The scanning transmission electron microscope (STEM) has been used to study the structure of the freeze-dried ciliary and flagellar protein dynein without the use of stain or fixative (Johnson and Wall, 1983). This technique, which uses a very low electron dosage, also allows the determination of mass from the electron scattering intensities over a particle.

The newest advance in TEM is the imaging of cells and macromolecules that are embedded in amorphous ice at very low (liquid nitrogen) temperatures. These specimens are unfixed and unstained, so the contrast arises directly from differences in density of the cell components (proteins and nucleic acids appear dark, lipids and water light). Instrumental or computer techniques are usually needed to enhance the low inherent contrast of these frozen-hydrated images (see Fig. 3e). The use of electron cryomicroscopy together with the development of computational techniques is beginning to approach the level of resolution of crystallographic techniques (see DeRosier, 1997)

Figure 3 illustrates application of electron microscopy to a membrane channel. Channels are protein structures that provide passage of small molecules across biological membranes. Fig. 3a is an electron micrograph of a negatively stained mitochondrial outer membrane, and Fig. 3e is a reconstruction of a preparation embedded in vitreous ice without stain. Other details of this figure are discussed below in relation to computer-aided reconstruction (see below).

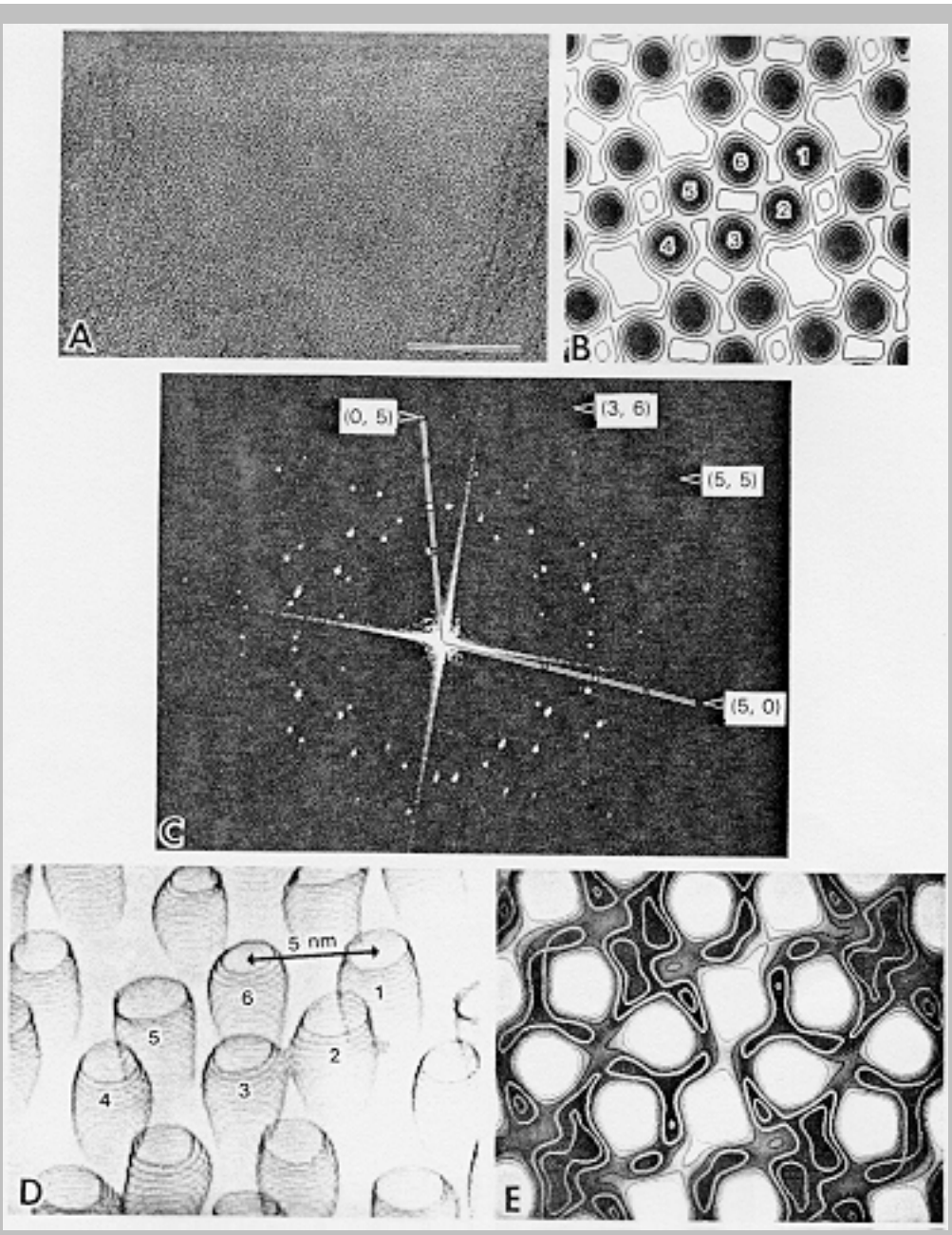


Fig. 3 Electron crystallography of a membrane channel. (a) Electron micrograph of a negatively stained mitochondrial outer membrane from the fungus *Neurospora* crassa (scale bar, 100 nm). (b) The two-dimensional repeat motif of one of the membrane arrays. (c) Optical diffraction of micrograph taken with coherent illumination from a laser. (d) Three-dimensional reconstruction of the array. (e) The projected density of the same membrane array embedded in vitreous ice without stain. (a) and (c) are reproduced from C.A. Mannella, *The Journal of Cell Biology*, by copyright © permission of Rockefeller University Press; (b), (d), and (e) courtesy of C. A. Mannella.

Scanning Electron Microscopy In the *scanning electron microscope* (SEM) a beam of electrons is focused to form a small diameter probe that is scanned across the specimen in a process similar to that used to produce television images (see Fleger et al., 1993). This probe interacts with atoms in the specimen causing them to emit a variety of signals. The microscope collects and displays electrons scattered from the surface of the object (secondary electrons). Generally, the specimen has to be dried and coated with metal. The metal increases the electrons scattering and also acts as a conductor to avoid

accumulation of charges.

The SEM produces images with a stereoscopic appearance because curved portions of the object reflect more electrons than flat areas. The SEM is very useful in observing the surfaces of three-dimensional objects. However, it is typically restricted in resolution to above 5 to 10 nm. The principles involved in forming an image are shown in Fig. 4. Secondary electrons are produced from sample-beam interaction but their escape from the specimen and subsequent detection depends on the surface geometry of the specimen as shown in Fig. 4. More escape from projections than from flat surfaces. The differences in the resulting voltage levels in the detector produce the image.

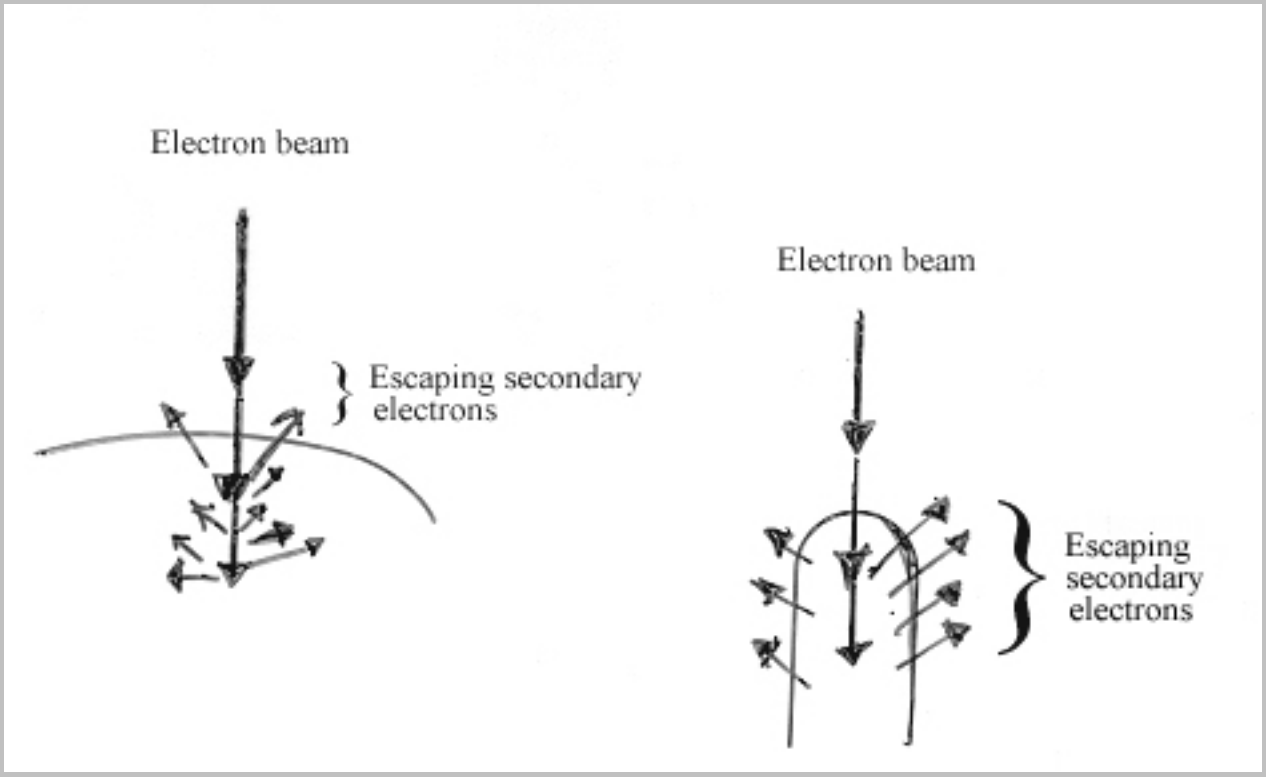


Fig. 4 Secondary electron escape, edge effects and image production.

More recently, high-resolution SEM (HRSEM) has been used as an effective tool to study subcellular detail (see Allen and Goldberg, 1993). These instruments approach the resolution of TEM (approximately 1 nm) by rastering the specimen with a very small (0.7 to 1 nm), but extremely intense spot, about 1,000 times brighter than conventional sources. With this technique the specimen is placed within the magnetic field of the final lens (rather than below as in conventional SEM). The beam interacts with the surface of the observed object and the signal of secondary electrons can be collected efficiently with a minimum of aberration. The voltages used are relatively low (a few kV). As the beam penetrates only the top 10 nm of the specimen, the image is restricted to a shallow portion of the surface. The material is either fixed or rapidly frozen, the water is generally removed or substituted. Although uncoated specimens can be used, generally they are coated with a metal coat, optimally chromium or tantalum at a thickness of 1 to 12 nm.

Computer processing Computer processing of TEM images (referred to as *tomography*) permits

reconstruction of three-dimensional structures. The analysis is more direct for periodically repeating units, such as those in two-dimensional crystals formed in membranes, which allow the application of crystallographic methods. The images of untilted and tilted specimens are first digitized. The three-dimensional reconstruction of the two-dimensional crystal is done with Fourier transforms (FTs). The three-dimensional FT of a two-dimensional crystal (in the *xy* plane in real space) is an array of lines normal to the plane of the crystal (Fig. 5) (Amos et al., 1982). Variations in the specimen density in the *z* direction in real space result in modulation of intensity along each *z* line. The two-dimensional FT of a projection image of the crystal represents a central section, that is, a slice through the origin of the three-dimensional FT with the angle of the slice equal to the tilt angle of the specimen in the EM. By combining many projection transforms (corresponding to different tilt and azimuthal angles), the three-dimensional Fourier volume is filled in, except for a missing cone defined by the maximum tilt angle (typically 50-60). The projection transforms are brought to a common phase origin (equivalent to aligning the images in real space) and the inverse transformation is performed, yielding the three-dimensional volume in real space.

Fig. 3a (Mannella, 1982) shows an electron micrograph of a two-dimensional crystal produced by concentrating the proteins in an outer membrane preparation by partially removing the lipid with phospholipase. The optical diffraction pattern from the micrograph is shown in Fig. 3c. The crystals are formed by the so-called mitochondrial porin or voltage-dependent anionic channel (VDAC). The pores are black when filled with stain (Fig. 3b) and white when filled with water (Fig. 3e). Fig. 3d is a three-dimensional reconstruction of the negatively stained crystals showing the channel structure.

Unordered molecules on an EM grid, which are viewed generally with negative staining or after freezing pose a different challenge, since the analysis is necessarily much more complex. The EM images are recorded with and without tilting of the specimen to provide three-dimensional information, as done for the two-dimensional crystals. The computer three-dimensional reconstruction of the nonperiodic structures is done in real space by tomography. Again, multiple projection images are used; these may be a "conical series," as shown in Fig. 6 (Radermacher et al., 1986), in which there is a constant large tilt angle and the complete range of azimuthal angles is covered. After careful alignment of the images and prefiltering in Fourier space to enhance high-resolution details, the reconstruction requires back-projecting the density at each point in each image into appropriate strips in the three-dimensional volume (Fig. 7, Frank et al., 1985) A flow diagram of the reconstruction procedure is shown in Fig. 8 (Radermacher et al., 1986).

Figure 9a and b show 50s ribosomal subunits tilted by 50 and untilted, respectively. A reconstruction of the ribosomes and various ribosomal subunits is shown in Fig. 10 (Frank et al., 1988).

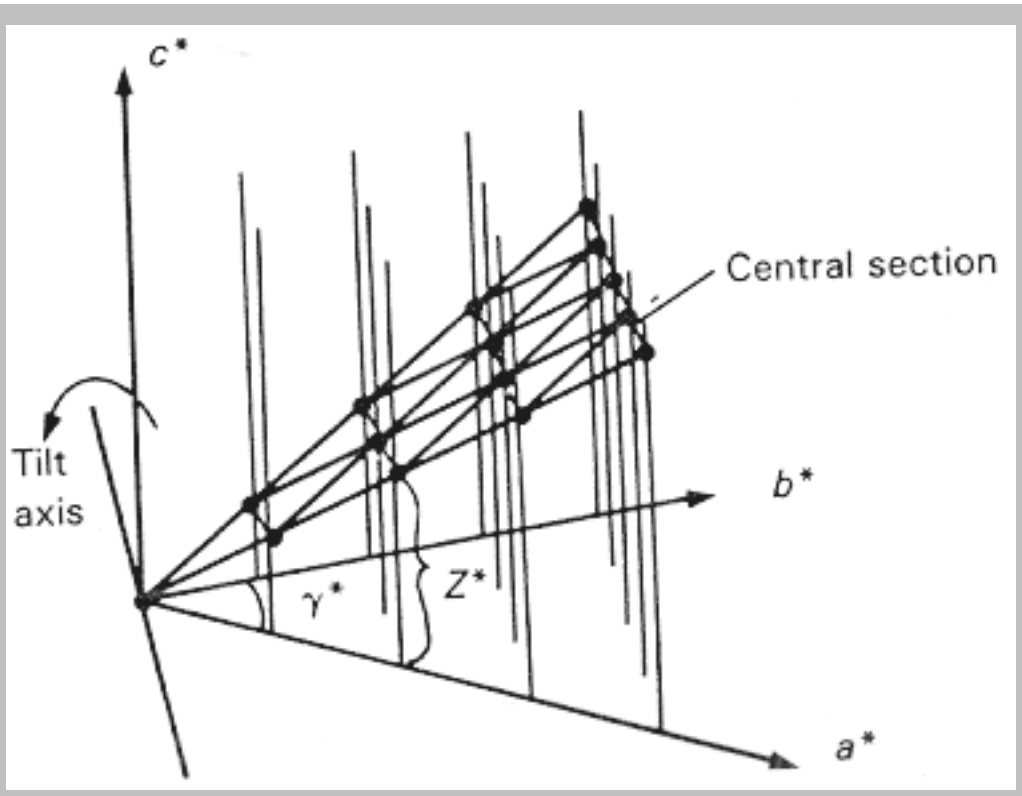


Fig. 5 Schematic diagram showing the transform of a two-dimensional crystal. The transform takes the form of a number of lattice lines extending perpendicular to the plane of the crystal. Each micrograph contains an image that, when Fourier-transformed, gives the values of amplitude and phase at points along the lattice lines where the central section (perpendicular to the viewing direction) intersects them (Amos et al., 1982). Reproduced from *Progress in Biophysics and Molecular Biology*, *39*, L. A. Amos, R. Henderson, and P. N. T. Unwin, 3-D structure determination by electron microscopy of 2-D crystals, Copyright &copy1982, Pergammon Journals Ltd.

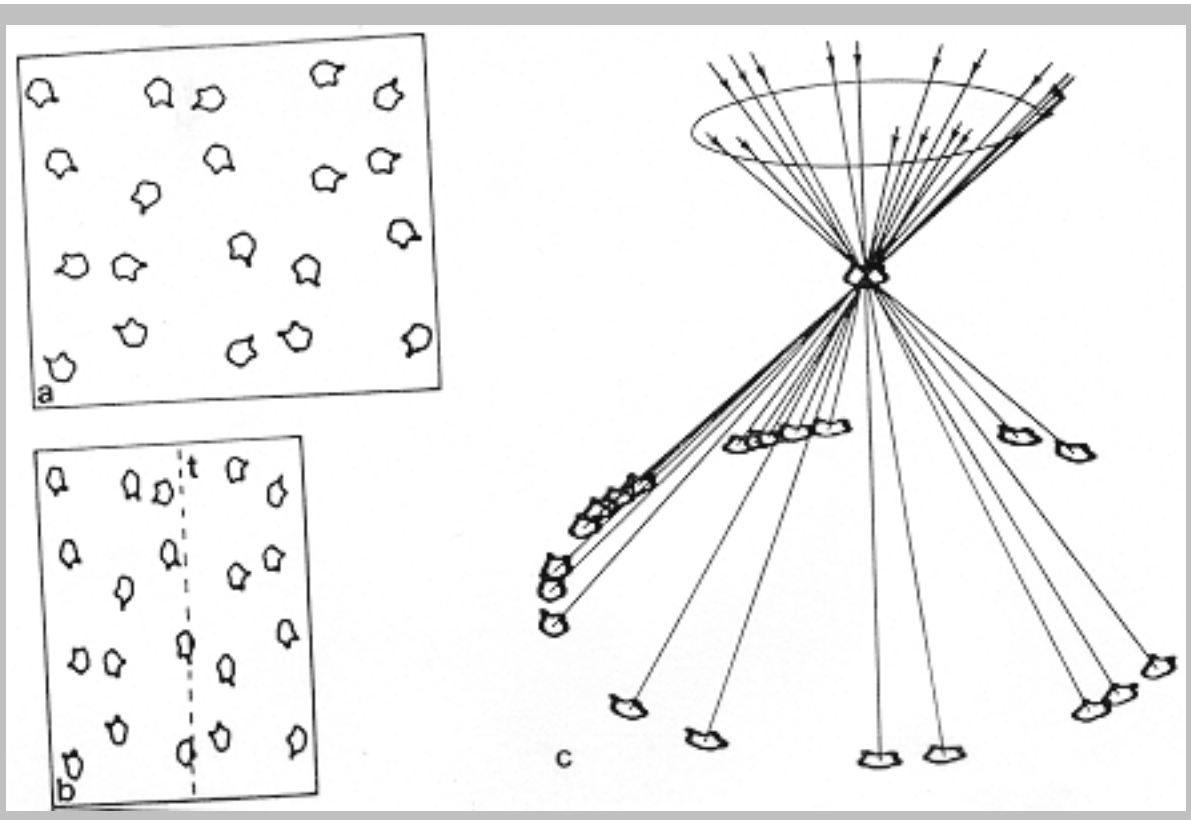


Fig. 6 Basic principle of the reconstruction scheme. (a) View of a specimen with randomly oriented 50s particles lying flat in the plane of the specimen. (b) Projection of the specimen in (a), tilted by 50. The images that can be extracted from the tilted image (b) form the conical tilt series shown in (c), equivalent to a tilt series of a single particle with random projection directions, all lying on the surface of a cone. Reproduced with permission from M. Radermacher, et al., *Journal of Microscopy*, 146:113-136. Copyright &copy1986 Royal Microscopical Society, Oxford, England.

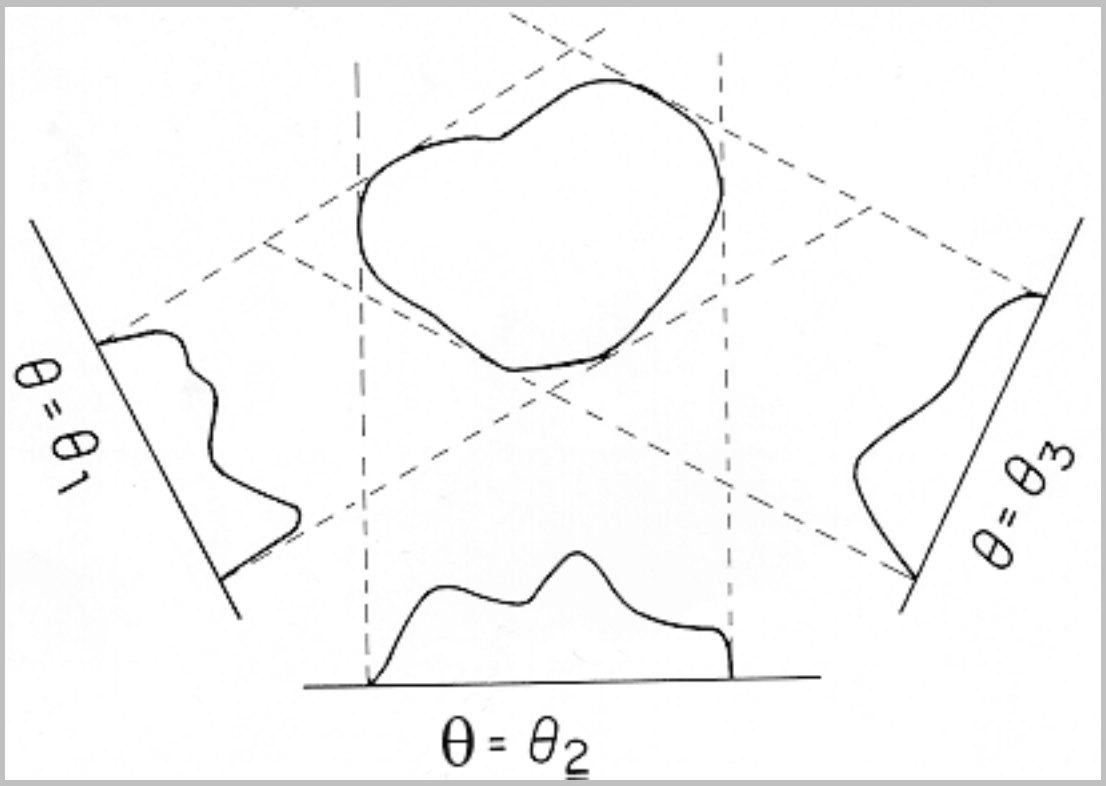


Fig. 7 Illustration of the back-projection method of three-dimensional reconstruction. In any plane perpendicular to the tilt axis, the optical density profiles measured along corresponding lines of the projections are smeared out into the direction of projection and are added up to form a slice of the three-dimensional object to be reconstructed. Reproduced with permission from J. Frank, et al., *New Methodologies in Studies of Protein Configuration*. Copyright &copy1985 Van Nostrand Reinhold, New York, NY.

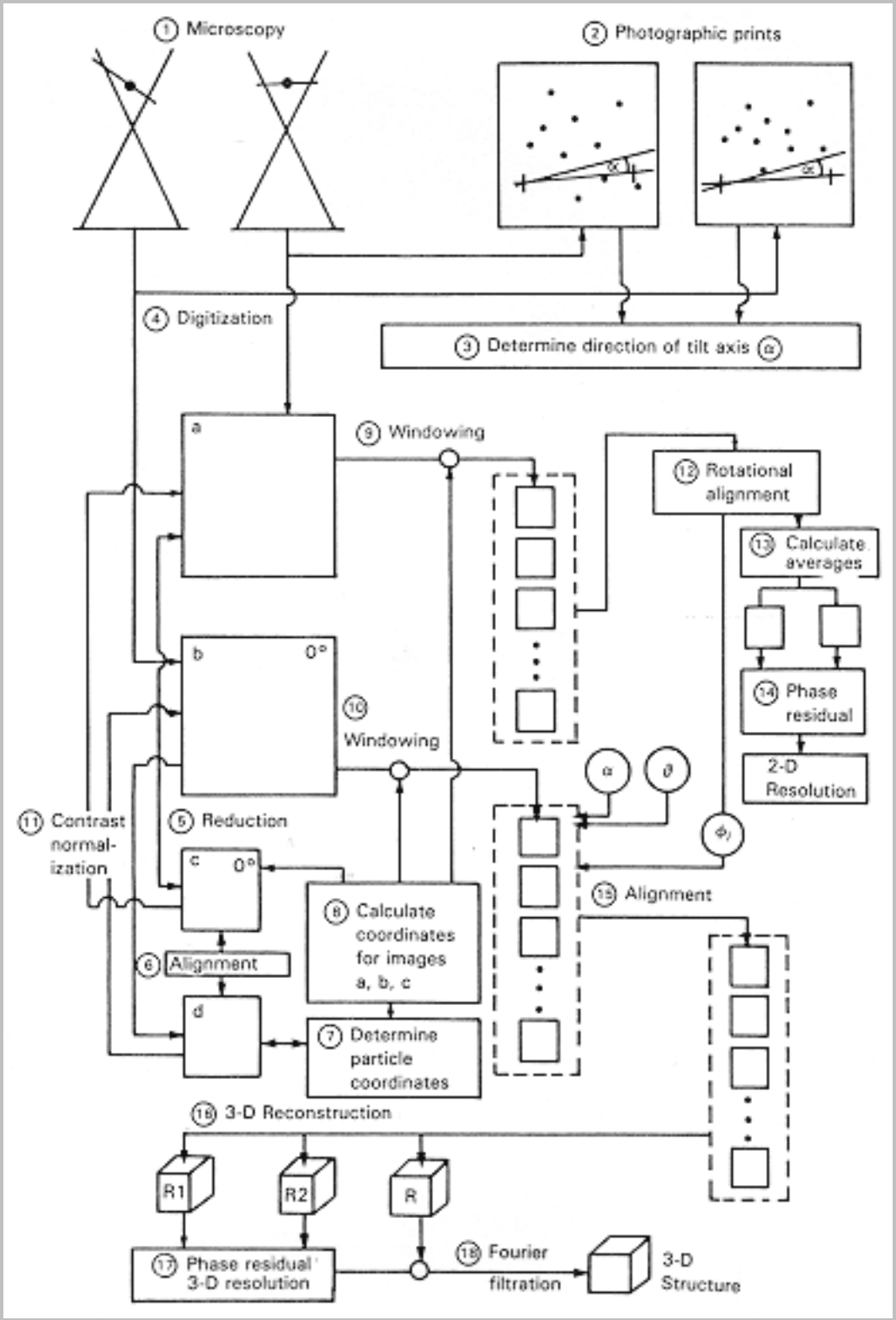


Fig. 8 Flow diagram outlining the complete reconstruction procedure. Reproduced with permission from M. Radermacher, et al., *Journal of Microscopy*, 146:113-136. Copyright &copy1986 Royal Microscopical Society, Oxford, England.

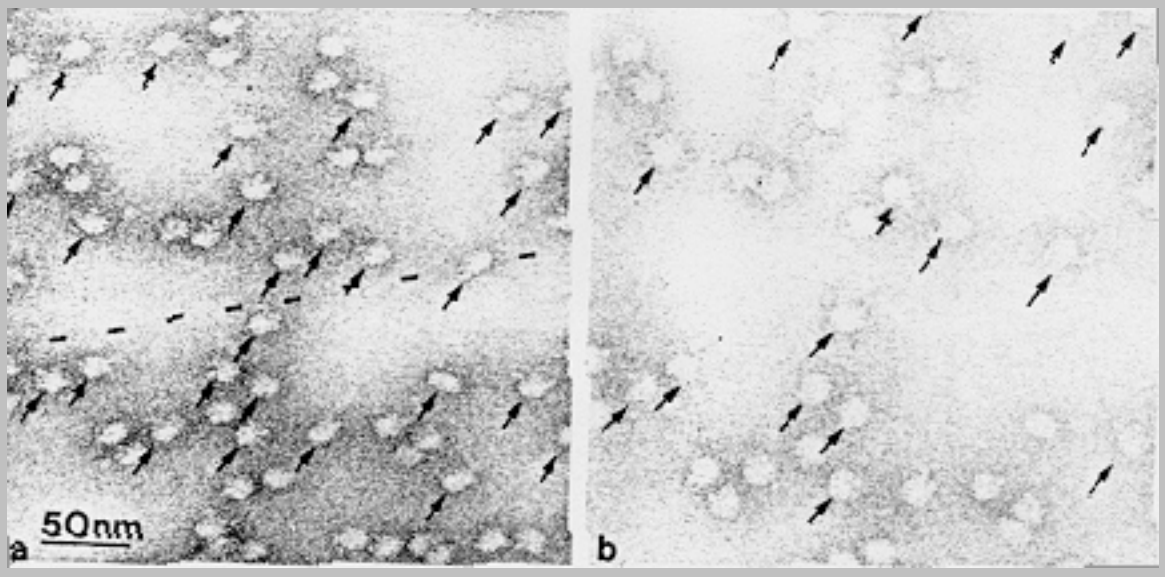


Fig. 9 Portion of the particle fields. (a) Projection of the specimen tilted by 50; the particles initially selected are marked at the lower left corner. (b) The same field without tilt; the particles finally used are marked. Only the area common to both micrographs and lying in the underfocus range in the tilted view (as determined by optical diffraction) was used for evaluation to avoid errors due to the sign change of the transfer function at low spatial frequencies. Average densities calculated in areas showing only carbon foil were used to normalize the contrast of the images. Reproduced with permission from Radermacher et al., *Journal of Microscopy*, 146:113-136. Copyright &copy1986 Royal Microscopical Society, Oxford, England.

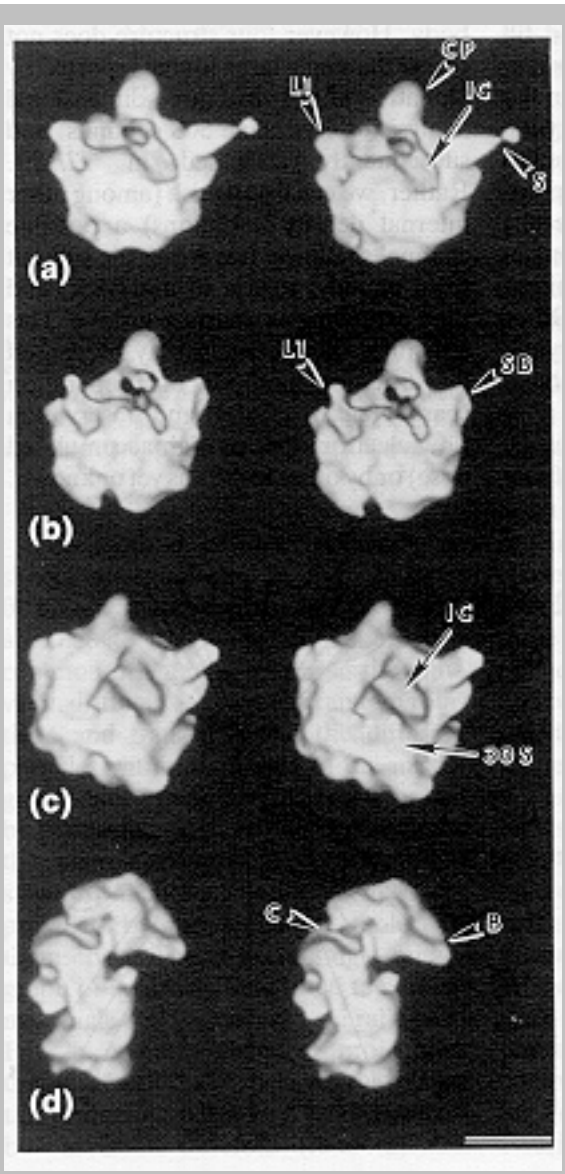


Fig. 10 Gallery of ribosomes and ribosomal subunits reconstructed with the new technique, presented as stereo pairs (scale bar, 10 nm). (a) 50S ribosomal subunit of *E. coli*, oriented with its interface surface toward the viewer. (b) 50S ribosomal subunit of *E. coli* depleted of L7/L12 proteins. Orientation as in (a). (c) 705 monosome of *E. coli* oriented so that the 505 domain matches position with (a) and (b). (d) 405 ribosomal subunit from rabbit reticulocytes in a lateral-view orientation. Reproduced from *Trends Biochm. Sci.* vol.13, Frank et al.,pp.123-127, copyright &copy1988, with permission from Elsevier Science.

Scanning probe microscopy

Scanning probe microscopy is based on entirely different principles. The microscope passes a probe close to the specimen surface and collects spatial information.

The *scanning tunneling microscope* (STM) has been applied to the study of biological surfaces to a very limited extent, since it is a relatively new application. However, its potential is considerable since it allows observations with atomic resolution (see Zadzinski, 1989). The method requires very thin molecular layers attached to a conductive substrate or alternatively coated with a conducting surface. A

metal tip is brought close to the surface and scans at moderate voltages (in contrast to those in electron microscopes), in the range of 2 mV to 2 V. Electrons tunnel between the tip and the conducting surface. A feedback system maintains the current constant by changing the height of the tip (alternatively, the current can be allowed to vary and the distance maintained constant). The image is a map of the tip height in relation to the lateral position of the probe. The resolution is generally very good because invariably some small protuberance projects out of the tip giving atomic resolution.

The *atomic force microscope* (AFM) can be used for imaging as well as micromanipulation (see Hoh and Hansma, 1992). The AFM scans the surface with a sharp tip attached to a soft cantilever. Deflections of the tip, which correspond to the surface topography are recorded optically from the cantilever displacement (Alexander et al., 1989). Various recent technologies have been used for the fabrication of the necessary devices. To date, the resolution of the system using biological specimen is in the range of 1 to 50 nm.

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

B. Isolation of Cell Components

The isolation of cell components has also led to enormous progress in our understanding of the molecular organization of cells. Basically, this approach depends on rupturing the cell in a medium that is compatible with preservation of the integrity of the organelle under study, followed by physical separation of the various cell components. Generally, the medium is a solution that is isosmotic or hyperosmotic in relation to the cell interior; chilled sucrose solutions are often used.

Cells and tissues are usually disrupted mechanically. Bacteria and plants present unique problems because of their tough protective capsules or walls. The capsules or walls can be broken down by enzymatic digestion, or can be prevented from forming by use of special techniques such as using a mutant with a defective capsule. Without such coverings, homogenization can be relatively gentle. For example, with the homogenization device shown in Fig. 11, up-and-down motion of the plunger forces the cells to squeeze through the small space between plunger and vessel wall. The resulting shear breaks down the plasma membrane and some of the cytoskeleton. The homogenizer can be maintained in a ice bath to dissipate the heat generated and to minimize the effect of hydrolytic enzymes.

The most widely used methods for large-scale separations involve centrifugation. The homogenate is placed in a plastic or heavy-walled test tube and then centrifuged. The rotation generates a centrifugal force. which increases with the speed of rotation and the radius of the rotor. The rate of sedimentation of a spherical particle is given by Stokes' law, shown in Eq. (1):

$$\frac{dx}{dt} = \frac{KG(d_p - d_m)r^2}{\eta} \qquad (1)$$

In this equation, x is the displacement in cm, t is time in seconds, G is the force exerted on the particle in gravitational units, d_p, is the density of the particle (g/cm^3) and d_m that of the medium, r is the radius of the particle in cm, and is the viscosity of the medium in poises, K corresponds to 2/9. The centrifugal force is a function of the rotations per minute (rpm) of the rotor as represented in Eq. (2), where L is the radius of the rotor in cm.

$$G = 11.17L\frac{rpm}{1000}^2 \qquad (2)$$

Equation (1) shows that particles differing in r and d_p, could be isolated by sedimenting them sequentially increasing the speed with each centrifugation, as done in *differential centrifugation*. In contrast, when d_p, is lower than the density of the medium ($d_p < d_m$), dx/dt would be negative and the particle would he displaced upward.

In differential centrifugation, after each centrifugation, samples are separated into distinct fractions: a *pellet* of packed sedimented particles and a *supernatant* containing unsedimethed material. The larger or denser particles sediment in earlier centrifugations. This process is illustrated in Fig. 11. Differential centrifugation cannot separate cell components into pure fractions in a single series of centrifugations, because particles will sediment according to their position in the tube rather than just size or density. However, repeated resuspensions and resedimentations can produce a satisfactory level of purity. The effect of position in the tube on isolation can be avoided by layering the homogenate on top of a layer of a denser solution. This process approximately equalizes the distance of travel for all particles. Layers or gradients can be produced by varying the concentration of sucrose or of a polymer (e.g., dextran or Ficoll) in the layers, with the denser layers in the bottom.

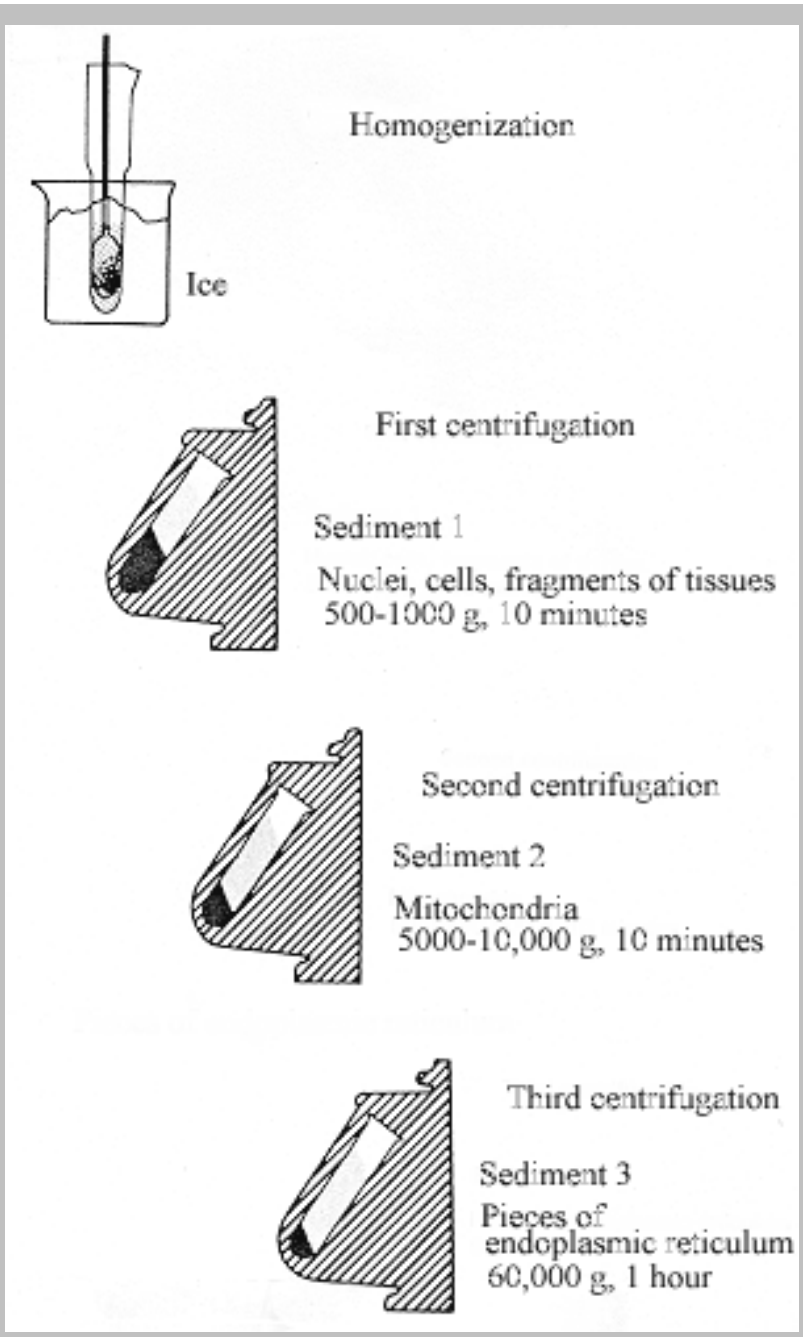


Fig. 11 Diagrammatic representation of differential centrifugation.

Density gradient centrifugation can provide high resolution. As a particle approaches a layer of identical density ($d_p = d_m$) the rate of sedimentation decreases [Eq. (1)] and eventually, at equilibrium, sedimentation stops. With equilibrium centrifugation the separation will depend exclusively on the density, not the size of the particle. However, in many cases the centrifugation need not be carried out until equilibrium. Not surprisingly, density gradient centrifugation has been found most useful for separating organelles of approximately the same size but different densities for example, separating lysosomes from mitochondria.

Centrifugation with a two-phase system has also been found useful. Particles lighter than the bottom layer are rejected and remain at the interface.

Rapidly spinning rotors generate considerable heat, therefore centrifugation techniques require refrigeration. In addition, to reach high speeds the friction has to be decreased by use of a partial vacuum around the rotor.

Subcellular particles can be separated by taking advantage of their surface properties. Many aqueous solutions containing two polymers, such as methylcellulose and dextran, separate out into phases. If the solutions have been mixed with subcellular components, viruses, or macromolecules, these substances will tend to favor one of the two phases or the interface between the two.

Organelles and other particles can also be sorted out by flow cytometry (see Böck et al., 1997) because a variety of fluorescent dyes stain organelles specifically (e.g. rhodamine for mitochondria). With this technique, the flow of medium containing the particles is delivered by a nozzle and broken into individual droplets by a vibrator, each droplet containing a single particle (this can be achieved by arranging a sufficiently dilute suspension). Depending on the fluorescence, the droplets are given either a positive or negative electrical charge. They are then separated by a strong electric field and collected according to charge. This approach has considerable promise, particularly if used after more conventional techniques, combining bulk isolation with higher precision.

The isolation techniques have made available large quantities of material for study by standard biochemical techniques. However, in some cases, lack of purity and possible disruption of native organelles still pose a problem.

The microscopic and biochemical techniques discussed have individual advantages and disadvantages and have been used together to produce a complex picture of cellular organization.

C. Cell Cultures

The use of cell cultures opened a new phase in the study of cells. There are very few sections in this book in which at least some of the experiments cited were not carried out with cells in culture.

Cells to be cultured are first dissociated from the tissue, most commonly by using proteolytic enzymes to digest the proteins that anchor them to other cells or extracellular matrix. Alternatively, fibroblasts can be allowed to grow out of a piece of tissue or explant that is subsequently removed. The cells are placed on plates and provided with appropriate conditions, such as temperature, nutrients, and serum, that probably provides growth factors. Most cells require an appropriate attachment surface to grow and reproduce until they become confluent. The contact between cells arrests their replication (*contact inhibition*) and the cells form a monolayer. The isolated cells display many of the characteristics of their differentiated state.

Cultures derived directly from a tissue are referred to as *primary cultures*. When *subcultured*, that is, removed from the original culture and cultured in a new medium at a lower concentration, the cells continue to divide. The cells can be maintained by repeated subculturing to form a cell line. However, all normal vertebrate cells can undergo only a finite number of divisions after which the cultures become

senescent and eventually die. In contrast, transformed or malignant cells do not have this limitation and are often said to be immortalized. If maintained under the appropriate conditions, they continue to proliferate to a much higher concentration than normal cells, since they do not exhibit contact inhibition. Some transformed cells will also grow in suspension without an attachment surface. Cells can be transformed by infection with oncogenic viruses or treatment with certain chemicals. Alternatively, malignant cells can be cultured directly from tumors. One of the most commonly used cell lines, the HeLa cell line, was originally derived from cervical cancer cells from a single patient. Cells can be stored frozen at liquid nitrogen temperature in the presence of cryoprotective agents. By preventing crystal formation, these agents minimize the damage produced by freezing.

Cells from recognized clones can be obtained from the American Type Culture Collection (Rockville, Maryland) or the Human Genetic Mutant Cell Repository, located at the Coriell Institute for Medical Research (Camden, New Jersey) and have been cataloged (e.g., Catalog of Cell Lines and Hybridoma 1988, American Type Culture Collection).

D. Manipulation of Cells

Micromanipulation techniques have a long history. Parts of cells have been removed or moved, dyes or exogenous macromolecules have been injected into cells. Laser beams have been used in microsurgery (Berns et al, 1969). Several new techniques have introduced new prospectives to these approaches.

There are alternative to to the introduction of components by micronjection into cells. Streptolysin O is pore-forming toxin that can permeabilize cells to proteins as large as 100 kDa. The permabilization can be reversed in the presence of Ca^{2+}-calmodulin and and intact microtubules (Walev et al., 2001). Resealed cells were found to be viable and were capable of proliferation and endocytosis. An entirely different approach is provided by a class of peptides called *penetratins* (see Derossi et al., 1998; Schwarze et al., 2000; Lindgren et al., 2000). These peptides enter cells freely and can carry with them whatever compounds have been attached to them covalently, even iron nanoparticles 40 nm in diameter (see Schwarze et al., 2000)! In practice, they can be linked either by chemical reactions or genetic manipulation (Prochiantz, 1996). Other polypeptides have been shown to have similar properties. Penetratins are throught to interact with lipids to form inverted vesicles with the hydrophilic groups inside and the hydrophobic groups outside (Derossi et al., 1994,; Berlose et al., 1996). This approach is promising and may provide a way of introducing a number of macromolecule into cells. Biologically, among the proteins that are transferred in this manner are *homeoproteins*. Homeoproteins are transcription factors involved in developmental steps (e.g., see Gehring, 1987). The transfer of these proteins could transfer positional information between cells during development.

Cell-permeable peptides have also been used in the study of protein interactions. The penatrins were linked to intracellular inhibitors, peptides containing protein interaction domains. By competing with the endogenous proteins containing the same domain they block their biological function (e.g., see May et al., 2000). More recently, this approach has been used to define the pathways of export from the nucleus of particular mRNAs (see Chapter 5), using cell-permeable peptides constructed to interfere with interactions

between known adapter and receptor proteins (Gallouzi and Steiz, 2001).

The use of single beam laser *optical gradient traps*, also called *optical tweezers*, holds considerable promise (Ashkin and Dziedizic, 1987). Optical tweezers allow experimenters to control of position to an accuracy of about 10 nm. They also allow forces in the order of 10^{-8} dynes. Optical tweezers can trap particles ranging in size between 15 nm to 25 µm and thus manipulate macromolecules in solution as well as organelles and whole cells.

Rays of light are refracted at the surface of a particle. Each passing photon, by virtue of its momentum, gives the particle a minuscule "kick" which can displace it. A single Gaussian laser with a Gaussian transverse intensity profile produces a stable configuration close to the beam focus and acts as a trap (Block, 1990). Optical tweezers have allowed, for example, measuring minute forces (e.g., see Simmons et al., 1996). The principles involved in optical tweezers are depicted in fig.12 (Block, 1992) In this figure the solid arrows represent the refracted light rays. The open arrows represent the reactive forces produced by conservation of momentum imparting a momentum equal and opposite to the momentum change of the rays.

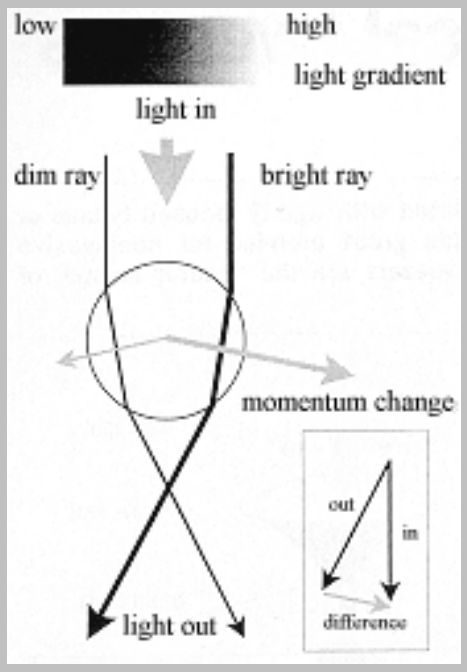


Fig. 12 A parallel beam of light with a gradient in intensity (depicted by the shaded area) impinges on a spherical lens. Two representative rays are drawn (black arrows). The rays are bent as shown. As shown by the vector diagram the rays pull the sphere towards the brighter light. In the absence of a gradient the sphere is "trapped". From Block, 1992. Reproduced by permission from Nature 386:779-787, copyright &copy1997 MacMillan Magazines Ltd.

Go to Part 3

REFERENCES

Search the textbook

Back to Part 2

II. TECHNIQUES OF MOLECULAR BIOLOGY

A. Recombinant DNA Techniques

The study of the macromolecules involved in cell function has been revolutionized by the development of recombinant DNA technology. Combinations of unrelated genes can be created, cloned and amplified. Furthermore, the production of complementary DNA (cDNA, discussed below), its cloning to produce large amounts of material and subsequent sequencing have allowed the study of proteins that could not be readily studied before.

Constructing new DNA

Novel DNA can be produced by the use of techniques in which a piece of DNA (referred to as *donor DNA*) is cut in pieces and then combined with other DNAs. *Restriction enzymes* or endonuclease that can cut up DNA molecules into smaller pieces are found in a variety of prokaryotes. They recognize specific base sequences in double-helical DNA and cleave them in such a way that the new ends are single stranded and complementary to each other; that is, two complementary strands are cut in slightly different positions, producing staggered ends (see Fig. 13a). The same restriction enzyme can be used to cut *vector*-DNA, creating staggered ends with precisely the same sequence as the donor DNA. These so called sticky ends allow the restricted fragments to be combined with the vector DNA. Vectors are phages or plasmids independently replicating nonchromosomal DNA. Treatment with DNA ligase will then join the two kinds of DNA, the vector and the donor piece (Fig. 13b). Similarly, a small, artificially synthesized DNA piece can be covalently joined to either vector or donor DNA.

The covalent joining of a DNA fragment to a DNA vector permits *cloning* of the fragment, that is, the production of many identical copies. DNA can be introduced into cells by a variety of techniques, including infection with a virus containing the donor DNA. The modified DNA replicates in host cells. A commonly used host for cloning is *Escherichia coli*, the intestinal bacterium that has already provided us with a wealth of genetic and biochemical information.

A method (unfortunately unpublished at this time) has been developed for the delivery of DNA by producing 25 nm balls containing compacted DNA and positively charged peptides. These particles appear to enter cells readily and will be transferred into the nucleus (see http://www.cgsys.com). Supposedly, this technique compares favorably with the use of liposomes or virus vectors to transfer the genetic information.

The cells containing the donor DNA can be selected by various procedures. Most simply, the DNA is ligated to a vector containing a marker gene, such as antibiotic resistance. This allows recombinant organisms to be selected by growing cells in the presence of antibiotic where only the cells expressing the marker survive. Alternatively, cells from each clone can be tested for the desired DNA sequence by *Southern blotting* (using a labelled complementary DNA; see below) or by assaying for a protein coded for by the new DNA, either by enzymatic assay if the protein is an enzyme or with a specific antibody (*Western blotting*; see below).

A similar technique can be used to replace a gene in a vector using restriction enzymes and ligase. This approach has been found particularly powerful when applied to yeast.

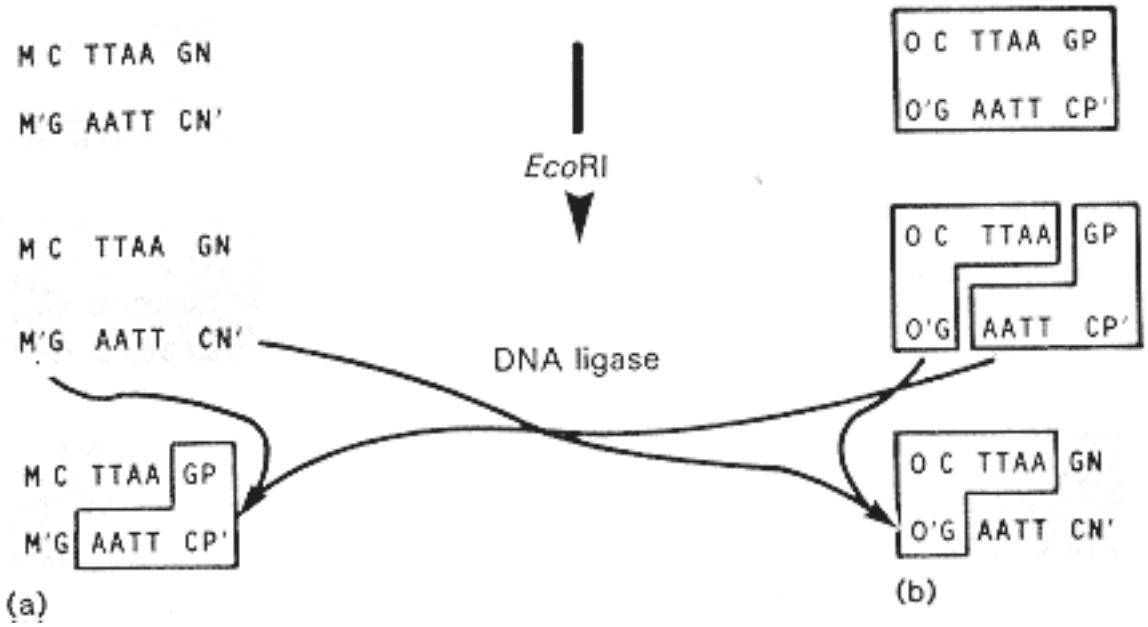


Fig. 13 Production of a novel DNA. Only the staggered ends are shown. (a) Production of staggered ends by endonuclease. (b) Joining of the two distinct DNAs by DNA ligase.

Selected genes can be cloned from the DNA representing the complete genome of a cell type. The DNA is fragmented by shearing or by use of restriction endonucleases and is separated out by electrophoresis to select pieces about 20 kilobases (kb) long. When the DNA pieces are linked to vectors, such as lambda phage DNA, and the infective phage is reconstituted in vitro, they can be used to infect *E. coli* and replicate repeatedly to form a so-called *genomic library*. A similar technique departing from the mRNA of a cell, followed by the production of cDNA and cloning is referred to as an *expression library*.

Infection and subsequent lysis of *E. coli* cells on a plate by a dilute suspension of phage produce plaques, each corresponding to the progeny of a single phage. When a sheet of nitrocellulose is applied to the plate, some of the DNA from lysed cells sticks to the sheet and forms a replica. After denaturation of the DNA with alkali, specific DNA sequences can be identified by hybridization to radioactive DNA or RNA *probes* (Southern or Northern blotting, see next section). The hybridization sites can be recognized by autoradiography and the corresponding plaques still present in the original plate can be picked out and grown to form millions of DNA clones.

Amplification by synthesis of DNA using the *polymerase chain reaction* (PCR) with a selected DNA serving as a template provides an alternative to cloning (Saiki et al., 1988). Single genomic sequences as large as 2 kb have been amplified more than 10 million times with a high-specificity, heat-resistant DNA polymerase extracted from a thermophilic bacterium. A thermostable polymerase is advantageous, since newly synthesized strands have to be separated (generally done by heat) before the next round of replication. A continuous flow PCR system (Kopp et al., 1998) permits very fast amplification. The sample is heated or cooled by flowing continuously and repeatedly over sections of a device where each section is maintained at constant temperature corresponding to melting, primer annealing and primer extension temperatures.

DNA probes can be produced from the corresponding messenger RNA (mRNA). The mRNA can be isolated from cells that produce predominantly a single protein (e.g., reticulocytes. which synthesize hemoglobin). Alternatively, the mRNA can be extracted from polysomes isolated by precipitation with antibodies against the protein in the process of being synthesized. Then complementary DNA, cDNA, can be synthesized using reverse transcriptase and the mRNA as a template. Reverse transcriptase is an enzyme found in retrovirus, where it synthesizes a DNA template complementary to genomic RNA during replication of its genome. The cDNA can then be cloned as already described or used to produce the corresponding protein.

Site-specific mutagenesis has obvious advantages over conventional mutagenesis, since the latter is a random rather than a directed process. Altering single nucleotides in isolated DNA permits synthesis of the modified DNA. In practice, this can be accomplished by first synthesizing a small oligonucleotide piece complementary to a short sector of the modified DNA containing the nucleotide change. The rest of the strand can then be synthesized by DNA polymerase which uses the small oligonucleotide as a primer. Site-specific mutagenesis has been used to modify proteins in a controlled and systematic manner in the study of mechanisms of catalysis and the relationship between protein structure and function.

Homologous recombination between exogenous DNA and a target locus can be used to place foreign DNA at precise sites in the chromosome (see Capecchi, 1989). Replacing a DNA sector pin-points a mutation precisely (producing a mutation by a process referred to as *gene targeting*). This technique can also be used to remove a gene to produce a *null* mutation, with a single gene missing (*knockout* mutation).

In homologous recombination there are two possible alternatives (see Capecchi et al., 1989). A sequence can be incorporated in the DNA using *sequence replacement* or *sequence insertion* linear vectors. With the sequence replacement technique, the linear replacement vector contains portions homologous to the wild-type gene bracketing the replacement sector. When the homologous segments (genomic and vector segments) are paired, recombination events may occur so that the genomic sequence is replaced by the vector sequence. In the insertion technique, in contrast, the linear vector is engineered so that loci that are adjacent in the genetic map of the genome are at the ends of the vector and separated by the new sequences. When the homologous sequences are paired to the genomic sequences, the entire vector is

incorporated so that part of the DNA remains as a duplicate.

Because the recombinations are rare, it becomes necessary either to have selection markers incorporated into the vector (e.g., resistance to drug) or enrichment techniques (e.g., PCR). The identification of correctly modified cells depends on amplifying specifically the recombinant DNA fragments by PCR (Kim and Smithies, 1988). This is achieved as follows. The target chromosomal sequence can be represented by A--B and the exogenous DNA as X--Y, where the letters represent DNA ends. The recombinant fragment will be A--Y. If only the primers for A and those for D are present during the PCR, the primer for A will replicate a sector corresponding to AB and the primer for Y will replicate the XY segment producing single stranded DNA at a linear rate (they can only start replicating at one end). However, the recombinant (A+Y) can synthesize a double stranded DNA with exponential amplification and therefore outpace the production of the other fragments (see diagram).

The application of homologous recombinations techniques have been used to eliminate genes from specific regions of the brain in mice (see Chapter 22). These studies used the phage P1-derived *Cre/loxP* recombination system (Tsien et al, 1996a). The Cre recombinase of the phage catalyzes the recombination between 34 base pairs *loxP* recognition sequences (Sauer and Henderson, 1988) in vitro or in vivo and the recombination does not require any other protein factors. The loxP sequences can be inserted in the genome of embryonic stem cells by homologous recombination so that they flank one or more exons of a gene of interest (referred to as a "floxed gene"). Mice homozygous for the floxed gene are crossed to a second mouse containing a *Cre* transgene under control of a cell-type specific transcriptional promoter (see Mayford et al., 1995). In the homozygous progeny containing both the floxed and the *Cre* transgene, the floxed gene will be removed by the Cre/loxP recombination and only in specific cells. The technique was used to eliminate the NMDAR1 gene in the CA1 pyramidal cells of the hippocampus of mice (Tsien et al, 1996b). In other experiments the NR2B subunit of the NMDA receptor was overexpressed in the forebrain of mice (see Chapter 22) (Tang et al., 1999) using the CaM-kinase-II promoter which is tissue specific (see Mayford et al., 1995; Tsien et al., 1996a).

Note also that the use of antisense RNA or DNA permits the manipulation of genomic DNA as discussed below.

An emerging technique allows modifying DNA using *introns* (see Chapter 1), referred to as group II introns, which can insert themselves into DNA (see Michel and Ferrat, 1995; Lambowitz et al., 1999). After removal from pre-mRNAs, the introns are incorporated into double stranded DNA and transcribed in reverse to form genomic DNA, a reaction requiring a protein with reverse transcriptase, RNA splicing and DNA endonuclease activity and encoded by the introns. The insertion takes place at sites of 14 nucleotides by base pairing interactions. The site of insertion can therefore be controlled by modifying the introns so that specific genes can be targeted (Guo et al., 2000), since introns can be inserted into any DNA with the appropriate target site. Additional DNA can be introduced in the intron. The intron can be delivered to human cells in culture using plasmids.

Gene function can be also manipulated inside cells using synthetic triplex-forming oligonucleotides (TFOs) (see Vasquez and Wilson, 1998). Intermolecular triplexes can form at the DNA double helix that are purine rich in one strand (Moser and Dervan, 1987; Cooney et al., 1988). The third strand is located in the major groove of the DNA and binds to the purine rich duplex by forming specific hydrogen bonds. The in vitro transcription of RNA polymerase was found to be inhibited by the third RNA strand (Morgan and Wells, 1968). Apparently, triplex formation interferes with transcription-factor binding or the initiation complex. In addition, the triplex can be used to produce site specific damage by attaching covalently to TFOs reagent moeities. The TFO has to be delivered into cells to be effective and a variety of techniques have been devised (see Vasquez and Wilson, 1998). TFOs have been shown to produce specific mutations in somatic cells of intact mice (Vasquez et al., 2000)

Another technique using *hammerhead* ribozymes (ribozymes are RNAs with enzymatic activity) that cleave mRNAs at specific sites (see Uhlenbeck, 1987, Haseloff and Gerlach, 1988) has been used to block the expression of certain genes. A dimeric ribozyme construct, called *maxizyme* (Kuwabara et al., 1998) has a sensor arm that can recognize target sequences and has been used to inactivate specific mRNAs (e.g., the inactivation of *BCR-ABL*-mRNA responsible for *myalogenous leukemia*, see Tanabe et al., 2000).

In *transfection*, the genetic information contained in a vector is incorporated in the host's DNA. Transfection of fertilized eggs can lead to the production of stable *transgenic* organisms that will pass the newly acquired gene from generation to generation.

Gene-silencing has been useful in determining the function of specific genes. However, since the function of some of these genes may be essential for the cell's viability, it has become important to generate cell lines or mice that are conditional mutants (see Porter, 1998; Hudson et al., 2002). Temperature sensitive mutants have been useful in this respect (e.g. see Chapter ll). One of the ways of producing a conditionally null gene is to keep the cell viable by introducing a transgene whose expression can be controlled experimentally (e.g., by introducing a construct with a promoter that can be induced or repressed) .

Recombinant DNA techniques have revolutionized genetics, the study of gene expression, and our understanding of the functioning of protein molecules by making it possible to produce or modify genes, change their genetic environment and change proteins. Used to produce large amounts of proteins and other compounds, recombinant DNA has entirely changed the study of their physiological role and therapeutic use.

Use of antisense RNA and DNA

The function of a gene can be studied by the introduction of an RNA or a single stranded DNA complementary to the mRNA of the target gene into the cytoplasm of cells. This *antisense* molecule can base pair to the mRNA so that it cannot be translated.

There are several ways in which this can be accomplished. Antisense RNA can be synthesized in vitro using RNA polymerase from phages and then microinjected into cells. Similarly, vectors can be constructed to produce high levels of antisense RNA. These can be plasmids containing the target gene in a backward orientation. It has been possible to produce transgenic mice with these plasmids. Alternatively, single-stranded DNA oligonucleotides can be synthesized with the sequence in the vicinity of the initiation site (the AUG codon). The hybridization of the oligonucleotides to the mRNA will block the initiation of translation. There is evidence of endogenous antisense RNA carrying out regulatory functions physiologically.

Double stranded RNA (dsRNA) has been found to silence genes (see Hammond et al., 2001). This silencing is post-transcriptional and takes place in a mechanism in which dsRNA triggers degradation of homologous mRNA in the cytoplasm (see below). However, at least in plants interactions between homologous DNA and RNA sequences can silence genes by inducing DNA methylation (see Fagard and Vaucheret, 2000). In addition, RNA-induced chromatin modifications have been observed in plants (see Matzke et al., 2001). Although so far there is no evidence of this latter effect in animals, it would be surprising if the phenomenon were restricted to plants alone.

In the nematode *Caenorhabditis elegans*, specific *double stranded mRNA* (dsRNA) was found to block selected genes specifically (Fire et al., 1998). dsRNA has similar effects in other organisms (e.g., Waterhouse et al., 1998; Sanchez-Alvarado and Newmark, 1999). Furthermore, the effect was observed by injecting the dsRNA in the body cavity of the nematode. Apparently, this molecule had no trouble crossing cellular barriers and affects a variety of differentiated cells. In addition, the gene silencing is also transmitted to the the worms progeny. Genetic studies have revealed that the systemic transmission of the RNA interference (RNA_i)in *C. elegans* requires the expression of a number of genes. One of these encodes a protein, SID-1 which has a putative transmembrane domain (Winston et al., 2002). Surprisingly only a few molecules of dsRNA are required per cell, suggesting the presence of a catalytic or amplification mechanism. How the dsRNA acts in *Caenorhabditis elegans* is still not entirely clear. In experiments using *Drosophila* cells in culture transfected with specific dsRNA (Hammond et al., 2000), the cells exhibited loss of function phenotype with a decrease in the corresponding mRNA. Extracts of the transfected cells were found to contain a nuclease which degrades transcripts homologous to the double-stranded RNA. Apparently, the dsRNA is degraded into short antisense RNA segments [the so-called short interfering RNAs (siRNAs)], complementary to the targeted mRNA (Hamilton and Baulcombe, 1999; Zamore et al., 2000) in a process that is ATP dependent. Both strands of the dsRNA are cut into segments 21-23 nucleotides in length, in an RNaseIII-like reaction. The targeted mRNA is cleaved only within the region of identity with the dsRNA at sites 21-23 nucleotides apart. The short segments used for targeting are thought to be incorporated into a ribonuclease complex and to serve as a guide for the degradation (Hammond et al., 2000). The endonuclease (*Dicer*) has helicase activity and contains a dsRNA binding domain and a PAZ domain (e.g., see Bernstein et al., 2001). The PAZ domain is a region of 110 amino acids involved in the production of siRNA (see Ceruti et al., 2000). In a second step, the antisense siRNA, homologous to the suppressed gene, guides another RNase complex, the *RNA-*

induced slicing complex (RISC), to cleave the homologous single stranded mRNAs approximately in the middle of the region paired with siRNA (e.g., Elbashir et al., 2001) .

The use of RNA interferences techniques has been extended by an approach that allows massive screening of the genome of *Caenorhabditis elegans* whose sequence is known. Such screens are made possible by feeding the worms with bacteria, each kind expressing double-stranded RNA corresponding to the DNA sequence present in the genome of this nematode. (Fraser et al., 2000). The extensive RNA_i library (16,757 RNAs) created in one study is reusable for other studies of gene function in *C. elegans* (Kamath et al., 2003). In one study (Kamath et al., 2003), the mutant phenotypes for 1,722 genes were identified. Genes of similar functions were found to share similar transcription profiles and to be clustered in distinct very large regions of individual chromosomes. An RNA_i inactivation screen of 5,690 *Caenorhabditis elegans* genes for genes involved in longevity (Lee et al., 2003) uncovered a mutation in the mitochondrial leucyl-tRNA synthetase gene which was found to impair mitochondrial function and was found responsible for a longer-lifespan. The findings suggest a complex coupling of metabolism and longevity. A similar screen was carried out to disrupt the expression of worm genes involved in normal fat storage using the entire available library. The study identified 305 gene inactivations that cause reduced body fat and 112 gene inactivations that cause increased fat storage (Ashrafi et al., 2003).Many of the nematode genes have mammalian homologues supplying a tool for the study of human function and disease.

The possibility of a similar effect of dsRNA in mammalian tissues is intriguing and it has been proposed that the effects ascribed to antisense RNA may be from a contaminating dsRNA (e.g., Hunter, 1999). However, in mammalian cells or in animals intracellular injection of dsRNA is often used as a control in antisense experiments and no comparable results have been reported. It would be interesting to observe what would happen with an extracellular injection in whole tissues or organisms.

The normal function of dsRNA is likely to be the suppression *transposons* (Tabara et al., 1999; Ketting et al., 1999). Transposons are mobile DNA elements which may contain several genes including those required for transposition and may insert copies of themselves all over the genome. Transposons produce dsRNA. This post-transcriptional gene silencing may be also important in defense from virus invasion or in the regulation of gene expression.

Recognition techniques

DNA and fragments obtained by restriction endonuclease treatment of DNA can be separated by polyacrylamide or agarose gel electrophoresis (see below). The positions of the bands can be detected by using autoradiography when the DNA has been labelled (e.g., with ^{32}[P]) or alternatively by staining with dyes such as the fluorescent ethidium bromide.

Specific base sequences can be recognized by hybridization techniques in which double-stranded DNA is denatured and the resulting single strand is annealed to a probe. Such a probe is a specific sequence of

DNA, usually radiolabelled so that its presence, and hence that of complementary sequences, can be recognized by autoradiography. In the laboratory, the transfer of denatured DNA bands onto nitrocellulose sheets before annealing, proved most practical. This technique is known as *Southern blotting*. The same technique applied to the recognition of RNA sequences is referred to as *Northern blotting*. Recognition of a protein by using a specific antibody as a probe is referred to as *Western blotting*.

A novel approach uses a device capable of identifying individual DNA strands with single-base resolution (Howorka et al., 2001). The biosensor consists of a DNA oligonucleotide covalently attached to the the lumen of an α-hemolysin (α HL) nanopore. When a voltage is imposed across the pore and single-stranded DNA molecules bind to the attached DNA strand, the ionic current flowing through a nanopore is reduced. The technique is capable of detecting single nucleotide differences in sectors up to 30 nucleotides in length.

A technique that has revolutionized the study of gene expression involves the use of so called *gene or DNA chips* or *DNA microarrays* (e.g., see Gerhold et al., 1999). In this approach, DNA fragments are arrayed at high density on a solid support (Schena et al., 1995). This array permits probing the mRNA of cells by hybridization and identifying active genes from whole genomes (e.g., DeRisi et al., 1997). DNA microarrays can also be used to study protein-DNA interactions (see Ren et al., 2000). In practice, the DNA from specific genes can be delivered to glass microscope slides with high speed robotics to form microarrays. As many as 2,400 yeast open reading frames (supposedly coding for proteins) can be placed on a single slide (e.g., Lashkari et al., 1997). The differential expression of the genes is evaluated by hybridization to cDNA labelled with fluorescent nucleotide analogs (e.g., derived from mRNA using inverse transcriptase and then amplified with PCR, e.g., see Spellman et al., 1998). A laser scanning device is used to measure the fluorescence, representing the extent of hybridization, and then the relative intensity is expressed in pseudo color (e.g, see Schena et al., 1995; Schena et al., 1996; Lashkari et al., 1997). Where two different states are to be compared two sets of fluorescent cDNA probes can be used, seen as red or green. When the two probes bind to the same DNA, the combination will show as yellow (e.g., Shalon et al., 1996; DeRisi et al., 1997). A clustering alogarithm developed by Eisen et al. (1998), allows arranging genes which are apparently coregulated. This provides information on the transcription of genes (for example, underlying cell cycle regulation). These mass-produced microarrays are a new tool for examining in detail gene expression. Studies have been carried out on the human genome with this technology, monitoring the expression of over 1,000 human genes (Schena et al., 1996). Similarly, the temporal program of gene expression accompanying the metabolic shift from fermentation to respiration, was studied using DNA microarrays containing almost all of the genes of *Saccharomyces cerevisiae* (Shalon et al., 1996; DeRisi et al., 1997). Another study implicated 800 yeast genes in the yeast's cell cycle (Spellman et al., 1998). The expression of 650 genes cycling as the consequence of circadian rhythms (Panda et al., 2002) has been identified with this approach in mammals (see Chapter 15).

DNA microarrays have also been used to differentiate B-cell lymphomas by examining the expression of

approximately 17,500 genes (Alizadeh et al., 2000) heralding possible important medical applications that can be used in diagnosis and treatment. In this case the DNA microarrays are referred to as *Lymphochip* and have been also used to define the changes in gene expression that are responsible for the immune system responses (e.g., Rogge et al., 2000) or that cause autoimmune diseases (Glynne et al., 2000). Other recent applications of DNA-microarrays consist in studies of genes expressed in human melanomas (Bittner et al., 2000) and those resulting in metastasis in mice and human melanomas (Clark et al., 2000). Four different childhood leukemias were distinguished by hybridization of 63 RNAs samples of four types of the tumors to DNA chips. A computer based alogarithm (Artificial Neuronal Network, ANN) was set up to recognize and categorize the resulting patterns. The system was able to classify 20 unknown cases of cancer (Khan et al., 2001).

Reverse-transcription and the polymerase chain reaction (RT-PCR) have been combined in a powerful technique that generates cDNA from mRNA transcripts and amplifies the resulting cDNAs (e.g., see Dukas et al., 1993). After gel electrophoresis, the location of the DNAs in the gel can be identified with dyes such as SYBR green (Morin and Smith, 1995) or TaqMan (e.g., see Schmittgen et al., 2000). These procedures allow the quantitative evaluation of gene expression in real time, even with very small amounts of RNA.

DNA microarrays (Ren et al., 2000) can also be used to study protein-DNA interactions. How can you detect the binding of a protein to the DNA? A genetic approach to study the binding of proteins to DNA and RNA is discussed in Section IIB, an extension of the two-hybrid technique of detecting protein-protein binding. This section will discuss biochemical aproaches. Apart from the older ultracentrifugation techniques that can detect a shift in molecular size by changes in sedimentation rates, two techniques have been predominantly used. In the *gel retardation* technique, the binding of protein to DNA fragments can be recognized by the slowing down of the migration of the fragment in a polyacrylamide gel. The slowing down results from the increased size of the complex containing the protein and the DNA. This procedure has been very useful in recognizing in vitro binding. Another technique known as *footprinting* demonstrates binding by virtue of the protective effect of the complexed protein. When the DNA is exposed in vitro to an endonucleases, the sectors which bind the protein are not spliced by the enzymes so that they do not appear when DNA fragments are examined by gel electrophoresis. The missing segments leave an empty space in the gel, the so called footprint. An alternative technique uses dimethylsulfate which cleaves DNA before G residues instead of enzymes. Again proteins binding to DNA can serve as shield. The chemical technique has the advantage of permitting use in intact cells thereby offering insights on in situ DNA binding.

An extension of the microarray techniques produces cells which express a defined DNA. Different cDNAs are placed as microarrays on the slide on which cells are grown. Cells on the spots containing the DNA are transfected. Thousands of cell clusters transfected with a defined DNA provide cells in which a gene product is either overproduced or inhibited. Together with other techniques, the availability of these cell microarrays provide a massive approach to detecting the function of specific genes (Ziauddin and Sabatini, 2001; see Wu et al., 2002). Transfected cell microarrays expressing 192 different cDNAs

demonstrated the involvement of proteins in tyrosine kinase signalling, apoptosis and cell adhesion (Ziauddin and Sabatini, 2001)

A powerful method that permits identifying and isolating DNA fragments binding to a known protein is the *chromatin immunoprecipitation* (CHIP) technique (Dedon et al., 1991). With this approach the DNA-protein complex is first cross-linked in situ using formaldehyde. Following homogenization of the cells, the preparation is sheared to solubilize the DNA and the complex is precipitated using an antibody for the protein. After harvesting the complex, the cross-linking is reversed (e.g., with heat). Most frequently the DNA is subjected to PCR amplification to facilitate identification.

How can specific DNA sequences be recognized in cells? Nucleic acid probes can be used in the techniques of *in situ hybridization* (e.g., see Trask, 1991). The DNA can be exposed temporarily to a very alkaline pH to separate the strands. The specific sites can then be recognized with an appropriate probe that will hybridize to the site under study. Modified nucleotides can be used to provide either a target to antibodies (attached to a fluorescent dye or gold particle, see Section IIB, below) or to some other molecule that can be recognized such as biotin that can be recognized by its binding to Texas red-avidin (e.g., O'Keefe et al., 1992). The techniques in which fluorescent probes are used for in in situ hybridization is known as *fluorescence in situ hybridization* (FISH). In situ localization of DNA may be also carried out using sequence-specific DNA-binding proteins. The proteins can be identified by immunological techniques either at the light or EM level. Similarly a green fluorescent protein-lac repressor fusion protein was used using the reporter gene technique(Robinett et al., 1996).

The recognition of transcriptional events is facilitated by the use of *reporter genes*. A piece of DNA coding for a protein that is easily recognizable is attached to the gene under study. Favorite reporter genes have been the *lacZ* gene of *E. coli* which codes for β-galalactosidase or the gene for chloramphenicol acetyl transferase of *E. coli* or, alternatively, the firefly gene coding for luciferase. Most recently *green fluorescent protein* (GFP) has gained favor (see section IIB, *Uses of the green fluorescent protein*).

Go to Part 4

REFERENCES

Search the textbook

Back to Part 3

B. Recognition and Separation of Proteins

Proteins are the basic units of structure and function of cells. The recognition and localization of proteins are therefore crucial to the understanding of cell organization. In addition, the ultimate measure of gene expression is the production and covalent modification of proteins. mRNA abundance, detectable using the DNA microarrays does not necessarily correlate with the production of proteins (see Gygi et al., 1999).

Use of antibodies

Injection of an antigen into an animal elicits the production of antibodies originating from different antibody-producing cells, each responding to a different *epitope* (i.e. region of the antigen molecule). However, a single antibody producing cell and its progeny (i.e., a clone) will synthesize only a *monoclonal* antibody, specific for one or a few closely related epitopes. Techniques that take advantage of these properties have been developed using *myeloma* cells. *Multiple myeloma* is a malignancy of antibody-producing cells. Many cells (a clone) can be generated from the proliferation of a single myeloma cell. In practice, antibody-producing cells are fused to myeloma cells. Lymphocytes and plasma cells are obtained from the spleen of a mouse that has been immunized against an antigen. They are then fused to myeloma cells by special procedures. The hybrid cells (*hybridoma cells*) are immortal and continue to proliferate in vitro, in contrast to normal cells such as nonfused cells from the spleen of the immunized mouse. The hybrid cells can be selected from the unfused malignant cells if the myeloma cells used in the fusion had a biochemical defect. Only the fused cells containing the wild-type genome of the spleen cells can survive. In the standard techniques, the defect is absence of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). The cells generated from one of these fused cells will produce a clone of identical cells producing the monoclonal antibody. Monoclonal antibodies can be used to recognize specific groups in proteins and, when incorporated in affinity columns, to aid in the isolation of a specific protein from a mixture (see below).

Antibodies can also be produced by other means. These procedures are simpler and have proven very practical. When injected into mice, PCR-amplified DNA open-reading frames (ORFs) noncovalently linked to a eukaryotic promoter and terminator, produce antibodies specific for the encoded protein (Sykes and Johnston, 1999). Monoclonal antiodies can also be produced by display technology. In the immune system the rearrangement of V gene segments produces the repertoire of antibodies, each capable of interacting with one antigen. These gene segments can be amplified using PCR. When these genes are cloned in phages (although other viruses, yeast, and bacteria can also be used) the variable antibody domains are displayed at the surface, so that they can be selected by antigen coated plates or columns

containing the antigen (see Winter et al., 1994).

In *immunoprecipitation* antibodies cross link antigen molecules so that they form large insoluble complexes and can be readily isolated. In studying the location of specific components of cells, either heterogeneous or monoclonal antibodies are conjugated to markers. For light microscopy, a fluorescent dye is frequently used. Alternatively, an antibody to a specific protein is used (primary antibody) and the fluorescent dye is attached to an antibody (secondary antibody) that recognizes the constant region of the primary antibody. Submicroscopic markers, such as ferritin or colloidal gold, are used with electron microscopy. Immunological techniques have provided valuable information about cell structure and function. To see a light micrograph using immunofluorescence click here.

Immunological methods can be used to measure the amount of a specific protein present in an extract. One technique has combined PCR with conventional immuno-detection methods (Immuno-PCR). More recently, another technique has combined immunoprecipitation with T7 RNA polymerase amplification (IDAT). In Immuno-PCR, a linker molecule with binding affinity for DNA and an antibody (Ab) is used to attach a marker DNA molecule specifically to an antigen-Ab complex to form a specific antigen-Ab-DNA conjugate. After precipitation of the complex, the attached marker DNA can be amplified by PCR (Sano et al., 1992) and eventually measured. This technique has a sensitivity 10^5 higher than the more conventional antigen detection systems. IDAT is 10^9 more sensitive than conventional immunological methods. It consists of attaching double stranded oligonucleotides containing the T7 promoter to antibodies against the antigen being studied. The RNA is amplified with T7 RNA polymerase using radioactive nucleotides and the radioactive product can be used to quantify the procedure (Zhang et al., 2001). Immuno-PCR and IDAT can be used to monitor proteins, lipids, and metabolites and in the case of IDAT, at the single-cell level.

Uses of fluorescent proteins

The use of fluorescent probes including fluorescent proteins and low molecular weight probes has been throughly discussed recently (Zhang et al., 2002). The small molecules form a fluorescent covalent complex with any intracellular protein to which a reactive group has been genetically fused. Some of the most useful techniques will be discussed in this section.

The jelly fish *Aequora victoria* emits blue light. It contains two photoactive proteins. The protein *aequorin* is bioluminescent and emits blue light when complexed to Ca^{2+} and the fluorescence is proportional to the concentration of Ca^{2+}. Aequorin can be microinjected into cells or introduced into cells by cDNA transfection (see Section IIA, above). An additional tool is provided by the targeting of aequorin to specific compartment of the cell (see Brini et al., 1999) by introducing in the cDNA codes for targeting the protein to specific localities.

A second protein, the *green fluorescent protein* (GFP) absorbs blue light and emits green light (Chalfie et al., 1994). In the intact organism the two, aequorin and GFP, act together to produce the green light of the

jelly fish. GFP has been used in a variety of experiments because it is stable and easily visualized microscopically (for a review see Lippincott-Schwartz et al., 2001). GFP-cDNA attached to a promoter provides a useful reporter gene. Chimeric DNA coding for a protein and GFP serves as a useful marker to trace the fate of newly synthesized proteins. It has been used, for example, to examine the fate of proteins leaving the ER (Presley et al., 1997).

The major disadvantage of the approach is that GFP can interfere with the function of its fusion partner (see for example Doyle and Bolstein, 1996, showing that actin-GFP fails to function appropriately). Fortunately, this may be the exception rather than the rule (see Wouters et al., 2001)

GFP-fusion proteins containing targeting information (in the form of an amino acid domain such as a signal sequence) can be produced by manipulation of cDNA. In this way, the GFP can be targeted to specific locations. This approach can identify the location of proteins or structures in a living cell without further manipulation. The necessity of producing a new cDNA makes it relatively easy to subject it to site-directed mutagenesis. Mutants of GFP are available that differ in excitation and emission peaks so that several proteins can be tagged in the same cell (Ormo et al., 1996; Heim and Tsien, 1996; Heim et al., 1994; see Matz et al., 1999).

Many fluorescent indicators derived from GFP are currently used. Some respond to pH (e.g., Llopis et al., 1998), others to Ca^{2+} (e.g., Nakai et al., 2001) and Cl^- (e.g., Jayaraman et al., 2000) concentrations. Any of these can be targeted to specific intracellular compartments.

GFP attached to repressor molecules has permitted identifying the position of operators in the study of gene expression in living cells (see Chapter 3).

Like any other fluorescent probe, GFP and GFP mutants can be used in *fluorescent recovery after photobleaching* (FRAP) studies. One added feature is that the bleaching is reversible in GFP mutants with two excitation peaks. When the bleaching is carried out at the longer wavelength, illumination at the shorter wavelength reestablished the fluorescence (Dickson et al., 1997).

Techniques capable of detecting biological ligands such as Ca^{2+} or cAMP take advantage of the *fluorescence resonance energy transfer* (FRET) (see Wouters et al., 2001, box 1) between different mutants of GFP (for other applications of FRET, see Chapter 4). FRET can take place between two chimerically connected GTP variant proteins, from shorter to longer wavelength (such as the transfer of energy from blue emitting protein to green or yellow emitting mutant proteins). The excitation of the donor nonradiatively excites the recipient and the latter emits a fluorescence at a characteristic wavelength. This transmission requires that the two variants of GTP be appropriately positioned. In principle, the GTP-protein pairs can be bridged by protein domains that change in conformation when they bind a biological ligand. These chimeric proteins can be used as indicators of the ligand. They can be introduced into cells via transfection of the modified gene. The chimera can also contain targeting sequences that direct them to specific locations (e.g., in the mitochondrial matrix or the interior of

vesicles of the endoplasmic reticulum). For example, calmodulin or calmodulin domains were connected to GTP-protein pairs in such a way that the distance or geometry between the pairs was altered by Ca^{2+} binding. The chimeric proteins could therefore be used as calcium indicators (Miyawaki et al., 1997; Romoser et al., 1997). The introduction of a targeting sequence directed the protein to the lumen of the endoplasmic reticulum (Miyawaki et al., 1997).

Many more applications of GFP technology have been developed in recent years (e.g., see next section). Adding the appropriate targeting signal to GFP, has made it possible to direct GFP to specific intracellular compartments (e.g., see De Giorgi et al., 1999). The fate of an RNA transcript (Beach et al., 1999) has been followed by constructing a plasmid containing the DNA coding for an RNA-binding protein fused to GFP.

Time dependent events can be followed in cells using a mutant (E5) of a red fluorescent protein (drFP583) isolated from corals (*Anthozoa*). E5 was found to change in fluorescence from green to red in a time dependent manner (Terskikh et al., 2000). Transgenes in which E5 was controlled by the promoter of heat shock protein or the Xenopus Otx-2 promoter were microinjected into *Caenorhabditis elegans* and *Xenopus laevis* embryo cells respectively. These experiments demonstrated that E5 could be used to monitor activation and down-regulation of target promoters.

Other protein probes

In addition to the DNA and antibody probes already discussed, other techniques are available. *Affinity* probes can be used in the study of binding sites to specific ligands in enzymes or other proteins. Affinity labels resemble the natural ligand, but they bind covalently. *Photoaffinity* labels can be used in a similar manner. They are generally analogues of the natural ligand, usually azido compounds that are stable in the absence of light. When photolyzed by exposure to light of the appropriate wavelength, they form highly reactive groups that bind to proteins.

The recognition of special binding domains in proteins (e.g., see below and Chapter 6: discussion, and Table 2) permits the binding of probe molecules (e.g., proteins attached to the GFP) to proteins (see Kavran et al., 1998). The specificity of binding depends on the selection of the appropriate probe molecule. This technique has been used to pinpoint the location of certain phosphoinositides in cells using proteins containing the PH domain (see Gillham et al., 1999).

Separation techniques

Many methods have been used to isolate, identify, and purify proteins. Some of these stand out for their usefulness or frequency of use. Integral proteins, embedded in membranes require the use of detergents for their extraction. The detergents bind to the hydrophobic portions of the lipids through their hydrocarbon chains and interact with water through their polar groups. The use of amphipols promises a new era in the study of membrane proteins. Amphipols are polymers with alternating hydrophilic and

hydrophobic side chains (Tribet et al., 1996). They can solubilize integral membrane proteins by surrounding the hydrophobic protein domains with a polar exterior. In aqueous solutions, the amphipols bound integral proteins studied are in their native state (bacteriorhodopsin, a bacterial photosynthetic reaction center, cytochrome b6f, and porin) . In at least the case of diacylglycerol kinase, an integral protein, amphipols have been shown to support enzyme activity normally requiring polar lipids such as cardiolipin (Gorzelle et al., 2002).

Antibodies can be used to precipitate (*immunoprecipitate*) specific proteins. On incubation of a mixture with a specific antibody, the antigen is bound. Multivalent antigens and multivalent antibodies form large complexes, which precipitate. With the exception of monoclonal antibodies, serum contains a variety of antibodies for different determinants in the antigen molecule. Precipitation can also be produced by adding *Staphylococcus* A cells. These cells contain at their surface, a protein that binds the constant region of most antibodies, producing very large aggregates. Obviously, specific antibodies cannot be produced unless a pure protein is available first to serve as a specific antigen.

The techniques used for the purification of proteins are varied. Some of the classical approaches depend on the solubility of proteins under a variety of conditions and concentrations of salts (such as ammonium sulfate) or binding of proteins to columns.

In ion-exchange chromatography, proteins are separated according to their charge. An ion exchanger, either an organic cation or anion, is bound to supportive material. The proteins separate out by binding differentially to the ion exchanger. Since the charge of the protein depends on its degree of ionization, the protein charge, and hence the degree of separation, depends on pH.

Many other techniques are based on separation of proteins by size. Among many others, they include gel filtration, density gradient centrifugation, and sodium dodecyl sulfate-polyacryl-amide gel electrophoresis (SDS-PAGE).

In gel filtration, separation of proteins depends on passage of the soluble mixture through a column containing hydrated carbohydrate beads. The smaller the protein, the larger the portion of the water in which it will be distributed, so the larger molecules will migrate faster through the gel. The rate of passage of various protein molecules of known size allows a molecular weight calibration that can be used to size other proteins.

In electrophoresis, proteins are separated in an electric field because of their charge. When a polyacrylamide gel is used, it acts as a restrictive matrix that also separates proteins by their size. In SDS-PAGE, the proteins are denatured and reduced (to eliminate sulfhydryl bridges) so that the component polypeptides are separated. Furthermore, they are coated with the detergent SDS, which is negatively charged. Ideally, this coating is evenly distributed, so that the proteins will separate electrophoretically by size only. The protein bands are stained or otherwise made visible (e.g., by autoradiography if they were radioactively labeled).

Affinity chromatography is potentially one of the most powerful techniques. In this technique a specific ligand (e.g., a cofactor, substrate, or antibody) is attached to a matrix. When an extract containing proteins is passed through the column, only the targeted protein remains in the column and can be subsequently eluted (e.g., by free ligand).

In high-performance liquid chromatography (HPLC) the separation uses narrow and long columns of tightly packed glass or plastic beads coated with a thin layer of stationary phase. The fluid phase is forced through at high pressures. The procedure is very rapid. However, the size of the samples is necessarily limited.

Proteins that specifically bind to other proteins can be identified by an ingenuous approach referred to as *interaction cloning* (Blanar and Rutter, 1992). With this technique, the cDNA of a known protein is modified by adding the coding sequence for the phosphorylation site of heart muscle kinase. The composite protein is then expressed in *E. Coli* and after purification and labelling with [^{32}P], it serves as a probe for a λgt11 cDNA expression library from eukaryotic cells under study. The *E.coli* plaques containing the unknown protein binding the radioactive probe can then be detected and the λgt11-cDNA isolated and cloned (see Section IIA). In the study of Blanar and Rutter, the purification of the probe was carried out by immunoprecipitation with a monoclonal antibody to an epitope added to the probe protein.

Gene expression and identification of proteins

The identification of proteins expressed under different conditions (for example, during development or in disease) using microarrays holds considerable promise (see Emili and Cagney, 2000; Kodadek, 2002, Weinberger et al., 2002). This approach is similar to the one using DNA microarrays (see above). A study of protein binding using protein microarrays is discussed later (see below). In general, the possibility of creating protein microchips has been beset by several difficulties. Whereas it is possible to amplify mRNA by the use of reverse-transcriptase polymerase reaction, no such amplification device is available for proteins. Furthermore, the proteins must be immobilized in their native conformation with their active sites exposed. In addition, the proteins are extremely heterogenous so that it is difficult to develop a surface to bind them all. The use of antibody microarrays to detect the presence of specific proteins (e.g., de Wildt, et al., 2000) is probably one of the most general techniques available. In another approach (Fung et al., 2001), proteins with differing surface chemistries are immobilized in arrays taking advantage of these differences (e.g., surface charge, metal affinity, etc.). The bound proteins are then examined with time-of-flight mass spectrometry (TOF MS).

C. Recognizing the Function of Proteins

In situ protein inactivation

The technique of *chromatophore-assisted laser inactivation* (CALI) allows for the selective inactivation of proteins (see Wang and Jay, 1996). CALI requires conjugating a dye (generally malachite green) to a

non-blocking antibody. Laser light at 620 nm (for this particular dye) is used to inactivate the protein by generating short lived hydroxyl radicals (psec). Generally, neighboring proteins remain unaffected. In contrast to proteins at the cell surface, intracellular proteins can only be studied by microinjecting the dye-conjugated antibody. The technique is not without its problems. The distance between dye and target protein may be too great for effective inactivation, or the protein may be particularly insensitive to hydroxyl radicals. Furthermore, in the case of an abundant protein (e.g., tubulin or actin), the possibility of inactivating all of the molecules present is remote. However, recovery by diffusion from other parts of the cell or de novo synthesis may be relatively fast. Therefore, a negative result does not necessarily indicate a lack of involvement in a function. The effect might also be indirect. In short, data obtained with this approach (as with any other) must be carefully evaluated.

Use of dominant inhibitory proteins

Dominant-inhibitory mutant proteins suppress the activity of the wild-type proteins. Therefore the expression of an inhibitory mutant can be used to delineate the biochemical function of the normal protein. The mutant gene can be introduced by transfection (e.g., see Feig and Cooper, 1988). This approach has been used extensively in the study of the function of certain proteins such as the Ras-family GTPases (see Feig et al., 1999). In general, as many as 6,000 studies of signaling molecules such as kinases, phosphatases and transcription factors have used dominant-inhibitory proteins.

A more direct approach involves the removal of a specific protein. The protein is targeted for degradation by the proteasomes. In the proteasomal degradation pathway, substrate specificity of the E_3-complex (such as the SCF complex) is determined by F-box proteins which serve as receptors for the substrates (see Deshaies, 1999) (see Chapter 15). Therefore, the introduction of a specific interaction domain in the F-box proteins (using the appropriate techniques, see above) allows binding of the targeted protein to E_3-complex and hence its degradation (Zhou et al., 2000)

D. Binding properties

The determination of the rate and binding of components, particularly proteins, has become increasingly important. Since protein-protein interactions play such an important role in function, determining these interaction may provide a basis for mapping their function (e.g. see Vidal and Legrain, 1999; Flores et al., 1999). The role of protein-protein interactions are many. Enzymatic reactions have frequently been found to involve complexes, and hence binding of proteins to other proteins. For example, RNA polymerase II can function as a complex of 12 subunits. However, further studies found that it is comprised of 55 subunits to reconstitute the native transcriptional activity (see Koleske and Young, 1994). Regulatory signals appear to involve protein complexes organized around scaffolding proteins (e.g., Tsunoda et al., 1997). In addition, where enzyme and substrate are both proteins, the two may be associated in a stable manner (e.g., Krek et al., 1995).

Generally, the studies of binding of proteins to other proteins, have used biochemical techniques. Three

new approaches, genetic and biophysical, have been used. More recently, the binding of proteins to other proteins or molecules has begun to be studied by microarray technology (MacBeath and Schreiber, 2000) that produces robotically small spots of immobilized proteins on glass slides treated with an aldehyde containing silane reagent. The aldehydes react with primary amines in the proteins. The proteins attach in various orientations so that the reactive groups of some of the molecules are not shielded from reagents or other proteins. With this technique, one protein present as 1600 spots per cm^2 can be tested for binding to other proteins or other chemicals labeled with a fluorescence probe. A similar technique was used to examine the interaction of proteins representing 5800 open reading frames of yeast with other proteins and phospholipids. Many new calmodulin- and phospholipid-interacting proteins were identified (Zhu et al., 2001). Various protein-protein binding domains are discussed in Chapter 6. Many interacting proteins contain several proline residues(e.g., SH3, WW). Other proteins contain a proline residue in their critical sequences (e.g., EH, WW and several others) (see Kay et al., 2000). The interactions between two proteins generally involve short segments, for example, in antibody-antigen reactions the epitope generally is composed of only 4 to 7 amino acid residues (Geysen et al., 1985). Similar conclusions were reached for *Src homology* (SH) 2 and 3 domains, *phosphotyrosine binding domains* (PTB), *postsynaptic density/disc-large/ZO1* (PDZ), WW domains, *Eps15 homology* (EH) domains and 14-3-3 proteins where they generally recognize sequences of 3 to 9 amino acids (see Cohen et al, 1995; Reuther and Pedergast, 1996; Di Fiore et al., 1997; Pawson and Scott, 1997; Shoelson, 1997). SH2, SH3 and the Pleckstrin homology domain (PH) are found in all eukaryotic organisms, except for SH2 that is not found in yeast.

One of the genetic approach (Fields and Song, 1989), the so called *two-hybrids technique*, takes advantage of the fact that gene activators have two independent domains. One domain binds DNA (DB) and the other is responsible for the activation (AD). If hybrid proteins are produced, one protein (e.g., Y) attached to the activating segment(AD-Y) and the other (e.g., X) to the DNA-binding domain (DB-X), the two will be able to activate the gene only if they bind to each other (see diagram). In the original study, the domains of the GAL4 protein of *Saccharomyces cerevisiae* were used and the hybrid proteins were produced by transferring vectors containing the appropriate coding DNAs into a yeast strain lacking GAL4. The yeast expressing GAL4 were selected and the plasmids isolated. The use of growth selection markers (e.g. Leu1 and HIS3) rather than Gal4p, has allowed the use of the powerful growth selection (e.g., Durfee et al., 1993; Vojtek et al., 1993). The two hybrid technique can be modified to recognize RNA-protein binding (see diagram) and DNA-protein binding (for more detail click here).

An extension of the two-hybrid protocol permits identifying DNA encoding proteins capable of binding to DB-X from DNA libraries. DB-X was referred to as "bait" (e.g., Chien et al., 1991). Plasmids are constructed to encode two hybrid proteins. One hybrid consists of the DB domain of the yeast protein Gal4p fused to the test protein. The other hybrid consists of the Gal4p activation domain fused to protein sequences encoded by a library of genomic DNA fragments (referred to as "prey"). Binding of the test protein and a protein encoded by one of the library plasmids leads to transcriptional activation of a reporter gene containing a binding site for GAL4. Use of the appropriate cDNA libraries in the activation domain plasmid could extend this technique to any system. This approach was used to identify mammalian proteins that bind to the proteins Jun or Fos (Chevray and Nathans, 1992) and to isolate

cDNAs that encode a protein that is recruited to the c-Fos serum response element (SRE) (Dalton and Treisman 1992). Two-hybrid analyses in yeast can also be carried out by introducing different "baits" and "preys" in the same diploid cells by mating (e.g., see Uetz et al., 2000).

In an attempt to characterize the functions and the relationships of proteins, a massive yeast (*Saccharomyces cerevisiae*) two-hybrid screen was undertaken (Uetz et al., 2000; see also CURAGEN and Fields et al., 2000). 6,000 yeast colonies were screened. Each expressed a different "prey" molecule (predicted from the yeast genomic sequence) fused to an activation domain and strains expressing 192 different "baits" (fused to a DNA-binding domain) were mated to each member of the "prey" colonies (the *array* method). Alternatively, the "prey" cells were mixed and mated to each different "bait" colony (the *library* method). The library method has the disadvantage that competition between proteins can mask interactions. 957 probable interactions involving 1004 proteins were detected. The function of some proteins could be deduced from the partner they were interacting with. New interactions were discovered, particularly in relation to the cell cycle. For example, the cyclin dependent kinase, Cks1, was found to interact with three different B-cyclins, so that Cks1 may have a role in activating Cdc28 kinase and initiate the cell cycle (see Chapter 8). Cdc28 (Cdc2) is the cyclin-dependent kinase of and Cks1/Suc1 is a regulatory protein associated with Cd28. In addition to these, the study uncovered a multitude of other interactions including those of proteins involved in meiotic recombination.

A modification of the two-hybrid technique involves the use of counterselection markers and has been called the *reverse two-hybrid system* (see Vidal, 1997). The expression of these markers are lethal under certain conditions. For these cases, the selection is for the dissociation rather than binding (e.g., Leanna and Hannick, 1996; Vidal et al., 1996). The technique can be used to screen for mutations that block specific associations (e.g., see Shi et al., 1996) and for low molecular weight regulators that block the association of proteins (e.g., Huang and Schreiber, 1997).

The two-hybrid system is extremely powerful. Two-hybrid screening using a library of random genomic fragments (Flores et al., 1999) was able to identify contacts between individual polypeptides of polymerase III and its interaction with TFIIIC. Similarly, a large scale two-hybrid analysis (Walhout et al., 2000) of 27 proteins involved in vulval development of *Caenorhabditis elegans* produced a protein interaction map of this system. The two-hybrid system provides an answer for in vivo interactions and allows the assay of a multitude of of coding sequences rather simply. It should be noted, however, that false positive or false negative results do take place (see Vidal and Legrain, 1999) and the two hybrid technique requires additional validations.

Although the yeast two-hybrid method has been extremely productive in detecting interactions between protein molecules, the proteins under study must be present in the nucleus. Consequently, the method is unsuited for the study integral proteins which are present in membranes. Integral proteins carry out fundamental functions such as solute transport, signaling, channels, electron transport, etc. Furthermore, approximately 30 % of all proteins coded by the genome are thought to be integral proteins . It is therefore important to be able to determine which proteins interact with integral proteins. Several methods

have been developed to allow these studies see Stagljar and Fields, 2002).

The *ubiquitin-based split-protein sensor* (USPS) (Johnsson and Varshasky, 1994) method is not limited to proteins present in the nucleus. Protein constructs containing ubiquitin are cleaved in vivo by ubiquitin-specific proteases (UBPs) which recognize the folded conformation of ubiquitin. Independently from UBPs, ubiquitin itself can be split into amino-terminal (Nub) and carboxy-terminal fragments (Cub). The two fragments remain attached without a covalent bond and the dimer remains functional. In USPS, a reporter protein attached to Cub will be cleaved by UBP when the two ubiquitin fragments assemble. The technique uses a mutationally altered Nub unable to attach to Cub. However, if the two ubiquitin fragments are fused to interacting proteins, Nub and Cub will be in close contact, activating the release of the reporter protein from Cub by the action of the UBPs. The reporter protein can be detected in a variety of ways (e.g., immunoprecipitation and Western blot analysis of the cleaved reporter protein). Alternatively, the reporter protein can be an enzyme, e.g., Ura3p which would enable yeast cells to form colonies on media lacking uracil (Dunnwald et al., 1999) or a transcription factor, which would activate reporter genes (Stagljar et al., 1998). USPS provides a generally applicable assay for in vivo protein interactions which makes it possible to monitor a protein-protein interaction as a function of time, at the natural sites of this interaction in a living cell.

The *reverse Ras recruitment system* (RRS) takes advantage of the properties of the Ras GTPase system (see Chapter 7) in yeast (Hubsman et al., 2001). Ras is present in the plasma membrane. The yeast Ras-guanyl-exchange factor Cdc25 present in the membrane interacts with Ras and stimulates its guanyl nucleotide exchange and ultimately produces cell growth. A temperature sensitive mutant (cdc25-2) grows at 23º but not at 36º. In the RRS method, a membrane protein is expressed in the membrane of yeast (X). Its possible interaction partner (Y) is fused to a mammalian Ras that is cytoplasmic but binds to the membrane and is able to function with Cdc25-2 . An interaction between X and Y and hence Cdc25-2 and the mammalian Ras permits growth of the cdc25-2 mutant at the non-permissive temperature. In a screening method to detect proteins interacting with X, a cDNA library is expressed as a fusion with the mammalian Ras. The use of this technique is limited by the fact that the fusion of membrane-associated proteins to mammalian Ras will also lead to growth even if there is no interaction between these proteins.

Another technique based on the yeast two-hybrid method uses inactivation of G-protein signaling (see Chapter 7) (Ehrhard et al., 2000). The G-protein constituted by αβ and γ subunits is attached to a receptor (Stet2). In the G-protein technique, an integral protein under study (X) interacts with a soluble protein (Y) attached to the γ subunit of the G-protein. The interaction between the integral protein and the soluble protein would disrupt the G-protein by sequestering the G β subunit which remains attached to the γ subunit bound to Y. Colonies in which interactions occurred can be detected by examining the sensitivity of the yeast cells to α factor. The cells should stop growing.

Other macromolecular interactions where proteins bind to DNA or RNA are as important as protein-protein interactions. In fact, DNA-protein interactions are responsible for the control of gene expression. Some of the biochemical techniques for studying DNA-protein interactions are discussed in Section IIA.

A number of variations of the original two-hybrid approach have been developed for the study of nucleic acid-protein interactions (see Vidal and Legrain, 1999) (for more details click here).

Chromatin immunoprecipitation (e.g., see Orlando, 2000) and DNA microarrays (Ren et al., 2000) have been used in the study of the interactions between DNA and proteins. In immunoprecipitation formaldehyde is used to crosslink the proteins to the DNA. Then the crosslinked material is precipitated immunologically. The DNA microarray technique follows the same general concept used for studying DNA-RNA binding.

Biophysical techniques have also proven very useful. The use of *surface plasmon resonance* (SPR) has become common (commercialized by Pharmacia, Uppsala, Sweden, as BIAcore). The technique is a real time optical detection system (see O'Shannessy et al., 1993). In this technique, one of the interactants is immobilized on a dextran-coated gold surface. The second interactant is then injected across the surface and the interaction is monitored optically continuously.

The technique is based on the fact that when monochromatic light is polarized and incident on a thin metal film-liquid interface, a component of the incident light momentum (the *evanescent wave*) penetrates a distance of about one wavelength into the less dense medium. The evanescent wave interacts with free oscillating electrons (the *plasmons*) in the metal surface. When plasmon resonance occurs, energy from the incident light is lost to the metal film, decreasing the reflected light intensity. The resonance occurs only at a precise angle of incident light and this angle depends on the refractive index of the medium. The refractive index is altered by the interaction of macromolecules and detected as a change in resonance angle (a protein that is no longer in solution, i.e., it is bound, no longer has an effect on the refractive index of the solution).

The fluorescence of a single molecule bound to a surface component can be detected microscopically using *total internal reflection* (discussed above). The technique has been applied using a fluorescent ATP analog bound to myosin (Funatsu et al., 1995). The ATP analog bound to a single myosin immobilized on a microscope slide is excited by a laser beam. The method depends on the total internal reflection of the fluorescence between the interface of the slide and the solution. The light penetrates the solution for a fraction of a wavelength, exciting primarily a molecule in contact with the surface rather than in the bulk solution (as long as the concentration of the fluorescent molecule is kept low). A stationary fluorescent spot indicates binding of ATP (or ADP) to the attached myosin. Disappearance of the spot indicates dissociation from myosin of either ATP or the ADP produced by hydrolysis. A similar total internal reflection technique (Tokunaga et al., 1997) was used with a single fluorescently labelled myosin subfragment-1 attached to a mechanical probe (Kitamura et al., 1999)

This approach was also used to follow the exocytosis of synaptic vesicles labeled with a fluorescent lipid in goldfish retinal bipolar neurons (Zenisek et al., 2000). The synaptic terminal attaches tightly to a coverslip. A thin layer of cytosol beneath the plasma membrane is illuminated selectively and then fluorescence emission reaching the microscope objective is collected. The light reflected from the interface between glass and terminal produces a field that excites any fluorescent molecule within a 100

nm of the site (Steyer and Almers, 2000) (*evanescent-field microscopy*). The brightness of a fluorescently labeled vesicle increases as it approaches this site. At the fusion, the fluorescence is incorporated into the plasma membrane.

E. Sequencing of Proteins and Computer Analysis

Techniques are available for sequencing proteins; in fact, the methodology has been automated. In practice it is easier to sequence DNA because of the capacity to multiply the DNA either through cloning or PCR, so that the amino acid sequence is most frequently deduced from the nucleotide sequence. The methodology has been standardized to the extent that it is possible to commercially contract for deriving sequencing information from purified compounds. Conversely, it is possible to synthesize peptides or polynucleotides.

A known sequence of a protein can be compared with others by the use of software and databases presently available (e.g., see Devereux et al., 1984). Similarly, it is possible to search for particular motifs in proteins (or nucleic acids) through computer searches. One of the interesting applications of this approach is the deduction of function by identifying amino acid sequence domains that are in common with proteins of known function. Presently, a major protein data bank is reachable at www.rcsb.org. Several up-to-date sequence and structure data banks are listed in Kreil et al. (1999) and in addition,. For a discussion see NPS@"network protein sequence analysis (2000).

The genome sequence of some organisms is now known and the time is approaching when the complete human genomic sequence will be known. The advent of *DNA microarrays* or *chips* (see discussion above) permits the study of the expression of most if not all of the genes of an organism. However, very little is known about the function of the gene products. *Mass spectrometry* and sequence database searching have been very useful tools in these studies.

Recent advances in protein characterization via mass spectrometry allow linking these DNA sequences to the proteins in functional complexes as had never been possible before (for an orientation, see Lamond and Mann, 1997; Kuster and Mann, 1998). For example, use of the DNA and protein data bases in conjunction with mass spectrometry have allowed the characterization of the entire spliceosome complex containing as many as 46 distinct proteins (Neubauer et al., 1998).

Mass spectrometry is a technique capable of weighing individual molecules by converting them into ions and subsequently measuring their trajectories in response to electric or magnetic fields. Until the advent of electronspray techniques (see Fenn et al., 1989), mass spectroscopy was difficult to apply to biological molecules because of their fragility. The spray techniques make use of high electric fields to desorb ions from small charged droplets of solution into gas. After partial protease digestion, component peptides are individually processed. Usually, each is randomly cleaved at the peptide bonds by collision with a gas stream, usually He, forming smaller peptides. In mass spectrographs of these smaller pieces, the fragments will be in order of mass (expressed as mass/z, where z is the charge of the molecule, usually

H^+). Analysis of the differences in mass of the different peptides permits deducing the amino acid sequence. The methodology for the identification of peptides from mass spectrographic data has been worked out (see Mann and Wilm, 1994). In *tandem mass spectroscopy* (Biemann and Scoble, 1987) two consecutive mass analyzers are used. The first generates and selects the ion (polypeptide + H^+). This is followed by the fragmentation in a separate chamber and the analysis of the products by a second mass spectrometer. Each peak is generated by the selected precursor. Refining of these techniques allows sequencing proteins from as little as 5 ng samples obtained from polyacrylamide gels (see Wilm et al., 1996).

Electrospray-tandem mass spectroscopy has been used in conjunction with microcapillary liquid chromatography and chemical reagents that have been called *isotope-coded affinity tags* (ICAT) to measure the differences in protein expression in *Saccharomyces cerevisiae* under different metabolic conditions (see Gygi et al., 1999). The ICAT reagent has specificity toward sulfhydril reagents and contains a biotin tag. With this technique, a reduced protein sample is tagged with light reagent and another sample, representing a different metabolic state is tagged with heavy (deuterated) reagent. After the two samples are mixed they are enzymatically cleaved to produce peptides. The peptides are then isolated by avidin chromatography (avidin binds to biotin present in the ICAT, selectively and with extremely high affinity). The isolated peptides are then separated and analyzed by microcapillary liquid chromatography and electrospray-tandem spectroscopy. The ratio of heavy versus light component provides a measure of relative expression of a protein.

Go to Part 5

REFERENCES

Search the textbook

Back to Chapter 1

REFERENCES

Alexander, S., Hellemans, L., Marti, O., Schneir, J., Elings, V. and Hansma, P. K. (1989) An atomic-resolution atomic-force microscope implemented using an optical lever, *J. Appl. Phys.* 65:164-167.

Alizadeh, A.A., Eisen, M.B., Davis, R.E., Ma, C., Lossos, I.S., Rosenwald, A., Boldrick, J.C., Sabet, H., Tran, T., Yu, X., Powell, J.I., Yang, L., Marti, G.E., Moore, T., Hudson, J. Jr, Lu, L., Lewis, D.B., Tibshirani, R., Sherlock, G., Chan, W.C., Greiner, T.C., Weisenburger, D.D., Armitage, J.O., Warnke, R., Levy, R., Wilson, W., Grever, M.R., Byrd, J.C., Botstein, D., Brown, P.O. and Staudt, L.M. (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling, *Nature* 403:503-511. (MedLine)

Allen, T.D. and Goldberg, M.W. (1993) High resolution SEM in cell biology, *Trends in Cell Biol.* 3:205-209.

Amos, L. A., Henderson, R. and Unwin, P. N. T. (1982) 3-D determination by electronmicroscopy of 2-D crystals, *Prog. Biophys. Mol. Biol.* 39:183-231.

Ashkin, A. and Dziedzic, J.M. (1987) Optical trapping and manipulation of viruses and bacteria, *Science* 235:1517-1520.(Medline)

Ashrafi, K., Chang, F.Y., Watts, J.L., Fraser, A.G., Kamath, R.S., Ahringer, J. and Ruvkun, G. (2003) Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes, *Nature* 421:268-272. (MedLine)

Beach, D.L., Salmon, E.D. and Bloom, K. (1999) Localization and anchoring of mRNA in budding yeast, *Curr. Biol.* 9:569-578.(Medline)

Benz, R. (1985) Porin from bacterial and mitochondrial outer membranes. *CRC Crit. Rev. Biochem.* 19:145-190.(Medline)

Berlose, J.-P., Convert, O., Derossi, D., Brunissen, A. and Chassaing, G. (1996) Conformational and associative behaviours of the third helix of antennapedia homeodomain in membrane-mimetic environments, *Eur. J. Biochem.* 242:372-386.(Medline)

Berns, M. W., Olson, R. S. and Rounds, D. E. (1969) In vitro prodiction chromosomal lesion with an Argon laser microbeam, *Nature* 221:74-75.(Medline)

Bernstein, E., Caudy, A.A., Hammond, S.M. and Hannon, G.J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference, *Nature* 409:363-366. (MedLine)

Biemann, K. and Scoble, H.A. (1987) Characterization by tandem mass spectrometry of structural modifications in proteins, *Science* 237:992-998.(Medline)

Bittner, M., Meltzer, P., Chen, Y., Jiang, Y., Seftor, E., Hendrix, M., Radmacher, M., Simon, R., Yakhini, Z., Ben-Dor, A., Sampas, N., Dougherty, E., Wang, E., Marincola, F., Gooden, C., Lueders, J., Glatfelter, A., Pollock, P., Carpten, J., Gillanders, E., Leja, D., Dietrich, K., Beaudry, C., Berens, M., Alberts, D. and Sondak, V. (2000) Molecular classification of cutaneous malignant melanoma by gene expression profiling, *Nature* 406:536-540. (MedLine)

Blanar, M.A. and Rutter, W.J. (1992) Interaction cloning: identification of a helix-loop-helix zipper protein that interacts with c-Fos, *Science* 256:1014-1018.(Medline)

Block, S.M. (1990) Optical tweezers: a new tool for biophysics, in *Noninvasive Techniques in Cell Biology* (Foskett, J.K. and Grinstein, S. eds.) pp. 375-402. Wiley-Liss, New York.

Block, S.M. (1992) Making light work with optical tweezers, *Nature* 360:493-495.(Medline)

Böck, G., Steinlein, P. and Huber, L.A. (1997) Cell biologists sort things out: analysis and purification of intracellular organelles by flow cytometry, *Trends in Cell Biol.* 7:499-503

Boppart, S.A., Brezinski, M.E., Bouma, B.E., Tearney, G.J. and Fujimoto, J.G. (1996) Investigation of developing embryonic morphology using optical coherence tomography, *Dev Biol.* 177:54-63. (MedLine)

Brini, M., Pinton, P., Pozzan, T. and Rizzuto, R. (1999) Targeted recombinant aequorins: tools for monitoring [Ca^{2+}] in the various compartments of a living cell, *Microsc. Res. Tech.* 46:380-389.(Medline)

Buckley, J. K. and Porter, K. R. (1967) Cytoplasmic fibril in living cultured cells. A light and electron microscope study, *Protoplasma* 64:349-390.(Medline)

Capecchi, M.R. (1989) Altering the genome by homologous recombination, *Science* 244:1288-1292.(Medline)

Cerutti, L., Mian, N. and Bateman, A. (2000) Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain, *Trends Biochem. Sci.* 25:481-482. (MedLine)

Chalfie, M., Tu, Y., Euskirschen, G., Ward, W.W. and Prasher, D. (1994) Green fluorescent protein as a marker for gene expression, *Science* 263:802-805.(Medline)

Chevray, P.M. and Nathans, D. (1992) Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun, *Proc. Natl. Acad. Sci. USA* 89:5789-5793.(Medline)

Chien, C.T., Bartel, P.L., Sternglanz, R. and Fields, S. (1991) The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest, *Proc. Natl. Acad. Sci. USA* 88:9578-9582.(Medline)

Chong, J.A. and Mandel, G. (1997) in *The Two Hybrid System*, Bartel, P.L. and Fields,S., eds., Oxfored University Press, New York, N.Y., pp. 289-297.

Chong, J.A., Tapia-Ramirez, J., Kim, S., Toledo-Aral, J.J., Zheng, Y., Boutros, M.C., Altshuller, Y.M., Frohman, M.A., Kraner, S.D. and Mandel, G. (1995) REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons, *Cell* 80:949-957.(Medline)

Chu, S., DeRisi, J., Eisen, M., Mulholland, J., Botstein, D., Brown, P.O. and Herskowitz, I. (1998) The transcriptional program of sporulation in budding yeast, *Science* 282:699-705.(Medline)

Clark, E.A., Golub, T.R., Lander, E.S. and Hynes, R.O. (2000) Genomic analysis of metastasis reveals an essential role for RhoC, *Nature* 406:532-535. (MedLine)

Cohen, G.B., Ren, R. and Baltimore, D. (1995) Modular binding domains in signal transduction proteins, *Cell* 80:237-248.(Medline)

Cooney, M., Czernuszewicz, G., Postel, E.H., Flint, S.J. and Hogan, M.E. (1988) Site-specific oligonucleotide binding represses transcription of the human c-myc gene in vitro, *Science* 241:456-459. (MedLine)

CURAGEN and Fields, S. et al. (2000) Yeast genome protein-protein interactions :http://curatools.curagen.com.

Dalton, S. and Treisman. R. (1992) Characterization of SAP-1, a protein recruited by serum esponse factor to the c-fos serum response element, *Cell* 68:597-612.(Medline)

Davidson, D. and Baldock, R. (2001) Bioinformatics beyond sequence: mapping gene function in the embryo, *Nature Rev. Genet.* 2:409-417. (MedLine)

de Wildt, R.M.T., Mundy, C.R., Gorick, B.D. and Tomlinson, I.M. (2000) Antibody arrays for high-

throughput screening of antibody-antigen interaction, *Nature Biotechnol.* 18:989-994. (MedLine)

Dedon, P.C., Soults, J.A., Allis, C.D. and Gorovsky, M.A. (1991) A simplified formaldehyde fixation and immunoprecipitation technique for studying protein-DNA interactions, *Anal. Biochem.* 197:83-90.(Medline)

De Giorgi, F., Ahmed, Z., Bastianutto, C., Brini, M., Jouaville, L.S., Marsault, R., Murgia, M., Pinton, P., Pozzan, T., and Rizzuto, R. (1999) Targeting GFP to organelles, *Methods Cell Biol.* 58:75-85.(Medline)

Denk, W. and Svoboda, K. (1997) Photon upmanship: why multiphoton imaging is more than a gimmick, *Neuron* 18:351-357.(Medline)

Denk, W., Strickler, J.H.and Webb, W.W. (1990) Two-photon laser scanning fluorescence microscopy *Science* 248:73-76.(Medline)

DeRisi, J.L., Iyer, V.R. and Brown, P.O. (1997) Exploring the metabolic and genetic control of gene expression on a genomic scale, *Science* 278:680-686.(Medline)

De Rosier, D.J. (1997) Electron cryomicroscopy: who needs crystals anyway?, *Nature* 386:26-27.(Medline)

Derossi, D., Joliot, A.H., Chassaing, G. and Prochiantz, A. (1994) The third helix of the antennapedia homeodomain translocates through biological membranes, *J. Biol. Chem.* 269:10444-10450.(Medline)

Derossi, D., Chassaing, G., Prochiantz, A. (1998) Trojan peptides: the penetratin system for intracellular delivery, *Trends in Cell Biol.* 8:84-87.(Medline)

Deshaies, R.J. (1999) SCF and Cullin/Ring H2-based ubiquitin ligases, *Annu. Rev. Cell Dev. Biol.* 15:435-467. (MedLine)

Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for VAX, *Nucleic Ac. Res.* 12:387-395.(Medline)

Dickson, R.M, Cubitt, A.B., Tsien, R.Y. and Moerner, W.E. (1997) On/off blinking and switching behaviour of single molecules of green fluorescent protein, *Nature* 388:355-358(Medline)

Di Fiore, P.P., Pelicci, P.G. and Sorkin, A. (1997) EH: a novel protein-protein interaction domain potentially involved in intracellular sorting, *Trends Biochem. Sci.* 22:411-413.(Medline)

Doyle, T. and Bolstein, D. (1996) Movement of yeast cortical actin cytoskeleton visualized in vivo, *Proc.*

Natl. Acad. Sci. USA 93:3886-3891.(Medline)

Dukas, K., Sarfati, P., Vayasse, N. A. and Pradayrol, L. (1993) Quantitation of changes in expression of multiple genes by simultaneous polymerase chain reaction, *Anal. Biochem.* 215:66-72.

Dunnwald, M., Varshavsky, A. and Johnsson, N. (1999) Detection of transient in vivo interactions between substrate and transporter during protein translocation into the endoplasmic reticulum, *Mol. Biol. Cell* 10:329-344. (MedLine)Durfee, T., Becherer, K., Chen, P.L., Yeh, S.H., Yang, Y., Kilburn, A.E., Lee, W.H. and Elledge, S.J. (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit, *Genes Dev.* 7:555-569.(Medline)

Ehrhard, K.N., Jacoby, J.J., Fu, X.Y., Jahn, R. and Dohlman, H.G. (2000) Use of G-protein fusions to monitor integral membrane protein-protein interactions in yeast, *Nature Biotechnol.* 18:1075-1079. (MedLine)

Eisen, M.B., Spellman, P.T., Brown, P.O. and Botstein, D. (1998) Cluster analysis and display of genome-wide expression patterns, *Proc. Natl. Acad. Sci. USA* 95:14863-14868.(Medline)

Elbashir, S.M., Lendeckel, W. and Tuschl, T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs, *Genes Dev.* 15:188-200. (MedLine)

Emili, A.Q. and Cagney, G. Large-scale functional analysis using peptide or protein arrays, *Nature Biotechnol.* 18:393-397. (MedLine)

Fagard, M. and Vaucheret, H. (2000) Systemic silencing signal(s), *Plant Mol. Biol.* 43(2-3):285-293. (MedLine)

Feig, L.A. (1999) Tools of the trade: use of dominant-inhibitory mutants of the Ras-family GTPases, *Nature Cell Biol.*1:E25-E27.(Medline)

Feig, L.A. and Cooper, G.M. (1988) Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GTP, *Mol. Cell Biol.* 8:3235-3243.(Medline)

Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F., Whitehouse, C.M. (1989) Electrospray ionization for mass spectrometry of large biomolecules, *Science* 246:64-71.(Medline)

Fields, S. and Song, O.-k. (1989) A novel genetic system to detect protein-protein interactions, *Nature* 340:245-246.(Medline)

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998) Potent and

specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, *Nature* 391:806-814.(Medline)

Fleger, S. L., Heckman, J. W., Jr. and Klomparens, K. L. (1993) *Scanning and Transmission Electron Microscopy. An Introduction*, W.H. Freeman and Co., New York.

Flores, A., Briand, J.F., Gadal, O., Andrau, J.C., Rubbi, L., Van Mullem, V., Boschiero, C., Goussot, M., Marck, C., Carles, C., Thuriaux, P., Sentenac, A. and Werner, M. (1999) A protein-protein interaction map of yeast RNA polymerase III, *Proc. Natl. Acad. Sci. USA* 96:7815-7820.(Medline)

Frank, J., Verschoor, A. and Wagenknecht, T. (1985) Computer processing of electron microscopic images of single macromolecules. In *New Methodologies in Studies of Protein Configuration* (Wu, T. T., ed.), p. 36. Van Nostrand Reinhold, New York.

Frank, J., Verschoor, A., Wagenknecht, T., Radermacher, M. and Carazo, J.-M. (1988) A new noncrystallographic image-processing technique reveals the architecture of ribosomes, *Trends Biochem. Sci.* 13:123-127.

Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M. and Ahringer, J. (2000) Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference, *Nature* 408:325-330. (MedLine)

Funatsu, T., Harada, Y., Tokunaga, M., Saito, K. and Yanagida, T. (1995) Imaging of single fluorescent molecules and individual ATP turnovers by myosin molecules in aqueous solution, *Nature* 374:555-559.(Medline)

Fung, E.T., Thulasiraman, V., Weinberger, S.R. and Dalmasso, E.A. (2001) Protein biochips for differential profiling, *Curr. Opin. Biotechnol.* 12:65-69. (MedLine)

Gallouzi, I.-E. and Steitz, J.A. (2001) Delineation of mRNA export pathways by the use of cell-permeable peptides, *Science* 294:1895-1901. (MedLine)

Gehring, W.J. (1987) Homeo boxes in the study of development, *Science* 236:1245-1452. (MedLine)

Geysen, H.M., Barteling, S.J. and Meloen, R.H. (1985) Small peptides induce antibodies with a sequence and structural requirement for binding antigen comparable to antibodies raised against the native protein, *Proc. Natl. Acad. Sci. USA* 82:178-182.(Medline)

Gerhold, D., Rushmore, T., Caskey, C.T. (1999) DNA chips: promising toys have become powerful tools, *Trends Biochem. Sci.* 24:168-173.(Medline)

Gillham, H., Golding, M.C., Pepperkok, R. and Gullick, W.J. (1999) Intracellular movement of green fluorescent protein-tagged phosphatidylinositol 3-kinase in response to growth factor receptor signaling, *J. Cell Biol.* 146:869-880. (MedLine)

Glynne, R., Akkaraju, S., Healy, J.I., Rayner, J., Goodnow, C.C. and Mack, D.H. (2000) How self-tolerance and the immunosuppressive drug FK506 prevent B-cell mitogenesis, *Nature* 403:672-676. (MedLine)

Gorzelle, B.M., Hoffman, A.K., Keyes, M.H., Gray, D.N., Ray, D.G. and Sanders, C.R. (2002) Amphipols can support the activity of a membrane enzyme, *J. Am. Chem. Soc.* 124:11594-11595. (MedLine)

Guo, H., Karberg, M., Long, M., Jones, J.P. 3rd, Sullenger, B., Lambowitz, A.M. (2000) Group II introns designed to insert into therapeutically relevant DNA target sites in human cells, *Science* 289:452-457. (MedLine)

Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H. and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags, *Nature Biotechnol.*17:994-999. (MedLine)

Haggie, M., Vetrivel, L. and Verkman, A.S. (2001) Diffusion of metabolic enzymes in mitochondria and the cytoplasm measured by fluorescence photobleaching, *Biophys. J.* (Annual Meeting Abstracts) 80 : 280a.

Hamilton A.J. and Baulcombe, D.C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants, *Science* 286:950-952. (MedLine)

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells, *Nature* 404:293-296. (MedLine)

Hammond, S.M., Caudy, A.A. and Hannon, G.J. (2001) Post-transcriptional gene silencing by double-stranded RNA, *Nature Rev. Genet.*. 2:110-119. (MedLine).

Haseloff, J. and Gerlach, W.L. (1988) Simple RNA enzymes with new and highly specific endoribonuclease activities, *Nature* 334:585-591. (MedLine)

Heim R. and Tsien, R.Y (1996) Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer, *Curr. Biol.* 6:178-182.(Medline)

Heim, R., Prasher, D.C.and Tsien, R.Y. (1994) Wavelength mutations and posttranslational autoxidation of green fluorescent protein, *Proc. Natl. Acad. Sci. USA* 91:12501-12504.(Medline)

Hoffman, R. and Gross, L. (1975) Modulation contrast microscopy, *Appl. Opt.* 14:1169-1176.(Medline)

Hoh, J.A. and Hansma, P.K. (1992) Atomic force microscopy for high-resolution imaging in cell biology, Trends in Cell Biol. 2:208-213.

Howorka, S., Cheley, S. and Bayley, H. (2001) Sequence-specific detection of individual DNA strands using engineered nanopores, *Nature Biotechnol.* 19:636-639. (MedLine)

Huang, J. and Schreiber, S.L. (1997) A yeast genetic system for selecting small molecule inhibitors of protein-protein interactions in nanodroplets, *Proc. Natl. Acad. Sci. USA* 94:13396-13401.(Medline)

Hubsman, M., Yudkovsky, G. and Aronheim A. (2001) A novel approach for the identification of protein-protein interaction with integral membrane proteins, *Nucleic Acids Res.* 29:E18. (MedLine)

Hudson, D.F., Morrison, C., Ruchaud, S. and Earnshaw, W.C. (2002) Reverse genetics of essential genes in tissue-culture cells; 'dead cells talking', *Trends in Cell Biol.* 12:281-287.

Hunter, C.P. (1999) A touch of elegance with RNAi, *Curr Biol.* 9:R440-R442.(Medline)

Jayaraman, S., Haggie, P., Wachter, R.M., Remington, S.J. and Verkman, A.S. (2000) Mechanism and cellular applications of a green fluorescent protein-based halide sensor, *J. Biol. Chem.* 275:6047-6050. (MedLine)

Johnson, K. A. and Wall, J. S. (1983) Structure and molecular weight of the dynein ATPase. *J. Cell Biol.* 96:669-678.(Medline)

Johnsson, N. and Varshavsky, A. (1994) Split ubiquitin as a sensor of protein interactions in vivo, *Proc. Natl. Acad. Sci. USA* 91:10340-10344. (MedLine)

Kachar, B. (1980) Asymmetric illumination contrast: a new method of image formation in video light microscopy, *Science* 227:766-768.

Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D.P., Zipperlen, P. and Ahringer, J. (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi, *Nature* 421:231-237. (MedLine)

Kavran, J.M., Klein, D.E., Lee, A., Falasca, M., Isakoff, S.J., Skolnik, E.Y. and Lemmon, M.A. (1998) Specificity and promiscuity in phosphoinositide binding by pleckstrin homology domains, *J. Biol. Chem.* 273:30497-30508. (MedLine)

Kay, B.K., Williamson, M.P. and Sudol, M. (2000) The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains, *FASEB J.* 14:231-241.(Medline)

Ketting, R.F., Haverkamp, T.H., van Luenen, H.G. and Plasterk, R.H. (1999) Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD, *Cell* 99:133-141.(Medline)

Khan, J., Wei, J.S., Ringner, M., Saal, L.H., Ladanyi ,M., Westermann, F., Berthold, F., Schwab, M., Antonescu, C.R., Peterson, C. and Meltzer, P.S. (2001) Classification and diagnostic prediction of cancers using gene expression profiling and artificial neural networks, *Nature Med.* 7:673-679. (MedLine)

Kim, H.S. and Smithies, O. (1988) Recombinant fragment assay for gene targetting based on the polymerase chain reaction, *Nucleic Acids Res.* 16:8887-8903.(Medline)

Kitamura, K., Tokunaga, M.,. Iwane, A.H. and Yanagida, T. (1999) A single myosin molecule moves along an actin filament with regular steps of 5.3 nanometres, *Nature* 397:129-134. (MedLine)

Kodadek T. (2002) Development of protein-detecting microarrays and related devices, *Trends Biochem. Sci.* 27:295-300. (MedLine)

Koleske, A.J. and Young, R.A. (1994) An RNA polymerase II holoenzyme responsive to activators, *Nature* 368:466-469.(Medline)

Kopp, M.U., de Mello, A.J. and Manz, A. (1998) Chemical amplification: continuous-flow PCR on a chip, *Science* 280:1046-1048.(Medline)

Kreil, D.P. and Etzold, T. (1999) DATABANKS - a catalogue database of molecular biology databases *Trends Biochem. Sci.* 24:155-157. (MedLine) see also BioMedNet

Krek, W., Xu, G. and Livingston, D.M. (1995) Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint, *Cell* 83:1149-1158.(Medline)

Kuster, B. and Mann, M. (1998) Identifying proteins and post-translational modifications by mass spectrometry, *Curr. Opin. Struct. Biol.* 8:393-400.(Medline)

Kuwabara, T., Warashina, M., Tanabe, T., Tani, K., Asano, S. and Taira, K. (1998) A novel allosterically trans-activated ribozyme, the maxizyme, with exceptional specificity in vitro and in vivo, *Mol. Cell* 2:617-627. (MedLine)

Lambowitz, A.M., Caprara, M.G., Zimmerly, P.S. and Perlman, P.S. (1999) Group I and group II

ribozymes as RNPs: Clues to the past and guides to the future, in *The RNA World*, Gesteland, R.F., Cech, T.R. and Atkins, J.F. eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2nd edition, pp. 451-485.

Lamond, A.I. and Mann, M. (1997) Cell biology and the genome projects--a concerted strategy for characterizing multiprotein complexes by using mass spectrometry, *Trends in Cell Biol.* 7:139-142.

Lashkari, D.A., DeRisi, J.L., McCusker, J.H., Namath, A.F., Gentile, C., Hwang, S.Y., Brown, P.O. and Davis, R.W. (1997) Yeast microarrays for genome wide parallel genetic and gene expression analysis, *Proc. Natl. Acad. Sci. USA* 94:13057-13062.(Medline)

Leanna, C.A. and Hannink, M. (1996) The reverse two-hybrid system: a genetic scheme for selection against specific protein/protein interactions, *Nucleic Acids Res.* 24:3341-3347.(Medline)

Lee, S.S., Lee, R.Y., Fraser, A.G., Kamath, R.S., Ahringer, J. and Ruvkun, G. (2003) A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity, *Nature Genet.* 33:40-48. (MedLine)

Lindgren, M., Hallbrink, M., Prochiantz, A. and Langel, U. (2000) Cell-penetrating peptides, *Trends Pharmacol. Sci.*21:99-103. (MedLine)

Lippincott-Schwartz, J., Snapp, E. and Kenworthy, A. (2001) Studying protein dynamics in living cells, *Nature Rev. Mol. Cell Biol.* 2:444-456. (MedLine)

Llopis, J., McCaffery, J.M., Miyawaki, A., Farquhar, M.G. and Tsien, R.Y. (1998) Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins, *Proc. Natl. Acad. Sci. USA* 95:6803-6808. (MedLine)

Luby-Phelps K. (2000) Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area, *Int. Rev. Cytol.* 192:189-221. (MedLine)

MacBeath, G. and Schreiber, S.L. (2000) Printing proteins as microarrays for high-throughput function determination, *Science* 289:1760-1763. (MedLine)

Mann, M. and Wilm, M. (1994) Error-tolerant identification of peptides in sequence databases by peptide sequence tags, *Anal. Chem.* 66:4390-4399.(Medline)

Mannella, C. A. (1982) Structure of the outer mitochondrial membrane: ordered arrays of porelike subunits in outer-membrane fractions from Neurospora crassa mitochondria, *J. Cell Biol.* 94:680-687.(Medline)

Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov. M.L. and Lukyanov, S.A. (1999) Fluorescent proteins from nonbioluminescent *Anthozoa* species, *Nature Biotechnol.* 17:969-973. (MedLine)

Matzke, M.A., Matzke, A.J., Pruss, G.J. and Vance, V.B. (2001) RNA-based silencing strategies in plants, *Curr. Opin. Genet. Dev.* 11:221-227. (MedLine)

May, M.J., D'Acquisto, F., Madge, L.A., Glockner, J., Pober, J.S. and Ghosh, S. (2000) Selective inhibition of NF-κB activation by a peptide that blocks the interaction of NEMO with the I κB kinase complex, *Science* 289:1550-1554. (MedLine)

Mayford, M., Wang, J., Kandel, E.R. and O'Dell, T.J. (1995) CaMKII regulates the frequency-response function of hippocampal synapses for the production of both LTD and LTP, *Cell* 81:891-904.(Medline)

Michel, F. and Ferat, J.L. (1995) Structure and activities of group II introns, *Annu. Rev. Biochem.* 64:435-461. (MedLine)

Minton, A.P. (2001) The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media, *J. Biol. Chem.*276:10577-10580. (MedLine)

Miyawaki, A., Lopis, J, Helm, R., McCaffery, J.M., Adams, J.A., Ikura, M. and Tsien, R.Y. (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin (1997) *Nature* 388:882-887.(Medline)

Montgomery, M.K., Xu, S. and Fire, A, (1998) RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*, *Proc. Natl. Acad. Sci. USA* 95:15502-15507. (MedLine)

Morgan, A.R. and Wells, R.D. (1968) Specificity of the three-stranded complex formation between double-stranded DNA and single-stranded RNA containing repeating nucleotide sequences, *J. Mol. Biol.* 37:63-80. (MedLine)

Morin, P.A. and Smith, D.G. (1995) Nonradioactive detection of hypervariable simple sequence repeats in short polyacrylamide gels, *Biotechniques* 19:223-228. (MedLine)

Moser, H.E. and Dervan, P.B. (1987) Sequence-specific cleavage of double helical DNA by triple helix formation, *Science* 238:645-650. (MedLine)

Nakai, J., Ohkura, M. and Imoto, K. (2001) A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein, *Nature Biotechnol.* 19:137-141. (MedLine)

Neubauer, G., King, A., Rappsilber, J., Calvio, C., Watson, M., Ajuh, P., Sleeman, J., Lamond, A. and Mann M. (1998) Mass spectrometry and EST-database searching allows characterization of the multi-protein spliceosome complex, *Nature Genet.* 20:46-50.(Medline)

NPS@: network protein sequence analysis (2000) *Trends Biochem. Sci.* 25:147-150.(Medline)

O'Keefe, R.T., Henderson, S.C. and Spector, D.L. (1992) Dynamic organization of DNA replication in mammalian cell nuclei: spatially and temporally defined replication of chromosome-specific α-satellite DNA sequences, *J. Cell Biol.*116:1095-1110.(Medline)

Orlando, V. (2000) Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation, *Trends Biochem. Sci.* 25:99-104. (MedLine)

Ormo M., Cubitt, A.B, Kallio K., Gross, L.A., Tsien, R.Y. and Remington, S.J. (1996) Crystal structure of the *Aequorea victoria* green fluorescent protein, *Science* 273:1392-1395(Medline)

O'Shannessy, D.J., Brigham-Burke, M., Soneson, K.K. and Hensley, P. (1993) Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance: use of non-linear squares analysis methods, *Analyt. Biochem.* 212:457-468.(Medline)

Ovadi, J. and Srere, P.A. (2000) Macromolecular compartmentation and channeling, *Int. Rev. Cytol.* 192:255-280. (MedLine)

Pagliaro, L. and Taylor, D.L. (1992) 2-Deoxyglucose and cytochalasin D modulate aldolase mobility in living 3T3 cells, *J. Cell Biol.* 118:859-863. (MedLine)

Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S. and Hogenesch, J.B. (2002) Coordinated transcription of key pathways in the mouse by circadian clock, *Cell* 109: 307-320.

Pawson, T. and Scott, J.D. (1997) Signaling through scaffold, anchoring, and adaptor proteins, *Science* 278:2075-2080.(Medline)

Pool, R. (1988) Near-field microscopes beat the wavelength limit, *Science* 241:25-26.(Medline)

Porter, A. (1998) Controlling your losses: conditional gene silencing in mammals, *Trends Genet.* 14:73-79. (MedLine)

Presley, J.F., Cole, N.B., Schroer, T.A., Hirschberg, K., Zaal, K.J.M. and Pippincott-Schwartz, J. (1997) ER-to-Golgi transport visualized in living cells, *Nature* 389:81-85.(Medline)

Prochiantz, A. (1996) Getting hydrophilic components into cells: lessons from homeopeptides, *Curr. Opin. Neurobiol.* 6:629-634.(Medline)

Radermacher, M., Wagenknecht, T., Verschoor, A. and Frank, J. (1986) Three-dimensional reconstruction from a single-exposure, random conical tilt series applied to 505 ribosomal subunit of Escherichia coli, *J. Microsc.* 146:113-136.(Medline)

Ren, B., Robert, F., Wyrick, J.J., Aparicio, O., Jennings, E.G., Simon, I., Zeitlinger, J., Schreiber, J., Hannett, N., Kanin, E., Volkert, T.L., Wilson, C.J., Bell, S.P. and Young, R.A. (2000) Genome-wide location and function of DNA binding proteins, *Science* 290:2306-2309. (MedLine)

Reuther, G.W. and Pendergast, A.M. (1996) The roles of 14-3-3 proteins in signal transduction, *Vitam. Horm.* 52:149-175.(Medline)

Robinett, C.C., Straight, A., Li, G., Willhelm, C., Sudlow, G., Murray, A. and Belmont, A.S. (1996) In vivo localization of DNA sequences and visualization of large-scale chromatin organization using Lac operator/repressor recognition, *J. Cell Biol.* 135:1685-1700.(Medline)

Rogge, L., Bianchi, E., Biffi, M., Bono, E., Chang, S.Y., Alexander, H., Santini, C., Ferrari, G., Sinigaglia, L., Seiler, M., Neeb, M., Mous, J., Sinigaglia, F. and Certa U. (2000) Transcript imaging of the development of human T helper cells using oligonucleotide arrays, *Nature Genet.* 25:96-101. (MedLine)

Romoser, V., Hinkle, P.M. and Persichini, A. (1997) Detection in living cells of Ca^{2+}-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. *J. Biol. Chem.* 272:13270-13274.(Medline)

Ross, K. F. A. (1967) *Phase Contrast and Interference Biology for Cell Biologists*. St. Martin's Press, New York.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Ehrlich, H. A. (1988) Primer directed enzymatic amplification of DNA with thermostable DNA polymerase, *Science* 239:487-491.(Medline)

Sanchez-Alvarado, A. and Newmark, P.A. (1999) Double-stranded RNA specifically disrupts gene expression during planarian regeneration, *Proc. Natl. Acad. Sci. USA* 96:5049-5054.(Medline)

Sano, T. , Smith, C. L. & Cantor, C. R. (1992) Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates *Science* 258: 120-122. (MedLine)

Sauer, B. and Henderson, N. (1988) Site-specific DNA recombination in mammalian cells by the Cre

recombinase of bacteriophage P1, *Proc. Natl. Acad. Sci. USA* 85:5166-5170.(Medline)

Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray, *Science* 270:467-470.(Medline)

Schena, M., Shalon, D., Heller, R., Chai, A., Brown, P.O. and Davis, R.W. (1996) Parallel human genome analysis: microarray-based expression monitoring of 1000 genes, *Proc. Natl. Acad. Sci. USA* 93:10614-10619.(Medline)

Schmittgen, T.D., Zakrajsek, B.A., Mills, A.G., Gorn, V., Singer, M.J. and Reed, M.W. (2000) Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods, *Anal. Biochem.* 285:194-204. (MedLine)

Schwarze, S.R., Hruska, K.A. and Dowdy, S.F. (2000) Protein transduction: unrestricted delivery into all cells? *Trends Cell Biol.* 10:290-295. (MedLine)

SenGupta, D.J., Zhang, B., Kraemer, B., Pochart, P., Fields, S. and Wickens, M. (1996) A three-hybrid system to detect RNA-protein interactions in vivo, *Proc. Natl. Acad. Sci. USA* 93:8496-8501.(Medline)

Shalon, D., Smith, S.J. and Brown, P.O. (1996) A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization, *Genome Res.* 6:639-645.(Medline)

Sharpe, J., Ahlgren, U., Perry, P., Hill, B., Ross, A., Hecksher-Sorensen, J., Baldock, R. and Davidson, D. (2002) Optical Projection Tomography as a Tool for 3D Microscopy and Gene Expression Studies, *Science* 296:541-545. (MedLine)

Shaw, S.L., Salmon, E.D. and Quatrano, R.S. (1995) Digital photography for the light microscope: results with gated, video rate CCD camera and NIH-software, *BioTechniques* 19:946-955.(Medline)

Shaw, S.L., Yeh, E., Bloom, K. and Salmon, E.D. (1997) Imagining GTP fusion proteins in *Saccharomyces cerevisiae*, *Current Biol.* 7:701-704.(Medline)

Shih, H.M., Goldman, P.S., DeMaggio, A.J., Hollenberg, S.M., Goodman, R.H. and Hoekstra, M.F. (1996) A positive genetic selection for disrupting protein-protein interactions: identification of CREB mutations that prevent association with the coactivator CBP, *Proc. Natl. Acad. Sci. USA* 93:13896-13901.(Medline)

Shoelson, S.E. (1997) SH2 and PTB domain interactions in tyrosine kinase signal transduction, *Curr. Opin. Chem. Biol.* 1:227-234.(Medline)

Simmons, R.M., Finer, J.T., Chu, S. and Spudich, J.A. Quantitative measurements of force and displacement using an optical trap, *Biophys. J.* 70:1813-1822.(Medline)

Spellman, P.T., Sherlock, G., Zhang, M.Q., Iyer, V.R., Anders, K., Eisen, M.B., Brown, P.O., Botstein, D., Futcher, B., Spellman, P.T., Sherlock, G., Zhang, M.Q., Iyer, V.R., Anders K., Eisen, M.B., Brown, P.O., Botstein, D., Futcher, B. (1998) Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization, *Mol. Biol. Cell* 9:3273-3297.(Medline)

Stagljar, I. and Fields, S. (2002) Analysis of membrane protein interactions using yeast-based technologies, *Trends Biochem. Sci.* 27:559-563. (MedLine)

Stagljar, I., Korostensky, C., Johnsson, N. and te Heesen, S. (1998) A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins *in vivo*, *Proc. Natl. Acad. Sci. USA* 95:5187-5192. (MedLine)

Steyer J.A. and Almers, W. (2000) Tracking single secretory granules in live chromaffin cells by evanescent-field fluorescence microscopy, *Biophys. J.* 76:2262-2271.

Steyer, J.A. and Almers, W. (2001) A real-time view of life within 100 nm of the plasma membrane, *Nature Rev. Mol. Cell Biol.* 2:268-275. (MedLine)

Sykes, K.F. and Johnston, S.A. (1999) Linear expression elements: a rapid, in vivo, method to screen for gene functions, *Nature Biotechnol.* 17:355-359. (MedLine)

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A. and Mello, C.C. (1999) The rde-1 gene, RNA interference, and transposon silencing in *C. elegans Cell* 99:123-132.(Medline)

Tanabe, T., Kuwabara, T., Warashina, M., Tani, K., Taira, K. and Asano, S. (2000) Oncogene inactivation in a mouse model, *Nature* 406:473-474. (Medline)

Tang, Y.P., Shimizu, E., Dube, G.R., Rampon, C., Kerchner, G.A., Zhuo, M., Liu, G. and Tsien, J.Z. (1999) Genetic enhancement of learning and memory in mice, *Nature* 401:63-69.(Medline)

Terskikh, A., Fradkov, A., Ermakova, G., Zaraisky, A., Tan, P., Kajava, A.V., Zhao, X., Lukyanov, S., Matz, M., Kim, S., Weissman, I. and Siebert, P. (2000) "Fluorescent Timer": protein that changes color with time, *Science* 290:1585-1588. (MedLine)

Tokunaga. M., Kitamura, K., Saito, K., Iwane, A.H. and Yanagida, T. (1997) Single molecule imaging of fluorophores and enzymatic reactions achieved by objective-type total internal reflection fluorescence microscopy, *Biochem. Biophys. Res. Commun.* 235:47-53.(Medline)

Trask, B.J. (1991) Gene mapping by *in situ* hybridization, *Curr. Opin. Genet. Dev.* 1:82-87.(Medline)

Tribet, C., Audebert, R. and Popot, J.L. (1996) Amphipols: polymers that keep membrane proteins soluble in aqueous solutions, *Proc. Natl. Acad. Sci. USA* 93:15047-15050. (MedLine)

Tsien, J.Z., Chen, D.F., Gerber, D., Tom, C., Mercer, E.H., Anderson, D.J., Mayford, M., Kandel, E.R. and Tonegawa, S. (1996a) Subregion- and cell type-restricted gene knockout in mouse brain, *Cell* 87:1317-1326.(Medline)

Tsien, J.Z., Huerta, P.T. and Tonegawa, S. (1996b) The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory, *Cell* 87:1327-1338.(Medline)

Tsunoda, S., Sierralta, J., Sun, Y,, Bodner, R., Suzuki, E., Becker, A., Socolich, M. and Zuker, C.S. (1997) A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade, *Nature* 388:243-249.(Medline)

Uetz, P., Giot, L., Cagney, G., Mansfield, T.A., Judson, R.S., Kight, J.R., Lockshon, D., Narayan, V., Srinivasan, M., Pocart, P., Qureshi-Emili, A., Li, Y., Goodwin, B., Conover, D., Kalbfleish, T., Vijayadomar, G., Yang, M., Johnston, M., Fields, S. and Rothberg, J.M. (2000) A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*, *Nature* 403:623-627.(Medline)

Uhlenbeck, O.C. (1987) A small catalytic oligoribonucleotide, *Nature* 328:596-600. (MedLine)

Vasquez, K.M. and Wilson, J.H. (1998) Triplex-directed modification of genes and gene activity, *Trends Biochem. Sci.* 23:4-9. (MedLine)

Vasquez, K.M., Narayanan, L. and Glazer, P.M. (2000) Specific mutations induced by triplex-forming oligonucleotides in mice, *Science* 290:530-533. (MedLine)

Verkman, A.S. et al. (2001) Diffusion measurements by photobleaching recovery methods, in *Methods in Cellular Imaging* , Oxford University Press, (Periasamy, A., ed.)pp. 112-127.

Verkman A.S. (2002) Solute and macromolecule diffusion in cellular aqueous compartments (2001) *Trends Biochem. Scie.* 27:27-33. .

Vidal, M. (1997) in *The Yeast Two-Hybrid System*, Bartel, P.L. and Fields, S. eds., Oxford University Press, New York, N.Y. pp. 109-147.

Vidal, M. and Legrain, P. (1999) Yeast forward and reverse 'n'-hybrid systems, *Nucleic Acids Res.* 27:919-929.(Medline)

Vidal, M., Braun, P., Chen, E., Boeke, J.D., Harlow, E. (1996) Genetic characterization of a mammalian protein-protein interaction domain by using a yeast reverse two-hybrid system, *Proc. Natl. Acad. Sci. USA* 93:10321-10326.(Medline)

Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf, *Cell* 74:205-214.(Medline)

Walev, I., Bhakdi, S.C., Hofmann, F., Djonder, N., Valeva, A., Aktories, K. and Bhakdi, S. (2001) Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O, *Proc. Natl. Acad. Sci. USA.* 98:3185-3190. (MedLine)

Walhout, A.J., Sordella, R., Lu, X., Hartley, J.L., Temple, G.F., Brasch, M.A, Thierry-Mieg, N. and Vidal, M. (2000) Protein interaction mapping in C. elegans using proteins involved in vulval development, *Science* 287:116-122.(Medline)

Wang, F.-S. and Jay, D.G. (1996) Chromatophore-assisted laser inactivation (CALI): probing protein function *in situ* with a high degree of spatial and temporal resolution, *Trends in Cell Biol.* 6:442-445.

Waterhouse, P.M., Graham, M.W. and Wang, M.B. (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA, *Proc. Natl. Acad. Sci. USA* 95:13959-13964.(Medline)

Waterman-Storer, C.M., Shaw, S.L. and Salmon, E.D. (1997) Production and presentation of digital movies, *Trends in Cell Biol.* 7:503-506.

Weinberger, S.R., Dalmasso, E.A. and Fung, E.T. (2002) Current achievements using ProteinChip Array technology, *Curr. Opin. Chem. Biol.* 6:86-91. (MedLine)

White, J. G. and Amos, W. B. (1987) Confocal microscopy comes of age, *Nature* 328: 183-184.

White, J. G., Amos, W. B. and Fordham, M. (1987) An evaluation of confocal versus conventional imaging of biological structures by fluorescence microscopy. *J. Cell Biol.* 105:41-48.(Medline)

Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T. and Mann, M. (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry, *Nature* 379:466-469.(Medline)

Winston, W.M., Molodowitch, C. and Hunter, C.P. (2002) Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1, *Science* 295:2456-2459. (MedLine)

Winter, G., Griffiths, A.D., Hawkins, R.E. and Hoogenboom, H.R. (1994) Making antibodies by phage display technology, *Annu. Rev. Immunol.* 12:433-455. (MedLine)

Wouters, F.S., Verveer, P.J. and Bastiaens, P.I. (2001) Imaging biochemistry inside cells, *Trends Cell Biol.* 11:203-211. (MedLine)

Wu, R.Z., Bailey, S.N. and Sabatini, D.M. (2002) Cell-biological applications of transfected-cell microarrays, *Trends in Cell Biol.* 12:485-488.

Zadzinski, J. A. N. (1989) Scanning tunneling microscopy with applications to biological surfaces, *BioTechniques* 7:174-187.(Medline)

Zamore, P.D., Tuschl, T., Sharp, P.A. and Bartel, D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals, *Cell* 101:25-33. (MedLine)

Zenisek, D., Steyer, J.A. and Almers, W. (2000) Transport, capture and exocytosis of single synaptic vesicles at active zones, *Nature* 406:849-854.

Zhang, B., Kramer, B., Sengupta, D., Fields, S. and Wickens, M. (1997) in *The Yeast Two-Hybrid System*, Bartel, P.L. and Fields, S. eds., Oxford University Press, New York, N.Y. pp.298-315.

Zhang, H.T., Kacharmina, J.E., Miyashiro, K., Greene, M.I. and Eberwine, J. (2001) Protein quantification from complex protein mixtures using a proteomics methodology with single-cell resolution, *Proc. Natl. Acad. Sci. USA* 98:5497-5502. (MedLine)

Zhang, J., Campbell, R.E., Ting, A.Y. and Tsien, R.Y. (2002) Creating new fluorescent probes for cell biology, *Nature Rev. Mol. Cell Biol.*3:906-918. (MedLine)

Zhou, P., Bogacki, R., McReynolds, L. and Howley, P.M. (2000) Harnessing the ubiquitination machinery to target the degradation of specific cellular proteins, *Mol. Cell* 6:751-756. (MedLine)

Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T., Mitchell, T., Miller, P., Dean, R.A., Gerstein, M. and Snyder, M. (2001) Global analysis of protein activities using proteome chips, *Science* 293:2101-2105. (MedLine)

Ziauddin, J. and Sabatini, D.M. (2001) Microarrays of cells expressing defined cDNAs, *Nature* 411:107-110. (MedLine)

2. Gene Expression and Differentiation

Suggested Reading
Web Resources
References
Back to List of Chapters

Everybody now knows that genetic information in the form of the sequence of nucleotide triplets in the DNA determines the structure and function of cells by specifying the nature of the proteins present in the cell. This is done in two steps which are now reasonably well understood. In the first step, the nucleotide sequence of one of the two DNA strands is *transcribed* onto that of mRNA, hence this process is known as *transcription*. The newly transcribed mRNA is *complementary* to one of the DNA that served as its template; the nucleotides in the newly formed RNA "complement" those of the DNA via specific hydrogen bonds between G and C, and between A and U. In the second step, the mRNA sequence is converted into specific sequences of amino acids in proteins where triplet bases of the RNA code for each individual amino acid. This latter process is known as *translation*. One of the most useful techniques of molecular biology is the synthesis of DNA complementary to mRNA (*cDNA*). cDNA can be synthesized in vitro with mRNA as its template, using the enzyme reverse transcriptase derived from retroviruses. Some of the uses of cDNA in the study of transcription and gene expression will be encountered in this chapter.

Gene expression determines the state of differentiation of cells and has a role in the regulation of cell activity in various physiological states. Gene activation or repression caused by differentiation is stable and, where the differentiated cell can divide, inheritable. When gene expression is under the control of physiological signals, it is transient and easily reversed. Despite these differences, many of the events surrounding these two different kinds of regulation via gene expression are common; discussion of the two will necessarily intertwine. These two aspects are discussed in this chapter and the following chapter.

Cells at different periods during the life cycle may differ significantly in structure and function. In fact, a multicellular organism possesses different kinds of cells, each type specialized to perform a specific task. These cells are said to be *differentiated*. The different kinds of cells differ from each other in that each kind expresses only a small portion of the genetic information of the *genome*. How can we explain these differences? One of the possible explanations is that through differentiation, part of the genome has been lost. As we shall see, this is generally not the case. However, in nematodes and some insects differentiation involves *chromosome diminution*, where part of the genetic complement is actually lost. In the ciliated protozoan *Oxytricha nova* internal DNA sequences are eliminated (Klobutcher et al., 1984). In addition, gene rearrangements occur in mammalian lymphocytes in the production of immunoglobulins (Hood et al., 1985)

I. DIFFERENTIATION

How do cells diverge during differentiation? Two kinds of experiments have helped us understand what happens. The first case that will be discussed involves a test of the genetic content of nuclei from

differentiated cells. This was done taking advantage of the oocytes of the African clawed frog, *Xenopus laevis* (Gurdon, 1978). These oocytes and their nuclei are rather large, allowing for relatively easy micromanipulation. In each experiment the nucleus of the oocyte was first inactivated by a short exposure to ultraviolet light. The nuclei from differentiated cells were then placed in the enucleated oocytes. Were these nuclei able to replace the normal nuclei of the oocytes to produce a variety of differentiated cells? If so, the transplanted nuclei must have all the genetic information needed by the organism. A loss of genome would be reflected in an inability to produce a particular kind of specialized tissue. Originally, the nuclei of intestinal epithelial cells of feeding tadpoles were used. Later, nuclei from a variety of differentiated cells in culture (Laskey and Gurdon, 1970) were used. In a number of cases, the transplants that contained only the transplanted nuclei (these were marked by using diploid cells containing a single nucleolus) produced an advanced tadpole stage possessing all specialized tissues. The similar and more spectacular experiments with mammalian cells provide a more satisfying answer (Campbell et al., 1996; Wilmut et al., 1997). In this study, cells derived from adult mammary glands induced to become quiescent were the source of the nucleus transferred to an enucleated unfertilized egg. In this case a live normal lamb was produced. The identity of the donor nucleus and that of the clone were later confirmed. DNA isolated from frozen tissue of the donor, the cells derived from it and those of the clone (the lamb Dolly) are identical (Ashworth et al., 1998 ; Signer et al., 1998). Asworth et al. examined 10 microsatellites, short stretches of DNA that vary from individual to individual. Singer et al. used a fingerprinting technique where DNA fragments are sorted out by size. More recently Wakayama et al. (1998) were able to extend the findings to more manageable animals where several offsprings were produced. They injected cumulus cell nuclei into enucleated oocytes of mice and, after implantation, produced viable mice in about 2 to 3% of the cases. Cumulus cells are differentiated granulosa cells that surround eggs. No less important is the cloning of pigs (Ohnishi et al., 2000) which may have applications in xenotransplantation by assuring a supply of genetically modified animals to be used to replace malfunctioning human organs.

The same principle has been shown in plants, where specialized cells can give rise to complete plants (e.g., Vasil and Hildebrandt, 1965). Therefore, differentiation does not involve the loss of genetic material. What could it possibly involve?

Frequently, important biological questions can be answered by using simpler systems. The second set of experiments to be discussed has a bearing on this question and used the unicellular organism called *Dictyostelium discoideum*. During its life *Dictyostelium* assumes different forms. As a haploid ameba, it feeds on bacteria by engulfing them in a process known as *phagocytosis*, and then digesting them. Other organisms feed by using similar processes. When times are tough and the *Dictyostelium* has run out of bacteria to engulf, the amebae aggregate to form a multicellular, migrating slug. Eventually, the slug settles down and forms a stalk and a hat, each made up of different kinds of cells. The cells in the hat are spore cells. The dispersion and germination of these spores reinitiates the cycle by producing the ameboid form of the cell. The cycle from vegetative to stalk and hat is completed in about 2 to 2 1/2 days. The various stages are represented in Fig. 1A (Raper, 1940).

Aside from the genes needed for survival (the so-called *housekeeping genes*), 300 genes are thought to be involved in this function (Kay, 1992). How does this process work? Different portions of the genome

could be transcribed at different stages. This idea can be examined in experiments using what is called a run-on assay. The nuclei of each stage are isolated and allowed to transcribe the DNA by providing them with all necessary precursors of RNA. Radioactively labelled inorganic phosphate [$^{32}P_i$] is also added to estimate the newly synthesized mRNA. The amount of $^{32}P_i$ incorporated into RNA can be measured by a process called autoradiography, where the material is held close to a photographic plate. The radioactive disintegrations (proportional to the RNA present) will darken the film.

The individual kinds of mRNA can be recognized by their ability to bind the DNA that is complementary to it (cDNA, mentioned above). Each individual mRNA will only bind to the sequence in the DNA identical to the genes that were transcribed. The DNA pieces corresponding to specific genes are immobilized in spots. The size and density of the dot shows the RNA corresponding to specific genes. A modern version of this technique is the use of the socalled *gene chips*. The results of this experiment are shown on Fig. 1B. The gene in question is listed on the left. The various stages from which the nuclei were isolated are shown along the top. V stands for vegetative phase, A for aggregation stage, the formation of an aggregate tip is T, the slug phase is S, and C stands for the final "hat" stage. As can be seen, different genes are transcribed differently depending on the stage. The differences are particularly noticeable when the earliest stage (V) is compared to later stages, but differences are evident between any two stages. These experiments indicate that the various differentiated states result from selecting the genes that are transcribed. In *Dictyostelium* the control can also be at the level of translation, for example, in controlling the stability of mRNA (Hildebrandt and Nellen, 1992). Other studies indicate that the translational control may result from the presence of repressors acting on mRNA, for example, in determining the developmental fate of two daughter cells (referred to as asymmetric expression) in the embryonic development of *Drosophila* (Okabe et al., 2001). Regardless of mechanism the controls result in differential *gene expression*.

In recent years, transcription analysis using DNA microarrays (see Chapter 1) has provided a good deal of evidence on gene activity (referred to as the *transcriptosome*). However, the final answer requires understanding what proteins are actually translated (*proteome*) and which ones are active (*metabolome*) and these questions are beginning to be adressed (see Oliver et al., 1998).

The combination of microarray studies of mRNA (which reveals mRNA expression) and ICAT-mass spectrometry analysis (which reveals protein expression, i.e. *protemics*; see Chapter 1) has proven very revealing and was applied to the enzymes involved in the regulation of the galactose pathway in *Saccharomyces cerevisiae* (Ideker et al., 2001). I nterestingly, there is little correlation between the level of an enzyme and that of the mRNA coding for it, suggesting that the amount of a specific mRNA is not a good measure of phenotypic expression.

RNA localization at specific sites in oocytes, embryos and somatic cells has a central role in producing cellular asymmetry, cellular polarity and in determining cell fate during development (see Bashirullah et al., 1998; Kloc et al., 2002). RNA localization is responsible for (1) formation of cell lineages during embryogenesis by restricting the presence of RNA to certain cells, (2) production of morphogens during

development, (3) segregation of RNAs to specific sites, and (4) production of a high concentration of protein at a specific location. For these reasons it can be considered a primary event in differentiation and embryogenesis. Some of the mechanisms which produce RNA localization are discussed in Chapter 3 and Chapter 10.

After terminal differentiation, most cell types are generally quiescent. This static state is maintained through mechanisms that regulate the activity of the E2F proteins (see Chapter 8). In terminally differentiated muscle cells (myotubes), most E2F proteins were found to be cytoplasmic (Gill and Hamel, 2000) (E2F4 was partitioned between cytoplasm and nucleus). However, when overexpressed by transfection, the E2Fs entered the nucleus. At these levels of overexpression, cyclins A and E were induced and protein cell-cycle inhibitors were suppressed, followed by entrance of the cells into S-phase. These findings argue for a mechanism by which cells remain quiescent through control of the movement of the E2F proteins.

A new aspect of this question was introduced by techniques which allow to test the production of specific mRNAs inside single cells. In these techniques fluorescence in situ hybridization is used with oligomer probes tagged with a fluorophore at multiple sites. The study of cultured cells with this approach revealed that gene expression of each individual cell differs significantly (Levsky et al., 2002). Whether this difference remains over a long time period would be of considerable interest.

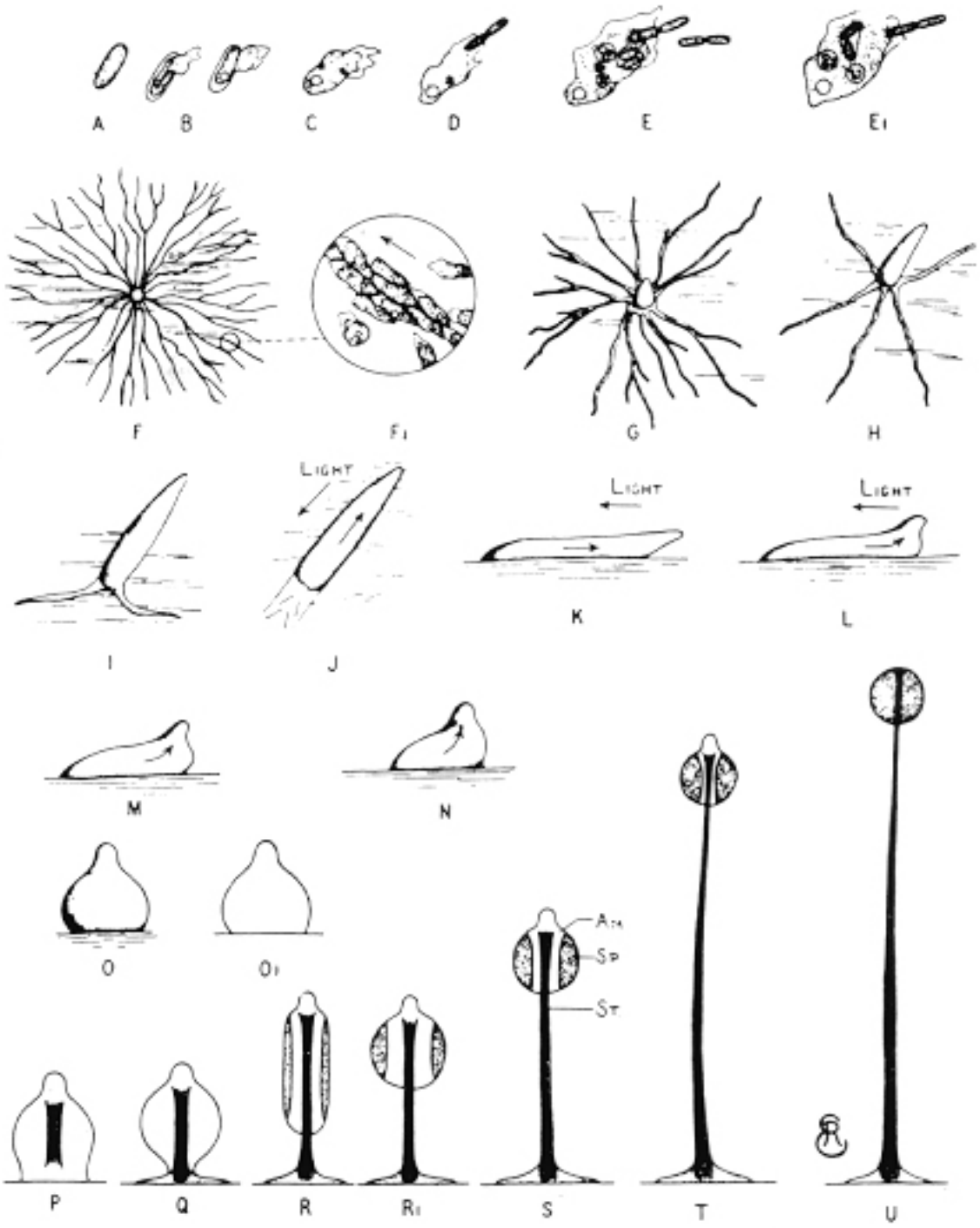
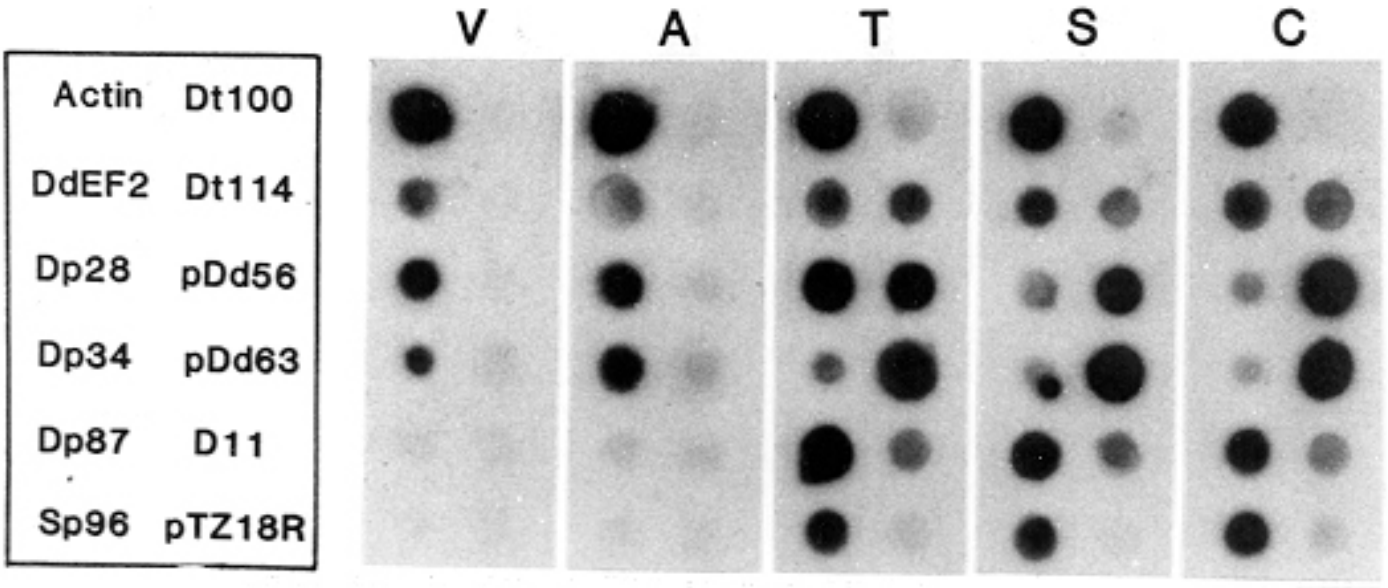


Fig. 1
A. Diagrammatic representation of the developmental cycle of *Dictyostelium discoideum*. A-C spore germination; D-E1, vegetative stage, myxameba ingesting and digesting bacteria; F, developing pseudoplasmodium; F1, detail of pseudoplasmodial stream; G-I, progressive aggregation of myxamebae; J-K migrating pseudoplasmodium; I-O cessation of migration and formation of rounded body; O1 longitudinal section of O; P-U , progressive stages in sorocarp formation in longitudinal section; Am, undifferentiated myxamebae; St, stalk; and Sp, spores. The cycle is normally completed in 2 to 2.5 days. From Raper, 1940, reproduced by permission.



B. Temporal pattern of transcription in *Dictyostelium discoideum*. Labelled RNA transcribed by isolated nuclei were hybridized with a DNA probe at the specified stage. The spots represent the radioactivity of the RNA complexed to the cDNA probes. Modified from Morio et al., 1991. Reproduced by permission.

II. SPECIFICATION, DIFFERENTIATION AND TRANSDIFFERENTIATION

The experiments just discussed indicate that in *Dictyostelium* differentiation takes place by controlling which genes are transcribed. The same principles apply for a multicellular organism. During development, cells destined to be part of different tissues will diverge in their capabilities. The restriction of developmental fate accompanying cell differentiation during embryogenesis has been divided into two stages (Slack, 1991): *specification* and *determination*. Specification refers to the ability of a tissue to differentiate when isolated from extracellular clues, by being placed in a neutral environment such as an artificial medium. Determination refers to the ability of cells to differentiate when placed in another region of the embryo. Either of the two phenomena indicate that cells have attained a stable state not readily reversed.

How can gene expression become restricted during embryogenesis? Since all cells have the same genomic complement, the gene expression initially must be in response to cytoplasmic determinants. In fact, segregation of cytoplasm components in early cleavage is uneven. In some cases, an uneven distribution is likely to require attachment of the determinants to cytoskeletal elements. Because this kind of specification depends on the cytoplasm alone it has been called *autonomous specification*. Cells can also be committed by clues in the environment originating from surrounding cells, often referred to as *conditional specification*. Specification can also occur before cells are separated out; the cells are interconnected and are said to form a *syncytium.* This kind of specification is referred to as *syncytial.* Most invertebrates are specified autonomously, vertebrates use both autonomous and conditional patterns, and only insects use syncytial specification.

How can these determinants affect gene expression? This is still a question that needs more information

and is one of the most important questions being asked at this time in the study of development. In a differentiated cell, some of the expressed genes must correspond to those needed for the cell's survival, the so-called *housekeeping genes*; others reflect the specialized task of the cell. For example, reticulocytes are engaged in the synthesis of an inordinate amount of hemoglobin. These cells are the precursors of red blood cells, which use the hemoglobin in the transport of oxygen and carbon dioxide. In addition to selecting which genes are expressed, the changes accompanying differentiation must be inheritable, since cells of a particular lineage retain the pattern of expression of their predecessors. Differentiation must involve the selection of some genes for expression and others for repression.

Heritable states of gene activity not explained by mutations or changes in gene sequences are referred to as *epigenetic phenomena*. Several cases will be discussed later such as parental imprinting (Section V). Among the many known epigenetic phenomena several have been studied, such as X chromosome inactivation (in the female only one X chromosome is functional), nucleolar dominance (only the rRNA genes from one parent are transcribed in inter-specific hybrids), transcriptional silencing at the HM mating-type loci in *Saccharomyces cerevisiae* and telomeric silencing (transcriptional repression of genes proximal to the telomeres). Heterochromatin is a condensed chromatin state that is inactive (see Cavalli and Paro, 1998a). In some cases, stable and heritable inactivation of transcription is easily recognized at a higher order of chromatin structure such as the condensed *heterochromatic* sequences around centromere regions. However, many of the proteins found in heterochromatin complexes are also involved in other aspects of silencing. *Chromo-domain* proteins (see Cavalli and Paro, 1998a) have been identified in some systems. These are proteins involved as part of silencing complexes involved in heritable gene repression. They are thought to shield from change *epigenetic* (i.e. acquired) propagated active chromosomal environments as well as silenced domains (Cavalli and Paro, 1998a). Silencing and activation appears to depend on the acetylated state: hyperacetylation of H3 and H4 histone being associated with activation (see Section VI) (e.g., Ekwall et al., 1997; Cavalli and Paro, 1999). The chromo-domain proteins are discussed in more detail in Section VI D. In the development of *Drosophila*, the Fab-7 DNA element can be switched to an active or a repressed mode during embryogenesis (Cavalli and Paro, 1998b, 1999). Various aspects of differentiation are discussed in the next sections of this chapter.

During embryonic development from the fertilized egg, the cells are progressively restricted in their differentiation options. However, differentiated cells have been found to be able to change from one type of differentiated cells to another, either when accompanied or unaccompanied by cell division. This phenomenon has been called *transdifferentiation* (see Slack and Tosh, 2001). The term *metaplasia* refers to the general phenomenon of switching from one cell type to another and includes the change of one kind of stem cells to another kind. As discussed below (Section IX) stem cells from specialized tissues are capable of changing into other cell types.

Presumably, transdifferentiation involves the alteration of transcription factors that control the gene expression. During normal differentiation, the genes the encode transcription factors capable of forcing a developmental path have been called *master control genes* . A case in point is that of the MyoD family of factors that can force the differentiation into muscle cells from a variety of cells in culture (e.g., Weintraub, 1993).

Transdifferentation has been shown in cell culture of pancreatic cells where the cells changed to liver cells after treatment with dexamethasone (Dex) (an adrenal steroid hormone analog) without cell division, a transition which induced the liver transcription factors LETF C/EBP β (Shen et al., 2000). The exocrine pancreatic cells expressed the appropriate enzymatic markers and Dex induced a variety of liver markers (Shen et al., 2000). Liver and pancreas are formed by neighboring regions of the endoderm. However, the transdifferentiation occurs in cells that are not always closely related. For example, the bone marrow stem cells are able to be converted into myoblasts, neurons, glial cells and heptic epithelium.(see Slack and Tosh, 2001).

Oligodendrocyte precursor cells derived from optic nerves of newborn rats were induced to develop into neurons as well as oligodendrocytes or astrocytes (Kondo and Raff, 2000. Neurons are not normally derived from oligodendrocyte precursor cells, since the differentiation pathways diverge at an earlier step. Protein markers for oligodendrocyte precursor cells, oligodendrocytes, astrocytes and neurons were identified immunologically to monitor the various stages. However, the possibility that some undetected stem cells were present in the cultures cannot be completely discarded. A similar demonstration was carried out with lymphoid committed progenitor cells from mice which normally develop only into lymphocytes. They were redirected by cytokines to become myeloid cells (Kondo et al., 2000.

Some of the evidence for transdifferentiation has been questioned because of the finding that adult stem cells spontaneously fuse to embryonic stem cells and acquire the characteristics of the embryonic stem cells (Ying et al., 2002; Terada et al., 2002; see Wurmser and Gage, 2002). However, some experiments clearly are not vitiated by these findings since cultures derived from single cells do transdifferentiate (e.g., Toma et al., 2001; Woodbury et al., 2000; Galli et al, 2000) . In addition, cell fusion is a rare event (1 in $5x\ 10^5$ to 1 in 10^4) whereas the putative transdifferentation has been found to occur with a high frequency (7-57%) (see Wurmser and Gage, 2002)

III. PROGRAMMED DEATH

In a multicellular organism, *programmed cell death* (PCD) plays a role in natural development, the maintenance of the organism and at the end of the immune response (Ellis et al., 1991; Jacobson et al., 1997). The role of programmed death in development is of utmost importance in sculpting the structure of metazoan organisms. This topic recently reviewed (Baehrecke, 2002) will not be discussed here. The *apoptotic cascade* (see below) is the best understood PCD. However, there are alternative programs (see Section F below).

Unscheduled *apoptosis* is responsible for a number of disorders such as Parkinson's disease and Alzheimer's. The neurodegenerative disorder, Huntington's disease, may involve components active in apoptosis (see Ona et al., 1999). On the other hand, a failure of apoptosis can lead to DNA damage that contributes to cancer (see Thompson, 1995, Evan and Littlewood, 1998). Apoptosis plays an important role in the immune system which produces lymphocytes in response to a pathogen. After the elimination

of the pathogen the excess lymphoid cells must be destroyed (Abbas, 1996). A variety of diseases can result from a failure of apoptosis to eliminate these cells. The autoimmune lymphoproliferative syndrome, ALPS, is a rare lymphoproliferative condition where patients frequently develop autoimmune diseases (Canale and Smith, 1967; Avila et al., 1999). In some cases, the failure can be traced to a mutation in the gene involved in apoptosis (see below).

Since apoptosis is one of the mechanisms involved in the body defenses against malignancy, inducing apoptosis in malignant cells can be used as a pharmacological tool. Cytotoxic ligands such as the *tumor necrosis factor-related-apoptosis inducing ligand* (TRAIL) induces apoptosis in tumor cells, supposedly with minimal effects on normal cells (e.g. see Kiechle and Zhang, 2002). The *second mitochondria-derived activator of caspases* (SMAC), a potent antagonist of *inhibitor of apoptosis proteins* (IAP) is also effective and it might be mimicked by peptides derived from SMAC (Fulda et al., 2002)

Apoptosis occurs in well defined steps (e.g., Kerr et al., 1971). The chromatin of the cell condenses and the cytoplasm contracts. After this the nuclear envelope degenerates, membrane whorls are formed, followed by the formation of autophagic vacuoles. Neighboring cells participate in phagocytizing the dying cells (e.g., macrophages, in mammals), originally thought to be a secondary event. Recent findings indicate that in the nematode *Caenothabditis elegans*,the engulfing cells have a role in assuring that when apoptosis is initiated by CED-3 caspase, it proceeds to the death of the engulfed cell (Reddien et al., 2001; Hoeppner et al., 2001). Cells may self-initiate apoptosis (under conditions of stress) or may undergo apoptosis following chemical signals activating certain surface receptors (see Sections IIIA and IIIB). As cells proceed through the active phase of apoptosis (see Mills et al., 1999), they detach from the extracellular matrix (ECM). The actin is reorganized to form a ring at the cortex and microtubules are disassembled. Myosin II-powered contraction (see Chapter 24) at the cortex produce a blebbing stage with repeated protrusions and retractions. The cells then condense to form a shrunken mass due to disassembly of F-actin. Basically, the process seems to be a well ordered destruction and the formation of a small "package", without the release of material to the neighboring cells (which otherwise could, for example, elicit an immune response).

Many factors can activate apoptosis including DNA damage, topoisomerase inhibition, hormones, growth factors deprivation and certain receptor mediated ligands such as *tumor necrosis factor* (TNF) that protect against malignancy and other unwanted events (Green and Martin, 1995).Apoptosis is initiated in endothelial cells when they detach from the *extracellular matrix* (ECM) (this kind of apoptosis is called *anoikis*).

Until recently, cell death had been examined in most detail in the developing nervous and the immune systems, however, it plays a prominent role in the development and maintenance of most tissues, and the process is now being examined in all its aspects.

A. The Processes of Apoptosis

Apoptosis is controlled by genes -- different genes generally controlling the apoptosis of distinct cells. Some of the genes essential for apoptosis code for proteases, the *cysteinyl aspartate specific proteinases,* or *caspases* (see Chapter 15, Thornberry et al., 1997a; Cryns and Yuan, 1998 , Thornberry and Lazebnik, 1998; Wolf and Green, 1999). In *Caenothabditis elegans,* a nematode used in many genetic studies, CED-3 and CED-4 execute apoptosis. In mammals, caspases are the homologs of CED-3 and fourteen caspases have been identified (see Thornberry and Lazebnik, 1998; Nakagawa et al., 2000). While the caspases which are responsible for the degradation of proteins, nucleases are involved in the breakdown of DNA.

In mammals, the final step, disposal of the dead cells, appears to proceed by phagocytosis (see Savill and Fadok, 2000) carried out by macrophages and requires the transfer of phosphatidylserine to the outer leaflef of the plasma membrane (see Henson et al., 2001). Without a disposal system, the debris are potentially toxic to cells and might elicit inappropriate immunological responses. The phagocytosis is specific for apoptotic cells, suggesting that the apoptotic cells possess a signal recognized by the phagocytes. *Milk fat globule-EGF-factor 8 (MFG-E8)* glycoprotein was found to be secreted by macrophages. MFG-E8 specifically binds aminophospholipids such as (e.g., phosphatidylserine) present at the surface of cells undergoing apoptosis (Hanayama et al., 2002). In vitro, phagocytes were found to engulf apoptotic cells when MFG-E8 was present.

The adhesion molecule CD31 (also called *platelet-endothelial cell adhesion molecule-1*, PECAM-1) present in healthy leukocytes was found to promote the detachment of cells from the macrophages (Brown et al. 2002). CD31 is thought to exert its effect in the detachment of healthy cells via a repulsion signal lacking in apoptotic cells.

The nucleases

During apoptosis breakdown of DNA has a characteristic pattern (Wyllie, 1980) to produce a so-called *DNA-ladder*. The term "DNA-ladder" refers to the appearance of the bands on gels of the hydrolysis products of DNA accompanying apoptosis. One of the nucleases found in mammals is referred to as *caspase activated DNase* (CAD or CPAN) (Enari et al., 1998 ; Halenbeck et al., 1998, Liu et al., 1998), a basic protein of 40 kDa with a nuclear localization signal at its carboxyl end. At rest, the nuclease is present in an inactive form in the cytoplasm of cells. A very acid protein of 29 to 30 kDa, probably present as a dimer binds and inhibits CAD and is therefore known as ICAD (Enari et al., 1998). Both CAD and ICAD were isolated from mouse cells and are generally present as a complex. Activation of CAD is produced by cleavage of ICAD catalyzed by caspase-3 (Enari et al., 1998). The liberated CAD cleaves the chromosomal DNA (see Nagata, 2000). Hyperexpression of ICAD blocks the chromatin changes of apoptosis (Sakahira et al., 1998) but does not change other steps of apoptosis (e.g., loss of mitochondrial oxidative phosphorylation). Another nuclease has has been found in the nematode worm *Caenorhabditis elegans* (Wu et al., 2000), NUC-1.

Mice cells lacking CAD still have a significant degradation of DNA. A search for another nuclease lead to another enzyme, *endoG*, activated by apoptotic stimuli and responsible for DNA fragmentation in

fibroblasts of embryonic mice lacking CAD (Li et al., 2001). EndoG does not require caspase activation, is released from mitochondria and is then translocated to the nucleus. A homologue of human mitochondrial endoG was found in *Caenorhabditis elegans* where it also localizes to mitochondria (Parrish et al., 2001).

Although caspases, DNases and lamin protease (Takahashi et al., 1996; Orth et al., 1996) play a role in the nuclear events, the mechanism that induce chromosome condensation in vitro is thought to be independent of these enzymes (Samejima et al., 1998). Chromatin condensation is inhibited by wheat germ agglutinin (WGA) indicating that it involves a cytoplasmic factor. WGA inhibits the transport of proteins into nuclei (see Chapter 5). A nuclear factor called *Acinus* (Sahara et al., 1999) was found to induce chromatin condensation after being activated by caspase-3 cleavage in permeabilized cells. Immunodepletion (i.e., removal by adding the corresponding antibody, see Chapter 1) of Acinus blocked the condensation of the chromosomes in vitro. In addition, transfection with antisense (see Chapter 1) acinuL cDNA delayed chromatin condensation in intact human cells. The mitochondrial factor, AIF, was also found to have a role in chromatin condensation (see below).

The caspases

Experiments with mice lacking caspase genes (see Zheng, 1999) provide us with some understanding of their importance. Mice lacking caspase 1 are deficient in its responses to cytokines. However, caspase 8 knockout mice exhibit early embryonic death and caspases 3 and 9, perinatal death.

The caspases can be either *initiators*, activated by self cleavage, or *effectors*, activated downstream from the initiators. Initiator caspases can act either on downstream caspases or on mitochondria (see below). In the latter case they release many factors that magnify the response.

On the basis of substrate specificity the caspases have been divided into three groups. Group I include caspases 1, 4 and 5. Group II includes caspases 2, 3 and 7 and group III caspases 6, 8, 9 and 10 (Thornberry et al., 1997b). Studies with caspase-deficient mutants reveal that the caspases are involved in apoptosis in a tissue and stimulus dependent manner (e.g., see Los et al., 1999). These observations indicate that individual caspases have unique properties that respond to different apoptotic signals.

The caspases are present as inactive procaspases and have to be activated by proteolysis (e.g., the proteolytic cleavage by initiator caspases such as caspase-9; see Thornberry and Lazebnik, 1998). In addition, some of the components of the apoptotic machinery are segregated. Members of the Bcl-2 family (see Section IIID) are located in the outer mitochondrial compartment, the nuclear envelope and the ER (Krajewski et al., 1993). Caspase-12 (see below) is localized in the ER (Nakagawa et al., 2000) and is released in response to stress to the ER. Immunofluorescence microscopy (see Chapter 1) localizes endogenous caspase-2 mostly in the Golgi cisternae and in the nucleus (Mancini et al., 2000) and it cleaves a Golgi protein, *golgin-160*. Golgins are a family of proteins that have been found to be antigens

for several autoimmune conditions. Some golgins function in vesicular traffic (see Chapter 11) (e.g., Gleeson et al., 1996; Sonnichsen et al., 1998; Nakamura et al., 1997). Therefore, elements of the Golgi apparatus are likely to act in apoptotic signaling and caspase-2 may be activated in response to signal or signals stemming from secretory functions.

The genetic studies of the nematode *Caenorhabditis elegans* have provided us with an outline of how the process takes place by one of the control mechanisms. In *C.elegans*, at least 14 genes are involved in programmed death (see Ellis et al., 1991). The role of three genes is reasonably well understood: *ced-3* (which encodes a caspase) and *ced-4* (encoding an activator of CED3) are required, whereas *ced-9* inhibits the action of the other two (Shaham and Horvitz, 1996). CED4, CED3 and CED9 can form multiprotein complexes (Chinnayan et al., 1997 ; Wu et al., 1997) at the plasma membrane. It is possible that they recruit pro-caspases at this location. This model visualizes CED4 as an adaptor that provides an attachment site for the other proteins. Similarly, in yeast, CED4, cross-links CED9 and CED3 (Jacobson, 1997; Goldstein, 1997). In *C. elegans* apoptosis requires the gene egl-1. Egl-1 negatively regulates the ced-9 gene and apparently EGL-1 (the protein coded by egl-1) initiates apoptosis by binding to and directly inhibiting the activity of CED-9 (Conradt and Horvitz, 1998).

Mammalian analogs for *ced3*, *ced9* and *ced4* (Zou et al., 1997) have been found. The cell death suppressor *Bcl-2* (see Section D) is analogous to CED9 and the *apoptotic activating factor 1*, Apaf-1, a 130 KDa protein, is equivalent to CED4. Members of the Bcl-2 family (see Section IIID) act as inhibitors either by interacting with Apaf-1 or blocking the release of cytochrome *c* (see below).

Programmed death is probably controlled by multiple mechanisms. Evidence from experiments using *C.elegans* in which ced-3, ced-4 and ced9 are overexpressed in cells originally programmed to survive, show that these three can act independently (Shaham and Horvitz, 1996). In addition, studies of the mammalian systems, Liu et al. (1996) have shown that caspase can be activated in vitro by adding dATP (the deoxyribose version of ATP). Cytochrome *c* was found to be one of the proteins whose release from mitochondria is required for apoptosis. Another fraction contained Apf-1 and was capable of binding cytochrome *c* and still another was found to be procaspase-9 (Li et al., 1997; Srinivasula et al., 1998). The mixture of the three presumably activates caspase-3, and subsequently caspase 3 can proteolytically activate other members of the caspase family. Cytochrome *c*, Apaf-1 and procaspase-9 are present in a complex referred to as an *apoptosome* (e.g., Li et al., 1997; Zou et al., 1999; Saleh et al., 1999). The apoptosome is a wheel-like particle with 7-fold symmetry (Acehan et al., 2002).Presumably a cascade is initiated by the release of cytochrome *c* from mitochondria. Cytochrome *c* binds to Apaf-1 in the presence of dATP or ATP, inducing the oligomerization of Apaf-1. The oligomeric complex forms the apoptosome with procaspase-9 and initiates the processing of procaspase-9 which in turn activates procaspase-3. Another mitochondrial factor capable of inducing apoptotic responses from the nuclei has been called the *apoptosis-inducing factor* (AIF) (Susin et al., 1996, 1999a) and is a flavoprotein. The AIF was also found to induce caspase-independent peripheral chromatin condensation (Susin, et al., 1999a). Microinjection of AIF into intact cells induces condensation of chromatin, and a collapse of the mitochondrial supposed transmembrane potential, and exposure of phosphatidylserine in the plasma membrane (which serves as a

target for phagocytosis; see below). It also induces purified mitochondria to release cytochrome *c* and caspase-9. These effects are independent of its ability to induce apoptotic changes. AIF is released from mitochondria in response to death stimuli (see Daugas et al., 2000). Genetic inactivation of AIF prevents the apoptosis of embryonic stem cells triggered by serum deprivation or the apoptosis required for mouse embryonic morphogenesis (Joza et al., 2001). The mitochondrial proteins released during apoptosis appear to be present in the intramitochondrial space. In addition to cytochrome *c* and AIF, other mediators of apoptosis are released by mitochondria (Kuida et al., 1996; Susin et al., 1999b). Normally located in the intermembrane mitochondrial space, caspase-9 is found primarily in nuclei after treatment of a neuronal cell line with apoptotic inducers (Krajewski et al., 1999), as shown with immuno-gold techniques (see Chapter 1). In addition, in ischemic brains, it is found in the nucleus only in cells undergoing apoptosis (Krajewski et al., 1999). This suggests that this caspase may be involved in triggering the nuclear events of apoptosis. The involvement of mitochondria is also discussed in section Section III C.

Internal and external signals

In mammals apoptosis can be initiated by internal signals or by extracellular signals such as death inducing cytokines (death factors). Activation of Fas, a receptor of the tumor necrosis factor (TNF) at the cell surface initiates apoptosis (see Nagata 1997). In Fas-mediated apoptosis, *death-induced signaling complex* (DISC) is formed in the cytoplasm. DISC contains Fas, a *Fas-associated death domain* (FADD) and procaspase-8. DISC generates active caspase-8 which subsequently activates procaspase-3 (Kischkel et al., 1995). The role of external signals on apoptosis is discussed in more detail in Section III B.

The power of apoptosis initiated from internal signals can be illustrated by the response to superoxide radicals (O_2^-). Superoxide dismutases (SODs) are enzymes that remove these radicals (see Section IVA). Cancer cells have very low SOD activity (e.g., Marklund et al., 1982) but much increased metabolism suggesting that they have a high retention of superoxide (e.g., Shulyakovskaya et al., 1993). Estrogen derivatives have been found to destroy human leukemia cells while sparing norman lymphocytes by blocking SOD and triggering an apoptotic cascade (Huang et al., 2000a). Apoptosis triggered by death factors has many similarities to the apoptotic process of *Caenorhabditis elegans* (Horvitz, 1999).

ER stress (see Chapters 10 and Chapter 7), such as protein misfolding and accumulation of exported proteins in the ER, for example in response to brefeldin A, (which blocks the ER to Golgi transport) or tunicamycin (which blocks glycosylation in the ER), increases the synthesis of ER resident protein chaperones regulated at the level of transcription (Welihinda et al., 1999) and in addition, induces apoptosis by releasing caspase-12 from the ER (Nakagawa et al., 2000).

Incorrect pairing or damage of nucleotides in DNA can lead to either repair (see Kolodner and Marsischky, 1999) or apoptosis (see Li, 1999). The mismatches are recognized in eukaryotes by heterodimeric complexes of MSH6-MSH2 or MSH3-MSH2, proteins of the MutS family (see Kolodner

and Masischky, 1999). Mispaired nucleotides arising from errors in DNA duplication or recombination events are detected by MSH6-MSH2 and repaired (see Kolodner and Masischky, 1999). A number of other proteins have also been implicated in mismatch repair. MutS proteins can also recognize chemical damage to the DNA (e.g., caused by chemotherapy) so that apoptosis is activated via p53 or the p53-related gene, p73 (Gong et al., 1999; see Li, 1999). p53 (see Chapter 8) is involved in blocking cell division and sometimes in initiating apoptosis. The MutS proteins are probably able to trigger these effects by undergoing a conformational change when they bind to the mismatch and then recruiting other proteins needed for repair or apoptosis.

p53 has many effects. However, the best understood ones are those involved in cell cycle arrest and in apoptosis (see Prives and Hall, 1999). The role in blocking cell division is discussed in Chapter 8. The balance that determines the level of p53 is very important. Loss of p53 frequently leads to carcinogenesis. On the other hand, its arrest of cell division in normal cells requires careful regulation, since the function of some cells requires their continual multiplication .

p53 has an important role in apoptosis triggered by DNA damage, oncogene activation and hypoxia (Soengas et al., 1999; see Vousden and Vande Woude, 2000). The related proteins p63 and p73 are needed for mouse embryo fibroblasts to undergo apoptosis in response to DNA damage in the presence of p53 (Flores et al., 2002). Apoptosis induced by the activation of mitogenic oncogenes requires p53 and involves Caspase-9 and its cofactor Apaf-1. p53 may act via Bax, responsible for the release of cytochrome *c* from mitochondria. p53 can act in apoptosis stimulating death receptor signaling and the mitochondrial pathway.

p53 activity is controlled by many mechanisms (see Vousden, 2000). One of these depends on the activity of Mdm. Mdm2 is an E_3 ligase (see Chapter 15) which is responsible for the proteasomal degradation of p53 as well as its own via the ubiquitination pathway (Vousden and Vande Woude, 2000). In addition, p53 activates the expression of Mdm2. These activities create a negative feedback loop. p53 has been shown to use microtubules to aid nuclear localization, required for its action. Apparently, the movement of proteins in the cytoplasm is frequently aided by microtubules and motor proteins. Mdm2 function can be inhibited by phosphorylation of Mdm2 or p53. Mdm2 is maintained inactive by its localization in the nucleolus. ARF binds to Mdm2 and keeps it in the nucleus thereby increasing the level of p53. Stress activates ARF via the action of the kinase DAPK (Raveh et al, 2001).

p53 activates several genes with a role in apoptosis. A gene, *p53 upregulated modulator of apoptosis* (*PUMA*) is activated by p53. PUMA-α and -β (encoded by the *PUMA* gene) bind to Bcl-2 (see below) and activate apoptosis by releasing cytochrome *c* from mitochondria (Nakano and Vousden, 2001).

Disturbances of the p53 system can lead to malignancy. For example, malignant melanoma cells have been found to bypass the control of p53 by blocking the trascription of the Arf gene (Soengas et al., 2001), possibly by a mechanism where the gene is inactivated epigenetically by methylation and deacetylation.

RB, a protein which blocks cell division (see Chapter 8), is cleaved by caspases during apoptosis initiated by tumor-necrosis factor-α but not that triggered by DNA damage, confirming the presence of two separate pathways . In a RB germ line mutation (Rb-MI) in mice, where RB cannot be cleaved, (Chau et al., 2002), fibroblasts expressing Rb-MI protein are protected from apoptosis induced by the tumor-necrosis factor-α type I receptor (TNF α RI) but not that induced by DNA damage.

The effect of the death supressor Bcl-2 (see Section IIID) , that acts through mitochondria (Newmeyer et al., 1994) (see Section III C), differs with cell type (see Scaffidi et al., 1999).

Signals from 'death' receptors (see Section III B) known as TNFR1/Fas on the cell surface require the presence of Bid. Bid, a protein of 22 kDa, is a member of the proapoptotic Bcl-2 family (see Section III D). It is activated as a result of its cleavage by caspase 8. The activated Bid is transferred to mitochondria where it releases cytochrome *c* which in turn activates caspases 9 and 3. Wild-type mice die from hepatocellular apoptosis and haemorrhagic necrosis when injected with an antibody directed against Fas(which acts as Fas-ligand). In contrast mice deficient in Bid generally survive (Yin et al., 1999). Bid deficient cultured hepatocytes with anti-Fas antibody showed a much reduced apoptotic reaction.

Generally, apoptosis is independent of transcriptional events. However, under some circumstances, apoptosis is transcription dependent and requires the activation of death genes (e.g., Miyashita and Reed, 1995; Kasibhatla et al., 1998; Le-Niculescu et al., 1999).

The interactions responsible for apoptosis can be complex and involve different cellular compartments. We have seen that the release of cytochrome *c* from mitochondria can initiate apoptosis. The transcription factor TR3 (also called nur77 or NGFI-B) was found to have a role in apoptosis [for example, overexpression of a dominant-negative TR3 or antisense TR3-mRNA (see Chapter 1) inhibit apoptosis]. Apparently TR3 initiates apoptosis when it is translocated from the nucleus to the mitochondria where it triggers the release of cytochrome *c* (Li et al., 2000c). In fact, the DNA binding domain of TR4 required for its transcriptional activity is unnecessary to trigger apoptosis. The TR3 protein belongs to the steroid-thyroid hormone-retinoid receptor superfamily of transcription factors and can be expressed by a variety of stimuli (see e.g., Kastner et al., 1995). As noted above, Mdm2, which has a role in controlling the concentration of p53, is maintained inactive by its localization in the nucleolus which in turn depends on binding to ARF.

The heat shock proteins (HSPs) (also discussed in Chapters 7 and 15) can influence whether apoptosis takes place via their direct interaction with key components of the apoptotic cascade. This is not surprising since they have a key role in the folding, assembly and cellular distribution of proteins (see Saibil et al., 2000).

Hsp60 favors the proteolytic activation of caspases. Apparently, procaspase-6 and procaspase-3 are present in a complex also containing Hsp60. Hsp60 is able to accelerate the maturation of procaspase-3

(Xanthoudakis et al., 1999). The complex, which also contains Hsp10, is present in the mitochondrial fraction. The presence of the pro-apoptotic agent, staurosporine, was shown to induce the activation of mitochondrial procaspase-3 and its dissociation from the Hsps which are released from mitochondria together with mitochondrial intermembrane proteins including cytochrome *c* and adenylate kinase (Samali et al., 1999). Two other HSPs (Hsp27 and Hsp70) have been found to favor the inhibition of apoptosis rather than its activation. Hp27 binds to cytochrome *c* after it is released (Bruey et al., 2000) preventing its interaction with Apaf-1. In contrast, Hsp70 blocks apoptosis by binding to Apaf-1 preventing the recruitment of procaspase-9 to the apoptosome (Beere et al., 2000; Saleh et al., 2000). The actual physiological role of the regulation by the Hsps is still an open question.

B. Death Receptors

In addition to the apoptotic signals generated within the individual cells, mammals have an additional system which responds to external signals. The system consists of death receptors such as CD95 (Fas) of the tumor-necrosis factor (TNF) receptor family present at the cell surface (see Nagata, 1997; Locksley et al., 2001). The death receptors are involved in a variety of tasks. These include killing activated mature T cells at the end of an immune response and targeting cells that are virus infected or malignant, as well as inflammatory cells in immunoprivileged sites (that is not accessible to immunological defences), such as the eye. Other receptors are involved in activating transcription factors NF-κB and AP-1 that favor proinflammatory genes, genes modulating the immunological responses and, in some cases, apoptosis.

Upon binding the ligand, the TNF-receptor forms a trimer and now can attach to the adaptor protein FADD/MORT1. FADD/MORT1 contains a *death effector domain* (DED) which probably binds to the DED of caspase-8 molecules. The complex of Fas-FADD and caspase-8 has been referred to as the *death inducing signalling complex* (DISC) (Kischkel et al., 1995). In the complex, several interacting caspase-8 molecules self-activate proteolytically (e.g., see Yang et al., 1998) initiating the caspase cascade (see Villa et al., 1997). Another protein, FLASH (Imai et al., 1999), activates with caspase-8. Cross linking studies show that FLASH is first recruited to the Fas (CD95) receptor and FLASH facilitates the effects of the overexpression of CD95.

The pathway differs with cell type. Fas mediated apoptosis in type I cells (e.g., hepatocytes) is initiated by large amounts of active caspase-8 formed at the DISC complex followed by activation of caspase-3. In contrast, in type II (e.g., lymphocytes) cells DISC (see above) activates caspase-8 slowly and induces the apoptogenic activity of mitochondria instead. The latter activates both caspase-8 and caspase-3. Consequently, only type II cells can block apoptosis when Bcl-2 or Bcl-x(L) are overexpressed . A number of other apoptosis-inhibiting or -inducing stimuli are effective only in type II cells, showing that their action is in the mitochondrial branch of the Fas pathway.

In anoikis (the apoptosis of endothelial cells when they detach from the ECM) , Fas, mediates the death signal when bound to FasL (see Nagata and Goldstein, 1995; Aoudjit and Fuori, 2001). The endothelial cells become sensitized to respond to Fas-mediated apoptosis when exposed to injuries or specific stimuli

such as the disruption of contact with the ECM. Apparently binding to integrins, which mediates the interaction with ECM, blocks apoptosis (see Frisch and Ruoslahti, 1997). The attachment to the ECM regulates the level of Fas and the *cellular FLICE-inhibitory protein* (c-FLIP), an antagonist of caspase-8.

Type II-ALPS a condition that leads to autoimmune diseases, can be traced to a mutation in the gene controlling caspase-10 (Wang et al., 1999b). Possibly the defective caspase-10 interferes with the death receptors probably by binding to FADD and caspase-8.

C. Role of Mitochondria

Mitochondria have a central role in initiating apoptosis (e.g., see Desagher and Martinou, 2000). A role of mitochondria in apoptosis is also discussed in Section IIIA. This role has been recognized for some time (Green and Reed, 1998). Three possible trigger mechanisms seem to be operating including: (a) release of proteins that activate caspases, (b) disruption of mitochondrial function such as interference with electron transport or oxidative phosphorylation, and (c) changes in redox potentials of the cell.

The involvement of mitochondria in apoptosis was first revealed by in vitro studies in which nuclear condensation that could be inhibited by Bcl-2 required the presence of mitochondria (Newmeyer et al., 1994). Other in vitro experiments demonstrated that caspase activation required the release of cytochrome *c* from mitochondria (Liu et al., 1996). In vivo or in vitro, cytochrome *c* is released by mitochondria during apoptosis and this release is blocked by the presence of Bcl-2 in these organelles (Yang et al., 1997; Kluck et al., 1997). An alteration in cytochrome *c*, precedes the steps of apoptosis (Varkey et al., 1999). A change in the conformation of the cytochrome *c* is shown by the availability of an epitope not normally exposed. Caspase activity was found to be necessary and sufficient to cause this alteration.

Some members of the Bcl-2 family of proteins are apparently found in the mitochondrial outer membrane although that may not be their original residence (see Reed et al., 1998). GFP-Bcl-2 and GFP-Bcl-XL, the fluorescent hybrids of these proteins and the green fluorescent proteins (see Chapter 1), colocalize with a mitochondrial marker. In contrast, GFP-Bax resides in the cytoplasm until an apoptotic signal causes its insertion in mitochondria (e.g., Wolter et al., 1997).

Other studies also show that the pro-apoptotic protein, Bax, is localized in the cytoplasm and is translocated into mitochondria after receiving an apopotic signal. After withdrawal of the cytokine interleukin-3 (IL-3), monomeric Bax was found to be translocated into mitochondria where it appears as a dimer (Gross et al., 1998) and initiates apoptosis. This process is blocked by the Bcl-2-protein. Artificial dimerization mimicks this activation suggesting that the dimerization is upstream to the mitochondrial transfer.

As already mentioned, several observations suggest that there may be multiple mechanisms of triggering apoptosis. At least one of the actions of the proteins of the Blcl-2 family appears to be by opening the mitochondrial *permeability transition pore*, PTP, a very large channel of the inner mitochondrial

membrane (see Reed et al., 1998; Marzo et al., 1998; Lemasters et al., 1998) (for a review on mitochondrial channels, Campo et al., 1998). Bax binding to the permeability transition pore and the adenine nucleotide translocator have been implicated in initiating apoptosis (Marzo et al., 1998). The opening of the PTP allows the passage of very large molecules. Subsequent osmotic swelling of the inner mitochondrial space and rupture of the outer membrane would release cytochrome *c*, since this cytochrome is localized in the intramembrane space between the inner and the outer mitochondrial membranes (the structure of mitochondria is discussed in Chapter 16). However, in some cases of apoptosis the mitochondria appear to be morphologically normal, suggesting that a swelling of the matrix and rupture of the outer membrane is not taking place.

The formation in the outer membrane of a large channel could also release cytochrome *c* (Green and Reed, 1998). The proteins of the Bcl-2 family have been shown to form channels in artificial bilayers (Kelekar and Thompson, 1998). Therefore, it would be entirely possible that Bax and Bak could induce cytochrome *c* leakage by forming channels. However neither Bak nor Bax by themselves can release cytochrome *c* (Shimizu et al., 1999). A vesicular reconstitution of outer membrane components implicate the BH3-domain peptide of Bid in the activation of monomeric Bax to produce membrane openings large enough for the passage of very large (2 MDa) dextran molecules in the presence of cardiolipin (Kuwana et al., 2002). The activity was blocked by antiapoptotic Bcl-x(L). In contrast to these mechanisms, the leakage of the cytochrome could be the consequence of opening of channels that are normally part of the outer membrane. One of the most abundant component of the outer membrane is the *voltage dependent anion channel*, VDAC (also called *mitochondrial porin*). If VDAC were induced to open, molecules in the intramembrane space small enough to pass through the pore could then be released. In liposome vesicles (see Chapter 4) reconstituted with VDAC, Bcl-X_L induces closure of the channels (Shimizu et al., 1999). In contrast, Bax and Bak induce opening. The leakage of the cytochrome was monitored using fluorescently labelled cytochrome *c* enclosed in the vesicles. An interaction of VDAC and Bax or Bak was demonstrated by using mitochondria deficient in VDAC. In these mitochondria, the addition of Bax did not release cytochrome *c*. However, complementing the mutants with human VDAC restored the ability of Bax to release the cytochrome.

A new ion channel, active during apoptosis (*mitochondrial apoptosis-induced channel*, MAC), has been reported from patch-clamping of mammalian mitochondria and reconstituted proteoliposomes formed from mitochondrial outer membranes (Pavlov et al., 2001). Unlike VDAC or the *translocase outer membrane* (TOM) channel, MAC is not voltage dependent. Its conductance is greater than the channels formed by Bax in artificial membranes. The size of the channel at its highest conductance state allows the efflux of cytochrome c and other large proteins. The channel is active in the presence of proapoptotic Bax in the mitochondrial outer membrane (e.g. in proteoliposomes prepared from the outer mitochondrial membrane of cells undergoing apoptosis) and less active in the presence of the antiapoptotic Bcl-2.

D. Anti- and Pro-Apoptotic Factors

As might be expected because of their role in apoptosis, caspases are highly regulated. Some proteins

facilitate the action of caspases whereas others block them. A tight regulation of apoptosis is important because inappropriate cell death is responsible for the destruction of healthy tissue as in neurodegenerative and autoimmune diseases. Survival factors block apoptosis. *bcl-2* was the first mammalian gene found to be capable of blocking apoptosis. However, a variety of other related gene products have been found (Hale et al., 1996; Adams and Cory, 1998) and at least eighteen members of this family of proteins have been identified (see Gross et al., 1999). These proteins share a number of common domains, the so-called *Bcl-2 homology domains* (BH1, BH2, BH3 and BH4). In this protein family, the action of anti-apoptotic proteins (e.g., Bcl-xL) is analogous to that of Bcl-2. In contrast, pro-apoptotic proteins of the same family (e.g., Bax, Bak) favor cell death (Boise et al., 1993; Oltvai et al., 1993). A third group of these proteins (e.g., Bcl-xS and BAD) are inactive by themselves but favor cell death induced by other components (Boise et al., 1993; Yang et al., 1995). The anti-apoptotic proteins have four BH domains, the pro-apoptotic proteins have three BH domains whereas members of the third group have a single BH domain (Gross et al., 1999). Some of the proteins are derived from alternative splicing of the same gene (e.g., Boise et al., 1993).

Monoclonal antibodies that are specific for known epitopes of anti-apoptotic and pro-apoptotic proteins, reveal novel conformational states (Hsu and Youle, 1997; Griffiths et al., 1999) under a physiological condition. The conformational changes expose certain epitopes only after activation. These changes in conformation are likely to play a role in controlling apoptosis.

The pro-apoptotic action of Bax apparently requires homodimerization and the formation of heterodimers with Bcl-2 . Overexpressed Bax favors apoptosis induced by cytokine deprivation and blocks the protective effects of Bcl-2 (Oltvai et al., 1993)

BAD dimerizes with Bcl-2 and more strongly to Bcl-xL. It is capable of reversing the anti-apoptotic activity of Bcl-xL. Apparently when BAD dimerizes to Bcl-xL, it displaces Bax, thereby restoring apoptotic activity (Yang et al., 1995). These observations suggest that the apoptotic pathways may involve complex interactions of many of these proteins (e.g., see Tao et al., 1998).

BAD can be phosphorylated by protein kinase A, anchored to mitochondria (Harada et al., 1999) with the result that apoptosis is blocked. The Ca^{2+}-activated protein phosphatase *calcineurin* (see Chapter 7) induces apoptosis by dephosphorylating BAD (Wang et al., 1999a). The Ca^{2+}-induced dephosphorylation of BAD is followed by dissociation from the protein 14-3-3 and translocation to mitochondria where Bcl-xL resides. In hippocampal neurons the whole process can be initiated by L-glutamate which triggers the Ca^{2+} influx.

Just as extracellular stimuli can initiate apoptosis, some such as insulin-like growth factor 1 (IGF1) and neurotrophins, can suppress apoptosis. Binding of ligands to their surface receptors can trigger the activation of several kinases (e.g., Fruman et al., 1998; Yano et al., 1998). These kinases activate a serine/threonine kinase known as Akt or PKB. Phosphorylation of Akt substrates: the Bcl2 family member BAD (Datta et al., 1997; del Peso et al., 1997) and Caspase 9 (Cardone et al., 1998) suppresses

pro-apoptotic function. Since Akt is translocated into the nucleus in response to survival factors (Andjelkovic et al., 1997) a role in transcription was suspected. The transcriptional factor of the *forkhead* family (FH), FKHRL1 has been found to be a target of Akt phosphorylation resulting in retention of the transcription factor in the cytoplasm and subsequent failure to transcribe. Withdrawal of the survival factor resulted in dephosphorylation, entry into the nucleus and the induction of genes involved in apoptosis (Brunet et al., 1999).

The inhibition of caspases is needed for normal function and development and also takes place during viral infections. Accordingly, caspase inhibitors can be viral or cellular (see Stennicke et al., 2002). Some of the inhibitors are endogenous to cells, the *inhibitors of apoptosis proteins* (IAPs). Others are encoded by virus, cowpox virus CrmA and baculovirus p35. Eight human IAPs have been found so far (see Stennicke et al, 2002) and more are unlikely, given the information from the completion of the human genome project. The importance of IAPs is shown by cell death following loss of IAP function in *Drosophila* (e.g., Rodriguez et al., 2002). The lack of effect of knockout mutations of some mammalian IAPs suggests that in mammals IAPs are present with a high degree of redundancy. IAPs bind and neutralize pro-apoptotic caspases, thereby blocking apoptosis (see Salvesen and Duckett, 2002). They block the entry of substrates to the active sites of caspases. This block can be overcome by proteins which bind IAPs at their amino terminal (e.g., Wang et al., 1999d). The binding motif is referred to as the *IAP-binding motif* (IBM). The *X-chromosome linked IAP* (XIAP), prominent in the regulation of apoptosis (see Holcik and Korneluk, 2001) has been found to inhibit caspases 3, 7 and 9 (see Deveraux and Reed, 1999). IAPs contain one or more *baculovirus IAP repeats* (BIRs). However, the flexible domain preceding the BIR sector is thought to be responsible for the inhibitory action (e.g., Huang et al., 2001). The BIR region has a role in binding to the caspase and might have a role in stabilizing the inhibitory interaction. The catalytic Cys residue is not involved in the tight binding. In contrast to the inhibitors of the Bcl-2 family which block only the mitochondrial branch of apoptosis, the IAPs block both mitochondrial and death receptor mediated apoptosis. The IAPs and the proteins which block their action are crucial for the regulation of apoptosis (see Deveraux and Reed, 1999; Miller, 1999). Besides their inhibitory role, IAPs are also involved in other functions such as receptor mediated signaling and ubiquitination (see Holcik et al., 2001). In *Drosophila*, the activity of IAPs is blocked by binding to the proteins Reaper, Grim and Hid (Wang et al., 1999c; Goyal et al., 2000). In mammals, the protein Smac/DIABLO is functionally the equivalent of Reaper, Grim and Hid of *Drosophila*. In addition to cytochrome *c*, the protein Smac/DIABLO (Du et al., 2000; Verhagen et al., 2000) is also released from mitochondria. As a homodimer, Smac/DIABLO binds to all the IAPs that were tested and eliminates their inhibitory activity (Chai et al., 2000). However, the role of Smac/DIABLO is not likely to be unique. The deletion of the corresponding gene does not seem to have an effect on proliferation, survival and apoptosis (Okada et al., 2002). A possible interpretation is that this protein is only one of the molecules involved in the inactivation of IAPs.

Ubiquitination has also been shown to have a role in regulation of IAPs. IAPs contain a carboxy-terminal *really interesting new gene* (RING) finger, a zinc binding domain characteristic of the E_3 ubiquitin protein ligases . This motif is required for blocking apoptosis (Wilson et al., 2002). The *Drosophila* IAP1

(DIAP1) RING finger was found to be responsible for the ubiquitination of itself and the *Drosophila* initiator caspase Dronc. Although ubiquitination may lead to degradation, the ubiquitination of Dronc did not lead to degradation. Similar results were obtained with mammalian systems where the monubiquitination of caspases was found to be mediated by IAPs (e.g., Huang et al., 2000b).

The *Drosophila* IBM proteins Grim and Reaper were also found to decrease DIAP1 levels by favoring its degradation via ubiquitination and also by suppressing global protein synthesis (e.g., Yoo et al., 2002; Holley et al., 2002) . In essence, by reducing IAP levels these two mechanisms, together lower the threshold for apoptosis

E. Developmental Role of Apoptosis

In development, apoptosis is precisely controlled. The same cells die at a characteristic time and the process occurs in well defined steps. Apoptosis functions in the sculpting of the nervous system. In moths, during metamorphosis, cells that are no longer needed undergo apoptosis. In some vertebrates as many as 85% of certain neuron populations die during enbryogenesis. Several morphogenesis events, such as the delineation of digits in the limb bud of the chick, are ruled by programmed death in which retinoic acid and bone morphogenic protein (BMP) play a prominent role (Rodriguez-Leon et al., 1999). Apoptotic processes remove thymocyte that bear T-cell receptors capable of recognizing self-antigens and, in addition, other cells that are no longer needed or may become harmful.

The role of apoptosis in sculpting the nervous system of vertebrates can be examined from its complementary facet, i.e what is needed to prevent apoptosis. Survival of the cells in both the peripheral and the central nervous system depends on an interplay between various *neurotrophins* and activity (see Oppenheim, 1991). Neurotrophins are protein growth factors needed for the maintenance and development of neurons (see Chapter 6). During development the presently understood scenario suggests an interaction of various neurotrophins that follow different signaling pathways to determine the life and death of the neurons (see Majdan and Miller, 1999). In at least some neurons, the *cyclic adenosine monosphate (cAMP) response element* (CREB) (see Chapter 7) mediates the survival of neurons.

During the development of the nervous system the extracellular matrix and gradients of neurotrophic factors play a prominent role in targeting the growing axon terminals. However, misdirected axons are supposedly no longer supported by neurotrophic factors and are subject to apoptosis (see Pettmann and Henderson, 1998). Neuronal terminals axons are directed to their final destination by intermediate targets (e.g., Tessier-Lavigne et al., 1988). The intermediate targets can also support the survival of migrating axons (Wang and Tessier-Lavigne, 1999). Growing spinal commissural axon terminals (which cross over from one side of the spinal cord to the other) pass over a group of cells (the *spinal-floor plate*) which constitutes the intermediate target (see Van Vactor and Flanagan, 1999). Floor plate-conditioned medium permits the survival of these neurons (Wang and Tessier-Lavigne, 1999). Conditioned medium corresponds to the medium remaining after removal of the cells. When the axons grow away from the location of the plate, they require both the conditioned medium and neurotrophins suggesting that the

migration takes place in steps each determining survival and apoptosis.

In addition to stimulation by the neurotrophins, the activity of the neurons is also important for the survival of the neurons. For example, blockage of either pre- or post-ganglionic transmission (see Chapter 22) produced greater neuron apoptosis (Maderdrut et al., 1988). This is also true in other neurons of the peripheral or central nervous systems (Franklin and Johnson, 1991) and neurons in culture (e.g., Meyer-Franke et al., 1998). In fetal or early neonatal development of rats pharmacological blockade of NMDA glutamate receptors (see Chapter 22) for only a few hours triggered apoptosis of neurons, suggesting that glutamate stimulation of NMDA receptors have a role in neuronal survival (Ikonomidou et al., 1999).

Stimulation may increase the release of neurotrophic factors (e.g., brain derived neurotrophic factors (BDNF), Ghosh et al., 1994) thereby increasing the basal stimulation of the neurotrophin alone. Alternatively, neurotrophins and neural activity could interact by activating the same targets.

The initiation or prevention of apoptosis are closely associated with synaptic terminals (e.g., Ivins et al., 1998). Accordingly, apoptosis can be activated at synaptic terminals. Apoptosis involves the glutamate receptors and receptors for neurotrophic factors in pre- and post-synaptic terminals (see Mattson and Duan, 1999). Activation of receptors for neurotrophic factors in synaptic terminals protects from apoptosis whereas activation of glutamate receptors in postsynaptic spines can initiate apoptosis (somewhat in contradiction to the behavior just described).

F. Alternative Programmed Cell Death

Alternative pathways of cell death have been uncovered upon interference with the apoptotic pathway (e.g., mutations, pharmacological agents, inhibition by IAP or some viral proteins) and occur in cell cultures as well as in vivo (see Leist and Jäättelä, 2001). In addition, some features that are characteristic of apoptosis also occur with alternative deaths (Chung et al., 2000), e.g., the translocation of phosphatidylserine to the outer plasma membrane leaflet (see Henson et al., 2001). Some of these programs resemble apoptosis at least morphologically (apoptotic-like PCD), others differ (e.g., no chromosome condensation) and resemble *necrotic* events (necrotic-like PCD; cell death that does not follow the apoptotic steps). Apoptotic-like PCD can take place without activation of effector caspases. Cell death also can occur after caspase activation and death receptor activation without following the apoptotic steps (e.g., Leist et al., 1997; Holler et al., 2000).

Some of the alternative death programs can be triggered by death receptors and mitochondrial events and are mediated by non-caspase proteases (see Leist and and Jäättelä., 2001). However, some of the effects can be initiated by the caspase pathway. The death receptor induced cell death depend on the kinase activity of the *receptor-interacting protein* (RIP) (Holler et al., 2000) and the production of *reactive oxygen radicals* (ROS) (e.g., Vercammen et al., 1998). Another pathway involves mitochondria and is also mediated by ROS. Defects in the apoptotic cytochrome *c* and AIF programs may also lead to necrotic cell death.

A form of programmed death occurring during development and distinct from apoptosis has been described and been named *paraptosis* (Sperandio et al., 2000). It differs from apoptosis in its different characteristic morphology and biochemistry, and its lack of response to apoptosis inhibitors. This form of cell death depends on an alternative caspase-9 activity that is Apaf-1-independent. Insulin-like growth factor I receptor (IGFIR) induces a form of nonapoptotic programmed cell death as shown by the occurrence of cytoplasmic vacuolation and resistance to apoptotic inhibitors. Paraptosis involves transcription and de novo protein synthesis. Furthermore, microarray screening (see Chapter 1) comparing gene expression of apoptotic cells and IGFIR-induced cells demonstrates distinct gene expression.

The relative importance of the alternative death programs is still difficult to assess since the information is relatively recent.

IV. AGING

Aging can be considered to represent a form of differentiation: the progressive change in phenotype that occurs in all the living (Faragher and Kipling, 1998; Johnson et al., 1999). The concept that aging corresponds to a developmental progression is supported somewhat by studies of aging in rodents. Goyns et al. (1998) used rat heart, brain and liver, and, in the study of Lee et al., 1999, mouse muscle . They found that aging is accompanied by differential gene expression, a decrease of expression some of the genes that are active and an increase in some others. However, the proportion of affected genes (about 1 to 2%) is small. Most significantly, a high proportion of these were those responsible for metabolic and biosynthetic activities. The studies used hybridization techniques (similar to the approach represented in the experiment of Fig. 1) with oligonucleotide arrays representing many genes (as many as 10,000 to 14,000 in the study of Goyns et al. and 6437 genes in the study of Lee et al.). These arrays are the so-called *gene* chips (see Chapter 1). The mRNA produced at any one time is identified by its ability to hybridize to a specific gene. In mice, aging can be prevented by limiting caloric intake. Under these conditions (Lee et al., 1999), the changes in gene expression were prevented. Various lines of research have indicated the involvement of many possible mechanisms in senescence that are discussed in this section. Whether one or more play a predominant role remains to be established. The fact that the doubling potential of individual cells in a culture differs even when derived from the same clone, (e.g., Smith and Whitney, 1980) suggests that there are multiple factors determining senescence. The role of telomere shortening in aging is discussed in Section VII.

In unicellular organisms, phenotypes corresponding to aging eventually culminate in cell death. The subject of many studies, *Saccharomyces cerevisiae* cells, undergo many morphological and physiological changes during their lifespan (e.g., Jazwinski, 1993) and their gene expression is a function of the age of the cells (e.g., Egilmez et al., 1989). Furthermore, they can divide only a limited number of times before dying (e.g., see Muller et al. 1980). Therefore, they are comparable to many other cells in this respect and can serve as a model of aging. In multicellular organisms, aging has been generally considered as a consequence of cell death because damaged cells cannot be replaced fast enough to maintain function.

However, apoptosis (Section III) is not likely to play a role in aging. This is indicated by the absence of overlap of genes involved in the two processes (Hengartner, 1997). However, in mice *bcl2* gene knock-out mutations, which favor apoptosis, produce functional failures that do resemble the phenotype accompanying aging (Veis et al., 1993).

A. Oxidative Damage

Reactive oxygen species (ROSs) such as superoxide anion, hydrogen peroxide and hydroxyl radicals are generated by metabolism and they are thought to create cumulative cell damage (Harman, 1981). The majority of the ROSs originate in mitochondria, at Complex I (NADH dehydrogenase) and III (ubiquinone-cytochrome *c* reductase) (see Chapter 16), but mostly at complex III (Turrens, 1997). Apparently, the free radical semiquinone [.Q^-] is an intermediate in the regeneration of CoQ. [.Q^-] transfers electrons to molecular oxygen forming a superoxide radical (see Finkel and Holbrook, 2000). In support of this view, human fibroblasts maintained in culture live longer at low oxygen pressure (Packer and Fuehr, 1977) and in general, genes that extend lifespan have been linked to features that decrease the level of ROS (see Finkel and Holbrook, 2000). For example, transgenic *Drosophila* carrying genes encoding antioxidants live longer (e.g. Orr and Sohal, 1994, Parkes et al., 1998a,b). In the study of Parkes et al., only the motor neurons were targeted. However, in some cases, mice mutated to lack glutathione peroxidase and *superoxide dismutase* (SOD) (SOD1, SOD2 and SOD3), do not show an obvious aging phenotype (Ho et al., 1996; Reaume et al., 1996), although some have serious defects that shorten their lifespan (Melov et al., 1998). Superoxide dismutases are enzymes that catalyze the conversion of two superoxide anions into hydrogen peroxide and oxygen (see Fridovich, 1995). Nevertheless, genetic inactivation of the mitochondrial form of superoxide dismutase in mice (Melov et al., 1998), results in dilated cardiomyopathy, hepatic lipid accumulation and early neonatal death. In addition, treatment with the SOD mimetic Mn 5,10,15,20-tetrakis (4-benzoic acid) porphyrin (MnTBAP), rescues these mutant mice from these deficiencies and prolongs their survival. However, a central nervous system degeneration proceeds possibly because MnTBAP may be unable to cross the blood brain barrier.

More recently, mice lacking the gene coding for the $p66^{shc}$ protein have been shown to have heightened resistance to agents causing oxidative damage (Migliaccio et al., 1999). Furthermore, the mice live 30% longer. $p66^{shc}$ is a variant of $p52^{shc}$/$p46^{shc}$ (Migliaccio et al., 1997), produced by alternative splicing. $p52^{shc}$/$p46^{shc}$ is a cytoplasmic factor involved in transmitting mitogenic signals from surface receptors to Ras (see Chapter 7) (Pelicci et al., 1992). These results suggest that oxidative damage can be an important contributing factor in aging and senescence.

Perhaps for the reasons outlined above, the life span of some animals can be extended by decreasing metabolism either by caloric restriction (e.g., rodents) or some other means of slowing down metabolism such as lowering the ambient temperature (e.g., in *Drosophila*).

In the nematode *Caenorhabditis elegans*, a mutation in the mitochondrial leucyl-tRNA synthetase gene

was found to impair mitochondrial function and to be responsible for a longer-lifespan (Lee et al., 2003). The findings suggest a complex coupling between metabolism and longevity.

There is evidence that premature senescence can also occur because of stresses (see von Zglinicki, 2002) such as oxidative stress (Touissaint et al., 2000) via effects on telomere shortening.. Mild stress has been found to increase telomere shortening and decrease replicative lifespan probably a consequence of unrepaired nucleotide damage (see von Zglinicki, 2002) and requires DNA replication. As discussed in Section VII, telomere shortening is associated with aging. In essence, telomeres count cell divisions and also the accumulation of mutations. Telomere shortening is therefore thought to depend at least in part on a balance between oxidative stress and antioxidant effects.

B. Genome Instability

The accumulation of genetic changes such as point mutations (Szilard, 1959), loss of repeated DNA sequences such as ribosomal DNA (Streheler, 1986), loss of chromosomes or chromosomal rearrangements are thought to be causes of aging. In fact, in mice an accumulation of mutations has been found. In these studies, transgenic lacZ reporter genes, present in chromosomally integrated plasmids (Dolle et al., 1997) were used as markers. The changes affected liver (which has high rate of mitosis) more than brain (which has a low rate of mitosis). A relatively large fraction of the mutations involved genome rearrangements.

A role of DNA damage in aging is supported by studies carried out with mice. Mutations of the XPD gene were found to produce premature aging and reduced life expectancy suggesting an important role in these events (de Boer et al., 2002). This gene encodes a DNA helicase with a role in both DNA repair and transcription. Whether the effect is solely due to DNA defects and not indirect is still unclear.

In budding yeast (*Saccharomyces cerevisiae*), the aging in the mother cell after budding, follows changes in the ribosomal DNA (rDNA)(Sinclair and Guarente, 1997), initially present in 100 to 200 tandem copies. In the middle of the lifespan of the cell, copies of the rDNA are removed from the chromosomes and remain as extrachromosomal circular copies (*extrachromosomal rDNA circles*, ECRs) which are distributed mostly to the mother cell after replication. The increase in ECRs is followed by fragmentation of the nucleolus (Sinclair et al., 1997), the site of rDNA transcription. Genetic data support the view that the ECRs play a role in aging in yeast. Yeast cells mutated in the *sgs1* gene accumulate ECRs more rapidly, leading to a shorter life span. The artificial creation of ECRs by genetic manipulation also shortens the life span. The *sgs1* gene encodes a DNA helicase (an enzyme that separates the DNA strands). *sgsl* corresponds to the *Werner's syndrome* (*WRN*) gene in humans. Mutations in *WRN* in humans result in Werner's syndrome, a disease with symptoms resembling premature aging.

Direct studies of Werner syndrome have provided clues of how aging is related to events occurring at the nucleolus. Werner syndrome is produced by a mutation of a single gene *WRN* (Epstein and Motulsky, 1996 ; Yu et al., 1996). The syndrome produces a reduced life span and premature phenotypic changes

resembling aging. The *WRN* gene has been cloned and encodes a DNA helicase (see Yu et al., 1996; Gray et al., 1997; Suzuki et al., 1997). Both the *sgs1*-protein of yeast and the WRN protein accumulate in the nucleolus (for human cells Gray et al., 1998; Marciniak et al., 1998). In yeast, ERCs form more readily when *sgs1* is not functional. However, there may be a difference between the two kinds of cells in this respect because there is no evidence in human cells linking DNA-circles to aging (Johnson et al., 1999).

As discussed in Section VII, in human cells in culture, telomeres shorten with each cell division (Harley et al., 1990) because they lack telomerase. Therefore, this could serve as a signal for growth arrest and eventual cell death. Certain cultured human cells can be reactivated by telomerase which lengthens the telomeres (Bodnar et al., 1998; Vaziri and Benchimol, 1998; Kiyono et al. 1998) extending their normal replicative limits without disturbing their phenotype to resemble malignant cells (Jiang et al., 1999; Morales et al., 1999). Furthermore there is a correlation between telomere length and decrease in capacity to proliferate (Effros, 1998). On the other hand, telomerase knockout mice have normal phenotype over several generations (Blasco et al., 1997; see also Wright and Shay, 2000). In addition, telomere shortening is not universal. Budding yeast while undergoing aging (see above) do not exhibit telomere shortening (D'mello and Jazwinski, 1991). Ironically, however, yeast has also provided the evidence that shows that progressive telomere shortening (in yeast produced by the mutation in one gene, *EST1*) can limit the cells' lifespan (Lundblad and Szostak, 1989). These results suggest that telomere-shortening may have a role in aging in some organisms.

What determines replicative senescence in rodents? Rodents repair DNA damage much less efficiently that human cells (Hart and Setlow, 1975) and are more susceptible to oxidative stress (Kapahi et al., 1999. Current thought (see Shay and Wright, 2001) suggests that eventually these effects accumulate to produce senescence (as discussed in Sections A, B and C). The interpretation of results pertaining to aging (i.e., replicative senescence) obtained with cell cultures has been called into question by more recent reports. Rat Schwann cells (Mathon et al., 2001) and oligodendrocyte precursor cells from postnatal rat optic nerve (Tang et al., 2001) were shown to proliferate indefinitely if prevented from differentiating. This phenomenon is distinct from malignancy since these cells maintain the checkpoints of cell division lacking in transformed cells which also can multiply indefinitely. These results suggest that in rodents cellular aging may depend on the presence or absence of external signals.

C. Extrachromosomal Genome Instability

The possibility that the accumulation of mutations in mitochondrial DNA may contribute to aging has been considered (see Lee et al., 1997; Wallace et al., 1998). This possibility is given credence by the fact that the mutation rate of mitochondrial DNA is 10 to 20 times that of nuclear DNA. However, it has been estimated that the mutated fraction of the genome must climb to more than 50-80% to be deleterious at least in human muscle (Sciacco et al., 1994). A variety of mutations in mitochondrial DNA increase with age (see Melov et al., 1995; Lee et al., 1997) and in individual cells a sizable fraction of the total DNA appears to be mutant (Schwarze et al., 1995; Brierley et al., 1998). More importantly, the main control

region for the duplication of mitochondrial DNA was found to have frequent point mutations in older subjects (Michikawa et al., 1999). In addition, mitochondrial electron transport function deteriorates with age (Boffoli et al., 1994; Kopsidas et al., 1998). Furthermore skeletal muscle fibers with a high level of deletions were found to be deficient in cytochrome *c* oxidase. Defective electron transport could lead to secondary effects such as accumulation of free radicals.

The possible role of oxidative damage of mitochondrial DNA in aging has been the target of several investigations (e.g., see Richter et al., 1988; Beckman and Ames, 1996). The levels of deoxyguanosine (dG) (8-oxo-7, 8-dihydro-2'-deoxyguanosine) in the mitochondrial or nuclear DNA were used as a measure of oxidative damage. Generally, dG was found to be present at a higher level in mitochondrial than in nuclear DNA. However, more recent experiments suggest that the level of dG is comparable in the two system. Supposedly, the oxidative damage is produced during the procedure needed to isolate mitochondria (Anson et al., 2000). However, a recent study (Barja and Herrero, 2000) in which the DNA was examined without isolation of either nuclei or mitochondria, shows an inverse relationship between the longevity of the species and the level of dG in the mitochondria of the heart and brain of several mammals. In contrast the level of nuclear dG was found to be lower than in mitochondria and to vary little with longevity. Therefore, it would seem prudent to consider this question still unresolved.

D. Genes Involved in Aging

The finding that mutations of single genes extend or limit the lifespan of some organisms has raised the hope that the mechanism controlling aging may be relatively simple. We have already seen that in yeast and in humans aging is strongly affected by mutations in the *sgs1* and *WRN* genes respectively (see above). In the nematode *Caenorhabditis elegans*, mutations in the *age-1* and *daf-2* genes extend the lifespan in adulthood 2 to 4 fold (Friedman and Johnson, 1988; Kenyon et al., 1993`; Larsen et al., 1995; Tissenbaum and Ruvkin, 1998) (see Johnson et al., 1999 for discussion). *daf-2* and *age-1* encode components of an insulin-like signaling pathway. The effect is mediated by DAF-2 through the AGE-1 phosphatidylinositol-3-OH kinase and DAF-16 transduces these signals. *daf-16* encodes a transcriptional activator that is a target of negative regulation by the PI 3-kinase-mediated signal cascade. Mutations at the *klotho* (*kl*) gene in mice (Kuro-o et al., 1998) shortens lifespan and cause the deterioration of a number of functions that correlate with aging.

In *Drosophila*, InR corresponds to daf-2 of *Caenorhabditis elegans* and the mammalian insulin receptors. Mutations in the insulin-like pathway, either in the genes coding for InR or CHICO also prolong life span (Tatar et al., 2001; Clancy et al., 2001). *chico* is the gene coding for an insulin receptor substrate that functions in an insulin-like growth factor signaling pathway. Apparently, the mutation of the gene that codes for InR, which prolongs life, acts through a deficiency in juvenile hormone. Daf-2 has also been implicated in contributing to the life span in *Caenorhabditis elegans* (Gems et al., 1998). A study of heterozygous knockout mice (null mutants are not viable) has revealed that igf1r(+/-) (which codes for IGF-1R) mice live on average 26% longer and exhibit a greater resistance to oxidative stress (Holzenberger et al., 2003).

Recently, the silencing protein, Sir2p, has been found to function in longevity. A role of Sir2p in silencing transcription, cell cycle progression, radiation resistance and genomic stability has been known for some time. Deletion of the *SIR2* gene shortens the life span, whereas an extra copy of the gene lengthens it (Kaeberlein et al., 1999). The *SIR2* gene homologs have been found in many organisms (Brachmann et al., 1995) so that its role may be universal. Sir2p has been found to be a nicotinamide-adenine nucleotide (NAD)-dependent histone deacetylase (Imai et al., 2000), providing a link between metabolism and silencing, since histone deacetylation results in gene repression (see below). NAD's key role in metabolism is illustrated in Fig. 2 of Chapter 14. A decrease in metabolism would be expected to decrease the level of NAD.

Restriction of caloric intake has been found to extend life-span in many organisms (e.g., see Weindruch et al., 1986). However, the mechanism for this effect has remained obscure. Caloric restriction can be mimicked in *Saccharomyces cerevisiae* by limiting the availability of glucose, or by mutating proteins in the cAMP-dependent protein kinase pathway (which is activated by glucose) resulting in a significant extension in life-span (Lin et al., 2000). In yeast lifespan is measured by the number of cell divisions before cell senescence. Longevity could be blocked by mutations in *SIR2* (the gene for the silencing protein Sir2p) or NPT1 (a gene required for the synthesis of NAD, the oxidized form of nicotinamide adenine dinucleotide). These findings suggest that aging depends on the silencing gene and the action is linked through the mediation of NAD, possibly by the production of reactive oxygen species produced during respiration.

How could Sir2p produce longevity (see Guarente, 2000 for an in depth discussion)? Caloric restriction could increase the level of NAD thereby enhancing Sir2p activity. In *Saccharomyces cerevisiae*, Sir2p is thought to promote longevity through its silencing of rDNA, preventing the formation of rDNA circles (ECRs) which are thought to trigger senescence (discussed above). In other eukaryotes there is no evidence of ECRs. However, inappropriate gene expression (blocked by silencing) could be responsible for senescence.

In *Drosophila* mutations of the gene *Indy* (I’m not dead yet) were found to double the adult life span (Rogina et al., 2000). The protein coded by *Indy* resembles the mammalian sodium dicarboxylate cotransporter which is active in the transport of Krebs cycle intermediates. It has been suggested that this protein may be operating in controlling intermediate metabolism and therefore the mutations may mimic the effect of caloric restriction.

V. ROLE OF DNA METHYLATION

Methylation occurs in vivo in the 5' position of cytosine (see Fig. 2) at CpG sites. The methylation does not interfere with the pairing of the bases and DNA can be replicated semiconservatively even when methylated. However, the newly formed DNA strands would not be methylated by the replicative process itself. The presence of a *maintenance methylase* can, however, transmit the methylation to the newly formed DNA because the enzyme methylates a GpC sequence only when paired to methylated GpC in the

complementary strands (G and C are complementary). Therefore, the machinery for producing heritable changes by methylation of DNA exists at least in mammals.

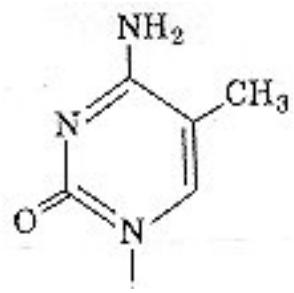


Fig. 2 5'-methylcytosine residue.

The biochemistry of methylation and de-methylation is reasonably well understood. Three methyltransferases have been found so far (Bestor et al., 1988; Okano et al., 1998). In addition, a specific demethylase has been recognized and cloned (Bhattacharya et al., 1999).

Methylation is responsible for the silencing of the genes on the inactive X chromosome (see Monk, 1986) and is thought to have a complex role in differentiation. A variety of observations have shown that, in early development, inactive genes are methylated (Cedar, 1988). Furthermore, housekeeping genes, which are always expressed, are not methylated (e.g., Bird et al., 1985). Conceivably, methylation-demethylation events could control gene expression and a general correlation exists between methylation and repression (e.g., Siegfried and Cedar, 1997). It should be recognized, however, that indirectly methylation can either increase or decrease transcription depending on whether the methylation is at positive or negative regulatory elements. Not surprisingly, several human diseases are the results of abnormal methylation (see Jones and Takai, 2001). Recent research indicate that methylated sites in the DNA serve as sites of assembly for repressor complexes with histone deacetylation activity (see Section VIC, below).

Although a general correlation exists between methylation and repression (e.g., see Siegfried and Cedar, 1997; Jones and Takai, 2001) some experiments suggest that methylation has a much more subtle role (see below). Methylation has a specific kind of control of gene expression in the case of *parental imprinting*. The expression of certain genes depends on whether the gene is inherited from the male or the female parent. The next section will examine parental imprinting.

A. Parental Imprinting

Parental imprinting has been observed in many organisms including mammals, plants, insects and yeast. Imprinting plays an important role in embryogenesis. In mice, the genes from the male parent seem to be more significant for the development of extra-embryonic tissues, whereas the maternal genes seem to play a more significant role in the development of the embryo proper.

What could possibly be the role of parental imprinting? One important consequence of parental imprinting is that parthenogenesis is not possible and consequently the genes from both mother and father are necessary for normal embryonic development (De-Groot and Hochberg, 1993), thereby preserving genetic diversity. Imprinting also maintains the diploid state in dividing cells (Tycko, 1994). At any rate, a number of imprinted genes have been recognized, as many as sixteen in mice and fourteen in humans (De Souza et al., 1997).

The involvement of methylation in imprinting has been shown by a variety of experiments. One case discussed here (Swain et al., 1987) involves transgenic mice (see Chapter 1) in which the transgenic gene, composed of a variety of DNA fragments, was found to be *autosomally* inherited (i.e., not carried by the X or Y chromosome). Demonstration of the presence of a specific mRNA indicates that the gene is being expressed (although strictly speaking this would only be the case if the mRNA is also translated). The presence of the specific mRNA corresponding to the transgenic gene was demonstrated using an RNase-protection analysis. The rationale of this technique is similar to that used in DNA-footprinting, which detects the binding of proteins to DNA (see Chapter 1). In this case the protective effect is provided by the binding to antisense RNA (labelled with ^{32}P), i.e., to a complementary strand of RNA, synthesized in vitro from the transgenic DNA. The protection from RNAase digestion provided by binding to the anti-RNA can be recognized by gel electrophoresis. By this procedure, the protected segments will be excluded because of their larger size. Expression of the transgenic DNA occurred only in the heart. Crosses of transgenic to normal mice are shown in Table 1 in the first two columns. The results are tabulated in the next two columns. TG was only transmitted through the male parent. The methylation of the DNA of the offspring invariably corresponded to the failure to transmit TG.

Table 1 Influence of Parental Origin of the Transgene on its Subsequent Expression in Hearts of Transgenic Offspring

PARENTS		Status of Transgene Expression in TG Offspring	
FEMALE	**MALE**	**EXPRESSED**	**NOT EXPRESSED**
TG	Non-TG	0	42
Non-TG	TG	20	0
TG	TG	6	9

TG, transgenic carrier.

Non-TG, nontransgenic carrier.

From, Swain et al., 1987. Reproduced by permission.

The role of methylation in genomic imprinting is well established. Mice unable to methylate DNA because of a mutation in the gene coding for methyltransferase have an altered imprinting (Li et al., 1993). However, methylation does not always correlate with repression. More recent studies indicate that imprinting can also take place through chromatin boundary elements (insulators) that block the promoter of the imprinted gene (Hark et al., 2000; Bell and Fesenfeld, 2000). A region upstream of the imprinted gene appears to be responsible and to be dependent on binding by a enhancer-blocking protein (CGTF, a zinc finger protein implicated in vertebrate boundary function). The binding is abolished by DNA methylation.

B. Methylation and Gene Expression

Parental imprinting that involves methylation of inactive genes could be regarded as a special case of differentiation. Does it have a role in gene expression accompanying differentiation in other cases? In mammals, the pattern of DNA methylation differs depending on the developmental stage. After fertilization, a wave of demethylation removes most of the methyl groups in the DNA. However, after implantation, methylation is reestablished (Monk et al., 1987; Kafri, 1991) except that the CpGs at the promoter region of housekeeping genes remain demethylated, probably by a specific demethylase (Frank et al., 1991). In later developmental stages many tissue specific genes are demethylated (e.g., Kafri et al., 1993). On the other hand, methylation is associated with long term silencing of genes (Li et al., 1993; Beard et al., 1995).

The role of methylation in differentiation could be tested by examining the methylation state of expressed and non-expressed genes. We already saw that a general correlation exists and the mechanism by which methylation is responsible for repression is now reasonably well understood (see below). However, the answer is not simple. In many cases a gene is not expressed when methylated (e.g., Benvenisty et al., 1985). However, in some cases extensively methylated genes were found to be transcribed (e.g., Vedel et al., 1983).

One kind of experiment (Yisraeli et al., 1986) provides us with additional information about the involvement of methylation in gene expression. In these experiments methylated and unmethylated α-actin gene from skeletal muscle was incorporated into cells by transfection. Methylation was carried out in vitro. When introduced into fibroblasts, which normally do not produce α-actin, the expression of the methylated gene was inhibited. In contrast, when transfected into myoblasts where the α-actin is inducible, the methylation had no effect. Examining the α-actin DNA after transfection showed that in

myoblasts the expression of the gene was accompanied by demethylation in a pattern similar to that present in myoblasts in vivo. Other experiments are completely in harmony with these findings. DNA methylation *per se* is not sufficient for repression. Injection of methylated or unmethylated DNA into *Xenopus* oocyte nuclei (Kass et al., 1997) showed that both were transcriptionally active. After a longer incubation, the methylated DNA was converted into an inactive form that ceased to be DNase I hypersensitive and became unable to bind to RNA polymerase. These results suggest that methylation acts indirectly and other factors must come into play. Taken together, they suggest that (a) DNA methylation in some cases but not in others prevents gene expression and, (b) methylation itself is under the control of other factors as also indicated by the observation that methylated DNA is transcriptionally repressed only when in a nucleosome (Buschhausen et al., 1997; Kass et al., 1997).

The importance of methylation in gene expression in mice has been clearly demonstrated. The use of DNA microarrays makes it possible examine the expression of genes representing a large part of the genome by permitting testing mRNA hybridizing to a very large number of DNA spots. The effect of the absence of methylation could be tested using this technique. This test was carried out by deleting one of the genes essential to maintain methylation, Dnmt1 (coding for a maintenance methyltransferase), in cultured mice fibroblasts (Jackson-Grusby et al., 2001) causing demethylation and a uniform p53-dependent cell death (see Chapter 8 and Section III above). Microarray analysis shows abnormal behavior of 10% of genes (mostly an increase in expression), many involved in cell-cycle-control, growth factor signal transduction and expression of retroelements.

As we saw, in vertebrates and mammals in particular, the methylation of DNA seems to play a role in gene expression. However, methylated DNA regions have not been found in *Drosophila* (e.g., Urieli-Shoval, et al., 1982), the nematode *Caenorhabditis elegans* (Simpson et al, 1986) and *Saccharomyces cerevisiae* (e.g., Proffitt et al., 1984), at least as seen with present technology. However, more recently, methylation has been found in to take place *Drosophila* but only in the early stages of embryonic development (Lyko et al., 2000). The reasons for these differences are still obscure.

Histones have also been found to be methylated. In the fungus *Neurosopora* (Tamaru et al., 2001) and plants (Jackson et al., 2002), mutations of a methyltransferase or in lysine 9 of the histone H3 prevents DNA methylation. This finding, indicates that DNA methylation depends on histone methylation.

VI. HISTONES AND CHROMATIN STRUCTURE

From studies of prokaryotes and current knowledge of transcriptional regulation, it would seem most likely that the binding of proteins to specific DNA sectors (such as promoter sites) is involved in the repression accompanying differentiation. However, considering the complexity of organization of chromatin, structural factors must also play a role.

A large part of the transcriptionally inactive chromatin is condensed and is referred to as *heterochromatin* (see John, 1999) (the non-condensed chromatin being referred to as *euchromatin*). Heterochromatin is

easily visualized with microscopic techniques. The distribution of heterochromatin differs in the various differentiated cell types (see Leitch, 2000) in agreement that the notion that inactivation of different genes must accompany differentiation.

An analyses of the distribution of active and inactive nuclear zones makes use of *fluorescence in situ hybridization* (FISH) usually in conjunction with confocal microscopy. These studies found that chromosomes and genes are located in discrete zones that have been called *chromosome territories* (e.g., Manuelidis et al. 1988; see Haaf and Schmid, 1991). In particular, DNA replication, RNA transcription and processing occur in groups scattered throughout the nucleus (Sadoni et al., 1999). Potentially active genes are located in the periphery of chromosome territories where transcription and RNA splicing is thought to take place (Zirbel et al., 1993; Verschure et al., 1999).

Classical studies of polytene and lampbrush chromosomes also implicate non-condensed segments of the chromosomes in transcription and condensed segments in repression. The involvement of chromosomal structure is not surprising, because the DNA in condensed chromosomes is compacted and unlikely to be accessible to the complex machinery required for transcription. Lampbrush chromosomes are meiotically paired chromosomes of amphibian oocytes. They form chromosome loops covered with large amounts of newly transcribed RNA. *Polytene* (multistranded) chromosomes are present in salivary glands of fly larvae. These chromosomes grow to enormous size by replicating DNA as many as a thousand times without cell divisions. Transcribed regions are non-condensed and are part of swollen puffs. An involvement of the loops and puffs in transcription is demonstrable by growing the larvae in the presence of [^{3}H] uridine, which is incorporated into RNA (Lamb and Danehoff, 1979).

The results discussed in Sections A and B show that many factors are involved in gene expression, which is probably regulated by a multiplicity of mechanisms.

A. The Histones and Other Nuclear Proteins

Besides this general arrangement into condensed and non-condensed states, how is chromatin organized? A discussion of the composition and structural arrangement of chromatin is necessary because of its role in gene expression. The DNA of multicellular eukaryotic cells is compacted about 10,000 fold from a DNA thread. Unfortunately, most of our knowledge comes from disrupting the original organization. The DNA of chromatin is associated with proteins, both the *histones* and the so-called *non-histone* proteins.

Not all eukaryotic cells have histones. The dinoflagellates package the DNA with basic proteins unrelated to histones (Vernet et al., 1990). Furthermore, in mammals, the majority of the DNA of sperms is compacted by interaction with protamines.

Electron micrographs of lysed nuclei show the chromatin to be arranged in fibers 100 nm in diameter (see Thoma et al., 1979). These can unravel to form 30 nm threads (Fig. 3, the top structure), which can be unraveled further to reveal bead-like structures, the *nucleosomes* (Kornberg and Lorch, 1999). 80% of the

DNA in the nucleus is present in nucleosomes (Noll, 1974). Current models favor the folding of the 30 nm fiber in loops anchored to the nuclear matrix or scaffold composed of the nonhistone proteins (Pienta et al., 1991), including *lamins*. The lamins line the nucleoplasmic surface of the nuclear envelope.

The nucleosomes contain octamer cores of histones, two of each kind: H2A, H2B, H3 and H4. The *core histones* are small, containing 102 to 105 amino acids arranged with a globular head and a tail at the amino-terminal end. The globular domains are probably responsible for organizing the DNA of the nucleosomes. Approximately 146 base pairs of the DNA are wound around the histone cores. H1, present in higher eukaryotes, is suspected of having a role in chromatin condensation (Clark and Kimura, 1990) and gene repression during differentiation. H1 histones are elongated molecules with a globular head. They are thought to be responsible for the folding of the chromatin into the 30 nm thread by binding with the globular head to a specific site on one side of the nucleosomes and with the tail end electrostatically to the DNA between the nucleosomes. A model showing the relationship between the extended form of the thread and the 30 nm fiber is shown in Fig. 3 (Thoma et al., 1979). The model is based on the structure of chromatin as seen in various concentrations of salt.

A histone binding protein, HIRA, has been found to be needed for the assembly of nucleosomes on non-replicating DNA but with no role during DNA replication (Ray-Gallet et al., 2002).

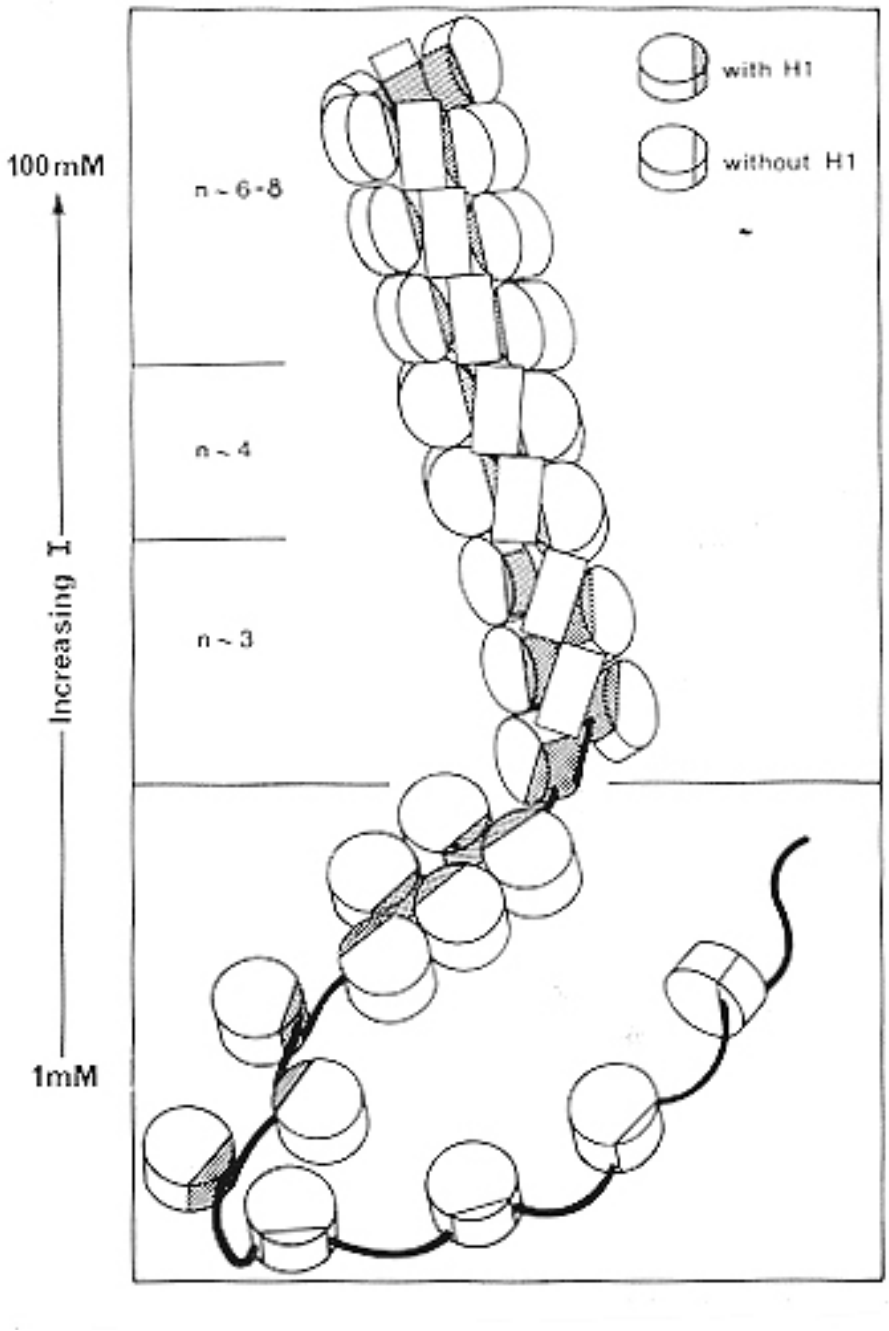


Fig. 3 Idealized drawing of structures formed by chromatin with increasing ionic strength. The bottom zig-zag of nucleosomes (bottom left) closes to form helices with increasing numbers of nucleosomes. In the absence of H1 (bottom right), no zig-zag or order was found. Reproduced from Thoma et al., (1979) *The Journal of Cell Biology* 83:413-427, by copyright ©1979 permission of The Rockefeller University Press.

A high resolution (2.8 Å) X-ray diffraction study of nucleosomes (Luger et al., 1997) shows that each core histone is arranged in a domain formed by three-helices (the histone fold) and two tails (Rhodes, 1997). The DNA is attached to H3 extensions where it enters or leave the nucleosome (Rhodes, 1997). The phosphate backbone of the DNA makes contact with the protein chains. In addition, 14 arginine side chains of the protein make contact with the minor grooves of the DNA which face the protein. This arrangement allows channels through which the histone tails pass and leaves the major grooves of the DNA accessible to other components. The tails make contact with neighboring nucleosomes. Fig. 4A

shows half of the structure of the nucleosome core. One turn of the DNA superhelix is shown (73 base pairs). Fig. 4B shows the histone octamer with the DNA removed.

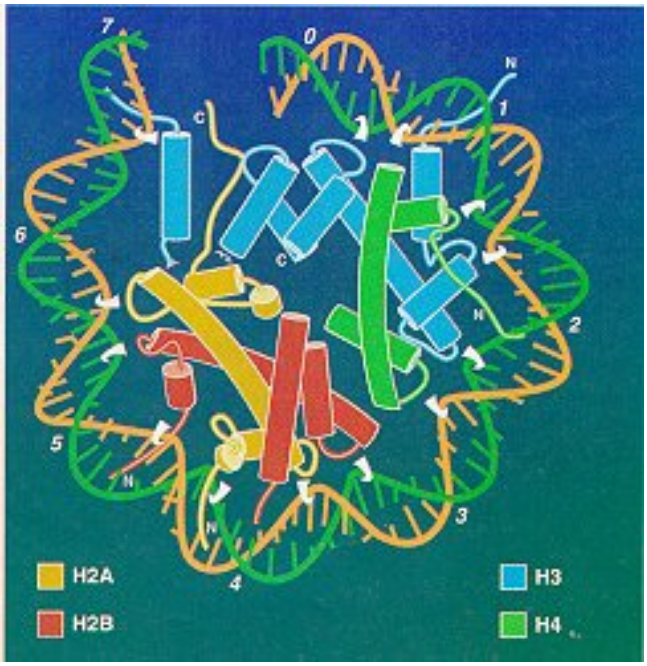
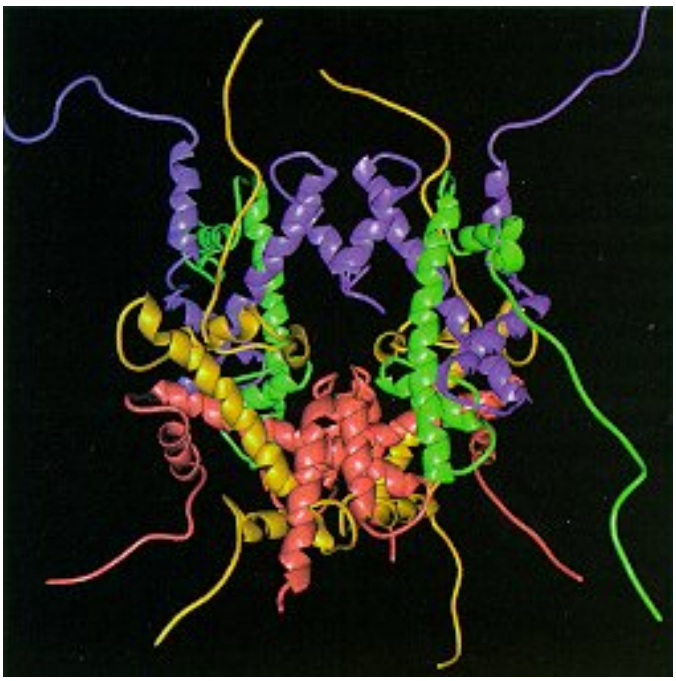


Fig. 4
A. Structure of the nucleosome as solved by Luger et al., 1997. The different histones are indicated by a different color as shown in the figure. The helical regions of the proteins are represented as cylinders. The center of the nucleosome is indicate by 0 above the DNA superhelix and terminates at site 7. Points of contact between DNA and protein are shown by white hooks.



B. The structural tails of histones. The structure of the histone octamer with the DNA removed. The view is down the superhelix axis and the different histones are color coded.

The acetylation of the histone tails has been associated with transcriptional activity (see section VIB, below). Acetyltransferases and deacetylases have been found associated with certain transcription factors (Tsukiyama and Wu, 1997). Because the tails interact with neighboring nucleosomes, the acetylation may disturb the higher order of the chromatin (van Holde et al., 1996).

The DNA in nucleosomes is protected from slight hydrolysis catalyzed by DNase I (pancreatic DNase). In contrast, the stretches of DNA between nucleosomes, the linker DNAs, are digested by the DNase. This suggests that the nucleosomal DNA is protected by its structural arrangement. Part of the linker DNA is digested even by very low concentrations of nucleases; these are the *nuclease-hypersensitive* sites.

The sensitivity of DNA to DNase has been frequently used to examine whether the DNA is complexed to protein. Resistance to digestion has been interpreted as shielding provided by the protein binding. In the case of complex structures such as chromosomes, the inaccessibility could also be from the compactness of the chromatin. When cells were exposed to DNase I, active genes were found to be preferentially digested. In contrast, inactive genes are DNase I resistant. In these experiments, DNA containing the globin gene from embryonic and adult chick red blood cells were compared. The cDNA derived from the mRNA was used to recognize the DNA of the gene by hybridization. Embryonic DNA from active genes was digested by the enzyme, whereas inactive genes were protected (Weintraub and Groudine, 1976). The hypersensitive sites, which are even more sensitive than those discussed, are thought to be necessary for transcription, perhaps representing upstream regulative elements such as promoters or enhancers.

A direct involvement of histones in gene expression involving differentiation has also been demonstrated in a variety of experiments. One set of experiments studied the genes controlling the 5S RNA system (Wolffe and Brown, 1988). 5S RNA is part of the large ribosomal subunit. This RNA is only 120 nucleotide pairs in length and, unlike other ribosomal RNA, is transcribed by *RNA polymerase III* (RNAPIII). In *Xenopus laevis* there are two kinds of multigene families that code for 5S RNA. In the oocytes, a large number of 5S RNA are synthesized that are coded by the oocyte-5S multigene family and also coded by the somatic-5S multigene family. After the embryo has developed, the oocyte-5S RNA is repressed and the somatic-5S genes remain active. The latter can be considered the end state of the differential expression of these two genes. Transcription of the 5S genes involves a promoter element (called *internal control region* or ICR), which binds the transcription factor IIIA (TFIIIA). TFIIIA, ICR and two other factors form a transcription complex. While this complex remains attached, RNAPIII transcribes the 5S RNA gene and the transcription terminates at a termination sequence. In the absence of a transcription complex, the polymerase does not recognize the gene. RNAPIII can also transcribe isolated chromatin. When the chromatin is isolated from somatic cells, the oocyte-5S RNA genes remain repressed. Transcription factors can activate the transcription of the oocyte genes, but only if H1 histone is removed. Similarly, addition of the H1 histone to chromatin previously depleted of protein, reestablished the repressed state with a stoichiometry of one H1 molecule per nucleosome. The oocyte-5S RNA genes in the repressed state are therefore thought to be associated with nucleosomes and H1 histone. These

results implicate histones, and H1 histone in particular, in repression. In addition to the interaction with nucleosomes and H1 histone, the state of compaction of the DNA almost certainly has a role. DNA that is less compacted will be more ready to exchange nucleosomes and H1 histone.

The stability of the repressed state, and the presence of stable transcription complexes in somatic 5S RNA genes even when not being transcribed, may explain the maintenance of the differentiated state in non-dividing cells where transcription factors and nucleosome structures are in competition.

What is the role of nucleosomes in controlling gene expression? In the experiments discussed above, histones were implicated in the repression of non-expressed genes, such as the 5S-oocyte genes in somatic cells. Furthermore, active genes were frequently found in DNase-sensitive sites, which are generally nucleosome free. However, transcription in vitro is not blocked by the presence of a few nucleosome octamers, and longer nucleosomal arrays only slow down transcription. In contrast, in vitro binding of a nucleosome to a promoter region blocks initiation (e.g., Knezetic and Luse, 1986).

The blocking of transcription by nucleosomes at promoter sites suggests models in which transcription complexes and regulative factors compete with histones for sites on the promoter. The binding may also be functionally irreversible. The first component to bind will either repress or activate in a permanent way. This is referred to as *pre-emptive competition*. Such competition is supported by observations in vitro. When added first, the tissue specific factors can block the formation of nucleosomes. Many other cases with similar conclusions have been described. In addition, in vivo, the presence of promoters and enhancers of active genes at hypersensitive DNA domains suggests that these domains are not occupied by nucleosomes.

A binding competition model is represented in Fig. 5 (Adams and Workman, 1993) for a case in which a transcription factor can displace a nucleosome present at a promoter site. An arrangement of nucleosomes in an extended configuration of chromatin is shown in panel A. The transcription factor displaces the H1 histone (panel B). The binding of several factors destabilizes the histone core of the nucleosome (panel C). The open DNA sector containing the promoter can now be transcribed.

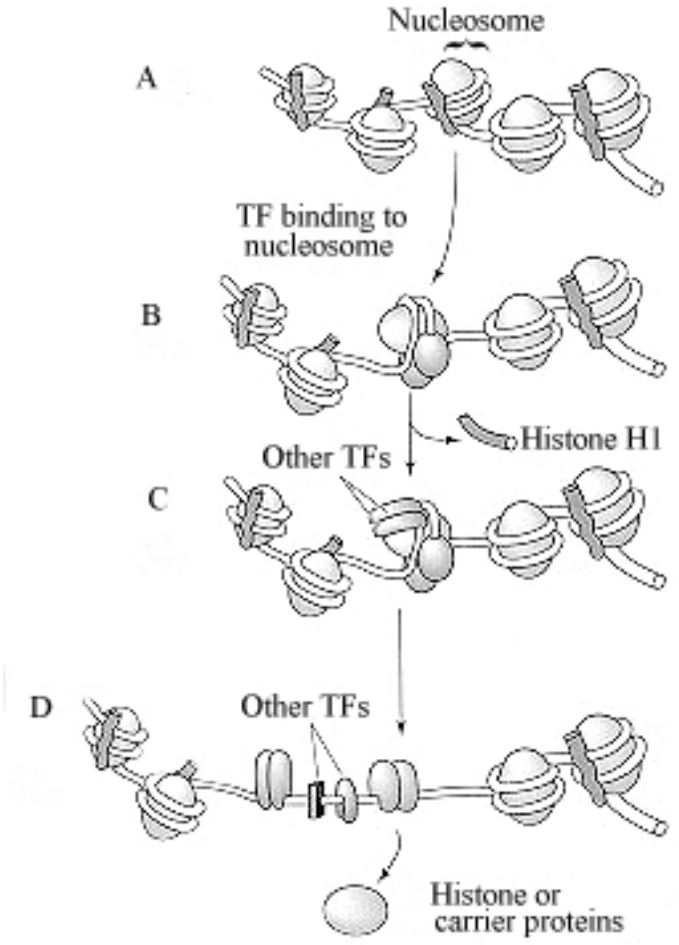


Fig. 5 Competition model where a transcription factor activates a promoter region by displacing the nucleosome (after Adams and Workman). Reproduced from Gilbert, 1994 by permission.

Although this model is attractive, other observations suggest not only a more complex pattern but probably multiple mechanisms. Promoters have been found within and between nucleosomes (Simpson, 1991) and, furthermore, nucleosomes and histone H1 are present in the majority of transcribed genes (Morse, 1992). In addition, activators and components of the transcription machinery (e.g., the preinitation complex) have been found in silent sites (Sekinger and Gross, 2001). The results suggest that the silencers function by blocking a step downstream of the preinitiation complex recruitment and not by displacing transcription factors. In addition, activation of silent promoter chromatin occurs wihout histone displacement and changes in histone acetylation, both associated with gene activation (see Section B, below)

Recent studies in yeast have shown that in vivo, mRNA is synthesized by an RNAPII holoenzyme, a megadalton-sized complex containing, in addition to the polymerase, general transcription factors and additional regulatory proteins (Carey, 1995). This complex contains SWI and SNF gene products (Wilson et al., 1996). These proteins have been shown to function as global gene regulators and are capable of remodeling chromatin (see below). Therefore, this polymerase carries its own disrupter of nucleosomal structure. These observations suggest that organization of the DNA into nucleosomes, per se, does not exclude transcription.

A role of chromatin structure in repression and activation of gene is undeniable. The unravelling of repressed chromatin is necessary for activating gene expression (e.g., Kingston et al., 1996; Fesenlfeld, 1996). This regulation involves enzyme complexes that require an expenditure of energy .

A complex (member of the ISWI family discusssed below) has been found able to remodel chromatin, and also function in chromatin assembly, the *chromatin-accessibility complex* (CHRAC). Powered by ATP hydrolyses, it produces regular arrays of nucleosomes with even spacing (Varga-Weisz et al., 1997). Included in the complex are several known proteins: the ATPase ISWI (that is also part of the *nucleosome remodeling factor*, NURF) and *topoisomerase II*. Topoisomerase II is an enzyme that produces reversible cuts in the DNA double helix in order to unravel it. The presence of proteins that can also associate with other complexes, suggests that the chromatin remodelling machinery is modular in nature.

There are a variety of ATP-utilizing remodeling factors which are able to change the histone-DNA contacts to activate (e.g. see Travers, 1999; Varga-Weisz and Becker, 1998; Vignali et al., 2000) or repress (see Tyler and Kadonaga, 1999) specific genes.The chromatin remodeling complexes contain an ATPase subunit and subunits responsible for regulation and specificity (see Tyler and Kadonaga, 1999). At this time three distinct families of ATPase subunits are recognized: the SWI/SNF2 , the Mi-2/CHD and the ISWI family. However, there are probably others since many proteins in sequence data bases resemble these proteins.

The energy generated by the hydrolysis of ATP is used to change the position of the histone octamer in relation to the DNA (e.g., Lorch et al., 1999) so that histone-DNA bonds have to be disrupted and remade elsewhere. For animation models see: http://www.dundee.ac.uk/~taowenhu/Toh_show.htm. A possible mechanism is suggested by studies of the yeast complex RSC (related to the SWI/SNF complex). RSC as well as its isolated ATPase subunit Sth1 are DNA translocases. Each RSC molecule has a single Sth1 subunit. They use ATP hydrolysis to release a single DNA strand suggesting that Sth1 tracks along one strand of a DNA duplex (Saha et al., 2002). The remodelers are thought to pump waves of DNA helix with a twist. Supposedly, they move the histone octamer on the DNA by releasing it to reform a point of contact or interact with protein factors such as the *TATA binding protein* (TBP), or transcriptional activators. Nucleosomes containing nicks in precise locations were used to uncouple the alleged twist and translocation and they were found to be remodeled less efficiently than intact nucleosomes.

NURF has been found to catalyze the redistribution of nucleosomes in either direction. Movements occur on the same DNA thread without transfer to competing DNA. The progression is in increments of several base pairs. All core components migrate without loss during this process, suggesting that the nucleosome remain intact through the transfer (Hamiche et al., 1999). CHRAC and ISWI were also shown to induce nucleosome sliding of intact histone octamers (Längst et al., 1999).

The SWI/SNF complex has been shown to be important for transcriptional activation as well as repression. Inactivation of SWI/SNF remodeling complex affected 6% of the genes a shown using the gene expression DNA-chip procedure (see Chapter 1). The results show that SWI/SNF represses most of

the genes (Hostege et al., 1998) either directly or indirectly (203 gene products were increased by inactivating the gene, whereas 126 were decreased) . In addition, there are other indications that SWI/SNF and the related RSC complex are involved in repression. RSC is essential for repression of the *CHA1* gene (Moreira and Holmberg, 1999) and BRG1 and hBRM, human equivalents to SWI/SNF2, bind to the RB gene product and inhibit the activity of E2F (see Muchardt and Yaniv, 1999). BRG1 also represses the human *c-fos*gene (Murphy et al., 1999). A possible tie-up between repression of transcription and histone deacetylation is provided by the NuRD/Mi-2 complex (Knoepfler and Eisenman, 1999) which has both chromatin remodeling and histone deacetylation activities. Histone deacetylation often correlates with transcriptional repression (see Section VIB) . Interestingly, NuRD interacts with CpG-methylated DNA (Bird and Wolffe, 1999). DNA methylation is also associated with gene inactivation (see Section VB)

The high mobility group (HMG) proteins, a family of nuclear proteins, also induce changes in the architecture of chromatin and increase the transcription and replication from chromatin templates (e.g., Bustin, 1999). The HMG proteins are considered to belong to three major subfamilies, HMGA, HMGB and HMGN (see Bustin 2001a). Each one of these subfamilies has a distinct motif which induces a different change in their target and affect different processes. The HMGN proteins move throughout the nucleus at a rate suggesting that they are part of a multiprotein complex and that they associate to chromatin transiently (Phair and Misteli, 2000).

The HMGN proteins of approximately 10 kDa, specifically bind to the 147-base pair nucleosome core particle. They contain primarily three functional motifs: one is a bipartite NLS motif that allows transfer from the cytoplasm to the nucleus, another binds to the nucleosomes and a third corresponds to a chromatin unfolding domain (Trieschmann et al., 1995; Ding et al., 1997) . The interaction of the nucleosomal-binding domain with its target is regulated by post transcriptional modification of the HMGN protein such as acetylation (e.g., Bergel et al., 2000) and phosphorylation (Thomson et al., 1999). The interaction facilitates the accessibility of the nucleosomal DNA and therefore transcription and replication of the DNA (see Bustin, 2001b). The effect is on the chromatin and not on deproteinized DNA. In cells, HMGN colocalizes with nascent transcripts (Hock et al., 1998) and injection of antibodies against HMGNs blocks RNA synthesis (Einck and Bustin, 1983).

Covalent modifications of histone play a role in these structural rearrangements as discussed in the next section.

B. Posttranslational Modification of Histones

Posttranslational modification of histones has a role in gene expression (e.g., see Wolffe and Hayes, 1999; Strahl and Allis, 2000). Histone modification may alter chromatin structure and the accessibility of specific genes. The histone modification is thought to have a role in signaling by allowing the histones to act as specific receptors to mediate the recruitment of proteins or protein complexes that act on downstream function (see Strahl and Allis, 2000). The activating machinery of transcription has been

linked to coactivator-mediated acetylation (e.g. Spencer et al., 1997) and methylation (e.g., Rea et al., 2000) of histones. Other modifications include phosphorylation (Sassone-Corsi et al., 1999; Thomson et al., 1999) and ubiquitination (Robzyk et al., 2000; Pham and Sauer, 2000). The molecular dynamics underlying these modifications and their significance in the regulation of gene expression are discussed in more detail in Chapter 3.

Acetylation of core histones (Kingston and Narlikar, 1999) occurs in all animals and plants that have been studied (Csordas, 1990). The acetylation occurs at the lysines of the amino-terminal tails that are exposed to the outside of the nucleosomes. Hyperacetylation of the tails leads to changes in nucleosome structure (Oliva et al., 1990, Garcia-Ramirez et al., 1995), reduces the association of histone H1 with chromatin (Reeves et al., 1985), and renders nucleosomal DNA more accessible to transcription (Lee et al., 1993). Furthermore, there is a general correlation between the degree of histone acetylation and transcriptional activity of specific domains (Jeppesen and Turner, 1993; Khochbin and Wolffe, 1993; Kingston and Narlikar, 1999). Hyperacetylated histones occur precisely at active transcriptional domains (Hebbes et al., 1994), just as hypoacetylated domains are present at repressed domains (Braunstein et al., 1993). Deacetylases have been shown to produce transcriptional repression of specific genes. Acetylation-deacetylation may, therefore, be part of the mechanisms of transcriptional regulation (see Imhof and Wolffe, 1998; Struhl, 1998). In addition to their effect on the histones, acetylation of transcription factors favors their activation (e.g., Boyes et al., 1998; Hung et al., 1999).

Exactly how acetylation disrupts the structure of chromatin is not clear, although by all indications several mechanisms are probably responsible. The neutralization of the positive charges of the histone tails may weaken the interaction between histone and DNA. The hyperacetylated tails might serve as docking sites for transcriptional activators (see Lee et al., 1993; Vattese-Dadey, 1996) or chromatin remodeling complexes (Georgel et al., 1997). Histone acetyltransferases and transcription regulators have been found to contain bromodomains (see Winston and Allis, 1999) which bind to acetyl-lysines. In addition, hypoacetylated histone tails mediate contacts between adjacent nucleosome producing a higher order (Luger et al., 1997). Therefore, acetylation is likely to disrupt this interaction.

Another modification of histones consists in the conjugation of ubiquitin with core histone H2A, H2B and H3 (e.g., see Wolffe and Hayes, 1999). 10% of the total ubiquitin in HeLa cells has ben estimated to be conjugated to histones (Carlson and Rechsteiner, 1987). The ubiquinated histones are stable in vivo (e.g., Wu et al., 1981) and the incorporation into nucleosome is likely to alter nucleosomal structure (e.g., Vassilev et al., 1995; Baarends et al., 1999). Furthermore, ubiquitination has been correlated with increased transcriptional activity (e.g., Davie and Murphy, 1990; Davie et al., 1991).

As discussed above, DNA methylation requires the methylation of histone H3.

C. Role of DNA Methylation in Acetylation-Deacetylation of Histones

We saw in Section VB that the methylation of DNA appears to have a role in gene repression and that this role is likely to be indirect. Furthermore, DNA methylation represses transcription only when present in a nucleosome (Buschhausen et al., 1997; Kass et al., 1997). Generally methylated chromatin templates are underacetylated. Furthermore, methylated silenced transgenes can be reactivated by inhibitors of HDAC or of DNA methylation (Pikaart et al., 1998; Eden et al., 1998). These observations strongly suggest that the methylated sites are involved in repression by facilitating the acetylation of the corresponding histones perhaps by serving as docking sites for HDACs. Present evidence favors this view: methylated DNA of chromatin binds the transcriptional repressor MeCP2 complexed to Sin3 and HDAC. MeCP1 and MeCP2 are methyl-CpG-binding proteins that interact specifically with methylated DNA and mediate transcriptional repression. The repression conferred by MeCP2 and methylated DNA can be relieved by inhibiting HDAC thereby facilitating the remodelling of chromatin and transcriptional activation (Jones et al., 1998; Nan et al. 1998).

D. Chromatin Domains

In eukaryotes, the genome is separated into distinct regions which permit an independent regulation within each region (see Bell et al., 2001). In agreement with this notion, the use of RNA interferences (RNA_i) techniques (see Chapter 1) has allowed massive screening of the genome of the nematode *Caenorhabditis elegans* whose sequence is known (Kamath et al., 2003). The mutant phenotypes for 1,722 genes were identified. Genes of similar functions were found to be clustered in distinct very large regions of individual chromosomes and to share similar transcription profiles.

Boundaries have been found in all organisms tested (see Gerasimova and Corces, 2001; West et al., 2002). DNA elements known as *insulators* are thought to mark the borders of chromatin domains to block *enhancer* or *silencer* elements (see Chapter3). Enhancers are regulatory DNA sequences which enhance the transcription genes. Silencers are DNA sequences that block the expression of genes. In fission yeast, these regions have been found to be defined by site-specific histone H3 (see above) methylation which determine euchromatic and the silent heterochromatic domains within a 47-kilobase region of the mating-type locus (Noma et al., 2001). H3 methylated at Lys3 (and Swi6, a chromodomain protein) is localized in the 20 kb heterochromatic region. However, H3 methylated at Lys4 is localized in the surrounding euchromatic region. Two inverted repeats flanking the silent section serve as insulators that mark the borders between heterochromatin and euchromatin. Deletion of these insulator elements allow the methylation of H3 Lys9 methylation and Swi6 to the neighboring regions and preventionof the H3 Lys4 methylation of the heterochromatic region.

Boundaries and insulators are suspected to function as matrix and scaffold attachment regions located at the bases of chromatin loops attached to the nuclear envelope (Hart and Laemmli, 1998). In fact, perinuclear localization was found to produce transcriptionally silent chromatin (Andrulis et al., 1998). Surprisingly, chromatin boundaries have been found be associated with transport proteins (Cse1p, Mex67p, and Los1p)(see Chapter 5) of the nucleopore complex (NPC) (Ishii et al., 2002). The block of heterochromatin spreading depends on tethering to the Nup2p receptor of the nuclear pore complex.

Potential docking sites are also provided by components of the nuclear lamina proteins (see Vlcek et al., 2001).

E. The Chromodomain Proteins

The *chromo* domain is a protein motif first found in the *Drosophila heterochromatin associated protein 1* (HP1) and *Polycomb* (PC) protein. Both proteins have a role in silencing the heterochromatin regions. A variety of proteins which contain the chromo domain (Koonin et al., 1995) have been found. Other domains often present in nuclear proteins with a role in chromatin organization are also known. Although the chromo domain has been found most frequently in repressed chromatin, it also occurs in activating proteins. In at least some cases the interaction of a chromo-domain protein with chromatin appears to be mediated by RNA (Akhtar et al., 2000).

The PC protein is part of a large complex of similar proteins (of the Polycomb group, PCG). Proteins of the PCG are bound to about 100 domains of the polytene chromosomes of *Drosophila* (see Alkema et al. 1997; Strutt and Paro, 1997). The PCG proteins interact with polycomb group response elements (PREs) in target genes. PREs have been shown to permit the inheritance of silent transcriptional states. Different PCG protein complexes function at different sites (Busturia and Bienz, 1993; Chan et al., 1994). Mutations in the PC chromo domain prevent this protein from binding to its target sites. In addition, the whole PCG-complex disassembles.

The silent state of heterochromatin is in part brought about by the interaction of a histone methylase (SUV39H1) which methylates the lysine residue 9 in the tail region of H3 (e.g., Lachner et al., 2001).. The *heterochromatin protein 1* (HP1) binds to the methylated site. The repetition of this process produces large silenced sectors (see also Chapter 3)

Chromo-domain proteins are just beginning to be understood and hopefully new research will clarify their role in more detail.

VII. THE TELOMERES

The ends of eukaryotic chromosomes, the *telomeres*, meaning end parts, contain both proteins and DNA and have special properties. Telomeres function to protect chromosomes from incomplete replication (e.g., Kirk et al., 1997), end to end fusion (van Steensel et al., 1998) and loss (Sandell and Zakian, 1993). The DNA of telomeres is bound to proteins that form a protective cap (see Lee et al., 1993; Grunstein, 1997b; Baumann and Cech, 2001). Large *t loops* have been identified at the ends of chromosomes using EM and shadow casting, after isolation of the DNA (Griffith et al., 1999). These loops are thought to contain telomeric DNA and TRF2, a telomeric protein required for the protection of the mammalian chromosome. Cells unable to duplicate the DNA of telomeres experience progressive telomere shortening until the telomeric DNA arrives at a critical length after which the chromosomes are unstable and the cells fail to divide.

Telomeres are important in many other ways as well. The presence of telomeres alters transcription, replication, and the chromatin structure of adjacent domains (Zakian, 1995; Shore, 1995). In somatic cells, telomere tract shortening is associated with senescence and is thought to block oncogenesis (Harley, 1995). In normal mammalian somatic cells, telomeres shorten with successive cell divisions (Cooke and Smith, 1986; Harley et al., 1990). Telomerase, responsible for maintaining the length of the DNA of telomeres (see below), undetectable in somatic cells, makes an appearance in many tumors, suggesting that telomere elongation may be part of carcinogenesis (de Lange, 1994). The size of the telomeres in either tumor or germ cells has a constant average value, not an inordinately long size.

Telomeres are needed for the complete replication of DNA. This is because a replicating strand is initiated by the synthesis of a short RNA segment, or primer, using the existing DNA strand as a template. The DNA polymerase can then attach nucleotides at the 3' end of the primer. When the synthesis of the strand is completed, the RNA primer is removed. This removal would leave the ends of the DNA incomplete and the DNA would be shortened with each replication. The telomeres prevent this difficulty.

In most eukaryotes the telomeres correspond to several repeat sequences, in humans repeats of TTAGGG (Henderson, 1995). The telomeres are added to the end of the chromosomes by a process unrelated to semiconservative replication. A ribonucleoprotein acting like reverse transcriptase, *telomerase*, is responsible for their addition. The RNA component of telomerase contains a sequence that is complementary to the sequence being synthesized in the telomere (Greider and Blackburn, 1989). When the DNA replicates and the primer at the 5' end is removed, this is of no significance because the missing region (part of the telomere) has no genetic information and can be reformed by telomerase. These observations suggest that telomerase has a role in continued cell division and tumorogenesis: its lack leading to senescence, its presence, together with other factors, leading to what is referred to as *immortalization* (see Sedivy et al., 1998).

The presence of telomerase alone does not appear to be sufficient for tumorigenesis, in view of the observation of Blasco et al. (1997) that mice lacking telomerase activity (knockout mice) are still subject to tumorigenesis. In fact, these studies also revealed that telomerase is not needed for maintaining telomeres in somatic cells that multiply continuosly (e.g., skin or gut), or in gametogenesis. These experiments are still too recent to be completely understood (for a discussion see Lansdorp, 1997).

Telomerase is held in nucleolar sites and during the cell cycle is released at the time expected for telomere replication .The localization of telomerase has been followed using chimeras of green fluorescent protein (GFP) and telomerase reverse transcriptase, the catalytic subunit of the RNP telomerase complex and in addition, confocal microscopy (Wong et al., 2002). In tumor and transformed cells, telomerase dissociates from nucleoli at all stages of the cell cycle. Transfection of the simian virus 40 genome into a primary cell line also releases the telomerase from the nucleoli. In contrast, ionizing radiation induces the reassociation of telomerase with nucleoli in both primary and transformed cells, possibly to prevent inappropriate repairs in DNA breaks.

Is telomere shortening involved in senescence? This question is also discussed in Section IV. Although other factors play a role, the answer to this question is probably yes (e.g., see Lundblad and Blackbrun, 1989). The gene for telomerase has been cloned. The expression of this gene by transfection (see Chapter 1) and the accompanying introduction of telomerase activity, was found sufficient for telomere elongation and for allowing cell divisions to proceed at least in retinal pigmented cells and fibroblasts (Bodnar et al., 1998; Vaziri and Benchimol, 1998). In addition, the frequency of senescence in primary cultures of human cells was compared between cells with telomerase and those without. The cells with the longer telomeres did not undergo senescence. However, in at least some cell types, such as keratinocytes or mammary epithelial cells, "immortalization" requires the inactivation of Rb and $p16^{INK4a}$, that normally block cell division in the G1 phase (Kiyono et al., 1998) (see Chapter 8) or the presence of two oncogenes (simian virus 40 large-T oncogene and oncogenic allele of H-*ras*) (Hahn et al., 1999).

A new perspective has been introduced by more recent observations. Overexpression of *telomere protection factor* (TRF2), a protein which binds to the telomere ends has been found to delay senescence in human primary cultures (Karlseder, et al., 2002). TRF2 overexpression increased the rate of telomere shortening in the cells. In addition, TRF2 protected shortened telomeres from fusion and repressed chromosome-end fusions, possibly explaining the effect of TRF2 in delaying senescence. However, the effect could also be ascribed in part to a protective effect which would delay further shortening.

How is telomerase regulated to produce the ends of appropriate length? A simple negative-feedback mechanism dependent on a "telomere size sensor" could account for the constant size of the tract in tumor or germ cells. Evidence for such a mechanism has been presented (Marcand et al., 1997). A protein of *Saccharomyces cerevisiae* Rap1p has been shown to block telomere elongation (Kyrion et al., 1992; see Shore, 1997). Supposedly, the number of Rap1 proteins bound to the telomere is proportional to telomere length (Marcand et al., 1997). Rap1 binds to specific sites in the telomere's DNA presumably blocking the action of the telomerase. A similar protein (hRap1) has been identified in human cells (Li et al., 2000b). hRap1 is located at telomeres, and affects telomere length. However, it differs from the yeast variety in that it does not bind DNA directly but the binding is bridged by TRF2. TRF1 and TRF2 are proteins that bind to the TTAGGG repeats found in human chromosome ends (Broccoli et al., 1997). The regulation of the telomere may result from higher order structure by blocking the action of telomerase (see Griffith et al., 1999), possibly the *t loops* mentioned above.

A yeast two-hybrid test (see Chapter 1) identified a protein interacting with human TRF1: *TRF1-interacting, ankyrin-related ADP-ribose polymerase* or *tankyrase* (Smith et al., 1998) Tankyrase contains 24 ankyrin repeats (characteristic of many protein-binding proteins, see Sedgwick and Smerdon, 1999) responsible for the binding to TRF1. In the presence of tankyrase, TRF1 dissociates from DNA presumably allowing telomerase to proceed.

The length of the telomeres, however, does not seem to be the only factor in determining telomere function. The incorporation of certain mutant DNA sequences in telomeres of the budding yeast

Kluyveromyces lactis produces telomere defects, misshapen cells, increased DNA content and defects in cell division (Smith and Blackburn, 1999). The cells are stabilized again when a few wild type terminal repeats are added to the DNA ends of the telomeres. The malfunctions appear to be the result of the telomere uncapping and not excessive telomere length.

VIII. MOLECULAR INTERACTIONS IN DIFFERENTIATION

As we saw in our general discussion, differentiation can be considered the selection of a specific pathway from several possibilities represented in the cell's genome (Hadorn, 1965). Some of the aspects of this process have been studied in detail with genetic approaches in *Drosophila*. The commitment to a pathway is controlled by *selector* genes, many of which are *homeotic* genes that contain a *homeobox* of 170 nucleotide pair sequences. The proteins coded by the homeobox can bind to specific DNA sequences and act on enhancers or silencers, thereby activating or inhibiting the transcription of specific genes.

Repression takes place in two stages. Initially, there is a reversible repression of short duration, followed by an inheritable long-range repression.

In *Drosophila*, *Ubx* is a gene that specifies the development of thoracic and abdominal cells (Lewis, 1978). Two other genes have been found to control segmentation: *Hunchback (Hb)* and *fushi tarazu (ftz)*. Appropriate development will take place where the *Ubx* is activated in the *Ubx*-developmental domains. However, it must be repressed on the cells that are destined not to express *Ubx*. The control is exerted by *Hb* and *ftz.* The protein coded by *ftz* acts as an activator of *Ubx*, whereas that coded by *Hb* acts as a repressor (Müller and Bienz, 1992). In effect the two are in competition, with *Ubx* being expressed in some cells and remaining non-expressed in others. The interaction between repression and activation seems to be universal in the control of segmentation and homeotic genes in early embryos (Stanojevic et al., 1991; Hoch et al., 1992).

The interaction between the proteins coded by *ftz* and *Hb* results in repression in the appropriate set of cells. However, long-range inheritable repression of *Ubx* requires the function of an additional gene, *Polycomb*, *Pc* (Müller and Bienz, 1991). Repression is thought to require complexes of three or more proteins and, in this case, Pc protein is thought to be a component of the repressor complex (Müller and Bienz, 1991; Zhang and Bienz, 1992). The presence of *Hb*-protein may be initially required for the expression of *Pc*. To be effective the repressor complex must be stable and capable of reforming on DNA after each round of duplication.

Pc protein is present in a multimeric complex (Franke, et al., 1992) and is not a histone (Paro, 1990). As might be expected from its action as a repressor, Pc protein is present in all tissues of the embryo except where *Ubx* is expressed. The action of Pc must be indirect, because it does not bind to DNA but to chromatin. This is not surprising, because a complex of several proteins is responsible for repression, as already mentioned.

The interaction between the genes *bicoid* and *caudal* in *Drosophila* is important in its development because it determines the pattern of segmentation. It is also particularly interesting because the interaction is posttranscriptional. The bicoid protein (bcd), which contains a homeodomain, distributes with an anterior-to-posterior gradient in *Drosophila* embryos. bcd binds DNA and activates target genes at different threshold concentrations. Caudal (cd) protein (which also contains a homeodomain) has a gradient opposite from the bicoid gradient. The bcd protein homeodomain binds a caudal response element in the cd-mRNA and represses its translation, producing an opposing gradient in cd (Dubnau and Struhl, 1996 ; Rivera-Pomar et al., 1996).

Activation and the maintenance of selector gene expression are likely to depend on several mechanisms, each responding to separate signals. Cell-to-cell interactions, perhaps in the form of secretion by adjacent cells, undoubtedly play a role. Cell-to-cell interactions also permit coordinate responses of groups of cells so that many cells can differentiate simultaneously. In fact, groups of cells can differentiate while dissociated cells do not (Gurdon, 1988). Sustained activation is thought to require positive *autoregulation* (Garcia-Bellide, 1975; Heemskerk, et al., 1991). Once cells are differentiated, reversible activation-deactivation processes are still needed for the actual transcriptional regulation which may require physiological signals. These experiments have allowed us to begin examining differentation in terms of the interaction of specific proteins coded by certain genes.

IX. STEM CELLS AND PLASTICITY

One of the topics that has become prominent in recent years is that of *stem cells*. Stem cells are cells that remain multipotent in embryonic or in adult tissues. Embryonic stem cells (ES) can differentiate into a multitude of cell types (Brook and Gardner, 1997). They could therefore be a source of differentiated cells that could be used to replace diseased or damaged tissues. In addition, the use of ES cells has permitted producing specific mice mutations where DNA is introduced in the ES cells first and then the cells are delivered into mice (see Joyner, 1991). Stem cells have also been shown to be present in the adult hematopoietic system of mice where one cell is capable of reconstituting the whole blood system (Osawa et al., 1996). However, it has become increasingly apparent not only that other tissues contain stem cells, but also that stem cells may not be restricted in differentiation to their tissue of origin (e.g., Jackson et al., 1999; Gussoni et al., 1999). This topic is still controversial and has been recently reviewed (see Verfaille, 2002). Mouse stem cells from the adult central nervous system can differentiate into cells of other organs (Bjornson et al., 1999; Clarke, 2000; Rietze et al., 2001). Cells from adult bone marrow have been shown to be capable of differentiating into cells with many of characteristics of neurons (Woodbury et al., 2000; Brazelton et al., 2000; Mezey et al., 2000). Cells from the dermis of mice and humans (Toma et al., 2001), when cultured in the presence of growth factors, differentiated into neurons, glia, smooth muscle and adipocytes. Apart from opening new avenues for the study of differentiation, the significance of the possible applications of this knowledge is staggering (see McKay, 2000). These could include cell-replacement therapy, applicable for example to brain diseases (see Snyder et al., 1995; Cameron and McKay, 1999) or liver damage (Alison et al., 2000). Most recently, bone marrow cells delivered to the site of myocardial damage was shown to generate new myocardium in mice (Orlic et al., 2001).

ES cells have unusual proliferative properties (see Smith, 2001). They proliferate indefinitely (Suda et al., 1987), do not require the presence of serum, and are not subject to contact inhibition or anchorage dependence. They resemble malignant cells in these respects. In fact, if injected into adult mice, under certain conditions they produce teratocarcinomas.

ES cells can be maintained in the undifferentiated states in the presence of the *leukemia inhibitory factor* (LIF) (e.g., Williams et al., 1988) or related cytokines. They all act through the same receptor, gp130 (Yoshida et al., 1994). The cytokine binding to gp130 results in the recruitment and activation of the transcription factor STAT3 mediated by the JAK kinase (see Chapter 7) (see Burdon et al. 1999). ES cell division is not regulated by the RB system (see Chapter 8) and the G1/S transition appears to be activated by cyclin E/CDK2 (see Chapter 8) (see Burdon et al., 2002). .

A topic closely related to the findings just described is that of plasticity and reprogramming accompanying adult urodele amphibian regeneration. These animals (e.g., the newt) can regenerate limbs and other structures (see Tsonis, 1996). During regeneration, cartilage, connective tissues and muscle cells lose their differentiated characteristics and become blastema cells forming a mesenchymal growth zone. Proliferation, differentiation and morphogenesis of these cells reform the missing limb (see Brockes and Kumar, 2002). The possibility that this phenomenon may be induced in mammals is of great interest even if current examples are limited (e.g., regeneration of myelinated peripheral nerves).

Appendix
For a basic review of transcription and translation click here

SUGGESTED READING

Genomic potential in differentiation

Di Bernardino, M.A. (1987) Genomic potential of differentiated cells analyzed by nuclear transplantation *Am. Zool.* 27:623-644.

Chromatin structure and gene expression

El-Osta, A. and Wolffe, A.P. (2000) DNA methylation and histone deacetylation in the control of gene expression: basic biochemistry to human development and disease, *Gene Expr.* 9:63-75. (Medline)

Kornberg, R.D. (1999) Eukaryotic transcriptional control, *Trends Cell Biol.*9:M46-49. (Medline)

Patterton, D. and Wolffe, A.P. (1996) Developmental roles for chromatin and chromosomal structure, *Dev. Biol.* 173:2-13. (Medline)

Varga-Weisz, P.D. and Becker, P.B. (1998) Chromatin-remodeling factors: machines that regulate? *Curr. Opin. Cell Biol.* 10:346-353. (Medline)

Strahl, B.D. and Allis, C.D. (2000) The language of covalent histone modifications, *Nature* 403:41-45. (Medline)

West, A.G., Gaszner, M. and Felsenfeld, G. (2002) Insulators: many functions, many mechanisms, *Genes Dev.* 16:271-288. (MedLine)

Wolffe, A. P. (1995) *Chromatin, Structure and Function*, Second Ed., Academic Press, New York, pp.312.

Telomeres

Sedivy, J.M. (1998) Can ends justify the means?: telomeres and the mechanisms of replicative senescence and immortalization in mammalian cells, *Proc. Natl. Acad. Sci. USA* 95:9078-9081. (Medline)

Shay, J.W. and Wright, W.E. (2001) When do telomeres matter? *Science* 291:839-841. (MedLine)

Molecular aspects

Davie, J.R. and Chadee, D.N. (1998) Regulation and regulatory parameters of histone modifications, *J. Cell Biochem.* Suppl. 30-31:203-213. (Medline)

El-Osta, A. and Wolffe, A.P. (2000) DNA methylation and histone deacetylation in the control of gene expression: basic biochemistry to human development and disease, *Gene Expr.* 9:63-75. (Medline)

Imhof, A. and Wolffe, A.P. (1998) Transcription: gene control by targeted histone acetylation, *Curr. Biol.* 8:R422-424. (Medline)

Lin, C.Q. and Bissell, M.J. (1993) Multi-faceted regulation of cell differentiation by extracellular matrix, *FASEB J.* 7:737-743. (Medline)

Morse, R.H. (1992) Transcribed chromatin, *Trends in Biochem. Sci.* 17:23-26. (Medline)

Siegfried, Z. and Cedar, H. (1997) DNA methylation: a molecular lock, *Curr. Biol.* 7:R305-R307. (Medline)

Wade, P.A. and Wolffe, A.P. (1997) Chromatin: histone acetyltransferases in control, *Current Biol.* 82-84. (Medline)

Aging

Faragher, R.G. and Kipling, D. (1998) How might replicative senescence contribute to human ageing? *BioEssays* 20:985-991. (Medline)

Programmed death

Alnemri, E.S. (1999) Hidden powers of mitochondria, *Nature Cell Biol.* 1:E40-E42. (Medline)

Desagher, S. and Martinou, J.-C. (2000) Mitochondria as the central control point of apoptosis, *Trends Cell Biol.* 10:369-377. (Medline)

Gumienny, T.L., Lambie, E., Hartwieg, E., Horvitz, H.R. and Hengartner, M.O. (1999) Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline, *Development* 126:1011-1022. (Medline)

Hengartner, M.O. (2000) The biochemistry of apoptosis, *Nature* 407:770-776. (Medline)

Krammer, P.H. (2000) CDC95's deadly mission in the immune system, *Nature* 407:789-795. (Medline)

Raff, M. (1998) Cell suicide for beginners, *Nature* 396:119-122. (Medline)

Reed, J.C., Jurgensmeier, J.M. and Matsuyama, S. (1998) Bcl-2 family proteins and mitochondria, *Biochim Biophys Acta* 1366:127-137 (Medline)

Vaux, D.L. and Korsmeyer, S.J. (1999) Cell death in development, *Cell* 96:245-254. (Medline)

Stem Cells

McKay, R. (2000) Stem cells--hype and hope,*Nature* 406:361-364. (Medline)

WEB RESOURCES

Hendzel, M. (2001) The Cell Nucleus: Free teaching and study materials. Contemporary review materials. Movies of dynamics in the cell nucleus. Listing of resources on the Web. http://www.cellnucleus.org

Lovell-Badge, R. 2001, Stem cell therapy and research, Mill Hill Essays

Kirkwood, T. 2001 The genes that control ageing, Mill Hill Essays

REFERENCES

Search the textbook

APPENDIX

Review notes

Transcription

The polymerase binds to the transcription complex at the promoter site. Separation of the DNA strands occurs and the initiation process begins. This is then followed by transcription. As shown in Fig. R1, transcription occurs in the 5'→3' direction, antiparallel to the 3'→5' strand of the template DNA.

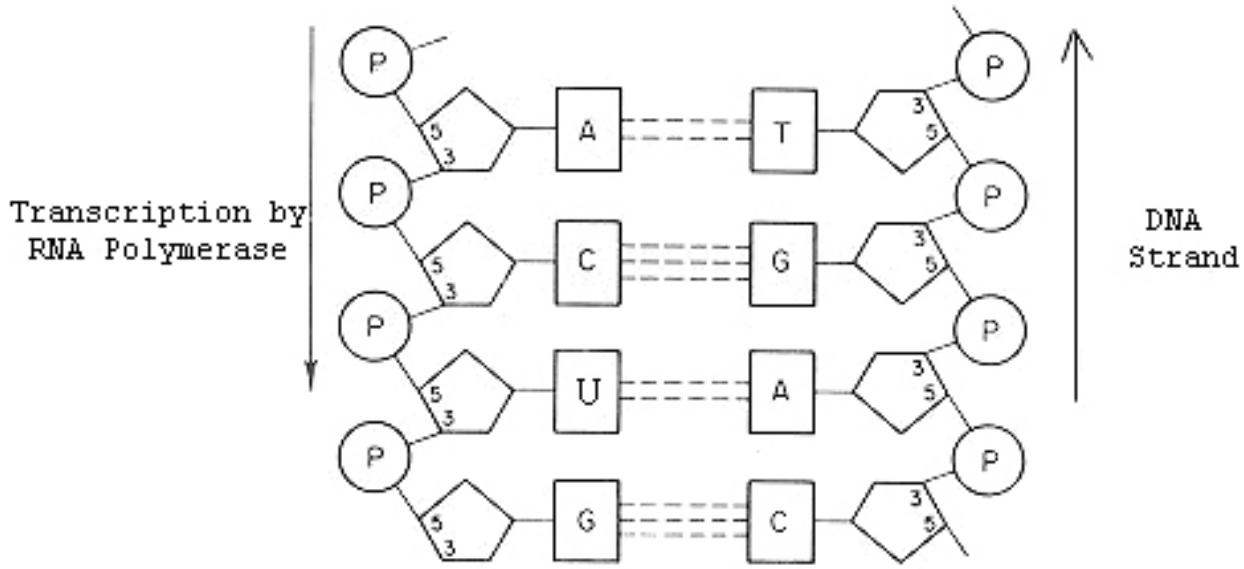
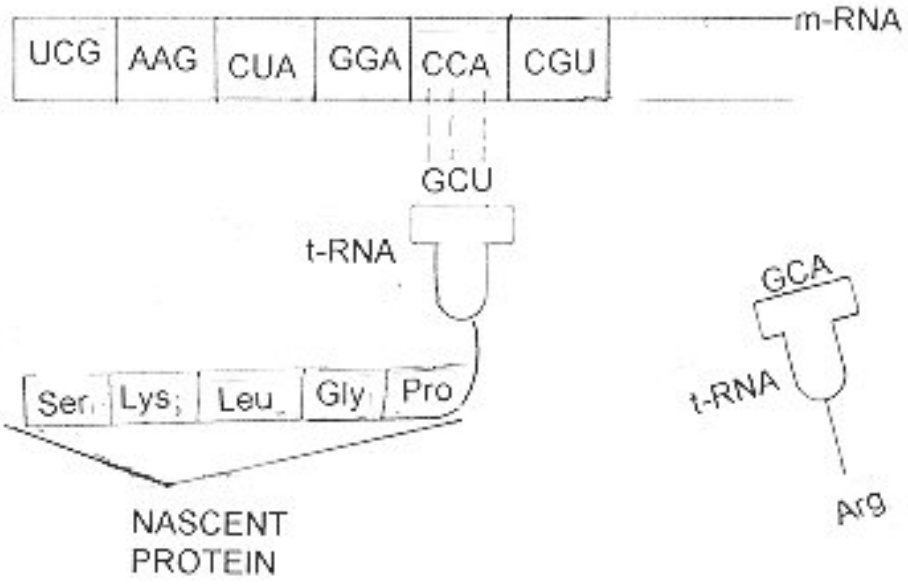


Fig. R1 Antiparallel transcription of RNA on a DNA strand. The broken lines indicate hydrogen-bonds.

Translation

The mRNA is read from the 5' end to the 3' end, one base triplet at a time. The appropriate amino acid is attached to its tRNA, which contains the triplet complementary to that in the mRNA. The amino acid is attached to the peptide so that the polypeptide grows one amino acid at a time. At the termination codon, the ribosomes and the finished polypeptide are released. A diagrammatic representation is shown in Fig. R2A. As shown in part B of the figure, the process takes place at ribosome sites. Several ribosomes are attached to each mRNA and they advance a triplet at a time.

A.


m-RNA
UCG
AAG
CUA
GGA
CCA
CGU
GCU
t-RNA
Ser
Lys
Leu
Gly
Pro
NASCENT
PROTEIN
GCA
t-RNA
Arg

B.

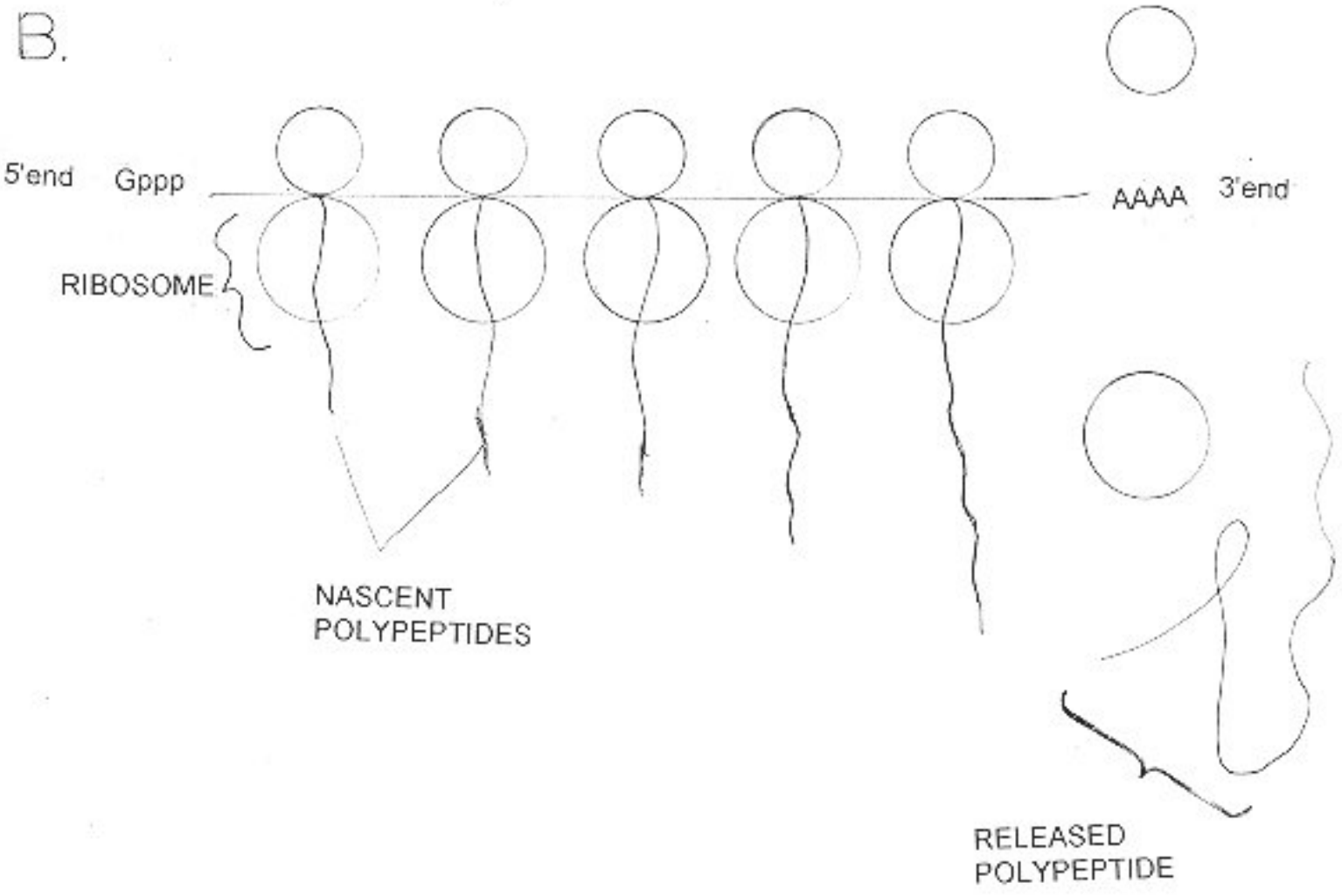

5'end
Gppp
AAAA
3'end
RIBOSOME
NASCENT
POLYPEPTIDES
RELEASED
POLYPEPTIDE

Fig. R2 Process of translation where the triplet code corresponds to amino acids. (A) The triplet is recognized by tRNA containing the corresponding amino acid so that the polypeptide grows one amino acid at a time. The involvement of the ribosomes is shown in part (B) of the figure.

Back to Chapter 2

Back to Chapter 2

REFERENCES

Abbas, A.K. (1996) Die and let live: eliminating dangerous lymphocytes, *Cell* 84:655-657.(Medline)

Acehan, D., Jiang, X., Morgan, D.G., Heuser, J.E., Wang, X. and Akey, C.W. (2002) Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation, *Mol. Cell* 9:423-432. (MedLine)

Adams, J.M. and Cory, S. (1998) The Bcl-2 protein family: arbiters of cell survival, *Science* 281:1322-1326.(Medline)

Adams, C.C. and Workman, J.L. (1993) Nucleosome displacement in transcription, *Cell* 72: 305-308(Medline)

Akhtar, A., Zink, D. and Becker, P.B. (2000) Chromodomains are protein-RNA interaction modules, *Nature* 407:405-409. (MedLine)

Alkema, M.J., Bronk, M., Verhoeven, E., Otte, A., van't Veer, L.J., Berns, A. and van Lohuizen, M. (1997) Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian polycomb complex, *Genes Dev.* 11:226-240. (MedLine)

Alison, M.R., Poulsom, R., Jeffery, R., Dhillon, A.P., Quaglia, A., Jacob, J., Novelli, M., Prentice, G., Williamson, J., and Wright, N.A. (2000) Hepatocytes from non-hepatic adult stem cells, *Nature* 406:257. (MedLine)

Andjelkovic, M., Alessi, D.R., Meier, R., Fernandez, A., Lamb, N.J., Frech, M., Cron, P., Cohen, P., Lucocq, J.M. and Hemmings, B.A. (1997) Role of translocation in the activation and function of protein kinase B, *J. Biol. Chem.* 272:31515-31524.(Medline)

Andrulis, E.D., Neiman, A.M., Zappulla, D.C. and Sternglanz, R. (1998) Perinuclear localization of chromatin facilitates transcriptional silencing, *Nature* 394:592-595. (MedLine)

Anson, R.M., Hudson, E. and Bohr, V.A. (2000) Mitochondrial endogenous oxidative damage has been overestimated, *FASEB J.* 14:355-360. (MedLine)

Aoudjit, F. and Vuori K. (2001) Matrix attachment regulates Fas-induced apoptosis in endothelial cells. A role for c-flip and implications for anoikis, *J. Cell Biol.* 152:633-644. (MedLine)

Ashkenazi, A. and Dixit, V.M. (1998) Death receptors: signaling and modulation, *Science* 281:1305-1308.(Medline)

Ashworth, D., Bishop, M., Campbell, K., Colman, A., Kind, A., Schieke, A., Blott, S., Griffin, H., Haley, C., McWhir, J. and Wilmut, I. (1998) DNA microsatellite analysis of Dolly, *Nature* 394:328.(Medline)

Avila, N.A., Dwyer, A.J., Dale, J.K., Lopatin, U.A., Sneller, M.C., Jaffe, E.S., Puck, J.M. and Straus, S.E. (1999) Autoimmune lymphoproliferative syndrome: a syndrome associated with inherited genetic defects that impair lymphocytic apoptosis--CT and US features, *Radiology* 212:257-263.(Medline)

Baarends, W.M., Hoogerbrugge, J.W., Roest, H.P., Ooms, M., Vreeburg, J., Hoeijmakers, J.H. and Grootegoed, J.A. (1999) Histone ubiquitination and chromatin remodeling in mouse spermatogenesis, *Dev. Biol.* 207:322-333.(Medline)

Baehrecke, E.H. (2002) How death shapes life during development, *Nature Rev. Mol. Cell Biol.* 3:779-787. (MedLine)

Barja, G. and Herrero, A. (2000) Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals, *FASEB J.* 14:312-318.(MedLine)

Bashirullah, A., Cooperstock, R.L. and Lipshitz, H.D. (1998) RNA localization in development, *Annu. Rev. Biochem.* 67:335-394. (MedLine)

Baumann, P. and Cech, T.R. (2001) Pot1, the putative telomere end-binding protein in fission yeast and humans, *Science* 292:1171-1175. (MedLine)

Bell, A.C., West, A.G. and Felsenfeld, G. (2001) Insulators and boundaries: versatile regulatory elements in the eukaryotic genome, *Science* 291:447-450. (MedLine)

Bergel, M., Herrera, J.E., Thatcher, B.J., Prymakowska-Bosak, M., Vassilev, A., Nakatani, Y., Martin, B. and Bustin, M. (2000) Acetylation of novel sites in the nucleosomal binding domain of chromosomal protein HMG-14 by p300 alters its interaction with nucleosomes, *J. Biol. Chem.* 275:11514-11520. (MedLine)

Burdon, T., Chambers, I., Stracey, C., Niwa, H. and Smith, A. (1999) Signaling mechanisms regulating self-renewal and differentiation of pluripotent embryonic stem cells, *Cells Tissues Organs* 165:131-143. (MedLine)

Burdon, T., Smith, A. and Savatier, P. (2002) Signalling, cell cycle and pluripotency in embryonic stem cells (2002) *Trends in Cell Biol.* 12:432-438.

Bustin, M.(1999) Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins, *Mol. Cell. Biol.* 19:5237-5246. (MedLine)

Bustin, M.(2001a) Revised nomenclature for high mobility group (HMG) chromosomal proteins, *Trends Biochem. Sci.* 26:152-153. (MedLine)

Bustin, M.. (2001b) Chromatin unfolding and activation by HMGN chromosomal proteins, *Trends Biochem. Sci.* 26:431-437. (MedLine)

Busturia, A. and Bienz, M. (1993) Silencers in abdominal-B, a homeotic Drosophila gene, *EMBO J.* 12:1415-1425. (MedLine)

Beard, C., Li, E. and Jaenisch, R. (1995) Loss of methylation activates Xist in somatic but not in embryonic cells, *Genes Dev.* 9:2325-2334.(Medline)

Beckman, K.B. and Ames, B.N. (1996) Detection and quantification of oxidative adducts of mitochondrial DNA, *Methods Enzymol.* 264:442-453.(Medline)

Beere, H.M., Wolf, B.B., Cain, K., Mosser, D.D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R.I., Cohen, G.M. and Green, D.R. (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the apaf-1 apoptosome, *Nature Cell Biol.* 2:469-475. (MedLine)

Bell, A.C. and Felsenfeld, G. (2000) Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene, *Nature* 405:482-485. (MedLine)

Benvenisty, N., Mencher, D., Meyuhas, O., Razin, A. and Reshef, L. (1985) Sequential changes in DNA methylation patterns of the rat phospho*enol*pyruvate carboxykinase gene during development, *Proc. Nat. Acad. Sci. USA* 82:267-271.(Medline)

Bestor, T., Laudano, A., Mattaliano, R. and Ingram, V. (1988) Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases, *J. Mol. Biol.* 203:971-283.(Medline)

Bhattacharya, S.Y., Ramchandi, S., Cervoni, N. and Szyf, M. (1999) A mammalian preotein with specific demethylase activity for mCpG DNA, *Nature* 397:579-583.(Medline)

Bird, A.P.and Wolffe, A.P. (1999) Methylation-induced repression--belts, braces, and chromatin, *Cell* 99:451-454.(Medline)

Bird, A., Taggart, M., Frommer, M., Miller, O.J. and Macleod, D. (1985) A fraction of the mouse genome that is derived from islands of nonmethylated CpG-rich DNA, *Cell* 40:91-99.(Medline)

Bjornson, C.R., Rietze, R.L., Reynolds, B.A., Magli, M.C. and Vescovi, A.L. (1999) Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo, *Science* 283:534-537. (MedLine)

Blasco, M.A., Lee, H.-W., Hand, M.P., Samper, E., Landsdorp, P.M., DePinho, R.A. and Greider, C.W. (1997) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA, *Cell* 91:25-34.(Medline)

Bodnar, A.G., Ouelette, M., Frolkis, M., Holt, S.E., Chiu, C., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S. and Wright, W.E. (1998) Extension of life span by introduction of telomerase in normal human cells, *Science* 279:349-352.(Medline)

Boffoli, D., Scacco, S.C., Vergari, R., Solarino, G., Santacroce, G. and Papa, S. (1994) Decline with age of the respiratory chain activity in human skeletal muscle, *Biochim. Biophys. Acta.* 1226:73-82.(Medline)

Boise, L.H. and Thompson, C.B. (1996) Hierarchical control of lymphocyte survival, *Science* 274:67-68.(Medline)

Boise, L.H., González-García, M., Postema, C.E., Ding L., Lindsten, T., Turka, L.A., Mao, X., Nuñez, G. and Thompson, C.B. (1993) *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death, *Cell* 74:597-608.(Medline)

Boyes, J. and Bird, A. (1991) DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein, *Cell* 64:1123-1134.(Medline)

Boyes, J., Byfield, P., Nakatani, Y. and Ogryzko, V. (1998) Regulation of activity of the transcription factor GATA-1 by acetylation, *Nature* 396:594-598.(Medline)

Brachmann, C.B., Sherman, J.M., Devine, S.E., Cameron, E.E., Pillus, L. and Boeke, J.D. (1995) The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability, *Genes Dev.* 9:2888-2902. (MedLine)

Braunstein, M., Rose, A.B., Holmes, S.G., Allis, C.D. and Broach, J.R. (1993) Transcriptional silencing in yeast is associated with reduced nucleosome acetylation, *Genes Dev.* 7: 592-604.(Medline)

Brazelton, T.R., Rossi, F.M., Keshet, G.I. and Blau, H.M. (2000) From marrow to brain: expression of neuronal phenotypes in adult mice, *Science* 290:1775-1779. (MedLine)

Brierley, E.J., Johnson, M.A., Lightowlers, R.N., James, O.F., Turnbull, D.M. (1998) Role of mitochondrial DNA mutations in human aging: implications for the central nervous system and muscle, *Ann. Neurol.* 43:217-223.(Medline)

Broccoli, D., Smogorzewska, A., Chong, L. and de Lange, T. (1997) Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2, *Nature Genet.* 17:231-235. (MedLine)

Brockes, J.P. and Kumar, A. (2002) Plasticity and reprogramming of differentiated cells in amphibian regeneration, *Nature Rev. Mol. Cell Biol.* 3:566-574. (MedLine)

Brook, F.A. and Gardner, R.L. (1997) The origin and efficient derivation of embryonic stem cells in the mouse, *Proc. Natl. Acad. Sci. USA.* 94:5709-5712. (MedLine)

Brown, S., Heinisch, I., Ross, E., Shaw, K., Buckley, C.D. and Savill, J. (2002) Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment, *Nature* 418:200-203. (MedLine)

Bruey, J.M., Ducasse, C., Bonniaud, P., Ravagnan, L., Susin, S.A., Diaz-Latoud, C., Gurbuxani, S., Arrigo, A.P., Kroemer, G., Solary, E. and Garrido, C. (2000) Hsp27 negatively regulates cell death by interacting with cytochrome c, *Nature Cell Biol.* 2:645-652. (MedLine)

Brunet, A., Bonni, A.M., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J. and Greenberg, M.E. (1999) Akt promotes cell survival by phosphorylating and inhbiting a forkhead transcription factor, *Cell* 96:857-868.(Medline)

Buschhausen, G., Wittig, B., Graessmann, M. and Graessmann, A. (1987) Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene, *Proc. Natl. Acad. Sci. USA* 84:1177-1181.(Medline)

Cameron, H.A. and McKay, R.D. (1999) Restoring production of hippocampal neurons in old age, *Nature Neurosci.* 2:894-897. (MedLine)

Campbell, K.H., McWhir, J., Ritchie, W.A. and Wilmut, I. (1996) Sheep cloned by nuclear transfer from a cultured cell line, *Nature* 380:64-66.(Medline)

Campo, M.L., Tedeschi, H., Muro, C. and Kinnally, K.W. (1998) The mitochodrial membrane channels and their pharmacology, in *Ion Channel Pharmacology* Soria, B. and Ceña, V., Oxford University Press, Oxford, New York, Tokyo pp.321-342.

Canale, V.C. and Smith, C.H. (1967) Chronic lymphadenopathy simulating malignant lymphoma, *J. Pediatr.* 70:891-899.(Medline)

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S., Reed JC (1998) Regulation of cell death protease caspase-9 by phosphorylation, *Science* 282:1318-1321.(Medline)

Carey, M.F. (1995) A holistic view of the complex, *Curr. Biology* 9:1003-1005.(Medline)

Carlson, N. and Rechsteiner, M. (1987) Microinjection of ubiquitin: intracellular distribution and metabolism in HeLa cells maintained under normal physiological conditions, *J. Cell Biol.* 104:537-546.(Medline)

Cavalli, G. and Paro, R. (1998a) Chromo-domain proteins: linking chromatin structure to epigenetic regulation, *Curr. Opin. Cell Biol.* 10:354-360.(Medline)

Cavalli, G. and Paro, R. (1998b) The Drosophila Fab-7 chromosomal element conveys epigenetic inheritance during mitosis and meiosis, *Cell* 93:505-518.(Medline)

Cavalli, G. and Paro, R. (1999) Epigenetic inheritance of active chromatin after removal of the main transactivator, *Science* 286:955-958.(Medline)

Cedar, H. (1988) DNA methylation and gene activity, *Cell* 53:3-4.(Medline)

Chai, J., Du, C., Wu, J-W., Kyin, A., Wang, X and Shi, Y. (2000) Structural and biochemical basis of apoptotic activation by Smac/DIABLO, *Nature*855-862.

Chan, C.S., Rastelli, L. and Pirrotta, V. (1994) A Polycomb response element in the Ubx gene that determines an epigenetically inherited state of repression, *EMBO J.* 13:2553-2564. (MedLine)

Chau, B.N., Borges, H.L., Chen, T.T., Masselli, A., Hunton, I.C. and Wang, J.Y. (2002) Signal-dependent protection from apoptosis in mice expressing caspase-resistant Rb, *Nature Cell Biol.* 4:757-765. (MedLine)

Chinnayan , A.M., O'Rourke, K., Lane, B.R. and Dixit, V.M. (1997) Interaction of CED-4 with CED-3 and CED-9: A molecular framework for cell death, *Science* 275:1122-1126.(Medline)

Chung, S., Gumienny, T.L., Hengartner, M.O. and Driscoll, M. (2000) A common set of engulfment genes mediates removal of both apoptotic and necrotic cell corpses in *C. elegans*, *Nature Cell Biol.* 2:931-937. (MedLine)

Clancy, D.J., Gems, D., Harshman, L.G., Oldham, S., Stocker, H., Hafen, E., Leevers, S.J. and Partridge L. (2001) Extension of life-span by loss of CHICO, a *Drosophila* insulin receptor substrate protein, *Science* 292:104-106. (MedLine)

Clark, D.J. and Kimura, T. (1990) Electrostatic mechanism of chromatin folding, *J. Mol. Biol.* 211:883-896.(Medline)

Clarke, D.L., Johansson, C.B., Wilbertz, J., Veress, B., Nilsson, E., Karlstrom, H., Lendahl, U. and Frisen, J. (2000) Generalized potential of adult neural stem cells, *Science* 288:1660-1663. (MedLine)

Conradt, B. and Horvitz, H.R. (1998) The *C. elegans* protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9, *Cell* 93:519-529. (MedLine)

Cooke, H.J. and Smith, B.A. (1986) Variability of telomeres of the human X/Y pseudoautosomal region, *Cold Spring Harbor Symp. Quant. Biol.* LI:213-219.(Medline)

Cryns, V. and Yuan, J. (1998) Proteases to die for, *Genes Dev.* 12:1551-1570.(Medline)

Csordas, A. (1990) On the biological role of histone acetylation, *Biochem. J.* 265:23-38.(Medline)

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. and Greenberg, M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery, *Cell* 91:231-241.(Medline)

Daugas, E., Nochy, D., Ravagnan, L., Loeffler, M., Susin, S.A., Zamzami, N. and Kroemer, G. (2000) Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis, FEBS Lett. 476:118-123. <a href="http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd="Retrieve&db=PubMed&list_uids=10913597&dopt=Abstract"">(MedLine)

Davie, J.R. (1998) Covalent modifications of histones: expression from chromatin templates, *Curr. Opin. Genet. Dev.* 8:173-178.(Medline)

Davie, J.R. and Murphy, L.C. (1990) Level of ubiquitinated histone H2B in chromatin is coupled to ongoing transcription, *Biochemistry* 29:4752-4757.(Medline)

Davie, J.R., Lin, R. and Allis, C.D. (1991) Timing of the appearance of ubiquitinated histones in developing new macronuclei of *Tetrahymena thermophila*, *Biochem. Cell Biol.* 69:66-71.(Medline)

de Boer, J., Andressoo, J.O., de Wit, J., Huijmans, J., Beems, R.B., van Steeg, H., Weeda, G., van der Horst, G.T., van Leeuwen, W., Themmen, A.P.N., Meradji, M. and Hoeijmakers, J.H.J. (2002) Premature aging in mice deficient in DNA repair and transcription, *Science* 296:1276-1279. (MedLine)

del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., Nunez, G. (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt, *Science* 278:687-689.(Medline)

De-Groot, N. and Hochberg, A. (1993) Gene imprinting during placental and embryonic development, *Mol. Reprod. Dev.* 36:390-406.(Medline)

de Lange, T. (1994) Activation of telomerase in human tumor, *Proc. Natl. Acad. Sci. USA* 91:2882-2885.(Medline)

Desagher, S. and Martinou, J.-C. (2000) Mitochondria as the central control point of apoptosis, *Trends Cell Biol.* 10:369-377. (MedLine)

De Souza, A.T., Yamada, T., Mills, J.J. and Jirtle, R.L. (1997) Imprinted genes in liver carcinogenesis,*FASEB J.* 11:60-67.(Medline)

Deveraux, Q.L. and Reed, J.C. (1999) IAP family proteins--suppressors of apoptosis, *Genes Dev.* 13:239-252. (MedLine)

Ding, H.F., Bustin, M. and Hansen, U. (1997) Alleviation of histone H1-mediated transcriptional repression and chromatin compaction by the acidic activation region in chromosomal protein HMG-14, *Mol. Cell. Biol.* 17:5843-5855. (MedLine).

D'Mello, N.P. and Jazwinski, S.M. (1991) Telomere length constancy during aging of *Saccharomyces cerevisiae*, *J. Bacteriol.* 173:6709-6713.(Medline)

Dolle, M.E., Giese, H., Hopkins, C.L., Martus, H.J., Hausdorff, J.M. and Vijg, J. (1997) Rapid accumulation of genome rearrangements in liver but not in brain of old mice, *Nature Genet.* 17:431-434.(Medline)

Du, C., Fang, M., Li, Y., Li, L. and Wang, X. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition, *Cell* 102:33-42. (MedLine)

Dubnau, J. and Struhl, G. (1996) RNA recognition and translational regulation by a homeodomain protein, *Nature* 379:694-699.(Medline)

Eden, S., Hashimshony, T., Keshet, I., Cedar, H. and Thorne, A.W. (1998) DNA methylation models histone acetylation, *Nature* 394:842.(Medline)

Effros, R.B. (1998) Replicative senescence in the immune system: impact of the Hayflick limit on T-cell function in the elderly, *Am. J. Hum. Genet.* 62:1003-1007.(Medline)

Egilmez, N.K., Chen, J.B. and Jazwinski, S.M. (1989) Specific alterations in transcript prevalence during the yeast life span, *J. Biol. Chem.* 264:14312-14317. (Medline)

Einck, L. and Bustin, M. (1983) Inhibition of transcription in somatic cells by microinjection of antibodies to chromosomal proteins, *Proc. Natl. Acad. Sci. USA* 80:6735-6739. (MedLine)

Ekwall, K., Olsson, T., Turner, B.M., Cranston, G. and Allshire, R.C. (1997) Transient inhibition of histone deacetylation alters the structural and functional imprint at fission yeast centromeres, *Cell* 91:1021-1032.(Medline)

Ellis, R.E., Yuan, J. and Horvitz, H.R. (1991) Mechanisms and functions of cell death, *Annu. Rev. Cell Biol.* 7:663-698.(Medline)

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD, *Nature* 391:43-50. (Medline)

Epstein, C.J. and Motulsky, A.G. (1996) Werner syndrome: entering the helicase era, *BioEssays* 18:1025-1027.(Medline)

Evan, G. and Littlewood, T.(1998) A matter of life and death, *Science* 281:1317-1322. (Medline)

Fadok, V.A. and Henson, P.M. (1998) Apoptosis: Getting rid of the bodies, *Curr. Biol.* 8:R693-R695.(Medline)

Faragher, R.G. and Kipling, D. (1998) How might replicative senescence contribute to human ageing? *BioEssays* 20:985-991.(Medline)

Fenselfeld, G., Chromatin unfolds (1996), *Cell* 86:13-19.(Medline)

Finkel, T. and Holbrook, N.J. (2000) Oxidants, oxidative stress and the biology of ageing, *Nature* 408:239-247. (MedLine)

Flores, E.R., Tsai, K.Y., Crowley, D., Sengupta, S., Yang, A., McKeon, F. and Jacks, T. (2002) p63 and p73 are required for p53-dependent apoptosis in response to DNA damage, *Nature* 416:560-564. (MedLine)

Frank, D., Keshet, I., Shani, M., Levine, A., Razin, A., Cedar, H. (1991) Demethylation of CpG islands in embryonic cells, *Nature* 351:239-241.(Medline)

Franke, A., DeCamillis, M., Zink, D., Cheng, N., Brock, H.W. and Paro, R. (1992) *Polycomb* and *polyhomeotic* are constituents of a multimeric protein complex in chromatin of *Drosophila melanogaster*, *EMBO J.* 11:2941-2950.(Medline)

Franklin, J.L. and Johnson, E.M., Jr (1992) Suppression of programmed neuronal death by sustained elevation of cytoplasmic calcium, *Trends Neurosci.* 15:501-508.(Medline)

Fridovich, I. (1995) Superoxide radical and superoxide dismutases, *Annu. Rev. Biochem.* 64:97-112. (MedLine)

Friedman, D.B. and Johnson, T.E. (1988) Three mutants that extend both mean and maximum life span of the nematode, *Caenorhabditis elegans*, define the age-1 gene, *J. Gerontol.* 43:B102-109.(Medline)

Frisch, S.M. and Ruoslahti, E. (1997) Integrins and anoikis, *Curr. Opin. Cell Biol.* 9:701-706. (MedLine)

Fruman, D.A., Meyers, R.E. and Cantley, L.C. (1998) Phosphoinositide kinases, *Annu. Rev. Biochem.* 67:481-507.(Medline)

Fulda, S., Wick, W., Weller, M. and Debatin, K.M. (2002) Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo, *Nature Med.* 8:808-815. (MedLine)

Galli, R., Borello, U., Gritti, A., Minasi, M.G., Bjornson, C., Coletta, M., Mora, M., De Angelis, M.G., Fiocco, R., Cossu, G. and Vescovi, A.L. (2000) Skeletal myogenic potential of human and mouse neural stem cells, *Nature Neurosci.* 3:986-391. (MedLine)

Garcia-Bellido, A. (1975) Genetic control of wing disc development in *Drosophila*. in *Cell Patterning*, Porter, R. and Elliot, K. ed., Elsevier, Amsterdam 29:161-182.(Medline)

Garcia-Ramirez, M., Rocchini, C. and Ausio, J. (1995) Modulation of chromatin folding by histone acetylation, *J. Biol. Chem.* 270: 17923-17928.(Medline)

Gems, D., Sutton, A.J., Sundermeyer, M.L., Albert, P.S., King, K.V., Edgley, M.L., Larsen, P.L. and Riddle, D.L. (1998) Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis elegans Genetics* 150:129-155. (MedLine)

Georgel, P.T., Tsukiyama, T. and Wu, C. (1997) Role of histone tails in nucleosome remodeling by Drosophila NURF, *EMBO J.* 16:4717-4726.(Medline)

Gerasimova, T.I. and Corces, V.G. (2001) Chromatin insulators and boundaries: effects on transcription and nuclear organization, *Annu. Rev. Genet.* 35:193-208. (MedLine)

Ghosh, A., Carnahan, J. and Greenberg, M.E. (1994) Requirement for BDNF in activity-dependent survival of cortical neurons, *Science* 263:1618-1623.(Medline)

Giannakakou, P., Sackett, D.L., Ward, Y., Webster, K.R., Blagosklonny, M.V. and Fojo T. (2000) p53 is associated with cellular microtubules and is transported to the nucleus by dynein, *Nature Cell Biol.* 2:709-717. (MedLine)

Gilbert, S.E. (1994) *Developmental Biology*, Fourth Edition, Sinauer Associates Inc., Sunderland, MA.

Gill, R.M. and Hamel, P.A.(2000) Subcellular compartmentalization of E2F family members is required for maintenance of the

postmitotic state in terminally differentiated muscle, *J. Cell Biol.* 148:1187-1202. (MedLine)

Gleeson, P.A., Anderson, T.J., Stow, J.L., Griffiths, G., Toh, B.H. and Matheson, F. (1996) p230 is associated with vesicles budding from the trans-Golgi network, *J. Cell Sci.* 109:2811-2821. (MedLine)

Goldstein, P. (1997) Controlling cell death, *Science* 275:1081-1082.(Medline)

Gong, J.G., Costanzo, A., Yang, H.Q., Melino, G., Kaelin, W.G. Jr., Levrero, M. and Wang, J.Y. (1999) The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage, *Nature* 399:806-809. (MedLine)

Gorovsky, M.A., Pleger, G.L., Keevert, J.B. and Hohmann, C.A. (1973) Studies on histone fraction F2A1 in macro and micronuclei of *Tetrahymena pyriformis*, *J.Cell Biol.* 57:773-791.(Medline)

Goyal, L., McCall, K., Agapite, J., Hartwieg, E. and Steller, H. (2000) Induction of apoptosis by Drosophila reaper, hid and grim through inhibition of IAP function, *EMBO J.* 19:589-597. (MedLine)

Goyns, M.H., Charlton, M.A., Dunford, J.E., Lavery, W.L., Merry, B.J., Salehi, M. and Simoes, D.C. (1998) Differential display analysis of gene expression indicates that age-related changes are restricted to a small cohort of genes, *Mech. Ageing Dev.* 101:73-90.(Medline)

Gray, M.D., Wang, L., Youssoufian, H., Martin, G.M., Oshima, J. (1998) Werner helicase is localized to transcriptionally active nucleoli of cycling cells, *Exp. Cell Res.* 242:487-494.(Medline)

Green D.R. and Martin, S.J. (1995) The killer and the executioner: how apoptosis controls malignancy, *Curr. Opin. Immunol.* 82:694-703.(Medline)

Green, D.R. and Reed, J.C. (1998) Mitochondria and apoptosis, *Science* 281:1309-1312.(Medline)

Greider, C.W. and Blackburn, E.H. (1989) A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis, *Nature* 337:331-337.(Medline)

Griffiths, G.J., Dubrez, L., Morgan, C.P., Jones, N.A., Whitehouse, J., Corfe, B.M., Dive, C., Hickman, J.A. (1999) Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis, *J. Cell Biol.* 144:903-914. (Medline)

Griffith, J.D., Comeau, L., Rosenfield, S., Stansel, R.M., Bianchi, A., Moss, H. and de Lange, T. (1999) Mammalian telomeres end in a large duplex loop, *Cell* 97:503-514.(Medline)

Gross, A., Jockel, J., Wei, M.C. and Korsmeyer, S.J. (1998) Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis, *EMBO J.* 17:3878-3885.(Medline)

Gross, A., McDonnell, J.M. and Korsmeyer, S.J. (1999) BCL-2 family members and the mitochondria in apoptosis, *Genes Dev.* 13:1899-1911.(Medline)

Grunstein, M.(1997a) Histone acetylation in chromatin structure and transcription, *Nature* 389:349-352.(Medline)

Grunstein, M. (1997b) Molecular model for telomeric heterochromatin in yeast, *Curr. Opin. Cell Biol.* 9:383-387.(Medline)

Guarente, L. (2000) Sir2 links chromatin silencing, metabolism, and aging, *Genes Dev.* 14:1021-1026. (MedLine)

Gurdon, J.B. (1978) *Gene Expression During Cell Differentiation*, Scientific Publication Division, Carolina Biological Supply Co., Burlington, N.C., pp.32.

Gurdon, J.B. (1988) A community effect in animal development, *Nature* 336:772-774.(Medline)

Gussoni, E., Soneoka, Y., Strickland, C.D., Buzney, E.A., Khan, M.K., Flint, A.F., Kunkel, L.M. and Mulligan, R.C. Dystrophin expression in the mdx mouse restored by stem cell transplantation, *Nature* 401:390-394. (MedLine)

Haaf, T. and Schmid, M. (1991) Chromosome topology in mammalian interphase nuclei, *Exp. Cell Res.* 192:325-332. (MedLine)

Hadorn, E. (1965) Problems of determination and transdetermination, in *Genetic Control of Differentiation*, Upton, New York 18:148-161.

Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W. and Weinberg RA (1999) Creation of human tumour cells with defined genetic elements, *Nature* 400:464-468.(Medline)

Hale, A.J., Smith, C.A., Sutherland, L.C., Stoneman, V.E., Longthorne, V.L., Culhane, A.C., Williams, G.T. (1996) Apoptosis: molecular regulation of cell death, *Eur. J. Biochem.* 236: 1-26.(Medline)

Halenbeck, R., MacDonald,H. Roulston, A., Chen, T.T., Conroy, L. and Williams, L.T. (1998) CSPAN a human nuclease regulated by the caspase-sensitive inhibitor DFF45, *Curr. Biol.* 8:537-540.(Medline)

Hamiche, A., Sandaltzopoulos, R., Gdula, D.A. and Wu, C. (1999) ATP-dependent histone octamer sliding mediated by the chromatin remodeling complex NURF, *Cell* 97:833-842.(Medline)

Hanayama, R., Tanaka, M., Miwa, K., Shinohara, A., Iwamatsu, A. and Nagata, S. (2002) Identification of a factor that links apoptotic cells to phagocytes, *Nature* 417:182-187. (MedLine)

Harada, H., Becknell, B., Wilm, M., Mann, M., Huang, L.J., Taylor, S.S., Scott, J.D. and Korsmeyer, S.J. (1999) Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinases A, *Mol.Cell* 3:413-422.(Medline)

Hark, A.T., Schoenherr, C.J., Katz, D.J., Ingram, R.S., Levorse, J.M. and Tilghman, S.M. (2000) CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus, *Nature* 405:486-489. (MedLine)

Harley, C.B. (1995) Telomeres and aging, in *Telomeres* (Backburn E. and Greider, C., eds.) Cold Spring Harbor Laboratory Press, N.Y., p. 247-264.

Harley, C.B., Futcher, A.B. and Greider, C.W. (1990) Telomeres shorten during aging of human fibroblasts, *Nature* 345:458-460.(Medline)

Harman, D. (1981) The aging process, *Proc. Natl. Acad. Sci. USA* 78:7124-718.

Hart, R.W. and Setlow, R.B (1974) Correlation between deoxyribonucleic acid excision-repair and life-span in a number of mammalian species, *Proc. Natl. Acad. Sci. USA* 71:2169-2173. (MedLine)

Hart, C.M. and Laemmli, U.K. (1998) Facilitation of chromatin dynamics by SARs, *Curr. Opin. Genet. Dev.* 8:519-525. (MedLine)

Hebbes, T.R., Thome, A.W. and Crane-Robinson, C. (1988) A direct link between core histone acetylation and transcriptionally active chromatin, *EMBO J.* 7:1395-1402.(Medline)

Hebbes, T.R., Clayton, A.L., Thorne, A.W. and Crane-Robinson, C. (1994) Core histone hyperacetylation and transcriptionally active chromatin, *EMBO J.* 13:1823-1830.(Medline)

Heemskerk, J. DiNardo, S., Kostriken, R., and O'Farrell, P.H. (1991) Multiple modes of *engrailed* regulation in the progression towards

cell fate determination, *Nature* 352:404-410.(Medline)

Henderson, E. (1995) Telomere DNA structure, in *Telomeres*, Blackburn, E.H. and Greider, C.W.,ed. (1996) Cold Spring Harbor Laboratory Press, pp. 11-34.

Hengartner, M.O. Genetic control of programmed cell death and aging in the nematode *Caenorhabditis elegans*, *Exp. Gerontol.* 32:363-374.(Medline)

Henson, P.M., Bratton, D.L. and Fadok, V.A. (2001) The phosphatidylserine receptor: a crucial molecular switch? *Nature Rev. Mol. Cell Biol.* 2:627-633. (MedLine)

Hildebrandt, M. and Nellen, W. (1992) Differential antisense transcription from Dictyostelium *EB4* gene locus: implications on antisense-mediated regulation of mRNA stability, *Cell* 69:197-204.(Medline)

Ho, Y.S., Magnenat, J.L., Bronson, R.T., Cao, J., Gargano, M., Sugawara, M. and Funk, C.D. (1997) Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia, *J. Biol. Chem.* 272:16644-16651.(Medline)

Hoch, M., Gerwin, N., Taubert, H. and Jäckle, H. (1992) Competition for overlapping sites in the regulatory region of the *Drosophila* gene *krüpple*, *Science* 256:94-97.(Medline)

Hock, R., Wilde, F., Scheer, U. and Bustin, M. (1998) Dynamic relocation of chromosomal protein HMG-17 in the nucleus is dependent on transcriptional activity, *EMBO J.* 17:6992-7001. (MedLine)

Hoeppner, D.J., Hengartner, M.O. and Schnabel, R. (2001) Engulfment genes cooperate with ced-3 to promote cell death in *Caenorhabditis elegans Nature* 412:202-206. (MedLine)

Holcik, M. and Korneluk, R.G. (2001) XIAP, the guardian angel, Nature Rev. Mol. Cell Biol. 2:550-556. (MedLine)

Holcik M, Gibson H, Korneluk RG. (2001) XIAP: Apoptotic brake and promising therapeutic target, *Apoptosis* 6:253-261. (MedLine)

Holler, N., Zaru, R., Micheau, O., Thome, M., Attinger, A., Valitutti, S., Bodmer, J.L., Schneider, P., Seed, B. and Tschopp, J. (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule, *Nature Immunol.* 1:489-495. (MedLine)

Holley, C.L., Olson, M.R., Colon-Ramos, D.A. and Kornbluthm S. (2002) Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition, *Nature Cell Biol.* 4:439-444. (MedLine)

Holstege, F.C.P., Jennings, E.G., Wyrick, J.J., Lee, T.I., Hengartner, C.J., Green, M.R., Golub, T.R., Lander, E.S. and Young, R.A. (1998) Dissecting the regulatory circuitry of a eukaryotic genome *Cell* 95:717-728.(Medline)

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Geloen, A., Even, P.C., Cervera, P. and Le Bouc, Y. (2003) IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice, *Nature* 421:182-187. (MedLine)

Hood, L., Kronenberg, M. and Hunkapillar, T. (1985) T cell antigen receptor and the immunoglobulin supergene family, *Cell* 40:225-229.(Medline)

Horvitz, H.R. (1999) Genetic control of programmed cell death in the nematode *Caenorhabditis elegans Cancer Res.* 59(7 Suppl):1701s-1706s.(Medline)

Hsu Y.-T. and Youle, R.J. (1997) Nonionic detergents induce dimerization among members of the Bcl-2 family, *J. Biol. Chem.* 272:13829-13834.(Medline)

Huang, P., Feng, L., Oldham, E.A., Keating, M.J. and Plunkett, W. (2000a) Superoxide dismutase as a target for the selective killing of cancer cells, *Nature* 407:390-395. (MedLine)

Huang, H., Joazeiro, C.A., Bonfoco, E., Kamada, S., Leverson, J.D. and Hunter, T. (2000b) The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7, *J. Biol. Chem.* 275:26661-26664. (MedLine)

Huang, Y., Park, Y.C., Rich, R.L., Segal, D., Myszka, D.G. and Wu, H. (2001) Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain, *Cell* 104:781-790. (MedLine)

Hung, H.L., Lau, J., Kim, A.Y., Weiss, M.J. and Blobel, G.A. (1999) CREB-Binding protein acetylates hematopoietic transcription factor GATA-1 at functionally important sites, *Mol. Cell Biol.* 19:3496-3505.(Medline)

Ideker, T., Thorsson, V., Ranish, J.A., Christmas, R., Buhler, J., Eng, J.K., Bumgarner, R., Goodlett, D.R., Aebersold, R. and Hood, L. (2001) Integrated genomic and proteomic analyses of a systematically perturbed metabolic network, *Science* 292:929-934. (MedLine)

Ikonomidou, C., Bosch, F., Miksa, M., Bittigau, P., Vockler, J., Dikranian, K., Tenkova, T.I., Stefovska, V., Turski, L., Olney, J.W. (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain, *Science* 283:70-74. 283:70-74.(Medline)

Imai, Y., Kimura, T., Murakami, A., Yajima, N., Sakamaki, K. and Yonehara, S, (1999) The CED-4-homologous protein FLASH is involved in Fas-mediated activation of caspase-8 during apoptosis, *Nature* 398:777-785.(Medline)

Imai, S., Armstrong, C.M., Kaeberlein, M. and Guarente, L. (2000) Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase, *Nature* 403:795-800. (MedLine)

Imhof, A. and Wolffe, A.P. (1998) Transcription: gene control by targeted histone acetylation, *Curr. Biol.* 8:R422-424.(Medline)

Ishii, K., Arib, G., Lin, C., Van Houwe, G. and Laemmli, U.K. (2002) Chromatin boundaries in budding yeast: the nuclear pore connection, *Cell*109:551-562. (MedLine)

Ivins, K.J., Bui, E.T. and Cotman, C.W. (1998) β-amyloid induces local neurite degeneration in cultured hippocampal neurons: evidence for neuritic apoptosis, *Neurobiol. Dis.* 5:365-378. (MedLine)

Jackson, K.A., Mi, T. and Goodell, M.A. (1999) Hematopoietic potential of stem cells isolated from murine skeletal muscle, *Proc. Natl. Acad. Sci. USA* 96:14482-14486. (MedLine)

Jackson, J.P., Lindroth, A.M., Cao, X. and Jacobsen, S.E. (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase, *Nature* 416:556-560. (MedLine)

Jackson-Grusby, L., Beard, C., Possemato, R., Tudor, M., Fambrough, D., Csankovszki, G., Dausman, J., Lee, P., Wilson, C., Lander, E. and Jaenisch, R.(2001) Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation, *Nature Genet.* 27:31-39. (MedLine)

Jacobson, M.D. (1997) Apoptosis: BCl-related proteins get connected, *Curr. Biol.* 7:R277-R281.(Medline)

Jacobson, M.D., Weil, M. and Raff, M.C. (1997) Programmed cell death in animal development, *Cell* 88:347-354.(Medline)

James, C., Gschmessiner, S., Fraser, A. and Evan, G.I. (1997) CED-4 induces chromatin condensation in *Schizosaccharomyces pombe* and is inhibited by direct physical association with CED-9, *Current Biol.* 7:246-252.(Medline)

Jazwinski, S.M. (1993) The genetics of aging in the yeast *Saccharomyces cerevisiae*, *Genetica* 91:35-51.(Medline)

Jeppesen, P. and Turner, B.M. (1993) The inactive X-chromosome in female mammals is distinguished by a lack of histone H-4

acetylation, a cytogenetic marker for gene expression, *Cell* 74: 281-289.(Medline)

Jiang, X.R., Jimenez, G., Chang, E., Frolkis, M., Kusler, B., Sage, M., Beeche, M., Bodnar, A.G., Wahl, G.M., Tlsty, T.D. and Chiu, C.P. (1999) Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype, *Nature Genet.* 21:111-114.(Medline)

John, B, (1988) *The Biology of Heterochromatin*, ed. Verma, R.S., Cambridge University Press, Cambridge, U.K.

Johnson, F.B., Sinclair, D.A. and Guarente, L. (1999) Molecular biology of aging, *Cell* 96:291-302.(Medline)

Jones, P.A. and Takai, D. (2001) The role of DNA methylation in mammalian epigenetics, *Science* 293:1068-1070. (MedLine)

Joyner, A.L. (1991) Gene targeting and gene trap screens using embryonic stem cells: new approaches to mammalian development, *BioEssays* 13:649-656. (MedLine)

Joza, N., Susin, S.A., Daugas, E., Stanford, W.L., Cho, S.K., Li, C.Y., Sasaki, T., Elia, A.J., Cheng, H.Y., Ravagnan, L., Ferri, K.F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y.Y., Mak, T.W., Zuniga-Pflucker, J.C., Kroemer, G. and Penninger, J.M. (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death, *Nature* 410:549-454. (MedLine)

Kaeberlein, M., McVey, M. and Guarente, L. (1999) The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms, *Genes Dev.* 13:2570-2580. (MedLine)

Kafri, T,, Ariel, M., Brandeis, M., Shemer, R., Urven, L., McCarrey, J., Cedar, H. and Razin, A. (1992) Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line, *Genes Dev.* 6:705-714.(Medline)

Kafri, T,, Gao, X. and Razin, A. (1993) Mechanistic aspects of genome-wide demethylation in the preimplantation mouse embryo, *Proc. Natl. Acad. Sci. USA.* 90:10558-10562.(Medline)

Karlseder, J., Smogorzewska, A. and de Lange T. (2002) Senescence induced by altered telomere state, not telomere loss, *Science* 295:2446-2449. (MedLine)

Kapahi, P., Boulton, M.E. and Kirkwood, T.B. (1999) Positive correlation between mammalian life span and cellular resistance to stress, *Free Radic. Biol. Med.* 26:495-500. (MedLine)

Kasibhatla, S,, Brunner, T., Genestier, L., Echeverri, F., Mahboubi, A. and Green, D.R. (1998) DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1, *Mol. Cell* 1:543-551.(Medline)

Kastner, P., Mark, M. and Chambon, P. (1995) Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* 83:859-869. (MedLine)

Kass, S.U., Landsberger, N., Wolffe, A.P. (1997) DNA methylation directs a time-dependent repression of transcription initiation, *Curr. Biol.* 7:157-165.(Medline)

Kay, R.R. (1992) Cell differentiation and patterning in *Dictyostelium*, *Curr. Opin. Cell Biol.* 4:934-938.(Medline)

Kelekar, A. and Thompson, C.B. (1998) Bcl-2-family proteins: the role of the BH3 domain in apoptosis, *Trends Cell Biol.* 8:324-330.(Medline)

Kennedy, B.K., Austriaco, N.R. Jr., Zhang, J. and Guarente, L.Mutation in the silencing gene SIR4 can delay aging in *S. cerevisiae*, *Cell* 80:485-496.(Medline)

Kenyon, C., Chang, J., Gensch, E., Rudner, A. and Tabtiang, R. (1993) A *C. elegans* mutant that lives twice as long as wild type, *Nature*

366:461-464.(Medline)

Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1971) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics, *Br. J. Cancer* 26:239-257.(Medline)

Khochbin, S. and Wolffe, A.P. (1993) Developmental regulation of butyrate inducible transcription of *Xenopus* histone H1 promoter, *Gene* 128:173-180.(Medline)

Kiechle, F.L. and Zhang, X. (2002) Apoptosis: biochemical aspects and clinical implications, *Clin. Chim. Acta.* 326:27-45. (MedLine).

Kingston, R.E. and Narlikar, G.J. (1999) ATP-dependent remodeling and acetylation as regulators of chromatin fluidity, *Genes Dev.* 13:2339-2352.(Medline)

Kingston, R., Bunker, C. and Imbalzano, A.N. (1996) Repression and activation by multiprotein complexes that alter chromatin structure, *Genes Dev.* 10:905-920.(Medline)

Kirk, K.E., Harmon, B.P., Reichardt, I.K., Sedat, J.W. and Blackburn, E.H. (1997) Block in anaphase chromosome separation caused by a telomerase template mutation, *Science* 275:1478-1481.(Medline)

Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H. and Peter, M.E. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor, *EMBO J.* 14:5579-5588.(Medline)

Kiyono, T., Foster S.A., Kopop, J.J., McDougall, J.K., Salloway, D.A. and Klingelhutz. A.J. (1998) Both Rb/p16^{INK4a} inactivation and telomerase activity are required to immortalize human epithelial cells, *Nature* 396:84-88.(Medline)

Klobutcher, L.A., Jahn, L.L. and Prescott, D.M. (1984) Internal sequences are eliminated from genes during macromolecular development in the ciliated protozoan *Oxytricha nova*, *Cell* 36:1045-1055.(Medline)

Kloc, M., Zearfoss, N.R. and Etkin, L.D. (2002) Mechanisms of subcellular mRNA localization, *Cell* 108:533-544. (MedLine)

Kluck, R.M., Bossy-Wetzel, E., Green, D.R., and Newmeyer, D.D. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis, *Science* 275:1132-1136.(Medline)

Knezetic, J.A. and Luse, D.F. (1986) The presence of nucleosomes on a DNA template prevents initiation by RNA polymerase II in vitro, *Cell* 45:95-104.(Medline)

Knoepfler, P.S. and Eisenman, R.N. (1999) Sin meets NuRD and other tails of repression, *Cell* 99:447-450.(Medline)

Kolodner, R.D. and Marsischky, G.T. (1999) Eukaryotic DNA mismatch repair, *Curr. Opin. Genet. Dev.* 9:89-96. (MedLine)

Kondo, T. and Raff, M. (2000) Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells, *Science* 289:1754-1757. (MedLine)

Kondo, M., Scherer, D.C., Miyamoto, T., King, A.G., Akashi, K., Sugamura, K. and Weissman, I.L. (2000) Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines, *Nature* 407:383-386. (MedLine)

Koonin, E.V., Zhou, S. and Lucchesi, J.C. (1995) The chromo superfamily: new members, duplication of the chromo domain and possible role in delivering transcription regulators to chromatin, *Nucleic Acids Res.* 23:4229-4233. (MedLine)

Kopsidas, G., Kovalenko, S.A., Kelso, J.M. and Linnane, A.W. (1998) An age-associated correlation between cellular bioenergy decline

and mtDNA rearrangements in human skeletal muscle, *Mutat. Res.* 421:27-36.(Medline)

Kornberg, R.D. (1999) Eukaryotic transcriptional control, *Trends Cell Biol.*9:M46-49.(Medline)

Kornberg, R.D. and Lorch, Y. (1999) Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome, *Cell* 98:285-294.(Medline)

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M.J., Fenton, W. and Reed, J.C. (1993) Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes, *Cancer Res.* 53:4701-4714.(Medline)

Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. and Flavell, R.A. (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice, *Nature* 384:368-372.(Medline)

Kuro-o, M., Matsumura, Y., Aizawa, H., Kawaguchi, H., Suga, T., Utsugi, T., Ohyama, Y., Kurabayashi, M., Kaname, T., Kume, E., Iwasaki, H., Iida, A., Shiraki-Iida, T., Nishikawa, S., Nagai, R., Nabeshima, Y.I. (1997) Mutation of the mouse klotho gene leads to a syndrome resembling ageing, *Nature* 390:45-51.(Medline)

Kuwana, T., Mackey, M.R., Perkins, G., Ellisman, M.H., Latterich, M., Schneiter, R., Green, D.R. and Newmeyer, D.D. (2002) Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane, *Cell* 111:331-342. (MedLine)

Kyrion, G., Boakye, K.A. and Lustig, A.J. (1992) C-terminal truncation of RAD1 results in the deregulation of telomere size, stability and function *Saccharomyces cerevisiae*, *Mol. Cell. Biol.* 12:5159-5173.(Medline)

Kyrion, G., Liu, K., Liu, C. and Lustig, A.J. (1993) RASP1 and telomere structure regulate telomere position effect in *Saccharomyces cerevisiae*, *Genes Dev.* 7:1146-1159.(Medline)

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. and Jenuwein, T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins, *Nature* 410:116-20. (MedLine)

Lamb, M.M.and Daneholt, B. (1979) Characterization of active transcription units in Balbiani rings of *Chironomus tentans*, *Cell* 17:835-848.(Medline)

Längst, G., Bonte, E.J., Corona, D.F. and Becker, P.B. (1999) Nucleosome movement by CHRAC and ISWI without disruption or trans-displacement of the histone octamer, *Cell* 97:843-852.(Medline)

Lansdorp, P.M. (1997) Lessons for mice without telomerase, *J. Cell Biol.* 139:309-312.(Medline)

Larsen, P.L., Albert, P.S. and Riddle, D.L. (1995) Genes that regulate both development and longevity in *Caenorhabditis elegans* , *Genetics* 139:1567-1583.(Medline)

Laskey, R.A. and Gurdon, J.B. (1970) Genetic content of adult somatic cells tested by nuclear transplantation from cultured cells, *Nature* 228:1332-1334.(Medline)

Lee, C.-K., Klopp, R.G., Weindruch, R. and Prolla, T.A. (1999) Gene expression profile of aging and its retardation by caloric restriction, *Science* 285:1390-1393.(Medline)

Lee, D.Y., Hayes, J.J., Pruss, D. and Wolffe, A.P. (1993) A positive role of histone acetylation in transcription factor access to nucleosome DNA, *Cell* 72: 73-84.(Medline)

Lee, M.S., Gallagher, R.C., Bradley, J. and Blackburn, E.H. (1993) *In vivo* and *in vitro* studies of telomeres and telomerase, *Cold Spring Harb. Symp. Quant. Biol.* 58:707-718.(Medline)

Lee, C.M,. Weindruch, R. and Aiken, J.M. (1997) Age-associated alterations of the mitochondrial genome, *Free Radic. Biol. Med.* 22:1259-1269.(Medline)

Lee, S.S., Lee, R.Y., Fraser, A.G., Kamath, R.S., Ahringer, J. and Ruvkun, G. (2003) A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity, *Nature Genet.* 33:40-48. (MedLine)

Leist, M., Single, B., Castoldi, A.F., Kuhnle, S. and Nicotera, P. (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis, *J. Exp. Med.* 185:1481-1486. (MedLine)

Leist, M. and Jäättelä, M. (2001) Four deaths and a funeral: from caspases to alternative mechanisms, *Nature Rev. Mol. Cell Biol.* 2:589-598. (MedLine)

Leitch, A.R. (2000) Higher levels of organization in the interphase nucleus of cycling and differentiated cells, *Microbiol. Mol. Biol. Rev.* 64:138-152. (MedLine)

Lemasters, J.J., Nieminen, A.L., Qian, T., Trost, L.C., Elmore S.P., Nishimura, Y., Crowe, R.A., Cascio, W.E., Bradham, C.A., Brenner, D.A., Herman, B. (1998) The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy, *Biochim. Biophys. Acta* 1366:177-196.(Medline)

Le-Niculescu, H., Bonfoco, E., Kasuya, Y., Claret, F.X., Green, D.R. and Karin, M. (1999) Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death, *Mol. Cell Biol.* 19:751-763.(Medline)

Levsky, J.M., Shenoy, S.M., Pezo, R.C. and Singer, R.H. (2002) Single-cell gene expression profiling, *Science* 297:836-840. (MedLine)

Lewis, E.B. (1978) A gene complex controlling segmentation in *Drosophila*, *Nature* 276:565-570.(Medline)

Li, E., Beard, C. and Jaenisch, R. (1993) Role of DNA methylation in genomic imprinting, *Nature* 366:362-365.(Medline)

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade, *Cell* 91:479-489.(Medline)

Li, G.-M. (1999) The role of mismatch repair in DNA damage-induced apoptosis, *Oncol. Res.* 11:393-400. (MedLine)

Li, M., Ona, V.O., Guegan, C., Chen, M., Jackson-Lewis, V., Andrews, L.J., Olszewski, A.J., Stieg, P.E., Lee, J.P., Przedborski, S. and Friedlander, R.M.. (2000a) Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model, *Science* 288:335-339. (MedLine)

Li, B., Oestreich, S. and de Lange, T. (2000b) Identification of human Rap1: implications for telomere evolution, *Cell* 101:471-483. (MedLine)

Li, H., Kolluri, S.K., Gu, J., Dawson, M.I., Cao, X., Hobbs, P.D., Lin, B., Chen, G., Lu, J., Lin, F., Xie, Z., Fontana, J.A., Reed, J.C. and Zhang, X (2000c) Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3, *Science* 289:1159-1164. (MedLine)

Li, L.Y., Luo, X. and Wang, X. (2001) Endonuclease G is an apoptotic DNase when released from mitochondria, *Nature* 412:95-99. (MedLine)

Lin, R., Cook, R.G. and Allis, C.D. (1991) Proteolytic removal of core histone amino termini and dephosphorylation of histone H1 correlate with the formation of condensed chromatin and transcriptional silencing during *Tetrahymena* macronuclear development, *Gen. Dev.* 5:1601-1610.(Medline)

Lin, S.J., Defossez, P.A. and Guarente L. (2000) Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*, *Science* 289:2126-2128. (MedLine)

Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c, *Cell* 86:147-157.(Medline)

Liu, X., Zou, H., Slaughter, C. and Wang, X. (1997) DFF, a herodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis, *Cell* 89:175-184.(Medline)

Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W.T., and Wang, X. (1998) The 40 kDa subunit DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis, *Proc. Natl. Acad. Sci. USA* 95:8461-8466.(Medline)

Locksley, R.M., Killeen, N. and Lenardo, M.J. (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology, *Cell* 104:487-501. (MedLine)

Lorch, Y., Zhang, M. and Kornberg, R.D. (1999) Histone octamer transfer by a chromatin-remodeling complex, *Cell* 96:389-392.(Medline)

Los, M., Wesselborg, S. and Schulze-Osthoff, K. (1999) The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice, *Immunity* 10:629-639. (MedLine)

Luger, K., Mäder, A.W., Richmond, R.K., Sargent, D.F. and Richmond, T.J. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution, *Nature* 389:251-260.(Medline)

Lundblad, V. and Szostak, J.W. (1989) A mutant with a defect in telomere elongation leads to senescence in yeast, *Cell* 57:633-643.(Medline)

Lyko, F., Ramsahoye, B.H. and Jaenisch, R. (2000) DNA methylation in *Drosophila melanogaster*, *Nature* 408:538-539.

Maderdrut, J.L., Oppenheim, R.W. and Prevette, D. (1988) Enhancement of naturally occurring cell death in the sympathetic and parasympathetic ganglia of the chicken embryo following blockade of ganglionic transmission, *Brain Res.* 444:189-194.(Medline)

Majdan, M. and Miller, F.D. (1999) Neuronal life and death decisions functional antagonism between the Trk and p75 neurotrophin receptors, *Int. J. Dev. Neurosci.* 17:153-161.(Medline)

Mancini, M., Machamer, C.E., Roy, S., Nicholson, D.W., Thornberry, N.A., Casciola-Rosen, L.A. and Rosen, A. (2000) Caspase-2 is localized at the Golgi complex and cleaves golgin-160 during apoptosis, *J. Cell Biol.* 149:603-612. (MedLine)

Manuelidis, L. and Borden, J. (1988) Reproducible compartmentalization of individual chromosome domains in human CNS cells revealed by in situ hybridization and three-dimensional reconstruction, *Chromosoma* 96:397-410. (MedLine)

Marcand, S. Gilson, E. and Shore, D. (1997) A protein counting mechanism for telomere length in yeast, *Science* 275:986-991.(Medline)

Marciniak, R.A., Lombard, D.B., Johnson, F.B. and Guarente, L. (1998) Nucleolar localization of the Werner syndrome protein in human cells, *Proc. Natl. Acad. Sci. USA* 95:6887-6892.(Medline)

Marklund, S.L., Westman, N.G., Lundgren, E. and Roos, G. (1982) Copper- and zinc-containing superoxide dismutase, manganese-containing superoxide dismutase, catalase, and glutathione peroxidase in normal and neoplastic human cell lines and normal human tissues, *Cancer Res.* 42:1955-1961. (MedLine)

Martin, L.J., Price, A.C., Kaiser, A., Shaikh, A.Y. and Liu, Z. (2000) Mechanisms for neuronal degeneration in amyotrophic lateral sclerosis and in models of motor neuron death, *Int. J. Mol. Med.* 5:3-13. (MedLine)

Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J.M., Susin, S.A., Vieira, H.L.A., Prevost, M.C., Xie, Z., Matsuyama, S., Reed, J.C. and Kroemer, G. (1998) Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis, *Science* 281:2027-2031.(Medline)

Mathon, N.F., Malcolm, D.S., Harrisingh, M.C., Cheng, L. and Lloyd, A.C. (2001) Lack of replicative senescence in normal rodent glia, *Science* 291:872-875. (MedLine)

Mattson, M.P. and Duan, W. (1999) "Apoptotic" biochemical cascades in synaptic compartments: roles in adaptive plasticity and neurodegenerative disorders, *J. Neurosci. Res.* 58:152-166. (MedLine)

McKay, R. (2000) Stem cells--hype and hope,*Nature* 406:361-364. (MedLine)

Melov, S., Shoffner, J.M., Kaufman, A. and Wallace, D.C. (1995) Marked increase in the number and variety of mitochondrial DNA rearrangements in aging human skeletal muscle, *Nucleic Acids Res.* 23:4122-4126.(Medline)

Melov, S., Schneider, J.A., Day, B.J., Hinerfeld, D., Coskun, P., Mirra, S.S., Crapo, J.D. and Wallace, D.C. (1998) A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase, *Nature Genet.* 18:159-163.(Medline)

Meyer-Franke, A., Wilkinson, G.A., Kruttgen, A., Hu, M., Munro, E., Hanson, M.G. Jr., Reichardt, L.F. and Barres, B.A. (1998) Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons, *Neuron* 21:681-693.(Medline)

Mezey, E., Chandross, K.J., Harta, G., Maki, R.A. and McKercher, S.R. (2000) Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow, *Science* 290:1779-1782. (MedLine)

Michikawa, Y., Mazzucchelli, F., Bresolin, N., Scarlato, G. and Attardi, G. (1999) Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication, *Science* 286:774-779.(Medline)

Migliaccio, E., Mele, S., Salcini, A.E., Pelicci, G., Lai, K.M., Superti-Furga, G., Pawson, T., Di Fiore, P.P., Lanfrancone, L. and Pelicci, P.G. (1997) Opposite effects of the $p52^{shc}$/$p46^{shc}$ and $p66^{shc}$ splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway, *EMBO J.* 16:706-716.(Medline)

Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P.P., Lanfrancone, L. and Pelicci, P.G. (1999) The p66shc adaptor protein controls oxidative stress response and life span in mammals, *Nature* 402:309-313.(Medline)

Miller, L.K. (1999)An exegesis of IAPs: salvation and surprises from BIR motifs, *Trends Cell Biol.* 9:323-328. (MedLine)

Mills, J.C., Stone, N.L. and Pittman, R.N. (1999) Extranuclear apoptosis. The role of the cytoplasm in the execution phase, *J. Cell Biol.* 146:703-708.(Medline)

Miyashita, T. and Reed, J.C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene, *Cell* 80:293-299.(Medline)

Monk, M.. (1986) Methylation and the X chromosome, BioEssays 4:204-208. (MedLine)

Monk, M., Boubelik, M. and Lehnert, S. (1987) Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development, *Development* 99:371-382.(Medline)

Morales, C.P., Holt, S.E., Ouellette, M., Kaur, K.J., Yan, Y., Wilson, K.S., White, M.A., Wright, W.E. and Shay, J.W. (1999) Absence of cancer-associated changes in human fibroblasts immortalized with telomerase, *Nature Genet.* 21:115-118.(Medline)

Moreira, J.M.A. and Holmberg, S. (1999) Transcriptional repression of the yeast CHA1 gene requires the chromatin-remodeling

complex RSC, *EMBO J.* 18:2836-2844.(Medline)

Morio, T., Ozaki, T., Takeuchi, I. and Tasaka, M. (1991) Transcriptional regulation of cell-type-enriched genes during development in *Dictyostelium discoideum* analyzed by nuclear run-on assays, *Develop. Growth and Diff.* 33:293-298.

Morse, R.H. (1992) Transcribed chromatin, *Trends in Biochem. Sci.*17:23-26.(Medline)

Muchardt, C. and Yaniv, M. (1999) The mammalian SWI/SNF complex and the control of cell growth, *Semin. Cell Dev. Biol.* 10:189-195.(Medline)

Muller, I., Zimmermann, M., Becker, D. and Flomer, M. (1980) Calendar life span versus budding life span of *Saccharomyces cerevisiae*, *Mech. Ageing Dev.* 12:47-52.(Medline)

Müller, J. and Bienz, M. (1991) Long range repression conferring boundaries of *Ultrabithorax* expression in *Drosophila* embryo, *EMBO J.* 10:3147-3156.(Medline)

Müller, J. and Beinz, M. (1992) Sharp anterior boundary of homeotic gene expression conferred by the *fushi tarazu* protein, *EMBO J.* 10:3147-3155.(Medline)

Murphy, D.J., Hardy, S. and Engel, D.A. (1999) Human SWI-SNF component BRG1 represses transcription of the c-fos gene, *Mol. Cell. Biol.* 19:2724-2733.(Medline)

Nagata, S. (1997) Apoptosis by death factor, *Cell* 88:355-365.(Medline)

Nagata, S. (2000) Apoptotic DNA fragmentation, *Exp. Cell Res.* 256:12-18. (MedLine)

Nagata, S. and Golstein, P. (1995) The Fas death factor, *Science* 267:1449-1456. (MedLine)

Nakagawa, T., Zhu, H., Morishima, N., Li E., Xu, J., Yankner, B.A. and Yuan, J. (2000) Caspase-12 mediates endoplasmic reticulum-specific apoptosis and cytotoxicity by amyloid-β, *Nature* 403:98-103.(Medline)

Nakamura, N., Lowe, M., Levine, T.P., Rabouille, C. and Warren, G. (1997) The vesicle docking protein p115 binds GM130, a cis-Golgi matrix protein, in a mitotically regulated manner, *Cell* 89:445-455. (MedLine)

Nakano, K. and Vousden, K.H. (2001) *PUMA*, a novel proapoptotic gene induced by p53, *Mol. Cell* 7:683-694.

Nan, X., Ng, H.H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N., Bird, A. (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex, *Nature* 393:386-389.(Medline)

Newmeyer, D.D., Farschon, D.M. and Reed, J.C. (1994) Cell-free apoptosis in Xenopus egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria, *Cell* 79:353-364.(Medline)

Noll, M. (1974) Subunit structure of chromatin, *Nucleic Acid Res.* 1:1573-1578.(Medline)

Noma, K., Allis, C.D. and Grewal, S.I. (2001) Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries, *Science* 293:1150-1155. (MedLine)

Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T., Hanada, H. and Perry, A.C. (2000) Pig cloning by microinjection of fetal fibroblast nuclei, *Science* 289:1188-1190. (MedLine)

Okabe, M., Imai, T., Kurusu, M., Hiromi, Y. and Okano, H. (2001) Translational repression determines a neuronal potential in

Drosophila asymmetric cell division, *Nature* 411:94-98. (MedLine)

Okada, H., Suh, W.K., Jin, J., Woo, M., Du, C., Elia, A., Duncan, G.S., Wakeham, A., Itie, A., Lowe, S.W., Wang, X. and Mak, T.W. (2002) Generation and characterization of Smac/DIABLO-deficient mice, *Mol. Cell. Biol* 22:3509-3517. (MedLine)

Okano, M., Xie, S. and Li, E. (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases, *Nature Genet.*19:219-220. (MedLine)

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S.M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D.M., Leri, A. and Anversa, P. (2001) Bone marrow cells regenerate infarcted myocardium, *Nature* 410:701-705. (MedLine)

Oliva, R. Bazett-Jones, D.P., Locklear, L and Dixon, G.H. (1990) Histone hyperacetylation can induce unfolding of the nucleosome core particle, *Nucleic Ac. Res.* 18:2739-2747.(Medline)

Oliver, S.G., Winson, M.K., Kell, D.B. and Baganz, F. (1998) Systematic functional analysis of the yeast genome, *Trends Biotechnol.* 16:373-378.(Medline)

Oltvai, Z.N., Milliman, C.L.and Korsmeyer, S.J. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death, *Cell* 74:609-619.(Medline)

Ona, V.O., Li, M., Vonsattel, J.P.G., Andrews, L.J., Khan, S.Q., Chung, W.M., Frey, A.S., Menon, A.S., Li, X.-J., Stieg, P.E., Yuan, J., Penney, J.B., Young, A.B., Cha, J.-H.J. and Friedlander, R.M. (1999) Inhibition of caspase-1 slows disease progression in mouas model of Huntington's disease, *Nature* 399:263-267.(Medline)

Oppenheim, R.W. (1991) Cell death during development of the nervous system, *Annu. Rev. Neurosci.* 14:453-501.(Medline)

Orr, W.C. and Sohal, R.S. (1994) Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster Science* 263:1128-1130.(Medline)

Orth, K., Chinnaiyan, A.M., Garg, M., Froelich, C.J. and Dixit, V.M. (1996) The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A, *J. Biol. Chem.* 271:16443-16446.(Medline)

Osawa, M., Hanada, K., Hamada, H. and Nakauchi, H. (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell, *Science* 273:242-245. (MedLine)

Osborne, B.A. (1996) Apoptosis and the maintenance of homoeostasis in the immune system, *Curr. Opin. Immunol.* 8:245-254.(Medline)

Packer, L. and Fuehr, K. (1977) Low oxygen concentration extends the lifespan of cultured human diploid cells, *Nature* 267:423-425. (MedLine)

Parkes, T.L., Elia, A.J., Dickinson, D., Hilliker, A.J., Phillips, J.P. and Boulianne, G.L. (1998) Extension of *Drosophila* lifespan by overexpression of human SOD1 in motorneurons, *Nature Genet.* 19:171-174.(Medline)

Parkes, T.L., Kirby, K., Phillips, J.P. and Hilliker, A.J. (1998b) Transgenic analysis of the cSOD-null phenotypic syndrome in Drosophila, *Genome* 41:642-651.(Medline)

Pavlov, E.V., Priault, M., Pietkiewicz, D., Cheng, E.H., Antonsson, B., Manon, S., Korsmeyer, S.J., Mannella, C.A. and Kinnally, K.W. (2001) A novel, high conductance channel of mitochondria linked to apoptosis in mammalian cells and Bax expression in yeast, *J. Cell Biol.* 155:725-731. (MedLine)

Parrish, J., Li, L., Klotz, K., Ledwich, D., Wang, X. and Xue, D. (2001) Mitochondrial endonuclease G is important for apoptosis in *C.*

elegans Nature 412:90-94. (MedLine)

Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T. and Pelicci, P.G. (1992) A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction, *Cell* 70:93-104.(Medline)

Petit, P.X., Susin, S.A., Zamzami, N., Mignotte, B. and Kroemer, G. (1996) Mitochondria and programmed cell death: back to the future, *FEBS Lett.* 396:7-13.(Medline)

Pettmann, B. and Henderson, C.E. (1998) Neuronal cell death, *Neuron* 20:633-647.(Medline)

Phair, R.D. and Misteli, T. (2000) High mobility of proteins in the mammalian cell nucleus, *Nature* 404:604-609. (MedLine)

Pham, A.-D. and Sauer, F. (2000) Ubiquitin-activating/conjugating activity of TAFII250, a mediator of activation of gene expression in *Drosophila Science* 289:2357-2360. (MedLine)

Pienta, K.J., Getzenberg, R.H. and Coffey, D.S. (1991) Cell structure and DNA organization, *CRC Crit. Rev. Eukaryotic Gene Expr.* 1:355-385.(Medline)

Pikaart, M.J., Recillas-Targa, F. and Felsenfeld, G. (1998) Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators, *Genes Dev.* 12:2852-2862.(Medline)

Porter, A.G., Ng, P. and Jänicke, R.U. (1997) Death substrates come alive, *BioEssays* 19:501-507.(Medline)

Prives," C. and Hall, P.A. (1999) The p53 pathway, *J. Pathol.* 187:112-126. (MedLine)

Proffitt, J.H., Davie, J.R., Swinton, D. and Hattman, S. (1984) 5-Methylcytosine is not detectable in *Saccharomyces cerevisiae* DNA, *Mol. Cell. Biol.* 4:985-988. (MedLine)

Raper, K.B. (1940) Pseudoplasmodium formation and organization in Dictyostelium discoideum, *J. Elisha Mitchell Soc.* 56:241-182.

Raveh, T., Droguett, G., Horwitz, M.S., DePinho, R.A. and Kimchi, A. (2001) DAP kinase activates a p19ARF/p53-mediated apoptotic checkpoint to suppress oncogenic transformation, *Nature Cell Biol.* 3:1-7. (MedLine)

Ray-Gallet, D., Quivy, J.P., Scamps, C., Martini, E.M., Lipinski, M. and Almouzni, G. (2002) HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis, *Mol. Cell* 9:1091-1100. (MedLine)

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M. and Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D. and Jenuwein, T. (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases, *Nature* 406:593-599. (MedLine)

Reaume, A.G., Elliott, J.L., Hoffman, E.K., Kowall, N.W., Ferrante, R.J., Siwek, D.F., Wilcox, H.M., Flood, D.G., Beal, M.F., Brown, R.H. Jr, Scott, R.W. and Snider, W.D. (1996) Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury, *Nature Genet.* 13:43-47.(Medline)

Reddien, P.W., Cameron, S. and Horvitz, H.R. (2001) Phagocytosis promotes programmed cell death in *C. elegans*, *Nature* 412:198-202. (MedLine)

Reed, J.C., Jurgensmeier, J.M. and Matsuyama, S. (1998) Bcl-2 family proteins and mitochondria, *Biochim Biophys Acta* 1366:127-137.(Medline)

Rhodes, D. (1997) The nucleosome core all wrapped up, *Nature* 389:231-233.(Medline)

Richter, C., Park, J.W. and Ames, B.N. (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive, *Proc. Natl. Acad. Sci. USA* 85:6465-6467.(Medline)

Rietze, R.L., Valcanis, H., Brooker, G.F., Thomas, T., Voss, A.K. and Bartlett, P.F. (2001) Purification of a pluripotent neural stem cell from the adult mouse brain, *Nature* 412:736-739. (MedLine)

Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W.J. and Jäckle, H. (1996) RNA binding and translational suppression of bicoid, *Nature* 379:746-749.(Medline)

Robzyk, K., Recht, J., Osley, M.A. (2000) Rad6-dependent ubiquitination of histone H2B in yeast, *Science* 287:501-504. (MedLine)

Rodriguez-Leon, J., Merino, R., Macias, D., Ganan, Y., Santesbatan, E. and Hurle, J.M. (1999) Retinoic acid regulates programmed cell death through BMP signalling, *Nature Cell Biol.* 1:125-126.(Medline)

Rodriguez, A., Chen, P., Oliver, H. and Abrams, J.M. (2002) Unrestrained caspase-dependent cell death caused by loss of Diap1 function requires the Drosophila Apaf-1 homolog, Dark, *EMBO J.* 21:2189-2197. (MedLine)

Rogina, B., Reenan, R.A., Nilsen, S.P. and Helfand, S.L. (2000) Extended life-span conferred by cotransporter gene mutations in *Drosophila*, *Science* 290:2137-2140. (Medline)

Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D,. Goto, J., O'Regan, J.P., Deng, H.X., et al. (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis, *Nature* 362:59-62. (MedLine)

Rudolph, L.R., Chang, S., Lee, H.-W., Blasco, M., Gottlieb, G.J., Greider, C. and DePinho, R.A. (1999) Longevity, stress response, and cancer in aging telomerase-deficient mice, *Cell* 96:701-712.(Medline)

Sadoni, N., Langer, S., Fauth, C., Bernardi, G., Cremer, T., Turner, B.M. and Zink, D. (1999) Nuclear organization of mammalian genomes. Polar chromosome territories build up functionally distinct higher order compartments, J. Cell Biol. 146:1211-1226. (MedLine)

Saha, A., Wittmeyer, J. and Cairns, B.R. (2002) Chromatin remodeling by RSC involves ATP-dependent DNA translocation, *Genes Dev.* 16:2120-2134. (MedLine)

Sahara, S., Aoto, M., Eguchi, Y., Imamoto, N., Yoneda, Y. and Tsujimoto, Y. (1999) Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation, *Nature* 401:168-173.(Medline)

Saibil, H. (2000) Molecular chaperones: containers and surfaces for folding, stabilising or unfolding proteins, *Curr. Opin. Struct. Biol.* 10:251-258. (MedLine)

Sakahira, H., Enari, M. and Nagata, S. (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis, *Nature* 391:96-99.(Medline)

Saleh, A., Srinivasula, S.M., Acharya, S., Fishel, R. and Alnemri, E.S. (1999) Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation, *J. Biol. Chem.* 274:17941-17945. (MedLine)

Saleh, A., Srinivasula, S.M., Balkir, L., Robbins, P.D. and Alnemri, E.S. (2000) Negative regulation of the apaf-1 apoptosome by hsp70, *Nature Cell Biol.* 2:476-483. (MedLine)

Salvesen, G.S. and Duckett, C.S. (2002) IAP proteins: blocking the road to death's door, *Nature Rev. Mol. Cell Biol.* 3:401-410. (MedLine)

Samali, A., Cai, J., Zhivotovsky, B., Jones, D.P. and Orrenius, S. (2000) Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of jurkat cells, *EMBO J.* 18:2040-2048. (MedLine)

Samejima, K., Tone, S., Kottke, T.J., Enari, M., Sakahira, H., Cooke, C.A., Durrieu, F., Martins, L.M., Nagata, S., Kaufmann, S.H. and Earnshaw, W.C. (1998) Transition from caspase-dependent to caspase-independent mechanisms at the onset of apoptotic execution, *J. Cell Biol.* 143:225-239.(Medline)

Sandell, L.L. and Zakian, V.A. (1993) Loss of a yeast telomere: arrest, recovery, and chromosome loss, *Cell* 75:729-739.(Medline)

Sassone-Corsi, P., Mizzen, C.A., Cheung, P., Crosio, C., Monaco, L., Jacquot, S., Hanauer, A. and Allis, C.D. (1999) Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3, *Science* 285:886-891. (MedLine)

Savill J. and Fadok, V. (2000) Corpse clearance defines the meaning of cell death, *Nature* 407:784-788. (MedLine)

Scaffidi, C., Schmitz, I., Zha, J., Korsmeyer, S.J., Krammer, P.H. and Peter, M.E. (1999) Differential modulation of apoptosis sensitivity in CD95 type I and type II cells, *J. Biol. Chem.* 274:22532-22538.(Medline)

Schwarze, S.R., Lee, C.M., Chung, S.S., Roecker, E.B., Weindruch, R. and Aiken, J.M. (1995) High levels of mitochondrial DNA deletions in skeletal muscle of old rhesus monkeys, *Mech. Ageing Dev.* 83:91-101.(Medline)

Sciacco, M., Bonilla, E., Schon, E.A., DiMauro, S. and Moraes, C.T. (1994) Distribution of wild-type and common deletion forms of mtDNA in normal and respiration-deficient muscle fibers from patients with mitochondrial myopathy, *Hum. Mol. Genet.* 3:13-19.(Medline)

Sedivy, J.M. (1998) Can ends justify the means?: telomeres and the mechanisms of replicative senescence and immortalization in mammalian cells, *Proc. Natl. Acad. Sci. USA* 95:9078-9081.(Medline)

Sedgwick, S.G. and Smerdon, S.J. (1999) The ankyrin repeat: a diversity of interactions on a common structural framework, *Trends Biochem. Sci.* 24:311-316.(Medline)

Sekinger, E.A. and Gross, D.S. (2001) Silenced chromatin is permissive to activator binding and PIC recruitment, *Cell* 105:403-414. (MedLine)

Shaham, S. and Horvitz, H.R. (1996) Developing *Caenorhabditis elegans* neurons may contain both cell-death protective and killer activities, *Genes Dev.* 10:578-591.(Medline)

Shay, J.W. and Wright, W.E. (2001) When do telomeres matter? *Science* 291:839-841.

Shen, C.N., Slack, J.M. and Tosh, D. (2000) Molecular basis of transdifferentiation of pancreas to liver, *Nature Cell Biol.* 2:879-887. (MedLine)

Shimizu, S., Narita, M. and Tsujimoto, Y. (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC, *Nature* 399:483-487.(Medline)

Shore, D. (1997) Telomere length regulation: getting the measure of chromosome ends, *Biol. Chem.* 378:591-597. (MedLine)

Shulyakovskaya, T., Sumegi, L. and Gal, D. (1993) *In vivo* experimental studies on the role of free radicals in photodynamic therapy. I.Measurement of the steady state concentration of free radicals in tumor tissues of mice, *Biochem. Biophys. Res. Commun.* 195:581-587. (MedLine)

Siegfried, Z. and Cedar, H. (1997) DNA methylation: a molecular lock, *Curr. Biol.* 7:R305-R307.(Medline)

Signer, E.N., Dubrova, Y.E., Jeffreys, A.J., Wilde, C., Finch, L.M.B., Wells, M. and Peaker, M. (1998) DNA fingerprinting of Dolly, *Nature* 394:329-330.(Medline)

Simpson, R.T. (1991) Nucleosomes positioning; occurrence, mechanisms and functional consequences, *Prog. Nucleic Ac. Res. Mol. Biol.*40:143-184.(Medline)

Simpson, V.J., Johnson, T.E. and Hammen, R. F. (1986) *Caenorhabditis elegans* DNA does not contain 5-methylcytosine at any time during development or aging, *Nucleic Acids Res.* 14: 6711-6719. (MedLine)

Sinclair, D.A. and Guarente, L. (1997) Extrachromosomal rDNA circles--a cause of aging in yeast, *Cell* 91:1033-1042.(Medline)

Sinclair, D.A., Mills, K. and Guarente, L. (1997) Accelerated aging and nucleolar fragmentation in yeast sgs1 mutants, *Science* 277:1313-1316.(Medline)

Slack, J.M.W.(1991) *From Egg to Embryo: Regional Specification in Early Development*, Cambridge University Press, New York.

Slack, J.M. and Tosh, D. (2001) Transdifferentiation and metaplasia--switching cell types, *Curr. Opin. Genet. Dev.* 11:581-586. (MedLine)

Smith, C.D. and Blackburn, E.H. (1999) Uncapping and deregulation of telomeres lead to detrimental cellular consequences in yeast, *J. Cell Biol.* 145:203-214.(Medline)

Smith, J.R. and Whitney, R.G. (1980) Intraclonal variation in proliferative potential of human diploid fibroblasts: stochastic mechanism for cellular aging, *Science* 207:82-84. (MedLine)

Smith, S., Giriat, I., Schmitt, A. and de Lange, T. (1998) Tankyrase, a poly(ADP-ribose) polymerase of human telomeres, *Science* 282:1484-1487.(Medline)

Smith, A.G. (2001) Embryo-derived stem cells: of mice and men, *Annu. Rev. Cell Dev Biol.* 17:435-462. (MedLine)

Snyder, E.Y., Taylor, R.M. and Wolfe, J.H. (1995) Neural progenitor cell engraftment corrects lysosomal storage throughout the MPS VII mouse brain, *Nature* 374:367-370. (MedLine)

Soengas, M.S., Alarcón, R.M., Yoshida, H., Giaccia, A.J., Hakem, R., Mak, T.W. and Lowe, S.W. (1999) Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition, *Science* 284:156-159. (MedLine)

Soengas, M.S., Capodieci, P., Polsky, D., Mora, J., Esteller, M., Opitz-Araya, X., McCombie, R., Herman, J.G., Gerald, W.L., Lzabnikm Y.A., Cardón-Cardó, C. and Lowe, S.W. (2001) Inactivation of the apoptotic effector *Apaf-1* in malignant melanoma, *Nature* 409:207-210.

Sohal, R.S., Ku, H.H., Agarwal, S., Forster, M.J. and Lal, H. (1994) Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse, *Mech. Ageing Dev.* 74:121-133.(Medline)

Sonnichsen, B., Lowe, M., Levine, T., Jamsa, E., Dirac-Svejstrup, B. and Warren, G. (1998) A role for giantin in docking COPI vesicles to Golgi membranes, *J. Cell Biol.* 140:1013-1021. (MedLine)

Spencer, T.E., Jenster, G., Burcin, M.M., Allis, C.D., Zhou, J., Mizzen, C.A., McKenna, N.J., Onate, S.A., Tsai, S.Y., Tsai, M.-J. and O'Malley, B.W. (1997) Steroid receptor coactivator-1 is a histone acetyltransferase, *Nature* 389:194-198.(Medline)

Sperandio, S., de Belle, I. and Bredesen, D.E. (2000) An alternative, nonapoptotic form of programmed cell death, *Proc. Natl. Acad. Sci. USA* 97:14376-14381. (MedLine)

Stanovejic, D., Small, S. and Levine, M. (1991) Overlapping gradients of transcriptional activators and repressors specify a pair-rule stripe in the *Drosophila* embryo, *Science* 254:1385-1387.

Stennicke, H.R., Ryan, C.A. and Salvesen, G.S. (2002) Reprieval from execution: the molecular basis of caspase inhibition, *Trends Biochem. Sci.*27:94-101. (MedLine)

Strahl, B.D. and Allis, C.D. (2000) The language of covalent histone modification, *Nature* 403:41-45.(Medline)

Strehler, B.L. (1986) Genetic instability as the primary cause of human aging, *Exp. Gerontol.* 21:283-319.(Medline)

Struhl, K. (1998) Histone acetylation and transcriptional regulatory mechanisms, *Genes Dev.* 12:599-606.(Medline)

Strutt, H. and Paro, R. (1997) The polycomb group protein complex of Drosophila melanogaster has different compositions at different target genes, *Mol. Cell. Biol.* 17:6773-6783. (MedLine)

Suda, Y., Suzuki, M., Ikawa, Y. and Aizawa, S. (1987) Mouse embryonic stem cells exhibit indefinite proliferative potential, *J. Cell Physiol.* 133:197-201. (MedLine)

Sun, Z.W. and Allis, C.D. (2002) Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast, *Nature* 418:104-108. (MedLine)

Susin, S.A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M. and Kroemer, G. (1996) Bcl-2 inhibits the mitochondrial release of an apoptogenic protease, *J. Exp. Med.* 184:1331-1341.(Medline)

Susin, S.A., Loreenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jocotot, E., Constantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. and Kroemer, G. (1999a) Molecular characterization of mitochodrial apoptosis-inducing factor, *Nature* 397:441-446.(Medline)

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., Prevost, M.C., Alzari, P.M. and Kroemer, G. (1999b) Mitochondrial release of caspase-2 and -9 during the apoptotic process, *J. Exp. Med.* 189:381-394.(Medline)

Suzuki, N., Shimamoto, A., Imamura, O., Kuromitsu, J., Kitao, S., Goto, M. and Furuichi, Y. (1997) DNA helicase activity in Werner's syndrome gene product synthesized in a baculovirus system, *Nucleic. Acids Res.* 25:2973-2978.(Medline)

Swain, J.L., Stewart, T.A. and Leder, P. (1987) Parental legacy determines methylation and expression of autosomal transgene: a molecular mechanism for parental imprinting, *Cell* 50:719-727.(Medline)

Szilard, L. (1959) On the nature of the aging process, *Proc. Natl. Acad. Sci. USA* 45:30-45.

Takahashi, A., Alnemri, E.S., Lazebnik, Y.A., Fernandes-Alnemri, T., Litwack, G., Moir, R.D., Goldman, R.D., Poirier, G.G., Kaufmann, S.H., Earnshaw, W.C. (1996) Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 β-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis, *Proc. Natl. Acad. Sci. USA* 93:8395-8400.(Medline)

Tamaru, H. & Selker, E. U. (2001) A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa Nature* 414: 277-283. (MedLine)

Tang, D.G., Tokumoto, Y.M., Apperly, J.A., Lloyd, A.C. and Raff, M.C. (2001) Lack of replicative senescence in cultured rat oligodendrocyte precursor cells, *Science* 291:868-871. (MedLine)

Tao, W., Kurschner, C. and Morgan, J.I. (1998) Bcl-xS and bad potentiate the death suppressing activities of bcl-xL, bcl-2, and A1 in yeast, *J. Biol. Chem.* 273:23704-23708.(Medline)

Tatar, M., Kopelman, A., Epstein, D., Tu, M.P., Yin, C.M. and Garofalo, R.S. (2001) A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function, *Science* 292:107-110. (MedLine)

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D.M., Nakano, Y., Meyer, E.M., Morel, L., Petersen, B.E. and Scott, E.W. (2002) Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion, . *Nature* 416:542-545. (MedLine)

Tessier-Lavigne, M., Placzek, M., Lumsden, A.G., Dodd, J. and Jessell, T.M. (1988) Chemotropic guidance of developing axons in the mammalian central nervous system, *Nature* 336):775-778.(Medline)

Thoma, F., Koller, T. and Klug, A. (1979) Involvement of histone H1 in the organization of the nucleosome and the salt dependent superstructures of chromatin, *J. Cell Biol.* 83:403-427.(Medline)

Thompson, C.B. (1995) Apoptosis in the pathogenesis and treatment of disease, *Science* 267:1456-1462.(Medline)

Thomson, S., Clayton, A.L., Hazzalin, C.A., Rose, S., Barratt, M.J. and Mahadevan, L.C. (1999) The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase, *EMBO J.* 18:4779-4793. (MedLine)

Thornberry, N.A. and Lazebnik, Y. (1998) Caspases: enemies within, *Science* 281:1312-1316.(Medline)

Thornberry, N.A., Rosen, A. and Nicholson, D.W. (1997a) Control of apoptosis by proteases, *Adv. Pharmacol.* 41:155-177.(Medline)

Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T. and Nicholson, D.W. (1997b) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis, *J. Biol. Chem.* 272:17907-17911. (MedLine)

Tissenbaum, H.A. and Ruvkun, G. (1998) An insulin-like signaling pathway affects both longevity and reproduction in *Caenorhabditis elegans Genetics*148:703-717.(Medline)

Toma, J.G., Akhavan, M., Fernandes, K.J., Barnabe-Heider, F., Sadikot, A., Kaplan, D.R. and Miller, F.D. (2001) Isolation of multipotent adult stem cells from the dermis of mammalian skin, *Nature Cell Biol.* 3:778-784. (MedLine)

Toussaint, O., Medrano, E.E. and von Zglinicki, T. (2000) Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes, *Exp. Gerontol.* 35:927-945. (MedLine)

Travers, A. (1999) An engine for nucleosome remodeling, *Cell* 96:311-314.(Medline)

Trieschmann, L., Postnikov, Y.V., Rickers, A. and Bustin, M. (1995) Modular structure of chromosomal proteins HMG-14 and HMG-17: definition of a transcriptional enhancement domain distinct from the nucleosomal binding domain, *Mol. Cell. Biol.* 15:6663-6669. (Medline)

Troy, C.M., Stefanis, L., Prochiantz, A., Greene, L.A.and Shelanski, M.L. (1996) The contrasting roles of ICE family proteases and interleukin-1beta in apoptosis induced by trophic factor withdrawal and by copper/zinc superoxide dismutase down-regulation, *Proc. Natl. Acad. Sci. USA* 93:5635-5640. (MedLine)

Tsonis, P.A. (1996) *Limb Regeneration*, Cambridge University Press, Cambridge, UK.

Tsukiyama, T. and Wu, C.(1997) Chromatin remodeling and transcription, *Curr. Opin. Genet. Dev.* 7:182-191.(Medline)

Turrens, J.F. (1997) Superoxide production by the mitochondrial respiratory chain, *Biosci. Rep.* 17:3-8. (MedLine)

Tycko, B. (1994) Genomic imprinting: mechanism and role in human pathology, *Am. J. Pathol.* 144:431-443.(Medline)

Tyler, J.K. and Kadonaga, J.T. (1999) The "dark side" of chromatin remodeling: repressive effects on transcription, *Cell* 99:443-446.(Medline)

Urieli-Shoval, S., Gruenbaum, Y., Sedat, J. and Razin, A. (1982) The absence of detectable methylated bases in *Drosophila melanogaster* DNA, *FEBS Lett.* 146:148-152. (MedLine)

van Holde, K. and Zlatanova, J. (1996) What determines the folding of the chromatin fiber, *Proc. Natl. Acad. Sci. USA* 93:10548-10555.(Medline)

van Steensel, B., Smogorzewska, A. and de Lange, T. (1998) TRF2 protects human telomeres from end-to-end fusions, *Cell* 92:401-413.(Medline)

Van Vactor, D. and Flanagan, J.G. (1999) The middle and the end: slit brings guidance and branching together in axon pathway selection, *Neuron* 22:649-652.(Medline)

Vanfleteren, J.R. and De Vreese, A. (1996) Rate of aerobic metabolism and superoxide production rate potential in the nematode Caenorhabditis elegans, *J.Exp.Zool.* 274:93-100.(Medline)

Varga-Wiesz, P., Wilm, M., Bonte, E., Dumas, K., Mann, M. and Becker, P.B. (1997) Chromatin-remodelling factor CHRAC contains the ATPases ISWI and topoisomerase II, *Nature* 388:598-602.(Medline)

Varga-Weisz, P.D. and Becker, P.B. (1998) Chromatin-remodeling factors: machines that regulate? *Curr. Opin. Cell Biol.* 10:346-353.(Medline)

Varkey, J., Chen, P., Jemmerson, R. and Abrams, J.M. (1999) Altered cytochrome c display precedes apoptotic cell death in *Drosophila*, *J. Cell Biol.* 144:701-710.(Medline)

Vasil, V. and Hildebrandt, A. C. (1965) Differentiation of tobacco plants from single isolated cell in microcultures, *Science* 150:889-892.

Vassilev, A.P., Rasmussen, H.H., Christensen, E.I., Nielsen, S. and Celis, J.E. (1995) The levels of ubiquitinated histone H2A are highly upregulated in transformed human cells: partial colocalization of uH2A clusters and PCNA/cyclin foci in a fraction of cells in S-phase. *J. Cell Sci.* 108:1205-1215.(Medline)

Vattese-Dadey, M., Grant, P.a., Hebbes, T.R., Crane-Robinsion, C., Allis, C.A. and Workman, J.C. (1996) Acetylation of histone H4 plays a primary role in enhancing trancription factor binding to nuclesosomal DNA, *EMBO J*15:2508-2518.(Medline)

Vaux, D.L. (1993) Toward an understanding of the molecular mechanisms of physiological cell death, *Proc. Natl. Acad. Sci. USA* 90:786-789.(Medline)

Vaziri, H. and Benchimol, S. (1998) Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span, *Curr. Biol.* 8:279-282.(Medline)

Vedel, M., Gomez-Garcia, M., Sala, M. and Sala-Trepat, J.M. (1983), Changes in methylation pattern of albumin and α-fetoprotein genes in developing rat liver and neoplasia, *Nucleic Ac. Res.* 11:4335-4354.(Medline)

Veis, D.J., Sorenson, C.M., Shutter, J.R. and Korsmeyer, S.J. (1993) Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair, *Cell* 75:229-240.(Medline)

Vercammen, D., Brouckaert, G., Denecker, G., Van de Craen, M., Declercq, W., Fiers, W. and Vandenabeele P. (1998) Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways, *J. Exp. Med.* 188:919-930. (MedLine)

Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, R.L., Simpson, R.J. and Vaux, D.L. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins, *Cell* 102:43-53. (MedLine)

Vernet, G., Sala-Rovira, M., Maeder, M., Jacques, F. and Herzog, M. (1990) Basic nuclear proteins of the histone less eukaryote *Gythecodinium cohnii* (Pyrrhophyta):two dimensional electrophoresis and DNA binding properties, *Biochim. Biophys. Acta* 1048:281-289.(Medline)

Verfaille, C.M. (2002) Adult stem cells: assigning the case for pluripotency, *Trends in Cell Biol.* 12:502-508.

Verschure, P.J., van Der Kraan, I., Manders, E.M. and van Driel, R. (1999) Spatial relationship between transcription sites and chromosome territories, *J. Cell Biol.* 147:13-24. (MedLine)

Vignali, M., Hassan, A.H., Neely, K.E. and Workman, J.L. (2000) ATP-dependent chromatin-remodeling complexes, Mol. Cell. Biol. 20:1899-910. (MedLine)

Villa, P., Kaufmann, S.H. and Earnshaw, W.C. (1997) Caspases and caspase inhibitors, *Trends Biochem. Sci.* 22:388-393.(Medline)

Vlcek, S., Dechat, T. and Foisner, R. (2001) Nuclear envelope and nuclear matrix: interactions and dynamics, *Cell. Mol. Life Sci.* 58:1758-1765. (MedLine)

von Zglinicki, T. (2002) Oxidative stress shortens telomeres, *Trends Biochem. Sci.* 27:339-344. (MedLine)

Vousden, K.H. (2000) p53. Death star, *Cell* 103:691-694. (MedLine)

Vousden, K.H. and Vande Woude, G.F. (2000) The ins and outs of p53, *Nature Cell Biol.* 2:E178-180. (MedLine).

Wakayama, T., Perry, A.C.F., Zuccotti, M., Johnson, K.R. and Yaganimachi, R. (1998) Full-term development of mice from enucleated oocytes infected with cumulus nuclei, *Nature* 394:369-374.(Medline)

Wallace, D.C., Brown, M.D., Melov, S., Graham, B. and Lott, M. (1998) Mitochondrial biology, degenerative diseases and aging, *Biofactors* 7:187-190.(Medline)

Wang, H. and Tessier-Lavigne, M. (1999) *En passant* neurotrophic action of an intermediate axonal target in the developing mammalian CNS, *Nature* 401:765-769.(Medline)

Wang, H.G., Pathan, N., Ethell, I.M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T.F. and Reed, J.C. (1999a) Ca^{2+}-induced apoptosis through calcineurin dephosphorylation of BAD, *Science* 284:339-343.(Medline)

Wang, J., Zheng, L., Lobito, A., Chan, F.K., Dale, J., Sneller, M., Yao, X., Puck, J.M., Straus, S.E. and Lenardo, M.J. (1999b) Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II, *Cell* 98:47-58.(Medline)

Wang, S.L., Hawkins, C.J., Yoo, S.J., Muller, H.A. and Hay, B.A. (1999c) The Drosophila caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID, *Cell* 98:453-463. (MedLine)

Wang, S.L., Hawkins, C.J., Yoo, S.J., Muller, H.A. and Hay, B.A. (1999d) The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID, *Cell* 98:453-463. (MedLine)

Weindruch, R., Walford, R.L., Fligiel, S. and Guthrie, D. (1986) The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake, *J. Nutr.* 116:641-654. (MedLine)

Weintraub, H. and Groudine, M. (1976) Chromosomal subunits in active genes have altered configuration, *Science* 193: 848-856.(Medline)

Weintraub, H. (1993) The MyoD family and myogenesis: redundancy, networks, and thresholds, *Cell* 75:1241-1244. (MedLine)

Welihinda, A.A., Tirasophon, W. and Kaufman, R.J. (1999) The cellular response to protein misfolding in the endoplasmic reticulum, *Gene Expr.* 7:293-300.(Medline)

West, A.G., Gaszner, M. and Felsenfeld, G. (2002) Insulators: many functions, many mechanisms, *Genes Dev.* 16:271-288. (MedLine)

Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A. and Gough, N.M.(1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells, *Nature* 336:684-687. (MedLine)

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H. (1997) Viable offsprings derived from fetal and adult mammalian cells, *Nature* 385:810-813.(Medline)

Wilson, C.J., Chao, D.M., Imbalzano, A.N., Schnitzler, G.R., Kingston, R.E. and Young, R.A. (1996) RNA polymerase holoenzyme contains SWI/SNF regulators involved in chromatin remodeling, *Cell* 84:235-244.(Medline)

Wilson, R., Goyal, L., Ditzel, M., Zachariou, A., Baker, D.A., Agapite, J., Steller, H. and Meier, P. (2002) The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis, *Nature Cell Biol.* 4:445-450. (MedLine)

Winoto, A. (1997) Cell death in the regulation of immune responses, *Curr. Opin. Immunol.* 9:365-370.(Medline)

Winston, F. and Allis, C.D. (1999) The bromodomain: a chromatin-targeting module? *Nature Struct. Biol.* 6:601-604. (MedLine)

Wolf, B.B. and Green, D.R. (1999) Suicidal tendencies: apoptotic cell death by caspase family proteinases, *J. Biol. Chem.* 274:20049-20052.

Wolffe, A.P. and Brown, D.D. (1988) Developmental regulation of two 5*S* ribosomal RNA genes, *Science* 241:1626-1632.(Medline)

Wolffe, A.P. and Hayes, J.J. (1999) Chromatin disruption and modification, *Nucleic Acids Res.* 27:711-720.(Medline)

Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G. and Youle, R.J. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis, *J. Cell Biol.* 139:1281-1292.(Medline)

Wong, J.M., Kusdra, L. and Collins, K. (2002) Subnuclear shuttling of human telomerase induced by transformation and DNA damage, *Nature Cell Biol.* 4:731-736. (MedLine)

Woodbury, D., Schwarz, E.J., Prockop, D.J. and Black, I.B. (2000) Adult rat and human bone marrow stromal cells differentiate into neurons, *J. Neurosci. Res.* 61:364-370. (MedLine)

Wright, W.E. and Shay, J.W. (2000) Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology, *Nature Med.* 6:849-851. (MedLine)

Wu, R.S., Kohn, K.W. and Bonner, W.M. (1981) Metabolism of ubiquitinated histones, *J. Biol. Chem.* 256:5916-5920.(Medline)

Wu, D., Wallen, H.D., Inohara, N. and Nuñez, G. (1997) Interaction and regulation of the *Caenorhabditis elegans* death protease by

CED-3, CED-4 and CED-9, *J. Biol. Chem.* 272:21449-21454.(Medline)

Wu, Y.C., Stanfield, G.M. and Horvitz, H.R. (2000) NUC-1, a *Caenorhabditis elegans* DNase II homolog, functions in an intermediate step of DNA degradation during apoptosis, *Genes Dev.* 14:536-548. (MedLine)

Wurmser, A.E. and Gage, F.H. (2002) Stem cells: Cell fusion causes confusion, *Nature* 416:485-487. (MedLine)

Wyllie, A.H.(1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation, *Nature* 284:555-556.(Medline)

Xanthoudakis, S., Roy, S., Rasper, D., Hennessey, T., Aubin, Y., Cassady, R., Tawa, P., Ruel, R., Rosen, A. and Nicholson, D.W. (1999) Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis, *EMBO J.* 18:2049-2056. (MedLine)

Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B. and Korsmeyer, S.J. (1995) Bad, a heterodimeric partner for Bcl-X_L and Bcl-2, displaces Bax and promotes cell death, *Cell* 80:285-291

Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.-I, Jones, D.P. and Wang, X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked, *Science* 275:1129-1132.

Yang, X., Chang, H.Y. and Baltimore, D. (1998) Autoproteolytic activation of pro-caspases by oligomerization, *Mol. Cell.* 1:319-325.(Medline)

Yang, Y., Fang, S., Jensen, J.P., Weissman, A.M. and Ashwell, J.D. (2000) Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli, *Science* 288:874-877. (MedLine)

Yano, S., Tokumitsu, H. and Soderling, T.R. (1998) Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway, *Nature* 396:584-587.(Medline)

Yin, X.-M., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K.A. and Korsmeyer, S.J. (1999) Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis *Nature* 400:886-891.(Medline)

Ying, Q.L., Nichols, J., Evans, E.P. and Smith, A.G. (2002) Changing potency by spontaneous fusion, *Nature* 416:545-548. (MedLine)

Yisraeli, Y., Adelstein, R.S., Melloul, D., Nudel, U., Yaffe, D. and Cedar, H. (1986) Muscle specific activation of a methylated chimeric actin gene, *Cell* 46:409-416.(Medline)

Yoo, S.J., Huh, J.R., Muro, I., Yu, H., Wang, L., Wang, S.L., Feldman, R.M., Clem, R.J., Muller, H.A. and Hay, B.A. (2002) Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms, *Nature Cell Biol.* 4:416-424. (MedLine)

Yoshida, K., Chambers, I., Nichols, J., Smith, A., Saito, M., Yasukawa, K., Shoyab, M., Taga, T. and Kishimoto T. (1994) Maintenance of the pluripotential phenotype of embryonic stem cells through direct activation of gp130 signalling pathways, *Mech. Dev.*45:163-171. (MedLine)

Yu, C.E., Oshima, J., Fu, Y.H., Wijsman, E.M., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S., Martin, G.M., Mulligan, J. and Schellenberg, G.D. (1996) Positional cloning of the Werner's syndrome gene, *Science* 272:258-262.(Medline)

Zakian, V.A. (1995) Telomeres: beginning to understand the end, *Science* 270:1601-1607.(Medline)

Zhang, C.C. and Bienz, M. (1992) Segmental determination in *Drosophila* conferred by hunchback (*hb*) a repressor of the homeotic gene Ultrabithorax (*Ubx*), *Proc. Natl. Acad. Scie. USA* 89:7511-7515.(Medline)

Zheng, T.S., Hunot, S., Kuida, K. and Flavell, R.A. (1999) Caspase knockouts: matters of life and death, *Cell Death Differ.* 6:1043-

1053. a
href="http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=10578172&dopt=Abstract">(MedLine)

Zirbel, R.M., Mathieu, U.R., Kurz, A., Cremer, T. and Lichter, P. (1993) Evidence for a nuclear compartment of transcription and splicing located at chromosome domain boundaries, *Chromosome Res.* 1:93-106. (MedLine)

Zou, H., Henzel, W.J., Liu, X., Lutschg, A. and Wang, X. (1997) Apat-1, a human protein homologous to C.elegans CED-4, participates in cytochrome c-dependent activation of caspase-3, *Cell* 90:405-413.(Medline)

Zou, H., Li, Y., Liu, X. and Wang, X. (1999) An APAF-1-cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9, *J. Biol. Chem.* 274:11549-11556. (Medline)

3. Gene Expression in Differentiated Cells

Back to List of Chapters

In the previous chapter we saw how cells differentiate. Any one kind of differentiated cell becomes specialized by selecting for expression different sets of genes from the same genetic complement. In cells which are already differentiated, gene expression will also vary with physiological or environmental conditions.

The control of gene expression in differentiated cells is complex because it includes various events taking place during transcription and translation. In addition to synthetic events, the degradation of macromolecules, such as mRNA and proteins, also plays a significant role. In order to understand the exquisite regulation of these systems, it is necessary to understand some of these processes at the molecular level. This chapter will present our current understanding of some of the machinery of eukaryotic transcription (the production of RNA) in Section I. Section II discusses complexes that repress transcription. Section III addresses the processing of the transcribed RNA. Translation (the production of proteins) is discussed in Section IV. Transcriptional and posttranscriptional regulative events are the subject of the last section (Section V), which uses some specific examples to illustrate the complexity of the regulation.

I. TRANSCRIPTION

Eukaryotic transcription is carried out by three separate RNA polymerases (RNAP; frequently abbreviated as Pol II). RNAPI transcribes the precursors of several ribosomal RNAs (5.8S, 18S and 28S where the number corresponds to the sedimentation coefficient, S; roughly speaking the larger the number, the larger the macromolecule). RNAPIII transcribes an additional ribosomal RNA, 5S RNA, and all tRNAs. RNAPII transcribes the mRNA encoding proteins and the RNA of the small ribonucleoprotein particles. In vitro, transcription of eukaryotic DNA by RNAPII alone is initiated at random sites. Initiation at the proper site requires the presence of accessory proteins. These include as many as seven *general transcription factors* (GTFs). Transcription factors (TFs) are numbered to identify the RNAP with which they interact and a letter to distinguish one from the other (e.g., TFIIA). A highly ordered assembly of GTFs, RNAPII and promoter DNA of the expressed gene are required for initiation of transcription of mRNA.

In addition to transcription, the production of mature mRNA requires several other processing reactions. The primary transcript, the RNA encoded by the DNA of an entire gene, is spliced so that parts of the molecule are removed (see IIIA). At the 5' end of each molecule a 7-methyl guanosine residue cap is attached to the RNA by triphosphate linkage (Fig. 1), a reaction catalyzed by a specific guanylyl transferase enzyme. The 3' end of the mRNA is cleaved downstream from the AAUAAA sequence and then a long run of A [a poly(A) tail] is attached to the 3' ends. The maturation processes depend on the carboxy-terminal of the polymerase (McCracken et al., 1997a; (Hirose and Manley, 1998). Apparently RNAPII forms a complex with processing factors active in the maturing processes including the capping of the mRNA (McCracken et al., 1997b).

The various processes occurring during transcription are discussed in this section.

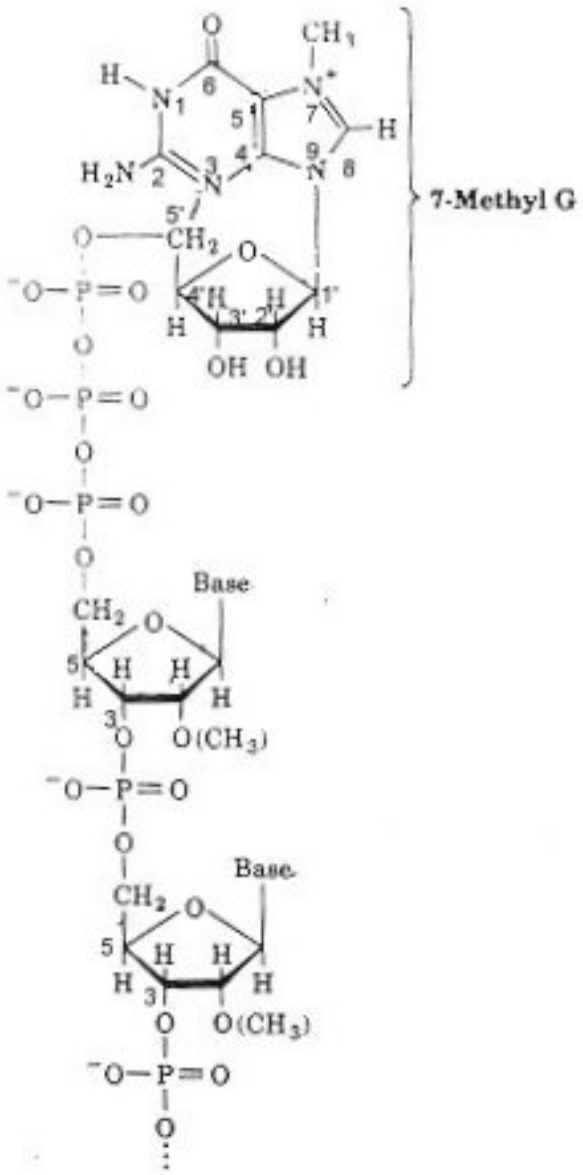


Fig. 1 Structure of mRNA cap. During processing of eukaryotic mRNA a cap is added at the 5' end. The cap is composed of a guanosine residue with a methyl group at position 7. The cap is bound covalently to the 5' end of the mRNA by a 5'-5' triphosphate bridge.

A. Proteins and DNA Interactions

A variety of proteins including transcriptional factors (Section B), activators and proteins involved in silencing (Section D) act by binding to DNA motifs. How can we detect binding of proteins to DNA? There are many ways in which binding can be detected. Electrophoretic migration in a polyacrylamide gel is a function of molecular size, where the gel acts as a sieve: the larger the molecule, the slower the migration. Therefore, DNA fragments would migrate more slowly if bound to a protein. This is illustrated in Fig. 2. Another approach, discussed in the previous chapter, involves *footprinting*. When DNA is

exposed to a disruptive procedure, such as a special chemical treatment or exposure to endonuclease, the region of the DNA which binds to a protein is protected from breakdown. The protected piece will not be fragmented and will not appear in the gel (which excludes large pieces) after electrophoresis. In practice, the portions of the DNA exposed to the treatment in the presence of protein, is compared to that which was treated in the absence of protein. The DNA fragments will appear as bands. When the corresponding sectors of the gels are compared, an empty space will appear where the DNA was protected by the protein binding (see Chapter 1). The various procedures have identified binding sites in the DNA and the construction of maps showing their location.

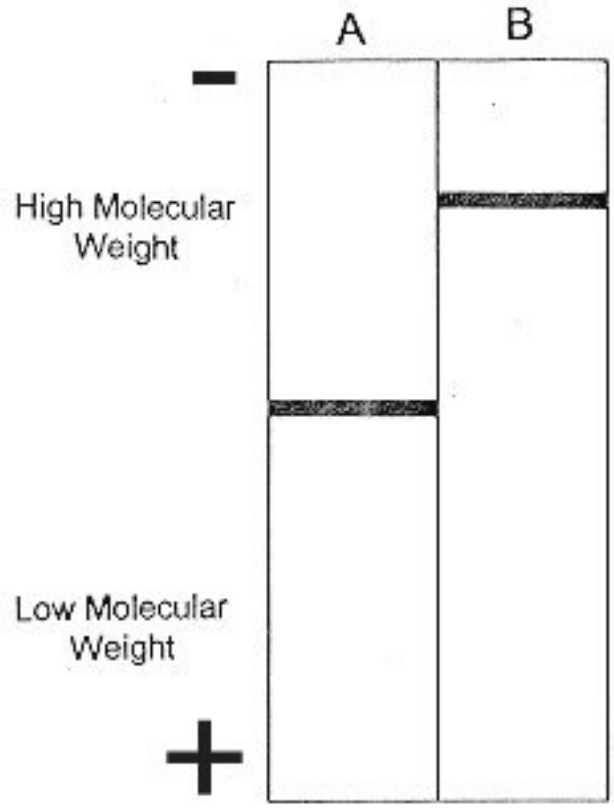


Fig. 2 Diagram showing the assay for a protein capable of binding DNA using an electrophoretic gel. (A). purified DNA fragment and (B). DNA fragment + binding proteins. The DNA can be visualized if radioactive by autoradiography or some other procedure and will appear as a band as shown.

What are the proteins which bind to these specific DNA sectors? One of the possible ways of isolating these proteins takes advantage of the binding ability of the sequences of promoter or enhancer sites in a technique called *affinity chromatography*, illustrated in the diagram of Fig. 3. The appropriate oligonucleotide segments implicated in promoter or enhancer activity and thought to bind protein can be synthesized (e.g., oligonucleotides containing GC motifs). These sectors are then covalently bound to the beads of conventional columns (part A). During the passage of the extract through the column, the proteins which remain bound to the oligonucleotide are retained (part B). They can then be released by special treatments (part C). Other proteins will not be retained. This method has allowed the isolation of many transcription factors. Once isolated, the protein can be sequenced. The production of the appropriate cDNA permits cloning and the production of the protein in quantity, for example, in an *E.coli* host (see

Chapter 1).

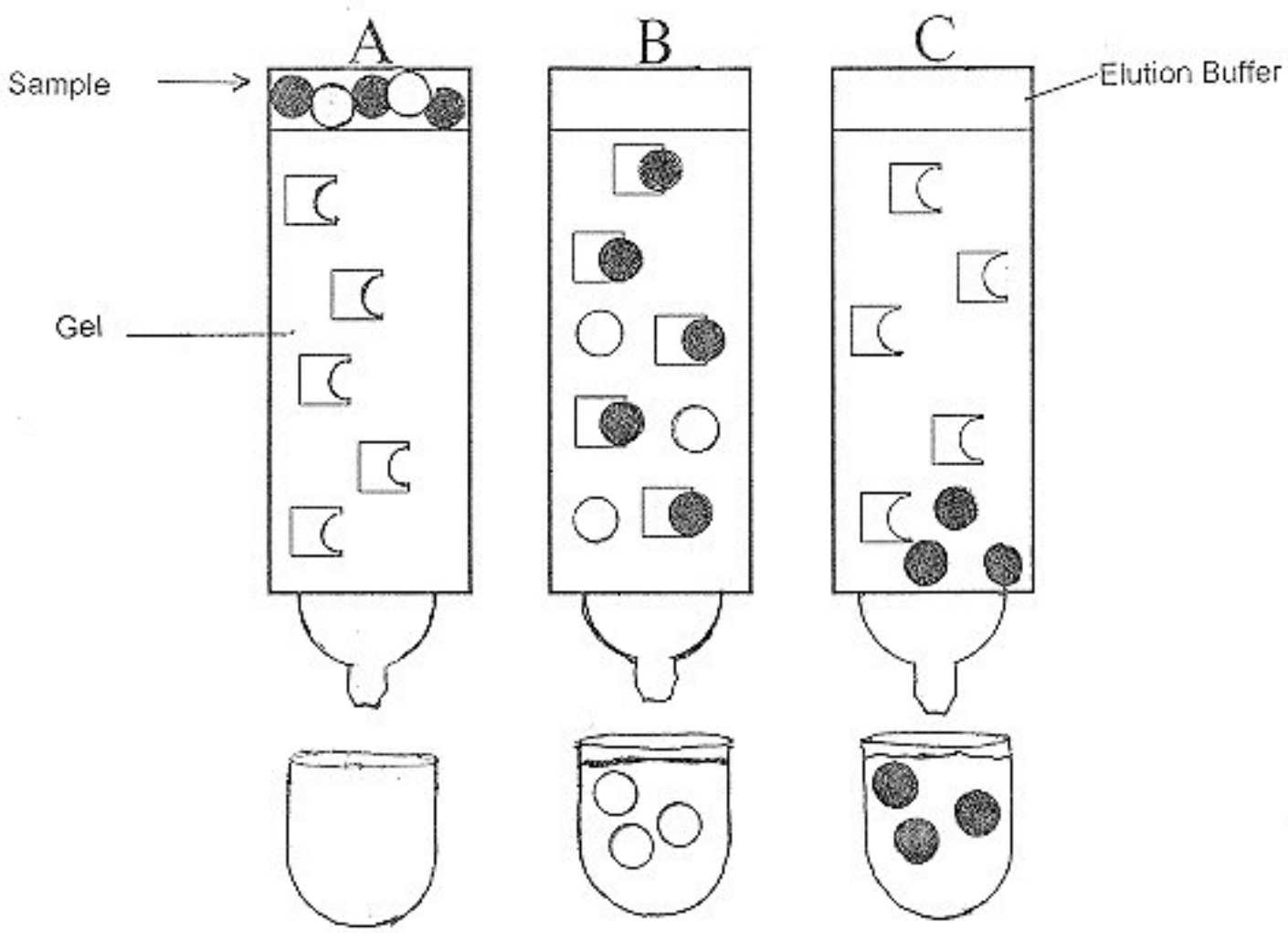


Fig. 3 Affinity column using oligonucleotides covalently bound to the column's matrix.

A. Sample placed in column
B. Retention of ligand and passage of other proteins
C. Collection of bound protein, e.g., introducing free oligonucleotides in solution which compete with the oligonucleotides attached to the column.

Other techniques have been developed more recently to examine protein-DNA interactions. These are discussed in Chapter 1. They include the use of DNA microarrays or modifications of the two hybrid technique.

B. Transcription Factors

As already discussed, in vitro experiments have revealed that in addition to RNAPII as many as seven *general transcription factors* (GTFs) are required for transcription of mRNA. TFIID, which is needed for initiation of transcription, binds the TATA box of many promoters. It is a complex which includes a

TATA binding protein (TBP), as well as eight other subunits known as *TBP-associated factors* (TAFs) (see Orphanides et al., 1996). The assembly of the transcription machinery proceeds in an ordered process. TFIID, TBP and associated factors first form a complex. Other GTFs and RNAPII are then incorporated, forming a pre-initiation complex. In the presence of all four ribonucleotide triphosphates, RNAPII, in the pre-initiation complex, can then initiate transcription at the start site (Zawell and Reinberg, 1993). The number of GTFs required may differ from promoter to promoter site, and there is some evidence that the exact complement needed depends on the structure of the DNA (Parvin and Sharp, 1993). The GTFs also mediate the promoter melting (DNA unwinding) and the transition between initiation and elongation of the RNA (see Orphanides et al., 1996).

TFIID has the DNA-specific binding capacity to recognize promoters and to mediate regulatory signals from upstream activators (see Burley and Roeder, 1996). Part of the complex, $TAF_{II}250$, possesses two tandem bromodomain modules that bind selectively to acetylated histone H4 peptides (Jacobson et al., 2000). Acetylation-deacetylation is thought to be part of the mechanisms of transcriptional regulation, where acetylation is thought to make nucleosomal DNA more accessible to transcription (see Chapter 2). Bromodomains are modules of approximately 110-amino-acid present in many chromatin-associated proteins (Dhalluin et al., 1999). The crystal structure of the double bromodomain shows two four-helix bundles with a highly asymmetric surface charge distribution. Each bundle contains a structure ideally suited for recognition of diacetylated histone H4 tails. The activation of the appropriate gene could then proceed as follows. First a histone acetyltransferase (HAT) containing activator is recruited. After recruitment, the amino terminal histone tails of the nucleosomes are acetylated. The acetylated tails then recruit TFIID via the bromodomains of $TAF_{II}250$. The role of $TAF_{II}250$ may be more immediate since this complex has been shown to have HAT-activity (see Sterner and Berger, 2000)

TFs generally contain two domains which operate independently. One domain binds DNA, the other activates transcription (the TAD domain). The independence of the two functions has been shown by producing hybrid proteins, where the DNA binding site of one protein is combined with the activation site of another. The resulting protein is fully active but now the biological signal activates the wrong gene. Many of the DNA binding domains are now well recognized and are shown in Fig. 4.

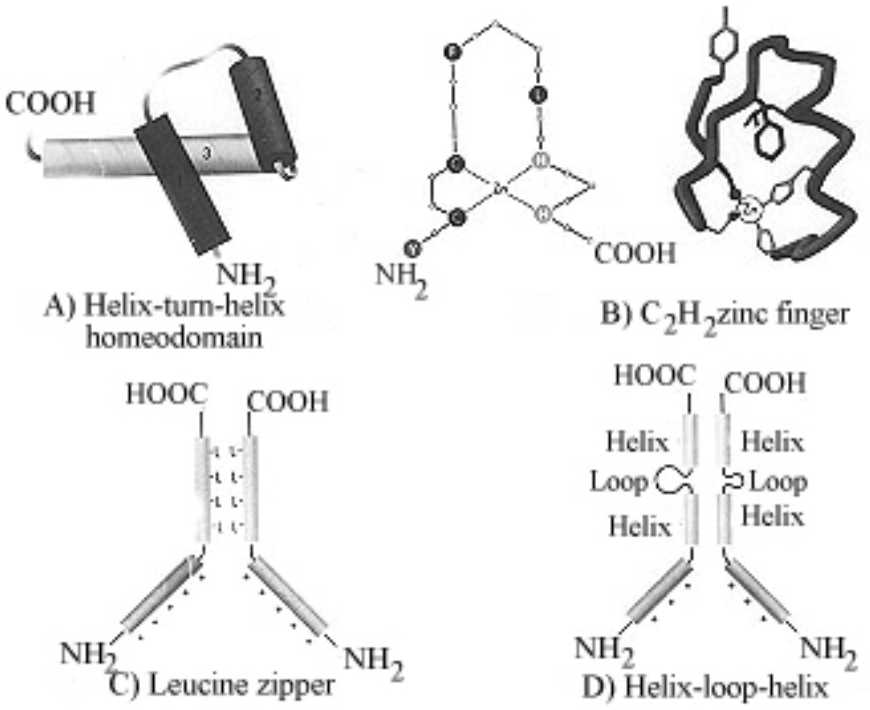


Fig. 4 Structural models of DNA-binding domains. A) The helix-turn-helix motif found in several prokaryotic regulatory proteins and in the homeodomain proteins of eukaryotes. Helix 1 and 2 lie on top of helix 3 and are able to make contact with other proteins. In some homeodomain proteins there is a fourth helix, B) the zinc finger. The finger shown here has two cysteines (C) and two histidines (H) coordinate around a molecule of Zn. The diagram on the left is represented as a structural model on the right. The zinc fingers of steroid receptors have four cysteines coordinating on Zn. C) The leucine zipper formed by a dimer. At the carboxyl terminal every seventh amino acids is leucine (L). The interaction between the two proteins is thought to be by hydrophobic bonding. The amino acid terminal region is rich in positive amino acids which are thought to interact with DNA. D) Helix-loop-helix proteins. The proteins contain two helices connected by a loop. Supposedly these also form dimers by hydrophobic interactions. A third helical portion has positive residues that can interact with DNA. From Watson et al., 1992, reproduced by permission.

The mechanisms by which transcription factors increase transcription are still unknown; they are presumed to function in stabilizing the binding between a transcription complex containing RNAP and the DNA.

The RNA polymerase, TBP and various transcription factors (e.g., see Conaway and Conoway, 1993) constitute the minimal, basic machinery for transcription. In addition, activator complexes attaching upstream of the TATA box, speed up the transcription either by direct binding (e.g., Roberts et al., 1993) or via a mediator (Kim et al., 1994). TFIID is thought to be recruited to the promoter by activators. TFIID binds the TATA nucleotide sequence. This is because the TFIID complex includes TBP and TAFs which bridge DNA-specific-activators and the basic transcriptional machinery.

Although transcription factors are most frequently involved in activation of gene expression, they also can serve as repressors. Others can act either as activators or repressors depending on individual conditions (see Latchman et al., 2001). The TFs act by binding either coactivators or corepressor proteins. An

example of this double function is provided by studies of the transcriptional factor Pt-1. Pt-1 is required for the development of the pituitary gland and is involved in the regulation of the genes coding for growth hormone, prolactin and thyrotropin (see Ryan and Rosenfeld, 1997). The factor binds both the growth hormone and the pituitary promoters. In lactotropes (anterior pituitary prolactin-secreting cells), Pit-1 activates the pituitary promoter and in addition, represses the growth hormone promoter by allowing the binding of the corepressor N-CoR (Scully et al., 2000). Similar functions are discussed in Chapter 7 for steroid and thyroid hormones.

Generally, transcription factors are rapidly degraded by the ubiquitin-proteasome system (see Thomas and Tyers, 2000). The effectiveness of a transcription factor correlates highly with its rate of degradation, suggesting a link between the two activities. A connection between the two processes is farther supported by the finding that mutations in the TAD domain of the transcription factor produce parallel changes in transcription activation and degradation (Salghetti et al., 2000). A direct link was demonstrated in a study of VP16. VP16 is an activator of the transcription of five virally encoded genes of herpes simplex virus type 1 . A transcription factor containing the VP16 TAD signals ubiquitination through the met30 ubiquitin-ligase. In addition met30 is needed for V16 TAD to activate transcription. In the absence of a met30, a construct of VP16 fused to ubiquitin activates transcription, demonstrating that ubiquitination is needed for transcriptional activity (Salghetti et al., 2001). These findings suggest that that ubiquitination regulates TAD function by serving as a dual signal for activation and activator destruction.

Several cofactors (see Näär et al., 2001) have been implicated in the regulation of the general transcriptional machinery, such as the *universal stimulatory activity* (USA) found in extracts and which contains negative and positive cofactors. Some of these interact directly with TBP. A set of RNAP II coactivator complexes, *Mediators*, are sequence specific activators that function in recruiting RNAP II to the promoter region of specific target genes. Some activators may act through Mediator complexes. A number of coactivators have been found to be large multifunctional complexes. They function as adaptors mediating activator recruitment of the transcriptional apparatus. Some are chromatin-remodeling or -modifying enzymes.

Gene expression has been found to be regulated by remodeling of chromatin, which may take place by acetylation, phosphorylation or methylation of histones (e.g., see Section II, below and Chapter 2), or chromatin remodeling enzymes which depend on the hydrolysis of ATP (see Section VA, below). Surprisingly, covalent modifications of the coactivators, including acetylation, phosphorylation and methylation (e.g., of p300/CBP; two related peptides), have also been found to act in transcriptional regulation (see Näär et al., 2001; Gamble and Freedman, 2002). Coactivators may activate a variety of transcription factors (e.g., CBP/p300, DRIP-ARC-TRAP) or to have very specific effects (e.g., p160/SRC1). The modification of cofactors has been found to be carried out at least in part by the same enzymes involved with the covalent modification of histones.

C. Eukaryotic Promoters

In prokaryotes, the promoter sequence, at the beginning of the operon, is the site at which transcription is

initiated by binding RNAP. Similarly, in eukaryotes, RNAP II promoters are needed for the accurate and efficient transcription of mRNA (Dynan and Tynan, 1985). How could these sequences be identified? As knowledge of the base sequence of eukaryotic DNA expanded, it was natural to look for recurring base sequence motifs in the DNA of the region upstream from the structural genes (see Maniatis et al., 1987). Many such regions with special recurring motifs were identified. But how many of these are actually promoters? How can promoter function be demonstrated? Generally, a suspected promoter region has been studied by mutagenesis of the DNA piece and introduction into a living cell. This is most frequently done by attaching the DNA to a *plasmid vector* (see Chapter 1). A plasmid is a piece of DNA that is capable of dividing independently from the chromosomes. The transfer of DNA into living cells using vectors (frequently of viral origin) is known as *transfection*. Most often a *reporter* gene is inserted downstream from the suspected sequence. The reporter gene is one that is easily recognizable by a simple assay, for example, an enzyme activity producing a color reaction. A favorite has been the *lacZ* gene of *E. coli*, which codes for the enzyme β-galactosidase and hydrolyses lactose to its component monosaccharides. In the laboratory, this enzyme also catalyzes the hydrolysis of artificial substrates to colored products. Another useful reporter gene is that coding for the green fluorescent protein (GFP) discussed in Chapter 1. A block in the transcription of the reporter gene produced by a mutation in the suspected promoter region, is good evidence that it functions as a promoter. This strategy is illustrated in Fig. 5.

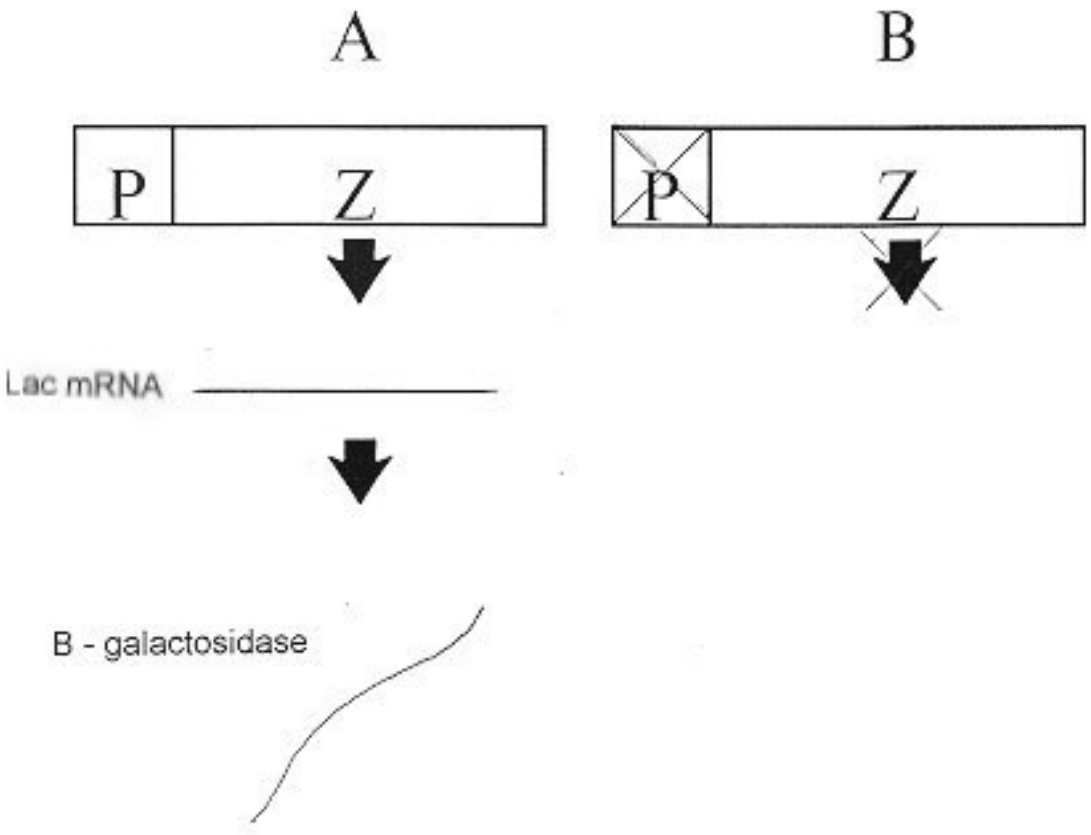


Fig. 5 Strategy used to assign promoter function to a DNA segment. The segment to be tested (P in the figure) attached to a reporter gene (Z) is used to transfect a normal cell. If P is a promoter it will activate Z (part A). Conversely, a mutation in P could block the activation.

Typical eukaryotic promoters contain an AT region (the *TATA box*) and one or more sequences containing 8 to 12 base pair elements called the *upstream promoter elements* (UPE), such as CCAAT and GGCG. Apparently, the TATA box insures accurate initiation of transcription, whereas the UPE increases the rate of transcription. The TATA box is typically 25 to 30 base pairs upstream from the site of transcription, and the UPE are upstream from this.

We have seen that eukaryotic RNAPs require the presence of transcription complexes which include many accessory proteins and the *promoter* region of DNA. Promoters for RNAPIII are 50 nucleotide long within the 5S gene. The promoters for RNAPI and II, in contrast, are upstream 5' to the gene.

A possible model for interaction between enhancer, promoters and DNA is shown in Fig. 6. Enhancers are discussed in the next section.

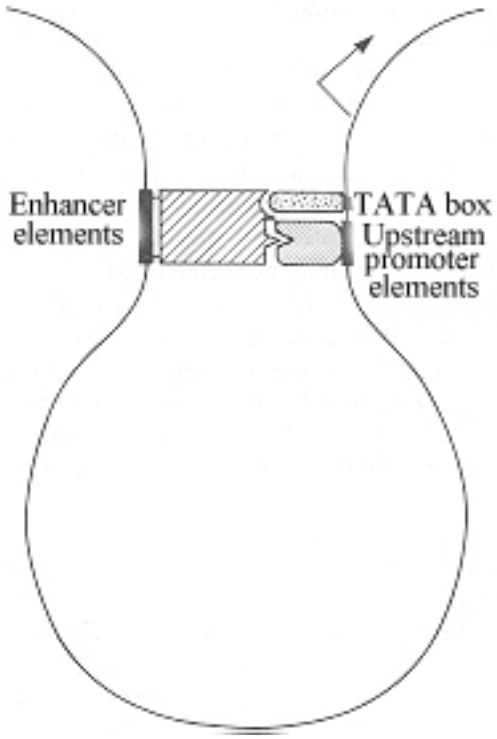


Fig. 6 The looping model of enhancer mediated transcription regulation. The interaction between enhancer and the proteins allows the initiation of transcription. Reproduced from *Biochimica et Biophysica Acta*, vol. 989, Marriott and Brady, Enhancer function in viral and cellular regulation, pp. 97-110, copyright &copy1989, with permission from Elsevier Science.

D. Enhancers and Other Elements

Animal viruses have been useful models of gene behavior. They can grow inside cells in culture and they borrow the entire array of the cell's machinery for their own duplication. Aside from a few proteins, they are basically constituted of a simple genetic DNA complement with as few as 5,000 base pairs. Therefore, they are simpler to study and have given us much insight into gene expression. Study of simian virus 40 (SV40) showed that transcription could also be regulated by regions distant from the gene, even

thousands of base pairs away (see Marriott and Brady, 1989). These DNA regions were called *enhancers*. Typically, enhancers contain 50 to 150 base pairs. They are composed of duplicated sequences in tandem. Apparently, the effect of enhancers does not depend on any single sequence motif. Synthetic oligonucleotides, stringing together repeats of oligonucleotides 18 to 20 base pairs in length, can functionally replace enhancers (see Zenke et al, 1986). Enhancers play a role in differentiation and in responding to hormonal or environmental signals. The immunoglobulin genes expressed at high levels in B cells of the immune system involve enhancers (Gillies et al., 1983). In contrast, in other cells not expressing the immunoglobulin genes, the enhancers serve to block transcription.

Enhancers have been found to come in two different forms, either modular or non-modular. The modular enhancers can respond to different activators allowing for a variety of distinct patterns of gene expression in which each activator makes a distinct contributions to transcription (see Struhl, 1991). Non-modular enhancers have an all-or-none function by acting as on-or-off switches where the “on” switch is turned on only when all activators are present.

Certain activators may be present only in one cell type and during a particular developmental stage. Many activators can act on a single gene. In addition, the activators can be arranged in different combinations. The enhancer sequences of DNA contain multiple binding sites for activators. Before transcription may proceed, chromatin which was previously silenced must be remodeled to permit access to the transcriptional machinery. Activators organize the recruitment of many proteins such as chromatin-remodeling enzymes as well as proteins involved in the assembly of the core transcription machinery. The pattern generally recognized proceeds by a mechanism in which activators first recruit chromatin-remodeling enzymes followed by the assembly of the preinitiation complex (PIC) which is constituted by RNA polymerase II and general transcription factors. Remodeling of chromatin may take place by the covalent modification of nucleosomal histones by acetylation, phosphorylation or methylation (e.g., see Chapter 2) or by enzymes which change the chromatin structure directly and require the hydrolysis of ATP (see Section V A, below).

In many cases, gene transcription was found to require the assembly of transcription factors, activators and DNA complexes referred to as *enhanceosomes* (see Merika and Thanos, 2001). Our present scenario on how enhanceosomes function is based in part on studies of the human interferon-β IFN-β enhancer. The IFN-β enhancer is a 65-base pair region upstream and adjacent to the promoter of the IFN-β gene. This enhancer is non-modular and initiates the transcription of the gene coding for IFN-β following viral infection. The assembly of the *enhanceosome* activates transcription by recruiting the histone acetyltransferase proteins, *CREB binding protein* (CBP) and p300/CBP-associated factors (PCAF)/GCN5 (proteins with histone acetyltransferase activity). As discussed below, histone acetyl transferases (HATs) activate and histone deacetylases (HADACs) repress genes (see Section II). In addition to modifying histones (see Chapter 2), these components acetylate, HMGI(Y), the architectural component required for enhanceosome assembly. The enhanceosome assembly occurs in well defined steps (Agalioti et al., 2000). The stability of the complex is enhanced by acetylation at lysine-71 of HMGI(Y). The disassembly is automatic upon acetylation of HMGI(Y) (at lysine-65) which inhibits its ability to bind DNA (Munshi et

al., 2001). A similar role of enhanceosomes is suspected for several other mammalian genes see (Merika and Thanos, 2001) .

However, a surprising amount of variation has been found in the details of the assembly of the necessary proteins (see Fry and Peterson, 2002) . The most striking difference is that found for the activation of the human α_1-antitrypsin gene (α_1-*AT*) which involves the region upstream from the α_1-*AT* gene containing nucleosomes (e.g., the region upstream of the IFN-β gene involved in activation is free of nucleosomes). One activator protein and two general transcription factors are bound to the nucleosomal promoter region. When differentiation of the cell is induced, RNA polymerase II and general transcription factors are assembled in the PIC, in contrast to the general pattern, in absence of remodeling enzymes (Soutoglou and Talianidis, 2002). However, a SWI/SNF and two HAT enzymes are recruited to the promoter region after PIC assembly and the promoter-associated nucleosomes are disrupted before transcription.

In reality, gene expression is controlled by a complex network which is not completely understood (see Müller, 2000). A variety of *cis* elements such as the enhancers and silencers play a role. Silencers are genes which block the expression of other genes. Enhancers and silencers may reflect the structural organization of the chromatin (see below). In addition to these elements, insulator elements (see Chapter 2) have been identified which block interactions between *cis*-regulatory elements (Bell and Felsenfeld, 1999; Udvardy, 1999). These insulators are thought to have a role in separating active and and inactive genes that are expressed differentially. Insulator DNAs can work in a dominant fashion to block enhancer-promoter interactions over long distances. An additional set of regulatory elements has been identified that permit bypassing insulators (Zhou and Levine, 1999). This element, the promoter targeting sequence (PTS), permits enhancers to overcome the blocking effects of insulators.

E. Structural Factors

Both single nucleosomes and high order chromosome structures are thought to inhibit transcription by a variety of mechanisms, such as steric hindrance of *trans*-acting factors binding or distortion of target binding sites. However, transcription can occur despite these inhibitions, suggesting that transcription factors can contend with either nucleosomes or folding.

In vitro, nucleosomes inhibit transcription in many cases (e.g.,Owen-Hughes and Workman, 1994). The inhibition can be reversed by a variety of mechanisms (see Edmondson and Roth, 1997): (1) transcription factors can bind newly replicated DNA before nucleosomes are formed, (2) regulatory factors may bind to sites in the nucleosomes and disrupt them, and (3) specialized disruptors may open the nucleosomes.

The concept that structural factors have a role in gene expression, namely that the disruption of the compacted form of chromatin is necessary for gene activation, is supported by experiments carried out in vivo. The *lac* repressor binding of direct repeats of the *lac* operator has allowed observing the organization of chromatin in vivo (see Belmont and Straight, 1998). GFP-repressor fusion proteins (see Chapter 1) can be used at the light microscope level. The method, combined with gene amplification has

allowed studies of living cells (Robinett et al., 1996). In these studies repeats of the lac operator were placed in vector constructs used for transfection (see Chapter 1). These sites, incorporated into the chromatin of the host cells, could then be labeled by the lac repressor-GFP chimeras that require detection of the fluorescence of this protein. Using electron microscopy, the gene amplification regions were shown to correspond to 100 nm fibers. GFP-lac repressor constructs made it possible to carry out in vivo observations of the operator-tagged chromosomal DNA either when integrated by a vector (Chinese hamster ovary cells) or homologous recombination (yeast).Transcriptional activation and chromatin organization were studied using this methodology (Tumbar et al., 1999). *Lac* operator repeats inserted in the chromosomes were targeted with a VP16-lac repressor fusion protein containing an VP16 acidic activation domain. This targeting resulted in increased transcription, localized histone hyperacetylation, and recruitment of at least three different histone acetyltransferases. These events are all associated with the activation of genes (see Chapter 2). The chromatin structure unfolded to form 25-40 μm fibers. The unfolding was propagated on a large-scale over hundreds of kilobase pairs. These changes occurred even when transcription was inhibited, suggesting that recruitment of the transcriptional machinery is responsible for the changes in chromatin structure. In the experiments of Tsukamoto et al. (2000) a similar system was used with GFP. Genes in specifics location in the chromatin were activated and the actual production of a specific protein was demonstrated.

As we have seen in Chapter 2, a variety of complexes have been found which regulate gene expression by modulating nucleosomal structure. Among these SWI/SNF, RSC, *Brahma* (BRM) and *Imitation Switch* (ISWI) (see Emerson, 2002). HATs and HDACs by modifying the histone components of nucleosomes also affect their structure (see Chapter 2, Section VIB; e.g., Hassan et al., 2001). Remodeling complexes as well as the complexes which modify histones covalently exhibit great specificity. Apparently, the specificity is provided by the association with other proteins, some of them DNA binding proteins (see Emerson, 2002)

The role of nucleosomes and histones in gene expression is discussed in some detail in Chapter 2, Section VI.

F. Mediator and Repression Complexes

In mammalian cells certain hormone receptors (discussed in Chapter 7) are activators (see also Mangelsdorf and Evans, 1995). In the case of the thyroid hormone receptor (TR) the activation requires a mediator present in the *TR-associated protein complex* (TRAP) (e.g., Fondell et al., 1999; Ito et al., 1999). Mediator complexes have been studied for some time in yeast (see Hampsey et al., 1998) and more recently in mammalian systems (e.g., Rachez et al., 1997; Boyer et al, 1999; Näär et al., 1999; Ito et al., 1999, Ryu et al., 1999; Jiang et al., 1999). The mammalian complexes are homologous to those in yeast.

Mediator complexes are huge. They contain from nine to twenty peptides and their mass ranges from 600 kDa to 1,000 kDa. Surprisingly, different activators can interact with the same mediators. In addition, many of the subunits of different mediators are the same (see, Parvin and Young, 1998; Ito et al., 1999).

However, aside from the shared subunits, the other subunits of each mediator are different. The mixing and matching allows for the needed variation in mediators. The size of the mediator complexes may reflect the need for interactions with different activators. Apparently some of the mediators only act on chromatin and not naked DNA (see Rachez et al., 1999; Näär et al., 1999) while others increase the rate of transcription on naked DNA (e.g., Boyer et al., 1999; Ryu et al., 1999).

Repression complexes have been found to have a major role in the silencing of genes in eukaryotes. The complex of Ssn6 and Tup1 (see Smith and Johnson, 2000) has been studied in some detail. These two proteins belong to a family of proteins represented in all eukaryotes studied. One Ssn6 molecule and four Tup1 molecules (all phosphoproteins) combine to form an elongated complex of 440 kDa (e.g., Redd et al., 1997). The repression induced by this complex is very efficient and may involve many genes. In *Saccharomyces cerevisiae*, the complex represses more than 150 genes (as much as 3% of the genome). The phenotype expressed by Tup1 and Ssn6 mutants is the result of inappropriate expression of these genes (DeRisi et al., 1997). The specific repression of a gene or set of genes can occur even if the activators are present.

Generally, Ssn6-Tup1 binding to DNA is mediated by sequence specific DNA-binding proteins which bind to operator sites upstream from the gene. The presence of mediator proteins is similar to the mixing and matching of the mediators of transcription discussed above. Although the mechanism of this inhibition is still not known two possibilities seem most likely: an alteration in chromatin structure and interference with the assembly of the transcription complexes (see Smith and Johnson, 2000).

The repression generally blocks genes that are essential to the survival of the cell under different conditions. Therefore, it is important to consider the mechanisms that remove the block. The most likely mechanism involves the inactivation of the DNA-binding proteins that attach to the Ssn6-Tup1 complex by blocking its transcription (if the protein has a high rate of turnover) (e.g., Zitomer et al., 1997). Another mechanism involves phosphorylation of the mediating proteins. The protein Crt1 becomes hyperphosphorylated when DNA is damaged and becomes unable to attach to the DNA (Huang et al., 1998). Similarly, when glucose levels are low, the protein Mig1 is phosphorylated and removed from the nucleus, thereby de-repressing the genes that are responsible for glucose metabolism (e.g., Treitel et al. 1998).

II. DYNAMICS OF ACTIVATION AND REPRESSION

A variety of covalent modifications of core histones have been found (e.g., see Chapter 2). Coactivator-mediated acetylation and methylation of histones have been found to be part of the activation machinery of transcription. Other modifications include phosphorylation (Sassone-Corsi et al., 1999 ; Thomson et al., 1999) and ubiquitination (Robzyk et al., 2000; Pham and Sauer, 2000).

Many proteins that regulate transcription have *histone acetyltransferase* (HAT) or *histone deacetylase* (HDAC) activity or the ability to recruit the appropriate acetyl-active enzymes (Kingston and Narlikar,

1999). HATs are involved in activation, whereas HDACs are involved in repression. Acetylation is thought to take place when a HAT (e.g., the yeast protein GCN5p, Brownell et al., 1996) is targeted to specific sites on the DNA by other protein molecules with which it forms a complex. This binding produces a selective disruption of the nucleosomal structure. In contrast, the HDACs are part of multicomponent complexes and may be required for full repression.

Many HATs are also coactivators of transcription and are recruited to promoters by activators after the latter attach to DNA. Conversely, certain histone deacetylases are transcriptional corepressor that are recruited to chromatin by repressor protein bound to DNA (see Nakayama et al., 2001). Methylation of the histones leads to deacetylation during gene silencing (Bannister et al., 2001; Lachner et al., 2001). HDACs have been found to be associated with chromatin remodelling complexes with DNA dependent ATPase activity (e.g., see below; Wade et al., 1998; Tong et al., 1998; Xue et al., 1998). Transcriptional repressors exert their effect by recruiting deacetylases to chromatin sites (e.g., Alland et al., 1997; Jones et al., 1998).

Ubiquitin has been found in the core histones H2A, H2B and H3 (e.g., see Wolffe and Hayes, 1999).The ubiquinated histones are stable in vivo (e.g., Wu et al., 1981). In contrast to polyubiquitination (which leads to protein degradation, see Chapter 15), monubiquitination has been found to be linked to gene activation (e.g., Strahl and Allis, 2000). The monoubiquination of H1 by $TAF_{II}250$ was found to be a step necessary for specific gene expression during *Drosophila* development (Pham and Sauer, 2000). TAF_{II}, a component of the general transcription factor TFIID (see Chapter 3), was found to be a histone-specific ubiquitin-activating/conjugating enzyme (Pham and Sauer, 2000) (see Chapter 15).

Present information indicates that methylation, phosphorylation and acetylation of the core histones all play a role in the molecular events underlying activation and repression. For example, heterochromatin protein 1 (HP1) mediates gene silencing; loss of HP1 eliminates gene silencing and overexpression increases silencing (see Eissenberg and Elgin, 2000).The silencing depends on a histone methylase. HP1 can bind with high affinity to histone H3 methylated at lysine 9. The results suggest that first a ‘methyl marker' is placed on histone H3 and this marker is recognized by HP1 (e.g., Bannister et al., 2001; Lachner et al., 2001). Although HP1 is a component of condensed chromatin, its presence is not static. (Fluorescence recovery after photobleaching of a GFP-HP1 fusion protein shows that HP1 of murine T cells is highly mobile in euchromatin or heterochromatin and its activity is increased by activation of the T cells (Festenstein et al., 2003). A similar study with Chinese hamster ovary (CHO) cells (Cheutin et al., 2003) also show that the binding of HP1 to chromatin is dynamic. The exchange correlates with the level of condensation of the chromatin and depends on the histone methyltransferase Suv39h. Maintenance of the heterochromatic (i.e., inert state) requires methyltransferases (Rea et al., 2000). A specific antibody can detect methylation of lysine 9 in histone H3 (Nakayama et al., 2001). The methylation was found to depend on a methyltransferase but also requires histone deacetylase. This appears to be followed by HP1 (or homologs) binding to produce silencing. Another mechanism appears to function in reverse and to involve phosphorylation. The phosphorylation of serine-10 facilitates acetylation of lysine-14 (Cheung et

al., 2000; Lo et al., 2000). This suggests the existence of on and off states. In the off-state lysine-9 is methylated and lysine-14 is deacetylated and in the on-state serine-10 is phosphorylated and lysine-14 is acetylated. In addition, methylation of lysine-9 inhibits methylation of serine-10 and viceversa (Rea et al., 2000). The interactions between acetylation and phosphorylation has been elucidated further by the isolation of a histone H3 serine-10 kinase complex (Lo et al., 2001). Its histone kinase subunit referred to as *Snf1* was identified. Snf1 and the acetyltransferase Gcn5 were found to function in sequence. These observations provide further support to the idea that transcription is controlled by coordinated patterns of histone modification.

Transcriptional cofactors are also regulated by covalent modifications as indicated above. For example, the coactivator *CREB-binding protein* (CBP) when phosphorylated is recruited to certain promoters (Zanger et al., 2001). In contrast, methylation of CBP blocks the binding to the *cAMP response element binding protein* (CREB) (Xu et al., 2001) (see Chapter 7 for a discussion of CREB and CBP)

III. PROCESSING OF THE PRIMARY TRANSCRIPT

Presently available information, primarily from in vitro experiments, allow the following reconstructions for the synthesis of mRNA in eukaryotes. The mRNA synthesized by the transcriptional machinery is much larger than the mature mRNA which is translated during protein synthesis. The large primary transcript has been called *heterogeneous nuclear RNA* (hnRNA). hnRNAs contain both coding and non-coding components. These are transcripts of the coding regions of the gene called *exons* and the non-coding regions called *introns*. The hnRNAs are associated with specific binding proteins to form *heterogeneous ribonucleoproteins* (hnRNAPs). Twenty different hnRNAPs have been recognized so far, ranging in molecular weight from 31 to 120 kDa (e.g., Dreyfuss et al., 1993). After processing, the newly formed mRNAs are translocated to the cytoplasm. The resulting cytoplasmic mRNAs can then be translated. During processing the hnRNA is first *spliced*. In this process the non-coding sections of the hnRNAs are cut away and the portions of the spliced coding mRNA are joined together.

In eukaryotic cells, the nucleolus corresponds to the multiple ribosomal RNA genes in a specialized chromatin region which carries out ribosomal gene transcription, rRNA processing and ribosomal subunit assembly (e.g., see Miller and Beatty, 1969). In addition, the nucleolus is the site of processing and assembly of non-ribosomal RNAs. These include the maturation of the signal recognition particle RNA (SRP) (see Pederson, 1998; Pederson and Politz, 2000), tRNA, U6 small nuclear RNA, telomerase RNAs and some mRNAs.

What are the sites of actively transcribed genes? One of the possible ways of examining this question is to turn to classic biological materials. These are well known because they could be studied in detail even before the EM technology became available, because they are clearly visible with the light microscope: *polytene chromosomes* and *lampbrush chromosomes* and their loops.

Lampbrush chromosomes are meiotically paired chromosomes of amphibian growing eggs. They are very

active in producing RNA and they form chromosome loops covered with large amounts of newly formed hnRNA bound to hnRNP proteins. *Polytene* (multistranded) chromosomes are present in salivary glands of fly larvae. The chromosomes grow to enormous size by replicating DNA, as many as a thousand times without cell divisions. The chromatids of these chromosomes are perfectly aligned so that different regions of the chromosomes can be readily distinguished morphologically. The dark bands seen in these chromosomes correspond to sites of condensed DNA. Transcribed regions are non-condensed and are part of swollen puffs. These are the sites of transcription, demonstrable by growing the larvae in the presence of [^{3}H] uridine, which is incorporated into RNA (Lamb and Daaneholt, 1979). The large puffs in *Chironomus* are called *Balbiani rings*. In addition to studies carried out with the light microscope, chromatin and nascent transcripts can be examined with the transmission electron microscope after spreading on a grid. The EM reveals the chromatin threads, RNA threads attached to it and granules. Together with other approaches, these EM studies examined the role of the various components in transcription.

EM studies of *Drosophila* embryo, reveal nascent hnRNA transcripts, seen at active gene sites as smooth fibrils about 5 nm thick. These have been referred to as *perichromatin fibers* (e.g., see Fakan, 1994). An electronmicrograph is shown in Fig. 7A (Beyer and Osheim, 1988). Part B of the figure represents a tracing of the active chromatin and part C is a map of this same region. In B and C, the dashed line represents the chromatin fiber and the full lines the ribonucleoprotein fibers. The dots represent particles. The events have been largely identified using in vitro systems. The position of these fibrils along the chromatin fiber are in general progressively longer, beginning with the first discernible fibril. This is what we would expect from the very active transcription of a gene where several polymerase molecules progress along the DNA coding thread. The transcription will be sequential because there is a start and a stop point of synthesis. The various RNA lengths represent different stages of transcription, so that from one end of the DNA fiber the perinuclear fibers become increasingly longer. However, there is some irregularity in this pattern. Some of the transcripts are shorter than expected from their position where splicing has occurred (see discussion of splicing in the next section). In some cases, the perinuclear fibers contain loops (see 15 and 28 in Fig. 7, part B). The loops reflect the lariat formation that accompanies splicing (see below). Ribonucleoprotein particles, about 25 nm in diameter, appear at various sites on the transcripts. These sites have been shown to correspond to specific locations in the transcript using in vitro systems. The spliceosomes form the larger particles, approximately 40 nm in diameter, from the smaller particles. The shortening of the perinuclear fibers and the presence of spliceosomes show that, in vivo, splicing is cotranscriptional. It is interesting to note, however, that it need not be because exogenous hnRNA has been shown to be spliced posttranscriptionally (Green et al., 1983), as discussed later.

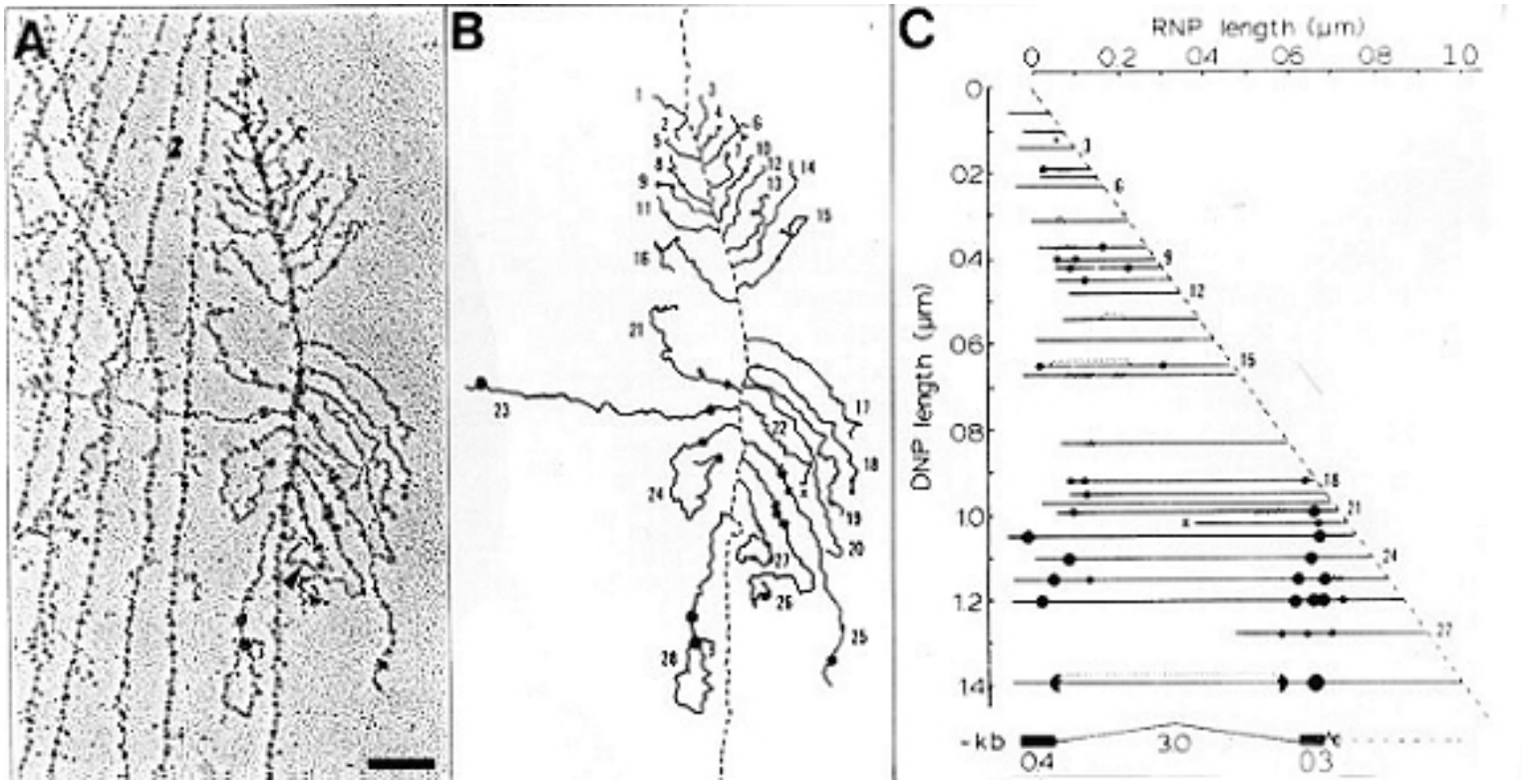


Fig. 7 A. Electronmicrograph of *Drosophila* embryo transcription unit. Arrowhead indicates possible small particle at site of proposed intron excision. Bar represents 0.2 µm. B. Interpretive tracing of micrograph. Dashed line: template chromatin; full lines: RNA transcript; (●) RNP particles; x: presumed broken transcripts. Transcript numbering starts at initiation end of gene. C. RNA-transcript map. Spacing of the fibrils on the vertical axis corresponds to their position on the chromatin template. Transcripts are shown as straight lines, with corresponding sequences approximately aligned (5 termini to the left). The dashed line represents the best fit least-square line of the unbroken transcripts. (●) RNP particles of three sizes. The hatched regions are projections traced as hairpin loops. The dotted line over the schematic fibrils represents loop structures and connects points of intramolecular contact. At the bottom is shown the proposed exon-intron structure of the transcripts and approximate lengths in kb. (■) Exons; (thin lines) intron; (....) difficult to assign as either introns or exons. The numbers are approximate lengths in kilobases. From Beyer and Osheim, 1988, reproduced by permission.

Other approaches support this interpretation which associates the splicing apparatus to the nascent mRNA. Immunofluorescence studies (see Chapter 1) show a localization of the snRNAPs and hnRNAP (Piñol-Roma et al., 1989) in the loops of lampbrush chromosomes recognized as sites of intense transcription. hnRNP-proteins and snRNPs are present in nascent RNA transcripts produced by RNAP II (Wu et al., 1991; Amero et al., 1992). Similarly, immunoelectronmicroscopy has shown the presence of hnRNPs, snRNAPs and nascent mRNA in perichromatin fibers (Fakan et al., 1984).

The observation, with the EM and the in vitro systems, indicates that, in vivo, all the components required for splicing (see next section), such as snRNP must be associated with nascent RNA transcripts.

A. Splicing

As already discussed, for most eukaryotic genes the primary transcript is much larger than the mRNA. This is because the DNA has coding regions (*exons*) and in-between regions (*introns*). The segments corresponding to the introns are removed by a process known as splicing. Splicing must involve various distinct tasks. Every exon must be located in the primary transcript, then the introns must be removed and the appropriate coding sequence must be joined precisely and in the correct order. We have seen that splicing requires hnRNPs, snRNPs and splicing factors.

Normally, splicing is cotranscriptional, however, it can occur postranscriptionally. In one of the early studies this was demonstrated when pre-mRNA from the β-globin sequence was synthesized in vitro using bacteriophage RNP. After injecting the pre-mRNA into *Xenopus* oocytes, the mRNA was recovered and was found to have been accurately spliced (Green et al., 1983). Clearly, transcription and splicing can take place as two separate processes.

The demonstration that transcription and splicing can occur independently suggests an experimental approach to determine what components are needed for processing. Synthetic pre-mRNA could be added to extracts and the processing of the RNA followed by gel electrophoresis. By adding them one at a time to a partial mixture, the role of each protein fraction could be tested for its ability to produce either mRNA or partially spliced products. A number of necessary factors have been identified in this way.

This approach revealed the need for interactions of hnRNA with the spliceosomes and each of their components (Reed, 1990). Many aspects of the biochemistry of splicing have been examined. The mechanism of splicing through lariat formation is shown in diagrammatic form in Fig. 8. The reaction is basically a transesterification where a phosphate bond between intron and exon is swapped, so that two exons are connected directly and the intron is detached in the form of a lariat. Splicing of some pre-rRNAs, mitochondrial and chloroplast pre-mRNA, and tRNA do not require a spliceosome. These RNAs self-splice by a similar mechanism in which the RNA itself functions as an enzyme.

Segments of the same mRNA can be spliced in alternative ways, giving rise to different proteins from the same gene. This has been referred to as *alternative splicing* (e.g., Lees-Miller et al., 1990). One third of human genes are thought to give rise to alternatively spliced mRNA (Lander et al., 2001)

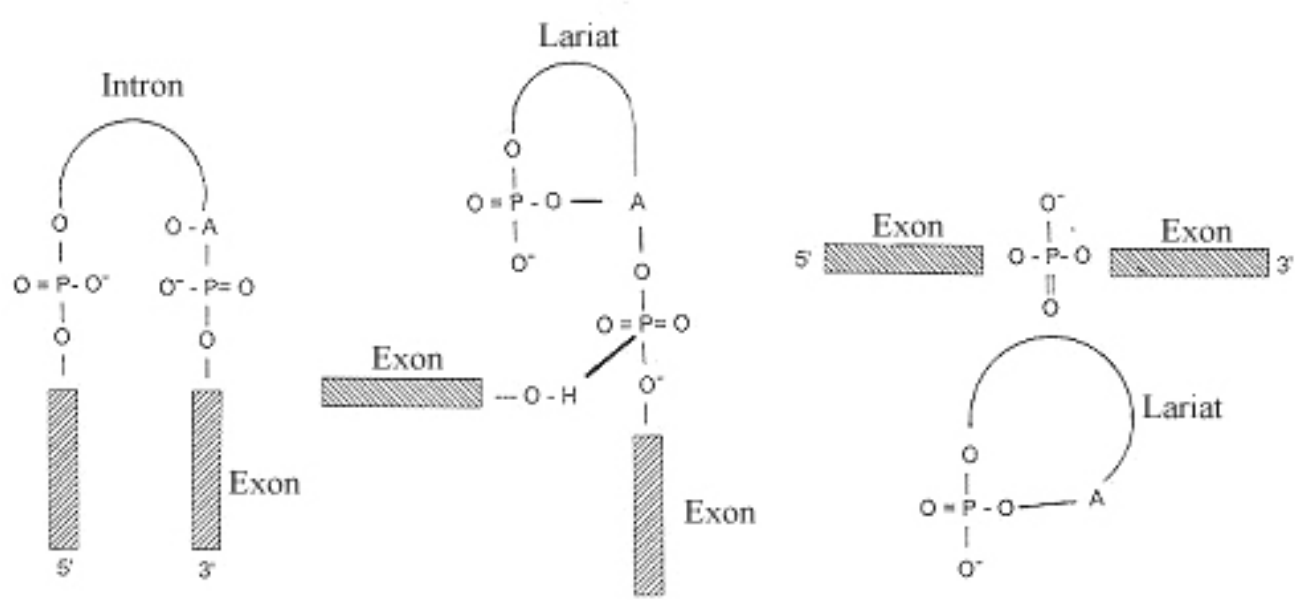


Fig. 8 Mechanism of splicing via lariat formation.

Splicing of hnRNA requires *spliceosome*s, large ribonucleoprotein assemblies containing a large number of proteins (e.g., Lamm and Lamond, 1993) and *small nuclear RNAs* (snRNAs) (see Section A below). Neubauer et al. (1998) characterized the entire mammalian spliceosome complex using mass spectroscopy in conjunction with DNA and protein sequences data bases (see Chapter 1). All components of the spliceosomes, including *small nuclear ribonucleoprotein particles* (snRNPs) and splicing factors, are found in the nucleoplasm.

The snRNPs of the spliceosome complex include U1, U2, U5 and U4/U6 (see Will and Lührmann, 1997). During the assembly of the spliceosomes, the pre-mRNA and the RNA components of the snRNPs interact. All sliceosomal proteins share common structure and mechanisms of assembly. Mammalian U1 snRNP consists of ten different proteins and an RNA consisting of 165 nucleotides. Seven of the proteins, the Sm proteins are shared by all U snRNPs. The other three are specific to U1. The snRNPs of the spliceosome bind sequentially to the pre-mRNA. U1 is bound first followed by U2, U2/U6 and U5 (see Raker et al., 1999) . A reconstruction at 10 Å resolution using cryo-electron microscopy (Stark et al, 2001) allows assigning seven Sm proteins as doughnut shaped elements and arriving at a model for U1 snRNP which agrees with a circular structural model of Sm protein arrangement (Kambach et al., 1999).

The localization of hnRNAPs and snRNAPs in the cell can be studied using immunofluorescence (see Chapter 1). Both hnRNAPs Piñol-Roma, 1989; Ghetti et al., 1991) and snRNAPs (Spector et al., 1983) are distributed throughout the nucleus. However, there are special regions with greater fluorescence. In the case of splicing factors, there are 20 to 50 spots of high fluorescence. Similar results have been obtained when the site of snRNA was identified using hybridization techniques with the appropriate DNA probe (Carmo-Fonseca et al., 1991). With this technique, two complementary single strands of DNA or RNA can be annealed to form a helical structure. This can be carried out with two complementary DNA strands or a DNA and an RNA strand. One of the two can be used as a probe, for example, if labelled with

a radioactive isotope (usually [^{32}P], recognized by autoradiography). The regions in which the splicing factors are present in high concentration have been called *speckles* and they are about 0.5 to 1.5 μm in diameter (e.g., see Misteli and Spector, 1997). In nuclei of some cells, the snRNP proteins are localized in small structures first observed in 1903 by Ramon-y-Cajal, called the *coiled bodies* (Lamond and Carmo-Fonseca, 1993) because of their appearance as coiled webs.

The EM reveals that the speckles contain *interchromatin granule clusters* (IGC) in which each granule is approximately 20 to 25 nm in diameter. Perichromatic fibers extend from the IGCs and are also found in the nucleoplasm. We have seen that these fibers represent nascent transcripts.

Although transcription occurs at the sites of perichromatic fibers, splicing factors can be found predominantly in the IGCs (Jiménez-García and Spector, 1993) and not in the perichromatic fibers (Wansink et al., 1993). The IGC could therefore represent a storage site for splicing factors from which they would have to be recruited. The translocation of splicing factors to the sites of transcription has been shown in the case of viral and plasmid transcription (e.g., Jiménez-García and Spector, 1993). The translocation of splicing factors to the active sites and their exit when the sites become inactive, has also been demonstrated (Baurén et al., 1996).

What determines the shuttling of splicing factors between the speckles and the transcription sites? A short amino acid motif of some splicing factors (*SR-proteins*), is sufficient to target the proteins to the speckles (Hedley et al., 1995). This motif is present in a serine-arginine rich domain, the RS-domain that is heavily phosphorylated. Four kinases have been shown to act specifically on SR-proteins and at least two of them have been shown to be responsible for the intranuclear localization of the proteins (e.g., Colwill et al., 1996). Furthermore, in at least some cases, SR-proteins and some snRNP proteins have to be phosphorylated to function in spliceosomes. In addition, dephosphorylation is probably required for the splicing reaction and the disassembly of spliceosomes (see Mermoud et al., 1992; Misteli and Spector, 1997). An involvement of phosphorylation in recruitment to the transcriptional sites would imply that splicing factors must be released from the IGCs and then returned when no longer needed. The return to IGCs depends on phosphatase activity (Misteli and Spector, 1996). Similarly, overexpression of the a kinase that localizes to speckles, becomes more diffusely distributed when phosphorylated (Colwill et al., 1996) and overexpression of the kinase displaces SR-proteins from the ICGs. The role of phosphorylation and dephosphorylation as a regulatory mechanism is a recurring theme in our discussions (see Chapter 13).

B. Control of Macromolecular Traffic by The Nuclear Envelope

Proteins are synthesized in the cytoplasm and mRNAs are transcribed and processed in the nucleus. Therefore, the nuclear envelope exerts an all important role in transcriptional control (by selecting the proteins entering the nucleus which may have a role in gene expression) and post-transcriptional control (by selecting the mRNA molecules leaving the nucleus). Proteins need *nuclear localization signals*

(NLSs) to be translocated into the nucleus (see Chapter 5). RNA export also requires some sort of signal sequence. However, many aspects of the role of the nuclear envelope are still to be explored in detail and, except for a few cases, this aspect cannot be clearly evaluated with the information presently available.

The general role of the nuclear envelope in regulating macromolecular traffic is an important component of gene expression. This can be demonstrated with an example: the role of translocation through the nuclear envelope in the regulation by steroid hormones. The hormone binds to the glucocorticoid receptor (GR) protein present in the cytoplasm. The binding induces a dissociation of the receptor subunits. The receptor subunit binding the hormone and now released from the complex can enter the nucleus and bind the *hormone response element* (HRE) in the DNA, which can now activate transcription of the downstream DNA sector. A model summarizing these observations is shown in Fig. 9.

hnRNPs have been found to accompany mRNAs as they exit the nucleus. Besides their role in splicing and in many regulatory functions (see Krecic and Swanson, 1999), they have a role in the localization of the mRNA in the cytoplasm, its translation and its turnover (see Shyu and Wilkison, 2000; see also Chapter 15).

In metazoan organisms, (e.g., HeLa cells, mouse and *Xenopus*), a protein, ALY, (equivalent to Yra1p in yeast, see Chapter 5), has been found to be recruited to mRNP complexes during spliceosome assembly. ALY shuttles between the nucleus and the cytoplasm. In the nucleus, it co-localizes with splicing factors (Zhou et al., 2000). ALY does not bind to already spliced mRNA or to hnRNP. Yra1p has been shown to bind to Mex67p, which is part of the mRNA export complex (Strässer and Hurt, 2000).

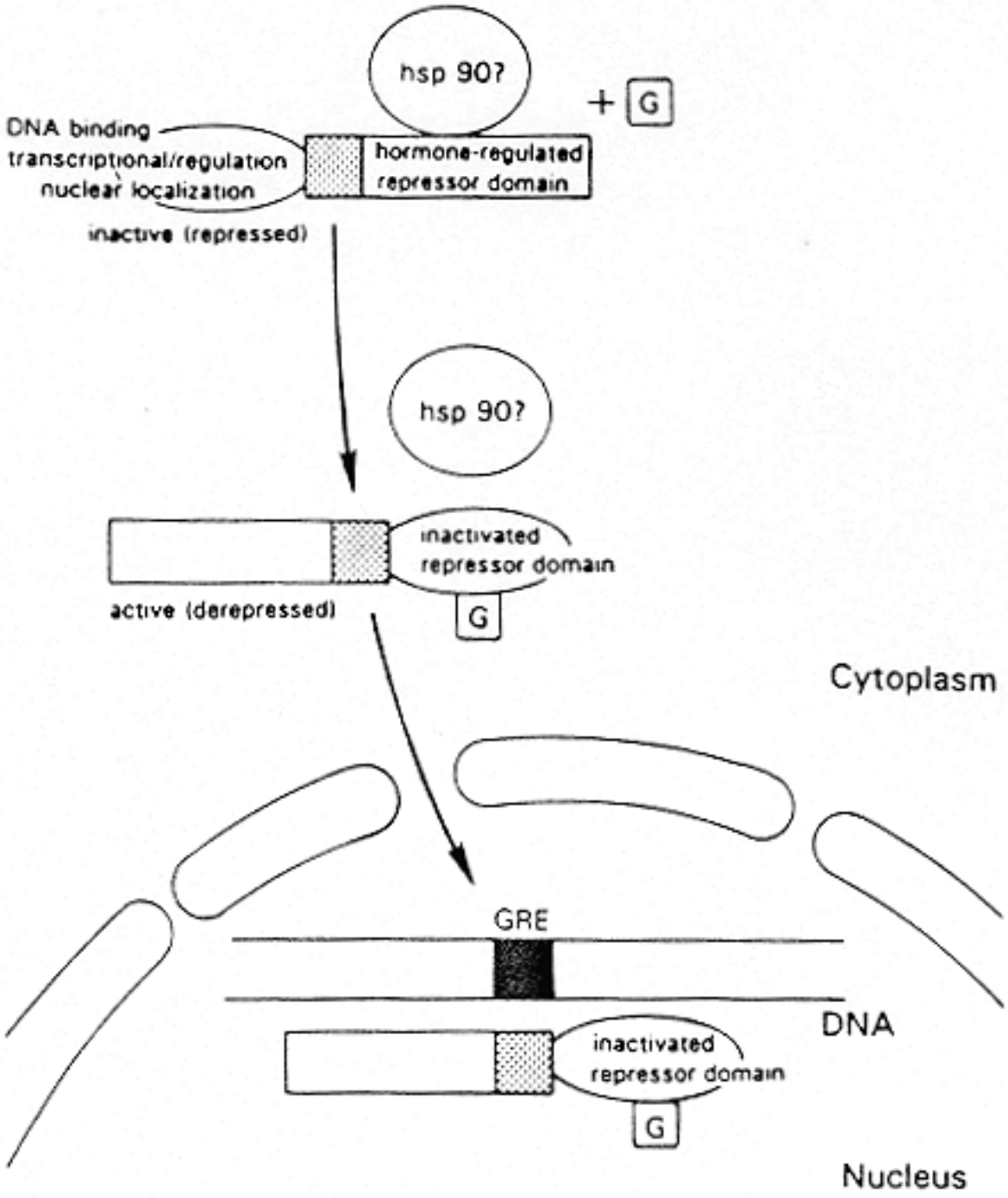


Fig. 9 A model showing the presence of the GR in the cytoplasm bound to hsp90 which has been hypothesized to be part of the unit. hsp90 is a protein which is part of the so-called *heat shock proteins*. These proteins are thought to have far reaching roles in cell biology, including important functions in the folding of proteins. When the hormone (G) binds to the GR, hsp90 is released and the GR is in its active form, which can enter the nucleus and bind to the GRE.

C. Interactions

As we saw, the production of mRNA and its translocation from the nucleus to the cytoplasm occurs in a number of steps which include transcription (initiation, elongation and termination), capping of the 5' end,

removal of introns, cleavage at the 3' end, polyadenylation and after release from the chromatin, the translocation into the cytoplasm. These processes are in series and tightly coupled physiologically and physically (see Maniaitis and Reed, 2002). In part these connections are arrived at by tethering to each other the machines responsible for each step. For example, RNA polymerase II and the RNA processing machinery are coupled. Similarly, the coupling of transcription to splicing is produced by tethering splicing factors to the transcriptional machinery. Likewise, the splicing of mRNA and its translocation are also coupled by attaching the nascent mRNA to a export factor Yra1p and transferring it to the receptor (TAP/Mex67) (see Chapter 5) and its delivery to the nuclear pore complex (see Chapter 5 , Section I)

D. Noncoding RNA

A variety of RNAs do not function as mRNA, tRNAs or rRNAs. These have been referred to as *noncoding RNAs* (ncRNAs) (small RNAs in bacteria) (see Storz et al., 2002). NcRNAs have been found to have a role in transcriptional regulation, chromosome replication, RNA processing and modification, mRNA stability and translation, as well as protein degradation and translocation. The sizes of ncRNAs varies depending on their function. Those associated with development in the nematode *Caenorrhabditis elegans*, *Drosophila* and mammals have been found to be 21 to 25 nucleotides in length. The translational regulators in bacteria are 100 to 200 nucleotides in length and those involved in gene silencing in eukaryotes are larger than 10,000 nucleotides. NcRNAs may target RNA or DNA by direct base pairing., my mimicking the structure of other nucleic acids or as part of a larger RNA-protein complex .

E. RNA Localization

RNA localization at specific sites is involved in differentiation and embryogenesis (see Chapter 2). In addition, it determines cellular asymmetry and cellular polarity (see Kloc et al., 2002), and has a role in synaptic plasticity. It produces a high concentration of protein at a specific cellular locations (e.g., actin at the leading edge of cells) and segregates RNAs to specific sites (e.g., in regions of a neuron). During embryogenesis, it has a role in the production of morphogens and the formation of cell lineages by restricting the presence of RNA to certain cells. The localization results from mechanisms which include vectorial transport out of the nucleus, cytoplasmic transport to targeted sites by the cytoskeleton, local entrapment at particular cytoplasmic sites and local stability accompanied by the general degradation of the RNA at other sites.

Targeting sequences in the mRNAs 3'-untranslated region (UTR) serve as signal sequences for their localization. In other cases, RNA-binding proteins have a role in the targeting. (see Kloc et al., 2002). The binding of these proteins may associate the RNA with a variety of other proteins to produce large ribonucleoprotein (RNP) particles which are transported to their targets (e.g., Barbarese et al., 1995). When the RNA arrives at its destination it is anchored by proteins or in some cases other RNA molecules (e.g., Heasman et al., 2001). (see also Chapter 10)

The regulation of the translation of localized mRNAs has a central role in synaptic plasticity and hence in

memory and learning (see Richter, 2001; Steward and Schuman, 2001). Synaptic activation through the N-methyl-D-aspartate receptor (NMDAR) can activate the translation of mRNAs. For example, the synthesis of the α subunit of the calcium-calmodulin-dependent kinase II (CAMKII-α), required for learning and memory (Mayford et al., 1996), is stimulated by the *brain-derived neurotrophic factor* (BDNF) which functions in synaptic plasticity.

The finding that many of the localization proteins also have a nuclear function (see Farina and Singer, 2002) suggests that these proteins have multiple function. In addition, it has been shown that certain localization proteins may associate with specific transcripts.

IV. TRANSLATION

In many cases, regulation of gene expression occurs during transcriptional processes. However, many experiments indicate that regulation of gene expression can also occur after transcription, that is, *posttranscriptionally*. Some of this regulation occurs during the process of translation. The present section will review present knowledge of the mechanics of protein synthesis.

Three phases are involved in translation: initiation, elongation and termination. The details of translation are still not understood completely. However, models can be constructed which summarize our present understanding. Two of them are outlined below. A model of initiation showing some of the presently known details is shown in Fig. 10 (Merrick, 1992). Fig. 12 (Merrick, 1992) addresses elongation. Although Figs. 11 and 12 have a wealth of detail, not easy to follow, they are well worth careful study because they summarize an enormous number of findings.

Initiation (see also Pain, 1996) involves the formation of a complex between the initiation factor eIF2, GTP and tRNA-Met [indicated as 3a] (1), with 40S subunit of ribosomes complexed to eIF-6 and eiF-3 [indicated as 3 and 4C respectively] (2). This complex (3) can then bind to the m^7G cap of mRNA at the 5' end of the mRNA (4), where it scans the mRNA in the 5'-3' direction until it reaches the AUG initiation codon (5). The factors are then released (6), GTP is hydrolyzed and the 60S subunit joins the mRNA where the Met-tRNA is held at the initiation codon, to form the initiation complex (7).

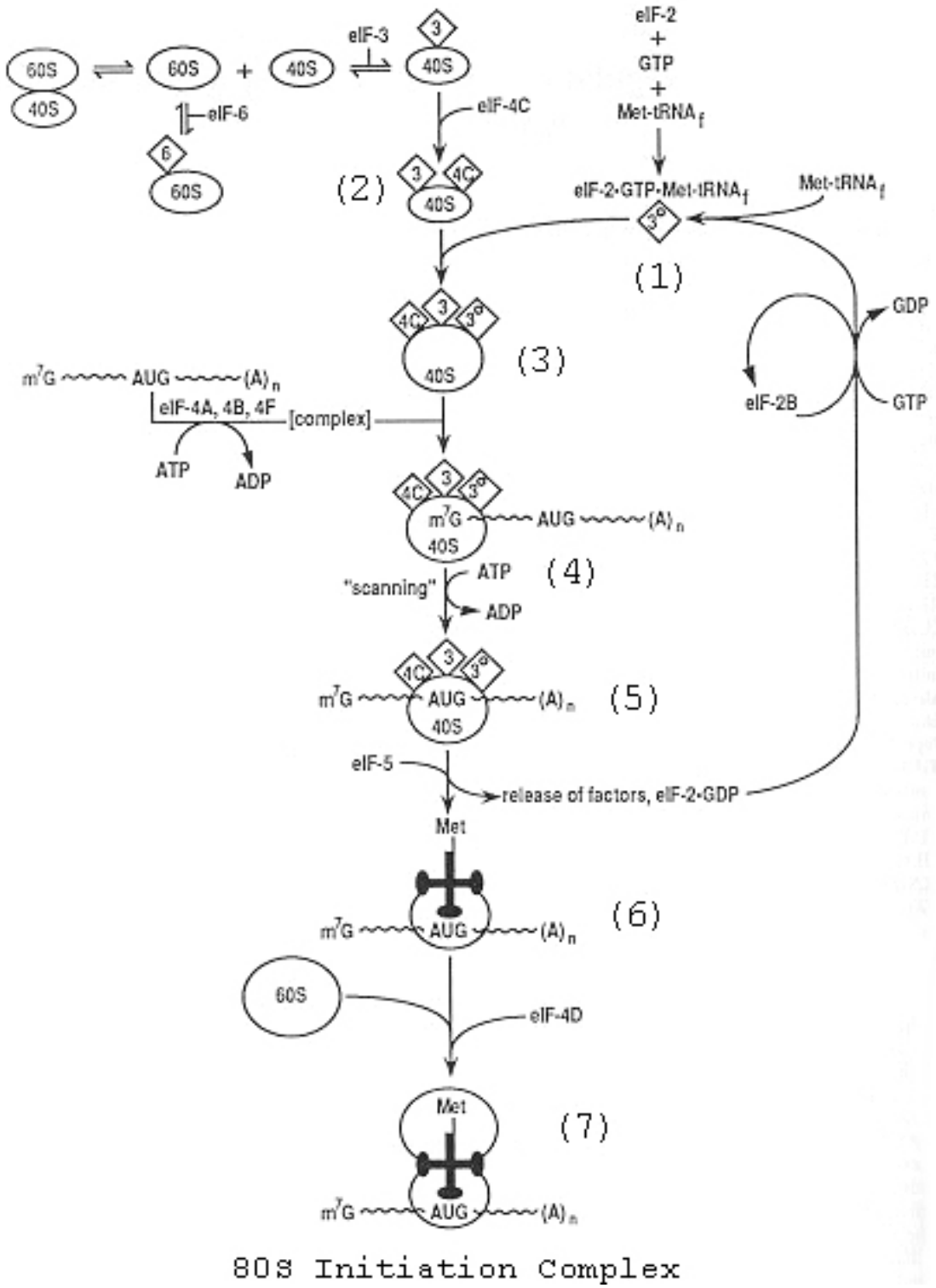


Fig. 10 Model for formation of 80S initiation complexes in eukaryotes. Reproduced from Merrick, W.C. (1992) *Microbiol. Rev.* 56:291-315.

Different initiation factors are part of complexes which act at different steps. At least 24 of the eIF polypeptides have been *cloned* (Rhoads, 1993). Cloning via the isolation of cDNA is discussed in the Appendix. Those of the eIF-1 class are thought to be involved in the pleiotropic stimulation of the assembly of the translational complex.

The model of elongation of Fig. 11 incorporates some of the features known from studies of prokaryotes. Elongation requires a triplet code where a combination of three bases (a *codon*) generally corresponds to one amino acid. Each tRNA species corresponds to a single amino acid and has a triplet complementary to that present in the coding mRNA (an *anticodon*). In this model (starting with 1), E is the exit site, P is the peptidyl-tRNA site and A is the site where the incoming aminoacyl-tRNA will bind. EF, followed by the appropriate number or symbol, indicates the involvement of an elongation factor. The amino acyl-t-RNA attaches to the codon in site A (2 and 3). The tRNA-peptide at site P is transferred to the aminoacyl-tRNA in site A and a peptide bond is formed between the aminoacyl-tRNA and the peptidyl-tRNA. In step 4, the 3 end of the deacetylated tRNA is now shifted to the E site without moving the anticodon. The peptidyl tRNA chains grows by one amino acid without moving. Translocation (step 4 step 1) moves the mRNA by one codon so that a new codon is at the A site. For the step immediately following initiation, the same model is proposed. However, in this case the Met-tRNA acts as the peptidyl-tRNA shown in this model.

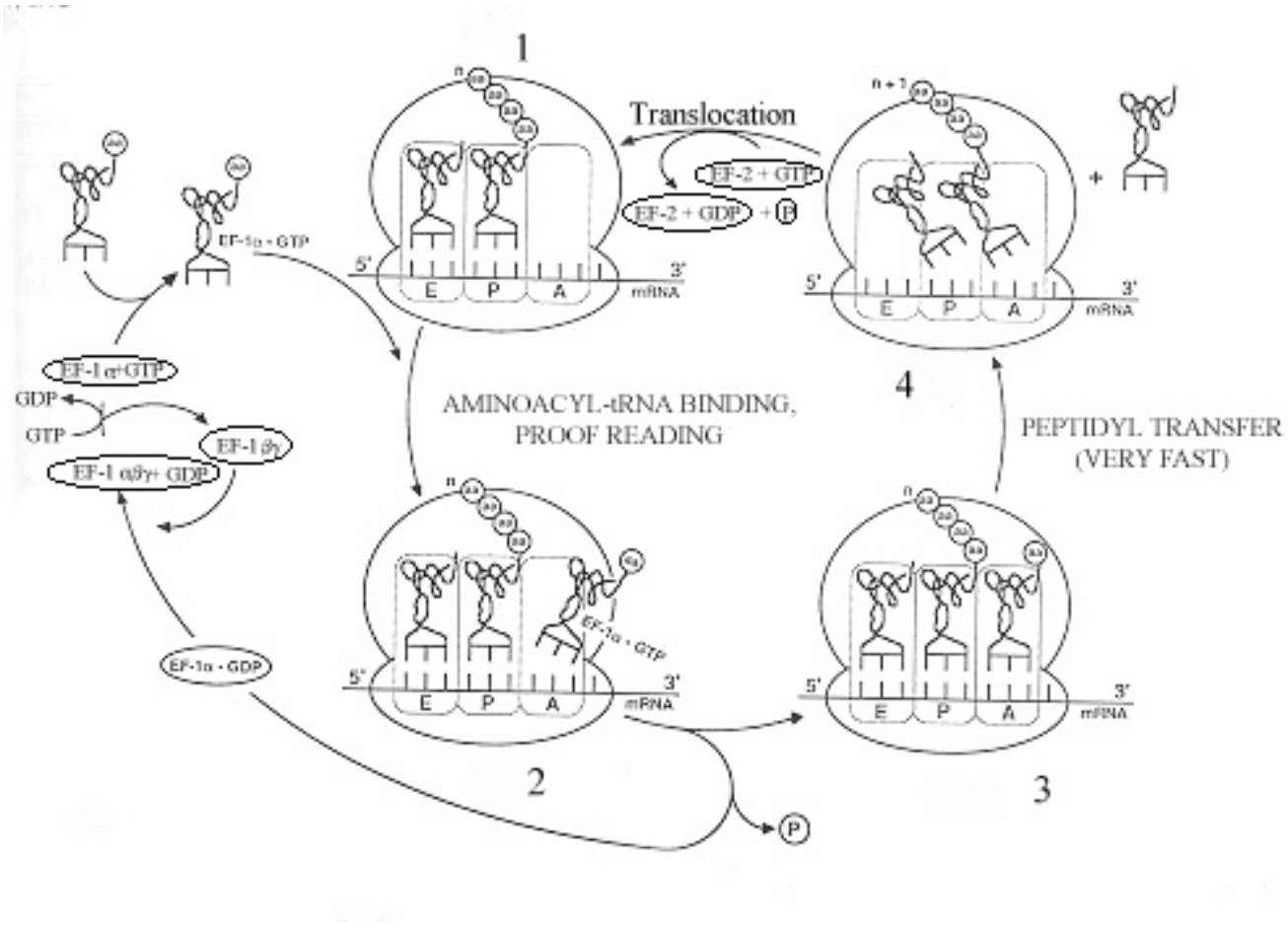


Fig. 11 Model for the elongation cycle in eukaryotes. The scheme is patterned after the "half-site" model of Moazed and Noller. E is the exit site, P is the peptidyl-tRNA site and A is the site for the binding of incoming aminoacyl-tRNAs. The slow steps appear to be the binding at P and the translocation. Reproduced from Merrick, W.C. (1992) *Microbiol. Rev.* 56:291-315.

Termination involves one of the stop codons (UAA, AGA and UAG) and a *release factor*, RF. RF binds GTP, recognizes the stop codon and induces the hydrolysis of the aminoacyl linkage and the GTP.

For simplicity, the previous discussion ignored in most respects the ribosome. However, the ribosome represents the translational engine. In fact, protein synthesis may be carried out by ribosomes in the absence of factors or GTP under non-physiological conditions, indicating that the necessary machinery is totally within ribosomes (Pestka, 1969; Gavrilova and Spirin, 1976).

In bacteria, the ribosome is about 2.5 MDa and is constituted by more than 50 different proteins and 3 RNA molecules representing over 4500 nucleotides (Hill et al., 1990; Matheson et al., 1995). The two subunits are denoted by their sedimentation constant, S. Peptide synthesis involves complex interactions in the ribosome. The smaller subunit (in bacteria 30S, 40S in eukaryotes) of the ribosome binds mRNA and the anticodon end of the tRNAs, the larger subunit (in bacteria 50S, 60S in eukaryotes) binds to the amino acid end of the tRNAs and catalyzes the formation of the peptide bond. After the peptide bond is formed, *elongation factor G* (EF-G) binds to the ribosome. The peptidyl-tRNA is then moved by one codon of the mRNA (see Wilson and Noller, 1998).

Clearly, the process requires movement. *Escherichia coli* 70S ribosomes in the various stages were examined by cryo-electron microscopy and three-dimensional reconstructions (Frank and Agrawal, 2000). The binding of EF-G and the GTP hydrolysis produce a ratchet-like rotations of the small subunit relative to the large subunit. The translocation occurs in two steps. First GTP binds to EF-G, followed by rotation of the subunits and opening of a channel occupied by mRNA. This is then followed by GTP hydrolysis and advance of the mRNA/(tRNA) complex in the direction of the rotation of the 30S subunit. In addition, many other conformational changes take place within the two subunits.

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

V. REGULATION

Regulation of gene expression by transcriptional and posttranscriptional mechanisms is so important to the physiology of the cell that a discussion of these topics will recur throughout the textbook. Despite a wealth of knowledge, there are still many unanswered questions.

A technique making use of *fluorescence in situ hybridization* (FISH) and computational microsopy (Levsky et al., 2002) has allowed the simultaneous study of the expression of many genes inside single cells using oligomer DNA probes tagged at multiple sites. . The study of 11 genes in serum-stimulated cultured mammalian cells showed that gene expression differed in the individual cells and revealed interesting features such as coordinate expression of α and γ actin during serum activation and a differential expression level of one of two alleles. This approach is providing a completely different way of looking at gene expression. However, more conventional observations will be discussed in the section that follows.

A. Transcriptional Regulation

In this section, coordinate expression of transcriptional regulation is discussed first, including one specific case will be addressed in detail. This is then followed by a discussion of specific transcriptional regulatory mechanisms. Some of the elements of activation and repression hve been presented in Section II, above. A discussion of transcriptional regulation will be addressed again in Chapter 7 in relation to intracellular signals.

Coordinate expression

The transcription of many genes is frequently coordinated. DNA-microchip technology (see Chapter 1) has allowed examining in detail the coordinate expression of related genes in eukaryotes over several hours (e.g., see Niehrs and Pollet, 1999). In this so-called *synexpression*, there is a close correlation between function and expression. However, in contrast to the operons of bacteria that are transcribed as polycistronic mRNAs, synexpression takes place by the coordinate activation of genes in different chromosomes. The sequence of transcriptional events accompanying the metabolic shift from fermentation to respiration, was studied using DNA microarrays containing almost all of the genes of *Saccharomyces cerevisiae* (DeRisi et al., 1997). The shift produced the coordinate enhancement and decline in the transcription of hundreds of genes. The rest of this section will discuss the coordinate regulation of much smaller group of genes: those that control the enzymes responsible for galactose

metabolism by the *GAL4* gene in the yeast, *Saccharomyces cerevisiae.*

The control of galactose metabolism in *Saccharomyces cerevisiae* by galactose represents a small sample of how some of these coordinated systems operate. In yeast, galactose is generally metabolized after conversion to glucose-6-phosphate in a pathway depicted in Fig. 12 (Johnston, 1987). In this representation, the genes coding for specific enzymes are listed on top of the enzyme. In the schematic representation of the pathway below these listings, Gal and Glu correspond to galactose and glucose respectively, and P is phosphate. Melibiose is first hydrolyzed to its two component monosaccharides extracellularly. After transfer into the cells mediated by a *permease*, Galactose is then metabolized in four separate steps (2-5) to produce Glucose-6-phosphate which is subsequently broken down through the reactions of the glycolytic pathway, as indicated. Except for the *Gal5* which is unregulated, the expression of the genes of this pathway is highly regulated. The presence of galactose induces the complete pathway. The model of gene regulation which explains most of the data presently available, is represented in Fig. 13 (Johnston, 1987). In this figure, each line represents a different chromosome. The various GALs correspond to the genes indicated in Fig. 12. *Gal1*, *Gal7* and *Gal10* are present in the same chromosome and have separate promoters so that their transcription is separate. *Gal2* and *Mel1* are present in other chromosomes. The expression of all these genes is activated by a protein encoded by *Gal4*, as indicated by the stippled arrows. The GAL4 protein (shown in the stippled circle) binds to the DNA upstream of these genes. Regulation, however, depends on an additional gene. *Gal80* encodes a protein which binds directly to GAL4 protein, inactivating its effect, as indicated by the stippled bar. Inducer binding of GAL80 protein prevents its inhibition of GAL4. Empty arrows in this figure indicate the direction of the transcription, solid arrows indicate the production of the protein indicated.

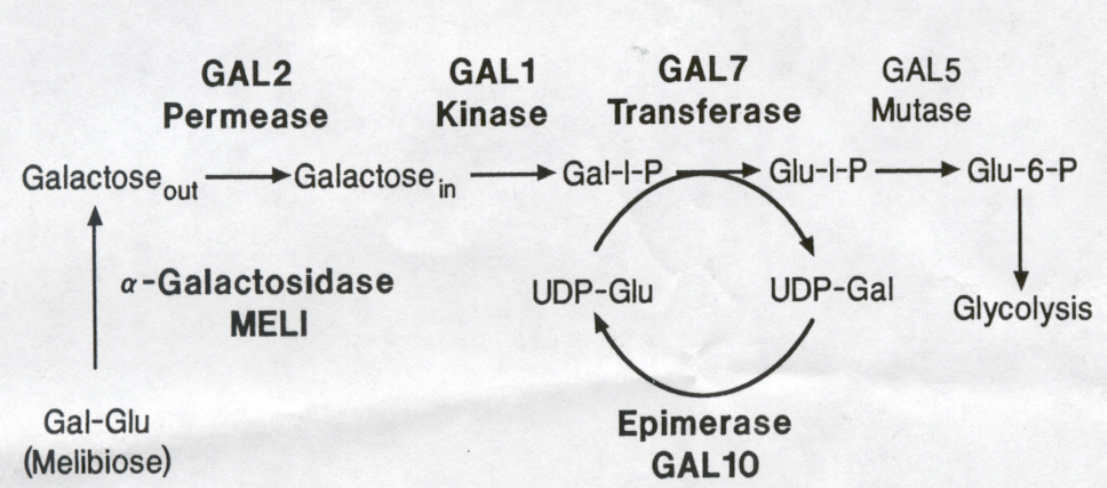


Fig. 12 Pathway of galactose utilization. The enzymes are galactokinase, encoded by *GAL1*, galactose-1-phosphate uridyltransferase, encoded by *GAL7*, uridine diphosphoglucose 4-epimerase encoded by *GAL10*, phosphoglucomutase encoded by *GAL5* and α-galactosidase, encoded by *MEL1*. The genes controlled by galactose are shown in bold face. Reproduced from Johnston, M. (1987) *Microbiol. Rev.* 51:458-476.

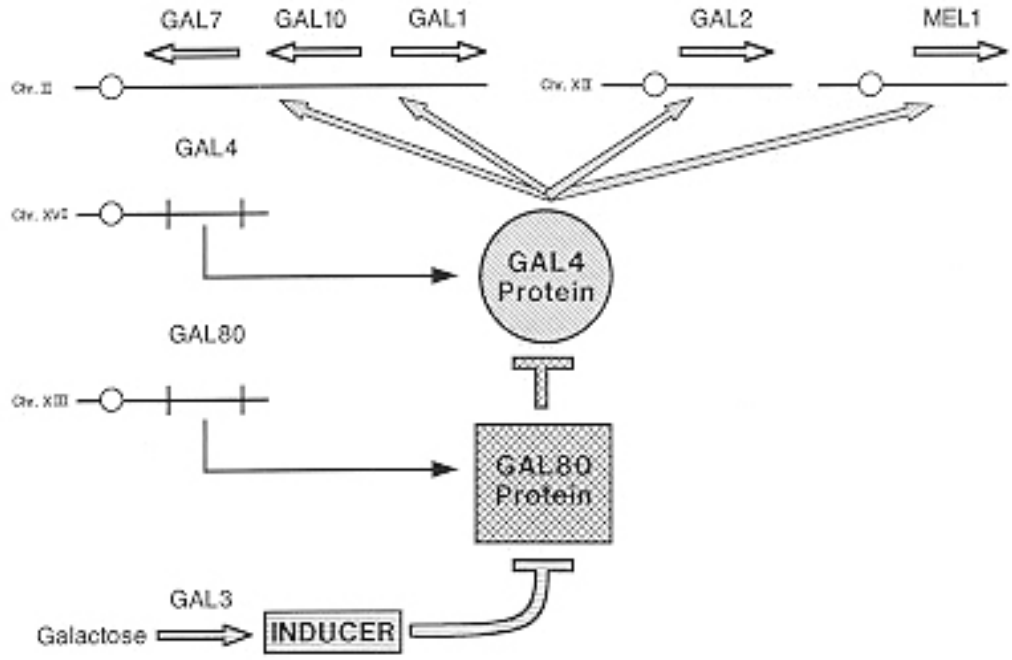


Fig. 13 Components of the *GAL* gene regulatory circuit. Bold lines with arrows denote stimulation of activity; those with bars denote inhibition of activity. Reproduced from Johnston, M. (1987) *Microbiol. Rev.* 51:458-476.

The DNA sectors which bind the GAL4 protein (Giniger et al., 1985), are the so called *upstream activation sequences* (UAS, in this case UAS_G) which correspond to enhancers of higher eukaryotes (Guarante, 1984). The binding of GAL4 protein to the UAS has been shown using footprint analysis with dimethylsulfate (DMS) similar to that described (Chapter 1). The gel electrophoresis patter of the UAS_G of the Gal^+ DNA is compared to that of a Gal^- mutant. The sectors protected by binding to the GAL4 protein are missing from the gel. The various DNA sectors were identified using radioactive probes complementary to the DNA sequences adjacent to UAS_G (Giniger et al., 1985).

The GAL4 protein has been found to contain various domains. One of these binds GAL80 protein. Another contains a nuclear localization sequence (NLS) which allows it to enter the nucleus. A Zn-finger domain binds DNA and a very acidic domain acts in transcription activation (Gill and Ptashne, 1987).

The GAL4 protein is present constitutively. GAL80 binds GAL4 only in the absence of an inducer. The interaction has been shown with UAS_G-DNA bound to Sepharose beads. In the presence, but not in the absence of GAL4 protein, GAL80 attaches to the beads (Lue et al., 1987). A model which would explain the molecular events controlling the pathway are depicted in the model of Fig. 14. In part A of this representation, GAL4 binds UAS of each of the genes involved in the pathway. As shown in part B, when GAL80 binds the inducer, it detaches from GAL4, which can now activate the transcription by mediating the attachment of the TATA box binding factor (TBF) to the promoter.

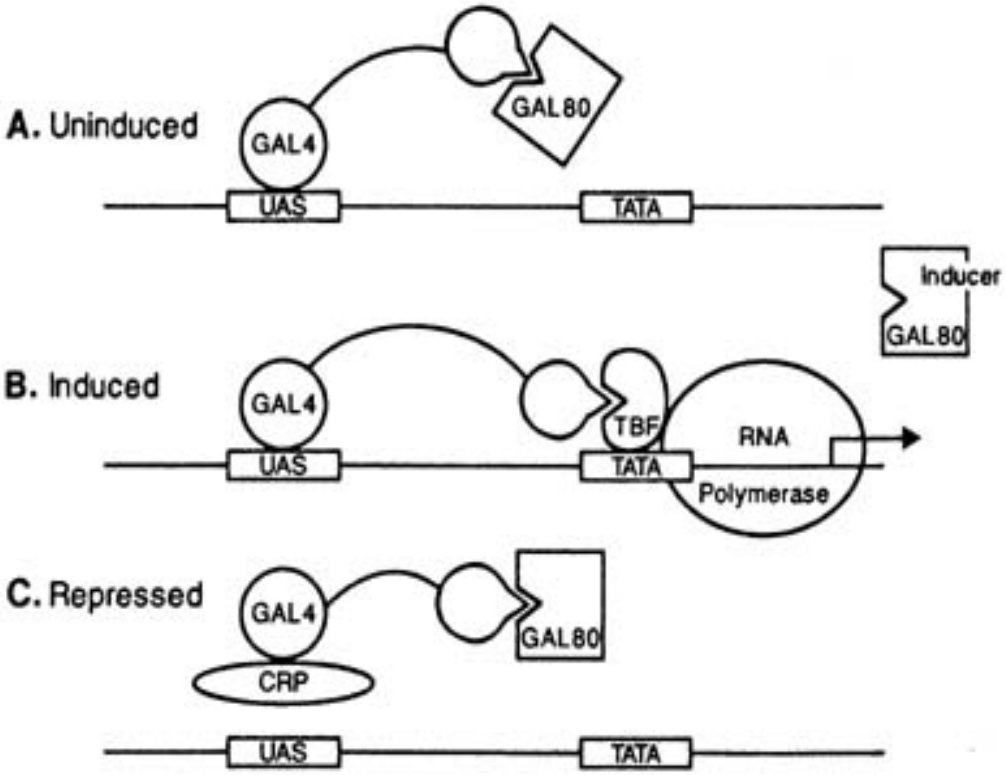


Fig. 14 Model for the mechanism of action of GAL4 protein. TBF, TATA box binding factor, UAS, upstream activation sequence. Reproduced from Johnston, M., (1987) *Microbiol. Rev.* 51:458-476.

The discussion in the next section will concentrate in some of the details involved in transcription.

A quick glance at various regulatory devices

Factors which regulate transcription, recognize elements called *response elements* (RE) which can be present in promoters or enhancers. For example, the glucocorticoid hormone receptor acts by binding to the glucocorticoid response element (GRE). Activation of a gene may involve the recognition of a promoter or enhancer by a transcription factor, so that transcription can be initiated. In some cases, there are several genes with common REs. Conversely, a single gene may be regulated by several REs, each corresponding to a different promoter. In contrast, repression may result from binding the target DNA sequence by a repressor protein, blocking the accessibility of the promoter to the transcriptional machinery.

The basic transcriptional machinery frequently requires sequence specific factors which bind to the protomoter/enhancer elements. A family of proteins have been identified which act as coactivators of transcription. They do not bind with the promoter elements but become part of the transcriptional initiation complexes. They connect the specific DNA-binding factors to the basic transcriptional machinery. These include the CREB (CREB=cAMP regulated enhancer binding) binding protein (CBP and p300). These proteins are the target of many regulative effects, such as the regulation by signal induced protein kinases (see Janknecht and Hunter, 1996). However, transcription factors and other

proteins are not alone in regulating eukaryotic transcription. Histones and their organization in nucleosomes are of fundamental importance (see Section ID, Section II).

For transcription to occur, the remodeling of repressed chromatin must take place. Chromatin transitions are apparently carried out by so-called *chromatin remodeling machines* (CRMs). All these complexes contain subunits homologous to DNA-dependent ATPases. They all carry out ATP-dependent perturbation of chromatin (Cairns, 1998). Their mode of action differs. The *nucleosome remodeling factor* (NURF) facilitates the ATP dependent binding of transcription factors to the chromatin (Tsukiyama and Wu, 1996). The *chromatin accessibility complex* (CHRAC), a complex of five proteins (Varga-Weisz et al., 1997), contributes to nucleosome spacing and makes chromatin more accessible. The *ATP-dependent chromatin assembly and remodeling factor* (ACF) consisting of two subunits (Acf1 and ISWI) contributes to the assembly of nucleosome arrays and facilitates the binding of activators to chromatin (Ito et al., 1997; Ito et al., 1999). The ISWI subunit is related to helicases and is responsible for the ATPase activity in which 2 to 4 molecules of ATP are hydrolyzed per base pair during nucleosome formation. The nucleosome remodeling involves the histone tails of the nucleosomes (Georgel et al., 1997). ACF was found to assemble nucleosomes in organized arrays ((Fyodorov and Kadonaga, 2002). SWI-SNF and related complexes (see Kingston et al., 1996) act in restructuring chromatin and are thought to play a role in transcriptional activation and the control of the cell cycle. In a model system, SWI-SNF has been found to displace nucleosomes along the DNA to another position (Whitehouse et al., 1999). The functioning of the CRMs is still being evaluated; each is thought to act in a distinct manner (Cairns, 1998).

RNA polymerase II has been been found to be associated with the gene regulator proteins SWI/SNF that are active in chromatin remodeling as discussed above (Wilson et al., 1996). The CREB binding protein CBP is also involved with the action of the polymerase. Apparently its role is that of an adaptor for many transcription factors and its action is thought to be mediated by its recruitment of histone acetyltransferases (HATs) (see Section II) to the promoter (Yang et al., 1996). In addition, CBP itself has been found to have HAT activity (Bannister and Kouzarides, 1996). The acetylation of histones has a role in gene expression (see Chapter 2).

Phosphorylation of histones plays a role in gene activation (see Chapter 2 and Section II). Growth factor activation via *mitogen activated proteins* (MAPs) phosphorylates H3. The phosphorylation corresponds to the H3 histone of *fos* and *myc* genes of stimulated cells, as detected by an antibody specific for phosphorylated Ser-10 of H3 (Chadee et al., 1999). *myc*, *fos* and *jun* genes are protoncogenes with a role in the regulation of cell growth. When overexpressed or hyperactivated they cause uncontrolled cell proliferation. Stimulation of the Ras-MAPK signaling pathway in oncogene-transformed cells also results in increased amounts of phosphorylated histone H1 (Chadee et al., 1995). Ras-GTP binding proteins (see Chapter 7) are responsible for transducing the signal from receptor tyrosine kinases to the nucleus to stimulate proliferation.

The treatment of mouse fibroblast with various growth factors or protein synthesis inhibitors results in the

transient phosphorylation of H3 and HMG14 (see Thomson et al., 1999). Apparently, the growth factors trigger a kinase cascade (the ERK pathway) which activates p42/p44, an activity mediated by GTP-binding proteins Ras and Raf (see Chapter 7). In mammalian cells, the phosphorylation of H3 associated with transcription is unique to a small set of genes (e.g., see Chadee et al., 1999; Sassone-Corsi et al., 1999).

How phosphorylation of the histones can affect gene expression is still an open question. Phosphorylation may act by adding to the histones negative groups that disrupt the interaction of the H3 tails and the negatively charged DNA backbone. Alternatively, phosphorylation could promote gene activation by serving as a site for recruitment of transcription factors. Some transcription factors contain SH2 domains which correspond to phosphotyrosine binding domains (see Hunter, 2000). We have seen one mechanism where histone phosphorylation serves in marking a histone lysine to facilitate its acetylation (see Section II).

Methylation of histones also play a role in gene activation as discussed in Section II (Chen et al., 1999). The p160 family of coactivators, are responsible for the transcriptional activation mediated by nuclear hormone receptors. A coactivator, *coactivator-associated arginine methyltransferase 1* (CARM1) can methylate histone H3 in vitro. In addition, a mutation of CARM1 was found to reduce both methyltransferase and coactivator activities.

B. Posttranscriptional Regulation

Posttranscriptional regulation includes a variety of controls including the degradation of mRNA and proteins. Some of these are discussed in this section to provide some insights into the diversity of mechanisms that have been discovered. In some cases, many reactions, usually in the same pathway, are regulated by the same mechanism. In other cases, only one or a few related genes are regulated by the same mechanism.

General regulation of translation

The global control of protein synthesis is necessarily linked to physiological conditions and in particular to the availability of the supply of the amino acids. The control of the synthesis of specific proteins also takes place. The rate limiting process is frequently the initiation of translation, so that in these cases regulation occurs at this step (see Pain, 1996). The most frequent mechanism for the regulation of protein synthesis is the phosphorylation and dephosphorylation of translational components, mostly initiation (eukaryotic initiation factor, eIF) or elongation factors (eukaryotic elongation factors, eEF) (e.g., see Manzanella et al., 1991). In some cases the phosphorylation activates the factor, while in other cases phosphorylation can blocks initiation.

Genetic manipulation of one of the eiFs (eIF-4E) has shown that this initiation factor is involved in the regulation of growth and cell proliferation. eIF-4E is the protein responsible for the recognition of the

m^7GpppN cap at the 5' end of almost all eukaryotic mRNA and it facilitates the melting of the secondary structure of 5'UTRs (see Pain, 1996). Cells transfected with vectors containing the gene for this protein grow rapidly and form tumors (Rhoads, 1991). Conversely, the expression of RNA antisense for the mRNA of eIF-4E (i.e., the RNA complementary to the normal mRNA, which would therefore inactivate the latter by specifically binding it) decreases protein synthesis and cell proliferation. The phosphorylation of this factor enhances its activity and promotes protein synthesis (Rhoads et al., 1991).

A family of regulators that bind eIF4E were recently discovered (Pause et al., 1994). PHAS/4E-BP1 inhibits the eIF-4E-dependent mRNA translation by blocking the binding of eIF4-E to eIF4-G (e.g., Mader et al., 1995). The latter interaction is required for efficient interactions of the 40S ribosomal subunit to the mRNA. The effect of PHAS/4E-BP1 is most pronounced in the translation of mRNAs with highly structured 5'UTRs (Manzella et al., 1991) rather than being global.

The regulation by several agents (e.g., insulin-like growth factor and interleukin 2) promotes the dissociation of PHAS/4EBP1, whereas those that increase cAMP increase the binding. The effect of insulin is most likely to be in response to a phosphorylation of PHAS/4E-BP1 and the subsequent release of eIF4E. The phosphorylation and dephosphorylation of PHAS/4E-BP1 is the most likely mechanism for the action of other agents as well.

The effect of eiF-4E and the control exerted by some genes is very general. However, the regulation may be much more specific, sometimes due to the presence of highly structured 5'UTRs in the mRNA (see Pain, 1996). However, the control of individual genes and that of an entire pathway may be very similar because a single gene may affect a variety of other genes.

Regulation of specific genes

The presence of specific mRNA and its availability to the translational machinery has a role in gene expression. In some cases, a pattern of repression-derepression similar to that occurring at the gene level has been found. In these cases, repressors or activators bind to specific sectors of the mRNA, blocking or facilitating translation. In other cases, the regulation is more complex.

One of the best studied mechanisms is the translational regulation of the eukaryotic *initiation factor 2* (eIF2). Regulation involves the phosphorylation of the α-subunit of eIF2. The phosphorylation downgrades translational initiation either in general or at specific steps (see Wek 1994, De Haro et al., 1996).

The eIF-2 kinases have their kinase catalytic domain in common. However, they contain unique regulatory regions adjacent to the catalytic domain, permitting regulation by different physiological signals (see Wek, 1994).

The phosphorylation of eIF-2α was first found in reticulocyte lysates deprived of hemin (see Chen and

London, 1995). The absence of hemin activated the eIF-2α kinase, the *heme-regulated inhibitor* (HRI). The *double-stranded RNA-activated kinase* (PKR) is constitutive in reticulocytes and inducible by interferon in other cells. PKR is activated by double stranded (i.e., viral) RNA in the presence of ATP (Mathews and Schenk, 1991). This kinase is part of the cell's defense against viral infection. Association of PKR with double stranded RNA results in autophosphorylation and consequent activation. In yeast (*Saccharomyces cerevisiae*), the *general control nonrepressible* kinase (GCN2) is activated by uncharged tRNA during amino acid starvation (Hinnebusch, 1993) and purine shortage (Rolfes and Hinnebusch, 1993). There are also indications that other eIF-2α kinases exist (Olmsted et al., 1993) and may play a regulatory role.

Gene specific translational control by the phosphorylation of eIF-2α is still not well understood. The regulation of GCN4 by GCN2-protein kinase depends on the relative rates of the translation of *upstream open reading frames* (uORFs) in the GCN4-mRNA (see below).

In this section, a detailed discussion will be limited to three cases which are reasonably well understood. These are the systems responsible for the regulation of intracellular iron, tubulin and amino acid metabolism in yeast, the latter mediated by GCN4 protein.

Regulation of iron

Iron is a vital component of living cells. Hemoglobin contains iron and so do the cytochromes which are responsible for the electron transport in mitochondrial oxidative phosphorylation. Not surprisingly, various components involved in Fe storage or transport, or the synthesis of Fe-containing compounds, are regulated by the availability of Fe. Ferritin is an intracellular protein of animal, plant, fungal and bacterial cells responsible for binding and storing Fe (Theil, 1987). Aminolevunilic acid (ALA) is one of the precursors of porphyrins needed for cytochromes and hemoglobin. Transferrin is a protein which carries iron in the blood and is taken up by cells through endocytosis mediated by the transferrin receptor (TfR). Accordingly, the presence of Fe in the cell favors the synthesis of ferritin and ALA synthase, but inhibits that of the TfR responsible for the uptake of Fe and which is needed in lesser amounts at high Fe concentrations.

The mRNAs corresponding to the proteins which are needed in larger amounts in the presence of iron (ferritin, ALA), have a 35-nucleotide region (iron response element, IRE) in the 5' untranslated region of the mRNA. This region is thought to form a stem-loop structure as shown in Fig. 15. A ferritin repressor (FRP) has been shown to bind to the IRE in vitro (Leibold and Munro, 1988). In contrast to the case of ferritin, the TfR is needed in larger amounts in the absence of iron to make up for its low concentration. The TfR-mRNA contains several copies of the IRE, similar but not identical to that of ferritin, in the 3' untranslated region. In this case binding of the repressor stabilizes the mRNA, so that the synthesis of the transferrin receptor is enhanced (Casey et al., 1988).

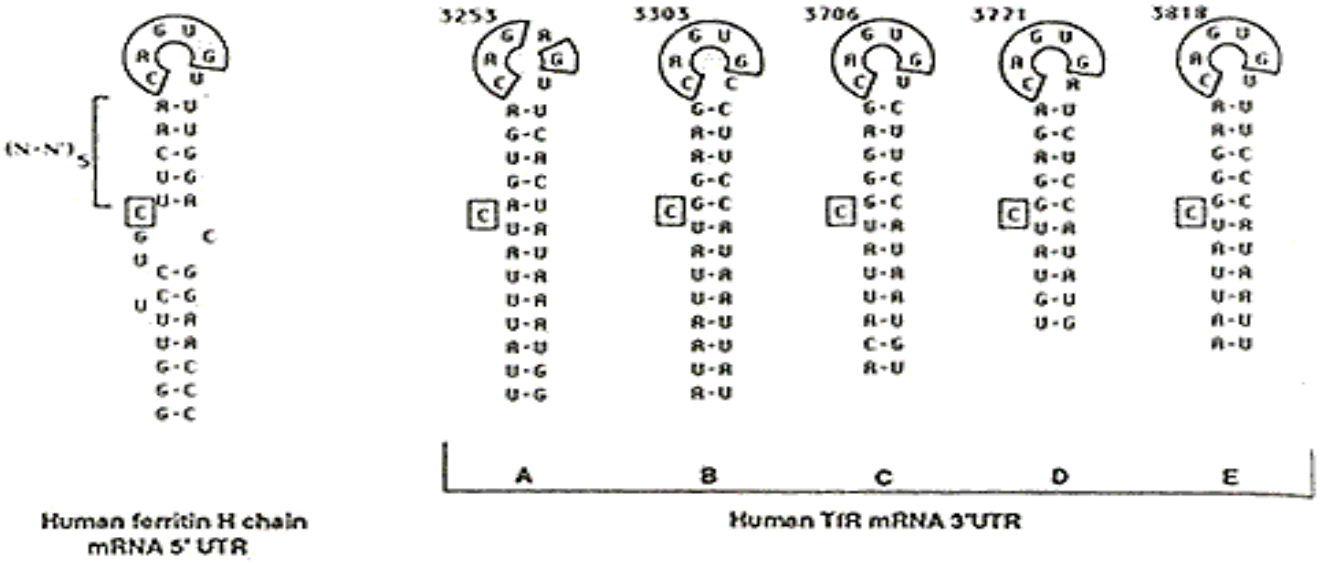


Fig. 15 Similarities between the IRE of the 5UTR of ferritin mRNA and sequence elements present in the 3UTR of the transferrin receptor (TfR) mRNA. Reproduced with permission from Casey, J.L., Henze, M.W., Koeller, D.M., Caughman, S.W., Rouault, T.A., Klausner, R.D., and Harford, J.B. *Science* 240:924-928, copyright &copy1988 American Association for the Advancement of Science.

Tubulin regulation

The regulation of the synthesis of the tubulin, the subunits of microtubules, follows a mechanism in which the stability of mRNA depends on events accompanying translation. Microtubules are structures which participate in a spectrum of cellular functions. Together with intermediate filaments and actin, they are basic structural elements determining cell shape and growth. Furthermore, they participate in vital cell functions, such as cell movement, the formation of the mitotic spindle and the movement of chromosomes during mitosis. The basic units of tubulin are the heterodimers of α and β tubulin. Microtubules are in rapid, dynamic steady state with the free tubulin dimers (e.g., Kirschner and Mitchison, 1986). During cell differentiation, one or more of the 6 to 7 genes that encode either subunit are activated (Cleveland, 1987). However, the rate of synthesis of tubulin needed to maintain the appropriate concentration is determined by the concentration of free tubulin dimers by a negative feedback mechanism at the posttranscriptional level.

The control of the tubulin synthesis by the tubulin level can be demonstrated by microinjection of tubulin into cultured cells (Cleveland et al., 1983), or in experiments in which the cells are treated with drugs, such as colchicine (which depolymerizes microtubules and therefore increases the concentration of free

tubulin). Increased free tubulin dimers decrease the amount of tubulin being synthesized. The regulation is posttranscriptional: the presence of a nucleus is not required. Analyses of tubulin mRNA levels show that the regulation can be accounted for by decreases in mRNA stability (e.g., Caron et al., 1985). The stability of the tubulin-mRNAs is regulated by the level of the free heterodimer (Gay et al., 1987) which apparently depends on the presence of four amino acids in β-tubulin. As few as 13 nucleotides artificially added at the 5' end of mRNAs coded for another protein, were found to confer β-tubulin sensitivity (Yen et al., 1988a). In addition, the regulation occurs only when the translation goes past the first 41 codons of the mRNA, as indicated by experiments carried out with truncated mRNAs, and requires the association of the mRNA to ribosomes to form polysomes.

These results suggest that the four terminal amino acids of β-tubulin bind to the free tubulin heterodimer as it emerges from the large ribosomal subunit during translation. This binding would activate a nuclease. This model is summarized in Fig. 16 (Yen et al., 1988b). As shown in the figure, unpolymerized tubulin subunits bind directly (or activate a factor which binds) to the nascent amino terminal nascent tetrapeptide (Met-Arg-Glu-Ile) of β-tubulin. This binding activates the RNase present in the adjacent ribosomes to degrade the mRNA attached to the ribosome. In the figure, MREI denotes the amino terminal of the β-tubulin polypeptide. As collected in Table 1, a similar mechanism is thought to operate in other systems (Merrick, 1992).

Generally, all mRNA is unstable. Typically in *E. coli*, mRNA has a half life of about 2 minutes, in yeast 10 to 20 minutes and in mammalian cells several hours. Because they are subject to regulation, the mRNAs of the same organism have different half lives.

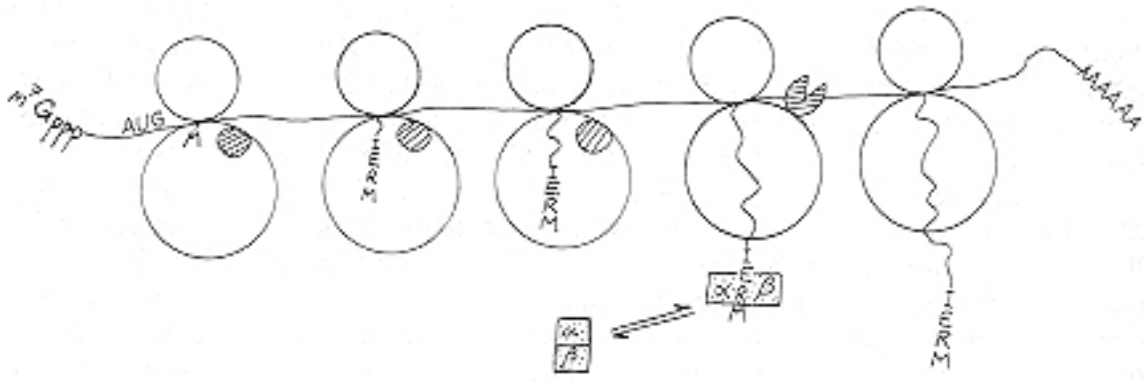


Fig. 16 Proposed model for autoregulated instability of β-tubulin mRNA. Reproduced with permission from *Nature*, Yen, T.J., Machlin, P.S. and Cleveland, D.W. 334:580-585, copyright &copy1988 MacMillan Magazines Ltd.

Table 1 mRNA Instability Regulated by Translation From Merrick et al., 1992, reproduced by permission.

mRNA	Reference
histone mRNA	Baumbach et al., 1987 Graves et al., 1987. Sive et al., 1984
c-myc	Linial et al., 1985
c-fos	Kruijer et al., 1984
transferrin receptor	Müllner and Kühn, 1988
Mat α1 gene in yeast	Parker and Jacobson, 1990
GM--CSF	Shaw et al, 1992

Synthesis of amino acids

GCN4 is a gene which regulates the biosynthetic pathway of amino acids in yeast. It activates more than 30 genes coding for enzymes involved in the biosynthesis of 10 different amino acids. *GCN4* expression increases during amino acid starvation. Conversely, when amino acid levels increase, *GCN4* expression decreases. During these changes, the level of GCN4 mRNA remains the same. The control is therefore translational.

The control by GCN4 involves a subtle interplay of translational events, in which the initiation complex and the CGN4 mRNA untranslated 5 portion of 590 bases play a role. The initiation complex contains Met-tRNA, where Met is the amino acid which initiates peptide synthesis. The mRNA region contains four small *open reading frames* (ORFs), referred to as ORF1 to ORF4. An open reading frame is a sequence of codons with a start and a terminator codon which could potentially code for a polypeptide. As shown in Fig. 17 (Abastado et al., 1991), the 40S subunit of ribosomes scans from the 5' end of the mRNA. They initiate translation when they arrive at either ORF-1 or ORF-2. The complete translational machinery, including an assembled ribosome, is bound at this site. At termination of the reading of each ORF, the translational machinery disassembles, but the 40S ribosomal subunit continues scanning in the 5' to 3' direction. When amino acids are present in abundance, the eIF-2.GTP.Met-tRNA complex reforms readily and reinitiation starts at ORF4 (line 1 of Fig. 18). However, the scanning of the 40S subunit cannot recognize the AUG initiation codon of GCN4, because the complex does not have enough time to reassemble after leaving ORF4. Therefore, GCN4 cannot be translated. Without GCN4, the synthesis of new amino acids is inhibited. When eIF-2.GTP.Met-tRNA is formed slowly (low level of amino acids,

line 2), the scanning ribosome bypasses ORF-3 and ORF-4 because the initiating machinery fails to assemble in time. However, the complex has enough time to reform and initiate translation at the AUG codon of GCN4. The increased, newly synthesized GCN4, will therefore activate the synthesis of new amino acids. This mechanism, based on the rate of separate translational events, is thought to allow eight-fold fluctuations of GCN4 between starvation (high GCN4) and high amino acid concentration (low GCN4). Line 3 of Fig. 17 illustrates one of the key pieces of evidence for this model. In accordance with the model, when an extra piece of RNA is inserted before ORF4, CGN4 is not produced, even under starving conditions. This is because the initiation complex has enough time to reform at ORF4.

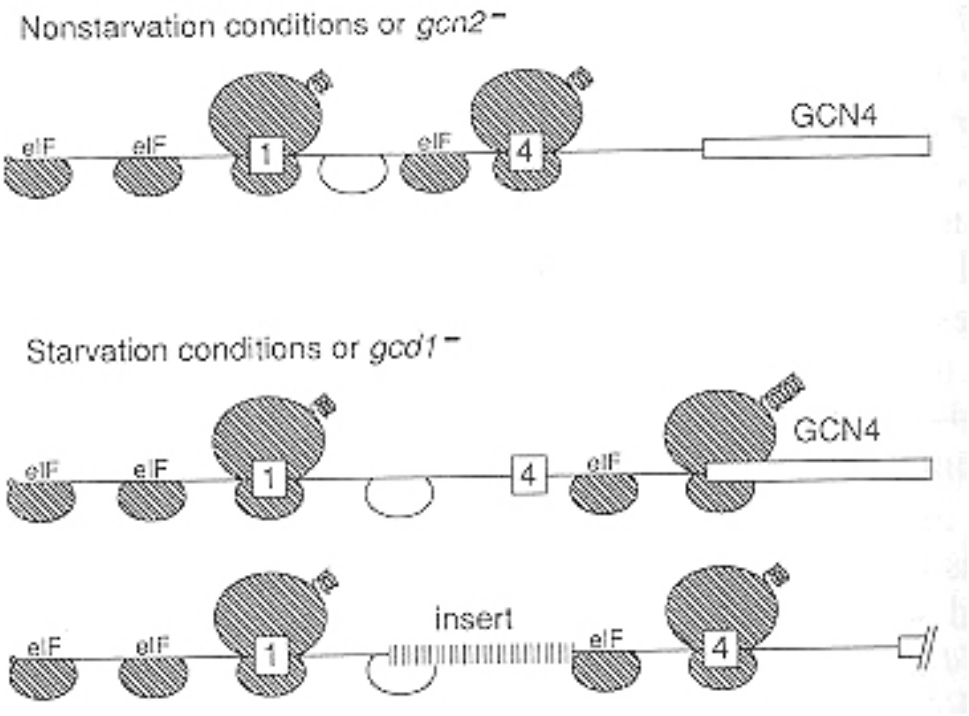


Fig. 17 Model for *GCN4* translational control. The beginning of *GCN4* mRNA coding sequences all indicated as boxes. 40S subunits containing eIFs and 80S translating ribosomes are crosshatched. Ribosomes translate ORF1 and the 40S subunits resume scanning. Under repressing conditions (non-starving, line 1), initiation factors are readily reassembled and reinitiation occurs at ORF4. After ORF4 translation, no reinitiation occurs at *GCN4*., because the ribosomes have dissociated from the mRNA. Under repressing conditions (starvation, line 2), the reassembly of the 40S initiation complex is slower and many subunits cannot initiate at ORF 4. However, they are ready when they reach *GCN4*. When the distance between ORF 1 and 4 is increased (line 3) reinitiation at ORF4 is possible even under depressing conditions so that *GCN4* is never translated. From Abastado et al., 1991. Reproduced by permission.

GCN4 is also controlled by the *general control nonrepressible* kinase (GCN2) which phosphorylates eIF-2α. The positive regulatory role of GCN2 on the amino acid synthetic pathway is one of the few cases of translational control of the expression of a gene now partially understood. GCN2 is activated by uncharged tRNA during amino acid starvation (Hinnebusch, 1993). The phosphorylation of eIF-2 stimulates the translation of GCN4-mRNA (Dever et al., 1992). How can phosphorylation of eIF-2, involved in initiation of translation globally, specifically control the translation of a specific gene? In the case of *GCN4*-mRNA in yeast, the regulation is through the uORFs. The phosphorylation of eIF-2 reduces the activity of eIF-2. The resulting delay of the assembly of the translational-ribosome complex at

the uORFs downstream from uORF1, permits the translation of the GCN-4 protein.

RNA editing

After transcription, certain RNAs are edited, i.e., nucleotides are deleted, inserted or replaced. The most far-reaching alterations are the consequence of editing mitochondrial RNA and, in one extreme case, as much as half of the mRNA is changed (see Simpson and Thiemann, 1995). Generally, the editing in nuclear transcripts is much more limited (see Scott, 1995) and may involve a single base. In mRNAs coded by nuclear genes, editing precedes splicing.

Frequently editing is necessary for the RNA to function; the editing is required to produce initiation and termination codons or eliminate internal frameshifts. Frameshift mutation removes a single base in the mRNA and would lead to misreading of the rest of the mRNA (the codons are base triplets, removing one base shifts the reading so that subsequent codons would be different). Some of the editing is needed to maintain a necessary, three-dimensional structure. For example, in tRNAs, function depends on a precisely formed three dimensional cloverleaf conformation. In other cases, the editing is to change an internal amino acid in the corresponding protein. The occurrence of these events has been detected in experiments in which the genomic sequence did not correspond to the cDNA sequence (mirroring that of the RNA rather than the gene).

Recently, editing in two mammalian mRNAs has been shown to permit the production of two distinct proteins from the same gene. This type of editing may be under developmental or physiological control. One case corresponds to a plasma lipoprotein, apolipoprotein B (apoB), which occurs in two forms: apoB-100 (of 512 kDa) and apoB-48 (of 241 kDa). The two apoB forms are involved in the transport of cholesterol and triglycerides in the blood. Another case corresponds to the glutamate-gated channels present at synapses.

ApoB-100 is produced mostly by the translation of the mRNA encoded by the genomic sequence in the liver. ApoB-48 is produced by the translation of an mRNA from the same gene, but edited to produce the shorter protein by substituting a stop codon (UAA) for a glutamine codon (CAA). The editing is tissue specific and is under physiological control, responding to nutrients and hormones (Baum, 1990).

The glutamate receptors (gluR) fall into three separate subgroups, depending upon the activating pharmacological agonists: the N-methyl-D-aspartate (NMDA), the α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) and kainate (KA) classes. So far, 28 gluR genes have been identified and others are known to exist. The AMPA receptors differ in the ion selectivity. The mRNA of the AMPA and KA classes are edited by substitution of a glutamine codon (CAG) with an arginine codon (CGG) in a portion coding for the channel segment of the protein (Sommer et al., 1991). Apparently, the presence of arginine blocks the passage of Ca^{2+}. The control of Ca^{2+} permeability, in turn, is likely to have important physiological consequences (for example, Ca^{2+} is a second messenger). The editing is tissue specific within the nervous system, and it has been proposed that it plays a role in the regulation of expression during the establishment of excitatory circuits in the developing nervous system. Several

studies have suggested that glutamate receptors are involved in learning and memory (see Hollmann and Hienamann, 1994).

In at least some cases, the enzyme involved in the editing recognizes a double strand locus in the RNA (e.g., Burns et al., 1997) formed by the hybridization of sequences in the intron to complementary sites in a nearby exon.

RNA interference

The events known as RNA interference have gained prominence in our understanding of posttranscriptional regulation (e.g., Zamore, 2002; Plasterk, 2002). The best understood phenomenon is the RNA interference involving double stranded RNA (dsRNA). dsRNA degrades homologous mRNA with the same sequence as one of the strands of dsRNA (see e.g., Sharp, 2001). This process proceeds through the production of *short interfering RNAs* (siRNAs) and is thought to provide a mechanism to remove defective RNA and protect cells from virus that use dsRNA to replicate, transposons and repetitive sequences (see Zamore et al., 2000). Some of the details of this phenomenon has been discussed in Chapter 1. Gene silencing via RNA interference also has been shown to have a role during the development of the nematode *Caenorhabditis elegans*, *Drosophila melanogaster* and other animals (e.g., Pasquinelli et al., 2000) through the production of *small temporal RNAs* (stRNAs; also known as microRNAs, miRNAs), which contain sequences complementary to specific target mRNAs. Unlike dsRNA, stRNAs are encoded by the genome of the developing cells and repress but do not degrade the target mRNA (e.g.,Olsen and Ambros, 1999).

Despite their different origin and probable role, there are many similarities between the siRNA and the stRNA systems. Both act through small RNAs (21-23 nucleotides in length) produced from longer RNAs. Both involve the ribonuclease Dicer, which produces the smaller pieces of RNA. However, whereas in siRNA Dicer acts on dsRNA, in stRNA, it acts on RNA coded by the genes *let-7* or *lin-4*) (Hutvàgner et al, 2001; Grishok et al., 2001).

Following their formation, the siRNAs are incorporated in the RNA-induced silencing complex (RISC) which targets homologous RNA for degradation (Hammond et al., 2000; Nykänen et al., 2001) . The process proceeds in steps: (1) an ATP dependent processing in of the double-stranded RNA into siRNAs, followed by (2) incorporation of siRNAs into a 360 kDa protein-RNA complex and (3) an ATP-dependent unwinding of the siRNA duplex, and finally (4) an ATP-independent recognition and cleavage of the RNA target. A similar system has been found in plants (Tang et al., 2003).

stRNAs block the translation of mRNAs by pairing with the 3' untranslated regions (UTR) of these RNAs (e.g. Olsen and Ambrose, 1999). They have also been found in plants (e.g., Hamilton and Baulcombe, 1999; Reinhart et al., 2002; Park et al. 2002). They are thought to control gene expression by base pairing with mRNAs. MiRNAs are part of a 550 kDa ribonucleoprotein complex (Mourelatos et al., 2002) containing many molecules of miRNAs.

The studies of RNA interference are relatively new and the implication of these events are only beginning to be understood.

SUGGESTED READING

Eukaryotic Transcriptional Controls

Beato, M. (1991) Transcriptional controls by nuclear receptors, *FASEB J5:2044-2051*. (Medline)

Edmondson, D.G. and Roth, S.Y. (1996) Chromatin and transcription, *FASEB J.* 10:1173-1182. (Medline)

Hartzog, G.A. and Winston, F. (1997) Nucleosomes and transcription: recent lessons from genetics, *Curr. Opin. Genet. Dev.* 7:192-198. (Medline)

Janknecht, R. and Hunter, T. (1996) Transcriptional control: versatile molecular glue, *Current Biology* 6: 951-954. (Medline)

Johnston, M. (1987) A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*, *Microbiol. Rev.* 51:458-476. (Medline)

Lemon, B. and Tjian, R. (2000) Orchestrated response: a symphony of transcription factors for gene control, *Genes Dev.* 14:2551-2569. (MedLine)

Struhl, K. (1996) Chromatin structure and RNA polymerase II connection: implication for transcription, *Cell* 84:179-182. (Medline)

Tsukiyama, T. and Wu, C. (1997) Chromatin remodeling and transcription, *Curr. Opin. Genet. Dev.* 7:182-191. (Medline)

Wolffe, A.P. and Pruss, D. (1996)Targeting chromatin disruption: transcription regulators that acetylate histones, *Cell* 84:817-819. (Medline)

Posttranscriptional Controls

Bass, B.L. (1993) RNA editing: New uses for old players in the RNA world, in *RNA World*, eds. Gesteland, R.F. and Atkins, J.F., Cold Spring Harbor Laboratory Press pp. 383-418.

Baum, C.L., Teng, B.-B.and Davidson, N.O. (1990) Apoliprotein B messenger RNA editing in the rat

liver: modulation by fasting and refeeding a high carbohydrate diet, *J. Biol. Chem.* 265: 19263-19270. (Medline)

Casey, J.L., Henze, M.W., Koeller, D.M., Caughman, S.W., Rouault, T.A., Klausner, R.D., and Harford, J.B. (1988) Iron-responsive elements: regulatory RNA sequences that control mRNA levels of translation, *Science* 240:924-928. (Medline)

Merrick, W.C. (1992) Mechanism and regulation of eukaryotic protein synthesis, *Microbiol. Rev.* 56:291-315. (Medline)

Pain, V.M. (1996) Initiation of protein synthesis in eukaryotic cells, *Eur. J. Biochem.* 236:747-771. (Medline)

Plasterk, R.H. (2002) RNA silencing: the genome's immune system, *Science* 296:1263-1265. (MedLine)

Sharp, P.A. (2001) RNA interference--2001, *Genes Dev.* 15:485-490. (MedLine)

Yen, T.J., Machlin, P.S. and Cleveland, D.W. (1988) Autoregulated instability of β-tubulin mRNAs by recognition of the nascent amino terminus of β-tubulin, *Nature* 334:580-585. (Medline)

Zamore, P.D. (2002) Ancient pathways programmed by small RNAs, *Science* 296:1265-1269. (MedLine)

WEB RESOURCES

Hendzel, M. (2001) The Cell Nucleus: Free teaching and study materials. Contemporary review materials. Movies of dynamics in the cell nucleus. Listing of resources on the Web. www.cellnucleus.org

Online Macromolecular Museum: A plug-in file called Chime is required to view the exhibits. Instructions are available at this site.

Institute for Molecular Biology, Jean, Germany RNA World Website, facilitates searching for specific information on RNA, including tutorials meant for the non-specialist.

REFERENCES

Search the textbook

Back to Chapter 3

REFERENCES

Abastado, J.-P., Miller, P.F., Jackson. B.M. and Hinnebusch, A.G. (1991) Supression of ribosomal reinitiatiation at upstream open reading frames in amino acid-starved cells forms the basis for *GCN4* translational control, *Mol. Cell. Biology* 11:486-496.(Medline)

Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T. and Thanos, D. (2000) Ordered recruitment of chromatin modifying and general transcription factors to the IFN-face="symbol">b promoter, *Cell* 103:667-678. (MedLine)

Alland, L., Muhle, R., Hou, H. Jr., Potes, J., Chin, L., Schreiber-Agus, N. and DePinho, R.A. (1997) Role for N-CoR and histone deacetylase in Sin-3-mediated transcriptional repression, *Nature* 387:49-55.(Medline)

Amero, S.A., Raychaudhuri, G., Cass, C.L., Van Venzooij, W.J., Habets, W.J., Krainer, A.R. and Beyer, A.L. (1992) Independent deposition of heterogenous nuclear ribonucleoprotein and small ribonucleoprotein particles at sites of transcription, *Proc. Natl. Acad. Sci. USA* 89:8409-8413.(Medline)

Bannister, A.J. and Kouzarides, T. (1996) The CBP co-activator is a histone acetyltransferase, *Nature* 384:641-643.(Medline)

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C. and Kouzarides, T. (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain, *Nature* 410:120-124. (MedLine)

Barbarese, E., Koppel, D.E., Deutscher, M.P., Smith, C.L., Ainger, K., Morgan, F. and Carson, J.H. (1995) Protein translation components are colocalized in granules in oligodendrocytes, *J. Cell Sci.*108:2781-2790. (MedLine)

Baum, C.L., Teng, B.-B. and Davidson, N.O. (1990) Apoliprotein B messenger RNA editing in the rat liver: modulation by fasting and refeeding a high carbohydrate diet, *J. Biol. Chem.* 265: 19263-19270.(Medline)

Baumbach, L.L, Stein, G.S. and Stein, J.L. (1987) Regulation of human histone gene expression: transcriptional and post-transcriptional control in the coupling of histone mRNA stability with DNA

replication, *Biochemistry* 26:6178-6187.(Medline)

Baurén, G., Jiang, W.-Q., Bernholm, K., Gu, F. and Wieslander, F. (1996) Demonstration of a dynamic, transcription dependent organization of pre-mRNA splicing factors in polytene nuclei, *J. Cell Biol.* 133:929-942.(Medline)

Bell, A.C. and Felsenfeld, G. (1999) Stopped at the border: boundaries and insulators, *Curr. Opin. Genet. Dev.* 9:191-198.(MedLine)

Belmont, A.S. and Straight, A.F. (1998) In vivo visualization of chromosomes using lac operator-repressor binding, *Trends Cell Biol.* 1998 8:121-124. (MedLine).

Beyer, A.L. and Osheim, Y.N. (1988) Splice site selection, rate of splicing, and alternative splicing on nascent transcripts, *Genes Dev.* 2:754-765.(Medline)

Boyer, T.G., Martin, M.E.D., Lees, E., Ricciardi, R.P. and Berk, A.J. (1999) Mammalian Srb/mediator complex is targeted by adenovirus E1A protein, *Nature* 399:276-279.(Medline)

Brownell, J.E., Zhou, J., Ranalli, T., Kobayashi, R., Roth, S.Y. and Allis, C.D. (1996) *Tetrahymena* histone acetyltransferase A: a homolog to yeast GCN5 linking histone acetylation to gene activity, *Cell* 84: 843-851. (Medline)

Burley, S.K. and Roeder, R.G. (1996) Biochemistry and structural biology of transcription factor IID (TFIID), *Annu. Rev. Biochem.* 65:769-799. (MedLine)

Burns, C.M., Chu, H., Rueter, S.M., Hutchinson, L.K., Canton, H., Sanders-Busch, E. and Emerson, R.B. (1997) Regulation of serotonin-2C receptor G-protein coupling by RNA editing, *Nature* 387:303-308.(Medline)

Cairns, B.R. (1998) Chromatin remodeling machines: similar motors, ulterior motives, *Trends Biochem. Sci.* 23:20-25.(Medline)

Carmo-Fonseca, M., Tollervey, D., Pepperkok, R., Barabino, S.M.L., Merdes, A., Brunner, C., Zamore, P.D., Green, M.R., Hurt, E. and Lamond, A.I. (1991) Mammalian nuclei contain foci which are highly enriched in components of pre-mRNA splicing machinery, *EMBO J.* 10:195-206.(Medline)

Caron, J., Jones, A.L., Rall, L.B. and Kirschner, M.W. (1985) Autoregulation of tubulin synthesis in enucleated cells, *Nature* 317:648-650.(Medline)

Casey, J.L., Henze, M.W., Koeller, D.M., Caughman, S.W., Rouault, T.A., Klausner, R.D. and Harford,

J.B. (1988) Iron-responsive elements: regulatory RNA sequences that control mRNA levels of translation, *Science* 240:924-928.(Medline)

Chadee, D.N., Taylor, W.R., Hurta, R.A., Allis, C.D., Wright, J.A., and Davie, J.R.(1995) Increased phosphorylation of histone H1 in mouse fibroblasts transformed with oncogenes or constitutively active mitogen-activated protein kinase kinase *J. Biol. Chem.* 270, 20098-20105.(Medline)

Chadee, D.N., Hendzel, M.J., Tylipski, C.P., Allis, C.D., Bazett-Jones, D.P., Wright, J.A.and Davie, J.R. (1999) Increased ser-10 phosphorylation of histone H3 in mitogen-stimulated and oncogene-transformed mouse fibroblasts, *J. Biol. Chem.* 274:24914-24920.(Medline)

Chen, J.J. and London, I.M. (1995) Regulation of protein synthesis by heme regulated eIF-2α kinase, *Trends Biochem. Sci.* 20:105-108.(Medline)

Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W. and Stallcup, M.R. (1999) Regulation of transcription by a protein methyltransferase, Science 284:2174-2177.

Cheung, P., Tanner, K.G., Cheung, W.L., Sassone-Corsi, P., Denu, J.M. and Allis, C.D. (2000) Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation, *Mol. Cell* 5:905-915. (MedLine)

Cheutin, T., McNairn, A.J., Jenuwein, T., Gilbert, D.M., Singh, P.B. and Misteli, T. (2003) Maintenance of stable heterochromatin domains by dynamic HP1 binding, *Science* 299:721-725. (MedLine)

Cleveland, D.W. (1987) The multitubulin hypothesis revisited, what have we learned? *J. Cell Biol.* 104:381-383.(Medline)

Cleveland, D.W., Pittenger, M.F. and Feramisco, J.R. (1983) Elevation of tubulin levels by microinjection suppresses new tubulin synthesis, *Nature* 305:738-740.(Medline)

Colwill, K., Pawson, T., Andrews, B., Prasad, J., Manley, J.L., Bell, J.C. and Duncan, P.I. (1996) The clk/sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution, *EMBO J.* 15:265-275.(medline)

Conaway, R.C. and Conoway, J.W. (1993) General initiation factors for RNA polymerase II, *Annu. Rev. Biochem.* 62:161-190.(Medline)

De Haro, C., Méndez, R. and Santoyo, J. (1996) The eiF-2α kinases and the control of protein synthesis, *FASEB J.* 10:1378-1387.(Medline)

DeRisi, J.L., Iyer, V.R. and Brown, P.O. (1997) Exploring the metabolic and genetic control of gene expression on a genomic scale, *Science* 278:680-686. (MedLine)

Dever, T.E., Feng, L, Wek, R.C., Cigan, A.M., Donahue, T.E. and Hinnebusch, A.G. (1992) Phosphorylation of initiation factor 2α by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast, *Cell* 68:585-596.>(Medline)

Dhalluin, C., Carlson, J.E., Zeng, L., He, C., Aggarwal, A.K. and Zhou, M.M. (1999) Structure and ligand of a histone acetyltransferase bromodomain, *Nature* 399:491-496. (MedLine)

Dreyfuss, G., Matunis, M.J., Piñol-Roma, S. and Burd, C.G. (1993) hnRNP proteins and the biogenesis of mRNA, *Ann. Rev. Biochem.* 62:289-321.(Medline)

Dynan, W.S. and Tjian, R. (1985) Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins, *Nature* 316:774-777.

Edmondson, D.G. and Roth, S.Y. (1998) Interactions of transcriptional regulators with histones, *Methods* 15:355-364. (MedLine)

Eissenberg, J.C. and Elgin, S.C. (2000) The HP1 protein family: getting a grip on chromatin, .*Curr. Opin. Genet. Dev.* 10:204-210. (MedLine)

Emerson, B.M. (2002) Specificity of gene regulation, *Cell* 109:267-270.

Fakan, S. (1994) Perichromatic fibrils are *in situ* forms of nascent transcripts, *Trends Cell Biol.* 4:86-90.

Fakan, S., Leser, G. and Martin, T.E. (1984) Ultrastructural distribution of nuclear ribonucleoproteins as visualized by immunochemistry on thin sections, *J. Cell Biol.* 98:358-363.(Medline)

Farina, K.L. and Singer, R.H. (2002) The nuclear connection in RNA transport and localization, *Trends Cell Biol.* 12:466-472.

Festenstein, R., Pagakis, S.N., Hiragami, K., Lyon, D., Verreault, A., Sekkali, B. and Kioussis, D. (2003) Modulation of heterochromatin protein 1 dynamics in primary mammalian cells, *Science* 299:719-721. (MedLine)

Fondell, J.D., Guermah, M., Malik, S. and Roeder, R.G. (1999) Thyroid hormone receptor-associated proteins and general positive cofactors mediate thyroid hormone receptor function in the absence of the TATA box-binding protein-associated factors of TFIID, *Proc. Natl. Acad. Sci. USA* 96:1959-1964.(Medline)

Frank, J. and Agrawal, R.K. (2000) A ratchet-like inter-subunit reorganization of the ribosome during translocation, *Nature* 406:318-322. (MedLine)

Fry, C.J. and Peterson, C.L. (2002) Transcription: unlocking the gates to gene expression, *Science*< 295:1847-1848.

Fyodorov, D.V. and Kadonaga, J.T. (2002) Dynamics of ATP-dependent chromatin assembly by ACF, *Nature* 418:897-900. (MedLine)

Gamble, M.J. and Freedman, L.P. (2002) A coactivator code for transcription, *Trends Biochem. Sci.* 27:165-167. (MedLine).

Gay, D. A., Yen, T.J. Lau, J.T.Y. and Cleveland, D.W. (1987) Sequences that confer β-tubulin autoregulation through modulated mRNA stability reside within exon 1 of β-tubulin mRNA, *Cell* 50:671-679.(Medline)

Georgel, P.T., Tsukiyama, T. and Wu, C. (1997) Role of histone tails in nucleosome remodeling by *Drosophila* NURF, *EMBO J.* 16:4717-4726.(Medline)

Ghetti, A., Piñol-Roma, S., Michael, W.M., Morandi, C. and Dreyfuss, G. (1992) hnRNPI, the polypyrimidine tract-binding protein: distinct nuclear localization and association with hnRNAs, *Nucleic Acids Res.* 20:3671-3678.(Medline)

Gill, G. and Ptashne (1987) Mutants of GAL4 protein altered in activation function, *Cell* 51:121-126.(Medline)

Gillies, S.D., Morrison, S.L., Oi, V.T. and Tonegawa, S. (1983) A tissues specific enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene, *Cell* 33:717-728.(Medline)

Giniger, E., Varnum, S. and Ptashne, M. (1985) Specific DNA binding of *GAl4*, a positive regulatory protein of yeast, *Cell*: 40:767-774.(Medline)

Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G. and Mello, C.C. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C.. elegans* developmental timing, *Cell* 106:23-34. (MedLine)

Graves, R.A., Pandey, N.B., Chodchoy, N. and Marzluff, W.F. (1987) Translation is required for regulation of histone mRNA degradation. *Cell* 48:615-626.(Medline)

Gavrilova, L.P> and Spirin, A.S. (1971) Stimulation of "non-enzymatic" translocation of ribosomes by p-

chloromercuribenzoate, *FEBS Lett.* 17:324-326.

Green, M.R., Maniatis, T. and Melton, D.A. (1983) Human β-globin pre-mRNA synthesized in vitro is accurately spliced in *Xenopus* oocyte nuclei, *Cell* 32: 681-694.(Medline)

Guarante, L. (1984) Yeast promoters; positive and negative elements, *Cell* 36:799-800.

Hamilton, A.J. and Baulcombe, D.C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants, *Science* 286:950-952. (MedLine)

Hammond, S.M., Bernstein, E., Beach, D. and Hannon, G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells, *Nature* 404:293-296. (MedLine)

Hampsey, M. (1998) Molecular genetics of the RNA polymerase II general transcriptional machinery, *Microbiol. Mol. Biol. Rev.* 62:465-503.(Medline)

Hassan, A.H., Neely, K.E., Vignali, M., Reese, J.C. and Workman, J.L. (2001) Promoter targeting of chromatin-modifying complexes, *Front. Biosci.* 6:D1054-D1064. (MedLine)

Hedley, M.L., Amrein, H. and Maniatis, T. (1995) An amino acid sequence motif sufficient for subnuclear localization of an arginine/serine-rich splicing factor, *Proc. Natl. Acad. Sci. USA* 92:11524-11528.(Medline)

Hill, W.E., Moore, P., Dahlberg, A., Schlessinger, R., Garrett, R. and Warner, J. (1990) *The Ribosome: Structure Function and Evolution*, American Society of Microbiology, Washington DC.

Hinnebusch, A.G. (1993) Gene-specific translational control of the yeast *GCN4* gene by phoshorylation of eukaryotic initiation factor 2, *Mol. Microbiol.* 10:215-223.(Medline)

Hirose, Y. and Manley, J.L. (1998) RNA polymerase II is an essential mRNA polyadenylation factor, *Nature* 395:93-96.(Medline)

Hollmann, M. and Heinemann, S.H. (1994) Cloned glutamate receptors, *Ann. Rev. Neurosci.* 17:31-108.(Medline)

Huang, M., Zhou, Z. and Elledge, S.J. (1998) The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor, *Cell* 94:595-605. (MedLine)

Hunter, T. (2000) Signaling--2000 and beyond, *Cell* 100:113-127. (MedLine)

Hutvàgner, G., McLachlan, J., Pasquinelli, A.E, Balint, E., Tuschl, T. and Zamore, P.D. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA, *Science* 293:834-838. (MedLine)

Ito, T., Bulger, M., Pazin, M.J., Kobayashi, R. and Kadonaga, J.T. (1997) ACF, an ISWI-containing and ATP-utilizing chromatin and remodeling factor, *Cell* 90:145-155.(Medline)

Ito, T., Levenstein, M.E., Fyodorov, D.V., Kutach, A.K., Kobayashi, R. and Kadonaga, J.T. (1999) ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly, *Genes Dev.* 13:1529-1539. (MedLine)

Ito, M., Yuan, C.X., Malik, S., Gu, W., Fondell, J.D., Yamamura, S., Fu, Z.Y., Zhang, X., Qin, J. and Roeder, R.G. (1999) Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators, *Mol. Cell* 3:361-370.(Medline)

Jacobson, R.H., Ladurner, A.G., King, D.S. and Tjian, R. (2000) Structure and function of a human TAFII250 double bromodomain module, *Science* 288:1422-1425. (MedLine)

Janknecht, R. and Hunter, T. (1996) Transcriptional control: versatile molecular glue, *Current Biology* 6: 951-954.(Medline)

Jiang Y.-W., Veschambre, P., Erdjument-Bromage, H., Tempst P, Conaway, J.W., Conaway, R.C. and Kornberg, R.D. (1998) Mammalian mediator of transcriptional regulation and its possible role as an end-point of signal transduction pathways, *Proc. Natl. Acad. Sci. USA* 95:8538-8543.(Medline)

Jiménez-García, L.F. and Spector, D.L. (1993) In vivo evidence that transcription and splicing are coordinated by a recruiting mechanism, *Cell* 73:47-59.(Medline)

Johnston, M., (1987) A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*, *Microbiol. Rev.* 51:458-476.(Medline)

Jones, P.L., Vennstra, G.J.C., Wade, P.A., Vermaak, D., Kass, S.U., Lansberger, N., Strouboulis, J., Wolffe, A.P. (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription, *Nature Gen.* 19:187-191.(Medline)

Kambach, C., Walke, S., Young, R., Avis, J.M., de la Fortelle, E., Raker, V.A, Lührmann, R., Li, J. and Nagai, K. (1999) Crystal structures of two Sm protein complexes and their implications for the assembly of the spliceosomal snRNPs, *Cell* 96:375-387. (MedLine)

Kim, Y.J., Bjorklund, S., Li, Y., Sayre, M.H. and Kornberg, R.D. (1994) A multiprotein mediator of

transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II, *Cell* 77:599-608.(Medline)

Kingston, R.E. and Narlikar, G.J. (1999) ATP-dependent remodeling and acetylation as regulators of chromatin fluidity, *Genes Dev.* 13:2339-2352.(Medline)

Kingston, R.E., Bunker, C.A. and Imbalzano, A.N. (1996) Repression and activation by multiprotein complexes that alter chromatin structure, *Genes Dev.* 10:905-920. (MedLine)

Kirschner, M.W. and Mitchison, T.J. (1986) Beyond self assembly: from microtubules to morphogenesis, *Cell* 45:329-342.(Medline)

Kloc, M., Zearfoss, N.R. and Etkin, L.D. (2002) Mechanisms of subcellular mRNA localization, *Cell* 108:533-544. (MedLine)

Krecic, A.M. and Swanson, M.S. (1999) hnRNP complexes: composition, structure, and function, *Curr. Opin. Cell Biol.* 11:363-371. (MedLine)

Kruijer, W., Cooper, J.A., Hunter, T. and Verma, I. (1984) Platelet derived growth factor induces rapid but transient expression of the c-fos gene and protein, *Nature* 312:711-716.(Medline)

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. and Jenuwein, T. (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins, *Nature* 410:116-120. (MedLine)

Lamb, M.M.and Daneholt, B. (1979) Characterization of active transcription units in Balbiani rings of *Chironomus tentans*, *Cell* 17:835-848.(Medline)

Lamm, G.M. and Lamond, A.I. (1993) Non-snRNP protein splicing factors, *Biochim. Biophys. Acta* 1173:247-265.(Medline)

Lamond, A.I. and Carmo-Fonseca, M. (1993) The coiled body, *Trends in Cell Biol.* 3:198-204.

Lander, E.S. et al. (2001) Initial sequencing and analysis of the human genome, *Nature* 409, 860 -921. (MedLine)

Latchman, D.S. (2001) Transcription factors: bound to activate or repress, *Trends Biochem. Sci.* 26:211-213. (Medline)

Lees-Miller, J.P., Goodwin, L.O. and Helfman, D.M.(1990) Three novel troposmyosin isoforms are expressed from the rat α-tropomyosin gene through the use of alternative promoters and alternative RNA

processing, *Mol. Cell. Biol.* 10:1729-1742.(Medline)

Leibold, E.A. and Munro, H.N. (1988) Cytoplasmic protein binds *in vitro* to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light-subunit mRNAs, *Proc. Natl. Acad. Sci.* 85: 2171-2175.(Medline)

Levsky, J.M., Shenoy, S.M., Pezo, R.C. and Singer, R.H. (2002) Single-cell gene expression profiling, *Science* 297:836-840. (MedLine)

Linial, M., Gunderson, N. and Groudine, M. (1985) Enhanced transcription of c-myc in bursal lymphoma cells requires continuous protein synthesis, *Science* 230:1126-1131.(Medline)

Lo, W.S., Trievel, R.C., Rojas, J.R., Duggan, L., Hsu, J.Y., Allis, C.D., Marmorstein, R. and Berger, S.L. (2000) Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14, *Mol Cell.* 5:917-926. (MedLine)

Lo, W-S., Duggan, L., Tolga Emre, N.C., Belotserkovskya, R., Lane, W.S, Shiekhattar, R. and Berger, S.L. (2001) Snf1SSa histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription, *Science* 293:1142-1146. (MedLine)

Lue, N.F., Chasman, D.I., Buchman, A.R. and Kornberg, R.D. (1987) Interaction of *GAL4* and *GAL80* gene regulatory proteins in vitro, *Mol. Cell. Biol.* 7:3446-3451.(Medline)

Mader, S., Lee, H., Pause, A. and Sonenberg, N. (1995) The translational initiation factor eIF-4E binds to a common motif shared by the translational factor eIF-4γ and the translational repressor 4E-binding protein, *Mol.Cell. Biol.* 15:4990-4997.(Medline)

Mangelsdorf, D.J. and Evans, R.M. (1995) The RXR heterodimers and orphan receptors, *Cell* 83:841-850.(Medline)

Maniatis, T., Goodbourn, S. and Fischer, J.A. (1987) Regulation of inducible and tissue-specific gene expression, *Science* 236:1237-1245.(Medline)

Maniatis, T. and Reed, R. (2002) An extensive network of coupling among gene expression machines, *Nature* 416:499-506. (MedLine)

Manzella, J.M., Rychlik,W., Rhoads, R.E., Hershey, J.W.B. and Blackshear, P.(1991) Insulin induction of ornithine decarboxylase. Importance of mRNA secondary structure and phosphorylation of eucaryotic initiation factors eIF-4B and eiF-4E, *J. Biol. Chem.* 266:2383-2389.(Medline)

Marriott, S.J. and Brady, J.N. (1989) Enhancer function in viral and cellular regulation, *Biochim. Biophys. Acta* 989:97-110.(Medline)

Matheson, A., Dennis, P., Davies, J. and Hill, W. (1995) *Frontiers in Translation*, National research Council Canada, Otawa, Canada.

Mathews, M.B. and Schenk, T. (1991) Adenovirus-associated RNA and translational control, *J. Virol.* 65:5657-5662.

Matunis, E.L., Matunis, M.J., and Dreyfuss, G. (1993) Association of individual hnRNP proteins and snRNP with nascent transcripts, *J. Cell Biol.* 121:219-228.(Medline)

Mayford, M., Bach, M.E., Huang, Y.Y., Wang, L., Hawkins, R.D. and Kandel, E.R. (1996) Control of memory formation through regulated expression of a CaMKII transgene, *Science* 274:1678-1683. (MedLine)

McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S.D., Wickens, M. and Bentley, D.L. (1997a) The C-terminal domain of RNA polymerase II couples mRNA processing to transcription, *Nature* 385:357-361(Medline)

McCracken, S,, Fong, N., Rosonina, E., Yankulov, K., Brothers, G., Siderovski, D., Hessel, A., Foster, S., Shuman, S., Bentley, D.L. (1997b) 5'-Capping enzymes are targeted to pre-mRNA by binding to the phosphorylated carboxy-terminal domain of RNA polymerase II, *Genes Dev.* 11:3306-3318.(Medline)

Merika, M. and Thanos, D. (2001) Enhanceosomes, *Curr. Opin. Genet. Dev.*11:205-208. (MedLine)

Mermoud, J.E., Cohen, P. and Lamond, A.I. (1992) Ser/Thr-specific protein phosphatases are required for both catalytic steps of pre-mRNA splicing, *Nucleic Acid Res.* 20:5263-5269.(Medline)

Merrick, W.C. (1992) Mechanism and regulation of eukaryotic protein synthesis, *Microbiol. Rev.* 56:291-315.(Medline)

Miller, O.L. Jr and Beatty, B.R. (1969) Visualization of nucleolar genes, *Science* 164:955-957.(Medline)

Misteli, T. and Spector (1996) Serine/threonine phosphatase 1 modulates the subnuclear distribution of pre-mRNA splicing factors, *Mol. Biol. Cell* 7:1559-1572.(Medline)

Misteli, T. and Spector, D.L. (1997) Protein phosphorylation and the nuclear organization of pre-mRNA splicing, *Trends in Cell Biol.* 7:135-138.

Mortillaro, M.J., Blencowe, B.J., Wei, X., Nakayasu, H., Du, L., Warren, S.L., Sharp, P.A. and Berezney, R. (1996) A hyperphosphorylated form of large subunit of RNA polymerase II is associated with splicing complexes and the nuclear matrix, *Proc. Natl. Acad. Sci USA* 93:8253-8257.(Medline)

Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M. and Dreyfuss, G. (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs, *Genes Dev.* 16:720-728. (MedLine)

Müller J. (2000) The benefits of selective insulation, *Curr. Biol.* 10:R241-R244. (MedLine)

Müllner, E.W. and Kühn, L.C. (1988) A stem-loop in 3' untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm, *Cell* 53:815-825.(Medline)

Munshi, N., Agalioti, T., Lomvardas, S., Merika, M., Chen, G. and Thanos, D. (2001) Coordination of a transcriptional switch by HMGI(Y) acetylation, *Science* 293:1133-1136. (MedLine)

Näär, A.M., Beaurang, P.A., Zhou, S., Abraham, S., Solomon, W. and Tjian R (1999) Composite co-activator ARC mediates chromatin-directed transcriptional activation, *Nature* 398:828-832.(Medline)

Näär, A.M., Lemon, B.D. and Tjian, R. (2001) Transcriptional coactivator complexes, *Annu. Rev. Biochem.* 70:475-501. (MedLine)

Nakayama, J.-i, Rice, J.C., Strahl, B.D., Allis, C.D. and Grewal, S.I. (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly, *Science* 292:110-1133. (MedLine)

Nan, X., Campoy, F.J. and Bird, A. (1997) MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin, *Cell* 88:471-481.(Medline)

Neubauer, G., King, A., Rappsilber, J., Calvio, C., Watson, M., Ajuh, P., Sleeman, J., Lamond, A. and Mann M. (1998) Mass spectrometry and EST-database searching allows characterization of the multi-protein spliceosome complex, *Nature Genet.* 20:46-50. (Medline)

Niehrs, C. and Pollet, N. (1999) Synexpression groups in eukaryotes, *Nature* 402:483-487.(Medline)

Nykänen, A., Haley, B. and Zamore, P.D. (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway, *Cell* 107:309-321. (MedLine)

Olmsted, E.A., O'Brien, L., Henshaw, E.C. and Panniers, R. (1993) Purification and characterization of eukaryotic initiation factor ((eIF)-2α kinases from Ehrich ascites tumor cells, *J. Biol. Chem.* 268:12552-12559.(Medline)

Olsen, P.H. and Ambros, V. (1999) The lin-4 regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation, *Dev. Biol.* 216:671-680. (MedLine)

Onate, S.A., Tsai, S.Y., Tsay, M.J. and O'Malley, B.W. (1995) Sequence and characterization of a coactivator for steroid hormone receptor superfamily, *Science* 270:1354-1357.(Medline)

Orphanides, G., Lagrange, T. and Reinberg, D. (1996) The general transcription factors of RNA polymerase II, *Genes Dev.* 10:2657-2683. (MedLine)

Owen-Hughes, T. and Workman, J.L. (1994) Experimental analysis of chromatin function in transcriptional control, *Crit. Rev. Eukaryot. Gene Expr.* 4:403-441.(Medline)

Pain, V.M. (1996) Initiation of protein synthesis in eukaryotic cells, *Eur. J. Biochem.* 236:747-771.

Park, W., Li, J., Song, R., Messing, J. and Chen, X. (2002) CARPEL FACTORY, a Dicer Homolog, and HEN1, a Novel Protein, Act in microRNA Metabolism in *Arabidopsis thaliana Curr. Biol.* 12:1484-1495. (MedLine)

Parker, R. and Jacobson, A. (1990) Translation and a 42-nucleotide segment within the coding region of the mRNA encoded by the Mat α1 gene are involved in promoting rapid mRNA decay in yeast, *Proc. Natl. Acad. Sci. USA* 87:2780-2784.(Medline)

Parvin, D.P. and Sharp, P.A. (1993) DNA topology and a minimal set of basal factors for transcription by RNA polymerase II, *Cell* 73:533-540.(Medline)

Parvin, J.D. and Young, R.A. (1998) Regulatory targets in the RNA polymerase II holoenzyme, *Curr. Opin. Genet. Dev.* 8:565-570.(Medline)

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Muller, P., Spring, J., Srinivasan, A., Fishman, M., Finnerty, J., Corbo, J., Levine, M., Leahy, P., Davidson, E. and Ruvkun, G (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA, *Nature* 408:86-89. (MedLine)

Pause, A., Belsham, G.J., Gingras, A.-C., Donzé, O., Lin, T.-A., Lawrence, J.C., Jr., Sonenberg, N. (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function, *Nature* 371:762-767.(Medline)

Pederson, T. (1998) The plurifunctional nucleolus, *Nucleic Acids Res.* 26:3871-3876.(Medline)

Pederson, T. and Politz, J.C. (2000) The nucleolus and the four ribonucleoproteins of translation, *J. Cell Biol* 148:1091-1096. (MedLine)

Pestka S.(1969) Studies on the formation of transfer ribonucleic acid-ribosome complexes. VI. Oligopeptide synthesis and translocation on ribosomes in the presence and absence of soluble transfer factors, *J. Biol. Chem.* 244:1533-1539. (MedLine)

Pham, A-.D. and Sauer, F. (2000) Ubiquitin-activating/conjugating activity of TAFII250, a mediator of activation of gene expression in *Drosophila Science* 289:2357-2360. (MedLine)

Piñol-Roma, S., Swanson, M.S. and Gall, J.G. and Dreyfuss, G. (1989) A novel heterogeneous nuclear RNP protein with a unique distribution on nascent transcripts, *J.Cell Biol.* 109:2575-2587.(Medline)

Plasterk, R.H. (2002) RNA silencing: the genome's immune system, *Science* 296:1263-1265. (MedLine)

Rachez, C., Lemon, B.D., Suldan, Z., Bromleigh, V., Gamble, M. and Naar, A.M., Erdjument-Bromage, H., Tempst, P. and Freedman, L.P. (1999) Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex, *Nature* 398:824-828.(Medline)

Raker, V.A., Hartmuth, K., Kastner, B. and Lührmann, R. (1999) Spliceosomal U snRNP core assembly: Sm proteins assemble onto an Sm site RNAnonanucleotide in a specific and thermodynamically stable manner, Mol. Cell Biol. 19:6554-6565. (MedLine)

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M. and Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D. and Jenuwein, T. (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases, *Nature* 406:593-599. (MedLine)

Redd, M.J., Arnaud, M.B. and Johnson, A.D. (1997) A complex composed of tup1 and ssn6 represses transcription in vitro, *J. Biol. Chem.* 272:11193-11197. (MedLine)

Reed, R. (1990) Protein composition of mammalian spliceosomes assembled *in vitro, Proc. Natl. Acad. Sci. USA* 87:8031-8035.(Medline)

Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B. and Bartel, D.P. (2002) MicroRNAs in plants, *Genes Dev.* 16:1616-1626. (MedLine)

Rhoads, R.E. (1991) Protein synthesis, cell growth and oncogenesis, *Current Opin. Cell Biol.* 3:1019-1024.(Medline)

Rhoads, R.E. (1993) Regulation of eukaryotic protein synthesis by initiation factors, *J. Biol. Chem.*

268:3017-3020.(Medline)

Richter, J.D. (2001) Think globally, translate locally: what mitotic spindles and neuronal synapses have in common, *Proc. Natl. Acad. Sci. USA* 98:7069-7071. (MedLine)

Roberts, S.G., Ha, I., Maldonado, E., Reinberg, D. and Green. M.R. (1993) Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation, *Nature* 363:741-744.(Medline)

Robinett, C.C., Straight, A., Li, G., Willhelm, C., Sudlow, G., Murray, A. and Belmont, A.S. (1996) In vivo localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/repressor recognition, *J. Cell Biol.*135:1685-1700. (MedLine)

Robzyk, K., Recht, J., Osley, M.A. (2000) Rad6-dependent ubiquitination of histone H2B in yeast, *Science* 287:501-504. (MedLine)

Rolfes, R.J. and hinnebusch, A.G. (1993) Translation of the yeast transcriptional activator *GCN4* is stimulated by purine limitation: implications for activation of protein kinase GCN2, *Mol. Cell. Biol.* 13:5099-5111.(Medline)

Ryan, A.K. and Rosenfeld, M.G. (1997) POU domain family values: flexibility, partnerships, and developmental codes, *Genes Dev.* 11:12071225. (MedLine)

Ryu, S., Zhou, S., Ladurner, A.G. and Tjian, R. (1999) The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1, *Nature* 397:446-450.(Medline)

Salghetti, S.E., Muratani, M., Wijnen, H., Futcher, B. and Tansey, W.P. (2000) Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis, *Proc. Natl. Acad. Sci. US* 97:3118-3123. (MedLine)

Salghetti, S.E., Caudy, A.A., Chenoweth, J.G. and Tansey, W.P. (2001) Regulation of transcriptional activation domain function by ubiquitin, *Science* 293:1651-1653. (MedLine)

Sassone-Corsi, P., Mizzen, C.A., Cheung, P., Crosio, C., Monaco, L., Jacquot, S., Hanauer, A. and Allis, C.D. (1999) Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3, *Science* 285:886-891. (MedLine)

Scott, J. (1995) A place in the world for RNA editing, *Cell* 81:833-836.(Medline)

Scully, K.M., Jacobson, E.M., Jepsen, K., Lunyak, V., Viadiu, H., Carriere, C., Rose, D.W., Hooshmand,

F., Aggarwal, A.K. and Rosenfeld, M.G. (2000) Allosteric effects of Pit-1 DNA sites on long-term repression in cell type specification, *Science*290:1127-1131. (MedLine)

Sharp, P.A. (2001) RNA interference--2001, *Genes Dev.* 15:485-490. (MedLine)

Shaw, S.N., Schmidt, A. and Marcus, A. (1992) A conserved AU sequence from the 3' untranslated region of FM-CSF mRNA mediates selective mRNA degradation, *Cell* 46:659-667.

Shikama, N., Lyon, I. and La Thangue, N.B. (1997) The p300/CBP family: integrating signals with transcription factors, *Trends Cell Biol.* 7:230-236.

Shyu, A.-B. and Wilkinson, M.F. (2000) The double lives of shuttling mRNA binding proteins, *Cell* 102:135-138.(Medline)

Simpson, R.T. and Thiemann, O.H. (1995) Sense from nonsense: RNA editing in mitochondria of kinetoplastid protozoa and slime molds, *Cell* 81:837-840.(Medline)

Sive, H.L., Heintz, N. and Roeder, R.G. (1984) Regulation of human histone gene expression during the HeLa cell cycle requires protein synthesis, *Mol. Cell. Biol.* 4:2723-2734.(Medline)

Smith, R.L. and Johnson, A.D. (2000) Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes, *Trends Biochem. Sci.* 25:325-330. (MedLine)

Sommer, B., Köhler, M., Sprengel, R. and Seeburg, P.H. (1991) RNA editing in brain controls a determinant of ion flow in glutamate-gated channels, *Cell* 67:11-19.(Medline)

Soutoglou, E. and Talianidis, I. (2002) Coordination of PIC assembly and chromatin remodeling during differentiation-induced gene activation, *Science* 295:1901-1904. (MedLine)

Spector, D.L., Schrier, W.H., Busch, H. (1983) Immunoelectron microscopic localization of snRNPs, *Biol. Cell.* 49:1-10.(Medline)

Spencer, T.E., Jenster, G., Burcin, M.M., Allis, C.D., Zhou, J., Mizzen, C.A., McKenna, N.J., Onate, S.A., Tsai, S.Y., Tsai, M.-J. and O'Malley, B.W. (1997) Steroid receptor coactivator-1 is a histone acetyltransferase, *Nature* 389:194-198.(Medline)

Stark, H., Dube, P., Lührmann, R. and Kastner, B. (2001) Arrangement of RNA and proteins in the splicesomal U1 small nuclear ribonucleoprotein particles, *Nature* 409:539-542.

Sterner, D.E. and Berger, S.L. (2000) Acetylation of histones and transcription-related factors, *Microbiol.*

Mol. Biol. Rev. 64:435-459. (MedLine)

Steward, O. and Schuman, E.M. (2001) Protein synthesis at synaptic sites on dendrites, *Annu. Rev. Neurosci.* 24:299-325. (MedLine)

Storz, G. (2002) An expanding universe of noncoding RNAs, *Science* 296:1260-1263. (MedLine)

Strahl, B.D. and Allis, C.D. (2000) The language of covalent histone modification, *Nature* 403:41-45.(Medline)

Strässer, K. and Hurt, E. (2000) Yra1p, a conserved nuclear RNA-binding protein, interacts directly with Mex67p and is required for mRNA export, *EMBO J.* 19:410-420. (MedLine)

Struhl, K. (1991) Mechanisms for diversity in gene expression patterns, *Neuron* 7:177-181. (MedLine)

Struhl, K. (1999) Fundamentally different logic of gene regulation in eukaryotes and prokaryotes, *Cell* 98:1-4. (MedLine)

Tang, G., Reinhart, B.J., Bartel, D.P. and Zamore, P.D. (2003) A biochemical framework for RNA silencing in plants, *Genes Dev.*17:49-63. (MedLine)

Theil, E.C. (1987) Ferritin: structure, gene regulation, and cellular function in animals, plants and microorganisms, *Ann. Rev. Biochem.* 56:289-315.(Medline)

Thomas, D. and Tyers, M. (2000) Transcriptional regulation: Kamikaze activators, *Curr. Biol.* 10:R341-343. (MedLine)

Thomson, S., Clayton, A.L., Hazzalin, C.A., Rose, S., Barratt, M.J. and Mahadevan, L.C. (1999) The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase, *EMBO J.* 18:4779-4793. (MedLine)

Tong, J.K., Hassig, C.A., Schnitzler, G.R., Kingston, R.E. and Schreiber, S.L. (1998) Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex, *Nature* 395:917-921.(Medline)

Treitel, M.A., Kuchin, S. and Carlson, M. (1998) Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in *Saccharomyces cerevisiae Mol. Cell Biol.* 18:6273-6280. (MedLine)

Tsukamoto, T., Hashiguchi, N,. Janicki, S.M., Tumbar, T., Belmont, A.S. and Spector, D.L . (2000) Visualization of gene activity in living cell, *Nature Cell Biol.* 2::871-878. (MedLine)

Tsukiyama, T. and Wu, C. (1996) Purification and properties of an ATP-dependent nuclesome remodeling factor, *Cell* 83:1011-1020.(Medline)

Tumbar, T., Sudlow, G. and Belmont, A.S. (1999) Large-scale chromatin unfolding and remodeling induced by VP16 acidic activation domain, *J. Cell Biol.* 145:1341-1354. (MedLine)

Udvardy A. (1999) Dividing the empire: boundary chromatin elements delimit the territory of enhancers, *EMBO J.* 18:1-8. (MedLine)

Varga-Weisz, P. D., Wilm, M., Bonte, E., Dumas, K., Mann, M. and Becker, P. B (1997) Chromatin-remodelling factor CHRAC contains the ATPases ISWI and topoisomerase II, *Nature* 388:598-602.(Medline)

Wade, P.A., Jones, P.L., Vermaak, D. and Wolffe, A.P. (1998) A multiple subunit Mi-2 histone deacetylase from Xenopus laevis cofractionates with an associated Snf2 superfamily ATPase, *Curr. Biol.* 8:843-846.(Medline)

Watson, J.D., Gilman, M., Witowski, J. and Zoller, M. (1992) *Recombinant DNA*, Scientific American Books, New York, see ch 9.

Wek, R.C. (1994) eiF-2 kinases: regulators of general and gene specific translational initiation, *Trends Biochem. Sci.* 19:491-496.

Whitehouse, I., Flaus, A., Cairns, B.R., White, M.F., Workman, J.L. and Owen-Hughes, T. (1999) Nuclosome mobilization catalyzed by yeast SWI/SNF complex, *Nature* 400:784-787.(Medline)

Will, C.L. and Lührmann, R. (1997) Protein functions in pre-mRNA splicing, *Curr. Opin. Cell Biol.* 9:320-328. (MedLine)

Wilson, K.S. and Noller, H.F. (1998) Molecular movement inside the translational engine, *Cell* 92:337-349. (MedLine)

Wilson, C.J., Chao, D.M., Imbalzano, A.N., Schnitzler, G.R., Kingston, R.E. and Young, R.A. (1996) RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling, *Cell* 84:235-244.(Medline)

Wolffe, A.P. and Hayes, J.J. (1999) Chromatin disruption and modification, *Nucleic Acids Res.* 27:711-720.(Medline)

Wolffe, A.P. and Pruss, D. (1996) Targeting chromatin disruption: transcription regulators that acetylate

histones, *Cell* 84:817-819.(Medline)

Wu, Z.A., Murphy, C., Callan, H.G. and Gall, J.G. (1991) Small nuclear ribonucleoproteins and heterogeneous nuclear ribonucleoproteins in the amphibian germinal vesicle: loops, spheres, and snurposomes, *J. Cell Biol.* 113:465-483. (MedLine)

Xu, W., Chen, H., Du, K., Asahara, H., Tini, M., Emerson, B.M., Montminy, M. and Evans, R.M. (2001) A transcriptional switch mediated by cofactor methylation, *Science* 294:2507-2511. (MedLine)

Xue, Y., Wong, J., Moreno, G.T., Young, M.K., Cote, J. and Wang, W. (1998) NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities, *Mol. Cell* 2:851-861..(Medline)

Yang, X.J., Ogyzko, V.V., Mishikawa, J., Howard, B.H. and Nakatani, Y. (1996) A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A, *Nature* 382:319-324.(Medline)

Yen, T.J., Gay, D.A., Patcher, J.S. and Cleveland, D.W. (1988a) Autoregulated changes in stability of polyribosome bound β-tubulin mRNAs are specified by the first 13 translated nucleotides, *Mol. Cell. Biol.* 8:1224-1235.(Medline)

Yen, T.J., Machlin, P.S. and Cleveland, D.W. (1988b) Autoregulated instability of β-tubulin mRNAs by recognition of the nascent amino terminus of β-tubulin, *Nature* 334:580-585.(Medline)

Zamore, P.D. (2002) Ancient pathways programmed by small RNAs, *Science* 296:1265-1269. (MedLine)

Zamore, P.D., Tuschl, T., Sharp, P.A. and Bartel, D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals, *Cell* 101:25-33. (MedLine)

Zanger, K., Radovick, S. and Wondisford, F.E (2001) CREB binding protein recruitment to the transcription complex requires growth factor-dependent phosphorylation of its GF box, *Mol. Cell.* 7:551-558. (MedLine)

Zawell, L. and Reinberg, D. (1993) Initiation of transcription by RNA polymerase II: a multi-step process, *Prog. Nucleic Acid. Res. Mol. Biol.* 44:549-561.(Medline)

Zenke, M.T., Grundstrom, H., Matthes, M., Winterith, C., Schatz, A., Wilderman, A. and Chambon, P. (1986) Multiple sequence motifs are involved in SV40 enhancer function, *EMBO. J.* 5:387-397.(Medline)

Zhou, J. and Levine, M. (1999) A novel cis-regulatory element, the PTS, mediates an anti-insulator

activity in the *Drosophila* embryo, *Cell* 99:567-575. (MedLine)

Zhou, Z., Luo, M.J., Straesser, K., Katahira, J., Hurt, E. and Reed, R. (2000) The protein Aly links pre-messenger-RNA splicing to nuclear export in metazoans, *Nature* 407:401-405. (MedLine)

Zitomer, R.S., Limbach, M.P., Rodriguez-Torres, A.M., Balasubramanian, B., Deckert, J. and Snow, P.M. (1997) Approaches to the study of Rox1 repression of the hypoxic genes in the yeast *Saccharomyces cerevisiae*, *Methods* 11:279-288. (Medline)

4. The Plasma Membrane

Scientists have been fascinated with the plasma membrane from the very first observations of living cells. At the interface between cytoplasm and its environment, the plasma membrane has an important role in maintaining the integrity of the cell. From first thinking that the plasma membrane was mostly made up of lipids with proteins of secondary importance, our knowledge has grown to include proteins embedded in the membrane -- glycoproteins whose carbohydrate branches provide an external cover for the cell. The plasma membrane has proven to be more than a barrier to the passage of solutes. Its machinery is actively involved in the transport of ions. It mediates responses to external stimuli and to certain hormones, as well as interactions between cells and the extracellular medium. In addition, it interacts with elements of the cytoskeleton. The present chapter will begin with a discussion of the components and properties of the plasma membrane.

All major organelles and internal vesicles are bounded by membranes that in some respect resemble the

plasma membrane. They separate the cell's interior into compartments and have many specialized functions that range from controlling the passage of macromolecules to energy transduction, as is the case of the inner mitochondrial membranes and the thylakoid membranes of chloroplasts. The intracellular membranes are the topic of the next chapter.

Many details of the plasma membrane and the internal membranes will be interspersed throughout the textbook.

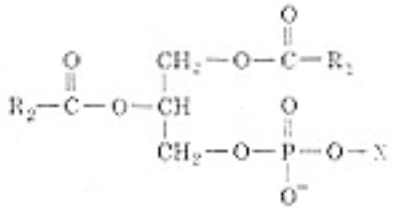
I. THE PLASMA MEMBRANE

As in any other structure, the components of membranes determine their properties and functional significance. In this chapter lipids will be discussed first, followed by proteins, with the rest of the chapter focusing on various properties of the plasma membrane.

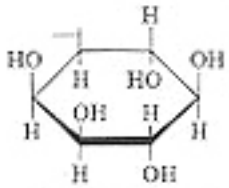
A. The Lipids of the Plasma Membrane

The glycerophospholipids, the major components of the plasma membrane, are esters of glycerol, where one glycerol is conjugated to two fatty acids and one phosphate. Their general formula is represented in Fig. 1A. The groups covalently attached to the phosphate (X in Fig. 1A) are listed in Fig. 1B. Some are positively charged, one has no net charge and two have no charge at all. They are all, however, polar. Although they have no net charge, the distribution of electrons between the component atoms is uneven, making part of the molecule positive and part negative. Because of their polarity they can interact with other polar molecules, such as water. The actual shape of the molecule is quite distinct from the generalized diagram of Fig. 1A, because the R groups are very long hydrocarbon chains, some containing double bonds. A more reasonable representation is shown in part C of Fig. 1.

A.



B.

Name of X—OH	Formula of —X	Name of Phospholipid
Water	—H	Phosphatidic acid
Ethanolamine	$-CH_2CH_2NH_3^+$	Phosphatidylethanolamine
Choline	$-CH_2CH_2N(CH_3)_3^+$	Phosphatidylcholine (lecithin)
Serine	$-CH_2CH(NH_3^+)COO^-$	Phosphatidylserine
myo-Inositol		Phosphatidylinositol
Glycerol	$-CH_2CH(OH)CH_2OH$	Phosphatidylglycerol
Phosphatidylglycerol	$-CH_2CH(OH)CH_2-O-P(=O)(O^-)-O-CH_2$	Diphosphatidylglycerol (cardiolipin)

C.

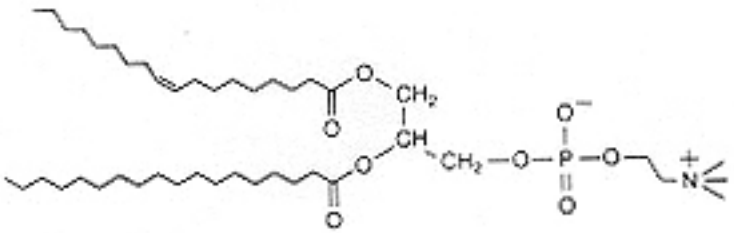


Fig. 1 Representation of the structure of phosphoglycerides.

A. Generalized structure. The R groups represent the hydrocarbon chains of fatty acids and X represents the head group shown in part B.

B. Tabulation of the phospholipids. The first column contains the name of the head group (the X of part A). The second column shows the corresponding formula, and the third lists the name of each phospholipid.

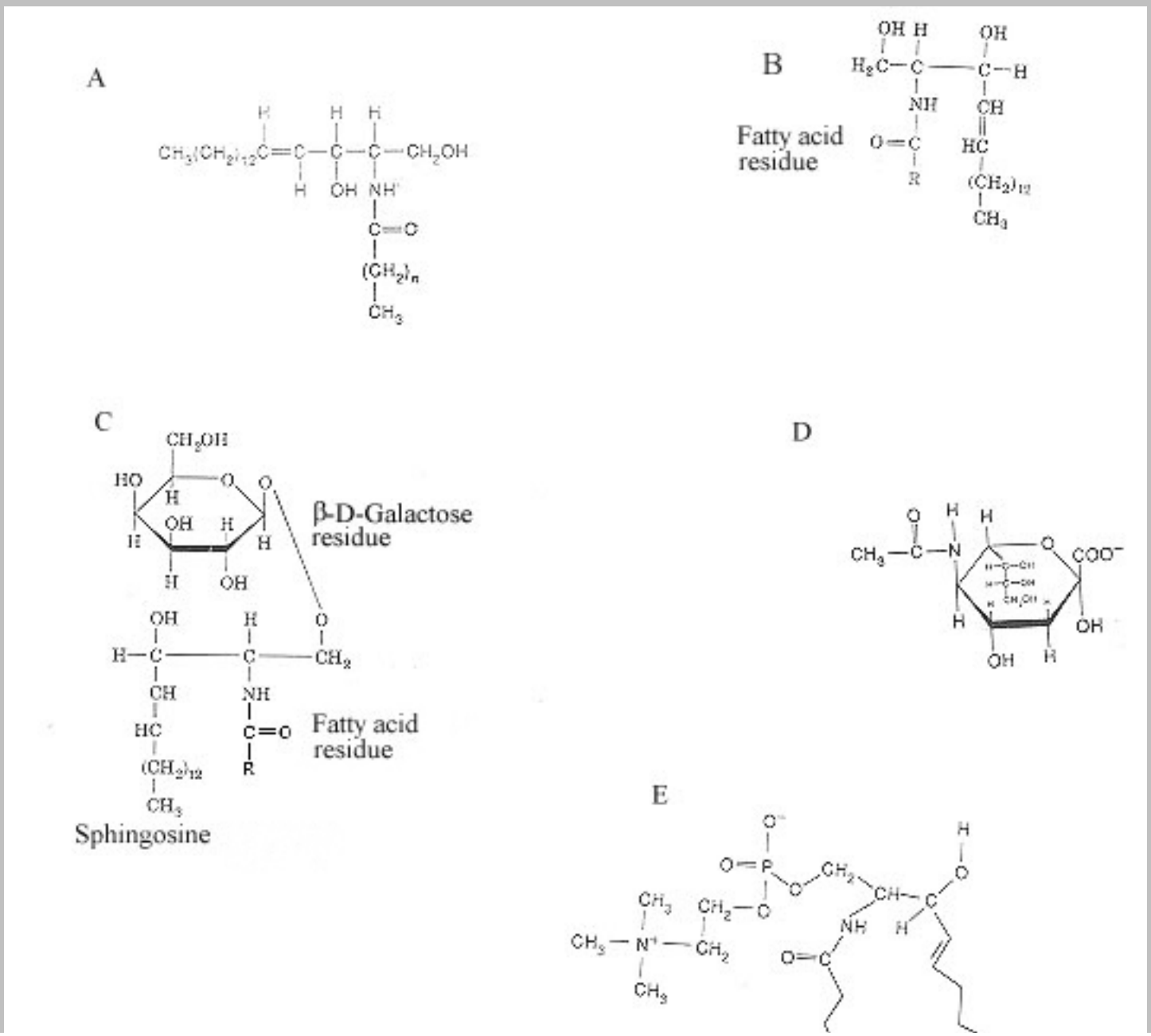
C. Diagram of phosphatidylcholine, showing the head charges. Note that the unsaturated bond introduces a kink in the molecule.

A second class of lipids are the *sphingolipids*. Instead of glycerol, s*phingosine* is the common component (Fig. 2A). Some sphingolipids have an N-acyl fatty acid attached to the sphingosine: these are *ceramides* (Fig. 2B). Others, in addition to the fatty acid, have a sugar such as glucose or galactose attached to the

first carbon, as in the case of a *galactosecerebroside* (Fig. 2C). Some of these are sulfated in the C3 position. In *gangliosides*, several sugars are attached to form a carbohydrate chain. At least one of these is *N*-acetylneuraminidate, also called sialic acid (Fig. 2D), which has a net negative charge. Again, many of these sphingolipids are charged, however, all have polar groups. In these lipids both sphingosine and fatty acids possess long hydrophobic chains, as shown for sphingomyelin in Fig. 2E.

The polar end of the lipids interact with water, whereas the hydrophobic hydrocarbon chains excluded from the water phase, tend to interact with other hydrophobic groups either in other lipids or proteins. Therein hangs a tale.

The importance of sphingolipids in cell function is shown by the effect of *fumosins*. Fumosins are toxins produced by fungi that resemble the base backbone of sphingolipids. Fumosin B_1 are inhibitors of ceramide synthase. Disruption of sphingolipid metabolism by this fumosin affects cell-cell interactions, the activity of protein kinases and cell growth and viability (see Merrill et al., 1996).



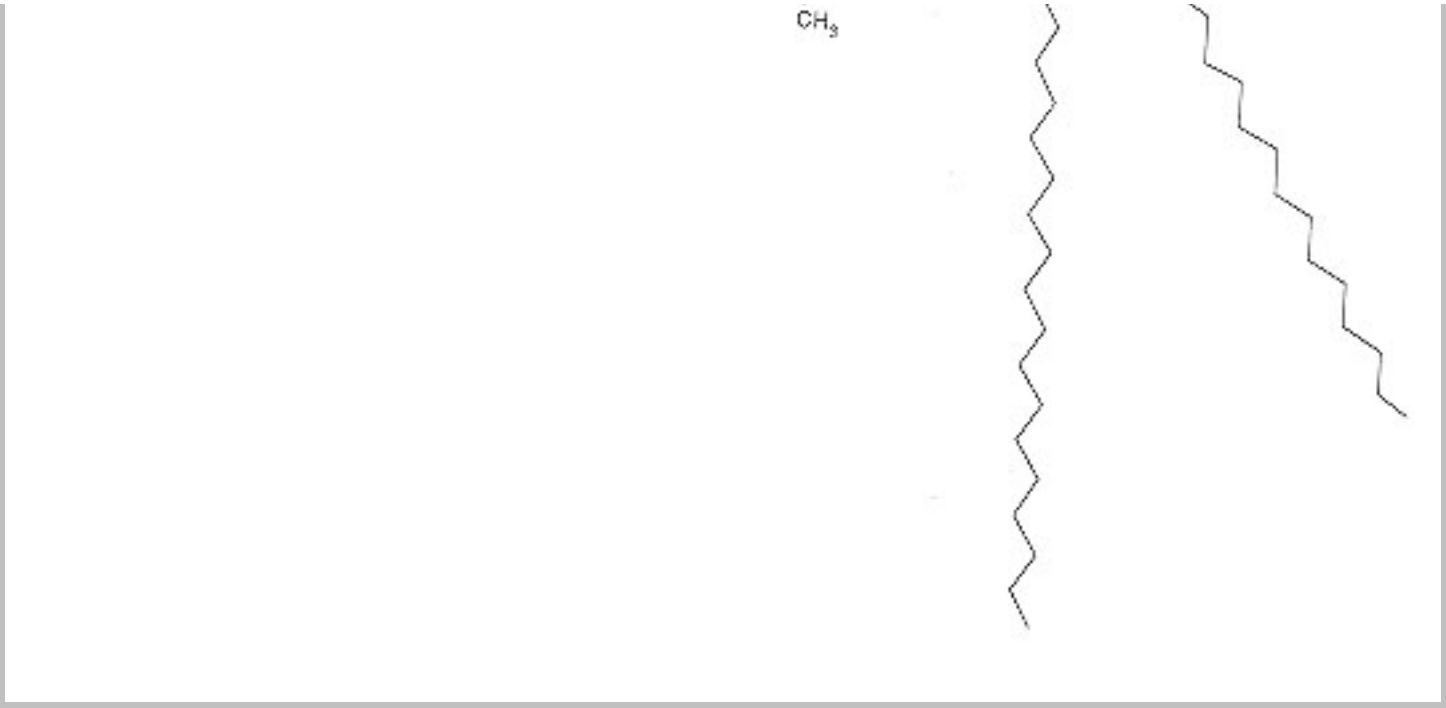


Fig. 2 Sphingolipids

A. Sphingosine
B. Ceramide
C. Galactosecerebroside
D. Sialic acid (N-acetylneuraminidate)
E. Sphingomyelin

The proportion of the lipids in the plasma membrane(25 mol % PC, 15 mol % PE, 30 mol% cholesterol, 10 mol % sphingolipids and 5% PS) is different than that of the internal membranes. In the endoplasmic reticulum, it is 60 mol % PC, 25 mol % PE and 10 mol % PI. The composition changes along the stacks of the Golgi until it acquires that of the plasma membrane is (see Keenan and Morré, 1970; Fleischer, et al. 1974). The symmetry of the endoplasmic reticulum is also changed to the asymmetric distribution of the plasma membrane (see Section IV). These differences are in agreement with the current view that the membranes are translocated and altered biochemically, starting from the endoplasmic reticulum until they reach the external cell membrane (see Chapter 10).

B. The Lipid Bilayer

The early concept that the lipid at the periphery of the cell is organized predominantly as a bilayer is still valid. This model can be represented as shown in Fig. 3. The lines represent hydrophobic chains of the phospholipid molecules and the circles represent the polar, hydrophilic portions. The major factor in determining structure is the ability of water molecules to squeeze out the nonpolar hydrocarbon chains so that they are held together in micelles or lamellae. The presence of the hydrophobic domains of the hydrocarbon chains of the lipids in a water medium, favors a structural arrangement of the surrounding water molecules, because of the high affinity of water molecules for each other (i.e., in the presence of

the nonpolar lipid side chains the arrangement of the whole system will be less random, ΔS would be negative, see Chapter 12). This property favors excluding the lipid from the water to form lipid lamellae or micelles. The lateral forces holding the structure together, which correspond to the van der Waals forces of hydrophobic bonding, also play a role. In addition, depending on the nature of the molecules, the charges of the polar groups may also be involved. The bimolecular model of membrane structure was originally based mostly on the results of two kinds of experiments: one studied the permeability of certain cells to nonelectrolytes, the other determined the composition of the membrane of mammalian red blood cells.

Mammalian red blood cells have been very useful in the study of membranes because they are readily available, uniform in population, and have no internal organelles. They lyse osmotically to form the so-called red blood cell *ghosts*. When placed in a medium of low solute concentration (high water concentration), they swell. Water will enter until at a critical volume the internal contents leak out. The ghosts are almost pure plasma membranes and can be isolated by simple procedures.

Other cells, such as internodal cells of the freshwater algae *Chara* or *Nitella*, were also found to be very useful for studying the permeability of the plasma membrane because of their size. These multinucleated cells are large, as long as 1 cm, therefore, it is relatively easy to manipulate them and withdraw samples for analysis. They have also been favored in the study of electrical events and cell movement. Data on the permeability of *Nitella* to nonelectrolytes have been analyzed as shown in Fig 4 (Collander, 1954). In this figure, the permeability of the cells multiplied by the square root of the molecular weight ($PM^{1/2}$) is shown on the ordinate. The abscissa represents the oil-water partition coefficient, a measure of the solubility of the nonelectrolyte in oil divided by its solubility in water. This coeffient can be obtained experimentally for each substance by equilibrating a water phase containing the nonelectrolyte with an identical volume of oil (or, in some cases, organic solvents). The ratio of concentrations of the nonelectrolyte in the two phases is the partition coefficient. Each point in Fig. 4 corresponds to a different nonelectrolyte. The product $PM^{1/2}$ was used, rather than the permeability constant P, because a smaller molecule is expected to enter the plasma membrane faster for steric reasons. This method of plotting corrects for differences in molecular size, following a theoretical treatment that is based on several assumptions (Davson and Danielli, 1952). In this treatment, a molecule of solute is considered to detach itself from the water phase and move into the lipid phase. It travels through the hydrophobic layer in steps, each with a characteristic energy of activation. Then it must enter the water phase again from the lipid.

Fig. 4 shows that there is a correlation between the permeability and the partition coefficient, indicating that substances that are more soluble in oil than in water penetrate the plasma membrane more readily. Similar data are available for the red blood cell. The correlation between the permeability constant and the solubility in oil suggests that the solute molecules have to dissolve in a lipid layer in order to pass through.

The significance of these observations is more readily apparent when the results (in this case for the red blood cell) are compared to the values calculated for a hypothetical cell enclosed by a water layer of

approximately the thickness of the plasma membrane (Table 1). In Table 1, the solute under discussion is listed in column 1. k corresponds to the diffusion coefficient in water (column 2). The calculated permeability constant (k') for a water layer is listed in column 3 and the permeability constant calculated from experimental results, in column 4. In columns 5 and 6, the permeability is expressed as the time to reach 90% of equilibrium (Jacobs, 1952), a parameter that is inversely proportional to the permeability constant. In contrast to observations with the red blood cells (column 6), equilibration in the model system (column 5) is almost instantaneous and the system is unable to distinguish one solute molecule from another.

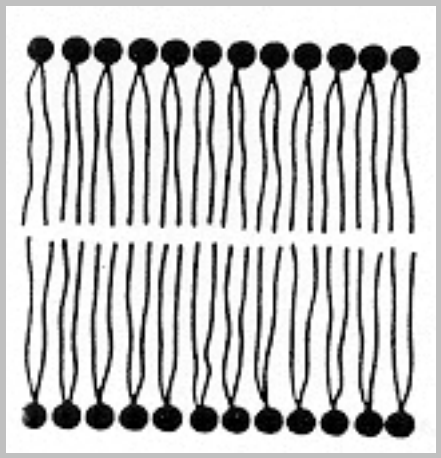


Fig. 3 Diagrammatic representation of phospholipid molecules in a bilayer arrangement. The lines represent the hydrocarbon chains of the lipids and the circles are the polar ends immersed in water.

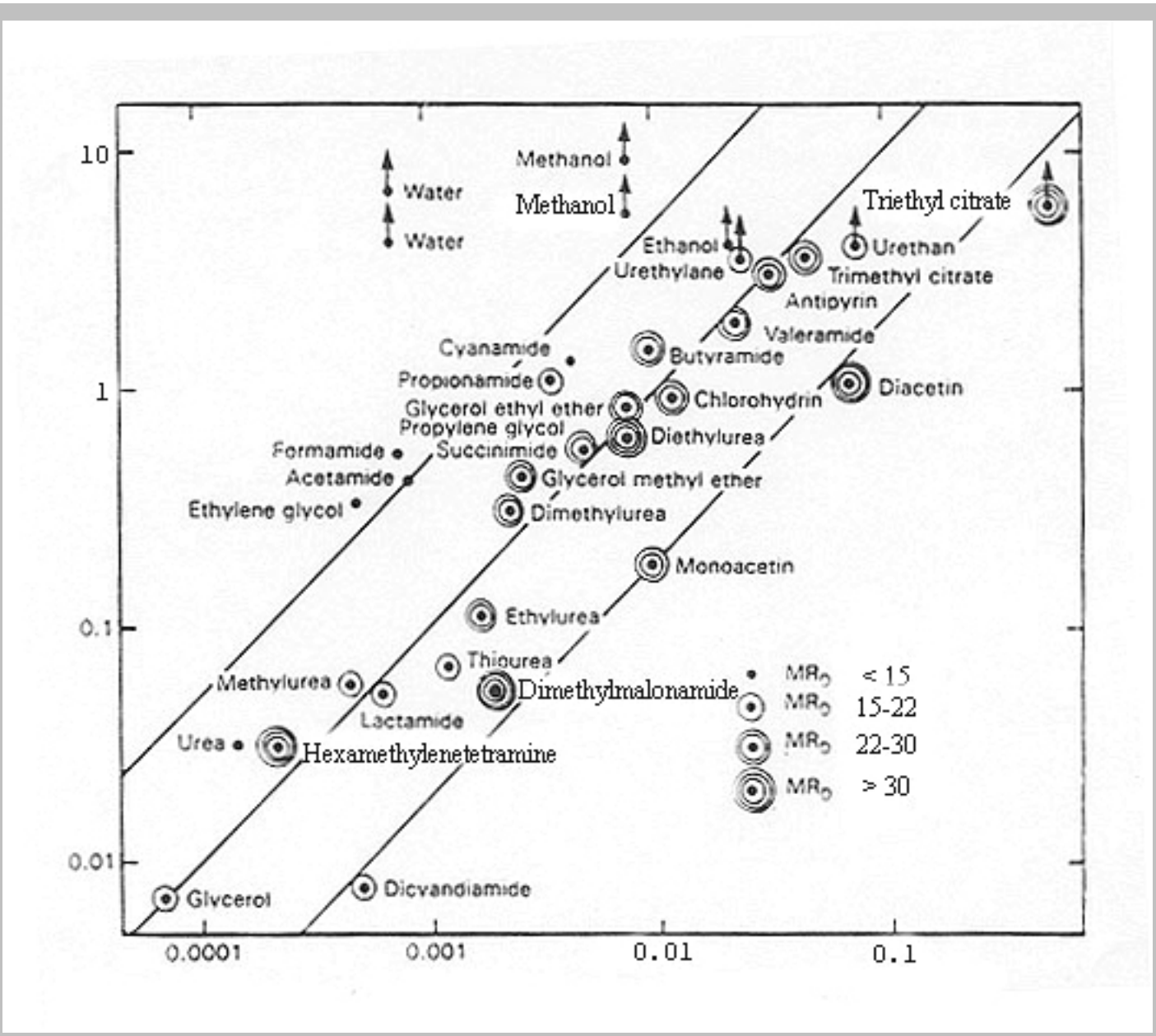


Fig. 4 Permeability of *Chara* cells plotted against oil-water partition coefficient. Ordinate: permeability (cm/h) x $M^{1/2}$. Abscissa: oil-water partition coefficient. The two scales are logarithmic. Reprinted with permission from Collander (1954).

The permeability data can be most readily interpreted by assuming that solutes must traverse a continuous lipid layer before they enter the cell. In addition, the known properties of lipids and the results of the study of Gorter and Grendel (1925) strongly suggest that the predominant organization of this lipid layer is as a bilayer. Gorter and Grendel used a Langmuir trough, which is represented in Fig. 5. In this figure, the bottom rectangle represents a space filled with water below air. When the phospholipids are placed in the trough, they arrange themselves so that their hydrophilic ends are immersed in the water and their hydrophobic chains stick out in the air. When the lipid molecules are close to each other they form a continuous monomolecular lipid sheath. The surface of the trough available per lipid molecule can easily be calculated from the number of lipid molecules added to the surface and the dimensions of the trough. The force exerted on the system by compression can be

measured. Data obtained by varying the position of the movable end of the trough can be plotted as shown in Fig.6, where the pressure is shown on the ordinate and the surface divided by the lipid molecules is shown on the abscissa. As the pressure is increased the surface area per lipid molecule decreases, until a limiting value is reached. At this point no further compression is possible and the monolayer buckles (not shown). The limiting value shown in the abscissa corresponds to the area occupied by one lipid molecule when the lipid molecules are maximally packed as they would be in a continuous monolayer.

Table 1 Calculations of the Time Required for 90% Equilibration of the Penetrants Listed in Column 1[a]

(1)	(2)	(3)	(4)	(5)	(6)
Times for 90% equilibrations (s)					
Solute	**k in water**	**k' in model (cm^2/s)**	**k' in cells (cm^2/s)**	**Model**	**Cells**
Urea	1.18×10^{-5}	11.8	1.94×10^{-4}	0.76×10^{-5}	0.5
Glycerol	0.83×10^{-5}	8.3	1.50×10^{-6}	1.08×10^{-5}	67
Mannitol	0.58×10^{-5}	5.8	0	1.55×10^{-5}	> 2 days
Sucrose	0.43×10^{-5}	4.3	5.5×10^{-20}	2.09×10^{-5}	∞

[a]The calculations assume the membrane thickness to be 10 nm, the red blood cell volume to be 30×10^{-12} cm^3, and the surface area to be 77×10^{-8} cm^2. k is the diffusion coefficient and k' the calculated pemeability constant.

Based on Jacobs (1952). Reproduced by permission.

Gorter and Grendel extracted the lipids from a known number of red blood cells and placed them in a

Langmuir trough. The minimal area of the monomolecular film at its limiting value was then determined, as described above. The area occupied by the surfaces of the red blood cells from which the lipid has been extracted, can be calculated approximately from the dimensions of one cell multiplied by the number of cells. The total area of the compressed monomolecular film divided by the area of the cells was found to be approximately 2, indicating that enough lipid was extracted for two monomolecular layers, that is, a bilayer. The results qualitatively support the notion that the lipid components of the membrane are approximately present as a bilayer.

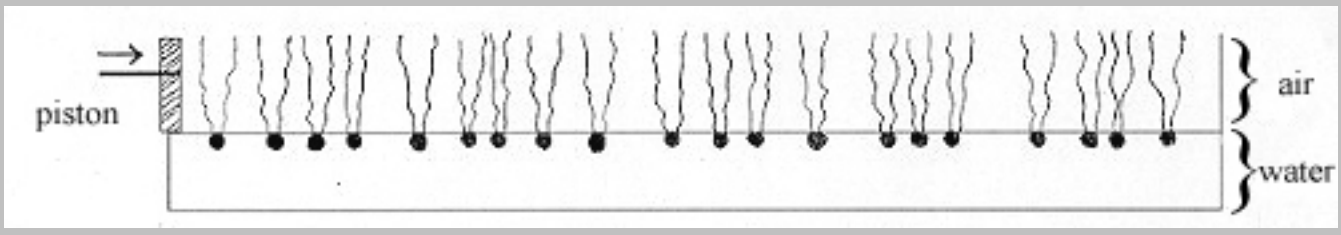


Fig. 5 Langmuir trough.

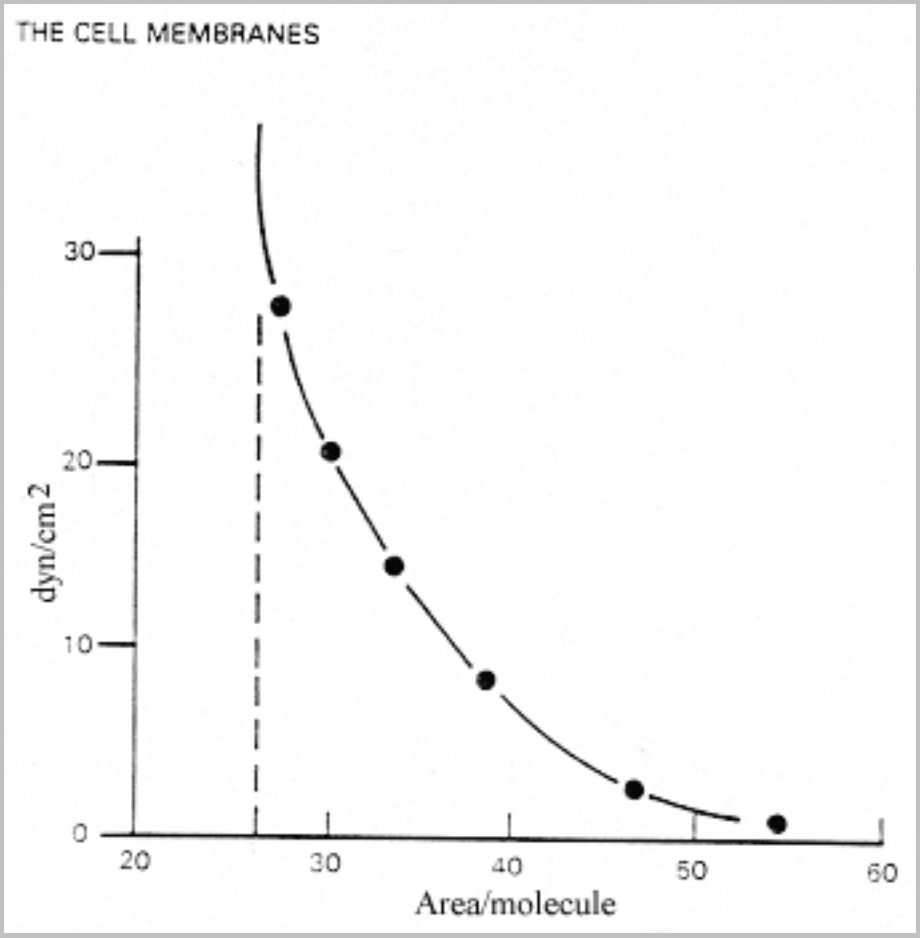


Fig. 6 Representation of data obtained from compression of the lipid layer in a Langmuir trough.

II. PROTEINS IN THE PLASMA MEMBRANE

The plasma membrane contains proteins as well as lipids. A current view of how proteins are distributed

in the plasma membrane of the red blood cell is shown in Fig. 7A (Fowler, 1986). Some of the evidence on which this model is based, will be discussed later. In this diagram, the lipid bilayer is represented by crosshatching. Several proteins extend across the membrane and are arranged so that one part of the protein molecule is present on the outer surface of the membrane and another is on the inner, cytoplasmic face. The hairlike processes shown in the diagram on the outer surface, represent the carbohydrate portion of the glycoproteins. Although only two kinds of proteins are shown traversing the membrane, several others not shown in the diagram are present in lesser amounts. Proteins that are embedded in the bilayer are known as *integral* membrane proteins; they can generally be removed from the membrane when the lipid is disrupted and replaced by detergents. The anionic transporter (also known as *Band 3*) represents the major protein of the membrane. Generally, the proteins are numbered according to the position of the corresponding bands on sodium dodecyl sulfate (SDS) gel electrophoresis that is used to separate them.

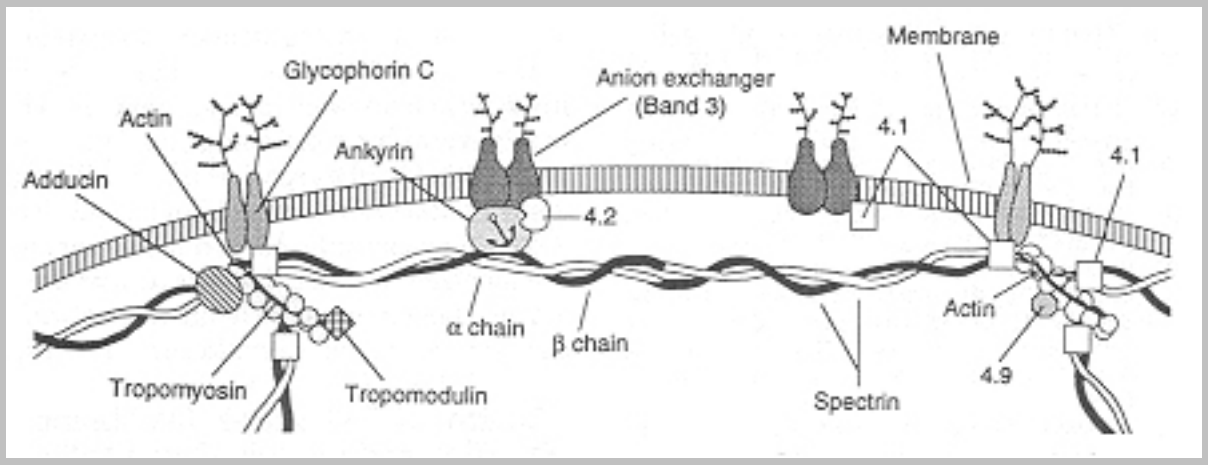
At least part of the *Band 3* protein is anchored to the network of filaments and fibers under the plasma membrane by *ankyrin*, a protein with a molecular mass of 215 kDa. This network, present throughout the cytoplasm, is particularly dense next to the membrane and is known as the *cytoskeleton*. It can be isolated after the cell membrane is disrupted by detergents. In this case, the filaments contain, among other proteins, *spectrin* and *actin*. Actin, discussed several times in this book, is part of the contractile apparatus of muscle and is involved in movement in other cells, generally in combination with myosin, and is also a determinant of the cells' architecture. In fact, the red blood cell contains myosin and arguments have been made for a role of actomyosin in the contraction of this cell.

As might be expected, different cells have different arrangements. However, some of the proteins seem to have similar characteristics and interactions. Many of them are concentrated in membrane domains which may confer some rigidity to the system and restrict the two dimensional diffusion. Fig. 7B (Luna and Hitt, 1992) is a model of the arrangement in striated muscle, and Fig. 7C (Luna and Hitt, 1992) represents the interactions thought to occur in focal contacts where fibroblasts make contact with the substratum.

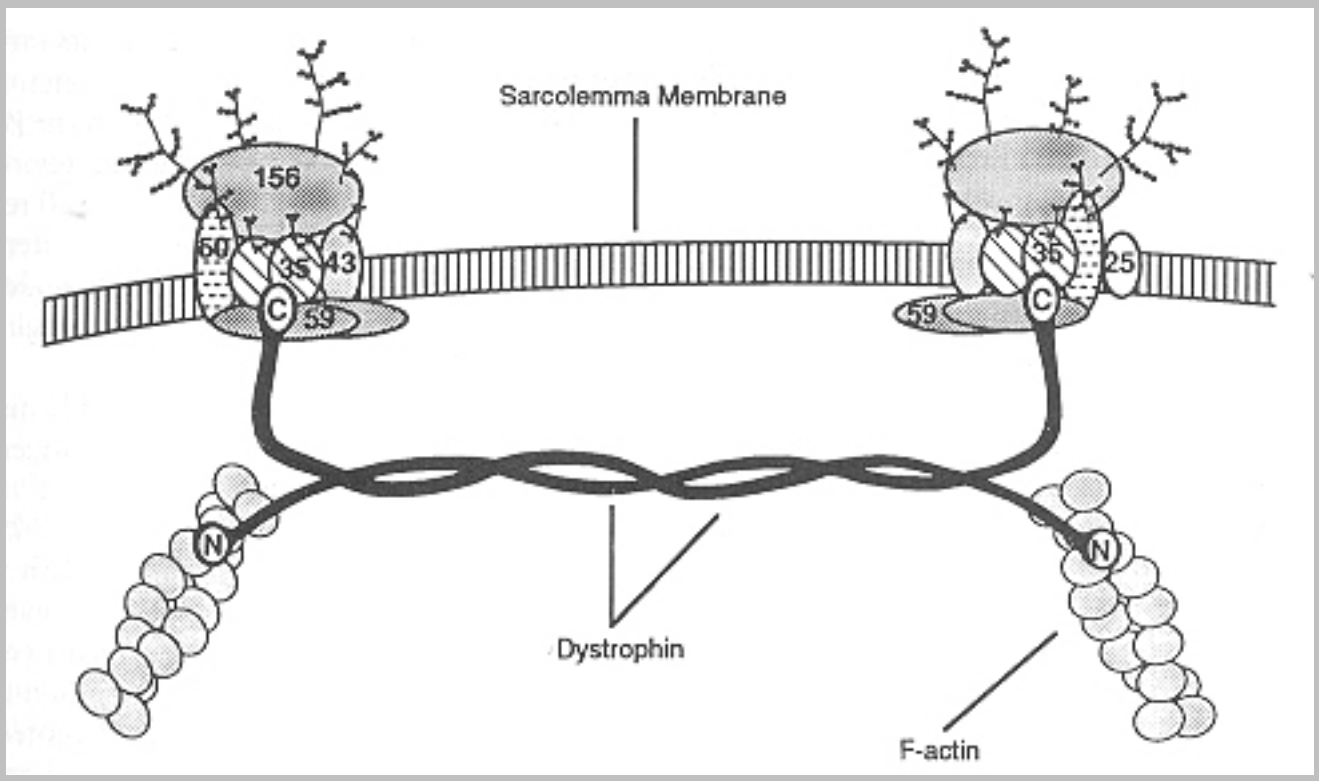
Proteins that mediate linkage of the cytoskeleton to the plasma membrane have a common domain of approximately 300 amino acids. This domain is now called the FERM domain (the initials of four proteins that serve as linkers between the cytoskeleton and the plasma membrane) (see Christi et al., 1998). The domain was first identified in band 4.1 of human red blood cells (Leto and Marchesi, 1984). The function of FERM in serving as a linker is shown not only for the band 4.1 protein (that binds various proteins of the plasma membrane as shown in Fig. 7 and some phospholipids), but also for other proteins that link transmembrane proteins to the actin cytoskeleton in a variety of cells.

Proteins that are anchored in specific areas of the plasma membrane may play an important role in the function of the cells. At synapses, receptors that bind specifically to neurotransmitters are clustered by anchoring proteins. Glutamate is the most common excitatory neurotransmitter in the mammalian brain. Glutamate receptors in postsynaptic cells are localized in clusters by their binding to special proteins containing a specific amino acid motif (e.g., Dong et al., 1997)

Fig. 7 Probable arrangement of integral proteins with cytoplasmic fibrous elements from Fowler, 1986 and Luna and Hitt, 1992 (reproduced with permission from Science 258:955-964. Copyright © 1992 American Association for the Advancement of Science). Reproduced with permission.

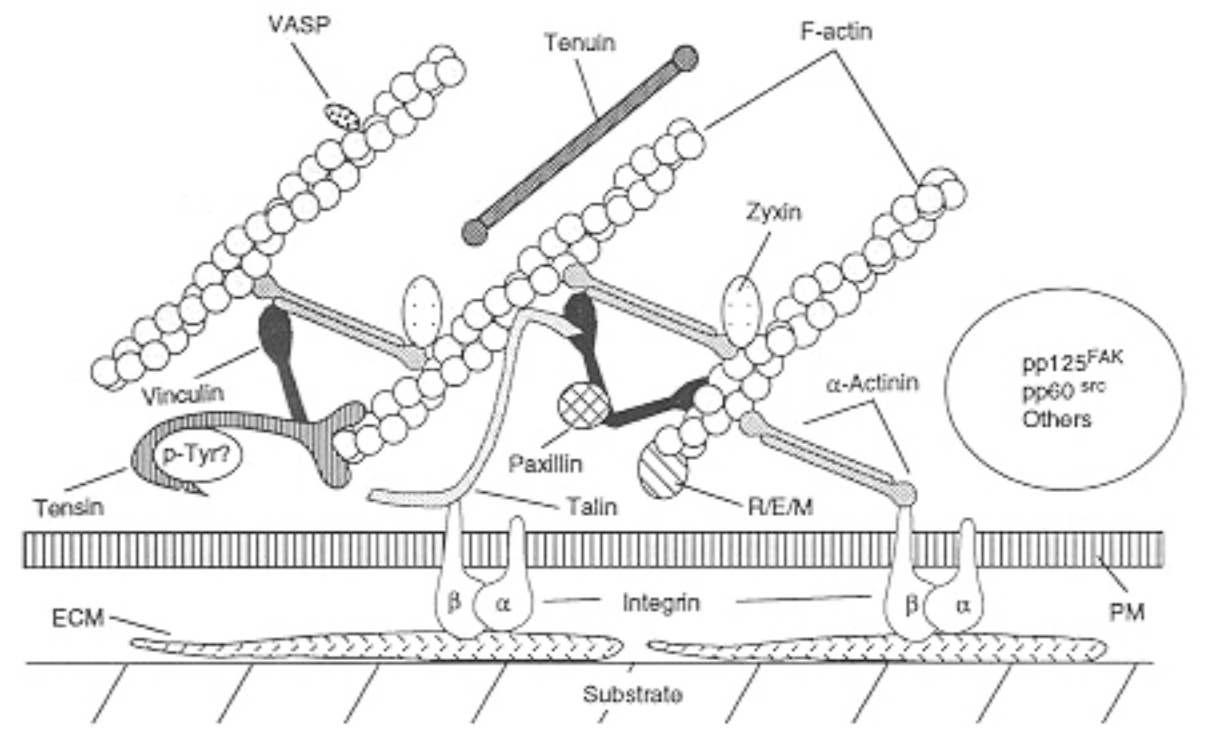


A. Organization of the membrane proteins and cytoskeletal filaments in the human red blood cell. The proteins of the membrane have been identified primarily by SDS gel electrophoresis. The molecular weights (from SDS-PAGE) in kDa are as follows. Spectrin: 260 and 225, ankyrin: 215, adducin: 105 and 100, *Band 3* (the anion exchanger): 90 to 100, protein 4.1: 78, protein 4.2 (pallidin): 72, dematin 48: glycophorin C: 25, actin: 43 and tropomyosin: 29 and 27.



B. Working model for the dystrophin-glycoprotein complex of skeletal muscle. The interactions are

speculative and based on the known location of the proteins in relation to the membrane.



C. Working model of the protein-protein interactions in focal adhesions of fibroblasts determined by in vitro binding experiments and immunolocalization. Most associations have not been verified in vivo. Abbreviations are as follows. ECM: extracellular matrix, PM: plasma membrane, p-Tyr-: unknown phosphotyrosine-containing protein, R/E/M member of the radixin/ezrin/moesin family, VASP: vasodilator-stimulated phosphoprotein.

The pattern of the cytoskeletal elements closely associated with the red blood cell membrane, has been known for some time from extraction and binding studies. A clear demonstration of the undisturbed structural pattern has been shown in experiments in which the meshwork of fibers has been isolated intact from the ghosts after detergent treatment (Liu et al., 1987). An electron microscopic examination of the spread-out network after negative staining, reveals the organization shown in Fig. 8 (Liu et al., 1987). The individual components labeled in the figure were identified by selective extraction of the isolated network. The globules containing ankyrin are also likely to contain *Band 3* protein, because the two are generally extracted together. As shown, the fibers form a hexagonal lattice (Fig. 8B and C). The network at lower magnification suggests that it is a continuous structure lining the membrane surface.

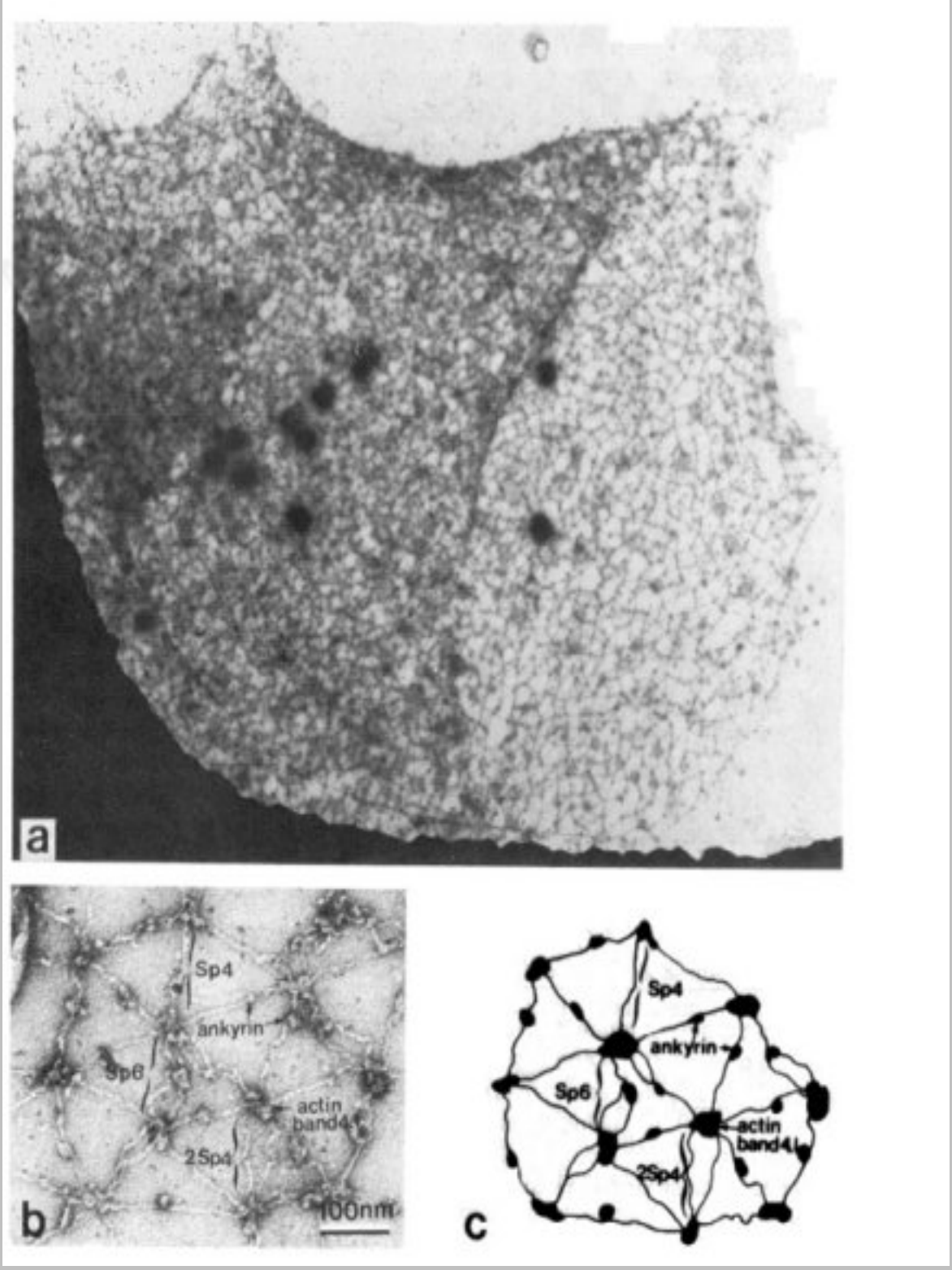


Fig. 8 Spread membrane skeleton examined by negative-staining electron microscopy. (a) A large area of a spread meshwork, revealing the marginal region of the exposed bottom layer of the skeleton. (b) A higher magnification of the spread meshwork reveals the hexagonal lattice of junctional complexes, presumably containing short F-actin and band 4.1, cross-linked by spectrin tetramers (*Sp4*), three-armed spectrin molecules (*Sp6*), and double spectrin filaments (*2 Sp4*). Globular structures of ankyrin (or ankyrin-containing complexes) are attached to spectrin filaments at the ankyrin-binding site, i.e., 80 nm from the distal end of spectrin. (c) Tentative assignment of these structural elements in a schematic diagram. Reproduced from *Journal of Cell Biology*, Liu et al., by copyright © permission.

The arrangement of membrane proteins at the surface, as depicted in Fig. 7, offers the possibility of specific interactions between cells or between the cell surface and extracellular components, for which there is considerable information. Furthermore, the presence of proteins traversing the membrane makes it possible for the cell to respond readily to outside stimuli. The interconnection between membrane proteins and cytoskeletal elements suggests that cell membrane elements can produce changes in the shape of the cell and movements at the cell surface.

The location of protein molecules across the plasma membrane suggests that, at least in some cases, they may operate as channels for the passage of water and certain solutes or, alternatively, mediate other forms of transport of solutes. In contrast to the permeability model already discussed, these molecules would not have to pass through the hydrocarbon layer of the phospholipid. There are indications that certain specialized transport processes take place through such protein channels. Protein channels are likely to play a role in the passage of water and some non-electrolytes through the membrane. Some of these channels will be discussed in Chapter 19.

In addition to the integral proteins and those more loosely associated with the membrane, a third variety is covalently attached to lipids or fatty acids (see Resh, 1999; Bijlmakers and Marsh 2003), as summarized in Table 2 (Gennis, 1989). Some eukaryotic membrane proteins are myristylated or palmitylated, i.e., covalently attached to myristic acid (tetradecanoic acid) or palmitic acid (hexadecanoic acid). Some of the proteins associated with signaling are attached via the S-group of cysteine to a palmitoyl (C16) moiety that serves as an anchor, for example in the case of rhodopsin. Similarly, farnesylation (C15), geranylation (C20), prenylation or the attachment of other fatty acids such as myristic, stearic, oleic and arachidonic acids at the carboxy end of several proteins provide hydrophobic anchors used in protein targeting and in signal transduction. The small Rab and Ras GTPases (see Chapter 7) involved in membrane signaling are prenylated (see Seabra, 1998). The fatty acid site may attach via hydrophobic interactions to the the phospholipid bilayer, usually on the cytoplasmic side. Another group of proteins is attached postranslationally to the cell surface by covalent linkage to glycosylphosphatidylinositol (GPI) at the carboxy terminal of the proteins. The portion of the GPIs acting as a protein anchor is shown in Fig. 9 (Medof et al., 1996). It contains a glycan composed of a phosphoethanol amine (EthN), three mannoses (Man) and a nonacetylated glucosamine (GlcN) attached to an inositol phospholipid. Some of the proteins attached to lipid elements are associated with specialized membrane areas referred as rafts.

The fatty acid component of these proteins can serve as a regulated targeting device. This is shown in the case of *recoverin* , a calcium sensor protein of retinal rods. This protein contains an acyl group, such as a myristoyl group, and it binds two calcium ions per molecule. After binding calcium, recoverin is translocated from the cytoplasm to the disc membrane (Zozulya and Stryer, 1992; Dizhoor et al., 1993) where it regulates the life-time of photoexcited rhodopsin. In the absence of bound Ca^{2+}, the merystoyl residue is sequestered in a hydrophobic domain of recoverin. Ca^{2+} induces the extrusion of the fatty acid so that it can interact with the lipid bilayer (Ames et al., 1977).

Table 2. Examples of membrane proteins linked to lipids

I. Prokaryotic
    1. Bacterial outer membrane lipoproteins (E. coli)
    2. Penicillinase (B. licheniformis)
    3. Cytochrome subunit of reaction center

II. Eukaryotic
    A. Myristylated Proteins
        1. $p60^{src}$
        2. Catalytic subunit of cAMP protein kinase
        3. NADH-cytochrome b_5 reductase
        4. α-subunit of guanine nucleotide binding protein
    B. Palmitylated Proteins -- *generally cytoplasmic face*
        1. $p21^{ras}$
        2. G glycoprotein of vesicular stomatitis virus
        3. HA glycoprotein of influenza virus
        4. Transferrin receptor (mammalian)
        5. Rhodopsin
        6. Ankyrin
    C. Proteins with glycosyl-phosphatidylinositol anchor
        1. Thy-1 glycoprotein
        2. Variant surface glycoprotein of trypanosomes
        3. Acetylcholinesterase -- *outer face*
        4. 5'-Nucleotidase
        5. Alkaline phosphatase
        6. Neural cell adhesion molecule (N-CAM 120)

From Gennis 1989. Reproduced by permission.

Myristic acid - a tetradecanioic acid

Myristic acid is added cotranslationally on terminal glycine.

Palmitic acid - a hexadecanoic acid

Palmitic acid is in the middle of the protein, close to the membrane spanning segment. It is added post-translationally to cysteine (as a thioester) or serine and threonine (as OH esters).

Myristic acid is attached cotranslationally (i.e., while the protein is being synthesized) to the amino terminal of glycine. In contrast, palmitic acid is attached posttranslationally by thioester linkage to cysteine or a hydroxyester linkage to serine or threonine.

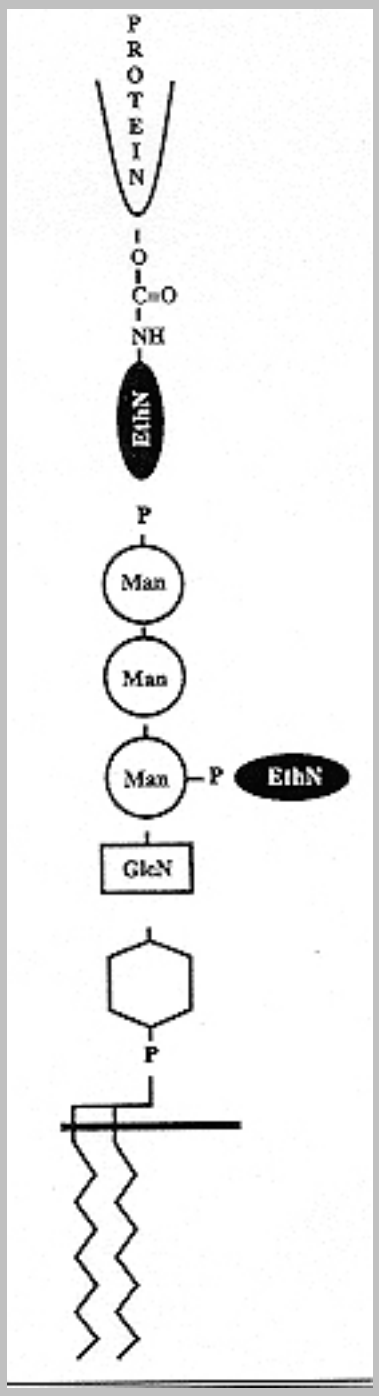
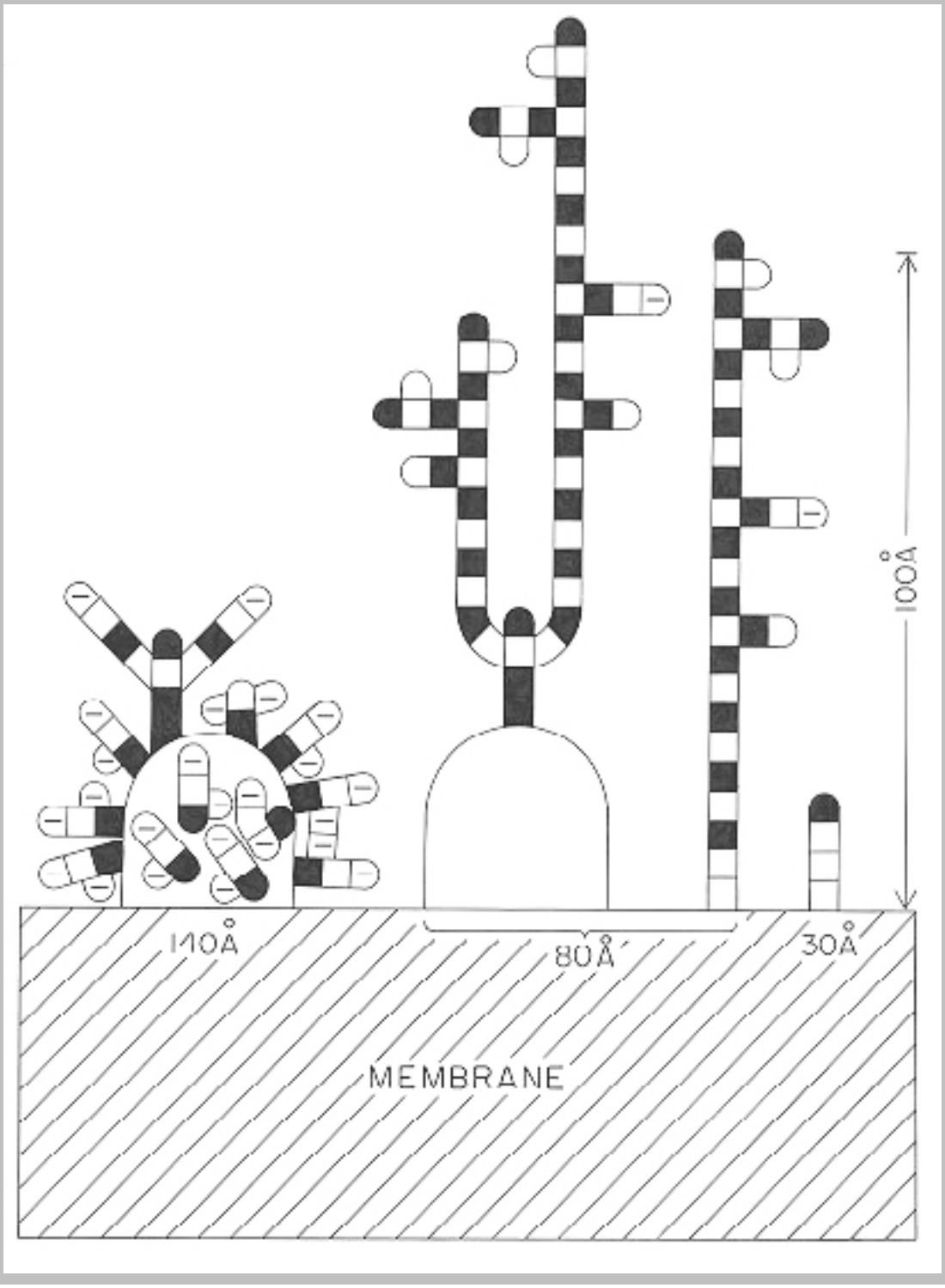


Fig. 9 Structure of mammalian GPI anchors. The EthN-P branching is present in 5-15% of the anchors. From Medof et al, 1996. Reproduced by permission.

The carbohydrate components, whether attached to the lipid or to the protein, are probably significant in biological interactions (e.g., see Chapter 6). They form a special domain that can interact with the extracellular environment.

Information about the carbohydrate components is most complete in the case of the red blood cell (

Viitala and Jarnefelt, 1985). A summary for purposes of illustration is shown in Fig. 10, which represents two of the glycoproteins and two of the glycolipids that project from the bilayer into the cell's exterior. The globular structures represent the part of the proteins that juts out of the bilayer. The chains represent the carbohydrate present. In the diagram, the black portions represent hexosamines (N-acetylglucosamines and N-acetylgalactosamines). The open portions represent hexoses such as fucose, galactose, mannose, or glucose. Alternatively, the open portions represent sialic acid when a minus sign (indicating its negative charge) is shown. The scale on the right indicates the reach of the arms from the membrane surface, and the scale along the horizontal axis indicates the calculated distance between like molecules. Clearly, the surface is densely populated by carbohydrate structures, some of them highly charged, and this coat must alter significantly the properties of the surface.



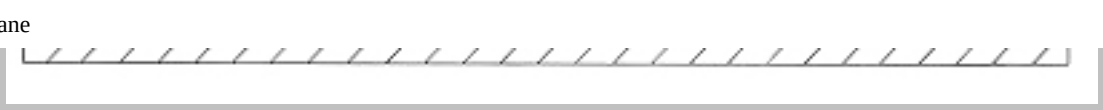


Fig. 10 Four carbohydrate components of the red blood cell surface. The first two represent the integral proteins glycophorin A and *Band 3*. The second two represent polylactosamine ceramide and globoside. The blackened areas represent hexosamines (N-acetylglucosamine and N-acetylgalactosamine). The blank areas represent hexoses (fucose, galactose, glucose, and mannose). The minus signs indicate sialic acid. The scale of the projections is shown on the right. Distances indicated on the horizontal axis correspond to the calculated average distances between like molecules.

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

III. COUPLING BETWEEN CELLS

Electrical techniques and the microinjection of fluorescent molecules have demonstrated that cells in many tissues are interconnected; they are said to be *coupled*. When a current is passed through a microelectrode inserted in one cell, a current is detected in the adjoining cell. The signal would not be detectable at these current levels if the two electrodes were in the bathing medium or the cells were insulated from each other. When one of the cells is damaged, this electrical coupling can no longer be observed. As discussed later (e.g., Chapter 19, 21 and 22), electrical currents in plasma membranes pass through protein coated channels.

Several experiments indicate that cells are uncoupled by increases in the concentration of Ca^{2+} or H^{+}, which normally are at very low levels (Peracchia and Peracchia, l980a, b). Calcium ions may play a role in response to cell injury. The Ca^{2+} concentration of the extracellular fluid is much greater than that inside the cell, so that an increase in the internal concentration could readily occur when the membrane is damaged.

The concentration of H^{+} or Ca^{2+} inside cells is very low (of the order of 10^{-7} M). Therefore, either H^{+} or Ca^{2+} can readily serve as signals. In fact, Ca^{2+} is one of the major internal signals of the cell (as discussed in Chapter 7). The H^{+} concentration of the cell is a sensitive signal that can vary in response to metabolic conditions. For example, acidity increases with the production of CO_2 or lactic acid. Whether the channels are opened or closed, would therefore be subject to fine tuning in the cell. The channels have also been found to be sensitive to changes in electrical potential (Obaid et al., 1983, Moreno et al., 1994a). In mammalian gap junctions, parameters of voltage sensitivity have been shown to vary according to the gap junction protein that is expressed. These results suggest that there are many regulative events which could control whether cells are coupled or not.

A fluorescent substance microinjected into a cell will spread into adjacent cells to which they are coupled, provided the molecule is small enough. Microinjection of uncharged proteins of different sizes provided further information (Schwartzmann et al., 1981). The probes were obtained by varying degrees of proteolysis of the same glycoprotein. Probes above a certain critical size failed to pass from one coupled cell to the other, suggesting that a pore controls the exchanges. In mammalian cells, the size is approximately 1.6 to 2 nm and in insect salivary cells it is 2 to 3 nm.

Coupling has been found at *gap junctions*. When cells are close to each other they might be held tightly by special structures. In some cases, the electron microscope reveals regular clear spaces or gaps, which

are traversed by structures at regular intervals, as shown in Fig. 11 (Peracchia and Dulhunty, 1976). These are the gap junctions widely present in many animal cells (see Goodenough et al., 1996, Simon and Goodenough, 1998).

In tangential views of the gap junctions (Fig. 12) (Peracchia and Dulhunty, 1976), either negative staining or freeze fracture shows these regular arrays. With negative staining (see Chapter 1), a dense material is allowed to distribute throughout the water spaces, so that the water spaces appear dark and the structures white. In freeze fracture, the specimens are frozen rapidly at low temperatures and fractured; the structures are subsequently exposed by sublimation and then coated so that a replica is examined. The fracture frequently occurs between two leaflets of the a bilayer. This exposes the two inner surfaces and the integral proteins within. Since the protein remains intact it is generally present in one of the two leaflets of the bilayer. The other leaflet would then exhibit a corresponding indentation. The *connexons*, appear in hexagonal patterns and correspond to the structures bridging the gap junctions. Each connexon constitutes a half of a channel spanning the gap junction and the plasma membranes of the two adjacent cells. The regular array of the connexons, shown by the membrane faces, seems to be characteristic of the uncoupled state. When coupled, the connexons appear to be in a more disordered arrangement. As is visible in some of the electron micrographs, the connexons are made up of particles in a regular hexagonal arrangement. When protein extracts from gap junction membranes are separated out with SDS gel electrophoresis, proteins referred to as *connexins* have been identified. The molecular weights of proteins of the connexin family range from 26 to 60 kDa. In the mouse, fourteen connexin genes (indicated by Cx followed by a number corresponding to the molecular weight in kDa) have been identified and six more are suspected. Connexins have four transmembrane domains with both carboxy and amino terminals in the cytoplasm.

In plants, the *plasmodesmata*, channels that provide a pathway between cells, may perform similar functions. However, they are morphologically quite different from gap junctions (see Overall and Blackman, 1996). A membrane cylinder, the *desmotubule*, runs through the channel. The desmotubule is continuous with the membrane of the ER of the connected cells. The plasmodesmata are thought to have an important role in the protein trafficking needed for the regulation of gene expression requiring interactions between cells (see Ding, 1998).

Channels connecting invertebrate cells are composed of a protein family of *innexins* (invertebrate connexins) (see Phelan et al., 1998), unrelated to the vertebrate *connexin* family.

Because of the regularity of the structures, packed pellets of the membranes containing gap junctions can be studied with X-ray diffraction techniques. Fig. 13 corresponds to the interpretation of combined X-ray diffraction and electron microscopy studies (Makowski et al., 1977). Each connexon appears to be formed by two basic units, one from each membrane, and each formed by six subunits. The close juxtaposition of the units from the two membranes show a continuous and patent central channel. More recently, connexons have been studied by cryoelectron microscopy with image reconstruction techniques (see Chapter 1). Two dimensional crystals of a truncated Cx43 have been examined. The connexons were found to have a ring of transmembrane α-helices lining the aqueous channel. A second ring is in close

contact with the lipids (Unger et al., 1997). For the assembly of a whole channel from two connexons in separate plasma membranes, the two must be closely apposed. This requires a rotation of 30º between connexons, so that the six protrusions on one connexon fit exactly into the six indentations of the other connexon (Perkins et al., 1998).

Connexons tend to assemble in the plasma membrane as closely packed hexagonal arrays in macular domains. These maculae are enriched in cholesterol (Malewicz et al., 1990). In addition, X-ray diffractions patterns of isolated gap junctions suggest an interaction between the cytoplasmic leaflet of the membrane bilayers and the connexins (Makowski et al., 1984). Each gap junction channel formed by two connexons may be constituted by a single type of connexin (a homomeric channel) or various kinds of connexins (a heteromeric channel) (e.g., Jiang et al., 1996; Stauffer, 1995). Adjacent cells can be connected by connexons that are either identical or composed of different connexins (e.g., Konig and Zampighi, 1995). Since there are a variety of connexins and these can be expressed in the same cell, a variety of distinct channels can be created from the various possible combinations. However, heterotypic channels are formed only from certain combinations of connexins (see Elfgang et al., 1995; White et al., 1995). Differences in the permeability properties of channels formed by the different kinds of connexins has been demonstrated (e.g., Elfgang et al., 1995; Cao et al., 1998) including that of the second messengers cAMP and cGMP (Bevans et al., 1998).

In the cells of the African frog, *Xenopus laevis*, coupling occurs when two cells form contacts. We have also seen that closing can be induced by Ca^{2+}. Therefore, it should be possible to follow the details of closing and opening. The openings can be monitored electrically using two electrodes. The resistance between the two electrodes would decrease when the cells become coupled, and increase when uncoupled. In this study, both opening and closing were found to take place in steps (Lowenstein et al., 1978). These steps are characteristic of channel opening and closing.

If the connexons are channels responsible for the coupling between cells, it is reasonable to expect some structural changes accompanying opening and closing. Gap junctions isolated either in the presence or the absence of Ca^{2+} should correspond to the two states. Careful analysis of electron micrographs (Unwin and Zampighi, 1980) suggests that in the coupled state there is a central channel; this channel can be closed by rotation and sliding of the subunits of the connexons in relation to each other, as shown in Fig. 14.

The implications of cell coupling for regulatory phenomena are far reaching, although at the moment they are difficult to evaluate precisely. However, cell coupling is thought to have a role in embryonic development and metabolic regulation.

Coupling of cells does play a significant role in differentiation, as demonstrated with antibodies to the connexon protein (Warner et al., 1984). Antibodies to the major protein of rat liver gap junctions were injected into early embryos of the frog *Xenopus*. Injection into cells in the eight-cell stage interfered with both electrical coupling and dye coupling of the injected cell and its progeny, resulting in substantial

developmental defects. Although these experiments show unequivocally that gap junctions play a role, the actual mechanism by which they exert their effect is still unknown.

Transfer of metabolites between cells may play an important role in metabolism. A possible role is shown by intracellular injections of glycolytic intermediates into confluent cultured pancreatic cells. Injection into adjacent cells can reduce the oxidized pyridine nucleotides of the connected cells (Kohen et al., 1979), indicating that metabolites generated by one cell can be used by another. Studies of metabolic regulation may therefore have to consider the metabolite pool of several cells. Ca^{2+} waves which serve as signals can also be transmitted from cell to cell (see Chapter 7) via connexon channels, probably by allowing the passage of the second messenger IP_3.

Information on the role of connexons in physiology and, consequently disease, is provided by human mutations and the availability of *knockout* (KO) mutations in mice. KO mutations are those that remove a gene. Charcot-Marie-Tooth disease is the result of 90 different Cx32 mutations in humans (see Bone et al., 1997) that produce progressive degeneration of peripheral nerves. Some of these mutations are inactivating, while others alter the properties of the channels (Bruzzone et al., 1994; Ressot et al., 1998). Cx32 KO mice have similar, but not identical, phenotypes in relation to the nervous system. They also have serious metabolic deficiencies in hepatocytes which have a vastly reduced gluconeogenesis (Nelles et al., 1996), possibly caused by the failure to communicate intracellular inositol (1,4,5) triphosphate, a second messenger responsible for Ca^{2+} release. These mice also have a high incidence of tumors (Temme et al., 1997).

Mutations in Cx26 result in hearing loss in humans (Kelsell et al., 1997). In mice the mutation is lethal probably by interfering with the transport of glucose through the connexons during embryogenesis (Gabriel et al., 1998). Cx40 KO mice have a partial conduction block in the His-Purkinje cells of the heart (Simon et al., 1998; Kirchhoff et al., 1998), whereas Cx43 mutations produce a defective development of the conus region (Reaume et al., 1995). Cx46 KO mice develop cataracts (Gong et al., 1997).

Most isolated connexins are phosphoproteins. The control of connexin phosphorylation by second messengers (e.g., Saez et al., 1990) and the changes in channel activity accompanying phosphorylation-dephosphorylation (Moreno et al., 1994b), suggest that the phosphorylative state of connexins has a regulatory role. Phosphorylation generally favors connexon assembly. The effect on conduction depends on the connexin and the site of phosphorylation. As discussed in several chapters of this book, phosphorylation-dephosphorylation events occupy a central position in the regulation of enzyme activity [see Chapter 13, Section IV and V) and Chapter 14), transcription factors, molecules involved in the regulation of cell division (Chapter 8) and various events that are part of regulative cascades of cells (Chapter 7)]

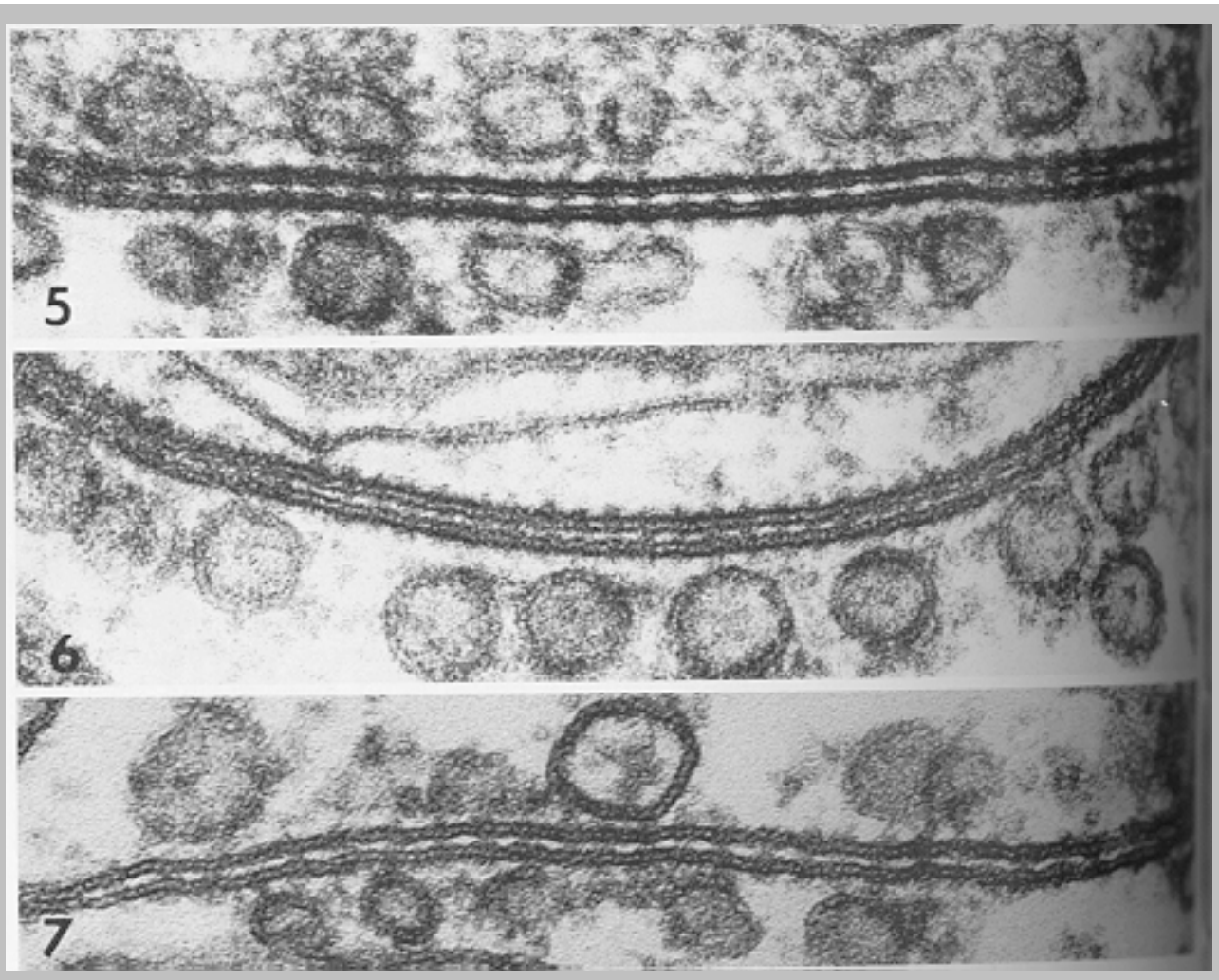


Fig. 11 Electron micrograph showing the "gap" seen between membranes of apposing cells at the site of a gap junction. Bar corresponds to 100nm. Reproduced from *The Journal of Cell Biology* by copyright © permission of The Rockefeller University Press.

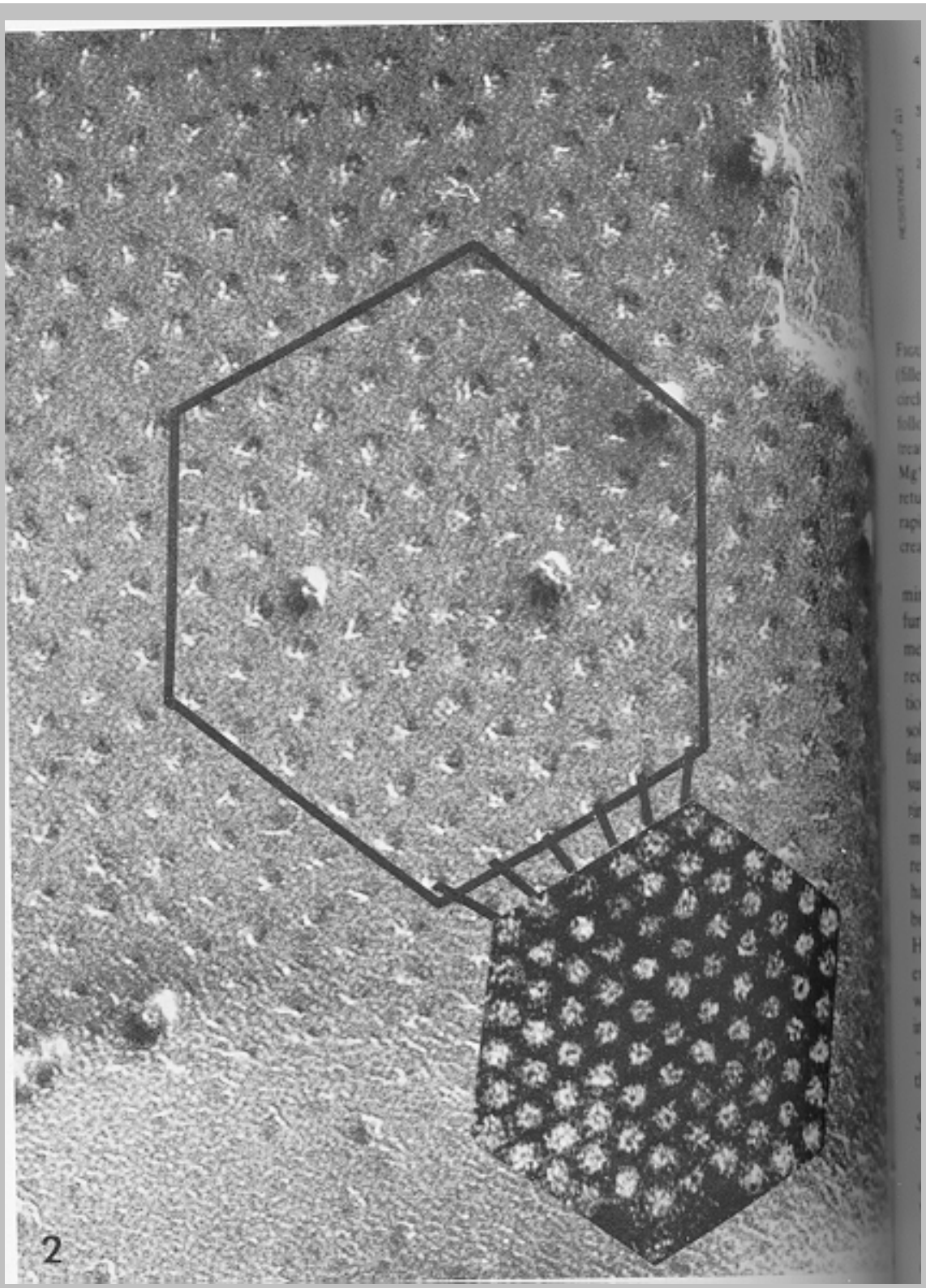


Fig. 12 Comparison of freeze-fracture and negative staining of junctions from control preparations. Face P of a fractured junction is shown. Most of the particles have been fractured away with the external membrane leaflet, leaving a fairly regular hexagonal array of pits. A few pits are occupied by particles. (Inset) Fragment of an isolated PTA negatively stained junction. The unit cell dimension of the arrays is about 20 nm in the freeze-fractured junction and 15 to 15.5 nm in the negatively stained one. Notice the difference between the two hexagonal areas, which contain the same number of particles at the same magnification. Bar corresponds to 48 nm. Reproduced from *The Journal of Cell Biology* by copyright © permission of The Rockefeller University Press.

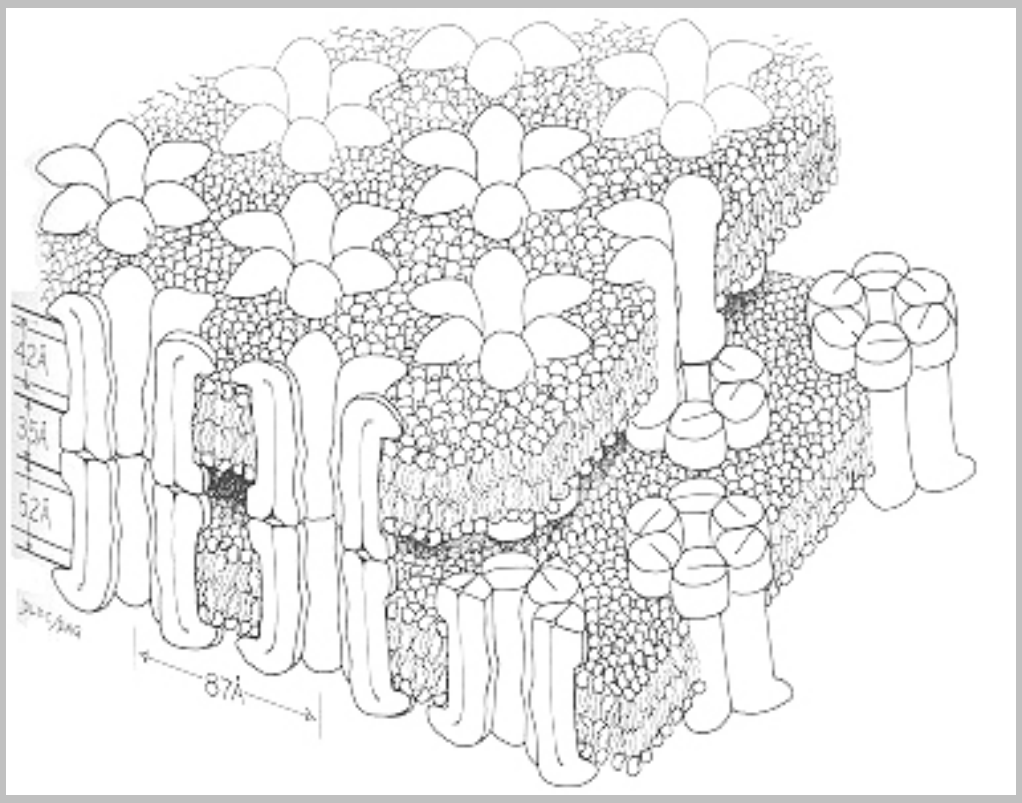


Fig. 13 Schematic representations of connexons in a gap junction, based on x-ray diffraction and electron microscopy studies (Makowski et al., 1977). Reproduced from *The Journal of Cell Biology* by copyright © permission of The Rockefeller University Press.

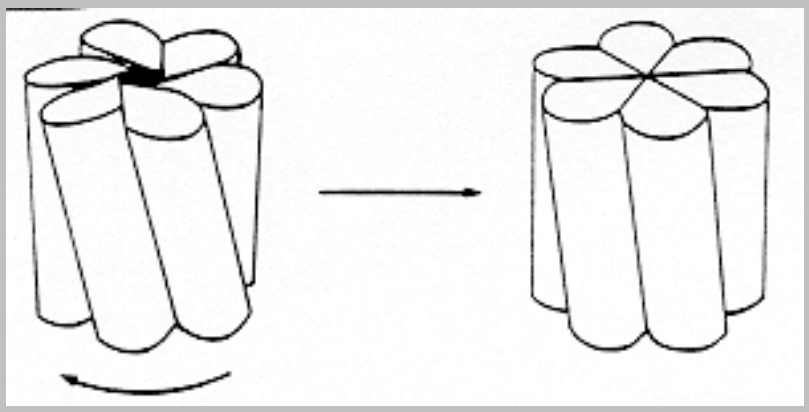


Fig. 14 Model of the connexon, depicting the transition from the "open" to the "closed" configuration. Closure on the cytoplasmic face (uppermost) is thought to take place by the sliding of subunits against each other accompanied by a clockwise rotation at the base (Unwin and Zampighi, 1980). Reproduced with permission from *Nature* 283:545-549 copyright &copy1980 Macmillan Magazines Ltd.

IV. ASYMMETRY OF THE MEMBRANE

How are the various components organized in the cell membrane? Most biological structures are highly organized. Their high state of organization is responsible for their ability to function. It would be surprising if the plasma membrane were not also highly structured. This section will concentrate on the

general organization of the plasma membrane. Specialized structures are present at special locations (e.g., the synapse) and will be discussed later.

The components of the two faces of the plasma membrane differ. This has been referred to as the *asymmetry* of the plasma membrane. The membrane also has considerable organization in relation to the plane of the membrane.

How can you distinguish whether a compound is present on one face of the membrane? One way is to use radioactively labelled reagents that react only with components at either the outer or the inner surface. Reactions with components at the outer surface would be favored when a polar reagent is used. This chemical would have difficulty passing through the membrane because of its low hydrophobicity. Polar groups can be attached to reagents which are non-polar. If a hydrophobic compound is used, it will label groups in both surfaces. When the polar reagent is introduced first and the hydrophobic compound second, components of the external surface will be labelled with the polar reagent and only the internal groups will be labelled by the hydrophobic reagent. Groups which are in the middle of the membrane, that is, in a hydrophobic environment, will react with the appropriate hydrophobic compound. After equilibration between the medium and the membrane and removal of any excess, this compound could be activated by light, a so-called *photoaffinity label*.

Proteins have also been used to probe the location of membrane components. Proteins cannot pass through the plasma membrane because of their size, therefore, proteolytic or lipolytic enzymes can only hydrolyze components at the external surface of cells. Similarly, antibodies can only bind to antigens at the external cell surface.

These techniques are particularly powerful when used with different preparations, such as intact cells and leaky cells (for example, produced by detergents or other techniques). In addition, plasma membranes can be made to form vesicles. Depending on the conditions of the isolation, these vesicles may have the same polarity as the intact membrane: the outside remains outside. The polarity, however, can be reversed using different procedures, so that the inner face is now external and becomes accessible to chemical or other manipulation.

These approaches have led us to a complex picture of membrane organization which will be discussed in the rest of the chapter.

A. Bilayer Asymmetry

The phospholipids of the plasma membrane are arranged as a bilayer. This bilayer of the plasma membrane conceivably could be composed of two identical lipid leaflets. However, all indications are that the two leaflets differ in composition; that is, the bilayer is asymmetric.

Results of the digestion of red blood cells with lipases are shown in Table 3 (Rennoij et al., 1976). The hydrolytic attack of phospholipases and sphingomyelinases is shown in Fig. 15 and can serve to identify

the lipids in the external leaflet. In the experiments of Table 3, the phospholipid population indicated in column 1 is shown as a percentage of the total in column 2. The results of the digestion with phospholipase (column 3), sphingomyelinase (column 4) or both (column 5) are shown as percentages of the individual phospholipids hydrolyzed. The results indicate that all of the sphingomyelin is at the external surface since it is all hydrolyzed. A substantial portion, about 50%, of the lecithin [phosphatidylcholine (PC)] also appears to be hydrolyzed, and more of it is available for hydrolysis (about 60%) when sphingomyelinase is present, suggesting that the hydrolysis of sphingomyelin exposes more PC to the enzyme. Only small portions of the phosphatidylserine and phosphatidylethanolamine (PS and PE, respectively) are hydrolyzed, indicating that these two phospholipids are predominantly in the inner leaflet. Other studies have shown that phosphatidylinositol (PI) is located in the inner leaflet (Gascard et al., 1991). As we will see later, compounds derived from PI act as second messengers (see Chapter 7).

Results obtained with red blood cell ghosts and inside-out vesicles, are entirely comparable to those just discussed. The bilayers of other eukaryotic cells also appear asymmetric, although there are individual differences as shown by the data collected in Table 4 (Deveaux, 1991).

Table 3 Nonhemolytic degradation of phospholipids by phospholipases in intact red blood cell

Phospholipids	Phospholipid composition (%)	Phospholipase A_2 (%)	Sphingomyelinase (%)	Phospolipase A_2 + sphingomyelinase (%)
Lysolecithin	5	--	0	--
Sphingomyelin	12	0	100	100
Lecithin	42	48	0	62
Phosphatidylserine[a]	16	0	0	6
Phosphatidylethanol-amine	25	8	0	20
Total phospholipid	100	22	12	44

[a]Including phosphatidylinositol

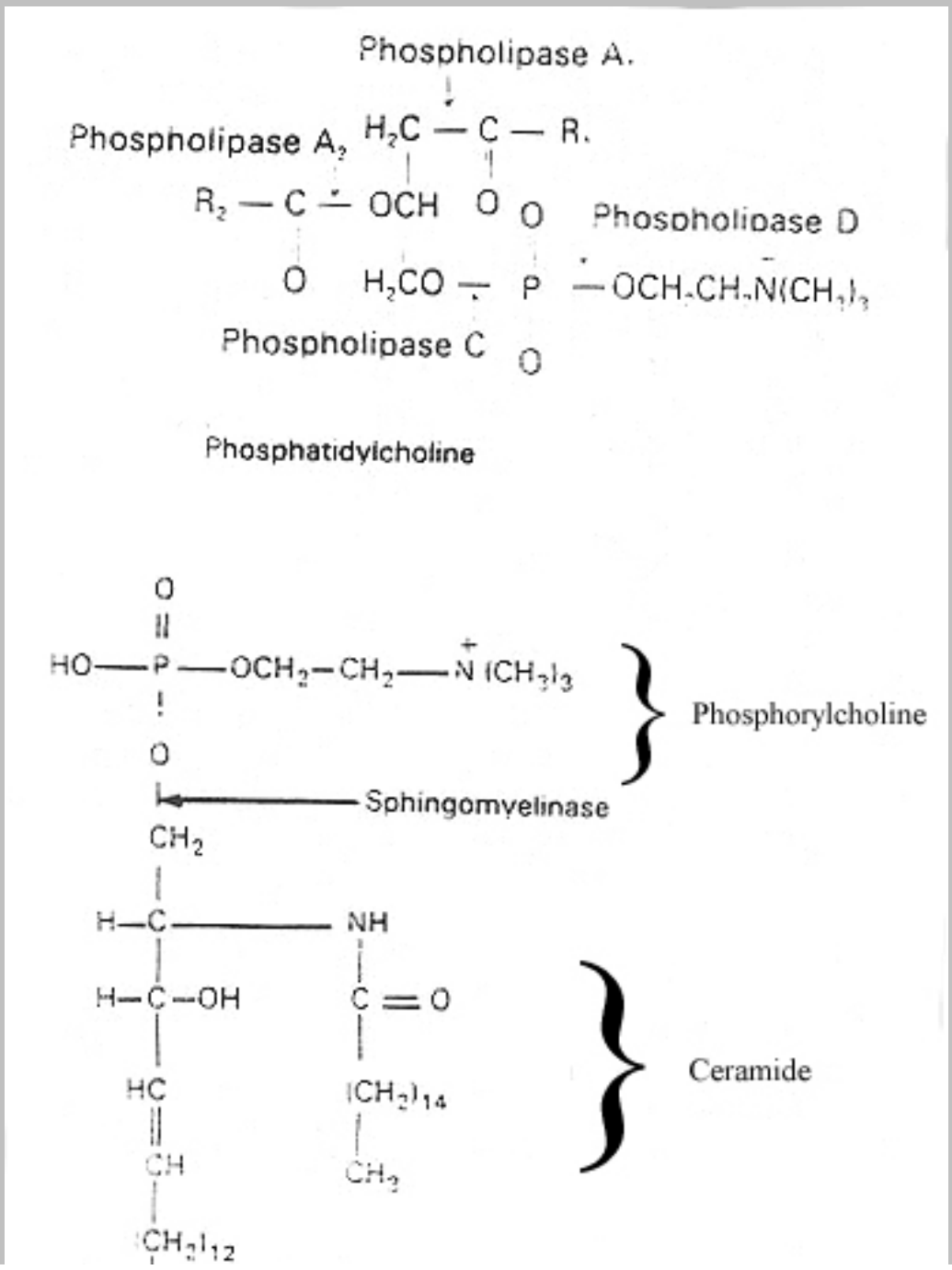
Reprinted with permission from W. Rennoij, et al., European Journal of Biochemistry, 61:53-58. Copyright &copy1976 Springer-Verlag, New York, N.Y.

Table 4 Quantitative Results on the Phospholipid Asymmetry in the Plasma Membrane of Eukaryotic Cells

	% in outer layer				
cell type	**PC**	**SM**	**PE**	**PS**	**PI**
red blood cells (man)	76 78	82 79	20 21	0 8	
red blood cells (rat)	62	100	20	6	
platelet (man)	62 45	93	54 20	6 9	34 16
platelet (pig)	40	91	34	6	
kidney brush order (rabbit)	34	80	23	15	
intestinal brush order (rabbit)	26		28		
heart sarcolemma	43	93	25	0	
embryo fibroblast			34	17	
embryo myoblast (chick)			66	46	
brain synaptosomes (mouse)			10-15	20	
LM fibroblast (mouse)			4-6	5	
hepatocytes (rat)					

	bile canalicular surface	85	63	50	0	0
	contiguous surface	82	0	0	14	0
	sinusoidal surface	85	66	55	0	0
Krebs II ascites (mouse)		51	46	45	20	30

From Devaux 1991. Reproduced by permission.



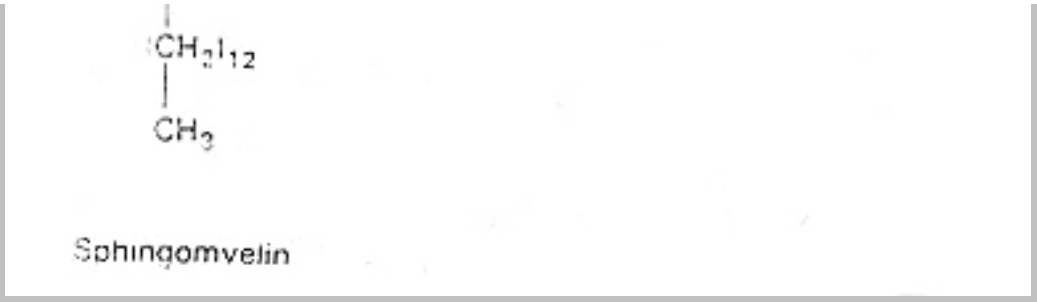


Fig. 15 Hydrolytic points of attack of enzymes.

Does the unequal distribution of phospholipids in the bilayer fulfill some important functions? Undoubtedly that is the case, however, at this time these are not clear. PE appears to have a role in maintaining the conformation of some membrane proteins (Bogdanov et al., 1996). Furthermore, PE aggregates tend to form concave surfaces because of the small head size of this phospholipid (see de Kruijff, 1997), suggesting a role of PE in maintaining the integrity of the bilayer since this phospholipid is predominantly on the inner (concave) leaflet of the bilayer. A role of lipids in membrane curvature has been suggested recently for endocytosis (Chapter 9). In endocytosis, vesicles are formed from invaginations of the the plasma membrane so that the inner leaflet becomes external and the outer leaflet becomes internal in relation to the vesicle. The enzyme lysophosphatic acid acyl transferase (endophilin I) was found to play a role in endocytosis at synapses (Schmidt et al., 1999). The formation of phosphatidic acid (two acyl chains) from lysophosphatic acid (one acyl chain) and arachidonoylCoA in the cytoplasmic leaflet of the plasma membrane catalyzed by this enzyme may play a role in the formation of vesicles by altering the membrane curvature to facilitate invagination during endocytosis (see Scales and Scheller, 1999). This change in the nature of the phospholipid would increase the surface area of the inner plasma membrane leaflet.

The asymmetry of the phospholipids in the plasma membrane is no longer maintained during programmed cell death (apoptosis) (Martin et al., 1995). The presence of phosphatidylserine at the cell surface is recognized by macrophage which phagocytize the cells (Fadok et al., 1992). The phagocytic cells express a surface receptor which specifically binds to PS (Fadok et al., 2000).

B. Orientation of the Membrane Proteins

We had a preview of the organization of a plasma membrane in Fig. 7A. As shown in that diagram, some proteins span the membrane while others are attached to proteins that span the membrane. How do we know this representation corresponds to the actual arrangement in the membrane? There were many hints that this is the case. When red blood cell ghosts were isolated, some proteins were found to be removed relatively easily by changing the ionic strength of the medium or adding chemicals which chelate Ca^{2+} or Mg^{2+}. Others were extremely difficult to detach: their extraction required the use of detergents. The first set corresponded to proteins on the surfaces of the bilayer, the second set to those proteins embedded in the membrane, the integral membrane proteins. Many of these were found to span the membrane.

Proteins can be shown to traverse the membrane if they can be shown to be accessible to reagents from either side. In one set of experiments (Whiteley and Berg, 1974), the external amino groups of the proteins of intact red blood cells were covalently bound to a polar reagent labelled with [^{3}H]. The polarity of the reagent prevented its entry into the cell. A second nonpolar reagent labelled with [^{14}C], which also reacted with the amino groups of proteins, was added subsequently. This reagent labelled the proteins containing the amino groups which could not be reached by the polar compound, primarily in the inner surface of the cells. The two radioactive labels can be counted simultaneously by scintillation counting. Using this technique, the solution containing the label is mixed with a fluor, a substance which emits light when excited by radioactive disintegrations. The wavelengths at which the light is emitted differs with each radioactive isotope because the amount of energy released differs. When the proteins were separated out by SDS-gel electrophoresis (which separates the component peptides by molecular weight), in some cases both [^{3}H] and [^{14}C] were found in the same band. Does this double labelling represent the labelling of two different proteins of the same molecular weight, one internal and one external, or a single molecule traversing the membrane? The two alternatives are shown in Fig. 16 A and B, respectively. One possible way of distinguishing between these two alternatives is to subject the intact cell to digestion by a proteinase after treatment with the two reagents. If a sufficiently large portion of the molecule sticks out into the medium, it will be cleaved. If the labels were on two separate molecules of identical molecular weight only one of the two, the external protein, would decrease in size (Fig.16C). In contrast, if both labels appear at bands representing peptides of lesser size (15 D), the two labels were originally on different ends of the same molecule (Fig. 16B). The experiments found the second alternative (Fig. 16 D) to be right. Therefore, the protein which had been labelled by both labels spans the membrane (Fig. 16B).

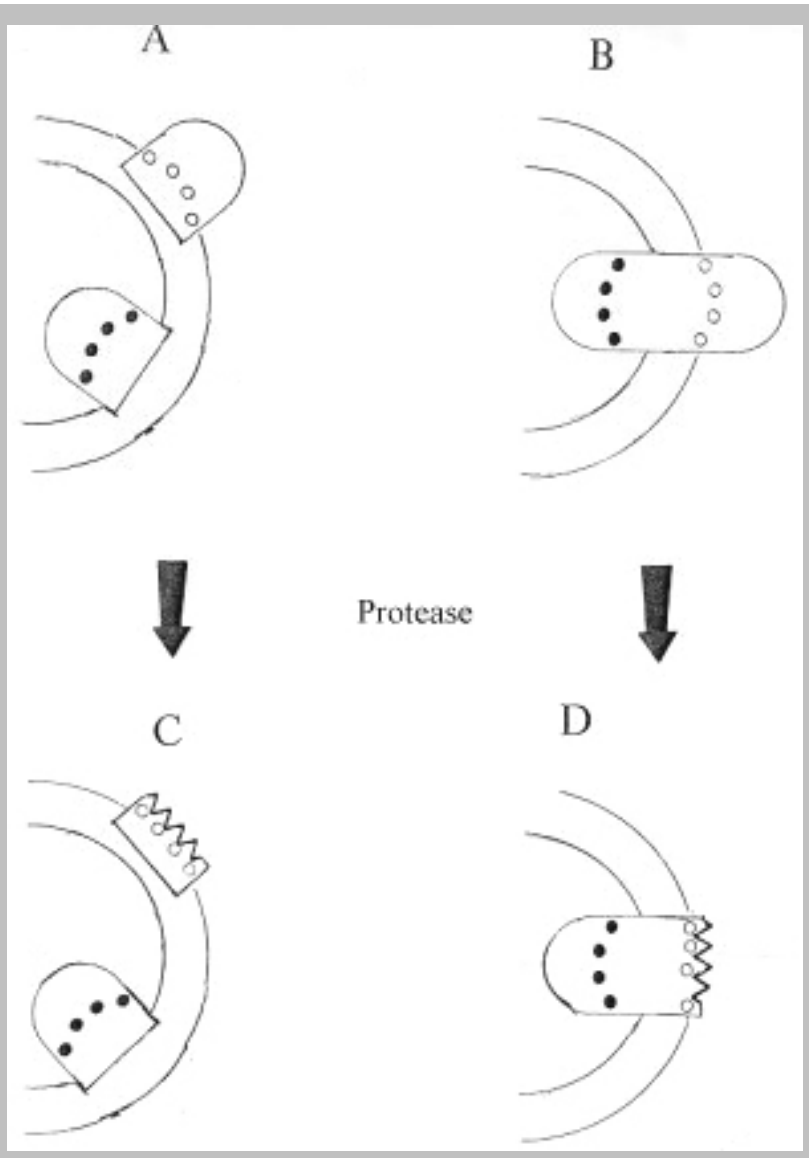


Fig. 16 Possible interpretations of results obtained using a polar reagent (○) and then a nonpolar reagent (●) in which the labels for both appear in the same band on an SDS gel. A. Two molecules of identical molecular weight, one facing the outside, the other facing the inside. B. Both labels on the same integral protein. C. When two separate molecules are involved (part A) after protease treatment only the external protein should be cleaved. D. In the case of a protein spanning the membrane, both labels should be found in a single band of lower molecular weight.

Some insights into the arrangement of plasma membrane components can be gained by chemical treatment and enzyme digestion, as already discussed. The details of this arrangement are generally referred to as the *topology* of a membrane protein. Our knowledge of the topography of many integral proteins has also been augmented by finding the location of binding sites and active groups of integral proteins involved in ion transport. Recently, these approaches have been supplemented by pinpointing sites with monoclonal antibodies (Ovchinnikov, 1987), that is, antibodies that react with specific amino acid sequences. Increased knowledge of the properties of proteins has also made it possible to predict their arrangement in the membrane.

The technique of cloning the DNA (see Chapter 1) that codes for either the Na^+, K^+-ATPase (Shull et al,

1985) or the Ca^{2+} ATPase (e.g., MacLennan et al., 1985), has permitted sequencing of the DNA and the deduction of the amino acid sequence of these two proteins (see Cantley, 1986). Knowledge of the hydrophilic and hydrophobic properties of the amino acid side chains allows use of a scale, the *hydropathy* index, in which positive values indicate hydrophobicity. Plotting this index, averaged for polypeptide segments as a function of their position in the chain (Kyte and Doolittle, 1982), permits the construction of models. These models, supplemented by other information such as the position of the phosphorylated amino acid or the ATP binding site, predict the location of the segments in the phospholipid bilayer and its two faces.

Profiles for the Na^+, K^+-ATPase are shown in Fig. 17 (Shull et al., 1985). In these representations, the polypeptides are shown from the amino terminal on the left to the carboxyl terminal on the right. Hydrophobicity is plotted upward. The shaded parallelograms indicate areas of *homology* (i.e., amino acid sequence similarity); P* is the phosphorylation site and A* is the ATP binding site. Clearly, the two profiles are very similar and suggest that the two proteins resemble each other, as also shown by their biochemical properties. A tentative reconstruction of the Na^+, K^+-ATPase is presented in Fig. 18 (Cantley, 1986). The numbered hydrophobic portions in Fig. 18 correspond to those shown in Fig. 17 and are thought to traverse the bilayer (indicated by the shaded portion) as α-helices. Figure 17 also shows sites of proteolytic cleavage that indicate the side of the membrane corresponding to the location of the polypeptide segment. The cation indicated (Na^+ or K^+) denotes that the proteolytic effect depends on whether Na^+ or K^+ is bound to the transport sites. As discussed later, the conformation of the transport proteins changes during transport. This change in conformation exposes some sectors of the protein to attack by proteases. In the case of Na^+, K^+-ATPase, the conformational changes are induced by Na^+ or K^+ binding to specific groups.

The hydrophobic domains of sufficient length are frequently assumed to be intramembrane domains and generally assumed to correspond to α-helices. A configuration in an α-helix exposes the hydrophobic side groups of the amino acids for interactions with a hydrophobic environment. However, these assumptions may not always be correct. There is much evidence for other conformations, for example, in the case of the acetylcholine receptor, a channel protein of synapses or the voltage dependent anion channel of the outer membrane (VDAC or mitochondrial porin). VDAC has been found to predominantly form β-barrels. An EM study carried out with tubular crystals of *Torpedo* postsynaptic membranes embedded in ice, revealed only one helical rod per subunit of the acetylcholine receptor (Unwin, 1993), at the center of the receptor, rather than the four predicted from hydrophobicity plots. Similarly, Fourier-transform infrared (FTIR) spectroscopy (Görne-Tschelnokow et al., 1994) of the internal membrane portion of the receptor showed as much alpha-helical as beta-structure embedded in the bilayer.

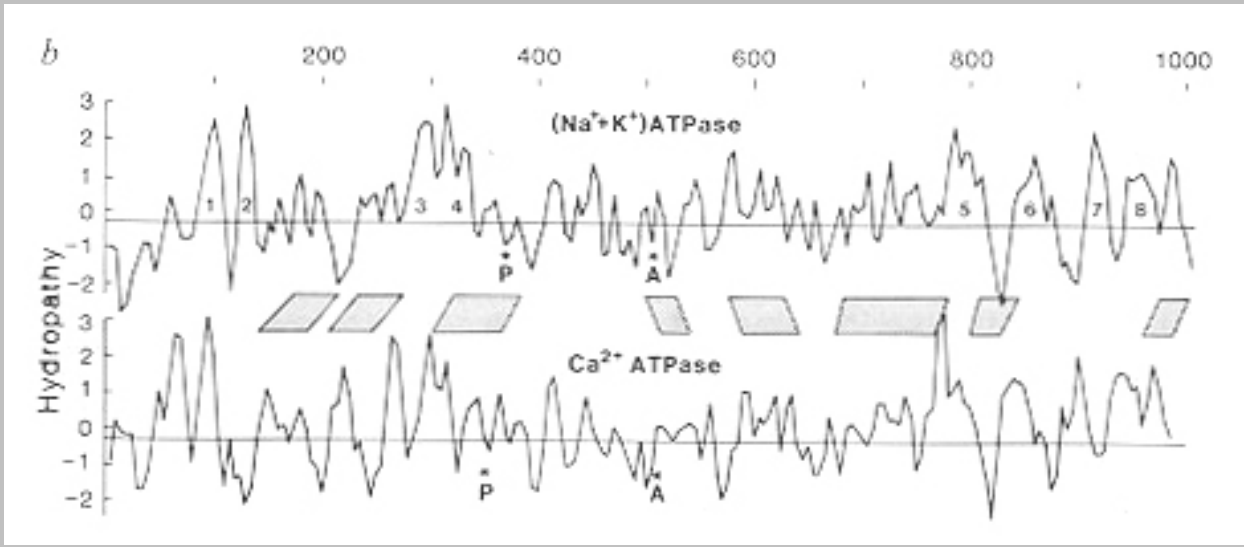


Fig. 17 Comparison of amino acid homology and hydropathy plots of sheep kidney Na^+, K^+-ATPase α-subunit and rabbit cardiac Ca^{2+}-ATPase (Shull et al., 1985). Reproduced with permission from *Nature* 316:691-695, copyright &copy1985 Macmillan Magazines Ltd.

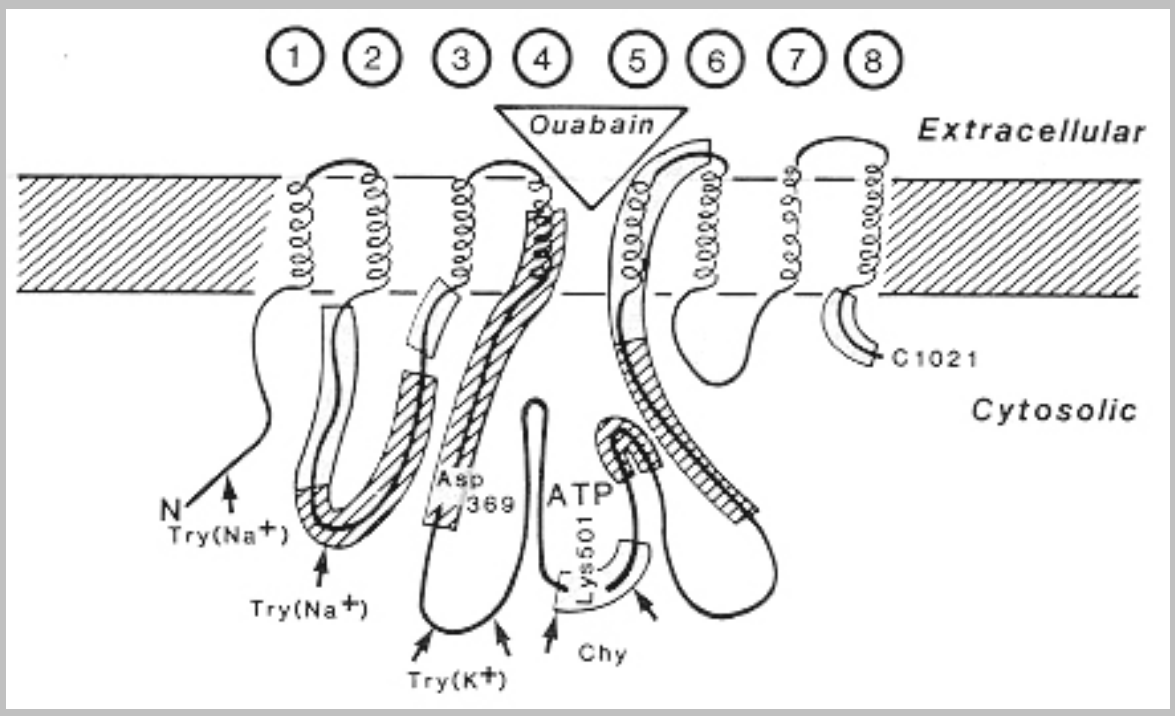


Fig. 18 A possible model for folding of the Na^+, K^+-ATPase catalytic subunit across the plasma membrane. Regions in the sequence where trypsin (Try) and chymotrypsin (Chy) cleave the native protein from the cytosolic side of the membrane are indicated by arrows. The sites indicated by (Na^+) and (K^+) are the primary trypsin cleavage sites when either sodium or potassium is bound to the transport sites. The hydrophobic regions proposed to be transmembrane are designated by the circled numbers one through eight. The aspartate residue at position 369 in the sheep kidney accepts phosphate as an intermediate in ATP hydrolysis and lysine 501 has been implicated in the ATP binding site by affinity labeling. Regions with sequence homology to sarcoplasmic reticulum Ca^{2+}-ATPase are indicated by open boxes and regions with homology to both Ca^{2+}-ATPase and bacterial K^+-ATPase are indicated by shaded boxes. The triangle indicates the binding site for ouabain, an inhibitor of Na^+, K^+-ATPase. Reproduced from *Trends in Neuro*

Sciences, L.Cantley, 9:1-3, copyright &copy1986, with permission from Elsevier Science Publishers, London.

Understanding the arrangement of a protein in a membrane requires supplementing the information obtained through hydrophobicity plots with other independent information that can pinpoint the location of specific segments. Hydrophobicity alone can be misleading, as already discussed in the case of the acetylcholine receptor channel. In addition, hydrophobic segments exist in soluble proteins (in the interior of the folded polypeptide).

Integral proteins have been classified on the basis of how many times the polypeptide spans the membrane. *Monotopic* proteins, although associated hydrophobically with the membrane, do not span the membrane entirely. They are relatively rare. *Bitopic* proteins cross the membrane once, and *polytopic* proteins cross it more than once (such as the protein represented in Fig. 18).

Our knowledge of the structure and function of integral proteins will certainly be refined with more information. The possibility of producing mutations in the cDNA and, therefore amino acid substitutions in the transport proteins, should play an important role in future progress.

V. THE MOBILITY OF MEMBRANE COMPONENTS

The lipid bilayer is considered to be a fluid matrix in which protein molecules are able to move (Singer and Nicolson, 1972). Note however, that when directed to specific components (see Section VIIA) the answers point to the presence of specific domains with distinct properties that differ from this simple picture. In addition to moving in the plane of the membrane, components such as phospholipid molecules could rotate in position or conceivably flip-flop from one interface to the other, or from one lipid leaflet to the other.

The most graphic demonstration of membrane fluidity and protein mobility in the membrane was provided by using different antibodies labelled with distinct fluorescent dyes. One antibody was prepared against the membrane antigens of mouse cells and the other against antigens from human cells. When one of the antibodies was added to the appropriate cells, it distributed uniformly, indicating that the antigens were evenly distributed. After fusion of the human and mouse cells, the antigens, each originally in its corresponding domain, rapidly intermixed (Frye and Edidin, 1970).

The rate of diffusion in the membrane has also been studied through the use of spin-labelled components. Synthetic lipids containing a radical are incorporated into membranes. The labelled molecules are paramagnetic because an electron of the radical is unpaired. The *electron paramagnetic resonance* (EPR) spectrum of the spin label indicates the fluidity of the layer and, in this case, is consistent with fluidity of the lipid membrane. The intensity of the spectrum is also a function of the concentration of the label: because of spin-spin interactions, the higher the concentrations, the less distinct the lines. This fact leads to experiments in which the diffusion of components in the plane of the membrane is followed solely by using EPR. A spot of labelled lipid is placed in a model system (such as a multilayer of lipid). Diffusion

of the label away from the concentrated spot increases the sharpness of the lines, permitting calculation of diffusion coefficients. The results obtained with this and other techniques are listed in Table 5 (Marsh, 1975) and Table 6 (Edidin, 1974; Marsh, 1975). The diffusion coefficients are generally higher than 1 x 10^{-8} cm^2/s (for comparison, note that the diffusion coefficient of ribonuclease in water is about this value). From the values of the diffusion coefficient, the viscosity of the membrane can be calculated; it corresponds to about 1 to 6 poises (similar to motor oil). These experiments show that diffusion of the lipids in the plane of the membrane takes place at a fairly high rate. What can be said about the rate of flip-flop of a lipid from one leaflet of the bilayer to the other? These experiments require a somewhat different approach.

One technique used in the study of flip-flop depends on the reduction of a spin label. When reduced, the label is no longer paramagnetic and, therefore, cannot be detected. When a reducing agent which does not enter vesicles is used, such as ascorbate, only the probe in outer leaflet of the bilayer will be affected. After an initial reduction of the label, any subsequent reduction must be the result of a flip-flop from the inner layer and can be used to measure of the rate of flip-flop. In model systems, this rate has generally been found to be extremely low, on the order of hours. However, experiments with vesicles derived from *Electrophorus electricus*, using the same method, suggest very rapid flip-flop, with a half-time of a few minutes. Such rapid flip-flop would make it extremely difficult to understand the asymmetry of the lipids in biological membranes just discussed.

Other experiments also support the notion that flip-flop can be relatively rapid in the plasma membrane. In these experiments (Morrot et al., 1989) spin labeled analogs of the natural lipids were used. These compounds have short β-chains bearing the nitroxide radical. This modification made them partially water soluble, so they could be easily introduced into the outer leaflet of the red blood cell plasma membrane. The amount remaining in the outer leaflet can be determined directly by removing it with albumin and then measuring the amount extracted. Serum albumin has the property of binding to hydrophobic compounds; these lipids are held loosely by the outer leaflet and can be easily removed. The rate of disappearance from the outer leaflet is shown in Fig. 19. Not only is the exchange rapid, but it differs for the different phospholipids. The rate of exchange shown in the graph can be compared to the composition of the inner leaflet of the membrane shown in the bar diagram (determined using phospholipases, Table 3). The rate of translocation from the outer to the inner leaflet is highest for those phospholipids preferentially localized in the inner membrane leaflet. The specificity of the transfer system suggests that special translocation mechanisms are involved and the rate of flip-flop may be driven by these special mechanisms. The preferential translocation of inner leaflet phospholipids suggests that these mechanisms are also responsible for the asymmetry of the membrane.

Table 5 Lipid Lateral Diffusion Coefficients in Phospholipid Bilayers and Membranes (Determined by Spin Label Measurements)

Bilayer/membrane	D (cm^2/s) x 10^{-8}
Dipalmitoyl PC, 50°C	3
Egg PC, 25°C	2
Egg PC: cholesterol (4:1), 40°C	12
Sarcoplasmic reticulum membrane, 37°C	6
E. coli membrane, 40°C	3
Liver microsomes, 30°C	11

Source: Marsh (1975). Reprinted by permission from Essays in Biochem. 11:139-180, copyright &copy1975 The Biochemical Society, London.

Experiments studying flip-flop suggest the involvement of enzymes. Non-specific enzymes which facilitate flip-flop have been proposed for the endoplasmic reticulum, in photoreceptor disc membranes and some cell plasma membranes. An aminophospholipid translocase that transports one molecule of the phospholipid per ATP hydrolyzed has been studied and is thought to have a role in the maintenance of the asymmetry. However, a precise mechanism capable of dynamically preserving this organization is presently difficult to visualize.

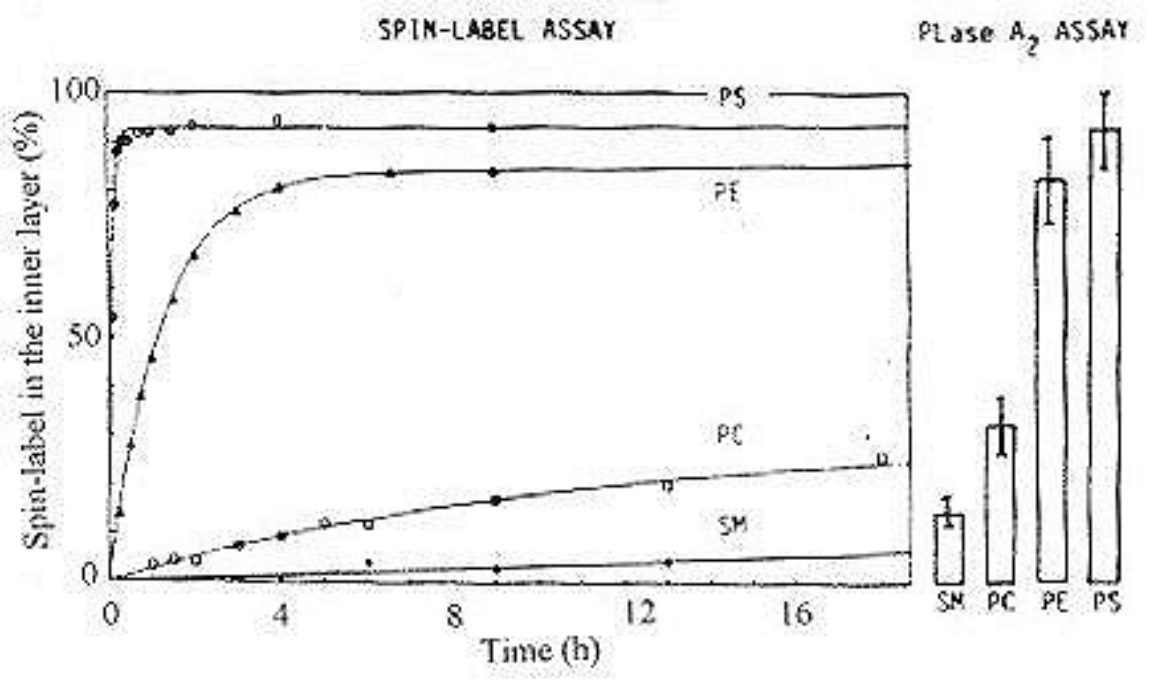


Fig. 19 Spontaneous passage from the outer monolayer to the inner leaflet of spin-labeled analogues of the major phospholipids in red blood cells at 37°C. PC, º ; PS, Δ ; PE, ♦ ; and SM, • . From Morrot et al., 1989. Reproduced by permission.

The presence of two distinct leaflets may correspond to only part of the organization of the phospholipid bilayers. The proportion of the phospholipids making up each of the leaflets corresponds closely to that predicted by a model in which the phospholipid heads are packed in a superlattice-like arrangement (Virtanen et al., 1998 ; Someharju et al., 1999).

The motion of proteins in the membrane has also been studied, usually by techniques that depend on photobleaching. When pigments or chromophore-labeled proteins are exposed to light of sufficient intensity, the energy absorbed produces chemical changes so that the molecule is bleached. The recovery of the system from photobleaching can be monitored with time by measuring the pigment's absorption of a nondamaging light beam (either of low intensity or of short duration). This technique is discussed in more general terms below. The earlier studies involved rhodopsin, the visual pigment in the rods of the retina (Cone, 1972). Rhodopsin, in situ, is dichroic when the electric vector of the light is parallel to the surface of the disk membranes of the rods, indicating that the chromatophores are parallel to the surface of the disk membranes. This is so because 11-cis-retinal, the chromatophore of rhodopsin, is dichroic. Dichroism is a consequence of absorbing light differently depending on its polarization; in this case, absorption is strongest when the electric vector is parallel to the long conjugated chain of the molecule. However, when the rods are viewed end-on, they are not dichroic, indicating that there is no preferred orientation of the chromatophore in relation to the long axis of the rod. When the rods are bleached with a beam of end-on polarized light, the rhodopsin becomes dichroic because it is bleached differentially. Disappearance of the dichroism should indicate the rate of rotation. The results are shown in Fig. 20 (Cone, 1972). The upper line indicates the dichroism ratio (absorbance of a parallel beam/absorbance of a perpendicular beam) of the rods. The lower line represents identical experiments in which the rhodopsin

was immobilized by the fixative glutaraldehyde, which cross-links the molecules. The unfixed rods recover rapidly with a recovery time of about 20 µs, whereas the fixed rods do not recover.

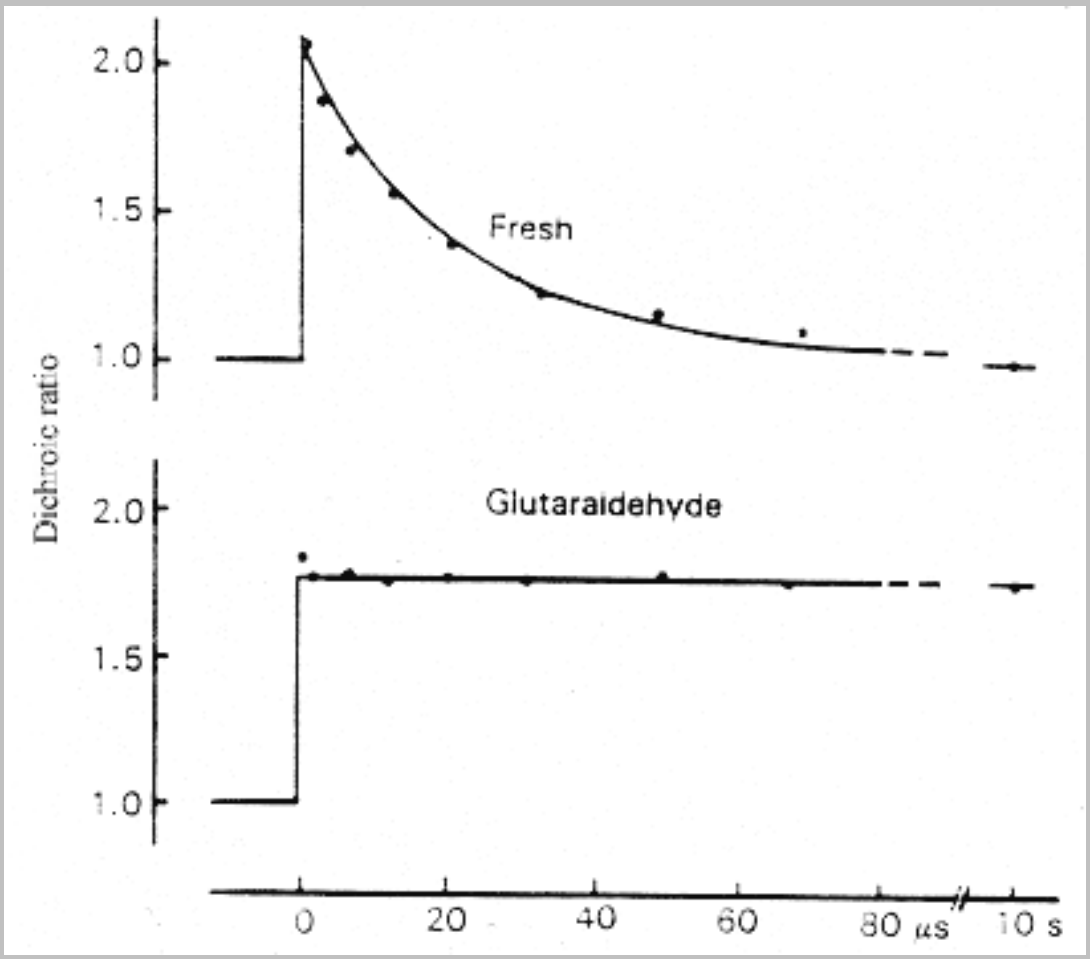


Fig. 20 Dichroic ratios plotted as functions of time after the actinic flash (Cone, 1972). Reproduced with permission from *Nature* 236:39-43, copyright &copy1972 Macmillan Magazines Ltd.

Table 6 Diffusion of Lipids in Natural Membranes and Their Extracted Lipids

Membranes	$D(cm^2/s) \times 10^8$	Temperature (°C)	Method
E. coli	1.8	31	NMR
Sciatic nerve	0.5	31	NMR
Sciatic nerve lipids	0.8	31	NMR

Sarcoplasmic reticulum	0.6	8	NMR
	1.8	25	NMR
	1.0	50	NMR
	2.5	25	ESR[a]
	6.0	40	ESR[a]
Liver microsomes	9.5	20	ESR[b]
	11.0	30	ESR[b]
	13.7	40	ESR[b]
Sarcoplasmic reticulum lipids	0.4	31	NMR
	10.0	40	ESR[a]
Electroplax membrane	≥ 0.1	33	NMR
Plasma membrane	$D(cm^2/s) \times 10^9$	Method	Label
Cultured rat muscle fiber	1-2	Spread of a florescent spot	Florescent antibody fragment
Amphibian disks	4-5	Randomization of bleached rhodopsin molecules	Retinol

[a]Labeled lecithin
[b]Labeled fatty acid

Source: Edidin (1974). Reproduced, with permission from the Annual Review of Biophysics and Bioengineering, Volume 3. Copyright &copy1974 by Annual Reviews Inc.

Similarly, the diffusion of rhodopsin can be measured by bleaching it with a large spot of light illuminating the retinal rod (Poo and Cone, 1974). The return of the rhodopsin to the bleached spot (which

can be monitored by following the absorption of light of the appropriate wavelength in the same location) provides a value for its diffusion coefficient, which was found to be approximately 3-5 X 10^9 cm^2/s.

The idea that proteins diffuse freely in the plane of the membrane cannot be entirely correct. Receptors at the cell surface are known to be at specific locations or patches. For example, at chemical synapses or at neuromuscular junctions we would expect a concentration of acetylcholine receptors. In fact, at least a fraction of a number of proteins have been found to be essentially immobile (see also Section VI, below). A number of mechanisms could account for the immobility. Surface interactions are clearly important, since the external domain of the glycoproteins has been shown to slow down their lateral diffusion. However, it is likely that the attachment of the integral proteins to cytoskeletal elements is the primary factor, as represented previously in Fig. 7 in the case of the red blood cell. In this figure, ankyrin, spectrin, and actin are shown to be involved in holding the *Band 3* protein (anion channel) immobile, and glycophorin is shown to bind the band 4.1 protein.

VI. PROTEIN-LIPID INTERACTIONS

Binding of proteins to lipids

A variety of proteins, including cytoskeletal elements, have been shown to bind to phospholipids. For example, antibodies specific to phosphatidylinositol 4,5-bisphosphate (PIP_2) co-precipitated several proteins bound to the phospholipid (Fukami et al., 1994). Other interactions were found by using light activated fatty acids, supposedly localized in the bilayer and labelling proteins (Niggli et al., 1990). One of the roles of phospholipid-binding domains is to mediate localization of proteins to the inner surface of the plasma membrane, such as proteins involved in signaling or proteins acting as anchors for cytoskeletal elements.

The binding of proteins to phospholipid is thought to require one of at least five kinds of domains (see Niggli, 2001, Bottomley et al., 1998). These include clusters of basic amino acids (such as lysine; e.g., ezrin) , *pleckstrin-homology* (PH) domains, amphipathic helices (e.g. profilin) and endonexin folds (C2 domains) (e.g., annexins).

PH domains of approximately 120 amino acids, present in some cytoskeletal and signaling proteins, represent a particular secondary or tertiary structure rather than a specific amino acid sequence. These domains possess a positive surface which can interact with phosphoinositides (see Bottomley et al., 1998; Lemmon and Ferguson, 2000). Notable among the PH-proteins are spectrin, dynamin and an isoform of myosin X (Berg et al., 2000). Myosin X has a tail domain with multiple PH domains. Some of the protein-lipid combinations act as second messengers in signaling pathways.

The C2 domain is a Ca^{2+}-binding motif of approximately 130 residues. Originally found in Ca^{2+}-dependent isoforms of protein kinase C , it has been identified in single and multiple copies in many signalling proteins that interact with cellular membranes (see Nalefski and Falke, 1996), some not

regulated by Ca^{2+} . This domain binds to a variety of ligands and substrates, such as phospholipids, inositol polyphosphates, and intracellular proteins.

The ERM family of proteins (part of the 4.1 superfamily) (see Mangeat et al., 1999) has a domain of approximately 300 amino acids the *4.1 ERM* (FERM) domain. ERM proteins are found at cell-cell adhesion sites, microvilli, and cleavage furrows, where actin filaments are densely associated with plasma membranes. They can bind to the plasma membrane either directly or indirectly through the FERM domain. In addition, they anchor actin filaments.

Protein-lipid interfaces

Although a good deal is known about protein structure, mostly from X-ray diffraction, much less is known about the interfaces between proteins and lipids. However, crystallographic data are available for highly ordered protein-lipid combinations (see Fyfe et al., 2001). Together with EPR data, they have shed considerable light on the organization of the interfaces. This is a very important topic because the activity of a variety of integral proteins depends on the presence of particular lipids (e.g., see Hoch, 1992; Dowhan, 1997).

EPR spectroscopy of spin-labelled lipids can be used to study the interaction of lipids and membrane proteins (e.g., see Borbat et al., 2001). The lipids interacting with the protein can be distinguished from the bulk lipids as long as they have a lower mobility. They are identified by the lipid-protein ratio dependence of the spectra or comparing the spectra of the lipids in the presence or absence of the protein component. EPR spectroscopy of integral proteins has demonstrated that the first layer of lipid molecules is highly restricted (so called annular lipids) (Marsh and Horvath, 1998).

The structure of several integral proteins, such as the bacterial photosynthesis reaction center (see Chapter 17), bacteriorhodopsin or cytochrome *c* oxidase are known in detail (for a discussion see Fyfe et al., 2001). Their hydrophobic surfaces in contact with the lipids are highly irregular. In the case of the bacterial reaction center, EPR spectroscopy suggests that its 11 transmembrane α-helices are surrounded by 30 to 35 restricted lipids (Marsh and Horvath, 1998). Diphosphatidyl glycerol (cardiolipin) has been shown to be present at the reaction center. The head groups interact through ionic interactions involving polar groups of a number of residues, the protein backbone, and bound water molecules. The acyl tails of the cardiolipin were found to be present along grooves in the α-helices forming the hydrophobic region of the molecules (see McAuley et al., 2000). Bacteriorhodopsin needs squalene and phosphatidyl glycerophosphate for normal activity (Joshi et al., 1998). The protein is present as a trimer, each monomer possessing seven transmembrane α-helices. An annulus of 18 tightly bound lipid chains is arranged around each monomer in the crystal (Luecke et al., 1999). The lipid molecules occupied most of the contact sites between each monomer. In the cases that could be modeled, the acyl chains were aligned with grooves in the protein hydrophobic surface.

Cytochrome *c* oxidase (see Chapter 16) is formed by 13 different subunits. The crystal structure at 2.3 Å

is thought to have 14 lipids per monomer (7 PGs, 3 pEs, 1 PC and two cardiolipins) (Mizushima et al., 1999) .

VII. MEMBRANE DOMAINS

The behavior of the plasma membrane components is far from uniform as revealed by a number of approaches. Studies of the lateral mobility of membrane components have revealed unsuspected complexities. Furthermore, there are plasma membrane domains which differ in composition.

Mobility of membrane components and membrane domains

Section V addressed the mobility of membrane components. As we saw the lipids studied move freely in the plane of the membrane. However, there are indications of restrictions of these movements for some lipids and proteins when the question is directed to specific molecules. In these cases, the mobility of membrane components has been studied primarily by four techniques. It is a tribute to workers in this field that all these techniques provide virtually the same answers.

Fluorescence depolarization following excitation with a flash or transient dichroism from the excitation of the dye in triplet state (e.g., Yguerabide, 1972), has provided information on the rotational motion of the molecules labelled with a fluorescent probe (e.g., eosin TC) (e.g., Cherry et al., 1980; Tsuji et al., 1988).

Fluorescence recovery after photobleaching (FRAP) is a technique using a two step approach. Components are labelled with fluorescent dyes or attached to fluorescently labelled antibodies. A spot is then bleached irreversibly with a pulse of intense laser light. The return of fluorescence is then measured to give an estimate of the diffusion of the fluorophores labelled components in the bleached area which allows calculating the fraction of diffusible proteins and the diffusion coefficient. This technique can only provide a statistical answer since hundreds or thousands of molecules are involved. The use of FRAP is not free of difficulties. Reversible photobleaching, such as triplet-state recovery, does take place in some cases without representing a diffusion phenomenon. In these cases, the fluorophore enters a triplet state which slowly decays back to the ground state (e.g., Song et al., 1996). There are also other poorly understood phenomena that can lead to a reversal of photobleaching seen as fluctuations in the molecular fluorescence emission (e.g., see Haupts et al., 1998).

Single-particle tracking (SPT) is carried out after labelling a specific component using a fluorescent particle or an antibody-coated colloidal gold particle. With this technique, the motion of individual structures or a few molecules can be followed. The gold particles are viewed in dark field and the light scattered by each object appears as a bright dot. The motion of the particles can be random and non-random, and careful analysis is required to distinguish between the two (see Saxton, 1993; Simson et al., 1995).

Optical tweezers allow trapping a particle (see Svoboda and Block, 1994) by optical forces (see Chapter

1). Then particle can be moved by moving either the beam or the microscope stage. Displacement of the particle requires only weak forces until the particle encounters an obstacle. The distance between barriers has been called the *barrier-free path* (BFP). Further studies can be carried out by exerting a force on the barrier.

The various techniques basically provide the same information and lead to very similar or identical models. For these reasons, the present discussion will make no attempt of arranging the information chronologically.

SPT revealed that the lipid components move freely (Lee et al., 1993). However, the integral proteins vary considerably in behavior. A fraction is free to move randomly, while another remains immobile. However, a large proportion of the proteins are retained in small domains. For example, cadherins, transferrin receptors and epidermal growth factor receptors, are retained in domains 300 to 600 nm in diameter for 3 to 30 s (Kusumi et al., 1993). Similar results were obtained with the laser trap (Edidin et al., 1991; Sako and Kusumi, 1995). In one of the studies (Edidin et al., 1991), the temperature was varied and the BFPs were found to increase with temperature. The confined particles moved within a domain at rates suggesting free diffusion. They could proceed farther in consecutive hops from one domain to another. These were thought of as encountering a "fence" (conforming to the *fence model*) over which they could occassionally jump. Trapping forces of 0.1 pN allowed for about half of the particles to exit the compartment. Other particles had a slower diffusion coefficient and could not be moved even with forces as high as 0.8, pN suggesting that they were attached to the cytoskeleton (i.e. they were "tethered"; this model is called the *tether model*).

The results suggesting *fenced* domains can be explained by a model in which a cytoskeleton network is closely apposed to the membrane (such as the one in the red blood cell, see Section II) (Kusumi et al., 1993, 1995) and blocks free exchanges of large molecules for steric reasons. The escape from the fence could be produced by thermally driven local fluctuations or dissociation of some of the components. The effective size of the "fence" would then depend on the molecular size of the component being examined. A stationary state is explained by a binding to the cytoskeleton (acting as a *tether*) (Sako and Kusumi, 1995). However, membrane domains that behave in a similar manner have been found for proteins that supposedly cannot interact with the cytoskeleton. For example, a lipid-linked isoform of the neural cell adhesion molecule (NCAM) was found to be confined to a similar sized domain (280 nm in diameter for 8 s) (Simson et al., 1995; Sheets et al., 1995; Simson et al., 1998). These observations may indicate interactions with proteins associated with the cytoskeleton rather than with the cytoskeleton itself. We should not lose sight, however, that lipid or fatty acid anchors could be linked or attached in some way to cytoskeletal elements (e.g., see Chapter 6).

Some studies were directed toward specific transmembrane proteins. Some of these studies were carried out with the red blood cell ghosts about which a good deal is known (see Section II). An involvement of the membrane cytoskeleton in *Band 3* movement was shown with FRAP (Sheetz et al., 1980). Mutants in which this network is lacking, exhibit a lateral movement of *Band 3* protein that is 50 times faster than in

the wild type. Experiments using both rotational and translational diffusion data (Tsuji et al., 1988), identified in red blood cell ghost membranes at least three populations of *Band 3* molecules with high, low, and no rotational mobilities. These populations are in steady state and the proportions of each class are strongly temperature dependent. The immobilization was consistent with binding to the cytoskeleton and specifically ankyrin. Ankyrin links the *Band 3* protein to the spectrin fibers of the cytoskeleton. Tomishige et al. (1998) used SPT with colloidal gold and optical tweezers in the study of *Band 3* proteins in red blood cell ghosts. For most particles, they found corralling for about 350 ms in a mesh 110 nm in diameter where the particles could hop from one compartment to another. These particles could be moved by exerting force on the cytoskeleton and would then continue hopping in the next compartment. Mild trypsin treatment, which removes the cytoplasmic domain of *Band 3*, increased the hopping by a factor of six. Another fraction (about 30%) was not mobile and only showed oscillatory motions characteristic of spectrin bridged by ankyrin. Furthermore they returned to the original position when moved with optical tweezers as spectrin does. These findings are consistent with the *fence* model and cytoskeletal immobilization (i.e. the *tether* model) of transmembrane molecules.

The involvement of the cytoskeleton in movement of E-cadherin, was put also to a test (Sako et al., 1998) using SPT and optical tweezers with cultured epithelial mouse cells. E-cadherin is a transmembrane recognition protein involved in Ca^{2+}-dependent cell-to-cell interactions (e.g., see Takeichi, 1991). The carboxy domain of the molecule is in the cytoplasm and binds to catenin that provides a bridge to actin filaments. Certain mutants of the cytoplasmic end of E-cadherin would, therefore, be incapable of interacting with the cytoskeleton. The extracellular domain of the E-cadherin and E-cadherin mutants was attached to gold or latex particles. Wild type and E-cadherin constructs (produced by transfection in mouse cultured cells, see Chapter 1) modified only in their cytoplasmic domain, were examined. The translational movements of the molecule in the region of the cell outside of cell-to-cell contact regions were studied. A portion of the wild-type E-cadherin and a construct capable of interacting with catenin were shown to behave as if they were tethered. A portion of the wild-type molecules and mutants unable to interact with catenin were either free to move or were confined to small areas. These findings also conform to the *fence* and the *tether* models.

Specialized domains

In some cells, large portions of the plasma membrane differ significantly in composition and constitute separate domains. These cells, such as epithelial cells or neurons, are said to be polarized; that is, their surfaces that face different environments differ in composition and physiological role. The lipid components of the membranes of the axons and those of the cell bodies, appear to correspond to separate domains in some neurons. In these experiments, *liposomes* containing fluorescent lipids were fused with the axons. Liposomes (called *proteoliposomes* when the membranes contain proteins) are vesicles produced artificially from lipids. The fluorescent lipids moved freely in the membrane of the axon, however, they were excluded from the cell body and the dendrites (Kobayashi et al., 1992).

In epithelial cells, the surface facing the lumen is called *apical*. The surface facing the extracellular matrix is the *basal* surface, which is frequently physiologically indistiguishable from the *lateral* surface (see

Chapter 11). The two are often lumped together as the *basolateral* surface. The apical and the basolateral domains are separated by *tight junctions* and have distinct protein and lipid composition. The tight junctions are structures maintaining the membranes of the adjacent cells closely apposed so that the epithelial cells form continuous sheets.

Aside from the targeting of the different components to either apical or basolateral domain, the difference in composition must be maintained in some way by a barrier preventing the mixing of the membrane components. Tight junctions act to prevent the intermixing of the two intramembranous domains (also referred to as *fence function*; see van Meer and Simons, 1986; Rodriguez-Boulan and Nelson, 1989; Mellman, 1996). In addition, tight junctions function as selective permeability barriers to blocking the passage of most water-soluble substances from one side of the sheet to the other (referred to as *gate function* see Diamond, 1977).

In contrast to the polarized epithelial cells, the axonal domain and the somatic-dendritic domains of neurons are maintained without an obvious physical barrier. The lateral mobility measured with optical-tweezers (see Chapter 1) show that mobility of proteins in the proximal part of the axon is severely restricted (Winckler et al., 1999). This reduced mobility is eliminated by disruption of F-actin. The initial segments of the axon contain specialized cytoskeletal structures which appear as a coat under the plasma membrane (Peters et al., 1968). These elements may, therefore, account for the restrictive distribution either through a direct tether or fence mechanisms as discussed above.

Other evidence also indicates the presence of specialized areas frequently referred to as *rafts* (see Simons and Ikonen, 1997). In addition, the surfaces of cells frequently assume a flask conformation, 50 to 100 nm in diameter. These structures have been called *caveolae* (Yamada, 1955), meaning little caves, and *plasmalemmal vesicles* (Palade and Bruns, 1968). Caveolae (also discussed in Chapter 9) are thought to have a special role in signal transduction and endocytosis. They are considered to be specialized rafts. The caveolin present in caveolae is thought to function in recruiting additional caveolin molecules (see Song et al., 1997) and in assembling signaling complexes in an inactive form. Thus caveolae are thought to have a regulatory role in down-regulating some signals and also allowing the rapid deployment of functional signaling molecules in response to physiological needs (e.g., Li et al., 1995) (for a review see Okamoto et al., 1998).

As we shall see the data pertinent to rafts is somewhat contradictory. In our opinion, present information supports the presence of these specialized domains and it may be possible to reconcile the various findings (see below)

The proposal that rafts or special structures such as caveolae containing glycosyl-phosphatidyl-inositol (GPI)-anchored proteins, cholesterol and glycosphingolipids, are present in native membranes rests on several observation. When extracted with cold Triton X-100, these components form detergent insoluble complexes (see Simons and Ikonen, 1997). Furthermore, they appear to be present in clusters in some studies using antibodies.

Recent studies suggest the presence of an additional set of rafts distinct from those resistant to Triton X-100. These are Triton X-100-soluble but insoluble in the detergent Lubrol. In addition, they do not co-localize with the Triton-X insoluble raft markers (Chamberlain et al., 2001). They are associated with cholesterol dependent-clusters of 200 nm which are likely to be involved in exocytosis (see Lang et al., 2001).

Several studies suggest that the clustering of caveolar components is an artifacts. For example, studies using antibodies show that the caveolar proteins initially are not concentrated in caveolae; they enter these structures after cross-linking with polyclonal secondary antibodies (e.g., Mayor et al., 1994; Fujimoto, 1996). Furthermore, the GPI-anchored proteins are distributed in clusters in membrane fragments only after detergent treatment (Mayor and Maxfield, 1995). However, both detergent dependent (Sargiacomo et al., 1993) or detergent independent isolation (Smart et al., 1995b; Chang et al., 1994) find GPI proteins in a caveolar fraction. Futhermore, EM histochemistry shows alkaline phosphatase either clustered in structures corresponding to caveolae or diffusely distributed at the cell surface, depending on the cell type (Ide and Saito, 1980; Latker et al., 1987; Kobayashi and Robinson, 1991). Despite these validations, other approaches may also challenge the presence of clusters.

The outer leaflet of the apical plasma membrane contains glycosphingolipids and is poor in glycerolipids. Are these components of the apical membranes present in microdomains? Two entirely different approaches were used to study these microdomains in polarized *Madin-Darby canine kidney* (MDCK) cultured cells (Kenworthy and Edidin, 1998; Friedrichson and Kurzchalia, 1998) and *Chinese ovary* (CHO) cells (Varma and Mayor, 1998). The MDCK epithelial cells form polarized monolayers in culture and for that reason have been used intensively in studies where the polarity of the cells has to be maintained. The studies of Kenworthy and Edidin (1998) and those of Varma and Mayor (1998) used fluorescence resonance energy transfer (FRET) [see Chapter 1 for applications involving the green fluorescent protein (GFP)]. FRET involves the nonradiative transfer of energy from the excited state of a donor to an appropriate acceptor (e.g., see Clegg, 1995; Runnels and Scarlatta, 1995). The rate of energy transfer is inversely proportional to the sixth power of the distance, allowing measuring the proximity of the molecules. In the study of Kenworthy and Edidin (1998), FRET was measured as an increase in donor fluorescence after complete photobleaching of the acceptor fluorophor. The technique takes advantage of energy transfer between fluorescent antibodies attached to GPI-anchored nucleosidases in the apical surface of MDCK cells. They found considerable energy transfer between acceptors and donors, consistent with the prediction from randomly distributed molecules suggesting that these molecules are not clustered (the technique has a resolution of less then 10 nm). In the study of Varma and Mayor (1998), the energy transfer between the GPI-anchored folate receptors bound to a florescent folate analog was measured. With this technique, the net emission is accompanied by a loss of polarization, so that the loss of fluorescence anisotropy provides a sensitive assay of the transfer between like fluorophores (Runnels and Scarlata, 1995). The anisotropy was found to be concentration independent, consistent with the distribution of the receptor in *sub-pixel* domains, likely to be smaller than 70 nm in diameter (Varma and Mayor, 1998) and not randomly distributed throughout the membrane (which would be concentration

dependent). A *pixel* (picture element) is a single element of a digitized video picture.

Another study suggests that the clustering of GPI anchors does take place (De Angelis et al., 1998). GFP molecules brought in close proximity change in intensities of emission when excited at 395 nm as compared to the emission at 475 nm. The evidence indicates that GPI-anchored GFP does cluster. The weak signal was attributed to a loose and transient attachment. An alternative interpretation might be that the GPIs are not all adjacent to each other in the raft.

Chemical crosslinking in MDCK cells (Friedrichson and Kurzchalia, 1998) also support the notion that GPI-anchored forms (and not transmembrane forms) do cluster. In this study, cross-linking of *growth hormone* attached to the GPI-anchoring signal of the *decay-accelerating* factor was carried out. The clusters were found to contain at least 15 molecules and to require, for their integrity, the presence of cholesterol. Interestingly enough, making certain assumption about protein size, the value of 16 molecules is of the same order of magnitude as the 70 nm predicted from the study of Varma and Mayor (1998) as the maximum size of the clusters.

Another study (Pralle et al., 2000) suggests the presence of very small rafts in baby hamster kidney (BHK) cells. The local movement due to thermal position fluctuations of single membrane proteins was followed using a laser trap (see Chapter 1) to keep the fluorescent bead (attached to the raft protein) restricted to a small area (approximately 100 nm). Trasfection to produce certain protein constructs were used. Single particle tracking allowed calculating the diffusion coefficient. The diffusion of GPI-anchored or transmembrane proteins when embedded in the raft were found to be independent of the nature of the membrane anchor and much reduced compared to transmembrane proteins not trapped in the raft. The GPI-anchored proteins were never shown to leave the raft within a 10 min period. The experiments suggest that the rafts are cholesterol stabilized complexes approximately 26 nm in size.

In summary, most studies indicate that the GPI-anchored proteins are present in very small domains. Another finds a completely random distribution. It should be kept in mind that more information will emerge in the future.

Other considerations should be taken into account. Most of the studies showing that the components thought to be in rafts are dispersed or in small clusters, used cells transfected with the probe molecule (e.g., Mayor et al., 1994; Fujimoto, 1996, Mayor and Maxfield, 1995, 1998; Friedrichson and Kurzchalia, 1998; Kenworthy and Edidin, 1998; Varma and Mayor, 1998; Pralle et al., 2000). The studies showing the opposite used normal cells (e.g. Ide and Saito, 1980; Latker et al., 1987; Kobayashi and Robinson, 1991; Sargiacomo et al., 1993; Smart et al., 1995; Chang et al., 1994). It is conceivable that the components acquired by transfection are not incorporated into the normal machinery of the cell, possibly because the existing system is already occupied. It is also possible that forming new caveolae or specialized rafts in response to cross-linking and detergent may mimic the normal activation reactions of the cell. Generally, receptors are activated by dimerization or oligomerization (such as the cross linking by antibodies) (see Chapter 6) and conceivably the interaction with hydrophobic domains of the detergent used (usually Triton X-100) may imitate interactions with the lipid components of the membrane resulting in activation.

Rafts are thought to coalesce into larger structures (e.g., see Viola et al., 1999) and serve important physiological functions in the activation of T cells.

Phosphoinositides (PIs) and their phosphorylated derivatives (PIPs) are thought to be present in plasma membranes at specific locations. One such site corresponds to caveolae (Pike and Casey, 1996; Hope and Pike, 1996). Immunocytochemistry (see Chapter 1) identifies several locations containing phosphatidylinositol-4,5-bisphosphate (PIP_2). Some of these are around the nuclei. PIP_2 was also found localized at microfilaments (F-actin) bundles and at focal contacts (see Chapter 23), where α-actinin and vinculin are present (Fukami et al., 1994). α-actinin and vinculin are usually associated with actin and they are present in PIP_2-bound form. The presence at specific locations is thought to result from their highly localized sites of synthesis of these components (e.g., see Martin, 1997). The specialized sites are thought to play a role in signaling (see Chapter 7), the formation of vesicles in secretion or endocytosis (see Chapter 11) and the polymerization of actin (see Chapter 23).

Other chapters will discuss specialized spots in the plasma membrane containing special proteins. These include cell junctions and caveolae, as well as motor endplates and other synapses (Chapter 22). Some of the aspects of targeting to different cell surfaces are discussed in Chapters 10 and 11.

SUGGESTED READING

General references

Glaser, M. (1993) Lipid domains in biological membranes, *Current Opinion Struct. Biol.* 3:475-481.

Jacobson K., Sheets, E.D. and Simson, R. (1995) Revisiting the fluid mosaic model of membranes, *Science* 268:1441-1442. (Medline)

Gennis, R. B. (1989) *Biomembranes: Molecular Structure and Function*, Chapters 1 to 5. Springer-Verlag, New York.

Stein, W. D. and Lieb, W. R. (1986) *Transport and Diffusion Across Cell Membranes*, Chapters 1-3. Academic Press, New York.

Yeagle, P. (1987) *The Membrane of Cells*, p. 292. Academic Press, New York. See Chapters 1-7.

Membrane proteins

Guidotti, G. (1986) Membrane proteins: structure, arrangement, and disposition in the membrane. In *Membrane Physiology*, 2d ed. (Andreoli, T. E., Hoffman, J. F., Fanestil, D. D., and Schulz, S. G., eds.), pp. 45-58. Plenum Medical Book Co., New York.

Kleinfeld, A. M. (1987) Current views of membrane structure, *Curr. Top. Membr. Transp.* 29:1-27.

Schlessinger, J. (1983) Mobilities of cell membrane proteins: how are they modulated by the cytoskeleton? *Trends Neurosci.* 6:360-363.

Membrane Lipids

Deveaux, P.F. (1991) Static and dynamic lipid asymmetry in cell membranes, *Biochem.* 30:1163-1173.

Kurzchalia, T.V. and Parton, R.G. (1999) Membrane microdomains and caveolae, *Curr. Opin. Cell Biol.* 11:424-431. (Medline)

Thompson, T. E. and Huang, C. (1986) Composition and dynamics of lipids in biomembranes. In *Membrane Physiology*, 2d ed. (Andreoli, T. E., Hoffman, f. F., Fanestil, D. D. and Schultz, 5. G., eds.), pp. 25-44. Plenum Medical Book Co., New York.

Clusters

Parton, R.G. (1996) Caveolae and caveolins, *Curr. Opin. Cell Biol.* 8:542-548. (Medline)

Simons, K. and Ikonen, E.(1997) Functional rafts in cell membranes, *Nature* 387: 569-572. (Medline)

However, see Discussion and references in Section VI.

Gap junctions

Bruzzone, R., White, T.W. and Paul, D.L. (1996) Connections with connexins: the molecular basis of direct intercellular signaling, *Eur. J. Biochem.* 238:1-27. (Medline)

Unwin, P. N. T. (1987) Gap junction structure and the control of cell to cell communication. *CIBA Found. Symp.* 125:78-91. (Medline)

WEB RESOURCES

Data base on lipids: http://www.lipidat.chemistry.ohio-state.edu

Diwan,J.J., Lipids and Membranes:
http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/lipid.htm

REFERENCES

Search the textbook

Back to Chapter 4

REFERENCES

Ames, J.B., Ishima, R., Tanaka, T., Gordon, J.I., Stryer, L. and Ikura, M. (1997) Molecular mechanics of calcium-myristoyl switches, *Nature* 389:198-202.(Medline)

Berg, J.S., Derfler, B.H., Pennisi, C.M., Corey, D.P. and Cheney, R.E. (2000) Myosin-X, a novel myosin with pleckstrin homology domains, associates with regions of dynamic actin, *J. Cell Sci.* 113:3439-3451. (MedLine)

Bevans, C.G., Kordel, M., Rhee, S.K. and Harris, A.L. (1998) Isoform composition of connexin channels determines selectivity among second messengers and uncharged molecules, *J. Biol. Chem.* 273:2808-2816.(Medline)

Bijlmakers, M.J., Marsh, M. (2003) The on-off story of protein palmitoylation, *Trends Cell Biol.* 13:32-42. (MedLine),

Bogdanov, M., Sun, J. Kaback, H.R. and Dowhan, W. (1996) A phospholipid acts as a chaperone in assembly of a membrane transport protein, *J. Biol. Chem.* 271:11615-11618.(Medline)

Bone, L.J., Deschenes, S.M., Balice-Gordon, R.J., Fischbeck, K.H. and Scherer, S.S (1997). Connexin32 and X-linked Charcot-Marie-Tooth disease, *Neurobiol. Dis.* 4:221-230.(Medline)

Borbat, P.P., Costa-Filho, A.J., Earle, K.A., Moscicki, J.K. and Freed, J.H. (2001) Electron spin resonance in studies of membranes and proteins, *Science* 291:266-269. (MedLine)

Bottomley, M.J., Salim, K. and Panayotou, G. (1998) Phospholipid-binding protein domains, *Biochim. Biophys. Acta.* 1436:165-183. (MedLine)

Bruzzone, R., White, T.W., Scherer, S.S., Fischbeck, K.H. and Paul, D.L. (1994) Null mutations of connexin32 in patients with X-linked Charcot-Marie-Tooth disease, *Neuron* 13:1253-1260.(Medline)

Cantley, L. (1986) Ion transport systems sequenced, *Trends Neurosci.* 9:1-3.

Cao, F., Eckert, R., Elfgang, C., Nitsche, J.M., Snyder, S.A., Hulser, D.F., Willecke, K. and Nicholson, B.J. (1998) A quantitative analysis of connexin-specific permeability differences of gap junctions

expressed in HeLa transfectants and *Xenopus* oocytes, *J. Cell Scie.* 111:31-34.(Medline)

Chamberlain, L.H., Burgoyne, R.D. and Gould, G.W. (2001) SNARE proteins are highly enriched in lipid rafts in PC12 cells: implications for the spatial control of exocytosis, *Proc. Natl. Acad. Sci. USA* 98:5619-5624. (MedLine)

Chang, W.J., Ying, Y.S., Rothberg, K.G., Hooper, N.M., Turner, A.J., Gambliel, H.A., De Gunzburg, J., Mumby, S.M., Gilman, A.G. and Anderson, R.G. (1994) Purification and characterization of smooth muscle cell caveolae, *J. Cell Biol.* 126:127-138.(Medline)

Cherry, R.J., Nigg, E.A. and Beddard, G.S. (1980) Oligosaccharide motion in erythrocyte membranes investigated by picosecond fluorescence polarization and microsecond dichroism of an optical probe, *Proc. Natl. Acad. Sci. USA* 77:5899-5903.(Medline)

Christi et al. (1998) The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane, *Trends in Biochem. Scie.* 23:281-282.(Medline)

Clegg, R.M. (1995) Fluorescence resonance energy transfer, *Curr. Opin. Biotechnol.* 6:103-110.(Medline)

Collander, R. (1954) The permeability of *Nitella* cells to non-electroytes, *Physiol. Plant.* 7:420-445.

Cone, R. A. (1972) Rotational diffusion of rhodopsin in visual receptor membrane, *Nature, New Biol.* 236:39-43.(Medline)

Davson, H. and Danielli, J. F. (1952) *The Permeability of Natural Membranes*, 2d ed. Cambridge University Press, London.

De Angelis, D.A., Miesenbock, G., Zemelman, B.V. and Rothman, J.E. (1998) PRIM: proximity imaging of green fluorescent protein-tagged polypeptides, *Proc. Natl. Acad. Sci. USA* 95:12312-1236. (MedLine)

de Kruijff, B.(1997) Lipids beyond the bilayer, *Nature* 386:129-130.(Medline)

Devaux, P. and McConnell, H. M. (1972) Lateral diffusion in spin- labeled phosphatidylcholine multilayers, *Am. Chem. Soc. J.* 94:4475-4481.(Medline)

Devaux, P. F. (1990) The aminophospholipid translocase: a transmembrane lipid pump-physiological significance, *News Physiol. Scie.* 5:53-58.

Deveaux, P.F. (1991) Static and dynamic lipid asymmetry in cell membranes, *Biochem.* 30:1163-1173.(Medline)

Devaux, P.F. (1993a) Phospholipid translocation in the endoplasmic reticulum, in *Endoplasmic Reticulum*, ed. by Borgese, N. and Harris, J.R., Plenum Press, London in press, pp 273-285.(Medline)

Devaux, P.F. (1993b) Lipid transmembrane asymmetry and flip-flop in biological membranes and lipid bilayers, *Curr. Opin. Struct. Biol.* 3:489-494.

Diamond, J.M. (1977) Twenty-first Bowditch lecture. The epithelial junction: bridge, gate, and fence, *Physiologist* 20:10-18.(Medline)

Ding, B. (1998) Intercellular protein trafficking through plasmodesmata, *Plant Mol. Biol.* 38:279-310.(Medline)

Dizhoor, A.M., Chen, C.-K., Olshevskaya, E., Sinelnikova, V.V., Phillipov, P. and Hurley, J.B. (1993) Role of acetylated amino terminus of recoverin in Ca^{2+} dependent membrane interaction, *Science* 259:829-832.(Medline)

Dong, H., O'Brien, R.J., Fung, E.T., Lanahan, A.A., Worley, P.F. and Huganir, R.L. (1997) GRIP: a synaptic PDZ domain-containing protein that interacts with AMPA receptors, *Nature* 386:279-284.(Medline)

Dowhan, W. (1997) Molecular basis for membrane phospholipid diversity: why are there so many lipids? *Annu. Rev. Biochem.* 66:199-232. (MedLine)

Edidin, M. (1974) Rotational and translational diffusion in membranes, *Annu. Rev. Biophys. Bioeng.* 3:179-201.(Medline)

Edidin, M., Kuo, S.C. and Sheetz, M.P. (1991) Lateral movements of membrane glycoproteins restricted by dynamic cytoplasmic barriers, *Science* 254:1379-1382.(Medline)

Elfgang, C., Eckert, R., Lichtenberg-Fraté, H., Butterweck, A., Traub, O., Klein, R.A., Hülser, D.F. and Willecke, K. (1995) Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells, *J. Cell Biol.* 129:805-817.(Medline)

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., Henson, P.M. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages, *J. Immunol.* 148:2207-2216.(Medline)

Fadok, V.A., Bratton, D.L., Rose, D.M., Pearson, A., Esekewitz, R.A.B. and Henson, P.M. (2000) A receptor for phosphatidylserine-specific clearance of apoptotic cells, *Nature* 405:85-90.(Medline)

Fleischer, B., Zambrano, F. and Fleischer, S. (1974) Biochemical characterization of the Golgi complex of mammalian cells, *J. Supramol. Struct.* 2:737-750. (MedLine)

Fowler, V.M. (1986) An actomyosin contractile mechanism for erythrocyte shape transformations, *J. Cell Biochem.* 31:1-9.(Medline)

Friedrichson, T. and Kuzchalia, T.V. (1998) Microdomains of GPI-anchored proteins in living cells revealed by crosslinking, *Nature* 394:802-805.(Medline)

Frye, L.D. and Edidin, M.J. (1970) The rapid intermixing of cell surface antigens after the formation of mouse-human heterokaryons, *J. Cell Scie.* 7:319-335.(Medline)

Fujimoto, T. (1996) GPI-anchored proteins, glycosphingolipids, and sphingomyelin are sequestered to caveolae only after crosslinking, *J. Histochem. Cytochem.* 44:929-941.(Medline)

Fukami, K., Endo, T., Imamura, M., and Takenawa, T. (1994) α-Actinin and vinculin are PIP_2-binding proteins involved in signaling by tyrosine kinase, *J. Biol. Chem.* 269:1518-1522. (MedLine)

Fyfe, P.K., McAuley, K.E., Roszak, A.W., Isaacs, N.W., Cogdell, R.J. and Jones, M.R. (2001) Probing the interface between membrane proteins and membrane lipids by X-ray crystallography, *Trends Biochem. Sci.* 26:106-112. (MedLine)

Gabriel, H.D., Jung, D., Butzler, C., Temme, A., Traub, O., Winterhager, E. and Willecke, K. (1998) Transplacental uptake of glucose is decreased in embryonic lethal connexin26-deficient mice, *J. Cell Biol.* 140:1453-1461.(Medline)

Gascard, P., Tran, D., Sauvage, M.. Sulpice, J.C., Kikami, K., Takenawa, T., Claret, M. and Giraud, F. (1991) Asymmetric distribution of phosphoinositides and phosphatidic acid in the human erythrocyte membrane, *Biochim. Biophys. Acta* 1069:27-36.(Medline)

Gennis, R. B. (1989) *Biomembranes: Molecular Structure and Function*, Chapters 1 to 5. Springer-Verlag, New York

Gong, X., Li, E., Klier, G., Huang, Q., Wu, Y., Lei, H., Kumar, N.M., Horwitz ,J. and Gilula, N.B. (1997) Disruption of α3 connexin gene leads to proteolysis and cataractogenesis in mice, *Cell* 91:833-843.(Medline)

Goodenough, D.A., Goliger, J.A. and Paul, D.L. (1996) Connexins, connexons, and intercellular communication, *Annu. Rev. Biochem.* 65:475-502.(Medline)

Görne-Tschelnokow, Strecker, A., Kaduk, C., Naumann, D. and Hucho, F., (1994) The transmembrane domains of the nicotinic acetylcholine receptor containing α-helical and βα structures, *EMBO J.* 13:338-341.(Medline)

Gorter, E. and Grendel, F. (1925) On bimolecular layers of lipids on the chromocytes of the blood, *J. Exp. Med.* 41:439-443.

Hoch, F.L. (1992) Cardiolipins and biomembrane function, *Biochim. Biophys. Acta* 1113:71-133. (Medline)

Haupts, U., Maiti, S., Schwille, P. and Webb, W.W. (1998) Dynamics of fluorescence fluctuations in green fluorescent protein observed by fluorescence correlation spectroscopy, *Proc. Natl. Acad. Sci. USA* 95:13573-13578. (MedLine)

Hope, H.R. and Pike, L.J. (1996) Phosphoinositides and phosphoinositide-utilizing enzymes in detergent-insoluble lipid domains, *Mol. Biol. Cell* 7:843-81.(Medline)

Ide, C. and Saito, T. (1980) Electron microscopic histochemistry of ATPase and alkaline phosphatase activities in mouse digital corpuscles, *J. Neurocytol.* 9:207-218.(Medline)

Jacobs, M. H. (1952) The measurement of cell permeability with particular reference to the erythrocyte. In *Modern Trends in Physiology and Biochemistry* (Barron, E.S.G., ed.), pp. 149-171. Academic Press, New York.

Jiang, J.X. and Goodenough, D.A. (1996) Heteromeric connexons in lens gap junction channels, *Proc. Natl. Acad. Sci. USA* 93:1287-1291.(Medline)

Joshi, M.K., Dracheva, S., Mukhopadhyay, A.K., Bose, S. and Hendler, R.W. (1998) Importance of specific native lipids in controlling the photocycle of bacteriorhodopsin, *Biochemistry* 37:14463-14470. (MedLine)

Keenan, T.W., Morré, D.J. (1970) Phospholipid class and fatty acid composition of Golgi apparatus isolated from rat liver and comparison with other cell fractions, *Biochemistry* 9:19-25. (MedLine)

Kelsell, D.P., Dunlop, J., Stevens, H.P., Lench, N.J., Liang, J.N., Parry, G., Mueller, R.F. and Leigh, I.M. (1997) Connexin 26 mutations in hereditary non-syndromic sensorineural deafness, *Nature*387:80-83.(Medline)

Kenworthy, A.K. and Edidin, M. (1998) Distribution of a glycosylphosphatidylinositol-anchored protein at the apical surface of MDCK cells examined at a resolution of 100 Å using imaging fluorescence resonance energy transfer, *J. Cell Biol.* 142:69-84.(Medline)

Kirchhoff,S., Nelles, E., Hagendorff, A., Kruger, O., Traub, O. and Willecke, K, (1998) Reduced cardiac conduction velocity and predisposition to arrhythmias in connexin40-deficient mice, *Curr. Biol.* 8:299-302.(Medline)

Kobayashi, T. and Robinson, J.M. (1991) A novel intracellular compartment with unusual secretory properties in human neutrophils, *J. Cell. Biol.* 113:743-756.(Medline)

Kobayashi, T., Storrie, B., Simons, K. and Dotti, C.G. (1992) A functional barrier to movement of movement of lipids in polarized neurons, *Nature* 359:647-650.(Medline)

Kohen, E., Kohen, C., Thorell, B., Mintz, D. H. and Rabinovitch, A. (1979) Intracellular communication in pancreatic islet monolayer cultures: a microfluorometric study, *Science* 204:862-865.(Medline)

Konig, N. and Zampighi, G.A. (1995) Purification of bovine lens cell-to-cell channels composed of connexin44 and connexin50, *J. Cell Sci.* 108:3091-3098.(Medline)

Kusumi, A., Sako, Y. and Yamamoto, M. (1993) Confined lateral diffusion of membrane receptors as studied by single particle tracking (nanovid microscopy). Effects of calcium-induced differentiation in cultured epithelial cells, *Biophys. J.* 65:2021-2040.(Medline)

Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of protein, *J. Mol. Biol.* 157:105-132.(Medline)

Lang, T., Bruns, D., Wenzel, D., Riedel, D., Holroyd, P., Thiele, C. and Jahn, R. (2001) SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis, *EMBO J.* 20:2202-2213. (MedLine)

Latker, C.H., Shinowara, N.L., Miller, J.C. and Rapoport, S.I. (1987) Differential localization of alkaline phosphatase in barrier tissues of the frog and rat nervous systems: a cytochemical and biochemical study, *J. Comp. Neurol.* 264:291-302.(Medline)

Lee, G.M., Zhang, F., Ishihara, A., McNeil, C.L., Jacobson, K.A. (1993) Unconfined lateral diffusion and an estimate of pericellular matrix viscosity revealed by measuring the mobility of gold-tagged lipids, *J Cell Biol.* 120:25-35.(Medline)

Lemmon, M.A. and Ferguson, K.M. (2000) Signal-dependent membrane targeting by pleckstrin homology (PH) domains, *Biochem. J.* 350:1-18. (MedLine)

Leto, T.L. and Marchesi, V.T. (1984) A structural model of human erythrocyte protein 4.1, *J. Biol. Chem.*

259:4603-4608.(Medline)

Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J.E., Hansen, S.H., Nishimoto, I. and Lisanti, M.P. (1995) Evidence for a regulated interaction between heterotrimeric G proteins and caveolin, *J. Biol. Chem.* 270:15693-15701. (MedLine)

Liu, S. C., Derick, L. H. and Palek, J. (1987) Visualization of the hexagonal lattice in the erythrocyte membrane skeleton, *J. Cell Biol.* 104:527-536.(Medline)

Lowenstein, W. R., Kanno, Y. and Socolar, S. J. (1978) Quantum jumps of conductance during formation of membrane channels at cell-cell junctions, *Nature* 274:133-136.

Luecke, H., Schobert, B., Richter, H.T., Cartailler, J.P. and Lanyi, J.K. (1999) Structure of bacteriorhodopsin at 1.55 Å resolution, *J. Mol. Biol.* 291:899-911. (MedLine)

Luna, E. J. and Hitt, A. L. (1992) Cytoskeleton-plasma membrane interactions, *Science* 258:955-964.(Medline)

MacLennan, D. H., Brandl, C. J., Konczak, B. and Green, N. M. (1985) Amino-acid sequence of a Ca^{2+} and Mg^{2+}-dependent ATPase from rabbit muscle sarcoplasmic reticulum deduced from its complementary DNA sequence, *Nature* 316:696-700.(Medline)

Makowski, L., Caspar, D. L. D., Phillips, W. C. and Goodenough, D. A. (1977) Gap junction structures. II. Analysis of the x-ray diffraction data, *J. Cell Biol.* 74:629-645.(Medline)

Makowski, L., Caspar, D.L., Phillips, W.C. and Goodenough, D.A. (1984) Gap junction structures. V. Structural chemistry inferred from X-ray diffraction measurements on sucrose accessibility and trypsin susceptibility, *J. Mol. Biol.* 174:449-481.(Medline)

Malewicz, B., Kumar, V.V., Johnson, R.G. and Baumann, W.J. (1990) Lipids in gap junction assembly and function, *Lipids* 25:419-427.(Medline)

Mangeat, P., Roy, C. and Martin, M. (1999) ERM proteins in cell adhesion and membrane dynamics, *Trends Cell Biol.* 9:187-192. (MedLine)

Marsh, D. (1975) Spectroscopic studies of membrane structure, In *Essays in Biochemistry* (Campbell, P. N., and Aldridge, W. N., eds.), Vol. 11, pp. 139-180, Academic Press, New York. (MedLine)

Marsh, D. (1985) ESR spin label studies of lipid-protein interactions, in *Progress in Protein-Lipid Interactions* vol. 1 (eds. Watts, A. a nd De Pont, J.J. H.H.M.) Elsevier, Amsderdam, New York, pp. 143-

172.

Marsh, D. and Horvath, L.I. (1998) Structure, dynamics and composition of the lipid-protein interface. Perspectives from spin-labelling, *Biochim. Biophys. Acta.* 1376:267-296. (MedLine)

Martin, T.F. (1997) Phosphoinositides as spatial regulators of membrane traffic, *Curr. Opin. Neurobiol.* 7:331-338.(Medline)

Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M. and Green, D.R. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl, *J. Exp. Med.* 182:1545-1556.(Medline)

Mayor, S.and Maxfield, F.R. (1995) Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment, *Mol. Biol. Cell* 6:929-944.(Medline)

Mayor, S., Rothberg, K.G. and Maxfield, F.R. (1994) Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking, *Science* 264:1948-1951.(Medline)

McAuley,K.E., Paul, K., Fyfe, Ridge, J. P., Isaacs,N.W., Cogdell, R.J., and Jones, M.R. (2000) Structural details of an interaction between cardiolipin and an integral membrane protein, *Proc. Natl. Acad. Sci.USA* 96:14706-14711.

Medof, M.E., Nagarajan, S. and Tykocinski, M.L. (1996) Cell surface engineering with GPI-anchored proteins, *FASEB J.* 10:574-586.(Medline)

Mellman, I. (1996) Molecular sorting of membrane proteins in polarized and non-polarized cells, *Cold Spring Harb. Symp. Quant. Biol.* 60:745-752.(Medline)

Mizushima, T.et al. (1999) Structure of phospholipids in amembraen protein complex, bovine cytochrome *c* oxidase, *Acta Crystallogr.*, A55 (Suppl) Abstr. P06.04.069. (see Fyfe et al., 2001)

Moreno, A.P., Rook, M.B., Fishman, G.I and Spray, D.C. (1994a) Gap junction channels: distinct voltage-sensitive and -insensitive conductance states, *Biophys. J.* 67:113-119.(Medline)

Moreno, A.P., Saez, J.C., Fishman, G.I. and Spray, D.C. (1994b) Human connexin43 gap junction channels. Regulation of unitary conductances by phosphorylation, *Circ. Res.*74:1050-1057.(Medline)

Merrill, A.H. Jr., Liotta, D.C. and Riley, R.T. (1996) Fumosins: fungal toxins that shed light on sphingolipid function, *Trends in Cell Biol.* 6:218-223.

Morrot, G., Hervé, P., Zachowski, A., Pellmann, P. and Devaux, P. F. (1989) Aminophospholipid translocase of human erythrocytes: Phospholipid substrate specificity and effect of cholesterol, *Biochem.* 28:3456-3462.(Medline)

Nalefski, E.A. and Falke, J.J. (1996) The C2 domain calcium-binding motif: structural and functional diversity, *Protein Sci.* 5:2375-2390. (MedLine)

Nelles, E., Butzler, C., Jung, D., Temme, A., Gabriel, H.D., Dahl, U., Traub, O., Stumpel, F., Jungermann, K., Zielasek, J., Toyka, K.V., Dermietzel, R. and Willecke, K. (1996) Defective propagation of signals generated by sympathetic nerve stimulation in the liver of connexin32-deficient mice, *Proc. Natl. Acad. Sci. USA* 93:9565-9570.(Medline)

Niggli, V. (2001) Structural properties of lipid-binding sites in cytoskeletal proteins, *Trends Biochem. Sci.* 26:604-611. (MedLine)

Niggli, V., Sommer, L., Brunner, J. and Burger, M.M. (1990) Interaction in situ of the cytoskeletal protein vinculin with bilayers studied by introducing a photoactivatable fatty acid into living chicken embryo fibroblasts, *Eur. J. Biochem.* 187:111-117. (MedLine)

Obaid, A. L., Socolar, S. J. and Rose, B. (1983) Cell-to-cell channels with two independently regulated gates in series: analysis of functional conductance modulation by membrane potential, calcium and pH, *J. Membr. Biol.* 73:69-89.(Medline)

Okamoto, T., Schlegel, A., Scherer, P.E. and Lisanti, M.P. (1998) Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane, *J. Biol. Chem.* 273:5419-5422. (MedLine)

Ovchinnikov, Y. A. (1987) Probing the folding of membrane proteins, *Trends Biochem Scie.* 12:434-438.

Overall, R.L. and Blackman, L.M. (1996) A model of the macromolecular structure of plasmodesmata, *Trends in Plant Sci.* 1:307-311.

Palade, G.E. and Bruns, R.R. (1968) Structural modulations of plasmalemmal vesicles, *J. Cell Biol.* 37:633-649.

Peracchia, C. and Dulhunty, A. F. (1976) Low resistance junctions in crayfish, J. Cell Biol. 70:419-439.(Medline)

Peracchia, C. and Peracchia, L. L. (1980a) Gap junction dynamics: reversible effects of divalent cations, *J. Cell Biol.* 87:708-718.(Medline)

Peracchia, C. and Peracchia, L. L. (1980b) Gap junction dynamics: reversible effects of hydrogen ions, *J. Cell Biol.* 87:719-737.(Medline)

Perkins, G.A., Goodenough, D.A. and Sosinsky, G.E. (1998) Formation of the gap junction intercellular channel requires a 30 degree rotation for interdigitating two apposing connexons, *J. Mol. Biol.* 277:171-177.(Medline)

Peters, A., Proskauer, C.C. and Kaiserman-Abramof, I.R. (1968) The small pyramidal neuron of the rat cerebral cortex. The axon hillock and initial segment, *J. Cell Biol.* 39:604-619.(Medine)

Phelan, P., Bacon, J.P., Davies, J.A., Stebbings, L.A., Todman, M.G., Avery, L., Baines, R.A., Barnes, T.M., Ford, C., Hekimi, S., Lee, R., Shaw, J.E., Starich, T.A., Curtin, K.D., Sun Y.A. and Wyman, R.J. Innexins: a family of invertebrate gap-junction proteins, *Trends Genet.* 14:348-349.(Medline)

Pike, L.J. and Casey, L. (1996) Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains, *J. Biol. Chem.* 271:26453-26456.(Medine)

Poo, M. and Cone, R. A. (1974) Lateral diffusion of rhodopsin in the photoreceptor membrane, *Nature* 247:438-441.(Medline)

Pralle, A., Keller, P., Florin, E., Simons, K. and Horber, J.K. (2000) Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells, *J. Cell Biol.* 148:997-1008. (MedLine)

Reaume, A.G., de Sousa, P.A., Kulkarni, S., Langille, B.L., Zhu, D., Davies, T.C., Juneja, S.C., Kidder, G.M., Rossant, J. (1995) Cardiac malformation in neonatal mice lacking connexin43, *Science* 267:1831-1834.(Medline)

Rennoij, W., Van Golde, L. M. G., Zwaal, R. F. A. and VanDeenen, L. L. M. (1976) Topical asymmetry of phospholipid metabolism in rat erythrocyte membranes, *Eur. J. Biochem.* 61:53-58.(Medline)

Resh, M.D. (1999) Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins, *Biochimica et Biophysica Acta* 1451: 1-16. (MedLine)

Ressot, C., Gomes, D., Dautigny, A., Pham-Dinh, D. and Bruzzone, R. (1998) Connexin32 mutations associated with X-linked Charcot-Marie-Tooth disease show two distinct behaviors: loss of function and altered gating properties, *J. Neurosci.* 18:4063-4075.(Medline)

Rodriguez-Boulan, E. and Nelson, W.J. (1989) Morphogenesis of the polarized epithelial cell phenotype, *Science* 245:718-725.(Medline)

Runnels, L.W. and Scarlata, S.F., (1995) Theory and application of fluorescence homotransfer to melittin oligomerization, *Biophys. J.* 69:1569-1583.(Medline)

Saez, J.C., Nairn, A.C., Czernik, A.J., Spray, D.C., Hertzberg, E.L., Greengard, P. and Bennett, M.V. (1990) Phosphorylation of connexin 32, a hepatocyte gap-junction protein, by cAMP-dependent protein kinase, protein kinase C and Ca2+/calmodulin-dependent protein kinase II, *Eur. J. Biochem.* 192:263-273.(Medline)

Sako, Y. and Kusumi, A. (1995) Barriers for lateral diffusion of transferrin receptor in the plasma membrane as characterized by receptor dragging by laser tweezers: fence versus tether, *J. Cell Biol.* 129:1559-1574.(Medline)

Sako, Y., Nagafuchi, A,, Tsukita, S., Takeichi, M. and Kusumi, A. (1998) Cytoplasmic regulation of the movement of E-cadherin on the free cell surface as studied by optical tweezers and single particle tracking: corralling and tethering by the membrane skeleton, *J. Cell Biol.* 140:1227-1240.(Medline)

Sargiacomo, M., Sudol, M., Tang, Z. and Lisanti, M.P. (1993) Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells, *J. Cell Biol.* 122:789-807.(Medline)

Saxton, M.J. (1993) Lateral diffusion in an archipelago. Dependence on tracer size, *Biophys. J.* 64:1053-1062 Lateral diffusion in an archipelago. Single-particle diffusion, *Biophys. J.* 64:1766-1780.(Medline)

Scales, S.J. and Scheller, R.H. (1999) Lipid membranes shape up, *Nature* 401:123-124.(Medline)

Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A.V., Witke, W., Huttner, W.B.and Soling, H.D. (1999) Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid, *Nature* 401:133-141.(Medline)

Schwartzmann, G., Wiegandt, H., Rose, B., Zimmerman, A., Ben- Haim, D. and Lowenstein, W. R. (1981) Diameter of cell-to-cell junctional membrane channel as probed with neutral molecules, *Science* 213:551-553.

Seabra M.C. (1998) Membrane association and targeting of prenylated Ras-like GTPases, *Cell Signal.* 10:167-172.(Medline)

Sheets, E.D., Simson, R. and Jacobson. K. (1995) New insights into membrane dynamics from the analysis of cell surface interactions by physical methods, *Curr. Opin Cell Biol.* 7:707-714.(Medline)

Sheets, E.D., Lee, G.M., Simson, R. and Jacobson, K. (1997) Transient confinement of a glycerophosphatidylinositol anchored protein in the plasma membrane, *Biochemistry* 36:12449-

12458.(Medline)

Sheetz, M.P., Schindler, M. and Koppel, D.E. (1980) Lateral mobility of integral membrane proteins is increased in spherocytic erythrocytes, *Nature* 285:510-511.(Medline)

Shull, G. E., Schwartz, A. and Lingrel, J. B. (1985) Amino-acid sequence of the catalytic subunit of the (Na^+, K^+) ATPase deduced from a complementary DNA, *Nature* 316:691-695.(Medline)

Simon, A.M. and Goodenough, D.A. (1998) Diverse functions of vertebrate gap junctions, *Trends Cell Biol.* 8:477-483.(Medline)

Simon, A.M., Goodenough, D.A. and Paul, D.L. (1998) Mice lacking connexin40 have cardiac conduction abnormalities characteristic of atrioventricular block and bundle branch block, *Curr. Biol.* 8:295-298.(Medline)

Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes, *Nature* 387:569-572.(Medline)

Simson, R,, Sheets, E.D. and Jacobson, K. (1995) Detection of temporary lateral confinement of membrane proteins using single-particle tracking analysis, *Biophys. J.* 69:989-993.(Medline)

Simson, R., Yang, B., Moore, S.E., Walsh, F.S. and Jacobson, K.A. (1998) Structural mosaicism in the submicron scale in the plasma membrane, *Biophys. J.* 74:297-308.(Medline)

Singer, S. J., and Nicolson, G. L. (1972) The fluid mosaic model of the structure of cell membrane, *Science* 175:750-751.(Medline)

Smart, E.J., Ying, Y.S., Mineo, C. and Anderson, R.G. (1995) A detergent-free method for purifying caveolae membrane from tissue culture cells, *Proc. Natl. Acad. Sci. USA.* 92:10104-10108.(Medline)

Somerharju, P., Virtanen, J.A. and Cheng, K.H. (1999) Lateral organisation of membrane lipids. The superlattice view.Biochim Biophys Acta. 1440:32-48. (MedLine)

Song, L., Varma, C.A., Verhoeven, J.W. and Tanke, H.J. (1996) Influence of the triplet excited state on the photobleaching kinetics of fluorescein in microscopy, *Biophys. J.* 70:2959-2968. (MedLine)

Song, K.S., Tang, Z., Li, S. and Lisanti, M.P. (1997) Mutational analysis of the properties of caveolin-1. A novel role for the C-terminal domain in mediating homo-typic caveolin-caveolin interactions, *J. Biol. Chem.* 272:4398-4403. (MedLine)

Stauffer, K.A. (1995) The gap junction proteins beta 1-connexin (connexin-32) and beta 2-connexin

(connexin-26) can form heteromeric hemichannels, *J. Biol. Chem.* 270:6768-6772.(Medline)

Svoboda, K., Block, S.M. (1994) Biological applications of optical forces, *Annu. Rev. Biophys. Biomol. Struct.* 23:247-285.(Medline)

Takeichi, M. (1991) Cadherin cell adhesion receptors as a morphogenetic regulator, *Science* 251:1451-1455.(Medline)

Temme, A., Buchmann, A., Gabriel, H.D., Nelles, E., Schwarz, M. and Willecke, K.(1997) High incidence of spontaneous and chemically induced liver tumors in mice deficient for connexin32, *Curr. Biol.* 7:713-716.(Medline)

Tomishige, M., Sako, Y. and Kusumi, A. (1998) Regulation mechanism of the lateral diffusion of band 3 in erythrocyte membranes by the membrane skeleton, *J. Cell Biol.* 142:989-1000.(Medline)

Tsuji, A., Kawasaki, K., Ohnishi, S., Merkle, H. and Kusumi, A. (1988) Regulation of band 3 mobilities in erythrocyte ghost membranes by protein association and cytoskeletal meshwork, *Biochemistry* 27:7447-7452.(Medline)

Unger, V.M., Kumar, N.M., Gilula, N.B. and Yeage, M. (1997) Projection structure of a gap junction membrane channel at 7 Å resolution, *Nature, Struct. Biol.* 4:39-43.(Medline)

Unwin, N. (1993) Nicotinic acetylcholine receptor at 9 resolution, *J. Mol. Biol.* 229:1101-1124.(Medline)

Unwin, P. N. T. and Zampighi, G. (1980) Structure of the junction between communicating cells, *Nature* 283:545-549.(Medline)

van Meer, G.and Simons, K. (1986) The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells, *EMBO J.* 5:1455-1464.(Medline)

Varma, R. and Mayor, S. (1998) GPI-anchored proteins are organized in submicron domains in the cell, *Nature* 394:798-801.(Medline)

Viitala, J. and Jarnefelt, J. (1985) The red cell surface revisited, *Trends Biochem. Sci.* 10:392-395.

Viola, A., Schroeder, S., Sakakibara, Y. and Lanzavecchia, A. (1999) T lymphocyte costimulation mediated by reorganization of membrane microdomains, *Science* 283:680-682. (MedLine)

Virtanen, J.A., Cheng, K.H. and Somerharju, P. (1998) Phospholipid composition of the mammalian red

cell membrane can be rationalized by a superlattice model *Proc. Natl. Acad. Sci. USA.* 95:4964-4969. (MedLine)

Warner, A. E., Guthrie, S. C. and Gilula, N. B. (1984) Antibodies to gap junctional communication in early amphibian embryo, *Nature* 311:127-132.(Medline)

White, T.W., Paul, D.L., Goodenough, D.A. and Bruzzone, R. (1995) Functional analysis of selective interactions among rodent connexins, *Mol. Biol. Cell* 6:459-470.(Medline)

Whiteley, N. M. and Berg, H. C. (1974) Amidination of the outer and inner surfaces of the human erythrocyte membrane, *J. Mol. Biol.* 87:541-561.(Medline)

Winckler, B,, Forscher, P. and Mellman, I. (1999) A diffusion barrier maintains distribution of membrane proteins in polarized neurons, *Nature* 397:698-701.(Medline)

Yamada, E. (1955) The fine structure of the gall bladder epithelium of the mouse, *J. Cell Biol.* 1:445-458.

Yguerabide, J. (1972) Nanasecond fluorescence spectrocopy of macromolecules, *Methods in Enzymology* 26:498-578.(Medline)

Zozulya, S. and Stryer, L. (1992) Calcium-myristoyl protein switch, *Proc.Natl.Acad.Scie. USA* 89:11569-11573.(Medline)

5. Internal Membranes

The myriad of functions of the cell are mirrored by the various compartments inside the cell, each performing a separate task. These compartments are generally bounded by membranes that control their interactions with the bulk of the cytoplasm and in some cases serve as the site of specialized transducing functions. This chapter will examine the role of the nuclear envelope and the mitochondrial membranes in regulating exchanges. Chapter 16-18 will examine the role of the mitochondrial and chloroplast membrane systems in energy transduction.

I. THE NUCLEAR ENVELOPE AND THE NUCLEOPORE COMPLEX

By virtue of its location, enclosing the chromosomes, the nuclear envelope must play a central role in development and in the physiology of the cell. The envelope is important in controlling both the traffic of RNA produced by transcription and the passage in or out of the nucleus of the macromolecules required for its function, including the control of genetic expression.

Most of the cell's RNA is synthesized in the nucleus but used in the cytoplasm for the synthesis of protein. Again, the nuclear envelope must be a significant player. Although the major sites of translation are in the cytoplasm, protein synthesis was also found to take place in the nucleus at sites of transcription (Iborra et al., 2001). The significance of this finding is still unclear.

Studies carried out with cells in culture, show that transport through the nuclear envelope depends on the phase of cellular activity (Feldherr and Akin, 1990). Furthermore, the location of certain proteins, whether in the nucleus or in the cytoplasm, is thought to reflect the developmental stage of cells. This is indicated by experiments in which the fates of four proteins present in the egg nuclei of *Xenopus*, the African frog, were followed in early development, from cleavage to neurula, using monoclonal antibodies labelled with a fluorescent dye (Dreyer et al., 1982). In the early stages, the proteins became isolated in the cytoplasm and were shifted to the nucleus at stages specific for the individual proteins. These experiments suggest a significant role of these proteins and of the transport mechanism in development.

The nuclear envelope is made up of two membranes joined at the openings of the numerous *nucleopore complexes* (NPC). EM and fluorescence microscopy studies (Fricker et al., 1997) of mammalian cells in culture, have indicated unexpected complexity of the envelope. This structure has deep invaginations open to the cytoplasm, some complex and some even transecting the nucleus. The NPCs can move within the nuclear envelope as shown using green fluorescent protein (GFP)-tagged component of the NPC (a nucleoporin) of one cell (see Chapter 1) in a yeast mating assay. When the two nuclei fused during normal mating, the GFP label passed from donor to recipient nucleus very rapidly (Bucci and Wenter, 1997).

Determination of many structural details of the NPC required novel approaches and sophisticated image reconstruction techniques. Eight spokes of the NPC form a diaphragm extending into the lumen of the pore. A large particle, approximately 35 nm in diameter at the center of the lumen, is thought to play a role in transport (see below). Particles of approximately 22 nm are frequently attached to the cytoplasmic rim of the pore. The structural arrangement is shown in Fig. 1 (Unwin and Milligan, 1982) and Fig. 2 (Akey, 1989).

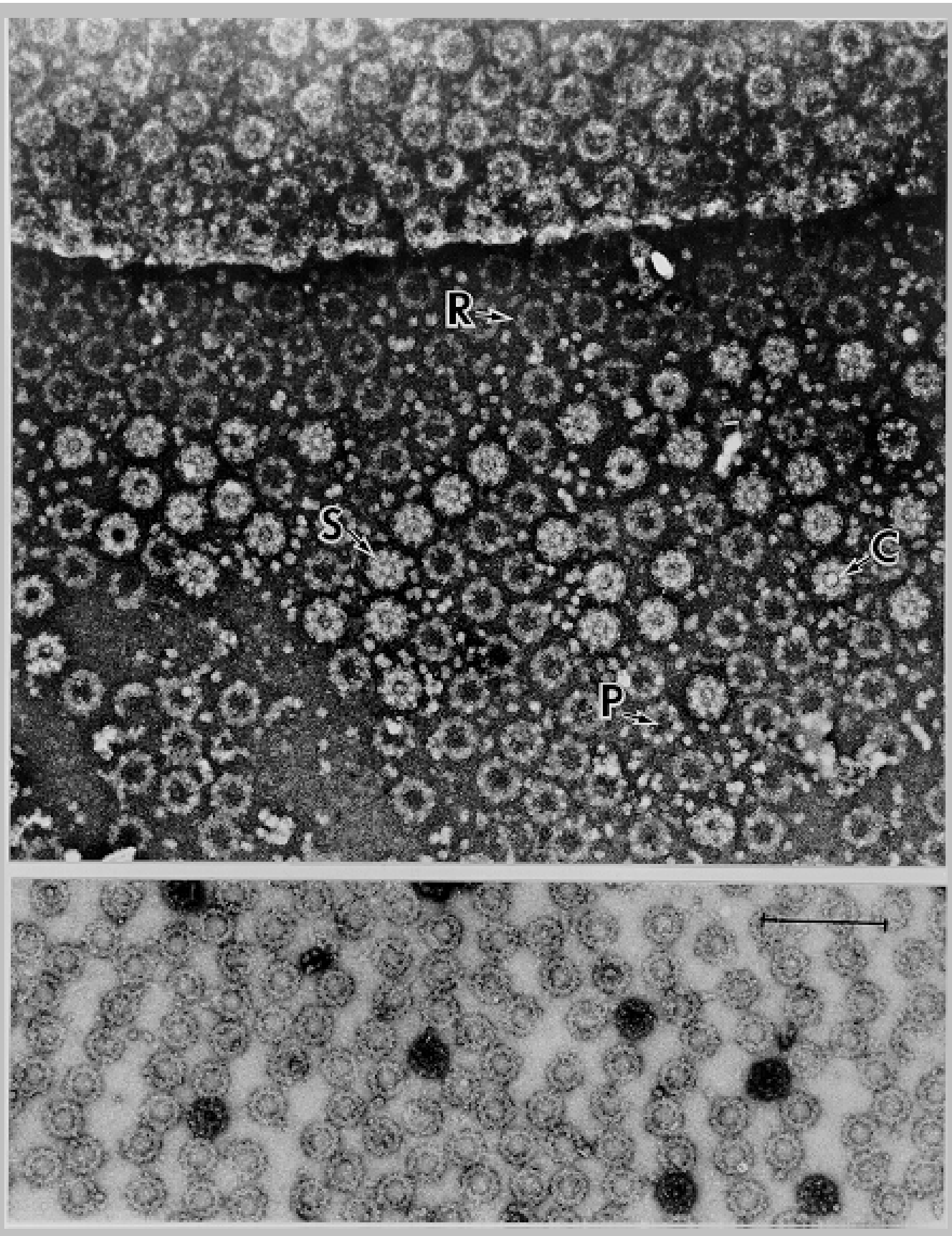


Fig. 1 Constituents of the nuclear envelope released after its partial detachment from a polylysine-treated carbon film in the presence of 0.1% Triton X-100. The constituents most obviously related to the pore complex are rings (R), central plug (C), spokes (S), and particles (P), occasionally observed around the rings. As the lower micrograph shows, the rings are sometimes obtained in large numbers by themselves. Uranyl acetate stain (bar corresponds to 300 nm). Reproduced from Unwin and Milligan, *The Journal of*

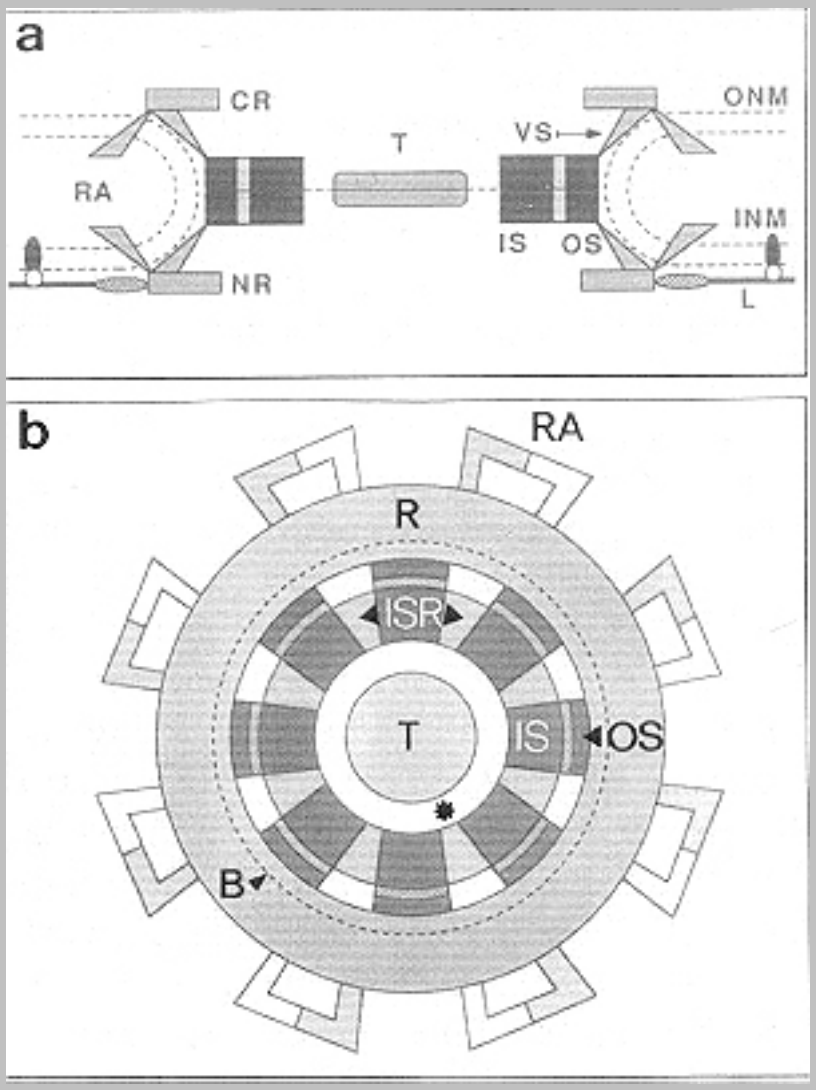


Fig. 2 Diagram of a nuclear pore complex (a) in central cross section and (b) seen from above. The major structural domains are indicated: inner spokes (IS), outer spokes (OS), vertical supports (VS), cytoplasmic coaxial ring (CR), nucleocytoplasmic coaxial ring (NR), and radial arms (RA). T indicates the transporter, which presumably has a central channel (not shown), and L the lamina attachment (Akey and Goldfarb, 1989). Reproduced from Unwin and Milligan, *The Journal of Cell Biology* by copyright © permission of the Rockefeller University Press.

An electronmicroscopic study of *Xenopus* oocyte nuclei using image analysis techniques (see Chapter 1) provides more detail and permits the reconstruction depicted in Fig. 3 (Hinshaw et al., 1992). In agreement with the previous diagram (Fig. 2), two peripheral rings, one cytoplasmic and one nucleoplasmic, are held together by a central spoke-like arrangement. There is little doubt that the central opening of the NPC is involved in the mediated transport of macromolecules (see below). However, this is not the only channel present. The 3D-image reconstruction indicates the presence of eight channels, each 10 nm in diameter, between adjacent spokes. These channels might be involved in the passive passage of solutes. For simplicity, Fig. 3 leaves out the transporter complex. A more recent reconstruction (Akey and Radermacher, 1993) differs in some of the details and, in addition, includes an

hour-glass shaped transporter complex filling the main channel (see the diagram of Fig. 4).

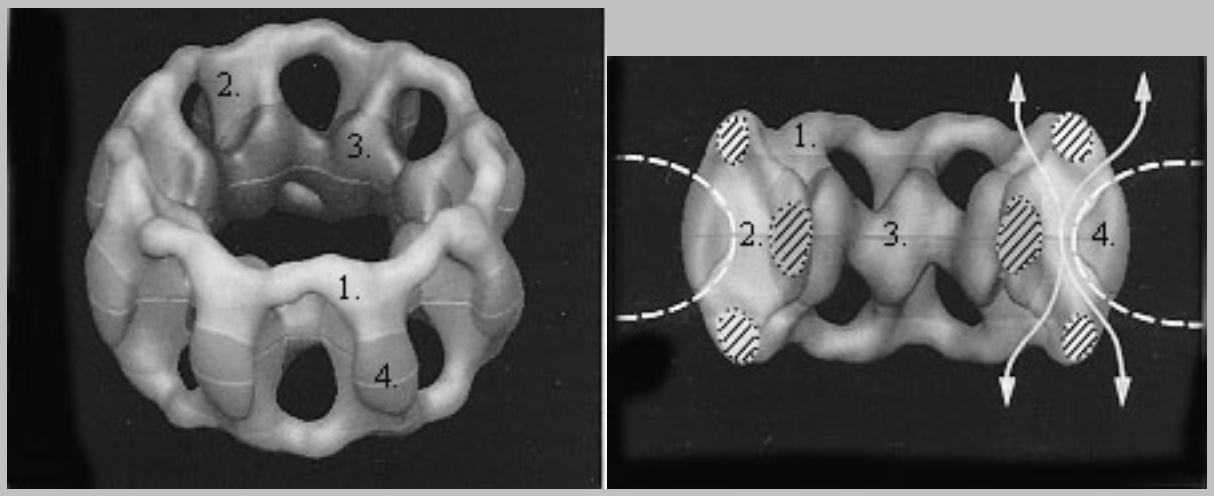


Fig. 3 Representation of the reconstruction of NPC. *1* indicates rings, *2* indicates the column subunits, *3* the annular subunits, and *4* the lumenal subunits. Drawn from Hinshaw et al., 1992. Reproduced by permission of Cell Press.

The structure of the NPC cannot be considered static. Averaging images during the transport of macromolecules, indicates that the central opening can expand during transport and the central transporter undergoes conformational changes during transport (Akey, 1990; Yang et al., 1998).

In amphibian oocytes, electron microscopy of quickly frozen and dried or critical point dried preparations (Jarnick and Aebi, 1991; Ris, 1991; Goldberg and Allen, 1992), reveals eight fibers 35-50 nm in length attached to the cytoplasmic ring and eight 50-100 nm fibers originating from the nucleoplasmic ring. The nucleoplasmic fibers are attached at their distal ends, forming a "basket" or "fish trap" (see Fig. 4). The "fish trap" appears to have different configurations, suggesting open and closed states (Jarnick and Aebi, 1991). Longer fibers, 3 to 6 nm in diameter (not shown in Fig. 4), extend from the basket into the nucleoplasm, in amphibian oocytes for as much as 1 μm (Cordes et al., 1993; Cordes, 1997; Zimowska et al., 1997), possibly forming a network of nuclear cables (Ris, 1997). The idea is emerging that these fibers may be involved in intranuclear transport.

The fibers on the cytoplasmic interface are difficult to see with conventional preparatory procedures. Their presence was previously deduced from the location of bound colloidal gold particles coated with nucleoplasmin in the process of being actively transported into the nucleus (Richardson et al., 1988). Furthermore, gold-labelled nucleoplasmin microinjected into *Xenopus* oocytes was shown to bind sequentially, first to the distant portion of the cytoplasmic filaments and then to the central channel (Panté and Aebi, 1996b). These filaments were therefore thought to be involved in the mediated transport process. However, nuclear import can occur without the filaments (Walther et al., 2002). In addition to these structures, a highly regular sheet of fibers forming a lattice were found attached to the nucleoplasmic rings of the NPC in the nuclear envelope isolated from oocytes of *Triturus* and *Xenopus* (Goldberg and Allen, 1992).

Most eukaryotic nuclei contain a distinct electron-dense layer, the *lamina*, between the inner membrane and adjacent chromatin elements. The lamina is absent in yeast. In some other eukaryotic cells the lamina is not prominent, but the presence of analogous elements is suspected. The lamina is thought to serve as a framework for the inner membrane of the nuclear envelope and to serve as an anchoring site for interphase chromosomes (Gerace et al., 1978). Similarly, the fibers of the lamina are thought to anchor the nuclear pore complexes (Gerace et al., 1978). The lamina is likely to disassemble reversibly during mitosis, possibly in a process regulated by phosphorylation-dephosphorylation cycles (Gerace et al., 1984). An additional lattice below the "fish trap" or "basket" arrangement has also been recently demonstrated. A diagram incorporating these features in a model are summarized in Fig. 4 (Rout and Wente, 1994).

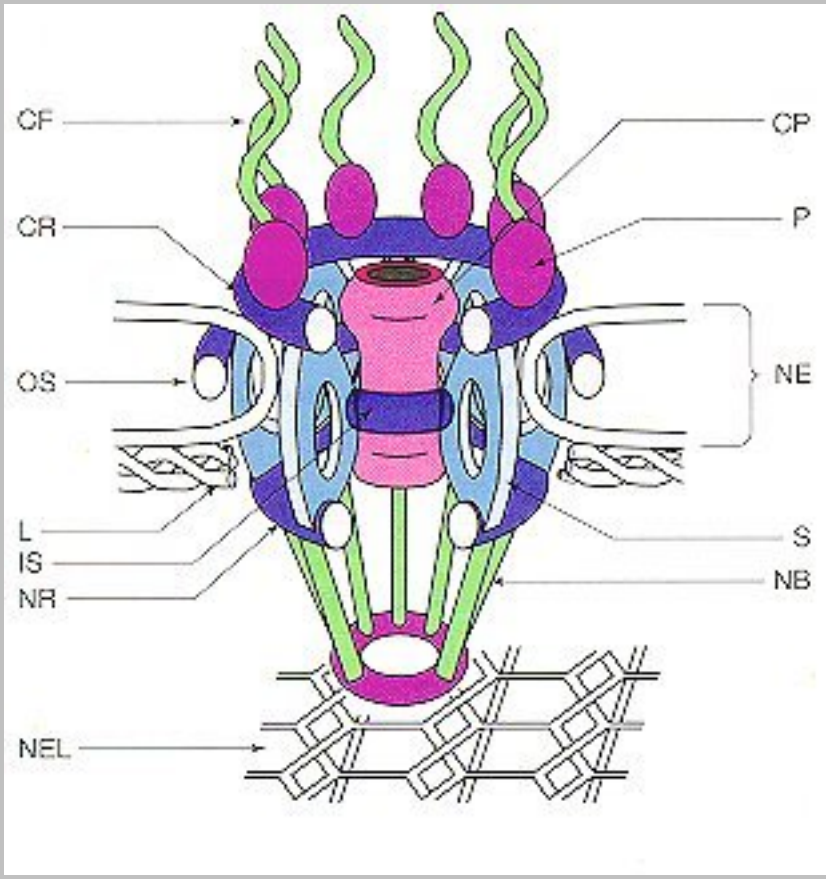


Fig. 4 A model representing the NPC drawn approximately to scale. The cytoplasmic face is on top. CF, cytoplasmic filament; P, cytoplasmic particles; CR, cytoplasmic ring; OS, outer spoke ring; IS, inner spoke ring; NR, nuclear ring; S, spoke; CP, central plug; NB, nuclear basket; NE, nuclear envelope; L, lamina; NEL, nuclear envelope lattice. Reproduced from *Trends in Cell Biology*, vol.4, Pores for thought: nuclear pore complex proteins, Rout, M.P. and Wente, S.R., pp.357-365, copyright &copy1994, with permission from Elsevier Science.

Studies of the yeast NPC (Yang et al., 1998; Fahrenkrog et al., 1998), using two vastly different preparatory techniques for electron microscopy, revealed similar features to those present in vertebrates as summarized in Fig. 4. Several differences were also found. The NPC of yeast is smaller. The study of

Yang et al. (1998) using frozen-hydrated specimen revealed that the inner ring is anchored to the membrane by another ring structure not previously described. The transporter is cylindrical and appears to be held in place by filaments. Furthermore, there are no lamina in yeast. Fahrenkrog et al. (1998) used a paraformaldehyde fixation and thin sectioning and immunogold techniques. The study revealed peripheral NPC structures: the cytoplasmic fibrils and the nuclear basket, and identified the location of some of the components (see below).

The NPC is readily isolated and many of the structural studies have been carried out on isolated, partially disrupted and reconstituted systems. However, progress in identifying the various macromolecules involved has been slow in vertebrate systems because the NPC is difficult to separate completely from nuclear and cytoplasmic contaminants. A protein is considered to be a nucleoprotein only if localized to the NPC using EM immunocyotochemical methods (see Chapter 1). Once identified, the proteins can be studied using the technology of molecular biology to produce and sequence cDNA. Each NPC probably contains as many as 30 to 50 different proteins, the *nucleoporins*, generally present in at least 8 copies each (see Paschal, 2002/a>) (see also below).

Knowledge of the components of the NPCs is crucial for our understanding of the function of the nuclear envelope. However, we should not lose sight of the fact that the NPCs must interface with components of the envelope itself. Although only a limited number of integral membrane proteins specific to the nuclear envelope have been recognized so far, Brr6p an integral protein coded by the essential gene BRR6, has been identified in the nuclear envelope (De Bruyn Kops and Guthrie, 2001). Brr6p depletion not only changes the distribution of nucleoporin but also affects the morphology of the nuclear envelope structure indicating that Brr6p is needed for the structural organization of NPCs. Furthermore, a BRR6 mutant has been found to be defective in the transport of mRNA and a protein containing a nuclear export signal.

Only relatively few nucleoporins have been identified in vertebrates. However, the number of those discovered in yeast had reached an excess of 30 in 1997 (see Doye and Hurt, 1997; Fabre and Hurt, 1997). The rapid progress is due to (a) the availability of biochemical procedures to isolate the NPCs in bulk, (b) the use of genetic screens to identify proteins interacting with known nucleoporins (*synthetic lethal screens*) and to isolate transport defective mutants, (c) the availability of information from the complete yeast genome. Synthetic lethal screens represent one of the most successful strategies. When a mutation in a single nucleoporin is tolerated by a cell, an additional mutation in a related component is lethal. The technique identifies proteins that actually bind to the original nucleoporin or are functionally related to it (see Fabre and Hurt, 1997).

A systematic mapping of the protein components of the NPC of *Saccharomyces cerevisiae* has been carried out by Rout et al. (2000). In this study, after a subcellular isolation of highly enriched NPCs, the proteins were separated by *high-performance liquid chromatography* (HPLC). The HPLC fractions were then subjected to SDS-PAGE separation. This was followed by the examination of each spot by trypsin digestion and mass spectroscopy. The determination of tryptic peptide masses (see Chapter 1) allowed identifying the open reading frames from the yeast genome data base (see Kuster and Mann, 1998). A

total of 174 proteins were identified and 40 appeared to be part of the NPC. These proteins were then localized in the isolated nuclear envelopes by immunofluorescence, subcellular fractionation and immmunolectronmicroscopy (see Chapter 1). In some cases, mild extractions had to be used to provide accessibility. Most docking proteins were found clustered around the openings or the transport channel.

A variety of NPC proteins have been found to have short repeat motifs. Recent tabulations with brief descriptions of structure and function can be found in review articles (Rout and Went, 1994; Bastos et al. 1995; Doye and Hurt, 1997; Fabre and Hurt, 1997).

Monoclonal antibodies and wheat germ agglutinin (WGA) that binds to the glycosylated portion of the glycoproteins, have permitted the identification of a group of NPC proteins with O-linked N-acetylglucosamine shown in Fig. 5. The glucosamines are attached at 10 to 20 different positions in the proteins, generally to serine or threonine. In mammalian nuclei there are at least eight of these groups attached to each protein molecule. Several supposedly O-glycosylated proteins have been detected in *S. cervisiae* (Sterne-Marr et al., 1992). These glycoproteins have been implicated in transport, because both monoclonal antibodies to these proteins and WGA block transport. Furthermore, when isolated nuclei are depleted of the O-linked glycoproteins, they become unable to carry out transport. Interestingly enough, cytoplasmic factors that bind to at least three of these proteins, are needed for mediated transport (Finlay et al., 1991). EM studies using WGA and immunological methods, show that glycoproteins are concentrated near the nuclear and cytoplasmic rings, suggesting that they may be related to the fibrils (Finlay et al., 1987, Snow et al., 1987).

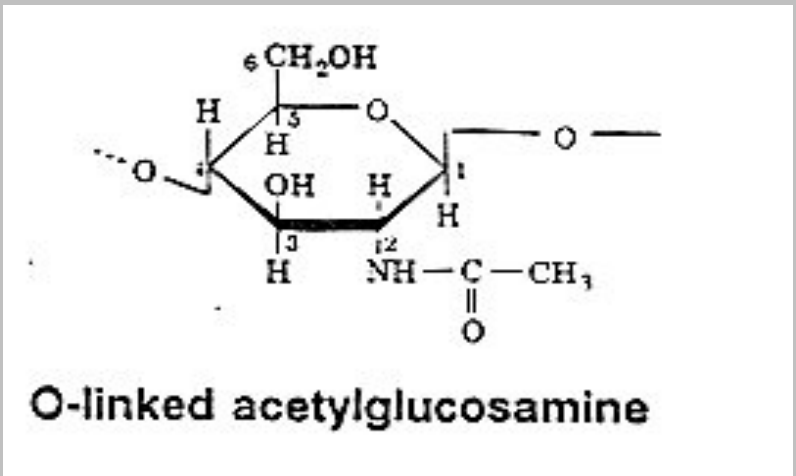


Fig. 5. O-linked acetylglucosamine.

Immunogold electron microscopy studies (see Chapter 1) have found several proteins associated with specific structures of the NPC. Cordes et al. (1997), have identified a polypeptide of approximately 270 kDa (p270) as a constituent of the intranuclear long filaments (see above) using a monoclonal antibody (not considered a nucleoporin). The human protein p270 is essentially identical to the protein Tpr reported by others to be located on the outer, i.e., cytoplasmic surface of NPCs (e.g. Byrd et al., 1994). This protein has extensive domains with coiled-coil confirmation. Present evidence suggests that this and

related proteins (e.g. Strambio-de-Castilla, 1999) are involved in intranuclear transport (see Zimowska et al., 1997; Shah et al., 1998). This suspicion is supported by the finding that the protein has also been found associated with importin β (Shah et al., 1998), a protein involved in the transport into nuclei (see below).

Electron microscopy studies of rat liver nuclear envelopes localized a 358-kDa protein (Nup358) at (or near) the tip of the cytoplasmic fibers of the NPC (Wu et al., 1995). Nup358 was shown to contain binding sites for two soluble nuclear transport factors: *importin* (*karyopherin*) and Ran-GTP (see Section C, below).

Immunoelectron microscopy using fragments of the CAN protein (now called Nup 214) showed association with the cytoplasmic side of the nuclear pore complex. This suggests that the protein is part of the short fibers that emanate from the cytoplasmic aspect of the nuclear pore complex. The protein was renamed Nup214 (nucleoporin of 214 kDa) (Kraemer et al., 1994).

Frequently, nucleoporins have been found to be present in complexes that can be isolated as such. The vertebrate p62 complex (p62, p58, p54 and p45) was the first to be characterized (e.g., Hu et al. 1996). p62, p58 and p54 have been shown to interact with cytosolic transport factors and are essential for nuclear protein import (Finlay et al., 1991). Immunogold electron microscopy (see Chapter 1) of isolated rat liver nuclear envelopes demonstrated that the p62 is present on both the nucleoplasmic and cytoplasmic sides of the NPC near the central gated channel involved in active transport of proteins and RNAs (Guan et al., 1995).

In yeast, Nsp1p (a homologue of p62) has been found in different complexes (the p62, Nup84p and Seh1p complexes). The Nsp1p was shown by immunogold electron microscopy, to be at the entry and exit sites of the central gated channel and at the terminal ring of the nuclear basket in three different complexes (Fahrenkro et al., 1998). These locations are NPC sites associated with cargo or transport ligands (e.g., see Görlich and Mataj, 1996). The results obtained with both rat liver nuclei and yeast, suggest that p62 and Nsp1p may be accompanying the cargo to the different sites.

Nup98, Nup93, Nup205 and Nup153 have been found to be associated with a region next to or at the nuclear basket (e.g., Sukegawa and Blobel, 1993; Radu et al., 1995b; Bastos et al., 1996; Grandi et al., 1997). Alteration in Nup98 and Nup153 (e.g., by injecting antibodies to Nup98, Powers et al., 1997) were found to inhibit RNA export (see Section E, below) without altering the capacity to import proteins (Bastos et al., 1996; Powers et al., 1997).

Much of our knowledge of how macromolecules are transferred through the nuclear envelope has been obtained by microinjecting macromolecules into either the cytoplasm or nucleus of intact cells, and by tracing their passage with the help of fluorescent labels (for light microscopy), radioactive labels (for autoradiography or other techniques), or colloidal gold (for electron microscopy). The large size of the

amphibian oocyte and its nucleus, such as that of the African frog, *Xenopus*, has made this cell the choice for micromanipulations, although the same principles seem to apply to other cells such as somatic mammalian cells. More recently, the use of "permeabilized cells" permitted more detailed studies (see below)

The next few sections (A to F) are dedicated to the transport of macromolecules in and out of the nucleus. As discussed in these sections, a good deal is known. However, the physical mechanism by which the various macromolecules and their receptors are translocated is still a mystery. Several possible transport mechanisms have been proposed for the active transport of macromolecules. The affinity gradient model proposes a transport path of increasing affinity (Ben-Efraim and Gerace, 2001). The model was inspired by the presence of a wide range of binding affinities of importin-β at distinct sites in the NPC for three nucleoporins. However, the transports through the NPCs is fully reversible so that the transport would be facilitated equally in either direction and not result in the accumulation of the cargo. In addition, kinetic measurements have indicated that high-affinity interactions are not needed for transport (Ribbeck and Görlich, 2001). The Brownian-affinity gate model (Rout et al., 2000) proposes that macromolecules attached to a receptor are more likely to entering the central channel because the interaction between receptor and nucleoporin favor the accumulation of cargo and receptor close to the channel entrance, possibly at the filaments, where the cargoes do accumulate. However, nuclear import can occur without the filaments (Walther et al., 2002). In addition, the model does not explain the transport of particles larger than the pore at rest or the passage of macromolecules. In the selective phase model (Ribbeck and Görlich, 2002) the phenylalanine repeats of the nucleoporins form an hydrophobic meshwork in the central channel which excludes hydrophilic molecules and binds to the FG repeats of the receptors (see below). Receptor binding to the FG repeats dissolve the meshwork which reforms behind the receptor. The permeability barrier was found to be disrupted reversibly by hexane diol whcih would interfere with the ability to interat with hydrophobic groups (Ribbeck and Görlich, 2002)

A. Passive Transport of Macromolecules

The passive passage of macromolecules into the nucleus seems to be controlled only by the size of the molecule. Microinjections of radioactively labelled dextrans have shown that dextrans of increasing size have increasing difficulty in entering (Fig. 6, Paine et al., 1975). The diameter of a pore with the permeability properties shown corresponds to 9 nm, approximately the size of a globular protein of 40-50 kDa. Fig. 6 shows the concentration of probe in the nucleus over that in the cytoplasm as a function of time for three probes of different sizes. The results are consistent with entry occurring passively through a channel. After equilibration, the apparent concentration of the probe molecules is higher in the nucleus, probably because of binding by the nuclear components. Virtually the same results were obtained with the microinjection of dextrans (Peters, 1984) and proteins (Reynolds and Tedeschi, 1984) into somatic mammalian cells.

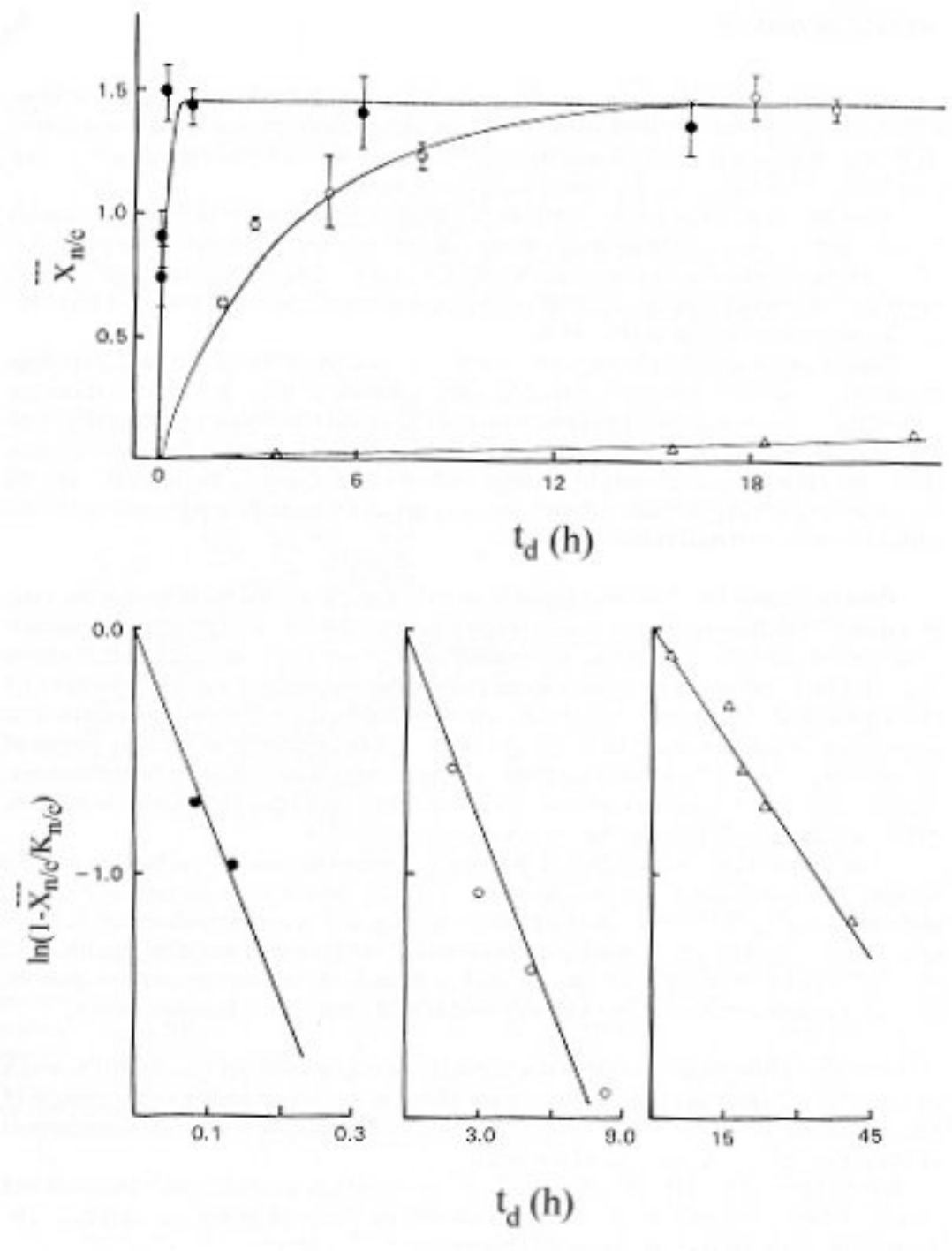


Fig. 6 (a) Time course of nuclear envelope permeation, expressed as average nuclear/cytoplasmic grain density as a function of diffusion time after microinjection, t_d, for 1.2 (•), 2.33 (O), and 3.55 (Δ) nm radius of ^{3}H-labeled dextrans. Vertical bars show standard error of the mean. (b) First-order exponential entry kinetics illustrated by plots for 1.2-, 2.33-, and 3.55 nm dextrans, respectively. K_{nc} is assumed to be 1.45. Note the different time scales on the abscissae (Paine et al., 1975). Reproduced with permission from *Nature* 254:109-114, copyright &copy1975 Macmillan Magazines Ltd.

The experiments discussed so far suggest that the nuclear envelope is very leaky to low molecular weight substances. However, there are indications that this is unlikely to be the case for at least some cells. For example, the electrical resistances of the envelope of *Drosophila* salivary gland nuclei (Lowenstein and

Kanno, 1962) and a variety of somatic nuclei (Lowenstein et al., 1966) are in the range of plasma membranes (1.5 Ω-cm^2). In addition, the nuclear envelope of several cells can be patch-clamped revealing a membrane a resistance of the patch in the GΩ-cm^2 range (see Bustamante, 1994) and in addition the activity of individual ion channels, inconsistent with the presence of a leaky membrane. A discussion of these factors is presented in Section F.

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

B. Active Import of Proteins into the Nucleus

In addition to the passive, sieve-like properties described above in section IA, special transport mechanisms involving the NPCs are also present. The traffic of macromolecules in and out of the nucleus via the NPCs is enormous. The movement in each NPC has been estimated to correspond to hundreds of proteins and ribonucleoprotein particles (RNP) per minute (Nigg, 1997). As we shall see, the macromolecules are either imported, exported or shuttled between the nucleus and the cytoplasm (see Table 1).

Much larger particles than protein molecules can be translocated. Cytoplasmic microinjection of colloidal gold coated with nucleoplasmin (Fig. 7) shows that the particles rapidly enter through the nucleopores (Feldherr et al., 1984). Figure 7a shows the colloidal gold in the cytoplasm just after injection and Fig. 7b and 7c show its entry into the nucleus through the nucleopore complex. Nucleoplasmin is a protein of 165 kDa extracted from the nucleus of *Xenopus* oocytes and is discussed in more detail in this section.

A special and complex role of the nucleopore in nuclear-cytoplasmic transport of proteins is suggested by a variety of observations. We saw that WGA and monoclonal antibodies to proteins derived from the nuclear envelope, block protein import into the nucleus. In addition, large particles such as colloidal gold, coated with a nuclear protein, can enter the nucleus even though they are larger than the nucleopore opening at rest.

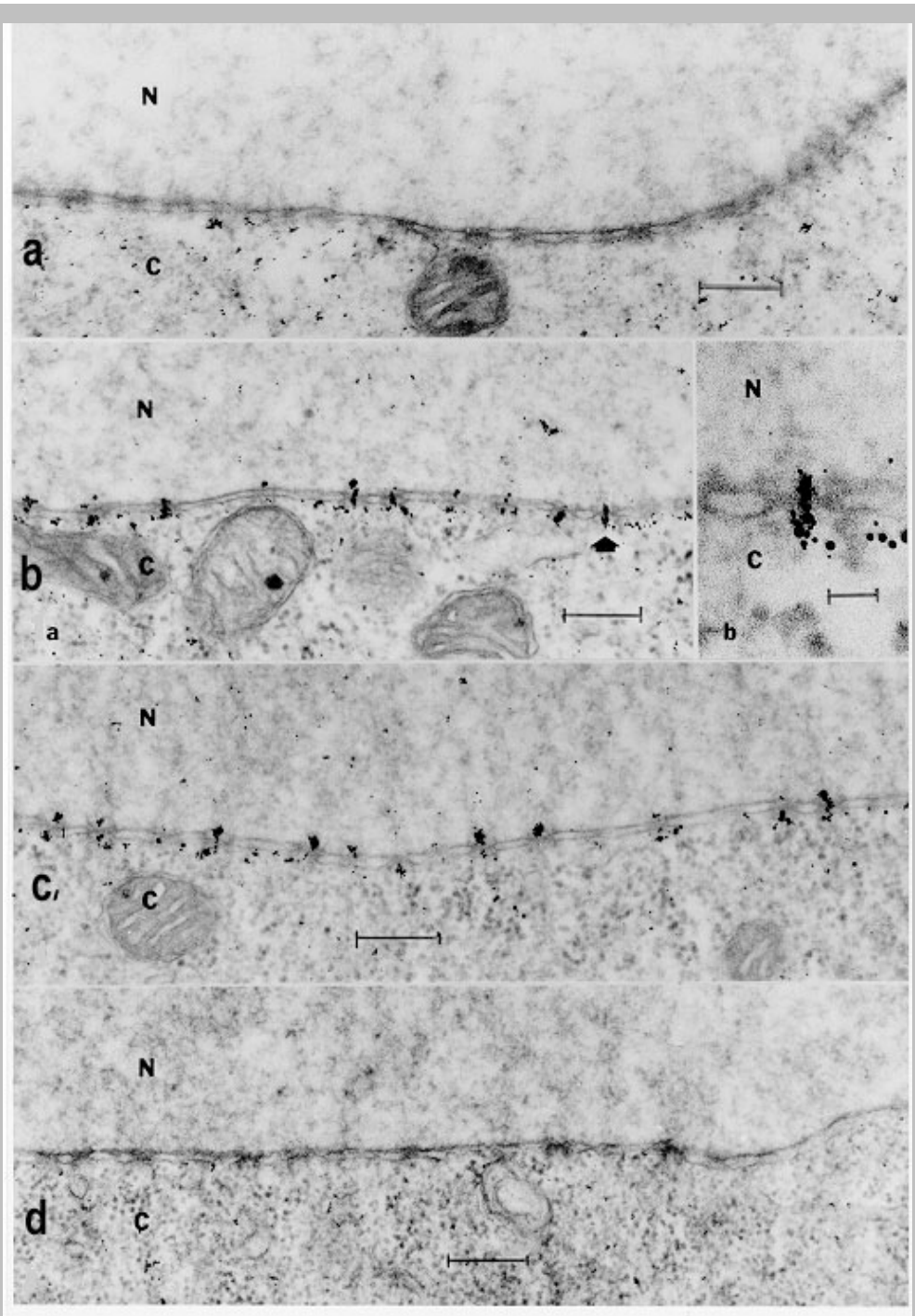


Fig. 7 Nucleoplasmin gold injection (a) 10 s after injection of gold-labeled nucleoplasmin. The gold is present only in the cytoplasm (C); the bar corresponds to 250 nm. (b) At 15 min after injection, gold is present in the cytoplasm and the nucleus (N). The accumulation can be seen next to and in the pore, bar corresponds to 250 nm (inset bar corresponds to 50 nm). (c) At 1 h after injection, the concentration in the nucleus is higher, bar corresponds to 250 nm. (d) Control: experiment in which the nucleoplasmin has been hydrolyzed. Gold is present only in the cytoplasm, bar corresponds to 250 nm. Reproduced from Feldherr et al., *Journal of Cell Biology*, by copyright © permission of The Rockefeller Press.

Other experiments offer clues to the nature of the transport mechanism (Dingwall et al., 1982; Dingwall and Laskey, 1986). Nucleoplasmin can be systematically cleaved with proteolytic enzymes. The protein, extracted from nuclei, is a pentamer. Each peptide of the pentamer possesses a tail segment (see Fig. 8).

Digestion produces a core pentamer segment missing some or all of the tail segments and, of course, the corresponding free tail segments. Any portion containing a tail, including a single tail segment, is capable of being rapidly transferred into the nucleus. The naked core segment, however, is not transferred in either direction. This has been demonstrated in experiments in which the nucleoplasmin was radioactively labeled and, after the appropriate hydrolysis with a protease, injected into the cytoplasm or the nucleus of the cells. The procedure and the results are shown in Fig. 8 (Dingwall and Laskey, 1986). These results suggest that the nucleoplasmin has a specific amino acid sequence that serves as a signal. Its role would be analogous to that of the signal sequences that determine the transfer of nascent polypeptide chains into the cisternae of the endoplasmic reticulum, or into the membrane structure itself. However, the signal sequences of the nucleus are not cleaved. Many other experiments have demonstrated a role of signal sequences which have been referred to as *nuclear localization sequences* (NLSs).

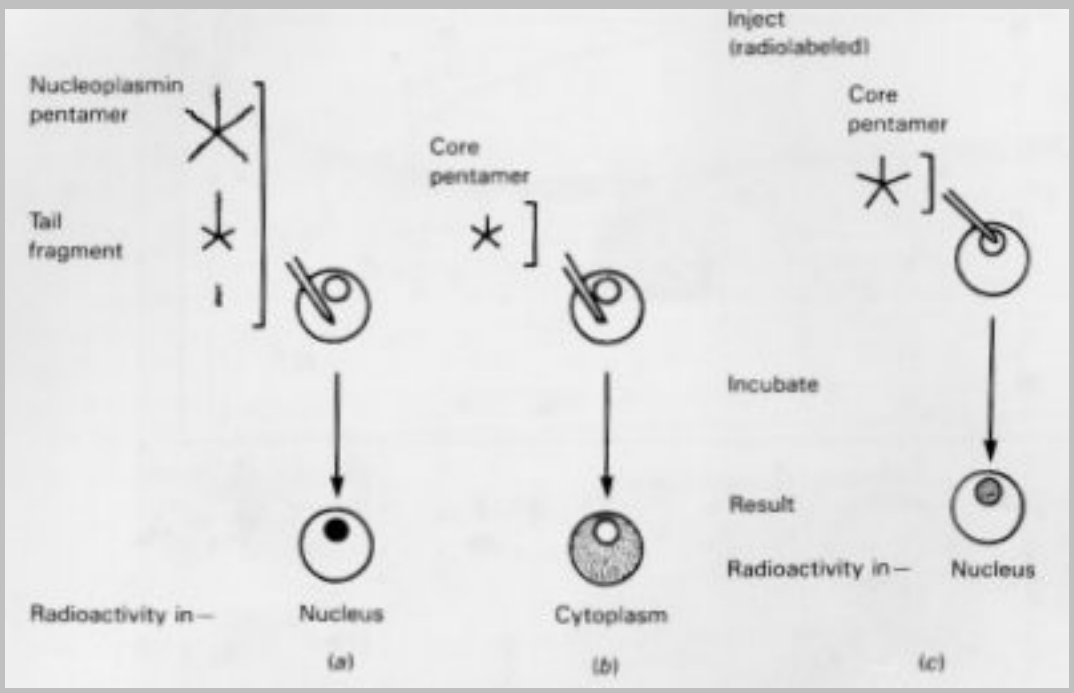


Fig. 8 Diagram illustrating the transport of nucleoplasmin molecules into the *Xenopus* oocyte nuclei. (a) The intact nucleoplasmin pentameter, a pentameter with a single intact subunit. The isolated "tail" fragment can accumulate in the nucleus after microinjection in the cytoplasm. (b) The nucleoplasmin "core" molecule cannot enter the nucleus after microinjection in the cytoplasm. (c) The core cannot reach the cytoplasm when microinjected in the nucleus (Dingwall and Laskey, 1986). Reproduced, with permission, from the *Annual Review of Cell Biology* 2, &copy1986 by Annual Reviews Inc.

The nuclear localization sequences

We saw that specific amino acid sequences determine whether a protein is localized in the nucleus. How can we identify these NLSs? The experimental design described in Fig. 8 provides a way of testing various possible sequences using synthetic peptides. An amino acid sequence can be covalently attached to a nonnuclear protein. Microinjection of the chimeric protein into the cytoplasm of an appropriate cell, such as an amphibian oocyte, will direct the entry of the protein into the nucleus only if the synthetic

portion corresponds to an NLS. This has been observed even for protein with an added NLS, such as ferritin, of 465 kDa and 9.4 nm diameter (Goldfarb et al., 1986; Lanford et al., 1986), or colloidal gold particles as large as 28 nm in diameter (Dworetzky et al., 1988), coated with a protein containing an NLS.

What constitutes an NLS? One kind of NLS is comprised of four to five basic amino acid residues in the large T antigen of a simian virus, SV40 (e.g., Garcia-Bustos et al., 1991). Other proteins, such as nucleoplasmin, have two basic amino acid domains separated by a 10 amino acid linker. This kind of NLS has been referred to as *bipartite*. Each domain resembles the large T antigen of SV40 and both are necessary for nuclear targeting. Approximately half of the nuclear proteins that have been sequenced have a bipartite NLS.

The properties of the bipartite NLS of nucleoplasmin were examined using nucleoplasmin mutants introduced into the cytoplasm of mammalian cells in culture (Robbins et al., 1991). Pyruvate kinase fused to mutant nucleoplasmin was injected into cells or alternatively introduced using a vector fused to the appropriate cDNA (see Chapter 1). An immunofluorescence method recognized the pyruvate kinase portion of the molecule. A mutation in only one domain did not abolish nuclear targeting. However, an additional similar mutation in the other domain would abolish targeting, indicating a cooperative involvement of the sites. Increasing the length of the spacer generally had no significant effect, but reducing the size of the linker decreased the transfer.

The two different signal systems, i.e., single and bipartite NLSs, involve the same receptors and individual pores are capable of recognizing and transporting proteins that contain different nuclear targeting sequences. In recent years, these NLSs have been referred to as the *classical* NLSs (see Weis et al., 1998).

When proteins are constructed with an increasing number of NLSs, the size of the nucleopore increases to a maximum of approximately 26 nm, and the rate of transfer also increases (Dworetzky et al., 1988).

Besides the two sets of NLS just discussed others are also present. Various signals involved in the transport of proteins across the NPC are listed below in Table 1 (Nigg et al., 1997).

A number of EM studies using colloidal gold coated with test molecules have implicated intermediate steps in the NPC-mediated transport. The results obtained with nucleoplasmin (Akey and Goldfarb, 1989), suggest that the protein is first bound predominantly to the periphery, possibly at the cytoplasmic filament sites (see Fig. 4). Subsequently, it is positioned at the center of the central particle. This structure, called *transporter* in this model, is thought to have a role in the translocation of macromolecules. Finally, the probe molecule passes through the transporter that is thought to have undergone a change to an open conformation. A summary of the various steps believed to be involved in nuclear mediated transport of proteins is represented in the model of Fig. 9 (Gerace, 1992).

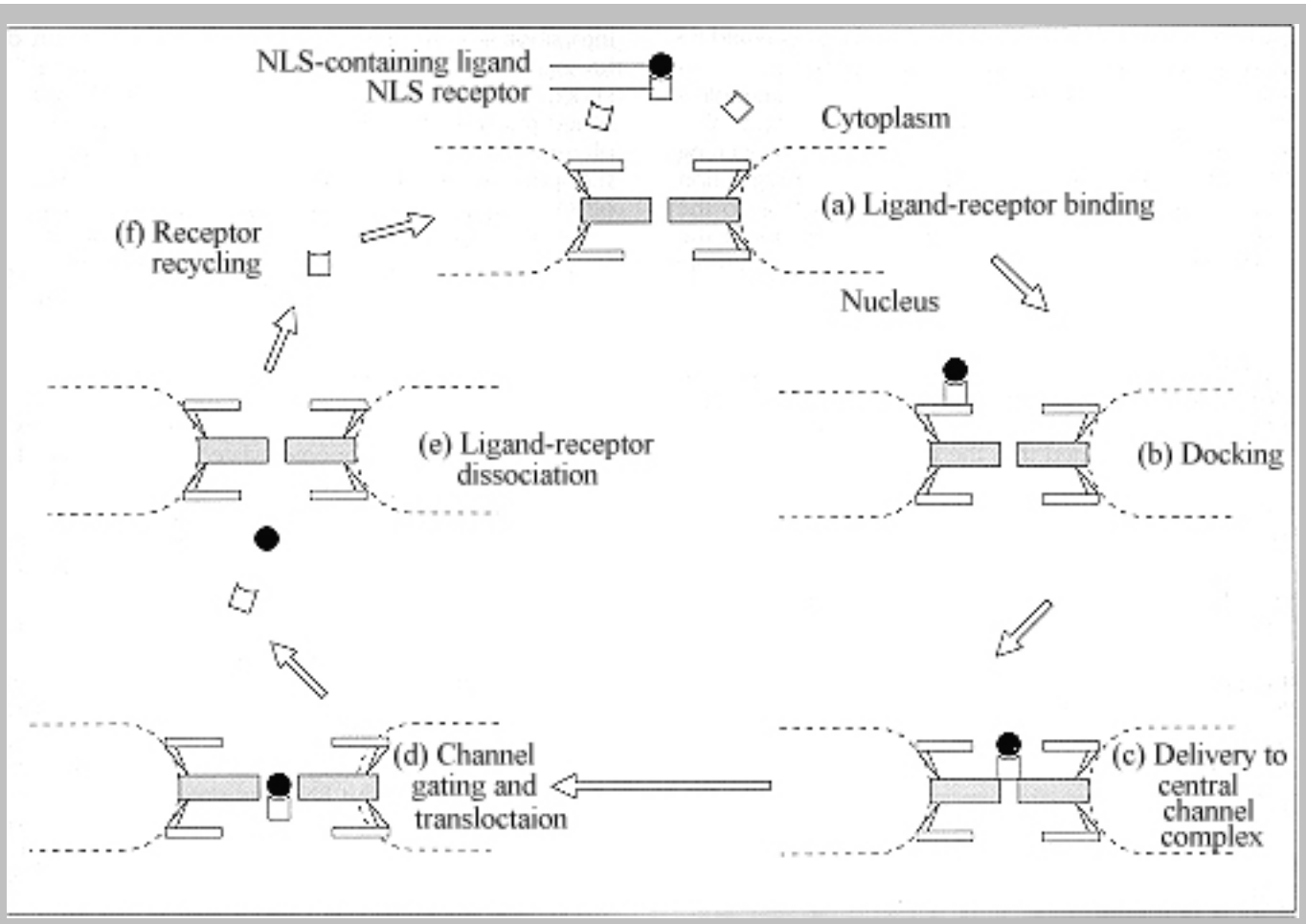


Fig. 9 A model representing some of our present understanding of the nuclear import of proteins. (a) First, the ligand containing NLS is bound by a cytoplasmic receptor. (b) The ligand-receptor complex docks at the cytoplasmic surface of the NPC. (c) The ligand-receptor complex is delivered to the central channel complex. (d) The central complex gates to allow the complex to be translocated. (e) The ligand and the receptor dissociate. (e) Finally, the receptor is recycled, presumably by leaving the nucleus. Reproduced from Gerace, 1992 by permission. (Available in the BioMedNet library at: http://biomednet.com/cbiology/cel)

The import machinery

How do NLSs provide the necessary information to trigger translocation? Evidence for the binding of the NLSs to specific receptors comes from the saturation of the protein import pathway when excess NLS sequences are microinjected into the cytoplasm (Goldfarb et al., 1986). Furthermore, competitive inhibition experiments of import or binding show that there are several pathways (e.g., Görlich et al., 1994; Weis et al., 1995). For example, SV40 and nucleoplasmin NLSs use the same receptors, but U snRNP (discussed below) does not compete (Michaud and Goldfarb,1991; Fischer et al., 1991).

Detailed studies of the molecular mechanisms of translocation require in vitro systems. Some studies use cells that have been rendered permeable by treatment with the detergent *digitonin*, and are said to be *"permeabilized"*. These cells are depleted of cytoplasmic components. Furthermore, they admit proteins added to the external medium that may be used to define the components needed for transport. These

generally have been derived from extracts from the cytoplasm of *Xenopus* eggs (see Görlich and Mattaj, 1996; Panté and Aebi, 1996a). In digitonin-treated cells, only four proteins were found to be absolutely required for the import of basic-type NLSs: *importin*-α, *importin*-β, *Ran* and *nuclear transport factor 2* (NTF2) (Moore and Blobel, 1993, 1994; Moroianu, 1995; Radu et al., 1995a). A heat shock protein, Hsp70, is involved in targeting and translocation, possibly as a chaperone to facilitate the formation of a receptor-NLS complex (Shulga et al., 1996).

Importin-α (also known as karyopherin α and in yeast as Kap60p and SP1), a protein of 60 kDa, was purified from *Xenopus* oocytes. Importin-α has been cloned and sequenced (Görlich et al., 1994) and corresponds to the 54/56 kDa protein purified from bovine red blood cells. This protein has been shown by immunofluorescence to be present in both the nucleus and the cytoplasm of mammalian cells (Imamoto et al., 1995a).

In *Sacchromyces cerevisiae*, there is only one importin α. Metazoan organism have several importin α-like proteins and these molecules have distinct specificities for NLS containing proteins. These variants of importin α apparently are expressed differentially in various tissues (Prieve et al., 1996; Nadler et al., 1997; Sekimoto et al., 1997; Nachury et al., 1998), suggesting that the nuclear import system may have a role in gene expression.

Importin-β (also known as karyopherin β and P97 and, in yeast, as Kap95p) is a 97 kDa protein originally purified from bovine red blood cells. Importin-α and importin-β are needed together for docking. The two together are bound to the nuclear envelope in an ATP and temperature independent process (Imamoto et al., 1995b,c). Importin-α is localized in both the cytoplasm and the nuclear envelope (Moroianu et al., 1995; Chi et al., 1995).

The NLS binds to the α-β heterodimer (e.g., Imamoto et al., 1995b). The α-component is responsible for binding the NLS, whereas the β component binds to the initial docking site (Görlich et al., 1996). The NLS-protein enters the nucleus along with importin-α, Ran and NTF2 (Moroianu et al., 1995; Görlich et al., 1995). Therefore, importin-α is the shuttling carrier of NLS-proteins. Since importin-β does not enter the nucleus, the α -β-complex must dissociate before the complex is transferred to the nuclear interior.

Ran, a small GTP-ase, and the 10 kDa protein NTF2 (also called p10) allow the import of docked proteins in cytosol-depleted cells. They enter the nucleus together with the NLS-protein (Moroianu et al., 1995). The Ran.GTP complex (but not Ran.GDP) directly binds to the NPC cytoplasmic filaments (e.g., see Fig. 4) (Wu et al., 1995; Yokoyama et al., 1995). GTP-binding proteins constitute a family of proteins that are involved in signal transduction and intracellular transport (see Chapter 7, 10 and 11).

Recent experiments suggest that the role of the GTPase and the hydrolysis of GTP might be in restoring the competence of the transport system after each transport event (see Steggerda and Paschal, 2002). In

an in vitro system using permeabilized cells, non-hydrolyzable nucleotide triphosphates were found to suffice for a single translocation of substrate in either direction where the export was mediated by the export receptor CRM1 (exportin 1) and the import by importin (karyopherin-β1). Similarly, single import events were found to occur in the absence of Ran GTPase. The energy needed is provided in the step of recycling receptors in the cytoplasm (Englmeier et al., 1999). Similar experiments were carried out by Ribbeck et al. (1999). They found that the translocation of *transportin* (karyopherin-β2) + substrate into the nucleus does not require either Ran or nucleotide triphosphates. Transportin is involved in the import of mRNA-binding proteins (see below). However, RanGTP is required in the nucleus to dissociate the tranportin-substrate complex and to re-export transportin. GTP hydrolysis is needed to restore the import competence of the re-exported transportin

NTF2 was identified after depletion with the nucleopore protein p62 (Paschal and Gerace, 1995). NTF2 is thought to be involved in the translocation of the NLS-protein from the initial docking site to the central gated channel or transporter (see section above) (Melchior and Gerace, 1995). NTF2 also binds to Ran.GDP, suggesting an involvement in triggering GTP hydrolysis and translocation across the channel.

Several proteins are very similar to karyopherin β, among these transportin that is also called karyopherin β2 (karyopherin β is now generally called β1). Other proteins have been found to belong to this family of proteins and are listed in a review of Wozniak et al., 1998). They include (Rout et al., 1997) karyopherin β3 (Kap121p/Pse1p) and β4 (Kap123p.). They are involved in the import into the nucleus of unassembled ribosomal proteins. They bind to RanGTP and copurify with NPCs (Rout et al., 1997). β4 interacts directly with ribosomal NLSs without the need for karyopherin α.

The presence of nine additional karyopherins of this family is suspected from the nucleotide sequences that code for them (see Fornerod, 1997). Proteins of the karyopherin β family are also involved in nuclear export. Crm1p (CAS in mammals) functions in the transport of proteins containing the leucine rich *nuclear export signal* (see Section D) (e.g., Stade et al., 1997).

The transport of proteins from the cytoplasm to the NPC apparently involves microtubules (Giannakakou et al., 2000) since overexpression of dynamitin (which binds to dynein) or microinjection of anti-dynein antibody prevents the entry of p53 indicating an involvement of dynein, a microtubular motor.

NPC components and import of proteins

What are the protein components of the NPC (the nucleoporins) required for the translocation? Genetic and biochemical studies carried out in yeast have begun to answer this question (e.g., Rout and Blobel, 1993; see Doye and Hurt, 1995; Davis, 1995). Mutations of some essential nucleoporins interfered with both protein import and export, suggesting that the two are coupled, or at least have parts of the pathway in common. Other mutant phenotypes revealed functional overlaps between some of the nucleoporins.

The nucleoporins generally contain peptide repeats of the FXFG (single letter code, X=any amino acid) sequence (vertebrates) or the GLFG sequence (yeast) separated by very hydrophilic spacers. It has been estimated that there are up to 30 phenylalanine repeats per nucleoporin and an estimated 10^4 per NPC; these are referred to as FG-motifs . The FG-motifs have been found to bind to transport receptors in vitro (e.g., Radu et al., 1995b; Rexach and Blobel, 1995; Iovine et al., 1995; Stutz et al., 1996). The FG motifs differ somewhat, allowing for the possibility that different FG motifs interact with different transported molecules. The structural bases for this interaction has been elucidate in some cases (Bayliss et al., 2000, 2002; Fribourg et al., 2001)

Some nucleoporins also have heptad repeats that favor an α-helical coiled-coil conformation. Some have motifs that favor protein-protein or protein nucleic acid binding (e.g., cysteine-zinc motifs). Others have octapeptide motifs characteristic of RNA-binding proteins. However, the significance of these motifs is not yet established.

The first step of the translocation is thought to correspond to a binding of the receptor-cargo complex to RanBP2 (Melchior et al., 1995), a protein localized in the cytoplasmic fibers (see Fig. 4) of the NPC (e.g., Wilken et al., 1995). RanBP2 is very large, with a molecular weight of 358 kDa. Four of its domains can bind RanGTP. There are also several FG repeats, a zinc-finger domain, a leucine-rich region, and a cyclophilin-like domain (e.g., Wu et al., 1995; Yokoyama et al., 1995). *Cyclophilin* is a chaperone that acts as a peptidyl propyl isomerase.

RanBP2 has been purified from rat liver nuclei (Delphin et al., 1997). Electron microscopy shows that it is a filamentous molecule about 36 nm in length with a diameter of about 5 nm in its extended form, suggesting that it is the major component of the cytoplasmic fibers of the NPC (35-50 nm in length; see section I and Fig. 4). RanBP2 binds to importin α an interaction that also involves RanGTP.

Shah et al. (1998) used immunoprecipitation to identify complexes of nucleoporins and importin subunits in *Xenopus* egg extracts. In addition, they were able to localize the components to regions of the nucleopore. Among several proteins tested (Nup62, Nup93, Nup98 or Nup214/CAN), only Tpr (a protein associated with intranuclear fibers) and Nup153 (associated with intranuclear "basket structures") were found in complexes with importin α and β. Nup153 binds to a complete import complex containing importin α and β consistent with an involvement in nuclear import. Importin β binds directly to Nup153. The GTP analog GMP-PNP disassembles both kinds of complexes. Apparently Nup153-importin β and Tpr-importin β complexes are present in intact nuclear pores.

Translocation involves movement through the NPC, across a considerable distance. The mechanism for this movement is unknown. Stepwise cyclic attachment and detachment of one of the components (e.g., importin-β) from sites across the NPC could produce the translocation. Possibly a GTP-GDP hydrolysis cycle would bind and release at each site (see Görlich and Mattaj, 1996).

Import of proteins associated with RNA

Ribosomal protein are first translocated into the nucleus and then transferred to the nucleolus. In the nucleolus, they are assembled into pre-ribosomal particles also containing the ribosomal RNA. Only a few signal sequences have been identified for the protein components. In yeast, the ribosomal protein L25 has a complex bipartite signal at the carboxy-terminal (Schaap et al., 1991). Receptors different from those previously described are involved (see above) (Rout et al., 1997; Schlenstedt et al., 1997). These receptors resemble the β1-transport factor and may also be involved in the transport of other ribosomal proteins. The GTP-protein Ran is also a participant in the translocation of these proteins.

Two pathways for nuclear import of mRNA-binding proteins have been described. As in the case of ribosomal protein, they are mediated by factors homologous to importin β, in this case, transportin (also called importin β2) that is also involved in the import of a subset of mRNA binding proteins (e.g., Fridell et al., 1997). Transportin, a 90 kDa protein, is somewhat related to importin-β. Despite their similarity to importin β, the ribosomal protein and the two mRNA-binding protein pathways, do not require importin α and the transport factors interact with the nucleopore components directly (e.g., Rout et al., 1997; Bonifaci et al., 1997).

The hnRNP A 1 (see below) wild type M9 (see below) import signal binds to transportin, whereas certain M9 mutants are unable to do so (Pollard et al., 1996). Transportin is clearly an NLS-binding protein, distinct from importin β. In vitro, incubation with excess M9 depletes the cytoplasmic extract. Proteins with the M9 NLS can no longer be transported into the nucleus. However, the transport of proteins with other NLSs are not affected. Transportin in the presence of Ran is able to mediate the transport of M9-proteins in an in vitro system of permeabilized HeLa cells (Izaurralde et al., 1997a). Because hnRNP A1 probably participates in mRNA transport, the role of transportin may be very significant. A protein with similar function, Kap104p, has been isolated from yeast (Aitchison et al., 1996). Kap104 can be extracted from the nucleus combined with two RNA binding proteins. In yeast, an additional pathway mediated by Np13p has also been found (Pemberton et al., 1997). The nuclear localization of A1, or a non-nuclear protein fused to M9, requires the ongoing RNA polymerase II transcription (Siomi et al., 1997).

The idea that different pathways may be involved in protein transport is supported by the observation that M9 containing proteins do not compete for import into the nucleus with UsnRNPs or proteins containing other NLSs (Izaurralde et al., 1997b).

An additional pathway, also involved in the import of mRNA-binding proteins, has been identified (Pemberton et al., 1997). The transport protein, Mtr10p, is involved in the translocation of the mRNA-binding protein Np13p.

C. Protein Export and Shuttles

Several studies indicate that import-export processes of the nuclear envelope require signal sequences

other than conventional (i.e., classic) NLSs. Nuclear localization of the shuttling hnRNP protein A1 is mediated by a nuclear localization signal of 38 amino acids at the carboxyl end of the molecule, rich in glycine, the so called *M9* domain (Siomi and Dreyfuss, 1995). Similar sequences were found in other RNA-binding proteins. Attaching this segment to proteins usually retained by the cytoplasm, induces their transport into the nucleus. M9 also serves as a *nuclear export signal* (NES). When fused to a protein normally retained in the nucleus, NES induces its export (Michael et al., 1995a). Therefore, a protein with the M9 segment can shuttle between nucleus and cytoplasm. Presumably, the binding of different receptors to different motifs in the M9 domain plays a role in the two different functions.

An additional sequence of approximately 40 amino acids, distinct from the M9 domain, mediates both import and export of hnRNP K proteins (Michael et al., 1997). This signal has been called *KNS* (K is for "hnRNP K", N stands for "nuclear and S for "shuttling"). hnRNP K has two NLSs, the conventional bipartite signal and, in addition, KNS. Removal of the bipartite NLS makes the import dependent on RNA polymerase II, suggesting that the two transport signals operate independently. The import pathway is distinct from that followed by either conventional NLSs or M9.

Many proteins, including some of those that bind to RNA, shuttle between the nucleus and the cytoplasm. The amino acid sequences of three shuttling proteins, the heat stable protein kinase inhibitor (PKI), Rev, and the protein A1 of hnRNP, have permitted the recognition of short sequences functioning as NESs. Attached to various proteins, the NESs mediate their translocation from the nucleus to the cytoplasm (Wen et al., 1995; Fischer et al., 1995; Michael et al., 1995b). The NES of PKI and Rev corresponds to a sequence of approximately 10 amino acids rich in leucine, entirely different from the 38-amino acid M9 domain. These observations suggest that there are distinct pathways for the export of proteins or RNAs.

A protein identified as an exportin, Msn5p (also called Kap142), functions in the nuclear export of two proteins in yeast (e.g., Kaffman et al., 1998; DeVit and Johnston, 1999).It also functions in the import of another cargo protein. Msn5p was isolated in a complex containing the *trimeric replication protein A* (RPA) (Yoshida and Blobel, 2001). RPA is required for DNA replication, DNA repair and recombination. Msn5p is required for RPA import into the nucleus as shown in yeast from which the corresponding gene has been deleted. Similarly, a new importin, importin 13 (Imp13), has been found to be involved in the import of several proteins into the nucleus (Mingot et al., 2001). Imp13 is also active in the export of an entirely different protein, the translation initiation factor eIF1A .Imp 13 also differs from other importins in relation to the discharge of eIF1A. Other importins functions are controlled by the RanGTPase system and allow binding the importin to its substrate and its subsequent release on opposite sides of the nuclear envelope. In contrast, Imp13 transport is regulated indirectly by RanGTP. The release of eIF1A from Imp13 depends on the binding of import substrates onto Imp13. It is likely that in the future other carrier proteins also may be found to move one cargo in one direction and another cargo in the opposite direction.

As discussed above, the GTP binding protein Ran is involved in the inward transport of proteins. It has also been shown to be involved in the export of mRNA from the nucleus as well (Schlenstedt et al., 1995).

In contrast to the NLSs and the NESs, other motifs act as *nuclear retention signals* (NRSs). The non-shuttling hnRNP proteins, such as hnRNP C1, are restricted to the nucleus because they contain an NRS of approximately 80 amino acids that can override the NES (Nakielny and Dreyfuss, 1996).

In order to continue the import of proteins into the nucleus, importin-α and -β must be recycled to the cytoplasm once the NLS-protein has been delivered in the nucleus. Importin-β is recycled much faster than importin-α, suggesting that they follow separate pathways (Görlich et al., 1995). Importin-α depends on its IBB domain (defined in Table 1) for entry into the nucleus (Görlich et al., 1996). However, when attached to another non-nuclear protein, IBB serves only as an entry signal; a different mechanism for translocation from the nucleus to the cytoplasm must be operative. There might be, for example, two different conformations of the importin.

The various signals involved in the transport of proteins and RNAs across the NPC are listed in Table 1 (Nigg, 1997).

Table 1 Signal sequences involved in transport across the pore. From Nigg, 1997. Reproduced by permission

(**a**) Import Cargo	
Proteins SV40 T antigen nucleoplasmin c-myc	Nuclear localization signals (NLS) P**KKKRK**V (monopartite NLS) **KR**PAATKKAGQA**KKKK** (bipartite NLS) **PAAKR**V**KLD**
RNAs U snRNAs 5S RNA	 2,2,7-trimethyl guanosine (m_3G) cap and Sm core proteins NLS on ribosomal protein L5 (?)

(**b**) Export cargo	
Proteins	Nuclear export signals (NES)
PKI (Rat)	**L**ALK**L**AG**L**D**I**
Rev (HIV-1)	**L**PP**L**ER**L**T**L**D
TFIIIA (Xenopus)	SLVLDKLTI
IκBα (Mouse)	QQLGQLTLENL
RNAs	hnRNP proteins such as hnRNP protein A1 (M9)
mRNAs	7-methyl guanosine (m7G) cap, recognized by cap-binding proteins (CBC)
U snRNAs	NES on TFIIIA or ribosomal protein L5 (?)
5S RNA	? (exported as periribosomal particles)
rRNAs (28S,18S,5.8S)	?
tRNAs	

(**c**) Shuttling proteins

Importin-α

Import signal (=importin β- binding domain)
RMRKFKNKGDTAELRRRRVEVSVELRKAKKDEQILKRRNV
(IBB domain)
export signal unknown

hnRNP protein A1 (rapidly shuttling)

import signal= export signal (M9)
N**QSSNFGPMK**GG**NFGG**RSS**GPYGGG**G Q**Y**FAKPRNQ**GGY**

nucleolin (slowly shuttling)

import signal= bipartite NLS: no export signal

The nomenclature can be confusing. If you wish to review it click "nomenclature"

As we saw in the case of protein import to the nucleus, binding of the NLS to cytoplasmic receptors is required. It would seem likely that a similar mechanism operates in the case of nuclear export of proteins to the cytoplasm. A protein of 110 kDa (called p110, CRM1 or *importin 1*) capable of binding NESs, has been implicated in the export (Fukuda et al., 1997; Fornerod et al., 1997; Ossareh-Nazari et al., 1997). A cytotoxin that blocks export (*leptomycin B*, LMB) binds to p110. Furthermore, export is disrupted in yeast with a mutation in the *crm1* gene.

In addition to proteins that shuttle between cytoplasm and nucleus, there are several instances in which the physiological role of a cytoplasmic protein can be fulfilled only if it is able to be transferred from the cytoplasm to the nucleus (e.g., see Chapter 7). These events can only occur if the translocation can be regulated. The protein will remain in the cytoplasm if, for example, the NLS is blocked either by complexing with other proteins or by intramolecular masking. In the first case, the detachment of the masking subunit and in the second, a conformational change in the molecule will expose the NLS and result in a shift to a nuclear localization. Phosphorylation of the proteins also regulates transport. Even the protein nucleoplasmin which we discussed earlier, is imported in the nucleus more rapidly when phosphorylated (Vancurova et al., 1995). The regulation of protein transport has been recently reviewed (Jans and Hübner, 1996).

As discussed above, the small GTPase Ran plays an essential role in the transport reactions. Our present understanding of the functioning of the RanGTP (see Ohno et al., 1998) in nuclear-cytoplasmic exchanges are based on the observations that: (a) RanGTP causes the dissociation of at least some import receptors-cargo complexes, (b) the concentration of RanGTP is likely to be high in the nucleus and low in the cytoplasm, and (c) two of the export receptors (CAS and CRM1) are bound tightly to their cargo only at high RanGTP concentration. These factors suggest a mechanism in which import takes place by the binding of the import cargo to its receptor in the cytoplasm (low concentration of RanGTP), followed by its release in the nucleus (high RanGTP). In contrast, export would occur by binding to the export receptor in the nucleus (high concentration) and release of the cargo in the cytoplasm after hydrolysis of the GTP. Various factors facilitating the hydrolysis are present in the cytoplasmic side of the nuclear envelope. In this model, the traffic would be directed and powered by the Ran hydrolysis of GTP.

D. RNA Transport

Export of RNAs from the nucleus exhibits a good deal of complexity and present information suggests the presence of multiple transport pathways. All RNAs are packaged as ribonucleoprotein particles, or RNPs. The RNAs are generally exported along with protein components, and the proteins are subsequently imported back into the nucleus (Piñol-Roma and Dreyfuss, 1992; see Nakielny and Dreyfuss, 1997).

RNA movement within the nucleus

Since RNA is synthesized in the nucleus, for our discussion, the story actually starts with its transcription, continues while RNA moves through the nucleoplasm and is brought to the NPCs where it is translocated. It ends with the arrival in the cytoplasm.

How can the pathway of RNAs through the cell nucleus be followed? Several methods have been used. The results seem to be contradictory. In one of these methods, the RNA in question was localized by hybridization to DNA probes labelled with a fluorescent label, such as fluorescein-avidin. Epstein-Barr virus (EBV)-infected cell types were used. The mRNA studied corresponded to EBV primary transcripts. Newly synthesized messages were found in discrete tracks in the nucleoplasm (Xing and Lawrence, 1991). Furthermore, the distribution of the transcripts were approximately the same after extraction of the nuclei. The extracted nuclei had lost 95% of their protein, DNA and phospholipids. This suggests that the RNA is present in stable subenvironments of the nuclear matrix. Other studies are not in agreement with this inference, although it is difficult to reach a final conclusion.

Other experiments suggest the presence of nuclear fibers involved in the movement of particles. The chromatin of insect salivary gland cells is multistranded (forming the so called *polytene chromosomes*), easily visualized even with the light microscope (also discussed in Chapter 2). The chromosomes contain *Balbiani rings*, forming loops that are actively transcribing mRNA. Electron microscope tomographic (see Chapter 1) studies of nuclei of midge salivary gland cells, observed Balbiani ring pre-mRNP particles in transit from the gene to the NPCs (Miralles et al., 2000). One-third of particles were found in contact with thin connecting fibers, which sometimes merged into large fibrogranular clusters. The interpretation of experiments using fluorescent probes is more complex and difficult to evaluate and do not suggest a role of fibers.

The movement of fluorescein-labeled oligodeoxynucleotides (oligos) inside the nucleus of cultured rat myoblasts was studied by fluorescence correlation spectroscopy (FCS) and fluorescence recovery after photobleaching (FRAP; see Chapter 4) (Politz et al., 1998). Supposedly, the oligos hybridize to RNA. Fluorescence correlation microscopy is a single-molecule detection technique based on the confocal principle (see Chapter 1). It is used to quantify molecular diffusion and concentration of fluorescent molecules with sub-micronresolution. The results indicate that the movement of a large portion of the oligos is at rates consistent with diffusion when complexed to RNA or in RNA-protein complexes. A subfraction of oligo(dT) (15%) moved over ten times more slowly, suggesting that this fraction is bound to very large macromolecular complexes.

In later experiments oligo(dT)s [which would complex with the poly(A) of mRNA] labeled with *caged* fluorescein were released at specific nuclear sites in rat myoblast cells by laser spot photolysis (Politz et al., 1999) and the spot of fluorescent hybridized oligo(dT) was tracked by fluorescence microscopy. Caged chemicals are unreactive complexes where the active component can be released by laser photolysis at a specific time and location. The movement was found to be in all directions with properties characteristic of a random diffusion. Three-dimensional imaging of live cells containing uncaged oligo(dT) suggests that the poly(A)-RNA could access most and perhaps all of the non-chromosomal space in the nucleus. Since the molecules being traced are hybridized to poly (A), the technique may miss a fraction of the mRNA where the poly (A) is shielded (e.g., bound to protein).

Some of these results may be reconciled. A small portion (15% in the study of Politz et al., 1998, 30% in the study of Miralles et al., 2000) could be attached to structures, possibly fibers, with the rest moving freely. The experiments with the caged oligo(dTs) as mentioned could have missed part of the mRNA (the portion attached to structures?). In addition, the results obtained with EBV primary transcripts may apply only to the viral RNA (the study of Xing and Lawrence, 1991).

Passage through the nucleopore complex

The ability of nuclei to export RNA and import the NLS-proteins specifically, poses an important question. Are the NPCs specialized, some being responsible for the transfer of proteins and perhaps others for RNA? The import of nucleoplasmin and export of RNA can be followed simultaneously with the electron microscope when the two are labeled with antibodies bearing gold particles of different sizes (Dworetzky and Feldherr, 1988). The two transfers appear to proceed through the same pore, even though they must take place in opposite directions. In addition, the mechanisms implicated in the export of RNA from the nucleus are likely to include components in common with those involved in the import of proteins: both can be blocked by antibodies to nucleoporins (Featherstone et al., 1988), the O-linked glycoproteins discussed above (however, see below).

Mehlin et al., (1992) have studied the transfer of mRNA directly, by examining the translocation through the nucleopore of a specific premessenger-RNP of the particles from the *Balbiani* rings of *Chironomus tentans*. In *Chironomus* each Balbiani ring is distinguished by numbering. Rings 1 and 2 correspond to genes 35-40 kb in length and encode secretory proteins of 1,000 kDa. The transcripts are packed in large particles about 50 nm in diameter (e.g., Lamb and Daneholt, 1979). The particles have been studied and analyzed using EM tomography (Mehlin et al., 1992) (see Chapter 1). The RNP particle is a thin ribbon bent into a ring-like structure. For the purpose of analysis, Mehlin et al. considered the structure to contain four domains, numbered 1 to 4. During the assembly, which is closely linked to the transcription, domain 1 is the first to be completed, implying that this domain is the 5' end of the RNA, since transcription begins with the synthesis of this end. The particle appears first to orient itself on the nucleopore in a specific manner. The ribbon is then unravelled and transported through the pore with the 5' end of the RNA leading (Fig. 10, Mehlin et al., 1992).

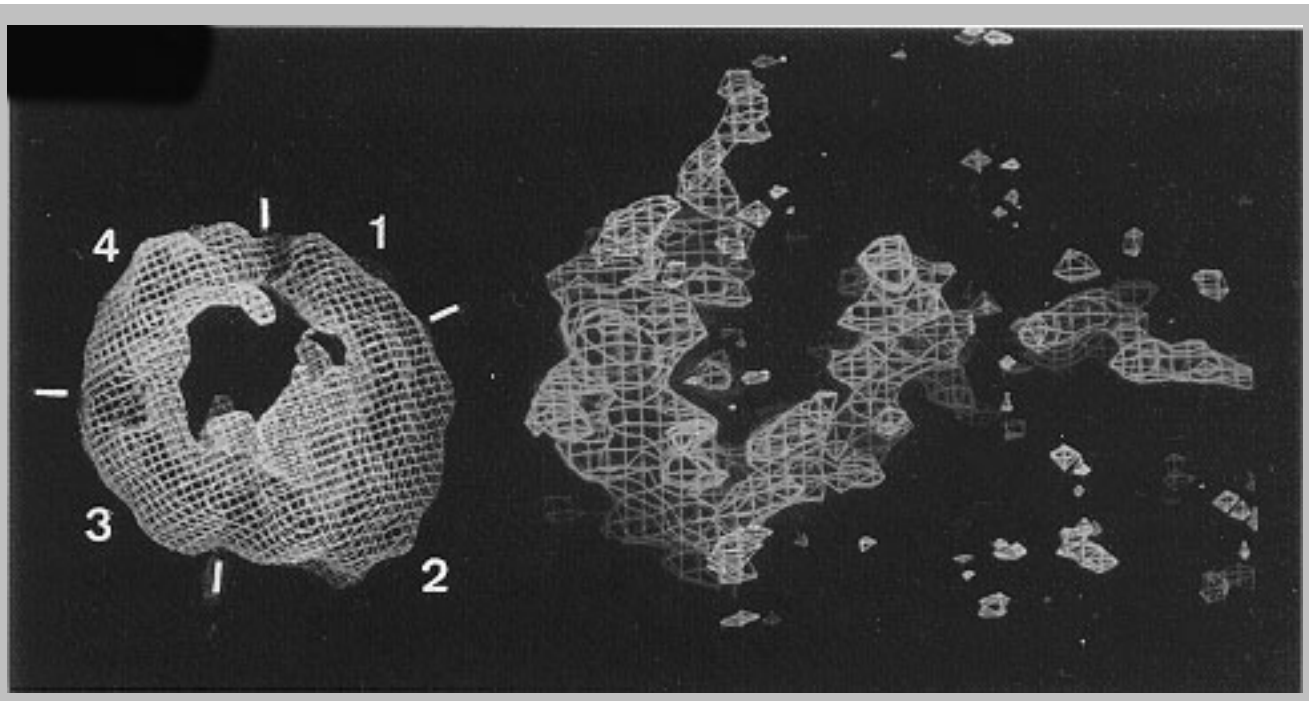


Fig. 10 Tomographic analysis of an RNP particle. An RNP particle in transit (right) is compared with a nucleoplasmic RNP (left). The solid shapes correspond to portions of the NPC. Reproduced from Mehlin et al., 1992 by permission.

Nuclear RNA export

Generally, RNAs are exported from the nucleus after adapter proteins bind to the nascent transcript and subsequently the adapters interact with receptor proteins. In order for export to occur, the RNA-adapter-receptor combination must interacts with NPC proteins. For example, the binding to adapter has been shown in *Saccharomyces cerevisiae*, where mRNAs are combined cotranscriptionally with factors required for export (Lei et al., 2001). This has been shown in various ways. Immunoprecipitation (see Chapter 1) of chromatin demonstrated that Npl3 was recruited to actively expressed genes. In addition, Npl3 has be found in a complex with the transcription machinery as indicated by its binding to RNA polymerase II. NP13 is an RNA-binding protein that shuttles in and out of the nucleus and is required for the export of mRNA (see Lee et al., 1996). Similarly, the mRNA export factor, Yra1, was also found to associated with chromatin cotranscriptionally. Mutations in the hnRNP Npl3 and TATA-binding protein (TBP) blocked mRNA export. The export of RNA is thought to follow alternative pathways as discussed below.

In the case of mRNA, only the mature form (i.e., the spliced form) can be exported, possibly because pre-mRNA is retained bound to splicesomal factors (Legrain and Rosbash, 1989) or alternatively, selection for export might depend on removal of nuclear retention signals (NRS) on a protein companion of the mature mRNA.

Multiple pathways

The study of retrovirus has helped elucidate some of the pathways of RNA export (see Cullen, 1998). Complex retroviruses (e.g., *human immunodeficiency virus type 1*, HIV-1) have been studied intensively. HIV-1 encodes REV, which acts as an adapter. REV binds to an RNA-target sequence found on all incompletely spliced RNAs, the *Rev response element* (RRE) (e.g. Malim et al., 1989, 1990). REV also possesses an NES (e.g., Fischer et al., 1995; Wen et al., 1995). The NES is the binding site for a heterodimer of CRM1-RanGTP (Fornerod et al., 1997a; Neville et al., 1997; Stade et al., 1997). The receptor CRM1 (*chromosome maintenance region 1*, exportin 1) supposedly is responsible for targeting the RNA-CRM1-RanGTP complex to the NPC where it binds to nucleoproteins including Nup214/Can (Fornerod et al., 1997b, Neville et al., 1997).

In contrast, at least some simple retroviruses (e.g., type D retrovirus), contain a domain in the RNA, the *constitutive transport element* (CTE) that permits nuclear export of unspliced Mason-Pfizer monkey virus (MPMV) (Bray et al., 1994) and can substitute for the REV-RRE system to mediate HIV-1 RNA export. Unlike to the REV system, the export of CTE is independent of CRM1 function as shown by failure to inhibit by the drug leptomycin B (which inactivates CRM1) (Otero et al., 1998). In addition, the nucleoporin (which follow the REV pathway) mutants which fail to associate with the NPC block REV but not CTE function (Bogerd et al., 1998; Zolotukhin and Felber, 1999).

Which of the two pathways are used by the various kinds of RNA? The role of the REV domain in the export of other RNAs was studied by cross-linking REV to albumen and microinjecting it into *Xenopus* oocyte nuclei in the presence of RNA labelled with [^{32}P]. High levels of REV NES were found to inhibit 5S rRNA and UsnRNA export but not that of mRNA (Fischer et al., 1995). Therefore, the results indicate a role of the REV pathway in the transport of the first two RNAs but not mRNA. With a similar experimental design, CTE containing RNA (e.g., MPMV-RNA) were found to block the export of mRNA, apparently by sequestering cellular mRNA export factors (Pasquinelli et al., 1997; Saavedra et al., 1997). Therefore mRNA is exported via the CTE pathway.

These experiments indicate two distinct RNA export pathways, one involving RRE and responsible for the export of RNA of complex viruses, 5S rRNA and UsnRNA. The second pathway, the CTE pathway, is active in the export of certain simple viruses and mRNA. Other studies provide additional information, Nup98 is a nuclear pore O-linked protein found in the oocytes of *Xenopus laevis*. Injection into *Xenopus oocyte* nuclei of antibodies to Nup98 block the export of snRNA, 5S RNA, large ribosomal RNAs and mRNA without affecting tRNA or protein import (Powers et al., 1997). Therefore, these result indicate a convergence of the two RNA export pathways just cited and an additional transport system responsible for tRNA export.

Studies of the competition between various RNAs are in agreement with the notion that there are distinct pathways. They suggest that the different classes of RNA are exported, at least in part, by the mediation of class-specific factors (Jarmolowski et al., 1994; Pokrywka et al., 1995), probably RNA-binding proteins acting as adapters. (Fischer et al., 1995). Several of these have been identified. For example, *exportin-t* binds tRNAs and in the presence of Ran-GTP mediates their tranfer to the cytoplasm (Arts et

al., 1998; Kutay et al., 1998).

CRM1 is the best understood mammalian receptor. It binds to cargo (e.g., Ossareh-Nazari et al., 1997) through the leucine rich NES of about ten amino acids of the adapter proteins (HIV-1 REV, PHAX abd Nmd3p).

The receptors and adapters of mRNAs are not as well understood. HnRNP A1 is thought to be an adapter which shuttles in-and-out of the nucleus by virtue of its M9 domain (see below). HnRNP A1 has been found to be involved in the export of dihydroxydfolate reductase-mRNA. Other shuttling proteins are suspected adapters (e.g., hnRNP K, SRP20, 9G8, ASF/SF2 and UAP56) (Huang and Steitz, 2001; Gatfield et al., 2001; see also Görlich and Kutay, 1999).

The yeast mRNA receptor Mex67p [TAP in mammals (e.g., Katahira et al., 1999)] uses the Yra1p protein (Aly/REF protein in mammals) as adapters (e.g., Luo et al., 2001). It was found to interact with NPC components and to be essential for nuclear export of CTE containing RNA (Segref et al., 1997). Mex67 acts in conjunction with Mtr2 with which it forms a complex (Santos-Rosa et al., 1998). The nuclear protein Yra1p has been shown to bind M67p and RNA. Mutants of YRA1 are impaired in nuclear poly(A)+ RNA export at restrictive growth conditions (Strässer and Hurt, 2000) (see also Chapter 3).

Human TAP specifically binds to wild-type CTE. CTE of the simian type D retroviruses recruits TAP. 372 amino acid residues of TAP containing leucine-rich repeats are responsible for binding to the CTE (Braun et al., 1999). The complex of TAP and the p15 protein (see below) can replace the Mex67p-Mtr2p complex in yeast. In mammalian cells, TAP usually detected in the nucleus, is also found in NPCs and shuttles between the nucleus and the cytoplasm. TAP binds mRNA in vivo, as evidenced by UV-crosslinking to poly(A)+ RNA in HeLa cells. TAP binds to the nucleoporin CAN/Nup214 through the FG-repeat domain. It also binds to a 15 kDa protein (p15) with homology to NTF2 . NTF2 is a nuclear transport factor (which associates with RanGDP). Two NLSs and one NES of TAP are involved in the shuttling as shown using microinjection techniques (Kang and Cullen, 1999).

Several proteins have been found to be involved with mRNA export from the nucleus. One of these is Gle1p which interacts with the protein cofactor Ri1p (Murphy et al., 1996). Gle2p (Rae1p in *S. pombe* or RAE1 in mammals) has been implicated in mRNA transport in *S. cerevisiae* (e.g. Murphy et al., 1996). Gle2p binds to the nuclear envelope component Nup116p through a short *Gle2p-binding motif* (GLEBS) also present in the human Nup98 protein (Bailer et al., 1998). In mammalian cells, RAE1 binds to a GLEBS-like NUP98 motif at the NPC through several domains as shown by binding studies and cross-linking experiments (Pritchard et al., 1999). Microinjection into the nucleus of *Xenopus* oocyte showed that RAE1 shuttles between nucleus and cytoplasm. The export is temperature dependent and does not require RanGTP. A variety of observations indicate a role of RAE1 in mRNA transport and RAE1 is most likely to be a shuttling transport factor involved in the translocation of mRNA (Pritchard et al., 1999). For example, docking of RAE1 to the nuclear envelope depends on new mRNA synthesis; overexpression of the GLEB-like motif blocks the binding of RAE1 to the NE and produces an

accumulation of poly(A) RNA in the nucleus, and these are reversed by the overproduction of RAE1.

The heterogeneous nuclear (hn) RNPs are a group of over 20 proteins (A to U) that bind pre-mRNA immediately after transcription (see Chapter 3). Some of the hnRNP proteins shuttle between nucleus and cytoplasm. Since they contain an RNA binding domain, they are thought to act as mediators of mRNA transport (see Izaurralde and Mattaj, 1995). hnRNPs have a role in transcriptional regulation, maintenance of telomere length, alternative pre-RNA splicing and processing of the 3'end (see Krecic and Swanson, 1999). They have been discussed in relation to splicing in Chapter 3. Their role in regulation of the fate of cytoplasmic mRNA is discussed in Chapter 15.

Several of hnRNP such as hnRNP A1 contain an M9 motif (see above) and are thought to act as adapters. The M9 of 38 amino acids binds to the receptor *transportin 1* (Trn1) which belongs to the importin β family known to shuttle hnRNP A1 (e.g., Siomi et al., 1997). Injection of hnRNP A1 into the nucleus of *Xenopus* oocytes competitively inhibits the export of mRNA, but does not interfere with the export of other kinds of RNA. These findings suggest that M9 containing proteins play a role in mRNA export from the nucleus. M9 is a motif that has been implicated in both nuclear import and export. Injection of a mutant hnRNP A1 protein lacking the M9 had no effect. In contrast, saturating levels of M9 had no effect on the import of either U snRNPs or proteins carrying a basic NLS sequence (Izaurralde et al., 1997b).

The pre-mRNA and the mRNA remain bound to proteins as long as the mRNA molecules are in the nucleus. The transport of RNAP A1 is one of the best understood. A1 binds with high affinity to RNA sequences resembling pre-mRNA 3' and 5' splice sites (e.g., Burd and Dreyfuss, 1994). A1 shuttles rapidly between nucleus and cytoplasm in an RNA polymerase II dependent manner (Piñol-Roma and Dreyfuss, 1991, 1992). In the cytoplasm, A1 is bound to poly(A)+-RNA suggesting that it plays a role in the export of the mRNA. Some hnRNP proteins, including A2 and K, also shuttle (see Michael et al., 1995). However, other hnRNP, such as hnRNP C1, C2 and U, are localized only in the nucleus and presumably have some other role. Not surprisingly, C1 has a NRS, probably responsible for the retention (Nakielny and Dreyfuss, 1996).

Eukaryotic transcripts of RNA polymerase II have a monomethyl cap that is added postranscriptionally (Izaurralde et al., 1994; 1995). This cap is required for export, as indicated by studies in which the monomethyl cap of pre-mRNAs was substituted with a trimethyl cap (Hamm and Mataj, 1990). On the other hand, a trimethyl cap and another motif in the core of the complex, are required for the reentry of U snRNP into the nucleus. The structure of these caps is shown in Fig. 11.

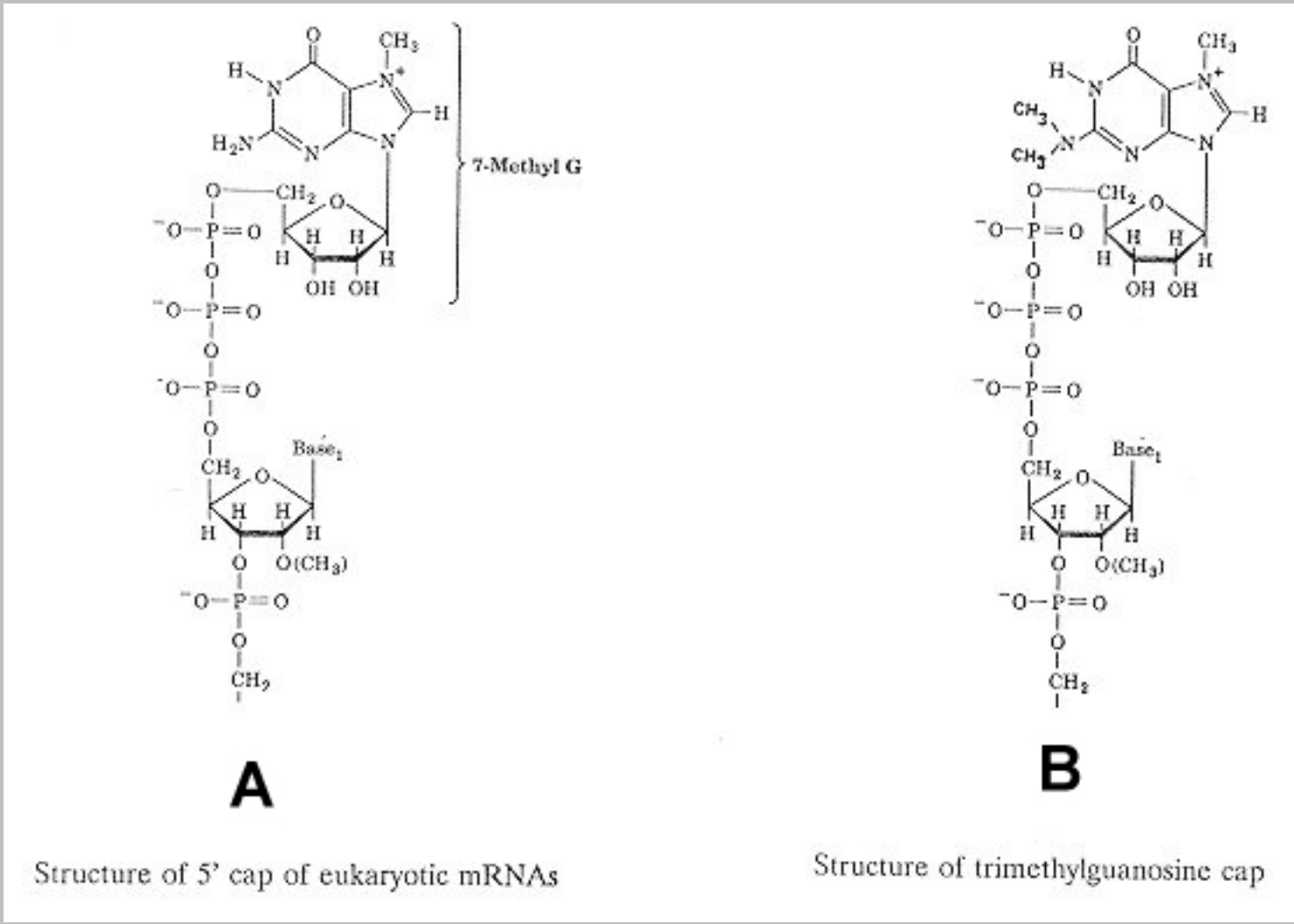


Fig. 11. Structure of RNA caps

Besides the hnRNPs proteins, other proteins also shuttle between the nucleus and the cytoplasm and accompany mRNA molecules when these are exported from the nucleus. In the cytoplasm, these proteins also have a role in the targeting of the mRNA to specific cellular locations as well as its translation and turnover (see Shyu and Wilkinson, 2000).

The *small nuclear ribonucleoproteins* (snRNPs) are cofactors essential for the splicing of pre-mRNA (Steiz et al., 1988) (see Chapter 3, section II and IIA). Their site of action is in the nucleus since splicing generally occurs during transcription. snRNPs contain snRNA and 6 to 10 snRNP proteins. The RNAP II-snRNAs are transcribed in the nucleus and subsequently migrate to the cytoplasm. In contrast, the protein components are synthesized and stored in the cytoplasm. After the RNA and protein components combine in the cytoplasm, the snRNPs-complexes are translocated into the nucleus. Obviously, the interaction between the two sets of macromolecules and their movements in and out of the nucleus have aroused considerable interest. Some proteins (Sm proteins) present in snRNPs are common to the various U snRNPs; others are specific for individual RNPs. The RNA components of the snRNPs have a unique cap structure, which is added co-transcriptionally at the 5' end of the RNA (see Fig. 11). The caps are required for snRNA export. Nuclear import of at least some of the snRNA requires the cap and binding to

at least one Sm protein. A nuclear *cap-binding protein* (CBP) complex composed of two proteins, CBP20 and CBP80, mediates the export of U snRNAs, one kind of snRNPs that possesses a trimethyl cap (Izaurralde et al., 1995).

Competition experiments show that the snRNP nuclear import pathway is different from that of NLS-carrying proteins. The translocation does not depend on importin α, although it involves both β (Palacios et al., 1997) and Ran (Palacios et al., 1996). A protein that might have the properties of a receptor for these proteins, has been identified (see Weis, 1998). The translocation of two U snRNPs (U1A and U2B") requires an unusually long and complex NLSs. In experiments with permeabilized HeLa cells, the transport of these two proteins was shown to be saturable and to require ATP (Hetzer and Mattaj, 2000). In contrast, it showed no competition with previously studied NLSs and the transport is not inhibited by nonhydrolyzable GTP analogues or a mutant of the GTPase Ran that is incapable of GTP hydrolysis.

As already indicated (Section D), the export of U snRNA requires the presence of a complex of two proteins, CBC80 and CBC20. In fact, CBC accompanies translocated RNP into the nucleus (Visa et al., 1996). Other proteins might also be involved (see Görlich and Mattaj, 1996).

As already discussed, ribosomal subunits are accumulated in the nucleoli. They are formed by a complex interaction of ribosomal proteins and RNAs (see Fatica and Tollervey, 2002). The 60S and 40S subunits are translocated from the nucleus separately. The size of the subunits (25-30 nm) may be the maximum allowed by the dimensions of the NPC. . Their export to the cytoplasm is energy dependent and requires CRM1/RanGTP. Export of the 60 S subunits requires the adaptor protein NMD3 which binds to only to mature and properly folded units. In yeast, the Ran-binding protein Yrb2p is needed for the export of the smaller subunit (Moy and Silver, 2002). Shuttling factors are released from the subunits in energy requiring steps (see Johnson et al., 2002).

E. Electrical Properties and Permeability to Small Ions vs the Transport of Macromolecules. Two Sets of Channels?

We saw that the nuclear envelope is involved in both the passive (Section IA), and active transport of macromolecules (Sections B to D). The passive entry and active transport of macromolecules seem to take place through the large central channel of the NPC. The involvement of the large channel was shown in the case of active transport of proteins (Section C) and RNAs (Section D). The common pathway for the transport of macromolecules whether passive or active is shown by the fact that a monoclonal antibody to the transmembrane glycoprotein Gp210 inhibits the uptake of nucleoplasmin as well as the entry of a 10 kDa dextran in cultured rat cells (Greber and Gerace, 1992). Gp210 is a vertebrate glycoprotein located in the NPC membrane domain (Gerace et al., 1992). Similarly, Ca^{2+}-depletion blocks both the transport of proteins containing an NLS as well as passive diffusion of a 10 kDa dextran in intact rat kidney and HeLa cells in culture (Greber and Gerace, 1995). ATP added to isolated rat liver nuclei increases the passive flux rate of transport of 64-kDa dextrans (Schindler and Jiang, 1986). An

effect of ATP (Rakowska et al., 1998) (which increases the height of the NPC and decreases the diameter of the opening) or Ca^{2+} depletion (Perez-Terzic, 1996) (the central plug occludes the central channel) suggest that these agents produce significant changes in NPC conformation.

The conformational changes of the NPCs have been found to associated with actin and myosin (Schindler and Jiang, 1986; Berrios and Fisher, 1986) and the passive transport of macromolecules also appears to depend on actin. Anti-actin or anti-myosin antibodies added to isolated rat liver nuclei significantly reduced the flux rate of fluorescently labeled 64-kDa dextrans (Schindler and Jiang, 1986). Phalloidin (a mushroom toxin which blocks actin depolymerization) and cytochalasin D (a fungal alkaloid that depolymerizes F-actin) inhibit the ATP stimulation of this transport.

Although macromolecules whether actively or passively transported may share a common pathway, small molecules and ions may follow a different route from the transport of macromolecules. As already mentioned (Greber and Gerace, 1995; Stehno-Bittel et al., 1995) Ca^{2+} has a role in the transport of macromolecules. In isolated nuclei of *Xenopus laevis* oocytes or nuclear ghosts (nuclei deprived of nucleoplasm), after depletion of Ca^{2+} by inositol 1,4,5-trisphosphate or calcium chelators, fluorescent molecules conjugated to 10-kilodalton dextran were shown to be unable to enter the nucleus, while in contrast small molecules (e.g. Mg^{2+} or the dye Lucifer Yellow of 500 Da) entered the nucleus even when Ca^{2+} was depleted (Stehno-Bittel et al., 1995). In a more recent study, isolated *Xenopus laevis* oocyte nuclei were exposed to media in which Ca^{2+} and ATP were varied (Shakin et al., 2001) . The electrical conductance of the nuclear envelope was found to be significant under conditions of low macromolecular permeability, suggesting that the ion permeability remains unaffected. Atomic force microscopy found many small pores in the periphery of the NPC only in the presence of ATP . The results suggest that ion flux occurs in these peripheral pores and that these channels are activated by Ca^{2+} and ATP. These results suggest that the passage of small molecules is through the small channels [probably the small channels shown in the structural reconstructions (see above) in the NPC whereas the macromolecules occur through the larger central channel. Both sets of channels can be opened or closed independently.

Several observation indicate a nuclear envelope which is rather impermeable, inconsistent with an open central pore of the NPC. For example, the electrical resistances of the envelope of *Drosophila* salivary gland nuclei (Lowenstein and Kanno, 1962) and a variety of somatic nuclei (Lowenstein et al., 1966) are in the range of plasma membranes (1.5 ω-cm^2). The nuclear envelope of several cells can be patch-clamped revealing a membrane a resistance of the patch in the Gω-cm^2 range (see Bustamante, 1994) inconsistent with the presence of open large channels. In addition, patch-clamping reveals the activity of individual small ion channels, which open and close. In patch-clamp techniques a patch pipette filled with a salt solution is fused to a small portion of the membrane under study. The voltage is maintained constant electronically. Under these conditions, channel openings are detected as deflections in current.

Patch-clamp studies found ATP increasing the permeability of the nuclear envelope to ions (Mazzanti et al., 1994; Assandri and Mazzanti, 1997), whereas Ca^{2+} decreased it (Assandri and Mazzanti, 1997). Note

however, that the effect of Ca^{2+} does not agree with the findings of Shakin et al., 2001. The reason for this descrepancy is not clear.

Experiments using patch-clamping also suggest that the number of open channels in the nuclear envelope may depend on the presence of actin. Cytochalasin was found to increase the number of active channels (Tonini et al., 1999). The authors suggest that the effects of Ca^{2+} and ATP are through actin and possibly myosin. In contrast, the results of Schindler and Jiang (1986) show a decrease in passive transport of a macromolecule in the presence of cytochalasin, suggesting again that the passive transport of macromolecules is distinct from that of low molecular weight compounds. However, the results of with cytochalasin are rather inconclusive since cytochalasin is not a specific reagent and may well interact with components other than actin.

Ca^{2+} has been found to have a role in nuclear functions (see below). Present information indicates that the cisternae of the nuclear envelope store Ca^{2+} that may be released to the nucleoplasm.. In isolated liver nuclei, the involvement of the nuclear envelope in the uptake and release of Ca^{2+} has been documented in experiments carried out with digital imaging and confocal microscopy using the Ca^{2+}-sensitive fluorescent probe Fura 2, (Gerasimenko et al., 1995) and where the nucleoplasmic Ca^{2+} was monitored with Fura 2 attached to dextran. The ATP-dependent uptake of Ca^{2+} into isolated liver nuclei and the release of the accumulated Ca^{2+} by IP_3 see Chapter 24) resulted from the transport of Ca^{2+} into the nuclear envelope cisternae and not into the nucleoplasm.. Changes in the external Ca^{2+} concentration produced changes in the nucleoplasmic Ca^{2+} concentration. Nevertheless, IP_3 and cyclic ADP-ribose (see Chapter 7, Section ID) produced transient intranuclear increases in Ca^{2+}. The release from the Ca^{2+} stores in or around the nuclear envelope appears to be directed into the nucleoplasm from where it can diffuse out through the permeable nuclear pore complexes.

Not surprising the machinery for the uptake and release of Ca^{2+} is present in the nuclear envelope. For example, immunocytochemistry techniques and confocal imaging showed the presence of ryodine receptor Ca^{2+} channels and Ca^{2+}-ATPase in the nuclear envelope. IP_3 (see Chapter 24) and inositol-1,3,4,5-tetrakisphosphate (IP_4) receptors have found in the nucleus suggesting a role of Ca^{2+}-signaling, since IP_4 is a second messenger . The IP_4 receptors (Humbert et al., 1996) and Ca^{2+}-ATPase were found in the outer membrane. The IP_3 and ryanodine receptors (see Chapter 24) are present in the inner nuclear membrane with their ligand-binding site facing the nucleoplasm (Gerasimenko et al., 1995). In intact nuclei, the Ca^{2+}-level determines whether ATP or IP_4 mediate the uptake of Ca^{2+} (Humbert et al., 1996). The Ca^{2+} is favored at lower Ca^{2+} concentrations whereas IP_4 is active at higher concentrations.

F. Regulation of Nuclear Transport

The nuclear transport machinery can be regulated in a variety of ways, providing mechanisms which may

affect many different cargoes (see Kaffman and O'Shea, 1999). The regulation may affect the cargo-receptor complex, for example by exposing or masking the NLS or NES. When the appropriate signal is exposed, the cargo can be moved in the direction specified by the domain. As we shall see control of the entry of some transcription factors follows this mechanism. Alternatively, the cargo or receptor-cargo complex may be tethered to cytoplasmic elements and unavailable for transport into the nucleus. For example, *cAMP-dependent protein* kinase (PKA) remains in the cytoplasm when attached to its regulatory subunit anchored to insoluble structures (see Chapter 7). The release of the PKA from its regulatory subunit permits its entry into the nucleus. However, the regulation may be on the NPC itself. This was dramatically demonstrated under different growth conditions (Feldherr, and Akin, 1991). Nucleoplasmin-coated colloidal gold particles were injected into the cells and their entry into the nucleus was observed in proliferating and growth-arrested cells using the electron microscopy. There was a sharp decrease in the uptake in growth-arrested cells for particles 110-270 Å in diameter with no effect on smaller particles, 50-80 Å in diameter. Assuming that the difference is caused by a change in the size of the opening, the functional diameters of the transport channels in the affected pores were estimated to be approximately to 110 to 130 Å in growth arrested cells and 230 Å in proliferating cells.

As in many other cases of regulation of cellular functions (e.g. see Chapter 13), phosphorylation plays a central role in the regulation of nuclear traffic. The regulation of nuclear import and export by the phosphorylative state was examined in digitonin-permeabilized cells (Kehelenbach and Gerace, 2000). The phosphatase inhibitors okadaic acid and microcystin inhibit transport mediated by the import receptors importin β and transportin (see above), without affecting the export receptor CRM1 (exportin 1) (see above). In addition, the protein kinase inhibitor staurosporine, reversed some of these effects suggesting that the inhibition of the import depend on an increase in phosphorylation of some component of the import machinery. However, substrate binding by the importin αβ complex and the association of the complex with the nucleoporins Nup358/RanBP2 and Nup153 are not changed by the phosphatase inhibitors. This finding indicates that the steps regulated by phosphorylation are subsequent to binding to these components.

In addition, to general effects of the phosphorylative states of undetermined nuclear components , phosphorylation and dephosphorylation of various transcription factors have been shown to activate or block their nuclear import or export signals (see Kaffman and O'Shea, 1999). Obviously, the entry of a transcription factor into the nucleus is essential for its function.

Ca^{2+} is thought to play a prominent role in gene expression (Hardingham et al., 1997; see Santella and Carafoli, 1996). Present information indicates that the cisternae of the nuclear envelope store Ca^{2+} that may be released to the nucleoplasm by Ca^{2+} release channels, the RyR or inositol 1,4,5-trisphosphate (IP_3) receptors (see Chapter 24) with potential effects on gene expression (e.g., Abrenica and Gilchrist, 2000). Furthermore, the uptake of Ca^{2+} is controlled by the second messengers cyclic ADP-ribose (see Chapter 7, Section ID) and inositol-1,3,4,5-tetrakisphosphate (IP_4) (see Chapter 7, Section IF).

II. THE MITOCHONDRIAL AND CHLOROPLAST MEMBRANES

With the exception of photosynthetic processes, the key reactions of the energy metabolism of most eukaryotes occur in mitochondria. The free energy from oxidation of substrates drives the synthesis of ATP. The reactions take place in the inner mitochondrial membrane and require the maintenance of ionic gradients. These events will be discussed in Chapters 16 and 18. Similarly, chloroplasts are involved in photosynthetic reactions in which internal membranes in the thylakoid vesicles play a role, as discussed primarily in Chapter 17.

Mitochondria are bounded by two sets of membranes. The outer membrane of mitochondria has as its major component the voltage dependent anion channel (VDAC), also known as mitochondrial porin. This channel, when reconstituted into bilayers, has large conductances (e.g., Colombini, 1979). For this reason, generally, it has been assumed that the outer membrane is extremely leaky. However, other results suggest that this conclusion may have to be reevaluated. The outer membrane can also be studied by patch clamping. With this technique, a pipette filled with a salt fuses with the membrane, isolating a membrane patch. When the voltage is maintained constant electronically, the opening or closing of a channel corresponds to discrete current traces. Calculations of the density of VDAC suggest that there are several hundred per patch. However, patch clamping of the outer membrane demonstrates very high patch membrane resistances (Moran et al., 1992; Kinnally and Tedeschi, 1994). These results suggest that VDAC are not open in their native state and environment.

Because of its role in energy transduction, several of the properties of the inner mitochondrial membrane will be addressed in a separate chapter. The inner mitochondrial membrane has been shown to be highly impermeable. The permeabilities of cell and mitochondrial inner membranes to non-electrolytes depend similarly on the oil-water partition coefficient (Tedeschi and Harris, 1955). ATP synthase and the dehydrogenases involved in oxidative phosphorylation are on the mitochondrial matrix side of the inner mitochondrial membrane. Therefore, a relatively impermeable barrier would block the passage of metabolites as well as ADP and ATP. These molecules are charged and would find it difficult to pass through an ordinary lipid bilayer. In mitochondria, as in cells, a system of transport proteins is responsible for these exchanges. The ADP/ATP transporter, which is needed to provide the energy generated by oxidative phosphorylation to the rest of the cell, is prominent among these. The various known transport systems are listed in Table 2 (Schoolwerth and LaNoue, 1985).

New perspectives have been introduced by evidence of interactions between inner and outer membranes. They are likely to be of great physiological significance because they involve the energy transducing system of mitochondria. Hackenbrock (1968) first described how inner and outer membranes make contact at specific sites. Results from many laboratories seem to implicate complexes between various inner and outer membrane components, including the adenine nucleotide translocator, VDAC, hexokinase, and creatine kinase. For example, the two kinases complex to VDAC. Some of these interactions are shown in Table 3 (Tedeschi and Kinnally, 1994). The sites of these interactions may well represent contact sites, as proposed by several investigators.

The importance of these associations between inner and outer membrane components, is shown by the fact that in some systems ATP synthesized in mitochondria (presumably translocated by the adenine nucleotide translocator) is used by hexokinase, in preference to the ATP in the medium.

Chloroplasts are also surrounded by two membranes constituting the chloroplast envelope. As in the case of mitochondria, the outer membrane is also thought to be leaky. Studies with isolated chloroplasts indicate easy passage of molecules below the range of 7 to 13 kDa (Flügge and Benz, 1984) through the outer membrane. As in mitochondria, the inner membrane appears to be the site of transport for metabolites (see Flügge and Heldt, 1991, Douce and Joyard, 1990). Inner and outer membranes are also joined in so called "contact sites" (Carde et al., 1982, Cremers et al., 1988).

III. TRANSPORT IN THE ER AND THE GOLGI APPARATUS

Many reactions take place in the lumen of the ER or the cisternae of the Golgi apparatus. Among these, the glycosylation, sulfation and phosphorylation of proteins, proteoglycans and sphingolipids (see Hirschberg and Snider, 1987). The precursors of these compounds originate in the cytoplasm and must be transported into lumen of these compartments (for the E.R., e.g., Perez and Hirschberg, 1985, 1986). These transport reactions were first detected in isolated vesicles (e.g., rat liver: Hirschberg and Snider, 1987, yeast: Abeijon et al., 1996) and were found to be against a concentration gradient, apparently by an antiporter mechanism. The substrate (e.g., GDP-fucose) is exchanged for the corresponding nucleotide monophosphate (e.g., GMP) (e.g., Capasso et al., 1984; Waldman and Rudnick, 1990). The transport has been studied in reconstituted systems (i.e., with proteoliposomes) and the use of mutants has demonstrated that translocation is required for the biochemical reactions, such as glycosylation (e.g., Abeijon, Brandli et al., 1988), to take place.

This chapter has presented some of the roles of internal membranes in cell function. Later chapters confirm and expand these themes.

Table 2. Mitochondrial metabolite transporters

Category	**Name**	**Substrates**	**Inhibitors**	
			Specific	**Sulfhydril reagent**
Electroneutral proton compensated	Phosphate	Phosphate, arsenate		Organic mercurials *N*-ethylmaleimide
	Glutamate	Glutamate	Avenaciolide	*N*-ethylmaleimide
	Pyruvate	Monocarboxylic acids, ketone bodies, branched-chain ketoacids	α-Cyano-3-hydro-xycinnamate	Organic mercurials *N*-ethylmaleimide
	Ornithine	Ornithine, citrulline, lysine		

Electroneutral anion exchange	Dicarboxylate	Phosphate, malate, succinate, oxalacetate	Butylmalonate, bathophenanthroline, iodobenzylmalonate, phenylsuccinate, phthaonate	Organic mercurials
	α-Ketoglutarate	Malate, α-ketoglutarate, succinate, oxalacetate	Phthalonate, bathophenanthroline, phenylsuccinate, butylmalonate	
	Tricarboxylate	Citrate, isocitrate, phosphoenolpyruvate, malate, succinate	1,2,3-Benzene-tricarboxylate, α-cetylcitrate, bathophenanthroline	
Neutral	Carnitine	Carnitine, acylcartinine	Sulfobetaines	Organic mercurials, N-ethylmaleimide
	Neutral amino acids	Neutral amino acids		Organic mercurials
	Glutamine	Glutamine		Organic mercurials
Electrophoretic	Glutamate/aspartate	Glutamate/aspartate	Glisoxepide (nonspecific)	
	Adenine nucleotide	ADP, ATP	Atractyloside, carboxyatractyloside, bongkreate, long chain acyl CoA, α-cetylcitrate	

Table 3. Interaction between various metabolically significant elements. From Tedeschi and Kinnally 1994, reproduced by permission.

Structure or enzyme	Binding to or Interaction
Contact sites	hexokinase, creatine kinase, VDAC
VDAC	hexokinase, glutathione transferase, adenine nucleotide translocator
Outer membrane	glycerol kinase
Matrix ATP	hexokinase, creatine phosphokinase

SUGGESTED READING

Nuclear Envelope.

Bustamante, J.O. (1994) Nuclear electrophysiology, *J. Membr. Biol.* 138:105-112. (Medline)

Dingwall, C. (1991) Transport across the nuclear envelope: enigmas and explanations, *BioEssays* 13:213-218. (Medline)

Mazzanti, M. (1998) Ion permeability of the nuclear envelope, *News Physiol. Sci.* 13:44-50.

Nakielny, S. and Dreyfuss, G. (1999) Transport of proteins and RNAs in and out of the nucleus, *Cell* 99:677-690. (Medline)

Pennisi, E. (1998) The nucleus revolving door, *Science* 279:1129-1131. (Medline)

Wente, S.R. (2000) Gatekeepers of the nucleus, *Science* 288:1374-1377. (Medline)

Chloroplasts and mitochondria

Flügge, U.-I. and Heldt, H. W. (1991) Metabolite translocators of the chloroplast envelope, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42:129-144.

Schoolwerth, A. C. and LaNoue, F. F. (1985) Transport of metabolic substrates in renal mitochondria. *Annu. Rev. Physiol.* 47: 143-171. (Medline)

WEB RESOURCES

Hendzel, M. (2001) The Cell Nucleus: Free teaching and study materials. Contemporary review materials. Movies of dynamics in the cell nucleus. Listing of resources on the Web. www.cellnucleus.org

Bickmore, W. et al. (2002) Nuclear Protein Data Base

REFERENCES

Search the textbook

Back to Chapter 5

REFERENCES

Abeijon, C., Mandon, E. C., Robbins, P. W. and Hirschberg, C. B. (1996) A mutant yeast deficient in Golgi transport of uridine diphosphate N-acetylglucosamine, *J. Biol. Chem.* 271:8851-8854.(Medline)

Abrenica, B. and Gilchrist, J. S. (2000). Nucleoplasmic Ca^{2+} loading is regulated by mobilization of perinuclear Ca^{2+}. *Cell Calcium* 28: 127-136. (MedLine)

Adam, S. and Gerace, L. (1991) Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import, *Cell* 66:837-847.(Medline)

Aitchison, J. D., Blobel, G. and Rout, M. P. (1996) Kap104p: a karyopherin in the nuclear transport of messenger RNA binding proteins, *Science* 274:624-627.(Medline)

Akey. C. W. (1989) Interactions and structure of the nuclear pore complex revealed by cryo-electron microscopy, *J. Cell Biol.* 109:955-970.(Medline)

Akey, C. W. (1990) Visualization of transport-related configurations of the nuclear pore transporter, *Biophys. J.* 58:341-355.(Medline)

Akey. C. W. and Goldfarb, D. S. (1989) Protein import through the nuclear pore complex is a multistep process, *J. Cell Biol.* 109:971-982.(Medline)

Akey, C,W, and Radermacher, M. (1993) Architecture of the *Xenopus* nuclear pore complex revealed by three-dimensional cryo-electron microscopy, *J. Cell Biol.* 122:1-19.(Medline)

Arts, G.J., Kuersten, S., Romby, P., Ehresmann, B. and Mattaj, I.W. (1998) Identification of a nuclear export receptor for tRNA, *Curr. Biol.* 8:305-314.(Medline)

Assandri, R. and Mazzanti, M. (1997) Ionic permeability on isolated mouse liver nuclei: influence of ATP and Ca^{2+}, *J. Membr. Biol.* 157:301-309.(Medline)

Bailer, S.M., Siniossoglou, S., Podtelejnikov, A., Hellwig, A., Mann, M. and Hurt, E. (1998) Nup116p and nup100p are interchangeable through a conserved motif which constitutes a docking site for the

mRNA transport factor gle2p, *EMBO J.* 17:1107-1119.(Medline)

Bastos, R., Panté, N. and Burke, B. (1995) Nuclear pore complex proteins, *Intern. Rev. Cytol.* 162B:257-302.(Medline)

Bastos, R., Lin, A., Enarson, M. and Burke, B. (1996) Targeting and function in mRNA export of nuclear pore complex protein Nup153, *J. Cell Biol.* 134:1141-1156.(Medline)

Bayliss, R., Littlewood, T. and Stewart, M. (2000) Structural basis for the interaction between FxFG nucleoporin repeats and importin-β in nuclear trafficking, *Cell*102:99-108. (MedLine)

Bayliss, R., Leung, S.W., Baker, R.P., Quimby, B.B., Corbett, A.H. and Stewart, M. (2002) Structural basis for the interaction between NTF2 and nucleoporin FxFG repeats, *EMBO J.* 21:2843-2853. (MedLine)

Ben-Efraim, I. and Gerace, L. (2001) Gradient of increasing affinity of importin β for nucleoporins along the pathway of nuclear import, *J. Cell Biol.* 152:411-417. (MedLine)

Berrios, M. and Fisher, P.A. (1986) A myosin heavy-chain-like polypeptide is associated with the nuclear envelope in higher eukaryotic cells, *J. Cell Biol.* 103:711-724.(Medline)

Bogerd, H.P., Echarri, A., Ross, T.M. and Cullen, B.R. (1998) Inhibition of human immunodeficiency virus Rev and human T-cell leukemia virus Rex function, but not Mason-Pfizer monkey virus constitutive transport element activity, by a mutant human nucleoporin targeted to Crm1, *J. Virol.* 72:8627-8635.(Medline)

Bonifaci, N., Moroianu, J., Radu, A. and Blobel, G. (1997) Karyopherin β2 mediates nuclear import of a mRNA binding protein, *Proc. Natl. Acad. Sci. USA* 94:5055-5060.(Medline)

Brandli, A.W., Hansson, G.C., Rodriguez-Boulan, E. and Simons, K. (1988) A polarized epithelial cell mutant deficient in translocation of UDP-galactose into the Golgi complex, *J. Biol. Chem.* 263:16283-16290.(Medline)

Braun, I.C., Rohrbach, E., Schmitt, C. and Izaurralde, E. (1999) TAP binds to the constitutive transport element (CTE) through a novel RNA-binding motif that is sufficient to promote CTE-dependent RNA export from the nucleus, *EMBO J.* 18:1953-1965.(Medline)

Bray, M., Prasad, S., Dubay, J.W., Hunter, E., Jeang, K.T., Rekosh, D. and Hammarskjöld, M.L. (1994) A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev-independent, *Proc. Natl. Acad. Sci. USA.* 91:1256-1160.(Medline)

Bucci, M. and Wente, S.R. (1997) In vivo dynamics of nuclear pore complexes in yeast, *J. Cell Biol.* 136:1185-1199.(Medline)

Burd, C.G. and Dreyfuss, G. (1994) RNA binding specifity of hnRNP A1: hnRNP A1 high-affinity binding sites in pre-mRNA splcing, *EMBO J.* 13:1197-1204.(Medline)

Bustamante, J.O. (1994) Nuclear electrophysiology, *J. Membrane Biol.* 138:105-112.(Medline)

Byrd, D.A., Sweet, D.J., Pante, N., Konstantinov, K.N., Guan, T., Saphire, A.C., Mitchell, P.J., Cooper, C.S., Aebi, U. and Gerace, L. (1994) Tpr, a large coiled coil protein whose amino terminus is involved in activation of oncogenic kinases, is localized to the cytoplasmic surface of the nuclear pore complex, *J. Cell Biol.* 127:1515-1526.(Medline)

Capasso, J.M. and Hirschberg,C.B. (1984) Mechanisms of glycosylation and sulfation in the Golgi apparatus: evidence for nucleoside sugar/nucleoside monophosphate and nucleotide sulfate/nucleoside monophosphate antiports in the Golgi apparatus membrane, *Proc. Natl. Acad. Sci. USA* 81:7051-7055.(Medline)

Carde, J.P., Joyard, J. and Douce, R. (1982) Electron microscopic studies of envelope membranes from spinach plastids, *Biol. Cell.* 44:315-324.

Chi, N.C., Adam, E.J.H., Adam, S,A. (1995) Sequence and characterization of cytoplasmic nuclear import factor p97, *J. Cell Biol.* 130:265-274.(Medline)

Colombini, M. (1987) Regulation of the mitochondrial outer membrane channel, VDAC, *J. Bioenerg. Biomembr.*, 19:309-320.(Medline)

Cordes, V.C., Reidenbach, S., Kohler, A., Stuurman, N., van Driel, R. and Franke, W.W. (1993) Intranuclear filaments containing a nuclear pore complex protein, *J. Cell Biol.* 123:1333-1344.(Medline)

Cordes, V.C., Reidenbach, S., Rackwitz, H.R. and Franke, W.W.(1997) Identification of protein p270/Tpr as a constitutive component of the nuclear pore complex-attached intranuclear filaments, *J. Cell Biol.* 136:515-529.(Medline)

Cremers, F.F.M., Voorhout, W.F., Van der Krift, T.P., Leunissen-Bijvelt, J.J.M. and Verlejj, A.J. (1988) Visualization of contact sites between outer and inner envelope membranes in isolated chloroplasts, *Biochim. Biophys. Acta* 933:334-340.

de Bruyn Kops A. and Guthrie, C. (2001) An essential nuclear envelope integral membrane protein, Brr6p, required for nuclear transport, *EMBO J.* 20:4183-4193. (MedLine)

DeVit, M.J. and Johnston, M. (1999) The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of *Saccharomyces cerevisiae*, *Curr. Biol.* 9:1231-1241. (MedLine)

Dingwall, C. and Laskey, R. A. (1986) Protein import into the cell nucleus, *Annu. Rev. Cell Biol.* 2: 367-390.(Medline)

Dingwall, C., Sharnick, S.V., Laskey, R.A. (1982) A polypeptide domain that specifies migration of nucleoplasmin into the nucleus, *Cell* 30:449-458. (MedLine)

Cullen, B.R. (1998) Retroviruses as model systems for the study of nuclear RNA export pathways, *Virology* 249:203-210.(Medline)

Davis, L.I. (1995) The nuclear pore complex, *Annu.Rev. Biochem.* 64:865-896.(Medline)

Delphin, C., Guan, T., Melchior, F. and Gerace, L. (1997) RanGTP targets p97 to RanBP2, a filamentous protein localized at the cytoplasmic periphery of the nuclear pore complex, *Mol. Biol. Cell* 8:2379-2390.(Medline)

Dingwall, C., Sharnick, S. V. and Laskey, R. A. (1982) A polypeptide domain that specifies the migration of nucleoplasmin into the nucleus, *Cell* 30:449-458.(Medline)

Douce, R. and Joyard, J. (1990) Biochemistry and function of the plastid envelope, *Annu. Rev. Cell Biol.* 6:173-216; see p.199-207.(Medline)

Doye, V. and Hurt, E.C. (1995) Genetic approaches to nuclear pore structure and function, *Trends Genet.* 11:235-241.(Medline)

Doye, V. and Hurt, E. (1997) From nucleoporins to nuclear pore complexes, *Curr. Opin. Cell Biol.* 9:401-411.(Medline)

Dreyer, C., Scholtz, E. and Hausen, P. (1982) The fate of oocyte nuclear proteins during early development of Xenopus laevis, *Wilhem Roux' Arch. Dev. Biol.* 191:228-233.

Dworetzky, S. I. and Feldherr, C. M. (1988) Translocation of RNA-coated gold particles through the nuclear pores of oocytes, *J. Cell Biol.* 106:575-584.(Medline)

Dworetzky, S. I., Lanford, R. E. and Feldherr, C. M. (1988) The effects of variations in the number and sequence of targeting signals on nuclear uptake, *J. Cell Biol.* 107:1279-1287.(Medline)

Englmeier, L., Olivo, J.C. and Mattaj, I.W. (1999) Receptor-mediated substrate translocation through the nuclear pore complex without nucleotide triphosphate hydrolysis, *Curr. Biol.* 9:30-41.(Medline)

Fabre E. and Hurt, E. (1997) Yeast genetics to dissect the nuclear pore complex and nucleocytoplasmic trafficking, *Annu. Rev. Genet.* 31:277-313.(Medline)

Fahrenkrog, B., Hurt, E.C., Aebi, U. and Pante, N. (1998) Molecular architecture of the yeast nuclear pore complex: localization of nsp1p subcomplexes, *J. Cell Biol.* 143:577-588.(Medline)

Fatica, A. and Tollervey D. (2002) Making ribosomes, *Curr. Opin. Cell Biol.* 14:313-318. (MedLine)

Featherstone, C., Darbly, M.K. and Gerace, L. (1988) A monoclonal antibody against the nuclear pore complex inhibits nucleo-cytoplasmic transport of protein and RNA in vivo, *J. Cell Biol*. 107:1289-1297.(Medline)

Feldherr, C. M. and Akin, D. (1990) The permeability of the nuclear envelope in dividing and nondividing cells, *J. Cell Biol.* 111:1-8.(Medline)

Feldherr, C. M., and Akin, D. (1991) Signal-mediated nuclear transport in proliferating and growth-arrested BALB/c 3T3 cells, *J. Cell Biol.* 115: 933-939. 9MedLine)

Feldherr, C. M., Kallenbach, E. and Schulz, N. (1984) Movement of a karyophilic protein through the nuclear pores of oocytes, *J. Cell Biol.* 99:2216-2222.(Medline)

Finlay, D. R., Newmeyer, D. D. Price, T. M. and Forbes, D. J. (1987) Inhibition of *in vitro* nuclear transport by a lectin that binds to nuclear pores, *J. Cell Biol.* 104:189-200.(Medline)

Finlay, D.R., Meier, E., Bradley, P., Horecka, J. and Forbes, D.J. (1991) A complex of nuclear pore proteins required for pore function, *J. Cell Biol.* 114:169-183.(Medline)

Fischer, U., Meyer, S., Teufel, M., Heckel, C., Lührmann, R. and Rautmann, G. (1994) Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA, *EMBO J*. 13:4105-4112.(Medline)

Fischer, U., Huber, J., Bolelns, W.C., Mattaj, I.W., and Lührmann, R. (1995) The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs, *Cell* 82:475-483.(Medline)

Flügge, U.-I. and Benz, R. (1984) Pore forming activity in the outer membrane of the chloroplast membrane, *FEBS Lett.*169:85-89.

Flügge, U.-I. and Heldt, H. W. (1991) Metabolite translocators of the chloroplast envelope, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42:129-144.

Fornerod, M., Ohno, M., Yoshida, M. and Mattaj, I.W. (1997a) CRM1 is an export receptor for leucine-rich nuclear export signals, *Cell* 90:1051-1060.(Medline)

Fornerod, M., van Deursen, J., van Ball, S., Reynolds, A., Davis, D., Murti, K.G., Fransen, J. and Grosveld, G. (1997b) The human homologue of of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and an novel nuclear pore component Nup88, *EMBO J.* 16:807-816.(Medline)

Fricker M., Hollinshead, M., White, N. and Vaux, D. (1997) Interphase nuclei of many mammalian cell types contain deep, dynamic, tubular membrane-bound invaginations of the nuclear envelope, *J. Cell Biol.* 136:531-544.(Medline)

Fribourg, S., Braun, I.C., Izaurralde, E. and Conti, E. (2001) Structural basis for the recognition of a nucleoporin FG repeat by the NTF2-like domain of the TAP/p15 mRNA nuclear export factor, *Mol. Cell* 8:645-656. (MedLine)

Fridell, R.A., Truant, R., Thorne, L., Benson, R.E. and Cullen, B.R. (1997)Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyophorin β, *J.Cell Scie.* 110:1325-1331.(Medline)

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M. and Nishida, E. (1997) CRM1 is responsible for intracellular transport mediated by the nuclear export signal, *Nature* 390:308-311.(Medline)

Garcia-Bustos, J., Heitman, J. and Hall, M. N. (1991) nuclear protein localization, *Biochim. Biophys. Acta* 1071:83-101.(Medline)

Gatfield, D., Le Hir, H., Schmitt, C., Braun, I.C., Kocher, T., Wilm, M. and Izaurralde, E. (2001) The DExH/D box protein HEL/UAP56 is essential for mRNA nuclear export in *Drosophila Curr. Biol.* 11:1716-1721. (MedLine)

Gerace, L. (1992) Molecular trafficking across the nuclear pore complex, *Current Opin. Cell Biol.* 4: 637-645.(Medline)

Gerace, L., Blum, A. and Blobel, G. (1978) Immunochemical localization of the major polypeptides of the nuclear pore complex-lamina fraction, *J. Cell Biol.* 79:546-566.(Medline)

Gerace, L., Ottaviano, Y. and Kondor-Koch, C. (1982) Identification of a major polypeptide of the nuclear pore complex, *J. Cell Biol.* 95:826-837.(Medline)

Gerace, L., Comeau, C. and Benson, M. J. (1984) Organization and modulation of nuclear lamina structure, *J. Cell Sci. Suppl* 1:137-160.(Medline)

Gerasimenko, O. V., Gerasimenko, J. V., Tepikin, A. V., and Petersen, O. H. (1995) ATP-dependent accumulation and inositol trisphosphate- or cyclic ADP-ribose-mediated release of Ca^{2+} from the nuclear envelope, Cell 80, 439-444. (MedLine)

Giannakakou, P., Sackett, D.L., Ward, Y., Webster, K.R., Blagosklonnym M.V. and Fojo T. (2000) p53 is associated with cellular microtubules and is transported to the nucleus by dynein, *Nature Cell Biol.* 2:709-717. (MedLine)

Goldberg, M.W. and Allen, T.D. (1992) High resolution scanning electron microscopy of the nuclear envelope: demonstration of a new, regular, fibrous lattice attached to the baskets of the nucleoplasmic face of the nuclear pores, *J. Cell Biol.* 119:1429-1440.(Medline)

Goldfarb, D. S., Gariépy, J., Schoolnik, G. and Kornberg, R. D. (1986) Synthetic peptides as nuclear localization signals, *Nature* 322:641-644.(Medline)

Görlich, D. and Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm, *Annu. Rev. Cell Dev. Biol.* 15:607-660. (MedLine)

Görlich, D. and Mattaj, I.W. (1996) Nucleocytoplasmic transport, *Science* 271:1513-1518.(Medline)

Görlich, D., Prehn, S., Laskey, R. and Hartmann, E. (1994) Isolation of a protein that is essential for the first step of nuclear protein import, *Cell* 79:767-778.(Medline)

Görlich, D., Vogel, F., Mills, A D., Hartmann, E. and Laskey, R. (1995) Distinct functions for the two importin subunits in nuclear protein import, *Nature* 377:246-248.(Medline)

Görlich, D., Henklein, P., Laskey, R.A. and Hartmann, E. (1996) A 41 amino acid motif in importin-α confers binding to importin-β and hence transit into the nucleus, *EMBO J.* 15:1810-1817.(Medline)

Grandi, P., Dang, T., Pane, N., Shevchenko, A., Mann, M., Forbes, D. and Hurt, E. (1997) Nup93, a vertebrate homologue of yeast Nic96p, forms a complex with a novel 205-kDa protein and is required for correct nuclear pore assembly, *Mol. Biol. Cell.* 8:2017-2038.(Medline)

Greber, U.F. and Gerace, L. (1992) Nuclear protein import is inhibited by an antibody to a lumenal epitope of a nuclear pore complex glycoprotein, *J. Cell Biol.* 116:15-30.(Medline)

Greber, U.F. and Gerace, L. (1995) Depletion of calcium from the lumen of endoplasmic reticulum

reversibly inhibits passive diffusion and signal-mediated transport into the nucleus, *J. Cell Biol.* 128:5-14.(Medline)

Grüter, P., Tabernero, C., von Kobbe, C., Schmitt, C., Saavedra, C., Bachi, A., Wilm, M., Felber, B.K. and Izaurralde, E. (1998) TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus, *Mol Cell* 1:649-659.(Medline)

Guan, T., Muller, S., Klier, G., Pante, N., Blevitt, J.M., Haner, M., Paschal, B., Aebi, U. and Gerace, L. (1995) Structural analysis of the p62 complex, an assembly of O-linked glycoproteins that localizes near the central gated channel of the nuclear pore complex, *Mol. Biol. Cell.* 6:1591-1603.(Medline)

Hackenbrock, C.R. (1968) Chemical and physical fixation of isolated mitochondria in low and high energy states, *Proc. Natl. Acad. Sci. USA* 61:598-605.(Medline)

Hamm, J. and Mattaj, I. W. (1990) Monomethylated cap structures facilitate RNA export from nucleus, *Cell* 63:109-118.(Medline)

Hamm, J., Darzynkiewicz, E., Tahara, S. M. and Mattaj, I. W. (1990) The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal, *Cell* 62:569-577.(Medline)

Hardingham, G.E., Chawla, S., Johnson, C.M. and Bading, H. (1997) Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression, *Nature* 385:260-265.

Hetzer, M. and Mattaj, I.W. (2000) An ATP-dependent, Ran-independent mechanism for nuclear import of the U1A and U2B" spliceosome proteins, *J. Cell Biol.*148:293-304.(Medline)

Hinshaw, J. E., Carragher, B. O. and Milligan, R. A. (1992) Architecture and design of the nuclear pore complex, *Cell* 69:1133-1142.(Medline)

Hirschberg, C.B. and Snider, M.D. (1987) Topography of glycosylation in the rough endoplasmic reticulum and the Golgi apparatus, *Annu. Rev. Biochem.* 56:63-88.(Medline)

Hu, T., Guan, T. and Gerace, L. (1996) Molecular and functional characterization of the p62 complex, an assembly of nuclear pore complex glycoproteins, *J. Cell Biol.* 134:589-601.(Medline)

Huang, Y. and Steitz, J.A. (2001) Splicing factors SRp20 and 9G8 promote the nucleocytoplasmic export of mRNA, *Mol. Cell* 7:899-905. (MedLine)

Humbert, J.P., Matter, N., Artault, J.C., Koppler, .P and Malviya, A.N. (1996) Inositol 1,4,5-trisphosphate receptor is located to the inner nuclear membrane vindicating regulation of nuclear calcium

signaling by inositol 1,4,5-trisphosphate. Discrete distribution of inositol phosphate receptors to inner and outer nuclear membranes, *J. Biol. Chem.* 271:478-485. (MedLine)

Iborra, F.J., Jackson, D.A. and Cook, P.R. (2001) Coupled transcription and translation within nuclei of mammalian cells, *Science* 293(5532):1139-1142. (MedLine)

Imamoto, N., Shimamoto, T., Takao, T., Tachibana, T., Kose, S., Matsubae, M., Sekimoto, T., Simonishi, Y. and Yoneda, Y. (1995a) *In vivo* evidence for involvement of a 58 kDa component of nuclear pore-targeting complex in nuclear protein import, *EMBO J.* 14:3617-3626.(Medline)

Imamoto, N., Tachibana, T., Matsubae, M. and Yoneda, Y. (1995b) A karyophilic protein forms as stable complex with cytoplasmic components prior to nuclear pore binding, *J. Biol. Chem.* 270:8559-8565.(Medline)

Imamoto, N., Shimamoto, T., Kose, S., Takao, T., Tachibana, T., Matsubae, M., Sekimoto, T., Shimonishi, Y. and Yoneda, Y. (1995c) The nuclear pore-targeting complex binds the nuclear pores after association with a karyophile, *FEBS Lett.* 368:415-419.(Medline)

Iovine, M.K., Watkins, J.L. and Wente, S.R. (1995) The GLFG repetitive region of the nucleoporin Nup116p interaction with Kap95p, an essential yeast nuclear import factor, *J. Cell Biol.* 131:1699-1713.(Medline)

Izaurralde, E. and Mattaj, I.W. (1995) RNA export, *Cell* 81:153-159.(Medline)

Izaurralde, E., Lewis, J., McGuigan, C., Jankowska, M., Darzynkiewicz, E. and Mattaj, I.W. (1994) A nuclear cap binding protein complex involved in pre-mRNA splicing, *Cell* 78:657.(Medline)

Izaurralde, E., Lewis, J., Gamberi, C., Jarmolowski, A., McGuigan, C. and Mattaj, I.W. (1995) A cap-binding protein complex mediating U snTNA export, *Nature* 376:709-712..(Medline)

Izaurralde, E., Kutay, U., von Kobbe, C., Mattaj, I.W., Görlich, D. (1997a) The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus, *EMBO J.* 16:6535-6547.(Medline)

Izaurralde, E., Jarmolowski, A., Beisel, C., Mattaj, I.W., Dreyfuss, G. and Fischer, U. (1997b) A role of M9 transport signal of hnRNP A1 in mRNA nuclear export, *J. Cell Biol.* 137:27-35.(Medline)

Jans, D.A. and Hübner, S. (1996) Regulation of protein transport to nucleus: central role of phosphorylation, *Physiol. Rev.* 76:651-685.(Medline)

Jarnick, M. and Aebi, U. (1991) Toward a more complete 3-D structure of the nuclear pore complex, *J. Struct.Biol.* 107:291-308.(Medline)

Jarmolowski, A., Boelens, W.C., Izaurralde, E. and Mataj, I.W. (1994) Nuclear export of different classes of RNA is mediated by specific factors, *J. Cell Biol.* 124:627-635.(Medline)

Johnson, A.W., Lund, E. and Dahlberg, J. (2002) Nuclear export of ribosomal subunits, *Trends Biochem. Sci.* 27580-585. (MedLine)

Kaffman, A. and O'Shea, E. K. (1999) Regulation of nuclear localization: a key to a door, i>Annu. Rev. Cell Dev. Biol. 15: 291-339. (MedLine)

Kaffman, A., Rank, N.M., O'Neill, E.M., Huang, L.S. and O'Shea, E.K. (1998) The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus, *Nature* 396:482-486. (MedLine)

Kang, Y. and Cullen, B.R. (1999) The human Tap protein is a nuclear mRNA export factor that contains novel RNA-binding and nucleocytoplasmic transport sequences, *Genes Dev.* 13:1126-1139.(Medline)

Katahira, J., Strasser, K., Podtelejnikov, A., Mann, M., Jung, J.U. and Hurt, E. (1999) The Mex67p-mediated nuclear mRNA export pathway is conserved from yeast to human, *EMBO J.* 18:2593-2609.(Medline)

Kehlenbach RH and Gerace L. (2000) Phosphorylation of the nuclear transport machinery down-regulates nuclear protein import in vitro, *J Biol Chem.* 275:17848-17856. (MedLine)

Kinnally, K.W. and Tedeschi, H. (1994) Mitochondrial channels: An integrated view, in *Molecular Biology of Mitochondrial Transport systems* (Forte, M. and Colombini, M., eds.) pp. 169-198. Springer Verlag, Berlin.

Kraemer, D., Wozniak, R.W., Blobel, G. and Radu, A. (1994) The human CAN protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm, *Proc. Natl. Acad. Sci. USA* 91:1519-1523.(Medline)

Krecic, A.M. and Swanson, M.S. (1999) hnRNP complexes: composition, structure, and function, *Curr. Opin. Cell Biol.* 11:363-371. (MedLine)

Kuster, B. and Mann, M. (1998) Identifying proteins and post-translational modifications by mass spectrometry, *Curr. Opin. Struct. Biol.* 8:393-400.(Medline)

Kutay, U., Lipowsky, G., Izaurralde, E., Bischoff, F.R., Schwarzmaier, P., Hartmann, E. and Görlich D

(1998) Identification of a tRNA-specific nuclear export receptor, *Mol. Cell* 1:359-369.(Medline)

Lamb, M. M. and Daneholt, B. (1979) Characterization of active transcription units in Balbiani rings of Chironomus tentans, *Cell* 17:835-848.(Medline)

Lanford, R. E., Kanda, P. and Kennedy, R. C. (1986) Induction of nuclear transport with a synthetic peptide homologous to the SV40 T antigen transport signal, *Cell* 46:575-582.(Medline)

Lee, M.S., Henry, M. and Silver, P.A. (1996) A protein that shuttles between the nucleus and the cytoplasm is an important mediator of RNA export, *Genes Dev.* 10:1233-1246. (MedLine)

Legrain, P. and Rosbash, M. (1989) Some *cis*- and *trans*-acting mutants for splicing target pre-mRNA to the cytoplasm, *Cell* 57:573-583.(Medline)

Lei, E.P., Krebber, H. and Silver, P.A. (2001) Messenger RNAs are recruited for nuclear export during transcription, *Genes Dev.* 15(14):1771-1182. (MedLine)

Lowenstein, W. R. and Kanno, Y. (1962) Some electrical properties of the membrane of a cell nucleus, *Nature* 195:462-464.

Lowenstein, W.R., Kanno, Y. and Ito, S. (1966) Permeability of the nuclear membrane, *Ann. N.Y. Acad. Sci.* 137:708-716.

Luo, M.L., Zhou, Z., Magni, K., Christoforides, C., Rappsilber, J., Mann, M. and Reed, R. (2001) Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly, *Nature* 413:644-647. (MedLine)

Malim, M.H., Hauber, S.-J., Le S.-Y., Maizel, J.V. and Cullen, B.R. (1989) The HIV-1 *rev trans*-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA, *Nature* 338:254-257.(Medline)

Malim, M.H., Tiley, L.S., McCarn, D.F., Rusche, J.R., Hauber, J. and Cullen, B.R. (1990) HIV-1 structural gene expression requires binding of the Rev *trans*-activator to its RNA target sequence, *Cell* 60:675-683.(Medline)

Mazzanti, M., Innocenti, B. and Rigatelli, M. (1994) ATP-dependent ionic permeability on nuclear envelope in in situ nuclei of *Xenopus* oocytes, *FASEB J.* 8:231-236.(Medline)

Mehlin, H., Skoglund, U. and Daneholt, B. (1991) Transport of Balbiani ring granules through nuclear pores in Chironomus tentans, *Exp. Cell Res.* 193:72-77.(Medline)

Mehlin, H., Daneholt, B. and Skoglund, U. (1992) Translocation of a specific premessenger ribonucleoprotein particle through the nuclear pore studied with electron microscope tomography, *Cell* 69:605-613.(Medline)

Melchior, F. and Gerace, G. (1995) Mechanism of nuclear protein transport, *Curr. Opin. Cell Biol.* 7:310-318.(Medline)

Melchior, F., Guan, T., Yokoyama, N., Nishimoto, T. and Gerace, L. (1995) GTP hydrolysis by Ran occurs at the nuclear pore complex in an early step of protein import, *J. Cell Biol.* 131:571-581.(Medline)

Michael, W.M., Choi, M. and Dreifuss, G. (1995a) A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear export pathway, *Cell* 83:415-422.(Medline)

Michael, W.M., Siomi, H., Choi, M., Pi&ntildeol-Roma, S., Nakielny, SD., Liu, Q. and Dreyfuss, G. (1995b) Signal sequences that target nuclear import and nuclear export of pre-mRNA binding proteins, *Cold Spring Harbor Symp. Quant. Biol.* 60:663-668.(Medline)

Michael, W.M., Eder, D.S. and Dreyfuss, G. (1997) The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein, *EMBO J.* 16:3587-3598.(Medline)

Michaud, N. and Goldfarb, D.S. (1991) Multiple pathways in nuclear transport: the import of U2 snRNP occurs by a novel kinetic pathway, *J. Cell Biol.*112:215-223.(Medline)

Mingot, J.M., Kostka, S., Kraft, R., Hartmann, E. and Gorlich, D. (2001) Importin 13: a novel mediator of nuclear import and export, *EMBO J.* 20:3685-3694. (MedLine)

Miralles, F., Öfverstedt, L.G., Sabri, N., Aissouni, Y., Hellman, U., Skoglund, U. and Visa, N. (2000) Electron tomography reveals posttranscriptional binding of pre-mRNPs to specific fibers in the nucleoplasm, *J. Cell Biol.* 148:271-282.(Medline)

Moore, M.S. and Blobel, G. (1993) The GTP-binding protein Ran/TC4 is required for protein import into the nucleus, *Nature* 365:661-663.(Medline)

Moore, M.S. and Blobel, G. (1994) Purification of a Ran-interacting protein that is required for protein import into the nucleus, *Proc. Natl. Acad. Sci. USA* 91:10212-10216.(Medline)

Moran, O., Sciancalepore, M., Sandri, G., Panfili, E., Bassi, R., Ballarin, C. and Sorgato, M.C. (1992) Ionic permeability of the mitochondrial outer membrane, *Eur. Biophys. J.* 21:311-319.(Medline)

Moroianu, J., Hijikata, M., Blobel, G. and Radu, A. (1995) Mammalian karyopherin $\alpha_1\beta$ and $\alpha_2\beta$

heterodimers: $\alpha_1$or α_2 subunit binds nuclear localization signal and β subunit interacts with peptide repeat-containing nucleoporins, *Proc. Natl. Acad. Sci. USA* 92:6532-6536.(Medline)

Moy, T.I. and Silver, P.A. (2002) Requirements for the nuclear export of the small ribosomal subunit, *J. Cell Sci.* 115:2985-2995. (MedLine)

Murphy, R. and Wente, S.R. (1996) An RNA-export mediator with an essential nuclear export signal, *Nature* 383:357-360.(Medline)

Murphy, R., Watkins, J.L. and Wente, S.R. (1996) GLE2, a *Saccharomyces cerevisiae* homologue of the *Schizosaccharomyces pombe* export factor RAE1, is required for nuclear pore complex structure and function, *Mol. Biol. Cell* 7:1921-1937.(Medline)

Nachury, M.V., Ryder, U.W, Lamond, A.I and Weis, K. (1998) Cloning and characterization of hSRP1 gamma, a tissue-specific nuclear transport factor, *Proc. Natl. Acad. Sci. USA* 95:582-587.(Medline)

Nadler, S.G., Tritschler, D., Haffar, O.K., Blake, J., Bruce, A.G. and Cleaveland, J.S. (1997) Differential expression and sequence-specific interaction of karyopherin alpha with nuclear localization sequences, *J. Biol. Chem.* 272:4310-4315.(Medline)

Nakielny, S. and Dreyfuss, G. (1996) The hnRNP C proteins contain a nuclear retention sequence that can override nuclear export signals, *J. Cell Biol.* 134:1365-1373.(Medline)

Nakielny, S. and Dreyfuss, G. (1997) Nuclear export of proteins and RNAs, *Curr. Opin. Cell Biol.* 9:420-429. (MedLine)

Neville, M., Stutz, F., Lee, L., Davis, L.I. and Rosbash, M. (1997) The importin-β family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export, *Curr. Biol.* 7:767-775.(Medline)

Nigg, E.A. (1997) Nucleoplasmic transport: signals, mechanisms and regulation, *Nature* 386:779-787.(Medline)

Ohno, M., Fornerod, M. and Mattaj, I.W. (1998) Nucleocytoplasmic transport: the lat 200 nanometers, *Cell* 92:327-336.(Medline)

Ossareh-Nazari, B., Bachelerie, F.and Dargemont, C. (1997) Evidence for a role of CRM1 in signal-mediated nuclear protein export, *Science* 278:141-144.(Medline)

Otero, G.C., Harris, M.E., Donello, J.E. and Hope, T.J. (1998) Leptomycin B inhibits equine infectious

anemia virus Rev and feline immunodeficiency virus rev function but not the function of the hepatitis B virus posttranscriptional regulatory element, *J. Virol.* 72:7593-7597.(Medline)

Paine, P. L., Moore, L. C. and Horowitz, S. B. (1975) Nuclear envelope permeability, *Nature* 254:109-114.(Medline)

Palacios, I., Weis, K., Klebe, C., Mattaj, I.W. and Dingwall, C. (1996) RAN/TC4 mutants identify a common requirement for snRNP and protein import into the nucleus, *J. Cell Biol.* 133:485-494.(Medline)

Palacios, I., Hetzer, M., Adam, S.A. and Mattaj, I.W. (1997) Nuclear import of U snRNPs requires importin β, *EMBO J.* 16:6783-6792.(Medline)

Panté, N. and Aebi, U. (1996a) Toward the molecular dissection of protein import into nuclei, *Curr. Opin. Cell Biol.* 8:397-406.(Medline)

Panté, N. and Aebi, U. (1996b) Sequential binding of import ligands to distinct nucleopore regions during their nuclear import, *Science* 273:1729-1732.(Medline)

Paschal, B.M. (2002) Translocation through the nuclear pore complex, *Trends Biochem. Sci.* 27:593-596. (MedLine)

Paschal, B.M. and Gerace, L.(1995) Identification of NFT2, a cytosolic factor for nuclear import that interacts with the nuclear pore complex protein p62, *J. Cell Biol.* 129:925-937.(Medline)

Pasquinelli, A.E., Ernst, R.K., Lund, E., Grimm, C., Zapp, M.L., Rekosh, D., Hammarskjöld, M.L. and Dahlberg, J.E. (1997) The constitutive transport element (CTE) of Mason-Pfizer monkey virus (MPMV) accesses a cellular mRNA export pathway, *EMBO J.* 16:7500-7510.(Medline)

Pemberton, L.F., Rosenblum, J.S. and Blobel, G. (1997) A distinct and parallel pathway of nuclear import of an RNA-binding protein, *J. Cell Biol.* 139:1645-1653.(Medline)

Perez, M.and Hirschberg, C.B. (1985) Translocation of UDP-N-acetylglucosamine into vesicles derived from rat liver rough endoplasmic reticulum and Golgi apparatus, *J. Biol. Chem.* 260:4671-4678.(Medline)

Perez, M.and Hirschberg, C.B. (1986) Topography of glycosylation reactions in the rough endoplasmic reticulum membrane, *J. Biol. Chem.* 261:6822-6830.(Medline)

Perez-Terzic, C., Pyle, J., Jaconi, M., Stehno-Bittel, L. and Clapham, D.E. (1996) Conformational states of the nuclear pore complex induced by depletion of nuclear Ca^{2+} stores, *Science* 273:1875-1877.(Medline)

Perez-Terzic, C., Jaconi, M. and Clapham, D.E. (1997) Nuclear calcium and the regulation of the nucleopore complex, *BioEssays* 19:787-792.(Medline)

Peters, R. (1984) Nucleo-cytoplasmic flux and intracellular mobility in single hepatocytes, *EMBO J.* 3:1831-1836.(Medline)

Pi&ntildeol-Roma, S. and Dreyfuss, G. (1991) Transcription-dependent and transcription-independent nuclear transport of hnRNP proteins, *Science* 253:312-314.(Medline)

Pi&ntildeol-Roma, S. and Dreyfuss, G. (1992) Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm, *Nature* 355:730-732.(Medline)

Pokrywka, N.J. and Goldfarb, D.S. (1995) Nuclear export pathways of tRNA and 40 S ribosomes include both common and specific intermediates, *J. Biol. Chem.* 270:3619-3624.(Medline)

Politz, J.C., Browne, E.S., Wolf, D.E. and Pederson, T. (1998) Intranuclear diffusion and hybridization state of oligonucleotides measured by fluorescence correlation spectroscopy in living cells, *Proc. Natl. Acad. Sci. USA* 95:6043-6048.(Medline)

Politz, J.C., Tuft, R.A., Pederson, T. and Singer, R.H. (1999) Movement of nuclear poly(A) RNA throughout the interchromatin space in living cells, *Curr. Biol.* 9:285-291.(Medline)

Pollard, V.W., Michael, W.M., Nakielny, S., Siomi, M.C., Wang, F. and Dreyfuss, G. (1996) A novel receptor mediated nuclear import pathway, *Cell* 86:985-994.(Medline)

Powers, M.A., Forbes, D.J., Dahlberg, J.E. and Lund, E. (1997) The vertebrate GLFG nucleoporin, Nup98, is an essential component of multiple RNA export pathways, *J. Cell Biol.* 136:241-250.(Medline)

Prieve, M.G., Guttridge, K.L., Munguia, J.E. and Waterman, M.L. (1996) The nuclear localization signal of lymphoid enhancer factor-1 is recognized by two differentially expressed Srp1-nuclear localization sequence receptor proteins, *J. Biol. Chem.* 271:7654-7658.(Medline)

Pritchard, C.E., Fornerod, M., Kasper, L.H. and van Deursen, J.M. (1999) RAE1 is a shuttling mRNA export factor that binds to a GLEBS-like NUP98 motif at the nuclear pore complex through multiple domains, *J. Cell Biol.* 145:237-254.(Medline)

Radu, A., Blobel, G. and Moore, M.S. (1995a) Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins, *Proc. Natl. Acad. Sci. USA* 92:1769-1773.(Medline)

Radu, A., Moore, M.S. and Blobel, G. (1995b) The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex, *Cell* 81:215-222.(Medline)

Rakowska, A., Danker, T., Schneider, S.W and Oberleithner, H. (1998) ATP-Induced shape change of nuclear pores visualized with the atomic force microscope, *J. Membr. Biol.* 163:129-136. (MedLine)

Rexach, M. and Blobel, G. (1995) Protein import into nuclei: association and dissociation reactions substrate, transport factors and nucleoporins, *Cell* 83:683-692.(Medline)

Reynolds C.R. and Tedeschi, H. (1984) Permeability of mammalian cell nuclei in living cells, *J. Cell Sci.* 70:197-207.(Medline)

Ribbeck, K. and Görlich, D. (2001) Kinetic analysis of translocation through nuclear pore complexes, *EMBO, J.* 20:1320-1330. (MedLine)

Ribbeck, K. and Gorlich, D. (2002) The permeability barrier of nuclear pore complexes appears to operate via hydrophobic exclusion, *EMBO J.* 21:2664-2671. (MedLine)

Ribbeck, K., Kutay, U., Paraskeva, E. and Görlich, D. (1999) The translocation of transportin-cargo complexes through nuclear pores is independent of both Ran and energy, *Curr. Biol.* 9:47-50.(Medline)

Richardson W. D., Mills, A. D., Dilworth, S. M., Laskey, R. A. and Dingwall, C. (1988) Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by a slower translocation through nuclear pores, *Cell* 52: 655-664.(Medline)

Ris, H. (1991) The three-dimensional structure of the nuclear pore complex as seen by high voltage electronmicroscopy and high resolution low voltage scanning electron microscopy, *EMSA Bull.* 21:54-56.

Ris, H. (1997) High-resolution field-emission scanning electron microscopy of nuclear pore complex, *Scanning* 19:368-375.(Medline)

Robbins, J., Dilworth, S. M., Laskey, R. A. and Dingwall, C. (1991) Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence, *Cell* 64:615-623.(Medline)

Rout, M.P. and Blobel, G. (1993) Isolation of yeast nucleopore complex, *J. Cell Biol.* 123:771-783.(Medline)

Rout, M.P. and Wente, S.R. (1994) Pores for thought: nuclear pore complex proteins, *Trends in Cell Biol.* 4: 357-365.

Rout, M.P., Blobel, G., and Aitchinson, J.D. (1997) A distinct nuclear import pathway used by ribosomal proteins, *Cell* 89:715-725.(Medline)

Rout, M.P., Aitchison, J.D., Suprapto, A., Hjertaas, K., Zhao, Y. and Chait B.T. (2000) The yeast nuclear pore complex. Composition, architecture, and transport mechanism, *J. Cell Biol.* 148:635-652. (MedLine)

Saavedra, C., Felber, B. and Izaurralde, E. (1997) The simian retrovirus-1 constitutive transport element, unlike the HIV-1 RRE, uses factors required for cellular mRNA export, *Curr. Biol.* 7:619-628.(Medline)

Santella, L. and Carafoli, E. (1997) Calcium signaling in the cell nucleus, *FASEB J.* 11:1091-1109. MedLine)

Santos-Rosa, H., Moreno, H., Simos, G., Segref, A., Fahrenkrog, B., Pante, N. and Hurt, E. (1998) Nuclear mRNA export requires complex formation between Mex67p and Mtr2p at the nuclear pores, *Mol. Cell. Biol.* 18:6826-6838..(Medline)

Schaap, P.J., van't Riet, J., Woldringh, C.L. and Raue, H.A. (1991) Identification and functional analysis of the nuclear localization signals of ribosomal protein L25 from *Saccharomyces cerevisiae*, *J. Mol. Biol.* 221:225-2370.(Medline)

Schindler, M. and Jiang L.-W. (1986) Nuclear actin and myosin as control elements in nucleocytoplasmic transport, *J. Cell Biol.* 102:859-862.(Medline)

Schlenstedt, G., Saavedra, C., Loeb, J.D.J., Cole, C.N. and Silver, P. (1995) The GTP-bound form of yeast Ran/TC4 homologue blocks nuclear protein import and appearance of poly(A)$^+$RNA in the cytoplasm, *Proc. Natl. Acad. Sci. USA*, 92:225-229.(Medline)

Schlenstedt, G., Smirnova, E., Deane, R., Solsbacher, J., Kutay, U., Görlich, D., Ponstingl, H.0 and Bischoff, F.R. (1997) Yrb4p, a yeast ran-GTP-binding protein involved in import of ribosomal protein L25 into the nucleus, *EMBO J.* 16:6237-6249.(Medline)

Schoolwerth, A. C. and LaNoue, F. F. (1985) Transport of metabolic substrates in renal mitochondria, *Annu. Rev. Physiol.* 47: 143-171.(Medline)

Segref, A., Sharma, K., Doye, V., Hellwig, A., Huber, J., Luhrmann, R. and Hurt, E. (1997) Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores, *EMBO J.* 16:3256-3271.(Medline)

Sekimoto, T., Imamoto, N., Nakajima, K., Hirano, T. and Yoneda, Y. (1997) Extracellular signal-dependent nuclear import of Stat1 is mediated by nuclear pore-targeting complex formation with NPI-1,

but not Rch1, *EMBO J.* 16:7067-7077.(Medline)

Shah, S., Tugendreich, S. and Forbes, D. (1998) Major binding sites for the nuclear import receptor are the internal nucleoporin Nup153 and the adjacent nuclear filament protein Tpr, *J. Cell Biol.* 141:31-49.(Medline)

Shahin, V., Danker, T., Enss, K., Ossig, R. and Oberleithner, H. (2001) Evidence for Ca^{2+}- and ATP-sensitive peripheral channels in nuclear pore complexes, *FASEB J* 15:1895-1901. (MedLine)

Shulga, N., Roberts, P., Gu, Z., Spitz, L., Tabb, M.M., Nomura, M. and Goldfarb, D.S. (1996) In vivo nuclear transport kinetics in *Saccharomyces cerevisiae*: a role for heat shock protein 70 during targeting and translocation, *J. Cell Biol.* 135:329-339.(Medline)

Shyu, A.-B. and Wilkinson, M.F. (2000) The double lives of shuttling mRNA binding proteins, *Cell* 102:135-138. (MedLine)

Siebrasse, J.P. and Peters, R. (2002) Rapid translocation of NTF2 through the nuclear pore of isolated nuclei and nuclear envelopes, *EMBO Rep.*3:887-892. (MedLine)

Siomi, H. and Dreyfuss, G. (1995) A nuclear-localization domain in the hnRNP A1 protein, *J. Cell Biol.* 129:551-560.(Medline)

Siomi, M.C., Eder, P.S., Kataoka, N., Wan, L., Liu, Q. and Dreyfuss, G. (1997) Transportin-mediated nuclear RNP proteins, *J. Cell Biol.* 138:1181-1192.(Medline)

Snow, C. M., Senior, A. and Gerace, L. (1987) Monoclonal antibodies identify a group of nuclear pore complex glycoproteins, *J. Cell Biol.* 104:1143-1156.(Medline)

Stade, K., Ford, C.S,, Guthrie, C. and Weis, K. (1997) Exportin 1 (Crm1p) is an essential nuclear export factor, *Cell* 90:1041-1050.(Medline)

Steggerda, S.M. and Paschal, B.M. (2002) Regulation of nuclear import and export by the GTPase Ran, *Int. Rev. Cytol.* 217:41-91. (MedLine)

Steiz, J. A., Black, D. L., Gerke, V., Parker, K. A., Krämer, A., Frendewey, D. and Keller, W. (1988) Functions of the abundant U snRNPs. in *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles* (Birnstiel, M., ed.) pp. 115-154, Springer Verlag, Heidelberg.

Stehno-Bittel, L., Perez-Terzic, C. and Clapham, D.E. (1995) Diffusion across the nuclear envelope inhibited by depletion of the nuclear Ca^{2+} store, *Science* 270:1835-1838.(Medline)

Sterne-Marr, R., Blevitt, J. M. and Gerace, L. (1992) O-linked glycoproteins of the nuclear pore complex interact with cytosolic factor required for nuclear pore import, *J. Cell Biol.* 116:271-280.(Medline)

Strambio-de-Castillia, C., Blobel, G. and Rout, M.P. (1999) Proteins connecting the nuclear pore complex with the nuclear interior, *J. Cell Biol.* 144:839-855.(Medline)

Strässer, K. and Hurt, E. (2000) Yra1p, a conserved nuclear RNA-binding protein, interacts directly with Mex67p and is required for mRNA export, *EMBO J.* 19:410-420. (MedLine)

Stutz, F., Izaurralde, E., Mattaj, J.W. and Rosbach, M. (1996) A role for nucleoporin FG repeat domains in export of human immunodeficiency virus type I Rev protein and RNA from the nucleus, *Mol. Cell. Biol.* 16:7144-7150.(Medline)

Sukegawa, J. and Blobel, G. (1993) A nuclear pore complex protein that contains zinc finger motifs, binds DNA, and faces the nucleoplasm, *Cell* 72:29-38.(Medline)

Tedeschi, H. and Harris, D. L. (1955) The osmotic behavior and permeability to non-electrolytes of mitochondria, *Arch. Biochem. Biophys.* 58:52-67.

Tedeschi, H. and Kinnally, K.W. (1994) Mitochondrial membrane channels, *Handbook of Membrane Channels*, Peracchia, C. ed., Academic Press, San Diego pp.529-548.

Tonini, R., Grohovaz, F., Laporta, C.A. and Mazzanti, M. (1999) Gating mechanism of the nuclear pore complex channel in isolated neonatal and adult mouse liver nuclei, *FASEB J.* 13:1395-1403.(Medline)

Unwin, P. N. T., and Milligan, R. A. (1982) A large particle associated with the perimeter of the nuclear pore, *J. Cell Biol.* 93:63-75.(Medline)

Vancurova, I., Paine, T.M., Lou, W., Paine, P.L.(1995) Nucleoplasmin associates with and is phosphorylated by casein kinase II, *J. Cell Scie.* 108:779-787.(Medline)

Visa, N., Izaurralde, E., Ferreira, J., Daneholt, B. and Mattaj, I.W. (1996) A nuclear cap-binding complex binds Balbiani ring pre-mRNA cotranscriptionally and accompanies the ribonucleoprotein particle during nuclear export, *J. Cell Biol.* 133:5-14.(Medline)

Waldman, B.C. and Rudnick, G. (1990) UDP-GlcNAc transport across Golgi membrane: electroneutral exchange for dianionic UMP, *Biochem.* 29:44-52.(Medline)

Walther, T.C., Pickersgill, H.S., Cordes, V.C., Goldberg, M.W., Allen, T.D., Mattaj, I.W. and Fornerod, M. (2002) The cytoplasmic filaments of the nuclear pore complex are dispensable for selective nuclear

protein import, *J. Cell Biol.* 158:63-77. (MedLine)

Weis, K. (1998) Importins and exportins: how to get in and out of the nucleus, *Trends in Biochem. Sci.* 23:185-189.(Medline)

Weis, K., Mattaj, I.W., and Lamond, A.I. (1995) Identification of snSRP1 α as a functional receptor for nuclear localization sequences, *Science* 268:1049-1052.(Medline)

Wen, W., Meinkoth, J.L. Tsien, R.Y. and Taylor, S.S. (1995) Identification of a signal of rapid export of proteins from the nucleus, *Cell* 82:463-473.(Medline)

Wilken, N., Senécal, J.-L., Scheeer, U. and Dabauville, M.-C. (1995) Localization of the Ran-GTP binding protein RanBP2 at the cytoplasmic side of nuclear pore complex, *Eur. J. Cell Biol.* 68:211-219.(Medline)

Wu, J., Matunis, M.J., Kraemer, D., Blobel, G. and Coutavas, E. (1995) Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin A homologous domain, and a leucine rich-region, *J. Biol. Chem.* 270:14209-14213.(Medline)

Xing, Y. and Lawrence, J. B. (1991) Preservation of specific RNA distribution within the chromatin-depleted nuclear substructure demonstrated by *in situ* hybridization coupled with biochemical fractionation, *J. Cell Biol.* 112:1055-1063.(Medline)

Yang, Q., Rout, M.P. and Akey, C.W. (1998) Three dimensional architecture of the isolated yeast nuclear architecture of the isolated yeast nuclear pore complex: functional and evolutionary implications, *Mol. Cell* 1:223-234.(Medline)

Yokoyama, N., Hayashi, N., Seki, T., Panté, N., Ohba, T., Nishii, K., Kuma, K., Hayashida, T., Miyata, T., Aebi, U., Fukui, M. and Nishimoto, T. (1995) A giant nucleopore portein that binds Tan/TC4, *Nature* 376:184-188.(Medline)

Yoshida, K. and Blobel, G. (2001) The karyopherin Kap142/Msn5p mediates nuclear import and export of different cargo proteins, *J. Cell Biol.* 152:729-739.

Zimowska, G., Aris, J.P. and Paddy, M.R. (1997) A Drosophila Tpr protein homolog is localized both in the extrachromosomal channel network and to nuclear pore complexes, *J. Cell Sci.* 110:927-944.(Medline)

Zolotukhin, A.S. and Felber, B.K. (1999) Nucleoporins nup98 and nup214 participate in nuclear export of human immunodeficiency virus type 1 Rev, *J. Virol.* 73:120-127.(Medline)

6. Chemical Signals: Receptors

Cells respond to signals. In a multicellular organism many of these signals are hormones and growth factors which cannot enter the cell readily. Either they are large, such as protein molecules (for example, insulin) or polar (for example, adrenaline). In some cases the response has to be rapid. Most of these chemicals bind specifically to integral proteins at the cell surface, the *receptors*. In addition, growing evidence indicates that in some cases both receptors and protein factors or fragments of the receptors are transferred to the interior of the cell where they exert their effects (see below). These findings suggest that there are multiple modes of action of the protein factors (see Wells and Marti, 2002). The binding of ligands to the surface recptors generally initiates a *cascade* of reactions that direct the cell to specific activities. Just a few molecules of a hormone, for example, can trigger a massive effect where hundreds or thousands of new molecules are produced by the cell's enzymes. Sometimes receptors capable of binding to the *polypeptide growth factors* (PGFs) and their ligands have been found in the nucleus (see Jans and

Hassan, 1998; Keresztes and Boonstra, 1999). Presumably, there must be a mechanism for the uptake of the PGFs and receptors, possibly via endocytosis (see Chapter 9). The internalized receptor may respond differently than the molecules of the same receptor remaining at the cell surface (e.g., Zhang et al., 2000). The translocation of the *fibroblast growth factor* (FGF) receptor into the nucleus has been found to depend on importin-β and to be involved in the regulation of cell proliferation (Reilly and Maher, 2001. Importin-β is a protein that has been implicated in the active transport of proteins into the nucleus (see Chapter 5). The presence of receptor for the *epidermal growth factor* (EGF) in the cell nucleus correlates with cell proliferation. It has been found associated with the promoter region of cyclin D1 and to act as a transcription activator (Lin et al., 2001). Cyclins are proteins that trigger various phases of cell division (see Chapter 8).

The proliferation and survival of normal cells are controlled by a variety of substances, including a group of polypeptide hormones, the PGFs or *mitogens*. The PGFs are a large family of regulatory peptides. Growth and development of cells in the immune and hematopoietic systems are under coordinated control of several polypeptide factors known as *cytokines*. This chapter is dedicated to the signals that involve events at the cell surface and includes the binding of ligands by their receptors at the surface, followed by cleavage of the receptors and targeting to the nucleus (see Chapter 7).

Steroid and thyroid hormones are also very important in growth and development. In contrast to the peptide factors, these hormones are small and relatively hydrophobic, and can go through the plasma membrane. They combine with intracellular receptors forming a complex which triggers the biological effect of the hormones. These hormones and the cascades of biochemical events which follow the binding to receptors, either at the cell surface or the cytoplasm, are discussed in Chapter 7.

Sction I concentrates on the receptors, sections II, III and IV of this chapter are concerned with the binding of factors to the receptors.

I. RECEPTORS AT THE CELL SURFACE

The transduction of signals at the cell surface is one of the roles of the receptors, generally integral proteins present at the cell surface. The receptors may be distributed throughout the plasma membrane or in specialized structures such as *coated pits* (see Chapter 9, Section IV) or *caveolae* (see Chapter 9, Section V). After binding a hormone or PGF, the activated receptors are thought to initiate a cascade of biochemical reactions in the cytoplasm. The intracellular signaling systems are the primary topic of Chapter 7 (Section I). Some of the receptors may be channels. Conformational changes in response to binding of a ligand could open or close them. These are known as *ligand-gated* channels. The activity of the channels would then, in turn, trigger intracellular events. Some of the channels, when open, allow the influx of certain ions (e.g., Ca^{2+}) or efflux of other ions (e.g., K^{+}).

In addition to acting as ligand-gated channels, at least one set of glutamate receptors in the central nervous system (see Chapter 22) also acts like conventional receptors. They produce a cascade (see

Chapter 7) of events (Hayashi et al., 1999) which culminate in the expression of *brain derived neurotrophic factor* (BDNF).

Some receptors have common amino acid domains. Others exhibit common features (such as domains that may traverse lipid bilayers), despite the absence of amino acid sequence-homology. The various kinds of receptors are displayed in Table 1 (Barnard, 1992a). Class 1 receptor subunits can be assembled into oligomers of like (*homomeric*) or unlike (*heteromeric*) subunits to form ion conducting channels when they bind transmitters such as neurotransmitters. Class 2 receptors have seven hydrophobic polypeptide domains thought to be transmembrane helices (consequently called *heptahelical receptors*). They generally correspond to monomers or homodimers requiring interaction with G-proteins (also known as the heterotrimeric GTP-binding proteins or GTPases) (see Chapter 7, Section II). Recently, however, other pathways not involving G-proteins have been implicated in the signaling pathway initiated by heptahelical receptors (see Hall et al., 1999). Class 3 receptors generally have single hydrophobic domains. The receptors interacting with growth factors (Section II) belong to this group. The ciliary neurotrophic factor receptor (CNFR) required by neurons for their survival, does not have transmembrane domains, but is anchored to a glycolipid.

Most commonly, the class 3 receptors (the receptors for the polypeptide growth factors discussed below in Section II) are tyrosine autokinases (*receptor tyrosine kinases*, RTKs), i.e., they phosphorylate their own tyrosine residues. The RTKs include approximately twenty subfamilies. The phosphorylation enhances receptor catalytic activity and/or the phosphorylated site serves as docking site for cytoplasmic signaling proteins, some involved in the production of second messengers (see Chapter 7, Section I). These include phosphatidyl inositol 3-kinase, phospholipase Cγ, (PLCγ), GTPase activating factor (GAP), (see Chapter 7), and other proteins. The amino acid sequences of proteins that bind to the phosphorylated sites, are the SH2 domains (see Table 2 and separate discussion) or the *phosphotyrosine binding* (PTB) domain (e.g., Pascal et al., 1994) (which can also bind to the unphosphorylated residue e.g., Zhou et al, 1995). The same receptor may bind different ligands, but at different binding sites.

Mutations of tyrosine-containing domains have allowed mapping the function of the various signaling molecules. For example, mutations in the phosphatidyl inositol 3-kinase (PI kinase) binding site of the *platelet derived growth factor* β *receptor* (PDGFβr) expressed in murine epithelial cells, blocks activation of the PI kinase and stops mitogenesis (Fantl et al., 1992). The pathways activated by the RTKs are schematically represented in Fig. 1 (Pazin and Williams, 1992). The details will be discussed in more detail in the rest of the chapter (see also Chapter 7, sections II, III, IV).

Table 1 Signal-transducing receptors of membranes: structural classification

Class	Subunit composition	Transduction system	Ligands

1.Channel-enclosing oligomers a. Extracellularly activated b. Intracellularly activated	Heteromeric or homomeric	Transmitter-gated ion channels	GABA glycine, Ach, glutamate $5HT_3$, ATP, cGMP, cAMP, cGMP, ATP, IP_3, Ca^{2+}
2. Seven-hydrophobic-domain polypeptides Superfamilies: I. Main superfamily II. Secretin, VIP, parathyroid hormone and calcitonin receptors III. Metabotropic glutamate receptors (via 2nd messenger)	Monomers or homodimers or post-translational heterodimers	Via a G protein A. Plus a diffusable messenger B. Acting directly on a channel C. After receptor cleavage by a polypeptide hormone acting as a site-specific protease (and not as a classical agonist) to form a self-activating receptor	A. All the small transmitters (except glycine); neuropeptides; odorants; certain cytokines (e.g. IL-8); lipid and related agonists (PAF, eicosaniods) B. Atrial muscarinic; neuronal α_1-adrenergic, etc. C. Thrombin is the only case so far known
3. Single-hydrophobic-domain polypeptides I. Containing one TM sequence II. glycolipid membrane anchor	Monomers or homodimers or post-translational heterototramers or native heterodimers or heterotrimers	The binding subunit itself is: A. a ligand-stimulated tyrosine kinase B. a ligand-stimulated guanylate cyclase C. Not of known enzymatic activity	All are polypeptides: A. Mitogenic growth factors; insulin B. Natriuretic peptides C. Neurotrophins; growth hormone, prolactin and many cytokines. Of type II, only CNTF-R is so far known

Abbreviations: TM, transmembrane domain; CNTF-R, ciliary neurotrophic factor receptor; IL, interleukin; PAF, platelet activating factor; VIP, vasoactive intestinal polypeptide.

From Barnard, 1992a. Reproduced by permission.

The heptahelical receptors (seven-transmembrane-span receptors, also known as G protein-coupled receptors, GPCRs) (Class 2 in Table 2) (see Pierce et al., 2002) are generally coupled to the heterotrimeric GTPase protein (see Chapter 7). In humans, these of receptors are represented by more than 1,000 proteins (see Bockaert and Pin, 1999) and they are activated by a variety of ligands. The

structure comprised of seven helical transmembrane domains, an extracellular amino-terminal and a cytoplasmic carboxy terminal are common to all of these proteins of (e.g., see Watson and Arkinstall, 1994). They are capable of combining with other GPCRs to form homo- or heterodimers with different GPCR. In addition, they also can combine through their carboxy-domain (cytoplasmic) directly with other proteins that possess a single transmembrane domain. The activation of the receptors is accompanied by changes in conformation (Ballesteros et al., 2001). The α subunit and the $\beta\gamma$ dimer act through the activation or inhibition of effectors (such as Ca^{2+} channels, adenylyl cyclase, cGMP phosphodiesterase, etc.) (see Pierce et al., 2002). The activation and inhibition of the GPCRs is regulated by the *arrestins* (see Perry and Lefkowitz, 2002).

Continuous stimulation of the receptors decreases their activity (*desensitization*). Desensitization can result from a variety of mechanisms. At the receptor level it is usually the consequence of receptor phosphorylation controlled by a negative-feedback loop (e.g., the receptor activates a protein kinase which in turn inactivates the receptor) or in response to second messengers activated by other receptors. Desensitization is discussed in more detail in Chapter 7

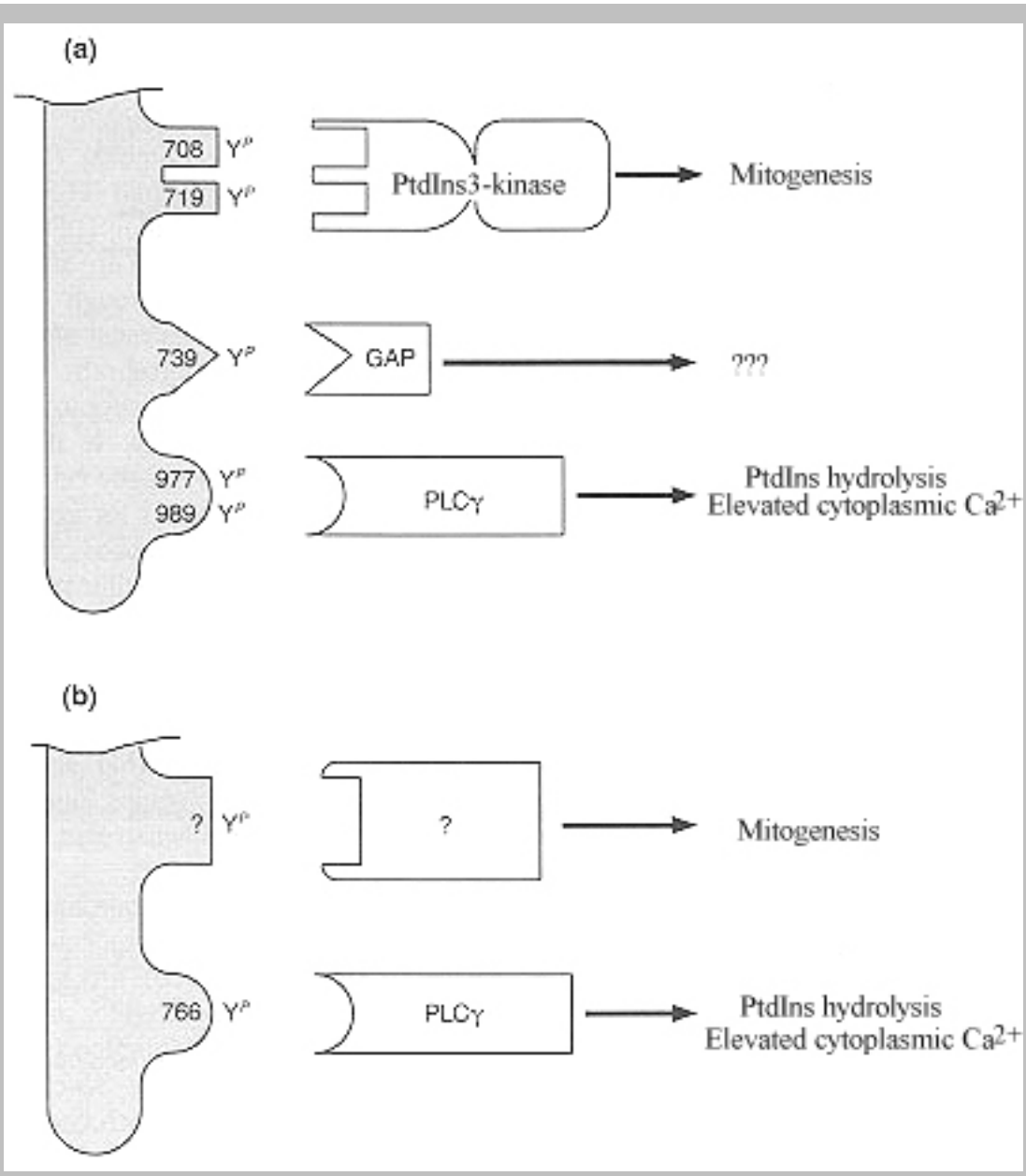


Fig. 1 Interactions of (a) PDGFβr and (b) FGFr with cytoplasmic signaling molecules. Numbers refer to specific phosphotyrosine residues (Y^p) in the cytoplasmic region of the murine PDGFβr and human FGFr-1. Each RTK phosphotyrosine-containing region binds a specific signaling molecule. The signaling molecules, which include GTPase activating protein (GAP) phospholipase C-γ (PLCγ) and phosphatidylinositol kinase (Ptdlns3-kinase), activate the indicated cellular responses. A question mark (?) indicates an unknown phosphotyrosine residue, signaling molecule or cellular response. Reproduced from *Trends in Biochemical Science*, vol.17, Pazin, M.J. and Williams, L.T., Triggering signal cascades by receptor tyrosine kinases, minireview, pp.374-378, copyright &copy1992, with permission from Elsevier Science.

A. Activation of the Receptors

What actually happens when a ligand binds to a receptor? Experiments with several growth factor receptors [those for *epidermal growth factor* (EGF), *platelet-derived growth factor* (PDGF) and insulin], revealed the surprising finding that bivalent antibodies to the receptors could frequently activate them in

the absence of hormone (Ullrich and Schlessinger, 1990). In contrast, monovalent antibodies had no effect. The receptors are likely to be free to move in the plane of the membrane (see Chapter 4). Therefore, they could be cross-linked by a bifunctional compound to form a dimer if they collided in the two dimensional framework of the membrane. A possible intepretation is that binding of receptor molecules to form a complex (receptor *oligomerization*) initiates signal transduction. Formation of a dimer has been shown to be part of the hormone induced activation mechanism for the human growth hormone (hGH) receptor and one of the tumor necrosis factor (TNF) receptors (see Wells, 1994 for a review). RTKs (see above) dimerize in response to the binding of their ligand or ligands (see van der Geer et al., 1994). hGH dimer formation is discussed in some detail below.

hGH regulates growth and metabolism in human tissues. This protein has a molecular weight of 22 kDa. Its receptor has 25 residues that span the plasma membrane. A 250 residue extracellular domain (referred to as hGHpp) contains the binding site for the hormone. In contrast, its cytoplasmic domain binds tyrosine kinase (Argentsinger et al., 1993) and presumably activates it. Activation involves hormone induced dimerization of the extracellular domains (Cunningham et al., 1991). The association between hormone and dimer is 1 to 1. In these experiments, a fixed amount of hGHpp (obtained from an *E. coli* gene expression system) was titrated by the addition of hGH by isothermal calorimetry. In this procedure, the temperature inside the calorimeter is kept constant by cooling or heating the preparation and the change in enthalpy (ΔH, see Chapter 12) recorded. The results are shown in Fig. 2 (Cunningham et al., 1991). After each addition, heat is released, until finally little or no deflection occurs with other additions. This would then correspond to the completion of the binding. Approximately 0.5 equivalents of the hormone (where the hGHpp concentration is 1) produce maximum binding. This corresponds to 2 hGHpp per hGH molecules. Similar conclusion can be reached by dissociating a hGHpp-hGH crystal and determining the concentrations of the two, using high-performance liquid chromatography (Cunningham et al., 1991).

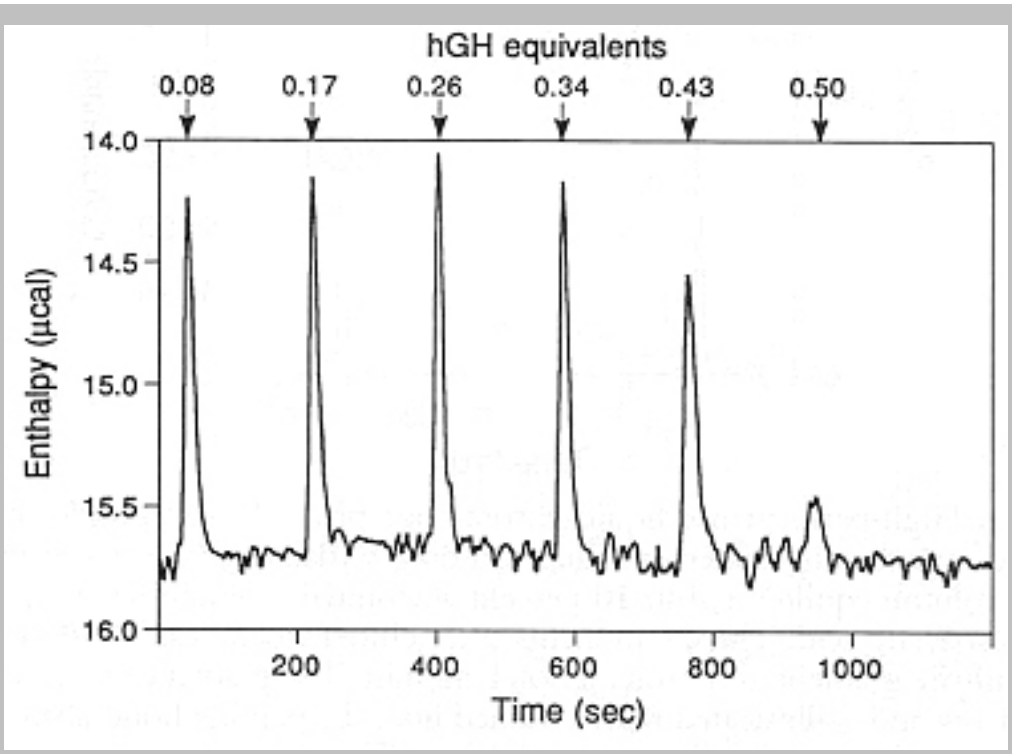


Fig. 2 Titration calorimetry of hGH with hGHpp. 4 µl of a solution containing the latter component were added over 8 s every 5 minutes. 1 equivalent corresponds to 20 nmoles of hGHpp. Reproduced with permission from Cunningham, B.C., Ultsch, M. de Vos, A.M., Mulkerrin, M.G., Clausner, K.R. and Wells, J.A. (1991) Dimerization of the extracellular domain of the human growth factor receptor by a single hormone molecule, *Science* 254:821-825. Copyright &copy1991 American Association for the Advancement of Science.

A model consistent with the data is shown in Fig. 3 (Wells, 1994) for the dimerization of hGH. The hormone has two different binding sites for the receptor molecules (marked as 1 and 2 in the diagram). The binding to receptor molecules is sequential. At high concentration of hormone, the probability of binding two receptor molecules on site 1 are high. This would prevent activation. At low concentration, the probability of the binding one receptor on site 1 and the other on site 2 is higher. This results in activation of the tyrosine autokinase activity of the receptor (known as JAK2; see see Chapter 7).

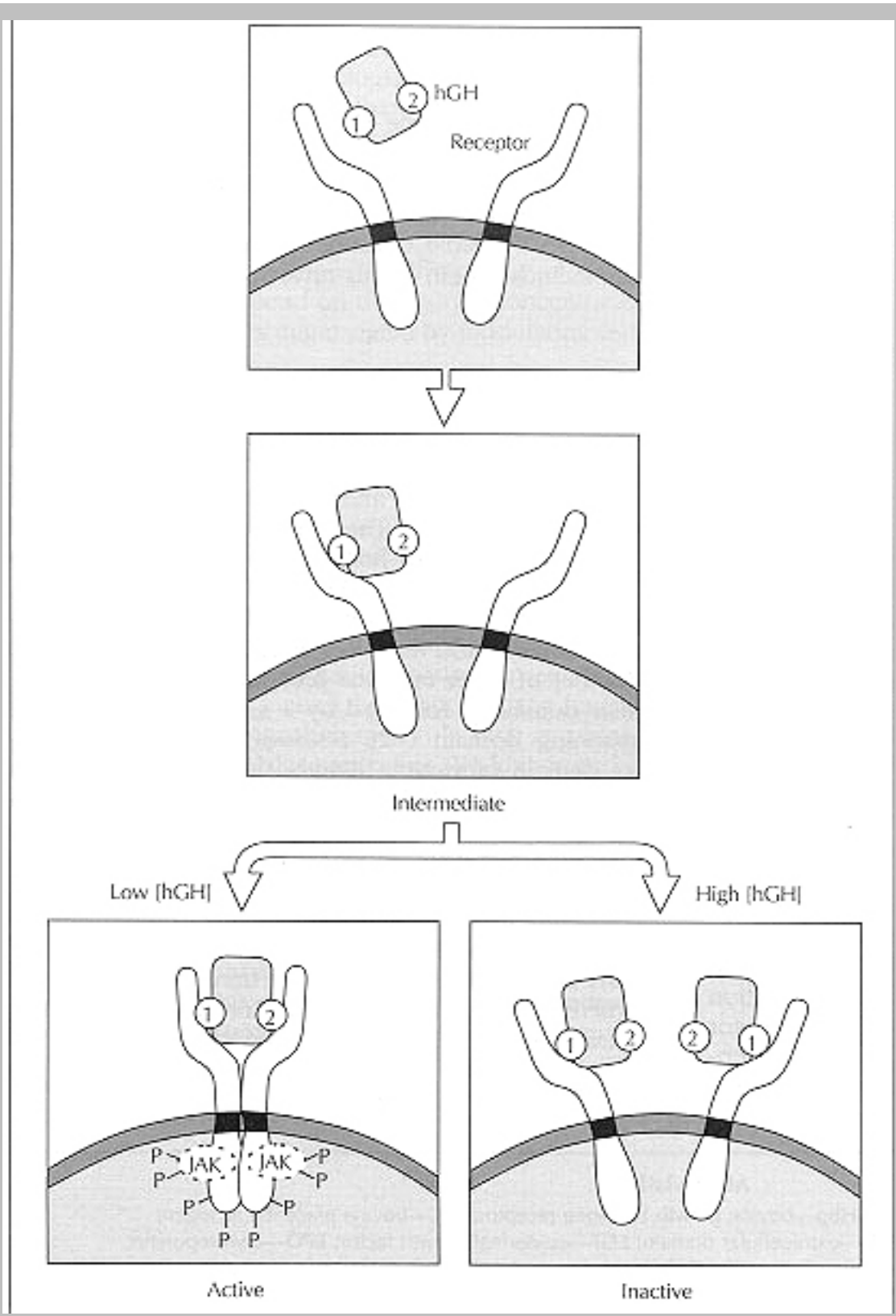


Fig. 3 Sequential dimerization mechanism for activation or inhibition of the hGH receptor. From Wells, 1994, reproduced by permission. (Available in the BioMedNet library at: http://biomednet.com/cbiology/cel)

The formation of a dimer of like subunits, known as *homodimerization*, is thought to be required for the action of a variety of cytokines. There is now evidence from reconstitution and cross-linking experiments

for the formation of dimeric complexes from unlike subunits (*heterodimeric complexes*) and, in some cases, tripartite complexes.

In the cases in which receptors heteroligomerize, the subunits are generally related. The different mixes of subunits allow for different forms of signaling (e.g., see Riese and Stern, 1998; Pinkas-Kramarski et al., 1998). For example, four proteins of the ErB RTK (EGFR) family are generally present as heterodimers. The various possible combinations of receptor subunits can bind different ligands [e.g., EGF, *neuregulins* (NRGs)], and, therefore, a single receptor can be activated by different extracellular ligands (see Moghal and Stenberg, 1999). NRGs are axon-associated survival signals needed for the development of oligodendrocytes. In addition to responding to different external chemical signals, the receptor subunits differ in their cytoplasmic domains so that they can bind different cytoplasmic signaling proteins and initiate distinct cascades.

Although oligomeric receptors are usually composed of related subunits, unrelated receptors can also interact at the surface of cells, for example, the γ chain of the $GABA_A$ receptor attaches to dopamine D5 receptor (a G-protein coupled receptor) resulting in the mutual inhibition of the pathways (see Liu et al, 2000).

Dimerization or oligomerization of receptors are not always the result of activation. In a number of cases, there are indications that some of the receptors are present as preformed dimers or oligomers before activation. This has been demonstrated using *fluorescence resonance energy transfer* (FRET) (see Chapter 1 and Chapter 4) and other techniques. FRET depends on the non-radiant transmission of energy between two fluorophores. The transmission is sharply dependent on the distance between fluorophores. Therefore, the energy transfer can take place only when the fluorescent dyes attached to the receptors are close together, as would occur with dimerization or oligomerization.

The receptors where oligomerization precedes activation include interleukin-2, EGF and erythropoietin receptors. Interleukins are factors that have a role in the immune response, inflammation and proliferation of T cells (see Section IIB). Epithelial growth factor is one of the growth factors discussed previously (see section IIA). Erythropoietin regulates proliferation and differentiation of erythroid cells (e.g., see Krantz, 1991). The hormone is a glycoprotein that binds to receptor which is a class 1 cytokine receptor (see Wells and de Vos, 1996).

The results obtained with FRET indicate that IL-2 receptor subunits are already assembled in resting cells in the absence of ligand (Damjanovich et al., 1997). The results obtained with EGF receptors (Gadella and Jovin, 1995) support previous studies that demonstrated the clustering of the receptors following binding of EGF. However, a subclass of receptors on quiescent cells are present in a predimerized or oligomerized state. The unliganded erythropoietin receptor is present as a dimer as shown by crystallographic data (Livnah et al., 1999).

A change in conformation upon activation of a receptor or receptor oligomers, has been suspected for

some time. Fluorescent binding complementation experiments (Remy et al., 1999) indicate that ligand binding to the erythropoietin receptor results in a conformational change that allows JAK (see Chapter 7) (the protein kinase that initiates the cascade after ligand binding) to be activated. The technique has been previously used to reassemble peptides into functional enzymes, as directed by oligomerization domains added to the fragments (Pelletier et al., 1998). The experiments are based on an assay where fragments of the enzyme dihydrofolate reductase (F[1,2] and F[3]) are fused to the erythropoietin receptor monomers. When the fragments are brought together by dimerization, they reassemble into an active enzyme molecule and bind with high affinity to a fluorescein conjugated inhibitor, methotrexate (fMTX). The complex remains bound, whereas free fMTX is actively transported out of the cells (e.g., Israel and Kaufman, 1993). Therefore, the fMTX fluorescent probe will bind to the functional dihyroxyfolate and the latter can be detected by its fluorescence. The results show that the fragments residing on the dimer are brought closer together by activation (Remy et al., 1999). However, when linkers of various lengths are attached to the receptors (to allow interactions of the peptide fragments that would be otherwise too distant in the non-activated receptors), the results confirm that they are present as dimers in their inactive form (Remy et al., 1999).

The assembly of complexes through specific protein-protein or protein-phospholipid interactions plays an essential role in signaling. The specific binding of recognition domains of various proteins to a single scaffold protein molecule, serves to bind together activated receptors and downstream effectors and provides a mechanism to ensure specificity and efficient transmittal (see Chapter 7 and Pawson and Scott, 1997; Tsunoda et al., 1998; Pawson and Nash, 2000). A specific example for this mechanism is shown below for the signal represented by the *transforming growth factors* (TGF)-β.

The role of these protein domains is summarized in Table 2. A discussion is presented separately. The components responsible for this scaffolding function have been referred to as *transducisomes*. In addition to the cytoplasmic domains presented in Table 2 or in the discussion there is some evidence for as many as 30 others (Bork at al., 1997). PH and FYVE are not the only domains that interacts with phosphatidyl compounds. The phosphorylation or hydrolysis of phosphatidylinositol compounds, elicited by signals, produce domains for the recruitment of proteins to membrane sites (e.g., see Rameh and Cantley, 1999; Fruman et al., 1999) and, in addition to proteins containing PH and FYVE, those with SH2 and PTB domains.

Table 2 Protein binding domains

Abbreviations	Domain name	Binding	Size (amino acids)	sample ref.

EH	**Eps homology**	**asparagine-proline-phenylalanine**	**approx. 110**	**Salcini et al., 1997**
ENTH	**NH_2-terminal homology**		**approx. 140**	**Hyman et al., 2000**
F-Box		**Skp1 and Skp1-like proteins**	**50**	**Kipreos and Pagano,2000**
FHA	**fork-head associated**	**phosphoserine, phosphothreonine**	**65-100**	**Li et al. 1999**
FYVE	**Fab1p, YOTB, Vab1p, EEA1**	**phosphatidyl inositol 3-phosphate**	**70**	**Wiedemann and Cockcroft, 1998** **Corvera and Czech, 1998**
PAS		**dimerization domain**	**approx. 270 amino acids**	**Huang et al., 1993**
PDZ	**postsynaptic density protein**	**serine-X-valine**	**approx. 90**	**Tsunoda et al., 1997**
PH	**pleckstrin homology**	**phosphoinositides**	**120**	**Lemmon and Ferguson, 2000**
PTB (PI)	**phosphotyrosine binding**	**phosphotyrosine and others**	**approx. 100**	**Zhou et al., 1995**
PX	**Phox homology**	**SH3 domain and phosphoinositides**	**80-125**	**Hiroaki et al., 2001**; **Cheever et al., 2001**
SH2	**src homology 2**	**phosphotyrosine**	**approx. 100**	**Pascal et al., 1994**
SH3	**src homology 3**	**proline rich sequences**	**approx. 60**	**Schlessinger, 1999**; **Larson and Davidson, 2000**
TPR	**Tetratricopeptide repeat**		**4**	**Gatto et al., 2000**
UIM	**ubiquitin interacting motif**	**ubiquitin**	**approx. 5-10**	**Hofmann and Falquet, 2001**

VHS	**Vps27p/Hrs/STAM**	**dileucine motif**	**153**	**Lohi and Lehto, 1998 ; Misra et al., 2000**
WW	**tryptophan-tryptophan binding**	**phosphoserine, phosphothreonine**	**35-40**	**Lu et al., 1999**
14-3-3		**phosphoserine, phosphothreonine binding**	**40-120**	**Aitken, 1996**

B. Processing of Receptors at the Surface

More recently, some of the the growth factor receptors have been found to be processed at the surface by proteolytic cleavage. One of the cases that has been studied is that of ErB-4, a member of the EGFR family, a receptor tyrosine kinase that regulates cell proliferation and differentiation activated by the ligand *heregulin* (HRG), a polypeptide growth factor. . The receptor is cleaved upon activation (Ni et al., 2001; Lee et al., 2002). First its ectodomain is cleaved by a metalloprotease. Then γ-secretase cleaves off the intracellular domain followed by its translocation into the nucleus.

Proteins of the *Delta-Serrate-Lag-2* (DSL)-ligand family of transmembrane proteins mediate cell-to-cell interactions and have a role in cell differentiation (see Section III B below) after binding to the Notch receptors of adjoining cells through direct contact. Notch receptors are type I integral membrane proteins (i.e. with the amino terminal in the extracellular or lumenal space). They also undergo cleavage steps (see Fortini, 2001; 2002), first in their maturation process in the Golgi apparatus, a step catalyzed by furin-like convertase. At the surface Notch binding to the DSL triggers the cleavage of the extracellular segment by metalloproteinases. The membrane attached carboxy-terminal segment is then cleaved by the γ secretase complex in the plasma membrane and released to the cytoplasm where it is translocated into the nucleus. A similar role of γ secretase in other forms of signaling involving cell surface and cell-adhesion molecules is strongly suspected (see Fortini, 2002).

II. POLYPEPTIDE GROWTH FACTORS AND CYTOKINES

PGFs and cytokines have a role in the proliferation and survival of normal cells. Some of these are discussed in the next two sections.

A. Growth Factors

Typically, PGFs increase the size and number of the cells they regulate. During embryonic development,

when growth is complete, the cells have become differentiated. PGFs also play an important role in repair and in tissues that are maintained by continued or intermittent cell turnover, such as epithelium and hematopoietic tissues. Each growth factor seems to have specific target cells or tissues. The amino acid sequences of several PGFs are known, either from amino acid sequence analysis or from cDNA studies. The present section will review some of the information available for three growth factors.

EGF binds to receptors that seem to move freely in the plane of the membrane. After binding, the receptors cluster, mainly over clathrin-coated pits (e.g., Hagler et al., 1979; Hopkins et al., 1981) and are taken up rapidly by endocytosis (Schlessinger et al., 1978). Like many receptors of PGFs, most, if not all, of the biological activity does not require internalization (nerve growth factor being a notable exception). Like many other PGF receptors, the EGF is a RTK, that is, it phosphorylates its own tyrosine residue (Hunter and Cooper, 1981) and induces the phosphorylation of its serine and threonine residues in reactions that are thought to be catalyzed by a separate enzyme, protein kinase C (Cochet et al., 1984; Iwashita and Fox, 1984). EGF increases phosphatidylinositol turnover, diacetylglycerol production, and Ca^{2+} influx. All these activities associated with second messengers are discussed in Chapter 7.

As mentioned above the EGFR is composed of a heterodimer containing two of four possible subunits. The EGF receptor has been found to serve as a mediator of different signal pathways (see Hackel et al., 1999; Moghal and Stenberg, 1999) blocking or facilitating other signaling pathways. For example, activation of the *bone morphogenic protein* (BMP) receptors or *transforming growth factor*- (TGF-) β (a serine/threonine kinase) is mediated by SMAD complexes which are translocated into the nucleus where they become transcriptionally active (see below). However, the simultaneous activation of EGFR blocks the nuclear translocation by inducing the phosphorylation of the linker region of two of the SMADs (see Kretzschmar and Massagué, 1998). The EGFR may also act in response to many stimuli, integrating information from different signaling pathways. For example, activated *G-protein coupled receptors* (GPCRs) activate the EFGR without the latter being bound by its ligand (e.g., see Daub et al., 1997). EGFR has been found in the nucleus where it acts as a transcription activator (see above). A transmembrane receptor tyrosine kinase belonging to the same family as EGFR, ErbB-4, inhibits cell proliferation. After activation by its ligand, *heregulin* (HRG) , the ErbB-4 external domain is cleaved by a metalloprotease. A second cleavage by γ-secretase releases the ErbB-4 intracellular domain from the membrane and facilitates its translocation to the nucleus (Ni et al., 2001). Inhibition of the γ-secretase was found to prevent growth inhibition by HRG.

Nerve growth factor (NGF) was the first PGF studied (Levi-Montalcini, 1954). NGF belongs to the family of neurotrophins that promote survival and development of many kinds of neurons (see Patapoutian and Reichardt, 2001). This family of growth factors in addition to NGF includes *brain derived neurotrophic factor* (BDNF) and *neurotrophins-3 and -4* (NT-3 and NT-4). They are synthesized as precursors or proneurotrophins. The immature forms of BDNF and NGF are secreted and cleaved to the mature form extracellularly by the serine protease plasmin and by selective matrix metalloproteinases (MMPs) (e.g., Edwards et al., 1988). However, these protoneurotrophins are active (Lee et al., 2001; see Chao et al., 2002). ProNGF binds to $p75^{NTR}$ (a neurotrophin receptor) thereby favoring programmed cell

death. The mature forms bind to receptor tyrosine kinases (see Patapoutian and Reichardt, 2001) and favor survival.. NGF affects central nervous system, sensory, and sympathetic neurons, as well as nonneuronal cells, such as chromaffin and mast cells. The NGF gene codes for a precursor protein containing 307 amino acids. The NGF is made up of two nonidentical monomers, 178 amino acids long. NGF has an essential maintenance role in early embryonic stages, as shown by the massive cell death which follows its removal by exposing the cells to NGF antibody. NGF, however, also has a supportive role in fully differentiated cells and directs growth and regeneration along its concentration gradient (Gundersen and Barrett, 1979).

The mechanism of action of NGF is not known in detail although considerable progress has been made (see Chapter 7). NGF may stimulate the expression of genes coding for proteins that affect cytoskeletal elements (e.g., Drubin et al., 1985) or the activation of oncogenes (Burnstein et al., 1985).

During differentiation, dendrites and axons grow out from the cell body of neurons. Axons, in particular, can grow to great lengths (the length of a nerve). The leading edge of a growing axon during development and regeneration is known as the *growth cone*. The NGF receptors are in the cell bodies and the growth cone. Binding of NGF to its receptor, results in a change in the affinity of the ligand for its receptor (Landreth and Shooter, 1980), followed by clustering (Levi et al., 1980) and endocytotic uptake (Calissano and Shelanski, 1980) (see Chapter 9). When taken up by the neuronal terminals, NGF is transported to the cell body (Hendry et al., 1974). This transport is essential, interference with this translocation kills the cell. The need for NGF at an intracellular location is also shown in experiments using variants unable to retain it (Eveleth and Bradshaw, 1992). These cells are unaffected by NSF. Once inside the cell, some of the NGF receptors are associated with the cytoskeletal elements and others are present in the nucleus.

Neurotrophins exert their effect on neurons by binding to receptor tyrosine kinases (trks) and a common neurotrophin receptor (p75). Trks are high-affinity receptors for *nerve-growth factor* (NGF) (trkA), *brain-derived neurotrophic factor* (BDNF) and neurotrophin-4/5 (trkB), and neurotrophin-3 (trkC). p75 is a low-affinity NGF receptor.

Neurotrophins play a significant role in developmental apoptosis (see Chapter 2). Survival depends on both the action of neurotrophins and on neuronal activity. The cells compete for the limiting concentration of NGF acting through the TrkA tyrosine kinase receptors (see Kaplan and Miller, 1997; Francis and Landis, 1999). In addition, other members of the same neurotrophin family are likely to play a role by binding to other receptors (Aloyz et al., 1998; Bamji et al., 1998). However, the responses point to a complex interplay between neurotrophin-stimulated survival, differentiation, and apoptosis. In one kind of neuron a neurotrophin may signal survival and in another apopotsis (e.g., see Kaplan and Miller, 1997).

The interaction between neurotrophins and neural activity may occur at different levels. In cortical neurons in culture, survival produced by KCl (which mimicks depolarization, see Chapter 22) acting

through the voltage gated Ca^{2+}-channels may be caused by an increase release of brain-derived neurotrophic factor (BDNF) (Ghosh et al., 1994). In addition, in cultured retinal cells, neuronal activity recruits TrkB receptors to the surface so that the neurons may respond to BDNF (Meyer-Franke et al, 1998) . In addition to increasing the release of neurotrophin and facilitating the response to the neurotrophin, the effect of neurotrophins and neuronal activity may be distinct but converge on the same target. Neurotrophin binding to TrkA activates several pathways including the phosphatidyl inositol 3-kinase (PI3-kinase, see Chapter 7) and the Ras-mitogen-activated protein kinase kinase (MEK)-mitogen activated protein (MAP) kinase pathways (see Kaplan and Stephens, 1994; Kaplan and Miller, 1997) (see Chapter 7). In contrast, depolarization acts through Ca^{2+} influx via L-calcium channels (Franklin et al., 1995). The Ca^{2+} influx can activated the calmodulin/calmodulin (see Chapter 7) dependent protein kinase pathway (Hanson and Schulman, 1992). This would then lead to convergence via the Ras-MEK-MAP kinase pathway (Rosen et al., 1994).

The targeting of growing axons (see McFarlane and Holt, 1997) is complex. Several growth factors including NGF, together with extracellular matrix components, chemoattractants and chemorepellants are thought to have a role.

Platelet-derived growth factor (PDGF) (Ross and Vogel, 1978; Ross et al., 1986) is produced by platelets, megakaryocytes, endothelium, or smooth muscle. PDGF is rapidly degraded after its release and, for this reason, it is thought to have a role at sites very close to its release. PDGF from human platelets has a molecular weight of approximately 30 kDA. Various peptide species ranging in molecular weight between 14 and 17 kDa and held together by disulfide bridges, make up the native PDGF molecule.

PDGF stimulates general protein synthesis and collagen synthesis in responsive cells and also stimulates the production of enzymes active in the hydrolysis of these proteins. So far, PDGF receptors have been demonstrated only in connective tissue components, such as fibroblasts, vascular smooth muscle, glial cells, and chondrocytes, where PDGF stimulates cell proliferation. PDGF also has many indirect effects, such as enhancement of erythropoiesis and production of vasoconstriction, both physiological reactions designed to make up for blood loss.

B. Cytokines

The cytokines have a complex role in regulating a variety of tissues including lymphoid, hemopoietic and endothelial cells. Each cytokine may act as a positive or a negative signal, depending on the type of target cell. The cytokines not only control several cell types, but their effects are multiple. In addition, different cytokines can act on the same cell to produce similar effects. Several cytokine genes have been isolated and their primary structures are now known (see Arai et al., 1990).

In the immune system, the cytokines mediate a host of functions. These include the recognition of self and nonself, elimination of foreign pathogens, neutralization of toxins and the destruction of tumor cells.

These interactions require specificity, in the form of cellular recognition of foreign antigens. Specificity is provided by components of the cell surface of T and B lymphocytes. Lymphocytes are circulating cells which are activated by antigens to produce antibodies. Macrophages, which can take up foreign material by *phagocytosis*, also play a crucial role in allowing the T-cells to recognize antigens and provide signals to activate both T and B cells. Some of these interactions are depicted in the diagram of Fig. 4 (Arai, et al., 1990). In this diagram, the factors released are indicated by the open arrows and their target or eventual response by the head of these arrows. The major source of cytokines are the T cells, although macrophages and B cells are also thought to be involved. Several of the cytokines are discussed in more detail below.

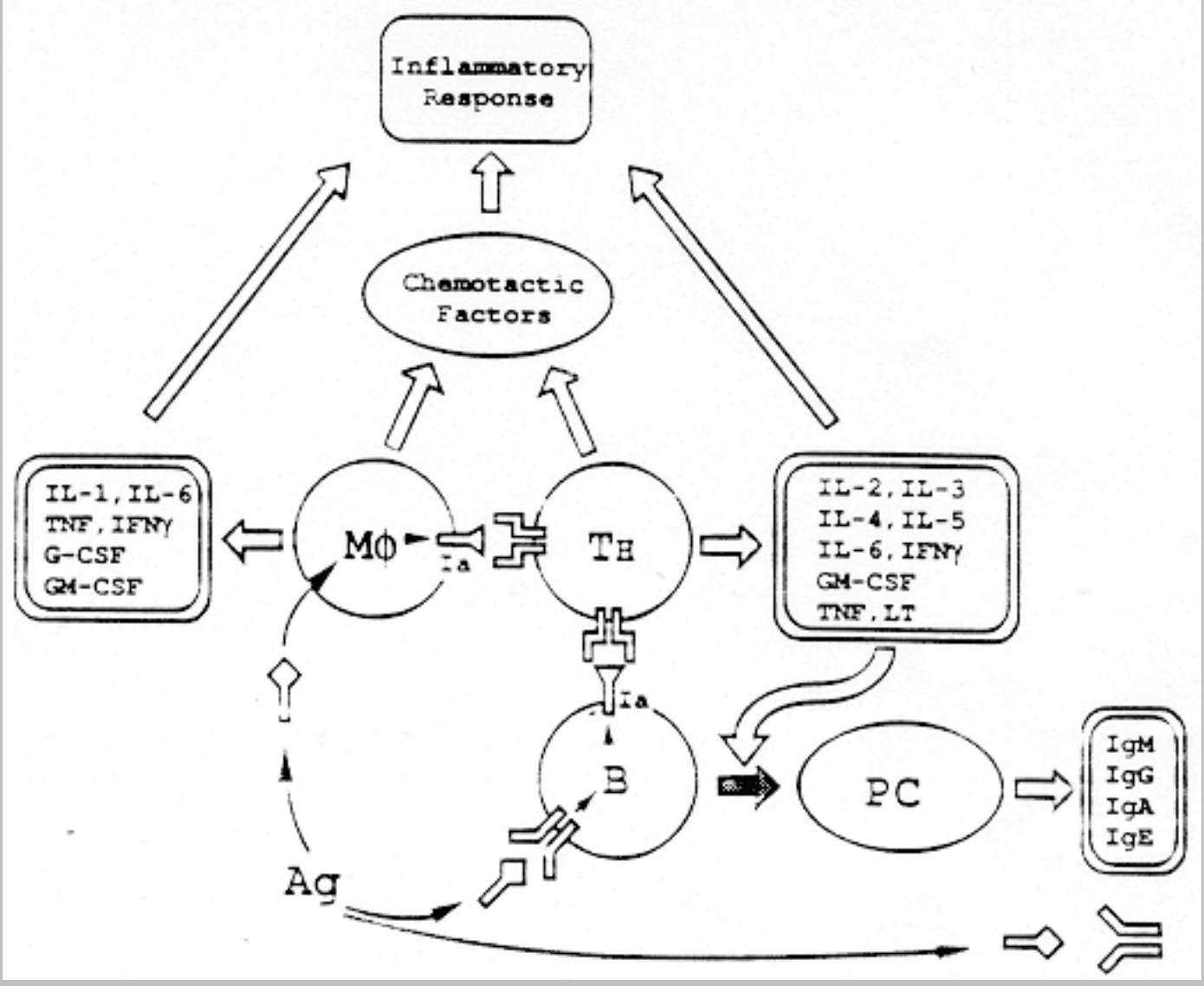


Fig. 4 Role of T cell-, B cell- and macrophage- derived cytokines in immune and inflammatory responses. PC = plasmacells; M = macrophages. IL is interleukin, TNF is tumor necrosis factor, IFN interferon, CSF colony-stimulating factor and LT, lymphotoxin. With permission, from the *Annual Review of Biochemistry*, vol.59, copyright &copy1990, by Annual Reviews Inc.

Like other growth factors, cytokines bind to plasma membrane receptors. Molecular cloning of many of

these receptors have allowed studies of the primary structure of these proteins. Unlike some of the PGF receptors, the cytokine receptors were not found to contain tyrosine kinase domains. In many cases, the receptor function, including the binding of ligand, requires the presence of additional proteins. Some of these auxiliary proteins are shared among different receptors.

The characteristics of the various receptors are shown in Fig. 5 (from Taga and Kishimoto, 1992). Various motifs are indicated by special symbols which are explained in the legend of the figure.

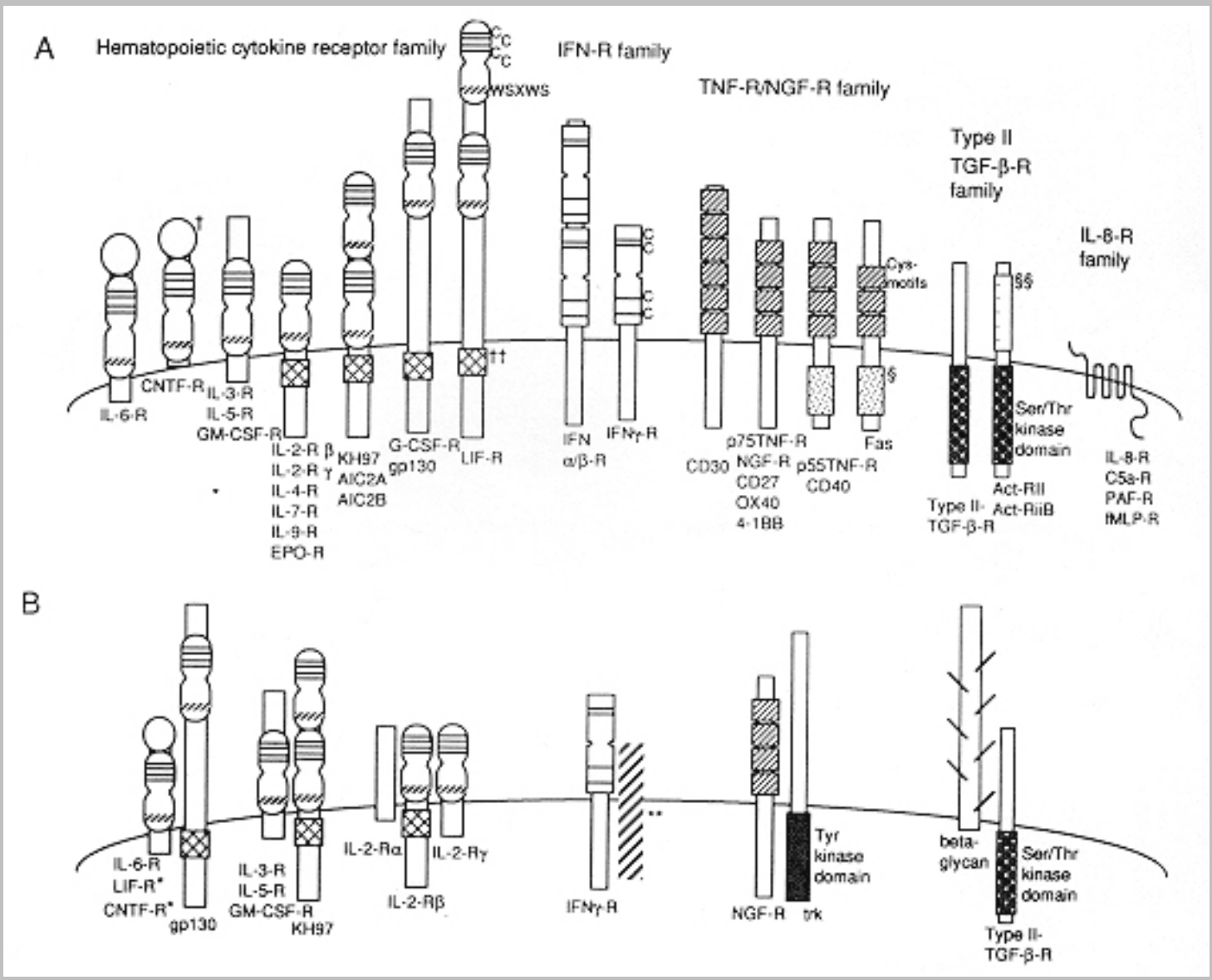


Fig. 5 Families of cytokine receptors. (A) Schematic depiction of cytokine receptor families. (B) Multi-chain receptor systems operating in cytokine receptor families. gp130 and KH97 are shared by several receptors as shown at bottom left. IL-2-Rα chain shows no structural similarity to other receptors. Like symbols (e.g., striping) indicate common domains. The abbreviations are as follows; CSF: colony stimulating factor, CNTF: ciliary neurotrophic factor, EPO: erythropoietin, GM-CSF: granulocyte macrophage CSF, IL: interleukin, LIF: leukemia inhibitory factor, NGF: nerve growth factor, TGF: transforming growth factor, TNF: tumor necrosis factor. Taga and Kishimoto, 1992. Reproduced by permission.

There are several families of cytokines. *Interleukins* (ILs) are hematopoietic cytokines with a role in helper T cell proliferation and B cell activation and proliferation. The cloning of cDNAs of receptors for interleukins (IL-R) found them to belong to the immunoglobulin (Ig) family (e.g., Sims et al., 1988).

Interferons (IFNs) constitute another group. IFNγ is secreted mostly by activated T-lymphocytes following exposure to an antigen or a mitogen. First recognized for their anti-viral activity, IFNs have a multiplicity of effects in the immunological system.

Tumor necrosis factors (TNFs) form a family of trimeric cytokines involved in a variety of cellular tasks, many associated with immunological reactions, programmed cell death, inflammation, bone and mammary gland homeostasis, neural development and other functions (see Locksley et al., 2001). Most members of this family have a narrower range of effects than TNFα (discussed below) and exhibit cell-type specificity. The *TNF receptors* (TNFRs) require two kinds of cytoplasmic adaptor proteins, *TNF receptor associated factors* (TRAFs) and *death domain* (DD) molecules. Each group of the two adaptors has several members allowing for a variety of responses by the activated receptors, depending on physiological conditions. The DD proteins are invoved in apoptosis (see Chapter 2). Several genetic diseases are associated with a failure in ligand receptor interactions. These include: *autoimmune lymphoproliferative sydrome* (ALPS), *TNF receptor-associated periodic syndrome* (TRAPS), *anhydrotic ectodermal dysplesia* (EDA), *familial expansile osteolysys* (FEO) and *X-linked hyper IgM syndrome* (see Locksley et al., 2001).

TNFα (see Baud and Karin, 2001) is produced by many cell types (e.g., macrophages, monocytes, fibroblasts) in response to a challenge such as inflammation, infection or injury. The responses include the activation of leukocytes and lymphocytes, fever, cell proliferation, differentiation and apoptosis (see Tracey and Cerami, 1993). Malfunction in TNFα pathway are thought to be implicated in Crohn's disease, rheumatoid arthritis, stroke, Alzheimer's disease and multiple sclerosis.(Balkwill et al., 2000).

When TNFα binds one of its two receptors (TNFR1 and TNFR2), signal transducers are recruited. and activate a minimum of three effectors. The activation of caspases and two transcription factors AP-1 and NF-κB, which follows, is the result of a complex network of cascades (see Baud and Karin, 2001). Although TNFα can initiate apoptosis (see Chang and Yang, 2000), some of the activated genes block TNFα-induced apoptosis (mostly dependent on NF-κB and requiring inhibition of RNA or protein synthesis).

NF-κB is a heterodimer of two subunits, p50 and RelA. The factor is kept in the cytoplasm in the form of a complex to the I-κB inhibitor, which is unable to be translocated into the nucleus (see Baldwin, 1996). Following the signal-coupled phosphorylation of I-κB (see Karin,1999), the inhibitor is degraded by the proteasome-system (see Chapter 15). The free NF-κB can now enter the nucleus. The system is regulated by a negative feed-back loop in which NF-κB induces the expression of I-κB. In the nucleus, NF-κB binds to κB enhancer elements. It recruits *CREB-binding protein* (p300/CBP) and the *CBP-associated*

factor (P/CAF) coactivators of targeted gene expression (e.g., Sheppard et al., 1999). Phosphorylation of the RelA subunit favors the interaction with the CBP (Zhong et al., 1998). CBP and p300 have acetyltransferase activity, implicated in acetylation of core histones and gene expression. The system is also regulated by acetylation. The acetylation of the RelA subunit blocks its association with p50. In turn, RelA can be deacetylated by histone deacetylase 3 (HDAC3) which facilitates its binding to to I-κB and exit from the nucleus (Chen et al., 2001).

The *transforming growth factors*-βs (TGF-βs) family of cytokines has a role in the control of cell growth, differentiation, matrix production and programmed cell death in a variety of cells (see Massagué, 1998; Whitman, 1998; Zhang and Derynck, 1999; see Massagué, 1998 for a tabulation). Disrupting their signaling pathway is responsible for a variety of malignancies and hereditary conditions (see Massagué, 1998). Members of the TGF-β family include *activins, inhibins, bone morphogenic protein* (BMP) and *Mullerian inhibiting substances*. The TGF-βs bind to receptors (TGF-β-R) are serine/threonine kinases, with one exception.

The TGF-β-Rs belong to two families: Type I and Type II. Type I and type II receptors are glycoproteins of approximately 55 kDa and 70 kDa respectively. TGF-β has to bind to both kinds of receptors. Apparently, Type II is bound first. Type I is then recruited and activated by phosphorylation. Experiments in which [^{125}]-labelled TGF-β1 was cross-linked to receptors suggest that the complex is constituted by two-βR-I and two βR-II (Yamashita et al., 1994). Both receptor types have to be phosphorylated in the complex for activation. Several studies have implicated other proteins in regulating the responses of the receptors (e.g., Chen et al., 1997a).

The effects of the ligands of the TGF-β family are mediated by proteins of 42 to 60 kDa called SMADs (see Heldin et al., 1997; Kawabata and Miyazono, 1999). Smads are members of the Mothers Against Dpp (MAD)-related family of proteins and are components of the serine/threonine kinase receptor signaling pathways. Other mediators may also exist. There are three kinds of SMADs: receptor-regulated (R-Smads), common-mediator Smad (co-SMAD) and the inhibitory SMADs (I-SMADs) (see Kawabata and Miyazono, 1999). Co-SMAD (SMAD4) is required for transducing signals for all TGF-β superfamily members and they form ligand-dependent complexes with phosphorylated R-SMADs. I-SMADs (e.g., SMAD6 and SMAD7) inhibit signaling by blocking receptor-mediated phosphorylation of R-SMADs. Nine SMADs have been found so far in vertebrates and several in *Drosophila* and *Caenorhabditis elegans* (see tabulation in Zhang and Derynck, 1999). R-SMADs are phosphorylated by specific type I receptors (e.g., see Wu et al., 2000). SMAD1, 5 and 8 are phosphorylated by the BMP receptors. SMADs 2 and 3 are phosphorylated by activin and TGFβ receptors. Different SMADs mediate different functions, for example, during embryonic development, SMAD2 induces dorsal mesoderm, whereas SMAD1 induces ventral mesoderm (e.g., Graff et al., 1996).

In more detail, the interaction between SMAD and (TGF)-βR is mediated by the *SMAD anchor for receptor activation*, SARA, that acts as a scaffold for the recruitment of SMD2 and SMAD3 to the TGF-β

receptor (Tsukazaki et al., 1998). SMAD2 and 3 are phosphorylated by type-I receptor kinases. The phosphorylated SMAD2 binds SMAD4 forming a trimer and dissociates from SARA, so that the detached complex is translocated to the nucleus. SARA contains a FYVE domain that has been shown to bind to phosphatidylinositol-3-phosphate and therefore can probably anchor to the inner plasma membrane leaflet. SARA determines the cellular localization of SMAD2 keeping non-activated SMAD2 in the cytoplasm. Tsukazaki et al., (1998) identified SARA from a bacterial expression library. The appropriate target protein can be recognized in the plaques in the bacterial plates produced by a phage vector containing the cDNA. The target proteins in the plaques will bind to the radiactive probe protein (a domain of SMAD2) used for screening. The phage vector and appropriate cDNA in the plaque can then be cloned (similar to the technique described by Watson et al., 1992, p.114).

In the nucleus SMADs activate genes (e.g., Heldin et al., 1997; Hoodless et al., 1996). In order to bind DNA effectively the SMADs bind to DNA-binding proteins, such as *forkhead activin signal transducer* (FAST) and many others (see Wrana, 2000). BMP might function through a similar activation pathway (see Hata et al., 2000). BMP requires a SMAD-binding element and the protein OAZ (*Olf-1-EBF associated zinc finger*). The gene activation mediated by SMADs is thought to result from the binding to DNA at promoter sites (e.g., Huang et al., 1995) or by interaction with transcription factors (e.g., Labbé et al., 1998; Chen et al., 1997b). Cell specific responses are the result of the presence or absence of certain SMAD partners. FAST and OAZ are not expressed in certain cells and therefore in these cells the pathway is silent. Other SMAD partners are ubiquitous and in these cases the specificity is thought to result from the input of other signaling pathways.

SMADs are degraded by the ubiquitin-proteasome system (see Chapter 15, Section on ubiquitination and Section on proteasomes). Specific E3 ligases have been identified, for example the E3, Smurf1, preferentially binds to BMP-regulated SMADs (e.g. Zhu et al., 1999). The activation of SMAD2 by TGFβ induces its degradation by the proteasomes (Lo and Massagué, 1999) and occurs when the SMAD2 is translocated to the nucleus. This suggests that the degadation pathway has a regulatory function.

The SMAD signalling system interacts with many other pathways in a complex manner. For example, epidermal growth factor (EGF) or hepatic growth factor (HEF) may trigger a pathway that phosphorylate the Smad directly through *mitogen activated protein kinase* (MAPK) (e.g., see de Caestecker et al., 1998)

As indicated in Fig. 5, in many cases, the complex of cytokine and receptor have to bind to accessory molecules to function. For example, when IL-6 binds IL-6-R, the complex is now capable of binding gp130, a 130 kDa signal transducing molecule, an integral membrane glycoprotein which also belongs to the hematopoietic cytokine receptor family (Taga et al., 1989; Hibi et al., 1990). The sharing of signal transducing components (such as gp130) may explain the tremendous overlap of the effects of different cytokines. The *glial-cell-line-derived neutrophilic factor* (GDNF) was initially isolated and cloned from a rat glial line. It enhances the survival of midbrain dopaminergic neurons. GDNF is related to the transforming growth factor β (TGF-β). However, in contrast to TGF-β that activates a serine/threonine receptor, the GDNF receptor (GDNFR-α) activates a protein tyrosine kinase receptor (Ret) (e.g., Jing et

al., 1996). GTNFR-α is attached to the outer plasma membrane leaflet by a glycosyl-phosphatidylinositol anchor.

Besides having common effects, each cytokine also has specific effects of its own, possibly because of unique mechanisms not shared with other ligands. The presence of a separate accessory molecule used in transducing the signals, may account for the divergence of effects of the different cytokines.

Many receptor proteins are also present in a water soluble form, rather than as integral plasma membrane proteins. What could be their function? They could, perhaps, be a reserve which could be integrated into the membrane structure. In contrast, they could sequester cytokines (see Mosley et al., 1989; Engelmann et al., 1989), thereby preventing their binding to the membrane receptors and, in effect, acting as inhibitors. The possibility of using this inhibitory capacity clinically is currently being examined.

Go to Part 2

REFERENCES

Search the textbook

III. INTERACTION WITH THE EXTRACELLULAR MATRIX AND WITH OTHER CELLS

In multicellular organisms, most cells are in contact with a highly complex matrix of proteins and other components, the extracellular matrix (ECM). Therefore, the fact that interactions between the cell surface and the ECM play an important biological role should not come as a surprise. Cell differentiation, development, and morphogenesis are all crucially dependent on the ECM. By all indications, the effect of the matrix components is mediated by binding at receptor sites on the cell surface.

The importance of ECM in cell proliferation can be illustrated by the observation that nontransformed cells are unable to proliferate without a solid support (Stoker et al., 1968), even in the presence of growth factors (Otsuka and Moskowitz, 1975). In contrast, transformed cells are free of these controls. The transformed cells are detected in test where they are able to grow in suspension or on soft agar.

The role of extracellular matrix is undoubtedly complex. There is evidence however, that part of the action of its components may be through a mechanism analogous to that of growth factors. The sequencing of the cDNA of extracellular proteins has revealed in these molecules amino acid sequences homologous to those of epidermal growth factor (EGF).

Transmission of a signal from the cell's surface to its interior is likely to require interaction of the signal with an integral membrane protein and, in many ways, resembles the action of hormones and growth factors which bind to surface receptors.

Benecke et al. (1978) showed that in culture absence of substratum inhibited mRNA and protein synthesis. This inhibition decreased with increasing degrees of cell transformation (Wittlsberger et al., 1981). The block in normal cells appeared to be in the G1-phase and to be ther result of interactions between cyclin, cyclin-dependent kinases (cln-cdk) (which are responsible for initiating cell division), cyclin inhibitors that block cell division (see Assoian, 1997, and Chapter 8) and the presence of substratum. Absence of a substratum prevented cell division despite the presence of mitogenic factors.

A. The Extracellular Matrix

The ECM contains collagen, glycoproteins, proteoglycans and glycosaminoglycans secreted by cells and interwoven into an organized network (Hay, 1984), that includes hyaluronic acid and the proteins fibronectin, laminin, entactin, and elastin.

Collagen

Collagen is formed intracellularly as procollagen, which has a higher molecular weight than the

extracellular collagen because it contains extensions at both the NH_2 and COOH terminals. Furthermore, a still higher molecular weight procollagen is produced by the translational system containing collagen mRNA.

Collagen is formed by three protein chains forming a triple helix (Fig. 6, Linsenmayer, 1991). There are various types of collagen. Type I is present in many adult connective tissues such as skin, bone, tendon and cornea. Type I collagen forms striated fibrils (in which the triple-helical arrangements are in register) 20 to 100 nm in diameter. The triple helix is formed by two different kinds of chains. Type I collagen is shown in Fig. 6.

Type II collagen is present in cartilage and is synthesized in chondrocytes. The fibrils are generally 10 to 20 nm in diameter. The chains of type II collagen are identical. This type of collagen has more hydroxylysine and glycosylation than type I.

Type III collagen is present primarily in loose connective tissue such as derma, blood vessel walls and placenta, as well as in the 50-nm fibers of reticular connective tissue. This collagen is composed of a single type of chain. The three chains making up the triple helices are held together by disulfide bonds. Accordingly, the amino acid composition is relatively rich in half cystines. Type III collagen has a higher glycine concentration than other collagens.

Types IV and V are collagens of the basement membrane. Type IV probably corresponds to two different chains rich in hydroxyproline and cysteine and with a good deal of glycosylation.

The structure of collagen, type I is represented in Fig. 6

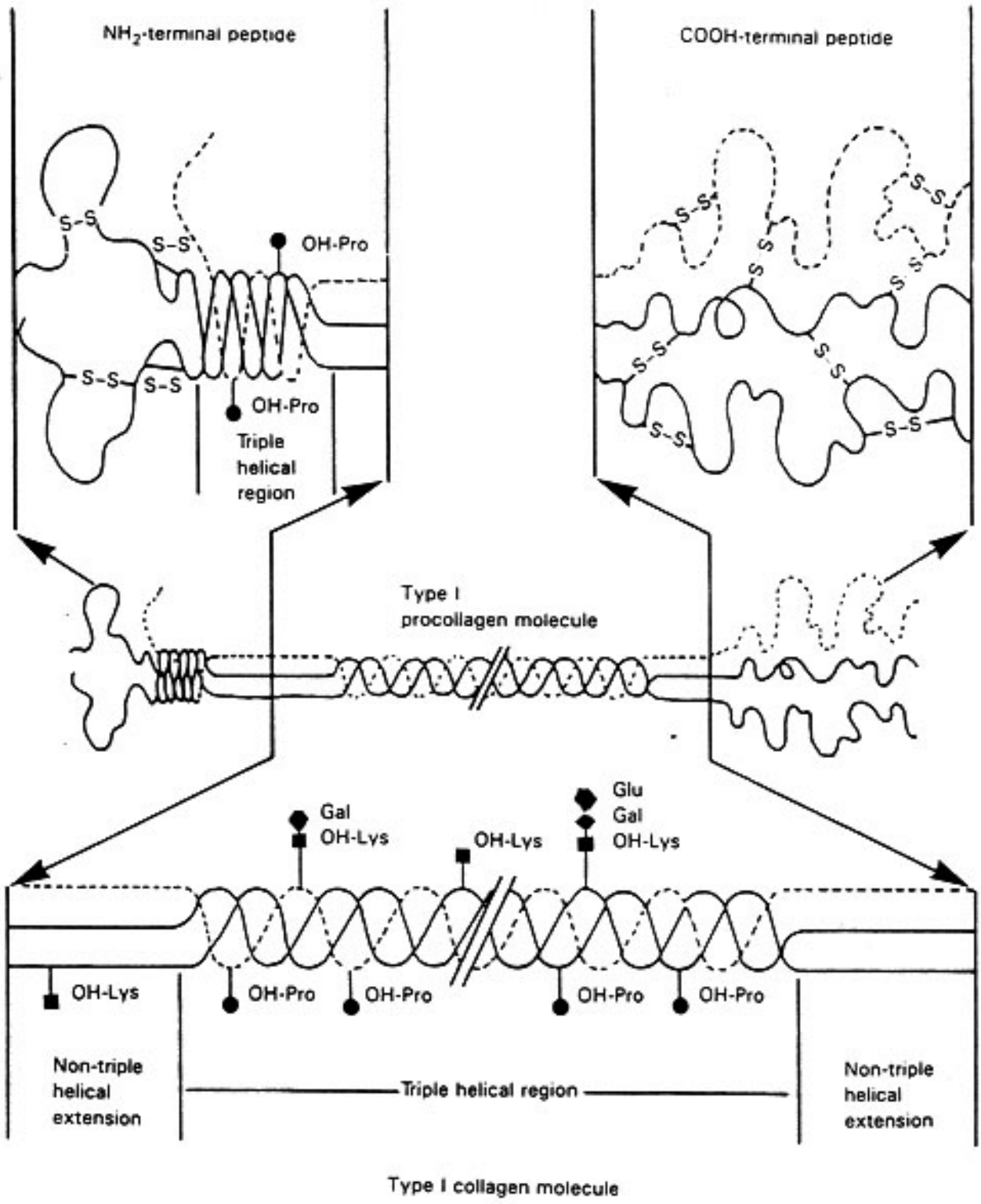


Fig. 6 Diagram of the major characteristics of the type I collagen molecule and its procollagen form. OH-PRO, hydroxyproline; OH-LYS, hydroxylysine; -S-S-, disulfide bonds; GAL, galactose; GLU, glucose. The NH_2-terminal propeptide and COOH-terminal propeptide show the two pieces that are cleaved from procollagen during its processing into a collagen molecule. Reproduced with permission from T. F. Linsenmayer, *Cell Biology of Extracellular Matrix*. Copyright &copy1981, Plenum Publishing Corporation.

Proteoglycans

Proteoglycans are complex macromolecules containing a protein core covalently bound to one or more glycosaminoglycan (GAG) chains (see Wight et al., 1991). Many have complex structures with domains similar to those of other protein families. These domains may confer special functional properties. Some of the proteoglycans (such as aggrecan) are matrix proteins. Others (such as the syndecans) are integral membrane proteins with large extracellular domains. The matrix proteoglycans create a water-filled matrix which is freely available to low molecular solutes but excludes large molecules. Their multiple negative charges attract cations, not only increasing hydration, but also serving as a selection device.

In cartilage, the protein sector of proteoglycan is 250 kDa in molecular weight and 300 nm in length. This core protein constitutes only 5 to 10% of the total mass, the rest being carbohydrate. In the extracellular matrix, the proteoglycan complexes attach along hyaluronic acid (HA) strands. Hyaluronic acid is a polymer in which the repeating unit is a disaccharide made up of D-glucuronic acid and N-acetyl-D-glucosamine. The binding site on the proteoglycan has a high affinity for long HA strands, as long as a decasaccharide or even longer. An electron micrograph of a negatively stained cartilage proteoglycan is shown in Fig. 7 (Hascall and Hascall, 1981) (diagrammatically represented on the right side of the figure) and in Fig. 8 (Hascall and Hascall, 1981). Several of the side groups shown in Fig. 7 are presented in more detail in Fig. 8. The arrangement of a proteoglycan network in collagen is shown in Fig. 9 (Hascall and Hascall, 1981).

Recent findings have implicated proteoglycans in the initial changes in atherosclerosis which appears to be initiated by the attachment of LDL to the negatively charged groups of proteoglycans (Skålen et al., 2002).

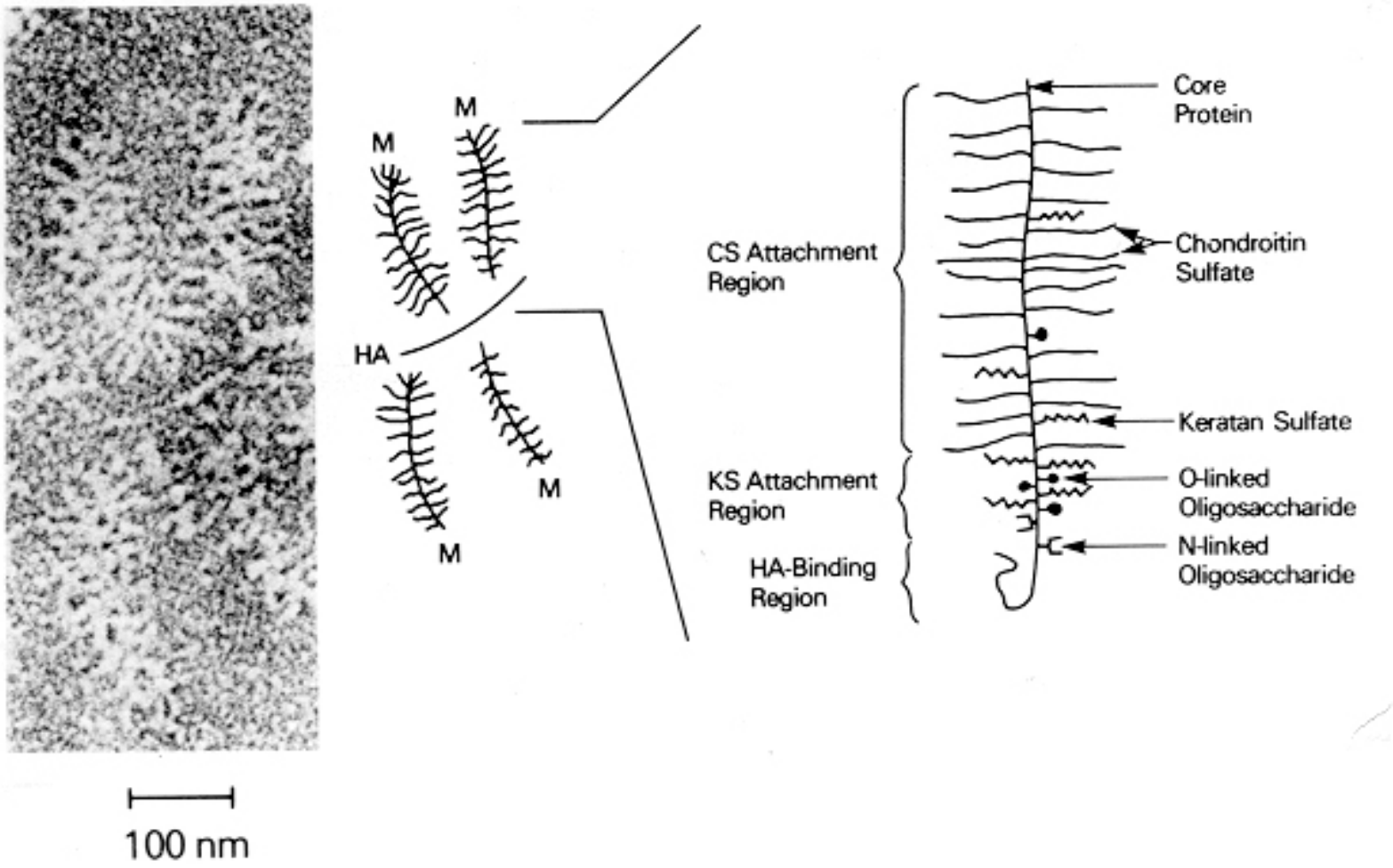


Fig. 7 Cartilage proteoglycan, monomer structure. Reproduced with permission from V. C. Hascall and G. K. Hascall, *Cell Biology of Extracellular Matrix*. Copyright &copy1981 Plenum Publishing Corporation.

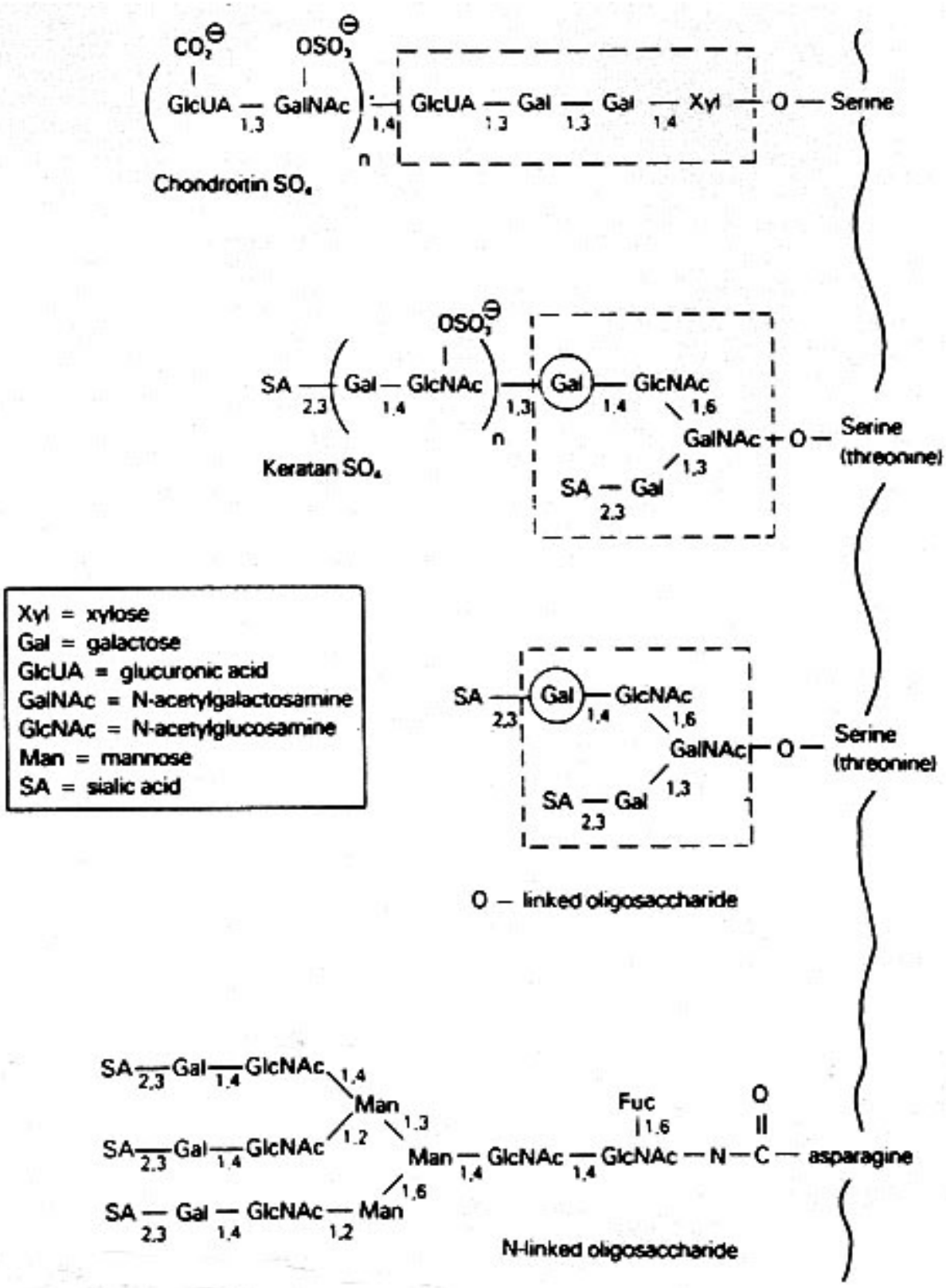


Fig. 8 Structures of the complex carbohydrates attached to cartilage proteoglycans. The repeating disaccharide structures of chondroitin sulfate and keratan sulfate are indicated, along with the specialized attachment regions by which the chains are covalently bound to the core protein (shown in the boxes). The 0-linked oligosaccharides are related to the linkage region structure for keratan sulfate as indicated by the circled, similarly located galactose residues. Reproduced with permission from V. C. Hascall and G. K. Hascall, *Cell Biology of Extracellular Matrix*. Copyright &copy1981 Plenum Publishing Corporation.

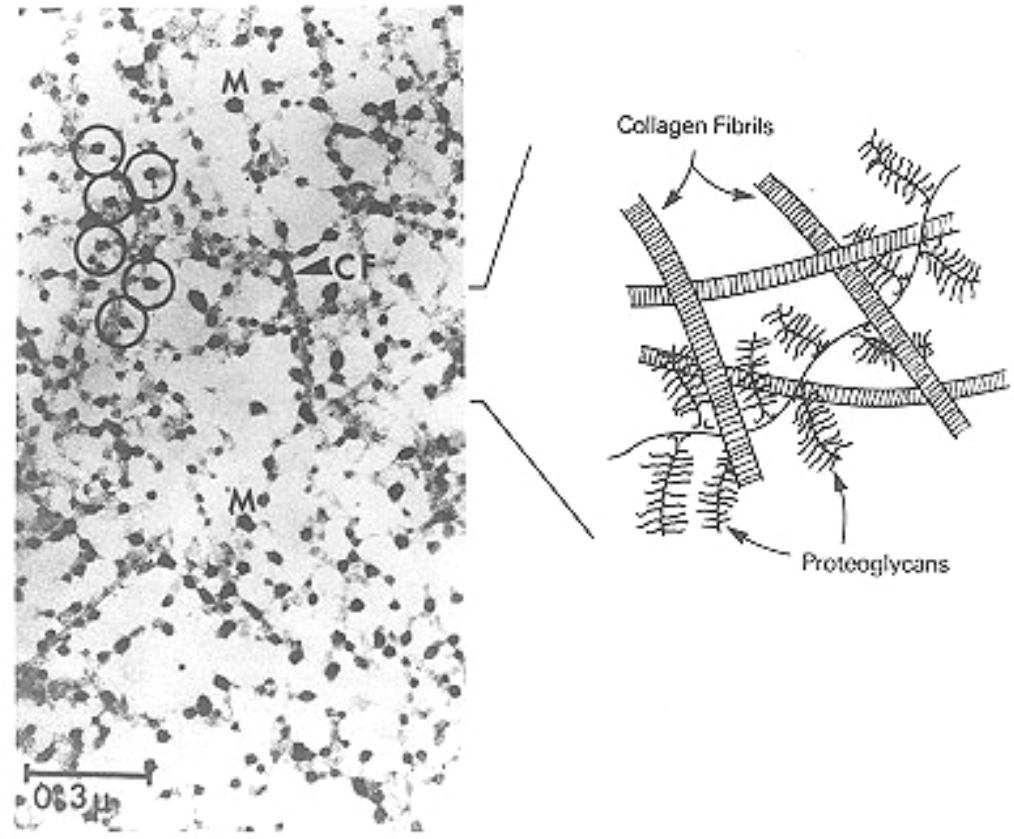


Fig. 9 Organization of structural elements of cartilage extracellular matrix. Matrix granules represent condensed proteoglycan monomers (M). The circles indicate the space that the expanded proteoglycans would occupy. The tissue is a composite structure of collagen fibrils with intertwining proteoglycan aggregates as shown schematically. Reproduced with permission from V. C. Hascall and G. K. Hascall, *Cell Biology of Extracellular Matrix*. Copyright &copy1981 Plenum Publishing Corporation.

Arrangement of cells in the matrix

The architecture of tissues can be very different. Many of the differences can be described in terms of the relationship of the cells to the ECM. Connective tissue cells, such as chondrocytes and dermal fibroblasts, are completely surrounded by the extracellular matrix. Endothelial cells may be separated from connective tissue by a specialized sheet, the basement membrane or lamina. Each cell may be completely surrounded by a basal membrane or the membrane may be on only one side of the cell.

The basement membranes primarily contain type IV collagen; heparan sulfate proteoglycans and laminin (see section B, below) are their major noncollagen components, and minor components may include fibronectin and entactin (Kefalides et al., 1979) (see below). The heparan sulfate proteoglycans in the basement membranes provide an anionic barrier to block the passage of proteins, which are generally negatively charged (Kamwar et al., 1980). Laminin is produced on one side of epithelial cells and therefore is localized on one side of these cells.

B. Cell Adhesion Proteins

The formation of tissues during development as well as their physiology and differentiation are crucially influenced by the interactions. These interactions may be between the ECM and cells or between cells.

Association of cells with the ECM

Cells bind to the ECM by interacting with matrix-molecules. The ability of some specialized glycoproteins to bind cells to other elements of the ECM are so striking that they are referred to as ECM-adhesion molecules. The adhesion glycoproteins affect cellular activity and regulate the formation of the matrix. These proteins can generally be shown to correspond to families of related proteins, such as fibronectins, chondronectins, thrombospondins, laminins, and tenascins -- each group with a more or less distinct tissue distribution and activity toward certain cell types. Proteins involved in cell adhesion, whether they mediate adhesion to the ECM or between cells (next section), are also referred to as *cell adhesion molecules* (CAMs). These proteins have dramatic effects on cell migration, morphology and metabolic activity, and play an important role in development, cell organization and repair. Schematic drawings of the ECM-adhesion glycoproteins are shown in Fig. 10. Their various domains are represented in Fig. 11 (Engel, 1991).

Many of the amino acid sequences of ECM adhesive proteins have been deciphered through DNA technology. Typically, ECM proteins are found to have repeat sections as well as regions of homology with other ECM proteins. Tenascin has EGF and fibronectin-like repeats, a region of homology with the globular domain of fibrinogen and a heptad repeat region (see Fig. 11). Heptad repeats are repeats of seven amino acids which tend to assume a coil-coil conformation.

Functional information is available only for some of the domains, despite knowledge of sequences. The presence of domains containing sequences in common with PGF, suggests that they activate growth and proliferation by binding to receptors in the plasma membrane just as the growth factors. Growth-promoting effect has been reported for specific components, such as laminin, thrombospondin, and tenascin. In the case of laminin, this function was localized to a specific sector with 25 EGF-like repeats.

Specific binding sites for growth factors have also been identified in several ECM proteins. These observations suggest that the ECM may serve to store and release PGFs in response to physiological stimuli. Fibroblast growth factor (FGF) has been found to be stored in the ECM.

Some of the ECM proteins have anti-adhesive effects. Tenascin, which promotes cell adhesion (but not spreading), blocks the effect of fibronectin.

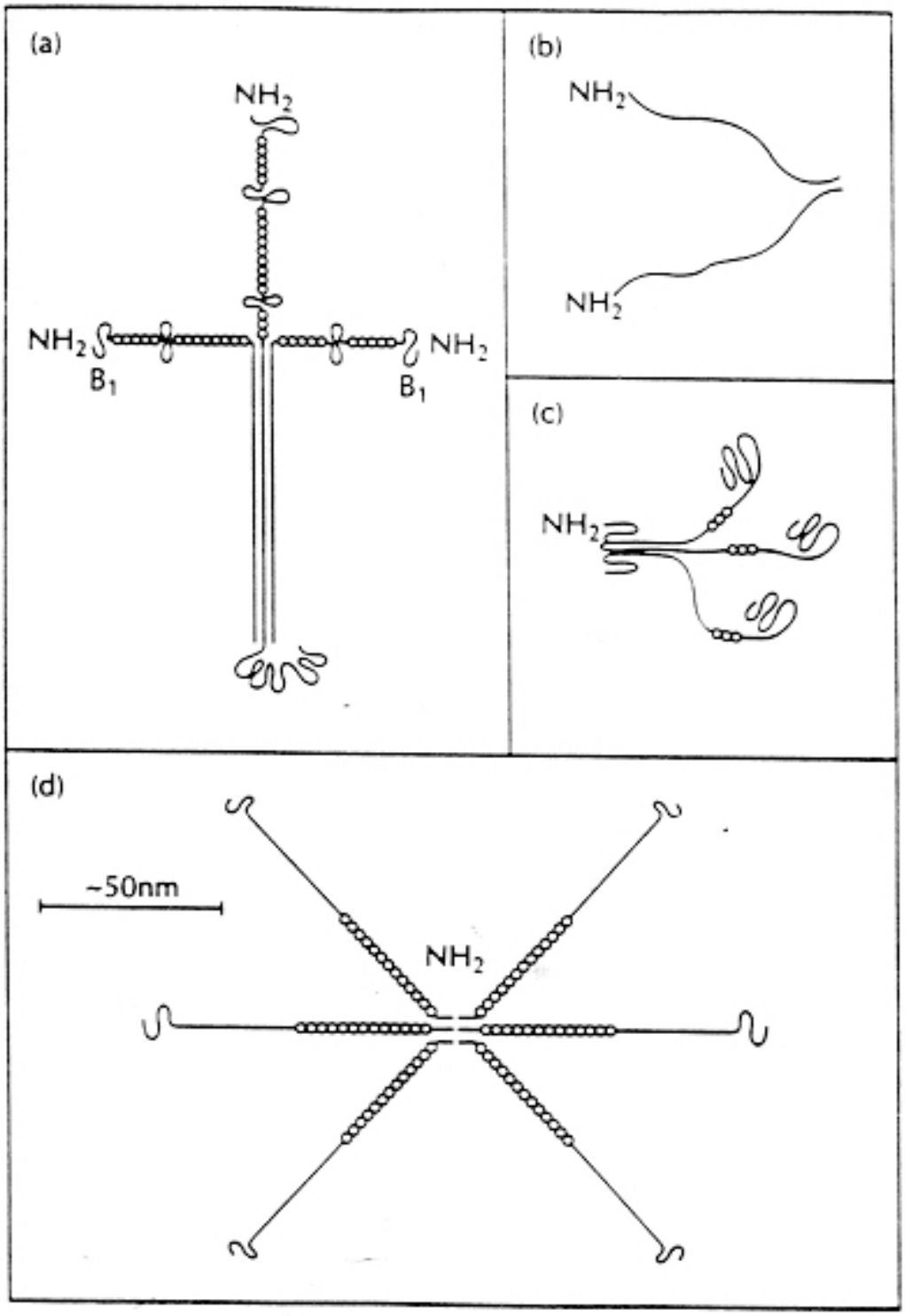


Fig. 10 Schematic drawing of (a) laminin, (b) fibronectin, (c) thrombospondin and (d) tenascin. The molecules are drawn approximately to scale. The circles indicate EGF-like domains. For other details see the more extensive diagram of Fig. 11. Reproduced from J. Engel (1991), *Current Opinion in Cell Biology*, 3:779-785. (Available in the BioMedNet library at:http://biomednet.com/cbiology/cel)

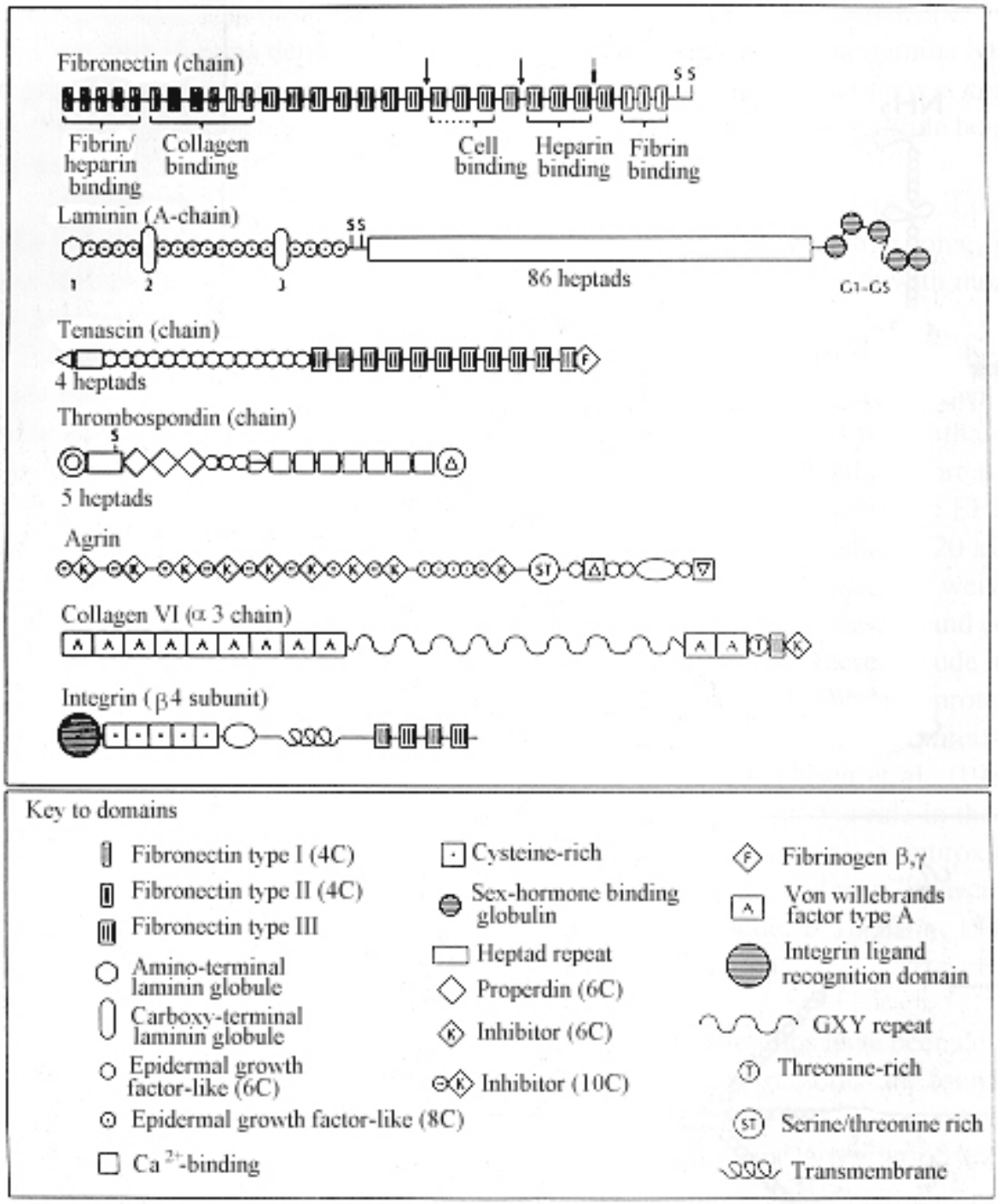


Fig. 11 Schematic representation of the various domains of some ECM-proteins, represented as single chains. See Engel, 1991 for more details. Reproduced from, J. Engel (1991), *Current Opinion in Cell Biology*, 3:779-785. (Available in the BioMedNet library at: http://biomednet.com/cbiology/cel))

The *fibronectins* (FNs) are a family of glycoproteins composed of two subunits 220-250 kDa with an acidic isoelectric point (pI = 5.5-6.0) which are joined by disulfide bridges. A protein is considered an FN if, in addition to these characteristics, it binds gelatin and cross-reacts immunologically with other FNs. The significance of the FNs is indicated by the fact that their quantity, location and, in some cases, structure vary with embryonic development (Wartiavaaren and Vaheri, 1980), disease (Akiyama and Yamada, 1983) and aging (Chandrasekharis et al., 1983).

FN is present in significant amounts at locations of tissue remodeling and cell migration, and in basement membranes. Experiments in which the effect of FN is blocked by FN antibodies suggest that FN has a role in determining the distribution of collagen (McDonald et al., 1982).

The various FN variants or isoforms, of which there can be as many as twenty subunits, are generated by alternative splicing of the same gene. FNs exists predominantly in two forms. The two FNs are very similar, but differ significantly in structure and activity. A soluble form is present in plasma and is predominantly produced in the liver. Another form, cellular FN, is secreted by fibroblasts and a wide variety of other cells; it forms an insoluble fibrillar network at the cell surface and in the extracellular matrix.

Table 3 (Yamada et al., 1984) summarizes some of the known activities of FN. The mechanism of these effects is still not clear. However, specific regions of the molecule, the binding domains, can be shown to bind specifically to various components, and this capacity may help explain how FN acts. The presence of different domains allows the FN to act as a bridge, so that one domain could attach to a component such as a cell and another domain of the same FN molecule could attach to a collagen fiber or some other element.

Table 3 Biological Activities of Fibronectin

Cell-to-substrate adhesion Attachment and spreading of cells on plastic or glass Cell attachment to collagen or fibrin
Cell-to-cell adhesion
Cell morphology Maintenance of flattened, fibroblastic shape and minimal numbers of cell surface microvilli Alignment of confluent fibroblasts in parallel arrays Promotion of actin microfilament bundle organization
Cell migration Stimulation of cell motility Haptotaxis

Stimulation or inhibition of cell differentiation
Nonimmune opsonic activity by microphage phagocytosis

The affinity of each domain can be demonstrated after limited digestion of the molecule by trypsin or another protease that leaves large fragments intact. The approach is summarized in Figure 12 (Yamada, et al., 1984). The ligand tested for binding is attached to an agarose bead in a column. FN (Method 1) or fragments of FN produced by protease treatment (Method 2), are allowed to bind to the attached ligand. Where the intact FN molecule is used, protease digestion follows the binding. The unattached sectors leave the column readily. The sector of the protein containing the binding domain remains attached to the ligand and can be eluted by special procedures. The information derived in these experiments is summarized in the fibronectin structure depicted in Figure 13 (Yamada et al., 1984). In this diagram, the proteolytic fragments are represented by boxes and the affinity of the domain for a specific component is written under the boxes. As shown in the diagram, fibronectin consists of two similar subunits held together by disulfide bonds. Corresponding domains of the two subunits are the same, although the structures differ slightly.

The FNs have a major role in the adhesion of fibroblasts, hepatocytes and endothelial cells to the ECM. The binding occurs at specific cell surface receptors (discussed also in the next section). The receptors were implicated in several different ways. Antibodies to cell surface components were tested for their ability to block binding to FN. If effective, they were used during the isolation procedure to identify the binding protein. Only the surface proteins binding the FN would be able to bind to the antibody (see Urushihara and Yamada, 1986). Chemical cross-linking of cell surface proteins to FN can also serve to identify FN receptors. The proteins can be cross-linked only if they are in close contact in intacts cells or membranes (Aplin et al., 1981). These experiments implicated a protein or proteins of 45 to 47 kDa. Other experiments identified a family of glycoproteins of 115 to 165 kDa, thought to be integral membrane proteins (e.g., Hasegawa et al., 1985;Knudsen et al., 1985). These are the *integrins* discussed in detail in the next section.

Another group of CAMs, the *laminin* family, has been found in the basal lamina of many tissues (see Beck et al., 1990). Basement membranes are highly organized thin extracellular matrix structures that separate epithelial cells from underlying stromal tissues. The basal membranes, viewed with the electron microscope, have an electron-dense region (lamina densa) and a more electron-transparent region (lamina lucida). The dense region contains a network of filaments (Kefalides et al., 1979) which include laminin. Laminin is composed of three B chains of 200 kDa and one A chain of 400 kDa. Electron microscopy reveals that laminin has a cruciform structure (Timpl, 1982) with two short arms, 36 nm in length, another short arm, 48 nm in length and a long arm, 77 nm in length (see Fig. 10).

Laminins can self-associate and also bind to heparin, cellular elastin and collagen. Several cell surface receptors have been implicated in laminin binding. These include a 67 kDa protein found in metastatic cells (von der Mark and Kühl) and a 66 kDa protein from skeletal muscle, aspartactin (Hall et al., 1988). In addition, members of the ubiquitous integrin family (discussed below) seem to serve as receptors for laminin (Gehlsen et al., 1988).

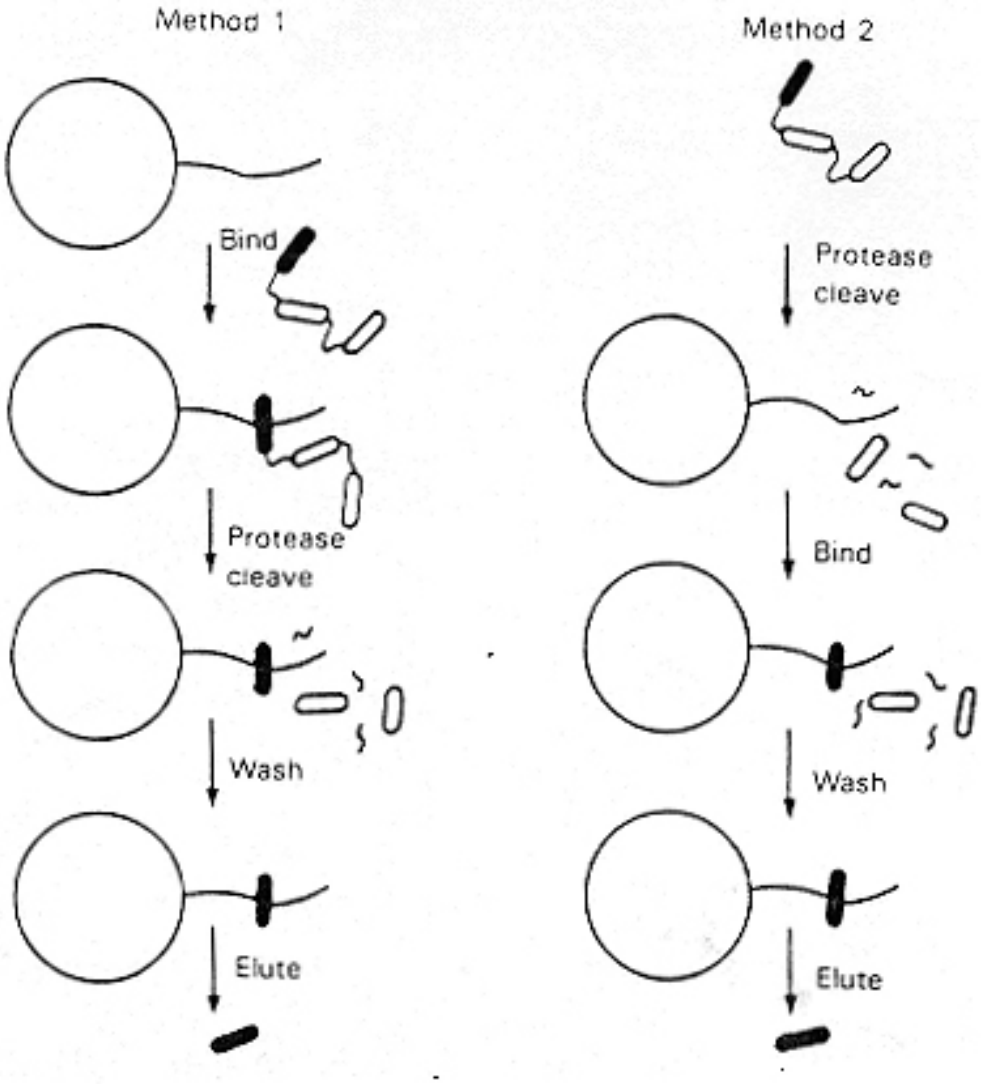


Fig. 12 Method for the isolation of functional domains from a binding protein. Circles at the left indicate agarose beads, with one of the attached ligand molecules depicted as extending to the right. Three protease-resistant domains are shown as black or shaded rodlike structures separated by flexible regions of polypeptide chain; the domain that binds specifically to the ligand is shown in black. Reproduced by permission from K. M. Yamada, et al., *The Role of Extracellular Matrix in Development*. Copyright &copy1984 Alan R. Liss, Inc.

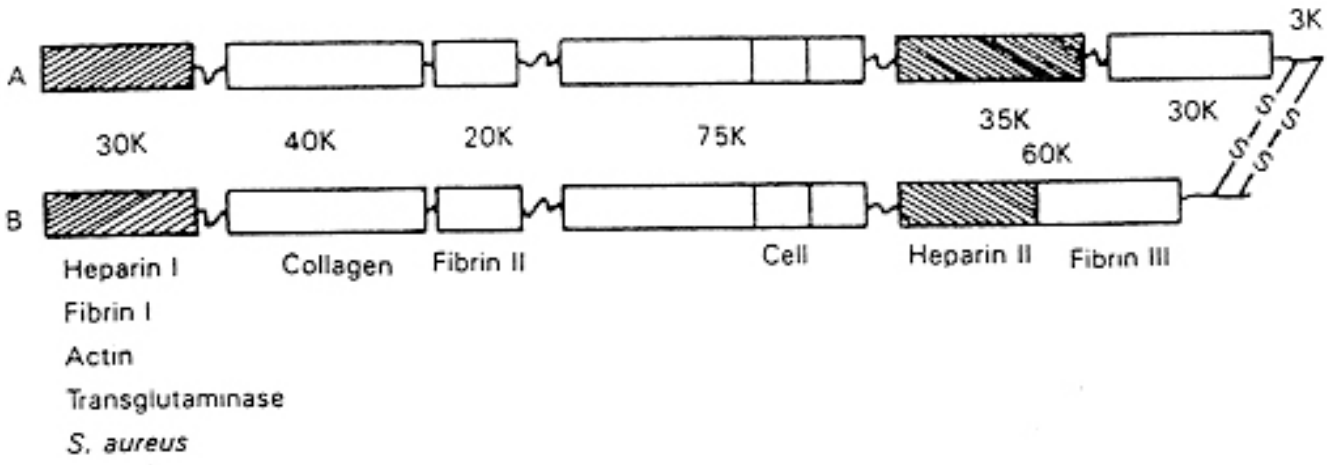


Fig. 13 Early map of the functional domains of human plasma fibronectin. Each rectangular box represents a protease-resistant functional domain of the molecule. The domains of the A and B subunits differ in at least one site near the carboxyl terminus. Sizes of the domains are indicated by the numbers; for instance, 30K means an apparent molecular weight of 30 kDa. The binding activities of each domain are listed underneath; some of these domains can bind to the same ligand and are numbered from amino terminus (left) to carboxyl terminus (right). Reproduced by permission from K. M. Yamada, et al., *The Role of Extracellular Matrix in Development*. Copyright &copy1984 Alan R. Liss, Inc.

Individual cell types can interact with more than one kind of adhesion molecule. Some of the activities of laminin overlap those of FN, but many are distinct.

Laminins are the first known ECM-adhesive molecules to appear in embryogenesis where they have been detected at the two-cell stage. The laminins have been shown to promote cell attachment, growth and differentiation, as well as promoting the extension of axons.

Tenascin (Fig. 10d) is one of the ECM adhesive proteins thought to play a role in the development of the nervous system. The six identical subunits of this glycoprotein are approximately 190-320 kDa (depending on the species) and are linked by disulfide bridges. Tenascin binds to ECM molecules such as FN, heparin and proteoglycans (see Chiquet-Ehrismann, 1990). It also binds to cell surface receptors such as syndecan (Salmivirta et al., 1991) and integrins (e.g., Bourdon and Ruoslahti, 1989).

As seen from these few examples, the interactions between the various elements of this system are of great complexity.

Cell-to-cell adhesion

Cells bind to other cells. The significance of cell-to-cell adhesion is illustrated by the classical finding that cells isolated from any one particular tissue and mixed with other unlike cells are targeted to the tissue of origin (e.g., Townes and Holtfreter, 1955). This finding can be explained by the differential adhesion hypothesis (Steinberg, 1963). This hypothesis proposes that each cell type has a specific adhesive capacity that allows it to attach only to like cells. The *cadherins* are adhesion molecules that mediate such attachments. The appearance of cadherin is developmentally regulated, the expression of specific cadherins

coinciding with morphogenic events (see Takeichi, 1991). Cells expressing two different kinds of cadherins are found to be sorted from one another (Nose et al., 1988). In addition, in vitro, cells expressing identical cadherins are also sorted out by a mechanism that apparently depends on the amounts of cadherin produced (Friedlander et al., 1989; Steinberg and Takeichi, 1994). These finding were recently confirmed in vivo (e.g., Godt and Tepass, 1998) in the case of ovary cells of *Drosophila melonagaster*. Besides its role in development, cell adhesion is also important in malignancy and the spread of malignant cells (metastasis). Metastasis requires the disruption of the attachment between cells.

The CAMs required for cell-to-cell adhesion have been classified into several families. Some of these bind like molecules (*homophilic* binding); others bind molecules unlike themselves (*heterophilic* binding). The adhesion molecules include cadherins, immunoglobulins (Ig), some integrins (which generally attach cells to extracellular matrix) and selectins (in lymphocytes).

Cadherins constitute a family of transmembrane glycoproteins which mediate Ca^{2+}-dependent cell-to-cell adhesion and are responsible for determining cell adhesion specificity for the majority of cell types (see Takeichi, 1991). Since these proteins were discovered independently in a number of laboratories, they were named repeatedly. Uvomorulin, L-CAM, cell-CAM 120/80 are all cadherins. All cell types that form solid tissues have cadherins, each with a specific homophilic binding capacity. The predominance of the role of cadherins is demonstrated in experiments in which cell layers were treated with cadherin antibodies. The cells failed to stick together and dispersed (see Takeichi et al., 1990). Presumably, the antibody competes with the cells for the binding of cadherin molecules. Conversely, cadherin-deficient cells acquire a Ca^{2+}-dependent cadherin-mediated cell-to-cell adhesion when transfected with cDNA coding for cadherin.

Different cadherins have different binding specificities. When cells expressing different cadherins are mixed, they reaggregate separately (Takeichi et al., 1981). Similarly, weakly aggregating cells transfected with different cDNAs, and therefore producing different kinds of cadherins, segregate out when mixed. Cells generally coexpress multiple classes of cadherins, producing a very large number of possible specificities.

An amino acid sequence of 113 amino acids at the amino-terminal of the cadherins determines their binding specificities (Nose et al., 1990). The cytoplasmic domain of cadherins, which includes the carboxy-terminal, is the most conserved region. Partial or complete deletion of this domain blocks cell-to-cell adhesion (e.g., Fujimori et al., 1990), implying that the conformation of the external portion depends on the cytoplasmic domain.

CAMs of the immunoglobulin superfamily have an important role in cell-cell and cell-ECM interactions. Their role in neuronal development and neuronal plasticity is well recognized. (see Chapter 22 and Fields and Ito, 1996).

CAM can interact with a variety of molecules at the cell surface or component of the ECM. They can function in cell recognition. The *receptor-like protein tyrosine phosphatases* (RPTPs) are similar in structure to CAMs. Like CAM they can bind to other like molecules. Their binding to ECM ligands

suggests a role in intracellular signalling (see Chapter 7).

RPTPβ on the surface of glial cells binds to *contactin* present at the surface of neurons. This interaction brings about the outgrowth of neuronal processes (Peles et al., 1995). Contactin is anchored via glycosylphosphatidylinositol (GPI) (see Chapter 4) to the outer leaflet of the plasma membrane with no cytoplasmic domain. However, it binds to integral proteins that, at least potentially, can carry a signal to the cytoplasmic side (Sakurai et al., 1997; Peles et al., 1997). The action of contactin is thought to depend on binding to other protein components of the membrane such as the 190 kDa transmembrane protein *contactin-associated protein*, Caspr (Peles et al., 1997).

The amino terminal of RPTPβ has a high homology to carbonic anhydrase (CAH) (see Peles et al., 1998). This sector is followed by a fibronectin type III repeat attached to a long extracellular spacer. A transmembrane region connects the spacer to two phosphatase domains in the cytoplasm. RPTPβ occurs in several isoforms, one of them water soluble. Isoforms of RPTPβ are found in glia and astrocytes, presumably having a role in neuronal migration. The external sector of RPTPβ has multiple domains capable of binding to many proteins such as the ECM protein *tenascin, pleiotrophin, heparin binding neurite promoting factor* and neuronal CAMs. Pleiotrophin is a heparin-binding neurite promoting factor that binds to phosphacan/RPTPβ.

The use of *Drosophila melonagaster* holds much promise for the study of cell-adhesion molecules and the understanding of their function in cell interaction and embryogenesis. The embryology and genetics of *Drosophila* are well known. The adhesion molecules isolated from this system have many similarities to those of vertebrates and have similar functions. Furthermore, the study of the molecular biology of cell-adhesion molecules and their surface receptors has been given a significant boost by the use of a line of *Drosophila* cells (Schneider-2, S-2) that grows predominantly unattached and rounded because of lack of adhesion molecules (see Hortsch and Bieber, 1991). Therefore, this line is ideal for testing the presence of an exogenous adhesion protein. The adhesion molecules can be introduced by transfection of the cells with cDNA coding for adhesion molecules. Several convenient vectors capable of transfecting *Drosophila* cells are available and many homophilic and heterophilic cell-to-cell interaction proteins have been identified using this approach.

C. Cell Surface Receptors

Interactions between cells through their surface receptors or with extracellular elements (or other signals) and the cell's interior, must also involve molecules bridging the plasma membrane, i.e., they must involve integral proteins. At the external face, they would bind extracellular elements to act as receptors. At the inner membrane face, they would transmit a chemical signal or bind to elements of the cytoskeleton. We saw that the binding domain of some extracellular elements resembles motifs present in growth factors. The binding of extracellular fibrous elements transmitted to the cell's interior can eventually affect gene expression. There is also some evidence that integral protein receptors at the cell surface can bind to those on the surface of another cell, resulting in a bidirectional signal (Holland et al., 1996). The potential of cell-to-cell interactions is shown by the role of Notch proteins. Notch proteins are transmembrane receptors that

function in neurogenesis. The notch ligand, Delta, is another integral protein present in nascent neuronal cells. Its binding to Notch prevents the cells from developing as neurons (see Weinmaster, 1997). Obviously, this mechanism is of great importance in the development of the nervous system (see Chapter 7).

Several cell surface receptors that bind extracellular matrix components directly, have been isolated. Some of these bind to collagen and laminin.

ECM proteins and conventional growth factors may act synergistically. The role of these factors in gene expression can be evaluated using DNA microarrays. The role of EGF in gene expression of cultured human embryonic kidney cells was examined after the attachment of the cells to the ECM proteins, laminin and FN (Yarwood et al., 2001). The DNA microarrays used represented 1,718 human genes. Generally EGF was found to act independently of ECM composition. However, clusters of EGF-regulated genes were found in which attachment of cells to a laminin or fibronectin substrata specifically modified gene expression.

The cell adhesion protein CD44 and its main ligand, the ECM component HA (see above) have a role in cytoskeletal rearrangement. They have been shown to trigger the formation of lamellopodia in mouse mammary epithelial cells in culture (see Chapter 23). CD44, a glycoprotein, is expressed on cell surfaces in several isoforms which are generated by alternative splicing of mRNA (see Chapter 3).

The formation of tissue patterns during embryogenesis may result from qualitative and quantitative differences in cell adhesion in time and space. The *neural cell adhesion molecule* (NCAM) has a number of properties that suggest it has such a role in neural tissue, a possibility supported by the presence of as many as 100,000 NCAM molecules per neuron. NCAMs are part of a family of integral membrane protein consisting of a single polypeptide chain. A site at the amino terminal allows one NCAM molecule to interact with another NCAM molecule. NCAM can also interact with the extracellular matrix components, FN in particular. The carboxyl terminal portion is the transmembrane component. A middle portion is attached to carbohydrate that contains polysialic acid of variable length, which modulates the binding: a decrease in length increases adhesion (Rutishauser and Goridis, 1986). NCAMs have been found to vary in quantity and quality within individual cells and from tissue to tissue. Furthermore, these variations occur spatially and chronologically with embryogenesis, suggesting that they may play a role in these events.

Integrins are the most ubiquitous family of bridging molecules (see Schwartz et al., 1995). Integrins are involved in binding to the cytoskeleton (see below). They are also involved in cell adhesion, so that they have a significant role in cell migration and growth (see Schwartz et al., 1995). Intracellular signals cause conformational changes in the integrin extracellular domain, changing its binding activity to ECM components. This has been referred to as "inside-out" signaling. Conversely, integrins can initiate cellular activities such as cell division, secretion or gene expression. They are also thought to be involved in the activations of certain ion translocases such as the Na^+/H^+ transport system (Schwartz et al., 1991), possibly by triggering second messengers, and to have a role in Ca^{2+}-homeostasis, possibly by an effect on Ca^{2+}-channels (Brass, 1985).

Integrin mediated signals controlled by the binding of extracellular matrix molecules, are involved in the growth regulatory pathway (e.g., see Schwartz, 1997), the same pathway used by growth factors and cytokines. The combined effect of growth factors and integrins appears to be synergistic. For example, cell adhesion enhances the autophosphorylation of the EGF and PDGF receptors brought about by binding their ligand (Cybulsky et al., 1994; Miyamoto et al., 1996). Either system alone is ineffective in most cases. They give a greater response when they are present together.

Integrins regulate gene expression by transcriptional and post-transcriptional mechanisms. Not surprisingly, binding of the extracellular domain of integrins by antibodies or matrix ligands activate the transcription of specific mRNAs (see Juliano, 1996). Integrins also act post-transcriptionally. Human platelets, which lack nuclei, can translate preformed mRNA when activated (Weyrich et al., 1998). Platelet integrin$\alpha_{IIb}\beta_3$ binding to fibrinogen is sufficient to activate new protein synthesis (Pabla et al., 1999). Block of integrin with antibody or peptides blocked this effect. Similarly the activation is missing in patients with *Glanzmann thrombasthemia* who have a defective integrin.

There are at least 11 different kinds of α chains and 6 β chains. Twenty distinct integrin heterodimers formed by noncovalent association of α and β subunits are now known (Springer, 1990; Quaranta, 1990). Generally, one kind of chain associates only with one kind of β chain; however, there are many exceptions. The number (sometimes as a subscript) following the Greek letter, indicates the class of the chain. The sequences of some of the integrin chains have been determined using cDNA techniques (e.g., Tamura et al., 1990). The extracellular ligands of integrin include matrix glycoproteins such as laminin, fibronectin and collagen, as well as the cell adhesion molecules 1CAM-1, 1CAM-2, and VCAM-1.

One of the significant connections bridged by integrins is the basal membranes or basal laminae separating epithelial cell sheets and tubes from other ECM components. Laminins have a binding domain for integrins (see Mecham, 1991). Integrins are also located around muscle, fat and Schwann cells.

Each integrin heterodimer recognizes several ECM proteins and each ECM protein binds to several integrins. The activity of integrin is controlled from inside the cell and integrins carry messages toward the inside the cell depending on the ECM molecule they bind (e.g., FN, laminins, collagens and vitronectin) (see Giancotti and Ruoslahti, 1999). Adherent cells (e.g., fibroblasts or epithelial cells) must be attached to the proper ECM component for survival (see below). Depending on the signal they may proliferate, exit the cell cycle or differentiate. Integrins were found to be needed for optimal activation by growth factors such as *platelet derived growth factor* (PDGF), *epidermal growth factor* (EGF) and *vascular endothelial growth factor* (VEGF).

For their action on the cytoplasmic face of the membrane, integrins associate with adapter molecules which directly bind cytoskeletal elements, protein kinases and growth factor receptors. Integrins, cluster in the plane of the membrane after binding to ECM elements. On the cytoplasmic side, actin filaments assemble into large *stress fibers* which favor further integrin clustering and form *focal adhesions* (see Chapter 23). Some of the integrins associate laterally with caveolin-1, normally associated with specialized plasma

membrane spots (see Chapter 9) . This is a very important association since the inhibition of caveolin expression blocks both the formation of focal adhesions and integrin signaling (e.g., Wary et al., 1998; Wei et al., 1999). For example, the inhibition blocks cell cycle progression which follows the activation of the tyrosine kinase Fyn or the ligand-induced *focal adhesion kinase* (FAK) activation.

Growth factors-mediated growth is favored by integrins via the activation of protein kinases such as the *Ras-extracellular signal regulated kinase* (ERK). Progression through the G1 phase of the cell cycle requires the sequential activation of *cyclin-dependent kinases* (Cdks) (see Chapter 8). Some of the Cdks have been shown to be activated by integrins. Others have been shown to require attachment to the ECM (see Giancotti and Ruoslahti, 1999).

In contrast to the effects triggered by attachment of cells to the ECM, the absence of attachment triggers programmed cell death (apoptosis) in many types of cells (e.g., Frisch and Francis, 1994).

The complexity of the integrin-mediated responses is illustrated by the effect of growth factors. These promote proliferation in endothelial cells when the cells attach to fibronectin through the α5β1 integrin (Mettouchi et al., 2001). In contrast, when the cells attach to laminin through α2 β proliferation is arrested. In the binding to FN, the GTP-binding protein Rac (see Chapter 7) is activated producing an accumulation of cyclin-D1 and progression from G to S. In contrast binding to laminin fails to activate Rac.

IV. RECEPTORS AND IMMUNITY

The cell surface also has a role in the production of antibodies in a manner somewhat related to its involvement in the action of growth factors.

Specific antibodies are produced by the immune system in response to proteins recognized as foreign, the *antigens*. *Antibodies* are proteins that can specifically bind a chemical moiety of the antigen, the *epitope*.

At present, there is considerable evidence that an antibody is produced by a process of clonal selection and activation. The theory of clonal selection assumes that each lymphocyte of an immunocompetent animal possesses a receptor at the cell surface that can bind only to the epitope. Each cell would have only one of these special receptors, so only a small portion of the lymphocytes could respond to one antigen. The binding of the antigen to the receptor stimulates the cell to divide, producing a clone of cells with the same receptor specificity. The progeny of this clone includes cells that synthesize and secrete antibody molecules of the same specificity as the receptor molecules.

The fact that the receptor to a given antigen is present in only a few cells before exposure to the antigen has been demonstrated in two different kinds of experiments. Experiment selected for destruction cells with the appropriate receptor. The other marked these cells so that they could be separated from the rest of the lymphocyte population. In the first kind of experiment, cells from mouse spleen were exposed to a ^{125}I-labeled antigen, *Salmonella* flagellin. Only 1 in 5000 cells could bind the antigen and, after incubation for 16 to 20 h, the high radioactivity of the label destroyed them. The remaining cells were tested for

immunological competence by injecting them into mice whose immunological systems had been destroyed by x-rays. Mice injected with the flagellin were incapable of producing the antibody, although they could produce an antibody to flagellin from a related strain of *Salmonella* (Ada and Byrt, 1969). Therfore, the results of this experiment are consistent with the theory of clonal selection, in that only a portion of the cells could bind the antibody and the rest of the cells were unable to produce the appropriate antibody. The results are shown in Table 4 (Ada and Byrt, 1969). The two different *Salmonella* strains are distinguished as SW1338 and SL871. Column 2 lists the pretreatment with the antigen, and columns 3 and 4 show the amount of antibody produced by the test mice.

Table 4 Antibody Titers of X-Irradiated Mice Injected with Two Serologically Distinct Flagellar Antigens 1 Day After Receiving Syngeneic Cells Pretreated with ^{125}I-Labeled or Unlabeled Antigen

		Mean antibody titer ($\log_2$) from groups of 10 mice after challenge with	
Experiment	**Antigenic pretreatment** per 1.3 x 10^7 cells	**SW1338**	**SL871**
1	0.5 µg SW1338, labelled	0.5	4.2
	0.5 µg SW1338, unlabelled	2.7	5.1
	5 µg SW1338, labelled	0.5	5.4
	5 µg SW1338, unlabelled	2.9	4.3
	No antigen	2.3	4.7
2	5 µg SL871, labelled	0.82	0.5
	5 µg SL871, unlabelled	1.6	4.8
	No antigen	1.1	4.2
3	5 µg SL871, labelled	2.0	1.1

	5 µg SL871, unlabelled	3.3	4.2
	No antigen	2.4	4.9

Reproduced with permission from *Nature* Ada, G.L. and Byrt, P. (1969) 222:1291-1292, copyright &copy1969, MacMillan Magazines Ltd.

The positive selection experiments were carried out with antigen labeled with a fluorescent dye. When the antigen was added to the spleen cells, some of the cells were able to bind the antigen. The cells were then sorted out by a cell sorter. The sorter functions by passing the suspension through a vibrator, which produces liquid droplets containing individual cells. A sensitive photocell can detect the fluorescent label when the cells are illuminated by a laser beam. The sorter imparts a charge to the fluorescent droplets so that labeled and unlabeled cells can be collected at metal plates with opposite charge. In this experiment, 0.1 to 3% of the mouse spleen cells were found to contain the fluorescent antigen. The sorter was able to collect cells that were fluorescent and more capable than other cells of producing immunity to the antigen when tested in the irradiated mice. The collection of labeled cells is shown in Table 5 (Julius, et al., 1972).

The formation of an antibody actually involves a complex interaction between at least two kinds of lymphocytes. The events are thought to begin with binding of the antigen by macrophages. After binding to surface receptors, the antigen is taken up by endocytosis (Pernis, 1985; Pernis and Axel, 1985) and partially hydrolyzed to shorter peptide fragments. The proteolytic fragments are transported back to the cell surface, where they become available to the well-characterized antigen receptor of neighboring T lymphocytes. The remaining steps in the production of antibody are complex and involve interactions between B and T lymphocytes.

Table 5 Enrichment of Antigen Binding Cells

Experiment	Strain	Priming antigen[a]	Staining technique	Before separation	Undeflected	Deflected	Enrichment factor
1	**BALB/cN**	KLH	Direct	0.1	0.1	40	400
2	**BAB/14**	KLH	Direct	0.1	0.1	52	520
3	**CWB**	H Albumin	Indirect	3.5	1	65	18.5
4	**CWB**	H Albumin	Indirect	1.3	0.1	55	42
5	**BALB/cN**	Human gamma globulin	Indirect	1	0.1	62	62

[a]KLH:Heyhole limpet hemocyanin; H albumin: human serum albumin

Reproduced with permission from M.H. Julis et al., *Proc. Nat. Acad. Sci*, 68:1934-1938, 1972.

SUGGESTED READING

Interactions of proteins with membranes

Shaw, G. (1996) The pleckstrin homology domain: an intriguing multifunctional protein module, *BioEssays* 18:35-46. (Medline)

Interaction between Cells

Takeichi, M. (1991) Cadherin cell adhesion receptors as a morphogenic regulator, *Science* 251:1451-1455. (Medline)

Interaction between Cells and the Cell Matrix

Ben-Ze'ev, A. (1991) Animal cell shape changes and gene expression, *BioEssays* 13:207-211.

Hardingham, T.E. and Fosang, A.J. (1992) Proteoglycans: many forms and many functions, *FASEB J.* 6:861-870. (Medline)

Hynes, R.O. (1990) *Fibronectins*, Springer-Verlag, New York, Berlin. Chapter 5: Interactions of fibronectins; Chapter 6: Structure of fibronectin; Chapter 8: Cellular adhesion and cell surface receptors.

Hynes, R.O. (1992) Integrins: Versatility, modulation and signaling in cell adhesion, *Cell* 69:11-25. (Medline)

Schwartz, M.A. (1997) Integrins, oncogenes and anchorage dependence, *J.Cell Biol.* 139:575-578. (Medline)

Receptor binding

Jans, D.A. and Hassan, G. (1998) Nuclear targeting by growth factors, cytokines, and their receptors: a role in signaling? *BioEssays* 20:400-411. (Medline)

Taga, T. and Kishimoto, T. (1992) Cytokine receptors and signal transduction, *FASEB J.* 7:3387-3396. (Medline)

Wells, J.A. (1994) Structural and functional basis for hormone binding and receptor oligomerization, *Curr.*

Opin. Cell Biol. 6: 163-173. (Medline)

Caveolae

Rothberg K.G., Heuser, J.E., Donzell, W.C., Ying, Y., Glenney, J.R. and Anderson, R.G.W. (1992) Caveolin, a protein component of caveolae membrane coats, *Cell* 68:673-682. (Medline)

Scaffolds, anchors and adaptors

Pawson, T. and Scott, J.D. (1997) Signaling through scaffold, anchoring, and adaptor proteins, *Science* 278:2075-2080. (Medline)

TGF-β and SMADs

Kawabata, M. and Miyazono, K. (1999) Signal transduction of the TGF-β superfamily by Smad proteins, *J. Biochem. (Tokyo)* 125:9-16. (Medline)

REFERENCES

Search the textbook

Back to Chapter 6

REFERENCES

Ada, G.L. and Byrt, P. (1969) Specific inactivation of antigen reactive cells with ^{125}I-labelled antigen, *Nature* 222:1291-1292.(Medline)

Aitken, A. (1996) 14-3-3 and its possible role in co-ordinating multiple signalling pathways, *Trends Cell Biol.*6:341-347.

Akiyama, S.K. and Yamada, K.M. (1983) Fibronectin in disease. In *Connective Tissue Diseases* (Wagner, B.M., Fleishmajer, P. and Kaufman, N., eds.) pp. 55-96. Williams & Wilkins, Baltimore.(Medline)

Aloyz, R.S., Bamji, S.X., Pozniak, C.D., Toma, J.G., Atwal, J., Kaplan, D.R. and Miller, F.D. (1998) p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors, *J. Cell Biol.* 143:1691-1703.(Medline)

Arai, K., Lee, F., Miyajima, A., Miyatake, S., Arai, N. and Yokota, T. (1990) Cytokines: coordinators of immune and inflammatory responses, *Ann. Rev. Biochem.* 59:783-836.(Medline)

Argentsinger, L.S., Campbell, G.S., Yang, X., Witthuhn, B.A., Silvennoien, O., Ihle, J.N. and Carter-Su, C. (1993) Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase, *Cell* 74: 237-244.(Medline)

Assoian, R.K. (1997) Anchorage-depedent cell cycle progression, *J. Cell Biol.* 136:1-4.(Medline)

Baldwin, A.S. Jr. (1996) The NF-κ B and I κB proteins: new discoveries and insights, *Annu. Rev. Immunol.* 14:649-683. (MedLine)

Balkwill, F., Foxwell, B. and Brennan, F. (2000) TNF is here to stay! *Immunol. Today* 21:470-471. (MedLine)

Ballesteros, J.A., Jensen, A.D., Liapakis, G., Rasmussen, S.G., Shi, L., Gether, U. and Javitch, J.A. (2001) Activation of the β 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6, *J. Biol. Chem.* 276:29171-29177. (MedLine)

Bamji, S.X., Majdan, M., Pozniak, C.D., Belliveau, D.J., Aloyz, R., Kohn, J., Causing, C.G., Miller, F.D. (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death, *J. Cell Biol.* 140:911-923.(Medline)

Barnard, E.A. (1992) Receptor classes and the transmitter-gated ion channels (minireview), *Trends in Biochem. Sci.* 17:368-374.(Medline)

Baud, V. and Karin, M. (2001) Signal transduction by tumor necrosis factor and its relatives, *Trends Cell Biol.* 11:372-377. (MedLine)

Beck, K., Hunter, I. and Engel, J. (1990) Structure and function of laminin: anatomy of a multidomain glycoprotein, *FASEB J.* 4:148-160.(Medline)

Benecke, B.-J., Ben-Ze'ev, A. and Penman, S. (1978) The control of mRNA production, translation and turnover in suspended and reattached anchorage depedent fibroblasts, *Cell* 14:931-939.(Medline)

Bockaert, J. and Pin, J.P. (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success, *EMBO J.* 18:1723-1729. (MedLine)

Bork, P., Schultz, J. and Ponting, C.P. (1997) Cytoplasmic signalling domains: the next generation, *Trends in Biochem. Scie.* 22:296-298.(Medline)

Bourdon, M.A. and Ruoslahti, E. (1989) Tenascin mediates cell attachment through an RGD-dependent receptor, *J. Cell Biol.* 108:1149-1155.(Medline)

Brass, L.F. (1985) Ca^{2+} transport across the platelet plasma membrane: a role for membrane glycoproteins IIb and IIIa, *J. Biol. Chem.* 260:2231-2236.(Medline)

Burnstein, D.E., Seeley, B.J. and Greene, L.A. (1985) Lithium ion inhibits nerve growth factor-induced neurite outgrowth and phosphorylation of nerve growth factor-modulated microtubule associated protein, *J. Cell Biol.* 101:862-870.(Medline)

Calissano, P. and Shelanski, M.L. (1980) Interaction of nerve growth factor with tight binding and pheochromocytoma cells. Evidence for sequestration, *Neuroscience* 5:1033-1039.(Medline)

Chandrasekharis, S., Sorrentino, J.A. and Millis, A.J.T. (1983) Interaction of fibronectin with collagen: age specific defect in biological activity of human fibroblast fibronectin, *Proc. Natl. Acad. Sci. USA.* 80:4747-4751.(Medline)

Chang, H.Y. and Yang, X. (2000) Proteases for cell suicide: functions and regulation of caspases,

*Microbiol. Mol. Biol. Rev.*64:821-846. (MedLine)

Chao MV and Bothwell M. (2002) Neurotrophins. To cleave or not to cleave, *Neuron* 33:9-12. (MedLine)

Cheever, M.L., Sato, T.K., de Beer, T., Kutateladze, T.G., Emr, S.D. and Overduin, M. (2001) Phox domain interaction with PtdIns(3)P targets the Vam7 t-SNARE to vacuole membranes, *Nature Cell Biol.* 3:613-618. (MedLine)

Chen, Y.G., Lie, F. and Massagué, J. (1997a) Mechanism of TGFβ receptor inhibition by FKBP12, *EMBO J.* 16:3866-3876.(Medline)

Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G. and Whitman, M. (1997b) Smad4 and FAST-1 in the assembly of activin-responsive factor, *Nature* 389:85-89.(Medline)

Chen, L.-f., Fischle, W., Verdin, E. and Greene, W.C. (2001) Duration of nuclear NF-κB action regulated by reversible acetylation, *Science* 293:1653-1657. (MedLine)

Chiquet-Erismann, R. (1990) What distinguishes tenascin from fibronectin? *FASEB J.* 4:2598-2604.(Medline)

Clurman, B.E., Sheaff, R.J., Thress, K., Groudine, M. and Roberts, J.M. (1996) Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation, *Genes Dev.* 10:1979-1990.(Medline)

Cochet, C., Gill, G.N., Meisenhelder, J., Cooper, J.A. and Eveleth, D.D. and Bradshaw, R.A. (1992) Nerve growth factor non-responsive pheochromocytoma cells. Altered internalization results in signalling dysfunction, *J Cell. Biol.* 117:291-299.(Medline)

Corvera, S. and Czech, M.P. (1998) Direct targets of phosphoinositide 3-kinase products in membrane traffic and signal transduction, *Trends Cell Biol.* 8:442-446.(Medline)

Cunningham, B.C., Ultsch, M. de Vos, A.M., Mulkerrin, M.G., Clausner, K.R. and Wells, J.A. (1991) Dimerization of the extracellular domain of the human growth factor receptor by a single hormone molecule, *Science* 254:821-825.(Medline)

Cybulsky, A.V., McTavish, A.J., and Cyr, M.D. (1994) Extracellular matrix modulates epidermal growth factor receptor activation in rat glomerular epithelial cells, *J. Clin. Invest.* 94:68-78.(Medline)

Damjanovich, S., Bene, L., Matko, J., Alileche, A., Goldman, C.K., Sharrow, S. and Waldmann, T.A. (1997) Preassembly of interleukin 2 (IL-2) receptor subunits on resting Kit 225 K6 T cells and their

modulation by IL-2, IL-7, and IL-15: a fluorescence resonance energy transfer study, *Proc. Natl. Acad. Sci. USA* 94:13134-13139.(Medline)

Daub, H., Wallasch, C., Lankenau, A., Herrlich, A. and Ullrich, A. (1997) Signal characteristics of G-protein-transactivated EGF receptor, *EMBO J.* 16:7032-7044. (MedLine)

de Caestecker, M.P., Parks, W.T., Frank, C.J., Castagnino, P., Bottaro, D.P., Roberts, A.B. and Lechleider, R.J. (1998) Smad2 transduces common signals from receptor serine-threonine and tyrosine kinases, *Genes Dev.* 12:1587-1592.(Medline)

Doria, M., Salcini, A.E., Colombo, E., Parslow, T.G., Pelicci, P.G. and Di Fiore, P.P. (1999) The eps15 homology (EH) domain-based interaction between eps15 and hrb connects the molecular machinery of endocytosis to that of nucleocytosolic transport, *J. Cell Biol.* 147:1379-1384.(Medline)

Drubin, D.G., Feinstein, S.C., Shooter, E.M. and Kirschner, M.W. (1985) Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly-promoting factors, *J. Cell Biol.* 101:1799-1807. (MedLine)

Durocher, D., Henckel, .J., Fersht, A.R. and Jackson, S.P. (1999) The FHA domain is a modular phosphopeptide recognition motif, *Mol. Cell* 4:387-394.(Medline)

Edwards, R.H., Selby, M.J., Garcia, P.D. and Rutter, W.J. (1988) Processing of the native nerve growth factor precursor to form biologically active nerve growth factor, *J. Biol. Chem.* 263:6810-6815. (MedLine)

Engel, J. (1991) Common structural motif in proteins of the extracellular matrix, *Curr. Opin. Cell Biol.* 3:779-785.(Medline)

Engelmann, H.D., Aderka, M., Rubinstein, M., Rotman, D. and Wallach, D. (1989) A tumor necrosis factor-binding protein purified to homogeneity from human urine protects cells from tumor necrosis factor toxicity, *J. Biol. Chem.* 264:11974-11980.(Medline)

Eveleth, D.D. and Bradshaw, R.A. (1992) Nerve growth factor nonresponsive pheochromocytoma cells: altered internalization results in signaling dysfunction, *J. Cell Biol.* 117:291-299.(Medline)

Fakuda, M. and Mikoshiba, K. (1996) Structure-function relationship of mouse Gap1^{m}. Determination of the inositol 1,3,4,5,tetrakiphosphate-binding domain, *J. Biol. Chem.* 271:18838-18842.(Medline)

Fantl, W.J., Escobedo, J.A., Martin, G.A., Turck, C.W., del Rosario, M., McCormick, F. and Williams, L.T. (1992) Distinct phosphotyrosine on a growth factor receptor binding to specific molecules that

mediate different signal pathways, *Cell* 69:413-423.(Medline)

Fields, R.D. and Ito, K. (1996) Neural adhesion molecules in activity-dependent development and synaptic plasticity, *Trends Neurosci.* 19:473-480.(Medline)

Fortini, M.E. (2001) Notch and presenilin: a proteolytic mechanism emerges, *Curr. Opin. Cell Biol.*(MedLine)

Fortini, M.E. (2002) γ-secretase-mediated proteolysis in cell-surface-receptor signalling, *Nature Rev. Mol. Cell Biol.* 3:673-684. (MedLine)

Francis, N.J. and Landis, S.C. (1999) Cellular and molecular determinants of sympathetic neuron development, *Annu. Rev. Neurosci.* 22:541-566.(Medline)

Franklin, J.L. and Johnson, E.M., Jr (1992) Suppression of programmed neuronal death by sustained elevation of cytoplasmic calcium, *Trends Neurosci.* 15:501-508.(Medline)

Friedlander, D.R., Mege, R.M., Cunningham, B.A. and Edelman, G.M. (1989) Cell sorting-out is modulated by both the specificity and amount of different cell adhesion molecules (CAMs) expressed on cell surfaces, *Proc. Natl. Acad. Sci. USA* 86:7043-7047.(Medline)

Frisch, S.M. and Francis, H. (1994) Disruption of epithelial cell-matrix interactions induces apoptosis, *J. Cell Biol.* 124:619-626. (MedLine)

Fruman, D.A., Rameh, L.E. and Cantley, L.C. (1999) Phosphoinositide binding domains: embracing 3-phosphate, *Cell*97:817-820.(Medline)

Fujimori, T., Miyatani, S. and Takeiehi, M. (1990) Ectopic expression of N-cadherin perturbs histogenesis in Xenopus, *Dev. Biol.* 110:97-104.(Medline)

Gadella, T.W. Jr and Jovin, T.M. (1995) Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation, *J. Cell Biol.* 129:1543-1558.(Medline)

Gatto, G.J. Jr., Geisbrecht, B.V., Gould, S.J. and Berg, J.M. (2000) Peroxisomal targeting signal-1 recognition by the TPR domains of human PEX5, *Nature Struct. Biol.* 7:1091-1095. (MedLine)

Gehlsen, K.R., Dillmer, L., Engvall, E. and Ruoslahti, E. (1988) The human laminin receptor is a member of the integrin family of cell adhesion receptors, *Science* 241:1228-1229 (correction, Science 245:342-343).(Medline)

Ghosh, A., Carnahan, J. and Greenberg, M.E. (1994) Requirement for BDNF in activity-dependent survival of cortical neurons, *Science* 263:1618-1623.(Medline)

Giancotti F.G. and Ruoslahti, E. (1999) Integrin signaling, *Science* 285:1028-1032. (MedLine)

Godt, D. and Tepass, U. (1998) *Drosophila* oocyte localization is mediated by differential cadherin-based adhesion, *Nature* 395:387-391.(Medline)

Graff, J.M., Bansal, A. and Melton, D.A. (1996) *Xenopus* Mad proteins transduce distinct subsets of signals for the TGFβ super family, *Cell* 85:479-487.(Medline)

Gundersen, R.W. and Barrett, J.N. (1979) Neuronal chemotaxis: chick dorsal-root axons turn toward high concentrations of nerve growth factor, *Science* 206:1079-1080. (MedLine)

Hackel, P.O., Zwick, E., Prenzel, N. and Ullrich, A. (1999) Epidermal growth factor receptors: critical mediators of multiple receptor pathways, *Curr. Opin. Cell Biol.* 11:184-189. (MedLine)

Hagler, H.T., McKanna, J.A. and Cohen, S. (1979) Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-43, *J. Cell Biol.* 81:382-395.(Medline)

Hall, D.E., Frazer, K.A., Hann, B.C and Reichardt, L.F. (1988) Isolation and characterization of a laminin-binding protein from rat and chick muscle, *J. Cell Biol.* 107:687-697.(Medline)

Hall, R.A., Premont, R.T. and Lefkowitz, R.J. (1999) Heptahelical receptor signaling: beyond the G protein paradigm *J. Cell Biol.* 145:927-932.(Medline)

Hanson, P.I. and Schulman, H. (1992) Neuronal Ca^{2+}/calmodulin-dependent protein kinases, *Annu. Rev. Biochem.* 61:559-601.(Medline)

Harrison, S.C. (1996) Peptide-surface association: the case of PDZ and PTB domains, *Cell* 86:341-343.(Medline)

Hasegawa, T., Hasegawa, E., Chen, W.T. and Yamada, K. M. (1985) Characterization of membrane-associated glycoprotein complex implicated in cell adhesion to fibronectin, *J. Cell Biochem.* 28:307-318.(Medline)

Hascall, V.C. and Hascall, G.K. (1981), Proteoglycan, in *Cell Biology of Extracellular Matrix*. pp. 39-63 Plenum Publishing Corporation, New York.

Hata, A., Seoane, J., Lagna, G., Montalvo, E., Hemmati-Brivanlou, A., Massagué, J. (2000) OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways, *Cell* 100:229-240.(Medline)

Hay, E.D. (1984) Cell-matrix interaction in embryo: cell shape. cell surface, and cell skeleton and their role in differentiation. In *The Role of Extracellular Matrix in Developmental* (Trelstad, R.L., ed.), pp. 1-31. Liss, New York.

Hayashi, T., Umemori, H., Mishina, M. and Yamamoto, T. (1999) The AMPA receptor interacts with and signals through the protein tyrosine kinase Lyn, *Nature* 397:72-76.(Medline)

Heldin, C.-H., Miyazono, K. and ten Dijke, P. (1997) TGF-β signalling from cell membrane to nucleus through SMAD proteins, *Nature* 390:465-471.(Medline)

Hemmings, B.A. (1977) PH domains - a universal membrane adapter, *Science* 275:1899.

Hendry, J.A., Stockel, K., Thoenen, H. and Iversen, L.L. (1974) The retrograde axonal transport of nerve growth factor, *Brain. Res.* 68:103-121.(Medline)

Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T. and Kishimoto, T. (1990) Molecular cloning and expression of an IL-6 signal transducer, gp130, *Cell* 63:1149-1157.(Medline)

Hiroaki, H., Ago, T., Ito, T., Sumimoto, H. and Kohda, D. (2001) Solution structure of the PX domain, a target of the SH3 domain, *Nature Struct. Biol.* 8:526-530. (MedLine)

Hofmann, K. and Falquet, L.(2001) A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems, *Trends Biochem. Sci.* 26:347-350. (MedLine)

Holland, S.J., Gale, N.W., Mbamalu, G., Yancopoulos, G.D., Henkemeyer, M. and Pawson, T. (1996) Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligand, *Nature* 383:722-725.(Medline)

Hoodless, P.A., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M.B., Attisano, L. and Wrana, J.L. (1996) MADR1, a MAD-related protein that functions in BMP2 signaling pathways, *Cell* 85:489-500.(Medline)

Hopkins, C.R., Boothroyd, B. and Gregory, H. (1981) Early events following the binding of epidermal growth factor to surface receptors on ovarian granulosa cells, *Eur. J. Cell Biol.* 24:259-265.(Medline)

Hortsch, M and Bieber, A.I. (1991) Sticky molecules in non-sticky cells, *Trends in Biochem. Sci.* 16:283-

287.(Medline)

Huang, Z.J., Edery, I. and Rosbash, M. (1993) PAS is a dimerization domain common to Drosophila period and several transcription factors, *Nature* 364:259-262. (Medline)

Huang, H.C., Murtaugh, L.C., Vize, P.D. and Whitman, M. (1995) Identification of a potential regulator of early transcriptional responses to mesoderm inducers in the frog embryo, *EMBO J.* 14:5965-5973.(Medline)

Hunter, T.C. (1984) C-kinasephosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity, *J. Biol. Chem.* 259:2553-2558.(Medline)

Hunter, T. and Cooper, J.A. (1981) Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A431 human tumor cells, *Cell* 24:741-752.(Medline)

Hyman, J., Chen, H., Di Fiore, P.P., De Camilli, P. and Brunger, A.T. (2000) Epsin 1 undergoes nucleocytosolic shuttling and its eps15 interactor NH_2-terminal homology (ENTH) domain, structurally similar to Armadillo and HEAT repeats, interacts with the transcription factor promyelocytic leukemia Zn^{2+} finger protein (PLZF), *J. Cell Biol* 149:537-546. (MedLine)

Irvine, R. (1998) Translocation, translocation, translocation....., *Curr. Biol.* 8:R557-R559.

Israel, D.I. and Kaufman, R.J. (1993) Dexamethasone negatively regulates the activity of a chimeric dihydrofolate reductase/glucocorticoid receptor protein, *Proc. Natl. Acad. Sci. USA* 90:4290-4294.(Medline)

Jans, D.A. and Hassan, G. (1998) Nuclear targeting by growth factors, cytokines, and their receptors: a role in signaling? *BioEssays* 20:400-411.(Medline)

Jing, S., Wen, D., Yu, Y., Holst, P.L., Luo, Y., Fang, M., Tamir, R., Antionio, L., Hu, Z., Cupples, R., Louis, J.-C., Hu, S., Altrock, B.W. and Fox, G.M. (1996) GDNF-induced activation of the Ret protein tyrosine kinase is mediated by GDNFR-α , a novel receptor for GDNF, *Cell* 85:1113-1124.(Medline)

Juliano, R. (1996) Cooperation between soluble factors and integrin-mediated cell anchorage in the control of cell growth and differentiation, *BioEssays* 18:911-917.(Medline)

Julius, M.H., Masuda, T. and Herzenberg, L.A. (1972) Demonstration that antigen binding cells are precursors of antibody producing cells after purification using a fluorescence activated sorter, *Proc. Natl. Acad. Sci. USA.* 69:1934-1938.(Medline)

Kamwar, Y.S., Linker, A. and Farquhar, M.G. (1980) Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion, *J. Cell Biol.* 86:688-693.(Medline)

Kaplan, D.R. and Miller, F.D. (1997) Signal transduction by the neurotrophin receptors, *Curr. Opin. Cell Biol.* 9:213-221.(Medline)

Kaplan, D.R. and Stephens, R.M. (1994) Neurotrophin signal transduction by the Trk receptor, *J. Neurobiol.* 25:1404-1417.(Medline)

Karin, M. (1999) How NF κ-B is activated: the role of the I κB kinase (IKK) complex, *Oncogene* 18:6867-6874. (MedLine)

Kavanaugh, W.M., Turck, C.W. and Williams, L.T. (1995) PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine, *Science* 268:1177-1179.(Medline)

Kawabata, M. and Miyazono, K. (1999) Signal transduction of the TGF-β superfamily by Smad proteins, *J. Biochem. (Tokyo)* 125:9-16.(Medline)

Kefalides, N.A., Alper, R. and Clark, C. C. (1979) Biochemistry and metabolism of basement membranes, *Int. Rev. Cytol.* 61: 167-228.(Medline)

Kennedy, M.B. (1995) Origin of PDZ (DHR,GLGF) domains, *Trends in Biochem. Sci.* 20:350.(Medline)

Keresztes, M. and Boonstra, J. (1999) Import(ance) of growth factors in(to) the nucleus, *J. Cell Biol.* 145:421-424.(Medline)

Kipreos, E.T. and Pagano, M. (2000) The F-box protein family, *Genome Biol.* 1:3002.1-3002.7. (MedLine)

Knudsen, K.A., Horwitz, A.F. and Buck, C.A. (1985) A monoclonal antibody identifies a glycoprotein involved in cell-substratum adhesion, *Exp. Cell Res.* 157:218-228.(Medline)

Kornau, H.C., Schenker, L.T., Kennedy, M.B. and Seeburg, P.H. (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95, *Science* 269:1737-1740.(Medline)

Krantz, S.B. Erythropoietin, *Blood* 77:419-434.(Medline)

Kretzschmar, M. and Massagué, J. (1998) SMADs: mediators and regulators of TGF- β signaling, *Curr.*

Opin. Genet. Dev. 8:103-111. (MedLine)

Labbé, E., Silvestri, C., Hoodless, P.A., Wrana, J.L. and Attisano, L. (1998) Smad2 and Smad3 positively and negatively regulate TGF beta-dependent transcription through the forkhead DNA-binding protein FAST2, *Mol. Cell* 2:109-120.(Medline)

Landreth, G.E. and Shooter, E.M. (1980) Nerve growth factor receptors on PC 12 cells: ligand-induced conversion from low to high-affinity states, *Proc. Natl. Acad. Sci. USA* 77:4751-4755.(Medline)

Larson, S.M. and Davidson, A.R. (2000) The identification of conserved interactions within the SH3 domain by alignment of sequences and structures, *Protein Sci.* 9:2170-2180. (MedLine)

Lee, H.J., Jung, K.M., Huang, Y.Z., Bennett, L.B., Lee, J.S., Mei, L. and Kim, T.W. (2002) Presenilin-dependent γ-secretase-like intramembrane cleavage of ErbB4, *J. Biol. Chem.* 277:6318-6323. (MedLine)

Lee, R., Kermani, P., Teng, K.K. and Hempstead, B.L. (2001) Regulation of cell survival by secreted proneurotrophins, *Science* 294:1945-1948. (MedLine).

Lemmon, M.A., Falasca, M., Ferguson, K.M. and Schellsinger, J. (1997) Regulatory recruitment of signalling molecules to the cell membrane by pleckstrin-homology domains, *Trends in Cell Biol.* 7:237-242.

Lemmon, M.A. and Ferguson, K.M. (2000) Signal-dependent membrane targeting by pleckstrin homology (PH) domains, *Biochem. J.* 350:1:1-18. (MedLine)

Levi, A., Shechter, Y., Neufeld, E.J. and Schlessinger, J. (1980) Mobility, clustering, and transport of nerve growth factor in embryonal sensory cells and sympathetic neuronal cell, *Proc. Natl. Acad. Sci.USA.* 77:3469-3473.(Medline)

Levi-Montalcini, R. (1954) Effect of mouse tumor transplantation in a mouse system, *Ann. N.Y. Acad. Sci.* 55:330-343.

Li, J., Smith, G.P. and Walker, J.C. (1999) Kinase interaction domain of kinase-associated protein phosphatase, a phosphoprotein-binding domain, *Proc. Natl. Acad. Sci. USA* 96:7821-7826.(Medline)

Lin, S.Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K.Y., Bourguignon, L. and Hung, M..C. (2001) Nuclear localization of EGF receptor and its potential new role as a transcription factor, *Nature Cell Biol.* 3:802-808. (MedLine)

Linsenmayer, T.F. (1991) Collagen, in *Cell Biology of Extracellular Matrix*, Hay, E.D., ed.) Plenum

Press, New York, pp.7-44.

Liu, F., Wan, Q., Pristupa, Z.B., Yu, X.M., Wang, Y.T. and Niznik, H.B. (2000) Direct protein-protein coupling enables cross-talk between dopamine D5 and γ-aminobutyric acid A receptors, *Nature* 403:274-280. (MedLine)

Livnah, O., Stura, E.A., Middleton, S.A., Johnson, D.L., Jolliffe, L.K. and Wilson I.A. (1999) Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation, *Science* 283:987-990.(Medline)

Lo, R.S. and Massagué, J. (1999) Ubiquitin-dependent degradation of TGF-β-activated Smad2, *Nature Cell Biol.* 1:472-478.

Locksley, R.M., Killeen, N. and Lenardo, M.J. (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology, *Cell* 104:487-501. (MedLine)

Lohi, O., and Lehto, V.-P. (1998) VHS domain marks a group of proteins involved in endocytosis and vesicular trafficking, *FEBS Lett.* 440: 255-257. (MedLine)

Lu, P.-J., Zhou, X.Z., Shen, M. and Lu, K.P. (1999) Function of WW domains as phosphoserine- or phosphothreonine-binding modules, *Science* 283:1325-1328.(Medline)

Majdan, M. and Miller, F.D. (1999) Neuronal life and death decisions functional antagonism between the Trk and p75 neurotrophin receptors, *Int. J. Dev. Neurosci.* 17:153-161.(Medline)

Massagué, J. (1998) TGF-β signal transduction, *Annu. Rev. Biochem.* 67:753-791.(Medline)

Mattsson, P.T., Vihinen, M. and Smith, C.I.E. (1996) X-linked agammaglobulinemia (XLA): a genetic tyrosine kinase disease, *BioEssays* 18:825-834.(Medline)

McDonald, J.A., Kelley, D.G. and Broekelmann, T.J. (1982) Role of fibronectin in collagen deposition: Fab' to the gelatin-binding domain of fibronectin inhibits both fibronectin and collagen organization in fibroblast extracellular matrix, *J. Cell. Biol.* 92: 485-492.(Medline)

McFarlane, S. and Holt, C.E. (1997) Growth factors: a role in guiding axons? *Trends in Cell Biol.* 7:424-430.(Medline)

Mecham, R. P. (1991) Laminin receptors, *Annu. Rev. Cell Biol.* 7:71-91.(Medline)

Mettouchi, A., Klein, S., Guo, W., Lopez-Lago, M., Lemichez, E., Westwick, J.K. and Giancotti, F.G.

(2001) Integrin-specific activation of Rac controls progression through the G_1 phase of the cell cycle, *Mol. Cell* 8:115-127. (MedLine)

Meyer-Franke, A., Wilkinson, G.A., Kruttgen, A., Hu, M., Munro, E., Hanson, M.G. Jr., Reichardt, L.F. and Barres, B.A. (1998) Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons, *Neuron* 21:681-693.(Medline)

Misra, S., Beach, B.M., Hurley, J.H. (2000) Structure of the VHS domain of human Tom1 (target of myb 1): insights into interactions with proteins and membranes,

Biochemistry 39:11282-11290. (MedLine)

Moghal, N. and Sternberg, P.W. (1999) Multiple positive and negative regulators of signaling by the EGF-receptor, *Curr. Opin. Cell Biol.* 11:190-196. (MedLine)

Mosley, B., Beckmann, P., March, C.J., Idzerda, R.L., Gimpel, S.D., VandenBos, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., Wignall, J.M., Smith, C., Gallis, B., Sims, J.E., Urdal, D., Widmer, M.B., Cosman, D. and Park, L.S. (1989) The murine interleukin-4 receptor: molecular cloning and and characterization of a secreted and membrane bound forms, *Cell* 59:335-348.(Medline)

Miyamoto, S., Teramoto, H., Gutkind, J.S. and Yamada, K.N. (1996) Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors, *J. Cell Biol.* 135:1633-1642.(Medline)

Ni, C.Y., Murphy, M.P., Golde, T.E. and Carpenter, G. (2001) γ-Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase, *Science* 294:2179-2181. (MedLine)

Nose, A., Nagafuchi, A. and Takeichi, M. (1988) Expressed recombinant cadherins mediate cell sorting in model systems, *Cell* 54:993-1001.(Medline)

Nose, A., Tsuji, K. and Takeichi, M. (1990) Localization of specificity determining sites in cadherin cell-adhesion molecules, *Cell* 61:147-155.(Medline)

Oppenheim, R.W. (1991) Cell death during development of the nervous system, *Annu. Rev. Neurosci.* 14:453-501.(Medline)

Otsuka, H. and Moskowitz, M. (1975) Arrest of 3T3 cells in G1 phase in suspension culture, *J. Cell Physiol.* 87:213-220.(Medline)

Pabla, R., Weyrich, A.S., Dixon, D.A., Bray, P.F., McIntyre, T.M., Prescott, S.M. and Zimmerman, G.A.

(1999) Integrin-dependent control of translation: engagement of integrin $\alpha_{IIb}\beta_3$ regulates synthesis of proteins in activated human platelets, *J. Cell Biol.* 144:175-184.(Medline)

Parise, L.V., Helgerson, S.L., Steiner, B., Nannizzi, L. and Phillips, D.R. (1987) Synthetic peptides from fibrinogen and fcollagen organization in fibroblast extracellular matrix, *J. Cell Biol.* 92:485-492.(Medline)

Pascal, S.M., Yamazaki, T., Singer, A.U., Kay, L.E. and Forman-Kay, J.D. (1994) Structural and dynamic characterization of the phosphotyrosine binding region of a Src homology 2 domain--phosphopeptide complex by NMR relaxation, proton exchange, and chemical shift approaches, *Biochemistry* 34:11353-11362. (MedLine)

Patapoutian, A. and Reichardt, L.F. (2001) Trk receptors: mediators of neurotrophin action, *Curr. Opin. Neurobiol.* 11:272-280. (MedLine)

Pawson, T. and Scott, J.D. (1997) Signaling through scaffold, anchoring, and adaptor proteins, *Science* 278:2075-2080.(Medline)

Pawson, T. and Nash, P. (2000) Protein-protein interactions define specificity in signal transduction, *Genes Dev.* 14:1027-1047. (MedLine)

Pazin, M.J. and Williams, L.T. (1992) Triggering signal cascades by receptor tyrosine kinases, minireview, *Trends in Biochem. Sci.* 17:374-378.(Medline)

Peles, E., Nativ, M., Campbell, P.L., Sakurai, T., Martinez, R., Lev, S., Clary, D.O., Schilling, J., Barnea, G., Plowman, G.D., Grumet, M. and Schlessinger, J. (1995) The carbonic anhydrase domain of receptor syrosine phosphatase β is a functional ligand for the axonal cell recognition molecule contactin, *Cell* 82:251-260.(Medline)

Peles, E., Nativ, M., Lustig, M., Grumet, M., Schilling, J., Martinez, R., Plowman, C.D. and Schlessinger, J. (1997) Identification of a novel-contactin-associated transmembrane receptor with multiple domains implated in protein-protein interactions, *EMBO J.* 16:978-988.(Medline)

Peles, E., Schlessinger, J. and Grumet, M. (1998) Multi-ligand interactions with receptor-like protein tyrosine phosphatase β: implications for intercellular signaling, *Trends Biochem. Sci.* 23:121-124.(Medline)

Pelletier, J.N., Campbell-Valois, F.X. and Michnick, S.W. (1998) Oligomerization domain-directed reassembly of active dihydrofolate reductase from rationally designed fragments, *Proc. Natl. Acad. Sci. USA* 95:12141-12146.(Medline)

Pernis, B. (1985) Internalization of lymphocyte membrane components, *Immunol. Today* 6:45-49.

Pernis, B. and Axel, R. (1985) A one and a half receptor mode for MHC-restricted antigen recognition by T lymphocytes, *Cell* 45:13-16..(Medline)

Perry, S.J. and Lefkowitz, R.J. (2002) Arresting developments in heptahelical receptor signaling and regulation, *Trends Cell Biol.* 12:130-138. (MedLine)

Pierce, K.L., Premont, R.T. and Lefkowitz, R.J. (2002) Seven-transmembrane receptors, *Nature Rev. Mol. Cell Biol.* 3:639-650. (MedLine)

Pinkas-Kramarski, R., Shelly, M., Guarino, B.C., Wang, L.M., Lyass, L., Alroy, I., Alimandi, M., Kuo, A., Moyer, J.D., Lavi, S., Eisenstein, M., Ratzkin, B.J., Seger, R., Bacus, S.S., Pierce, J.H., Andrews, G.C., Yarden, Y. and Alamandi, M.. (1998) ErbB tyrosine kinases and the two neuregulin families constitute a ligand-receptor network, *Mol. Cell. Biol.* 18(10):6090-6101. (MedLine)

Quaranta, V. (1990) Epithelial integrins, *Cell Diff. Dev.* 32:361-366.(Medline)

Rameh, L.E. and Cantley, L.C. (1999) The role of phosphoinositide 3-kinase lipid products in cell function, *J. Biol. Chem.* 274:8347-8350.(Medline)

Reilly, J.F. and Maher, P.A. (2001) Importin β-mediated nuclear import of fibroblast growth factor receptor. Role in cell proliferation, *J. Cell Biol.* 152:1307-1312. (MedLine)

Remy, I., Wilson, I.A. and Michnick, S.W. (1999) Erythropoietin receptor activation by a ligand-induced conformation change, *Science* 283:990-993.(Medline)

Riese, D.J. 2nd and Stern, D.F. (1998) Specificity within the EGF family/ErbB receptor family signaling network, *Bioessays* 20:41-48. (MedLine)

Rosen, L.B., Ginty, D.D., Weber, M.J. and Greenberg, M.E. (1994) Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras, *Neuron* 12:1207-1221.(Medline)

Ross, R., Raines, E.W. and Bowen-Pope, D.F. (1986) The biology of platelet-derived growth factor, *Cell* 46:155-169.(Medline)

Ross, R. and Vogel, A. (1978) The platelet-derived growth factor, *Review. Cell* 14:203-210.(Medline)

Rutishauser, U. and Goridis, C. (1986) NCAM. The molecule and its genetics, *Trends Genet.* 2:72-76.

Sakurai, T., Lustig, M., Nativ, M., Hemperley, J.J., Schlessinger, J., Peles, E. and Grumet, M. (1997) Induction of neurite outgrowth through contactin and Nr-CAM by extracellular regions of glial receptor tyrosine phosphatase β, *J. Cell Biol.* 136:907-918.(Medline)

Salcini, A.E., Confalonieri, S., Doria, M., Santolini, E., Tassi, E., Minenkova, O., Cesareni, G., Pelicci, P.G. and Di Fiore, P.P. (1997) Binding specificity and in vivo targets of the EH domain, a novel protein-protein interaction module, *Genes Dev.* 11:2239-2249.(Medline)

Salmivirta, M., Elenius, K., Vainio, S., Hofer, U., Chiquet-Ehrismann, R., Thesleff, I. and Jalkenen, M. (1991) Syndecan from embryonic tooth mesenchyme binds tenascin, *J. Biol. Chem.* 266:7733-7739.(Medline)

Schlessinger, J. (1994) SH2/SH3 signaling proteins, *Curr. Opin. Genet. Dev.* 4:25-30.(Medline)

Schlessinger, J., Shechter, Y., Willingham, M.C. and Pastan, I. (1978) Direct visualization of binding, aggregation, and internalization of insulin and epidermal growth factor on living fibroblastic cells, *Proc. Natl. Acad. Sci. USA* 75:2659-2663.(Medline)

Schwartz, M.A. (1997) Integrins, oncogenes and anchorage dependence, *J.Cell Biol.* 139:575-578

Schwartz, M.A., Lechene, C. and Ingber D.E. (1991) Insoluble fibronectin activates the Na/H antiporter by clustering and immobilizing integrin α5 β1, independent of cell shape, *Proc. Natl. Acad. Sci. USA* 88:7849-7853..(Medline)

Schwartz, M.A., Schaller, M.D. and Ginsberg, M.H. (1995) Emerging paradigms of signal transduction, *Annu. Rev. Cell Dev. Biol.* 11:549-599.(Medline)

Shaw, G. (1996) The pleckstrin homology domain - an intriguing multifunctional protein module, *BioEssays* 18:35-46.(Medline)

Sheppard, K.A., Rose, D.W., Haque, Z.K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M.G., Glass, C.K. and Collins T. (1999) Transcriptional activation by NF- κB requires multiple coactivators, *Mol. Cell Biol.* 19:6367-6378. (MedLine)

Sims, J.E., March, C.J., Cosman, D., Widmer, M.B., MacDonal, H.R., McMahan, C.J., Grubin, C.E., Wignall, J.M., Jackson, J.L. and Call, S.M. (1988) cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily, *Science* 241:585-589.(Medline)

Skålen, K., Gustafsson, M., Rydberg, E.K., Hultén, L.M., Wiklund, O., Innerarity, T.L. and Borén, J. (2002) Subendothelial retention of atherogenic lipoproteins in early atherosclerosis, *Nature* 417:750-754.

(MedLine)

Springer, T.A. (1990) Adhesion receptors of the immune system, *Nature* 346:425-434.(Medline)

Steinberg, M.S. (1963) Reconstruction of tissues by dissociated cells, *Science* 141:401-408.

Steinberg, M.S. and Takeichi, M. (1994) Experimental specification of cell sorting, tissue spreading, and specific spatial patterning by quantitative differences in cadherin expression, *Proc. Natl. Acad. Sci. USA* 91:206-209.(Medline)

Stoker, M., O'Neill, C. and Beirymans, S. (1968) Anchorage and growth regulation in normal and virus transformed cells, *Int. J. Cancer.* 3:683-693.(Medline)

Taga, T. and Kishimoto, T. (1992) Cytokine receptors and signal transduction, *FASEB J.* 7:3387-3396.(Medline)

Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T. and Kishimoto, T. (1989) Interleukin-6 triggers the association of its receptors with a possible signal transducer, gp130, *Cell* 58:573-581.(Medline)

Takeichi, M., Atsumi, T., Yoshida, C., Uno, K. and Okada, T.S. (1981) Selective adhesion of embryonic carcinoma cells and differentiated cells by Ca^{2+}-dependent sites, *Dev. Biol.*87:340.(Medline)

Takeichi, M. (1990) Cadherins: a molecular family important in selective cell-cell adhesion, *Annu. Rev. Biochem.* 59:237-252.(Medline)

Takeichi, M. (1991) Cadherin cell adhesion receptors as a morphogenic regulator, *Science* 251:1451-1455.(Medline)

Tamura, R.N., Rozzo, C., Stan, L., Chambers, J., Reichardt, L.F., Cooper, H.M. and Quaranta, V. (1990) Epithelial integrin $\alpha_6\beta_4$: complete primary structure of α_6 and variant forms of β_4, *J. Cell Biol.* 111:1593-1604.(Medline)

Timpl, R. (1982) Antibodies to collagens and procollagens, *Methods Enzymol.* 82:472-498.(Medline)

Townes, P.L. and Holtfreter, J. (1955) Directed movement and selective adhesions of embryonic amphibian cells, *J. Exp. Zool.* 128:53-120.

Tracey, K.J. and Cerami, A. (1993) Tumor necrosis factor, other cytokines and disease, *Annu. Rev. Cell Biol.* 9:317-343. (MedLine)

Tsukazaki, T., Chiang, T.A., Davison, A.F., Attisano, L. and Wrana, J.L. (1998) SARA, a FYVE domain protein that recruits Smad2 to the TGFβ receptor, *Cell* 95:779-791.(Medline)

Tsunoda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Socolich, M. and Zuker, C.S. (1997) A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade, *Nature* 388:243-249.(Medline)

Tsunoda, S., Sierralta, J. and Zuker, C.S. (1998) Specificity in signaling pathways: assembly into multimolecular signaling complexes, *Curr, Opin. Genet. Dev.* 8:419-422.(Medline)

Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity, *Cell* 61: 203-212.(Medline)

Urushihara, H. and Yamada, K.M. (1986) Evidence for involvement of more than one class of glycoprotein in cell interactions with fibronectin, *J. Cell Physiol.* 126:323-332.(Medline)

van der Geer, P., Hunter, T. and Lindberg, R.A. (1994) Receptor protein-tyrosine kinases and their signal transduction pathways, *Annu. Rev. Cell Biol.* 10:251-337. (MedLine)

von der Mark, K. and Kühl, U. (1985) Laminin and its receptor, *Biochim. Biophys. Acta* 823:47-160.(Medline)

Wartiavaaren, J. and Vaheri, A. (1980) Fibronectin in early mammalian embryogenesis, *Dev. Mamm.* 4:233-266.

Wary, K.K., Mariotti, A., Zurzolo, C. and Giancotti, F.G. (1998) A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth, *Cell* 94:625-634. (MedLine)

Watson AS. And Arkistall, A. (1994) *The G-protein Linked Receptor Fact Book*, Academic Press.

Watson, J.D., Gilman, M., Witkowski, J. and Zoller, M. (1992) *Recombinant DNA* Second edition, Scientific American Books, New York.

Wei, Y., Yang, X., Liu, Q., Wilkins, J.A. and Chapman, H.A. (1999) A role for caveolin and the urokinase receptor in integrin-mediated adhesion and signaling, *J. Cell Biol.* 144:1285-1294. (MedLine)

Weinmaster, G. (1997) The ins and outs of Notch signaling, *Mol. Cell Neurosci.* 9:91-102.(Medline)

Wells, J.A. (1994) Structural and functional basis for hormone binding and receptor oligomerization, *Curr. Opin. Cell Biol.* 6: 163-173.(Medline)

Wells J.A. and de Vos A.M. (1996) Hematopoietic receptor complexes, *Annu. Rev. Biochem.* 65:609-634.(Medline)

Wells, A.and Marti, U. (2002) Signalling shortcuts: cell-surface receptors in the nucleus? *Nature Rev. Mol. Cell Biol.* 3:697-702. (MedLine)

Weyrich, A.S., Dixon, D.A., Pabla, R., Elstad, M.R., McIntyre, T.M., Prescott, S.M. and Zimmerman, G.A. (1998) Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets, *Proc. Natl. Acad. Sci. USA* 95:5556-5561.(Medline)

Whitman, M. (1998) Smads and early developmental signaling by the TGFβ superfamily, *Genes Dev.* 12:2445-2462.(Medline)

Wiedemann, C. and Cockcroft, S. (1998) Vesicular transport. Sticky fingers grab a lipid, *Nature* 394:426-427.(Medline)

Wight, T.N., Heinegard, D.K. and Hascall, V.C. (1991) Collagen, in *Cell Biology of Extracellular Matrix*, Hay, E.D., ed.) Plenum Press, New York, pp.45-77.

Wittlsberger, S.K., Kleene, K. and Penman, S. (1981) Progressive loss of shape-responsive metabolic controls in cells with increasingly transformed phenotype, *Cell* 24:859-866.(Medline)

Wrana, J.L. (2000) Regulation of Smad activity, *Cell* 100:189-192.(Medline)

Wrana, J.L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.F. and Massagué, J. (1992) TGF β signals through a heteromeric protein kinase receptor complex, *Cell* 71:1003-1014.(Medline)

Wu, G., Chen, Y.G., Ozdamar, B., Gyuricza, C.A., Chong, P.A., Wrana, J.L., Massagué, J. and Shi, Y. (2000) Structural basis of Smad2 recognition by the Smad anchor for receptor activation, *Science* 287:92-97.(Medline)

Yaffe, M.B., Rittinger, K., Volinia, S., Caron, P.R., Aitken, A., Leffers, H., Gamblin, S.J., Smerdon, S.J. and Cantley, L.C. (1997) The structural basis for 14-3-3:phosphopeptide binding specificity, *Cell* 91:961-971.(Medline)

Yamada, K.M., Hayashi, M., Hirano, H., and Akiyama S.K. (1984) Fibronectin and cell surface

interactions. In *The Role of Extracellular Matrix in Development* (Trelstad, R.L., ed.), pp. 89-121, Liss, New York.(Medline)

Yamashita, H., ten Dijke, P. Franzén, P., Miyazono, K. and Heldin, C.-H. (1994) Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factors-β, *J. Biol. Chem.* 269:20172-20178.(Medline)

Yarwood, S.J. and Woodgett, J.R. (2001) Extracellular matrix composition determines the transcriptional response to epidermal growth factor receptor activation, *Proc. Natl. Acad. Sci. USA* 98:4472-4477. (MedLine)

Zhang, Y. and Derynck, R. (1999) Regulation of Smad signalling by protein associations and signalling crosstalk *Trends Cell Biol.* 9:274-279.(Medline)

Zhang, Y., Moheban, D.B., Conway, B.R., Bhattacharyya, A. and Segal, R.A. (2000) Cell surface Trk receptors mediate NGF-induced survival while internalized receptors regulate NGF-induced differentiation, *J. Neurosci.* 20:5671-5678. (MedLine)

Zhong, H., Voll, R.E. and Ghosh, S. (1998) Phosphorylation of NF- κ B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300, *Mol. Cell.* 1:661-671. (MedLine)

Zhou, M.-M., Ravichandran, K.S., Olejniczak, E.F., Petros, A.M., Meadows, R.P., Sattler, M., Harlan, J.E., Wade, W.S., Burakoff, S.J. and Fesik, S.W. (1995) Structure and ligand recognition of the phosphotyrosine binding domain of Shc, *Nature* 378:584-592. (MedLine)

Zhu, H., Kavsak, P., Abdollah, S., Wrana, J.L., Thomsen, G.H. (1999) A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation, *Nature* 400:687-693.(Medline)

7. Intracellular Signals and Cascades

As discussed in Chapter 6, a variety of chemical signals, neurotransmitters and hormones, act by binding cell surface receptors. These signals frequently require the mediation of internal signals, the *second messengers*. In the cytoplasm, the second messenger in turn induces the molecular changes that are responsible for the physiological action of the signal. The first section of this chapter will discuss the intracellular signals, followed by the role of GTP-binding proteins in this signaling system (Section II) and the hormone binding receptors found inside cells (Section III). The targets of the intracellular cascades are presented in Section IV. Section V discusses the role of light as a regulator in plants. Note that this topic is also considered from the point of view of metabolic regulation by the oxidation-reduction state of cytoplasmic components in Chapter 14. Section VI examines intracellular signals that originate from organelles rather than the cell surface.

I. INTRACELLULAR SIGNALS

Various chapters relate how changes in the intracellular concentration of Ca^{2+} serve as signals for responses involving secretion (e.g., see neurotransmitters in Chapter 22) and other biochemical functions. Ca^{2+} also induces contraction in muscle and other contractile systems (Chapters 23 and 24). As an activator of secretion, Ca^{2+} enters cells through channels in the plasma membrane. In the activation of contractile systems, the Ca^{2+} is released from the sarcoplasmic reticulum. Calcium as an internal messenger, a so-called *second messenger*, is discussed in Section A. In this role, Ca^{2+} is most frequently released from intracellular stores. The next few sections (B to F) discuss a variety of cytoplasmic signals.

A. Calcium

Ca^{2+} controls a host of cellular processes such as fertilization, secretion, contraction, proliferation and neural signaling (see Berridge et al., 2000; Bootman et al., 2001). It serves as an intracellular messenger for a whole class of extracellular stimuli and couples them to specific cellular responses. It differs significantly from other intracellular signals because of several unique features. Ca^{2+} is present in large reservoirs: the external medium and intracellular compartments (ER and mitochondria). Its release does not require synthetic reactions or the intervention of enzymes. Conversely, it cannot be metabolized. Therefore, very precise mechanisms must exist to control its concentration in the cytoplasm. When Ca^{2+} is released, its concentration next to the reservoirs can be extremely high so that the location of the target

is of the utmost importance in its response. Finally, Ca^{2+} is released in space and time as waves (see below). The frequency of these spikes contain information that provides the Ca^{2+}-signal with considerable specificity.

Ca^{2+} may act through specialized proteins such as calmodulin (CAM) or directly on calcium-activated enzymes. These proteins generally change in conformation upon binding Ca^{2+} (see Ikura, 1996). This group of proteins includes troponin C (which regulates striated muscle contraction), CAM, the S100 proteins and the myristoylated proteins (see Chapter 4) (e.g., recoverin) (see Braunewell and Guldelfinger, 1999). Other proteins bind Ca^{2+} with low affinity and do not undergo conformational changes upon binding Ca^{2+}. They are thought to act as Ca^{2+} buffers (e.g., paralbumin and calbindin).

Various interactions of Ca^{2+} and the proteins that mediate its effect are listed in Table 1 (Rasmussen and Barrett, 1984.

Table 1 Calcium Receptor Proteins

I. True receptor proteins
    A. Soluble homologous class (cytosol)
        1. Calmodulin-all cells
        2. Troponin C-skeletal and cardiac muscle
        3. Parvalbumin-skeletal muscle
        4. Myosin light chain
    B. Membrane bound
        1. Calmodulin
II. Calcium-activated enzymes without specific calcium receptor subunit
    A. Bound to mitochondrial membrane
        1. Glyceraldehyde phosphate dehydrogenase
        2. Mitochondrial substrate transport protein
    B. Mitochondrial matrix
        1. Pyruvate dehydrogenase
        2. α-Ketoglutarate dehydrogenase
    C. Cytosol
        1. Calcium-activated, phospholipid-dependent protein kinase

---

From H. Rasmussen and P.G. Barrett, *Physiological Review*, 64:940. Copyright &copy1984 The American Physiological Society.

Aside from its role as a second messenger and a trigger in cell movement, Ca^{2+} is required for chaperone function (see Chapter 15 and Chapter 10) in the ER (e.g., BiP, see Ivessa et al., 1995), membrane traffic between the ER and the Golgi (see Ivessa et al., 1995). Furthermore, the Ca^{2+}-depletion of the nuclear cisternae decreases the import of macromolecules into the nucleus (see Perez-Terzic et al., 1997), although some of the results contradict this view (see Strubing and Clapham, 1999).

The Ca^{2+} concentration in the cytoplasm and in intracellular stores will be addressed first. The release of Ca^{2+} into the cytoplasm, from either the external medium or the intracellular stores will follow. The release may be in the form of Ca^{2+} waves which have been recognized to play a major regulative role. The distinct function of calcium in different compartments is presented separately , followed by a discussion of calcium effectors, its role in the regulation oxidative metabolism and in gene expression.

Intracellular calcium

For Ca^{2+} to serve as an intracellular signal, its resting cytoplasmic concentration must be kept low, approximately 0.1 µM. As a signal, its concentration increases sharply; at specific locales in the cell, such as presynaptic neuronal terminal (Chapter 22), it may reach as much as 100 µM!

Structural aspects are very important in determining the concentration of Ca^{2+} at the effector site. For example, there is a close anatomical association between ER and mitochondria (see Chapter 16). This proximity plays a significant role in exposing mitochondria to Ca^{2+} concentrations that are very high. The mitochondrial Ca^{2+} of living HeLa cells has been estimated using a Ca^{2+}-sensitive photoprotein, aequorin (see Chapter 1), targeted to the outer face of the inner mitochondrial membrane (see below) (Rizzuto et al., 1998; Sibon et al., 2000). The opening of the inositol 1,4,5-triphosphate (IP_3)-gated channels of the ER (see Chapter 24), induced by histamine (that acts as an IP_3 agonist), was found to provide the inner mitochondrial surface to Ca^{2+} concentrations much higher than those present in the cytoplasm. The transmission of Ca^{2+} in waves (see below) can also increase the local Ca^{2+} concentrations to levels well above those in the bulk cytoplasmic phase.

Several transport systems maintain the low resting level of Ca^{2+}. At the plasma membrane, a transporter protein exchanges external Na^+ for internal Ca^{2+}. The Na^+ electrochemical gradient provides the driving force for the Ca^{2+} efflux (the Na^+ external concentration is much greater than inside and the membrane potential of the plasma membrane is negative inside). Powered by the hydrolysis of ATP, a Ca^{2+}-ATPase of the plasma membrane mediates the efflux of Ca^{2+} against its concentration gradient. In the cell's interior, a similar transport enzyme mediates the accumulation of Ca^{2+} in vesicular organelles such as the sarcoplasmic reticulum. In addition, mitochondria accumulate Ca^{2+} coupled to oxidative metabolism. The presence of cytoplasmic buffers also reduces the concentration of free Ca^{2+} in the cytoplasm (see Allbritton and Meyer, 1993).

The segregation of Ca^{2+} into vesicles and its release to serve as a signal, imposes several requirements. The stores must be capable of having a high content of Ca^{2+}. At the same time the calcium must be available for quick release. The storage capacity of cells is considerable, in the range of 0.3 to 1.5 millimoles per cell (Bastinutto et al., 1995). The high capacity is the result of the presence of Ca^{2+}-binding proteins. *Calsequestrin* is a binding protein isolated from striated muscle sarcoplasmic reticulum. It has high binding capacity (50 binding sites per molecule) of low affinity (K_d = 1 mM) (Campbell et al., 1983). A similar protein, *calreticulin*, has been found in the endoplasmic reticulum of most other cells. This protein has 20 to 40 low affinity binding sites and one high affinity site (Michalak et al., 1992, 1999; Krause et al., 1997). The low affinity sites make the Ca^{2+} readily available for release. A calcium binding protein with a high homology to calreticulin, CALNUC (also called *nucleobindin*), was found in the Golgi, predominantly in the cis-Golgi and the CGN (Lin et al., 1998).

The concentration of free calcium in the lumen of the sequestering organelle must be sufficiently high to permit a rapid release to the cytoplasm. However, it must be affordable in term of energy requirement for the translocation. The most likely concentration ranges in the ER is from 0.1 to 0.7 mM (see Meldolesi and Pozzan, 1998a,b), much greater than that of the cytoplasm (50-100 nM, see Hofer and Schulz, 1996).

Calcium release

The channels that release Ca^{2+} from intracellular stores are the *ryanodine receptor* (RyR) and the *inositol 1,4,5-trisphosphate* receptors (IP_3 receptors). Mammalian tissues express three isoforms of RyRs coded by different genes and several isoforms of IP_3 receptors (see Chapter 24).

The release of Ca^{2+} from intracellular stores is controlled by a variety of signals. Four of these are well recognized at this time and include the second messengers, inositol triphosphate (IP_3) (Berridge, 1993a; see Section F), Cyclic ADP-ribose (cADPR) (Lee et al., 1989), nicotinic acid dinucleotide phosphate (NAADP) (Lee and Aarhus, 1995) (see Section D) and sphingosine derivatives (see below), probably including sphingosine-1-phosphate (Ghosh et al., 1990).

The concentration of Ca^{2+} itself can serve as a signal for Ca^{2+} release. An increase in cytoplasmic Ca^{2+} releases Ca^{2+} from the internal stores. This is known as the Ca^{2+}-induced Ca^{2+} release (CICR) and will be discussed in relation to striated muscle cells (see Chapter 24). The role of this mechanism in other cells is not clear. Alonso et al. (1999) targeted aequorin (e.g., see below) to the ER of adrenal chromaffin cells so that they could monitor the Ca^{2+} concentration in the ER lumen. An increase in cytoplasmic Ca^{2+}, brought about by K^+ induced depolarization, released Ca^{2+} from the ER. The release which could be complete, depended on the Ca^{2+} in the ER and was increased by low concentrations of caffeine. In contrast $InsP_3$-producing agonists released 60-80% of the ER Ca^{2+}. Ryr caused complete and permanent emptying of the stores. Apparently, the ER offers a Ca^{2+}pool that can be discharged by Ryr, IP_3 or CICR.

In many nonexcitable cells, depletion of intracellular Ca^{2+} stores activates a Ca^{2+} influx pathway in the plasma membrane (see Parekh and Penner, 1997). This effect has been called *capacitative calcium entry*. A variety of Ca^{2+}-permeable channels in the plasma membrane underlie this influx. These channels are referred to as *store-operated Ca^{2+} channels* or *Ca^{2+} release-activated channels* (CRACs). The mechanisms by which the depletion of the stores activates CRACs is still unknown (see Putney and McKay, 1999). Some experiments suggest a novel mechanism , possibly corresponding to a direct mechanical interaction which requires a close apposition of the two players: the Ca^{2+}-channels of the PM and the ER. A mechanical interaction has been suggested for the coupling of T tubules and the terminal cisternae of the sarcoplasmic reticulum of muscle to release Ca^{2+} (see Chapter 24). Two sets of experiments support this concept. One set demonstrates a role played by the organization of the actin in the cortical cytoplasmic layer which separates the ER from the PM. The other is based on the dependence of Ca^{2+}-current of the PM on components of the membrane-fusion machinery of cells. A tight cortical layer interferes with the coupling, whereas disassembly of this layer re-establishes coupling (Patterson et al., 1999). These events have no effect on the Ca^{2+} release induced by IP_3, the second messenger that is recognized to trigger the Ca^{2+}-release from the ER (e.g. see above). The study of Yao et al. (1999) of *Xenopus* oocytes, used patch-clamping (see Chapter 22) to follow the Ca^{2+}-current (I_{soc}). In this study the entry of Ca^{2+} across the plasma membrane, I_{soc}, was measured in cell-attached patches, after Ca^{2+}-store depletion. The condition remained after patch-excision. I_{soc} was potentiated by the inhibition of Rho-GTPase and inhibited by expression of wild-type or constitutively active Rho. Rho regulates cytoskeletal rearrangements and membrane trafficking. In addition, dominant-negative mutants of SNAP-25 and botulinum neurotoxin (but not brefeldin A) inhibited I_{soc}. Botulinum neurotoxin is a protease which degrades molecules of the cell's membrane fusion machinery including SNAP-25. Brefeldin inhibits constitutive exocytosis by blocking the exit from the Golgi system. These results suggest that oocyte I_{soc} does not depends on second messengers but on SNAP-25, probably via a mechanism resembling exocytosis involving membrane channels or regulatory molecules. SNAP-25, the acronym of *synaptosome-associated protein of 25 kDa*, is a presynaptic membrane SNARE that has been clearly implicated in membrane fusion in neurons (e.g., see Fasshauser et al., 1997). The cell's molecular machinery for membrane fusion is discussed in Chapter 11.

CRAC channels are under negative feedback control by Ca^{2+} in the cytosolic side so that the channels are eventually inactivated when the concentration of Ca^{2+} is increased sufficiently (see Hoth and Penner, 1993). What is the function of CRACs? An obvious possibility is that they can aid in replenishing the intracellular Ca^{2+} stores. Furthermore, CRACs are though to have a role in the Ca^{2+}-oscillations (see Parekh and Penner, 1997).

The identity of many of the plasma membrane channels involved in the Ca^{2+} entry into cells is not known. The *transient receptor potential channels* (TRPs) of the plasma membrane are non-selective cationic channels which allow the influx of Ca^{2+} into cells. Generally, TRPs are regulated by phosphatidylinositol signaling pathways. Channel opening follows activation of phospholipase C.

Encoded by 20 different genes, TRPs are considered to be subdivided into three sub-families (see Harteneck et al., 2000; Clapham et al., 2001) . The channel subunits have six transmembrane domains and are likely to assemble into tetramers . Different combinations of subunits may assemble contributing to the variety of these channels. Their functions are largely unknown. However, at least one of them (LTRPC7) plays a very important role since overexpression (e.g., by transfection) or knockout of this channel is lethal (Nadler et al., 2001).

TRPs may also be involved in repletion of intracellular Ca^{2+} stores, receptor-mediated excitation and modulation of the cell cycle. Some of these channels may be involved in CRAC. TRP4 and CaT1, both TRP channels, are suspected to be involved in CRAC (Philipp et al., 2000; Freichel et al, 2001; Yue et al., 2001). When expressed in mammalian cells (rat basophilic leukemia cells), the protein CaT1 exhibits all the properties of a channel carrying the I_{SOC} current, as shown with patch clamping (Yue et al., 2001). The CAT1 channel is activated by low Ca^{2+} concentration and inactivated by high Ca^{2+} concentrations. Furthermore, it shares many other properties with I_{SOC}, such as ion selectivity, whole cell current kinetics and loss of selectivity in the absence of Ca^{2+}.

In excitable cells, the Ca^{2+} channels of the plasma membrane open in response to voltage, so that Ca^{2+} can enter the cells. They are regulated by the resting potential which depends on the opening or closing of K^+ channels and therefore by action potentials. Generally, Ca^{2+} is also released from intracellular stores. Although in most cells the ER functions as the major store for Ca^{2+}, an important role of mitochondria has become evident in the last few years (e.g., see Duchen, 1999). Mitochondrial Ca^{2+} has been found to have a very significant role in Ca^{2+}-storage, the regulation of metabolism, apoptosis and in Ca^{2+} oscillations.

Calcium oscillation and waves

A fascinating feature of the free Ca^{2+} concentration of cells is that it can oscillate at specific locations in the cytoplasm (see Thomas et al., 1996). Furthermore, the release of Ca^{2+} is followed by further release in adjacent areas. Therefore, Ca^{2+}-pulses travel through the cytoplasm in waves (see Jaffe, 1993; Fewtrell, 1993). Oscillations have been demonstrated using Ca^{2+}-sensitive fluorescent dyes (such as fura 2), the photo-emitting protein, aequorin, and Ca^{2+}-sensitive microelectrodes.

Oscillations insure that the Ca^{2+}-signal does not decay with distance and arrives full strength to its subcellular target (Rooney and Thomas, 1993). In the absence of propagating waves, the range of the full Ca^{2+} signal is limited to a few µm by dilution and binding to cytoplasmic Ca^{2+}-buffers (Allbritton and Meyer, 1993).

Oscillations begin when Ca^{2+} channels, in either the plasma membrane or in non-excitable cells in internal vesicles stores, open intermittently. The intracellular stores are released through activation of two

distinct channels present in intracellular vesicles. These channels are the receptors for the second messenger inositol 1,4,5-triphosphate (IP_3R) and ryanodine (RYR). The two receptors are very similar. Physiologically, the first is activated by IP_3 (discussed in Section F) whereas RYR is activated by cyclic ADP-ribose (see Section D). The release of Ca^{2+} from intracellular stores may parallel fluctuations in a second messenger, as has been shown for IP_3 (see Berridge, 1993 and Section F).

IP_3 travels more rapidly then Ca^{2+} and, therefore, might actually carry the signal for the spread of the Ca^{2+}-waves (Allbritton and Meyer, 1993). The raised Ca^{2+} concentration of the wave could also activate adjoining Ca^{2+}-release sites by itself (see CICR, discussed above). Either mechanism could result in the propagation of the waves over distances as long as 1 mm (e.g., oocytes or between cells through gap junctions, see below).

Any wave mechanism requires a distribution of release stores along the path of the waves. All experimental data indicate that the store coincide with the distribution of the ER (or SR in muscle) (see Thomas et al., 1996). However, mitochondria seem to be involved in the Ca^{2+} release and increasing the Ca^{2+}-wave amplitude, velocity and interwave period. These changes are favored by mitochondrial oxidizable substrates and can be blocked by either inhibitors of mitochondrial metabolism or of mitochondrial Ca^{2+} transport (Jouaville et al., 1995). In fact, mitochondria appear to be active participants in intracellular Ca^{2+}-signaling because of their ability to rapidly sequester or release Ca^{2+} (Babcock et al., 1997).

Calcium waves can propagate signals between cells coupled by gap junctions (see Chapter 4) because the second messenger IP_3 and the Ca^{2+} readily traverse the channels formed by the connexons (Saez et al., 1989). Synchronized Ca^{2+} oscillations have been observed in adjacent cells. Long range Ca^{2+} waves have been shown in confluent cultured astrocytes in response to the presence of the neurotransmitter glutamate (Cornell-Bell et al., 1990). Astrocytes are one type of neuroglia cells, the supporting cells of the nervous system. Gap junctions are a prominent feature of brain astrocytes. Astrocyte plasma membrane channels open in response to glutamate in the time frame of mseconds. The propagation of the Ca^{2+} waves is over distances of as long as 0.5 mm with a mean velocity of 19 µm per second, suggesting that they might play a significant role in signaling in the brain (Cornell-Bell et al., 1990). In general, glial cells respond to a variety of stimuli, such as neurotransmitters and neuromodulators, by waves of Ca^{2+} that propagate from cell to cell (see Verkhratsky et al., 1998). Not surprisingly, neuronal activity can trigger Ca^{2+} waves, e.g., in astrocytes in cultured slices of rat hippocampus, where astrocyte and neuronal networks are present in their normal tissue relationship (Dani et al., 1992). In addition, astrocytes interact with neurons so that astrocyte waves trigger elevation of Ca^{2+} in neurons (Nedergaard, 1994; Newman and Zahs, 1998). Most frequently, glia-to-neuron communication has been found to be sensitive to ionotropic glutamate receptor antagonists, suggesting a role of glutamate (see Verkhratsky et al., 1998).

Generally two patterns of oscillations are evident. In the case of *baseline transient oscillations*, the cytoplasmic Ca^{2+}-spikes return to a constant baseline level. The oscillations can be maintained for long periods. The amplitude of these pulses does not diminish with time and these oscillations last for relatively long periods (several minutes). In contrast, s*inusoidal oscillations* return to an elevated baseline. They are of higher frequency (1 per min) and only last a few minutes.

With increasing agonist, the baseline spikes increase in frequency (Putney and Bird, 1993) with a shorter latency period (Rooney et al., 1989). Their amplitude remains unchanged. The initial latent period and the frequency of the baseline spikes can serve as a signal (e.g., Cobbold et al., 1991). In sinusoidal oscillations, the average cytoplasmic Ca^{2+} is elevated with no effect on spike frequency or latency.

During the oscillations the Ca^{2+} concentration can be brought back to a lower level by a negative feed-back component, in this case thought to be provided by diacylglycerol (Bird et al., 1993) which is produced by phospholipase C, in the same reaction that generates IP_3 (which releases Ca^{2+})(see Section I F). Diacylglycerol inhibits the production of IP_3.

Speculation about the possible roles of oscillations is intriguing. One of the important features of Ca^{2+} spiking is the sharp threshold of its trigger. In such systems, subthreshold signals or noises are ineffective, and devices that can add up signals can be easily imagined (see Meyer and Stryer, 1991).

Ca^{2+} oscillations whose frequency is a function of agonist concentration can induce Ca^{2+} spikes that are additive in their effect, compared to a single large release (Meyer et al., 1992). Such additions have been demonstrated in the activation of energy production in mitochondria (see below) of hepatocyes in response to increased Ca^{2+} brought about by *vasopressin* (Hajnózky et al., 1995), the hormone that regulates blood pressure. The spikes of increased NADH and flavoprotein decayed more slowly than the Ca^{2+}-spikes so that integration could occur at frequencies above 0.5 min^{-1}. Maximal vasopressin produced a sustained increased Ca^{2+}, producing a single NADH transient because the Ca^{2+} is compensated by its uptake (Rizzuto et al., 1994) so that the large signals are tuned out. A variety of studies support the notion that frequency modulation has an important role as a signal in other functions as well. For example, it plays a role in the control of fluid secretion in insect salivary glands (Rapp and Berridge, 1981) and neuronal functions (Gu and Spitzer, 1995).

Ca^{2+} oscillations can raise the concentration of Ca^{2+} at specific sites without an effect on the overall cytoplasmic Ca^{2+}, permitting very specific, site determined Ca^{2+}-induced responses. Amplitude modulation has been shown in differential gene transcription in B lymphocytes, where low concentrations of Ca^{2+} activate one kind of pathway and much higher levels activate a different group of transcriptional regulators (Dolmetsch et al., 1997).

Different frequencies of Ca^{2+}-oscillations were shown to activate genes specifically. In these

experiments, the normal machinery that triggers the oscillation was by-passed. In the experiments of Dolmetsch et al. (1998) the oscillations were produced in thymocyte attached to a flow chamber. Treatment with a medium containing the drug *thapsigargin*, in the absence of Ca^{2+}, irreversibly opened calcium channels and depleted the cells of calcium (Zweifach and Lewis, 1993). The chamber was then flooded with solutions either depleted of Ca^{2+} or containing Ca^{2+} mimicking the natural Ca^{2+}-oscillations. The internal Ca^{2+} was monitored using a Ca^{2+}-sensitive dye. The activities of the transcription factors NF-AT, NF-κB and Oct/Oap were monitored using *lac*Z or luciferase reporter genes (see Chapter 1). The experiments observed the effect of Ca^{2+} either at constant or oscillating Ca^{2+} concentrations. The results showed that oscillations enhance the efficiency particularly at low Ca^{2+}-levels. Furthermore, different frequencies of oscillation activate genes preferentially.

In the experiments of Li et al. (1998) the Ca^{2+}-waves were controlled using a *caged* IP_3. IP_3 is the natural second messenger that triggers the release of Ca^{2+} from the internal cell's stores (see Fig. 10, above). The caged compound is an IP_3-analog trapped inside cells by ester hydrolysis (the esters shield polar groups to allow for diffusion into the cells). The release of the caged compound can then be controlled by illumination with UV light of various intensities and pulse durations. The gene expression elicited by repetitive flashes was found to be more effective than that produced by the release as a single pulse of long duration.

Distinct functions of calcium-pools

In neurons, the level of Ca^{2+} is controlled in part by voltage or ligand-gated channels that are responsible for adaptive changes in the nervous system. In any cell system, the effect of Ca^{2+} can be extremely localized because of the presence of Ca^{2+} buffers and the Ca^{2+}-sequesting mechanisms (e.g., Clapham, 1995; Meldolesi and Pozzan, 1998). In general, nuclear and cytoplasmic Ca^{2+}, at least under some conditions, constitute two separate pools (see Bustamante, 1994), although they can also vary in parallel. Nuclear microinjection of a non-diffusible calcium chelator can block increase in nuclear Ca^{2+} without interfering with cytoplasmic Ca^{2+} (Hardingham et al., 1997). This approach was able to demonstrate that the nuclear Ca^{2+} concentration controls the Ca^{2+}-activated gene expression mediated by the cAMP-response element (CRE), where the CRE-binding protein functions as Ca^{2+}-responsive transcription factor. Independently from the nucleus, cytoplasmic Ca^{2+} acts through the serum-response element (SRE).

Changes in the free Ca^{2+} in the ER lumen, have been found to have an effect on the synthesis and secretion of protein (see Sambrook, 1990) and the action of chaperones in the ER (Lodish et al., 1992; Corbett et al., 1999; see Corbett and Michalak, 2000). Chaperones are complexes involved in the folding of proteins in the ER lumen and in the cytoplasm (see Chapter 15 and Chapter 10).

The Ca^{2+} concentration inside the ER has been estimated (Miyawaki et al., 1997) using fluorescence

measurements of either blue-fluorescent protein or protein constructs of green-fluorescent protein (GFP), (see Chapter 1) fused to CAM and the CAM-binding peptide M13. These were targeted to the ER of HeLa cells by transfection with the appropriate cDNA. The free Ca^{2+} inside the ER of Hela cells was estimated under a variety of conditions. When the Ca^{2+} stores were full the free Ca^{2+} was in the 60-400 μM range. However, when the Ca^{2+} stores were depleted they were in the 1-50 μM range.

The Ca^{2+}-binding chaperone, calreticulin (discussed above) binds to other chaperones in a Ca^{2+}-dependent manner to regulate their function (see Baksh et al., 1995; Corbett et al., 1999). Calnexin is a similar protein with similar functions. Calreticulin and calnexin are involved in the folding of glycoproteins (see Helenius et al., 1997). In addition to its other functions, calreticulin regulates the Ca^{2+} transport of the ER (John et al., 1998). Overexpression of calreticulin has been shown to inhibit Ca^{2+} oscillations induced by IP_3.

The binding to glycoproteins required for chaperone activity is favored by high ER Ca^{2+} (Vassilakos et al., 1998). Furthermore, the binding of calreticulin to protein disulfide isomerase (PDI) is blocked at high ER-Ca^{2+} concentration increasing the chaperone activity of PDI (Primm et al., 1996) .

Calreticulin is also implicated the regulation of gene expression (Michalak et al., 1996) and cell adhesion (Opas et al., 1996) apparently indirectly (see Kaufman, 1999). The regulation of cell adhesion is via an increase in the transcription of the vinculin gene, since both vinculin protein and mRNA levels are increased in cells overexpressing calreticulin and decreased in cells expressing a diminished amount of calreticulin. Vinculin is a protein involved with actin in the formation of fibers in the cytoskeleton (e.g., see Chapter 11 and Chapter 24).

Calcium effectors

As shown in Table 1, one of the signaling systems involving Ca^{2+} is mediated by CAM. At elevated Ca^{2+} concentrations, each molecule of CAM binds four Ca^{2+} ions. The binding produces a conformational change in the CAM so that it can activate various CAM-regulated enzyme systems. Many Ca^{2+}-binding proteins such as CAM or troponin C form a helix-loop-helix configuration, where the Ca^{2+}-binding site is in between the two helices. This conformation has been called the *EF hand* (see Kretsinger, 1987).

CAM is present in all eukaryotic cells where it plays an important role in growth, proliferation and movement. Vertebrate CAM is a single polypeptide of 16.7 kDa which has four EF hands. Its affinity to Ca^{2+} expressed as a K_d is in the range of $5x10^{-7}$ to 10^{-6}. The binding of CAM to effector proteins raises the affinity of CAM to Ca^{2+} ten-fold (Peersen et al., 1997). The two domains of CAM change in conformation in the presence of Ca^{2+}. In its absence the two helices of each E-F hands are close together. Without binding Ca^{2+} the molecule can open slightly to expose hydrophobic patches, possible allowing the molecule to bind to target proteins (see Swindells and Ikura, 1996). With increases in Ca^{2+}

concentration, the Ca^{2+} is bound in each loop of CAM by seven ligands. In addition, the conformation changes to a greater extent and exposes hydrophobic domains.

The effectors that bind to CAM and are responsible for the physiological function can be grouped in six classes. Some effectors (a) bind irreversibly to CAM, (b) associate with CAM only in the absence of Ca^{2+} (e.g., neuromodulin and neurogranin; they have different functions from those in the presence of Ca^{2+} or serve as storage forms) (see Jurado et al., 1999), (c) form low affinity complexes with CAM unless Ca^{2+} is present (Mamar-Bachi and Cox, 1987; Kincaid and Vaughn, 1986), (d) bind to Ca^{2+} which inhibits their function (e.g., some members of the G-protein-receptor kinases, see Iacovelli et al., 1999) or (e) are activated by CAM when it binds Ca^{2+} (e.g., CAM-dependent protein kinases).

Ion channels, such as Ca^{2+}-activated K^+ channels (Xia et al., 1998), *N*-methyl-D-aspartate receptor channels (Ehlers et al., 1996) or Ca^{2+}-channels, are regulated by Ca^{2+} binding to CAM. CAM regulates the activity of Ca^{2+} channels though inactivation (see Eckert and Chad, 1984) or facilitation (see Noble and Shimoni, 1981). Apparently, CAM mediates both signals that open and those that close the channels (e.g., Zühlke et al., 1999; Qin et al., 1999).

Frequently, effectors controlled by CAM are involved in some way in protein phosphorylation (see below) (CAM dependent adenylyl cyclases, phosphodiesterases, protein kinases and the protein phosphatase calcineurin). In addition CAM regulates the Ca^{2+} pump, ion channels, the ryanodine receptor (see Chapter 24) and isoforms of (1,4,5)-triphosphate receptor (see below) (see Chin and Means, 2000).

A subfamily of EF-hand Ca^{2+}-sensors, the *calcium-myristoyl switch proteins* act as switches (Zozulya and Stryer, 1992; Dizhoor et al., 1993). This subfamily includes *recoverin*, *guanylyl cyclase-activating protein* (GCAP), *frequenin*, *visinin* and *neurocalcin*. They contain a covalently attached myristoyl or related N-acyl group (see Chapter 4). In vertebrate rod outer segments (see Section IIA, below), in the dark and at high Ca^{2+}, recoverin and GCAP are inhibitors of rhodopsin kinase and guanylate cyclase, respectively. With light, the lowering of Ca^{2+} activates these enzymes (Olshevskaya et al., 1997). Ca^{2+}-binding of recoverin induces the binding of myristoylated but not of unmyristoylated protein to rod outer segment membranes. Presumably, the myristoyl group is extruded, enabling recoverin to insert this chain into a lipid bilayer membrane and become an active participant in Ca^{2+} signaling (Zozulya and Stryer, 1992, Tanaka et al., 1995). However, GCAP does not function through a calcium-myristoyl switch (Olshevskaya et al., 1997).

A *Saccharomyces cerevisiae* homolog of frequenin was found to stimulate a phosphatidylinositol-4-OH kinase (Hendricks et al., 1999). Since the phosphoinositides (see Section I F, below) have been found to have a significant role in cellular function (e.g., De Camilli et al., 1996), the activation of this enzyme could have far reaching consequences in the functioning of cells.

Calcium and Ras signalling

Some of the functions of Ca^{2+} result from the activation of the small GTPase Ras (see Section IIB below) (e.g., see Rosen et al., 1994) . Ras is localized in the inner leaflet of the plasma membrane and depends for its activation on the presence of GTP exchange factors (GEFs), GTPase activating proteins (GAPs) and Ras-guanine-relasing factors (RasGRF). The action of Ca^{2+} on Ras may be through these proteins (see Cullen and Lockyer, 2002). Through the activation of Ras, the concentration Ca^{2+} regulates a myriad of pathways, among which the extracellular-signal- regulated kinase (ERK)/mitogen activated protein kinase (MAPK) (e.g., Chao et al., 1992).

Regulation of oxidative metabolism

Ca^{2+} regulates mitochondrial oxidative-phosphorylation (see McCormack et al., 1990; Robb-Gaspers et al., 1998; Duchen, 1999). Chapter 14 describes how mitochondrial oxidative-phosphorylation is thought to vary inversely with ATP/ADP and NADH/NAD^+ ratios. As noted in that chapter, increased Ca^{2+} also speeds up several reactions of the tricarboxylic acid (TCA) cycle.

Indeed, in some tissues, such as cardiac muscle, many physiological conditions increase the rate of oxidative phosphorylation without changing the ADP/ATP and the NAD/NADH ratios by stimulating the mitochondrial dehydrogenases (e.g., Hansford, 1991, Harris and Dass, 1991). However, changes in cytoplasmic Ca^{2+} parallel these increases (e.g., Unitt et al., 1989). Mitochondrial pyruvate dehydrogenase (PDH), NAD^+-isocitrate dehydrogenase (ICDH) and 2-oxoglutarate dehydrogenase (OGDH) are activated by Ca^{2+} (see Denton and McCormack, 1980; McCormack and Denton, 1986). These are key metabolic reactions. PDH is the point of entry of products of glycolysis into the oxidative pathway. PDH is inactive when phosphorylated and active when dephosphorylated. Ca^{2+} activates the phosphatase that dephosphorylates PDH.

Free mitochondrial Ca^{2+} can generally override all other regulatory mechanisms. A role of mitochondrial Ca^{2+} has been found in many vertebrate tissues tested. To have an effect on these enzymes localized in the mitochondrial matrix, the mitochondrial internal concentration must be sufficiently high. PDH and OGDH are activated by 0.05 to 3 µM Ca^{2+} and ICDH by an order of magnitude higher (Rutter, 1990). Does the mitochondrial matrix Ca^{2+} concentration suffice to stimulate the activity of these enzymes?

The mitochondrial Ca^{2+} transport systems regulate the internal Ca^{2+}. Under physiological conditions the Ca^{2+} concentration in the mitochondrial matrix is thought to mirror that of the cytoplasm (e.g., Crompton, 1990). Experiments with isolated mitochondria loaded with Ca^{2+} indicators (McCormack et al., 1989) suggest that the regulative mechanism would come into play maximally when the extramitochondrial concentration reaches 0.8 to 1.2 µM (expected in maximally stimulated cells).

The concentration of free Ca^{2+} in mitochondria of intact cells has been estimated with a variety of techniques. In one set of experiments, cell lines were transfected to express the apoprotein of aequorin, a Ca^{2+} sensitive photoemitting protein targeted to mitochondria (Rizzuto et al., 1992), by adding the appropriate nucleotide sequence to the DNA so that the protein contained a mitochondrial signal sequence (see Chapter 10). The aequorin was then activated by incubating the cells with its prosthetic group (coelenterazine) and used as a Ca^{2+} indicator. In additional experiments, cells were loaded with the Ca^{2+} sensitive dye indo-1 and the cytoplasmic signal was quenched using Mn^{2+} so that only the mitochondrial component would be detected (Miyata et al., 1991). Together, these experiments indicate that in intact cells the resting Ca^{2+} level in the mitochondrial matrix is about 0.1 µM and is increased maximally to 1 to 5 µM by agents that increase cytoplasmic Ca^{2+}.

The relationship between the Ca^{2+} uptake by mitochondria and metabolic activity has been shown more directly in intact HeLa cells and primary cultures of skeletal myotubes (Jouaville et al., 1999). In these experiments, the Ca^{2+} was monitored via the targeted aequorin discussed above. The mitochondrial and cytosolic ATP levels were monitored using specifically targeted chimeras of the ATP-dependent photoprotein luciferase. The light emitted by the luciferin-luciferase reaction is a function of ATP concentration. Upon exposure of the cells to histamine, the increase in mitochondrial Ca^{2+} increased ATP production and, furthermore, the effect continued even after the agonist was washed away, showing a role of Ca^{2+} in producing a prolonged metabolic activation in stimulated cells.

In bovine chromaffin cells from the adrenal medulla, mitochondrially targeted aequorin proteins (Montero et al., 2000), show that half of the chromaffin cell mitochondria accumulate rapidly millimolar Ca^{2+} (as high as 1.5 mM) upon stimulation of cells with acetylcholine, caffeine or high concentrations of potassium ions. Such large transients are possible because of the localization of some of the mitochondria close to Ca^{2+}-channels of the plasma membrane or of the ER. Because of the large capacity of mitochondria to store Ca^{2+}, these results also indicate a major role of mitochondria in signaling for the release of catechol amines from the adrenal medulla.

Calcium and gene expression

The mechanisms by which Ca^{2+} regulates gene expression are complex and sometimes follow a very indirect path. Some of the aspects of Ca^{2+} regulation are just beginning to be elucidated so that a clear picture is still not available. However, much progress has been made.

The myocyte enhancer factor-2 (MEF2) family of transcription factors have been found to have a central position in the regulation gene expression by Ca^{2+} (see McKinsey et al., 2002). They have a key role in the activation of genes controlling cell division, differentiation and apoptosis. MEF2s are part of the mitogenic signaling pathway. MEF2s are involved in *c-jun* expression in response to *epidermal growth factor receptor* (EGFR), lipopolysacccharide receptor, CD28 co-stimulatory receptor of T lymphocytes and the G-protein coupled receptors (see McKinsey et al., 2002). Four different *MEF2 genes* (*MEF*2A, -

B, C and D) are expressed at different embryonic stages and in adulthood. The various proteins are very similar at the amino terminal which contains a MADS domain. The MADS domain is neeed for dimerization, binding to DNA and association with transcriptional cofactors which control specific target genes. The carboxy-terminal is needed for transcriptional activation.. The involvement of Ca^{2+} is generally through posttranslational mechanisms.

The ability to control such diverse functions rests on interactions with other proteins. The actions of MEF2s are distinct, depending on tissue, developmental stage and physiological state. For example, a Ca^{2+}-dependent action of MEF2 has been found to be as a survival factor that blocks apoptosis in neurons (Mao et al., 1999; Okamato et al., 2000). In contrast, in thymocytes, MEF2 is a Ca^{2+}-dependent pro-apoptotic factor where it activates the nuclear hormone receptor TR3 (see Chapter 2) which in turn triggers cytochrome *c*-activated apoptosis (e.g., Woronicz et al., 1995).

As we have seen repeatedly (e.g., see Chapter 13) phosphorylation and dephosphorylation of proteins plays an important role in their functional regulation. The effect of Ca^{2+} on protein kinases or phosphatases can have far reaching effects on transcription. For example, the activation of immune response genes requires prolonged Ca^{2+} exposure (Timmerman et al., 1996). The NF-AT_cs, are transcription factors involved in the immune response and present in the cytoplasm in a form capable of activating transcription of the appropriate genes (Flanagan et al., 1991). However, they can only enter the nucleus when *calcineurin*, a serine/threonine phosphatase activated by binding Ca^{2+} and calmodulin, dephosphorylates them exposing two *nuclear localization sequences* (NLSs) (Beals et al., 1997; see Crabtree, 1999). These sequences allow the import of the proteins into the nucleus. Calcineurin comes into play after sustained, low amplitude Ca^{2+} spikes. However, when calcineurin activity decreases, the NF-AT proteins are phosphorylated again and exported via Crm1, an export receptor that recognizes the *nuclear export sequence* (NES) (e.g., Kehlenbach et al., 1998; Zhu and McKeon, 1999). Apparently, calcineurin also blocks the export in the absence of phosphatase activity by competing with Crm1 for binding to the NF-AT NES (Zhu and McKeon,1999). Ca^{2+} and calcineurin also play a role in the induction of apoptosis (see Chapter 2).

In addition to targeting transcription factors of the NF-AT family, calcineurin can activate MEF2. In the case of one kind of MEF2, after dephosphorylation NF-AT is translocated into the nucleus where it binds to MEF2. NFAT stimulates MEF2-dependent transcription by favoring the recruitment of the p300 coactivator to the MEF2-response element (Youn et al., 2000). This kind of mechanism is important in T-thymocyte apoptosis and in regulating slow twitch fiber genes in skeletal muscle. In neurons, calcineurin and MEF2 protect from Ca^{2+}-induced apoptosis. Calcineurin dephosphorylates MEF2D in response to electrical depolarization of cells (Mao and Wiedmann, 1999) and thereby enhances the DNA binding activity of MEF2A and therefore maximizes its transactivation capability. In the absence of depolarization, MEF2D is phosphorylated and targeted for caspase-mediated apoptotic degradation (Li et al., 2001). It is not clear at this time whether this mechanism is present in other cell types.

In some cases, the effect Ca^{2+} is mediated by CAM. The *calcium-calmodulin dependent protein kinase* (CAMK) phosphorylates MEF2D increasing its transcriptional activity (Blaeser et al., 2000). CAMK is activated mostly by high-amplitude transient Ca^{2+} spikes. However, in the case of other MEF2s the effect of CAMK is likely to be through an intermediary factor probably by interfering with histone deacetylase (HDAC)-MEF2 interaction (HDACs act as transcriptional repressors) (see Chapter 2 and Lu et al., 2000). HDACs repress transcription by deacetylating the amino terminal tails of core histones, whereas histone acyltransferases (HACs) acetylate the tails and have the opposite effect. Phosphorylation of HDACs creates a docking site for the chaperone protein 14-3-3 which disrupts HDAC-MEF2 complexes. Furthermore the binding to the chaperone masks the NLS of HDAC and exposes the NES causing the exit of HDACs from the nucleus. The freed MEF2 can associate with the p300 coactivator and the MEF2-dependent genes are activated. The associations between MEF2 and HATs and MEF2 and HDACs are probably mutually exclusive.

The involvement of Ca^{2+} in transcription may be more direct as in the case of the production of *Prodynorphin*. Prodynorphin is an opioid peptide involved in memory and pain (Weisskopf et al., 1993; Naranjo et al., 1991). The gene encoding prodynorphin is repressed as long as it binds the *downstream-regulatory-element antagonist modulator* (DREAM) through the *downstream-regulatory element* (DRE) (Carrión et al., 1999). DREAM was also found to repress transcription of *cfos*. DREAM has four Ca^{2+} binding sites (EF-hand motifs). In the presence of Ca^{2+}, DREAM can no longer bind the DRE thereby failing to repress the gene.

Ca^{2+} influx through Ca^{2+}- channels in the plasma membrane couples neuronal electrical activity and the biochemical events that eventually impinge on gene expression. Different patterns of Ca^{2+} can produce different patterns of gene expression. (see Finkbeiner and Greenberg, 1998). In part, the electrical properties of cells are a reflection of alternative splicing of mRNA coding for channel proteins (e.g., Xie et al., 1998) such as the calcium- and voltage-activated potassium channels coded by *Slo*. Some hormonal changes affect the electrical properties of cells by changing the proportion of the proportion of Slo transcripts containing an exon referred to as "STREX". Depolarization of GH3 pituitary cells decreases the splicing of the STREX exon in BK potassium channel transcripts through the action of Ca^{2+}-CAM-dependent protein kinases (CaMKs). CaMK IV-responsive RNA element (CaRRE), mediates the alternative splicing of ion channel pre-mRNAs (Xie et al., 2001). .

B. Sodium Influx

Fertilization of oocytes or, in somatic cells, the binding of growth factors to their appropriate receptors activates the influx of Na^{+}. This influx is thought to stimulate the growth of cells. Growth factors that stimulate Na^{+} influx include epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), fibroblast growth factor (FGF), thrombin, and insulin. Although the influx and the growth have been strongly linked (see below), it is not at all clear that Na^{+} can be considered a second messenger.

The entry of Na^+ takes place by a Na^+-H^+ exchange mediated by a transport protein or antiporter. Consequently, the cytoplasm becomes alkaline, as shown in several experiments by using pH-sensitive dyes delivered intracellularly (Cassel et al., 1983).

The growth factors acting on the G_0 or G_1 stage of cell replication (i.e., the arrested state or the period preceding DNA synthesis) stimulate an Na^+ influx that has been found to be inhibited by amiloride (Leffert and Koch, 1985). The Na^+ influx ranges from 5 to 20 mol/l0^6 cells per minute, a 1.5- to 5-fold elevation from the basal level. Mitogen-activated Na^+ influx bursts have been observed seconds after the addition of the factor, and the uptake persists 2 to 60 min. There is a good correlation between incorporation of tritiated thymidine and initial Na^+ influx rates. Furthermore, amiloride blocks both the mitogen-activated Na^+ influx and the DNA synthesis. However, the time courses of the two events are so different, minutes in the case of Na^+ and hours in the case of DNA synthesis, that it is difficult to consider them as being directly linked.

These observations support the idea that the Na^+ influx directly or indirectly links the growth factors to their physiological effects. However, there is no indication that the intracellular Na^+ concentration is changed, and the effect may be indirect.

C. Free Radicals and Nitric Oxide

Many extracellular ligands act by either generating or requiring the presence of free radicals or derived compounds. In biological systems, free radicals are oxygen or nitrogen-based compounds with unpaired electrons. These free radicals can generate many reactive species and only some of these are free radicals. Because of the variety of compounds generated by free radicals, the terms reactive oxygen species (ROS) or reactive nitrogen species (RNS) are used (Lander et al., 1997).

ROSs and RNSs have a critical role as antiviral, antibacterial and antitumor agents. Nitric oxide (NO) can act a second messenger in generating cGMP. ROSs and RNSs function in the regulation of many important biological processes such as differentiation, growth,ion transport, transcription, neuromodulation, apoptosis and many other physiological processes through the action of cytokines, growth factors and hormones (see Dröge, 2002; Cooper et al., 2002). Some of the effects of the redox states in metabolism via specific enzymes or transcription factors are discussed in Chapter 14.

The free radicals do not act by a specific receptor mediated mechanism but rather by specific chemical reactions. For example, they can generate redox-active molecules that can covalently modify selected targets such as thiols and iron. Table 2 lists sources of ROSs and RNSs, their targets and the endogenous scavengers that can react with them.

Table 2 Sources, targets and scavengers of free radicals or derived species . From Lander et al., 1997, reproduced by permission.

Reactive species	Sources	Direct targets	Endogenous scavengers
ROS	NAD (P) H oxidases	Transcription factors	Glutathione
ROS	Xanthine oxidase	G-proteins	Thioredoxin
ROS	Cyclooxygenase	Ion channels	Superoxide dismutase
ROS	Catecholamine oxidation	Cyclooxygenase	Catalase
ROS	Ionizing radiation	Lipids	Bel-2
NOS	Nitric oxide synthase (type I-III)	Guanylyl cyclase Protein kinases and phosphatases Ribonucleotide reductase nitric oxide synthase	α-tocopherol α-lipoate

The scavengers can regulate the level of the ROS or RNS and the concentration of the scavanger themselves also depends on the the ROSs and RNSs. The complexity of the effects can be illustrated by the regulation of the NF-B transcription factor. With low levels of glutathione disulfide, NF-B binding to DNA is inhibited but high levels inhibit signal transmission (see Dröge et al., 1994). In turn, the protein tyrosine kinases that trigger signal cascades for NF-B activation are redox-regulated.

NO was recognized only recently as the active agent responsible for the activity of the so-called endothelial-derived relaxing factor of vascular smooth muscle. This agent is involved in signal transduction in the brain, regulation of tone in the vascular system of mammals and the cytotoxicity of stimulated macrophages. In the nervous system, NO is likely to play a role in the efficacy of neuronal communication at synapses, and has a role in neurotransmitter release (see Chapter 22). Under physiological conditions NO can be interconverted to different redox forms with distinct chemistry (Stamler et al., 1992). Because NO can diffuse rapidly through cell membranes, it can act not only as a second messenger but also as a neurotransmitter (see Chapter 22). The signal generated by one cell can

be transmitted to many other unconnected cells, although the action is limited by its rapid degradation; its half-life has been estimated to be 1 to 5 s (Ignarro, 1990).

NO is synthesized by endothelial cells, macrophages, and brain neuronal cells. NO synthase (NOS) converts arginine to citrulline and NO (Moncada et al., 1989). Different NOS isoforms have been found. Several have been cloned. Some appear to be constitutive, others are considered cytokyne-inducible. When constitutive, the activity may be regulated by the Ca^{2+}-CAM system (Bredt and Snyder, 1990). Other NOS are transcriptionally induced. All NOSs bind heme, FMN, FAD, NADPH and CAM and require tetrahydrobiopterin as a cofactor.

NO reacts with a variety of important biological molecules including thiol groups and heme and non-heme iron (see Table 3, Pantopoulos et al., 1994). NO is thought to activate guanylate cyclase in brain and blood vessels by binding to the heme iron of this enzyme. This binding produces a cascade of subsequent reactions initially mediated by cGMP and has effects on the synthesis and release of other second messengers. cGMP activates protein kinases. When released from macrophages NO shuts off a variety of basic metabolic functions of the target cells (e.g., DNA synthesis and mitochondrial respiration, see Pantopoulos et al., 1994).

One of the major physiological roles of NO is in iron regulation. Iron regulatory protein (IRP, also called IRE-binding protein, IRE-BP) is considered the regulator of iron in cells (see Chapter 3). When IRP binds the iron response element (IRE) in mRNA, in iron deficiency, it serves as a translational inhibitor (e.g., Emery-Goodman et al., 1993). In macrophages, when NOS is activated, the production of NO coincides with IRE binding (Drapier et al., 1993; Weiss et al., 1993). In transfection experiments, ferritin synthesis can be shown to be regulated by NO. For example, an increase in NO decreases ferritin biosynthesis and the translation of reporter mRNA containing the IRE in the 5 position.

NOS activity has been found in mitochondria (e.g., Ghafourifar and Richter, 1997).Furthermore, NO binds to cytochrome oxidase (Complex IV; see Chapter 16), the terminal complex of oxidative phosphorylation (e.g., Torres et al., 1995) thereby inhibiting the respiration. It has been proposed that NO has a role in regulating mitochondrial respiration (e.g., Beltrán et al., 2000) at physiological oxygen concentrations. However, prolonged exposure to NO ultimately leads to cell death (e.g., Beltrán et al., 2000). More recently, nitric oxide has been found to induce the biogenesis of mitochondria in a variety of cells (Nisoli et al., 2003). The effect required cGMP.

Table 3 Examples of NO Targets

Groups reactive towards NO within proteins	Examples
Free radicals	Ribonucleotide reductase

Thiol groups	NMDA receptor
Haem Fe	Haemoglobin Guanylate cyclase
Fe-S clusters	Mitochondrial aconitase NADH:ubiquinone oxidoreductase NADH:succinate oxidoreductase
Fe in other form	Ferritin

From Pantopoulos et al., 1994 reproduced by permission

D. Cyclic ADP-Ribose and Nicotinic Acid Dinucleotide Phosphate

Cyclic ADP-ribose (cADPR) (Lee et al., 1989) and nicotinic acid dinucleotide phosphate (NAADP) (Lee and Aarhus, 1995,) are two related second messengers. For a recent review of this topic, see Lee, 1997 and Patel et al., 2001. The structure of these two compounds is represented in Fig. 1. cADPR is derived from NAD and NAADP from NADP. NAADP was found to be active in invertebrates, vertebrates and plants (see Patel et al., 2001). To function as a signal, a molecule must be both synthesized and degraded. Pathways for the synthesis and breakdown of these compounds are known (e.g., Lee and Aarhus,1993, 1995; Takasawa et al., 1993b)

Both messengers activate the opening of Ca^{2+}-channels to release Ca^{2+} from intracellular stores, although cADPR directly or indirectly may open channels in the plasma membrane of neurons, since the presence of cADPR activates inward Ca^{2+}-currents (Currie et al., 1992). The Ca^{2+} stores responding to NAADP are thought to be different from those responding to IP_3 or cADPR (Clapper et al., 1987; Lee, 1995).

The action of cADPR on the opening of Ca^{2+} channels was fist demonstrated by the microinjection of cADPR into sea urchin eggs. Ca^{2+} is released even in the absence of external calcium, implicating internal stores. cADPR is as effective as inositol triphosphate (IP_3, discussed in Section F) in mobilizing intracellular Ca^{2+}. The Ca^{2+} releases induced by either IP_3 or cADPR are not additive in sea urchin egg

homogenates and have an inverse relationship, suggesting that they target the same Ca^{2+} stores. However, cADPR is thought to act through the ryanodine receptor, perhaps indirectly, while IP_3 binds to its own receptor (IP_3-receptor).

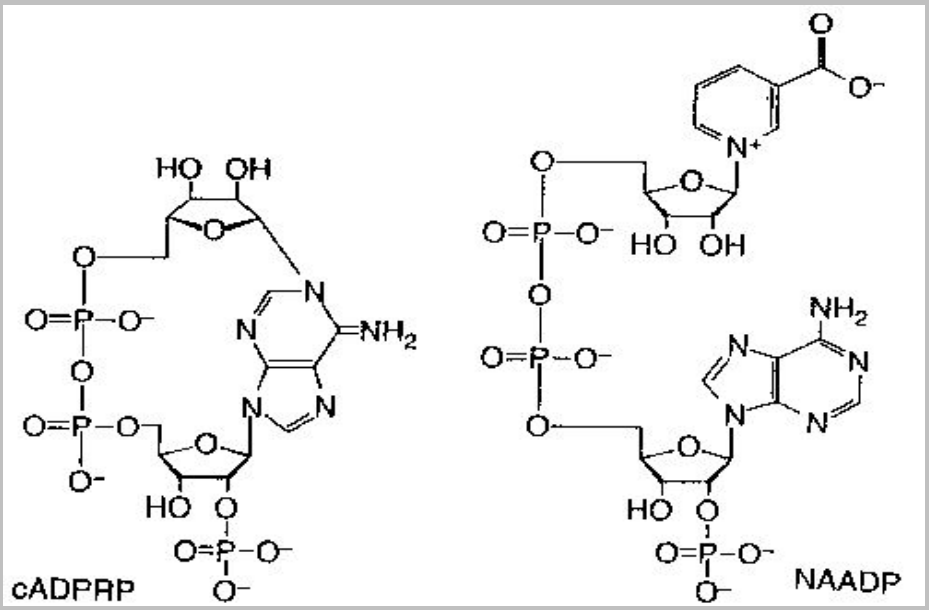


Fig. 1 Chemical structure of cyclic ADP-ribose (cADPR) and nicotinic acid dinucleotide phosphate (NAADP).

In isolated brain microsomes, cADPR also stimulates Ca^{2+} release (White et al., 1993). Microinjection of cGMP into sea urchin eggs induces Ca^{2+} release (Galione et al., 1993). The kinetics of this release suggest that the effect requires the synthesis of cADPR.

Present evidence indicating that cADPR acts as a second messenger includes its generation in pancreatic cells (Takasawa et al., 1993a) by glucose stimulation, as indicated in experiments using permeabilized cells.

What is the signal for the production or release of cADPR? The plasma membrane receptors responsible for the production of second messengers are not always known. NO (discussed in Section C, above) that can act as a second messenger or a neurotransmitter has also been shown to activate cADPR (Clementi et al., 1996; Willmott et al., 1996). Some studies have been carried out on the polypeptide hormone cholecystokinin (CCK) that produces calcium spiking in pancreatic cells. CCK is a duodenal mucosa hormone that stimulates secretion of pancreatic enzymes by acinar cells. Presumably, CCK acts through its plasma membrane receptor in the pancreatic cells. The Ca^{2+} spiking is mediated by either cADPR or IP_3 and, accordingly, either IP_3 or cADPR delivered to the interior of the cells, produce repetitive Ca^{2+} spikes. The cADPR antagonist 8-NH_2 cADPR blocks the cADPR induced spikes but not those induced by IP_3. The inhibitor can block the Ca^{2+}-spiking produced by CCK (Cancela and Petersen, 1998). The study of Cancela et al. (1998) used whole cell patch clamping to follow the Ca^{2+} current. In this

technique, the pipette that is used as an electrode connects to the interior of the cell. The pipette is used to record currents but also to deliver chemicals to the cell's interior. The intracellular level of glucose was found to regulate intracellular Ca^{2+} (Cancela et al., 1998) by blocking the cADPR effect and facilitating the IP_3 spiking. CCK had an effect regardless of the presence or absence of glucose, whereas 8-NH_2cADPR blocked the CCK-induced spiking only in the presence of glucose. This suggests that the hormone can act either through IP_3 or cADPR and that glucose controls the switch between pathways.

The properties of the NAADP-sensitive channels are distinct from those of other Ca^{2+}- release channels. In contrast to the other Ca^{2+}-release channels, the NAADP-sensitive channels were found to be sensitive to L-type channel antagonists and some K^+-channel blockers. In addition, the NAADP-channels have a unique property. They do not respond to increased Ca^{2+} in contrast to those sensitive to IP_3 and ryanodine which undergo CICR. They therefore are unsuited for direct involvement in the generation of Ca^{2+}-waves. In homogenates, NAADP in sub-threshold concentrations (less than 1 nM) blocks the release by high concentrations (0.5 μM) (e.g., see Aarhus et al., 1996). Presumably, this could be the result of the presence of two different binding sites. One would be of high affinity and lead to inactivation and the other would be of low affinity and lead to Ca^{2+} release (e.g., Patel et al., 2000). The two-binding site behavior may have significance in determining this pathway as a ‘one shot’ signal when the concentration of NAADP is sufficiently high (see Genazzani et al., 1996). When the NAADP changes slightly, the magnitude of the release following a higher concentration of the ligand will only be reduced gradually as more is bound to the high affinity sites (see Patel et al., 2000). Interestingly, NADP binding had higher affinity for the binding site than NADPH (although lower than that of NAADP), suggesting that NAADP signaling is regulated by the redox state of the cell (Billington and Genazzani, 2000).

E. Cyclic AMP

Cyclic AMP (cAMP), shown in Fig. 2, is one of the most studied second messengers. Generally, the binding of a variety of neurotransmitters or hormones to specific receptors at the cell surface activates adenylate cyclase (AC) on the inner face of the membrane. AC catalyzes the formation of the second messenger, cAMP, from ATP. In contrast, binding to an inhibitory receptor leads to inhibition of adenylate cyclase.

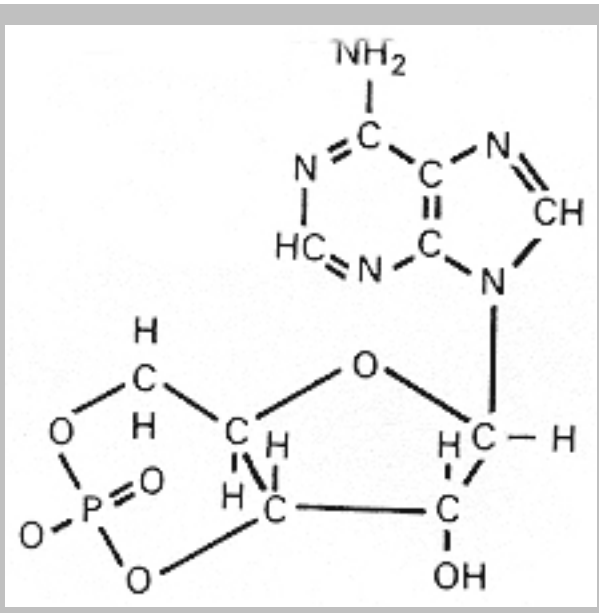


Fig. 2 Structure of cAMP.

cAMP activates the *cAMP-dependent protein kinases (PKAs)* that phosphorylate a plethora of intracellular proteins, which in turn are responsible for the biological response. Phosphorylation is one of the mechanisms controlling the activity of enzymes and many other biologically active proteins, such as transcription factors.

Several hormones with different receptors and target cells, such as *epinephrine*, *norepinephrine* and *prostaglandins*, share cAMP as the cytoplasmic messenger transmitting the signal to the cell's interior. *Adrenergic receptors* physiologically respond to *norepinephrine* or *epinephrine*, an effect mimicked by *sympathomimetic* drugs such as isoproterenol. Epinephrine and norepinephrine are released by the adrenal medulla and the sympathetic nervous system. Prostaglandins are hormones released by certain tissues and they have a role in the control of local circulation.

The formation of cAMP in response to the ligand binding actually involves three separate units, as discussed in the rest of this section.

The presence of two distinct elements in the system was shown in experiments where different cells were fused. There are several procedures that permit the fusion of cells. In some experiments, a cell normally responsive to prostaglandin was fused to a cell in which the AC was inactivated by chemical treatment. The fused cells were able to respond to both epinephrine and prostaglandin (Schulster et al., 1978). In contrast, cells which were not fused could not respond to epinephrine. Those containing the prostaglandin receptor could still respond to the prostaglandin. The rationale of these experiments is summarized in the diagram of Fig. 3. These results show that the hormone receptors are separate entities from the AC and either receptor or enzyme, or both, are free to move in the plane of the membrane.

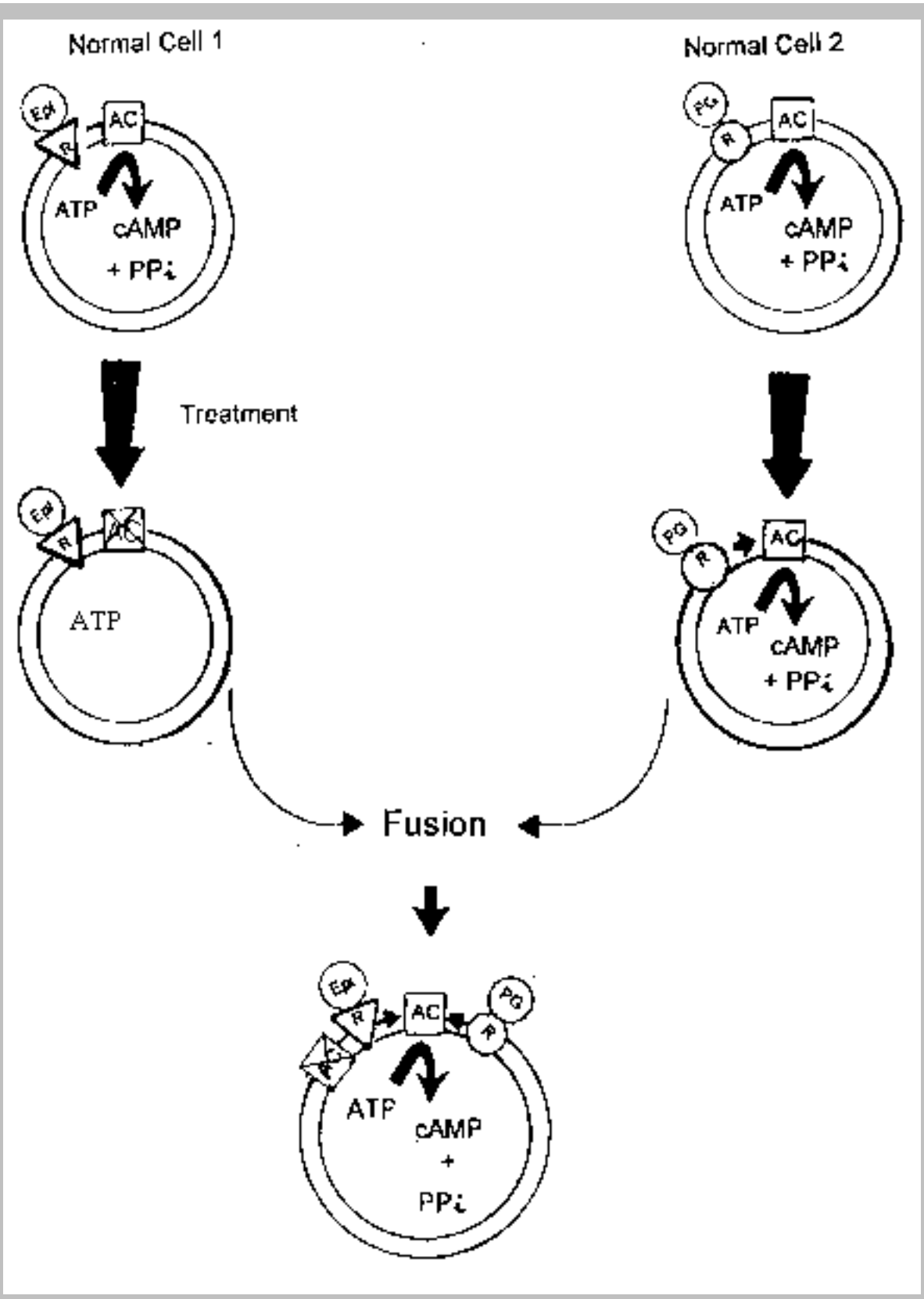


Fig. 3 Cells with membranes containing the epinephrine receptor but no AC will respond to epinephrine when fused to cells lacking the epinephrine receptor but containing AC.

The activation of AC requires the presence of GTP. A non-hydrolyzable analogue of GTP (e.g. 5'-guanylyl-diphosphonate) can activate AC even in the absence of a receptor. The need for a third component was demonstrated when membrane extracts of cells lacking AC were mixed with extracts of

cells lacking the receptor, but in the presence of the GTP analog (Ross and Gilman 1977). These results implicate a third component, linking the receptor and the enzyme.

The heterotrimeric GTP-binding protein or GTPase, also called G protein, is reponsible for the GTP dependence (not to be confused with the VSV coat glycoprotein also called G protein). The functioning of these three components is illustrated in Fig. 4. The model shows how the hormone binds the receptor and activates the G-protein, which exchanges the bound GDP with GTP (Step 1). The GTP-G-protein complex, in turn, activates AC so that cAMP is produced from ATP (Step 2). The G-protein returns to its resting state with the hydrolysis of GTP, leaving GDP bound to the protein (Step 3).

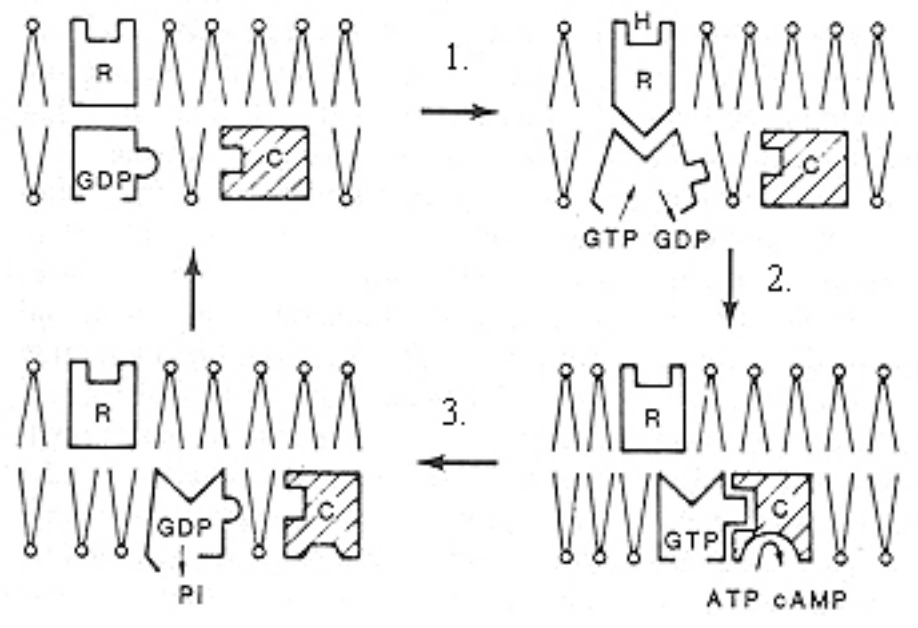


Fig. 4 Scheme of component interactions in the activation and inactivation of the adenylate cyclase system. A section of the cell membrane shows the three components (upper left). Phospholipid is represented by the polar heads with a pair of fatty acid tails interspersed between the components. When the neurotransmitter or hormone, H, binds to the receptor, R, HR interacts with the binding protein G to release GDP and to facilitate the tight binding of GTP (upper right). The G binding protein thus activated by GTP associates with the catalytic unit C to form the active enzyme complex GC (lower right). Hydrolysis of the GTP at the G protein site, with release of inorganic phosphorus (P_i), results in dissociation of G from C and cessation of enzyme activity (lower and upper left areas). R is arbitrarily drawn as limited to the outer layer of the cell membrane. There appears to be no evidence as yet that R transverses the entire membrane. Reproduced with permission from M. Schramm and Z. Selinger, *Science*, 225:1350-1356. Copyright © 1984 by the American Association for the Advancement of Science.

F. The Phospholipid System

In some cases, second messengers are generated by the hydrolysis of phospholipid components or by phosphorylation of a precursor by phosphonositide kinases. One of the systems that is best understood is the hydrolysis of a minor membrane component, phosphatidylinositol-4,5-bisphosphate (IP_2), by a

specific phospholipase C (see below). This reaction has been shown to generate two powerful second messengers, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). More recently, however, it has become obvious that many different phospholipases are activated by extracellular signals and that the hydrolysis of major components of the plasma membrane, such as phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, also generate second messengers. The phospholipids and the phospholipases that generate these messengers are shown in Fig. 5 (Liscovitch, 1992).

Some phosphatidylinositol phosphates mediate signals without being hydrolyzed by phospholipase C. They are generated by the phosphorylation of their phosphatidylinositol precursors catalyzed by *phosphoinositol-3-OH kinases* (PI3Ks). The PI3Ks constitute a family of enzymes that catalyzes the formation of phosphatidyl inositol 3,4,5-triphosphate from phosphatidyl inositol 4,5-bisphosphate. Their importance is indicated by the observations that activation of PI3Ks prevents apoptosis in several kinds of cells (see Franke et al., 1997), has been shown to transform chicken fibroblasts (Chang et al., 1997) and it prolongs the life of the soil worm *Caenorhabditis elegans* adults (Morris et al., 1997). Furthermore, products of PI3K activity (including PIP_3) have been found to be involved in a variety of events involving the plasma membrane and the cytoskeleton. They include: phagocytosis, exocytosis, endocytosis, and cytoskeletal organization (see Czech, 2000).

PI3K is made up of a catalytic subunit (p110) and a regulatory subunit (p85). After activation of receptors for a variety of factors, PI3K is translocated to the plasma membrane where it phosphorylates phosphatidyl inositol 4,5-bisphosphate. The resulting triphosphate activates a variety of other kinases such as Akt/PKB (a serine/threonine protein kinase), the *3-phosphoinositide-dependent protein kinase* (PDK) (involved in the phosphorylation of Akt/PKB) and *Bruton protein kinase* (Btk) (important in B cell development).

Phosphatidylinositol (3,4,5) trisphosphate participates in the cascade initiated by growth factor receptors, G-protein coupled receptors and cytokine receptors. Phosphatidylinositol-3-phosphate is involved in vesicle transport (Simonsen et al., 1998; Gaullier et al., 1998; Patki et al., 1998; Burd and Emr, 1998). It is required for the transport from the trans-Golgi to the lysosomes and in the endocytotic receptor mediated pathway involving the small GTPase Rab5 (see Section IIB).

In addition to their role as second messengers, the phosphoinositides are thought to play a pivotal role in the recruitment of cytoplasmic proteins needed for vesicle formation and budding at specific locations in the membranes (see Chapter 11). They also serve as foci for the polymerization of actin triggered by the Rho family of small GTPases (see Chapter 23). Phosphoinositides bind to proteins that function in actin assembly (e.g., see Chapter 23) such as the actin-capping protein profilin or gelsolin (see Toker, 1998). Phosphatidyl inositol 3,4-bisphosphate blocks profilin inhibition of polymerization and inactivates the actin-severing protein gelsolin (Hartwig et al., 1996). *Wortmannin*, a fungal metabolite that inhibits PIP3K, blocks PDGF-stimulated ruffling in fibroblasts (Wennstrom et al., 1994). PIP_2 has a role in membrane ruffling (see Honda et al., 1999). Ruffling depends on a particular rearrangement of actin filaments.

Enzymes and inositol derivatives of the inositide cycle have been shown to be present in the nucleus (see Maraldi et al., 1999). As we saw several of these compounds have been found to function as second messengers. Therefore, it is not surprising to find that they have a role in gene expression. Three genes which encode phospholipase C and two inositol polyphosphate kinases were found to contribute to the nuclear export of mRNA (York et al., 1999). These genes represent a signaling pathway from phosphatidylinositol 4,5-bisphosphate to inositol hexakisphosphate (IP6) (*IPK1*, *PLC1* and *IPK2*). One of these genes, encodes the inositol polyphosphate kinase (IPK1p) which was found located in proximity of the nuclear envelope and the NPCs. In addition, Ipk2p, a pleotropic kinase that is known to regulate a variety of yeast processes, was found to be an essential component of the ArgR-MCM1 transcription complex (Odom et al., 2000). This complex activates or represses genes involved in arginine production (e.g., Crabeel, et al., 1995; El Bakkoury et al., 2000). Ipk2 was found to exert its effect at the level of transcription.

A direct involvement of the nuclear compartment in signaling is not unexpected since many of the targets of the pathway have to be in the nucleus (e.g., transcription factors). Furthermore, as discussed in Chapter 6, some PGFs and their receptors have been found in the nucleus.

A schematic representation of the generation of IP_3, arachidonic acid (AA), DAG and PA is shown in Fig. 6. The stimulation shown follows mitogen stimulation. The sequence of events is actually very much dependent on the cell type. Arachidonic acid is generated by phospholipase A_2 (PLA_2) in the figure. DAG is generated by PI-phospholipase C (PLC) or by PLC from PC. Phosphatidic acid (PA) is generated by phospholipase D (PLD).

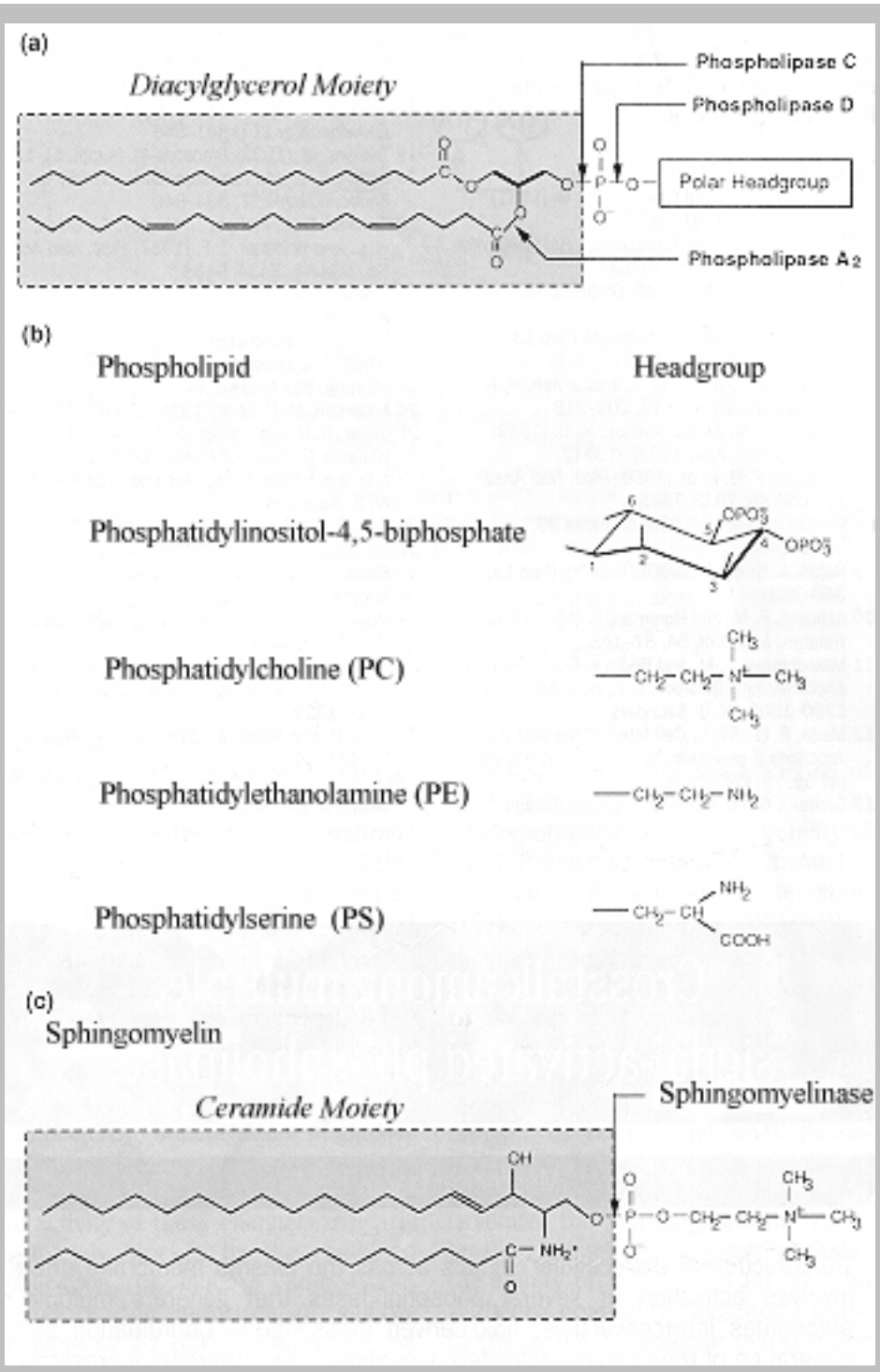


Fig. 5 Major cellular phospholipids and their breakdown by signal-activated phospholipases . Reproduced from *Trends in Biochemical Science*, vol.17, Liscovitch,M., Crosstalk among multiple signal activated phospholipases, pp.393-399, copyright &copy1992, with permission from Elsevier Science.

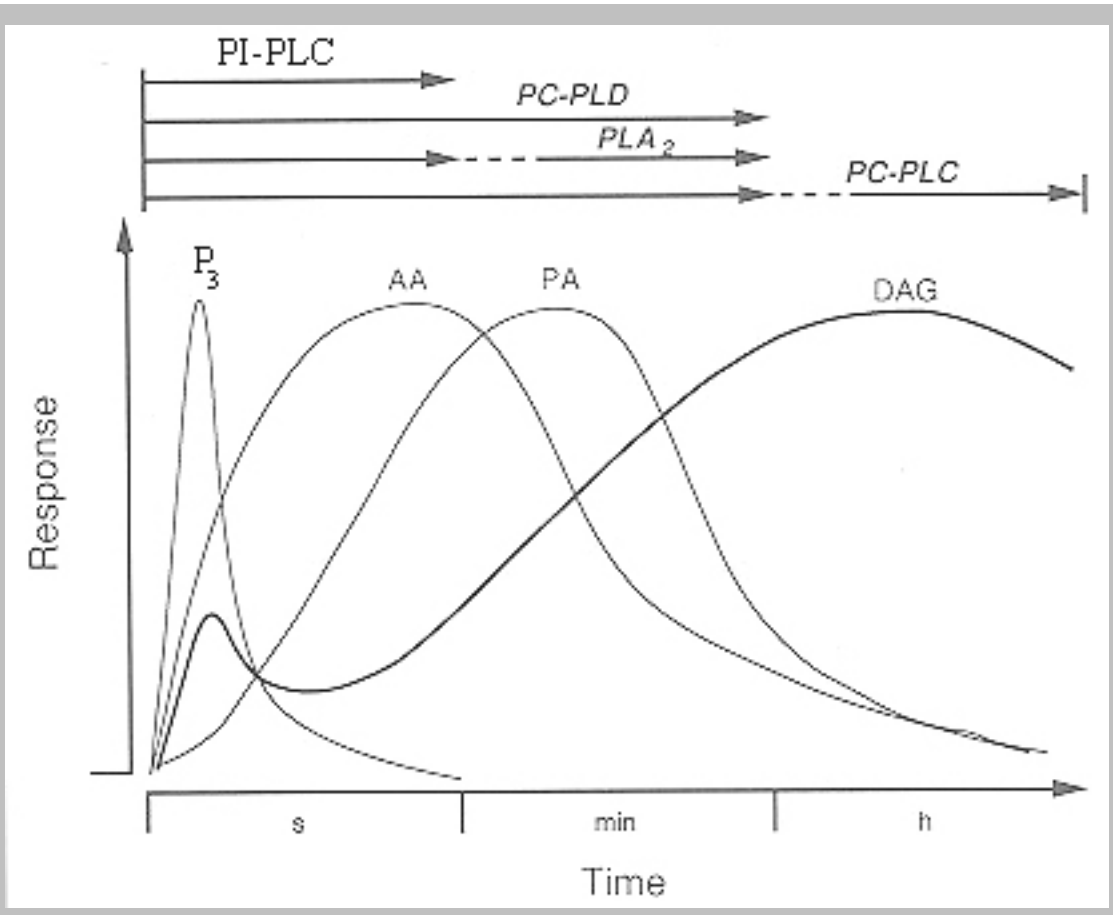


Fig. 6 Schematic representation of phospholipid-derived messengers produced by signal-activated phospholipases. Reproduced from *Trends in Biochemical Science*, vol.17, Liscovitch,M., Crosstalk among multiple signal activated phospholipases, pp.393-399, copyright &copy1992, with permission from Elsevier Science.

The inositol phosphate system activated by phospholipase C was the first involving phospholipids to be studied in detail and will be discussed in the rest of this section. Present evidence indicates the biochemical reactions shown in Fig. 7 (Nishizuka, 1984). Phospholipase C is activated by the binding of ligand to the receptor. The reaction catalyzed by the enzyme generates both IP_3 and DAG. IP_3 in turn activates the release of Ca^{2+} from intracellular stores. DAG, that is membrane component, stimulates protein kinase C (PKC), a multifunctional enzyme that phosphorylates a variety of proteins. DAG acts by increasing the affinity of this enzyme for Ca^{2+}. These two effects, the increase in Ca^{2+} and the activation of the kinase, mediate the physiological effects of the chemical signal.

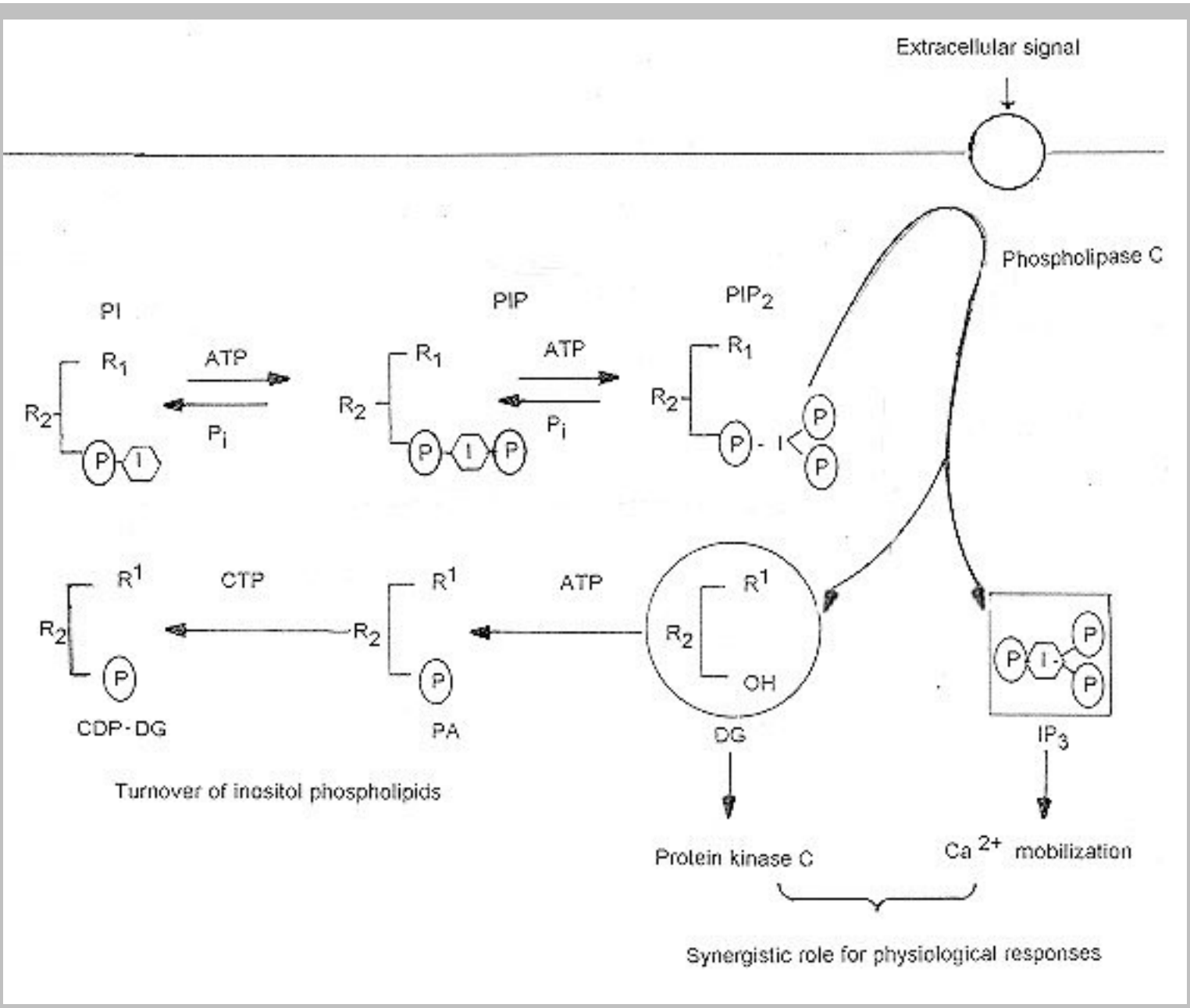


Fig. 7 Turnover of inositol phospholipids and signal transduction. Abbreviations: PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-biphosphate; DAG, diacylglycerol; IP_3, inositol triphosphate; PA, phosphatidic acid; PIP, phosphatidylinositol 4-phosphate. Reproduced with permission from Z. Nishizuka, *Science*, 225: 1365-1370. Copyright &copy1984 by the American Association for the Advancement of Science.

DAG has an important role in cell growth and differentiation (see Jackowski, 1996; Olson et al., 1993). The role of DAG in cell growth is shown by the observation that it is absolutely needed for regulation of the cell cycle. Growth factors can increase DAG in the nuclear membrane without affecting the DAG of the plasma membrane (Banfic et al., 1993; Divecha et al., 1991). The nuclear DAG is responsible for the effects on growth and differentiation. As shown in Fig. 7 (Nishizuka, 1992), DAG activates protein kinase C (PKC). The activated PKC is then translocated into the nucleus (Neri et al., 1994) and can then phosphorylate a variety of target molecules. Some of the isoforms of PKC have an absolute requirement for DAG. Diacylglycerol kinases (DGKs) decrease the level of DAG by converting it into phophatidic

acid. Topham et al. (1998) showed with immunofluorescence that DAG kinase-ζ shuttles between the cytoplasm and the nucleus. Apparently the regulation of the levels of DAG takes place by a mechanism in which PKC phosphorylates DAG kinase-ζ thereby inhibiting its nuclear entry (Topham et al., 1998). The phosphorylation by PKC takes place at the *myristoylated alanine-rich C-kinase substrate* (MARCKS) domain of DAG kinase-ζ which encodes a nuclear localization signal (see Chapter 5). The overexpression of DAG kinase-ζ was found to decrease the nuclear DAG and increased cell-doubling time where the cells accumulate in the Go/G1 stage.

In summary, the system works as follows. In response to growth factors, DAG kinase-ζ is phosphorylated by PKC. The phosphorylated DAG kinase-ζ is excluded from the nucleus and as a consequence the level of DAG is maintained high allowing for a high PKC activity.

The production of DAG in response to binding of thrombin in the case of platelets is shown in Fig. 8. Platelets can be activated by several factors. The platelet activator thrombin is a plasma protein that is formed during clotting and catalyzes the formation of fibrin. Thrombin also induces the release of serotonin, which is mediated by the production of DAG. This is shown in Fig. 8 (Sano et al., 1983), which shows the transient production of DAG from a radioactive precursor (shown as counts per minute) (curve 1) induced by thrombin. This is followed by the phosphorylation of the 40-kDa protein (presumably by PKC) (curve 2) which has a role in serotonin release and eventually the release of serotonin, the physiological response to the thrombin (shown as radioactivity of serotonin) (curve 3).

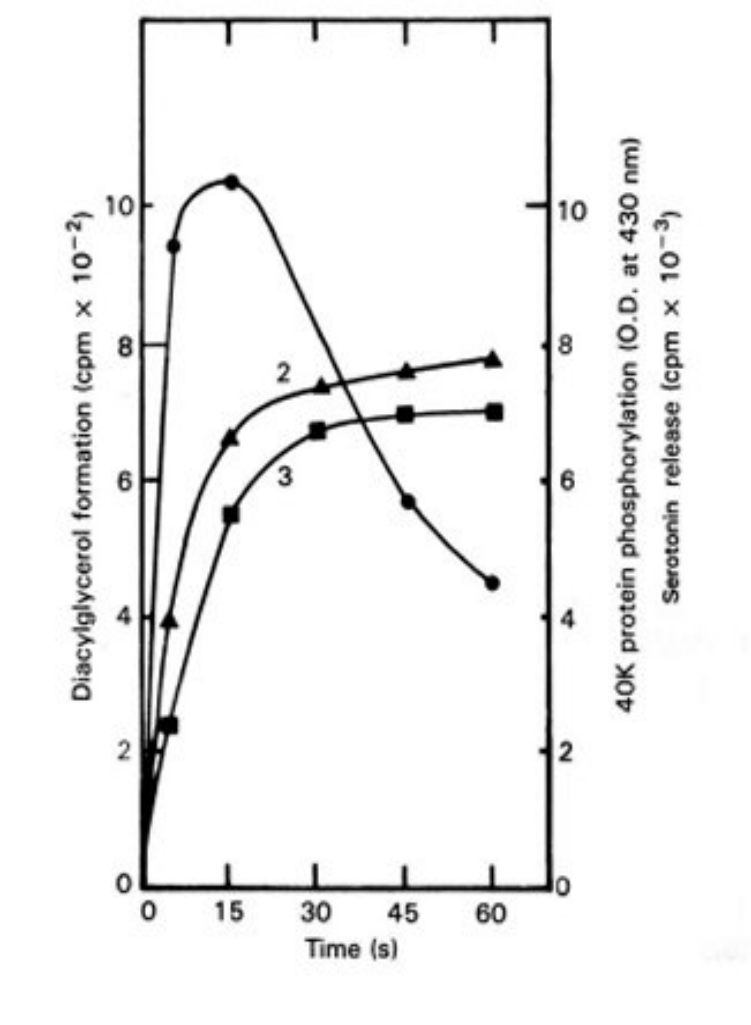


Fig. 8 Time courses for diacylglycerol formation (curve 1) expressed as counts per minute (cpm), 40 kDa protein phosphorylation (curve 2), and serotonin release in platelets (curve 3) stimulated by thrombin expressed as cpm. Reproduced with permission from K. Sano et al., *Journal of Biological Chemistry*, 258:2010-2013. Copyright &copy1983 American Society of Biological Chemists, Inc.

The dependence of PKC activity on the concentration of Ca^{2+} and diacylglycerol is shown in Fig. 9 (Kishimoto et al., 1980). At zero Ca^{2+}, any trace of free calcium has been removed by addition of the chelator, ethylene glycol (aminoethyl ether) tetraacetate (EGTA). Curves 3, 4, and 5 show the effect of adding each component alone: phospholipid, diolein, or neutral lipid, respectively. The effect is small, and phospholipid has the most effect. However, the effect is much greater when phospholipid is combined with the diolein, as shown by curve 1. The diolein can be replaced by neutral lipid, as shown by curve 2, although the effect is somewhat less in the latter case. The various DAGs can be regarded as acting by modifying the sensitivity of the kinase to Ca^{2+}.

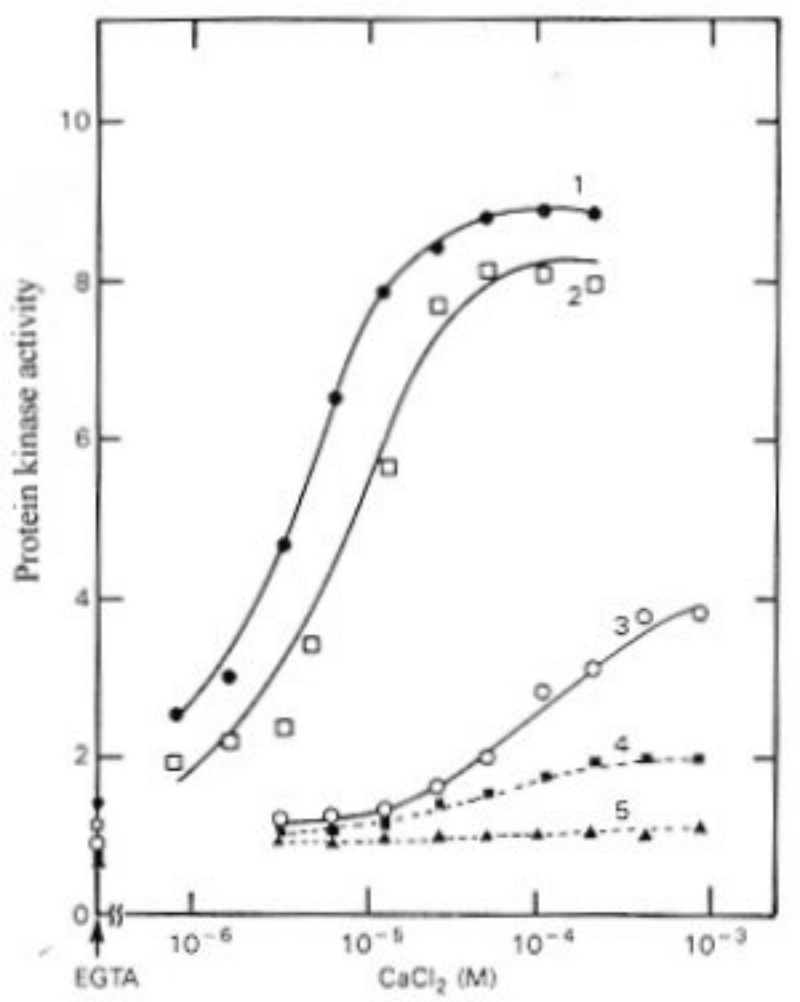


Fig. 9 Effects of neutral lipid and diolein on reaction velocity of protein kinase C at various concentrations of Ca^{2+}. Curve 1, diolein and phospholipid added; curve 2, neutral lipid and phospholipid added; curve 3, phospholipid alone; curve 4, diolein alone; curve 5, neutral lipid alone. Reproduced with permission from A. Kishimoto, et al., *Journal of Biological Chemistry*, 255:2273-2276. Copyright &copy1980 American Society of Biological Chemists, Inc.

In hepatocytes, the breakdown of PIP_2 to form DAG and PIP_3 is induced by α-adrenergic agonists and vasoactive peptides. The probable involvement of PIP_3 in the release of Ca^{2+} from internal stores, has been shown by the addition of exogenous PIP_3 after hepatocytes were made leaky by treatment with the detergent digitonin. The concentration of Ca^{2+}, $[Ca^{2+}]$ can be followed by using Ca^{2+}-sensitive electrodes or by monitoring the light absorption or fluorescence emission of a Ca^{2+}-sensitive dye. In the experiments discussed here, either the fluorescence of the dye Quin 2 or the electrode method was used.

Some of the results are shown in Fig. 10 (Joseph et al., 1984) where the Ca^{2+} concentration measured with the Ca^{2+}-sensitive electrode is expressed as pCa^{2+} (which corresponds to $-\log_{10}[Ca^{2+}]$) as a function of time. ATP and a system containing phosphocreatine and creatine kinase to regenerate ATP were present in the medium to provide energy needed for the Ca^{2+} transport system. At the times indicated by the arrows, IP_3 was added in various concentrations. After each addition there was a Ca^{2+} release. The

release was only temporary, because IP_3 is hydrolyzed and the $Ca^{2+}$is taken up by the Ca^{2+} transport system. Insert (b) in Fig. 11 simply shows that the electrode does not respond to IP_3 in the absence of hepatocytes. As shown at the right side of the figure, Ca^{2+} was added at various concentrations at the end of the experiment to provide the scale shown in the ordinate. The numbers shown at the arrows indicate the added Ca^{2+} in nanomoles. Experiments such as this allow the construction of a curve showing Ca^{2+} release as a function of IP_3 concentration (Fig. 11). The saturation suggests the presence of a receptor that binds IP_3.

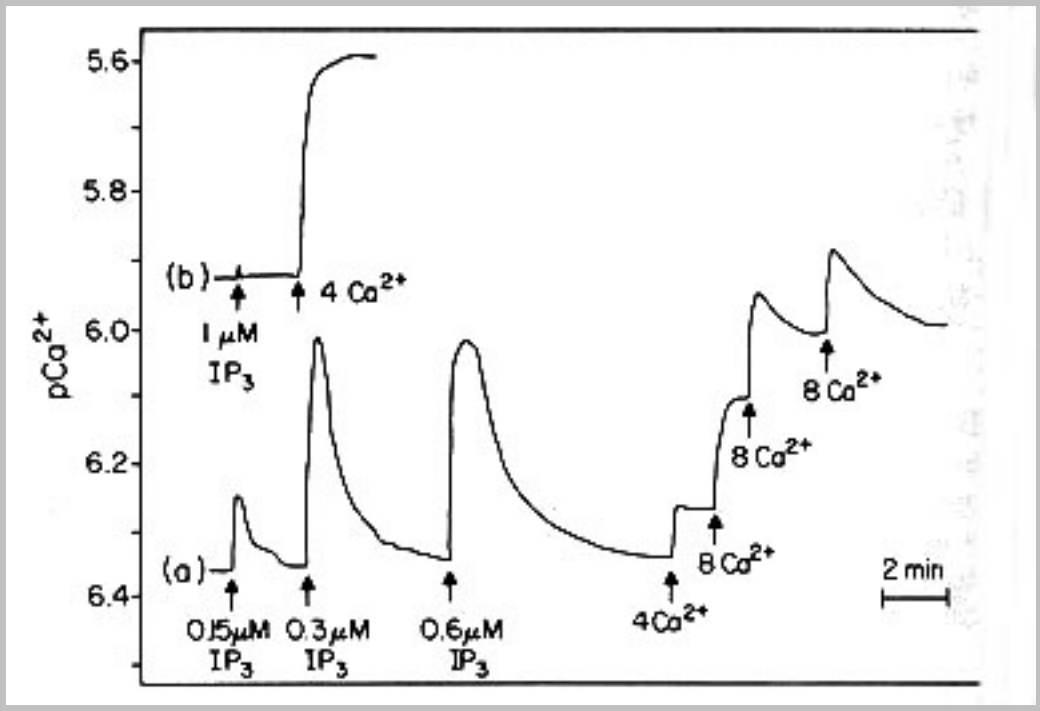


Fig. 10 Release of Ca^{2+} induced by IP_3 in saponin-permeabilized hepatocytes. Reproduced with permission from S. K. Joseph et al., *Journal of Biological Chemistry*, 259:3077-3081. Copyright &copy1984 American Society of Biological Chemists, Inc.

A fraction of the vesicles of the endoplasmic reticulum (analogous to the sarcoplasmic reticulum) are known to accumulate Ca^{2+}, but mitochondria are also capable of accumulating and releasing Ca^{2+}. Which one is involved? This question can be resolved by using oligomycin, which blocks the mitochondrial capacity to use ATP as an energy source. If mitochondria were involved the accumulation of Ca^{2+} (which requires energy) should not take place. When IP_3 is subsequently added, there should be no release of Ca^{2+}. These and other tests show that mitochondria are not involved, indicating that other internal stores are responsible.

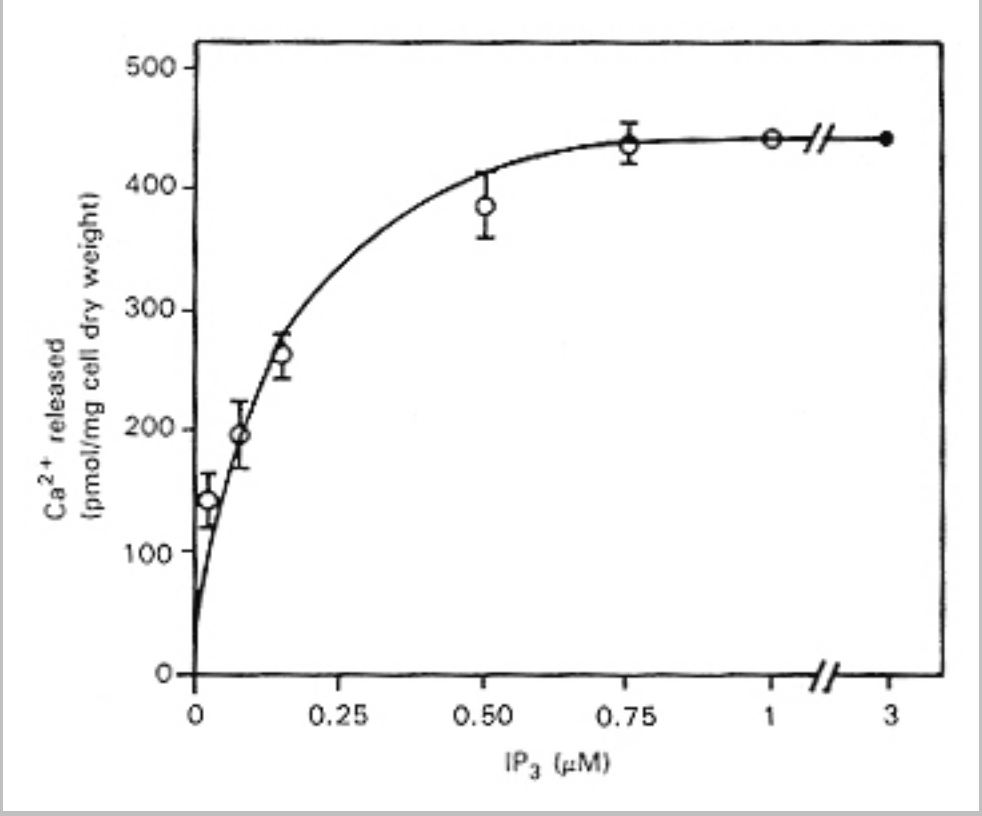


Fig. 11 Relationship between the amount of Ca^{2+} released and the concentration of added IP_3.
Reproduced with permission from S. K. Joseph, et al., *Journal of Biological Chemistry*, 259:3077-3081.

In summary, the results with permeabilized cells indicate that IP_3 releases Ca^{2+} from internal stores, primarily nonmitochondrial at low [Ca^{2+}].

The activation of protein kinase C and the mobilization of Ca^{2+}, together, probably are both essential for the IP_3-DAG system (Karbuchi et al., 1983).

Where is the Ca^{2+} stored? Where does IP_3 act? Three approaches have been used to define these sites using antibodies to proteins involved in Ca^{2+} release or storage. The drug, ryanodine, blocks Ca^{2+} release from the sarcoplasmic reticulum. From all indications, all biological chemicals we have examined so far act by binding a receptor. Receptors have been identified for both IP_3 and ryanodine. The synthesis of the appropriate cDNAs permitted the deduction of the amino acid sequence of these proteins (Furuichi et al., 1989, Takeshima, 1989). They are related proteins and both act as Ca^{2+} release channels. Ca^{2+} is generally sequestered by specialized proteins in vesicles such as *calsequestrin*, *calreticulin* or related proteins (see Treves et al., 1990; Krause et al., 1990). Why not, then, search for the Ca^{2+} organelle by using antibodies to these three kinds of proteins? Antibodies labelled with colloidal gold have been used to localize the proteins with electron microscopy. Results of these experiments have led to the concept that a specialized smooth vesicle, the *calciosome*, is responsible for the accumulation of Ca^{2+} (Volpe et

al., 1988; Hashimoto et al., 1988; Treves et al., 1990). Results obtained with all three probes using cerebellar Purkinje cells provide a complex picture (see Burgoyne and Cheek, 1991). Some membranes respond to one probe, some to another, and a third group of membranes binds to all three.

Sphingolipids and sphingolipid metabolites also have a role in signal transduction and behave as second messengers. They are involved in cell contact, as receptor components, and as anchors for proteins (see Hannun, 1996; Riboni et al., 1997; Spiegel and Merril, 1996; Spiegel et al., 1996). Ceramide, sphingosine, sphingosine-1-phosphate and sphingomyelin have been implicated in some of these functions. A number of extracellular agents (e.g., tumor necrosis factor, interferon-γ, NGF and Fas ligands) (Fas is one of the so-called death receptors of the TNF receptor gene superfamily, (see Chapter 2), cytotoxic agents (e.g., chemotherapeutic agents) and environmental stresses (e.g., heat shock, growth factor withdrawal) cause the activation of sphingomyelinases, which act on membrane sphingomyelin and release ceramide. The activation can result in cell cycle arrest, apoptosis or cell senescence. Paradoxically, they also function in cell proliferation (e.g., see Spiegel and Merrill, 1996; Spiegel et al., 1996). At least in vitro, ceramide activates a serine-threonine protein phosphatase. In cells, it regulates protein phosphorylation and many other targets (e.g., proteases, stress-activated protein kinases, and the RB protein). Many of effects of cytokines and stress inducers appear to correspond to changes in ceramide concentration (see Hannun and Luberto, 2000). Furthermore, ceramides have been implicated in triggering differentiation (e.g., Furuya et al., 1998; Jung et al., 1998). Ceramides are very hydrophobic and threfore likely to remain anchored to membranes. If this is correct, the targets of ceramide would have to be transported to the ceramide site. Although ceramides are generated at the plasma membrane by the action of sphingomyelinases, they are also appear to be produced in the ER (Bose et al., 1995) and possibly by the nuclear envelope (Tsugane et al., 1999, mitochondria (e.g., Shimeno et al., 1998) and endosomes or lysosomes (Heinrich et al., 1999)

The activation of sphingomyelinase and the cascades that follow are not well understood (see Pfeilschifter and Huwiler, 2000). They are thought to involve the activation of the stress-activated protein kinases (SAPKs, also called c-Jun NH_2-terminal kinases) (see below). One of the systems that has been studied in some detail is the activation of sphingomyelinase following binding of the *tumor necrosis factor* receptor (TNFR) by TNF-α (see Adam-Klages et al., 1998). The eventual targets of ceramide include the serine/threonine protein kinase, Raf, PKC-α and the protein kinase C, PKC-δ (see below) (Huwiler et al., 1996).

Sphingosine-1-phosphate, generally generated by sphingosine kinase in response to the action of certain growth factors (e.g., PDGF and NGF), has been shown to act by two distinct mechanisms. As a ligand of a receptor coupled through a heterodimeric GTPase, it acts at the cell membrane (Lee et al., 1998). In addition, independently from the receptor it activates in the cytoplasm signalling pathways involving phospholipase D, Ca^{2+} and the tyrosine phosphorylation of the *focal adhesion kinase* ($p124^{FAK}$), a cytosolic tyrosine kinase that localizes in focal adhesions. These pathways induce DNA synthesis, suppression of apoptosis, as well as other effects (see Van Brocklyn et al., 1998).

II. THE GTP BINDING PROTEINS

Cellular functions, including the signal transduction pathway (discussed in most of this chapter) require "on" and "off" switches. Many of these switches are GTP-binding proteins or G-proteins which include the heterotrimeric G-protein first discussed in Section IE, and the smaller GTP-binding proteins (the latter are usually referred to as GTPases). Many other physiological processes, such as the assembly of microtubules (see Chapter 23) and the conformational changes of *dynamin* associated with vesiculation (see e.g., Sweitzer and Hinshaw, 1998) (see Chapter 9), are also regulated by guanine nucleotide switches. The binding of guanosine 5'-triphosphate, GTP, provides an "on" signal. The hydrolysis of GTP by the G-proteins themselves the "off" signal.

A. The heterotrimeric G-proteins

The broad involvement of the heterotrimeric G-proteins in physiological functions is shown in Table 4 (Stryer and Bourne, 1986), which includes hormonal activation and also sensory transitions of eukaryotes. In the latter role, they control the opening of channels and thereby affect the membrane potential of the receptor.

The receptors coupled to the GTP-binding proteins form a very large family of proteins estimated to contain as many as 1,000 proteins (see Wess, 1997). These receptors have been termed the *heptahelical receptors* (see Hall et al., 1999) because they are thought to possess seven α-helical transmembrane segments (see Chapter 6). However, various physiological responses to heptahelical receptors do not involve G-proteins (see Hall et al., 1999).

Although a large number of receptors are coupled to heterotrimeric GTP-binding proteins, the responses to the activation of these diverse receptors is specific. How can this be accomplished? The answer probably lies in the fact that signaling complexes are assembled at the site of the receptor. This is illustrated by the case of the β_2 adrenergic receptor in neurons which activates channels in the heart and brain (e.g., Davare et al., 2001). The receptor is complexed to its effector, the class C L-type calcium channel Ca_v1, together with a G-protein, an adenylyl cyclase, cAMP-dependent protein kinase, and phosphatase PP2A. This is the package of components required for the activation and regulation of the channel. Electrophysiological recordings from hippocampal neurons show that the signal transduction from the receptor to the channel is highly localized.

Each of the G-proteins subunits α, β and γ can occur in various isoforms (there are 20 known αs, 6 βs and 12 γs). However, there are four main classes of G proteins of known function. G_s activates adenylyl cyclase, G_i inhibits adenylyl cyclase, G_t activates photoreceptor cGMP phosphodiesterase and G_q activates phospholipase C. These G-proteins also interact with other effector proteins (see Hamm, 1998). Table 5 (Stryer and Bourne, 1986) lists some of the G proteins.

The α, is 45 to 52 kDa in molecular weight; β, 35 to 36 kDa; and γ, approximately 8 kDa. The β and γ components are required for attachment of the α polypeptide, which is soluble even in the absence of detergent (Schuster et al, 1978). The βγ complex does not dissociate without denaturation. The α subunit alone can activate the cyclase (Cerione et al., 1984; May et al., 1985). However, at least for activation of the acetylcholine receptor, the β and γ subunits suffice to open the K^+ channel (Logothetis et al., 1987).

The cycle of activation and inactivation of G_s is shown in Fig. 12. (Stryer and Bourne, 1986). The receptors (RH in Fig. 12) have seven transmembrane helices and undergo a change in conformation when ligand-activated (see Wess, 1997). The activated receptor (R*) catalyzes the binding of GTP and dissociates the Gα from the βγ subunits (e.g., see (Lambright et al., 1994, 1996), as indicated in Fig. 12. Eventually, upon hydrolysis of GTP to GDP, the subunits reassemble into the complex. The activated α subunit undergoes a change in conformation, accompanied by an increase in affinity for the effector (Skiba et al., 1996).

$G\alpha_s$ activates adenylyl cyclase, $G\alpha_i$ inhibits the same enzyme and $G\alpha_t$ activates photoreceptor cGMP phosphodiesterase. A variety of additional targets of the α subunit have been identified by the two-hybrid screening of protein binding (see Chapter 1), some of the proteins that bind to α are *regulators of G protein signaling* or RGS proteins (e.g., see Berman and Gilman, 1998), others are probably effectors (see Hamm, 1998).

The free βγ complex activates a long list of effectors (see Clapham and Neer, 1997) including two phospholipase C enzymes, an adenylyl cyclase and several ion channels and protein kinases. The complex does not change in conformation but is probably activated by its release from the α subunit.

Mutational analysis of the Gβ domain that makes contact with effectors and α-GDP, showed that different regions were needed for different effectors although there were areas of overlap (Ford et al., 1998).

The structure of Gαβ γ and free Gβγ has been elucidated by crystallographic studies (see Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996). Free Gα binding GDP is different in conformation from Gα present in the heterotrimer. Gβ forms a β propeller (see Neer and Smith, 1996). The propeller structure is similar to other proteins such as haemopexin, methylamine dehydrogenase (Chen et al., 1992) or coronin (see de Hostos, 1999). Haemepexin is a serum glycoprotein that binds heme reversibly (Faber et al., 1995). Each blade of Gβ corresponds to a short four-stranded twisted β sheet. A central channel is lined with the edge of the inner strand of each blade containing unsatisfied H-donors and acceptors. These are likely to be hydrated. The blades of the G β propeller structure contain seven repetitions of a sequence usually bounded by Gly-His (GH) and Trp-Asp (WD) and separated by a variable length region. The WD repeating units are common themes and occur in as many as 50 unrelated proteins (Neer

et al., 1994) which are likely to assume a propeller conformation.

Table 4 Examples of Physiological Processes Mediated by G Proteins

Stimulus	Receptor	G protein	Effector	Physiological response
Epinephrine	β-Adrenergic receptor	G_s	Adenylate cyclase	Glycogen breakdown
Serotonin	Serotonin receptor	G_s	Adenylate cyclase	Behavioral sensitization and learning in *Aplysia*
Light	Rhodopsin	Transducin	cGMP phospho-diesterase	Visual excitation
IgE-antigen complexes	Mast cell IgE receptor	G_{PLC}	Phospholipase C	Secretion
fMet peptide	Chemotactic receptor	G_{PLC}	Phospholipase C	Chemotaxis
Acetylcholine	Musarinic receptor	G_K	Potassium channel	Slowing of pacemaker activity

Source: Stryer and Bourne (1986). Reproduced, with permission, from the *Annual Review of Cell Biology*, Volume 2, 1986 by Annual Reviews Inc.

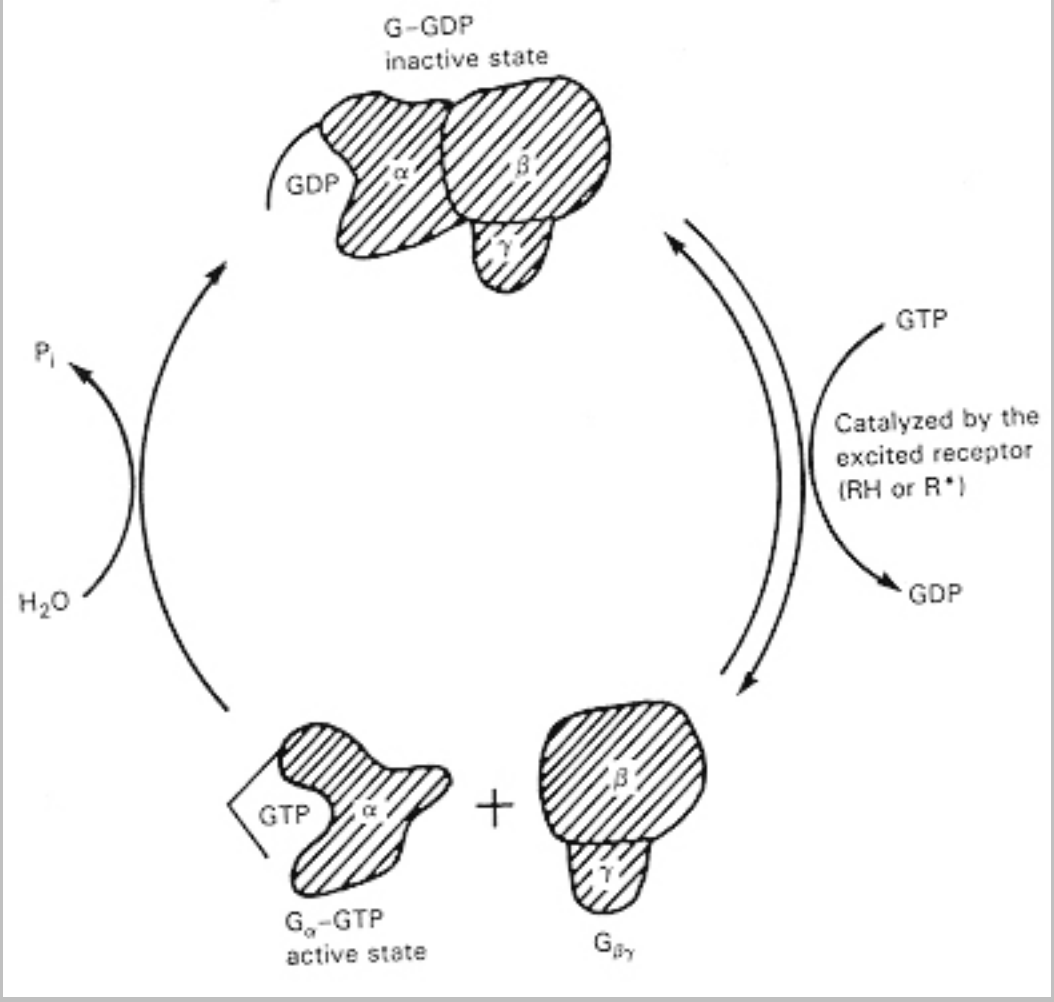


Fig. 12 Cycle of activation and inactivation of G_s (Stryer and Bourne, 1986*Annual Review of Cell Biology*, Volume 2, copyright &copy1986 by Annual Reviews Inc.

Perhaps one of the most exciting functional roles of GTP-binding heterotrimeric proteins is that of *transducin* (T) in visual reception in the retinal rods. Retinal rod cells function as single-photon receptors through the light-induced cis-trans isomerization of a single molecule of rhodopsin, which, by a cascade amplification mechanism, can block the entry of 10^6 moles of Na^+. At rest, the Na^+ channels of rod cells are kept open by the presence of cyclic GMP (cGMP), which results in a characteristic resting potential. In excitation, the cGMP is hydrolyzed by cGMP phosphodiesterase activated by a T-mediated system. The resulting block of the Na^+ channels produces a hyperpolarization that serves as a signal to the cell synapse to release neurotransmitter. In turn, this is followed by an action potential in the postsynaptic neuron (see Chapter 22). The fact that cGMP keeps the channel open has been shown by patch clamping of an excised piece of rod outer segment (Fesenko et al., 1985). In this technique (Chapter 22), a glass micropipette filled with a salt solution is fused to the membrane under study. At constant voltage, opening and closing of a channel are detected as discrete current deflections. These events are summarized in Fig. 13 (Stryer, 1986).

Table 5 Functions and Properties of Purified G Proteins

Functional and structural parameters	G_s	G_i	Transducin
Signal detector	β-Adrenergenic receptor, glucagon receptor, and many others	Muscarinic receptor, opiate receptor, and many others	Rhodopsin
Effector protein	Adenylate cyclase	Adenylate cyclase	cGMP phospho- diesterase Stimulation
Function	Stimulation	Inhibition	Stimulation
Subunit masses (kDa) α β γ	 45 and 52 35 and 36 8	 41 35 and 36 8	 39 36 8
Toxin susceptibility	Cholera	Pertussis	Pertussis and cholera
Location	Nearly all cells	Nearly all cells	Retinal rod outer segments

Source: Stryer and Bourne (1986). Reproduced, with permission, from the *Annual Review of Cell Biology*, Volume 2,

As shown in this figure, the photoexcited *trans*-rhodopsin serves as the activated receptor, R, which catalyzes the GTP-GDP exchange in the T-GTP. In turn, the T-GTP activates the phosphodiesterase. To permit rapid recovery, the activated rhodopsin is rendered ineffective by phosphorylation followed by combination with a molecule of *arrestin* (A). The complete cycle of this mechanism is represented in the scheme of Fig. 14 (Stryer and Bourne, 1986).

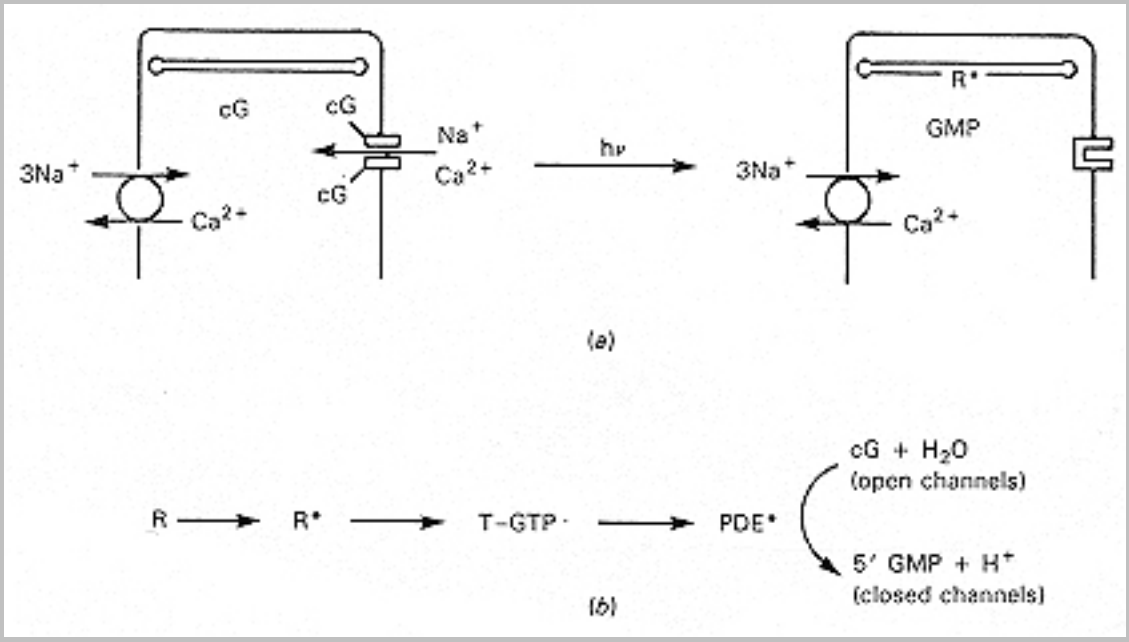


Fig. 13 Cyclic GMP controls sodium channels in the plasma membrane. (a) In the dark, a high level of cGMP in the cytosol opens sodium channels in the plasma membrane. Na^+ and Ca^{2+} enter the outer segment through these channels. Ca^{2+} is extruded in exchange for Na^+ by an Na^+/Ca^{2+} exchanger. On illumination, photoexcited rhodopsin triggers a cascade that results in the hydrolysis of cGMP to GMP. The lowered level of cGMP closes sodium channels. Ca^{2+} continues to be extruded from the outer segment by the exchanger. (b) Flow of information in the cGMP cascade R^* is photoexcited rhodopsin. T-GTP is the activated form of transducin, and PDE^* is the activated form of the phosphodiesterase (Stryer, 1986). Reproduced with permission, from the *Annual Review of Neuroscience*, Volume 9, copyright &copy1986 by Annual Reviews Inc.

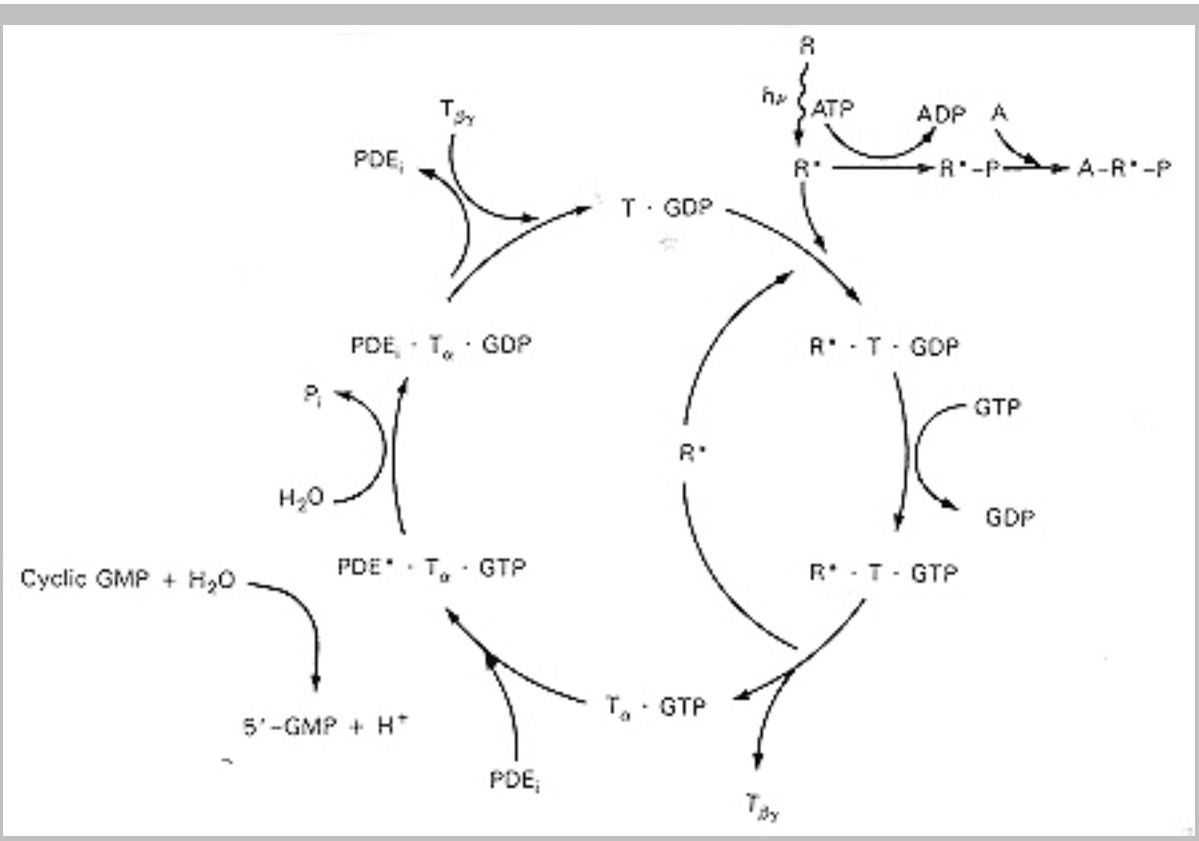


Fig. 14 Light-triggered transducin cycle of vertebrate photoreceptors. A, arrestin; PDE_i and PDE^*, inhibited and activated forms of cGMP phosphodiesterase; R and R^*, unexcited and photoexcited rhodopsin; T, transducin (Stryer and Bourne, 1986). Reproduced with permission, from the *Annual Review of Cell Biology*, Volume 2, copyright &copy1986 by Annual Reviews Inc.

The rapid inactivation of the rhodopsin (or other receptors) is mediated by the phosphorylation of the cytoplasmic carboxy terminal of the receptor by a specific kinase (see Krupnick et al.; Lefkowitz, 1998). Proteins of the arrestin family bind to the phosphorylated receptor so that it can no longer interact with the G-protein. The binding to the receptor is permitted by alterations in the conformation of arrestin that accompany the binding (see Granzin et al., 1998; Hirsch et al., 1999; Vishnivetskiy et al., 1999).

When a receptor is continually stimulated its activity decreases (*desensitization*). The desensitization can be the result of events affecting the receptor directly (receptor phosphorylation, effects of second messengers) and the *G-protein-coupled receptor kinase* (GRK)-arrestin system just discussed. Other slower mechanisms can be involved in desensitization including the regulation of receptor transcription, translation or degradation. The RGS family of proteins act as *GTPase activating proteins* (GAPs) of which there are several and are thought to act specifically on G-proteins (see Ross and Wilkie, 2000) by increasing the rate of GTP hydrolysis facilitating the inactivation after stimulation has stopped. Desensitization also results from internalization by endocytosis (e.g., Chuang and Costa, 1979; see also Feguson, 2001). A case that has been examined in some detail is the internalization which depends on the β-arrestin. Internalization and degradation occurs by a mechanism involving ubiquitination (Shenoy et al., 2001). The internalization also has an entirely different function by activating the MAP kinase

signaling cascade (Daaka et al., 1998).

B. The Small GTPases

The small GTPases are involved in a variety of functions as discussed in many other chapters of this book. The Ras-related superfamily of GTPases (see Bos, 1997) include Ras, Rho and Rab proteins, as well as the smaller ARF, Sar and Ran. One of the functions of the Rho-Rac family is the assembly of actin in response to external signals (see Hall, 1998 and Chapter 23) and directly or indirectly plays a role in smooth muscle contraction and cell shape changes (e.g., Uehata et al., 1997; Barrett et al., 1997; Hacker and Perrimont, 1998). Mutations of RhoA and Rac1 in polarized epithelial cells have been found to disrupt tight junctional structure (see Chapter 4) as well as gate (permeability properties of the tight junction) and fence (barrier to the passage of membrane components so that the membrane faces maintain their distinct composition) functions (e.g., Jou et al., 1998). However, these proteins have many other tasks, including involvement in membrane trafficking, transcriptional regulation, cell growth and apoptosis (see Narumiya, 1996; Van Aelst and D'Souza-Schorey, 1997).

Rab GTPases have been implicated in a variety of functions. Rab GTPases are modified post-translationally by attachment of geranylgeranyl moieties to carboxyl-terminal cysteine residues. This modification is necessary for their proper membrane targeting (see Seabra, 1998). Rab are involved in recruitment of specific effector molecules on cellular membranes (e.g., see Chapter 11) and have a role in docking and fusion of involving the SNARE machinery (see Chapter 11 and Olkkoken and Stenmark, 1997). In addition, they are involved in transport of proteins into the nucleus (see Chapter 5). Rab GTPases are also involved in the formation of vesicles during endocytosis and exocytosis. Rab5 is required for endocytosis. Other Rab proteins are involved in membrane fusion reactions, Rab3 in exocytosis (Jones et al., 1993) and Rab6 in exocytotic budding (see Bean and Scheller, 1997; Jones and Howell, 1997).

The way the Rab GTPases function in endocytosis is beginning to be understood. A protein, the *early endosomal autoantigen* (EEA1), was found to be required for endocytosis involving Rab5 (Mills et al., 1998). EEA1 contains a domain (FYVE) about 70 amino acids long that binds to phosphatidylinositol-3-phosphate (PI(3)P) (see Gaullier et al., 1998, and Patki et al., 1998) and Rab5-GTP (Simonsen et al., 1998). While EEA1 acts to hold the Rab5 and PI(3)P together, Rab5 and PI(3)P are anchored to the bilayer through their hydrophobic tails. EEAI is present only in early endosomes, providing a mechanism for specificity (Mu et al., 1995). Proteins with similar motifs are also thought to be involved in vacuolar protein sorting and vacuole function (see Burd and Emr, 1998).

Ras is located in the plasma membrane and responds to a variety of growth factors. Activation and inactivation of Ras is very important. Mutants of Ras that cannot be deactivated are tumorigenic. The molecular basis activation and inactivation are beginning to be elucidated (e.g., Scheffzek et al., 1997, Boriack-Sjodin et al., 1998). They require *guanine nucleotide exchange factors* (GEFs) for activation and

GTPase-activating proteins (GAP) for inactivation (see below).

GEFs directly activate Ras in response to extracellular stimuli (see Quilliam et al., 1995). GEFs favor the dissociation of GDP from the inactive GTP-binding proteins so that GTP can then bind and induce conformational changes and act as an "on" switch. Analogous GEFs regulate Ras-related proteins that serve other cellular functions. A family of proteins (*Dbl homology proteins*) is thought to function as GEFs for the Rho family of Ras-related proteins (see below). There are four main classes of GEFs (see Cherfils and Chardin, 1999). In contrast to the GEFs, GAPs increase the rate of hydrolysis of GTP and, therefore, favor the "off" position of the switch.

Another layer of regulation is provided by the *guanine nucleotide dissociation inhibitors* (GDI) that can inhibit either GEFs or GAPs. The GEFs are essential to the function of the GTP-switches because alone the latter exchange GDP and GTP very slowly (e.g., see Feig et al., 1994; Lenzen et al., 1998).

The various GTPases can interact in a complex manner. For example, Rho GTPases are required for the transformation of cells mediated by Ras (Qiu et al., 1995). Constitutively present Ras induces the inhibitor of the cyclin-dependent kinase $p21^{Waf1/Cip1}$ thereby blocking the cell cycle. However, the activation of Rho blocks the induction of the inhibitor, causing cell proliferation (Olson et al., 1998).

A GEF of Ras was found in yeast to be Cdc25 (i.e., a protein coded by a gene involved in cell division). Mammalian homologues were identified, such as, for example, *son of sevenless* (Sos) (named after a *Drosophila* gene) or $Cdc25^{Mm}$ (see Feig, 1994). Sos is a cytoplasmic protein, recruited to Ras when growth factors activate their receptor. GDP would dissociate very slowly without a reduction of affinity provided by Sos (see Lenzen et al., 1998). Sos increases the dissociation of GDP so that it detaches in less than a second, as required by its function. Boriack-Sjodin et al. (1998) using X-ray crystallography, demonstrated conformational changes of Ras induced by Sos. Sos blocks the entry of Mg^{2+} and the phosphate portion of GDP at one site and simultaneously opens the cavity of Ras, enclosing GDP, allowing its release.

A large family of GEFs activate Rho. These have Dbl (for *diffuse B-cell lymphoma*) homology domain (DH) followed by a Pleckstrin homology domain (PH) (see Van Aelst and D'Souza-Schorey, 1997). Other domains distinct for each of the individual regulators are also present. The GAPs (also called *regulators of G protein signaling*, RGS) contain a distinct domain, the *RGS box*) (e.g. see Popov et al., 1997; Faurobert and Hurley, 1997). A family of over 19 proteins contains the RGS domain. As discussed above they regulates the G-proteins by stimulating the GTPase activity.

The Rho family of small GTPases regulate a variety of cellular functions including actin rearrangement, regulation of transcription, cell cycle regulation, apoptosis and membrane trafficking (see Bishop and Hall, 2000). About 30 effector proteins that interact with members of this family have been identified. As discussed for other small GTPases, three, GEFs, GAPs and GDIs are such effectors. Rho regulation like

that of other small GTPases is then the result of the balance between all these effects (see Moon and Zheng, 2003). GDI (see Olofsson, 1999) also may have a role in their intracellular localization.

C. Interactions Between G-proteins and the Small GTPases

We saw that the heterotrimeric G-proteins are activated by ligand-binding receptors (generally a protein with seven transmembrane segments). Most frequently, they regulate the production of second messengers. The second messengers mediate the physiological effects that may be on enzymes or on gene expression. However, at least two, G_{12} and G_{13}, are involved in cell cycle progression and changes in the actin cytoskeleton through an entirely different pathway. The action is via the smaller GTPase, Rho. G_{13} activates a GAP containing the RGS-domain that also favors the dissociation of GDP from the small GTPases (Kozasa et al., 1998). The GAP acts to down regulate the heterotrimeric G-protein, but also is an effector by virtue of its acting as a GEF and therefore an activator of Rho. In this case, the activation of a receptor activates the heterotrimeric G-protein. This activation is amplified via the action of Rho which induces cytoskeletal rearrangements and gene activation (see Kozasa et al., 1998 and Hart et al., 1998).

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

III. RECEPTORS INSIDE THE CELL

In vertebrates, steroid and thyroid hormones coordinate various aspects of gene expression important in growth, development and normal function. They also have a regulatory role in ion transport and metabolism. These hormones are small, relatively hydrophobic and move freely across the plasma membrane. A variety of other ligands such as retinoids, vitamin D, prostanoids and farnesoids act through similar mechanisms. In addition, the activation pathways of some of these ligands are also regulated by metabolites and protein kinases (e.g., see Freedman, 1999). These receptors act as transcription factors, which have been discussed in Chapter 3). This section will discuss some of the mechanisms by which these hormones exert their effect on gene expression. Other hormones and polypeptide growth factors, previously discussed in Chapter 6, are polar and some of them are rather large so that, presumably, they cannot enter cells readily. As discussed in the present chapter and Chapter 6, these ligands exert their effects by binding to surface receptors, although there is evidence that some of them act intracellularly (see Chapter 6). In contrast, many of the effects of steroids and thyroid hormones are mediated by binding to specific intracellular receptor proteins. However, we should not lose sight of the fact that the steroids may act directly on channels or via receptors at the cell surface. These have been referred to as nongenomic effects (see below and Falkenstein et al., 2000).

The intracellular receptors are present either in the cytoplasm (e.g., the glucocorticoid receptor, GR) or the nucleus (e.g., thyroid hormone receptor, THR, estrogen and progesterone receptors, ER and PR, respectively; known collectively as *nuclear receptors*). The receptors bind to specific DNA stretches of target genes called the hormone response elements (HREs). The THRs are already bound to the HRE before interacting with the hormone (Samuels and Tsai, 1973). The intracellular receptor-hormone complexes bound to the HREs mediate the formation of transcriptional regulatory complexes. These complexes activate or repress transcription from nearby promoters (see Mangelsdorf et al., 1995; Lemon and Tjian, 2000).

Some of the sequences found most frequently in the nuclear responsive elements are displayed in Table 6 (Beato et al., 1989).

Table 6 Consensus Responsive Elements for Nuclear Receptors

1. GRE (+)	1 2 3 4 5 6 10 11 12 13 14 15 G G T A C A n n n T G T T C T
2. PRE	"
3. ARE	"
4. MRE	"
5. ERE	A G G T C A n n n T G A C C T
6. EcRE	A G G G T T n n n T G C A C T
6. TRE	T C A G G T C A - - - T G A C C T G A
7. RRE	"
8. GRE (-)	A T Y A C N n n n T G A T C W 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

G = glucocorticoid A = androgen R = retinoic P = progestin

M = mineral corticoid E = estrogen T = thyroid hormone

From Beato, 1989. Reproduced by permission.

The nuclear receptors nomenclature can be confusing. A unified nomenclature system has been proposed and a tabulation of the various receptors and their names are listed in a table (see Nuclear Receptors Nomenclature Committee, 1999).

Cloning the cDNA (see Chapter 1) of these receptors revealed a striking similarity in the sequences of very large regions of these molecules, indicating that they were part of a superfamily of transcription factors. This superfamily also includes the receptors of retinoic acid, vitamin D, vitamin A and the protooncogene v-

erb A (see Mangelsdorf et al., 1995). Retinoic acid is involved in the differentiation of cells.

The nuclear receptor superfamily includes more than an estimated 150 proteins (see Mangelsdorf, et al., 1995). Four classes of this superfamily have been recognized on the basis of dimerization and DNA binding properties. Two of these have been extensively studied. Class I includes receptors that act as ligand induced homodimers (e.g., steroid hormone receptors). The class II receptors such as the retinoic acid receptor (RAR), THR and others, bind DNA after forming a heterodimer with retinoid X-receptor (RXR) (see Mangelsdorf and Evans, 1995; Chambon, 1996). The heterodimer activates transcription of specific genes. The RAR and the RXRs have three isotypes each (α,β and γ). The various combinations of the isotypes produce a diversity of effects.

GR cDNA was the first to be studied. Its predicted amino acid sequence matched large regions of the v-*erb A* protooncogene (Weinberger et al., 1985). The cloning and sequencing of the cDNA from estrogen, progesterone, mineral corticoids, androgens and thyroxine receptors rapidly followed. Other proteins of the same superfamily as *erb A* were identified by screening cDNA libraries using DNA fragments corresponding to erb A or estrogen receptor DNA-binding domains (Giguère et al., 1987; Petkovich et al., 1987). A cDNA library is the DNA produced in vitro by reverse transcriptase using mRNA of cells as a template. The DNA-binding domains were used as probes capable of hybridizing to the cDNA complementary to it. The amino acid sequence of the protein can be determined from the DNA base sequence (see Chapter 1). Because the proteins bind hormone and DNA, we would expect each protein of this superfamily to have domains that can bind to both of these. In order to be bound to chromosomal DNA, the cytoplasmic receptors must also have a domain which would allow them to be targeted to the nucleus (see Chapter 5), the NLS. The various domains of the receptors are summarized in Fig. 15 (Beato et al., 1989). Highly homologous DNA binding regions of 66 to 68 amino acids are found close to the amino-terminal of the various receptor proteins. The common residues range from 98 to 40% in relation to GR. Eight of these residues correspond to so-called *"zinc fingers"* (see Beato et al., 1991) (Chapter 3). In this case, each contains four cysteines tetrahedrally coordinating a Zn ion, as proposed for transcription factor TPIII of *Xenopus laevis*. In TPIII, however, two cysteines and two histidines can coordinate each of the nine Zn ions (Miller et al., 1985). Two separate families based on the amino acid sequence of the DNA-binding domain are of particular interest. GR, androgen receptor, and the mineral corticoid receptor form one family. A larger family includes ER, THR, the receptor for vitamin D3, the receptors for retinoic acid, and several others of unknown function (referred to as *orphan receptors*).

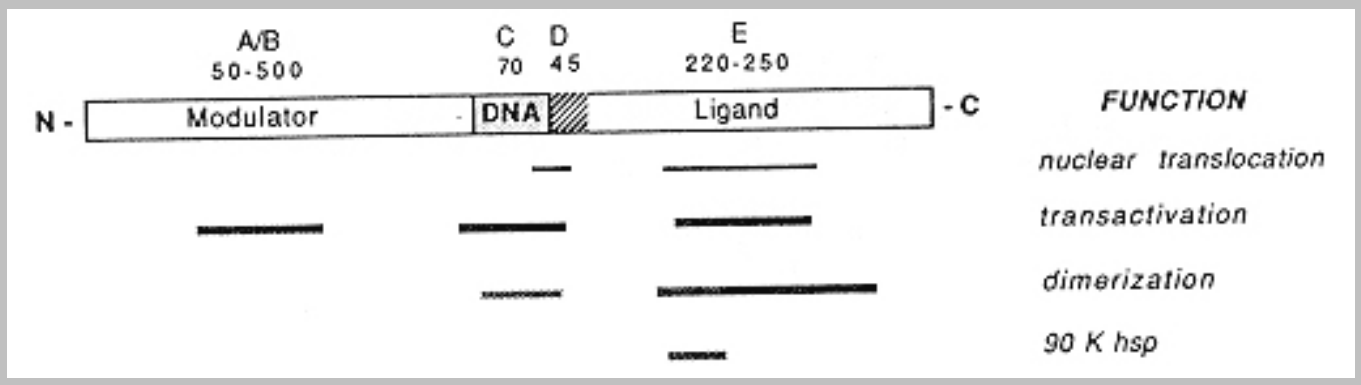


Fig. 15 General and functional organization of nuclear receptors. From Beato et al., &copy1989, reproduced by permission, copyright by Cell Press.

What is the role of the ligand? According to the classical view, ligand binding induces a conformational change in the receptor, which allows not only the translocation into the nucleus, but also binding to DNA. A direct in vitro test of the question, however, indicates that the hormone is not needed for binding to the DNA. This question was tested for GR using the *mouse mammary tumor virus* (MMTV), which contains the appropriate HRE. GR was bound to the MMTV in the absence of the hormone (Willmann and Beato, 1986). Later studies with the progesterone receptor (PR), ER and THR also showed that the hormone was not needed for DNA binding. In contrast, other information shows that in intact hepatocyes, the HRE of the rat tyrosine amino transferase gene is occupied by GR only after hormone administration (Becker et al., 1986). In these experiments the intact cells are exposed to dimethyl sulfate (DMS), which cleaves DNA that is not complexed to protein. Apparently, the protein shields the DNA from chemical or enzymatic attack. When the DNA is subsequently subjected to electrophoresis, the large, uncleaved segments are missing, leaving empty spaces on the gel (so called footprints; see Chapter 1). In contrast to DNAase used in a similar technique, DMS enters cells readily.

In summary, on one hand, in intact cells the GRs cannot bind to DNA without complexing to the hormone. On the other hand, in vitro, the receptor alone can bind to the DNA. How can the paradox be resolved? The two sets of findings can be reconciled by assuming that binding to the hormone is needed for the translocation into the nucleus. This could be the case, for example, if the receptor was sequestered in a complex with another protein, such as the heat shock protein hsp90. hsp90 is a component of a complex containing the inactive steroid receptor (Godowski and Picard, 1989). The GR, ER and PR are thought to be bound to hsp90, making them unavailable for DNA binding in the absence of the ligand (Groyer et al., 1987, Joab et al., 1984, Pratt et al., 1988). In accordance with this view, THR, which does not interact with hsp90, does not require the hormone for binding to DNA in vivo. A model that incorporates (a) the binding of hsp90 to GR, (b) the release of GR after binding of the hormone, (c) the translocation into the nucleus and (d) the binding to the GRE, is represented in Fig. 16. In the case of GR, besides allowing the receptor to enter the nucleus, the binding to the ligand is also responsible for the transcriptional regulation. The presence of two separate roles is demonstrated by the use of antiestrogenic compounds, which allow the dissociation of the receptor from hsp 90 and its transfer into the nucleus in the absence of the hormone (Damm et al., 1989). However, once in the nucleus the receptor fails to activate transcription because the hormone is missing. The dynamics of the GR have been followed by the use of GR-GFP constructs, where the green fluorescent protein is linked to the GR (see Chapter 1). GR-GFP remains in the cytoplasm in the absence of ligand. When the ligand is introduced, GR-GFP is translocated into the nucleus rapidly (less then 10 min) (Carey et al., 1996; Walker et al., 1999). A cell line with several GR binding sites allows following the dynamics of the GR binding in the presence of the ligand (McNally et al., 2000). Photobleaching (FRAP) (see (Chapter 4 and Chapter 10) and fluorescence loss after phobleaching (FLIP) of GR-GFP provide detailed information. With FRAP, GFP-GR attached to the chromatin arrays was bleached with a laser beam. The bleached spot was rapidly replaced by fluorescent molecules. The opposite design, FLIP, the GFP-GR elsewhere in the nucleus were continuously bleached. In this case, the GFP-GR in the arrays was replaced by the non-fluorescent molecules, indicating a dynamic exchanges of GR between the nucleoplasm and the chromatin.

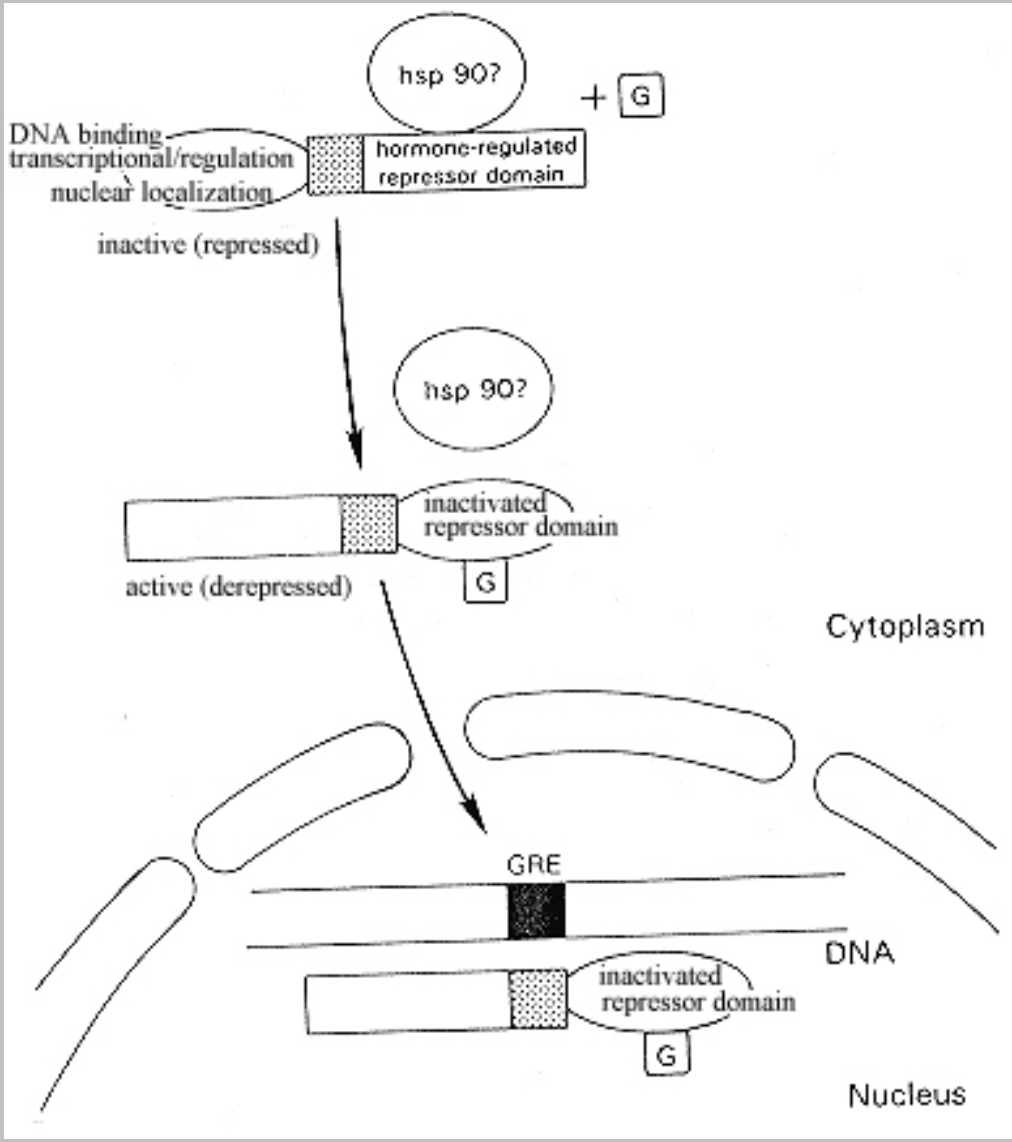


Fig. 16 Model showing the presence of GR in the cytoplasm bound to hsp90 in a repressed state, followed by its release after binding of G. The activated GR is then translocated into the nucleus, where it binds to the GRE site in chromosomal DNA.

Just as translocation into the nucleus of the receptor-hormone complex is part of the mechanism for activating transcription, removal from the nucleus will have the opposite effect. NGF1-B (also called NUR77) is an orphan receptor rapidly induced by nerve growth factor (NGF) (see Chapter 6). NGF1-B activates transcription through two distinct response elements (Wilson et al., 1991; Philips et al., 1997). In response to NGF, RXR forms heterodimers with NGF1-B and induces the phosphorylation of Ser105 of NGFI-B. The complex is then removed from the nucleus (see Katagiri et al., 2000) using nuclear export signals (see Chapter 5) within the NGFI-B molecule. This translocation decreases the transcriptional activity of the RXR-RAR complex.

THR is complexed to the corresponding HRE in the absence of hormone. The presence of the hormone, however, stimulates transcription 10 to 100-fold. As with other transcription factors, both the DNA binding domain and the activation domain of the receptors are needed for the transcriptional activation of target

genes. The binding of the sequence specific transcription factors frequently requires binding to other proteins such as the *TATA-box binding proteins* (TBF) and the *TATA associated factors* 5-5 (TAF). A family of mammalian bridging proteins such as the CREB binding protein (CBP) and p300 have been found necessary for the formation of transcriptional initiation complexes. These proteins are the target of many regulative signals (e.g., Chakravarti et al., 1996) and they are discussed in more detail below.

Hormone-dependent nuclear import of the receptors is very rapid ($t_{1/2}$= 5-10 min) (Picard and Yamamoto, 1987; Yang and DeFranco, 1994). In contrast, nuclear export is very slow (Madan and deFranco, 1993, Sackey et al., 1996). However, the dissociation of the receptor from the DNA is rapid ($t_{1/2}$=10 min) (Munck and Foley, 1976). The finding that in digitonin permeabilized mammalian cells the receptors remain in a subnuclear compartment after being released from the chromatin (Yang et al., 1997,) explains this discrepancy. The receptors are subsequently exported from the nucleus.

The intracellular receptors have also been shown to respond to signals and cascades, sometimes independently from their ligand. How can this be the case? Apparently the nuclear receptors associate with transcriptional *corepressors* and in later steps to *coactivators*. These proteins or protein complexes respond to signals other than the receptor's ligand (see Xu et al., 1999). Biochemical and yeast two-hybrid studies (see Chapter 1) have found proteins interacting with receptor protein through their ligand binding domains and, in some cases, through unrelated domains. These proteins have been termed the p160 coactivators (see Xu et al., 1999). One of the coactivators of steroid receptors has been found to contain RNA and to be part of a 600-700 kDa complex that can be isolated and immunoprecipitated (see Chapter 1) associated with the coactivator SRC-1 (Lanz et al., 1999).

Once the appropriate assembly of proteins has taken place, how do the appropriate DNA-sequences of the chromatin become accessible to these complexes? As we saw, acetylation of histones by acetyltransferases (HATs) seems to play a role in the activation of genes (see Chapter 2 or Wade and Wolffe, 1997). The coactivators have intrinsic HAT activity (Blanco et al., 1998; Sim et al., 1999). For example, SCR-1, a coactivator for many steroid receptor transcription factors, (Onate et al., 1995) is a HAT with a specificity for the H3 and H4 histones (Spencer et al., 1997). It also binds to p300/CBP-associated factor (PCAF) that also has HAT activity, possibly increasing its activity. The inverse is also true, corepressors have histone deacetylase (HADAC) activity (e.g., Heinzel et al., 1997).

In summary, in vertebrates, the picture that is emerging is that of silencing complexes that are recruited to the receptor in the absence of ligand and coactivators that are recruited to the receptors only in the presence of ligand. In addition, coactivators were found to have HAT activity, whereas corepressors have histone deacetylase (HADAC) activity. A similar pattern has been found in *Drosophila* for the system dependent on the hormone *ecdysone* (Tsai et al., 1999).

The transcriptional regulatory complexes associated with the intracellular receptor-hormone complexes (see above) are no longer active after hormonal withdrawal. The chaperones p23 and Hsp90 were found to inactivate receptor-mediated transcription as well as the activity of other transcriptional regulatory

complexes (Freeman and Yamamoto, 2002). The recognized role of chaperones is that of folding polypeptides (see Chapter 10) The transcriptional regulatory complexes are very stable in vitro but have a very rapid turnover in vivo. These results suggest that chaperones are involved in the disassembly of transcriptional regulatory complexes to respond to changes.

Some signal transduction pathways act via nuclear receptors independently from hormonal signals, as in the case of cyclin dependent kinases and growth factor pathways that activate *mitogen activated protein kinases* (MAPKs) (Trowbridge et al., 1997;Krstic et al., 1997) (see below). A MAPK phosphorylates the ER-β, which stimulates the recruitment of SRC-1 (see Freedman, 1999; Tremblay et al., 1999). Phosphorylation can also be inhibitory. The *peroxisome proliferator-activated receptor-γ* (PPAR-γ) is involved in adipocyte differentiation, insulin sensitivity, cell proliferation and inflammatory processes. Phosphorylation of PPAR-γ by MAPK decreases the affinity for its ligands (Shao et al., 1998).

We have seen how steroids activate intracellular receptors that act directly on transcription. This is not to say that they always function in this manner. Steroids also have regulatory effects that are independent of transcription. The steroid progesterone has been shown to act on the oxytocin receptor, inhibiting the effect of this peptide hormone. Progesterone has its effects at the cell surface as shown by its action even when attached to a protein that is unable to enter the cell (Grazzini et al., 1998). This inhibition is important in pregnancy by favoring uterine quiescence. Other less defined effects on receptors also take place (Wehling, 1997) and inhibitory and excitatory effects of progesterone on receptors of neurotransmitters have been reported (e.g., Valera et al., 1992). In addition, the estrogen receptor has been shown to activate protein kinases known to be activated by mitogens (see <a href=" #4a1">below) (Migliaccio et al., 1996) and the glucocorticoid receptor has been shown to interfere with the activation of responses to UV light and inflammatory responses (Caelles et al., 1997).

Proteins of unknown function were found to be very similar in amino acid sequence to those of the known nuclear receptor family. These were called orphan receptors on the expectation that they are able to respond to hormonal signals (e.g., see Willy and Mangelsdorf, 1998; Blumberg and Evans, 1998). As many as forty such proteins coded by different genes have been identified in humans. Many of the orphan receptors were cloned. How could their function be uncovered? An approach referred to as *inverse endocrinology* has opened a number of doors to our understanding (e.g., Heyman et al., 1992; Levin et al., 1992). The approach identifies the receptor by making use of a particular strategy. All known non-steroidal ligand activated nuclear receptors heterodimerize with retinoid X receptors (RXR) (9-*cis* retinoic acid receptors) for high-affinity DNA binding. The binding of an unknown ligand (present in an homogenate of cells) to a protein dimerizing with RXR can serve as a trap. The ligand would then be identified by sophisticated analytical techniques after extraction with organic solvents. The major tasks still remain of identifying the function and mechanism of the now known ligand. However difficult these tasks might be, conceptually the necessary approaches are straight forward. A variety of important signaling pathways have been identified in this way (see Blumberg et al.; Kliewer et al., 1999), such as the peroxisome proliferator-activated receptor mentioned above.

As already mentioned above, the action of the steroid may be through a separate mechanism from that

described, in the so-called nongenomic steroid actions (see Falkenstein et al., 2000), frequently through specific membrane receptors. Steroids have been shown to act directly on neurotransmitter receptors (e.g., Valverde et al., 1999). In addition, steroid receptors have been shown at the surface of cells as detected with antibodies raised to different epitopes of estrogen receptors (Nadal et al, 1998) where they act on K^+ (ATP) channels. The receptors at the cell surface are generated from the same gene as the conventional receptors already discussed (Razandi et al., 1999). The receptors at the cell surface may be involved in signaling cascades after interacting with G proteins stimulated pathways (Razandi et al., 1999). Some receptors may be different from the conventional steroid receptors. For example, neural excitability is affected by 17 β in α estradiol receptors in knockout mice mutants lacking intracellular receptors and in the presence of an anti-estrogen drug (Gu et al., 1999). The estrogen receptor present in the cytoplasm can also activate cell proliferation though a different pathway than the one involving direct interaction with the chromatin, but involving the ERK pathway (Migliaccio et al., 2000).

IV. TARGETS OF INTRACELLULAR SIGNALS

The effects of intracellular signals depend on their capacity to regulate specific cellular events such as biochemical reactions. The control may be directed at channels, metabolic pathways, intracellular receptors, or gene expression. In actuality, the impact of second messengers is generally extremely complex, not only because they have multiple targets (i.e., their regulation is pleiotropic), but because there is considerable "cross-talk" connecting pathways controlled by various second messengers.

As we have seen, some second messengers act on channels that they may open or close (some are regulated directly by neurotransmitters at the cell surface). These channels are said to be chemically gated. We have already encountered an example: the Ca^{2+}/Na^+ channel that opens in the presence of cGMP in visual reception (Section II). Many enzymes are regulated by phosphorylation and dephosphorylation reactions, so that signal-controlled protein kinases and protein phosphatase play a fundamental role in regulation. Similarly, the regulation of gene expression involves the phosphorylation of transcription factors and elements involved in translation. The next two sections will discuss primarily the regulation of enzyme activity and gene expression.

We should not loose sight that our knowledge is incomplete and new aspects of any biological problem surface every day. In addition to the mechanisms discussed in more detail below, new mechanisms in the control of enzymes and gene expression are likely. One of the important regulative systems in the development of neurons involve the protein *Notch* and its ligand Delta (see Weinmaster, 1997). The control by these two elements is very different from those described in this section, illustrating the multiplicity of mechanisms. The two are active in the interaction between cells discussed in Chapter 6. Both are transmembrane proteins that function in neurogenesis. The Notch protein is a receptor for the ligand Delta. When Delta binds to Notch, the Notch cells are prevented from developing into neurons (see Weinmaster, 1997). Apparently the inhibitory mechanism depends on the proteolytic release of the intracellular domain of Notch. In the nucleus, this fragment activates a member of the CSL family of DNA-binding proteins that acts on gene expression (Struhl and Addachi, 1998; Schroeter et al., 1998).

A. Activation-inactivation of Enzymes

Many enzymes are regulated by phosphorylation-dephosphorylation reactions. The phosphorylations are catalyzed by *protein kinases*. Dephosphorylations are catalyzed by *protein phosphatases* (PPs). The activity of these key enzymes is frequently controlled by second messengers.

Protein kinases

Phosphorylation alters the activity of enzymes (e.g., see Chapter 13). A summary of some of the intracellular signaling systems involving protein kinases is provided by Fig.17 (modified from Cohen, 1992). Many of the effects of cAMP depend on *cAMP-dependent protein kinases (PKAs)*, which phosphorylate a plethora of intracellular proteins (Cohen, 1988). *cGMP-dependent protein kinases (PKGs)* were originally thought to have a more restricted role, mostly in visual reception, the cerebellar region of the brain and smooth muscle. However, cGMP protein kinases have been more recently found to be involved in the mediation of many more signal pathways (Lincoln et al., 1995).

Ca^{2+} acting as a second messenger frequently requires calmodulin (Section IA). Upon binding Ca^{2+}, calmodulin undergoes a conformational change that allows it to activate many enzymes, including protein kinases.

DAG, as well as the products of hydrolysis catalyzed by PLA_2, lyso PC and cis-unsaturated fatty acids, activate *protein kinase C (PKC*), a multifunctional kinase.

Some protein kinases may phosphorylate a multiplicity of enzymes (*multifunctional kinases*), whereas others have a single substrate, such as glycogen phosphorylase kinase (Chapter 14), the myosin light chain kinase (Chapter 24) or elongation factor kinase. Each of the latter controls, respectively, only glucose metabolism, muscle contraction or protein synthesis.

In contrast, polypeptide hormones and growth factors bind to receptors that phosphorylate their own tyrosine residues, i.e., they act as tyrosine kinases. The Trk family of *tyrosine kinase receptors* are the signaling receptors for growth factors, such as neurotrophins, the nerve growth factors (see Barbacid, 1995). Other receptors are serine/threonine kinases and act in a similar manner (e.g., Pawson and Nash, 2000) The autophosphorylation of the receptor proteins is rapidly followed by the activation of *mitogen activated protein kinases* (MAPKs) which phosphorylate many intracellular proteins. The *cytokines* that control cells in the immune and hematopoietic systems (see Chapter 6) are not tyrosine autokinases. However, they activate separate tyrosine kinases.

The MAPKs represent a family of protein kinases associated with cell proliferation. They respond to many hormones and growth factors and play a very important role in differentiation and proliferation. They are organized in a series of phosphorylating reactions or cascades held together by scaffolding proteins. These scaffolds are largely responsible for the specificity of the responses (see below). The eventual targets of

these cascades of reactions are many cytoplasmic and nuclear proteins. They phosphorylate and activate transcription factors (see below) as well as several other proteins such as other kinases. The pathways permits extensive amplification and integration of signals.

A second group of protein kinases is activated by stress such as DNA damage or oxidative stress. These are the *stress activated protein kinases* (SAPKs)

The small guanine nucleotide-binding protein Ras is frequently required for MAPK activation. The MAP kinases are activated by a cascade of sequential phosphorylative events (e.g., see Elion, 1995). MAP kinase kinase kinase (MEKK) phosphorylates MAP kinase kinase (MEK) which finally phosphorylates MAP kinase at the tyrosine and threonine residues at the catalytic cleft of the enzyme. MAP kinases regulate many different functions in the same cell. In *Saccharomyces cerevisiae*, at least 6 MAP kinase cascades are present. The right target must be activated in response to a particular stimulus. These cascades preserve their separate identity even when they use common protein kinases probably because various components associate in assemblies (see section below). At least three of the six MAP kinases of yeast form multi-kinase complexes with a protein called Ste5. Stet5 is thought to function as a scaffold to link proteins that function sequentially in a cascade (see Elion, 1995). Similar findings with mammalian cells (Schaeffer et al., 1998; Whitmarsh et al., 1998) indicate that the mechanism is probably universal in eukaryotes. For example, the *c-Jun NH_2-terminal kinase* (JNK; also known as *stress-activated protein kinase*, SAPK) is activated by MAP kinases when cell are exposed to stresses. JNK is required for a number of processes, such as embryonic development, apoptosis, transformation and immune responses. A protein has been isolated that binds to several components of the JNK signaling pathway. This scaffold protein also favors the activation of the pathway.

One of the signaling pathways activated by MAPK involves pp90 ribosomal S6 kinases (RSKs). RSKs (also known as *MAP kinase-activated protein*, MPKAP kinases-1) are a family of proteins of 85 to 90 kDa. In mammals three isoforms are specifically phosphorylated by an *extracellular signal-regulated kinase* (ERK), in this case ERK-MAPK, only. Mutations of RSK-2 have been found to have a role in Coffin-Lowry syndrome (e.g., Manouvrier-Hanu et al., 1999) and X-linked disorders (e.g., Ronce et al., 1999) leading to mental retardation and skeletal malfunctions.

The RSKs are phosphorylated and partially activated by MAPKs (e.g., Zhao ete al., 1996). Although the MAPKs phosphorylation is needed for activation, phosphorylation by PDK1 (phosphoinositide-dependent kinase) fully activates the RSKs (Jensen et al., 1999; Richards et al., 1999). The RSKs, in turn, phosphorylate and activate a variety of transcription factors (see Frödin and Gammeltoft, 1999). The phosphorylation of histone H3 by RSKs suggests a direct effect on chromatin remodelling (Sassone-Corsi et al., 1999) (see Chapter 2). RSKs have also been implicated in the regulation of the cell cycle. They phosphorylate and down-regulate the $p34^{cdc2}$ inhibitory kinase Myt1 allowing for progression through G2/M phase in meiosis (Palmer et al., 1998) and mitosis (Wright et al., 1999). Metaphase arrest produced by the *cytostatic factor* (a factor that prevents mature but unfertilized oocytes from dividing) requires RSK-2 (Bhatt and Fherrel, 1999; ; Gross et al., 1999).

Other roles have been found for RSKs. RSK-2 and the protein kinase Akt (a phosphoinositide-3 kinase) phosphorylate and inactivate the proapoptotic protein BAD. RSK-2 is thought to activate *Bcl-2*, an anti-apoptotic gene (Bonni et al., 1999). Apoptosis, programed cell death, is discussed in Chapter 2.

Protooncogenes are generally genes involved in the regulation of cell proliferation. A mutation can convert them into agents of unregulated growth, i.e., malignancy. Therefore, any gene coding one of the components of the cascades culminating in cell division can be a protooncogene. Many protooncogenes code for tyrosine kinases that are involved in the early parts of these cascades.

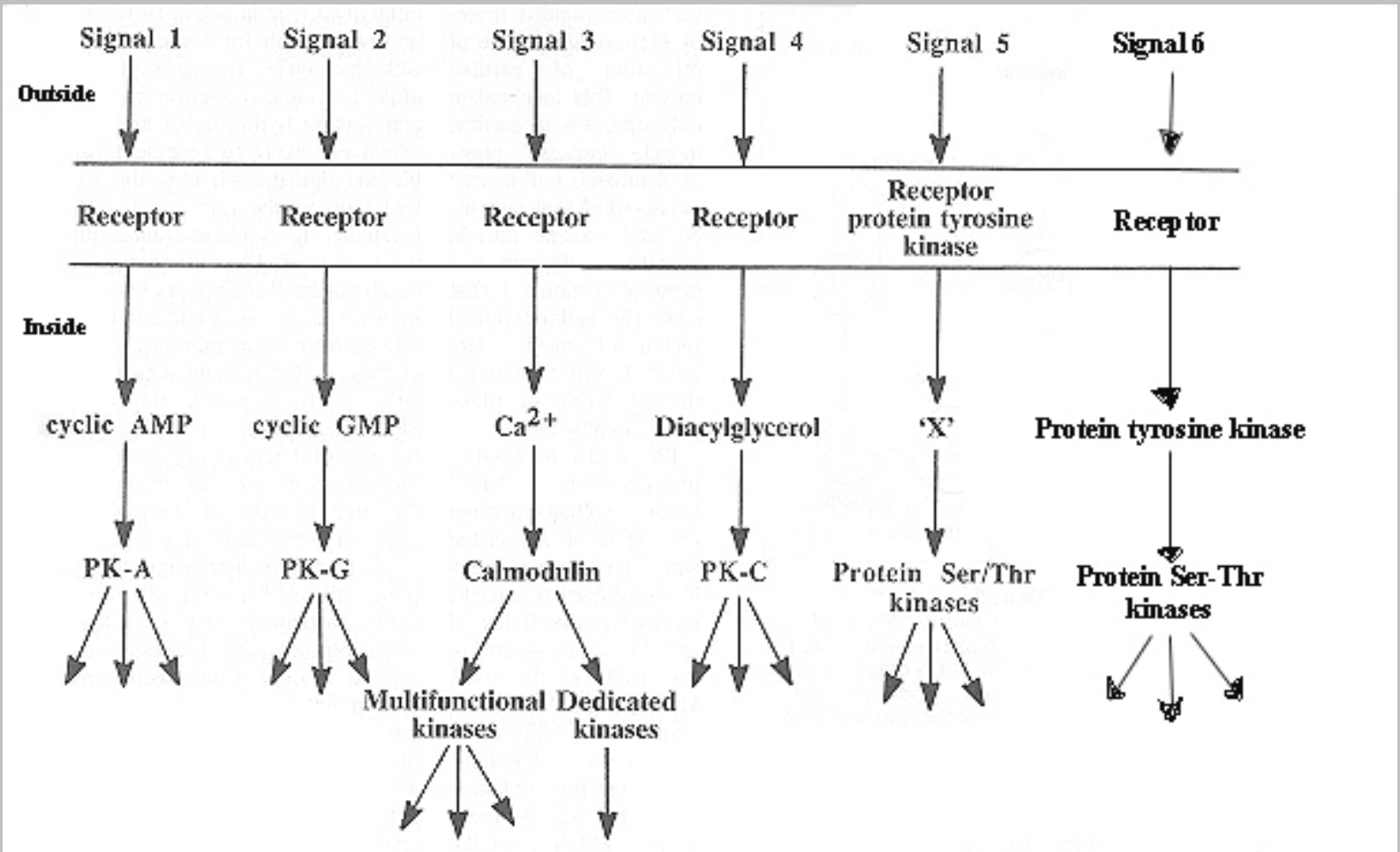


Fig. 17 Six of the principal signalling systems that operate in eukaryote cells. "X" is an hypothetical second messenger. Reproduced and modified from *Trends in Biochemical Science*, vol.17, Cohen,P., Signal integration at the level of protein kinases, protein phosphatases and their substrates, pp.408-417, copyright &copy1992, with permission from Elsevier Science.

Protein phosphatases

Regulation by dephosphorylation catalyzed by PPs, possibly also under the control of signals, represents the other side of the coin. By dephosphorylating proteins, the phosphatases will generally have an effect opposite from the protein kinases. An example will illustrate this principle. In mammalian muscle, the phosphorylation of glycogen phosphorylase activates the enzyme (Chapter 14). This phosphorylation is

induced by a cascade of reactions initiated by the binding of epinephrine to its receptor, and is mediated initially by cAMP. On the other hand, dephosphorylation catalyzed by protein phosphatase 1 (PP1) inactivates it (Cohen, 1989). PP1 itself can be blocked by several mechanisms.

There are at least three different kinds of phosphatases. The *protein tyrosine phosphatase* (PTP) family includes many enzymes (Fauman and Saper, 1996). The receptor-like phosphatases (see below) belong to this family. The cdc25 phosphatases dephosphorylate and thereby inactivate cyclin-dependent kinases responsible for initiating phases of the cell cycle. Another group of phosphatases, coded by the PPP and PPM gene families, dephosphorylate serine and threonine (see Barford, 1996).The PPP family includes Ca^{2+}-activated phosphatases and many other phosphatases. The PPM family includes Mg^{2+}-dependent phosphatases.

Genetic evidence in yeast indicates a very central role of PTPs because reversible tyrosine phosphorylation is the mechanism of regulation of the cell cycle of all cells (e.g., see Chapter 8 and Atherton-Fessler et al., 1993). There is also a good deal of circumstantial evidence linking individual PTPs with stages of development (e.g., den Hertog et al., 1993) and in the transduction of the signal response to antigen binding in T-cell receptors (Woodford-Thomas and Thomas, 1993).

Some PPs are cytoplasmic proteins. However, many PTPs are probably integral membrane proteins, as indicated by their structure (e.g., Mourey and Dixon, 1994). Therefore, they may be capable of responding directly to extracellular signals. They have been called *receptor-like tyrosine phosphatases* (RPTPs). The amino acid sequences suggest the presence of cytoplasmic and transmembrane domains. The domains thought to be extracellular are diverse, as would be expected if they were receptors capable of responding to different signals. These extracellular domains have sectors containing immunoglobulin motifs; some contain fibronectin III-like motifs and motifs similar to those of NCAM. The PTPase supergene family contains $Cys(X)_5Arg$ (where X stands for any amino acid). The cysteine residue is involved in catalysis and arginine is required to bind the phosphoryl group to the substrate (see Denu and Dixon, 1998). Other PP motifs are thought to have a role in subcellular localization, targeting and phosphorylation of the PPs, suggesting another layer of complexity. In some cases (see below and next section), both targeting and specificity depend on the binding of the PP to regulatory subunits.

RPTPs, like other protein tyrosine phosphatases, regulate the level of phosphotyrosine-containing proteins. RPTPs are type-I integral proteins. They contain one or two catalytic sites in the cytoplasmic domain (Mourey and Dixon, 1994). It is not known whether extracellular ligands regulate these phosphatases. Based on the crystal structure of murine RPTP, Bilwes et al. (1996) have proposed that the catalytic activity of this protein can be regulated downward by dimerization, possibly in response to ligand-binding.

The RPTPs are similar in structure to *cell adhesion molecules* (CAM) (see Chapter 6). Like CAM, they can bind to other like molecules. Some bind to proteins that are likely to determine their localization in cells and some are connected to cytoplasmic proteins, suggesting involvement in cellular communication (see Brady-Kalnay and Tonks, 1995). In this role they appear to be major players in the development of the

nervous system and in neuronal plasticity (e.g., see Chapter 22; Tessier-Lavigne and Goodman, 1996; Fields and Ito, 1996). The binding of RPTP β and CAMs to cell surface receptors can be stimulatory or inhibitory in cell adhesion and the growth of neuronal processes, depending on the receptors on the surface of the responding cell. In addition, sulfation or carbohydrate composition of RPTPs β can alter their affinity for other proteins (e.g., see Peles et al., 1998).

The type 1 (PP1) and type 2 (PP2) protein phosphatases belong to a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Their catalytic subunits can interact with many distinct regulatory subunits and the diversity of PP1 (Bollen, 2001) and PP2 (Janssens and Goris, 2001) functions rests on their interaction with at least 100 regulatory subunits. Therefore, a single phosphatase is capable of regulating very different functions, e.g. glycogen metabolism, muscle contraction and the cell cycle. The regulatory subunit provide specificity by targeting the catalytic unit to different parts of the cell and determining the interaction with specific substrates and permit regulation by different hormones and growth factors (Hubbard and Cohen, 1993; Janssens and Goris, 2001). Some of the regulatory subunits of PP1 have been shown to be affected by other agents that have a biological or pharmacological effects, such as Ca^{2+} and other chemicals (e.g. see MacKintosh and MacKintosh, 1994). An additional level of regulation is provided by the dependence of the activity of the regulatory subunits themselves on their phosphorylation state (e.g., Egloff et al., 1997). Different regulatory subunits of PP1 contain common motifs that allow them to bind to the phosphatase and the regulation is mutually exclusive (Egloff et al., 1997; Johnson et al., 1996). A similar situation is suspected for regulatory subunits of other PPs.

As we saw, many protooncogenes code for tyrosine kinases that are involved in the early parts of the signalling cascades. Consequently, some protein tyrosine phosphatases function as tumor suppressors. *PTEN* (*phosphatase and tensin homologue*, that also goes by other names: *MMAC1* and *TEP1*) is one of these genes. It is mutated or deleted in a variety of tumors such as glioblastoma, breast, prostate and kidney malignant tumor cell lines (see Li et al.,1997; Steck et al., 1997; Cantley and Neel, 1999). Germline mutations of *PTEN* were also found in Cowden disease (also known as *multiple hemartoma syndrome*) which exhibits increased breast and thyroid cancer (e.g., see Eng, 1998; Liaw et al., 1997) and Bannayan-Zonana syndrome (also known as Ruval-Riley-Smith syndrome), that exhibits a variety of clinical symptoms (Marsh et al., 1997). *PTEN* knockout mutations (see Chapter 1) in mice demonstrate an essential role of *PTEN* in embryogenesis since they produce lethality and defects in the cephalic and caudal regions and in placentation (Suzuki et al., 1998) as well as neoplasms in multiple organs (e.g., Podsypanina et al., 1999). PTEN is a tyrosine phosphatase whose active domain resembles that of dual-specificity phosphatases (DSPs), which also dephosphorylate serine/threonine residues (e.g. see Di Cristofano and Pandolfi, 2000).

Present indications are that PTEN blocks the phosphoinositide 3-kinase (PIP3-kinase) signaling pathway (see above) by dephosphorylating the 3 position of phosphoinositides (mainly phosphatidylinositol (3,4,5)-triphosphate (PIP_3). The job of PTEN appears to be keeping the level of PIP_3 low (Stambolic et al., 1998). The PI3-kinase pathway regulates cell growth and survival. In resting cells, PIP_3 is present in very low

amounts . Growth factors activate PI3-kinase. The accumulation of PIP_3 at the membrane, recruits PH-domain proteins (see Chapter 6). which bind to PIP_3 . The serine/threonine kinase Act (also called PCB) is activated by this system and proceeds to block apoptosis (see Chapter 2 and Datta et al., 1999).

PTEN overexpression reduced the phosphorylation of *focal adhesion kinase* (FAK) (see Section IF) (Gu et al., 1998). Apparently, phosphoinositides (see Section IF) are substrates of PTEN. Over expression of PTEN reduced insulin induced PIP_3 and transfection with inactive PTEN produced an accumulation of PIP_3 , even without insulin stimulation (Maehama and Dixon, 1998). Part of the effect of insulin is through the activation of *receptor substrate protein* (IRS). IRS activates PI3-kinase (PI3K) which generates the inositide second messengers (see Vanhaeseboeck et al., 1997).

Little is known on how PTEN is regulated, except for the observation that PTEN mRNA is regulated by transforming factor β (Li and Sun, 1997). The subcellular location of PTEN is probably carefully regulated. PTENs contain a PDZ-binding domain. PDZs are modular protein-protein interaction domains, consisting of 80-120 amino acid, generally involved in the formation of multiprotein complexes (see Chapter 6).

Just as dephosphorylation of the kinase substrates by PPases counters the effect of kinases, the kinases themselves can be the target of phosphatases. The protein phosphatase 2A (PP2A), a serine/threonine phosphatase, is the major kinase phosphatase of eukaryotic cells. Therefore, this phosphatase can down-regulate multiple kinase cascades. PP2A has been implicated in the regulation of cellular metabolism, DNA replication, transcription, mRNA splicing, cell-cycle, development and transformation (see Mayer-Jaeckel and Hemmings, 1994; Wera and Hemmings, 1995). As in the case of PP1, the variety of actions of PP2A are the result of its capacity to bind a wide variety of regulatory and targeting subunits. More than 30 kinases have been shown to be regulated by PP2A at least in vitro (see tables in Millward et al., 1999) and many kinases have been shown to form complexes with PP2A. The role of PP2A is so far reaching that many of its facets are still under examination.

Scaffolds, anchors, adaptors and other complexes

Many protein kinases and phosphatases have relatively broad substrate specificities. We have seen how regulatory subunits provide specificity. In addition, specificity is provided in part by precise intracellular recruitment (see Pawson and Scott, 1997; Pawson and Nash, 2000; Garrington and Johnson, 1999) so that repertoires of enzymes are organized in specific signaling pathways at specific sites (see Tsunoda et al., 1998). In part, the specificity is provided by the regulation of the activation of positioned enzymes. The organization and coordination ultimately depends on protein-protein and protein lipid-interactions. These interactions have been discussed in Chapter 6 (see discussion, also listing in Table 2, Chapter 6) An example of targeting is provided by the association with scaffold proteins such as A-kinase anchoring proteins (AKAP) (Klauck et al., 1996). In some cases, the recruited enzymes are retained in an inactive state (Faux and Scott, 1997) and released upon activation. In other cases, AKAPs place PKA holoenzymes at locations where they can respond to the changes in cAMP and where they might be close to their substrates.

Although the unique location of many AKAPs has been worked out (see Colledge and Scott, 1999) little is known about the targeting. For example, AKAPs of the S-AKAP84/D-AKAP1 family have a sequence that targets them to the mitochondrial outer membrane (e.g., Chen et al., 1997). The muscle mAKAP, is targeted to a perinuclear location in muscle cells by spectrin-like repeats (see Colledge and Scott, 1999). Some of the AKASPs are targeted to membranes by their association with fatty acid moieties. AKAP15/18 is targeted to the plasma membrane by its myristoylation at glycine 1 and palmitoylation of cysteines 4 and 5 (Fraser et al., 1998; Gray et al., 1998).

The targeting system can be extremely precise; AKAP79 and the protein *yotiao* localize to the neuronal postsynaptic membrane. AKAP79 has three polybasic targeting regions that interact with membrane phospholipids (Dell'Acqua et al., 1998). The protein *yatiao* is associated to *N*-methyl-D-aspartate (NMDA) receptors which function as ion channels (see Chapter 22) and enhances cAMP potentiation of channel currents (see Colledge and Scott, 1999).

These considerations indicate that the formation of a macromolecular assembly is capable of providing specificity of response, by limiting the possible interactions of the various elements. Adapter or scaffold molecules play a central role in forming these assemblies. In addition, they may also provide mechanisms for precise cross-talk between two systems as indicated by the role of the adapter protein *arrestin*, discussed below. Protein kinases have been shown to have special properties by being able to complex to the *docking site* on the substrate molecule. The presence of these substrate docking sites increases the specificity and the rate of the enzyme and it determines the localization of the kinase inside the cell (see Chapter 13), at least in some cases .

We have seen that adrenergic receptors acting through a G-protein, activate adenylate cyclase to produce the second messenger cAMP (Section IE). cAMP proceeds to activate a cascade of biochemical events. The adrenergic receptors themselves are phosphorylated by the *G-protein receptor kinase* (GRK). Arrestin inactivates the receptors by binding to the phosphorylated site. The inactive receptor-arrestin complexes are sequestered by clathrin coated pits (see Chapter 9), dephosphorylated and recycled to the cell surface or degraded (e.g., Zhang et al., 1997). Arrestin is responsible for recruitment of the clathrin to the complex. In addition, it activates a completely separate pathway by recruiting the *c-Src non-receptor tyrosine kinase* to the complex and stimulating the *mitogen activated protein* (MAP) kinase pathway (Luttrell et al., 1999). Arrestin, besides acting to assemble the components that silence the adrenergic receptors and the formation of coated endocytotic vesicles, also acts as an adapter for the cross-talk with a second separate pathway.

Caveolae (see Chapter 9) provide specialized microdomains. Their major membrane proteins, the caveolins provide a scaffold on which a variety of signalling molecules can assemble. These interacting proteins include G-proteins α subunits, Ha-Ras, Src family tyrosine kinases, endothelial nitrous oxide synthase, protein kinase C isoforms, EGF-receptors and similar tyrosine kinases (see Okamoto et al., 1998).

The scaffolding of proteins may associate enzymes with opposite effects to facilitate regulation. We have seen how PPs play a role opposite to that of protein kinases. The regulation occurs by a feed-back

mechanism where protein kinases, also controlled by phosphorylation, are the substrates of specific phosphatases. A Ca^{2+}-calmodulin dependent serine-threonine protein kinase was found to bind a phosphatase to form a complex of approximately 232 kDa (Westphal et al., 1998). The phosphatase dephosphorylates the kinase so that the PP functions as a negative regulator of the kinase signal. The target of the kinase is the nuclear protein CREB (seebelow) needed for the activation of the transcription of certain factors. A similar regulation of MAP kinases takes place by specific dephosphorylation of phospho-tyrosine and threonine residues, by the phosphatase MKP where the two molecules are present as a complex (Camps et al., 1998).

Role of Hsp90

In early studies, the *heat shock proteins*, Hsps, were found at high concentrations in cells that had undergone an exposure to high temperature. Their role as chaperones (see Chapter 15 and Chapter 10), in the folding of newly formed proteins, is well recognized now. However, until recently, little was known about the class of heat shock proteins 90 (Hsp90) (see Bakau, 1999; Mayer and Bakau, 1999>). In eukaryotes, these molecules have a 25 kDa amino terminal and a 50 kDa carboxy terminal domain separated by a charged region. The amino terminal has an ATPase domain (Prodromou et al., 1997). Hsp90 probably has a marginal role after a heat shock and does not function in the folding of the bulk of the newly formed proteins in yeast (e.g., Nathan et al., 1997). However, it is needed for the folding of a specific subset of proteins. The role of Hsp90 appears to be in the control of cell growth and differentiation (see Toft, 1999). The proteins requiring Hsp90 are often labile, have very complex folding patterns, or they have to be maintained in a particular conformation to insert a cofactor or other ligand (see Mayer and Bukau, 1999). Three components of the mitogen-activated protein (MAP) kinase pathway have been found to require Hsp90. Hsp90 and co-chaperones remain associated with the multiprotein complexes not only during maturation but also after they arrived at their native state.

B. Regulation of Gene Expression

As shown in Fig. 17, the response to external signals are mediated in the cytoplasm by second messengers and, directly or indirectly, by protein kinases. The kinases frequently trigger a cascade of events. In some cases, phosphorylation-dephosphorylation events exert their effect on transcriptional factors. Changes in gene expression in response to external signals may also take place at the level of mRNA splicing and translation. This section will discuss primarily the regulation of transcription and translation initiated by ligand-receptor binding at the cell surface.

Control of transcription

One important group of transcription factors, regulated by intracellular signals (see De Cesare et al., 1999), has conserved basic (B) and leucine zipper (Zip) domains (hence they are called *Bzip proteins*, see Chapter 3, Fig. 5). These domains control the dimerization required for DNA binding. The Bzip proteins have distinct domains for DNA binding, dimerization and transcriptional activation (Angel and Karin, 1991). Two families of the signal regulated transcription factors have been studied in detail: AP-1 and CREB

proteins. AP-1 proteins are a family of structurally related transcription factors of the Jun-Fos family. The signal transduction of AP-1 initially involves tyrosine kinases and phospholipid turnover (Cantley et al., 1991). Eventually, AP-1 is activated by PKC and in turn activates a number of genes. CREB is the so-called *cAMP-response element binding protein*. CREB proteins are cAMP-regulated (see Section IE) and have been found to induce gene transcription in response to activation of the PKA pathway (Karin, 1992). Two other groups of proteins within this family, the *c-AMP-responsive elements modulators* (CREMs) and the *activating transcription factor-1* (ATF-1), are also regulated by cAMP.

CREBs, CREMs or ATF-1 are frequently the final targets involved in cell proliferation and differentiation as well as signal transduction. These cAMP-responsive factors regulate many physiological functions such as memory and long term potentiation (Silva et al., 1998), circadian rhythms (Foulkes et al., 1997), pituitary function (Struthers et al., 1991) and spermatogenesis (see Sassone-Corsi, 1998). A variety of mitogens also operate with CREB and CREM as targets. These include *nerve growth factor* (NGF), *brain derived neurotrophic factor* (BDNF) and *epidermal growth factor* (EGF) which trigger a complex cascade (e.g., see Cohen, 1997). *Fibroblast growth factor* (FGF) and *tumor necrosis factor* (TNF) also activate a cascade of biochemical reactions that eventually phosphorylates CREB or ATF-1 (see Deak et al., 1998).

NGF and other neurotrophins support survival of neurons. In at least some neurons, CREB mediates the effect. Exposure of distal sympathetic neurons to NGF results in the phosphorylation of CREB at the transcriptional regulatory site of the protein (Riccio et al., 1997). Transmission of the signal requires the internalization of NGF and its receptor tyrosine kinase TrkA, as well as their transport from the nerve terminals (where endocytosis takes place) to the cell body. CREB-mediated gene expression is required for NGF-dependent survival (Riccio et al., 1999) and its activation was found to be effective even in the absence of NGF. In addition, the expression of Bcl-2 was activated by NGF in a CREB-dependent transcriptional mechanism. Bcl-2 is an inhibitor of apoptosis (see Chapter 2).

CREM and CREB contain in their amino terminal region, a phosphorylation box that is phosphorylated by protein kinases (Gonzalez and Montminy, 1989; de Groot et al., 1994) and two glutamine-rich domains flanking the phosphorylation sites (Laoide et al., 1993; Nakajima et al., 1997). In contrast, ATF-1 has only one glutamine rich domain. The phosphorylation site and at least one glutamine-rich region are needed for activation. In many cases, phosphorylation is required for the activation of CREB and CREM (on Ser 133 in CREB; Ser 117 in CREM). The phosphorylation allows them to interact with the coactivator *CREB-binding protein* (CBP) or p300 and converts them into powerful transcriptional activators. The complexes are thought to interact with the transcription mechanism (see Shikama et al., 1997). CBP and p300 have been implicated a number of cellular processes, such as cell proliferation, differentiation, DNA repair and apoptosis, by binding to a variety of transcription factors (see Shikama et al., 1997). Their action is complex. They are probably involved in recruitment of RNA polymerase II. Since they have acetyltransferase activity, they can acetylate histones thereby rearranging chromatin (see Chapter 2). They also acetylate activators and transcription factors (see Kouzarides, 1999). In addition, both CBP and p300 associate with other coactivators with acetyl-transferase activity (see Korzus et al., 1998; Xu et al., 1999).

The genes encoding CREB, CREM and ATF-1 produce many isoforms (see Montminy, 1997) by alternative splicing, alternative initiation codons and alternative intronic promoters (e.g., see Sassone-Corsi, 1995). Some of the isoforms of CREB or CREM are activators, while others are inhibitors of transcription. Repressors are formed generally by alternative splicing or insertion of premature stop codons. These forms lack some of the domains (e.g., one of the glutamine-rich regions) and lack the DNA-binding domain or the NLS. Repressor isoforms can also be produced by alternative translation-initiation sites.

An example of regulation of a transcription factor, CREB, in response to extracellular signals, is shown in Fig. 18 (Karin and Smeal, 1992). In this mechanism, PKA is directly stimulated by elevation of cAMP. The activation occurs by the binding of cAMP to the regulatory subunits, which release the catalytic subunits. These are transported into the nucleus, where they phosphorylate CREB and thereby activate this element and, subsequently, the appropriate genes (Nigg et al., 1985). PKA has frequently been found to be involved as shown in Fig. 18. (e.g., see Montminy, 1997). However, some signals (e.g., Sheng et al., 1991; de Groot et al., 1994; Ginty et al., 1994) act via different protein kinases to phosphorylate CREM and CREB at the same sites. After phosphorylation, these CREM and CREB interact with other coactivators which act on the transcriptional machinery (Montminy, 1997). In addition, some coactivators have been found that stimulate CREB and CREM without the need for phosphorylation (Fimia et al., 1999).

The activation of the target gene depends on binding to the cAMP-responsive element (CRE) of the gene consisting of eight nucleotide pairs (TGACGTCA), typically about 100 nucleotides from the TATA box. Apparently, the palindromic CRE can be separated into two CGTCA motifs on the same or opposite strands and they act cooperatively (Fink et al., 1988).

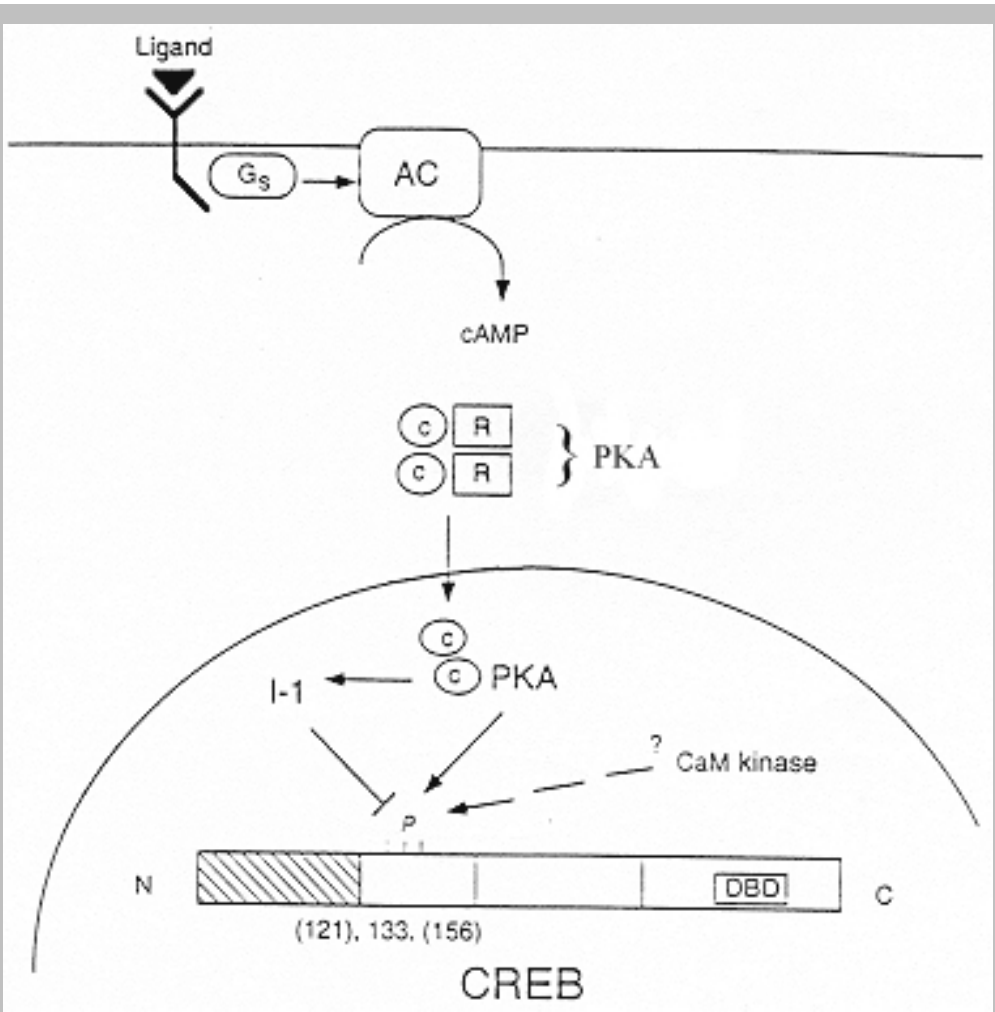


Fig. 18 The regulation of CREB activity in response to extracellular signals that elevate cAMP levels. The pathway that leads to the activation of PKA activity can be viewed as a simple two-component system, as shown. cAMP binds to the regulatory subunit of PKA (R), allowing the catalytic subunit (C) to dissociate and translocate to the nucleus, where it phosphorylates CREB on Ser133. Reproduced from *Trends in Biochemical Science*, vol.17, Karin,M. and Smeal,T., pp.418-422, copyright &copy1992, with permission from Elsevier Science.

Phosphorylation-dephosphorylation regulates transcription factors. However, the phosphorylation itself can either activate or block transcription. The mechanism by which phosphorylation regulates the binding to DNA may result from a conformation change, although electrostatic charge may play a role.

The details of transcription have been discussed in Chapter 3. They are summarized in Fig. 19 (Jackson, 1992). Transcription of specific genes is thought to be regulated by regulatory transcriptional factors and the corresponding domains in the DNA molecules. The transcription factors bind to elements flanking the transcription initiation site. They impart specificity and efficiency to the transcription catalyzed by RNA-polymerase II. The general transcription factor, TFIID, binds to the TATA box, directing the assembly of the transcriptional apparatus on the promoter DNA. This complex can function at low efficiency. The efficiency depends on sequence-specific DNA-binding regulatory factors such as Sp1, Fos and Jun proteins. The process can also be facilitated by binding of factors to the enhancer region, far away from the initiation site (occupied by X in the diagram). Growth factors and cytokines act indirectly on these

regulatory elements, as discussed above.

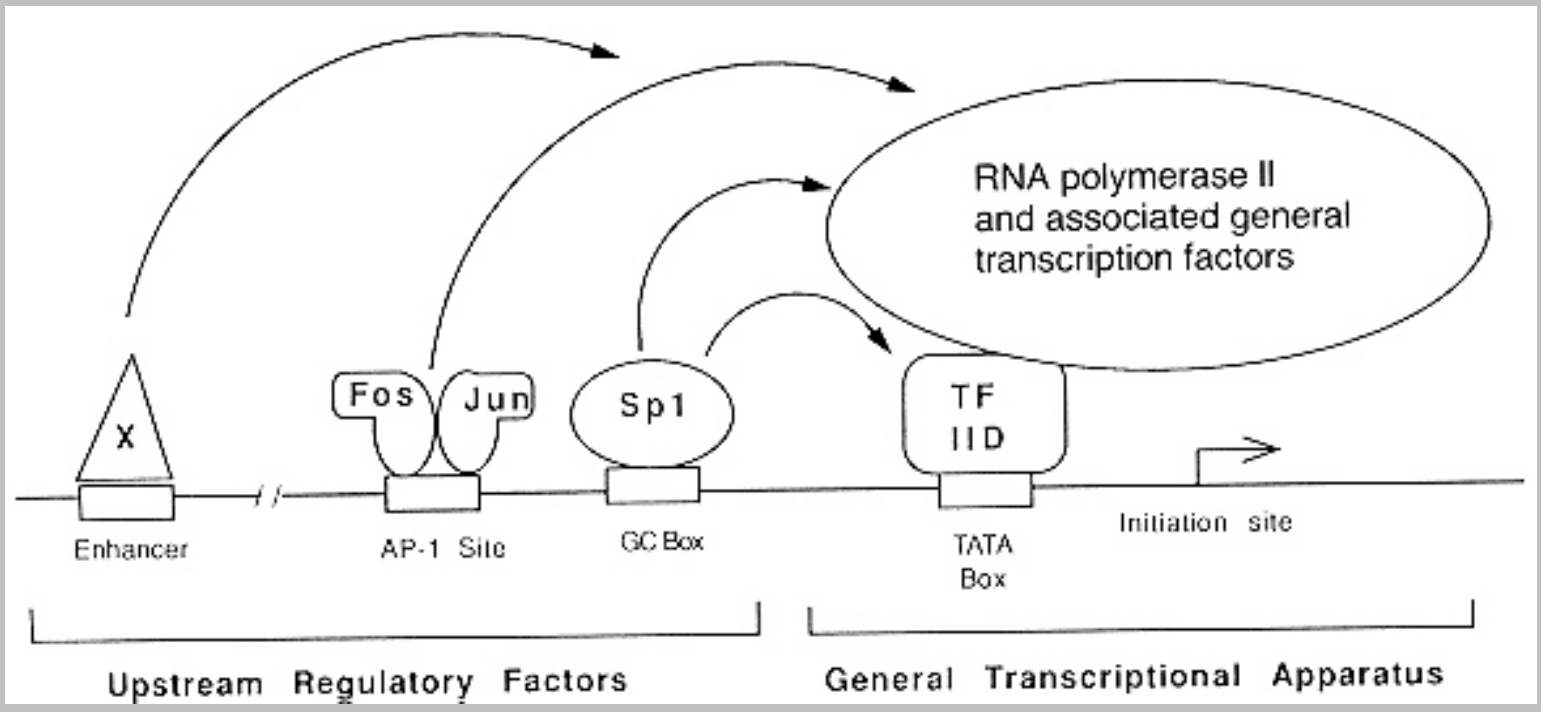


Fig. 19 Regulation of transcriptional activation. Reproduced from the *Trends in Cell Biology*,volume 2, Jackson, S.P., Regulating transcription factor activity by posphorylation, pp.104-108, copyright &copy1992,with permission from Elsevier Science.

First discovered in the study of the transcriptional activation induced by the interferons (IFN)-α and -γ, the JAK-STAT pathway has been found to be one of the most important signaling conduits from the receptors of cytokines and growth factors to the genes in the nucleus (see Schindler and Darnell, 1995). Typically, in this pathway, a member of the *Janus kinases* (JAKs) family is activated by the binding of the ligand (usually a cytokine) to a receptor associated with the kinase such as those of α, β and γ interferons (IFNs), interleukins (IL) (2 to 7, 10 to 13 and 15), erythropoietin, growth hormone, prolactin, thrombopoietin and others (see Leaman et al., 1996). The activation results in the phosphorylation of the JAK (two subunits dimerize and self-phosphorylate). In turn, the JAK phosphorylates a tyrosine of one of a family of cytoplasmic transcription factors, the *signal transducers and activators of transcription* (STAT) (see Darnell et al., 1994). The phosphorylation of the tyrosine in STATs is catalyzed by some receptors with intrinsic tyrosine kinase activity [e.g., the receptors of EGF, PDGF and colony-stimulating factor-1 (CSF-1)] rather than JAKs. Some of the STATs (1,3 and 5) also require the phosphorylation of a serine residue (e.g., Wen et al. 1995) thought to be catalyzed by members of the MAP kinase family (e.g., David et al., 1995). The methylation of an arginine residue of STAT1, catalyzed by the protein arginine methyl-transferase (PRMT), is also required for this activation (Mowen et al., 2001).

There are several mammalian JAKs. Phosphorylation of the kinases is the first of three phosphorylations before STAT activation. After activation of the receptors, the JAKs phosphorylate tyrosine sites of the *Src-homology 2* domain (SH2) of the receptor. These phosphorylated sites serve as docking sites for STATs. The STATS attached to the receptors are phosphorylated on tyrosine. This chain of events seems to be

general for the STATs pathway. STAT homo or heterodimes are capable of binding DNA.

The STATs, constitute a family of transcription factors (see Levy and Darnell, 2001). Inactive STATs are present in the cytoplasm or on the cytoplasmic face of the plasma membrane. The phosphorylated STATs dimerize by binding through the phosphotyrosine moieties of their *Src-homology 2* domain (SH2) (see Chapter 6). The dimers, after translocation into the nucleus, mediate the transcription of many different genes.

STATS are coded in mammals by seven genes. The genes are transcribed by differential splicing to increase the number of STATs. Different cytokines activate different subsets of STATs (reviewed in Schindler and Darnell, 1995).

Five of the seven known STATs are restricted as to cell type. The STATs bound to DNA interact with a number of DNA-binding proteins suggesting very varied responses. STAT activation is generally transient. Deactivation may be from dephosphorylation by protein tyrosinephosphatases (they remain activated in the presence of phosphatase inhibitors) or by degradation (inhibitors of proteasome activity prolong their activity). Other proteins block the STATs by binding receptor sites or JAK sites (*supressor of cytokine signaling*, SOCS) and also are able to initiate STAT degradation via the ubiquitin -proteasome pathway (Krebs and Hilton, 2001). The transcription of SOCS genes is increase by the level of STATs forming a negative feedback circuit. Naturally present truncated forms of STATs (generated by alternative splicing) have also been found to inhibit the activity of STATs.

Transcriptional responses require binding of a variety of factors to specific DNA elements. Effective initiation of transcription probably requires coordination in time and space of a variety of different factors. Some genes, such as, *c-fos* involve different DNA elements. Full activation requires coordinate activation of most or all of the elements (Hill and Treisman, 1995; Robertson et al., 1995)

A model incorporating our present knowledge is shown in Fig. 20. EGF, PDGF, CSF-1, and other receptors that utilize heterotrimeric G proteins are also thought to follow this mechanism.

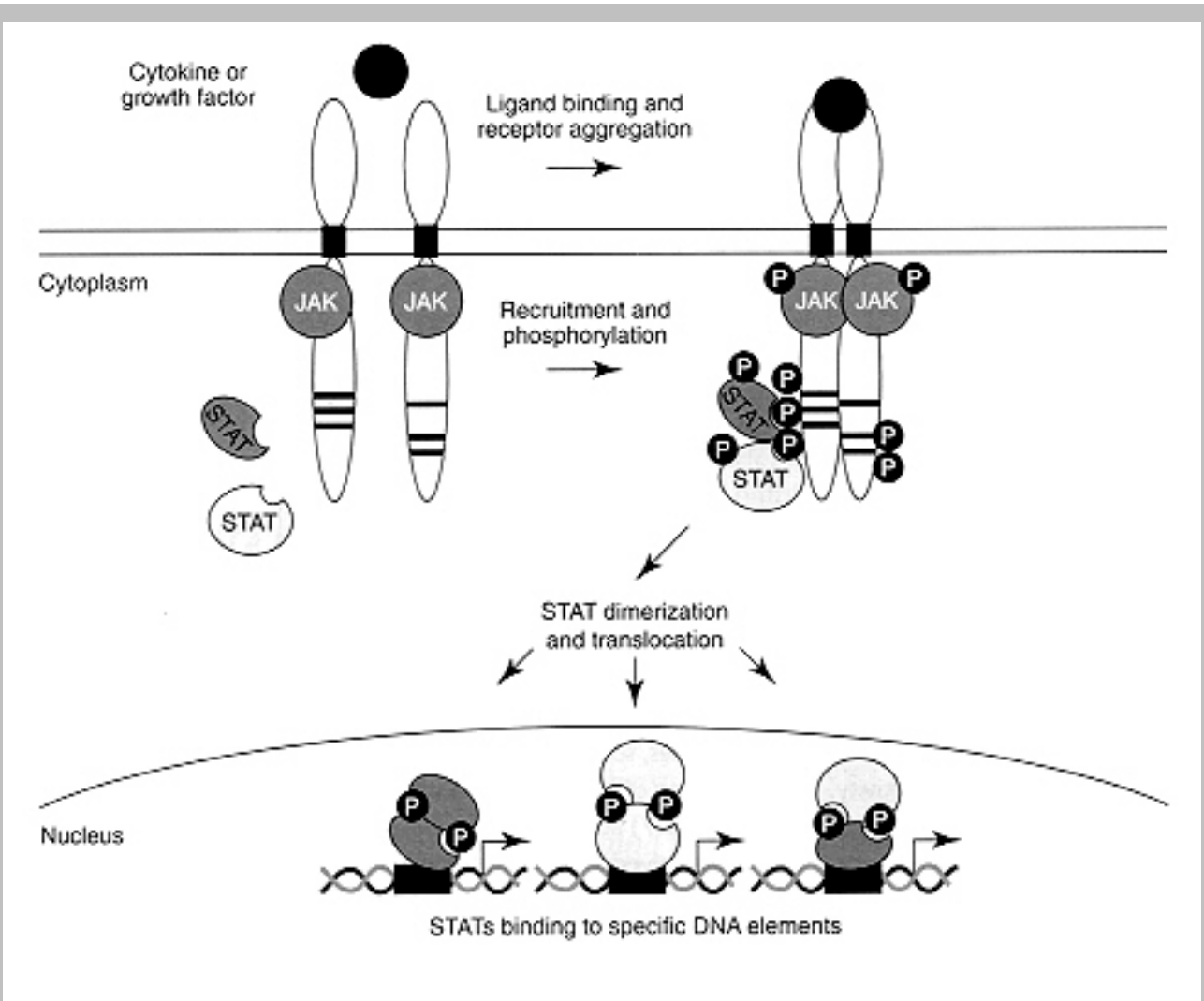


Fig. 20 A model of STAT activation by cytokine receptors. Ligand binding produces an aggregation of the receptors. JAK, bound to the receptor, is phosphorylated. The activated JAKs phosphorylate the receptor's tyrosines, providing docking sites for the STATs. The STATs are recruited by the receptor by binding their SH2 domains with the phosphorylated tyrosine of the receptors. The STATs are also phosphorylated and they dissociate from the receptors and form homo- or heterodimers by reciprocal interactions of their SH2 domains and the phosphorylated tyrosine residue. The STAT dimers are translocated to the nucleus, bind to response elements, and activate transcription of genes. JAK, Janus kinase; P, phosphate; STAT, signal transducer and activator of transcription. Reproduced from *Trends in Cell Biology*, vol.6, Briscoe et al., JAKs and STATs branch out, pp.336-340, copyright 1996, with permission of Elsevier Science.

As shown in Fig. 20, the activation of receptors is responsible for the expression of genes. The receptors frequently act through JAKs that, in turn, phosphorylate STAT transcription factors, recognized by promoter sites. Several inhibitors have been found that inhibit receptor phosphorylation and STAT activation, some by interacting with JAKs directly, possibly by acting as a negative feed-back regulator of cytokine action (e.g., Naka et al., 1997).

Cross-talk between the various pathways are know to occur. One example involves the action of the *growth hormone receptor* (GHR). GHR belongs to the cytokine receptor superfamily (Ihle et al., 1994). Its activation by growth hormone is followed by binding and activation of Jak2 (Argetsinger et al., 1993). The

resulting cascade involves the activation of several cytoplasmic proteins, such as the mitogen-activated protein (MAP) kinases and eventually involves the expression of specific genes. In addition, in response to GHR activation, Jak2 phosphorylates the receptor of the epidermal growth factor (EGFR) (that is an autokinase). Apparently this phosphorylation is needed to provide docking sites for components of the GH cascade (Yamauchi et al., 1997).

Cross-talk can result in enhancement (synergy) or diminution (antagonism). Cross-talk can take place through the coactivator molecules p300 and CBP. These large proteins interact with a variety of transcription factors through several domains and are thought to serve as a scaffold to bring several transcription factors in contact with the basal transcription machinery. In mice, deficiency in p300 or CBP shows that they are essential and in limited supply. In humans, deficiencies lead to a variety of malignancies (see Giles et al., 1998). Some of the negative interference between pathways may be the result of the competition for binding one of these two proteins (e.g., Kamei et al., 1996; Yao et al., 1998).

Cross talk between *leukemia inhibitory factor* (LIF) and *bone morphogenetic protein 2* (BMP2) has been studied in some detail (Nakashima et al., 1999). The two proteins are survival and differentiation factors for neurons. They induce separate transduction pathways that activate distinct transcription factors (STAT3 and Smad1, respectively). p300 facilitates the synergistic interaction of pathways activated by LIF and BMP2 in the differentiation of fetal neuroepithelial cells into astrocytes. The LIF cascade acts through STAT3 that activates the target genes (see Darnell, 1997; Heinrich et al., 1998). The BMP2 cascade acts through a Smad1-Smad4 complex (see Massaguè, 1998). The three proteins form a complex with p300 (Nakashima et al., 1999) as shown by immunoprecipitation and act synergistically in activating the promoter of *glial fibrillar acidic protein* (GFAP) by binding different DNA sites. The actual mechanism of activation is open to question. One possibility is which p300 that has histone acetyltransferase activity may favor gene transcription (see Chapter 2) or activate transcription factors (e.g., see Boyes et al., 1998; Hung et al., 1999).

Control of translation

Regulation may occur post-transcriptionally, as suggested by the observation that the synthesis of specific proteins does not reflect the relative abundance of mRNA molecules. Translational factors may be able to select mRNA molecules. The process of initiation of the translation is most likely to be regulated because generally it is rate limiting (e.g., Jagus et al., 1981). At least two of the initiation factors appear to be regulated. The eIF-4F initiation factor has three subunits that bind to the 5'-cap structure of eukaryotic mRNA (m^7pppX, where X is a nucleotide). Its probable involvement in regulation is shown by its ability to transform cells so that they continue dividing when eIF-4F is overexpressed in fibroblasts (Lazaris-Karatgzas et al., 1990). Similarly, when microinjected into quiescent fibroblasts, eIF-4F induces DNA synthesis (Smith et al., 1990a).

A variety of mitogenic factors have been shown to phosphorylate eIF-4F (at ser^{53}) (e.g., Frederickson et al., 1991) and other initiation factors. Similarly, a ribosomal subunit, S6, is phosphorylated by agents that

induce growth (Traugh and Pendergast, 1986).

A mechanism proposed for the control at the eIF-4E step is shown in Fig. 21 (Frederickson and Sonenberg, 1992). The binding of growth factors (A) activates several protein kinases (B) so that they phosphorylate eIF-4E. eIF-4E-P is now activated and initiates translation.

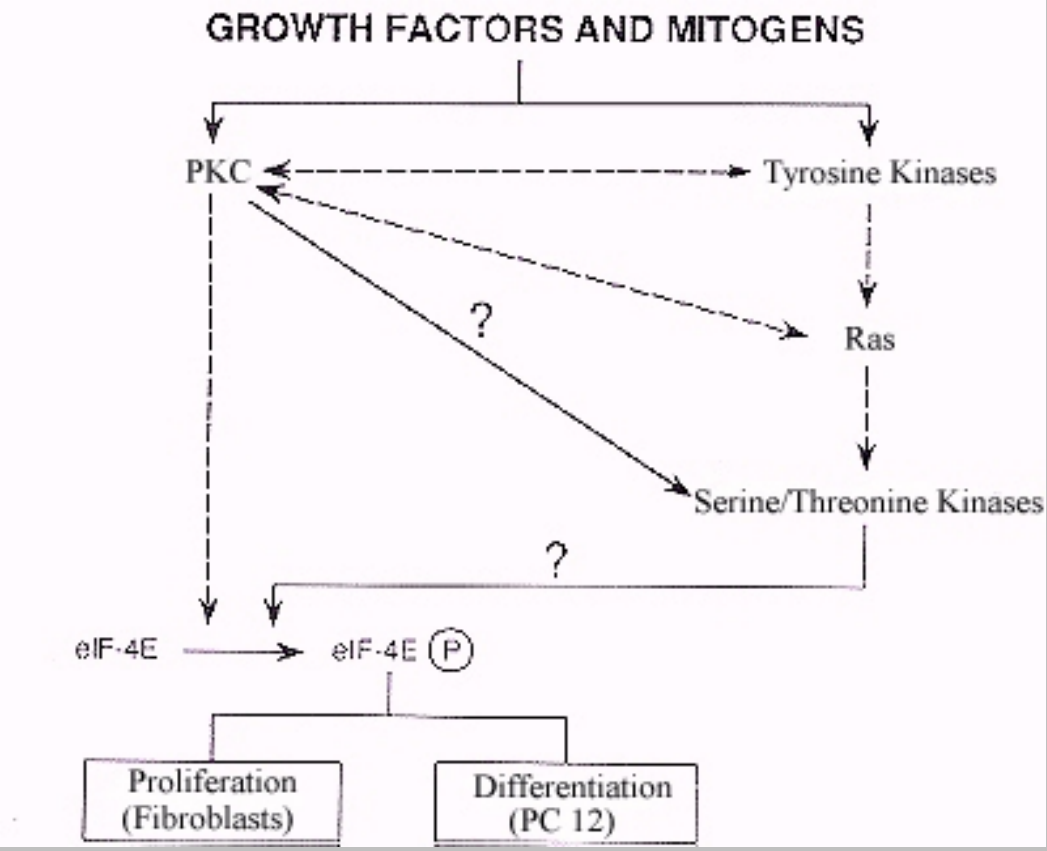


Fig. 21 Model for signal transduction to eIF-4E. The figure illustrates our current understanding of the relay of extracellular signals to eIF-4E, resulting in its phosphorylation. Both PKC-dependent and independent pathways are implicated. Broken lines depict possible indirect signalling sequences. Solid lines accompanied by a question mark indicate pathways for which there exists no definitive evidence, but which are nonetheless possible (from Frederickson and Sonenberg, 1992). Reproduced by permission.

C. Signaling Networks

The study of the signaling pathways has necessarily examined each pathway separately, although interactions between various pathways has been obvious even with limited information. More recently the concept has emerged that these pathways are interconnected and form complex signaling networks (see Jordan et al., 2000). Although the individual receptors and the eventual response of the cells may differ from cell type to cell type, the patterns of the networks are very similar.

The convergence of pathways (e.g., the same component may receive signals from various receptors at so-called *junctions*), allows for the integration of signals. The divergence of the pathways (at so-called *nodes*) gives rise to diverse effects from the same signal. In addition, the same element may be involved in either

function.

Adenylyl cyclases are capable of receiving signals from different sources (see, Pieroni et al., 1993) and the signals are integrated as reflected in the cAMP level. On the other hand, the protein kinase activated by cAMP can produce divergent effects for example, on gene expression, enzyme activity or channel activity. The receptor tyrosine kinases which direct growth factor signals through different pathways (see Schlessinger, 2000) are another example of a node.

Although complexes were mentioned, most of the sections presented above, concentrated on a relatively simple and static picture. In contrast, signaling proteins are frequently present in dynamic complexes and may be assembled in response to a signal. One such complex is that of the postsynaptic region below the neuronal membrane (see Chapter 22) in the central nervous system of vertebrates. The signaling complexes are assembled at the location of cytoskeletal elements (see Kennedy, 1997). As many as seventy seven proteins are associated with the NMDA receptor complex (see Chapter 22) in the mouse brain (Husi et al., 2000)! They include receptor molecules, adaptors, signaling and cytoskeletal proteins and several proteins of unknown function. Scaffold proteins targeted to various location of the cell, play a prominent role in the assembly of these complexes and a large number of AKAPs have been found (e.g. Edwards and Scott, 2000). AKAPs bind simultaneously a variety of protein kinases and phosphatases and sometimes actin (e.g., Westphal et al., 2000). Scaffold proteins are responsible for the recruitment of the components that constitute the nodes and junctions. Furthermore, their the assembly and disassembly depend on activity.

Another question of some importance is how signals that act by activating the same pathways (e.g., through receptor tyrosine kinases) can provide specificity (see Simon, 2000). In these cases, the strength and duration of the signal may play a role. In addition, the response may depend on the simultaneous activation of other pathways. Alternatively, cells may differ in their responses depending on their history (e.g., presence or absence of certain components).

V. LIGHT ACTIVATION IN PLANTS

Light not only provides energy in photosynthesis (see Chapter 17) but also regulates much of the life cycle of plants (e.g., see Fankhauser and Chory, 1997). Not surprisingly, they possess a complement of photoreceptors which regulate gene expression. Some of these photoreceptors have been intensely studied. Others such as those sensitive to UV-B light are yet to be identified. The nature of these photoreceptors and how the light signal is transduced to affect gene expression are questions of great interest. The involvement of photoreceptors in the control of clocks is discussed in Chapter 15.

Cryptochromes (CRY1 and CRY2) and the *non-phototrophic hypocotyl 1* (NPH1) respond to UV-A and blue light (e.g., Suarez-Lopez and Coupland, 1997). Five phytochromes have been identified [in *Arabidopsis*, phytochromes A to E (phyA to phyE)] and they are activated by red and far-red light (see Mathews and Sharrok, 1997).

The CRYs are pterin/flavin containing proteins (e.g., see Cashmore et al., 1999) which resemble in amino acid sequence repair photolyases. The phytochromes are homodimers of about 124 kDa. Each subunit is covalently bound to a linear tetrapyrrole chromatophore (also called *phytochromobilin*). Red light converts the phytochromes from an inactive form to an active form. The effect is reversible and far-red light converts them back in the inactive form. Photoisomerization between rings C and D of the chromatophore moiety activates by causing a conformational change in the holoprotein (see Quail, 1997). NPH1 is a 120 kDa protein (Huala et al., 1997) which binds non-covalently to flavin mononucleotide (Christie et al., 1998).

How is the light signal communicated dowstream? It would not be surprising to find biochemical cascades similar to those following the activation of other receptors already discussed. Emerging information is compatible with this thought. NPH1, is a serine-threonine protein kinase which autophosphorylates (Christie et al., 1998). phyA has been shown to autophosphorylate at serine and threonine residues in response to light (Yeh et al., 1998). Apparently, the *phytochrome kinase substrate-1* (PKS1) is phosphorylated by phyA (Fankhauser et al., 1999). PKS1 negatively regulates phytochrome signaling. The action of phyA and B requires a reaction partner *phytochrome-interacting factor-3* (PIF-3) (Ni et al., 1998, 1999). PIF3 and its binding to the activated phytochromes was found using a yeast two-hybrid screen. The interaction is terminated when the activation by light is stopped. What is known about PIF3 suggests a role in gene expression. The amino acid sequence of PIF3 has domains similar to the bHLH DNA-binding domain (see Chapter 3 and Chapter 15) of transcription factors and in addition a bipartite NLS domain (allowing it to be localized in the nucleus) (see Chapter 5). PIF3 also has a PAS domain required for interaction with other proteins. PIF3 binds to wild-type C-terminal domains of both phyA and phyB. Its localization into the nucleus when overexpressed suggests an interaction between the photoreceptor and a transcriptional regulator. Overexpression of PIF3 in the sense orientation increases light sensitivity (Ni et al., 1998). In contrast the antisense orientation decreases it. After activation, phyB is translocated into the nucleus as shown by GFP-phyB chimeras (Yamaguchi et al., 1999), arguing for an involvement of PIF3 (constitutively in the nucleus) and phyB (translocated into the nucleus in response to light) in the regulation of transcription by light. In contrast, phyB-GFP fluorescence was found throughout the cell in dark-grown seedlings. It is interesting to note that CRY1-GFP chimeras are also found to be localized to the nucleus (Cashmore et al., 1999Chapter 1.

In *Arabidopsis thaliana*, a component in the phyA pathway, SPA1 (for "suppressor of phyA-105"), (Hoecker et al., 1999) represses the activation of the phytochrome and is needed for the photosensory specificity of phytochrome A. SPA1 is a WD (tryptophan-aspartic acid)-repeat protein that is similar in amino acid sequence to protein kinases.

In addition to the effects discussed above, phytochromes also control Ca^{2+} permeability of plasma membrane and induce transient changes in the Ca^{2+} concentration in the cytoplasm (e.g. see Volotovski, 1998). In addition, the endoplasmic reticulum and vacuole act as intracellular Ca^{2+} stores under phytochrome regulation. The regulation of the release from intracellular stores may be indirect since phytochromes also stimulate the hydrolysis of phosphatidyl 4,5-bisphosphate, a precursor of inositol 1,4,5-

trisphosphate (see Section IF).

VI. SIGNALS FROM ORGANELLES

We have seen how chemical signals can be transmitted within the cell after binding of the ligand by receptors, either on the cell surface or in the cytoplasm. The binding is followed by a cascade of events some culminating in the activation of transcription of specific genes.

Organelles can also elicit transcriptional responses with changing physiological needs. These mechanisms are very important. The composition and size of organelles must be able to adjust to new conditions and needs (see Nunnari and Walter, 1996). An increase in secretory activity during differentiation leads to an increase in ER (Wiest et al., 1990g). An increase in the demand for energy in muscle produces an increase in mitochondria (Hood et al., 1994). These changes appear to reflect changes at the level of transcription (see Nunnari and Walter, 1996). As might be expected, some of the details resemble those of the pathways we already examined. However, some of the details are very different.

Agents inducing peroxisomes proliferation (e.g., clofibrate), for example, activate a transcription factor, *peroxisome proliferator activated receptor* (PPAR), which coordinates peroxisomal gene expression (Lee, 1995; Varanasi et al., 1996). These genes contain a PPAR response element (PPRE). Many ligands for PPAR-α are metabolized in peroxisomes (e.g., postaglandins). Several diseases caused by peroxisomes malfunction lead to an excess of transcription of the genes activated by this receptor (Wolfrum et al., 1999). Generally diseases of peroxisome malfunction can be traced to a mutation affecting a gene coding a peroxisomal enzyme (e.g. Refsum disease) or a mutation affecting a gene involved in the maintenance of the organelle (e.g., peroxisome-biogenesis disorder, PBD) (see Lazarow and Moser, 1995; Tabak et al., 1999). Several signal pathways are responsible for the maintainance of the multiple functions of the ER. The ER is the first compartment to receive secreted proteins (see <a href=" 10part1.html#I">Chapter 10). N-linked carbohydrates are added when the proteins enter the ER (see Helenius, 1994). In the ER lumen, the proteins are folded by resident chaperones. These chaperones are the so-called heat shock proteins (Hsps), such as BiP and accessory proteins (see Gething and Sambrook, 1992). The ER is also involved in the sequestration of Ca^{2+} (see Section I A, above and Chapter 21) and in the oxidations of a variety of chemicals such as pesticides and some endogenous substrates (Chapter 16).

ER proliferation is probably controlled by the production of ER enzymes. In the case of phenobarbital induced proliferation, the trigger is probably the excess production of the cytochrome P450 (Kanai et al., 1986) and other proteins that detoxify the drug. In most cases, ER proliferation requires both the synthesis of ER proteins and phospholipid components. These activities are probably regulated together (Cox et al., 1997). There are indications that some of the regulatory functions of the ER are achieved indirectly via activation of gene expression and interactions with the cytoskeleton (see above).

The study of the intracellular signal pathways have barely begun. Many of the aspects currently uncovered are involved in membrane homeostasis (such as sterol metabolism and caveolin which is closely associated

with cholesterol; see Chapter 9) and the mechanisms of folding of proteins. The common mediator, comparable to a second messenger, originates from *intramembrane proteolysis* (Rip). In Rip, the cleavage of proteins in the plane of the membrane liberates fragments which are translocated into the nucleus and regulate gene transcription (see Brown et al., 2000). Most known Rips involve transmembrane proteins of the ER or Golgi apparatus. The intramembrane proteolysis requires a protease, a polytopic membrane protein such as presenilin-1 (probably zinc metalloproteases, see Rudner et al., 1999). There are several examples of this kind of signal.

The regulation of the cholesterol level in animals, involves the cleavage of membrane-attached transcription factors, called *sterol regulatory element-binding proteins* (SREBPs) in the Golgi apparatus. This cleavage and release into the cytoplasm is triggered by a sterol-sensing protein, SCAP, which forms a complex with SREBPs in the ER. In sterol-depleted cells, SCAP is needed to transport SREBPs from ER to Golgi, where SREBPs are cleaved by a protease to form smaller molecules (Duncan et al., 1998). Sterols block this transport and abolish cleavage (see DeBose-Boyd et al., 1999). The SREBPs are transcriptional regulators activating the genes containing the *sterol-regulatory element* (SRE) in their promoters (Smith et al., 1990b). These genes encode enzymes of cholesterol and fatty acid synthesis (see Brown and Goldstein, 1997; Pahl and Bauerle, 1997a).

The accumulation of integral proteins in the ER elicits the *ER overload response* (EOR) (see Pahl and Bauerle,1997b). Some of the signals that activate EOR also activate the *unfolded protein response* (UPR) (see below and Chapter 10), but the two follow separate pathways. The EOR is involved in the activation of NF-κ-B, a protein which is responsible for initiating the transcription of interferons and cytokines during inflammation and immune responses. EOR is thought to be activated by an excess production of viral proteins and probably is not involved in normal biosynthesis of ER components. In contrast, the accumulation of unfolded protein in the ER induces UPR, the activation of genes which code for ER-resident enzymes that function in protein folding (see Chapman et al., 1998) of all eukaryotic cells, although most of our knowledge comes from *Saccharomyces cerevisiae*.

The activated genes have a common sequence in their promoters, the *unfolded protein response element* (UPRE) (Mori et al., 1992). The UPR also activates enzymes, playing a key role in phospholipid synthesis (see below). The UPR is initiated by a variety of stresses, such as inhibition of disulfide bonding with reducing agents, inhibition of glycosylation (e.g., by the drug tunicamycin) or expression of abnormal proteins that do not fold properly. A variety of abnormal conditions also initiate the UPR: a block of ER to Golgi transport (Liu et al., 1992), depletion of the Ca^{2+} in the ER (Li et al., 1993), inhibition of protein degradation (Bush et al., 1997) or overproduction of secretory or membrane proteins. The actual initiating signal is not unfolded protein unless they bind to BiP. One of the current ideas is that the ER responds to the amount of free BiP. For example, reduced levels of BiP induce the transcription of ER resident protein genes (Hardwick et al., 1990; Beh and Rose, 1995).

Unlike yeast UPR, mammalian UPR is similar to that reported for cholesterol homeostasis. The UPR is initiated by the transmembrane protein, ATF6. ATF6 is converted into a smaller water soluble molecule, by

proteolytic cleavage in ER-stressed cells. The water soluble segment is translocated into the nucleus where it acts as a transcription factor to express protein involved in the folding response (see Haze et al., 1999). However, mammals have also an additional pathway similar to that in yeast. An isoform of Ire1p is activated by UPR (see below). The proteolytically liberated cytoplasmic domain of Irep enters the nucleus and is thought to interact with a mammalian version of HAC1-mRNA (Niwa et al., 1999). Apparently, the proteolytic step requires the presence of presenilin-1 (see Niwa et al., 1999).

In *Saccharomyces cerevisiae* the response follows a distinct pathway. The IRE1 gene is required (Cox et al., 1993; Mori et al., 1993) for the synthesis of phospholipid (see below) and, under stress conditions, for the induction of the chaperone molecule, BiP, encoded by KAR2. IRE1 codes for the transmembrane receptor Ire1p/Ern1p which contains a Ser/Thr protein kinase and endoribonuclease activity. This protein transmits the UPR from the ER to the nucleus. Activation of Ire1p kinase induces its endoribonuclease activity to cleave unspliced HAC1 mRNA and generate exon fragments that are subsequently ligated by tRNA ligase (RLG1). The spliced HAC1 mRNA is efficiently translated. The amino terminal of Irep is in the ER lumen (or inner nuclear membrane) whereas the carboxy-terminal is in the nucleus or cytoplasm. A domain at the amino terminal is thought to be responsible for its activation in response to unfolded protein. A domain at the carboxy terminal acts as a kinase that initiates a cascade of events. Irep oligomerizes in the plane of the membrane and is phosphorylated by a neighboring Irep molecule (Shamu and Walter, 1996). The cascade of events that follows is unique (see below).

In yeast, a second component of the UPR, the basic-leucine zipper (see Chapter 3, Fig. 5) transcription factor (bZIP) Hac1p, is another element of the cascade (Cox and Walter 1996; Mori et al., 1996; Nikawa et al., 1996). Hac1p is induced by the UPR and is regulated by the alternative splicing of its mRNA, which replaces the carboxy terminal tail of Hac1p with a different peptide. A 252-nucleotide intron is present in the uninduced mRNA (*HAC1*^{u}mRNA). *HAC1*^{u}mRNA is transferred to the cytoplasm after transcription. However, no Hac1p^{p} protein is produced probably because the intron of this mRNA blocks translation. The splicing results in the shorter induced *HAC1*mRNA (*HAC1*^{i}mRNA). Hac1p specifically binds to UPRE in vitro and activates transcription in vivo. Hac1p could only be detected in cells that had an activated UPR, suggesting that its action is not controlled by a change in conformation but a change in concentration.

The splicing of the *HAC1*^{i}mRNA proceeds by a unique splicing pathway (Sidrauski et al., 1996) operating independently from the spliceosome (discussed in Chapter 3). The results are consistent with a model in which accumulation of unfolded protein activates Irep which is required for the induction of cleavage of *HAC1* mRNA. The ligation of *HAC1*mRNA is then a function of tRNA-ligase. Apparently the cleavage of *HAC*^{u} is catalyzed by Ire1p acting as an endonuclease (see Kawahara et al., 1998; Sidrauski and Walter, 1997). The ligating enzyme is coded in yeast by the gene *RLG1*.

The ER is the site of most lipid synthesis and production of membranes. The enzymes required for phospholipid synthesis are controlled in yeast by the levels of inositol (see Henry and Patton-Vogt, 1998). The promoters of genes coding enzymes of phospholipid biosynthesis (Carman and Henry, 1989) contain an element (UAS_{ino}) needed for their activation. Transcriptional regulation by the inositol involves the

transcriptional repressor *OPII* (White et al. 1991). The sequence of Opi1p reveals a leucine zipper domain. Two other transcription activators are also involved (Hirsch and Henry, 1986). The inositol response is regulated, along with the UPR, by either unfolded proteins or inositol level. UPRE mutants fail in the transcription of UAS_{ino} controlled genes. However, the mechanisms of these interactions are not clear at this time.

The proteolysis of membrane-bound precursors of signaling molecules is involved in the control of unsaturated fatty acid levels and at least in one case (SPT23) the regulation of fatty acid pools (Hoppe et al., 2000). Two transcription factors (SPT23 and MGA2) are present as inactive precursors in the ER membrane and the nuclear envelope. In this case, the proteolytic activation involves the ubiquitin-proteasome system and not membrane bound proteases. Yeast SPT23 and MGA2 correspond to NF-κB of mammals.

SUGGESTED READING

Calcium

Alvarez, J., Montero, M. and García-Sancho, J. (1999) Subcellular Ca^{2+} dynamics, *News Physiol. Sci.*, 14:161-168.

Ashley, R.H. (1995) Intracellular calcium channels, *Essays in Biochemistry* 30: 97-117. (Medline)

Berridge, M.J., Bootman, M.D. and Lipp, P. (1998) Calcium--a life and death signal, *Nature* 395:645-648. (Medline)

Berridge, M.J., Lipp, P. and Bootman, M.D. (2000) The versatility and universality of calcium signalling, *Nature Rev. Mol. Cell Biol.* 1:11-21.

Fankhauser, C. and Chory, J. (1999) Photomorphogenesis: Light receptor kinases in plants! *Curr. Biol.* 9:R123-126. (Medline)

McCormack, J.G. and Denton, R.M. (1994) Signal transduction by intramitochondrial Ca^{2+} in mammalian energy metabolism, *News in Physiol. Scie.* 9:71-76

Meldolesi, J. (2002) Rapidly exchanging Ca^{2+} stores: ubiquitous partners of surface channels in neurons, *News Physiol. Sci.* 17:144-449. (MedLine)

Meldolesi, J. and Pozzan, T. (1998) The heterogeneity of ER Ca^{2+} stores has a key role in nonmuscle cell signaling and function, *J. Cell Biol.* 142:1395-1398. (Medline)

Robb-Gaspers, L.D., Rutter, G.A., Burnett, P., Hajnoczky, G., Denton, R.M. and Thomas, A.P. (1998)

Coupling between cytosolic and mitochondrial calcium oscillations: role in the regulation of hepatic metabolism, *Biochim. Biophys. Acta* 1366:17-32. (Medline)

Rutter, G.A. and Rizzuto, R. (2000) Regulation of mitochondrial metabolism by ER Ca^{2+} release: an intimate connection, *Trends Biochem. Sci.* 25:215-221. (Medline)

Sanderson, M.J. (1996) Intracellular waves of communication, *News Physiol. Sci*. 11:262-269.

Thomas, A.P., Bird, G.S.J., Hajnóczky, G., Robb-Gaspers, L.D. and Putney, J.W. Jr. (1996) Spatial and temporal aspects of cellular calcium signaling, *FASEB J.* 10:1505-1517. (Medline)

GTP-binding proteins

Freissmuth, M., Casey, P. J., and Gilman, A. G. (1989) G proteins control diverse pathways of transmembrane signaling, *FASEB J.* 3:2125-2131. (Medline)

Lefkowitz, R.J. (1998) G protein-coupled receptors. III. New roles for receptor kinases and β-arrestins in receptor signaling and desensitization, *J. Biol. Chem.* 273:18677-18680. (Medline)

Stryer, L. (1986) Cyclic GMP cascade of vision, *Annu. Rev. Neurosci.* 9:87-119. (Medline)

Stryer, L. and Bourne, H. R. (1986) G proteins: a family of signal transducers, *Annu. Rev. Cell Biol.* 2:391-420. (Medline)

Steroid hormones and nuclear receptors

Xu, L., Glass, C.K. and Rosenfeld, M.G. (1999) Coactivator and corepressor complexes in nuclear receptor function, *Curr. Opin. Genet. Dev.* 9:140-147. (Medline)

Phosphoinositides

Toker, A. and Cantley, L.C. (1997) Signalling through the lipid products of phosphoinositide-3-OH kinase, *Nature* 387:673-676. (Medline)

Various second messengers

Berridge, M.J. (1993) The tale of two messengers, *Nature* 365:388-389. (Medline)

Lee, H.C. (1994) The signaling pathway involving cyclic ADP-ribose, cGMP and nitric oxide, *News In Physiol. Sci.* 9:134-137.

Phosphorylation-dephosphorylation

Darnell, J.E. Jr. (1997) STATs and gene regulation, *Science* 277:1630-1635. (Medline)

Ihle, J.N. (1996) STATS: signal transducers and activators of transcription, *Cell* 84:331-334. (Medline)

Neary, J.T. (1997) MAPK cascades in cell growth and death, *News in Physiol. Sci.* 12:286-293.

Jackson, S.P. (1992) Regulating transcription factor activity by phosphorylation, *Trends in Cell Biol.* 2:104-108.

Multimolecular signaling complexes

Pawson, T. and Scott, J.D. (1997) Signaling through scaffold, anchoring, and adaptor proteins, *Science* 278:2075-2080. (Medline)

Schillace, R.V. and Scott, J.D. (1999) Organization of kinases, phosphatases, and receptor signaling complexes, *J. Clin. Invest.* 103:761-765. (Medline)

Tsunoda, S., Sierralta, J. and Zuker, C.S. (1998) Specificity in signaling pathways: assembly into multimolecular signaling complexes, *Curr. Opin. Genet. Dev.* 8:419-422. (Medline)

Signaling in intracellular organelles

Chapman, R., Sidrauski, C. and Walter, P. (1998) Intracellular signaling from the endoplasmic reticulum to the nucleus, *Annu. Rev. Cell Dev. Biol.* 14:459-485. (Medline)

WEB RESOURCES

Calcium oscillations and waves (associated movies):

Calcium waves home page

G-protein-coupled receptors

Michael Sanderson laboratory: Intercellular calcium waves

Calcium waves in Retinal Glial Cells Newman, R.A and Zahs,K.R.

Diwan, J.J., Ca^{2+} signals, http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/casignal.htm

Outline of signaling cascades in Transpath

Signal transduction cascades, http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/signals.htm

Nuclear receptors

REFERENCES

Search the textbook

Back to Chapter 7

REFERENCES

Aarhus, R., Dickey, D.M., Graeff, R.M., Gee, K.R., Walseth, T.F. and Lee, H.C. (1996) Activation and inactivation of Ca^{2+} release by $NAADP^{+}$, *J. Biol. Chem.* 271:8513-8516. (MedLine)

Adam-Klages, S., Schwandner, R., Adam, D., Kreder, D., Bernardo, K. and Kronke, M. (1998) Distinct adapter proteins mediate acid versus neutral sphingomyelinase activation through the p55 receptor for tumor necrosis factor, *J. Leukoc. Biol.* 63:678-682.(Medline)

Allbritton, A.L. and Meyer, T. (1993) Localized calcium spikes and propagating calcium waves, *Cell Calcium* 14:691-697.(Medline)

Alonso, M.T., Barrero, M.J., Michelena, P., Carnicero, E., Cuchillo, I., Garcia, A.G., Garcia-Sancho, J., Montero, M. and Alvarez, J. (1999) Ca^{2+}-induced Ca^{2+} release in chromaffin cells seen from inside the ER with targeted aequorin, *J. Cell Biol.* 144:241-254.(Medline)

Angel, P. and Karin, M. (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation, *Biochim. Biophys. Acta* 1072: 129-157.(Medline)

Argetsinger, L.S., Campbell, G.S., Yang, X., Witthuhn, B.A., Silvernnoien, O., Ihle, J.N. and Carter-Su, C. (1993) Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase, *Cell* 74:237-227.(Medline)

Atherton-Fessler, S., Hannig, G. and Piwnica-Worms, H. (1993) Reversible tyrosine phosphorylation and cell cycle control, *Seminars in Cell Biology* 4: 433-442.(Medline)

Babcock, D.F., Herrington, J., Goodwin, P.C., Park, Y.B. and Hille, B. (1997) Mitochondrial participation in intracellular Ca^{2+} network, *J. Cell Biol.* 136:833-844.(Medline)

Bakau, B. (ed.) (1999) *Molecular Chaperones and Folding Catalysts-Regulation, Cellular Function and Mechanisms*, Harwood Academic Publishers, Amsterdam.

Baksh, S., Burns, K., Andrin, C. and Michalak, M. (1995) Interaction of calreticulin with protein disulfide isomerase, *J. Biol. Chem.* 270:31338-31244. (MedLine)

Banfic, H., Zizak, M., Divecha, N.and Irvine, R.F. (1993) Nuclear diacylglycerol is increased during cell proliferation in vivo, *Biochem. J.* 290:633-636.(Medline)

Barbacid, M. (1995) Neurotrophic factors and their receptors, *Curr. Opin. Cell. Biol.* 7:148-155.(Medline)

Barford, D. (1996) Molecular mechanisms of the protein serine/threonine phosphatases, *Trends Biochem. Sci.* 21:407-412.(Medline)

Barrett, K., Leptin, M. and Settleman, J. (1997) The Rho GTPase and a putative RhoGEF mediate a signaling pathway for the cell shape changes in *Drosophila* gastrulation, *Cell* 91: 905-915.(Medline)

Bastianutto, C., Clementi, E., Codazzi, F., Podini, P., De Giorgi, F., Rizzuto, R., Meldolesi, J. and Pozzan T. (1995) Overexpression of calreticulin increases the Ca^{2+} capacity of rapidly exchanging Ca^{2+} stores and reveals aspects of their lumenal microenvironment and function, *J. Cell Biol.* 130:847-855.(Medline)

Beals, C.R., Clipstone, N.A., Ho, S.N. and Crabtree, G.R. (1997) Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction, *Genes Dev.* 11:824-834.(Medline)

Bean, A.J. and Scheller, R.H. (1997) Better late than never: a role for rabs late in exocytosis, *Neuron* 19:751-754.(Medline)

Beato, M. (1989) Gene regulation by steroid hormones, *Cell* 56:335-344.(Medline)

Beato, M. (1991) Transcriptional controls by nuclear receptors, *FASEB J.* 5:2044-2051.(Medline)

Becker, P.B., Gloss, B., Schmid, W., Strähle, U. and Schütz, G. (1986) In vivo protein-DNA interactions in a glucocorticoid response element require the presence of the hormone, *Nature* 324:686-688.(Medline)

Beh, C.T. and Rose, M.D. (1995) Two redundant systems maintain levels of resident proteins within the yeast endoplasmic reticulum, *Proc. Natl. Acad. Sci. USA* 92:9820-9823.(Medline)

Beltrán, B., Mathur, A., Duchen, M.R., Erusalimsky, J.D. and Moncada, S. (2000) The effect of nitric oxide on cell respiration: A key to understanding its role in cell survival or death, *Proc. Natl. Acad. Sci. USA*97:14602-14607. (MedLine)

Berman, D.M., and Gilman, A.G. (1998) Mammalian RGS proteins: barbarians at the gate, *J. Biol. Chem.* 273:1269-1272.(Medline)

Berridge, M.J., Lipp, P. and Bootman, M.D. (2000) The versatility and universality of calcium signalling,

Nature Rev. Mol. Cell Biol. 1:11-21. (MedLine)

Berridge, M.J. (1993a) Cell signalling. A tale of two messengers, *Nature* 365:388-389.(Medline)

Berridge, M.J. (1993b) Inositol triphosphate and calcium signalling, *Nature* 361: 315-325.(Medline)

Berridge, M. J. and Irvine, R. F. (1989) Inositol phosphate and cell signalling, *Nature* 341:197-205.(Medline)

Bhatt, R.R., Ferrell, J.E. Jr. (1999) The protein kinase p90 rsk as an essential mediator of cytostatic factor activity, *Science* 286:1362-1365.(Medline)

Billington, R.A. and Genazzani, A.A. (2000) Characterization of $NAADP^+$ binding in sea urchin eggs, *Biochem. Biophys. Res. Commun.* 276:112-116. (MedLine)

Bilwes, A.M, den Hertog, J., Hunter, T. and Noel, J.P. (1996) Structural basis for inhibition of receptor protein-tyrosine phosphatase- by dimerization, *Nature* 382:555-558.(Medline)

Bird, G.St.J., Rossier, M.F., Obie, J.F.and Putney, J.W. Jr. (1993) Sinusoidal oscillations in intracellular calcium requiring negative feed-back by protein kinase C, *J. Biol. Chem.* 268: 8425-8428.(Medline)

Bishop, A.L. and Hall, A. (2000) Rho GTPases and their effector proteins, *Biochem. J.* 348:241-255. (MedLine)

Blaeser, F., Ho, N., Prywes, R. and Chatila, T.A. (2000) Ca^{2+}- dependent gene expression mediated by MEF2 transcription factors, *J. Biol. Chem.* 275:197-209. (MedLine)

Blanco, J.C., Minucci, S., Lu, J., Yang, X.J., Walker, K.K., Chen, H., Evans, R.M., Nakatani, Y. and Ozato, K. (1998) The histone acetylase PCAF is a nuclear receptor coactivator, *Genes Dev.* 12:1638-1651.(Medline)

Blumberg, B. and Evans, R.M. (1998) Orphan nuclear receptors--new ligands and new possibilities, *Genes Dev.* 12:3149-3155.(Medline)

Bollen, M. (2001) Combinatorial control of protein phosphatase-1, *Trends Biochem. Sci.* 26:426-431. (MedLine)

Bonni, A., Brunet, A., West, A.E., Datta, S.R., Takasu, M.A. and Greenberg, M.E. (1999) Cell survival promoted by the ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms, *Science* 286:1358-1362.(Medline)

Bootman, M.D., Lipp, P. and Berridge, M.J. (2001) The organisation and functions of local Ca^{2+} signals, *J. Cell Sci.* 114:2213-2222. (MedLine)

Boriack-Sjodin, P.A., Margarit, S.M., Bar-Sagi, D. and Kuriyan, J. (1998) The structural basis of the activation of Ras by Sos, *Nature* 394:337-343.(Medline)

Bos, J.L. (1997) Ras-like GTPases, *Biochim. Biophys. Acta* 1333:M19-M31.(Medline)

Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuks, Z. and Kolesnick, R. (1995) Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals, *Cell* 82:405-414. (MedLine)

Boyes, J., Byfield, P., Nakatani, Y. and Ogryzko, V. (1998) Regulation of activity of the transcription factor GATA-1 by acetylation, *Nature* 396:594-598.(Medline)

Brady-Kalnay, S.M. and Tonks, N.K. (1995) Protein tyrosine phosphatases as adhesion receptors, *Curr, Opin. Cell. Biol.* 7:650-657.(Medline)

Braunewell, K.H. and Gundelfinger, E.D. (1999) Intracellular neuronal calcium sensor proteins: a family of EF-hand calcium-binding proteins in search of a function, *Cell Tissue Res.* 295:1-12. (MedLine)

Bredt, D. S. and Snyder, S. H. (1990) Isolation of nitric oxide synthetase, a calmodulin requiring enzyme, *Proc. Natl. Acad. Sci. USA* 87:682-685.(Medline)

Briscoe, J., Kohlhuber, F. and Müller, M. (1996) JAKs and STATs branch out, *Trends in Cell Biol.* 6:336-340.

Brown, M.S. and Goldstein, J.L. (1997) The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor, *Cell* 89:331-340.(Medline)

Brown, M.S., Ye, J., Rawson, R.B. and Goldstein, J.L. (2000) Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans, *Cell* 100:391-8 39. (MedLine)

Burd, C.G. and Emr, S.D. (1998) Phosphatidylinositol (3)-phosphate signaling mediated by specific bindig of RING FYVE domains, *Mol. Cell* 2:157-162.(Medline)

Burgoyne, R. D. and Cheek, T. R. (1991) Locating intracellular calcium stores, *Trends in Biochem. Sci.* 16:319-320.(Medline)

Bush, K.T., Goldberg, A.L. and Nigam, S.K. (1997) Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance., *J. Biol. Chem.* 272:9086-9092.(Medline)

Bustamante, J.O. (1994) Nuclear electrophysiology, *J. Membr. Biol.* 138:105-112.(Medline)

Caelles, C., González-Sancho, J.M., Muñoz, A. (1997) Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway, *Genes Dev.* 11:3351-3364.(Medline)

Campbell, K. P. , MacLennan, D.H., Jorgensen, A.O. and Mintzer, M.C. (1983) Purification and characterization of calsequestrin from canine cardiac sarcoplasmic reticulum and identification of the 53,000 dalton glycoprotein, *J. Biol. Chem.* 258:1197-1204.(Medline)

Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U. and Arkinstall, S. (1998) Catalytic activation of the phosphatase MKP-3 by EKR2 Mitogen-activated protein kinase, *Science* 280:1262-1265.(Medline)

Cancela, J.M. and Petersen, O. (1998) The cyclic ADP ribose antagonist 8-NH2-cADP-ribose blocks cholecystokinin-evoked cytosolic Ca^{2+} spiking in pancreatic acinar cells, *Pflügers Arch.* 435:746-748.(Medline)

Cancela, J.M., Mogami, H., Tepikin, A.V. and Petersen, O.H. (1998) Intracellular glucose switches between cyclic ADP-ribose and inosotol trisphosophate triggering of cytosolic Ca^{2+} spiking, *Curr. Biol.* 8:865-868.(Medline)

Cantley, L.C. and Neel, B.G. (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/ACT pathway, *Proc. Natl. Acad. Sci. USA* 96:4240-4245.(Medline)

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S.(1991) Oncogenes and signal transduction, *Cell* 64:281-302.(Medline)

Carey, K.L., Richards, S.A., Lounsbury, K.M. and Macara, I.G. (1996) Evidence using a green fluorescent protein-glucocorticoid receptor chimera that the Ran/TC4 GTPase mediates an essential function independent of nuclear protein import, *J. Cell Biol.* 133:985-996.(Medline)

Carman, G.M. and Henry, S.A. (1989) Phospholipid biosynthesis in yeast, *Annu. Rev. Biochem.* 58:635-669.(Medline)

Carrión, A.M., Link, W.A., Ledo, F., Mellstrom, B. and Naranjo, J.R. (1999) DREAM is a Ca2+-

regulated transcriptional repressor, *Nature* 398:80-84. (MedLine)

Cashmore, A.R., Jarillo, J.A., Wu, Y.J. and Liu, D. (1999) Cryptochromes: blue light receptors for plants and animals, *Science* 284:760-765.(Medline)

Cassel, D., Rothenberg, P. Zhuang, Y., Deuel, T. F. and Glaser, L. (1983) Platelet derived growth factor stimulates Na/H exchange and induces cytoplasmic alkalinization in NR6 cells, *Proc. Natl. Acad. Sci. USA* 80:6224-6228.(Medline)

Cerione, R. A., Sibley. D. R., Codina, J., Benovic, J. L., Winslow, J., Neer. E. J., Birnbaumer, L., Caron, M. G. and Lefkowitz, R. J. (1984) Reconstitution of a hormone-sensitive adenylate cyclase system, *J. Biol. Chem.* 259:9979-9982.(Medline)

Chakvarti, D., LaMorte, V.J., Nelson, M.C., Nakiajima, T., Schulman, I.G., Juguilon, H., Montminy, M. and Evans, R.M. (1996) Role of CBP/P300 in nuclear receptor signalling, *Nature* 383: 99-103.(Medline)

Chambon P. (1996) A decade of molecular biology of retinoic acid receptors, *FASEB J.* 10:940-954. (MedLine)

Chang, H.W., Aoki, M., Fruman, D., Auger, K.R., Bellacosa, A., Tsichlis, P.N., Cantley, L.C., Roberts, T.M. and Vogt, P. K. (1997) Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase, *Science* 276:1848-1850.(Medline)

Chao, T.S., Byron, K.L., Lee, K.M., Villereal, M. and, Rosner, M.R. (1992) Activation of MAP kinases by calcium-dependent and calcium-independent pathways. Stimulation by thapsigargin and epidermal growth factor, *J. Biol. Chem.* 267:19876-19883. (MedLine)

Chapman, R., Sidrauski, C. and Walter, P. (1998) Intracellular signaling from the endoplasmic reticulum to the nucleus, *Annu. Rev. Cell Dev. Biol.* 14:459-485.(Medline)

Chen, L., Durley, R., Poliks, B.J., Hamada, K., Chen, Z., Mathews, F.S., Davidson, V.L., Satow, Y., Huizinga, E., Vellieux, F.M., et al. (1992) Crystal structure of an electron-transfer complex between methylamine dehydrogenase and amicyanin, *Biochemistry* 31:4959-4964.(Medline)

Chen, Q. Lin, R.Y. and Rubin, C.S. (1997) Organelle-specific targeting of protein kinase AII (PKAII). Molecular and in situ characterization of murine A kinase anchor proteins that recruit regulatory subunits of PKAII to the cytoplasmic surface of mitochondria, *J. Biol. Chem.* 272:15247-15257.(Medline)

Cherfils, J. and Chardin, P. (1999) GEFs: structural basis for their activation of small GTP-binding proteins, *Trends Biochem. Sci.* 24:306-311.(Medline)

Chin, D. and Means, A.R. (2000) Calmodulin: a prototypical calcium sensor, *Trends Cell Biol.* 10:322-328. (MedLine)

Christie, J.M., Reymond, P., Powell, G.K., Bernasconi, P., Raibekas, A.A., Liscum, E. and Briggs, W.R. (1998) *Arabidopsis* NPH1: a flavoprotein with the properties of a photoreceptor for phototropism, *Science* 282:1698-1701.(Medline)

Chuang, D.M. and Costa, E. (1979) Evidence for internalization of the recognition site of β-adrenergic receptors during receptor subsensitivity induced by (-)-isoproterenol, *Proc. Natl. Acad. Sci. USA* 76:3024-3028. (MedLine)

Clapham, D.E. (1995) Calcium signaling, *Cell* 80:259-268.(Medline)

Clapham, D.E. and Neer, E.J. (1997) G protein βγ subunits, *Annu. Rev. Pharmacol. Toxicol.* 37:167-203.(Medline)

Clapham, D.E., Runnels, L.W. and Strubing, C. (2001) The trp ion channel family, *Nature Rev. Neurosci.* 2:387-396. (MedLine)

Clapper, D.L., Walseth, T.F., Dargie, P.J. and Lee, H.C. (1987) Pyridine nucleotide metabolites stimulate calcium release from sea urchin egg microsomes desensitized to inositol trisphosphate, *J. Biol. Chem.* 262:9561-9568.(Medline)

Clementi, E., Riccio, M., Sciorati, C., Nistico, G. and Meldolesi, J. (1996) The type 2 ryanodine receptor of neurosecretory PC12 cells is activated by cyclic ADP-ribose. Role of the nitric oxide/cGMP pathway, *J. Biol. Chem.* 271:17739-17745.(Medline)

Cobbold, P.H., Sanchez-Bueno, A. and Dixon, C.J. (1991) The hepatocyte calcium waves, *Cell Calcium* 12:87-95.(Medline)

Cohen, P. (1988) Protein phosphorylation and hormone action, *Proc. Roy. Soc., London, Ser. B*, 234:115-144.(Medline)

Cohen, P. (1989) The structure and regulation of protein phosphatases, *Ann. Rev. Biochem.* 58:453-508.(Medline)

Cohen, P. (1992) Signal integration at the level of protein kinases, protein phosphatases and their substrates, *Trends in Biochem. Scie.* 17:408-417.(Medline)

Cohen, P. (1997) The search for physiological substrates of MAP and SAP kinases in mammalian cells,

Trends Cell Biol. 7:353-361.

Cohen, P. and Cohen, P.T.W. (1989) Protein phosphatases come of age, *J. Biol. Chem.* 264:21435-21438.(Medline)

Colledge, M. and Scott, J.D. (1999) AKAPs: from structure to function, *Trends Cell. Biol.* 9:216-221.(Medline)

Cooper, C.E., Paterl, R.P., Bookes, P.S. and Darley-Usmar, V.M. (2002) Nanotransducers in cellular redox signaling: modification of thiols by reactive oxygen and nitrogen species, *Trends Biochem. Scie.* 27:489-492.

Corbett, E.F. and Michalak, M. (2000) Calcium, a signaling molecule in the endoplasmic reticulum? *Trends Biochem. Sci.* 25:307-311. (MedLine)

Corbett, E.F., Oikawa, K., Francois, P., Tessier, D.C., Kay, C., Bergeron, J.J., Thomas, D.Y., Krause, K.H. and Michalak, M. (1999) Ca^{2+} regulation of interactions between endoplasmic reticulum chaperones, *J. Biol. Chem.* 274:6203-6211. (MedLine)

Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S. and Smith, A.J. (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling, *Science* 247: 470-473.(Medline)

Cox, J.S. and Walter, P. (1996) A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response, *Cell* 87:391-404.(Medline)

Cox, J.S., Shamu, C.E. and Walter, P. (1993) Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase, *Cell* 73:1197-1206.(Medline)

Cox, J.S., Chapman, R.E. and Walter, P. (1997) The unfolded protein response coordinates the production of endoplasmic reticulum protein and endoplasmic reticulum membrane, *Mol.Biol.Cell* 8:1805-1814.(Medline)

Crabeel, M., de Rijcke, M., Seneca, S., Heimberg, H., Pfeiffer, I. and Matisova, A. (1995) Further definition of the sequence and position requirements of the arginine control element that mediates repression and induction by arginine in *Saccharomyces cerevisiae Yeast* 11:1367-1380. (MedLine)

Crabtree, G.R. (1999) Generic signals and specific outcomes: signaling through Ca2+, calcineurin, and NF-AT, *Cell* 96:611-614. (MedLine)

Crompton, M. (1990) The role of Ca^{2+} in the function and dysfunction of heart mitochondria, in *Calcium and Heart*, ed.

Cullen, P.J. and Lockyer, P.J. (2002) Integration of calcium and Ras signalling, *Nature Rev. Mol. Cell Biol.* 3:339-348. (MedLine)

Currie, K.P., Swann, K., Galione, A. and Scott, R.H. (1992) Activation of Ca^{2+}-dependent currents in cultured rat dorsal root ganglion neurones by a sperm factor and cyclic ADP-ribose, *Mol. Biol. Cell* 3:1415-1425. (MedLine)

Czech, M.P. (2000) PIP2 and PIP3: complex roles at the cell surface, *Cell* 100:603-606.(Medline)

Daaka, Y., Luttrell, L.M., Ahn, S., Della Rocca, G.J., Ferguson, S.S., Caron, M.G. and Lefkowitz, R.J. (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase, *J. Biol. Chem.* 273:685-688. (MedLine)

Damm, K., Thompson, C.C. and Evans, R.M. (1989) Protein encoded by v-*erb*A functions as a thyroid-hormone receptor antagonist, *Nature* 339:593-597.(Medline)

Dani, J.W., Chernjavsky, A. and Smith, S.J. (1992) Neuronal activity triggers calcium waves in hippocampal astrocyte networks, *Neuron* 8:429-440.(Medline)

Darnell, J.E. Jr. (1997) STATs and gene regulation, *Science* 277:1630-1635.(Medline)

Darnell, J.E. Jr., Kerr, I.M. and Stark, G.R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins, *Science* 264:1415-1421.(Medline)

Datta, S.R., Brunet, A. and Greenberg, M.E. (1999) Cellular survival: a play in three Akts, *Genes Dev.* 13:2905-2927.(Medline)

Davare, M.A., Avdonin, V., Hall, D.D., Peden, E.M., Burette, A., Weinberg, R.J., Horne, M.C., Hoshi, T. and Hell, J.W. (2001) A β_2 adrenergic receptor signaling complex assembled with the Ca^{2+} channel $Ca_V1.2$, *Science* 293:98-101. (MedLine)

David, M., Petricoin, E. 3rd, Benjamin, C., Pine, R., Weber, M.J. and Larner, A.C. (1995) Requirement for MAP kinase (ERK2) activity in interferon α- and interferon β-stimulated gene expression through STAT proteins, *Science* 269:1721-1723. (MedLine)

DeBose-Boyd, R.A., Brown, M.S., Li, W.P., Nohturfft, A., Goldstein, J.L. and Espenshade, P.J. (1999) Transport-dependent proteolysis of SREBP: relocation of site-1 protease from Golgi to ER obviates the need for SREBP transport to Golgi, *Cell* 99:703-712.(Medline)

De Camilli, P., Emr, S.D., McPherson, P.S. and Novick, P. (1996) Phosphoinositides as regulators in membrane traffic, *Science* 271:1533-1539.(Medline)

De Cesare, D., Fimia, G.M. and Sassone-Corsi, P. (1999) Signaling routes to CREM and CREB: plasticity in transcriptional activation, *Trends Biochem. Scie.* 24:281-285.(Medline)

de Groot, R.P., Ballou, L.M. and Sassone-Corsi, P. (1994) Positive regulation of the cAMP-responsive activator CREM by the p70 S6 kinase: an alternative route to mitogen-induced gene expression, *Cell* 79:81-91.(Medline)

de Hostos, E.L., (1999) The coronin family of actin-associated proteins, *Trends in Cell Biol.* 9:345-350.(Medline)

Deak, M., Clifton, A.D., Lucocq, L.M. and Alessi, D.R. (1998) Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB, *EMBO J.* 17:4426-4441.(Medline)

Dell'Acqua, M.L., Faux, M.C., Thorburn, J., Thorburn, A. and Scott, J.D. (1998) Membrane-targeting sequences on AKAP79 bind phosphatidylinositol-4, 5-bisphosphate, *EMBO J.* 17:2246-2260.(Medline)

den Hertog, J., Pals, C.E.G.M., Peppelenbosch, M.P., Tertoolen, L.G.J., De Laat, S.W. and Kruijer,W. (1993) Receptor protein tyrosine phosphatase -activates pp60$^{c\text{-}src}$ and is involved in neuronal differentiation, *EMBO J.* 12: 3789-3798.(Medline)

Denton, R.M. and McCormack, J.G. (1980) On the role of the calcium transport cycle in heart and other mammalian mitochondria, *FEBS Lett.* 119: 1-8.(Medline)

Denu, J.M. and Dixon, J.E. (1998) Protein tyrosine phosphatases: mechanisms of catalysis and regulation, *Curr. Opin. Chem. Biol.* 2:633-641.(Medline)

Di Cristofano, A. and Pandolfi, P.P. (2000) The multiple roles of PTEN in tumor suppression, *Cell* 100:387-390. (MedLine)

Divecha, N., Banfic, H. and Irvine, R.F. (1991) The polyphosphoinositide cycle exists in the nuclei of Swiss 3T3 cells under the control of a receptor (for IGF-I) in the plasma membrane, and stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus, *EMBO J.* 10:3207-3214.(Medline)

Dizhoor, A.M., Chen, C.K., Olshevskaya, E., Sinelnikova, V.V., Phillipov, P. and Hurley, J.B. (1993) Role of the acylated amino terminus of recoverin in Ca^{2+}-dependent membrane interaction, *Science*

259:829-832.(Medline)

Dolmetsch, R.E., Lewis, R.S., Goodnow, C.C. and Healy, J.I. (1997) Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration, *Nature* 386:855-858.(Medline)

Dolmetsch, R.E., Xu, K. and Lewis, R.S. (1998) Calcium oscillations increase the efficiency and specificity of gene expression, *Nature* 392:933-936.(Medline)

Drapier, J.C. Hirling, H., Wietzerbin, J., Kaldy, P.and Kühn, L.C. (1993) Biosynthesis of nitric oxide activates iron regulatory factor in macrophages, *EMBO. J.* 12: 3643-3649.(Medline)

Dröge W. (2002) Free radicals in the physiological control of cell function, *Physiol Rev.* 82:47-95. (MedLine)

Dröge, W., Schulze-Osthoff, K., Milm, S., Galtar, D., Schenk, H., Eck, H.-P., Roth, S. and Gmunder, H. (1994) Functions of glutathione and glutathione disulfide in immunology and immunopathology, *FASEB J.* 8:1131-1138.(Medline)

Duchen, M.R. (1999) Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death, *J. Physiol.(London)* 516:1-17.(Medline)

Duncan, E.A., Dave, U.P., Sakai, J., Goldstein, J.L., Brown, M.S. (1998) Second-site cleavage in sterol regulatory element-binding protein occurs at transmembrane junction as determined by cysteine panning, *J. Biol. Chem.* 273:17801-17809.(Medline)

Eckert, R. and Chad, J.E. (1984) Inactivation of Ca channels, *Prog. Biophys. Mol. Biol.* 44:215-267.(Medline)

Edwards, A.S. and Scott, J.D. (2000) A-kinase anchoring proteins: protein kinase A and beyond, *Curr. Opin. Cell Biol.* 12:217-221. (MedLine)

Egloff, M.-P., Johnson, D.P., Moorhead, G.M., Cohen, P. and Barford, D. (1997) Structural basis for the recognition of regulatory subunits by the catalytic subunit of phosphatase 1, *EMBO J.* 16:1876-1887.(Medline)

Ehlers, M.D., Zhang, S., Bernhadt, J.P. and Huganir, R.L. (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit, *Cell* 84:745-755.(Medline)

El Bakkoury, M., Dubois, E. and Messenguy, F. (2000) Recruitment of the yeast MADS-box proteins, ArgRI and mcm1 by the pleiotropic factor ArgRIII is required for their stability, *Mol. Microbiol.* 35:15-

31. (MedLine)

Elion, E.A. (1995) Stet5: a meeting place for MAP kinases and their associates, *Trends in Cell Biol.* 5:322-327.

Emery-Goodman, A., Hirling, H., Scarpellino, L., Henderson, B. and Kühn, L.C. (1993) Iron regulatory factor expressed from recombinant baculovirus: conversion between the RNA-binding apoprotein and Fe-S cluster containing aconitase, *Nucleic Acid Res.* 21: 1457-1461.(Medline)

Eng, C. (1998) Genetics of Cowden syndrome: through the looking glass of oncology, *Int. J. Oncol.* 12:701-710.(Medline)

Faber, H.R., Groom, C.R., Baker, H.M., Morgan, W.T., Smith, A. and Baker, E.N. (1995) 1.8 Å crystal structure of the C-terminal domain of rabbit serum haemopexin, *Structure*.3:551-559.(Medline)

Falkenstein, E., Tillmann, H.C., Christ, M., Feuring, M. and Wehling, M. (2000) Multiple actions of steroid hormones--a focus on rapid, nongenomic effects, *Pharmacol Rev.* 52:513-556. (MedLine)

Fankhauser, C. and Chory, J. (1997) Light control of plant development, *Annu. Rev. Cell Dev. Biol.* 13:203-229.(Medline)

Fankhauser, C., Yeh, K.C., Lagarias, J.C., Zhang, H., Elich, T.D. and Chory, J. (1999) PKS1, a substrate phosphorylated by phytochrome that modulates light signaling in *Arabidopsis*, *Science*., 284:1539-1541.(Medline)

Fasshauer, D., Bruns, D., Shen, B., Jahn, R., and Brunger, A. T. (1997) A Structural change occurs upon binding of syntaxin to SNAP-25, *J. Biol. Chem.* 272: 4582-4590.(Medline)

Fauman, E.B. and Saper, M.A. (1996) Structure and function of the protein tyrosine phosphatases, *Trends Biochem. Sci.* 21: 413-417.(Medline)

Faurobert, E. and Hurley, J.B, (1997) The core domain of a new retina specific RGS protein stimulates the GTPase activity of transducin in vitro, *Proc. Natl. Acad. Sci. USA* 94:2945-2950.(Medline)

Faux, M.C. and Scott, J.D. (1997) Regulation of the AKAP79-protein kinase C interaction by Ca2+/Calmodulin, *J. Biol. Chem.* 272:17038-17044.(Medline)

Feig, L.A. (1994) Guanine-nucleotide exchange factors: a family of positive regulators of Ras and related GTPases, *Curr. Opin. Cell Biol.* 6:204-211.(Medline)

Ferguson, S.S. (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling, *Pharmacol. Rev.* 53:1-24. (MedLine)

Fesenko, E. E., Kolesnikov, S. S. and Lyubarsky. A. L. (1985) Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment, *Nature* 313:310-313.(Medline)

Fewtrell, C. (1993) Ca^{2+} oscillations in non-excitable cells, *Annu. Rev. Physiol.* 55:427-454. (MedLine)

Fields, R.D. and Ito, K. (1996) Neural adhesion molecules in activity-dependent development and synaptic plasticity, *Trends Neurosci.* 19:473-480.(Medline)

Fimia, G.M., De Cesare, D. and Sassone-Corsi, P. (1999) CBP-independent activation of CREM and CREB by the LIM-only protein ACT, *Nature* 398:165-169.(Medline)

Fink, J.S., Verhave, M., Kasper, S., Tsukada, T., Mandel, G. and Goodman, R.H. (1997) The CGTCA sequence motif is essential for biological activity of the vasoactive intestinal peptide gene cAMP-regulated enhancer, *Proc. Natl. Acad. Sci. USA.* 85:6662-6666.(Medline)

Finkbeiner, S. and Greenberg, M.E. (1998) Ca^{2+} channel-regulated neuronal gene expression, *J. Neurobiol.* 37:171-189. (MedLine)

Flanagan, W.M., Corthesy, B., Bram, R.J. and Crabtree, G.R. (1991) Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A, *Nature* 352:803-807.(Medline)

Ford, C.E., Skiba, N.P., Bae, H., Daaka, Y., Reuveny, E., Shekter, L.R., Rosal, R., Weng, G., Yang, C.S., Iyengar, R., Miller, R.J., Jan, L.Y., Lefkowitz, R.J. and Hamm, H.E. (1998) Molecular basis for interactions of G protein $\beta\gamma$ subunits with effectors, *Science* 280:1271-1274.(Medline)

Foulkes, N.S., Borjigin, J., Snyder, S.H. and Sassone-Corsi, P. (1997) Rhythmic transcription: the molecular basis of circadian melatonin synthesis, *Trends Neurosci.* 20:487-492.(Medline)

Fraser, I.D., Tavalin, S.J., Lester, L.B., Langeberg, L.K., Westphal, A.M., Dean, R.A., Marrion, N.V. and Scott, J.D. (1998) A novel lipid-anchored A-kinase anchoring protein facilitates cAMP-responsive membrane events, *EMBO J.* 17: 2261-2272.(Medline)

Frederickson R.M. and Sonenberg, N. (1992) Signal transduction and regulation of translation initiation, *Seminars in Cell Biol.* 3:107-115.(Medline)

Frederickson, R.M., Montine, K.S. and Sonenberg, N. (1991) Phosphorylation of eukaryotic initiation factor 4E is increased in Src-transformed cell lines, *Mol Cell Biol.* 11:2896-2900.(Medline)

Freedman, L.P. (1999) Increasing the complexity of coactivation in nuclear receptor signaling, *Cell* 97:5-8.(Medline)

Freeman, B.C. and Yamamoto, K.R. (2002) Disassembly of transcriptional regulatory complexes by molecular chaperones, *Science* 296:2232-2235. (MedLine)

Freichel, M., Suh, S.H., Pfeifer, A., Schweig, U., Trost, C., Weissgerber, P., Biel, M., Philipp, S., Freise, D., Droogmans, G., Hofmann, F., Flockerzi, V. and Nilius, B. (2001) Lack of an endothelial store-operated Ca^{2+} current impairs agonist-dependent vasorelaxation in TRP4-/- mice, *Nature Cell Biol.* 3:121-127. (MedLine)

Frödin, M. and Gammeltoft, S. (1999) Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction, *Mol. Cell. Endocrinol.* 151:65-77.(Medline)

Fry, M. (1994) Structure, regulation and function of phosphoinositide 3-kinases, *Biochim. Biophys. Acta.* 1226:237-268.(Medline)

Fu, X, Kessler, D.S., Velas, S.A., Levy, D.E. and Darnell, J.E., Jr.(1990) ISGF3, the transcriptional activator induced by inteferon consists of multiple interacting polypeptide chains, *Proc. Natl. Acad. Sci. USA* 87:8555-8559.(Medline)

Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. (1989) Primary structure and functional expression of the inositol 1,4,5-triphosphate-binding protein P_{400}, *Nature* 342:32-38.(Medline)

Furuya, S., Mitoma, J., Makino, A., Hirabayashi, Y. (1998) Ceramide and its interconvertible metabolite sphingosine function as indispensable lipid factors involved in survival and dendritic differentiation of cerebellar Purkinje cells,

J. Neurochem. 71:366-377. (MedLine)

Galione A, White,A., Willmott, N., Turner,M., Potter B.V. and Watson,S.P. (1993) cGMP mobilizes intracellular Ca^{2+} in sea urchin eggs by stimulating cyclic ADP-ribose synthesis, *Nature* 365: 456-459.(Medline)

Garrington, T.P. and Johnson, G.L. (1999) Organization and regulation of mitogen-activated protein kinase signaling pathways, *Curr. Opin. Cell Biol.* 11:211-218. (MedLine)

Gaullier, J.-M., Simonsen, A., D'Arrigo, A., Bremnes, B. and Stenmark, H. (1998) FYVE fingers bind PtdIns(3)P, *Nature* 394:432-433.(Medline)

Genazzani, A.A., Empson, R.M. and Galione, A. (1996) Unique inactivation properties of NAADP-sensitive Ca^{2+} release, *J. Biol. Chem.* 271:11599-11602. (MedLine)

Gething, M.J. and Sambrook, J. (1992) Protein folding in the cell, *Nature* 355:33-45.(Medline)

Ghafourifar, P. and Richter, C. (1997) Nitric oxide synthase activity in mitochondria, *FEBS Lett.* 418:291-296. (MedLine)

Ghosh, T.K., Bian, J. and Gill, D.L. (1987) Intracellular calcium release by sphingosine derivatives generated in cell, *Science* 248:1653-1656.(Medline)

Giguère, V. Hollenberg,S.M., Rosenfeld, M.G. and Evans, R.M. (1986) Functional domains of the human glucocorticoid receptor, *Cell* 46:645-652.(Medline)

Giguère, V., Ong, E.S., Segui, P. and Evans, R.M. (1987) Identification of a receptor for the morphogen retinoic acid, *Nature* 330: 624-629.(Medline)

Giles, R.H., Peters, D.J. and Breuning, M.H. (1998) Conjunction dysfunction: CBP/p300 in human disease, *Trends Genet.* 14:178-183.(Medline)

Ginty, D.D., Bonni, A. and Greenberg, M.E. (1994) Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB, *Cell* 77:713-725.(Medline)

Godowski, P.J. and Picard, D. (1989) Steroid receptors. How to be both a receptor and a transcription factor, *Biochem. Pharm.* 38:3135-3142.(Medline)

Gonzalez, G.A. and Montminy, M.R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133, *Cell* 59:675-680.(Medline)

Granzin, J., Wilden, U., Choe, H.W., Labahn, J., Krafft, B. and Buldt, G. (1998) X-ray crystal structure of arrestin from bovine rod outer segments, *Nature* 391:918-921.(Medline)

Gray, P.C., Johnson, B.D., Westenbroek, R.E., Hays, L.G., Yates, J.R. 3rd, Scheuer, T., Catterall, W.A. and Murphy, B.J. (1998) Primary structure and function of an A kinase anchoring protein associated with calcium channels, *Neuron* 20:1017-1026.(Medline)

Grazzini, E., Guillio, G., Mouillac, B. and Zingg, H.H. (1998) Inhibition of oxytocin receptor function by direct binding of progesterone, *Nature* 392:509-512.(Medline)

Greenberg, M.L. and Lopes, J.M. (1996) Genetic regulation of phospholipid biosynthesis in *Saccharomyces cervisiae, Microb.Rev.* 60:1-20.(Medline)

Gross, S.D., Schwab, M.S., Lewellyn, A.L. and Maller, J.L. (1999) Induction of metaphase arrest in cleaving *Xenopus* embryos by the protein kinase p90, *Science* 286:1365-1367.(Medline)

Groyer, A., Schweizer-Groyer, G., Cadepond, F., Mariller, M. and Baulieu, E.E. (1987) Antiglucocorticosteroid effects suggest why steroid hormone is required for receptors to bind DNA *in vivo* but not *in vitro*, *Nature* 328:624-626.(Medline)

Gu, X. and Spitzer, N.C. (1995) Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients, *Nature* 375:784-787.(Medline)

Gu, J., Tamura, M. and Yamada, K.M. (1998) Tumor suppressor PTEN inhibits integrin- and growth factor-mediated mitogen-activated protein (MAP) kinase signaling pathways, *J. Cell Biol.* 143:1375-1383.(Medline)

Gu, Q., Korach, K.S. and Moss, R.L. (1999) Rapid action of 17 β-estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors, *Endocrinology* 140:660-666. (MedLine)

Hacker, U. and Perrimon, N. (1998) DRhoGEF2 encodes a member of the Dbl family of oncogenes and controls cell shape changes during gastrulation in Drosophila, *Genes Dev.* 12:274-284.(Medline)

Hajnózky, G., Robb-Gaspers, L.D., Seitz, M.B. and Thomas, A.P. (1995) Decoding of cytosolic calcium oscillations in mitochondria, *Cell* 82:415-424.(Medline)

Hall, A. (1998) Rho GTPases and the actin cytoskeleton, *Science* 279:509-514.(Medline)

Hall, R.A., Premont, R.T. and Lefkowitz, R.J. (1999) Heptahelical receptor signaling: beyond the G protein paradigm *J. Cell Biol.* 145:927-932.(Medline)

Hamm, H.E. (1998) The many faces of G protein signaling, *J. Biol. Chem* 273:669-672.(Medline)

Hannun, Y.A. (1996) Functions of ceramide in coordinating cellular responses to stress, *Science* 274:1855-1859.(Medline)

Hannun, Y.A and Luberto, C.(2000) Ceramide in the eukaryotic stress response, *Trends Cell Biol.* 10:73-80. (MedLine)

Hansford, R.G. (1991) Dehydrogenase activation by Ca^{2+} in cells and tissues, *J. Bioenerg. Biomembr.* 23:823-854.(Medline)

Hardingham, G.E., Chawla, S., Johnson. C.M. and Bading, H. (1997) Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression, *Nature* 385:260-265.(Medline)

Hardwick, K.G., Lewis, M.J., Semenza, J., Dean, N. and Pelham, H.R. (1990) ERD1, a yeast gene required for the retention of luminal endoplasmic reticulum proteins, affects glycoprotein processing in the Golgi apparatus, *EMBO J.* 9:623-630.(Medline)

Harris, D.A. and Das, A.M. (1991) Control of ATP synthesis in the heart, *Biochem. J.* 280:561-573.(Medline)

Hart, M.J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W.D., Gilman, A.G., Sternweis, P.C. and Bollag, G. (1998) Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by $G\alpha_{13}$, *Science* 280:2112-2114.(Medline)

Harteneck, C., Plant, T.D. and Schultz, G. (2000) From worm to man: three subfamilies of TRP channels, *Trends Neurosci.* 23:159-166. (MedLine)

Hartwig, J.H., Kung. S., Kovacsovics, T., Janmey. P.A., Cantley, L.C., Stossel, T.P. and Toker A. (1996) D3 phosphoinositides and outside-in integrin signaling by glycoprotein IIb-IIIa mediate platelet actin assembly and filopodial extension induced by phorbol 12-myristate 13-acetate, *J. Biol. Chem.* 271:32986-32993.(Medline)

Hashimoto, S., Bruno, B., Lew, D. P., Pozzan, T., Volpe P. and Meldolesi, J. (1988) Immunocytochemistry of calcisomes in liver and pancreas, *J. Cell Biol.* 107:2523-2531.(Medline)

Haze, K., Yoshida, H., Yanagi, H., Yura, T. and Mori, K. (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress, *Mol. Biol. Cell* 10):3787-3799.(Medline)

Heinrich M., Wickel, M., Schneider-Brachert, W., Sandberg, C., Gahr, J., Schwandner, R., Weber, T., Saftig, P., Peters, C., Brunner, J., Kronke, M. and Schutze, S. (1999) Cathepsin D targeted by acid sphingomyelinase-derived ceramide, *EMBO J.* 18:5252-5263. (MedLine)

Heyman, R.A., Mangelsdorf, D.J., Dyck, J.A., Stein, R.B., Eichele, G., Evans, R.M. and Thaller, C. (1992) 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor, *Cell* 68:397-406.(Medline)

Heinrich, P.C., Behrmann, I., Muller-Newen, G., Schaper, F. and Graeve, L. (1998) Interleukin-6-type

cytokine signalling through the gp130/Jak/STAT pathway, *Biochem. J.* 334:297-314.(Medline)

Heinzel, T., Lavinsky, R.M., Mullen, T.M., Soderstrom, M., Laherty, C.D., Torchia, J., Yang, W.M., Brard, G., Ngo, S.D., Davie, J.R., Seto, E., Eisenman, R.N., Rose, D.W., Glass, C.K. and Rosenfeld, M.G. (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression, *Nature* 387:43-48. (MedLine)

Helenius, A. (1994) How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum, *Mol. Biol. Cell* 5:253-265.(Medline)

Helenius, A., Trombetta, E.S., Hebert, D.N. and Simons J.F. (1997) Calnexin, calreticulin and the folding of glycoproteins, *Trends in Cell Biol.* 7:193-200.

Hendricks, K.B., Wang, B.Q., Schnieders, E.A. and Thorner, J. (1999) Yeast homologue of neuronal frequenin is a regulator of phosphatidylinositol-4-OH kinase, *Nature Cell Biol.* 1:234-241.(Medline)

Henry, S.A. and Patton-Vogt, J.L. (1998) Genetic regulation of phospholipid metabolism: yeast as a model eukaryote, *Prog. Nucleic Acid Res. Mol. Biol.* 61:133-179.(Medline)

Hill, C.S. and Treisman, R. (1995) Differential activation of *c- fos* promoter elements by serum, lysophosphatidic acid, G protein and polypeptide growth factors, *EMBO J.* 14:5037-5047.(Medline)

Hirsch, J.P. and Henry, S.A. (1986) Expression of the Saccharomyces cerevisiae inositol-1-phosphate synthase (INO1) gene is regulated by factors that affect phospholipid synthesis, *Mol. Cell Biol.* 6:3320-3328.(Medline)

Hirsch, J.A., Schubert, C., Gurevich, V.V. and Sigler, P.B. (1999) The 2.8 Å crystal structure of visual arrestin: a model for arrestin's regulation, *Cell* 97:257-269.(Medline)

Hoecker. U., Tepperman, J.M., Quail, P.H. (1999) SPA1, a WD-repeat protein specific to phytochrome A signal transduction, *Science* 284:496-499.(Medline)

Hoppe, T., Matuschewski, K., Rape, M., Schlenker, S., Ulrich, H.D. and Jentsch, S. (2000) Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing, *Cell* 102:577-586.(Medline)

Hoth. M. and Penner, R. (1993)Calcium release-activated calcium current in rat mast cells, *J. Physiol.* (London)465:359-386.(Medline)

Hofer, A.M. and Schulz, I. (1996) Quantification of intraluminal free [Ca^{2+}] in the agonist-sensitive

internal calcium store using compartmentalized fluorescent indicators: some considerations, *Cell Calcium* 20:235-242.(Medline)

Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A.J., Frohman, M.A. and Kanaho, Y. (1999) Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation, *Cell* 99:521-532. (MedLine)

Hood, D., Balaban, A., Connor, M., Craig, E., Nishio, M., Rezvani, M. and Takahashi, M. (1994) Mitochondrial biogenesis in striated muscle, *Can. J. Appl. Physiol.* 19:12-48.(Medline)

Huala, E., Oeller, P.W., Liscum, E., Han, I.S., Larsen, E. and Briggs, W.R. (1997) *Arabidopsis* NPH1: a protein kinase with a putative redox-sensing domain, *Science* 278:2120-2123.(Medline)

Hubbard, M.J. and Cohen,P. (1993) On target with a new mechanism for the regulation of protein phosphorylation, *Trends Biochem. Sci.*18:172-177.(Medline)

Hung, H.L., Lau, J., Kim, A.Y., Weiss, M.J. and Blobel, G.A. (1999) CREB-Binding protein acetylates hematopoietic transcription factor GATA-1 at functionally important sites, *Mol. Cell Biol.* 19:3496-3505.(Medline)

Husi, H., Ward, M.A., Choudhary, J.S., Blackstock, W.P. and Grant, S.G. (2000) Proteomic analysis of NMDA receptor-adhesion protein signaling complexes, *Nature Neurosci.* 3:661-669. (MedLine)

Huwiler, A., Brunner, J., Hummel, R., Vervoordeldonk, M., Stabel, S., van den Bosch, H. and Pfeilschifter, J. (1996) Ceramide-binding and activation defines protein kinase c-Raf as a ceramide-activated protein kinase, *Proc. Natl. Acad. Sci. USA* 93:6959-6963.(Medline)

Iacovelli, L., Sallese, M., Mariggio, S. and de Blasi, A. Regulation of G-protein-coupled receptor kinase subtypes by calcium sensor proteins, *FASEB J.* 13:1-8. (MedLine)

Ignarro, L. J. (1990) Nitric oxide. A novel signal transduction mechanism for intracellular communication, *Hypertension* 16:477-483.(Medline)

Ihle, J.N. and Kerr, I.M.(1995) Jaks and Stats in signaling the cytokine receptor superfamily, *Trends in Genet.* 11:69-74.(Medline)

Ihle, J.N., Witthuhn, B.A., Quelle, F.W., Yamamoto, K., Thierfelder, W.E., Kreider, B. and Silvennoien, O. (1994) Signaling by the cytokine receptor superfamily, JAKs and STATS, *Trends in Biochem. Sci.* 19:222-227.(Medline)

Ikura, M. (1996) Calcium binding and conformational response in EF-hand proteins, *Trends Biochem. Sci.* 21:14-17. (MedLine)

Ivessa, N. E., De Lemos-Chiarandini, C., Gravotta, D., Sabatini, D. D. and Kreibich G. (1995) The Brefeldin A-induced retrograde transport from the Golgi apparatus to the endoplasmic reticulum depends on calcium sequestered to intracellular stores, *J. Biol. Chem.* 270:25960-25967.(Medline)

Jackowski, S. (1996) Cell cycle regulation of membrane phospholipid metabolism, *J. Biol. Chem.* 271:20219-20222.(Medline)

Jackson, S.P. (1992) Regulating transcription factor activity by phosphorylation, *Trends in Cell Biol.* 2:104-108.

Jaffe, L.F. (1993) Classes and mechanisms of calcium waves, *Cell Calcium* 14: 736-745.(Medline)

Jagus, R., Anderson, W.F.and Safer, B. (1991) Initiation of mammalian protein bioshynthesis, *Prog. Nucleic Acid Res. Mol. Biol.* 25:127-185.

Janssens, V. and Goris, J. (2001) Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling, *Biochem. J.* 353:417-439. (MedLine)

Jensen, C.J., Buch, M.B., Krag, T.O., Hemmings, B.A., Gammeltoft, S. and Frodin, M. (1999) 90-kDa ribosomal S6 kinase is phosphorylated and activated by 3-phosphoinositide-dependent protein kinase-1, *J. Biol. Chem.* 274:27168-27176.(Medline)

Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M.G., Binaut, N. Mester, J. and Baulieu, E.S. (1984) Common non-hormone binding component in non-tranformed chick oviduct receptor of four steroid hormones, *Nature* 308:850-853.(Medline)

John, L.M., Lechleiter, J.D. and Camacho, P. (1998) Differential modulation of SERCA2 isoforms by calreticulin, *J. Cell Biol.* 142:963-973. (MedLine)

Johnson, D.F.,Moorhead, G.. Caudwell, F.B., Cohen, P., Chen Y.H. and Chen, M.X. (1996) Identification of protein-phosphatase-1-binding domains on the glycogen and myofibrillar targetting subunits, *Eur. J. Biochem.* 239:317-325.(Medline)

Jones, S.M. and Howell, K.E. (1997) Phosphatidylinositol 3-kinase is required for the formation of constitutive transport vesicles from the TGN, *J. Cell Biol.* 139:339-349.(Medline)

Jones, S.M., Crosby, J.R., Salamero, J. and Howell, K.E. (1993) A cytosolic complex of p62 and rab6

associates with TGN38/41 and is involved in budding of exocytic vesicles from the trans-Golgi network, *J. Cell Biol.* 122:775-788.(Medline)

Jordan, J.D., Landau, E.M. and Iyengar, R. (2000) Signaling networks: the origins of cellular multitasking, *Cell* 103:193-200.

Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F. and Williamson. J. R. (1984) M\o-inositol 1 ,4,5-trisphosphate: a second messenger for the hormonal mobilization of intracellular Ca^{2+} in liver, *J. Biol. Chem.* 259:3077-3081.(Medline)

Jou, T.-S., Schneeberger, E.E. and Nelson, W.J. (1998) Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases, *J. Cell Biol.* 142:101-115.(Medline)

Jouaville, L.S., Ichas, F., Holmuhamedov, E.L., Camacho, P. and Lechleiter, J.D. (1995) Synchronization of calcium waves by mitochondrial substrates in *Xenopus laevis* oocytes, *Nature* 377:438-441. (MedLine)

Jouaville, L.S., Pinton, P., Bastianutto, C., Rutter, G.A. and Rizzuto, R. (1999) Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming, *Proc. Natl. Acad. Sci. USA* 96:13807-13812. (MedLine)

Jung, E.M., Griner, R.D., Mann-Blakeney, R. and Bollag, W.B. (1998) A potential role for ceramide in the regulation of mouse epidermal keratinocyte proliferation and differentiation, *J. Invest. Dermatol.* 110:318-323. (MedLine)

Jurado, L.A., Chockalingam, P.S. and Jarrett, H.W. (1999) Apocalmodulin, *Physiol. Rev.*79:661-682. (MedLine)

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.C., Heyman, R.A., Rose, D.W., Glass, C.K. and Rosenfeld, M.G. (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors, *Cell* 85:403-414.(Medline)

Kanai, K., Watanabe, J. and Kanamura, S. (1986) Quantitative analysis of smooth and rough endoplasmic reticulum proliferation in differentiating hepatocytes of midpostnatal mice treated with phenobarbital, *J. Ultrastruct. Mol. Struct. Res.* 97:64-72.(Medline)

Karin, M. (1992) Signal transduction from cell surface to nucleus in development and disease, *FASEB J.* 6:2581-2590.(Medline)

Karin, M. and Smeal, T. (1992) Control of transcription factors by signal transduction pathways: the beginning of the end, *Trends in Biochem. Scie.* 17:418-422.(Medline)

Katagiri, Y., Takeda, K., Yu, Z.X., J Ferrans, V., Ozato, K. and Guroff, G.(2000) Modulation of retinoid signalling through NGF-induced nuclear export of NGFI-B, *Nature Cell Biol.* 2:435-440. (Medline)

Kaufman, R.J. (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls, *Genes Dev.* 13:1211-1233. (MedLine)

Kawahara, T., Yanagi, H., Yura, T. and Mori, K. (1998) Unconventional splicing of HAC1/ERN4 mRNA required for the unfolded protein response, sequence-specific and non-sequential cleavage of the splice sites, *J. Biol. Chem.* 273:1802-1807.(Medline)

Kehlenbach, R.H., Dickmanns, A. and Gerace, L. (1998) Nucleocytoplasmic shuttling factors including Ran and CRM1 mediate nuclear export of NFAT in vitro, *J. Cell Biol.* 141:863-874.(Medline)

Kennedy, M.B. (1997) The postsynaptic density at glutamatergic synapses, *Trends Neurosci.* 20:264-268. (MedLine)

Kessler, D.S., Veals, S.A., Fu, X.Y., and Levy, D.E. (1990) Inteferon- regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator, *Genes Dev.* 4:1753-1765.(Medline)

Kincaid, R.L. and Vaughan, M. (1986) Direct comparison of Ca2+ requirements for calmodulin interaction with and activation of protein phosphatase, *Proc. Natl. Acad. Sci. USA* 83:1193-1197. (MedLine)

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover, *J. Biol. Chem.* 255:2273-2276.(Medline)

Klauck, T.M., Faux, M.C., Labudda, K., Langeberg, L.K., Jaken, S. and Scott, J.D. (1996) Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein, *Science* 271:1589-1592.(Medline)

Kliewer, S.A., Lehmann, J.M. and Willson, T.M. (1999) Orphan nuclear receptors: shifting endocrinology into reverse, *Science* 284:757-760.(Medline)

Kodaki, T., Hosaka, K., Nikawa, J.I. and Yamashita, S. (1991) Identification of the upstream activation sequences responsible for the expression and regulation of the *PMEI* and *PME2* genes encoding the enzymes of the phosphatidylethanolamine methylation pathway in *Saccharomyces cervisiae*, *J. Biochem.* 109:276-287.(Medline)

Korzus, E., Torchia, J., Rose, D.W., Xu, L., Kurokawa, R., McInerney, E.M., Mullen, T.M., Glass, C.K. and Rosenfeld, M.G. (1998) Transcription factor-specific requirements for coactivators and their

acetyltransferase functions, *Science* 279:703-707.(Medline)

Kouzarides, T. (1999) Histone acetylases and deacetylases in cell proliferation, *Curr. Opin. Genet. Dev.* 9:40-48.(Medline)

Kozasa, T., Jiang, X., Hart, M.J., Sternweis, P.M., Singer, W.D., Gilman, A.G., Loffalg, G. and Sternweis, P.C.(1998) p115 RhoGEF, a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$, *Science* 280:2109-2111.(Medline)

Kozutsumi,Y., Segal, M., Normington, K., Gething, M.-J. and Sambrook, J. (1988) The presence of malfolded protein in the endoplasmic reticulum signals induction of glucose-regulated, *Nature* 332:462-464.(Medline)

Krause, K.-H. and Michalak, M. (1997) Calreticulin, *Cell* 88:439-443.(Medline)

Krause, K.-H., Simmerman, H. K. B., Jones, L. R. and Campbell, K. P. (1990) Sequence similarity of calreticulin with a Ca^{2+}-binding protein that copurifies with an Ins(1,4,5) P_3-sensitive Ca^{2+} store in HL-60 cells, *Biochem. J.* 270:545-548.(Medline)

Krebs, D.L. and Hilton, D.J. (2001) SOCS proteins: negative regulators of cytokine signaling, *Stem Cells* 19:378-387. (MedLine)

Kretsinger, R.H. (1987) Calcium coordination and the calmodulin fold: divergent versus convergent evolution, *Cold Spring Harb. Symp. Quant. Biol.* 52:499-510.(Medline)

Krstic, M.D., Rogatsky, I., Yamamoto, K.R. and Garabedian, M.J. (1997) Mitogen-activated and cyclin-dependent protein kinases selectively and differentially modulate transcriptional enhancement by the glucocorticoid receptor, *Mol. Cell Biol.* 17:3947-3954.(Medline)

Krupnick, J.G. and Benovic, J.L. (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation, *Annu. Rev. Pharmacol. Toxicol.* 38:289-319.(Medline)

Lambright, D.G., Noel, J.P., Hamm, H.E. and Sigler, P.B. (1994) Structural determinants for activation of the α-subunit of a heterotrimeric G protein, *Nature* 369:621-628.(Medline)

Lambright, D.G., Sondek, J., Bohm, A., Skiba, N.P., Hamm, H.E. and Sigler, P.B. (1996) The 2.0 Å crystal structure of a heterotrimeric G protein, *Nature* 379:311-319.(Medline)

Lander, H.M. (1997) An essential role for free radicals and derived species in signal transduction, *FASEB J.* 11:118-124.(Medline)

Lanz, R.B., McKenna, N.J., Onate, S.A., Albrecht, U., Wong, J., Tsai, S.Y., Tsai, M.J. and O'Mailey, B.W. (1999) A steroid receptor coactivator, SRA, functions as an RNA and is present in a SRC-1 complex, *Cell* 97:17-27.(Medline)

Laoide, B.M., Foulkes, N.S., Schlotter, F. and Sassone-Corsi, P. (1993) The functional versatility of CREM is determined by its modular structure, *EMBO J.* 12:1179-1191.(Medline)

Lazaris-Karatzas, A., Montine, K.S., and Sonenberg, N. (1990) Malignant transformation of a eukaryotic initiation factor subunit that binds to the mRNA 5'cap binding protein, *Nature* 345:544-547.(Medline)

Lazarow, P.B. and Moser, H.W. (1995) In *Metabolic and Molecular Bases of Inherited Disease* (Scriver, C.R. et al., eds.), McGraw-Hill, New York, pp. 2287-2324.

Leaman, D.W., Leung, S., L., X. and Stark, G.R. (1996) Regulation of STAT-dependent pathway by growth factors and cytokines, *FASEB J.* 10:1578-1588.(Medline)

Lee, H.C. (1991) Specific binding of cyclic ADP-ribose to calcium-storing microsomes from sea urchin eggs, *J. Biol. Chem.* 266:276-281.(Medline)

Lee, H.C. (1997) Mechanisms of calcium signaling by cyclic ADP-ribose and NAADP, *Physiol. Rev.* 77:1133-1164.(Medline)

Lee H.C. and Aarhus, R. (1993) Wide distribution of an enzyme that catalyzes the hydrolysis of cyclic ADP-ribose, *Biochim.Biophys. Acta.* 1164: 68-74.(Medline)

Lee, H.C. and Aarhus, R. (1995) A derivative of NADP mobilizes calcium stores insensitive to inositol trisphosphate and cyclic ADP-ribose, *J. Biol. Chem.* 270:2152-2157.(Medline)

Lee, H.C., Walseth, T.F., Bratt, G.T., Hayes, R.N. and Clapper, D.L. (1989) Structural determination of a cyclic metabolite of NAD^+ with intracellular Ca^{2+}-mobilizing activity, *J. Biol. Chem.* 264:1608-1615.(Medline)

Lee, H.C., Galione, A. and Walseth, T.F. (1994) Cyclic ADP ribose: metabolism and calcium mobilizing function. in "Vitamins and Hormones", ed.Litwack, G., Academic Press, Orlando Fl. 48:199-254.(Medline)

Lee, S.S.-T., Pineau, T., Drago, J., Lee, E.J., Owens, J.W., Kroetz, D.L., Fernandez-Salguero, P.M., Westphal, H. and Gonzalez, F.J. (1995) Targeted disruption of the isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effect of peroxisome

proliferators, *Mol. Cell Biol.* 15:3012-3022.(Medline)

Lee, M.J., Van Brocklyn, J.R, Thangada, S., Liu, C.H., Hand, A.R., Menzeleev, R., Spiegel, S. and Hla, T. (1998) Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1, *Science* 279:1552-1555.(Medline)

Leffert, H. L. and Koch, K. S. (1985) Growth regulation by sodium ion fluxes. In *Control of Animal Cell Proliferation* (Boynton, A. L., and Leffert, H. L. eds.), Vol. 1. pp. 367-413. Academic Press. New York

Lefkowitz, R.J. (1998) G protein-coupled receptors. III. New roles for receptor kinases and β-arrestins in receptor signaling and desensitization, *J. Biol. Chem.* 273:18677-18680..(Medline)

Lemon, B. and Tjian, R. (2000) Orchestrated response: a symphony of transcription factors for gene control, *Genes Dev.* 14:2551-2569. (MedLine)

Lenzen, C., Cool, R.H., Prinz, H., Kuhlmann, J., Wittinghofer A (1998) Kinetic analysis by fluorescence of the interaction between Ras and the catalytic domain of the guanine nucleotide exchange factor $Cdc25^{Mm}$, *Biochemistry* 37:7420-7430.(Medline)

Levin, A.A., Sturzenbecker, L.J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A. and Grippo, J.F. (1992) 9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXR α, *Nature* 355:359-361.(Medline)

Levy, D.E. and Darnell, J.E. Jr. 2001) STATs: transcriptional control and biological impact, *Nature Rev. Mol. Cell Biol.* 3:651-662. 9MedLine)

Li, D.M. and Sun, H. (1997) TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor β, *Cancer Res.* 57:2124-2129.(Medline)

Li, W.W., Alexandre, S., Cao, X. and Lee, A.S. (1993) Transactivation of the grp78 promoter by Ca2+ depletion. A comparative analysis with A23187 and the endoplasmic reticulum Ca(2+)-ATPase inhibitor thapsigargin, *J. Biol. Chem.* 268:12003-12009.(Medline)

Li J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H. and Parsons, R. (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer, *Science* 275:1943-1947.(Medline)

Li, W.-H., Llopis, J., Whitney, M., Zlokarnik, G. and Tsine, R.Y. (1998) Cell-permeant caged $InsP_3$ ester shows that Ca^{2+} spike frequency can optimize gene expression, *Nature* 392:936-941.(Medline)

Li, M., Linseman, D.A., Allen, M.P., Meintzer, M.K., Wang, X., Laessig, T., Wierman, M.E. and Heidenreich, K.A.(2001) Myocyte enhancer factor 2A and 2D undergo phosphorylation and caspase-mediated degradation during apoptosis of rat cerebellar granule neurons, *J. Neurosci.* 21:6544-6552. (MedLine)

Liaw, D., Marsh, D.J., Li, J., Dahia, P.L., Wang, S.I., Zheng, Z., Bose, S., Call, K.M., Tsou, H.C., Peacocke, M., Eng, C. and Parsons, R. (1997) Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome, *Nature Genet.* 16:64-67.(Medline)

Lin, P., Le-Niculescu, H., Hofmeister, R., McCaffery, M.J., Jin, M., Hennemann, H., McQuistan, T., De Vries, L., Farquhar, M.G. (1998) The mammalian calcium-binding protein, nucleobindin (CALNUC), is a Golgi resident protein, *J. Cell Biol.* 141:1515-1527.(Medline)

Lincoln, T.M., Komalavilas, P., Boerth, N.J., MacMillan-Crow, L.A. and Cornwall, T.L. (1995) cGMP signaling through cAMP- and cGMP-dependent protein kinases, *Adv. in Pharmacol.* 34:305-322.(Medline)

Liscovitch, M. (1992) Crosstalk among multiple signal activated phospholipases, *Trends in Biochem. Scie.* 17:393-399.(Medline)

Liu, E.S., Ou, J.H and Lee, A.S. (1992) Brefeldin A as a regulator of grp78 gene expression in mammalian cells, *J. Biol. Chem.* 267:7128-7133.(Medline)

Lodish, H.F., Kong, N. and Wikström L. (1992) Calcium is required for folding of newly made subunits of the asialoglycoprotein receptor within the endoplasmic reticulum, *J. Biol. Chem.* 267:12753-12760. (MedLine)

Logothetis. D., Kurachi, Y., Galper. J., Neer, E. J. and Calpham, D. E. (1987) The subunits of GTP-binding proteins activate the muscarinic K channel in heart, *Nature* 325:321-326.(Medline)

Lu, J., McKinsey, T.A., Nicol, R.L. and Olson, E.N. (2000) Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases, *Proc. Natl. Acad. Sci. USA* 97:4070-4075. (MedLine)

Luttrell, L.M., Ferguson, S.S.G., Daaka, Y., Miller, W.E., Maudsley, S., Della Rocca, G.J., Lin, F.-T., Kawakatsu, H., Owada, K., Luttrell, D.K., Caron, M.G. and Lefkowitz, R.J. (1999) β-arrestin-dependent formation of β_2 adrenergic receptor-Src protein kinase complexes, *Science* 283:655-661.(Medline)

MacKintosh, C. and MacKintosh R.W. (1994) Inhibitors of protein kinases and phosphatases, *Trends in Biochem. Sci.* 19:444-448.(Medline)

Madan, A.P. and DeFranco, D.B. (1993) Bidirectional trasnsport of glucorticoid receptors across the nuclear envelope, *Proc. Natl. Acad. Sci. USA* 90:3588-3592.(Medline)

Maehama, T. and Dixon, J.E. (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate, *J. Biol. Chem.* 273:13375-13378.(Medline)

Mamar-Bachi, A. and Cox, J.A. (1987) Quantitative analysis of the free energy coupling in the system calmodulin, calcium, smooth muscle myosin light chain kinase, *Cell Calcium* 8:473-482. (MedLine)

Mangelsdorf, D.J. and Evans, R.M. (1995) The RXR heterodimers and orphan receptors, *Cell* 83:841-850. (MedLine)

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R.M. (1995) The nuclear receptor superfamily: the second decade, *Cell* 83:835-839. MedLine)

Manouvrier-Hanu, S., Amiel, J., Jacquot, S., Merienne, K., Moerman, A., Coeslier, A., Labarriere, F., Vallee, L., Croquette, M.F. and Hanauer, A. (1999) Unreported RSK2 missense mutation in two male sibs with an unusually mild form of Coffin-Lowry syndrome, *J. Med. Genet.* 36:775-778.(Medline)

Mao, Z. and Wiedmann, M. (1999) Calcineurin enhances MEF2 DNA binding activity in calcium-dependent survival of cerebellar granule neurons, *J. Biol. Chem.* 274:31102-31107. (MedLine)

Mao, Z., Bonni, A., Xia, F., Nadal-Vicens, M. and Greenberg, M.E. (1999) Neuronal activity-dependent cell survival mediated by transcription factor MEF2, *Science* 286:785-790. (MedLine)

Maraldi, N.M., Zini, N., Santi, S. and Manzoli, F.A. (1999) Topology of inositol lipid signal transduction in the nucleus, *J. Cell Physiol.* 181:203-217. (MedLine)

Marsh, D.J., Dahia, P.L., Zheng, Z., Liaw, D., Parsons, R., Gorlin, R.J. and Eng, C. (1997) Germline mutations in PTEN are present in Bannayan-Zonana syndrome, *Nature Genet.* 16:333-334.(Medline)

Massaguè, J. (1998) TGF-β signal transduction, *Annu. Rev. Biochem.* 67:753-791.(Medline)

Mathews, S. and Sharrock, R.A. (1997) Phytochrome gene diversity, *Plant Cell Environ.* 20:666-671.

May, D. C., Ross, E. M., Gilman. A. G. and Smigel, M. D. (1985) Reconstitution of catecholamine-stimulated adenylate cyclase activity using three purified proteins, *J. Biol. Chem.* 260:15829-15833.(Medline)

Mayer, M.P. and Bukau, B. (1999) The busy life of Hsp90, *Curr. Biol.* 9:R322-325.(Medline)

Mayer-Jaeckel, R.E. and Hemmings, B.A. (1994) Protein phosphatase 2A - a 'ménage à trois', *Trends in Cell Biol.* 4:287-291.

McCormack, J.G. and Denton, R.M. (1986) Ca^{2+} as a second messenger within mitochondria, *Trends in Biochem. Sci.* 11: 391-425.

McCormack, J.G., Browne, H.M. and Dawes, N.J. (1989) Studies on mitochondrial Ca^{2+}-transport using fura-2-loaded rat heart mitochondria, *Biochim. Biophys. Acta* 973:420-427.(Medline)

McCormack, J.G., Halestrap, A.P. and Denton, R.M. (1990) Role of Calcium ions in regulation of mammalian intramitochondrial metabolism, *Physiol. Rev.* 70: 391-425.(Medline)

McKinsey, M.A., Zhang, C.L. and Olson, E.N. (2002) MEF2:a calcium-dependent regulator of cell division, differentiation and death, *Trends Biochem. Scie.* 27:40-47.

McNally, J.G., Muller, W.G., Walker, D., Wolford, R., Hager, G.L. (2000) The glucocorticoid receptor: rapid exchange with regulatory sites in living cells, *Science* 287:1262-1265.(MedLine)

Meldolesi, J. and Pozzan, T. (1998a) The endoplasmic reticulum Ca^{2+}: a view from the lumen, *Trends in Biochem. Sci.* 23:10-14.(Medline)

Meldolesi, J. and Pozzan, T. (1998b) The heterogeneity of ER Ca^{2+} stores has a key role in nonmuscle cell signaling and function, *J. Cell Biol.* 142:1395-1398.(Medline)

Meyer, T. and Stryer, L. (1991) Calcium spiking, *Ann. Rev. Biophys. Biophys. Chem* 20: 153-174.(Medline)

Meyer, T., Hanson, P.I., Stryer, L. and Schulamn, H. (1992) Calmodulin trapping by calcium-calmodulin-dependent protein kinase, *Science* 256:1199-1202.(Medline)

Michalak, M., Milner, R.E., Burns, K. and Opas, M. (1992) Calreticulin, *Biochem. J.* 285:681-692.(Medline)

Michalak, M., Burns, K., Andrin, C., Mesaeli, N., Jass, G.H., Busaan, J.L. and Opas, M. (1996) Endoplasmic reticulum form of calreticulin modulates glucocorticoid-sensitive gene expression, *J. Biol. Chem.* 271:29436-29445. (MedLine)

Michalak, M., Corbett. E.F., Mesaeli, N., Nakamura, K. and Opas, M. (1999) Calreticulin: one protein,

one gene, many functions, *Biochem. J.* 344:281-292.(Medline)

Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E. and Auricchio, F. (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells, *EMBO J.* 15:1292-1300.(Medline)

Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Barone, M.V., Ametrano, D., Zannini, M.S., Abbondanza, C. and Auricchio, F. (2000) Steroid-induced androgen receptor-oestradiol receptor βSrc complex triggers prostate cancer cell proliferation, *EMBO J.* 19:5406-5417. (MedLine)

Millward, T.A., Zolnierowicz, S. and Hemmings, B.A. (1999) Regulation of protein kinase cascades by protein phosphatase 2A, *Trends Biochem. Sci.* 24:186-191.(Medline)

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M. and Tsien, R.Y. (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin, *Nature* 388:882-887. (MedLine)

Miyata, H., Silverman, H.S., Sollott, S.J., Lakatta, E.G., Stern, M.D. and Hansford, R.G. (1991) Measurements of mitochondrial free Ca^{2+} in living single rat cardiac myocytes, *Am. J. Physiol.* 261: (*Heart Circ. Physiol.* 30) H1123-1134.(Medline)

Moncada, S., Palmer, R. M. J. and Higgs, E. A. (1989) Biosynthesis of nitroxide from L-arginine-a pathway for the regulation of cell function and communication, *Biochem. Pharm.* 38:1709-1715.(Medline)

Montero, M., Alonso, M.T., Carnicero, E., Cuchillo-Ibañez, I., Albillos, A., García, A.G., García-Sancho, J. and Alvarez, J. (2000) Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca^{2+} transients that modulate secretion, *Nature Cell Biol.* 2:57-61.(Medline)

Montminy, M. (1997) Transcriptional regulation by cyclic AMP, *Annu. Rev. Biochem.* 66:807-822.(Medline)

Moon, S.Y. and Zheng, Y. (2003) Rho GTPase-activating proteins in cell regulation, *Trends Cell Biol.* 13:13-22. (MedLine)

Mori, K., Sant, A., Kohno, K., Normington, K., Gething, M.J. and Sambrook, J.F. (1992) A 22 bp cis-acting element is necessary and sufficient for the induction of the yeast KAR2 (BiP) gene by unfolded proteins, *EMBO J.* 11:2583-2593.(Medline)

Mori, K., Ma, W., Gething, M.J. and Sambrook, J. (1993) A transmembrane protein with a cdc2+/CDC28-related kinase activity is required for signaling from the ER to the nucleus, *Cell* 74:743-756.(Medline)

Mori, K., Kawahara, T., Yoshida H., Yanagi, H. and Yura, T. (1996) Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway, *Genes Cells* 1:803-817.(Medline)

Morris, J.Z., Tissenbaum, H.A.and Ruvkun, G.A. (1996) phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*, *Nature* 382:536-539.(Medline)

Mourey, R.J. and Dixon, J.E. (1994) Protein tyrosine phosphatases: characterization of extracellular and intracellular domains, *Curr. Opin. Gen Dev.* 4:31-39.(Medline)

Mowen, K.A., Tang, J., Zhu, W., Schurter, B.T., Shuai, K., Herschman, H.R. and David, M. (2001) Arginine methylation of STAT1 modulates IFN α/β-induced transcription, *Cell* 104:731-741. (MedLine)

Mu, F.T., Callaghan, J.M., Steele-Mortimer, O., Stenmark, H., Parton, R.G., Campbell, P.L., McCluskey, J., Yeo, J.P., Tock, E.P. and Toh, B.H. (1995) EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine "fingers" and contains a calmodulin-binding IQ motif, *J. Biol. Chem.* 270:13503-13511.(Medline)

Munck, A. and Foley, R. (1976) Kinetics of glucocorticoid-receptor complexes in rat thymus cells, *J. Steroid Biochem.* 7:1117-1122.(Medline)

Nadal, A., Rovira, J.M., Laribi, O., Leon-quinto, T., Andreu, E., Ripoll, C. and Soria, B. (1998) Rapid insulinotropic effect of 17beta-estradiol via a plasma membrane receptor, *FASEB J.* 12:1341-1348. (MedLine)

Nadler, M.J.S, Hermosura, M.C., Inabe, K., Perraud, A.L., Zhu, Q., Stokes A.J., Kurosaki, T., Kinet, J.P., Penner, R., Scharenberg, A.M. and Fleig, A. (2001) LTRPC7 is a Mg.ATP-regulated divalent cation channel required for cell viability, *Nature* 411:590-595. (MedLine)

Naka, T., Narazaki, M., Hirata, M., Matsumoto, T., Minamoto, S., Aono, A., Nishimoto, N., Kajita, T., Taga, T., Yoshizaki, K., Akira, S. and Kishimoto, T. (1997) Structure and function of a new STAT-induced STAT inhibitor, *Nature* 387:924-929.(Medline)

Nakajima, T., Uchida, C., Anderson, S.F., Parvin, J.D. and Montminy, M. (1997) Analysis of a cAMP-responsive activator reveals a two-component mechanism for transcriptional induction via signal-dependent factors, *Genes Dev.* 11:738-747.(Medline)

Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K.

and Taga, T. (1999) Synergistic signaling in fetal brain by STAT3-smad1 complex bridged by p300, *Science* 284:479-482.(Medline)

Naranjo, J.R., Mellström, B., Achaval, M. and Sassone-Corsi, P. (1991) Molecular pathways of pain: Fos/Jun-mediated activation of a noncanonical AP-1 site in the prodynorphin gene, *Neuron* 6:607-617.(Medline)

Narumiya, S. (1996) The small GTPase Rho: cellular functions and signal transduction, *J. Biochem* 120:215-228.(Medline)

Nathan, D.F., Vos, M.H. and Lindquist, S. (1997) In vivo functions of the *Saccharomyces cerevisiae* Hsp90 chaperone, *Proc. Natl. Acad. Sci. USA* 94:12949-12956.(Medline)

Nedergaard, M. (1994) Direct signaling from astrocytes to neurons in cultures of mammalian brain cells, *Science* 263:1768-1771.(Medline)

Neer, E.J. (1995) Heterotrimeric G proteins: organizers of transmembrane signals, *Cell* 80:249-257.(Medline)

Neer, E.J. and Smith, T.F. (1996) G protein heterodimers: new structures propel new questions, *Cell* 84:175-178.(Meldine)

Neer, E.J., Schmidt, C.J., Nambudripad, R. and Smith, T.F. (1994) The ancient regulatory-protein family of WD-repeat proteins, *Nature* 371:297-300.(Medline)

Neri, L.M., Billi, A.M., Manzoli, L., Rubbini, S., Gilmour, R.S., Cocco, L. and Martelli, A.M. (1994) Selective nuclear translocation of protein kinase C α in Swiss 3T3 cells treated with IGF-I, PDGF and EGF, *FEBS Lett.* 347:63-68.(Medline)

Newman, E.A. and Zahs, K.R. (1998) Modulation of neuronal activity by glial cells in the retina, *J. Neurosci.* 18:4022-4028.(Medline)

Ni, M., Tepperman, J.M. and Quail, P.H. (1998) PIF3, a phytochrome-interacting factor necessary for normal photoinduced signal transduction, is a novel basic helix-loop-helix protein, *Cell* 95:657-667.(Medline)

Ni, M., Tepperman, J.M. and Quail, P.H. (1999) Binding of phytochrome B to its nuclear signalling partner PIF-3 is reversibly induced by light, *Nature* 400:781-784.(Medline)

Nigg, E.A., Hiltz, H., Eppenberger, H.M. and Dutly, F. (1985) Rapid and reversible translocation of the

catalytic subunit of cAMP-dependent protein-kinase type II from the Golgi-complex to the nucleus, *EMBO J.* 4:2801-2806.(Medline)

Nikawa, J., Akiyoshi, M., Hirata, S. and Fukuda, T. (1996) *Saccharomyces cerevisiae* IRE2/HAC1 is involved in IRE1-mediated KAR2 expression, *Nucleic Acids Res.* 24:4222-4226.(Medline)

Nishizuka, Y. (1984) Turnover of inositol phospholipids and signal transduction, *Science* 225:1365-1370.(Medline)

Nisoli, E., Clementi, E., Paolucci, C., Cozzi, V., Tonello. C., Sciorati, C., Bracale, R., Valerio, A., Francolini, M., Moncada, S. and Carruba, M.O. (2003) Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide, *Science* 299:896-899. (MedLine)

Niwa, M., Sidrauski, C., Kaufman, R.J. and Walter, P. (1999) A role for presenilin-1 in nuclear accumulation of Ire1 fragments and induction of the mammalian unfolded protein response, *Cell* 99:691-702.(Medline)

Noble, S. and Shimoni, Y. (1981) The calcium and frequency dependence of the slow inward current 'staircase' in frog atrium, *J. Physiol. (London)* 310:57-75.(Medline)

Nuclear Receptors Nomenclature Committee (1999) A unified nomenclature system for the nuclear receptor superfamily, *Cell* 97:161-163.(Medline)

Nunnari, J. and Walter, P. (1996) Regulation of organelle biogenesis, *Cell* 84:389-394.(Medline)

Odom, A.R., Stahlberg, A., Wente, S.R. and York, J.D. (2000) A role for nuclear inositol 1,4,5-trisphosphate kinase in transcriptional control, *Science* 287:2026-2029. (MedLine)

Okamoto, T., Schlegel, A., Scherer, P.E. and Lisanti, M.P. (1998) Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane, *J. Biol. Chem.* 273:5419-5422.(Medline)

Okamoto, S., Krainc, D., Sherman, K. and Lipton, S.A. (2000) Antiapoptotic role of the p38 mitogen-activated protein kinase-myocyte enhancer factor 2 transcription factor pathway during neuronal differentiation, *Proc. Natl. Acad. Sci. USA.* 97:7561-7466. (MedLine)

Olkkonen, V.M. and Stenmark, H. (1997) Role of Rab GTPases in membrane traffic, *Int. Rev. Cytol.* 176:1-85.(Medline)

Olofsson, B. (1999) Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling, *Cell*

Signal 11:545-554. (MedLine)

Olshevskaya, E.V., Hughes, R.E., Hurley, J.B. and Dizhoor, A.M. (1997) Calcium binding, but not a calcium-myristoyl switch, controls the ability of guanylyl cyclase-activating protein GCAP-2 to regulate photoreceptor guanylyl cyclase, *J. Biol. Chem.* 272:14327-14333.(Medline)

Olson, E.N., Burgess, R. and Staudinger, J. (1993) Protein kinase C as a transducer of nuclear signals, *Cell Growth Differ.* 4:699-705.(Medline)

Olson, M.F., Paterson, H.F. and Marshall, C.J. (1998) Signals from Ras to Rho GTPases interact to regulate expression of $p21^{Waf1/Cip1}$, *Nature* 394:295-299.(Medline)

Onate, S.A., Tsai, S.Y., Tsai, M.-J. and O'Malley, B.W. (1995) Sequence and characterization of a coactivator for steroid hormone receptor superfamily, *Science* 270:1354-1357.(Medline)

Opas, M., Szewczenko-Pawlikowski, M., Jass, G.K., Mesaeli, N. and Michalak, M. (1996) Calreticulin modulates cell adhesiveness via regulation of vinculin expression, *J. Cell Biol.* 135:1913-1923. (MedLine)

Pahl, H.L. and Baeuerle, P.A. (1997a) Endoplasmic reticulum-induced signal transduction and gene expression, *Trends in Cell Biol.* 7:50-55.

Pahl, H.L. and Baeuerle, P.A. (1997b) The ER-overload response: activation of NF-κ B. *Trends Biochem. Sci.* 22:63-67.(Medline)

Palmer, A., Gavin, A.C. and Nebreda, A.R. (1998) A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1, *EMBO J.* 17:5037-5047.(Medline)

Pantopoulos, K., Weiss, G. and Hentze, M.W. (1994) Nitric oxide and the post-transcriptional control of cellular iron traffic, *Trends in Cell Biol.* 4: 82-86.(Medline)

Parekh, A. B., and Penner, R. (1997) Store depletion and calcium influx, *Physiol. Rev.* 77:901-930.(Medline)

Patel, S., Churchill, G.C., Sharp, T. and Galione, A. (2000) Widespread distribution of binding sites for the novel Ca^{2+}-mobilizing messenger, nicotinic acid adenine dinucleotide phosphate, in the brain, *J. Biol. Chem.* 275:36495-36497. (MedLine)

Patel, S., Churchill, G.C. and Galione, A. (2001) Coordination of Ca^{2+} signalling by NAADP, *Trends*

Biochem. Sci. 26:482-489. (MedLine)

Patki, V., Virbasius, J., Lane, W.S., Toh, B.H., Shpetner, H.S. and Corvera S (1997) Identification of an early endosomal protein regulated by phosphatidylinositol 3-kinase, *Proc. Natl. Acad. Sci.* USA 94:7326-7330.(Medline)

Patki, V., Lawe, D.C., Corvera, S., Virbasius, J.V. and Chawla, A. (1998) A functional PtDIns(3)P-binding motif, *Nature* 394:433-434.(Medline)

Patterson, R.L., van Rossum, D.B. and Gill, D.L. (1999) Store-operated Ca^{2+} entry: evidence for a secretion-like coupling model, *Cell* 98:487-499.(Medline)

Pawson, T. and Nash, P. (2000) Protein-protein interactions define specificity in signal transduction, *Genes Dev.* 14:1027-1047. (MedLine)

Pawson, T. and Scott, J.D. (1997) Signaling through scaffold, anchoring, and adaptor proteins, *Science* 278:2075-2080.(Medline)

Peersen, O.B., Madsen, T.S. and Falke, J.J. (1997) Intermolecular tuning of calmodulin by target peptides and proteins: differential effects on Ca^{2+} binding and implications for kinase activation, *Protein Sci.* 6:794-807. (MedLine)

Peles, E., Schlessinger, J. and Grumet, M. (1998) Multi-ligand interactions with receptor-like protein tyrosine phosphatase β: implications for intercellular signaling, *Trends Biochem. Sci.* 23:121-124.(Medline)

Perez-Terzic, Cc, Jaconi, M. and Clapham, D.E. (1997) Nuclear calcium and the regulation of the nuclear pore complex, *BioEssays* 19:787-792. (MedLine)

Petkovich, M., Brand, N.J., Krust, N.J., and Chambon, P. (1987) A human retinoic acid receptor belongs to the family of nuclear receptors, *Nature* 330:444-450.(Medline)

Pfeilschifter, J. and Huwiler, A. (2000) Ceramides as key players in cellular stress reponse, *News Physiol. Sci.* 15:11-15.

Philipp, S., Trost, C., Warnat, J., Rautmann, J., Himmerkus, N.,Schroth, G., Kretz, O., Nastainczyk, W., Cavalie, A., Hoth, M. and Flockerzi, V. (2000) TRP4 (CCE1) protein is part of native calcium release-activated Ca^{2+}-like channels in adrenal cells, *J. Biol. Chem.* 275:23965-23972. (MedLine)

Philips, A., Lesage, S., Gingras, R., Maira, M.H., Gauthier, Y., Hugo, P. and Drouin, J. (1997) Novel

dimeric Nur77 signaling mechanism in endocrine and lymphoid cells, *Mol. Cell Biol.* 17:5946-5951. (MedLine)

Picard, D. and Yamamoto, K.R. (1987) Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor, *EMBO J.* 6:3333-3340.(Medline)

Pieroni, J.P., Jacobowitz, O., Chen, J. and Iyengar, R. (1993) Signal recognition and integration by Gs-stimulated adenylyl cyclases, *Curr. Opin. Neurobiol.* 3:345-351. (MedLine)

Podsypanina, K., Ellenson, L.H., Nemes, A., Gu, J., Tamura, M., Yamada, K.M., Cordon-Cardo, C., Catoretti, G., Fisher, P.E. and Parsons, R. (1999) Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems, *Proc. Natl. Acad. Sci. USA* 96:1563-1568.(Medline)

Popov, S., Yu, K., Kozasa, T. and Wilkie, T.M, (1997) The regulators of G protein signaling (RGS) domains of RGS4, RGS10, and GAIP retain GTPase activating protein activity in vitro, *Proc. Natl. Acad. Sci. USA* 94:7216-7220.(Medline)

Pratt, W.B, Jolly, D.J., Pratt, D.V., Hollenberg, S.M., Giguère, A., Cadepon, F.M., Schweizer-Groyer, G. Cartelli, M.G., Evans, R.M. and Baulieu, E.E. (1988) A region in the steroid-binding domain determines formation of the non-DNA binding, as glucocorticoid receptor complex, *J. Biol. Chem.* 263: 267-273.(Medline)

Primm, T.P., Walker, K.W. and Gilbert, H.F. (1996) Facilitated protein aggregation. Effects of calcium on the chaperone and anti-chaperone activity of protein disulfide-isomerase, *J. Biol. Chem.* 271:33664-33669. (MedLine)

Prodromou, C., Roe, S.M., O'Brien, R., Ladbury, J.E., Piper, P.W. and Pearl, L.H. (1997) Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone, *Cell* 90:65-75.(Medline)

Putney, J.W., Jr. and Bird, G.S.J. (1993) The inositol phosphate- calcium signaling system in non-excitable cells, *Endocr. Rev.* 14:610-631.

Putney, J.W. Jr. and McKay, R.R. (1999) Capacitative calcium entry channels, *BioEssays* 21:38-46.(Medline)

Qiu, R.G., Chen, J., McCormick, F. and Symons, M. (1995) A role for Rho in Ras transformation, *Proc. Natl. Acad. Sci. USA* 92:11781-11785.(Medline)

Qin, N., Olcese, R., Bransby, M., Lin, T. and Birnbaumer, L. (1999) Ca^{2+}-induced inhibition of the cardiac Ca^{2+} channel depends on calmodulin, *Proc. Natl. Acad. Sci. USA.* 96:2435-2438.(Medline)

Quail, P.H. (1997) An emerging molecular map of the phytochromes, *Plant Cell Enviro.* 20:657-665.

Quilliam, L.A., Khosravi-Far, R., Huff, S.Y. and Der, C.J. (1995) Guanine nucleotide exchange factors: activators of the Ras superfamily of proteins, *BioEssays* 17:395-404.(Medline)

Rapp, P.E. and Berridge, M.J. (1981) The control of transepithelial potential oscillations in the salivary gland of *Calliphora Erythrocephala*, *J. Exp. Biol.* 93:119-132.

Razandi, M., Pedram, A., Greene, G.L. and Levin, E.R. (1999) Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ER α and ER β expressed in Chinese hamster ovary cells, *Mol. Endocrinol.*13:307-319. (MedLine)

Riboni, L., Viani, P., Bassi, R., Prinetti, A. and Tettamanti, G. (1997) The role of sphingolipids in the process of signal transduction, *Prog. Lipid Res.* 36:153-195.(Medline)

Riccio, A., Pierchala, B.A., Ciarallo, C.L. and Ginty, D.D. (1997) An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons, *Science* 277:1097-1100.(Medline)

Riccio, A., Ahn, S., Davenport, C.M., Blendy, J.A. and Ginty, D.D. (1999) Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons, *Science* 286:2358-2361.(Medline)

Richards, S.A., Fu, J., Romanelli, A., Shimamura, A. and Blenis, J. (1999) Ribosomal S6 kinase 1 (RSK1) activation requires signals dependent on and independent of the MAP kinase ERK, *Curr. Biol.* 9:810-820.(Medline)

Rizzuto, R.A., Simpson, W.M., Brini, M. and Pozzan, T. (1992) Rapid changes in mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin, *Nature* 358: 325-327.(Medline)

Rizzuto, R., Bastianutto, C., Brini, M., Murgia, M. and Pozzan, T. (1994) Mitochondrial Ca^{2+} homeostasis in intact cells, *J. Cell Biol.* 126:1183-1194.(Medline)

Rizzuto, R., Pinton, P., Carrington, W., Fay, F.S., Fogarty, K.E., Lifshitz, L.M., Tuft, R.A. and Pozzan, T. (1998) Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses, *Science* 280:1763-1776.(Medline)

Robb-Gaspers, L.D., Rutter, G.A., Burnett, P., Hajnoczky, G., Denton, R.M. and Thomas, A.P. (1998) Coupling between cytosolic and mitochondrial calcium oscillations: role in the regulation of hepatic metabolism, *Biochim. Biophys. Acta* 1366:17-32.(Medline)

Robertson, L.M, Kerppolla, T.K., Vendrell, M., Luk, D., Smeyne, R.J., Bocchiaro, C., Morgan, J.I. and Curran, T. (1995) Regulation of *c-fos* expression in transgenic mice requires multiple interdependent transciption control elements, *Neuron* 14:241-252.(Medline)

Ronce, N., Raynaud, M., Toutain, A., Moizard, M.P., Colleaux, L., Gendrot, C., Briault, S. and Moraine, C. (1999) Evidence for a new X-linked mental retardation gene in Xp21-Xp22: clinical and molecular data in one family, *Am. J. Med. Genet.* 83:132-137.(Medline)

Rooney, T.A. and Thomas, A.P. (1993) Intracellular calcium waves generated by Ins(1,4,5)P_3-dependent mechanism, *Cell Calcium* 14:674-690.(Medline)

Rooney, T.A., Sass, E.J. and Thomas, A.P. (1989) Characterization of cytosolic calcium oscillations induced by phenylephrine and vasopressin is single fura-2-loaded hepatocytes, *J. Biol. Chem.* 264:17131-17141.(Medline)

Rosen, L.B., Ginty, D.D., Weber, M.J. and Greenberg, M.E. (1994) Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras, *Neuron* 12:1207-1221. (MedLine)

Ross, E. M. and Gilman, A. G. (1977) Resolution of some components of adenylate cyclase necessary for catalytic activity, *J. Biol. Chem.* 252:6966-6989.(Medline)

Ross, E.M. and Wilkie, T.M. (2000) GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins, *Annu. Rev. Biochem* 69:795-827. (MedLine)

Rudner, D.Z., Fawcett, P. and Losick, R. (1999) A family of membrane-embedded metalloproteases involved in regulated proteolysis of membrane-associated transcription factors, *Proc. Natl. Acad. Sci. USA* 96:14765-14770.(Medline)

Rutter, G.A. (1990) Ca^{2+}-binding to citrate cycle dehydrogenases, *Int. J. Biochem.* 22:1081-1088. (MedLine)

Sackey, F.N.A., Hache, R.J.G., Reich, T., Kwast-Welfeld, J. and Lefebvre, Y.A. (1996) Determinants of subcellular distribution of glucocorticoid receptor, *Mol. Endocrinol.* 10:1191-1205.(Medline)

Saez, J.C., Connor, J.A., Spray, D.C. and Bennett, M.V.L. (1989) Hepatocyte gap junctions are permeable to the second messenger inositol 1,4,5-triphosphate and to calcium ions, *Proc. Natl. Acad. Sci. USA* 86: 2708-2712.(Medline)

Sambrook, J.F. (1990) The involvement of calcium in transport of secretory proteins from the endoplasmic reticulum, *Cell* 61:197-199. (MedLine)

Samuels, H.H. and Tsai, J.S. (1973) Thyroid hormone action in cell culture: demonstration of nuclear receptors in intact cells and isolated nuclei, *Proc. Natl. Acad. Sci.* 70:3488-3492.(Medline)

Sano, K., Takai, Y., Yamanashi, J. and Nishizuka. Y. (1983) A role of calcium-activated phospholipid-dependent protein kinase in human platelet activation, *J. Biol. Chem.* 258:2010-2013.(Medline)

Sassone-Corsi, P. (1995) Transcription factors responsive to cAMP, *Annu. Rev. Cell. Dev. Biol.* 11:355-377.(Medline)

Sassone-Corsi, P. (1998) CREM: a master-switch governing male germ cells differentiation and apoptosis, *Semin. Cell Dev. Biol.* 9:475-482.(Medline)

Sassone-Corsi, P., Mizzen, C.A., Cheung, P., Crosio, C., Monaco, L., Jacquot, S., Hanauer, A., Allis, C.D. (1999) Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3, *Science* 285:886-891.(Medline)

Schaeffer, H.J., Catling, A.D., Eblen, S.T., Collier, L.S., Krauss, A., Weber, M.J. (1998) MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade, *Science* 281:1668-1671.(Medline)

Scheffzek, K., Ahmadian, M.R., Kabsch, W., Wiesmuller, L., Lautwein, A., Schmitz, F. and Wittinghofer, A. (1997) The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants, *Science* 277:333-338.(Medline)

Schindler, C. and Darnell, J.E., Jr (1995) Transcriptional responses to polypeptide ligands: The JAK-STAT pathway, *Annu. Rev. Biochem.* 64: 621-651.(Medline)

Schlessinger, J. (2000) Cell signaling by receptor tyrosine kinases *Cell* 103:211-225.

Schramm, M. and Selinger. Z. (1984) Message transmission: receptor controlled adenylate cyclase system, *Science* 225:1350-1356.(Medline)

Schroeter, E.H., Kisslinger, J.A. and Kopan, R. (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain, *Nature* 393:382-386.(Medline)

Schulster, D., Orly, J., Seidel, G. and Schramm, M. (1978) Intracellular cyclic AMP production enhanced by a hormone receptor transferred from a different cell, *J. Biol. Chem.* 253:1201-1206.(Medline)

Seabra M.C. (1998) Membrane association and targeting of prenylated Ras-like GTPases, *Cell Signal.*

10:167-172.(Medline)

Shamu, C.E. (1997) Signal transduction: splicing together the unfolded-protein-response, *Curr. Biol.* 7:67-70.(Medline)

Shamu, C.E. and Walter, P. (1996) Oligomerization and phosphorylation of the IreIp kinase during intracellular signaling from the endoplasmic reticulum to the nucleus, *EMBO J.* 15:3028-3039.(Medline)

Shamu, C.E., Cox, J.S. and Walter, P. (1994) The unfolded-protein- response pathway in yeast, *Trends in Cell Biol.* 4:57-60.

Shao, D., Rangwala, S.M., Bailey, S.T., Krakow, S.L., Reginato, M.J. and Lazar, M.A. (1998) Interdomain communication regulating ligand binding by PPAR-γ, *Nature* 396:377-380.(Medline)

Sheng, M., Thompson, M.A. and Greenberg, M.E. (1991) CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases, *Science* 252:1427-1430.(Medline)

Shenoy, S.K., McDonald, P.H., Kohout, T.A. and Lefkowitz, R.J. (2001) Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and β-arrestin, *Science* 294:1307-1313. (MedLine)

Shikama, N., Lyon, J. and La Thangue, N.B. (1997) The p300/CBP family: integrating signals with transcription factors and chromatin, *Trends in Cell Biol.* 7:230-236

Shimeno, H., Soeda, S., Sakamoto, M., Kouchi, T., Kowakame, T. and Kihara, T. (1998) Partial purification and characterization of sphingosine N-acyltransferase (ceramide synthase) from bovine liver mitochondrion-rich fraction, *Lipids* 33:601-605. (MedLine)

Sidrauski, C. and Walter, P. (1997) The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response, *Cell* 90:1031-1039.(Medline)

Sidrauski, C., Cox, J.S. and Walter, P. (1996) tRNA ligase is required for regulated mRNA splicing in the unfolded protein response, *Cell* 87:405-413.(Medline)

Silva, A.J., Smith, A.M. and Giese, K.P. (1998) Gene targeting and the biology of learning and memory, *Annu. Rev. Genet.* 31:527-546.(Medline)

Simon, M.A. (2000) Receptor tyrosine kinases: specific outcomes from general signals, *Cell* 103:13-15. (MedLine)

Simonsen, A., Lippé, R., Christoforidis, S., Toh, B.-H., Murphy, C., Zerial, M. and Stenmark, H. (1998)

EEA1 links PI(3)K function to Rab5 regulation of endosome fusion, *Nature* 394:494-498.(Medline)

Skiba, N.P., Bae, H. and Hamm, H.E. (1996) Mapping of effector binding sites of transducin alpha-subunit using G alpha t/G alpha i1 chimeras, *J. Biol. Chem.* 271:413-424.(Medline)

Smith, M.R. Jaramillo, M.L., Liu, Y., Dever, T.E., Merrick, W.C. Kung, H. and Sonenberg, N. (1990a) Translation initiation factors induce DNA synthesis and transform NIH 3T3, *New Biol.* 2:648-654.(Medline)

Smith, J.R., Osborne, T.F., Goldstein, J.L. and Brown, M.S. (1990b) Identification of nucleotides responsible for enhancer activity of sterol regulatory element in low density lipoprotein receptor gene, *J. Biol. Chem.* 265:2306-2310.(Medline)

Sondek, J., Bohm, A., Lambright, D.G., Hamm, H.E. and Sigler, P.B. (1996) Crystal structure of a G-protein beta gamma dimer at 2.1 Å resolution, *Nature* 379:369-374.(Medline)

Spencer, T.E., Jenster, G., Burcin, M.M., Allis, C.D., Zhou, J., Mizzen, C.A., McKenna, N.J., Onate, S.A., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1997) Steroid receptor coactivator-1 is a histone acetyltransferase, *Nature* 389:194-198. (MedLine)

Spiegel. S.and Merrill, A.H. Jr. (1996) Sphingolipid metabolism and cell growth regulation, *FASEB J.* 10:1388-1397.(Medline)

Spiegel, S., Foster, D. and Kolesnick, R. (1996) Signal transduction through lipid second messengers, *Curr. Opin. Cell Biol.* 8:159-167.(Medline)

Stambolic, V., Suzuki, A., de la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P. and Mak, T.W. (1998) Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN, *Cell* 95:29-39.(Medline)

Stamler, J.S., Singel, D.J. and Loscalzo, J. (1992) Biochemistry of nitric oxide and its redox activated forms, *Science* 258: 1898-1902.(Medline)

Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H. and Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D.H., Tavtigian, S.V. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers, *Nature Genet.* 15:356-362. (MedLine)

Strubing, C. and Clapham, D.E. (1999) Active nuclear import and export is independent of lumenal Ca^{2+} stores in intact mammalian cells, *J. Gen. Physiol.* 113:239-248. (MedLine)

Struhl, G. and Adachi, A. (1998) Nuclear access and action of notch in vivo, *Cell* 93:649-660.(Medline)

Struthers, R.S., Vale, W.W., Arias, C., Sawchenko, P.E. and Montminy, M.R. (1991) Somatotroph hypoplasia and dwarfism in transgenic mice expressing a non-phosphorylatable CREB mutant, *Nature* 350:622-624.(Medline)

Stryer. L. (1986) Cyclic GMP cascade of vision, *Annu. Rev. Neurosci.* 9:87-119.(Medline)

Stryer. L., and Bourne, H. R. (1986) A family of signal transducers, *Annu. Rev. Cell Biol.* 2:391-420.(Medline)

Suarez-Lopez, P. and Coupland, G. (1997) Plants see the blue light, *Science* 279:1323-1324.(Medline)

Suzuki, A., de la Pompa, J.L., Stambolic, V., Elia, A.J., Sasaki, T., del Barco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., Fukumoto, M., Mak, T.W. (1998) High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice, *Curr. Biol.* 8:1169-1178.(Medline)

Sweitzer, S.M and Hinshaw, J.E. (1998) Dynamin undergoes a GTP-dependent conformational change causing vesiculation, *Cell* 93:1021-1029.(Medline)

Swindells, M.B. and Ikura, M. (1996) Pre-formation of the semi-open conformation by the apo-calmodulin C-terminal domain and implications binding IQ-motifs, *Nature Struct. Biol.* 3:501-504. (MedLine)

Tabak, H.F., Braakman, I. and Distel, B. (1999) Peroxisomes: simple in function but complex in maintenance, *Trends Cell Biol.* 9:447-453.(Medline)

Takasawa, S., Nata, K., Yonekura, H., and Okamoto, H. (1993a) Cyclic ADP-ribose in insulin secretion from pancreatic beta cells, *Science* 259: 370-373.(Medline)

Takasawa, S., Tohgo, A., Noguchi, N., Koguma, T., Nata, K., Sugimoto, T., Yonekura H and Okamoto, H. (1993b) Synthesis and hydrolysis of cyclic ADP-ribose by human leukocyte antigen CD38 and inhibition of the hydrolysis by ATP, *J. Biol. Chem.* 268:26052-26054.(Medline)

Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaskam, M., Hirose, T. and Numa, S. (1989) Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor, *Nature* 339:439-445.(Medline)

Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R. and Yamada, K.M. (1998) Inhibition of cell

migration, spreading, and focal adhesions by tumor suppressor PTEN, *Science* 280:1614-1617.(Medline)

Tan, Y., Rouse, J., Zhang, A., Cariati, S., Cohen, P. and Comb, M.J. (1996) FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2, *EMBO J.* 15:4629-4642.(Medline)

Tanaka, T., Ames, J.B., Harvey, T.S., Stryer, L. and Ikura, M. (1995) Sequestration of the membrane-targeting myristoyl group of recoverin in the calcium-free state, *Nature* 376:444-447.(Medline)

Tessier-Lavigne, M. and Goodman, C.S.(1996) The molecular biology of axon guidance, *Science* 274:1123-1133.(Medline)

Thomas, A.P., Bird, G.S.J., Hajnóczky, G., Robb-Gaspers, L.D. and Putney, J.W. Jr. (1996) Spatial and temporal aspects of cellular calcium signaling, *FASEB J.* 10:1505-1517.(Medline)

Timmerman, L.A., Clipstone, N.A., Ho, S.N., Northrop, J.P. and Crabtree, G.R. (1996) Rapid shuttling of NF-AT in discrimination of Ca2+ signals and immunosuppression, *Nature* 383:837-840.(Medline)

Toft, D.O. (1999) Control of hormone receptor function by molecular chaperones and folding catalysts, in *Molecular Chaperones and Folding Catalysts-Regulation, Cellular Function and Mechanisms*, Bakau, B. ed., Harwood Academic Publishers, Amsterdam pp.313-327.(Medline)

Toker A. (1998) The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate, *Curr. Opin. Cell Biol.* 10:254-261. (MedLine)

Topham, M.K., Bunting, M., Zimmerman, G.A., McIntyre, T.M., Blackshear, P.J. and Prescott, S.M. (1998) Protein kinase C regulates the nuclear localization of diacylglycerol kinase-ζ, *Nature* 394:697-700.(Medline)

Torres, J., Darley-Usmar, V. and Wilson, M.T. (1995) Inhibition of cytochrome c oxidase in turnover by nitric oxide: mechanism and implications for control of respiration, *Biochem. J.* 312:169-173. (MedLine)

Traugh, J.A. and Pendergast , A.M. (1986) Regulation of protein synthesis by phosphorylation of ribosomal protein S6 and aminacyl-tRNA synthetase, *Prog. Nucleic Acid Res. Mol. Biol.* 33:195-230.(Medline)

Tremblay, A., Tremblay, G.B., Labrie, F. and Giguère, V. (1999) *Mol. Cell*(Medline)

Treves, S., De Mattei, M., Lanfredi, M., Villa, A., Green, M., MacLennan, D. H., Meldolesi, J. and Pozzan, T. (1990) Calreticulin, a candidate for a calsequestrin-like function in Ca^{2+} -storage

compartments (calcisomes) of liver and brain, *Biochem. J.* 272:473-480.(Medline)

Trowbridge, J.M., Rogatsky, I. and Garabedian, M.J. (1997) Regulation of estrogen receptor transcriptional enhancement by the cyclin A/Cdk2 complex, *Proc. Natl. Acad. Sci. USA* 94:10132-10137.(Medline)

Tsai, C.C., Kao, H.Y., Yao, T.P., McKeown, M. and Evans, R.M. (1999) SMRTER, a Drosophila nuclear receptor coregulator, reveals that EcR-mediated repression is critical for development, *Mol. Cell.* 4:175-186. (MedLine)

Tsugane, K., Tamiya-Koizumi, K., Nagino, M., Nimura, Y. and Yoshida, S. (1999) A possible role of nuclear ceramide and sphingosine in hepatocyte apoptosis in rat liver, *J. Hepatol.* 31:8-17. (MedLine)

Tsunoda, S., Sierralta, J. and Zuker, C.S. (1998) Specificity in signaling pathways: assembly into multimolecular signaling complexes, *Curr. Opin. Genet. Dev.* 8:419-422.(Medline)

Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M. and Narumiya, S, (1997) Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension, *Nature* 389:990-994.(Medline)

Unitt, J.F., McCormack, J.G., Reid, D. MacMachlan, L.K. and England, P.J. (1989) Direct evidence for a role of mitochondrial Ca^{2+} in the regulation of oxidative phosphorylation in the stimulated rat heart. Studies using ^{31}P-NMR and ruthenium red, *Biochem. J.* 262: 293-301.(Medline)

Valera, S., Ballivet, M. and Bertrand, D. (1992) Progesterone modulates a neuronal nicotinic acetylcholine receptor, *Proc. Natl. Acad. Sci. USA* 89:9949-9953.(Medline)

Valverde, M.A., Rojas, P., Amigo, J., Cosmelli, D., Orio, P., Bahamonde, M.I., Mann, G.E., Vergara, C,. and Latorre, R. (1999) Acute activation of Maxi-K channels (hSlo) by estradiol binding to the β subunit, *Science* 285:1929-1931. (MedLine)

Van Aelst, L. and D'Souza-Schorey, C. (1997) Rho GTPases and signaling networks, *Genes Dev.* 11:2295-2322.(Medline)

Van Brocklyn, J.R., Lee, M.J., Menzeleev, R., Olivera, A., Edsall, L., Cuvillier, O., Thomas, D.M., Coopman, P.J.P, Thangada, S., Liu, C.H,, Hla, T. and Spiegel, S. (1998) Dual actions of sphingosine-1-phosphate: extracellular through the G_i-coupled receptor Edg-1 and intracellular to regulate proliferation and survival, *J. Cell Biol.* 142:229-240.(Medline)

Vanhaesebroeck, B., Leevers, S.J., Panayotou, G. and Waterfield, M.D. (1997) Phosphoinositide 3-

kinases: a conserved family of signal transducers, *Trends Biochem. Sci.* 22:267-272.(Medline)

Varanasi, U., Chu, R., Huang, Q., Castellon, R., Yeldani, A.V. and Reddy, J.K. (1996) Identification of a peroxisome proliferator-responsive element upstream of the human peroxisomal fatty acyl coenzyme A oxidase gene, *J. Biol. Chem.* 271:2147-2155.(Medline)

Vassilakos, A., Michalak, M., Lehrman, M.A. and Williams, D.B. (1998) Oligosaccharide binding characteristics of the molecular chaperones calnexin and calreticulin, *Biochemistry* 37:3480-3490. (MedLine)

Velazquez, L., Fellous, M., Stark, G.R. and Pellegrini, S. (1992) A protein tyrosine kinase in the interferon / signaling pathway, *Cell* 70:313-322.(Medline)

Verkhratsky, A., Orkand, R.K. and Kettenmann, H. (1998) Glial calcium: homeostasis and signaling function, *Physiol. Rev.* 78:99-141.(Medline)

Vishnivetskiy, S.A., Paz, C.L., Schubert, C., Hirsch, J.A., Sigler, P.B. and Gurevich, V.V. (1999) How does arrestin respond to the phosphorylated state of rhodopsin? *J. Biol. Chem.* 274:11451-11454.(Medline)

Volotovski, I.D. (1998) Ca^{2+} and intracellular signalling in plant cells: a role in phytochrome transduction, *Membr. Cell Biol.* 12:721-742.(Medline)

Volpe, P., Krause, K.-H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J. and Lew, D. P. (1988) "Calciosome," a cytoplasmic organelle, the inositol, 1,4,5-triphosphate-sensitive Ca^{2+} store of nonmuscle cells, *Proc. Natl. Acad. Sci. USA* 85:1091-1095.(Medline)

Wade, P.A. and Wolffe, A.P. (1997) Histone acteyltransferases in control, *Curr. Biol.* 7:R82-R84.(Medline)

Walker, D., Htun, H. and Hager, G.L. (1999) Using inducible vectors to study intracellular trafficking of GFP-tagged steroid/nuclear receptors in living cells, *Methods* 19:386-393.(Medline)

Wall, M.A., Coleman, D.E., Lee, E., Iniguez-Lluhi, J.A., Posner, B.A., Gilman, A.G. and Sprang, S.R. (1995) The structure of the G protein heterotrimer Gi $\alpha_1 \beta_1 \gamma_2$, *Cell* 83:1047-1058.(Medline)

Wehling, M. (1997) Specific, nongenomic actions of steroid hormones, *Annu. Rev. Physiol.* 59:365-393.(Medline)

Weinmaster, G. (1997) The ins and outs of Notch signaling, *Mol. Cell Neurosci.* 9:91-102.(Medline)

Weiss, G., Gossen, B., Doppler, W., Fuchs, D., Pantopoulos, K., Werner-Felmayer, G., Wachter, H. and Henze, M.W., Translational regulation via iron-responsive elements by nitric oxide/NO synthase pathway, *EMBO J.* 12: 3651-3657.(Medline)

Weisskopf, M.G., Zalutsky, R.A. and Nicoll, R.A. (1993) The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation, *Nature* 362:423-427.(Medline)

Wen, Z., Zhong, Z. and Darnell, J.E. Jr., (1995) Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation, *Cell* 82:241-250. (MedLine)

Wennstrom, S., Hawkins, P., Cooke, F., Hara, K., Yonezawa, K., Kasuga, M., Jackson, T., Claesson-Welsh, L. and Stephens,L. (1994) Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling, *Curr. Biol.* 4:385-393.(Medline)

Wera, S. and Hemmings, B.A. (1995) Serine/threonine protein phosphatases, *Biochem. J.* 311:17-29.(Medline)

Wess, J. (1997) G-protein coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition, *FASEB J.* 11:346-354.(Medline)

Westphal, R.S., Anderson, K.A., Menas, A.R. and Wadzinski, B.E. (1998) A signal complex of Ca^{2+}-calmodulin-dependent protein kinase IV and protein phosphatase 2A, *Science* 280:1258-1261.(Medline)

Westphal, R.S., Soderling, S.H., Alto, N.M., Langeberg, L.K. and Scott, J.D. (2000) Scar/WAVE-1, a Wiskott-Aldrich syndrome protein, assembles an actin-associated multi-kinase scaffold, *EMBO J.* 19:4589-4600. (MedLine)

White, M.J., Hirsch, J.P. and Henry S.A., (1991) The OPI1 gene of *Saccharomyces cerevisiae*, a negative regulator of phospholipid biosynthesis, encodes a protein containing polyglutamine tracts and a leucine zipper, *J. Biol. Chem.* 266:863-872.(Medline)

White, A.M., Watson, S.P. and Galione, A.(1993) Cyclic ADP-ribose-induced Ca2+ release from rat brain microsomes, *FEBS-Lett.* 318: 259-263.(Medline)

Whitmarsh, A.J., Cavanagh, J., Tournier, C., Yasuda, J., Davis, R.J. (1998) A mammalian scaffold complex that selectively mediates MAP kinase activation, *Science* 281:1671-1674.(Medline)

Wiest, D.L., Burkahrdt, J.K., Hester, S., Hortsch, M., Meyer, D.I. and Argon, Y. (1990) Membrane biogenesis during B cell differentiation: most endoplasmic reticulum proteins are expressed coordinately,

J. Cell Biol. 1501-1511.(Medline)

Willmann, T. and Beato, M. (1986) Steroid-free glucocorticoid receptor binds specifically to mouse mammary tumour virus DNA, *Nature* 324:688-691.(Medline)

Willmott, N., Sethi, J.K., Walseth, T.F., Lee, H.C., White, A.M. and Galione, A. (1996) Nitric oxide-induced mobilization of intracellular calcium via the cyclic ADP-ribose signaling pathway, *J. Biol. Chem.* 271:3699-3705.(Medline)

Willy, P.J. and Mangelsdorf, D.J. (1998) Nuclear orphan receptors: the search for novel ligands and signaling pathways, in *Hormones and Signaling* (O'Malley, B.W., ed.), vol. 1, pp.307-358, Academic Press, San Diego, CA.

Wilson, T.E., Fahrner, T.J., Johnston, M. and Milbrandt, J. (1991) Identification of the DNA binding site for NGFI-B by genetic selection in yeast, *Science* 252:1296-1300. (MedLine)

Wolfrum, C., Ellinghaus, P., Fobker, M., Seedorf, U., Assmann, G., Borchers, T. and Spener, F. (1999) Phytanic acid is ligand and transcriptional activator of murine liver fatty acid binding protein, *J. Lipid Res.* 40:708-714.(Medline)

Woodford-Thomas, T. and Thomas, M.L. (1993) The leukocyte common antigen, CD45 and other protein tyrosine phosphatases in hematopoietic cells, *Seminars in Cell Biology* 4:409-418.(Medline)

Woronicz, J.D., Lina, A., Calnan, B.J., Szychowski, S., Cheng, L. and Winoto, A. (1995) Regulation of the Nur77 orphan steroid receptor in activation-induced apoptosis, *Mol. Cell Biol.* 15:6364-6376. (MedLine)

Wright, J.H., Munar, E., Jameson, D.R., Andreassen, P.R., Margolis, R.L., Seger, R. and Krebs, E.G. (1999) Mitogen-activated protein kinase kinase activity is required for the G(2)/M transition of the cell cycle in mammalian fibroblasts, *Proc. Natl. Acad. Sci. USA* 96:11335-11340.(Medline)

Xia X.-M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J.E., Ishii, T., Hirschberg, B., Bond, C.T., Lutsenko, S., Maylie, J. and Adelman, J.P. (1998) Mechanism of calcium gating in small-conductance calcium-activated potassium channels, *Nature* 395:503-507. (Medline)

Xie, J.and McCobb, D.P (1998) Control of alternative splicing of potassium channels by stress hormones, *Science* 280:443-446. (MedLine)

Xie, J. and Black, D.L. (2001) A CaMK IV responsive RNA element mediates depolarization-induced alternative splicing of ion channels, *Nature* 410:936-939. (MedLine)

Xu, L., Glass, C.K. and Rosenfeld, M.G. (1999) Coactivator and corepressor complexes in nuclear receptor function, *Curr. Opin. Genet. Dev.* 9:140-147. (MedLine)

Yamaguchi, R., Nakamura, M., Mochizuki, N., Kay, S.A. and Nagatani, A. (1999) Light-dependent translocation of a phytochrome B-GFP fusion protein to the nucleus in transgenic *Arabidopsis*, *J. Cell Biol.* 145:437-445.(Medline)

Yamauchi, T., Ueiki, K., Tobe, K., Tamemoto, H., Sekine, N., Wada, M., Honjp, M., Takahashi, M., Takahashi, T., Hirai, H., Tushima, T., Akanuma, Y., Fujita, T., Komuro, I., Yasaki, Y. and Kadowaki, T. (1997) Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone, *Nature* 390:91-96.(Medline)

Yang. J. and Defranco, D.B. (1994) Diffferential role of heat shock proteins 70 in the *in vitro* nuclear import of glucocorticoid and simian virus 40 large tumor antigen, *Mol. Cell. Biol.* 14:5088-5098.(Medline)

Yang, J., Liu, J. and DeFranco, D.B. (1997), Subnuclear trafficking of glucocorticoid receptors in vitro: Chromatin recycling and nuclear export, *J. Cell Biol.* 137:523-538.(Medline)

Yao, T.P., Oh, S.P., Fuchs, M., Zhou, N.D., Ch'ng, L.E., Newsome, D., Bronson, R.T., Li, E., Livingston, D.M. and Eckner, R. (1998) Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300, *Cell* 93:361-372.(Medline)

Yao, Y., Ferrer-Montiel, A.V., Montal, M. and Tsien, R.Y. (1999) Activation of store-operated Ca^{2+} current in *Xenopus* oocytes requires SNAP-25 but not a diffusible messenger, *Cell* 98:475-485.(Medline)

Yeh, K.C. and Lagarias, J.C. (1998) Eukaryotic phytochromes: light-regulated serine/threonine protein kinases with histidine kinase ancestry, *Proc. Natl. Acad. Sci. USA.* 95:13976-13981.(Medline)

York, J.D., Odom, A.R., Murphy, R., Ives, E.B. and Wente, S.R. (1999) A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export, *Science* 285:96-100. (MedLine)

Youn, H.D., Chatila, T.A. and Liu, J.O. (2000) Integration of calcineurin and MEF2 signals by the coactivator p300 during T-cell apoptosis, *EMBO J.* 19:4323-4331. (MedLine)

Yue, L., Peng, J.B., Hediger, M.A. and Clapham, D.E. (2001) CaT1 manifests the pore properties of the calcium-release-activated calcium channel, *Nature*410:705-709. (MedLine)

Zhao, Y., Bjorbaek, C. and Moller, D.E. (1996) Regulation and interaction of pp90(rsk) isoforms with

mitogen-activated protein kinases, *J. Biol. Chem.* 271:29773-29779.(Medline)

Zhang, J., Barak, L.S., Winkler, K.E., Caron, M.G., Ferguson, S.S. (1997) A central role for β-arrestins and clathrin-coated vesicle-mediated endocytosis in β2-adrenergic receptor resensitization. Differential regulation of receptor resensitization in two distinct cell types, *J. Biol. Chem.* 272:27005-27014.(Medline)

Zhu, J. and McKeon, F. (1999) NF-AT activation requires supression of Crn 1-dependent export by calcineurin, *Nature* 398:256-260.(Medline)

Zozulya, S. and Stryer, L. (1992) Calcium-myristoyl protein switch, *Proc. Natl. Acad. Sci. USA* 89:11569-11573.(Medline)

Zühlke, R.D., Pitt, G.S., Deisseroth, K., Tsien, R.W. and Reuter, H. (1999) Calmodulin supports both inactivation and facilitation of L-type calcium channels, *Nature* 399:159-162.(Medline)

Zweifach, A. and Lewis, R.S. (1993) Mitogen-regulated Ca^{2+} current of T lymphocytes is activated by depletion of intracellular Ca^{2+} stores (1993), *Proc. Natl. Acad. Sci. USA.* 90:6295-6299.(Medline)

8. Regulation of the Cell Cycle

Suggested Reading
References

Back to List of Chapters

The very survival of life depends on cell division. How else could genetic information be transmitted from generation to generation or, during cell proliferation, from parent cells to daughter cells? The mechanisms involved in cell division must be precise. Any error, whether in the replication of DNA or the distribution of the genetic complement to the daughter cells, would have severe repercussion to the organism.

Cell division proceeds in sequential steps which set into motion machinery that is defined by information contained in the genome. Hence, the mechanisms involve sequential gene expression, and are relevant to the problem of the control of gene expression in general. The present chapter will focus on cell division and, in particular, regulative events. The mechanical aspects of mitosis will be covered in more detail in Chapter 23 (Section IV E).

I. STAGES OF THE CELL CYCLE

The various stages of the cell cycle (also referred to as mitotic cycle) were defined relatively early in the history of cell research. For cells that are continuously dividing, it has been most useful to express the time sequence of events diagrammatically by a circle, as in Fig. 1. The sequence begins with a silent gap period, G1, followed by a period during which new DNA is synthesized in a semiconserved fashion, called the S phase or period. Following that is another gap period, G2 , then the M phase, during which the actual cell division takes place. The processes of the M phase are not discussed in this chapter (see Chapter 23). After completing the process, the two daughter cells then return to G1 and can repeat the cycle for another round of cell division.

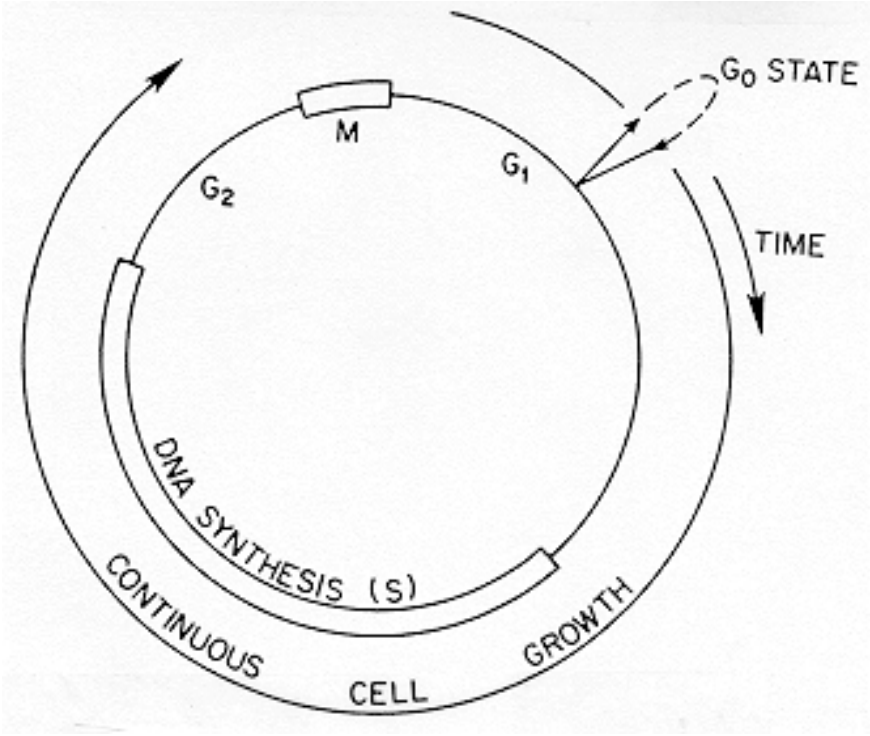


Fig. 1 The major features of the cell cycle of an animal cell in culture. The relative proportions of the cycle may vary considerably from one kind of cell to another, but the reproduction of every cell consists of growth coupled with DNA replication, followed by cell division. For example, a mammalian cell growing in culture with a generation time of 16 hours will have a G1 = 5 hours, S = 7 hours, G2 = 3 hours, and M = 1 hour. G_0 is the state into which cells are postulated to move when the cell cycle is arrested in G1 by various kinds of environmental conditions (from Prescott, 1976). Reproduced by permission.

Most differentiated cells do not divide continuously, they are thought to go from G1 to a special state, sometimes referred to as G_o. This is indicated Fig. 1 by the side branch labelled G_o. In vivo, some cells remain in this non-active state indefinitely.

The scheme of the cell cycle represented in Fig. 1 has been arrived at by several methods. The incorporation of radioactive label into the cell's DNA has provided some of the insights. When animal cells in culture, dividing asynchronously, are grown in a medium containing [^{3}H]-thymidine, the label is incorporated only into DNA during its synthesis. After a short exposure and after washing away the remaining [^{3}H]-thymidine, the fate of the synthesized DNA can be followed by autoradiography (see Chapter 1). The percentage of labelled cells (distinguished by the presence of AgCl grains) undergoing mitosis (recognized by the presence of condensed chromosomes) can then be plotted as a function of time, as in Fig. 2 (Prescott, 1976). As shown in the figure, after the introduction of the radioactive thymidine there is a delay before any of the dividing cells are labelled. This indicates that there is a separate stage, G2, that follows the synthesis and precedes the cell division (as indicated in Fig. 1). The length of time from addition of the labelled thymidine until the first appearance of the label in mitotic cells corresponds to the duration G2. After reaching a maximum, the percentage of radioactive mitotic cells decreases again. The time between the first appearance of the radioactivity and its subsequent disappearance in mitotic cells defines the duration

of the period during which DNA is synthesized, called the S period. As shown in Fig. 2, the S period can be represented as the time span enclosed by the two end points at which 50% of the dividing cells are labelled. Eventually, labelled mitotic cells appear again, marking the second round of duplication. For the second division, the rising phase of the plot on Fig. 2 is less sharp, reflecting a loss of synchrony in the cells because of individual differences. The radioactivity per cell will now be halved, because the DNA is semiconserved. This halving of radioactivity will not alter the pattern when it is expressed as a percentage of labelled cells. The time interval known as the generation time (GT in Fig. 2) begins when 50% of the cells are labelled during the first division and ends when 50% of the cells are labelled during the second division.

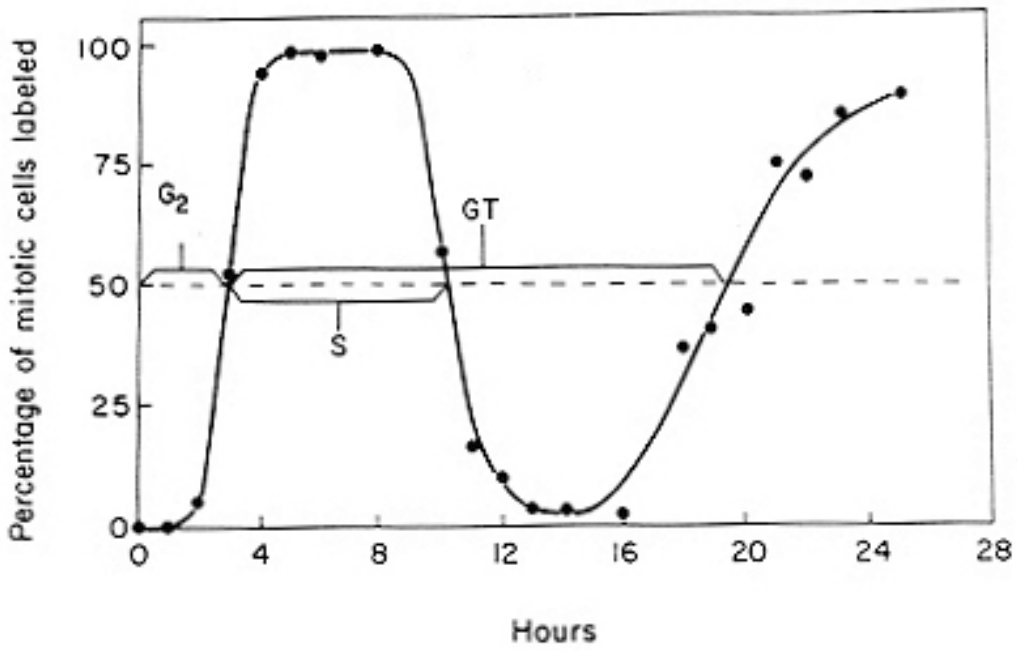


Fig. 2 Determination of the average length of the generation time, G2, S, and G1 periods, by the labelled mitosis method. See text (from Prescott, 1976). Reproduced by permission.

The G-periods between S phases have been so named because they appear as inactive "gaps". The time between the introduction of the label and its appearance in mitotic cells is the G2 phase (Fig. 1) and the period during which mitosis actually takes place is the M phase. The M phase must follow G2, and the period delineated by the M phase and the S phase is G1. The duration of the M phase can be calculated from the percentage of the cells that are in mitosis at any one time and the duration of the cycle. For example, for a 20 h cycle if 5% of the cells are in mitosis, M must last approximately 1 h. The duration of G1 can be calculated after subtracting the durations of the other three stages.

This cell-cycle scheme is very useful in organizing what we know about cell division. As might be expected from the complexity of living systems, an all-inclusive description of the orderly synthesis, assembly and movement would require a much more complex outline. Furthermore, there are small but significant differences in various organisms. In rapidly dividing cells, G1 may be lacking. In many cases the phases are not sharply defined but they overlap. Some of the G1 activities (in

preparation for S) may occur concurrently with G2 and M. Similarly, G2 may overlap with S. Obviously such overlaps would change the duration of each stage. Some of the variability can be illustrated with the example of two evolutionarily distinct yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, also known as the budding and the fission yeast, respectively. Even though the DNA content of these single-celled eukaryotes is only about five times greater than that of the bacterium *Escherichia coli*, they exhibit most of the features of other, more complex eukaryotes. Together with well understood genetics and ease of application of recombinant DNA technology, these cells are ideal for some studies and have been extensively used in the study of cell division.

The life cycle of the two is represented schematically in Fig. 3 (Forsburg and Nurse, 1991). These cells have both a haploid (one set of chromosomes) and a diploid (two sets of chromosomes) cycle. In their haploid state they can mate with a cell of opposite mating type, as long as the two cells are arrested in G1 by mating pheromones. A diploid cell can continue to divide. However, when the nutritional content of the medium is insufficient they undergo meiosis and produce haploid spores. *S. pombe* mate only if nutritionally deprived.

As shown in the diagram, *S. cerevisiae* lacks a clear separation between S, G2 and M. This is in part because the formation of a bud and migration of the daughter nucleus occur early in the cycle. *S. pombe*, on the other hand, follows the conventional stages already discussed.

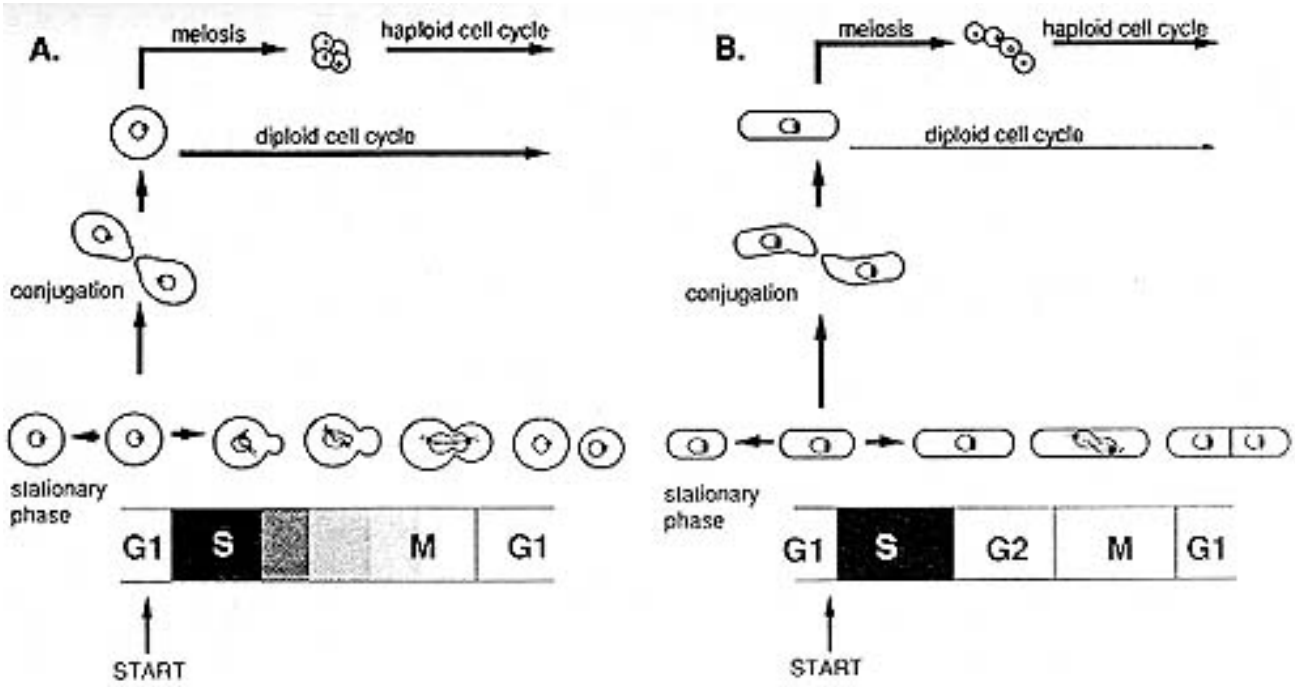


Fig. 3 A diagram of the life cycles of *S. cerevisiae* (A) and *S. pombe* (B). N indicates the haploid number of chromosomes. The top half of the figure shows a cartoon of conjugation and meiosis. The bottom half of this figure shows a cartoon of a haploid cell, and its position relative to the stages of the cell cycle. The stages are essentially similar in both haploid and diploid cells. Note that in *S. cerevisiae* the mitotic spindle forms very early and S and M phases overlap, while in *S. pombe* these phases are distinct. The *S. pombe* diploid is unlikely to undergo a mitotic cycle (indicated by the thinner lines). As with other fungi, the nuclear envelope remains intact. With permission, from the

Annual Review of Cell Biology, vol. 7, copyright &copy1991, by Annual Reviews Inc.

II. EVENTS UNDERLYING THE CELL CYCLE

Each stage of cell division is likely to require its own cadre of special enzymes and other proteins. Of the various mechanisms one might imagine for the control of cell division, transcriptional on and off switches of different genes operating sequentially seem worthy of consideration, although this aspect is far from being understood. Many of the growth factors (also called *mitogens*, because they induce cell proliferation) work through mechanisms that affect transcription. The cell cycle is the result of the delicate balance between the expression of genes favoring growth and cell proliferation (oncogenes) and tumor suppressor genes that block these activities.

Before they are committed to cell division, cells must leave the so-called resting state (G_0) and, after returning to the G1 phase, prepare for the synthesis of DNA.

Not surprisingly, many events regulating cell duplication take place at the G1-S interface, known as *Restriction point* in mammalian cells and *Start* in *S. cerevisiae*. This point in the cycle is thought to be the point of no return, after which the cells are committed to divide. In these cells, the G2-M and M-G1 interfaces are also regulated by similar mechanisms. However, *S. pombe* as well as oocytes and early embryos are primarily regulated at the G2-M interface.

In non-transformed mammalian cells, such as fibroblasts, G1 can be divided into various stages, all preparing for the S-phase. Except for the final G1 stage, all of these depend on the presence of one or more growth factors. The final G1 stage representing the final preparation for the DNA synthesis includes events such as the transfer of needed enzymes into the nucleus and the formation of the enzyme complex needed for DNA replication.

III. REGULATION OF THE CELL CYCLE

One of the striking features in cells that are continuously dividing is the cyclicity of the events. This is particularly true in early embryos where, by successive mitoses, a single cell generates a whole individual made up of thousands of cells. One of the possible ways of examining the mechanisms controlling the cell cycle is to look for a signal generated during the cycle that may trigger the cascade of events of cell division.

Ca^{2+} involved in so many regulatory cell functions (see Chapter 7), has been implicated also in the regulation of the cell cycle. Ca^{2+} has been found to be essential for the proliferation of eukaryotic cells (e.g., Means, 1994; Tokmakov et al., 2001). The effect of Ca^{2+} may be mediated by calmodulin (CaM) which was also found essential. Mouse cells in culture containing a vector expressing antisense calmodulin RNA were arrested in G1 (Rasmussen and Means, 1989). Similarly, addition of a monoclonal antibody (see Chapter 1) to calmodulin to permeabilized fibroblasts prevented the synthesis of DNA (Rasmussen and Rasmussen, 1995). The Ca^{2+}-

calmodulin-dependent phosphatase calcineurin is also thought to have some role in cell proliferation (see Means et al., 1999). <p>Ca²⁺ and calmodulin have been found to have a role in initiating the proliferative cycle of cells as well as in the G1/S, G2/M and the metaphase-anaphase transitions see (see Means et al., 1999; Lu and Means 1993). Calmodulin, and CaMKII were also found to be essential for the initiation of centrosome duplication (see Matsumoto and Maller, 2002). These findings suggest that Ca^{2+} and that the calmodulin,-CaMKII cascade are part of the signal required for centrosome duplication and subsequent cell division. Centrosomes have been found essential for cell division (see Section K, below)

Considering the key role of proteins in gene expression, it is of special interest to examine whether a new protein appears during the cell cycle. The degradation of proteins involved in the cell cycle is no less important as addressed in the various sections. below.

A. The Cyclins

Newly synthesized protein can be detected by introducing a radioactive amino acid such as [^{35}S] methionine. The proteins synthesized can be separated by molecular weight in polyacrylamide gels using SDS-gel electrophoresis (see Chapter 1). The gel acts as a sieve and the smaller polypeptides will migrate more rapidly than larger molecules. The denatured polypeptides are covered with negatively charged detergent so that the migration will be in the direction of the positive electrode. The position of the newly synthesized peptides can be seen using autoradiography (see Chapter 1) and will appear on a photographic film as a dark band. In such an experiment, the entire cell population has to be synchronized. For eggs of the sea urchin or clam, this synchronization can be readily obtained by introducing sperms which will fertilize the eggs.

In sea urchin eggs a protein was found to closely anticipate each division. This protein was promptly named *cyclin*. The results of an experiment are displayed in Fig. 4 (Evans et al., 1983). This figure represents results obtained from a suspension of eggs after fertilization (the 0 time on the abscissa) in a medium containing [^{35}S] methionine. The cyclin is shown by the circles and line A, whereas the triangles and line B represent another protein. The amount of each protein was estimated from the density of the bands (right hand ordinate) after autoradiography of the gel using SDS-PAGE (see Chapter 1). The percentage of cells which are dividing, also known as the cleavage index (open squares, the dashed line and left hand ordinate), identifies the time at which the cells divide. The results not only show that cyclin is synthesized before each cell division, but it is degraded at each mitosis. In contrast, the protein represented by line B is independent of the cell cycle.

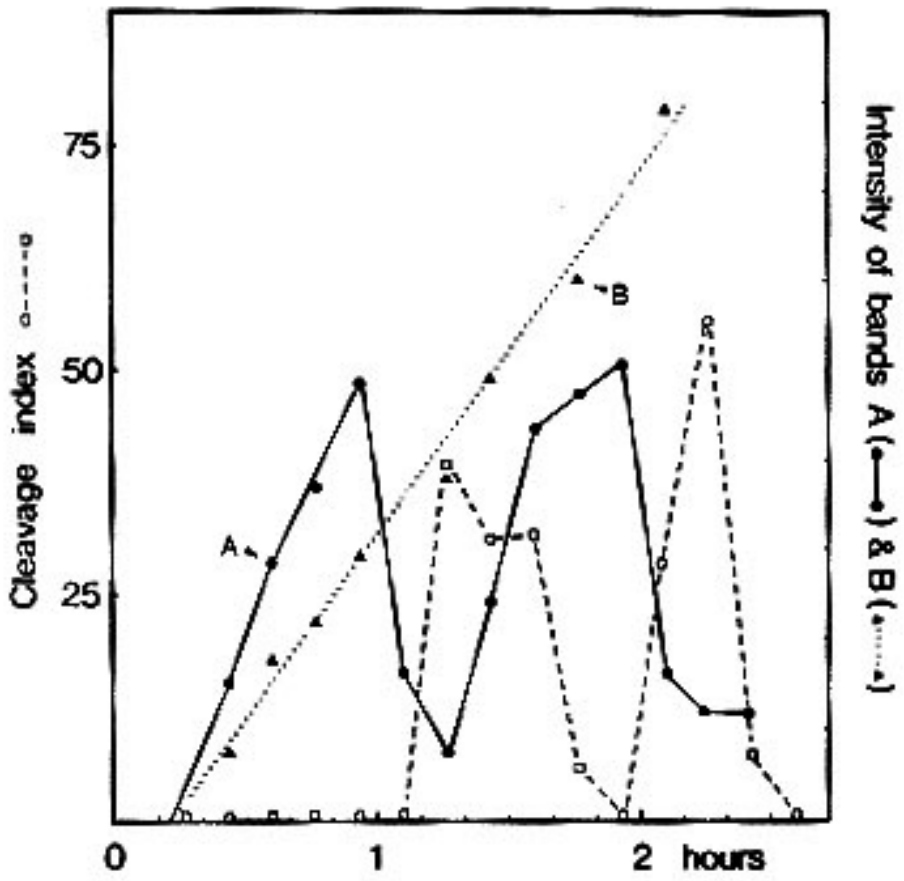


Fig. 4 Correlation of the level of cyclin with the cell division cycle in sea urchin eggs. The dashed lines and squares indicate the cleavage index (see text). The circles and full line (A) represent the intensity of the bands corresponding to cyclin and the triangles and dotted line (B) correspond to the intensity of another protein, which exhibits no cyclicity (from Evans et al., 1983). Reproduced by permission.

The results of Fig. 4 can be interpreted in two different ways. The degradation of cyclin following the initiation of cell division could occur while its synthetic rate remained constant. Alternatively, both the synthesis and the degradation could occur cyclically, with the synthesis obviously preceding the degradation. These two separate hypotheses can be tested. The synthetic ability of the cells can be followed by pulse label experiments. With this design, the eggs are exposed to the radioactive amino acid for a short time, after which the radioactivity is diluted by adding an excess of unlabelled amino acid. When pulse-label is introduced at different stages of the cycle, the results provide an estimate of the synthetic capacity of the system during each of these pulse periods. These experiments show that in sea urchin eggs the rate of cyclin synthesis is not greatly changed with the cell cycle, implicating degradation as the major regulatory factor. This is not true in somatic cells, where cyclin synthesis is generally under transcriptional control. In fertilized sea urchin, the mRNA for cyclin is a maternal contribution, so no new mRNA is immediately needed. The mRNA is in an inactive state until it is activated by fertilization. The maternal mRNA continues to be used in the early cell divisions. In mammalian somatic cells and *S. cerevisiae*, the concentration of cyclin mRNA is cycle-dependent (see below, e.g., see Fig.5).

Several cyclins are now known and some of these are listed in Table 1 (Lew and Reed, 1992). Their classification is based on their amino acid sequence.

Yeast cyclins

Many of the studies of cyclins were carried out with yeast. In *S. cerevisiae*, three proteins with some analogies to the known cyclins were identified. These proteins were found to be needed for Start. Elimination of one or two of the genes controlling these cyclins (*CLN1*, *CLN2* and *CLN3*) by insertional mutations (that is, mutations that incorporate a piece of foreign DNA and eliminate the wild type gene, e.g., see Chapter 1) is deleterious and delays *Start*. However, elimination of all three produces large amorphous cells that cannot proceed past G1. These findings indicate that the three genes that code for cyclins have a similar function, and this function is crucial for the process of cell division.

Do the yeast cyclins exhibit the same periodicity as those discussed for the sea urchin eggs? In such a study the cells must be dividing synchronously. In *S. cerevisiae* the cells can all be stopped at G1 in the presence of mating pheromone. When the pheromone is removed, the cells begin dividing synchronously. As we have seen before, proteins can be labelled by maintaining the cells in a medium containing radioactive amino acid. Each individual protein can be recognized and isolated using a specific antibody, which precipitates the antigen by crosslinking several molecules together (this procedure is called *immunoprecipitation*, see Chapter 1). Immunoprecipitation using CLN2-antibody, showed that CLN2 protein appears cyclically, peaking at the G1-S transition. Similar experiments were carried out with other cyclins.

Table 1 Cyclins in Yeast and Mammalian Cells. Reproduced from Lew and Reed, 1992. Reproduced by permission.

Cyclin	Species	Class	Cell Cycle Function
CLN1	*S. cerevisiae*	CLN	G1 (START)
CLN2		CLN	G1 (START)
CLN3		CLN	G1 (START)
HCS26			G1 (START)?
CLB5		B	G1 (START)?
CLB3		B	G1-S and G2-M?
CLB4		B	G1-S and G2-M?
CLB1		B	G2-M
CLB2			G2-M

CDC13 cig1 mcs2 puc1	*S. pombe*	B B CLN	G2-M G1 or G1-S M? ?
Cyclin A Cyclin B1 Cyclin B2 Cyclin C CyclinD1 Cyclin E	*H. sapiens*	A B B C D E	G1-S and G2-M G2-M G2-M ? ? G1 or G1-S
CYL1 CYL2 CYL3	*M. musculus*	D D D	? ? ?

Is the control of cyclins transcriptional? Transcriptional control would produce each cyclin mRNA at a different time during the cycle. Transcripts can be recognized by hybridization to cDNA probes (which are complementary to the mRNA) attached to nitrocellulose sheets (Northern blot, see Chapter 1). The results show that the amount of mRNA transcribed does indeed reflect the events of the cycle for CLN1 and CLN2. Careful comparison of the mRNAs shows that they slightly precede the synthesis of the cyclins. In contrast to these findings, the level of CLN3-RNA remains unchanged. For CLN1 and CLN2 these results indicate that the control is transcriptional. Furthermore, both the proteins and the mRNAs are degraded following the phase of the cycle that they trigger.

While CLN1, CLN2 and CLN3 act at the G1-S transition, the B-class cyclins have different roles. CLB1 accumulates during the S and G2 phases and CLB5 at G1. Other cyclins are thought to play a role in the earlier stages. A diagrammatic summary of the synthesis and degradation of the various cyclin-mRNAs in yeast is shown in Fig. 5 (Lew and Reed, 1992).

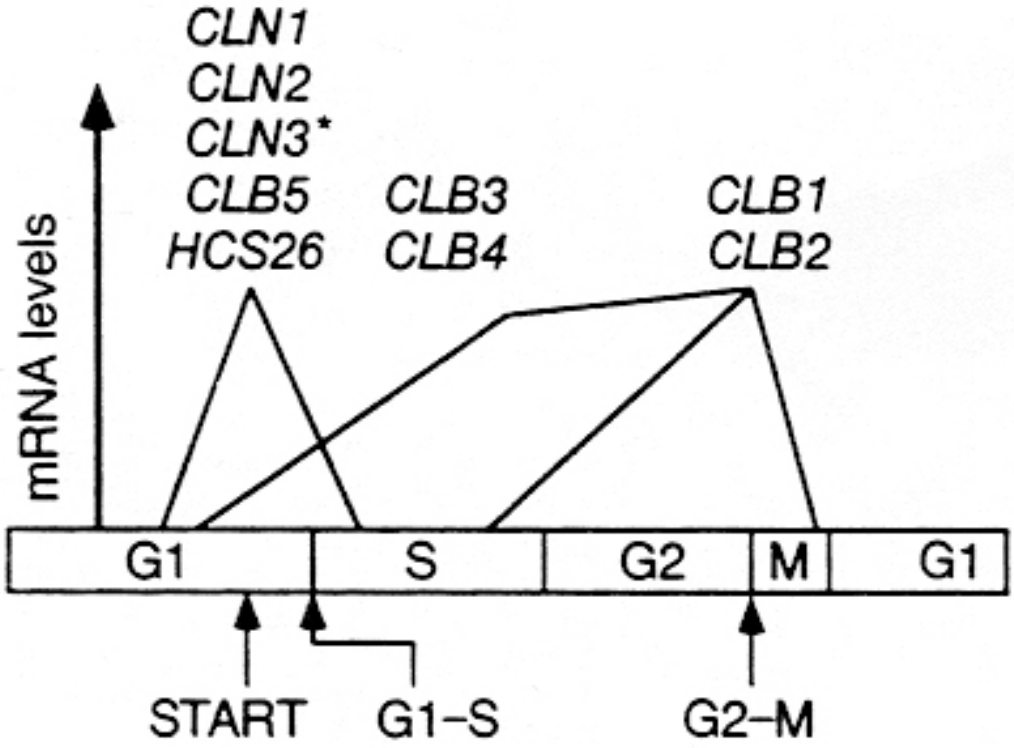


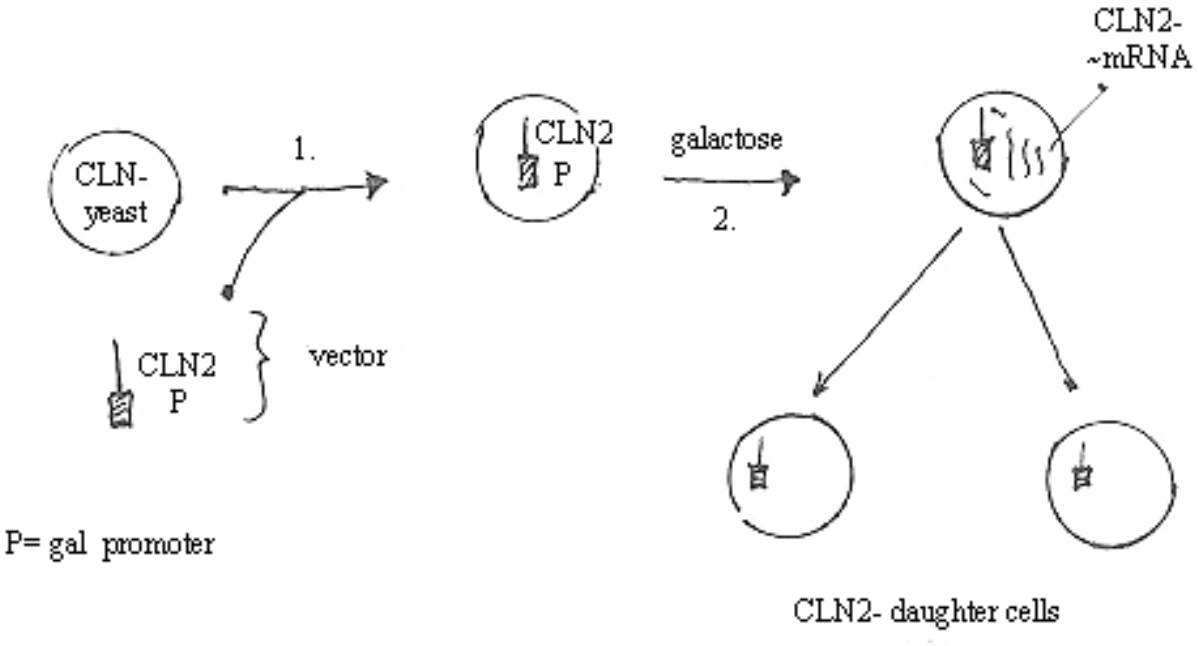
Fig. 5 Periodicity of cyclins during the cell cycle in *Saccharomyces cerevisiae*. Cyclins are grouped according to suggested times of action during the cell cycle and mRNA periodicity. As indicated by *, CLN3 is the exception to the rule in that its mRNA levels remain constant throughout the cell cycle. Reproduced from *Trends in Cell Biology*, vol.2, Lew, D.J. and Reed, S.I., A proliferation of cyclins, pp.77-80, copyright &copy1992, with permission from Elsevier Science.

Mammalian cyclins

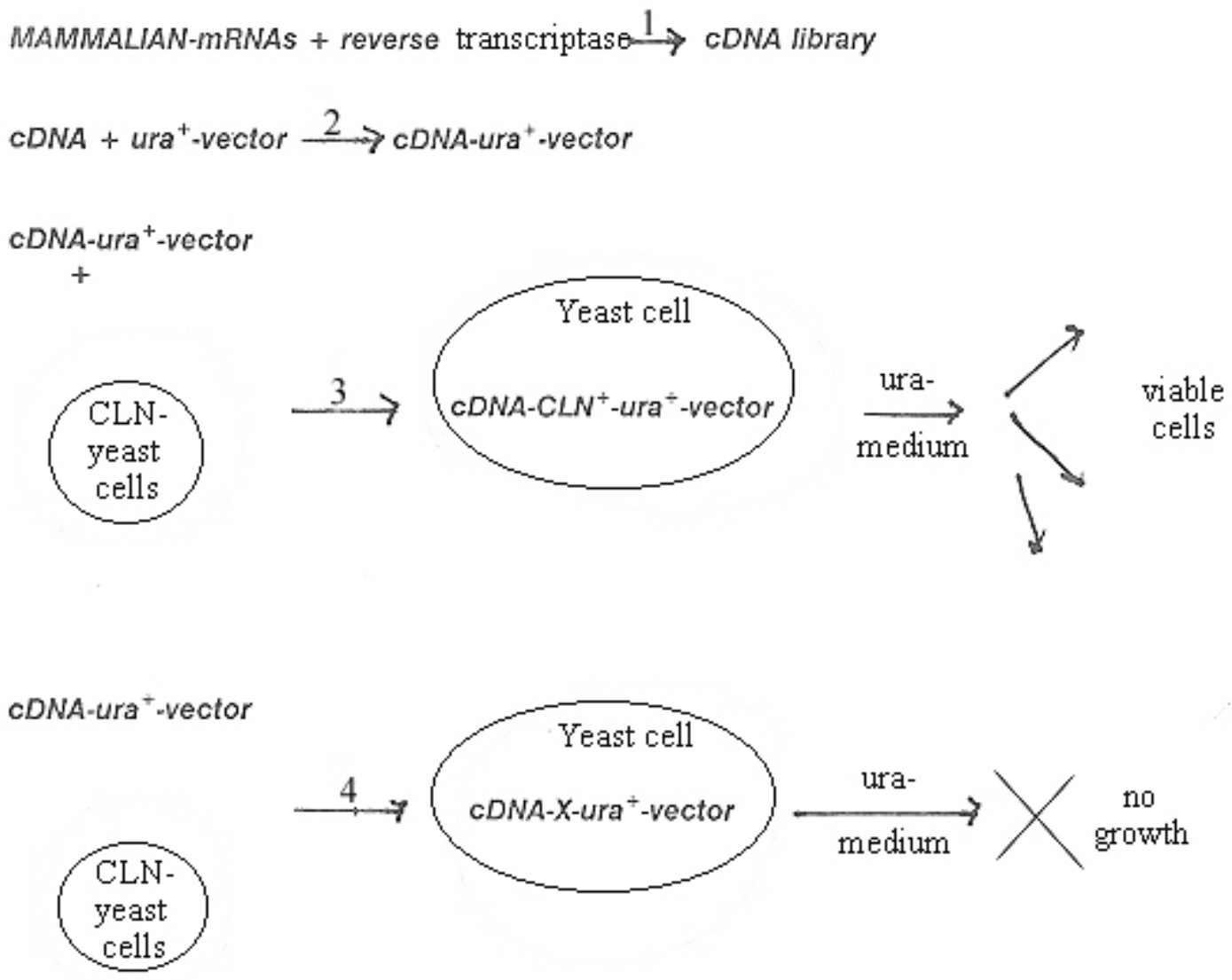
As already mentioned, the major control of mitosis in mammalian cells is at the G1-S interface. The early studies with mammalian cells, however, only identified G2-M cyclins. As summarized in Fig. 5, several cyclins active at the G1-S interface have been identified in yeast. Why not use this knowledge to study mammalian cyclins? Generally, proteins that have a universal role are highly conserved from organism to organism. Why not search for cDNAs of mammalian cells that restore function in yeast strains lacking *CLN1*, *CLN2* and *CLN3* (Lew et al., 1991; Xiong et al., 1991; Koff et al., 1991)? The experiment was carried out by first inactivating the *CLN* genes of a *S. cerevisiae* strain by insertional mutation (mutations that replace the wild type gene with foreign DNA, see Chapter 1). A human cDNA library in yeast expression vectors would then be used to attempt to restore function. A cDNA library is synthesized in vitro from the mRNA present in cells, therefore the cDNA is complementary to the mRNA (*c* stands for complementary) and contains the sequence information needed for proteins produced by the cell (see Chapter 1). The rationale for this experiment is simple. However, the details of the design must be more complex. Deletion of three G1-S CLN genes in yeast is lethal: the cells are unable to divide. How can you get cells to reproduce without these genes to provide cells that can be used for the experiment? Furthermore, how can you select for the cells which have incorporated the mammalian cDNA? The production of a yeast strain from the transformation would be extremely rare and would be confused with possible spontaneous

revertants. The task of bypassing these two problems would be difficult, if not impossible, without reaching into the bag of tricks of the molecular biologist. Expression of the CLN genes was put under the control of the experimenter by first fusing the *GAL1* promoter region to the *CLN2* coding region and then, using a vector, this DNA combination was incorporated into the yeast lacking the CLN genes. The transfected cells were able to grow in a galactose medium. However, they were *CLN*-less and unable to divide when placed into a glucose medium. The expression of the *CLN* provided by the vector could be turned on (when the cells have to be grown) or off (when they are to be tested for the *CLN* ability provided by the mammalian cDNA). Selection of cells containing components of the human cDNA library was obtained by combining the DNA with a yeast expression vector containing the *URA2* gene. This gene provides an enzyme which allows the cells to produce their own uracil. Then the transformed cells could be selected in a medium lacking uracil and containing glucose. In a glucose medium they will be able to grow only if they contain the mammalian equivalent of the CLN gene and the *URA2* gene. The experimental design used by Lew et al. (1991) is represented in Fig. 6.

Fig. 6 Experimental design to identify mammalian CLN genes by replacing the missing genes in CLN^- yeast.



A. How to grow cells missing the CLN yeast genes. (1) A vector containing the galactose promoter attached to CLN2 DNA transfects CLN^- cells. (2) In a galactose-containing medium the CLN2 is expressed to produce CLN2-mRNA, allowing the cells to divide.



B. Rescue of CLN$^-$ cells by mammalian cDNA. (1) Production of cDNA library from mammalian cell's mRNA. (2) Production of ura$^+$cDNA (3) transfection of CLN$^-$ with ura$^+$-cDNA; these cells will be viable if the cDNA contained the CLN$^+$ mammalian gene, (4) transfection with DNA containing ura$^+$ but no CLN$^+$: these cells will not grow because of the absence of CLN.

Three novel mammalian cyclin genes (C,D and E) were recognized in this way. Cyclin A was recognized in similar experiments; it reaches a maximum near the G2-M transition. In mammalian cells, the function of cyclin A has been examined by micro-injecting antisense cyclin A plasmids in rat fibroblasts in the G1 phase (Girard et al., 1991). The production of RNA that is antisense (that is, complementary) in relation to the mRNA of the target gene, in effect inactivates its translation supposedly by binding to the mRNA (see Chapter 1). The cells are then unable to replicate DNA. However, injecting cyclin A reestablished cell function. These experiments support a role of cyclin A either in the G1-S transition or in the S phase. Cyclin E-mRNA reaches a maximum near the G1-S transition. The physiological role of cyclin E was also examined by overexpressing cyclin E in fibroblasts by transfection with the appropriate cDNA (Ohtsubo and Roberts, 1993). In these cells, the duration of G1 and cell size are decreased and the cells are less dependent on serum, suggesting

a role of cyclin E in the G1-S transition. The D-cyclins (D1, D2 and D3) are probably the major factors responsible for cells to enter the S-phase. Expression of cyclin D1 speeds up the progression through the G1 phase (Quelle et al., 1993; Resnitzky et al., 1994). The microinjection of anti-cyclin D1 antibodies or antisense plasmids prevents cells from entering the S phase (Quelle et al., 1993, Baldin et al., 1993; Lukas et al., 1994).

Mice lacking the cyclin D1 gene have severe defects (Fantl et al., 1995; Sicinski et al., 1995). In contrast, strains in which the gene coding for cyclin D1 has been replaced by human cyclin E genes, have a normal phenotype. These results suggest the cyclin E is a downstream target of cyclin D (Geng et al., 1999).

Basically, D and E cyclins function in the early stages and are generally thought of as G1 cyclins (see Sherr, 1993). In contrast, cyclins A, B1 and B2 predominate in the S and M phases (see Norbury and Nurse, 1992).

B. The Cyclin-dependent Kinases

Early experiments were able to demonstrate the involvement of other factors in the control of cell division. The experiments were carried out by the fusion of HeLa cells in culture, at either G1 or G2 stages, to cells in the S-phase (Rao and Johnson, 1970). The fusion was induced by introducing a virus to the mixed cultures. The cells in G1 which fused to the S cells synthesized DNA earlier, as estimated by [^{3}H]thymidine incorporation (Fig. 7). Furthermore, the time at which the incorporation occurred was accelerated if 2 S nuclei were fused with G1 cells (Fig. 8). The presence of G2 nuclei did not delay the onset of DNA synthesis when G2 and G1 nuclei were fused (Fig. 9). The factor that promotes DNA synthesis has been referred to as the *S-phase promoting factor* (SPF). An additional factor must be present to promote the M-phase. Fusion of G2 to G1 or S cells induces an earlier onset of mitosis as shown in Fig. 10 for G1 nuclei. The proportion of G1 nuclei undergoing mitosis, as indicated by the mitotic index (the ratio of dividing cells/ all cells) in the ordinate, occurs earlier when G2 nuclei are fused to G1 nuclei. The greater the G2 dosage, the earlier the onset of mitosis. These results speak for a second factor, the *M-phase* or *maturation promoting factor* (MPF). The term "maturation" refers to the induction of mitosis in amphibian oocytes.

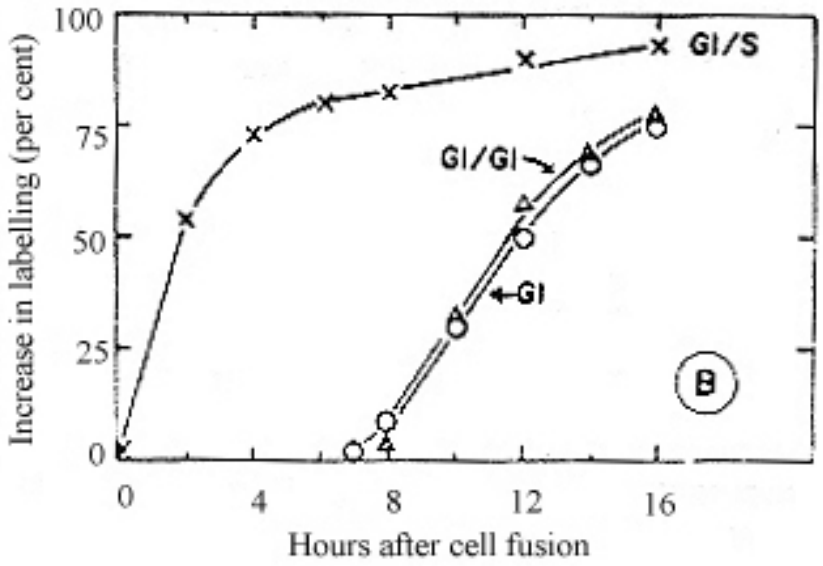


Fig. 7 Rate of induction of DNA synthesis determined from the labelling with [^{3}H]- thymidine in G1 nuclei of G1/S fused cells. Reproduced with permission from *Nature*, Rao, P.N. and Johnson, R.T.,225:159-164, copyright &copy1970 MacMillan Magazines Ltd.

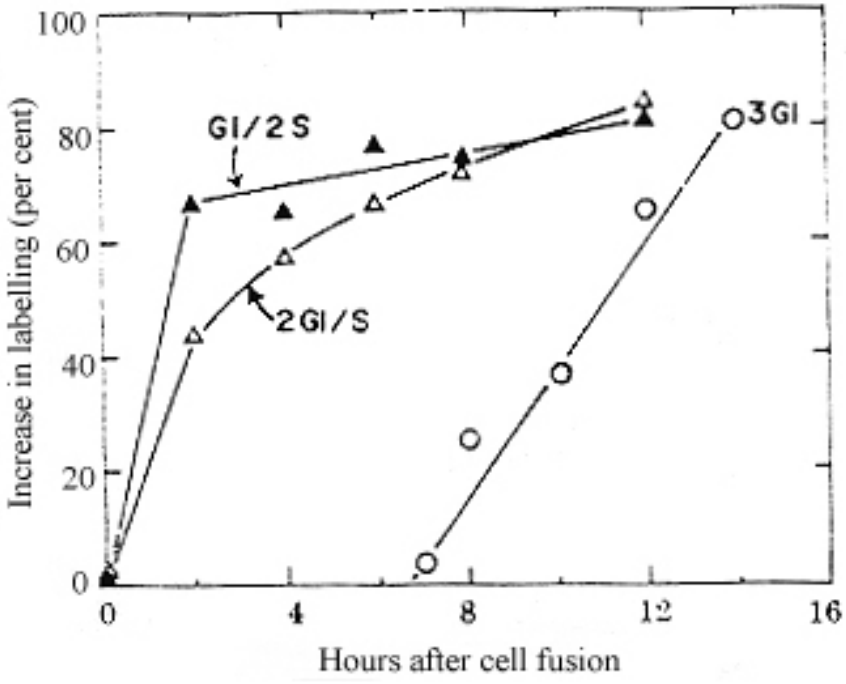


Fig. 8 Dosage effect on the induction of DNA synthesis in G1 nuclei in trinucleate cells. Reproduced with permission from *Nature*, Rao, P.N. and Johnson, R.T.,225:159-164, copyright &copy1970 MacMillan Magazines Ltd.

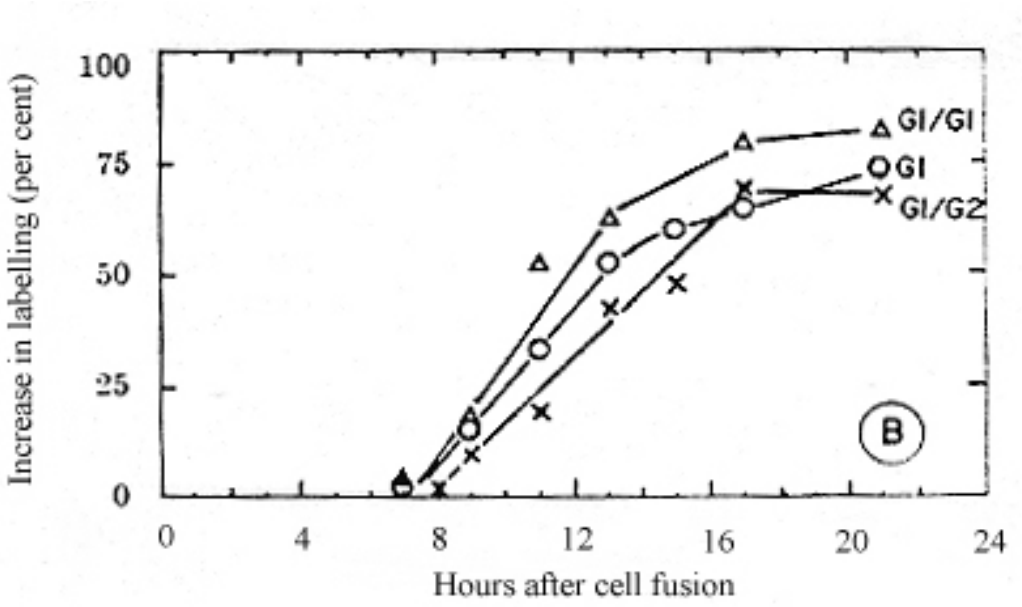


Fig. 9 Rate of induction of DNA synthesis comparing G1/G2, to G1/G2 and parent G1. Reproduced with permission from *Nature*, Rao, P.N. and Johnson, R.T.,225:159-164, copyright 1970 MacMillan Magazines Ltd.

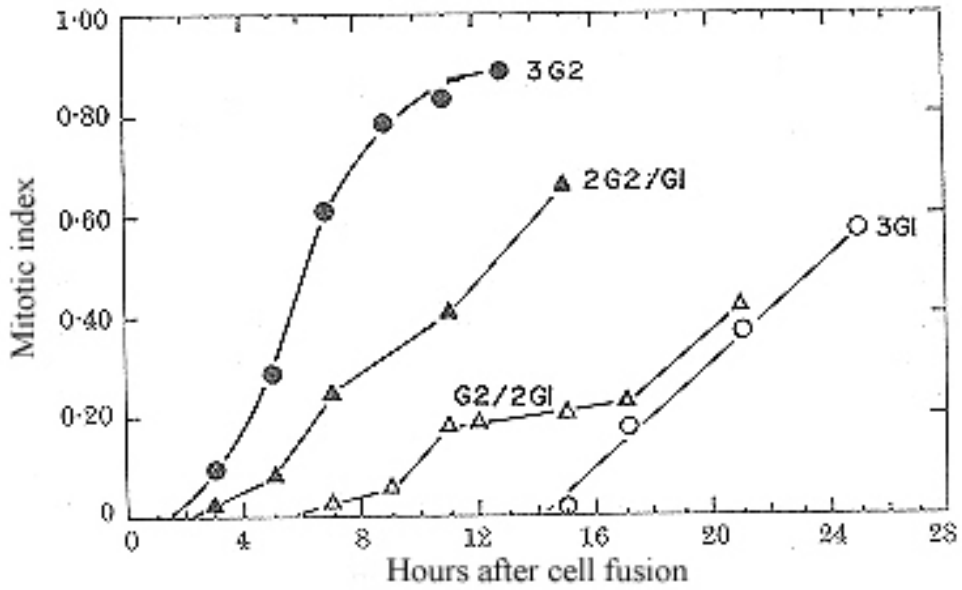


Fig. 10 Dosage effect of the G2 component on the rate of mitotic accumulation. Reproduced with permission from *Nature*, Rao,P.N. and Johnson,R.T.,225:159-164, copyright &copy1970 MacMillan Magazines Ltd.

Amphibian oocytes are arrested at the first meiotic division and remain at this stage until fertilization. After fertilization meiosis is completed and the egg begins a series of mitotic divisions. Extracts collected during the cell cycles of fertilized eggs, which contained MPF, were found to activate cell division in the arrested oocytes**.** The MPF was found to peak before each mitosis**.** The activity was assayed by microinjecting extracts into unfertilized oocytes and observing the activation of cell division (Masui and Markert, 1971). The extracts were prepared from fertilized

eggs at different stages of the cell cycle. The study of the extracts led to the extensive purification of the protein (Lohka et al., 1988), which was found to be a complex of a protein kinase and a cyclin. The kinase activity depends on the presence of a cyclin. The kinases that require binding to a cyclin are referred to as *cyclin dependent protein kinases* (CDKs).

The CDKs are activated at the transition points and presumably phosphorylate mitosis-specific substrates that have a key role in either initiating events, such as transcription or inactivating inhibitors of cell division (see next section). A model outlining some of the events in yeast is presented in Fig. 11A (Wittenberg et al., 1990). The models discussed here (for example, Fig. 11-12) incorporate the knowledge available at the time they were formulated. Many more elaborations (including changes in nomenclature) were introduced later. These are areas of active research and many more additions and modifications can be expected (e.g., consult Morgan, 1995 for a review of CDKs). In Fig. 11A, the CLN genes are periodically transcribed and a different cyclin (Cln) is produced depending on the stage of the cycle. The CLN-mRNA and the Clns are degraded. As shown for G1/S and G2/M, the formation of the complex of the active Cln (perhaps phosphorylated) with the protein kinase (p34 in Fig. 11A; also referred to as $p34^{cdc2}$; CDC2 is the gene coding for $p34^{cdc2}$, in budding yeast CDC28) activates the latter to phosphorylate a "mitotic" substrate. The substrate could correspond, for example, to transcription factors such as E2F. Protein phosphatases that hydrolyze the phosphate of the phosphorylated proteins under the control of cyclins also play a role in the cell cycle. Cdc25-phosphatase of the MPF (the $p34^{cdc2}$-cyclin B complex) allows the translocation $p34^{cdc2}$ into the nucleus and its activation of mitosis. The phosphatase CDC14 plays a key role in the departure from the M-phase (see below). In yeast, the data are consistent with the presence of a single CDK (p34 in Fig. 11A) activated at different parts of the cycle (mostly at the G1-S, G2-M transitions) by a different cyclin. In contrast, in animal cells there are different CDKs which are activated by different cyclins. This is shown in the diagram of Fig. 11B. A protein kinase homologous to the yeast $p34^{cdc2}$ is also present in mammalian cells and as indicated in the figure, it is essential to initiate the M-phase (see Nurse, 1990). $p34^{cdc2}$ is one of the components of MPF.

In normal animal cells, the centrosome reproduces only once per cell cycle. Then the two centrosomes separate and nucleate the mitotic spindle at the two poles. By ensuring a bipolar mitotic spindle, the cell avoids the misdirection of chromosomes that could lead to genetic instability (see Hinchcliffe and Sluder, 2001). Centrosome duplication requires the presence of active Cdk2-cyclin E in *Xenopus* (Hinchcliffe et al., 1999; Lacey et al., 1999) and in somatic human cells (Matsumoto et al., 1999). A protein kinase present in centrosomes which mediates centrosome duplication, has been found in *Caenorhabditis elegans*. However, this kinase is not needed for cell cycle progression. The absence of the corresponding gene (*zyg-1*) produces a monopolar spindle and the failure of duplication of the centrosome (O'Connell et al., 2001). The protein kinase, ZYG-1, acts at least one cell cycle before to each spindle assembly. In embryos, paternal ZYG-1 regulates duplication during the first cell cycle, and maternal ZYG-1 regulates subsequent duplications. In *Drosophila* embryos (Vidwans et al., 1999), Cdc25-phosphatase initiates mitosis and is also needed for daughter centriole assembly. Cdc20 by activating the APC initiates cyclin degradation and initiates transition from metaphase to anaphase (see below) and apparently also has a role in the separation of mother and

daughter centrioles. In addition, the stabilization of cyclins prevent exit from mitosis and the assembly of the daughter centrioles.

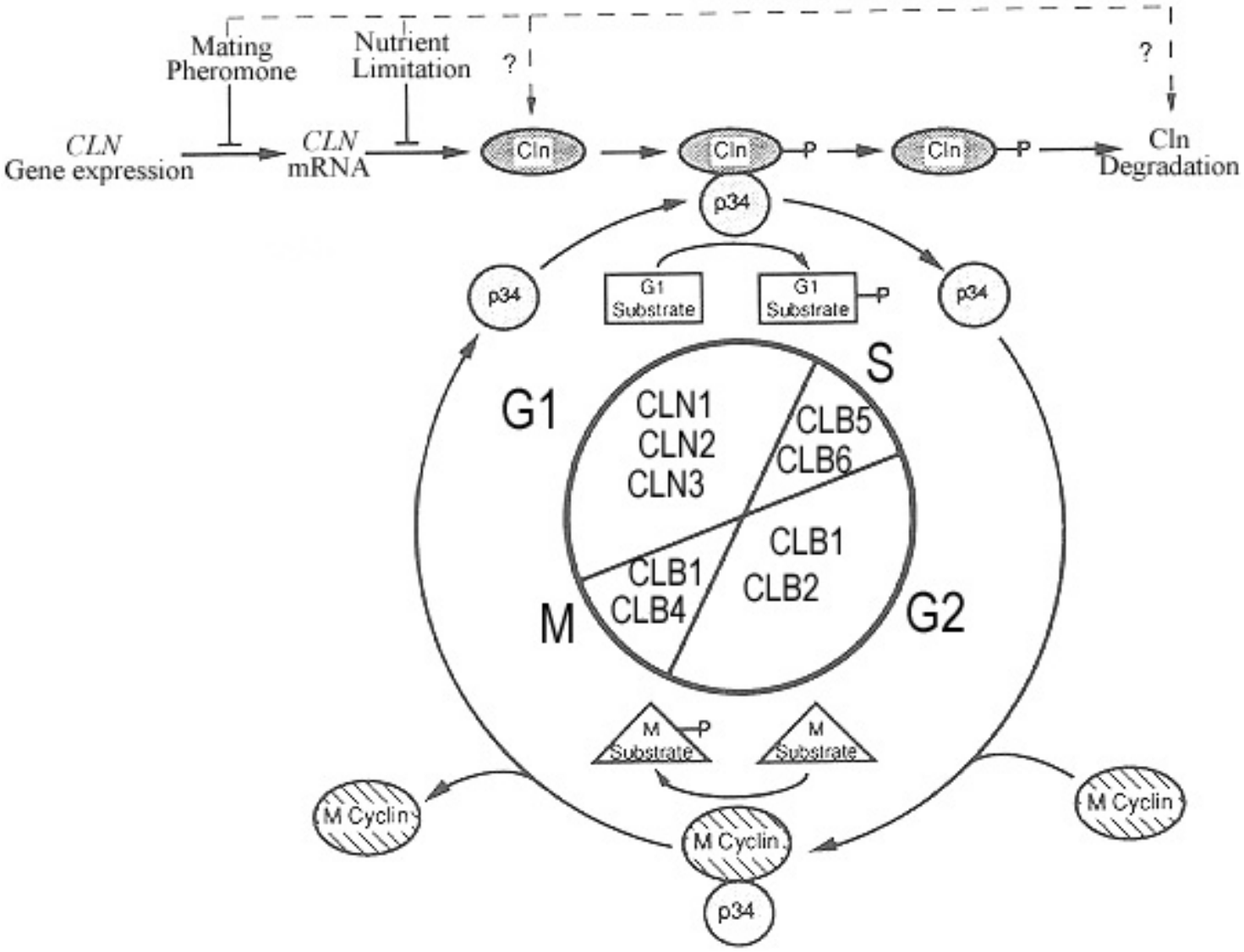


Fig. 11A A model of the regulation and role of cyclin proteins in yeast. Two transitions, G1/S and G2/M are represented. See text for details (from Wittenberg et al., 1990). Reproduced by permission.

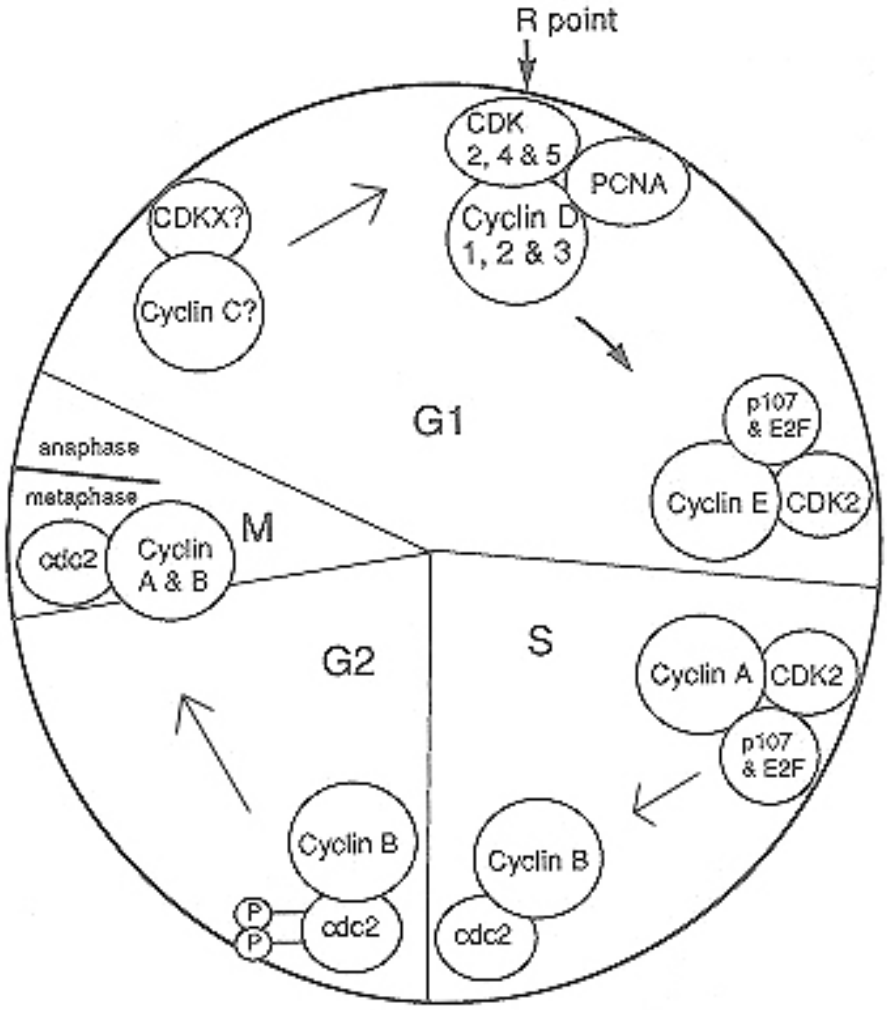


Fig. 11B A model summarizing the interactions between cyclins and the cyclin dependent kinases in multicellular animal cells. The R point is the restriction point. The diagram shows the stages of the cell cycle and the binding of the specified cyclins with the corresponding CDKs at each stage. cdc2 is kinase, p107 and E2F are proteins involved in transcription. Reproduced from *Trends in Biochemical Science*,vol.18, Pines, J., Cyclins and cyclin-dependant kinases:take your partners, pp.195-197, copyright &copy1993, with permission from Elsevier Science.

Binding of cyclin only partially activates the CDKs (see Morgan, 1995). The CDKs are subject to regulation by phosphorylation. They are fully activated only when phosphorylated at a threonine residue (Thr 161). They can be inhibited not only by dephosphorylation at threonine 161 but also by phosphorylation at tyrosine sites near the amino terminal.

In order to activate the appropriate genes, most CDK/cyclin complexes must be translocated into the nucleus. Therefore, the localization of the various cyclins and CDKs, whether cytoplasmic or nuclear, is likely to play a role in the regulation of the cell cycle (see Yang and Kornbluth, 1999).

What mechanisms determine the localization of these components? Apparently, a sequence of 42 amino acids the amino acid terminal region of cyclin B, the so-called *cytoplasmic retention sequence* (CRS), is necessary for a cytoplasmic localization of that cyclin (see Pines and Hunter

1994). However, the CRS sequence contains a nuclear export sequence (NES) (see Chapter 5) (Hagting et al., 1998; Toyoshima et al., 1998) suggesting that, in actuality, the cyclin is capable of cycling between nucleus and cytoplasm. The cytoplasmic localization of cyclin B1 during interphase is directed by an NES-dependent export. In addition, the use of leptomycin B, an inhibitor of nuclear export, was found to lead to an interphase nuclear accumulation (Toyoshima et al., 1998) suggesting that normally the localization is determined by a balance between import and export.

Although they have no obvious NLS domains, the mechanisms for the import of these complexes are beginning to be elucidated (Moore et al., 1999). Permeabilized mammalian cells were exposed to fluorescently labelled complexes of CDK2/cyclin E and Cdc2/cyclin B1. The nuclear import apparatus imports the complexes by direct interaction with the complexes. Whatever sequence is required for import is contained in the cyclin units. In contrast, the nuclear import of Cyclin A requires its CDK (Maridor et al., 1993g1). Cyclins E and B1 are imported by different mechanisms. Cyclin binds to the α subunit of importin-α and β. In contrast, cyclin B1 binds to importin-α alone.

Knowledge of what genes are activated during the various phases would go far in completing the story of the mitotic cycle. Little by little new information is being collected. However, new technology, namely DNA arrays and chromatin-immunoprecipitation, has permitted taking a giant leap in this area for the G1/S transition. This transition initiates the whole cycle and for this reason has attracted a good deal of attention. In yeast, as shown in Fig. 11A the cyclins *CLN1*, *CLN2*, *CLB5* and *CLB6* appear at G1/S transition (along with other proteins). Their arrival is driven transcriptionally (e.g., see Fig. 5) in late G_1. In addition, the CLN/CDK combination activates a variety of needed components such as transcription factors which initiate a cascade of biochemical events needed for the transition. The transcribed genes have been divided into two groups depending on the sequences motifs in their promoters. The SCB motif has been found in the promoters of *CLN1* and *CLN2* and in other genes. A second set has another motif, the MCB element. Two separate transcription factors, SBF and MCF, respectively, act on the two promoters (see Koch and Nasmyth, 1994). The two factors are heterodimers of Swi4 and Swi6 (SBF) and Mdp1 and Swi6 (MBF) (see Koch et al, 1993; Andrews and Herskowitz, 1989). Swi4 and Mbp1 are the components which bind DNA. Swi6 probably has a regulatory function (Primig et al., 1992 ; Dirick et al., 1992). Which are the genes activated in the G1/S transition? These genes can be identified by finding the DNA binding sites of SBF and MBF by immunoprecipitation with antibodies to SBF and MCF of chromatin (after chemical crosslinking) and using microarrays of most of the yeast genome. 200 probable targets were identified (Iyer et al., 2001). SBF was found to be primarily involved in budding as well as membrane and wall biosynthesis. In contrast, MBF was found to be involved in DNA replication and repair.

C. Inhibitors of Cell Division

Just as cyclins play a role as positive effectors of the cell cycle, other proteins block the cell cycle. As already discussed, in mammals progression through the cell cycle depends on several kinds of CDKs. These are constrained by *CDK-inhibitors* (CKIs) (see Morgan, 1995; Scherr and Roberts,

1995, 1999). In mammals, CKIs have been assigned to two different groups on the basis of their structure and target CDKs. The *inhibitors of CDK4* (INK4) block the catalytic subunits of CDK4 and CDK6 specifically. The Cip/Kip family of inhibitors are much less specific and interact with cyclin D, E and A-dependent kinases by binding to subunits of both cyclins and CDKs. In addition, they act as positive regulators of cyclin D-dependent kinases. The Cip/Kip family of inhibitors include three different gene products (p21, p27 and p35) with a broad range of specificity so that they are able to inhibit all of the G1 cyclin-CDK complexes and to some extent cyclin B-CDK (see Lees 1995). Some repressors of cell division such as as p53 (see below) act by a mechanism involving CKIs. In addition to the functions described in this section, certain CKIs have a role in producing senescence phenotype in mammalian cells (McConnell et al., 1998).

The inhibitors of the Cip/Kip family can also promote the function of the cyclin-CDK complexes. Cyclin D-CDK complexes are resistant to Cip/Kip inhibition. The inhibitors promote cyclin D-CDK assembly (LaBaer et al., 1997, Cheng et al., 1999). In addition, the Cip/Kip inhibitors target cdk4 and cyclin D1 to the nucleus, needed for cell cycle progression. This does not conflict with the role of the inhibitor to function as such. p21 at low concentrations favors the assembly of active kinase complexes. Inhibition takes place at higher concentrations.

RB-protein is a repressor protein involved in regulation. These repressors have been called *"pocket proteins"* (see Weinberg, 1995). They include several family members in addition to RB-protein (p107 and p130). Phosphorylation of pRb in mid-G1 is catalyzed by G1-phase CDKs. p107 is phosphorylated in mid- and late-G1-phase. The phosphorylation removes the repression. This section examines the involvement of three proteins: the RB-protein, p53, and p27.

RB-protein

There are three members of the RB-family of proteins: RB, p107 and p130. The three proteins are coded by separate genes (e.g., see Cobrinik et al., 1996) and they have different functions (see below). The RB gene product is a nuclear phosphoprotein of about 110 kDa which is present in all cells (referred to as pRb, p110RB or RB-protein). The RB gene earned its initials from its role in producing retinoblastoma. Retinoblastoma is a childhood cancer of the retina. Retinoblastoma cells lack the expression of normal p110RB and correspond to a mutation in both alleles of the RB gene. However, RB gene defects are also responsible for other carcinomas. Replacement of the missing functional RB gene by transfection with a retrovirus containing the gene, suppresses the tumorigenic potential of the cells (e.g., Bookstein et al., 1990). Besides possessing a domain capable of binding DNA, the protein can bind to transcription factors of the E2F family required for the S-phase to take place. These observations suggest a role of p110RB in regulating cell division by controlling the activity of E2F (see Weinberg, 1995).

A role of RB-protein can be examined using purified RB-protein or RB-antibody (Goodrich et al., 1991). These experiments were carried out using osteosarcoma cells which lack expression of the RB gene. The cell cycle of these cells can be synchronized using the drug *nocodazole*, which blocks

reversibly the formation of microtubules. The drug blocks in the M-phase, which requires microtubules for the formation of the mitotic spindle. When nocodazole is washed away, the cell resumes dividing. The synthesis of the DNA can be followed from the incorporation of bromodeoxyuridine, for which there is an antibody. When fixed cells are exposed to the the antibody labelled with a fluorescent probe, the fluorescence serves as a measure of DNA synthesis. Microinjection of RB-protein in early G1 inhibited cell division and the effect was blocked by the coinjection of RB-antibody. However, RB had no effect in late G1 or G1-S transition: injection of the RB-protein or the RB antibody at these later stages had no effect. These observations support a role of RB-protein in blocking cell division. One might presume, therefore, that the concentration of RB-protein decreases when cell division is induced. However, this is not true. Unlike the cyclins, the amount of RB-protein remains constant throughout the cell cycle. How could RB-protein function as an inhibitor of cell division? As we saw for other cases, RB-protein might be present in active and inactive forms. In many cases, the phosphorylative state of the protein determines its biological activity. Why not examine the phosphorylative state of RB-protein during the various cell cycle stages?

A study of the phosphorylative state of the RB-protein (Buchkovich et al., 1989) was carried out with human tumor HeLa cells. The cells were grown synchronously after they were isolated at G1. The HeLa cells' size and mass change as they progress through the mitotic cycle. Thus, cells in different stages can be separated by centrifugation, because their sedimentation velocity increases with each stage. The technique permits separating cells that are primarily in G1 and S. However, the cells in G2 and M remain together. In this experiment, aliquots of the cell fractions were incubated with $[^{32}P]$-P_i. After incubation, the cells were disrupted and the RB-protein was immunoprecipitated with an antibody, run on a gel (SDS-PAGE), and the location of the proteins recorded with fluorography. In fluorography (analogous to scintillation counting), the gel contains a fluor. The fluor emits light when excited by the radioactive disintegrations and the light emission is recorded on photographic film. The RB-protein was found to be relatively unphosphorylated at G1, the stage at which the control of cell proliferation is thought to take place, and becomes highly phosphorylated in the S phase and the G2/M phases. These results suggest that the unphosphorylated RB-protein is the molecule blocking the initiation of cell division and that phosphorylation of this protein releases the the block. Naturally, this conclusion raises a series of questions. What is responsible for the phosphorylation of RB-protein? How does RB-protein inhibit the cell cycle?

The phosphorylation of RB begins in late G1 and continues until the M-phase (e.g., Weinberg, 1995). RB was shown to be phosphorylated by CDKs in vitro (Taya et al., 1989). At least three different CDKs are involved in the phosphorylation of RB (see Taya, 1997). Cyclin E is thought to be one of the activators of a CDK responsible for the phosphorylation. However, the D-cyclins, the major factors responsible for cells to enter the S-phase are also involved. Inhibition of the cell cycle by RB-protein is complex, because this protein binds as many as seven nuclear proteins. At least one of these proteins is a transcription factor, E2F. E2F binds DNA and has been found in complexes containing RB-protein and cyclin A. This suggests that the formation of the complex blocks transcription. Present information supports a model in which cyclin D-dependent kinases initiate RB-protein phosphorylation at mid-G1 phase after which cyclin E-CDK2 are activated and further

phosphorylate the RB-protein (e.g., Lundberg and Weinberg, 1998). RB is maintained in a hyperphosphorylated form by cyclin A and cyclin B-dependent kinases until cells exit mitosis and then the RB-protein is returned to its hypophosphorylated form by a phosphatase (Ludlow et al., 1993). The RB-protein hyperphosphorylation in late G1 frees it from its association of the E2F transcription factors which permit the transcription of genes needed for DNA synthesis (e.g., see Dyson, 1998; Nevins, 1998). Among the trascribed genes are those coding for cyclin E and A and thymidylate kinase, needed for the G1/S transition (e.g., Pagano et al., 1992; Ohtsubo et al., 1995). Fig. 12 (Hollingsworth et al., 1993) summarizes some of these features. The arrow at the bottom of the figure indicates the stage of the cell cycle. Upon activation by an extracellular signal (a) the cyclin binds a protein kinase (b), which is activated by the binding and phosphorylates RB-protein (c). The phosphorylated RB-protein no longer binds to E2F that remains attached to the DNA, (d). The E2F begins transcribing the mRNA of a specific gene (e), and the cell is now able to proceed from G1 to S. The genes containing E2F-binding sites are crucial during the G1-phase.

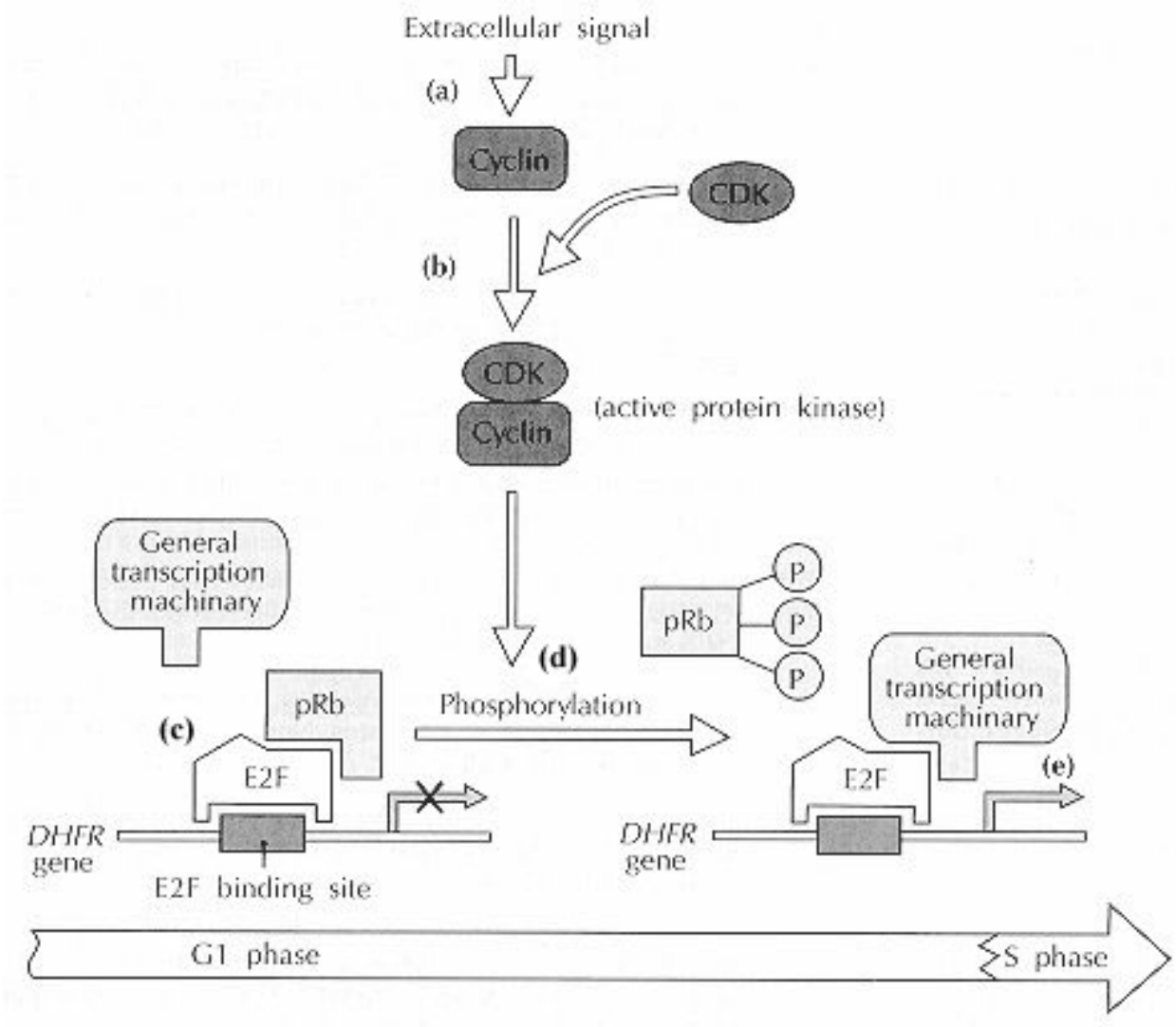


Fig. 12 A model for the interaction between E2F and RB-protein induced by the cyclins during the cell cycle. (a) G1 cyclins are synthesized in response to an environmental stimulus. (b) A complex between cyclins and cyclin dependent kinase (CDK) which activates the kinase. (c) phosphorylation of RB-protein (d) releases E2F for (e) transcription of appropriate mRNAS (e.g., corresponding to

activators of key reactions of cell division. From Hollingworth et al., 1993, reproduced by permission.(Available in the BioMedNet library at:http://biomednet.com/cbiology/cel)

However, the picture currently emerging is somewhat more complex than that outlined so far in this section. The key phases of cell division are regulated by the interaction of five proteins of the family of E2F transcription factors, composed of six members (E2F1-E2F6) (see Dyson, 1998) and the three proteins of the RB-family. The function of the proteins of the RB-family differs. In certain settings p107 and p130 perform growth-regulatory functions that are not fulfilled by pRB. (e.g., Cobrinik et al., 1996). Indeed, pRB and p107/p130 have different roles in their interaction with E2F (see Hurford et al., 1997).

RB-protein has other effects not involving CDKs but still blocking cell growth. RB specifically blocks the activation of the promoter of polymerase I, by binding the upstream binding factor (UBF) needed for the activation of transcription of the ribosomal DNA genes by polymerase I (see Cavanaugh et al., 1995). Similarly, RNA polymerase II (e.g., Weintraub et al., 1995) and III (White et al., 1996) are also repressed by RB. Therefore, RB-protein is likely to have a general effect in restraining protein synthesis, by blocking production of ribosomal and other small RNAs involved in translation, explaining further its anti-tumor effect. It is worth noting that RB can also act by activating transcription (see Section H, below). This is the case for the glucocorticoid-system that is not involved in proliferation, but in differentiation, so that this effect is not contrary to the usual role of RB. RB activates the glucocorticoid-receptor mediated transcription by binding to hBmr (Singh et al., 1995). hBmr is a gene that codes for a protein involved in regulating the activity of DNA-binding transcription factors.

Recent data indicate that RB protein has an active role in repression, by forming part of an assembly that remodels chromatin. Unlike the process silencing heterochromatin which affects large chromatin sectors, RB only blocks the genes that are needed to enter the S-phase (see Brehm and Kouzarides, 1999). Repression of RB protein depends on a complex constituted by E2F, RB-protein, the histone deactylase HDAC1 and the hSWI/SNF nucleosome remodeling complex, thereby inhibiting the transcription of genes for cyclins E and A (Zhang et al., 2000) (see Chapter 2). E2F and HDAC1 do not interact directly but require the presence of the RB-protein order to bind. HDAC1 in the presence of RB represses the cyclin E promoter. The HDAC1-RB combination catalyzes the removal of acetyl groups from core histones (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; Luo et al., 1998). Removal of the charged groups supposedly produces a tighter association between DNA and nucleosomes and blocks the access of the transcription factors (see Chapters 2 and 3 and Wolffe, 1997). Luo et al. (1998) were able to show changes in acetylation at a Gal4-dependent promoter after binding a chimera of Gal4-RB. Normally, Gal4 is resposible for activating the genes needed to metabolize galactose in yeast. An antibody specific for acetylated histone H3 was found to precipitate promoter DNA only when the Gal4 DNA-binding domain was present and not when the Gal4-RB fusion protein was expressed, indicating that RB deacetylated the histone. This technique of chromatin immunoprecipitation is referred to as CHIP (see Chapter 1). In addition, RB has been found to act through a mechanism similar to that producing heterochromatin

(see Chapter 2). RB binds to the complex of SUV39H1 and HP1 and represses the cyclin E promoter. RB is necessary to direct methylation of histone H3, and for binding of HP1 to the cyclin E promoter. Therefore, the SUV39H1-HP1 complex is involved in the repression of euchromatic genes by RB (Nielsen et al., 2001). However, in contrast to the silencing of heterochromatin, the events leading to repression of the cyclins is limited and may involve as little as one nucleosome.

The action of RB is not always through the recruitment of histone deacetylase and this protein can act directly on transcription factors. Experiments with trichostastin, an inhibitor of histone deacetylase, show that it blocks RB-repression in the cases in which RB is recruited to the promoter. However, in the cases where RB acts by inhibiting neighboring transcription factors, the drug has no effect, as for example in the case of p107, a member of the RB-family.

In epithelial cells, *transforming growth factor* β (TGFβ) is one of the regulators of the G1 to S transition (see Massague, 1996) by several mechanisms including the accumulation of CDKIs (e.g., see Sherr and Roberts, 1995). Cell-to-cell contact also acts by multiple mechanisms, including the increase of CDKIs (e.g., St. Croix et al., 1998). The CDK inhibitors prevent the phosphorylation of RB, thereby blocking cell division. Apparently both the contact inhibition and the effect of TGFβ are both mediated by a repression brought about by the RB-E2F complex (Zhang et al., 1999).

p53

The gene coding for p53 is highly conserved and, like RB, functions as an inhibitor of cell proliferation (Milner, 1984). The mutations of this gene are responsible for in vitro transformation of cells. Furthermore, the loss of function in the two alleles for p53 is implicated in the production of 50 to 55% of human tumors (see Levine 1997), such as colorectal carcinoma and human lung carcinoma. The regulation of p53 has been recently reviewed (see Giaccia and Kastan, 1998)

Activation of p53 can occur in response to a number of cellular stresses such as DNA damage, hypoxia and nucleotide deprivation. By blocking the division of cells and activating *programmmed cell death* (apoptosis) (see Chapter 2), p53 avoids genome instability that may produce multiple genetic alterations that result in tumor formation (see Hanahan and Weinberg, 2000).

In the absence of activation, p53 is present mostly at a low level and in an inactive state which activates transcription inefficiently. When p53 is activated in response to DNA damage, the p53 level rapidly increases and is more effective in binding DNA and activating transcription. Apparently, the increase in activity is in part a reponse to phosphorylation, de-phosphorylation and acetylation events on the p53 polypeptide (see Agarwal et al., 1998; Lakin and Jackson, 1999).

Several genes activated by signals involved in cell proliferation, checkpoint-arrest, DNA repair and apoptosis depend on p53 for their transcription (e.g., Di Leonardo et al., 1994; see Lakin and Jackson, 1999).

How does p53 affect cell division? p53 protein has been found to have a transcriptional activation domain at the amino terminal and it binds to specific DNA sequences. In addition, some p53 mutants which fail to block cell proliferation have lost the ability to bind. These observations suggest that p53 could activate genes capable of inhibiting cell growth or could repress genes needed for cell growth or cell cycle progression (see also below). Both possible modes of action have been shown. p53 binds to, and activates, the *growth arrest DNA damage* (GADD) gene, a growth suppressor gene. Both the binding site in p53 (Kastan et al., 1992) and the response element in the GADD gene have been identified. Furthermore, a high level of p53 represses a large number of promoter regions of genes regulating the cell cycle (e.g., Ginsberg et al., 1991).

The activation, synthesis and degradation of p53 are inextricably interwined. The level of p53 is a balance between its synthesis and its degradation. In undamaged dividing cells, p53 has a short half-life. In addition, p53 is largely inactive. However, after radiation damage, p53 is more stable, becomes activated and accumulates in the cell, followed by the expression of genes under its control.

The instability of p53 depends on Mdm2. The oncoprotein Mdm2, binds to the amino terminal of p53 and targets it for degradation by the ubiquitination pathway, where it functions as an E3 ubiquitin-protein ligase (Honda and Yasuda, 1999). The stability of p53 is regulated negatively by a feedback loop where Mdm2 decreases stability and the Mdm2 gene is activated in turn by p53. After treatment of cells with ionizing radiation, the action of Mdm2 is blocked by its phosphorylation in an ATM-dependent manner (e.g., Khosravi et al.,1999). ATM is discussed in more detail below. In a number of cellular responses to ionizing irradiation, the protein kinase, ATM, exhibits enhanced activity in all phases of the cell cycle (e.g., see Pandita, et al. 2000). Responding to DNA damage, Msm2 is phosphorylated by the protein kinases ATM and Chk2 (Khosravi et al.,1999). Since p53 is also activated by ATM, this protein kinase may promote p53 activity and stability by mediating the phosphorylation of both p53 and Msm2.

What protein kinase or kinases are responsible for the phosphorylation of p53? A *DNA-dependent protein kinase* (DNA-PK) that requires the presence of DNA breaks for its activity (e.g., Morozov et al., 1994), has been suspected as the trigger for the action of p53 in response to DNA damage. However, this is not likely, since cells lacking DNA-PK show no defect in the p53 block of the cell cycle (Huang et al., 1996). CHK2 (the homolog of the checkpoint kinase Cds1 of *Schizosaccharomyces pombe*, and RAD53/SPK1 of *Saccharomyces cerevisiae*, also called hCds1), phosphorylates tetrameric p53. The phosphorylation at Ser20 of p53 stabilizes it (Hirao et al., 2000; Chehab et al., 2000; Shieh et al., 2000). The human homolog of the *S. pombe* checkpoint kinase, Chk1 (hCHK1) also phosphorylates p53 in vitro at Ser20 (Shieh et al., 2000).

p53 also exerts its effect through CKIs; in mammals the proteins p16, p21 and p27 (e.g., Harper et al., 1993). Tumor cells lacking p21 (Waldman et al., 1996) continue synthesizing DNA without mitosis, arguing for the absence of a checkpoint between the S and the M phase (possibly at the

G2/M interface). p53 acts as a transcription factor for the *p21/WAF1* promoter (see Levine, 1997). The p53 protein acts on the gene *p21/WAF1*, in conjunction with another protein encoded by another tumor suppression gene, *ING1* (Garkavtsev et al., 1998). Neither protein alone can inhibit growth by themselves. $p33^{INGI}$ and p53 actually form a complex detected by immunoprecipitation (see Chapter 1).

Some of the p53 actions require the gene for *interferon regulatory factor, IRF1* (Tanaka et al, 1996), in addition to $p33^{INGI}$. Whether the interaction actually involves a binding is not known at this time.

The proliferation of eukaryotic cells requires maintenance of telomere length and function (see Chapter 2). Telomere dysfunction produces the activation of p53 leading to growth arrest (e.g., Chin et al., 1999) or apoptosis (Karlseder et al., 1999). Telomere loss occurs with cell division in telomerase deficient mice (Chin et al., 1999). It is overcome by a deficiency in p53 that leads to carcinogenesis.

Two genes related to the *p53* gene have also been found: *p73* and *p63* (p63 is also called p40, p51, KET, or p73L) (see Chen, 1999). These genes are expressed only in certain tissues. Each one of the corresponding proteins has several isoforms produced by alternative splicing of their mRNA (see Kaelin, 1999). The function of these additional molecules differ from that of p53. p73 is not induced by DNA damage and is not targeted for inactivation by viral oncoproteins. So far neither *p73* nor *p63* has been found to be mutated in human cancers. p73 is involved in apoptosis resulting from DNA damage (e.g., Gong et al., 1999) (see Section IIIJ). Both p73 and p63 have important developmental roles still under scrutiny. Mutations of *p63* produce defects in limb and skin development in mice (Yang et al., 1999; Mills et al., 1999) and cause the *ectrodactyly, ectodermal dysplasia and cleft lip* (EEC) syndrome in humans (Celli et al., 1999). *p73* mutations produce neurological and inflammatory defects (Yang et al., 2000).

The involvement of RB-protein and p53 in the control of cell proliferation is now well established, although much has to be learned about the underlying mechanism. The involvement of other as yet unknown suppressor genes is suspected. Many investigators are now examining DNA lesions in tumor cells in the hope of unmasking other inhibitory proteins.

Proteins coded by the INK4a gene

The gene INK4a has a central role in proliferation and tumorigenesis. Mutations of this gene have been implicated in a variety of cancers (see Kamb, 1995). The gene is capable of producing two different proteins by alternative splicing of the corresponding RNA (e.g., Quelle et al., 1995): $p16^{INK4a}$ and $p19^{ARF}$ (corresponding to $p14^{ARF}$ in human cells). $p16^{INK4a}$ is a CKI that inhibits the association between CDK4/6 and D cyclins, blocking the phosphorylation of RB and thereby the exit from G1 (Serrano et al., 1993). $p16^{INK4a}$ is also necessary for the apoptosis that follows the loss of contact in nontransformed epithelial cells (Plath et al., 2000). Mice lacking $p19^{ARF}$ develop

cancer (Serrano et al., 1996). This protein is involved in the arrest at G1 and G2 (Quelle et al., 1995), an effect that requires the presence of p53 (Kamijo et al., 1997). The mechanism is not known, but MDM2, which is involved in p53 degradation, has been implicated (e.g., Pomeranz et al., 1998). $p19^{ARF}$ appears to block the degradation (see Larsen et al., 1996; Zhang et al., 1998).

The human supressor gene $p14^{ARF}$ ($p19^{ARF}$ in mice) is activated by E2F-1 (Bates et al., 1998). Therefore, the inactivation of RB (that frees E2Fs) can supress cell proliferation by this alternative mechanism, providing one more line of defence against unregulated cell growth.

p27

p27 is a CKI specific for CDK2. p27 normally acts at the G1 stage. The absence of the p27-gene in mice, leads not only to increased cell proliferation in virtually every organ but also increased body size (e.g., Nakayama et al., 1996).

The structure of CDK2, in its various states, has been fully elucidated. The structure of the cyclin-CDK2-p27 complex has been recently reported (Russo et al., 1996) and accounts for the inhibition in molecular terms by binding cyclin, disrupting the structure of CDK2 and occupying the ATP binding site of the kinase.

Eventually, p27 is phosphorylated before becoming a substrate for the ubiquitin/proteasome machinery (e.g., Sheaff et al., 1997; Vlach et al., 1997). A yeast two-hybrid system (see Chapter 1) identified a protein interacting with $p27^{Kip1}$, a mouse protein coded by the *Jab1* gene (p38) (Tomada et al., 1999). Increased levels of p38 caused increased breakdown of $p27^{Kip1}$. The binding of the two proteins directed the translocation of $p27^{Kip1}$ from the nucleus to the cytoplasm. Mutants lacking Jab1 remained in the nucleus and were not degraded. Proteasome inhibitors blocked the transfer and the degradation, possibly because the proteasomes may control the degradation of a factor blocking protein export form the nucleus. The involvement of translocation from the nucleus in the control of proteins factors regulating the cell cycle may have general applicability.

Tumor viruses

Some DNA tumor viruses act through their binding of RB proteins. Tumor DNA viruses can disrupt the normal controls of cell division and induce uncontrolled proliferation when they *transform* cells. Much can be learned about normal cell division by studies of how *oncoproteins* exert their effect.

Several nuclear oncoproteins associated with tumor DNA-viruses have been found to interact with RB-protein. The regions of the oncoproteins interacting with RB-protein overlap with those needed for transformation. These results suggest that the protein generated from the DNA-virus acts by binding and inactivating the non-phosphorylated version of RB-protein in a manner analogous to the RB mutations leading to carcinogenesis.

D. Initiation of DNA Replication

In eukaryotes, the capacity to initiate DNA replication at G1 rests on the ordered assembly of multiprotein complexes at *origins of DNA replication*. Once these components are assembled DNA synthesis can proceed under the action of CDKs and the Cdc7 family of protein kinases. CDKs also have a role in the prevention of the formation of new initiation complexes (see Kelly and Brown, 2000).

In *Saccharomyces cerevisiae*, the chromosomal origins of replication, the *autonomously replicating sequences* (ARS) are modular, composed of short sequences distributed in a region of 100 to 200 base pairs (bp). One of these sequences, the A element, contains 11 bp AT-rich, *ARS consensus sequences* (ACSs). The ACS is a component of the binding site for the *origin recognition complex* (ORC). The ORC of budding yeast has been purified. It functions in recruiting replication factors to the origins of DNA replication (see Dutta and Bell, 1997).

Cdc6 in *Saccharomyces cerevisiae* (Cdc18 in *S.pombe*) has a central role in the initiation of DNA replication. High level of expression of Cdc18 initiates multiple rounds of DNA replication in the absence of mitosis (e.g., Muzi Falconi et al., 1996)

Six related proteins, the *minochromosome maintenance proteins* (MCM), also have a role in DNA replication. All six form a complex apparently involved in DNA unwinding (see Ishimi, 1997). Cdc45 (*Saccharomyces cerevisiae*) and analogs in other organisms are essential of DNA replication and associate with chromatin periodically.

After assembly, the initiation complexes are activated by the action of the CDKs and Cdc7-dbf4 kinase. Apparently, Cdc6/18 as well as the MCM proteins and various ORC subunits are phosphorylated by the kinases.

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

E. The Inactivation of the Cell Cycle Regulators

The timely removal of inhibitory proteins, the appearance of the cyclins and their activation of the appropriate kinases are responsible for the cell cycle. As with other biological regulators, removal of inhibitors, cyclins and kinases are as important as as their appearance.

The inactivation of the CDKs involves two interacting systems: the degradation of the cyclins and in yeast the activity of the kinase inhibitor Sic1 which binds to the CDKs and inhibits their activity (see Morgan, 1997; King et al., 1996). The degradation of the cyclins takes place through ubiquitination (Chapter 15, Section 2B) and the proteolytic machinery of the 26S proteasome. Two distinct entry pathways open the proteolytic pathway. One of these is involved in the G1-S transition and uses an E_2 known as CDC34 and involve the SCF complex (see below). The other underlies the onset of anaphase and the termination of mitosis and involves a complex of E_3, the *anaphase promoting complex* (APC/C) or *cyclosome*. The vertebrate APC is a multicomponent complex (see Morgan, 1999). The APC of yeast has 12 subunits and most are homologous to the vertebrate variety. Its activity follows the cell cycle.

First we will discuss the reactions involved in the proteolytic pathway. This will then be followed by a discussion of the regulation of the system.

The ubiquitination pathway

The details of the involvement of protein degradation in the cell cycle have recently been reviewed (King et al., 1996; Pagano, 1997; Townsley and Ruderman, 1998; Koepp et al., 1999). Cyclin degradation occurs via the ubiquitination pathway described in Chapter 15 (Section 2B). Ubiquitination involves three enzyme catalyzed processes. In an activating reaction, E_1 uses ATP to form a thiolester between itself and ubiquitin. The activated ubiquitin is then transferred to E_2. Some E_2s catalyze the formation of an isopeptide bond between ubiquitin and the ε-amino group of lysine in the substrate molecule. The degradation of other proteins require a second protein, E_3. Long polyubiquitin chains must be formed to direct proteins to the proteasomes. Polyubiquitination may require only E_2, although both E_2 and E_3 may be involved in forming more than one kind of ubiquitin-ubiquitin bond (Kalderon, 1996). An additional factor, E_4 (UFD2 in yeast) (Koegl et al., 1999), was found necessary for polyubiquitination.

Novel E_2s and E_3s involved in the ubiquitination of cyclin A and B and other mitotic proteins have been identified in yeast and in cell free systems, generally derived from clam or *Xenopus* eggs (Irniger et al.,

1995; Hershko et al., 1994; King et al., 1995; Sudakin et al., 1995; Tugendreich et al., 1995).

The cyclins preceding the M-phase are targeted for degradation by phosphorylation and ubiquitination by phosphoprotein-ubiquitin ligase E_3 complexes and the SCF ubiquitin ligase system (the complex is named after its components one of which is an F-box containing protein) (see Skowyra et al., 1997). The SCF complex, responsible for the specificity of the degradation (see Craig and Tyers, 1999) is composed of the subunits Skp1, Rbx1, Cdc53 (in yeast) or Cull (mammals) and any one of a large number of different *F-box proteins*. Specific F-box proteins determine the specificity by recruiting a particular set of substrates for ubiquitination to the core complex composed of Skp1, Rbx1, Cdc53 and the E_2 enzyme Cdc34. F-box proteins have a common F-box motif which links F-box proteins to Skp1 and the core complex and in addition a motif that binds to one of the various proteins to be degraded. Most SCF substrates are degraded in the early part of the cell cycle except for the mitotic regulator Wee1 which is degraded later. Wee1-like kinases inhibit CDK function by tyrosine phosphorylation and preventing entry into mitosis.

In contrast to the G1-S components, mitotic B-type cyclins and some S-phase cyclins such as cyclin A (see Table 1 and Fig. 11B) are ligated to ubiquitin by the APC/C (the cyclosome) (Sudakin et al., 1995). The APC/C favors the entry into anaphase and the termination of mitosis. Mutations of some of the subunits of APC/C block the ubiquitination and degradation of mitotic cyclins (Zachariae and Nasmyth, 1996). They also arrest cells in metaphase. The APC/C also mediates the degradation of proteins responsible for the assembly and disassembly of the mitotic spindle. The proteolysis involving APC/C starts at anaphase continues during G1 and is stopped by cyclin-dependent kinases. The sections below will discuss in more detail the G1-S transition and the metaphase-anaphase transition separately.

A large number of deubiquitination enzymes work in the opposite direction and play a significant regulatory role which is still to be fully evaluated. As an indication of their importance, present estimates suggest that more genes code for the deubiquitinating enzymes than for E_2. In addition, in budding yeast, interference with the deubiquitinating enzyme activity disrupts the physiology of the cells (e.g., Zhu et al., 1996).

Degradation requires the presence of specific amino acid sequences. For the G1 and S cyclins, A and B, this sequence is the *destruction box* of nine amino acids. The yeast G1 cyclins CLN2 and CLN3 contain a sequence with a similar mission, the *PEST-sequence* (see Deshaies, 1995). The destruction occurs after the cyclin containing the PEST-sequence is phosphorylated. Cdc28-protein kinase activity is required for phosphorylation (Lanker et al., 1996). Cdc34 kinase is required for cyclin destruction (Salama et al., 1994, Deshaies et al., 1995).

However, it should be recognized that ubiquitination need not lead to degradation in all cases. For example, an entirely different mechanism is involved when the transcription factor Met4p is ubiquitinated. A gene or genes regulated by Met4p are thought to delay the G1-S transition (e.g., Patton et al. 2000). This transition requires inhibition by SCF^{Met30}. Met30 is the substrate recognition F-box

protein subunit of SCF^{Met30} which specifically targets the transcription factor, Met4p for ubiquitination. However, although the transcriptional activity of Met4p is blocked by ubiquitination, Met4p is not degraded (Kaiser et al., 2000). Apparently, ubiquinated Met4p associates with target promoters which become unable to initiate transcription. Met4p appears to control its own activity by regulating the amount of assembled SCF^{Met30} ubiquitin ligase (Rouillon et al., 2000). The ubiquitination of a ribosomal protein (L28) also has an entirely different role. In this case, the ubiquitins are attached to Lys63 of the ribosomal protein, an association that does not affect its stability. In *Saccharomyces cerevisiae*, the level of phosphorylation of L28 was found to vary with the stages of the cell cycle (highest during the S-phase, lowest during G1 or Go). Mutants at the Lys63 site were found o be defective in ribosomal function. The ubiquitination is likely to increase the association between the ribosomes and the nascent mRNAs and facilitate protein synthesis (Spence et al., 2000).

M-G1 and the G1-S transitions

In somatic cells and yeast the cycle, a prolonged G1 is required to allow enough time for growth or differentiation. Cdk inactivation in late mitosis activates Cdh1-APC so that cyclin continue being maintained to a minimum level throughout G1. At least in yeast, the Cdc20-APC dependent destruction of Clb2 was found to be necessary for mitotic exit (Wäsch and Cross, 2002).

In mammalian cells, the CDK inhibitor p27 (discussed above), functions in the control of proliferation (see Sherr and Roberts, 1999). The entry into cell division requires its degradation which follows its phosphorylation by cyclin-CDK complexes (the SCF complex, see above). An F-box protein (see Craig and Tyers, 1999), SKP2 is the F-box protein of the ubiquitin-protein ligase that specifically recognizes phosphorylated p27 (Carrano et al., 1999) so that it is degraded by proteasomes.

In budding yeast, a single CDK, Cdc28 (also referred to as Cdc2 , p34 or $p34^{cdc2}$), combines with various cyclins (for the G1-S transition, CLN1, 2 and 3). The S-phase involves the cyclins CLB5 and CLB6, and the M-phase, CLB1 and CLB4. In higher eukaryotes, the equivalent cyclins are D and E during G1, cyclins E and A during S-phase and A and B during mitosis (see Table 1 and Fig. 11A and B).

The gene coding for the E_2, CDC34, is required for G1-S transition in budding yeast and it encodes a ubiquitinating conjugation step needed before initiation of the S-phase (Goebl et al., 1988). CDC34 is involved in the degradation of CLN2 and CLN3. In addition, it permits progression by mediating the degradation of the S-phase CDK inhibitor Sic1 (see Schwob et al., 1994) (see next section). The various substrates of CDC34 are degraded differentially during the cell cycle. The G1 cyclins rapidly turnover throughout the cycle in yeast (Willems, 1996) and so does Cyclin E in animal cells (Clurman et al., 1996). However, Sic1 is stable during G1 but is degraded upon entering the S-phase (Schwob et al., 1994; Donovan et al., 1994). The regulation of the stability of the substrates depends on the substrate specific phosphorylation which is required for CDC34-dependent ubiquitination. CLN2 and CLN3 are phosphorylated by Cdc28 (Deshaies et al., 1995; Yaglom et al., 1995).

The stability of Sic1 is controlled by a similar mechanism. CDK activity is needed for the multiubiquitination of Sic1. An important function of the G1 cyclins is to phosphorylate Sic1 to allow for the ubiquitination mediated by CDC34 (Tyers, 1996).

The degradation of the cyclins of G1 are needed before G2 cyclins can be effective. In yeast, the proteolysis of the G1 cyclins CLN1 and CLN2 requires the presence of the G2 cyclins CLB1, CLB2, CLB3 and CLB4, thereby providing a mechanism for coupling the two processes (Blondel and Mann, 1996).

The metaphase-anaphase transition

Anaphase is initiated by an increase in APC activity probably by its phosphorylation by CDKs and an increase in the level of an activator of APC, CDC20. In turn APC degrades cyclins and anaphase inhibitors. Proteolysis of cyclins involving the APC complex and inhibition of the CDKs by Sic1 interact for the exit from mitosis. In addition the protein phosphatase CDC14 plays an important role in these interaction.

The APC recognizes a destruction box with a minimum sequence of RXXLXXIXXN (see Yamano et al., 1998). in *Saccharomyces cerevisiae*, APC is activated by two proteins, CDC20 and Hct/Cdh1 (see Morgan 1999, for the names in other organisms) (e.g., see Fang et al., 1998a,b). These two activators are likely to confer different substrate specificity and are activated by separate mechanisms.

Initially, the APC is inactive because its activator, Cdh1, is inhibited by its phosphorylation mediated by cyclin B/CDK1. The CDC14 phosphatase is responsible for the dephosphorylation of Cdh1 (e.g., Zachariae et al., 1998; Jaspersen et al., 1999).

As mentioned above, inhibition of the CDKs by Sic1 is important for terminating the M-phase. The regulation of the events involve an interplay between the kinase inhibitor and the phosphatase CDC14. The level of Sic1 depends on the balance between its production and its degradation. Sic1 synthesis depends on the transcription of its mRNA. On the other hand, Sic1 is targeted for degradation by CDK1 phosphorylation (Skowyra et al., 1997; Feldman et al., 1997). The role of the phosphatase CDC14 is complex. CDC14 increases Sic1. This effect depends on the dephosphorylation and consequent activation of the transcription factor Swi5. This activation permits an increase in transcription of the Sic1 mRNA. In addition, CDC14 interferes with the degradation of Sic1 by removing the phosphate (thereby making it less susceptible to degradation) and by decreasing the CDK1 activity (Visintin et al., 1998). The two together, the accumulation of Sic1 and the activation of APC by activators, bring an end to mitosis.

As we saw, the presence and availability of the phosphatase CDC14, is of key importance in the regulation of mitosis by releasing cdh1 from its inactive state to activate ADC and by controlling the level of the CDK inhibitor Sic1. How is CDC14 regulated? The localization of CDC14 in the cell has shed more light on the mechanism controlling the arrest of mitotic cycle. Immunofluorescence studies

(Visintin et al., 1999: Shou et al., 1999) found CDC14 in the nucleolus during G1 and the S phase. At the beginning of anaphase, CDC14 spreads throughout the nucleus and, to a limited extent, the cytoplasm. Therefore the activation of CDC14 may correspond to its release from the nucleolus. A nucleolar protein Cfi1 (Tem1 in yeast) (which might be a phosphatase) binds to CDC14, suggesting that it might be the anchor which holds it to the nucleolus. In agreement with this notion, *cfi1* deletion mutants are unable to retain CDC14 in the nucleolus (Visintin et al., 1999, Shou et al., 1999) and the cells have difficulty in entering S phase. In *Saccharomyces cerevisiae*, the complex anchoring CDC14 to the nucleolus has been called RENT (Shou et al., 1999).

The sequestration in nucleolus may be a general regulatory mechanism. The tumor suppressor protein $p19^{Arf}$ activates p53 by sequestering its inhibitor Mdm2 in the nucleolus (see Weber et al., 1999).

F. Polo-like and Other Kinases

We have seen that cyclins and CDKs have a central role in cell division. Other protein kinases have been shown to be produced and degraded at specific transition points in the cell cycle, e.g., NIMA protein kinase (a kinase coded by the nimA gene) has its highest concentration at the end of G2 and the M-phase and has a role in the regulation of the cell cycle. The *polo-like kinases* (plks), like the CDKs are also members of the serine/threonine kinase family. They play a complex and often distinct role in the progression throughout mitosis (see Glover et al., 1996; 1998), including centrosome maturation, bipolar spindle formation, activation of APC and cytokinesis (i.e., the actual separation into two cells) (see Field et al., 1999). The plks may function in conjuction with the CDK system. plk can help maintain the mitotic state by phosphorylating the Cdc25 phosphatase that activates $p34^{cdc2}$ (Kumagai and Dunphy, 1996). MPF (the $p34^{cdc2}$-cyclin B complex) allows the translocation $p34^{cdc2}$ into the nucleus and its activation of mitosis. The nuclear translocation depends on the phosphorylation of the cyclin (e.g., Hagting et al, 1998). A protein kinase from *Xenopus* M-phase extract phosphorylates a serine residue in the middle of the nuclear export sequence of the cyclin. Apparently the kinase corresponds to a Polo-like kinase (homologous to plk-1). Antibodies to plk-1 block the kinase activity and an antiphosphate-serine147 antibody binds cyclin B1 only during the G2/M phase. Constitutive Plk1 stimulates the entry of cyclin B1 to the nucleus during phophase (Toyoshima-Morimoto et al., 2001).

plks can also act separately from CDKs in maintaining functional centrosomes and a mitotic spindle. The *polo*^{1} mutation in *Drosophila* have a disorganized centrosome and an abnormal microtubular organization (Sunkel and Glover, 1988). The loss of polo-like kinases results in monopolar mitotic spindles in other cases as well. For example, the microinjection of Plk1-antibodies into human cells, blocks the cells in mitosis with monopolar spindles and unseparated centrosomes (Lane and Nigg, 1996). Immunological cytochemistry (see Chapter 1) indicates that Plk1 is associated with the polar region of the spindle from prophase to metaphase (Golsteyn et al., 1995). The Plk1 of *S. cerevisiae* behaves differently from the other systems studied. The cells can form a spindle. Nevertheless, cell division is stopped when the chromosomes have separated and the spindle is elongated (Hartwell et al., 1973).

The polo kinase Cdc5 is needed for cytokinesis. HeLa cells transfected with either wild-type or mutant plk exhibit a failure of cytokinesis independently from its kinase activity (Mundt et al., 1997). The kinase probably favors APC activation and the cytokinetic pathway (Song and Lee, 2001).

G. Cohesion of the Chromatids

For the M-phase to take place in an orderly manner, the sister chromatids have to be held tightly together. Failure to maintain cohesion between each pair, produces serious malfunctions. Congenital aneupleudy is one of the causes of Down's syndrome and chromosome-segregation defects uncover recessive mutations which can produce tumors. When the sister kinetochores are attached to the spindle, forces are generated which would separate the chromatids (Losada et al., 1998) if it were not held firmly together. Cohesion is responsible for the alignement during metaphase, needed for the proper distribution of the chromosomes. Generally, separation is initiated at anaphase, when the elimination of the cohesiveness triggers the separation of the sister chromatids (see Nasmyth, 1999).

In *Saccharomyces cervisiae*, a complex of at least four subunits (Scc1, Scc3, Smc1 and Smc3), *cohesin*, is responsible for the cohesion (Michaelis et al., 1997; Guacci et al., 1997, Toth et al., 1999). Smc1 and Smc3 are members of the SMC family, which are putative ATPases with coiled-coil domains. Two other proteins are needed to establish cohesion (Toth et al., 1999; Uhlmann and Nasmyth, 1998; Skibbens et al., 1999).

The separation of the sister chromatids occurs after the disappearance of Scc1 and Scc3. Both the dissociation of Scc1 from the chromosomes and the separation of the sister chromatids depend on a *separin* protein (Esp1) (Ciosk et al., 1998).

Esp1 dissociates Scc1 from the chromosomes by stimulating its proteolysis (Uhlmann et al., 1999) thereby initiating the separation of the sister chromatids. Before this, Esp1 is held inactive by binding to the inhibitor Pds1 (Ciosk et al., 1998) until the inhibitor is ubiquitinated and then degraded at the metaphase-anaphase transition by the anaphase-promoting factor (APC) (Cohen-Fix et al., 1996). The activation of APC depends on CDC20 (see above).

H. Anchorage Requirement and the Cell Cycle

The dependence of cell growth on cell anchorage has been the subject of studies for many years. Benecke et al. (1978) showed that, in culture, absence of substratum inhibited mRNA and protein synthesis. This inhibition decreased with increasing degrees of cell transformation (Wittlesberger et al., 1981). The block in normal cells appeared to be in the G1-phase.

The cyclins that play a role in the G1 phase are indicated in Fig. 11B. In addition, cyclin inhibitors (CKI) are also involved and the various factors interact in a complex manner (see Assoian, 1997). Cyclin D1 is

the primary cyclin for several cell types. ECM and mitogens are jointly required to induce cyclin D1 expression. In the absence of substratum, quiescent cells in culture did not produce cyclin D1 mRNA even in the presence of mitogens. In addition, the production of cyclin D1 using preexisting mRNA is also blocked in the absence of a substratum. Cyclin E and CDK2, active in late G1, are also growth factor dependent and require attachement to the substratum. However, the induction is presumed to occur by decreasing the levels of CKIs. The induction of cyclin A, active in the S-phase, is also strongly dependent on cell anchorage. In this case, however, the mitogenic effect is through a mechanism in which RB or p107 are phosphorylated.

These effects of anchorage are profound because the lack of these enzyme activities precludes phosphorylation of pRb and p107 and the subsequent activation of E2F-dependent transcription. The effects of anchorage are mediated in some way by the cytoskeleton. Adhesion and dependence on the cytoskeleton appear to go hand in hand (Böhmer et al., 1996). For example, cytochalasin D, that disrupts the cytoskeleton, blocks the progress through G1. However, as is the case for anchorage, cytochalasin is totally without effect between the Go and the S-phase. Anchorage and the cytoskeleton are needed for the phosphorylation of Rb-protein.

The ECM and mitogenic growth factors can also act synergistically. Cell adhesion to substratum and the aggregation of integrins in focal contacts, activate several signalling molecules (see Bottazzi et al., 1997). Many of these signalling molecules are also activated by mitogenic growth factors. The extent of signaling and progression through the cell cycle is determined by the interaction between receptor tyrosine kinases and integrins. The molecular connections between these two pathways is still unknown.

I. Licensing Factors

In proliferating eukaryotic cells, DNA replication is confined to the S phase. In this process each sequence in the chromosomes is replicated only once. Consequently, DNA replication does not occur again until the segregation of the chromosomes in mitosis. The mechanisms for preventing additional replications during the mitotic cycle could be positive or negative. That is, a factor could induce the replication or, alternatively, the control could be through an inhibitor that prevents replication. The inactivation of the inhibitor would then initiate replication. The induction of DNA synthesis and mitosis by the S-phase promoting factor (SPF) and the maturation promoting factor (MPF), argues for a positive control, which has been referred to as a *replication licensing factor* (RLF).

Xenopus egg extracts support the initiation and synthesis of the entire DNA complement, which replicates only once. Rupture of the nuclear envelope, however, allows cells arrested in G2 to re-replicate with fresh extract (Blow and Laskey, 1988). This finding argues that the RLF licenses only in the absence of a functioning nuclear envelope during the M-phase. Such a mechanism would clearly allow only one replication per cycle.

SPF induces initiation of the "licensed" origins and terminates the license. The action of RLF and SLF is sequential. RLF cannot cross the nuclear envelope and it can license the DNA synthesis only during

mitosis. However, the SPF can initiate DNA replication on licensed DNA even in an intact nucleus. However, the sequential nature of the process is guaranteed by the fact the both activities occur at specific points in the cells cycle. RLF is abruptly activated after the metaphase-anaphase transition and is degraded at interphase (Blow, 1993). In contrast the SPF activity can be detected only in interphase (Blow and Nurse, 1990).

The RLF activity is found in two components, both needed for licensing, RLF-M and RLFB (Chong et al., 1995; Tada et al, 1999). RLF-M is a complex of six members of the MCM/P1 family (see Tye, 1999) such as MCM4 discussed below, originally identified in yeast. Inactivation of the MCM proteins in mammalian cells by microinjection with antibodies, blocks DNA replication in the S-phase (Kimura et al., 1994). All six assemble at the replication origin during the late stages of mitosis and G1 and form a pre-replicative complex (PreRC) (see Tye, 1999). Proteins in this family are: (1) localized in the nucleus in G1 and late mitosis in yeast (e.g., Hennessy et al., 1990), (2) present in a broad variety of eukaryotes ranging from plants to mammals (Chong et al., 1996, Kearsey et al., 1996), (3) required for the replication of DNA in higher eukaryotes (e.g., Todorov et al., 1994, Treisman et al., 1995), and (4) reassociated with chromatin only after permeabilization or breakdown of the nuclear envelope (e.g., Chong et al, 1995, Kubota et al., 1995). Only two contain nuclear localization sequences (NLSs) so that it is likely that the complex moves to the nucleus after being assembled. Two other proteins are involved: the *origin-recognition complex* (ORC) and Cdc6. The presence of CDKs acts in the opposite direction by blocking initiation.

One of the mechanisms that insures that DNA replication occurs only once per cell cycle rests in the spatially separation of key components such as MCM4 and template DNA. In yeast cells, MCM4 is exported from the nucleus when no longer needed. This process avoids re-replication. MCM is present in the nucleus in late mitosis and the G1 phase and cytoplasmic during the S-phase, the following G2 phase and early mitosis (Hennessy et al., 1990). The movements of MCM can be followed using MCM4 fused to green fluorescent protein (GFP) (see Chapter 1) (Labib et al., 1999). The MCM-GTP hybrid was found in the nucleus only in the absence of CDK activity. The inhibition by CDKs is probably indirect, the result of the activation of an inhibitor which requires the presence of CDKs (Mahbubani et al., 1997). When the CDKs are inactivated in the G2-phase, the MCM-GFP returns to the nucleus.

In mammalian cells, entry of the MCM proteins into the nucleus does not determine their binding to the chromatin. The binding requires a rupture of the nuclear membrane, suggesting the presence of another factor involved in the licensing, a *loading factor*, which cannot pass through the nuclear envelope (Madine et al., 1995).

In *Saccharomyces cerevisiae*, other experiments indicate that the cyclin B-Cdk complexes prevent replication during S, G2 and M phases - probably by blocking the transition of replication origins to a pre-replicative state (Dahmann et al., 1995). Experiments carried out with cell free extracts of *Xenopus* eggs, show that CDK2-cyclin E and A kinases negatively regulate DNA synthesis. Once the chromatin is assembled, CDK2 kinase is accumulated in the chromatin 100 fold. CDK2-cyclin E did not block the

association of the ORC complex with sperm chromatin, but prevented MCM3 from associating (Hua et al., 1997). A role of CDK2 in limiting the replication of DNA has been shown in rat fibroblasts, where inhibitors of cdc2 activity produce multiple rounds of replication in the absence of mitosis (Usui et al., 1991).

J. Sequential Processes Controlled by Checkpoints

As discussed above, the cell cycle seems to be regulated to a large extent by interactions between cyclins, CDKs, inhibitory proteins and their degradation. These proteins are instrumental in activating and deactivating specific sequential steps. A sequential order could also be imposed by the nature of the reactions. For example, reactive sites that permit the addition of the next component may be created by the previously synthesized protein. This process would introduce a well-defined sequential assembly pathway. Such endogenous sequential processes undoubtedly play a role in cell division. For example, aster formation requires the presence of functioning microtubule organizing centers. However, there may be special mechanisms to produce a sequential pattern. A temporal inhibition may take place so that a particular step is not activated until the preceding step is completed. In effect, these controls act as *checkpoints* and represent a block in some of the essential steps in the mitotic progression. The precise mechanism of these controls is not entirely clear at this time. They are of obvious importance in maintaining function and avoiding abnormalities that could lead to serious problems such as malignancy.

Completion of DNA replication and DNA damage

After one round of duplication, the re-initiation of DNA replication has to be blocked to maintain a single replication per cell cycle. CDKs have been implicated in blocking re-initiation in eukaryotes (see Kelly and Brown, 2000) by preventing the assembly of preinitiation complexes at the origins (see above). High CDK activity in the G2/M prevents the association of MCM proteins with the origin of duplication. In addition the CDKs phosphorylate Cdc6. In *Saccharomyces cerevisiae*, B-type CDKs block re-initiation via three inhibitory pathways: nuclear exclusion of MCM 2-7 complex, phosphorylation of the *origin recognition complex* (ORC) and inhibition of Cdc6 activity (Nguyen et al., 2001).

Checkpoints responsive to DNA damage stop the cell cycle either at G1 (G1-DNA-damage checkpoint) or just before mitosis (G2-DNA-damage checkpoint). The loss of checkpoints involved in blocking cell division because of DNA damage have been implicated in human cancers and genetic instability (e.g., see Hartwell and Kastan, 1994; Paulovich et al., 1997).

Cell fusion experiments (see section IIIB, above) show that when cells in the S phase and G2 phase are fused, the G2 nucleus is delayed in entering mitosis until the DNA duplication of the S-phase cell is completed (Rao and Johnson, 1970). These experiments highlight how checkpoints work. Similarly, interference of DNA synthesis, either by the introduction of specific inhibitors or mutational inactivation of enzymes involved in DNA synthesis, prevents mitosis.

How do incomplete DNA replication or damage prevent the completing of mitosis? The mechanisms and targets of these controls are still unclear. However, several patterns are beginning to emerge. Entry into mitosis requires dephosphorylation by the Cdc25-phosphatase of the MPF (the $p34^{cdc2}$-cyclin B complex) which allows the translocation $p34^{cdc2}$ into the nucleus and its activation of mitosis. Many of the checkpoints have been found to converge on this mechanism. $p34^{cdc2}$ (also referred to as Cdc2 and in budding yeast, Cdc28) is the protein kinase involved in initiating several phases of the cell cycle in *Saccharomyces cervisiae* and some steps in many other systems, including mammals (see Fig. 11A and 11B). At interphase, the complex is maintained inactive by kinases which phosphorylate $p34^{cdc2}$ (in *Schizosaccharomyces pombe*, *wee1* kinase). The cell cycle starts only after $p34^{cdc2}$ is dephosphorylated by the phosphatase Cdc25. Conversely, a block in the cell cycle at G2 persists as long as $p34^{cdc2}$ remains phosphorylated (see Elledge, 1996). Phosphorylation of $p34^{cdc2}$ prevents the activation of MPF (Nurse, 1990). The binding of Cdc25-phosphatase to a 14.3.3 protein blocks its action so that $p34^{cdc2}$ remains phosphorylated. DNA damage leads to the degradation of Cdc25 (Mailand et al., 2000) blocking the progression.

The *14-3-3 protein* family (see Aitken, 1996) (so named because of their two-dimensional migration pattern on DEAE-cellulose chromatography and starch gel electrophoresis) has seven known members in humans. Homologues have been found in plants, insects, amphibians and in the nematode *Caenorhabditis elegans* as well as in fission and budding yeast. The homologues in budding yeast are Rad24 and Rad25. The 14-3-3 proteins bind to phosphoserine of certain proteins such as Cdc25-phosphatase (e.g., Muslin et al., 1996). In fission yeast, the components of this checkpoint were identified by means of a genetic screen based on the sensitivity to radiation. Without a functional check point responding to DNA damage, mutant cells begin mitosis despite the presence of damaged DNA. Among other proteins, Chk1 (a serine-threonine kinase) and Rad24, were identified as involved in the block.

Chk1 and Rad24 act through the Cdc25-phosphatase. In both fission yeast and human cells, damaged DNA activates the protein kinase, Chk1. In turn, Chk1 phosphorylates the Cdc25-phosphatase creating a phosphoserine binding site for a 14.3.3 protein (e.g., see Peng et al., 1997). However, Cdc25-phosphatase is active whether bound to the 14.3.3 protein or not. Why then is the phosphorylated Cdc25-phosphatase able to block mitosis? The study of Lopez-Girona et al. (1999) provides the answer to this paradox. They found the Cdc25-phosphatase is mostly cytoplasmic and, in contrast, its substrate, cyclin B/$p34^{cdc2}$, is mostly nuclear. The localization of the Cdc25-phosphatase acts as a regulator of cell division. When the DNA is damaged, the phosphorylated Cdc25-phosphatase leaves the nucleus combined with the protein Rad24. Thereby Cdc25-phosphatase becomes separated spatially from its substrate, the cyclin B/$p34^{cdc2}$ kinase, which is nuclear, and the kinase remains phosphorylated and inactive. Therefore, the initiation of mitosis is blocked. In contrast, in the absence of DNA damage, the cell cycle is initiated when some Cdc25-phosphatase molecules are translocated to the nucleus. Presumably, the cell cycle is triggered when the phosphatase, Cdc25, overwhelms the effect of the kinase, *wee1*.

Two related protein kinases, either together or separately, appear to coordinate the signals following DNA damage: ATM (*ataxia telangiectasia-mutated*) and ATR (*ataxia telangiectasia and Rad3 related*) (see

Shiloh, 2001). The two are serine/threonine protein kinases of the phosphatidylinositol 3-kinase (PI3K) family. ATM functions mostly in response to DNA strand breaks and ATR in several different checkpoint responses. Although they follows similar pathways, their functions are quite different as shown by mutations. In humans, ATM mutation produces *ataxia telangiectasia*, a condition with multiple defects and progressive neurodegeneration. Mutation in the *ATR* gene in mice, which leads to early embryonic death, produces extensive chromosomal fragmentation.

In mammals, ATM, ATR and p53 are gene products that play a role in maintaining the integrity of the genome by controlling checkpoints (see Morgan and Kastan, 1997; Shiloh, 2001; Walworth, 2001). Although ATM and ATR were both found to act via the phosphorylation of p53, supposedly ATM responds to ionizing radiations, whereas ATR responds to UV radiation. ATR may also act independently. ATR can act by activating the Chk1 kinase by phosphorylation (see Liu et al., 2000). The p53 protein is an important player in the G1 DNA-damage check point (Kastan et al., 1991). However, p53 also makes a contribution to the G2-DNA damage checkpoint. p53, as discussed above, acts as an inhibitor of cell division. ATM is also involved in other cellular processes such as S phase and G2-M phase arrest and in radiosensitivity.

The kinases of the ATM family are needed for the checkpoint arrest following DNA damage or incomplete replication. Disruption of the *ATM* gene is responsible for causing a disease with cerebellar dysfunction, chromosomal instability and pre-disposition for cancer (Xu and Baltimore, 1996; Xu et al., 1996; Xu et al., 1999). ATM is part of a complex that contains BRCA1 (coded by the breast cancer gene 1, Brca1). *ATM*, Brca1 and Brca2 are tumor suppressor genes (see Kinzler and Voglestein, 1997). BRCA1 is required to maintain genetic stability by regulating the duplication of the centrosomes and provide a G2-M checkpoint (Xu et al., 1999). Therefore ATM has a dual role, one in the cell cycle and the other in cell-cycle arrest when DNA is defective (Xu and Baltimore, 1996). ATM phosphorylates BRCA1 after γ radiation (Cortez et al., 1999), although BRCA1 is also controlled independently of ATM (Scully et al., 1997). Presumably Brca1 and Brca2 are involved in the recombination repair after DNA damage (see Moynahan et al., 1999). Brca1 has also been implicated in transcription (e.g., Anderson et al., 1998; Kleiman and Manly, 1999). p53 is also be phosphorylated by ATM or by an ATM homologue (e.g., Canman et al., 1998; Lakin et al., 1999; Tibbets et al., 1999).

Mutations of the Brca1 gene are associated with breast and ovarian cancers (e.g., Miki et al., 1994). These mutations account for 45% of families with high incidence of breast cancer and for 80-90% of families with both breast and ovarian cancer. BRCA1 has several motifs characteristic of transcription factors and has been shown to function as a transcription factor (e.g., Monteiro et al., 1996): its carboxy-terminal region, fused to GAL4 DNA binding domain has been shown to activate transcription of GAL4 (see Chapter 1). BRCA1 has also been found to be present in a complex related to SWI/SNF, a complex with chromatin remodeling activity (see Chapter 2 and 3). The binding is to the BRG1, the ATPase subunit indispensable for its action in chromatin. BRCA1 mutants were unable to act as coactivators of transcription which also requires p53 (Bochar et al., 2000).

In the G1 arrest, the cyclin-dependent kinase inhibitor $p21^{WAF1/clp1/SA11}$ (e.g, Brugarolas et al., 1995) mediates the effect. p21 is transcriptionally regulated by p53 (Xiong et al., 1993). p53 also activates the transcription of other proteins such as 14-3-3σ (Hermeking et al., 1997) in response to DNA damage and blocks the cell cycle at G2 (Hermeking et al., 1997). In the presence of p53, Cdc25-phosphatase cannot activate $p34^{cdc2}$ because the phosphatase remains in a phosphorylated form, bound to 14-3-3 proteins. As we saw above, the phosphorylated phosphatase is tranferred to the cytoplasm and cannot act on the kinase.

Damage apoptosis (see White, 1996), whether induced by malignancy (e.g., see Hartwell and Kastan, 1994), radiation damage (e.g., Lowe et al., 1993), hypoxia (Graeber et al., 1996) or infection by certain viruses, genrally involves p53. ATM has also been implicated in triggering apoptosis via the phosphorylation of p73 (See Shaul,2000). p53 exerts its effect through several CKIs (such as p21). The inhibition is reversible if the defect is minor or of short duration. However, in the absence of a recovery the cell proceeds to *apoptosis* (see Chapter 2). Apoptosis is a systematic process of programmed cellular death. It takes place in well-defined steps in individual cells, including cytoplasmic condensation and formation of cytoplasmic protuberances, chromatin condensation and DNA fragmentation (see Wyllie et al., 1980). In addition to a role in cell-cycle arrested cells, apoptosis plays a role in organogenesis, tissue homeostasis and in the functioning of the immune system.

The role of 14-3-3σ in p53-controlled checkpoints was evaluated by producing knockout somatic cells (see Chapter 1) lacking the 14-3-3 protein. After DNA damage the cells were arrested at G2 but could not maintain the arrest and the cells died upon entering mitosis. The failure to block mitosis was caused by the absence of sequestration of cyclin B1 and $p34^{cdc2}$ (Chan et al., 1999b) by 14-3-3σ. Normally, the $p34^{cdc2}$-cyclin B1 complex is shuttled between nucleus and cytoplasm (see Yang, J. and Kornbluth, S., 1999) All aboard the cyclin train: subcellular trafficking of cyclins and their CDK partners, Trends Cell Biol. 9:207-210. Yang and Kornbluth, 1999). In the knockout mutants, the absence of 14-3-3σ allows the $p34^{cdc2}$/cyclin B1 to accumulate in the nucleus and eventually bypass the block.

The results of other studies, some with *Aspergillus nidulas* have shown other aspects of DNA damage checkpoints. In addition to the phosphorylation of $p34^{cdc2}$, the phosphorylation of a component (Cdh1 also called Hct1) of the anaphase promoting complex (APC) (Ye et al., 1996; Zachariae et al., 1996; Peters et al., 1996) is also involved. Phosphorylation of Hct1 block its incorporation into APC, eliminating the degradation of cyclins. Another check point, at least in *A. nidulans*, is the regulation of the mitosis-promoting NIMA protein kinase (Osmani and Ye, 1995, 1996). NIMA appears to be activated by $p34^{cdc2}$cyclin B (Ye et al., 1995). The presence of both protein kinases is necesary for the G2-M transition.

Mutations of *CHK2* have been shown to be responsible for the predisposition to cancer in individuals with *Li-Fraumeni syndrome* (Bell et al., 1999). This gene codes for checkpoint kinase CHK2 (Cds1 in

Schizosaccharomyces pombe or Rad53 in *Saccharomyces cerevisiae*). Studies of the CHK2-deficient mice indicate that this kinase acts upstream from p53. The mice had a defective CHK2 gene produced by gene targeting (see Chapter 1). These cells, have several defective checkpoint after irradiation (Hirao et al., 2000). The CHK2-deficient cells were embryonic mice stem cells lacking arrest in G1 (but having an arrest in G2) and apoptosis or thymocytes. The CHK2 absence failed to maintain G2 arrest in the stem cells and the deficient thymocytes were defective in DNA-damage apoptosis. The deficient cells were also defective in p53 stabilization (see discussion of p53 stability, above) and p53 dependent transcripts (such as that of p21). Normal function was reestablished by re-introducing the *CHK2* gene. CHK2 stabilizes p53 which has a central role in G1 arrest. Therefore, the absence of CHK2 produces an inability to enter apoptosis (which depends on p53) and the G2 damage checkpoint (not requiring p53).

Two genes related to the *p53* gene (see Section IIIC) have also been found: *p73* and *p63* (*p63* is also called *p40*, *p51*, *KET*, or *p73L*). The two genes are expressed only in certain tissues. Each one of the proteins has several isoforms produced by alternative splicing (see Kaelin, 1999). The p73 protein is involved in responses due to DNA damage. p73 has been found to be a target of non-receptor tyrosine kinase-cAbl following DNA damage (Gong et al., 1999). DNA damage activates c-Abl kinase activity (Kharbanda et al., 1995). Both cAbl and p73 act together in producing apoptosis (Agami et al., 1999; Yuan et al., 1999).

In addition to the checkpoints dependent on completion of functional DNA duplication, other checkpoints are under study. These include the block to the separation of chromosomes when the mitotic spindle is damaged or chromosomes fail to attach properly to the spindle, the anaphase to metaphase transition which requires: the completion of anaphase, the inhibition of cell division until they have reached a critical size, the prevention of a new round of DNA replication until completion of the M phase, and the requirement for DNA synthesis before the centrosome can duplicate. The centrosomes are required for the G1-S transition (Hinchcliffe et al., 2001) and the completion of cytokinesis (Piel et al., 2001). These finding suggest that there may be checkpoints linked to centrosome function.

When other DNA replication and damage checkpoints fail, another mechanism causes mitotic spindle defects and chromosome-segregation failures and can be considered an additional check-point (see Sibon et al, 2000). This check point results in inactivation of the centrosome with dissociation of the γ-tubulin ring complex.

Checkpoints of the mitotic apparatus

The capture of kinetochores by microtubules during mitosis is a random process (e.g., see Nicklas, 1997). The orderly distribution of the chromosomes requires that mitosis be delayed until all chromosomes are properly aligned at the spindle equator. The checkpoint responsible for this control is sensitive even to a single unattached kinetochore (Rieder et al., 1995). The mechanism by which an unattached kinetochore inhibits entry into anaphase is unknown. However, it appears to respond to the attachment of kinetochore to microtubules (Rieder et al., 1994) and the level of tension exerted on the kinetochore (Li and Nicklas,

1995). Immunofluorescence microscopy (see Chapter 1) using antibodies sensitive to phosphoproteins has demonstrated that kinetochore proteins are phosphorylated when the chromosomes are unattached and become dephosphorylated after the chromosomes attach to the spindle (Gorbsky and Ricketts, 1993; Nicklas et al., 1995). Misattached chromosomes remain phosphorylated. In order to identify kintechore phosphoproteins, this technique requires the extraction of soluble phosphoproteins from the cell sections and the inhibition of endogenous phosphatases which remove the phosphates from the phosphoproteins. Micromanipulation accompanied by immunofluorescence with the phosphoprotein antibodies demonstrates that tension obtained by micromanipulation causes kinetochore protein dephosphorylation, while relaxation causes rephosphorylation (Li and Nicklas, 1997). In view of these findings, it would not be surprising to find that protein kinases are associated with this checkpoint.

What is the mechanism of chromosome separation and what are the events underlying the checkpoint that block it? After DNA replications the sister chromatids are held together by *cohesin* complexes (see Nasmyth, 2001). The separation of the two sister chromatids to opposite spindle poles depends on the intervention of *separins* which mediate the release of cohesins from the chromatids. However, the activity of separins is blocked by a class of proteins known as *securins* (PTTG in vertebrates) . The separation of the chromosomes during the metaphase-to-anaphase transitions requires the degradation of the securins initiated by a subunit of APC (CDC20), an ubiquitin ligase. CDC20 is one of the target of the checkpoint. When the degradation is blocked, the chromosome cannot separate. We have already seen that APC is involved in the progression of cell division to allow the initiation of cell division (see Section IIIC).

How is CDC20 blocked? The protein Mad2 at unattached kinetochores binds and inhibits the activity of APC (see Shah and Cleveland, 2000). Other proteins are also present in unattached kinetochores such as Bub3, Mad1 and the mitotic kinases Bub1, MAP kinase and BubR1 (the mammalian Mad3) and may play a role in the checkpoint. A complex which has been called the *mitotic checkpoint complex* (MCC) (Sudakin et al., 2001) is composed of the hBubR1, hBub3, CDC20, and Mad2 checkpoint proteins in near equal stoichiometry. MCC inhibits APC/C. However, MCC is not assembled at kinetochores and is also present and active in interphase cells. Only APC/C isolated from mitotic cells was sensitive to inhibition by MCC suggesting the presence of an activator. The centromeres of misaligned chromosomes inactivate the APC by sequestering the protein CDC20, essential for APC function by forming a complex with MAD and BUB proteins. Without an active APC the progression is arrested

APC is also required for spindle disassembly and cytokinesis (see Hardwick, 1998). The mitotic cyclins (A and B in higher eukaryotic and CLB in budding yeast) function as activators of CDK1 (CDC28 in budding yeast), required for spindle formation and pre-anaphase processes (e.g., see King et al., 1994). They are degraded via APC ubiquitination.

The metaphase allignement of the chromosomes as well as the separation of the chromosomes require movement, indicating an involvement of motors. However, the motor proteins have been found to be needed for the spindle checkpoint itself. Three microtubule motors and some binding proteins have been found in kinetochores. CENP-E is a kinesin-like motor (plus-end directed) active in metaphase allignment

and checkpoint activity (e.g., Yao et al., 2000; Abrieu et al., 2000). CENT-E binds to spindle microtubules as well as kinetochore-associated checkpoint kinase BUBR1. CENP-E is involved in delivery of components from kinetochores to poles. Depletion of this motor leads to checkpoint activation (Yao et al., 2000).

Cytoplasmic dynein is the only minus-directed motor that might drive the poleward movement of the sister chromatids after anaphase (see Banks and Heald, 2001). The contribution of dynein at the knetochore for chromosome movement in prometaphase and anaphase was demonstrated by preventing the expression of AW10 or ROD, proteins needed for localizing dynein to the kinetochores. (Savoian et al., 2000). Dynein inhibitors were found to disrupt the alignment of kinetochores on the metaphase spindle equator and also chromatid-to-pole movements during anaphase A (Sharp et al., 2000). Zw10 and Rod not present in yeast, are also required for the spindle checkpoint in metazoan cells (e.g., Chan et al., 2000). Therefore, dynein may be the target of spindle-checkpoint regulators.

K. The Centrosomes

Centrosomes are present in most animal cells, but they are missing in higher plants, some meiotic cells, eggs and certain embryos. When present, centrosomes have been shown to be the major MT organizing centers (MTOCs) and are thought to have a role in spindle assembly. The assembly of MTs at MTOCs is also discussed in Chapter 23. Centrosomes play an important role in most cells (see Doxsey, 2001; Rieder et al., 2001). Their absence in some cells suggests that unrecognized components may fill a similar role. Many important questions about centrosomes are still at least in part unresolved including their precise function, the mechanism of centrosome duplication and assembly, as well as the regulation and mechanism of the centrosomal microtubule nucleation activity (see Andersen, 1999).

In non-dividing cells, the centrosomes are usually located in approximately the center of the cell near the nucleus. They are composed of two centrioles and pericentriolar material. Centrioles are cylindrical in shape and composed of nine sets of triplet microtubules. The centrioles are at right angle from each other. The centrioles contain several specific proteins such as centrin, cenexin and tektin. The α-β- tubulin subunits of the centrioles are modified, in one case by polyglutamylation (see Andersen, 1999; Bobinnec et al., 1998). Centrioles are involved in the assembly of other centrosome components. Most kinds of cells have a non-motile cilium formed from the oldest "mother" centriole (Roth et al., 1988). Basal bodies of cilia and flagella, responsible for their formation, are similar to centrioles.

Normally, microtubular nucleation occurs mainly at centrosomes. The MTs are anchored to the centrosome through their minus ends, whereas the plus ends are toward the cytoplasm. The microtubules formed inside the cell have 13 protofilaments (see Evans et al. 1985) (the number is variable when formed in vitro). The precision of the number of protofilaments in vivo, suggests the formation of MTs on a template. This template has been found to be the γ-tubulin ring complex (γTuRCs) (Stearns and Kirschner, 1994; Zheng et al., 1995). Small and large complexes are present in most cells with only small

complexes in budding yeast. Supposedly, the γ-tubulins are organized in a ring which can interact either longitudinally (the template model) or laterally with αβ-tubulins (the protofilament model) (see Erickson and Stoffler, 1996; Erickson, 2000).

Anchoring and nucleation are considered two different processes. The apical region of epithelial cells (Mogensen et al., 1997, 2000) has anchoring domains but the molecules involved in nucleation such as γ-tubulin or perincentrin are lacking. Pericentrin is involved in the recruitment of γTuRCs to centrosomes in vertebrate cells (Dictenberg et al., 1998). Only two proteins have been isolated at this time in the anchoring sites: ninein and centriolin.

In recent years, the role of centrosomes has had to be reevaluated. They have been found not to be essential for spindle formation in mammalian cells (Hinchcliffe et al., 2001; Khodjakov et al, 2000) in experiments in which centrosomes were removed either microsurgically or by laser ablation. Nevertheless, when present they have a dominant function in MT assembly (Heald et al., 1997). Rieder et al. (2001) suggest that their role may be more important for whole organisms since stable cell lines can be established in the absence of centrosomes (Debec et al., 1995). However, in *Drosophila* a mutant lacking centrosomin (a core component of centrosomes in *Drosophila*) can develop into an adult fly (Vaizel-Ohayon and Schejter, 1999; Megraw et al., 2001), despite the absence of centrosomes, a defective formation of astral microtubules and cytoskeletal defects during embryonic development. In mammals, centrosomes are required for formation of astral MTs and for positioning the mitotic spindle (Khodjakov and Rieder, 2001), although apparently alternative mechanisms exist (e.g., Megraw et al. 2001).

Centrosomes have been found to have a role in the checkpoints of the cell cycle. In the absence of centrosomes, somatic cells are arrested in G1 (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). In *Drosophila* embryos, mutations in the DNA-replication checkpoint produce an inactivation of centrosomes in mitosis (Sibon et al., 2000) preventing chromosome segregation.

In addition to their role in checkpoints, centrosomes are thought to anchor regulators of cellular functions such as cAMP-dependent protein kinase A (PKA) via A-kinase anchoring proteins (AKAPs) (see Diviani and Scott, 2001; Feliciello et al., 2001).

The replication of the centrosomes takes place during the G1-S phase transition and is completed before the cells enter mitosis, a process requiring cdk2 (see (Hinchcliffe and Sluder, 2001). After replication each daughter cell contains one old and one new centriole. In humans and mice, the centrins of somatic cells are centrin 2 and 3. In HeLa cells, RNA interference (RNA_i) (see Chapter 1) blocks the synthesis of centrin-2, a calcium binding protein of centrioles, and the centrioles are unable to duplicate during the cell cycle (Salisbury et al., 2002) . In the absence of centrin-2 synthesis the pair of centrioles separate, and bipolar spindles have only one centriole at each spindle pole. The cells can undergo division, however, without centrioles they do not separate by cytokinesis in subsequent cell cycles, they are multinucleated and die.

Although there are abundant observations of the details of the duplication of centrosomes (see Doxsey, 2001), the molecular mechanisms are still unresolved. The duplication of centrosomes was found to depend on the Ca^{2+}-CAM-CAMKII system (see above)

L. Regulation of Translation

We have emphasized how cell division depends on transcriptional events and the degradation of both mRNAs and certain proteins. However, in order to be effective in promoting cell division, mitogens must also increase the activity of the translational machinery. Accordingly, the production of rRNA and 5S RNA is decreased during quiescent periods and increased in response to growth factors (Johnson et al., 1974). The control might involve the RB gene that, in addition to its control of cell division, also inhibits rRNA and 5S RNA production (Cavanaugh et al., 1995; White et al., 1996). In addition to the recognized role of the RB gene, the *ncl-1* gene of the nematode *Caenorhabditis elegans* controls rRNA and 5S RNA synthesis by acting as a repressor of the transcription of ribosomal and 5S RNA (Frank and Roth, 1998). The expression or repression of this gene is, therefore, involved in the regulation.

Part of the action of mitogens is to trigger signals that converge on the 70 kDa S6 kinase ($p70^{S6K}$) (see Chou and Blenis, 1995). When the ribosomal subunit S6 is phosphorylated by $p70^{S6K}$, there is an increased translation of mRNA (Jefferies et al., 1997). In particular, the translation of mRNAs involved in cell-cycle progression is increased. Inactivation of $p70^{S6K}$ with injection of the appropriate antibodies (Lane et al., 1993) into cells, or by treatment with rapamycin, causes G1 cell arrest in many cell types (Chou and Blenis, 1995).

The activation of $p70^{S6K}$ is sequential by phosphorylation at three specific sites to produce a fully active kinase (Pullen and Thomas, 1997). 3-phosphoinositide-dependent protein kinase 1 (PDK1) is thought to be responsible for these phosphorylations (Alessi et al., 1997; Pullen et al., 1998).

The activation of $p70^{S6K}$ is triggered by mitogens apparently acting through phosphatidylinositol 3-kinase, protein kinase B and C and phospholipase C (see Chou and Blenis, 1995).

IV. SUMMARY

The cell cycle proceeds in steps controlled by mechanisms that have been highly conserved. In this process, genes are activated in a specific order. In mammalian cells, some of the initial steps of G1 depend on the presence of growth factors. The regulation of cell division involves an interplay between the signals that block cell division and those that activate the steps of the cell cycle. The activating signals, the cyclins, are synthesized and then degraded at the steps they control. The cyclins act by forming a complex with other proteins, and this complex has protein kinase activity. The phosphorylation can either activate or inhibit transcription factors. Further control is provided by the need to complete a step (e.g., DNA synthesis) before the next step is initiated (e.g., mitosis).

SUGGESTED READING

Brehm, A. and Kouzarides, T. (1999) Retinoblastoma protein meets chromatin, *Trends Biochem. Sci.* 24:142-145. (Medline)

Dyson, N. (1998) The regulation of E2F by pRB-family proteins, *Genes Dev.* 12:2245-2262. (Medline)

Elledge, S.J. (1996) Cell cycle checkpoints: preventing an identity crisis, *Science* 274:1664-1672. (Medline)

Heichman, K.A. and Roberts, J.M. (1994) Rules to replicate by, *Cell* 79:557-562. (Medline)

Hunter, T. and Pines, J. (1994) Cyclins and cancer II: cyclin D and CDK inhibitors come of age, *Cell* 79:573-582. (Medline)

Johnston, L.H. (1992) Cell cycle control of gene expression in yeast, *Trends in Cell Biol.* 2: 353-357.

King, R.W., Jackson, P.K. and Kirschner, M.W. (1994) Mitosis in transition, *Cell* 79:563-571. (Medline)

Lees, M. (1995) Cyclin dependent kinase regulation, *Current Opin. Cell Biol.* 7:773-780.

Morgan, D.O. (1995) Principles of CDK regulation, *Nature* 374:131-134. (Medline)

Murray, A. and Hunt, T. (1993) *The Cell Cycle. An Introduction.* Freeman and Co., New York, Chapters 5-8.

Nasmyth, K. (1999) Separating sister chromatids, *Trends Biochem. Sci.* 24:98-104. (Medline)

Nicklas, R.B. (1997) How cells get the right chromosomes, *Science* 275:632-637. (Medline)

Nurse, P. (1994) Ordering S phase and M phase in the cell cycle, *Cell* 79:547-550. (Medline)

Pagano, M. (1997) Cell cycle regulation by the ubiquitin pathway, *FASEB J.* 11:1067-1075. (Medline)

Prives, C. (1993) Doing the right thing: feedback control and p53, *Curr. Opin. Cell Biol.* 5:214-218. (Medline)

Romanowski, P. and Madine, M.A. (1996) Mechanisms restricting DNA replication to once per cell cycle. MCMs pre-replicative complexes and kinases, *Trends in Cell Biol.* 6:184-188.

Scherr, C.J., (1994) G1 phase progression: cyclin on clue, *Cell* 79:551-555.

REFERENCES

Search the textbook

ieder

Back to Chapter 8

REFERENCES

Abrieu, A., Kahana, J.A., Wood, K.W. and Cleveland, D.W. (2000) CENP-E as an essential component of the mitotic checkpoint *in vitro*, *Cell* 102:817-826. (MedLine)

Agami, R., Blandino, G., Oren, M. and Shaul, Y. (1999) Interaction of c-Abl and p73α and their collaboration to induce apoptosis, *Nature* 399:809-813. (Medline)

Agarwal, M. L., Taylor, W. R., Chemov, M. V., Chemova, O. B. & Stark, G. R. (1998) The p53 network, *J. Biol. Chem.* 273:1-4, (MedLine)

Aitken, A. (1996) 14-3-3 and its possible role in co-ordinating multiple signalling pathways, *Trends in Cell Biol.* 6:341-347.

Alessi, D.R., Kozlowski, M.T., Weng, Q.-P., Morice, N. and Avruch, J. (1997) 3-phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 kinase *in vivo* and *in vitro*, *Curr. Biol.* 8:69-81. (Medline)

Amon, A. (1999) The spindle checkpoint, *Curr. Opin. Genet. Dev.* 9:69-75. (MedLine)

Andersen, S.S.L. (1999) Molecular characteristics of the centrosome, *Int. Rev. Cytol.*187:51-109. (MedLine)

Anderson, S.F., Schlegel, B.P., Nakajima, T., Wolpin, E.S. and Parvin, J.D. (1998) BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A, *Nature Genet.* 19:254-256. (Medline)

Andrews, B.J. and Herskowitz, I. (1989) The yeast SWI4 protein contains a motif present in developmental regulators and is part of a complex involved in cell-cycle-dependent transcription, *Nature* 342:830-833. (MedLine)

Assoian, R.K. (1997) Anchorage-depedent cell cycle progression, *J. Cell Biol.* 136:1-4. (Medline)

Baldin, V., Lukas, J., Marcorte, M.J., Pagano, M. and Draetta, G. (1993) Cyclin D1 is a nuclear protein required for cell cycle progression in G1, *Genes Dev.* 7:812-821. (Medline)

Banks, J.D. and Heald, R. (2001) Chromosome movement: dynein-out at the kinetochore, *Curr. Biol.* 11:R128-131. (MedLine)

Bates, S., Phillips, A.C., Clark, P.A., Stott, F., Peters, G. Ludwig, R.I. and Vousden, K.H. (1998) $p14^{ARF}$ links the tumour supressors RB and p53, *Nature* 395:124-125. (Medline)

Bell, D.W., Varley, J.M., Szydlo, T.E., Kang, D.H., Wahrer, D.C., Shannon, K.E., Lubratovich, M., Verselis, S.J., Isselbacher, K.J., Fraumeni, J.F., Birch, J.M., Li, F.P., Garber, J.E. and Haber, D.A. (1999) Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome, *Science* 286:2528-2531. (MedLine)

Benecke, B.-J., Ben-Ze'ev, A. and Penman, S. (1978) The control of mRNA production, translation and turnover in suspended and reattached anchorage depedent fibroblasts, *Cell* 14:931-939. (Medline)

Blondel, M. and Mann, C. (1996) G2 cyclins are required for the degradation of G1 cyclins in yeast, *Nature* 384:279-282. (Medline)

Blow, J.J. (1993) Preventing re-replication of DNA in a single cell cycle: evidence for a Replication Licensing Factor, *J. Cell Biol.* 122:993-1002. (Medline)

Blow, J.J. and Laskey, R.A. (1988) A role of the nuclear envelope in controlling DNA replication within the cell cycle, *Nature* 332:546-548. (Medline)

Blow, J.J. and Nurse, P. (1990) A cdc2-like protein involve din the initiation of DNA replication in *Xenopus* egg extracts, *Cell* 62:855-862. (Medline)

Bobinnec, Y., Moudjou, M., Fouquet, J.P., Desbruyeres, E., Edde, B. and Bornens, M. (1998) Glutamylation of centriole and cytoplasmic tubulin in proliferating non-neuronal cells, *Cell Motil. Cytoskeleton* 39:223-232. (MedLine)

Bochar, D.A., Wang, L., Beniya, H., Kinev, A., Xue, Y., Lane, W.S., Wang, W., Kashanchi F. and Shiekhattar R. (2000) BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer, *Cell* 102:257-265. (MedLine)

Böhmer, R.-M., Sharf, E. and Assoian, R.K. (1996) Cytoskeletal integrity is required throughout the mitogen stimulation phase of cell cycle and mediates the anchorage-dependent expression of cyclin D1, *Mol. Biol. Cell* 7:101-111. (Medline)

Bookstein, R., Shew, Y.-P., Chen, P.-L., Scully, P. and Lee, W.H. (1990) Suppression of tumorigenicity of human prostate carcinoma cells by replacing mutated Rb gene, *Science* 247:712-715. (Medline)

Bottazzi, M. E. and Assoian, R.K. (1997) The extracellular matrix and mitogenic growth factors control of cyclin-dependent kinase inhibitors, *Trends Cell Biol.* 7:348-352.

Brehm, A. and Kouzarides, T. (1999) Retinoblastoma protein meets chromatin, *Trends Biochem. Sci.* 24:142-145. (MedLine)

Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J. and Kouzarides, T. (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription, *Nature* 391:597-601. (Medline)

Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T. and Hannon, G.J. (1995) Radiation-induced cell cycle arrest compromised by p21 deficiency, *Nature* 377:552-557. (Medline)

Buchkovich, K., Duffy, L.A. and Harlow, E. (1989) The retinoblastoma protein is phosphorylated during specific phases of the cell cycle, *Cell* 58:1097-1105. (MedLine)

Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B. and Siliciano, J.D. (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53, *Science* 281:1677-1679. (Medline)

Carrano, A.C., Eytan, E., Hershko, A. and Pagano, M. (1999) SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27, *Nature Cell Biol.* 1:193-199. (Medline)

Cavanaugh, A.H., Hempel, W.M., Taylor, L.J., Rogalsky, V., Todorov, G. and Rothblum, L.I. (1995) Activity of RNA polymerase I transcription factor UBF blocked by *Rb* gene product, *Nature* 374:177-180. (Medline)

Celli, J., Duijf, P., Hamel, B.C., Bamshad, M., Kramer, B., Smits, A.P., Newbury-Ecob, R., Hennekam, R.C., Van Buggenhout, G., van Haeringen, A., Woods, C.G., van Essen, A.J., de Waal, R., Vriend, G., Haber, D.A., Yang, A., McKeon, F., Brunner, H.G. and van Bokhoven, H. (1999) Heterozygous germline mutations in the p53 homolog p63 are the cause of EEC syndrome, *Cell* 99:143-153. (MedLine)

Chan, G.K., Schaar, B.T. and Yen, T.J. (1998) Characterization of the kinetochore binding domain of CENP-E reveals interactions with the kinetochore proteins CENP-F and hBUBR1, *J. Cell Biol.* 143:49-63. (Medline)

Chan, G.K., Jablonski, S.A., Sudakin, V., Hittle, J.C. and Yen, T.J. (1999a) Human BUBR1 is a mitotic checkpoint kinase that monitors CENP-E functions and kinetochores and binds the cyclosome/APC, *J.*

Cell Biol. 146:941-954. (Medline)

Chan, T.A., Hermeking, H., Lengauer, C., Kinzler, K.W. and Vogelstein, B. (1999b) 14-3-3σ is required to prevent mitotic catastrophe after DNA damage, *Nature* 401:616-620. (Medline)

Chan, G.K., Jablonski, S.A., Starr, D.A., Goldberg, M..L. and Yen, T.J. (2000) Human Zw10 and ROD are mitotic checkpoint proteins that bind to kinetochores, *Nature Cell Biol.* 2:944-947. (MedLine)

Chehab, N.H., Malikzay, A., Appel, M. and Halazonetis, T.D. (2000) Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53, *Genes Dev.* 14:278-288. (MedLine)

Chen, X. (1999) The p53 family: same response, different signals? *Mol. Med. Today.* 5:387-392. (MedLine)

Cheng, M., Olivier, P., Diehl, J.A., Fero, M., Roussel, M.F., Roberts, J.M. and Sherr, C.J. (1999) The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts, *EMBO J.* 18:1571-1583. (Medline)

Chin, L., Artandi, S.E., Shen, Q., Tam, A., Lee, S.L., Gottlieb, G.J., Greider, C.W. and DePinho, R.A. (1999) p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis, *Cell* 97:527-538. (Medline)

Chong, J.P.J., Mahbubani, M.H., Khoo, C.-Y. and Blow, J.J. (1995) Purification of an Mcm-containing complex as a component of the DNA replication licensing system, *Nature* 375:418-421. (Medline)

Chong, J.P.J., Thömes, and Blow, J.J. (1996) The role of MCM/P1 proteins in the licensing of DNA replication, *Trends in Biochem. Sci.* 21:102-106. (Medline)

Chou, M.M. and Blenis, J. (1995) The 70 kDa S6 kinase: regulation of a kinase with multiple roles in mitogenic signalling, *Curr. Opin. Cell Biol.* 7:806-914. (Medline)

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M. and Nasmyth, K. (1998) An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast, *Cell* 93:1067-1076. (Medline)

Cobrinik, D., Lee,M.H., Hannon, G. Mulligan, G., Bronson,R.T., Dyson,N., Harlow, E., Beach, D., Weinberg, R.A. and Jacks, T. (1996) Shared role of the pRB-related p130 and p107 proteins in limb development, *Genes Dev.*, 10:1633-1644. (MedLine)

Cohen-Fix, O., Peters, J.M., Kirschner, M.W. and Koshland, D. (1996) Anaphase initiation in

Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p, *Genes Dev.* 10:3081-3093. (Medline)

Clurman, B.E., Sheaff, R.J., Thress, K., Groudine, M. and Roberts, J.M. (1996) Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation, *Genes Dev.* 10:1979-1990. (Medline)

Cortez, D., Wang, Y., Qin, J. and Elledge, S.J. (1999) Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks, *Science* 286:1162-1166. (Medline)

Craig, K.L. and Tyers, M. (1999) The F-box: a new motif for ubiquitin dependent proteolysis in cell cycle regulation and signal transduction, *Prog. Biophys. Mol. Biol.* 72:299-328. (Medline)

Dahmann, C., Diffley, J.F.X. and Nasmyth, K.A. (1995) S-phase promoting cyclin-dependent kinases prevent re-replication by inhibiting transition of replication origins to a pre-replicative state, *Curr. Biol.* 5:1257-1269. (Medline)

Debec, A., Detraves, C., Montmory, C., Geraud, G. and Wright, M. (1995) Polar organization of γ-tubulin in acentriolar mitotic spindles of *Drosophila melanogaster* cells, *J. Cell Sci.*108:2645-2653. (MedLine)

Deshaies, R.J. (1995) The self-destructive personality of cell cycle in transition, *Curr. Opin. Cell Biol.* 6:781-789. (Medline)

Deshaies, R.J., Chau, V. and Kirschner, M. (1995) Ubiquination of the G1 cyclin cln2p by Cdc34-dependent pathway, *EMBO J.*14:303-312. (Medline)

Di Leonardo, A., Linke, S.P., Clarkin, K. and Wahl, G.M. (1994) DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts, *Genes Dev.* 8:2540-2551. (MedLine)

Dictenberg, J.B., Zimmerman, W., Sparks, C.A., Young, A., Vidair, C., Zheng, Y., Carrington, W., Fay, F.S. and Doxsey, S.J. (1998)Pericentrin and γ-tubulin form a protein complex and are organized into a novel lattice at the centrosome, *J. Cell Biol.*141:163-174. (MedLine)

Dirick, L., Moll, T., Auer, H. and Nasmyth, K. (1992) A central role for SWI6 in modulating cell cycle Start-specific transcription in yeast, *Nature* 357:508-513. (MedLine)

Diviani, D. and Scott, J.D. (2001) AKAP signaling complexes at the cytoskeleton, *J. Cell Sci.*114:1431-1437. (MedLine)

Donovan, J.D., Toyn, J.H., Johnson, A.L. and Johnston, L.H. (1994) P40SDB25, a putative CDK inhibitor, has a role in the M/G1 transition in *Saccharomyces cerevisiae*, *Genes Dev.* 8:1640-1653. (Medline)

Doxsey, S. (2001) Re-evaluating centrosome function. *Nature Rev. Mol. Cell Biol.* 2:688-698. (MedLine)

Dutta, A. and Bell, S.P. (1997) Initiation of DNA replication in eukaryotic cells, *Annu. Rev. Cell Dev. Biol.* 13:293-332. (MedLine)

Dyson, N. (1998) The regulation of E2F by pRB-family proteins, *Genes Dev.* 12:2245-2262. (MedLine)

Elledge, S.J. (1996) Cell cycle checkpoints: preventing an identity crisis, *Science* 274:1664-1672. (Medline)

Erickson, H.P. (2000) γ-tubulin nucleation: template or protofilament? *Nature Cell Biol.* 2:E93-E96. (MedLine)

"Erickson, H.P. and Stoffler, D. (1996) Protofilaments and rings, two conformations of the tubulin family conserved from bacterial FtsZ to α/β and γ tubulin, *J. Cell Biol.* 135:5-8. (MedLine)

Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D. and Hunt, T. (1983) Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division, *Cell* 33:389-396. (Medline)

Evans, L., Mitchison, T. and Kirschner, M. (1985) Influence of the centrosome on the structure of nucleated microtubules, *J. Cell Biol.* 100:1185-1191. (MedLine)

Ezhevsky, S.A., Nagahara, H., Vocero-Akbani, A.M., Gius, D.R., Wei, M.C. and Dowdy, S.F. (1997) Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D:Cdk4/6 complexes results in active pRb, *Proc. Natl. Acad. Sci. USA* 94:10699-10704. (Medline)

Fang, G., Yu, H. and Kirschner, M.W. (1998a) Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1, *Mol. Cell.* 2:163-171. (Medline)

Fang, G., Yu, H. and Kirschner, M.W. (1998b) The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation, *Genes Dev.* 12:1871-1883. (Medline)

Fantl, V., Stamp, G., Andrews, A., Rosewell, I. and Dickson, C. (1995) Mice lacking cyclin D1 are small and show defects in eye and mammary gland development, *Genes Dev.* 9:2364-2372. (Medline)

Feldman, R.M., Correll, C.C., Kaplan, K.B. and Deshaies, R.J. (1997) A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p, *Cell* 91:221-230. (Medline)

Feliciello, A., Gottesman, M.E. and Avvedimento, E.V. (2001) The biological functions of A-kinase anchor proteins, *J. Mol. Biol.* 308(2):99-114. (MedLine)

Field, C., Li, R. and Oegema, K. (1999) Cytokinesis in eukaryotes: a mechanistic comparison, *Curr. Opin. Cell Biol.* 11:68-80. (MedLine)

Forsburg, S.L. and Nurse, P. (1991) Cell cycle regulation in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, *Ann. Rev. Cell Biol.* 7:227-256. (Medline)

Frank, D.J. and Roth, M.B. (1998) *ncl-1* is required for the regulation of cell synthesis in *Caenorhabditis elegans*, *J. Cell Biol.* 140:1321-1329. (Medline)

Garkavtsev, I., Grigorian, I.A., Ossovskaya, V.S., Chernov, M.V., Chumakov, P.M. and Gudkov, A.V. (1998) The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control, *Nature* 391:295-298. (MedLine)

Geng. Y., Whoriskey, W., Park, M.Y., Bronson, R.T., Medema, R.H., Li, T., Weinberg, R.A. and Sicinski, P. (1999) Rescue of cyclin D1 deficiency by knockin cyclin E, *Cell* 97:767-777. (Medline)

Giaccia, A.J. and Kastan, M.B. (1998) The complexity of p53 modulation: emerging patterns from divergent signals, *Genes Dev.* 12:2973-2983. (MedLine)

Ginsberg, D., Mechta, F., Yaniv, M. and Oren, M. (1991) Wild-type p53 can down-modulate the activity of various promoters, *Proc. Natl. Acad. Sci.USA* 88:9979-9983. (Medline)

Girard, F., Strausfeld, U., Fernandez, A. and Lamb, N.J.C. (1991) Cyclin A is required for the onset of DNA replication in mammalian fibroblasts, *Cell* 67: 1169-1179. (Medline)

Glover, D.M., Ohkura, H. and Tavares, A. (1996) Polo kinase: the choreographer of the mitotic stage? *J. Cell Biol.* 135:1681-1684. (Medline)

Glover, D.M., Hagan, I.M. and Tavares, A.A. (1998) Polo-like kinases: a team that plays throughout mitosis, *Genes Dev.* 12:3777-3787. (MedLine)

Goebl, M.G., Yochem, J., Jentsch, S., McGrath, J.P., Varshavsky, A. and Byers, B. (1988) The yeast cell cycle gene CDC34 encodes a ubiquitin-conjugating enzyme, *Science* 241:1331-1335. (Medline)

Golsteyn, R., Mundt, K., Fry, A., and Nigg, E. (1995) Cell cycle regulation of the activity and subcellular localization of PLK1, a human protein kinase implicated in mitotic spindle function, *J. Cell Biol.* 129:1617-1628. (Medline)

Gong, J.G., Costanzo, A., Yang, H.-Q., Melino, G., Kaelin, W.G. Jr., Levrero, M. and Wang, J.Y.J. (1999) The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage, *Nature* 399:806-809. (Medline)

Goodrich, D.W., Wang, N.P., Qian, Y.W., Lee E.Y.-H.P. and Lee W.H. (1991) The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle, *Cell* 67:293-302. (Medline)

Gorbsky, G.J. and Ricketts, W.A. (1993) Differential expression of a phosphoepitope at the kinetochores of moving chromosomes, *J. Cell Biol.* 122:1311-1321. (Medline)

Gorbsky, G.J., Chen, R.H. and Murray, A.W. (1998) Microinjection of antibody to Mad2 protein into mammalian cells in mitosis induces premature anaphase, *J. Cell Biol.* 141:1193-1205. (Medline)

Graeber, T.G., Osmanian, C., Jack, T., Housman, D.E., Koch, C.J., Lowe, S.W. and Giacca, A.J. (1996) Hypoxia-mediated selection of cells with diminished apoptotic potentials in solid tumours, *Nature* 379:88-91. (Medline)

Guacci, V., Koshland, D. and Strunnikov, A. (1997) A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in *S. cerevisiae*, *Cell* 91:47-57. (Medline)

Hagting, A., Karlsson, C., Clute, P., Jackman, M. and Pines, J. (1998) MPF localization is controlled by nuclear export, *EMBO J.* 17:4127-4138. (Medline)

Hanahan, D. and Weinberg, R.A. (2000) The hallmarks of cancer, *Cell* 100:57-70. (MedLine)

Hardwick, K.G. (1998) The spindle checkpoint, *Trends Genet.* 14:1-4. (Medline)

Hardwick, K.G. and Murray, A.W. (1995) Mad1p, a phosphoprotein component of the spindle assembly checkpoint in budding yeast, *J. Cell Biol.* 131:709-720. (Medline)

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. (1993) The p21 CDK-interacting protein Cipl is a potent inhibitor of G1 cyclin-dependent kinases, *Cell* 75:805-816. (Medline)

Hartwell, L.H. and Kastan (1994) Cell cycle control and cancer, *Science* 266:1821-1828. (Medline)

Hartwell, L.H., Mortimer, R.K., Culotti, J. and Culotti M. (1973) Genetic control of the cell division in cycle in yeast V. genetic analysis of cdc mutants, *Genetics* 74:267-286.

He, X., Patterson, T.E. and Sazer, S. (1997) The *Schizosaccharomyces pombe* spindle checkpoint protein mad2p blocks anaphase and genetically interacts with the anaphase-promoting complex, *Proc. Natl. Acad. Sci. USA.* 94:7965-7970. (Medline)

Heald, R., Tournebize, R., Habermann, A., Karsenti, E. and Hyman, A. (1997) Spindle assembly in *Xenopus* egg extracts: respective roles of centrosomes and microtubule self-organization *J. Cell Biol.*138:615-628. (MedLine)

Hennessy, K.M., Clark, C.D. and Botstein, D. (1990) Subcellular localization of yeast CDC46 varies with the cell cycle, *Genes Dev.* 4:2252-2263. (Medline)

Hermeking, H., Lengauer, C., Polyak, K., He, T.C., Zhang, L., Thiagalingam, S., Kinzler, K.W. and Vogelstein, B. (1997) 14-3-3 σ is a p53-regulated inhibitor of G2/M progression, *Mol. Cell* 1:3-11. (Medline)

Hershko, A., Ganoth, D., Sudakin, V., Dahan, A., Cohen, L.H., Luca, F.C., Ruderman, J.V. and Eytan, E. (1994) Components of a system that ligates cyclin to ubiquitin and their regulation by the protein kinase cdc2, *J. Biol. Chem.* 269:4940-4946. (Medline)

Hinchcliffe, E.H. and Sluder, G. (2001) "It takes two to tango": understanding how centrosome duplication is regulated throughout the cell cycle, *Genes Dev.* 15:1167-1181. (MedLine)

Hinchcliffe, E.H., Thompson, E.A., Maller, J.L. and Sluder G. (1999) Requirement of Cdk-2-cyclin E activity for repeated centrosome reproduction in *Xenopus* egg extracts, *Science* 283:851-854. (Medline)

Hinchcliffe, E.H., Miller, F.J., Cham, M., Khodjakov, A. and Sluder, G. (2001) Requirement of a centrosomal activity for cell cycle progression through G1 into S phase, *Science* 291:1547-1550. (MedLine)

Hirao, A., Kong, Y.-Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S.J. and Mak, T.W. (2000) DNA damage-induced activation of p53 by the checkpoint kinase chk2, *Science* 287:1824-1827. (MedLine)

Hochstrasser, M. (1996), Ubiquitin-dependent protein degradation, *Annu. Rev. Genet.* 30:405-439. (Medline)

Hollingsworth, R.E. Jr., Chen, P-L. and Lee, W.-H. (1993) Integration of cell cycle control with

transcriptional regulation by the retinoblastoma protein, *Curr. Opin. Cell Biol.* 5:194-200. (Medline)

Honda, R. and Yasuda, H. (1999) Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53, *EMBO J.* 18:22-27. (MedLine)

Howell, B.J., Hoffman, D.B., Fang, G., Murray, A.W. and Salmon, E.D. (2000) Visualization of Mad2 Dynamics at Kinetochores, along Spindle Fibers, and at Spindle Poles in Living Cells, *J. Cell Biol.* 150:1233-1250. (MedLine)

Hoyt, M.A., Totis, L. and Roberts, B.T. (1991) *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function, *Cell* 66:507-517 (Medline)

Hua, X.H., Yan, H. and Newport, J. (1997) A role of Cdk3 kinase in negatively regulating DNA replication during S phase of the cell cycle, *J. Cell Biol.* 137:183-192. (Medline)

Huang, L.C., Clarkin, K.C. and Wahl, G.M. (1996) p53-dependent cell cycle arrests are preserved in DNA-activated protein kinase-deficient mouse fibroblasts, *Cancer Res.* 56:2940-2944. (MedLine)

Hurford, R.K. Jr, Cobrinik, D., Lee, M.H. and Dyson, N., (1997) pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes, *Genes Dev.* 11, 1447-1463. (MedLine)

Irniger, S., Piatti, S., Michaelis, C. and Nasmyth, K. (1995) Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast, *Cell* 81:269-278. (Medline)

Ishimi, Y. (1997) A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex, *J. Biol. Chem.* 272:24508-24513. (MedLine)

Iyer, VR, Horak, C.E., Scafe, C.S., Botstein, D., Snyder, M. and Brown, P.O. (2001) Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF, *Nature* 409:533-538.

Jablonski, S.A., Chan, G.K., Cooke, C.A., Earnshaw, W.C. and Yen, T.J. (1998) The hBUB1 and hBUBR1 kinases sequentially assemble onto kinetochores during prophase with hBUBR1 concentrating at the kinetochore plates in mitosis, *Chromosoma* 107:386-396. (Medline)

Jaspersen, S.L., Charles, J.F. and Morgan, D.O. (1999) Inhibitory phosphorylation of the APC regulator Hct1 is controlled by the kinase Cdc28 and the phosphatase Cdc14, *Curr. Biol.* 9:227-236. (Medline)

Jefferies, H.B.J., Fumagalli, S., Dennis, P.B., Reinhard, C., Pearson, R.B. and Thomas, G. (1997) Rapamycin supresses 5' TOP mRNA translation through inhibition of $p70^{S6K}$, *EMBO J.* 16:3693-3704. (Medline)

Johnson, L.F., Abelson H.T., Green, H. and Penam, S. (1974)Changes in RNA in relation to growth of the fibroblast. I. Amounts of mRNA, rRNA and tRNA in resting and growing cells, *Cell* 1:95-100.

Kaelin, W.G. Jr. (1999) The emerging p53 gene family, *J. Natl. Cancer. Inst.* 91:594-598. (Medline)

Kaiser, P., Flick, K., Wittenberg, C. and Reed, S.I. (2000) Regulation of transcription by ubiquitination without proteolysis: Cdc34/SCF^{Met30}-mediated inactivation of the transcription factor Met4, *Cell* 102:303-314. (MedLine)

Kalderon, D. (1996) Protein degradation, de-ubiquinate to decide your fate, *Curr. Biol.* 6:662-665. (Medline)

Kallio, M., Weinstein, J., Daum, J.R., Burke, D.J. and Gorbsky, G.J. (1998) Mammalian p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events, *J. Cell Biol.* 141:1393-1406. (Medline)

Kamb, A. (1995) Cell-cycle regulators and cancer, *Trends Genet.* 11:136-140. (Medline)

Kamijo, T., Zindy, F., Roussel, M.F., Quelle, D.E., Downing J.R., Ashmun, R.A., Grosveld, G., Sherr, C.J. (1997) Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF, *Cell* 91:649-659. (Medline)

Karlseder, J., Broccoli, D., Dai, Y., Hardy, S. and de Lange, T. (1999) p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2, *Science* 283:1321-1325. (Medline)

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B. and Craig, R.W. (1991) Participation of p53 protein in the cellular response to DNA damage, *Cancer Res.* 51:6304-6311. (Medline)

Kearsey, S.E., Maiorano, D., Holmes, E.C., and Todorov, I.T. (1996) The role of MCM proteins in the cell cycle control of genome duplication, *Bioessays* 18:183-190. (Medline)

Kelly, T.J. and Brown, G.W. (2000) Regulation of chromosome replication, *Annu. Rev. Biochem.* 69:829-880. (MedLine)

Kharbanda, S., Ren, R., Pandey, P., Shafman, T.D., Feller, S.M., Weichselbaum, R.R. and Kufe, D.W. (1995) Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents, *Nature* 376:785-788. (Medline)

Khodjakov, A. and Rieder, C.L. (2001) Centrosomes enhance the fidelity of cytokinesis in vertebrates and

are required for cell cycle progression, *J. Cell Biol.* 153:237-242. (MedLine)

Khodjakov, A., Cole, R.W., Oakley, B.R. and Rieder, C.L. (2000) Centrosome-independent mitotic spindle formation in vertebrates, *Curr. Biol.* 10:59-67. (MedLine)

Khosravi, R., Maya, R., Gottlieb, T., Oren, M., Shiloh, Y. and Shkedy, D. (1999) Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage, *Proc. Natl. Acad. Sci. USA.* 96:14973-14977. (MedLine)

Kim, S.H., Lin, D.P., Matsumoto, S., Kitazono, A. and Matsumoto, T. (1998) Fission yeast Slp1: an effector of the Mad2-dependent spindle checkpoint, *Science* 279:1045-1047. (Medline)

Kimura, H., Nozaki, N., Sugimoto, K. (1994) DNA polymerase alpha associated protein P1 a murine analog of yeast MCM3 changes its intranuclear distribution during DNA synthetic period, *EMBO J.* 13:4311-4320. (Medline)

King, R.W., Jackson, P.K. and Kirschner, M.W. (1994) Mitosis in transition, *Cell* 79:563-571. (Medline)

King, R.W., Peters, J.M., Tugendreich, S., Rolfe, M., Hieter, P. and Kirschner, M.W. (1995) A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B, *Cell* 81:279-288. (Medline)

King, R.W., Deshaies, R.J., Peters, J.-M. and Kischner, M.W. (1996) How proteolysis drives the cell cycle, *Science* 274:1652-1659. (Medline)

Kinzler, K.W. and Vogelstein, B. (1997) Cancer-susceptibility genes. Gatekeepers and caretakers, *Nature* 386:761, 763. (Medline)

Kleiman, F.E. and Manley, J.L. (1999) Functional interaction of BRCA1-associated BARD1 with polyadenylation factor CstF-50, *Science* 285:1576-1579. (Medline)

Koch, C. and Nasmyth, K. (1994) Cell cycle regulated transcription in yeast, *Curr. Opin. Cell Biol.* 6:451-459. (MedLine)

Koch, C., Moll, T., Neuberg M., Ahorn, H. and Nasmyth, K. (1993) A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase, *Science* 261:1551-1557. (MedLine)

Koegl, M., Hoppe, T., Schenkler, S., Ultich, H.D., Mayer, T.U. and Jentsch, S. (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly *Cell* 96:635-644. (Medline)

Koepp, D.M., Harper, J.W. and Elledge, S.J. (1999) How the cyclin became a cyclin: regulated proteolysis in the cell cycle, *Cell* 97:431-434. (Medline)

Koff, A., Cross, F., Fisher, A., Schumacher, J., Leguellee, K., Philippe, M. and Roberts, J.M. (1991) Human cyclin E, a new cyclin that interacts with two members of the *CDC2* gene family, *Cell* 66:1217-1228. (Medline)

Kubota, Y., Minura, S., Nishimoto, S., Takisawa, H. and Nojima, H. (1995) Identification of the yeast MCM3-related protein as a component of *Xenopus* DNA Replication Licensing Factor, *Cell* 81:601-609. (Medline)

Kumada, K., Nakamura, T., Nagao, K., Funabiki, H., Nakagawa, T. and Yanagida, M. (1998) Cut1 is loaded onto the spindle by binding to Cut2 and promotes anaphase spindle movement upon Cut2 proteolysis, *Curr. Biol.* 8:633-641. (Medline)

LaBaer, J., Garrett, M.D., Stevenson, L.F., Slingerland, J.M., Sandhu, C., Chou, H.S., Fattaey, A. and Harlow, E. (1997) New functional activities for the p21 family of CDK inhibitors, *Genes Dev.* 11:847-862. (Medline)

Labib, K., Diffley, J.F. and Kearsey, S.E. (1999) G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus, *Nature Cell Biol.* 1:415-422. (Medline)

Lacey, K.R., Jackson, P.K. and Stearns, T. (1999) Cyclin-dependent kinase control of centrosome duplication, *Proc. Natl. Acad. Sci. USA* 96:2817-2822. (Medline)

Lakin, N.D. and Jackson, S.P. (1999) Regulation of p53 in response to DNA damage, *Oncogene* 18:7644-7655. (MedLine)

Lakin, N.D., Hann, B.C. and Jackson, S.P. (1999) The ataxia-telangiectasia related protein ATR mediates DNA-dependent phosphorylation of p53, *Oncogene* 18:3989-3995. (Medline)

Lane, H. and Nigg, E. (1996) Antibody microinjection reveals and essential role for human polo-like kinase 1 (Plk1) in the functional maturation of functional centrosomes, *J. Cell Biol.* 135:1701-1713. (Medline)

Lane, H.A., Fernandez, A., Lamb, N.J.C. and Thomas, G. (1993) $p70^{S6K}$ function is essential for G1 progression, *Nature* 363:170-172. (Medline)

Lanker, S., Valdivieso, M.H. and Wittenberg, C. (1996) Rapid degradation of the G1 cyclin Cln2 is induced by CDK-dependent phosphorylation, *Science* 271:1597-1601. (Medline)

Larsen, C.J. (1996) $p16^{INK4a}$: a gene with a dual capacity to encode unrelated proteins that inhibit cell cycle progression, *Oncogene* 12:2041-2044. (Medline)

Lee, T.H. and Kirschner, M.W. (1996) An inhibitor of $p34^{cdc2}$/cyclin B that regulates G2/M transition in *Xenopus* extracts, *Proc. Natl. Acad. Sci. USA* 93:352-356. (Medline)

Lees (1995) Cyclin dependent kinase regulation, *Curr. Opin. Cell Biol.* 7:773-780. (Medline)

Levine, A.J. (1997) p53, the cellular gatekeeper for growth and division, *Cell* 88:323-331. (Medline)

Lew, D.J., Duli, V. and Reed, S.I., (1991) Isolation of three human cyclins by rescue of G1 cyclin (Cln) function in yeast, *Cell* 66:1197-1206. (Medline)

Lew, D.J. and Reed, S.I. (1992) A proliferation of cyclins, *Trends Cell Biol.* 2: 77-80.

Li, R. and Murray, A.W. (1991) Feedback control of mitosis in budding yeast, *Cell* 66:519-531. (Medline)

Li, X. and Nicklas, R.B. (1995) Mitotic forces control a cell-cycle checkpoint, *Nature* 373:630-632. (Medline)

Li, X. and Nicklas, R.B. (1997) Tension-sensitive kinetochore phosphorylation and the chromosome distribution checkpoint in praying mantid spermatocytes, *J. Cell Sci.*110:537-545. (Medline)

Li, Y., Gorbea, C., Mahaffey, D., Rechsteiner, M. and Benezra, R. (1997) MAD2 associates with the cyclosome/anaphase-promoting complex and inhibits its activity, *Proc. Natl. Acad. Sci. USA* 94:12431-12436. (Medline)

Liu, Q., Guntuku, S., Cui, X.-S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., Donehower, L.A. and Elledge, S.J. (2000) Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint, *Genes Dev.* 14:1448-1459. (MedLine)

Lohka, M.J., Hayes, M.K. and Maller, J.L. (1988) Purification of maturation-promoting factor, an intracellular regulator of early mitotic events, *Proc. Natl. Acad. Scie. USA*, 85: 3009-3013. (Medline)

Lopez-Girona, A., Furnari, B., Mondesert, O. and Russell, P. (1999) Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein, *Nature* 397:172-175. (Medline)

Losada, A., Hirano, M. and Hirano, T. (1998) Identification of Xenopus SMC protein complexes required

for sister chromatid cohesion, *Genes Dev.* 12:1986-1997. (Medline)

Lowe, S.W., Schmitt, E.M., Smith, S.W.. Osborne, B.A. and Jacks, T. (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes, *Nature* 362:847-849. (Medline)

Lu, K.P. and Means, A.R. (1993) Regulation of the cell cycle by calcium and calmodulin, *Endocr. Rev.* 14:40-58. (MedLine)

Ludlow, J.W., Glendening, C.L., Livingston, D.M. and DeCaprio, J.A. (1993) Specific enzymatic dephosphorylation of the retinoblastoma protein, *Mol. Cell. Biol.* 13:367-372. (Medline)

Lukas, J., Müller, H., Bartkova, J., Spitkovsky, D., Kjerulff, A.A., Jansen-Dürr, Strauss, M. and Bartek, J. (1994) DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G1, *J. Cell Biol.* 125:625-638. (Medline)

Lundberg, A.S. and Weinberg, R.A. (1998) Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes, *Mol. Cell. Biol.* 18:753-761. (Medline)

Luo, R.X., Postigo, A.A. and Dean, D.C. (1998) Rb interacts with histone deacetylase to repress transcription, *Cell* 92:463-473. (Medline)

Madine, M.A., Khoo, C-Y., Mills, A.D., Musahl, C. and Laskey, R.A. (1995) The nuclear envelope prevents reinitiation of replication by regulating the binding of MCM3 to chromatin in *Xenopus* egg extracts, *Curr. Biol.* 5:1270-1279. (Medline)

Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le Villain, J.P., Troalen, F., Trouche, D., Harel-Bellan, A. (1998) Retinoblastoma protein represses transcription by recruiting a histone deacetylase, *Nature* 391:601-605. (Medline)

Mahbubani, H.M., Chong, J.P.J., Chevalier, S., Thömmes, P. and Blow, J.J. (1997) cell cycle regulation of the replication licensing system: involvement of a Cdk-dependent inhibitor, *J. Cell Biol.* 136:125-135. (Medline)

Mailand, N., Falck, J., Lukas, C., Syljuasen, R.G., Welcker, M., Bartek, J. and Lukas, J. (2000) Rapid destruction of human Cdc25A in response to DNA damage, *Science* 288:1425-1429. (MedLine)

Maridor, G., Gallant, P., Golsteyn, R. and Nigg, E.A. (1993) Nuclear localization of vertebrate cyclin A correlates with its ability to form complexes with cdk catalytic subunits, *J. Cell Sci.* 106:535-544. (Medline)

Massague, J. (1996) TGFβ signaling: receptors, transducers, and Mad proteins, *Cell* 85:947-950. (Medline)

Masui, Y. and Markert, C.L. (1971) Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes, *J. Exp. Zool.* 177:129-146 (Medline)

Matsumoto, Y. and Maller, J.L. (2002) Calcium, calmodulin, and CaMKII requirement for initiation of centrosome duplication in *Xenopus* egg extracts, *Science* 295:499-502. (MedLine)

Matsumoto, Y., Hayashi, K. and Nishida, E. (1999) Cyclin-dependent kinase 2 (Cdk2) is required for centrosome duplication in mammalian cells, *Curr Biol.* 9:429-432. (Medline)

McConnell, B,B., Starborg, M., Brookes, S. and Peters, G. (1998) Inhibitors of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts, *Curr. Biol.* 8:351-354. (Medline)

Means, A.R. (1994) Calcium, calmodulin and cell cycle regulation, *FEBS Lett.* 347:1-4. (MedLine)

Means, A.R. (2000) Regulatory cascades involving calmodulin-dependent protein kinases, *Mol. Endocrinol.* 14:4-13. (MedLine)

Means, A. R., Kahl, C. R., Crenshaw, D. G., and Dayton, J. S. (1999) Traversing the cell cycle :the calcium/calmodulin connection, in *Calcium as a Cellular Regulator* (Carafoli, E. , and Klee, C., eds), Oxford University Press, New York, pp. 512-528.

Megraw, T.L., Kao, L.R. and Kaufman, T.C. (2001) Zygotic development without functional mitotic centrosomes, *Curr. Biol.* 11:116-120. (MedLine)

Michaelis, C., Ciosk, R. and Nasmyth, K. (1997) Cohesins: chromosomal proteins that prevent premature separation of sister chromatids, *Cell* 91:35-45. (Medline)

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W., et al. (1994) A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1, *Science* 266:66-71. (MedLine)

Mills, A.A., Zheng, B., Wang, X.J., Vogel, H., Roop, D.R. and Bradley, A. (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis, *Nature* 398:708-713. (MedLine)

Milner, J., (1984) Different forms of p53 detected by monoclonal antibodies in non-dividing and dividing cells, *Nature* 310: 143-145.

Monteiro, A.N., August, A. and Hanafusa, H. (1996) . Evidence for a transcriptional activation function of BRCA1 C-terminal region, *Proc. Natl. Acad. Sci. USA.* 93:13595-13599. (MedLine)

Mogensen, M.M., Mackie, J.B., Doxsey, S.J., Stearns, T. and Tucker, J.B. (1997) Centrosomal deployment of γ-tubulin and pericentrin: evidence for a microtubule-nucleating domain and a minus-end docking domain in certain mouse epithelial cells, *Cell Motil. Cytoskeleton* 36:276-290. (MedLine)

Mogensen, M.M., Malik, A., Piel, M., Bouckson-Castaing, V. and Bornens, M. (2000) Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein, *J. Cell Sci.* 113:3013-3023. (MedLine)

Morgan, D.O. (1999) Regulation of the APC and the exit from mitosis, *Nature Cell Biol.* 1:E47-53. (Medline)

Morgan, S.E. and Kastan, M.B. (1997) p53 and ATM: cell cycle, cell death, and cancer, *Adv. Cancer Res.* 71:1-25. (Medline)

Moore, J.D., Yang, J., Truant, R. and Kornbluth, S. (1999) Nuclear import of Cdk/Cyclin complexes: identification of distinct mechanisms for import of Cdk2/Cyclin E and Cdc2/Cyclin B1, *J. Cell Biol.* 144:213-224. (Medline)

Morgan, D.O. (1995) Principles of CDK regulation, *Nature* 374:131-134. (Medline)

Morgan, D.O. (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors, *Annu. Rev. Cell Dev. Biol.* 13:261-291. (Medline)

Morozov, V.E., Falzon, M., Anderson, C.W. and Kuff, E.L. (1994) DNA-dependent protein kinase is activated by nicks and larger single-stranded gaps, *J. Biol. Chem.* 269:16684-16688. (Medline)

Moynahan, M.E., Chiu, J.W., Koller, B.H. and Jasin, M. (1999) Brca1 controls homology-directed DNA repair, *Mol. Cell* 4:511-518. (Medline)

Mundt, K.E., Golsteyn, R.M., Lane, H.A. and Nigg, E.A (1997) On the regulation and function of human polo-like kinase 1 (PLK1): effects of overexpression on cell cycle progression, *Biochem. Biophys. Res. Commun.* 239:377-385. (MedLine)

Muslin, A.J., Tanner, J.W., Allen, P.M. and Shaw, A.S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine, *Cell* 84:889-897. (Medline)

Muzi Falconi, M., Brown, G.W. and Kelly, T.J. (1996) cdc18+ regulates initiation of DNA replication in

Schizosaccharomyces pombe Proc. Natl. Acad. Sci. USA 93:1566-1570. (MedLine)

Nakayama, K., Ishida, N., Shirave, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., Loh, D.Y. and Nakayama, K.-i. (1996), Mice lacking $p27^{Kip1}$ display increased body size, multiple organ hyperplasia and pituitary tumors, *Cell* 85:707-720. (Medline)

Nasmyth, K., Separating sister chromatids, *Trends Biochem. Sci.* 24:98-104. (Medline)

Nasmyth, K. (2001) Disseminating the genome: joining, resolving, and separating sister chromatids during mitosis and meiosis, *Annu. Rev. Genet.* 35:673-745. (MedLine)

Nelson, W.G. and Kastan, M.B. (1994) DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways, *Mol. Cell. Biol.* 14:1815-1823. (Medline)

Nevins, J.R. (1998) Toward an understanding of the functional complexity of the E2F and retinoblastoma families, *Cell Growth Differ.* 9:585-593. (Medline)

Nguyen, V.Q., Co, C. and Li, J.J. (2001) Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms, *Nature* 411:1068-1073. (MedLine)

Nicklas, R.B. (1997) How cells get the right chromosomes, *Science* 275:632-637. (Medline)

Nicklas, R.B., Ward, S.C. and Gorbsky, G.J. (1995) Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint, *J. Cell Biol.* 130:929-939. (Medline)

Nielsen, S.J., Schneider, R., Bauer, U.M., Bannister, A.J., Morrison, A., O'Carroll, D., Firestein, R., Cleary, M., Jenuwein, T., Herrera, R.E and Kouzarides, T. (2001) Rb targets histone H3 methylation and HP1 to promoters, *Nature* 412:561-565. (MedLine)

Norbury, C. and Nurse, P. (1992) Animal cell cycles and their control, *Annu. Rev. Biochem.* 61:441-470. (Medline)

Nurse, P. (1990) Universal control mechanism regulating onset of M-phase, *Nature* 344:503-508. (Medline)

O'Connell, K.F., Caron, C., Kopish, K.R., Hurd, D.D., Kemphues, K.J., Li, Y. and White, J.G. (2001) The *C. elegans* zyg-1 gene encodes a regulator of centrosome duplication with distinct maternal and paternal roles in the embryo, *Cell* 105:547-558. (MedLine)

Ohtsubo, M. and Roberts, J.M. (1993) Cyclin dependent regulation of G_1 in mammalian fibroblasts,

Science 259:1908-1912. (Medline)

Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M. and Pagano, M. (1995) Human cyclin E, a nuclear protein essential for the G1-to-S phase transition, *Mol. Cell. Biol.* 15:2612-2624. (Medline)

Osmani, S.A. and Ye, X.S. (1996) Cell cycle regulation in *Aspergillus* by two protein kinases, *Biochem. J.* 317:633-641. (Medline)

Osmani, S.A. and Ye, X.S. (1997) Targets of checkpoints controlling mitosis: lessons from lower eukaryotes, *Trends in Cell Biol.* 7:283-288.

Pagano, M. (1997) Cell cycle regulation by the ubiquitin pathway, *FASEB J.* 11:1067-1075. (Medline)

Pagano, M., Pepperkok, R., Verde, F., Ansorge, W. and Draetta, G. (1992) Cyclin A is required at two points in the human cell cycle, *EMBO J.* 11:961-971. (Medline)

Pandita, T.K., Lieberman, H.B., Lim, D.S., Dhar, S., Zheng, W., Taya, Y., Kastan, M.B. (2000) Ionizing radiation activates the ATM kinase throughout the cell cycle, *Oncogene* 19:1386-1391. (MedLine)

Patton, E.E., Peyraud, C., Rouillon, A., Surdin-Kerjan, Y., Tyers, M. and Thomas, D. (2000) SCF^{Met30}-mediated control of the transcriptional activator Met4 is required for the G_1-S transition, *EMBO J.* 19:1613-1624. (MedLine)

Paulovich, A.G., Toczyski, D.P. and Hartwell, L.H. (1997) When checkpoints fail, *Cell* 88:315-321. (Medline)

Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S. and Piwnica-Worms, H. (1997) Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216, *Science* 277:1501-1505. (Medline)

Peters, J.M., King, R.W., Hoog, C. and Kirschner, M.W. (1996) Identification of BIME as a subunit of the anaphase-promoting complex, *Science* 274: 1199-1201. (Medline)

Piel, M., Nordberg, J., Euteneuer, U. and Bornens, M. (2001) Centrosome-dependent exit of cytokinesis in animal cells, *Science* 291:1550-1553. (MedLine)

Pines, J. (1993) Cyclins and cyclin-dependent kinases: take your partners, *Trends in Biochem. Sci.* 18:195-197. (Medline)

Pines, J. and Hunter, T. (1994) The differential localization of human cyclins A and B is due to a

cytoplasmic retention signal in cyclin B, *EMBO J.* 13:3772-3781. (Medline)

Plath, T., Detjen, K., Welzel, M., von Marschall, Z., Murphy, D., Schirner, M., Wiedenmann, B. and Rosewicz, S. (2000) A novel function for the tumor suppressor p16(INK4a). Induction of anoikis via upregulation of the $\alpha_5\beta_1$ fibronectin receptor, *J. Cell Biol.* 150:1467-1478. (MedLine)

Pomerantz, J., Schreiber-Agus, N., Liegeois, N.J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H.W., Cordon-Cardo, C. and DePinho, R.A. (1998) The Ink4a tumor suppressor gene product, $p19^{Arf}$, interacts with MDM2 and neutralizes MDM2's inhibition of p53, *Cell* 92:713-723 (Medline)

Prescott, D.M. (1976) *Reproduction of Eukaryotic Cells,* Academic Press, New York and London, pp. 177.

Primig, M., Sockanathan, S., Auer, H. and Nasmyth, K. (1992) Anatomy of a transcription factor important for the start of the cell cycle in *Saccharomyces cerevisiae Nature* 358:593-597. (MedLine)

Pullen, N. and Thomas, G. (1997) The modular phosphorylation and activation of $p70^{S6K}$ by PDK1, *FEBS Lett.* 410:78-82. (Medline)

Pullen, N., Dennis, P.B., Andjelkovic, M., Dufner, A., Kozma, S.C., Hemmings, B.A. and Thomas, G. (1998) Phosphorylation and activation of $p70^{S6K}$by PDK1, *Science* 279:707-710. (Medline)

Quelle, D., Ashmun, R.A., Shurtleff, S.A., Kato, J.-y., Bar-Sagi, D., Roussel, M.F. and Scherr, C.J. (1993) Overexpression of mounse D-type cyclins accelerates G1 phase in rodent fibroblasts, *Genes Dev.* 7:1559-1571 (Medline)

Quelle, D.E., Zindy, F., Ashmun, R.A. and Sherr, C.J. (1995) Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest, *Cell* 83:993-1000. (Medline)

Rao, P.N. and Johnson, R.T. (1970) Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis, *Nature* 225:159-164. (Medline)

Rasmussen, C.D. and Means, A.R. (1989) Calmodulin is required for cell-cycle progression during G1 and mitosis, *EMBO J.* 8:73-82. (MedLine)

a name="rasmussen2"> Rasmussen, G. and Rasmussen, C. (1995) .Calmodulin-dependent protein kinase II is required for G1/S progression in HeLa cells, *Biochem. Cell Biol.* 73:201-207. (MedLine)

Resnitzky, D., Gossen, M., Bujard, H. and Reed, S.I (1994) Acceleration of thre G1/S phase transition by expression of cyclin D1 and E with an inducible system, *Mol. Cell Biol.* 14:1669-1679. (Medline)

Rieder, C.L., Schultz, A., Cole, R. and Sluder, G. (1994) Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle, *J. Cell Biol.* 127:1301-1310. (Medline)

Rieder, C.L., Cole, R.W., Khodjakov, A. and Sluder, G. (1995) The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores, *J. Cell Biol.* 130:941-948. (Medline)

Rieder, C.L., Faruki, S. and Khodjakov, A. (2001) The centrosome in vertebrates: more than a microtubule-organizing center, *Trends Cell Biol.*11:413-419. (MedLine)

Roberts, B.T., Farr, K.A. and Hoyt. M.A. (1994) The Saccharomyces cerevisiae checkpoint gene BUB1 encodes a novel protein kinase, *Mol. Cell. Biol.* 14:8282-8291. (Medline)

Roth, K.E., Rieder, C.L. and Bowser, S.S. (1988) Flexible-substratum technique for viewing cells from the side: some in vivo properties of primary (9+0) cilia in cultured kidney epithelia, *J. Cell Sci.* 89:457-466. (MedLine)<\a>

Rouillon, A., Barbey, R., Patton, E.E., Tyers, M. and Thomas D. (2000) Feedback-regulated degradation of the transcriptional activator Met4 is triggered by the SCF Met30 complex. *EMBO J.* 19:282-294. (MedLine)

Rowles, A., Chong, J.P.J., Brown, L., Howell, M., Evan, G.I. and Blow, J.J. (1996) Interaction between the Origin Recognition Complex and the replication licensing system in *Xenopus*, *Cell* 87:287-296. (Medline)

Russo, A.A., Jeffrey, P.D., Patten, A.K., Massagué. J. and Pavletich, N.P. (1996) Crystal structure of the $p27^{Kip1}$ cyclin-dependent-kinase inhibitor bound to cyclin A-Cdk2 complex, *Nature* 382:325-331. (Medline)

Salama, S.R., Hendricks, K.B. and Thorner, J. (1994) G1 cylin degradation : the PEST motif of yeast CLN2 is necessary but not sufficient for rapid protein turnover, *Mol. Cell Biol.* 14:7953-7966. (Medline)

Salisbury, J.L., Suino, K.M., Busby, R. and Springett, M. (2002) Centrin-2 is required for centriole duplication in mammalian cells, *Curr. Biol.* 12:1287-1292. (MedLine)

Savoian, M.S., Goldberg, M.L. and Rieder, C.L. (2000) The rate of poleward chromosome motion is

attenuated in *Drosophila zw10* and *rod* mutants, *Nature Cell Biol.* 2:948-952. (MedLine)

Schaar, B.T., Chan, G.K., Maddox, P., Salmon, E.D. and Yen, T.J. (1997) CENP-E function at kinetochores is essential for chromosome alignment, *J. Cell Biol.* 139:1373-1382. (Medline)

Schwob, E., Bohm, T., Mendenhall, M.D. and Nasmyth, K. (1994) The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae*, *Cell* 79:233-244. (Medline)

Scully, R., Chen, J., Ochs, R.L., Keegan, K., Hoekstra, M., Feunteun, J. and Livingston, D.M. (1997) Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage, *Cell* 90:425-435. (Medline)

Serrano, M., Hannon, G.J. and Beach, D. (1993) A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4, *Nature* 366:704-707. (Medline)

Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D. and DePinho, R.A. (1996) Role of the INK4a locus in tumor suppression and cell mortality, *Cell* 85:27-37. (Medline)

Shah, J.V. and Cleveland, D.W. (2000) Waiting for anaphase: Mad2 and the spindle assembly checkpoint, *Cell* 103:997-1000. (MedLine)

Sharp, D.J., Rogers, G.C. and Scholey, J.M. (2000) Cytoplasmic dynein is required for poleward chromosome movement during mitosis in Drosophila embryos, *Nature Cell Biol.* 2:922-930. (MedLine)

Shaul Y. (2000) c-Abl: activation and nuclear targets, *Cell Death Differ.* 7:10-6. (MedLine),

Sheaff, R.J., Groudine, M., Gordon, M., Roberts, J.M. and Clurman, B.E. (1997) Cyclin E-CDK2 is a regulator of p27Kip1, *Genes Dev.* 11:1464-1478. (Medline)

Sherr, C.J. (1993) Mammalian G1 cyclins, *Cell* 73:1059-1065. (Medline)

Sherr, C.J. and Roberts, J.M. (1995) Inhibitors of mammalian G_1 cyclin dependent kinases, *Genes Dev.* 9:1149-1163. (Medline)

Sherr, C.J. and Roberts, J.M. (1999) CDK inhibitors: positive and negative regulators of G1-phase progression, *Genes Dev.* 13:1501-1512. (Medline)

Shieh, S.-Y., Ikeda, M., Taya, Y. and Prives, C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2, *Cell* 91:325-334. (Medline)

Shieh, S.-Y., Ahn, J., Tamai, K., Taya, Y. and Prives, C. (2000) The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites, *Genes Dev.* 14:289-300. (MedLine)

Shiloh Y. (2001) ATM and ATR: networking cellular responses to DNA damage, *Curr. Opin. Genet. Dev.* 11:71-77. (MedLine)

Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W., Jang, J., Shevchenko, A., Charbonneau, H. and Deshaies, R.J. (1999) Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex, *Cell* 97:233-244. (Medline)

Sibon, O.C.M., Kelkar, A., Lemstra, W. and Theurkauf, W.E. (2000) DNA-replication/DNA-damage-dependent centrosome inactivation in *Drosophila* embryos, *Nature Cell Biol* 2:90-95. (Medline)

Sicinski, P., Donaher, J.L., Parker, S.B., Li, T., Fazeli, A., Gardner, H., Haslam, S.Z., Bronson, R.T., Elledge, S.J. and Weinberg, R.A. (1995) Cyclin D1 provides a link between development and oncogenesis in the retina and breast, *Cell* 82:621-630. (Medline)

Singh, P., Coe, J. and Hong, W. (1995) A role for retinoblastoma protein in potentiating transcriptional activation by the glucocorticoid receptor, *Nature* 374:562-565. (Medline)

Skibbens, R.V., Corson, L.B., Koshland, D. and Hieter, P. (1999) Ctf7p is essential for sister chromatid cohesion and links mitotic chromosome structure to the DNA replication machinery, *Genes Dev.* 13:307-319. (Medline)

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J. and Harper, J.W. (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex, *Cell* 91:209-219. (Medline)

Song, S. and Lee K. (2001) A Novel Function of *Saccharomyces cerevisiae CDC5* in Cytokinesis, *J. Cell Biol.* 152:451-470. (MedLine)

Spence, J., Gali, R.R., Dittmar, G., Sherman, F., Karin, M. and Finley, D. (2000) Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain, *Cell* 102:67-76. (MedLine)

St. Croix, B., Sheehan, C., Rak, J.W., Florenes, V.A., Slingerland, J.M. and Kerbel, R.S. (1998) E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(KIP1), *J. Cell Biol.* 142:557-571. (Medline)

Stearns, T. and Kirschner, M. (1994) In vitro reconstitution of centrosome assembly and function: the central role of γ-tubulin,*Cell*76:623-637. (MedLine)

Stueland, C.S., Lew, D.J., Cismowski, M.J. and Reed, S.I. (1993) Full activity of p34^{cdc28} histone H1 kinase activity is unable to promote entry into mitosis in check point-arrested cells of the yeast *Saccharomyces cerevisiae*, *Mol. Cell. Biol.* 13:3744-3755. (Medline)

Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hershko, J., Luca, F.C., Ruderman, J.V. and Hershko A (1995) The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis, *Mol. Biol Cell* 6:185-197. (Medline)

Sudakin, V., Chan, G.K. and Yen, T.J. (2001) Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2, *J. Cell Biol.* 154:925-936. (MedLine)

Sunkel, C.E. and Glover, D.M. (1988) *polo*, a mitotic mutant of *Drosophila* displaying abnormal spindle poles, *J. Cell Sci.* 89:25-38. (MedLine)

Tada, S., Chong, J.P.J, Mahbubani, H.M. and Blow, J.J. (1999) The RLF-B component of the replication licensing system is distinct from Cdc6 and functions after Cdc6 binds to chromatin, *Curr. Biol.* 9:211-214. (Medline)

Tanaka, N. et al., (1996) Cooperation of the tumour suppressor IRF-1 and p53 in response to DNA damage, *Nature* 382:816-818. (Medline)

Taya, Y. (1997) RB kinases and RB-binding proteins: new points of view, *Trends in Biochem. Sci.* 22:14-17. (Medline)

Taya, Y., Yasuda, H., Kamijo, M., Nakaya, K., Nakamura, Y., Ohba, Y. and Nishimura, S. (1989) *In vitro* phosphorylation of the tumor suppressor gene RB protein by mitosis specific histone H1 kinase, *Biochem. Biophys. Res. Comm.* 164:580-586. (Medline)

Taylor, S.S. and McKeon, F. (1997) Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage, *Cell* 89:727-735. (Medline)

Taylor, S.S., Ha, E. and McKeon, F. (1998) The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase, *J. Cell Biol.* 142:1-11. (Medline)

Tibbetts, R.S., Brumbaugh, K.M., Williams, J.M., Sarkaria, J.N., Cliby, W.A., Shieh, S.Y., Taya, Y., Prives, C. and Abraham, R.T. (1999) A role for ATR in the DNA damage-induced phosphorylation of p53, *Genes Dev.* 13:152-157. (Medline)

Tishler, R.B., Calderwood, S.K., Coleman, C.N. and Price, B.D. (1993) Increases in sequence specific DNA binding by p53 following treatment with chemotherapeutic and DNA damaging agents, *Cancer Res.*

53:2212-2216. (Medline)

Todorov, I.T., Pepperkok, R., Philipova, R.N., Kearsey, S.E., Ansorge, W. and Werner, D. (1994) A human nuclear protein with homology to a family of early S phase proteins is required for the entry into S phase and for cell division, *J. Cell Scie.* 107:253-265. (Medline)

Tokmakov, A.A., Sato, K.I. and Fukami, Y. (2001) Calcium oscillations in *Xenopus* egg cycling extracts, *J. Cell. Biochem.* 82:89-97. (MedLine)

Toyoshima-Morimoto, F., Taniguchi, E., Shinya, N., Iwamatsu, A. and Nishida, E. (2001) Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase, *Nature* 410:215-220. (MedLine)

Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleiffer, A. and Nasmyth, K. (1999) Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication, *Genes Dev.* 13:320-333. (Medline)

Toyoshima, F., Moriguchi, T., Wada, A., Fukuda, M. and Nishida, E. (1998) Nuclear export of cyclin B1 and its possible role in the DNA damage-induced G2 checkpoint, *EMBO J.* 17:2728-2735. (Medline)

Tomada, K., Kubota, Y. and Kato J-y. (1999) Degradation of the cyclin-dependent-kinase inhibitor $p27^{Kip1}$ is instigated by Jab1, *Nature* 398:160-165. (Medline)

Townsley, F.M. and Ruderman, J.V. (1998) Proteolytic ratchets that control progression through mitosis, *Trends Cell Biol.* 8:238-244. (Medline)

Treisman, J.E., Follette, P.J., O'Farrell, P.J. and Rubin, G.M. (1995) Cell proliferation and DNA replication defects in *Drosophila* MCM2 mutant, *Genes and Dev.* 9:1709-1715. (Medline)

Tugendreich, S., Tomkiel, J., Earnshaw, W. and Hieter, P. (1995) CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition, *Cell* 81:261-268. (Medline)

Tye, B.K. (1999) MCM proteins in DNA replication, *Annu. Rev. Biochem.* 68:649-686. (Medline)

Tyers, M. (1996) The cyclin-dependent kinase inhibitor p40SIC1 imposes the requirement for Cln G1 cyclin function at Start, *Proc. Natl. Acad. Sci. USA* 93:7772-7776. (Medline)

Uhlmann, F. and Nasmyth, K. (1998) Cohesion between sister chromatids must be established during DNA replication, *Curr. Biol.* 8:1095-1101. (Medline)

Uhlmann, F., Lottspeich, F. and Nasmyth, K. (1999) Sister-chromatid separation at anaphase onset is protmoted by cleavage of cohesin subunit Scc1, *Nature* 400:37-42. (Medline)

Usui, T., Yoshida, M., Abe, K., Osada, H., Isono, K. and Beppu, T. (1991) Uncoupled cell cycle without mitosis induced by a protein kinase inhibitor, K-25a, *J. Cell Biol.* 115:1275-1282. (Medline)

Vaizel-Ohayon, D. and Schejter, E.D. (1999) Mutations in centrosomin reveal requirements for centrosomal function during early *Drosophila* embryogenesis, *Curr. Biol.* 9:889-898. (MedLiner)

Vidwans, S.J., Wong, M.L. and O'Farrell, P.H. (1999) Mitotic regulators govern progress through steps in the centrosome duplication cycle, *J. Cell Biol.* 147:1371-1378. (Medline)

Visintin, R., Craig, K., Hwang, E.S., Prinz, S., Tyers, M. and Amon, A. (1998) The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation, *Mol. Cell.* 2:709-718. (Medline)

Visintin, R., Hwang, E.S. and Amon, A. (1999) Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus, *Nature* 398:818-823. (Medline)

Vlach, J., Hennecke, S. and Amati, B. (1997) Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27, *EMBO J.* 16:5334-5344. (Medline)

Waldman, T., Lengauer, C., Kinzler, K.W. and Vogelstein, B. (1996) Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21, *Nature* 381:713-716. (Medline)

Walworth, N.C. (2001) DNA damage: Chk1 and Cdc25, more than meets the eye, *Curr. Opin. Genet. Dev.* 11:78-82. (MedLine)

Wäsch, R. and Cross, F.R. (2002) APC-dependent proteolysis of the mitotic cyclin Clb2 is essential for mitotic exit, *Nature* 418:556-562. (MedLine)

Weber, J.D., Taylor, L.J., Roussel, M.F., Sherr, C.J. and Bari-Sagi, D. (1999) *Nature Cell Bio* 1:20-26. (Medline)

Weinberg, R.A. (1995) The retinoblastoma protein and cell cycle control, *Cell* 81:323-330. (Medline)

Weintraub, S.J., Chow, K.N.B., Luo, R.X., Zhang, S.H., He, S. and Dean, D.C.(1995) Mechanism of active transcriptional repression by the retinoblastoma protein, *Nature* 375:812-815. (Medline)

Weiss, E. and Winey, M. (1996) The *Saccharomyces cerevisiae* spindle pole body duplication gene MPS1

is part of a mitotic checkpoint, *J. Cell Biol.* 132:111-123. (Medline)

Wells, W.A. and Murray, A.W. (1996) Aberrantly segregating centromeres activate the spindle assembly checkpoint in budding yeast, *J. Cell Biol.* 133:75-84. (Medline)

White, E. (1996) Life, death and the pursuit of apoptosis, *Genes Dev.* 10:1-15. (Medline)

White, R.J., Trouche, D., Martin, K., Jackson, S.P. and Kouzarides, K. (1996) Repression of RNA polymerase III by retinoblastoma protein, *Nature*382:88-90. (Medline)

Willems, A.R., Lanker, S., Patton, E.E., Craig, K.L., Nason, T.F., Mathias, N., Kobayashi, R., Wittenberg, C. and Tyers, M. (1996) Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway, *Cell* 86:453-463. (Medline)

Wittlesberger, S.K., Kleene, K. and Penman, S. (1981) Progressive loss of shape-responsive metabolic controls in cells with increasingly transformed phenotype, *Cell* 24:859-866. (Medline)

Wittenberg, C., Sugimoto, K. and Reed, S.I. (1990) G1-specific cyclins of S. cerevisiae: cell cycle periodicity, regulation by mating pheromone, and association with $p34^{cdc28}$ protein kinase, *Cell* 62:225-237. (Medline)

Wolffe, A.P. (1997) Transcriptional control. Sinful repression, *Nature* 387:16-17. (Medline)

Wyllie, A.H., Kerr, J.F.R. and Currie, A.R. (1980) Cell death: The significance of apoptosis, *Intern. Rev. Cytol.*68:251-306. (Medline)

Xiong, Y., Connolly, T., Fuchter, A.B. and Beach, D.(1991) Human D-type cyclin, Cell 65:691-699. (Medline)

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. (1993) p21 is a universal inhibitor of cyclin kinases, *Nature* 366:701-704. (Medline)

Xu, Y. and Baltimore, D. (1996) Dual roles of ATM in the cellular response to radiation and in cell growth control, *Genes Dev.* 10:2401-2410. (Medline)

Xu, Y., Ashley, T., Brainerd, E.E., Bronson, R.T., Meyn, M.S. and Baltimore, D. (1996) Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma, *Genes Dev.* 10:2411-2422. (Medline)

Xu, X., Weaver, Z., Linke, S.P., Li, C., Gotay, J., Wang, X.-W., Harris, C.C., Ried, T. and Deng, C.-X.

(1999)Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells., *Mol. Cell* 3:389-395. (Medline)

Yaglom, J., Linskens, M.H., Sadis, S., Rubin, D.M., Futcher, B. and Finley, D. (1995) p34Cdc28-mediated control of Cln3 cyclin degradation, *Mol. Cell Biol.* 15:731-741. (Medline)

Yamano, H., Tsurumi, C., Gannon, J. and Hunt, T. (1998) The role of the destruction box and its neighbouring lysine residues in cyclin B for anaphase ubiquitin-dependent proteolysis in fission yeast: defining the D-box receptor, *EMBO J.* 17:5670-5678. (Medline)

Yang, J. and Kornbluth, S. (1999) All aboard the cyclin train: subcellular trafficking of cyclins and their CDK partners, *Trends Cell Biol.* 9:207-210. (Medline)

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R.T., Tabin, C., Sharpe, A., Caput, D., Crum, C. and McKeon, F. (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development, *Nature* 398:714-718. (MedLine)

Yang, A., Walker, N., Bronson, R., Kaghad, M., Oosterwegel, M., Bonnin, J., Vagner, C., Bonnet, H., Dikkes, P., Sharpe, A, McKeon, F. and Caput, D. (2000) p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours, *Nature* 404:99-103. (MedLine)

Yao, X., Abrieu, A., Zheng, Y., Sullivan, K.F. and, Cleveland, D.W. (2000) CENP-E forms a link between attachment of spindle microtubules to kinetochores and the mitotic checkpoint, *Nature Cell Biol.* 2:484-491. (MedLine)

Ye, X.S., Xu, G., Pu, R.T., Fincher, R.R., McGuire, S.L., Osmani, A.H. and Osmani, S.A. (1995) The NIMA protein kinase is hyperphosphorylated and activated downstream of $p34^{cdc2}$/cyclin B: coordination of two mitosis promoting kinases, *EMBO J.* 14:986-994. (Medline)

Ye, X.S., Fincher, R.R., Tang, A., O'Donnell, K. and Osmani, S.A. (1996) Two S-phase checkpoint system, one involving the function of both BIME and Tyr15 phosphorylation of $p34^{cdc2}$, inhibit NIMA and prevent premature mitosis, *EMBO J.* 15:3599-3610. (Medline)

Yuan Z.-M., Shioya, H., Ishiko, T., Sun, X., Gu, J., Huang, Y.Y., Lu, H., Kharbanda, S., Weichselbaum, R. and Kufe, D. (1999) p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage, *Nature* 399:814-817. (Medline)

Zachariae, W. and Nasmyth, K. (1996) TPR proteins required for anaphase progression mediate ubiquitination of mitotic B-type cyclins in yeast, *Mol. Biol. Cell* 7:791-801. (Medline)

Zachariae, W., Shin, T.H., Galova, M., Obermaier, B. and Nasmyth, K. (1996) Identification of subunits of the anaphase-promoting complex of *Saccharomyces cerevisiae, Science* 274:1201-1204. (Medline)

Zachariae, W., Schwab, M., Nasmyth, K. and Seufert, W. (1998) Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex, *Science* 282:1721-1724. (Medline)

Zhang, Y., Xiong, Y. and Yarbrough, W.G. (1998) ARF promotes MDM2 degradation and stabilizes p53: *ARF-INK4a* locus deletion impairs both the Rb and p53 tumor suppression pathways, *Cell* 92:725-734. (Medline)

Zhang, H.S., Postigo, A.A. and Dean, D.C. (1999) Active transcriptional repression by Rb-ELF complex mediates G1 arrest triggered by $p16^{INK4a}$, TGFβ, and contact inhibition, *Cell* 97:53-61. (Medline)

Zhang, H.S., Gavin, M., Dahiya, A., Postigo, A.A., Ma, D., Luo, R.X, Harbour, J.W. and Dean, D.C. (2000) Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF, *Cell* 101:79-89. (MedLine)

Zheng, Y., Wong, M.L., Alberts, B. and Mitchison, T. (1995) Nucleation of microtubule assembly by a γ-tubulin-containing ring complex, *Nature*378:578-583. (MedLine)

Zhu, Y., Carroll, M., Papa, F.R., Hochstrasser, M. and D'Andrea, A.D. (1996) DUB-1, a deubiquitinating enzyme with growth-suppressing activity, *Proc. Natl. Acad. Sci. USA* 93:3275-3279. (Medline)

9. Endocytosis

No discussion of cell structure can do justice to its dynamics. Cell components are in continuous motion and change. Even when cells appear to be in a steady state, membrane-enclosed vesicles are continuously formed at the cell surface, material is taken up and processed, new molecules are produced and components are broken down. The present chapter is concerned with the events of *endocytosis*. In endocytosis, cells ingest extracellular materials by trapping them in invaginations of the cell membrane, which then pinch off to form membrane-lined intracellular vesicles. Many of these events result in the trafficking of vesicles between the surface and the cell's interior and these topics will be treated in this chapter. Some of pertinent molecular details are presented in Chapter 11: they are common to other forms of intracellular transport. The trafficking between the site of synthesis and other parts of the cell will be discussed in Chapters 10 and 11. Since most of the trafficking involves a variety of intracellular vesicles and compartments, this account also concerns membranes.

Endocytosis includes a very broad spectrum of cellular activities including *pinocytosis* (the uptake of liquids) and *phagocytosis* (the uptake of large particles). There are several distinct endocytotic mechanisms (see Mukherjee et al., 1997; Lamaze and Schmid, 1995a). The discussion of this chapter includes the involvement of coated pits, (Sections IV) caveolae (Section V) and still other forms of endocytosis (Section VI). At this time, the relative importance of the various forms of endocytosis is not clear. There is evidence that clathrin mediated endocytosis is the major mechanism of fluid uptake in some cells (e.g., baby hamster kidney cells, BHKC; Griffiths et al., 1989). However, mechanisms not involving clathrin are thought to be significant in fluid uptake in other cells (e.g., Tran et al., 1987, Hewlett et al., 1994; see Sandvig and van Deurs, 1994). For example, fluid-phase endocytosis continues despite pharmacological blocks of clathrin mediated endocytosis (see Sandvig and Van Deurs, 1994)

In protozoans endocytosis has a role in feeding. Macrophages, specialized phagocytic cells of multicellular organisms, use endocytosis to remove foreign particulate material. This is then followed by digestion in the lysosomal system. However, endocytosis is not limited to specialized cells. All mammalian cells are thought to be capable of endocytotic uptake which typically proceeds at a prodigious rate. In mouse fibroblasts the amount of surface membrane taken up by endocytosis has been estimated to be 50% per hour (Pearse, 1975).

Endocytosis has several recognized roles, some already discussed. It permits internalization of receptors, nutrient uptake, antigen presentation, pathogen internalization and the maintenance of plasma membrane surface (see Riezman et al., 1997; Marsh and McMahon, 1999).

Endocytosis generally requires receptors. *Receptors* are integral membrane proteins that specifically bind a ligand with high affinity. The receptors involved in the endocytosis of growth factors and polypeptide hormones are the same ones that initiate the cascade of events which are responsible for the effect of the ligands (Chapters 6 and 7). Receptors and channels are taken up by endocytosis only after monoubiquitination (see below. Ubiquitination, especially in relation to degradation of proteins is also discussed in Chapter 15).

Monoubiquitination marks a protein at the cell surface for endocytosis in both *Saccharomyces cervisiae* and in mammals (see Hicke, 2001). The monoubiquitination of proteins of the endocytotic machinery also has a role in endocytosis. A short amino acid motif, the *ubiquitin-interacting motif* (UIM) at the carboxy termini of proteins is required as a signal to monoubiquitinate a protein and is also required for the recognition of the protein by the endocytotic machinery (Polo et al., 2002; Raiborg et al., 2002; Shih et al., 2002). See below for a discussion of ubiquitination.

In receptor-mediated endocytosis, after the specific binding of ligands to the surface receptors, other proteins and components present in the medium are taken up without any selectivity as part of the pinching-off process of endocytosis.

The purpose of receptor-mediated endocytosis of metazoans is not always clear. In some cases

endocytosis provides biologically important compounds to the interior of the cell. The *low density lipoprotein* (LDL) receptor system functions in the processing of cholesterol by the cell. *Transferrin* receptors function in iron metabolism and are responsible for the uptake of transferrin that carries iron. The physiological role of the receptors at the cell surface is the best understood. Their importance is shown by the failure of certain drugs, such as alkylamines or the antibiotic bacitracin, to block the mitogenic activity of *epidermal growth factor* (EGF), while greatly interfering with the movement of receptors to coated pits needed for endocytosis (Maxfield et al., 1979). However, the functioning of receptors after endocytosis is well documented (e.g., Sorkin et al., 1993; Zhang et al., 2000). In addition. in the case of the *nerve growth factor* (NGF), the receptors at the two different cellular locations appear to have different functions. Survival responses are activated by the receptors at the cell surface, whereas responses leading to differentiation were found to depend on their presence in endosomes (Zhang et al., 2000). Some regulative factors have been shown to exert their effect in the cell interior. Supposedly, endocytosis allows them to reach their target. The uptake of *nerve growth factor* (NGF) is likely to have a role in transporting the NGF to intracellular targets. There are indications that part of the activity of insulin and other factors may be exerted directly at intracellular sites such as the cell nucleus. Microinjection of insulin into *Xenopus* oocytes increases RNA and protein synthesis, indicating an intracellular receptor site (Miller, 1988). Furthermore, insulin (Harada et al., 1992) and insulin-like growth factor I (Peralta Soler et al., 1990) are translocated into the nucleus in intact cells and insulin-like growth factor-binding protein type 3 (IGFBP-3) has a nuclear localization (NLS)-like sequence (Radulescu, 1994). In addition, protein tyrosine kinases, which have a role in the signal transduction pathway involving receptors (Chapter 7) have been found in the nucleus (Wang et al., 1994).

Endocytosis and the physiological roles of peptide hormones are unavoidably linked because the two functions involve binding to the same receptors. In these cases the role of the endocytotic uptake of receptor and ligand is likely to be the regulation of the surface concentration of receptor required to provide its physiological response (see Katzmann et al., 2002). In fact, the removal of receptors from the cell surface by endocytosis after they are activated by binding to their ligand is part of the mechanism by which cells return to the unstimulated condition. The receptors can either be recycled or degraded by lysosomes (see below) or by the *proteasome* system (see Chapter 15). In addition, with changing conditions, channels and transporters can be regulated by a similar endocytotic pathway. This mechanism is physiologically significant as indicated by hypertension brought about by the failure of internalization of epithelial Na^+ channels (Snyder et al., 1995) (Liddle's syndrome). Furthermore, the inability to internalize the epidermal growth factor (EGF) leads to transformation (Wells et al., 1990; Vieira et al., 1996).

The various organelles involved in receptor mediated endocytosis have been defined by their kinetic relationships (see Mellman, 1996; Gruenberg, 2001). The uptake of material is typically initiated by formation of coated vesicles from coated pits, although other alternatives are emerging from more recent studies. After internalization, the chlathrin coated vesicles loose their coating, a process driven by a 70 kDa ATPase (Braell et al., 1984; Rothman and Schmid, 1986). The early endosomes may have a variety of shapes from tubular to granular. Some have are vesicles about 250-400 nm surrounded by tubules (50-

60 nm in diameter) (Gruenberg et al., 1989). The vesicles possess numerous invaginations which may have detached from the limiting membranes. Initially early endosomes have a high level of raft sphingomyelin and cholesterol as well as the raft-associated proteins caveolin-1 and flotillin-1. Their contents include cargo, recycling receptors and their bound ligands,and down regulated receptors. The late endosomes are multivesicular in appearance and have been referred to as *multivesicular bodies* (MVBs). The MVBs might be formed from invaginations of the endosomal membrane (e.g., see Hirsch et al., 1968). In yeast, the uptake of receptors culminating with their degradation in the vacuole (which corresponds to the lysosomes in mammals) involves as many as 50 genes. (see Katzmann et al., 2002).

Phosphoinositides, phosphorylated derivatives of phosphatidylinositol (PI), have been implicated in endocytosis and the MVB pathway. They have a role in the invagination involved in the formation of endosomes by recruiting the necessary protein components.One of the lipids, lysobisphosphatidic acid (LBPA) is present in the lumenal membranes of the MVBs (Kobayashi et al., 1998) and is thought to have a role in lumenal vesicle formation and the distribution of cholesterol

The early endosomes constitute the first step of sorting within the endosome system. In these endosomes, receptors and ligands are separated rapidly by acid pH. A vacuolar H^+-ATPase is responsible for the acidity (see Al Awqati, 1986). Subsequently, some of the receptors are recycled while others are to be degraded by the lysosomes system. The sorting in the early endosomes is likely to take place by a mechanism in which the recycled components are segregated in the tubular elements (possibly forming *recycling endosomes*) whereas the cargo to be degraded remains in the central vesicle (see below). No protein motifs have been found for recycling to the cell surface. Selection to the degradation pathways (see Mukherjee et al., 1997; Gruenberg, 2001) may require signal mediated sorting. Specialized lipid domains, the so-called rafts capable of interacting with certain proteins (e.g., GPI anchored proteins) may contribute to sorting (e.g., Mayor et al., 1998, Mukherjee and Maxfield, 2000) (see Chapter 4 and below). The transit through the recycling endosomes may be as long as 5 to 10 min (Schmid et al., 1988; Daro et al., 1996) A certain proportion of the recycling vesicles are thought to be transferred directly from the early endosomes to the plasma membrane where the vesicles fuse to the plasma membrane by exocytosis. In the nervous sytem, endocytosis plays a very important role in replacing the vesicles discharged during synaptic conduction (see Chapter 22)

A recycling route involves *recycling endosomes* (also called the *perinuclear recycling vesicles*) whose cargo contains only molecules to be recycled. The structures are distinct tubules 50-70 nm in thickness (Yamashiro et al., 1984; Gagescu et al., 2000) which frequently accumulate close to *the microtubule-organizing center* by a mechanism involving microtubules (Yamashiro et al., 1984). These structures may have originated from the tubules of the early endosomes.

Ligands and receptors destined for degradation follow a separate route. They are transferred to late endosomes and lysosomes in spheres (500 nm in diameter), in a process requiring functioning microtubules (Gruenberg et al., 1989). The *endosomal carrier vesicles/multivesicular bodies* (ECV/MVB) derived from early endosomes form the late endosomes. Late endosomes, have the

morphological characteristics of multivesicular bodies, can fuse with other late endosomes and are able to fuse to lysosomes. The latter initiates degradation of the endosomal contents. The passage from early endosomes to the degradation directed endosomes has been proposed to take place by a maturation process rather than by a transfer from compartment to compartment, since probe molecules are not diluted during the transition (Dunn and Maxfield 1992).

The compartments and mechanisms involved in the traffic of polarized epithelial cells is more complex. These cells have distinct apical and basolateral surfaces (see Chapter 11). The biosynthetic pathway must sort out the components in the TGN (see Rindler et al., 1984). Although in some cases the endosomes and the recycling compartments may be involve as well (e.g., Futter et al., 1995). Endocytosis takes place in each of these surfaces (see Mukherjee et al., 1997). Furthermore, in recycling or transcytosis the targeting must be to one of the two surfaces. Reflecting this organization, there are two separate sets of early endosomes: The apical early endosomes (AEE) and the basolateral early endosomes (BEE). However, the late endosomes and lysosomes are compartments shared by both systems. AEE contents can be recycled, some transcytose and others are delivered to the late endosomes and lysosomes. However, these two are the main recipients of basolateral contents. There is a recycling compartment common to both systems and BEE can recycle components to the basolateral surface or the apical surface. The presence of a common recycling compartment indicates the presence of some sorting mechanism in this compartment. Some of these exchanges depend on actin, others on microtubules and some on both these components (see Apodaca, 2001).

The progression of endocytosis from coated pits to endosomes can be followed in some cells by synchronizing the endocytotic events. The giant reticulospinal axon of the lamprey have proven very informative (see Brodin et al., 2000; Higgins and McMahon, 2002). When these cells are depleted of Ca^{2+} endocytosis is arrested. Reintroduction of Ca^{2+} reinitiates endocytosis. The changes can then be followed at various times with conventional transmission electron microscopy so that the time course of endocytotic events from coated pits to invaginated pits and pits with narrow necks can be followed. The use of mutants and blocking the effect of a protein by microinjection of either an antibody or a domain of the protein into cells have implicated various components in individual steps of endocytosis. The peptide domain blocks the effect of the native protein by competing with it. This approach can be illustrated by the study of a temperature sensitive mutation affecting the protein *dynamin* (*shibire* , ts-1) in *Drosophila*. This mutation arrests the process at the stage of in which coated pits have developed narrow necks and a collar (Koenig and Ikeda, 1989), implicating dynamin in the pinching off of the vesicles. Similarly, mutations in mice implicated *synaptojanin* in the uncoating of vesicles.

I. RECEPTOR-MEDIATED ENDOCYTOSIS

The process that has been studied in most detail is the *clathrin-mediated* endocytosis where receptors, ligands and extracellular fluid are captured by 100-150 nm diameter *coated pits* (see below). *Caveolae* (Section V) have been found to be involved in receptor mediated endocytosis, transcytosis and the transport of certain blood macromolecules (see Schnitzer, 1997) as well as viruses, toxins and

conformational altered proteins. Caveolae are also thought to contain signaling molecules (Liu et al., 1997). However, the present discussion will first be restricted to the better known system of coated pits and vesicles containing clathrin.

The receptors involved in the endocytotic uptake of ligands are transmembrane glycoproteins with the carbohydrate moiety attached to the amino terminal region. In principle, the arrangement is similar to that of other intrinsic proteins discussed in Chapter 4. All have residues such as phosphate, palmitate, or oligosaccharides that have been added posttranslationally.

In receptor-mediated endocytosis, the uptake of ligand occurs within minutes of binding to the receptor. In some cases, the receptors are preferentially located in coated pits, in others the receptors migrate to the coated pits after binding the ligand. The coated pits are indentations in the membrane in which the inner surface is lined with a fuzzy coat (see Fig. 1, Anderson, et al., 1977a). Most generally, the proteins taken up by endocytosis are in coated vesicles which lose their coat when they form *endosomes* (see Section IV). In many cases, the proteins are eventually digested by the lysosomal enzymes. *Lysosomes* are vesicles that contain a full array of hydrolytic enzymes capable of digesting materials taken up by endocytosis. The ways in which the endosomal contents and the lysosomal enzymes interact is presently under discussion (see Section VII, below).

The *low density lipoprotein* (LDL) receptor system which functions in the transport of cholesterol from the plasma into cells, was one of the first to be examined in detail and will be the first subject of our discussion.

II. THE LDL RECEPTOR FAMILY

A. The LDL Receptors

In mammals, cholesterol is transported in the bloodstream in spherical LDL particles, 22 nm in diameter, which originate in the liver. The particles are complexes containing a core of 1500 cholesterol molecules esterified to long-chain fatty acids which are covered by phospholipids, cholesterol, and protein (Goldstein and Brown, 1977).

The LDL receptors (LDLRs), lipoprotein molecules with a molecular mass of 164 kDa (Schneider et al., 1982), are synthesized in the endoplasmic reticulum when cholesterol is required. The LDL particles first bind to the LDLRs. Then they are internalized at the cell surface and eventually the receptor-LDL particle complexes are delivered to the lysosomes. In these organelles the protein component of the LDL particles is hydrolyzed and cholesterol is regenerated from the cholesteryl esters. Cholesterol in the cytoplasm inhibits the synthesis of the receptor molecules and thereby prevents an excessive accumulation. Apparently, the LDL receptors are recycled: they return to the surface where they cluster again in the coated pits (Anderson et al., 1977b, Goldstein et al., 1976).

What is the location of the LDLRs and what are the details of the LDL uptake? To answer these questions LDL was labelled with ferritin. Ferritin has a high iron content and therefore is visible with the electron microscope. In contrast to other systems, the LDL receptors are present in coated pits before the addition of LDL. This has been demonstrated by the binding of the ferritin-labelled LDL to cells that have been fixed and presumably cannot migrate. Between 50 and 80% of the receptors are clustered in 2% of the cell surface and have been shown to be present in coated pits by electron microscopy (Anderson et al., 1977a, Orci et al., 1978).

The location of coated pits coincides with the position of the underlying cytoplasmic stress fibers (Anderson et al., 1978) which have been shown in other studies to contain actin (see Chapter 23. In fact, clathrin, the major protein of coated vesicles, can bind actin. Since actin fibers are associated with movement, it is possible that they have a role in the internalization of vesicles.

Accumulation in the coated pits and binding of the ligand are two separate functions of the LDLRs since they are affected by different mutations. This conclusion is based on observations of patients with familial *hypercholesterolemia* (FH), a disease characterized by very high levels of cholesterol in the blood. One kind of FH can be traced to a mutation in which the receptors are unable to bind LDL; another kind involves a mutation in which the receptors are unable to be incorporated into coated pits. These observations suggest that the receptors have two separate sites: the site for binding LDL that faces the medium and the site that interacts with the coated pits which is presumably cytoplasmic (see discussion in section IVC). Some of the receptor molecules are likely to have other specialized sites responsible for their sorting out into various cellular compartments.

The sequence of events underlying the binding of LDL and endocytosis is illustrated in Fig. 1 (Anderson et al., 1977a) which summarizes the electron microscopic observations with the LDL-ferritin marker. Cultured fibroblasts were first incubated at 4ºC for 2 h and then, after extensive washing, were incubated for various lengths of time in the presence of ferritin-labeled LDL at 37ºC. Fig. 1 A-C shows that after incubation for 1 min the various configurations of early endocytosis are already present in coated pits. Fig. 1D shows a coated vesicle that is beginning to lose its coat after a 2 min incubation, and Fig. 1E shows a vesicle or endosome without a coat, also after a 2 min incubation. Fig. 1 F-H correspond to configurations in the formation of secondary lysosomes, that is, lysosomes formed by fusion of endosomes and newly formed primary lysosomes. In Fig. 1 F and G the amount of ferritin per vesicle seems to be greater than in the earlier stages, possibly indicating a fusion of various vesicles; Fig. 1H corresponds to a fully formed lysosome containing ferritin.

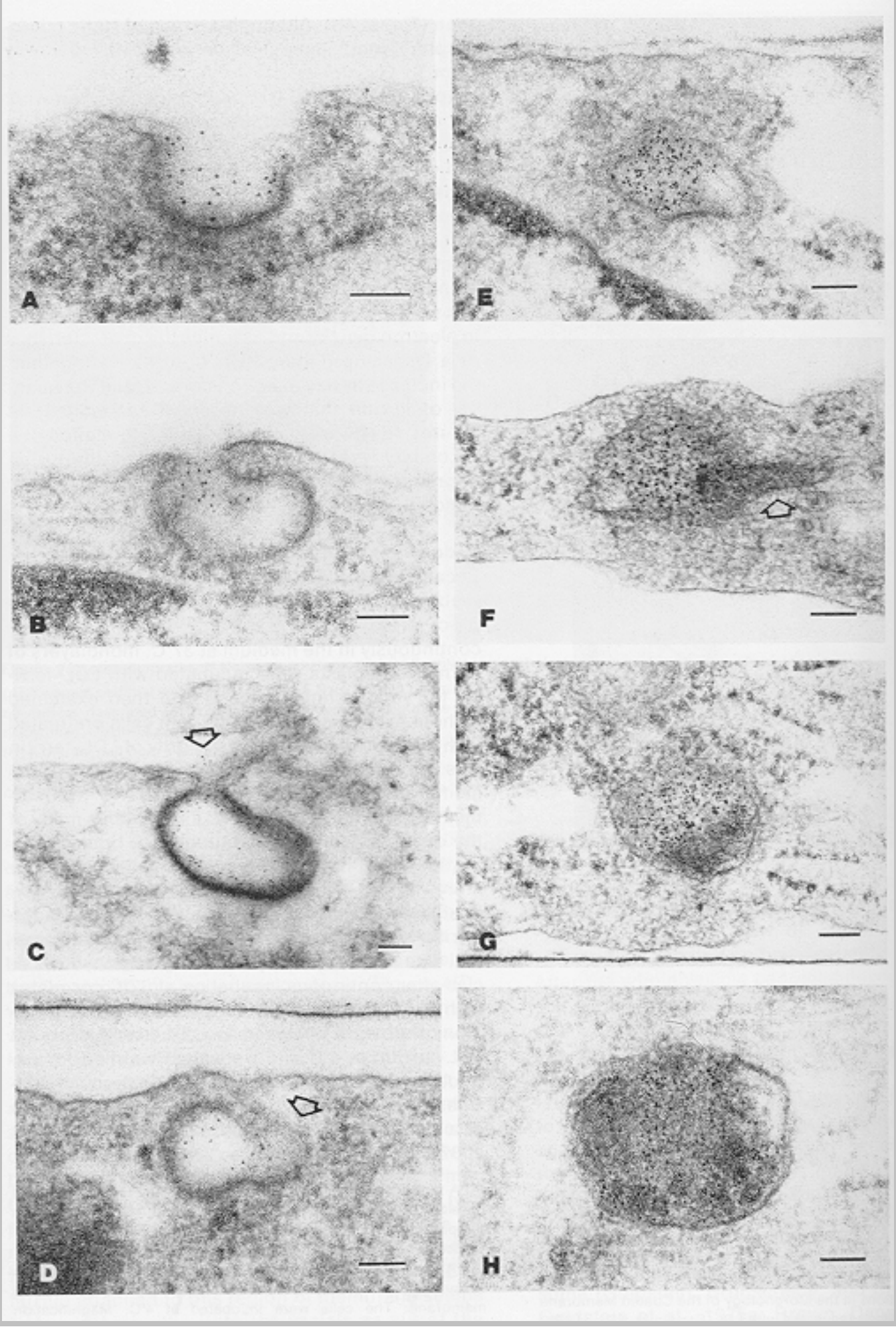

A
E
B
F
C
G
D
H

Fig. 1 Role of the coated endocytotic vesicle in the uptake of receptor-bound low-density lipoprotein in human fibroblasts. The bars represent 100 nm. See text. Reproduced with permission from Anderson, et al., (1977a), copyright © 1977 by Cell Press.

The general information derived from the electron microscopic studies has been confirmed biochemically in cultured fibroblasts after labelling LDL with the radioactive isotope [^{125}I]. The actual endocytotic uptake was distinguished from binding by adding heparin to the cells. Heparin releases only surface LDL and does not have an effect on the LDL inside the cells. Therefore, the [^{125}I]-labelled LDL that is released after heparin treatment can be assumed to be attached to the LDL receptor at the surface. Heparin, a glucosaminoglycan which acts as an anticoagulant, is produced by mast cells. The release of the LDL from the receptor is not the result of damage to the receptors because after incubation in heparin and its removal, the binding of LDL is the same as that of untreated cells. Fig. 2 (Goldstein, et al., 1976) shows that the surface-bound [^{125}I]-labeled LDL is taken up very rapidly, most within 5 min. However, the heparin-insensitive uptake of LDL continues linearly with time at a lower rate, suggesting that it corresponds to rapid uptake into cytoplasmic vesicles.

What is the fate of the LDL? Its later association with lysosomes, revealed by the electron microscopy, suggests a proteolytic breakdown. The proteolysis of LDL can be followed by measuring the radioactivity of trichloroacetic acid (TCA)-soluble cell extracts. Hydrolytic products of proteolysis, amino acids and low molecular weight peptides, are soluble in TCA. In contrast, protein is denatured by TCA and becomes insoluble. An increase in the TCA-soluble radioactivity of the extract serves as a measure of LDL hydrolysis. Fig. 3 (Goldstein et al., 1976) represents the degradation of the LDL at 37ºC after an initial binding at 4ºC. After the initial binding the labelled LDL is removed. The radioactivity of the bound protein disappears (curve 1) and most of it appears in a TCA soluble form (curve 2).

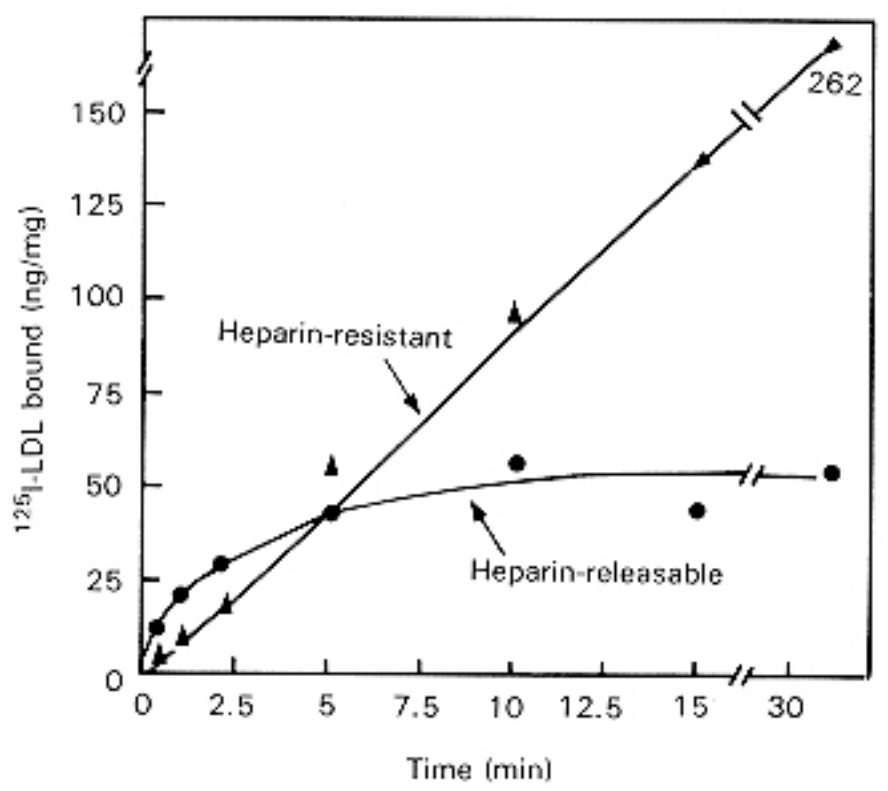


Fig. 2 Relation between heparin-releasable and heparin-resistant ^{125}1-labelled LDL binding at 37ºC at early time points. Reproduced with permission from J.L. Goldstein, et al., *Cell*, 7:85-95. Copyright © 1976 by Cell Press.

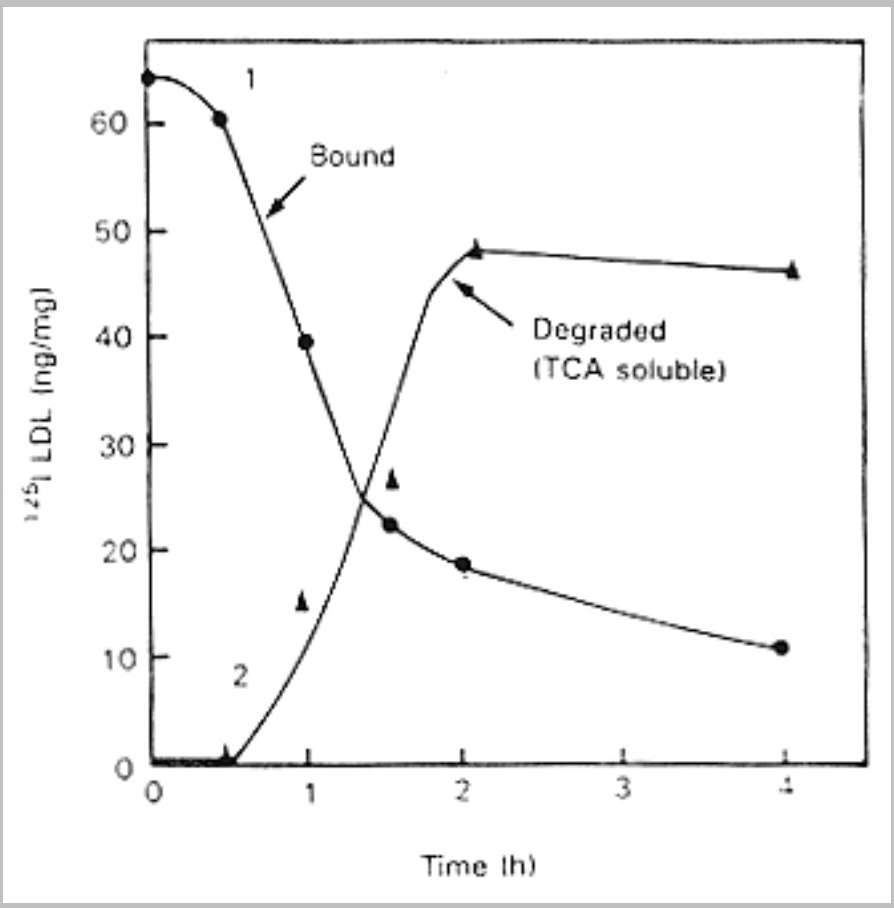


Fig. 3 Proteolytic degradation at 37ºC of ^{125}I-labeled LDL previously bound to normal fibroblasts at 4ºC. Reproduced with permission from, J.L. Goldstein, et al., *Cell*, 7:85-95. Copyright © 1976 by Cell Press.

B. Other Receptors of the LDLR family

LDLRs have been found to be part of a family of receptors. Nine have been recognized in mammals. They share common structural motifs and all have a role in the uptake of lipoproteins. However, many of them are involved in many other functions as revealed in knockout mice mutants or in human gene defects (see Nykjaer and Willnow, 2002, Gliemann, 1998). Members of the LDL receptor family have roles in neuronal migration, synaptic plasticity and vitamin metabolism. Their multiple functions may be the result of their ability to bind to a variety of ligands, their interaction with other proteins present at the cell surface (co-receptors), including seven-transmembrane-span receptors, glycosylphosphatidylinositol (GPI) anchored proteins and adhesion molecules, and in addition their interaction via their cytoplasmic tails with cytoplasmic adaptors . Two of these receptors are large (600 kDa): *LDL-receptor-related protein* (LRP) and *megalin* megalin (see Gliemann, 1998; Herz and Strickland, 2001). LRP is present in hepatocytes, macrophages, smooth muscle cells, and neurons. Megalin is present in various epithelia such as that of proximal kidney tubules and intestine. These two proteins bind numerous ligands at different combinations of sites.

LRPs function in lipid metabolism, the regulation of proteinases and proteinase inhibitors, activation of lysosomal enzymes, cellular signal transduction and neurotransmission, and recognizes at least 30 different ligands. In addition, a variety of cytoplasmic proteins bind to the tail of LRP and some of these proteins initiate endocytosis. LRP binds to remanents of chylomicron, and the lipases directly involved in the forming lipoproteins from triglyceride-rich chylomicrons. Chylomicrons are particles which carry primarily dietary cholesterol from the intestine to the liver. Some ligands first bind to heparan sulfate proteoglycans before being picked up by LRP. LRPs are involved in the removal of proteinase and proteinase inhibitor complexes and are important regulators of extracellular proteolytic activity including matrix metalloproteinases. LRPs are involved in sphingolipid activator protein (SAP) uptake which is required for activation of cerebrosidases, sphingomyelinases, glucosidases, and hexosaminidases in the lysosomes. In neurons, LRPs have been implicated in NMDA receptor function. They interact with *tissue plasminogen activator* (tPA). tPA expression is thought to have a role in synaptic plasticity in the brain.

In the kidney, magalin is involved in the uptake of low molecular plasma proteins that have been lost through the glomerulus such as plasma carriers that transport vitamins and ions (e.g. retinol binding proteins, vitamin A, vitamin D- binding proteins, tranferrin). In megalin knockout mutants, vitamins are excreted. In some cases, megalin ligands bind to *cubulin*, a peripheral protein of 460 kDa which binds to ligands but is unable to be internalized by itself without attaching to megalin.

III. OTHER RECEPTORS

A. Involvement in Endocytosis

Various proteins are taken up specifically by cells, as shown in Table 1 (Goldstein, et al., 1979); at the time of this compilation, 25 specific receptors involved in endocytosis had been recognized.

Table 2 (Brown, et al., 1983) lists some of the cell surface receptors that have been purified and characterized. The receptors have been generally identified by their ability to bind the appropriate ligand. The LDL receptors were extracted from cell membrane preparations of bovine adrenal cortex. Adrenal cells are particularly rich in these receptors because they use cholesterol in the synthesis of steroid hormones. It has been estimated that there are 100,000 receptor molecules per adrenal cell. Binding of [^{125}I]-labelled LDL was used as the assay of the receptor through the various steps of the fractionation procedures.

Table 1 Systems for Receptor-mediated Endocytosis of Proteins

			Fate of internalized protein	
Protein	**Cell Type**	**Internalization via coated pits and coated vesicles**	**Degraded in lysosomes**	**Other**
Transport proteins	Fibroblasts, smooth muscle cells, endothelial cells, adrenocortical cells,lymphocytes	Yes	Yes; cholesterol retained by cells	---
LDL		Yes		Delivered to yolk granules
Yolk proteins	Oocytes (chicken, mosquito)	Data not available	No	---
(phosvitin, lipovitellin)		Yes	Yes, vitamin B_{12} retained by cells	Iron retained by cells
Transcobalamin II	Kidney cells, hepatocytes, fibroblasts			
Transferrin	Erythroblasts, reticuloblasts		Data not available	

Protein hormones	Fibroblasts, 3T3 cells	Yes	Yes	---
Epidermal growth factor	Sympathetic ganglion cells	Data not available	Data not available	Carried in vesicles retrograde up to the axon
Nerve growth factor	Hepatocytes, hepatoma cells, lymphocytes, adipocytes, 3T3 cells	Data not available	Yes	Also delivered to Golgi apparatus and nuclei
Insulin	Leydig tumor cells, ovarian luteal cells	Data not available	Yes	---
Chorionic gonadotropin	Melanoma cells	Data not available	Data not available	Delivered to Golgi apparatus and melanosomes
β-Melanotropin				
Other proteins				
Asialoglycoproteins	Hepatocytes	Data not available	Yes	---
Lysosomal enzymes	Fibroblasts	Data not available	No	Delivered to lysosomes and Golgi-associated structures; enzymes remain active for many days
a_2-Macroglobulin	Fibroblasts, macrophages, 3T3 cells	Yes	Yes	---
Maternal immunoglobulins (lgG)	Fetal yolk sac, neonatal intestinal epithelial cells	Yes	No	Transferred intact in coated vesicles to basal surface of cells, where igG is discharged into fetal or neonatal circulation

Source: From Goldstein et al. (1979). Reprinted by permission from *Nature* 279:679-685, copyright © 1979 Macmillan Magazines Ltd.

Clathrin coated pits and vesicles have been shown to be involved in many cases of receptor-mediated endocytosis (see however, Section V and Section VI), although many of the details of the uptake and processing differ. We saw that the LDL receptors are located in the coated pits even before they bind LDL. Most or all receptors binding inert cargo such as LDL (i.e. not a signaling molecule) are internalized regardless of whether they are binding the ligand. Various receptors follow this pattern. However others, such as the EGF receptors, are distributed throughout the surface and cluster only after binding the ligand (Hagler, 1978). As we saw in Chapter 4, membrane components can diffuse two-dimensionally inside the membrane. The rate of the diffusional movement of receptors toward the coated pits is sufficiently rapid to account for the clustering (Bretscher and Pease, 1984, Hopkins, 1985), so that no special mechanism has to be invoked to explain the migration. In contrast to receptors binding inert cargo, signaling receptors require ligand binding for uptake. What allows the receptor proteins to be taken up by endocytosis when bound to their ligand? Many of the details are still unknown. Some of these aspects will be discussed in the section below (Section IV). The presence of short motifs of four or five amino acids in the cytoplasmic domain of the receptors seems to play a role (Collawn et al., 1990, 1991, Vaux, 1992). In the absence of these motifs the receptors do not initiate endocytosis when bound to the ligand. In the case of EGFRs, ligand binding cause the receptor to autophosphorylate (see Schlessinger and Ulrich, 1992). The phosphorylation results in a conformational change that exposes the motifs in the cytoplasmic domain required for coated pit targeting (Cadena et al., 1994). However, downstream receptor signaling (Lamaze and Schmid, 1995b) and at least in the case of other receptors, recruitment of clathrin to form coated pits (Grimes et al., 1996) are required. Apparently the binding of EGF to the receptor activates a kinase (SRC kinase) that phosphorylates the clathrin heavy chain (Wilde et al., 1999). This phosphorylation triggers the formation of vesicles and movement of clathrin to the interior of the cell. Without SRC kinase activity EGF endocytosis is delayed.

B. Fate of Ligand and Receptor

Many of the receptors recycle. In contrast to LDL which is hydrolyzed, the LDL receptor is rapidly returned to the surface and has a half-life as long as 15 h (Brown et al., 1981). As might be expected, blocking protein synthesis with cycloheximide does not have an immediate effect on LDL endocytosis. In contrast to the recycling of the LDL receptor, the EGF receptor is degraded in most tissues (Schlessinger et al., 1978) although it is recycled in the liver (Dunn and Hubbard, 1984).

Table 2 Cell Surface Receptors That Concentrate in Coated Pits

		Estimated molecular mass (kDa)		
Receptor	**Source**	**Subunit**	**Holoreceptor**	**Residues added posttranslationally**

LDL	Bovine adrenal cortex human fibroblasts	160	160	O-linked oligosaccharide; sulfate on N-linked oligosaccharides
Transferrin	Human leukemia cells	90	180 (disulfide linked dimers)	palmitate; phopshate on tyrosine and serine
Epidermal growth factor	Human A-431 cells	170	170	phopshate on tyrosine and serine
Insulin	Rat adipose tissue Rat liver Human placenta	90 125	350; 2 subunits of 90, 2 subunits of 125 (disufide linked)	phopshate on tyrosine and serine
Lysosomal enzyme	Bovine liver Rat chondrosarcoma	215	215	
Asialoglyco- proteins	Chicken liver rat liver	26 43,54,64	26 43, 54, 64	phosphate on serine
Fibroblast growth factor	Pituitary	15	15	

From M.S. Brown et al., *Cell* 32:663-667, Copyright &copy1983 by Cell Press, reproduced by permission.

As we saw for LDL, many of the proteins taken up by endocytosis are degraded. In contrast, yolk proteins are accumulate in yolk granules. NGF, which enters by endocytosis at the tip of the axon, accumulates in the cell body (Bradshaw, 1978). The fate of insulin differs from both of these cases: some of the insulin taken up by endocytosis is degraded and some remains intact inside the cell.

Both receptor and ligand may recycle, as is the case for *transferrin* (Bleil and Bretscher, 1982), a protein that functions in the transport of iron in organisms. After uptake of transferrin by endocytosis, the iron is removed from the transferrin in the endosome; the apotransferrin (i.e. transferrin stripped of iron) remains attached to the receptor and both are returned to the cell surface (Geuze, 1984).

Four possible pathways for the receptor-ligand complex are shown in Fig. 4 (Goldstein, et al., 1985).

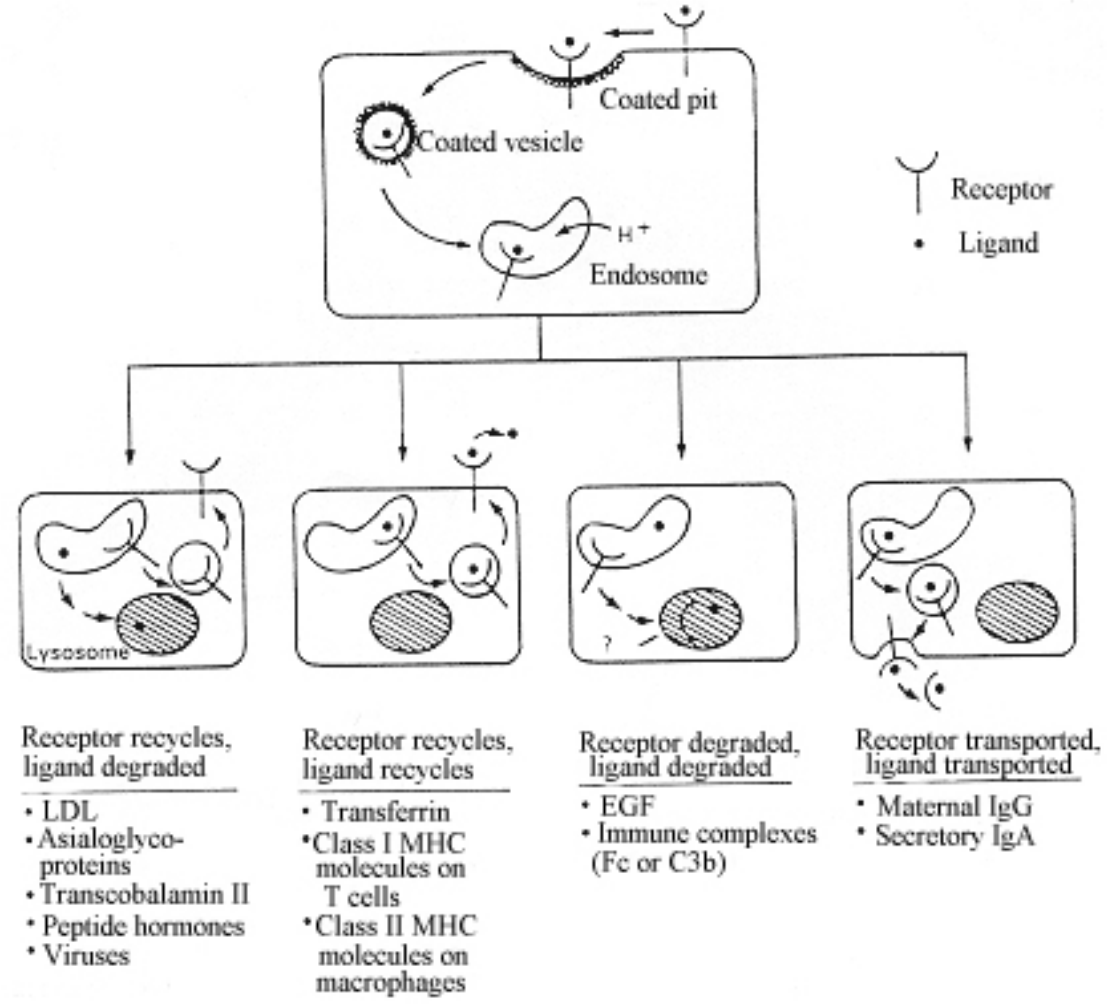


Fig. 4 Four pathways of receptor-mediated endocytosis. The initial steps (clustering of receptors in coated pits, internalization of coated vesicles, and fusion of vesicles to form endosomes) are common to the four pathways. After entry into acidic endosomes, a receptor-ligand complex can follow any of the four pathways shown. From Goldstein, et al., 1985. Reproduced, with permission, from the *Annual Review of Cell Biology*, Volume 1, copyright &copy1985 by Annual Reviews Inc.

C. Hydrolysis, Recycling, and Sorting

What is the fate of ligands and receptors which are not recycled? As we saw for LDL, after endocytosis the coated vesicles shed their coat and presumably fuse to form smooth larger vesicles, called endosomes. These in turn either fuse with the lysosomes or transfer the LDL to the lysosomes by means of transport vesicles (see section VII, below). The digestion of receptor then takes place in the lysosomes as indicated in Fig. 4.

As already indicated, in mammals the lysosomal degradation pathway is involved in the degradation of certain receptors such as growth hormone receptors (Strous et al., 1996). Treatment with NH_4Cl disrupts

lysosomal function and blocks the degradation of these receptors. Others, seem to be degraded by *proteasomes*. Proteasomes are huge (1,700 kDa!) multimolecular assemblies involved in the degradation of a variety of proteins (see Chapter 15). Proteasomes are implicated in the degradation of the *platelet-derived growth factor* (PDGF) receptors since inhibitors of proteasome function interfere with their degradation (Mori et al., 1995; Jeffers et al., 1997). A proteasomal pathway is suspected in down-regulating mammalian receptors by degrading their cytosolic domains (see Hicke, 1997).

Until now, for the degradation of yeast integral plasma membrane proteins, only the lysosomal pathway has been implicated (e.g. Berkower et al., 1994, Kölling and Kollenberg, 1994) (see Section C, Chapter 15, and section on the degradation of integral proteins in Chapter 15). For example, normal internalization and degradation occurs in mutants lacking proteasome function (Galan et al., 1994).

Ubiquitination (see also above) marks proteins for internalization and degradation by either the lysosomal system (e.g. Hicke and Riezman, 1996) or the proteasome (e.g. Strous et al., 1996). *Ubiquitin* (see Chapter 15) is a small protein (8.5 kDa) that has been found to tag proteins for proteolysis, although it is likely to have other functions as well. As little as one ubiquitin is necessary for internalization (Hicke and Rieezman, 1996) in yeast, whereas polyubiquitin with a minimum of four ubiquitin molecules is required for recognition by the proteasomes (Deveraux et al., 1994). Besides a role in internalization, ubiquitination is needed for sorting in the late endosomes and from the TGN to the lysosomes (vacuole in yeast) (see Rotin et al., 2000; Dupré et al., 2001). At least in *Saccharomyces cerevisiae* the downregulation of the membrane receptors and transporter proteins takes place by internalization signaled by monoubiquitination. The enzymes involved in ubiquitination, such as Cbl, an E3 or ubiquitin ligase recruited to phosphotyrosine motifs, may have some additional roles. Cbl ubiquitinates the epidermal growth factor receptor (EGF-R) at the cell surface. However, it remains bound throughout the endosomal pathway suggesting some other function (de Melker et al., 2001; Levkowitz et al., 1998).

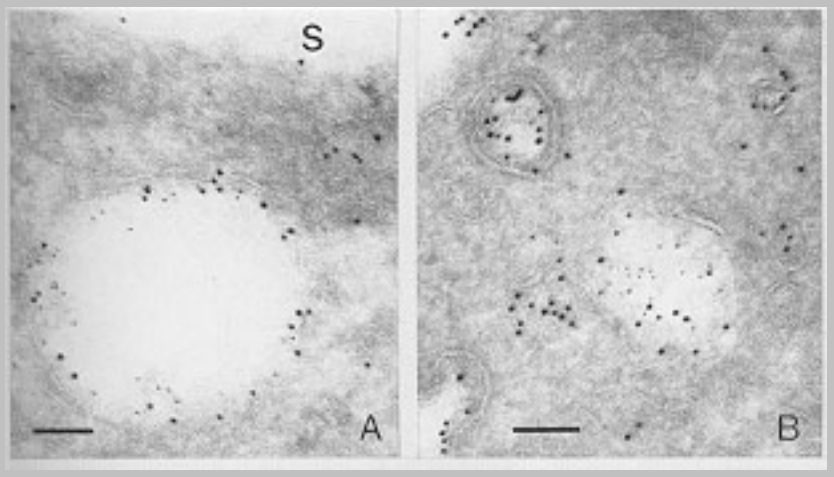
The targeting of the monoubiquitinated transmembrane proteins such as the cell surface receptors to the multivesicular bodies/lysosomal vesicles (or vacuole in yeast) involves several Vps proteins (Vps2, Vps20, Vps24, and Snf7). The Vps are transferred from the cytoplasm to endosomal membranes where they oligomerize into protein complexes, ESCRTs. (ESCRT-I ,II and III). ESCRT-III, a membrane associated complex. includes the AAA-type ATPase Vps4. The ESCRT complexes perform a cascade of events in which the cargoes are sorted out and delivered to the vacuole or lysosomes (Babst et al. 2002a and b).

Ligands and receptors may have separate fates (Fig. 4). It is difficult to imagine how this can take place unless there is some special mechanism for separating them and conveying them to different compartments. During endocytosis, the interior of the endosome becomes acidic. The acidity of the endosome has a role in detaching the ligand from the receptor. The increase in acidity has been shown for the endocytotic uptake of α_2-macroglobulin conjugated to fluorescein (Tycko and Maxfield, 1982). Fluorescein is a dye whose fluorescence varies with pH. After 20 min of endocytosis induced by the

labeled macroglobulin, the fluorescence of the dye in the vesicle indicated a pH of approximately 5. Experiments with isolated vesicles show that the internal acidification results from an H^+-pump powered by ATP hydrolysis (Galloway et al., 1983). Once separated, a special mechanism must be present to direct the two components to a different location. One of the systems that provides information on this process is that responsible for the uptake of asialoglycoprotein.

The asialoglycoprotein receptor system has been studied with the electron microscope after labeling with separate antibodies for ligands, receptors, and clathrin (Geuze et al., 1983). Clathrin is the major coat component of coated pits (see Sections IV below). *Asialoglycoproteins* are abnormal plasma glycoproteins that have been stripped of the sialic acid residue that normally covers the terminal galactose. The antibodies used in this study are visualized and distinguished from one another by coupling to colloidal gold particles of different sizes. The results indicate that after separation, ligands and receptors are segregated in a vesicle (*compartment of uncoupling receptor and ligand*, CURL; probably corresponding to early endosomes) containing tubular extensions. The receptors attach to the membranes of the tubules, whereas the ligands remain in the lumen. Presumably, budding of the tubules produces smaller vesicles, which return the receptors to the surface. Fig. 5A (Geuze, et al., 1983) shows that both ligand (coupled to the 5-nm particle) and receptor (coupled to the 8-nm particle) are present in a vesicle close to the cell surface. Fig.5B and C show how the ligand remains in the lumen of the CURL, whereas the tubules favor the receptors. In (B) the receptor is labeled with the 8-nm gold particle and in (C) the labeling is reversed.

The movement of receptor or receptor-ligand complex occurs through several compartments, with accurate sorting in each. As in the case of translocation into the nucleus, the targeting of newly synthesized proteins requires special domains (Chapter 5). The targeting of receptors may also have many functional domains for interaction not only with the ligand but also the various macromolecular species capable of redirecting it to a new target.



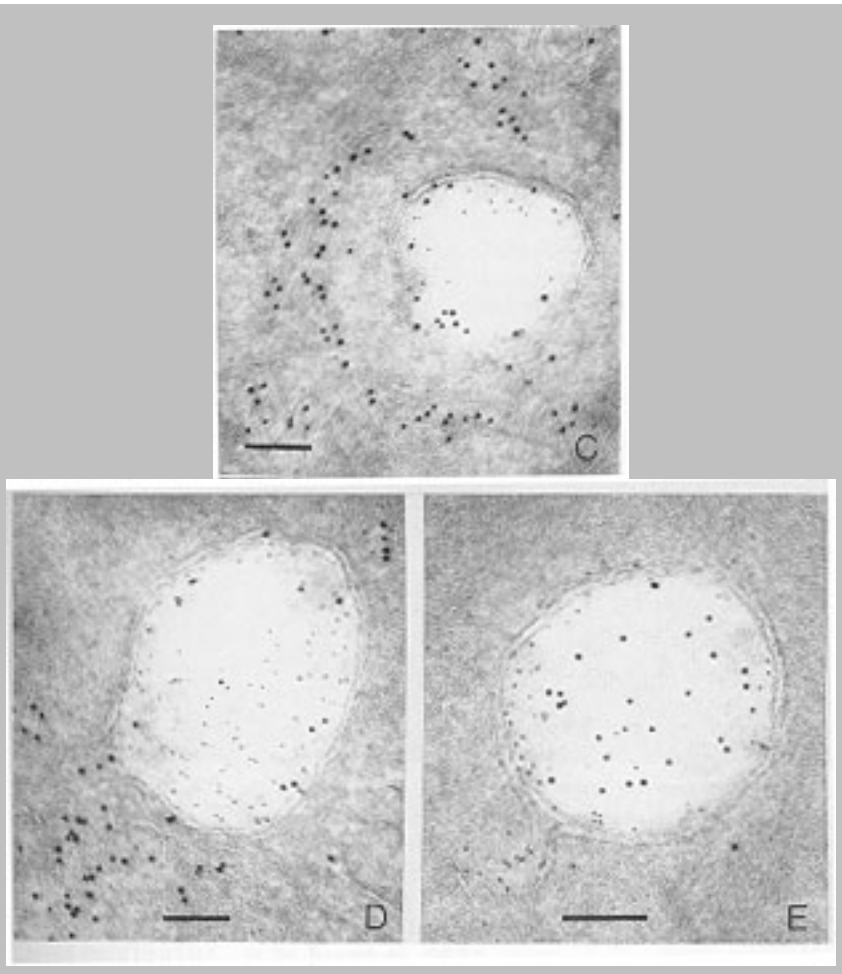


Fig. 5 Simultaneous demonstration of receptor and ligand in CURL and in a multivesicular body. The bars correspond to 100 nm. (a) Vesicle just beneath plasma membrane at the sinus (S), with ligand (5-nm gold) associated with receptor (8-nm gold). (b) Coated pit with receptor (8-nm gold) and ligand (5-nm gold) at upper left. The slightly tangential view of early endosomes shows a heterogeneous distribution of receptor in the vesicular portion and abundant receptor in associated tubules. Simultaneous demonstration of receptor and ligand in CURL and in a multivesicular body. (c) Early endosomes profile shows peripheral ligand (5-nm gold) and heterogeneous labeling of receptor (8-nm gold). Intense receptor labeling is present over the tubules adjacent to the vesicular portion of early endosome. (d) Free ligand (5-nm gold) can be seen in the lumen of the vesicular portion of early endosome, which also shows scarce and heterogeneous receptor (8-nm gold) labeling. Receptor labeling is intense over the connecting tubules. (e) Early endosome profile in which the receptor (5-nm gold) is located predominantly at the pole, where a tubule with heavy labeling of receptor is connected. Most of the ligand (8-nm gold) is present free in the vesicle lumen. Reproduced with permission from Geuze, et al., (1983), copyright &copy1983 by Cell Press.

IV. COATED PITS

Coated pits and coated vesicles have been found in virtually all nucleated animal cells (see for a review Takei and Haucke, 2001). The coated pits have a fuzzy cytoplasmic coat which, at higher resolution, can be shown to correspond to periodically spaced fibers. Electronmicrographs of freeze-fractured and deep etched preparations of vesicles (Heuser et al., 1988) reveal a regular lattice composed of many *clathrin* molecules.

A. Clathrin and Adaptor Complexes

While coated pits have been found to be involved in the uptake of proteins from the medium, coated vesicles have been implicated not only in endocytosis but also in intracellular membrane transport. Four different coat proteins are recognized, including two clathrin proteins and COPI and COPII. The coat proteins of intracellular transport are discussed in Chapter 11. Adaptor proteins (APs) are components of the clathrin coats (see below). Clathrin associated with AP-2 and AP180 are involved in endocytosis, whereas clathrin associated with AP-1 and AP-3 are involved in the transport from the TGN to the lysosomes (see Chapter 10 and 11). AP-4 has been found to be associated with the TGN. In the case of AP-1 and AP-3, the guanosine triphosphatase ADP-ribosylation factor 1 and possibly other proteins are required instead of AP180 (see Dell'Angelica, 2001). A major role of clathrin in endocytosis is attested by the observation that the introduction of anticlathrin antibody into living cells results in inhibition of both receptor-mediated endocytosis and fluid endocytosis (Doxsey et al., 1987) but not the transport accompanying secretion. There are indications, however, that in some cases endocytosis (including receptor-mediated endocytosis) proceeds by mechanisms not involving clathrin (see below)

Isolated coated vesicles are composed of the membrane components surrounded by a basket-like arrangement of protein, predominantly clathrin, a protein of 180 kDa. Clathrin itself is formed from three heavy and three light chains forming a so-called *triskelion* (see Fig. 6). The clathrin coat also contains the APs. APs have a role in the assembly and the attachment of clathrin to the plasma membrane (see Hirst and Robinson, 1998; Kirchhausen 1999; Kirchhausen, 2000). In addition, APs mediate the interaction with lipids. AP180 has been shown to aid in the assembly of clathrin in vitro and is needed for endocytosis and the maintenance of proper vesicular size in vivo (see McMahon, 1999; Ford et al., 2001).

As indicated, the various populations of clathrin coated vesicles have distinct APs. In addition to the APs, β-arrestin and arrestin-3 may act as adaptors (e.g., see Goodman et al., 1997) for the internalization of receptors coupled to the heterotrimeric GTP-binding proteins (see Chapter 7).

APs are dimers, and each monomer has four polypeptides called *adaptins*. Two of the adaptins are approximately 100 kDa in size, one of 50 kDa and another of 25 kDa. In vitro, in the presence of clathrin triskelia, the APs are attached to the terminal domain of the clathrin unit. After removal of clathrin from coated vesicles, the adaptins appear as blocks with the two carboxy-terminals of the larger subunits sticking out (Heuser and Keen, 1988).

B. Structure of Clathrin and Assembly

The structure of clathrin cages has been studied (see Smith and Pearse, 1999) with EM (Vigers et al., 1986; Smith et al., 1998) and fragments of clathrin have been the subject of crystallographic studies (ter Haar et al., 1998; Ybe et al., 1999). Vigers et al. examined tilt series of electron micrographs from unstained clathrin cages embedded in vitreous ice. The three-dimensional reconstructions of individual hexagonal barrels show details of the internal structure. Cryoelectron microscopy and single-particle

reconstruction (Smith et al, 1998) shows details of the packing of entire clathrin molecules as they interact to form a cage with two polyhedral layers. A triskelion hub is at the vortex of the cage (see Fig. 6). Three legs extend in different directions from the hub. Midway along each leg there is a bend corresponding to the proximal and distal domains. The amino-terminal of each of each clathrin heavy chain is adjacent to the distal domain. The carboxy-terminal is at the hub of the triskelion at the so-called *trimerization domain*. Each leg contributes two adjacent faces of the cage structure. The distal legs are positioned alongside on the underside of the second edge below the next hub where they can interacts with the APs inside the cage.

The crystal structure of a fragment of clathrin corresponding to the hub domain (see Fig. 6) has been elucidated (Ybe et al., 1999). It forms an elongated coil of α-helices. In addition, a 145-residue motif is repeated seven times along the filamentous leg. This motif is similar to that of other proteins involved in vacuolar protein sorting. The hub domain contains a light chain binding region and the portion mediating spontaneous clathrin heavy-chain polymerization. Light chains modulate polymerization negatively in vitro and may play a role in preventing the unphysiological assembly of cages in the cytoplasm (Ungewickell and Ungewickell, 1991). The structure of the amino-terminal portion (ter Haar et al., 1998) has also been studied by crystallography. It corresponds to a globular terminal domain forming a β seven-blade propeller joined to the leg by the linker domain which is in an α zig zag arrangement. The propeller domains form an inner polyhedral array in the coated vesicle that allows many binding sites for adaptor molecules (Smith et al., 1998). AP-2 form an inner shell of density in the EM map of the clathrin coat.

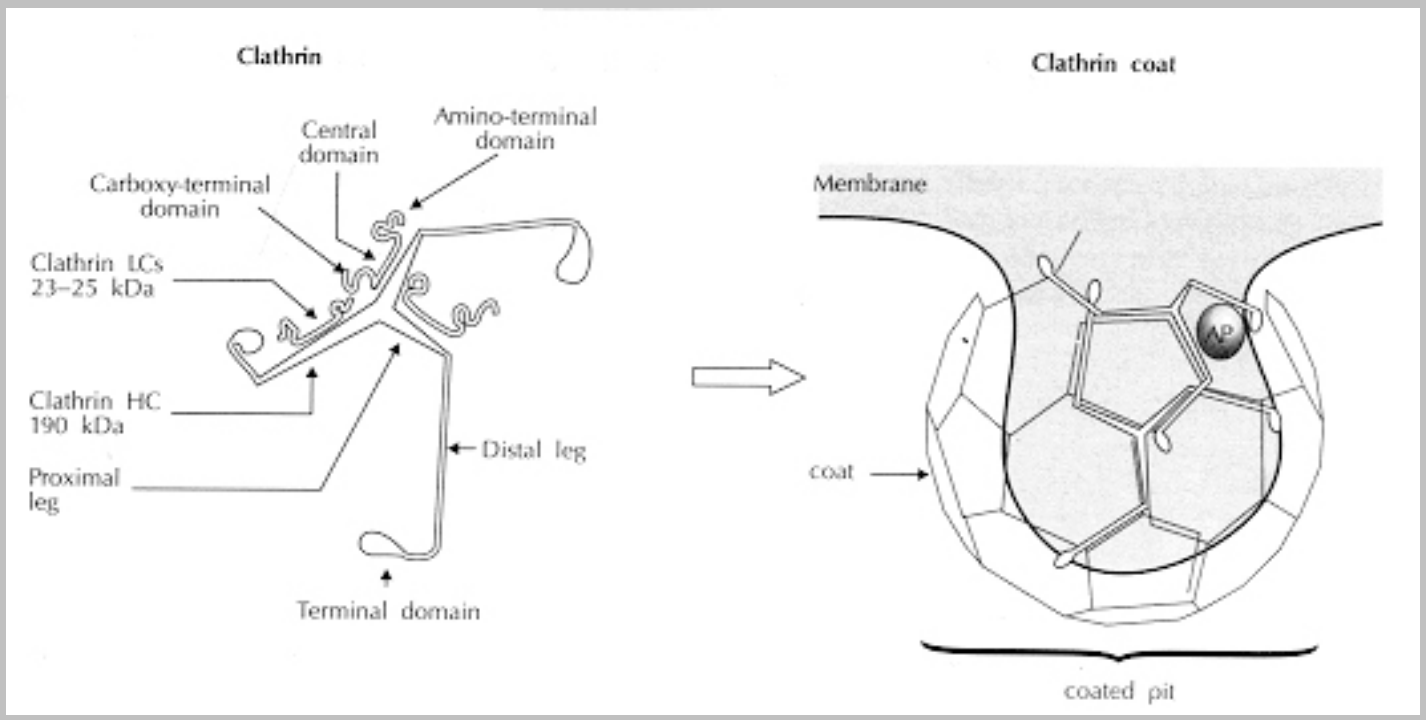


Fig. 6 Representation of the heavy (HC) and light chains (LC) of a clathrin trimer free in solution and within a clathrin lattice. The APs are located beneath the clathrin edges. From Kirchhuasen, 1993, reproduced by permission.

Clathrin can be readily removed from coated vesicles which bind it noncovalently. Under special conditions, free clathrin spontaneously assembles into a network of hexagons and pentagons similar to the basket-like arrangement present in coated vesicles.

In endocytosis, the clathrin coat is assembled in the cytoplasmic face of the plasma membrane, forming pits that pinch off to become vesicles. In cell cultures the production of clathrin coated vesicles takes about 1 minute (Marsh and Helenius, 1980; Gaidarov et al., 1999). However, at the synapse where speed is important the uptake can be much faster (De Camilli and Takei, 1996; Marks and McMahon, 1998).

Several clathrin-binding proteins have been shown to recognize the cytoplasmic domains of transmembrane proteins which are either cargo or receptors for soluble cargo. For soluble cargo molecules, the first step of the transport using vesicles can be considered the binding of cargo molecules to a transmembrane receptor. When preexisting pits are not involved, the receptors are then concentrated by binding to coat proteins needed for the budding of the vesicles. Cargo concentration and formation of a coat are probably coupled. After budding, the vesicles are transported, targeted and fused to the acceptor compartment.

The assembly of clathrin coat components is thought to first require a recruitment of the adaptor AP-2 and various accessory molecules molecules from the cytosol to the membrane (see Robinson, 1994; Cremona and De Camilli, 1997). The cytoplasmic tails of the receptors are thought to play a role in this recruitment (see discussion in Section IIIA). Subsequently, clathrin is thought to be bound to the AP complexes attached to the membrane. The β chains of AP-1 or AP-2 are thought to be sufficient for the interaction with clathrin and the formation of a coat. Among the needed accessory molecules required are the GTPase, dynamin, the amphysin dimer and synaptojanin 1. Synaptojanin, an inositol 5-phosphatase (e.g., McPherson et al., 1994), is a major presynaptic protein associated with endocytic coated intermediates (Ringstad et al., 1999). In neurons of synaptojanin 1 mutant mice, phosphatidyl inositol 4,5-bisphosphate (PIP_2) levels are increased and clathrin-coated vesicles accumulate in the cytomatrix-rich area that surrounds the synaptic vesicle cluster in nerve endings (Cremona et al., 1999). Other phosphoinositol metabolites are also is involved in endocytosis (e.g., see the role of endophilin, below). Dynamin (see below) oligomerizes into collar structures at the neck of the invaginated clathrin coated pits. A conformational change in dynamin was thought to produce the separation of the vesicle from its stalk, acting as a "*pinchase*" (Oh et al., 1998; Sweitzer and Hinshaw, 1998), however, other studies suggest an indirect regulatory role (see below). Another of these factors is Eps15, a protein associated with clathrin that binds to the α-adaptin subunit of AP-2 (e.g., Benmerah et al., 1998; Tebar et al., 1996). Another protein that binds to Eps15, one of the *epsins*, is also required for endocytosis of coated pit invagination of synaptic vesicles (Chen et al., 1998).

The epsins constitute a family of proteins which contain many binding domains. The *epsin amino-terminal homology* (ENTH) domain is present at the amino terminal and is structurally related to the VHS domain. Next to the ENTH domain is the *ubiquitin-interacting motif* (UIM). At the carboxy terminal many epsins contain motifs that bind to clathrin, accessory components and adaptors such as AP2 (see

Brett et al., 2002). Epsin binds to the EH-domain of proteins (see table 2 of Chapter 6) via two to four *asparagine*, *proline*, *phenylalanine* (NPF) motifs. The tripeptide Asp-Pro-Trp (DPW) binds to α-adaptin. and other motifs. The epsins are also thought to bind to specific cargoes (De Camilli et al., 2002; Shih et al., 2002) and might be considered adaptors. The UIM is required for ubiquitin binding and protein transport (Shih et al., 2002). Conjugation to ubiquitin has a role in marking a protein for endocytotic uptake and targeting (see above)

Phosphoinositol derivatives are linked to endocytosis. AP-2 and AP180 as well as epsin bind to phosphorylated metabolites of inositol such as phosphatidyl inositol bisphosphate (PIP_2) and phosphatidyl inositol trisphosphate (IP_3). PIP_3 binds to AP-2 when this complex is assembled into a coat structure (Gaidarov et al., 1996).

The assembly of the various components is regulated by phosphorylation. Dephosphorylation promotes the assembly of the components. Phosphorylation generally inhibits the assembly (see Wilde and Brodsky, 1996; Slepnev et al., 1998). For example, phosphorylation of the tyrosine signal residues blocks the interaction (Boll et al., 1996; Ohno et al., 1996). Changes in AP-2 can also be regulatory. The interaction of AP-2 with clathrin increases the strength of the binding to tyrosine-containing signals (Rapoport, et al., 1997), thereby favoring the assembly of receptors to partially assembled coated pits. Furthermore, AP-2 can be phosphorylated in vivo (Wilde and Brodsky, 1996). 3'-phosphorylated phosphoinositides increase the binding of AP-2 and tyrosine signals (Rapoport et al., 1997).

C. Sorting of Proteins

Several clathrin-binding proteins have been shown to recognize the cytoplasmic domains of transmembrane proteins which are either cargo or receptors for soluble cargo. There are also indications that the LDL receptor is recognized directly by clathrin (Kibbey, 1998). All three AP-complexes have been shown to recognize the sorting signals for transmembrane proteins Yxxϕ (x is any amino acid and ϕ a bulky hydrophobic amino acid, such as Leu, Ile, Phe, Val and Met) and the dileucine signals (see Kirchhausen, 1999). The tyrosine (Y) is present within a loop structure called a "tight turn" (Collawn et al., 1990; Eberle et al., 1991; Bansal and Gierasch, 1991). β-Arrestin and β-arrestin 2 bind to the cytoplasmic domain of ligand activated G-protein-coupled receptors and recruit them into the coated vesicles (see Kirchhausen, 1999). Other types of signal include a cluster of acid amino acids (Pond et al., 1995; Voorhees et al., 1995; Jones et al., 1995) and the di-lysine signal KKFF (Itin et al., 1995). The KKFF signal is present in the protein VIP36 that cycles between the plasma membrane and the Golgi, and the ER protein, ERGIC-53, that cycles in the same way when overexpressed. Ubiquitin added to lysine residues in plasma membrane proteins also serves as an internalization signal (Hicke and Riezman, 1996; Strous et al., 1996). Coat proteins may be involved in these processes as well. In addition, in the TGN pathway, GGAs (*Golgi-localized, γ-adaptin ear homology and ADP-ribosylation-factor-binding proteins*) recognize acid-cluster-dileucine motifs of sortilin (a multi-ligand receptor) and mannose-6-phosphate receptors (e.g., Nielsen et al., 2001; Puertollano et al., 2001). The μ2 subunit of AP-2 binds to the

tyrosine-sorting motifs (Owen and Evans, 1998; Bonifacino and Dell'Angelica, 1999). Subsets of the signals are responsible for later targeting to lysosomes, lysosomal-endosomal compartments, the TGN or the basolateral membrane in polar cells (see Mellman, 1996; Marks et al., 1997). Supposedly, they are recognized by complexes similar to the APs.

X-ray crystallography has also been used to examine the epidermal growth factor (EGF) receptor and the *trans*-Golgi protein TGN38, while attached to the signal recognition domain of the μ2 subunit of AP-2 (Owen and Evans, 1998). In the complex, the signal peptides acquire an extended conformation rather than the expected tight turn. The hydrophobic pockets of μ2 bind the tyrosine and leucine of the peptides.

D. Budding and Disassembly

The process of clathrin vesicle budding has been studied in vitro in systems consisting of broken cells, either mechanically disrupted or broken by freeze thawing. These systems have permitted examination of the biochemistry of pit and vesicle formation. Aside from its structural components, coated pit assembly requires ATP and cytosol (Smythe et al., 1989, Schmid and Smythe, 1991). Budding and vesicle formation involving clathrin differs significantly from that occurring in the TGN. The events of assembly and disassembly of clathrin coated structures during endocytosis can be outlined as shown in Fig. 7 (Schmid and Damke, 1995). An involvement of GTP (and therefore GTP-binding proteins such as dynamin) is shown in steps *3* and *4* of the diagram. An involvement of GTP-binding proteins Rho and Rac in receptor mediated endocytosis of tranferrin was demonstrated by the inhibition produced in HeLa cells transfected with a Rac or Rho mutants (Lamaze et al., 1996).

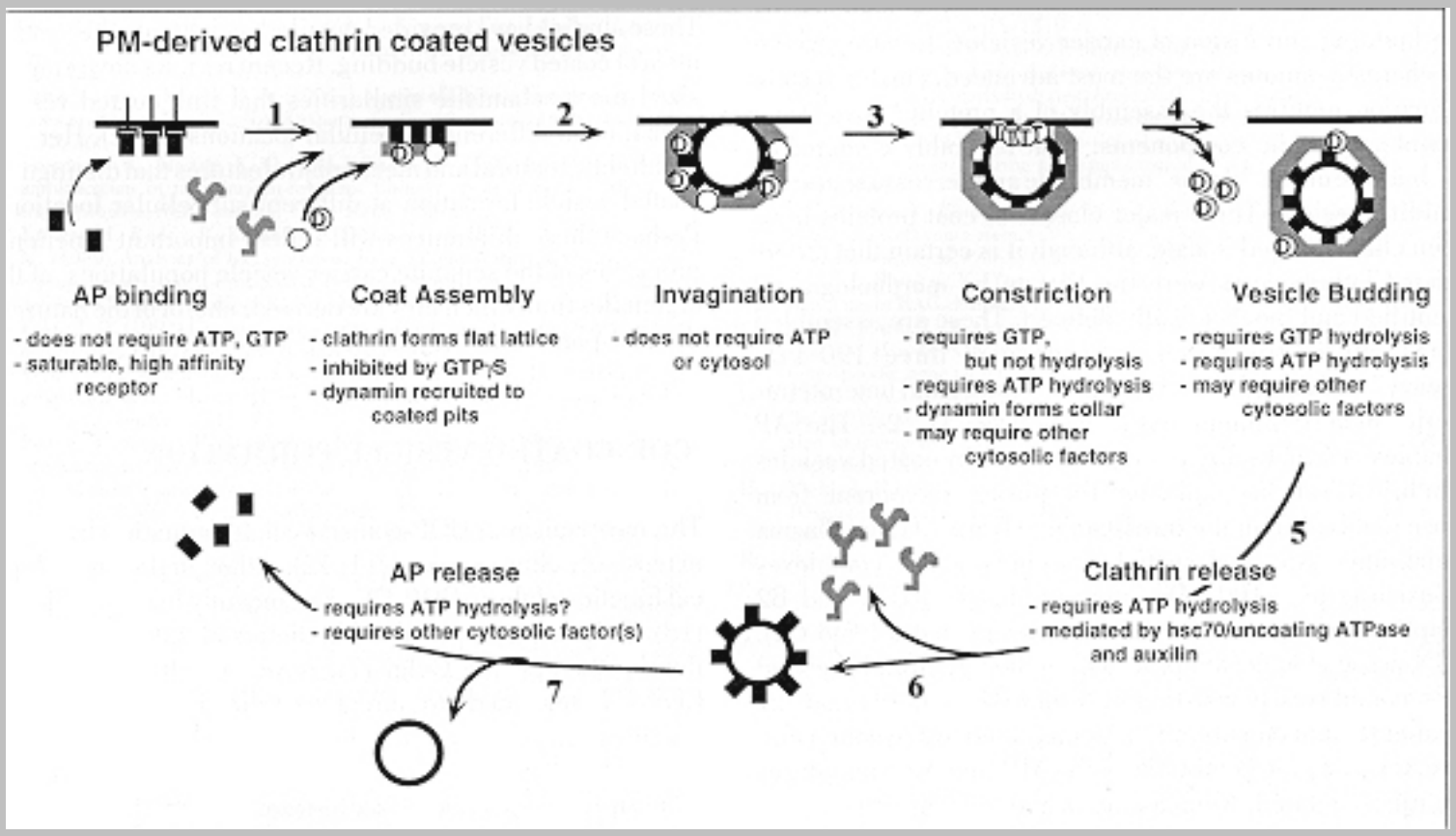


Fig. 7 Summary of events during formation of coated pits and clathrin-coated vesicles. The open circles represent the GTP-binding molecule dynamin. The letter D indicates that it binds GDP, and T indicates that it binds GTP. Modified from Schmid and Damke, 1995. Reproduced by permission.

The mechanics of vesicle excision are still not clear. A cytoplasmic GTPase, *dynamin*, is obviously involved (see Schmid et al., 1998). Dynamin is a member of a subfamily of GTPases (see van der Bliek, 1999a), present as a homotetramer in its native state (Muhlberg et al., 1997). Mutants with a defective dynamin have a defect in endocytosis. In addition, dynamin is found at the neck of invaginating coated pits (see Schmid et al., 1998). Dynamin is discussed in relation to the formation of intracellular cargo vesicles in Chapter 11. This GTPase is a homotetramer of 100 kDa. Dynamin has several domains (see Muhlberg et al., 1997). Binding to these domains regulates the GTPase activity by regulating the stability or the assembly of the oligomer (e.g., Warnock et al., 1996). Dynamin binds to microtubules, acidic phospholipids and phosphatidyl inositol 4,5 bisphosphate ($PI(4,5)P_2$) containing vesicles, oligomeric Src homology domain (SH)-containing proteins and the $\beta\gamma$ subunits of hereromeric G-proteins.

Dynamin can be found at the neck of tubular invaginations or buds in the process of pinching off vesicles. It may provide the force required for vesicle scission from tubules (Takei et al., 1998; Sweitzer and Hinshaw, 1988) as shown by the observation that purified recombinant dynamin binds to acidic lipid vesicles in a regular pattern to form helical tubes. The pinching off could result from constriction of the dynamin structures upon the addition of GTP (Oh et al., 1998; Sweitzer and Hinshaw, 1998), thereby producing vesicles. Alternatively, an elongation of the dynamin spiral (a spring-like conformational change) could cause the vesicle fission (Stowell et al., 1999). However, other observations suggest that the role is indirect. Dynamin in the presence of GTP, is not sufficient to produce vesicles in perforated cells (see Schmid, 1997), possibly because other components are also needed. The role of GTP hydrolysis may also be indirect (see van der Bliek, 1999b), where dynamin acts as a switch rather than a "pinchase".

Sever et al. (1999) produced two dynamin mutants. One of these was defective in its *GTPase effector domain* (GED). The GED is needed to increase the GTPase activity when the complex forms rings around lipid structures. The other dynamin mutant interferes with the self assembly. However the two were found to accelerate tranferrin mediated endocytosis of perforated cultured cells in the presence of a cytosolic fraction. These findings have been interpreted to negate a direct role of dynamin in the constriction of tubules and formation of vesicles. However, it is difficult to find fault with the experiments using synthetic liposomes and purified recombinant dynamin (Sweitzer and Hinshaw, 1998). It is conceivable that some component of the cytosolic extract used by Sever et al. (1999) compensates for the defect in the mutant dynamin.

Vertebrates have a minimum of three dynamin isoforms and more than 25 variants are produced in rats by alternative splicing (Cao et al., 1998). Dynamin-1 is neuron specific, dynamin-2 is expressed widely in various tissues but not in neurons and dynamin-3, expressed in testes and neurons, is also present in many other tissues. Experiments in which dynamin was attached to green fluorescent protein (see Chapter 1)

(Cao et al., 1998) show that the various variants localize to different sites inside the cell. Some dynamins localized in clathrin coated vesicles and others on vesicles not coated with clathrin. Apparently two very short amino acid domains in the middle of the molecule control their targeting. Dynamin-2 has a role in vesicle formation in clathrin dependent and independent pathways. In vitro addition of peptide-specific anti-dynamin antibodies to the assay mixture inhibited exocytic and clathrin-coated vesicle formation from the TGN (Jones et al., 1998). The microinjection of antibodies to dynamin-2 into cultured hepatocyes (Henley et al., 1998) inhibited clathrin-mediated endocytosis and induced the formation of long plasmalemmal invaginations with attached clathrin-coated pits. In addition, invaginations resembling caveolae (see the next section, below) accumulated at the plasma membrane and caveola-mediated endocytosis of labeled cholera toxin B was inhibited.

A second protein, *amphiphysin*, has been found to be involved in the recruitment of dynamin. Amphiphysin binds to both AP2 and dynamin. Disruption of the interaction between dynamin and amphiphysin (e.g., by recombinant amphiphysin SH3 domains that bind to dynamin) blocks recruitment of dynamin to coated pits and blocks endocytosis (Wigge et al., 1997; Shupliakov et al., 1997). In nerve, the interaction of the two is negatively regulated by phosphorylation of serine residues of dynamin (Slepnev et al., 1998).

A rapid endocytosis is of vital importance at synapses which require the recycling of vesicles in order to repackage synaptic vesicles (see Chapter 22). These vesicles would then be poised to carry out a new signal across the vesicle. One of the components required for endocytosis in nerve tissue is *endophilin I*. Depletion of endophilin I (Schmidt et al., 1999) in perforated cells blocks the formation of synaptic-like microvesicles. Similarly, the injection of antibodies to endophilin block clathrin-mediated endocytosis (Ringstad et al., 1999). Endophilin I is a lysophosphatic acid acyl transferase which catalyzes the formation of phosphatidic acid (two acyl chains) from lysophosphatic acid (one acyl chain) and arachidonoylCoA. Endophilin I is present in the cytoplasmic leaflet of the plasma membrane and may play a role in the formation of vesicles by altering the membrane curvature to facilitate invagination during endocytosis (see Scales and Scheller, 1999; Schmidt et al., 1999). Presumably this would be accomplished by increasing the surface area of the cytoplasmic leaflet (to become the external leaflet of the vesicle). See also Chapter 4 for a discussion of this question

At synapses, during endocytosis, clathrin and AP-2 have been shown to interact with elements present in the newly incorporated membranes following exocytosis (Gad et al., 1998). These include *synaptotagmin* which binds to AP-2 and is present in synaptic vesicles (Zhang et al., 1994). Synaptotagmins are Ca^{2+} and phospholipid-binding proteins present in all cells and tissues in membranes from which clathrin-AP2-coated vesicles are formed (e.g., Li et al., 1995; Sugita et al., 2001). They have been shown to serve as docking sites for AP2 at the plasma membrane (e.g., Haucke and de Camilli., 1999). Genetic experiments in mice, *Drosophila* and the nematode *Caenorhabditis elegans*, show that synaptotagmins are involved in both endo-and exocytosis (Littleton et al., 1994; Geppert et al., 1994; Jorgensen et al., 1995). In addition, to the interaction with synaptotagmin, we already saw that phosphoinositides bind to several proteins involved in endocytosis, e.g., the α subunit of AP-2 (Beck and Keen, 1991; Gaidarov and Keen 1999)

suggesting an involvement of these lipids in the mechanics of endocytosis.

No less important than the assembly of clathrin is its disassembly. The uncoating of the clathrin coated vesicles needed for subsequent processing proceeds by a process involving the chaperone-ATPase hsp70c (Ungewickell et al., 1995). Auxilin mediates the binding of hsp70 to the clathrin cage. Synaptojanin 1, a phosphatase degrades phosphatidylinositol (4,5)-bisphosphate, the latter being essential for assembly. Auxilins 1 and 2 recruit the chaperone Hsc70 and stimulate its ATPase activity to depolymerize clathrin (see Slepnev and de Camilli, 2000).

Apart of the special case of synaptic vesicles, which are recycled, what is the fate of uncoated vesicles and what is their relationship to clathrin coated vesicles? The fates of cholera toxin (CT) and α_2-macroglobulin (α_2m) added to fibroblasts in cell culture were followed simultaneously with the EM by labelling them with different size gold particles (7 nm and 15 nm in diameter respectively). Both ligands bind to specific cell surface receptors. The CT first bound to non-coated pits and then appeared in a network of tubules distinct from the Golgi apparatus. In contrast, the α_2m bound to coated pits was taken up by coated vesicles and then appeared in the network of tubules. Despite being packaged in distinct vesicles, the two macromolecules were found in the same compartments beginning with their appearance in the tubular network. Eventually both were present in the multivesicular bodies (which probably correspond to lysosomes). The results are summarized in Fig. 8 (Tran et al., 1987). In this figure, the % of the gold particles are shown (the ordinate) in relation to time (the abscissa).

New information has surfaced on mechanisms of endocytosis (see Kirchhausen,2000; Nichols and Lippincott-Schwartz, 2001). Studies taking advantage of mutants unable to form clathrin-vesicles have provided considerable evidence for clathrin-independent endocytosis (see Puri et al., 2001; Nichols et al., 2001; Lamaze et al., 2001). In addition, techniques that allow tagging proteins and lipids with fluorescent labels has introduced the possibility of visualizing the movement of these components (Pelkmans et al., 2001; Puri et al., 2001; Nichols et al., 2001).

Many of the non-clathrin based processes have been found to involve caveolin or lipid rafts. (see Nichols et al., 2001). Their role can be defined by interfering with their formation. Cholesterol deprivation (which may also have other effects) does not interfere with clathrin-mediated endocytosis but blocks the caveolin-linked pathways (e.g., Puri et al., 2001; Nichols et al., 2001. Caveolae and lipid rafts (section V) are discussed below.

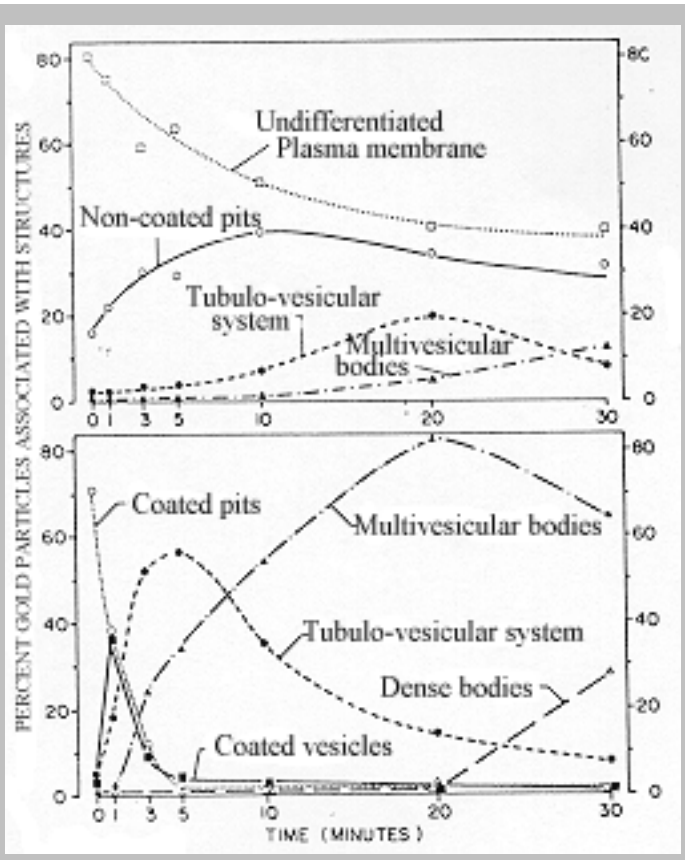


Fig. 8 Relationship of CT-gold (7-15 nm) (upper) and α_2m-gold (lower) with the various locations in fibroblasts. First incubation at 4ºC for two hours and then without labelled ligand at 22 ºC. Tran et al., 1987, reproduced by permission.

V. CAVEOLAE, RAFTS AND OTHER MEMBRANE PATCHES

Many studies have centered on coated pits. However, there are other specialized areas at the surface of cells. Some of these have the appearance of flasks 50 to 100 nm in diameter. These structures have been called *caveolae* (Yamada, 1955), meaning "little caves". They are probably the same structures as those described by Palade and Bruns (1958) for endothelial cells, which they called *plasmalemmal vesicles*. Their importance in endocytosis and receptor mediated signaling is just beginning to be appreciated (e.g., see Parton, 1996; Anderson, 1998). They are likely to constitute a system parallel to that of the clathrin coated pits and vesicles. With most standard EM techniques, caveolae and vesicles derived from caveolae appear uncoated. However, high resolution EM and freeze-deep etch techniques have shown the caveolae have a distinctive cytoplasmic coat of delicate filaments arranged as striations (Peters et al., 1982, Izumi et al., 1988). The striations appear as ridges or strands 6 to 10 nm in width and are seen most clearly after treatment with the detergent saponin (which presumably removes other proteins). The striped structures were enhanced by treatment with subfragment-1 (S1) of myosin or the mushroom toxin phalloidin, suggesting an involvement of actin. Actin is discussed in relation to endocytosis in Section IX and in relation to cell movement in later chapters (e.g., in Chapter 23 and Chapter 24, Section IA and Section IVA). Phalloidin and S1 bind to F-actin and phalloidin prevents its depolymerization. The distinct protein present in the caveolar membranes is *caveolin* (e.g. see Schnitzer et al. 1995b). Whether all uncoated

invaginations or vesicles can be considered part of the caveolar system is not clear. Caveolae have been isolated by a variety of methods, some in the absence of detergents (e.g., Smart et al., 1995b; Song et al., 1996).

As important as caveolae are likely to be, mice lacking caveolae (because of disruption of caveolin-1) have been shown to survive, although defective in nitric oxide and calcium signaling in the cardiovascular system. These mice exhibit defects in endothelium-dependent relaxation, contractility, maintenance of myogenic tone and exhibit a thickening of alvaeolar septa in the lungs (Drab et al., 2001). However, all of these defects would be in harmony with the suspected role of caveolae in organizing signaling pathways in the cell (see below).

Early studies suggested the presence of membrane patches rich in glycosphingolipids found to be detergent (such as Triton) insoluble. Structurally, these fractions were found to contain vesicles 50 to 100 nm in diameter, similar to caveolae. These domains were found to contain cholesterol, glycosphingolipids, *glycosylphosphatidylinositol*- (GPI)-linked proteins (see Chapter 4) and caveolin.

Some studies challenge the idea that the supposed caveolar components are present in clusters. The results of these studies suggest that at least some of the components are distributed randomly throughout the plasma membrane. They attribute the earlier results on artifacts produced by the use of detergent or cross-linking by antibodies. However, a very strong case can be made for the clustering of GPI-anchored proteins in caveolae (see discussion in Chapter 4).

Caveolin is a 21-24-kDa protein, the principal component of caveolar membranes. Unlike clathrin, the hydrophobicity plot of clathrin indicates a transmembrane sequence (Glenney, 1992), a conclusion supported by the accessibility of the protein in intact cells to chemicals from either the external or the cytoplasmic surface of the plasma membrane (see Chapter 4). The hydrophobic domain contains 33 amino acids and the carboxy- and amino-terminals are free in the cytoplasm. Recently, caveolin has been recognized as a family of proteins (see Parton, 1996) and the caveolin originally studied has been renamed caveolin-1. In mammals, there are three distinct caveolin genes coding for caveolin-1, -2 and -3. In addition, there are two different isoforms for caveolin 1, Cav-1α and Cav-1β produced by alternative initiation during translation. Caveolin-1 and 2 are most abundantly expressed in endothelial cells, smooth muscle cells, skeletal myoblasts, fibroblasts, and differentiated adipocytes (Schereret al. 1997). Caveolin-3 is expressed mostly in muscle (Tang et al., 1996). Purified caveolin homo-oligomers have the capacity to self-associate into caveolae-like structures (Sargiacomo et al., 1995). They bind cholesterol with 1:1 stoichiometry (Murata et al., 1995) and insert into model lipid membranes only in the presence of cholesterol. In addition, caveolin-1 also binds fatty acid (Trigatti et al., 1999). Caveolin 1 and 2 form hetero-oligomeric complexes of high molecular weight (14-16 monomeric units per oligomer) and localize to caveolae as shown by immunoelectron microscopy (Scherer et al., 1997).

Caveolin and cholesterol dynamics are clearly interrelated. The synthesis of caveolin depends on cholesterol concentration: caveolin mRNA levels in culture cells dropped to one-sixth of control levels

after treatment an inhibitor of cholesterol synthesis, or a cholesterol sequestering drug (e.g., Hailstones et al., 1998). On the other hand, increases in caveolin increase cholesterol transport Cultured cells transport new cholesterol to caveolae with a half-time of approximately 10 min (Smart et al., 1996). The cholesterol then rapidly flows from caveolae to non-caveolar membranes. Cholesterol moved out of caveolae even when the supply of fresh cholesterol from the ER was interrupted.

Some of the interactions are with the hydrophobic domain of caveolin, thought to traverse the membrane (Wary et al., 1996; Das et al., 1999). Another domain close to the insertion into the membrane (amino acids 80-100) binds to variety of signal transducing molecules (see Okamoto et al, 1998) including tyrosine kinase receptors, nitric oxide synthase and heterotrimeric G proteins. This domain, called the *caveolin scaffolding domain* was used as receptor to select *caveolin-binding peptide ligands* (Couet et al., 1997) from random sequences. Two similar caveolin-binding motifs were found [ϕXϕXXXXϕ and ϕXXXXϕXXϕ; ϕ can be any aromatic amino acid (Trp, Phy or Tyr)]. These motifs are present in most caveolae-associated proteins. The scaffolding domain is apparently not used in recruitment (Mineo et al. 1999) but is thought to have a role in modulating transduction (see Okamoto et al., 1998).

The role of the various caveolin-1 domains in targeting was studied systematically by introducing mutations in caveolin-cDNA followed by transfection of cells in culture. (Machleidt et al., 2000). The amino acid domain between amino acid 66 and 70 was found to be required for exit from the ER. The domain between amino acids 71 and 80 was found to control the incorporation of caveolin-1 into detergent resistant regions of the Golgi (presumably rafts). The domains of amino acids 91 to 100 and 134 to 154 were needed for oligomerization and exit from the Golgi apparatus. The fate of the caveolin mutants or wild type were followed using immunofluorescence (see Chapter 1).

Caveolae have a specific lipid composition, many lipid modified proteins, receptors and signal-transducing molecules. The lipid composition is high in glycosphingolipids, sphingomyelin and cholesterol. Proteins attached to either GPI or fatty acids (see Chapter 4) are enriched in caveolae. Mutations that remove the GPI-anchor (e.g., Ritter et al., 1995) or protein acylation (e.g., Robbins et al., 1995) redistribute the proteins to other environments. In the cell, caveolin is associated with cholesterol (e.g., Li et al., 1996) and cholesterol stabilizes caveolin oligomers (Monier et al., 1995) suggesting that the two must function together to provide a coat. The lipid core of caveolae forms in the transitional region of the Golgi (e.g., Lisanti et al., 1993). The proteins attached by GPI anchors and caveolin synthesized in the ER are incorporated into this lipid core (see Lisanti et al., 1993). Caveolae reach the cell surface via exocytotic vesicles (Dupree et al., 1993). Bidirectional ER-to-caveolae transport of cholesterol involves caveolin-1 (e.g., see Smart et al., 1994; 1996). Accordingly, caveolin-1 (then called VIP21) (see Glenney, 1992) was found in the Golgi, the plasma membrane and vesicular structures and appears to be involved in the machinery of vesicular transport (Kurzchalia et al., 1992).

Tracer studies have implicated uncoated vesicles in transcytosis of LDL (see Simionescu, 1983), serum albumin and other molecules across capillary endothelial cells (Ghitescu et al., 1986; Vasile et al., 1983).

Caveolae are also involved in the endocytotic uptake of macromolecules (Tran et al., 1987; Keller et al., 1992). Transcellular transport involves vesicles originating from caveolae (e.g., see Simionescu et al., 1975; Schnitzer et al., 1994, 1996) that may also form channels across cells (Simionescu et al., 1975). Caveolae are dynamic, actively interacting with the endocytic compartment including early-sorting endosomes (e.g., see Pol et al., 1999). A reconstituted cell-free system capable of forming vesicles from caveolae has been developed using endothelial plasma membranes (Schnitzer et al., 1996).

Caveolae have a role in clustering glycosylphosphatidylinositol-linked receptors. Unclustered and clustered GPI-proteins are thought to be in dynamic equilibrium (van Meer et al., 1987). The unclustered proteins are highly mobile in the plane of the membrane (Zhang et al., 1991). The mobile fraction can bind to transmembrane proteins and be internalized. These results suggest that the GPI-anchored proteins spend part of the time in caveolae and part of the time free; the latter can interact with receptors so that they might be internalized in coated pits (Anderson, 1993).

Components important in Ca^{2+} transport have also been found in caveolae (Fujimoto, 1992, 1993). Immunogold EM studies using antibodies specific to the inositol 1,4,5-triphosphate (IP_3) receptor and Ca^{2+}-ATPase revealed the presence of these proteins in smooth invaginations of the plasma membrane of a variety of cells. We saw in Chapter 7 that IP_3 receptors function as a Ca^{2+}-channel, at least inside the cell.

Caveolae are involved in signaling (e.g., Lisanti et al., 1995; see Okamoto et al., 1998; Razani et al., 2000). In at least some cases, glycosylphosphatidylinositol (GPI) anchored receptor proteins become associated with caveolae only after binding to their ligand (or an antibody) (Sevinsky et al., 1996).). Receptor and non-receptor tyrosine kinases have been found in caveolae (see Anderson, 1998, his table 1), e.g., by immunofluorescence. Caveolins bind to signaling molecules such as heterotrimeric G protein subunits, Src kinases, and Raf,. G-protein-coupled receptors such as inositol 1,4,5-trisphosphate receptors, endothelin, bradykinin, muscarinic acetylcholine, and adrenergic receptors. Caveolae are the principal location of *platelet derived growth factor* (PDGF) receptors at the surface of platelets and many caveolar proteins are phosphorylated when PDGF binds to its receptor suggesting that many proteins involved in the signaling cascade are also in caveolae (Liu et al., 1996). GTP-binding proteins (G-proteins) (Sargiacomo et al., 1993), receptors and effectors appear to be enriched in caveolae. In addition to this direct role, some of the lipids and lipid-anchored proteins are sources of signaling intermediaries for the formation of ceramide, inositol trisphosphate (IP_3) and inositol phosphoglycans. Many G-coupled receptors are localized or internalized at uncoated invaginations or vesicles presumed to be caveolae (Montesano et al., 1982, Raposo et al., 1987, 1989; Strosberg et al., 1991). A role of caveolae in signal transduction is also strongly supported by the observation that antibodies against different GPI-anchored proteins activate cells (e.g. Thompson et al., 1989). We saw in Chapter 6 that cross-linking by antibodies activates receptors.

Mutations of the gene coding for caveolin 3 have been implicated in limb-girdle muscular dystrophy and

in rippling muscle disease. Caveolin 3 truncation mutants have been found to inhibit signaling (Roy et al., 1999) and caveolin 3 point mutants have been found to inhibit H-Ras-dependent signaling. The inhibition is rescued by cholesterol addition (Carozzi et al., 2002). When activated H-Ras cause significant changes in cell shape and cytoskeletal organization.

What is the role of caveolae in signaling? The caveolins are thought to act as scaffolds for the assembly of signaling complexes. In this way the assembled complexes can be quickly deployed where needed. In addition, caveolae may have an inhibitory regulatory function in removing these complexes from the active pool (see Okamoto et al., 1998). However, in some cases the signaling molecules can be shown to be activated in caveolae so that the assembling may actually permit a more effective activation of the complexes (e.g., see Chambliss et al., 2000; Peiro et al., 2000).

The internalization of receptors requires protein kinase C (PKC)α (see Chapter 7) and serine/threonine phosphatase activity in caveolae (Smart et al., 1995a). A 90-kDa protein is the substrate for these enzymes. The carrier vesicles derived from caveolae retain much of their structure and can function as exocytotic vesicles (see Anderson, 1993).

Until relatively recently, the link between caveolae and intracellular vesicles was unclear because the pinching off of vesicles had not been observed in detail and caveolin containing vesicles had not been studied sufficiently. The formation of vesicles from caveolae by a pinching off process was observed in permeabilized cells (Schnitzer et al., 1996). Caveolae can be isolated after mechanical disruption of endothelial tissues and after GTP-induced formation of vesicles (Schnitzer et al., 1995a). They have been found to contain the machinery for receptor mediated endocytosis and transcytosis as well as vesicle budding, docking and fusion (see Schnitzer, 1995a). As in the case of clathrin mediated endocytosis, the pinching off of vesicles was found to require cytosolic dynamin (see above section) and the hydrolysis of GTP (also required for formation of trans-Golgi vesicles, Jones et al., 1998). In addition, dynamin was found to be concentrated in caveolae (Oh et al., 1998; Hendley et al. 1998). The emerging picture suggests that the caveolar system behaves in an entirely analogous way to that of coated pits as represented in Fig. 7.

The process of vesicle formation from caveolae has been studied by observing the endocytosis elicited by simian virus 40 (SV40) in monkey kidney normal cells (CV-1) in culture (Pelkmans et al., 2002). The movement of the various proteins was followed by tagging them with fluorescent proteins [green fluorescent protein (GFP) or yellow fluorescent protein (YFP); see Chapter 1]. After binding to the caveolae, SV40 was found to induce the disassembly of stress fibers. The actin and dynamin II were then recruited to the caveolae where their recruitment lead to actin "tail" formation. The formation of tails corresponds to an assembly of actin molecules to produce motion (see Chapter 24). Cholesterol and the phosphorylation of the proteins of caveolae by tyrosine kinases was found to be required for endocytosis to occur.

Anderson et al. (1992) have hypothesized that caveolae are active in the endocytotic uptake of low molecular weight substances, a process referred to as *potocytosis* (*poto* meaning to drink). A role of potocytosis in the transport of low molecular weight substances is primarily based on the study of the uptake of 5-methyltetrahydrofolate. The properties of the folate transport system have been studied in folate-depleted cells. Folate binds to a specific receptors. At 0ºC half of the receptors bind folate. At 37ºC, however, after 1 hour, internal and external receptors were found to bind folate. The two could be distinguished because externally bound folate is released by acid wash. Eventually, all the folate was inside the cell and accumulation stopped. The release into the cytoplasm required an acid pH and was mediated by an anion transporter. The process in one distinct from endocytosis: the folate receptors have never been found in endocytotic vesicles. The receptors are segregated in caveolae and the recycling of the receptors closely matches the sequestration of materials in the caveolae. The involvement of an anion carrier is based on the observation that the release is probenecid sensitive. Probenecid is a drug which blocks tubular secretion (i.e. active transport) of many anionic drugs. The transport is thought to follow the steps outlined by the model shown in Fig. 9 (Rothberg et al., 1990). In step 1, the folate molecules are bound to their receptors. In step 2, the caveola closes and in step 3 the caveolar interior is acidified, so that the folate molecules detach from the receptors. The folate molecules are transported to the cytoplasm in step 4, and the partially empty caveola opens again in step 5.

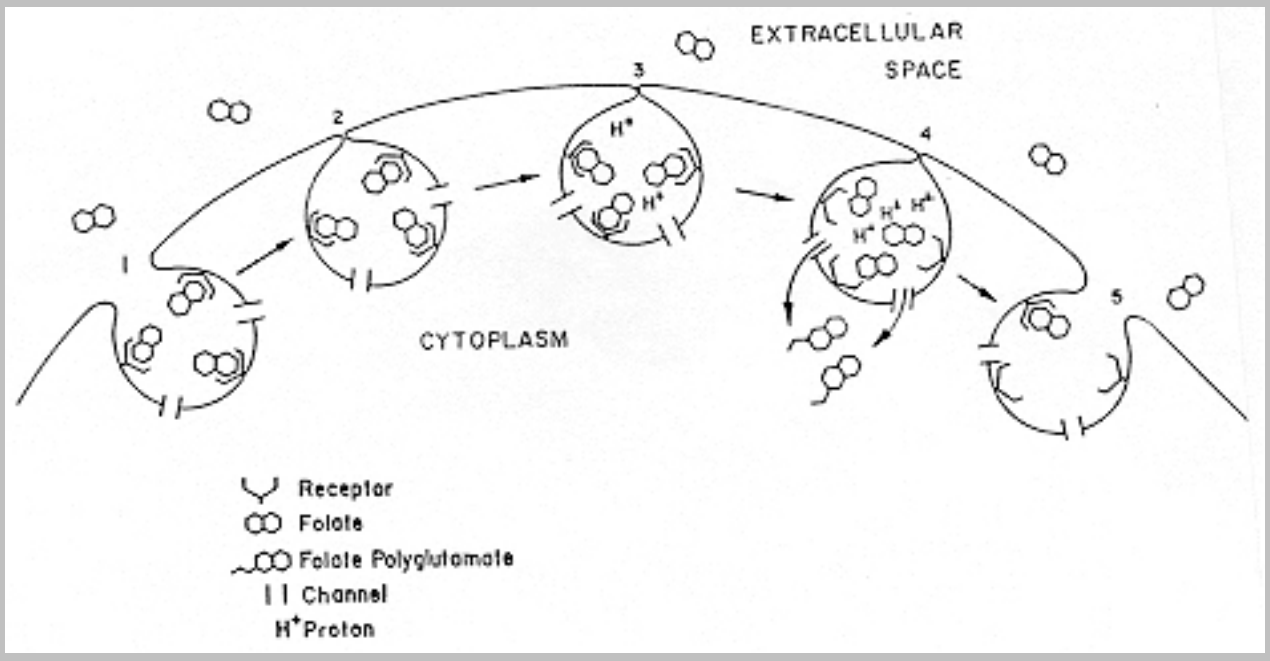


Fig. 9 Model of receptor coupled transmembrane transport for folate in caveolae. Reproduced from Rothberg et al., *Journal of Cell Biology* by copyright &copy1990. Reproduced by permission of The Rockefeller University Press.

Some of the clathrin-independent pathways carry lipids and GPI-anchored proteins to the Golgi apparatus (Nichols et al., 2001). In at least some cases, caveolae are not likely to be involved because the processes are dynamin independent (e.g., Vickery and von Zastrow, 1999; Roseberry and Hosey, 2000). In view of the involvement of GPI, rafts are thought to be reponsible for this transport (Puri et al., 2001; Nichols et al., 2001). To complicate this scenario even further, different raft markers follow different pathways.

Some GPI-anchored proteins cycle between the cell surface and the Golgi, others cycle to the endosomes (Nichols et al., 2001; Mayor et al., 1998).

A study of proteins anchored to GPI (the folate receptor, the decay accelerating factor and GFP-linked to GPI) taken up by endocytosis found them recycling to an endosomal compartment by a pathway not involving either clathrin, caveolae or the Golgi apparatus, (Sabharanjak et al., 2002). The transfer was mediated by tubular-vesicular elements distinct from those involved with the Golgi. The pathway was dependent on the GTPase Cdc42 .

In addition to the possible role of caveolae and rafts, other lipid specializations are thought to have a role in the localization of components. Late endosomes contain a internal membranes in their lumen. The distribution within these organelles is distinct. Some proteins are present in the internal membranes, whereas others are localized in the limiting membrane. The internal membranes form a specialized domain with a high content of lysobisphosphatidic acid 2-phosphate (LBPA) (Kobayashi et al., 1999). LBPA is also the antigen for human antibodies associated with the antiphospholipid syndrome. The membranes are responsible for the sorting of the receptors for insulin-like growth factor 2 and lysosomal enzymes. In addition, the LBPA-rich domain has a role in cholesterol transport. Cholesterol derived from LDL was found to be processed by late endosomes where cholesterol accumulates in the genetic disease Niemann-Pick type C (NPC) and in cells treated with drugs that mimic NPC (Kobayashi et al., 1999). Another lipid, phosphatidylinositol 3-phosphate has been found in limiting membranes of early endosomes and in the internal membranes of the multivesicular bodies (Gillooly et al., 2000). A variety of proteins containing the FYVE and the PX (PHOX) homology domains which bind to phosphatidylinositol 3-phosphate have been shown to be present in these endosomes and supposedly may be bound to the membranes .

VI. OTHER FORMS OF ENDOCYTOSIS

Phagocytosis, the uptake of large particles by cells, is most generally thought to be a property of phagocytic protozoa or phagocytic leukocytes of the mammalian immune system such as macrophages. In protozoans, phagocytosis has a nutritional role. In mammals, one of the important roles is in immunity (see Chapter 6 and Aderem and Underhill, 1999) and apoptosis (see Chapter 2). Cell surface receptors bind to the particle to initiate phagocytosis. In protozoans, the receptors involved in phagocytosis are thought to be lectin-like components (Cohen et al., 1994). Macrophages possess several phagocytic receptors that bind conserved motifs on pathogens (e.g., the mannose receptor). In addition, pathogens are recognized by receptors after coating of the particles with complement, Ig Fc or specific antibodies (*opsonization*). Receptors include members of the IgG Fc receptor family (Mellman et al., 1983), integrins (e.g. Isberg and Tran Van Nhieu, 1994) and lectins (Ezekowitz et al., 1991). The binding to receptors is followed by polymerization of actin in the cytoplasm close to the site of particle attachment (see May and Machesky, 2001). Various small GTPases (see Chapter 11) control this step. Phosphatidylinositol 3-kinase is recruited to the plasma membrane and is involved in pseudopodia extension and phagosome formation (e.g., Vieira et al., 2001). Basically the process takes place as

follows. First the receptor binds to its ligand. Other receptors are recruited and lead to the formation of a protrusion without intervention of actin. This is then followed by recruitment of cytoskeletal components (e.g., the Arp2/3 complex) which in turn recruit actin and form a pseudopod. Then, the actin network moves the pseudopod around the target and the pseudopod engulfs the bound particles in a large membrane enclosed vacuole, the *phagosome* (see Greenberg et al., 1990, 1991). The vacuole or phagosome taken up by the macrophage undergoes maturation, which involves sequential interactions with components of the endocytic pathway, and culminates with fusion to lysosomes (see Tjelle et al., 2000) followed by digestion (e.g. Desjardins et al., 1994). The membrane component is eventually recycled. Apparently, membrane elements of the endoplasmic reticulum (Gagnon et al., 2002) fuse with the plasma membrane to provide some of the needed membrane for the formation of the vacuole. The mediation of the ER is regulated by phosphatidylinositol 3-kinase.

Clathrin-dependent endocytosis is well recognized. We have also discussed evidence for an involvement of caveolae in endocytosis. Many observations indicate endocytosis also proceeds by different mechanisms.

Macropinocytosis (see Swanson and Watts, 1995) was first observed in macrophages where surface ruffles (see Chapter 23) formed endocytotic vesicles. Since then similar events have been observed in other cells. Macropinocytosis differs from clathrin-mediated endocytosis in that vesicles are formed only at margins of cells at sites of ruffling. The size of the vesicles varies but can be as large as 5 µm in diameter, whereas coated pits are restricted to 85-110 nm because of their clathrin coating. The formation of macropinocytotic vesicles can be stimulated by binding of some growth factors. The role of macropinocytosis is not clear, although it might possibly contribute to the immune response.

As in the case of phagocytosis, actin is likely to play a role in the formation of vesicles in macropincyotis. Ruffles are formed by outwardly directed actin filaments. The actin filaments are involved in the formation of the vesicles, possibly by enclosing the vesicles as clathrin does in the case of the endocytosis mediated by coated pits. At any rate, in a later step the vacuoles lose their actin coating and are indistinguishable from other endosomal compartments.

Endocytosis of activated receptors without the involvement of clathrin has also been observed.. Interleukin 2 receptors were found to become internalized in dominant-negative mutants of eps15 (Lamaze et al., 2001) in human (HeLa)cells, mouse fibroblasts transfected with genes encoding the IL2 receptor and lymphocytes. Expression of the mutants inhibits both constitutive and ligand induced receptor-mediated endocytosis (see Benmerah et al., 1999). The receptors were not present in clathrin coated structures but were present in detergent resistant membranes. Since lymphocytes do not have caveolae the receptors are probably internalized in membrane rafts (see Chapter 4).

The biochemical requirements for non-clathrin endocytosis have generally been found to be similar to those of the clathrin-dependent process. Clathrin-independent endocytosis is temperature dependent, requires ATP, and is sensitive to sulfhydryl reagents (Sandvig et al., 1991). Activated Rho, a small GTP-

binding protein, has been shown to stimulate pinocytosis when microinjected into *Xenopus* oocytes (Schmalzing et al., 1995), and Ras and Rho have a role in membrane ruffling in fibroblasts (e.g. Ridley et al., 1992).The endocytosis of the IL2 receptors requires the presence of dynamin and is also controlled by GTPases of the Rho family (Lamaze et al., 2001).

VII. ENDOSOMES AND LYSOSOMES: INTERACTIONS

The discussion of the fate of ligands and receptors after endocytosis (section IIIC) included the digestion of material present in endosomes by lysosomal hydrolytic enzymes. The properties of either endosomes or lysosomes differ depending on their stage during their cycle. Early endosomes close to the cell surface differ from late endosomes which interact with lysosomes. It has been assumed that these differences are the result of a maturation process, and that the late lysosomes and endosomes interact by fusion. The characteristics of the endosomal compartments have been discussed above.

Recently, a view has gained favor that endosomes and lysosomes form systems of stationary organelles akin to the Golgi stacks and tubular elements. Transport vesicles shuttle between the stationary elements (e.g. Storrie, 1988, Griffiths and Gruenberg, 1991). The stationary elements act as the relay stations: the endosomes receive endocytotic transport vesicles and deliver the contents, also in transport vesicles, to the lysosomes where digestion takes place. In part, the proposals find support from structural observations. The early endosomes of many kinds of cells are constituted of cisternae, tubules, and large vesicles exhibiting coated buds. Similarly, a complex lysosomal system of cisternae, tubules and large vesicles has been described. In the case of the transfer of material taken up by endocytosis (e.g., horse radish peroxidase) the process is discontinuous, and is mediated by transport vesicles possibly transported along microtubules. In addition, the properties of the vesicles derived from the endosome system are in harmony with this general concept. Early endosomes can fuse with each other in vitro, a fusion that is regulated by a GTP-binding protein which has been implicated in intracellular transport. Direct fusion between early and late endosomes does not take place in vitro. However, the apparent endosomal-transport vesicles fuse with late endosomes.

In the late endosomes or MVBs , some proteins are found in the internal membranes while others are present only in the outer membrane (Griffiths et al., 1988). The internal membranes contain a unique lipid, lysobisphosphatidic acid, forming a specialized domain that is thought to segregate several proteins including lysosomal enzymes (Kobayashi et al., 1998). An antibody to this lipid interferes with the structure and function of these organelles, for example it blocks the recycling of the mannose-6-phosphate receptor. The fusion of the outer membrane of the MVBs with the lysosomal membrane discharges the contents and the internal vesicles into the lysosome for degradation (Futter et al., 1996). In contrast, the proteins are not to be degraded, such as receptors that are recycled, remain in the limiting membrane of the MVB by being excluded from the inner vesicles.

The *sorting nexin* (SNX) proteins (see Worby and Dixon. 2002) are either cytoplasmic or membrane bound. This protein family functions in intracellular membrane traffic. The SNXs are needed for transport

to the lysosomes and recycling of endosomes. SNX3 is present in the early endosome and its action depends on its PX domain (Xu et al., 2001) which allows it to bind to phosphoinositides (see Chapter 11). Some of the SNXs contain only a PX domain. However, others contain protein-protein binding sequences (SH3 and TPR, see Table 2, Chapter 6), hydrophobic sequences that allow interaction with membranes and G-protein regulatory sequences. Some of the phospholipids are localized at particular membrane sites and can serve as targeting signals. Some of the SNXs are likely to have a role in sorting vesicles not originating from the plasma membrane.

Two-hybrid interaction demonstrated a binding between SNX1 and the intracellular portion of the receptor tyrosine kinase EGFR (Kurten et al., 1996). SNX1 was implicated in the ligand-induced EGFR degradation where it could play a role in sorting EGFR to lysosomes. Similar proteins in yeast the proteins *vacuolar sorting proteins* (Vps) were shown to be involved in vacuolar targeting in yeast. SNX1 also binds other receptors of the tyrosine kinase family such as PDGFR, the insulin receptor, the leptin receptor and the transferrin receptor (see Worby and Dixon. 2002).

The story of endocytosis is a fascinating one which has opened many windows to our understanding of cell processes. However, there are still many blank spots that will be the focus of future studies.

VIII. TRANSCYTOSIS

Epithelial cells form monolayers lining ducts and cavities in the body. They constitute tight barriers that allow selective transport through the cells themselves. In these cells, the surface of the plasma membrane in contact with the lumen (the *apical surface*) differs significantly from the rest of the surface (the *basolateral surface*). It has a unique protein composition and a high glycosphingolipid content. These cells are said to be *polarized* (see Chapter 11).

The solute-pumps of the kidney, intestine and other tissues operate to transport solutes across the cells. For example, in the cortical collecting ducts of the kidney, Na^+ enters the cells in the direction of its electrochemical gradient at the apical surface through Na^+-channels. Simultaneously, it is actively pumped out into the interstitial fluid by the Na^+, K^+-ATPase of the basolateral membrane. Na^+ traverses the cell by diffusion so that this transport results in a net flux of Na^+ from the lumen of the ducts to the interstitial fluid.

In addition to the transport of solutes, vesicle-mediated traffic carrying materials across the cells by *transcytosis* is continuous (see Simons and Wandiger-Ness, 1990). Generally, transcytosis occurs by receptor mediated endocytosis (see Schaerer et al., 1991; Sztul et al., 1991; Rodriguez-Boulan and Powell, 1992), followed by passage of vesicles across the cells and eventually delivery to the cell surface by exocytosis. The biosynthetic pathway which transports newly synthesized proteins to apical or basolateral surfaces is obviously related to the transcytotic system (see Chapter 11) although the connections between the two are not entirely clear.

One of the in vitro epithelial cell system most commonly used for studying these two pathways is that of Madin-Darby canine kidney (MDCK) cells because in culture they form polarized monolayers when grown on permeable filters. A cultured human intestinal adenocarcinoma cell line (Caco-2) also forms monolayers and has been used for similar studies. The MDCK cells do not normally express receptors for polymeric immunoglobulin (pIgR) or transferrin (TRs). When these two kinds of receptors are introduced by transfection with recombinant retrovirus vectors containing the appropriate cDNAs (see Chapter 1), pIgRs transport bound dimeric IgAs from the basolateral to the apical surfaces (Mostov and Simister, 1985) whereas TRs move in the opposite direction (e.g., Knight et al., 1995; Odorizzi et al., 1996).

The cytoplasmic regions of the pIgR contain domains which function as trafficking signals throughout the transcytotic pathway (Mostov et al., 1992; Casanova et al., 1990, 1991; Aroeti et al., 1993). These include the tyrosine and dileucine motifs recognized by the clathrin/AP2 system. However, basolateral targeting requires other residues (Odorizzi et al., 1996; Mellman, 1996).

As discussed above, transport across the cells can reach different targets. Do the various receptor mediated transports use the same pathway? The endosomes involved in the trafficking of pIgR and IgA also contain internalized TRs (Barroso and Sztul, 1994; Apodaca et al., 1994). However TRs are returned to the basolateral surface whereas pIgR proceeds to the apical surface (Barroso and Sztul, 1994; Apodaca et al., 1994).

The exocytotic vesicles that carry the TR to the basolateral surface, arise from clathrin-AP-1 coated buds associated with the endosomes (Futter et al., 1998). Clathrin-AP-1 coated buds and vesicles had previously only been found associated with the *trans-Golgi network* (TGN) (see Chapter 11). The various components were studied with gold labelling and electron microscopy. The apical transcytotic pathway of dIgA, pIgR at 20 º shows that these components internalized at the basolateral surface and were subsequently localized in interconnected vacuoles and tubules they share with internalized TR and *epidermal growth factor receptor* (EGFR) (Gibson et al., 1998). When transferred to 37º the vacuoles form long tubules in a process that depends on the cytoplasmic microtubular system. These long tubules then form distinct cup-shaped 100nm vesicles containing pIgR. The cup-shape vesicles carry dIgA and pIgR to the apical surface where they are exocytosized. In contrast, the fate of TRs and EGFR are different. TRs are selectively removed in 60 nm clathrin coated buds, whereas EGFR are removed by intraluminal vesicle of multivesicular bodies. EGFRs are ultimately targeted to the lysosomes. TRs are targeted to the basolateral surface. These results indicate that although these transported integral proteins share common early compartments eventually they are targeted separately.

In agreement with the notion of an initial common pathway, a sorting station is thought to operate in polarized cells next to the TGN. This station connects the apical and basolateral transport pathways and is involved in transcytosis and the recycling of proteins (e.g., Gibson et al, 1998; Futter et al., 1998) and lipids (e.g., vanIJzendoorn et al., 1997; van IJzendoorn and Hoekstra, 1998). Current thinking considers it a separate compartment, the Golgi *sub-apical compartment* (SAC) related to the endosomes. The SAC appears to be involved for either apical to basolateral transport or the basolateral to apical transport (see

van IJzendoorn and Hoekstra, 1999).

The evidence that has led to the formulation of this concept is as follows: (a) basolaterally derived proteins and proteins internalized from the apical surface colocalize to the same structures (e.g., Hughson and Hopkins, 1990; Knight et al., 1995), and, (b) the transcytotic marker polymeric immunoglobulin receptor (pIgR) which mediates basolateral to apical transcytosis of IgA and IgM (Apodaca et al., 1994) is picked up by endocytosis from the basolateral surface and is targeted to the SAC before delivery to the apical membrane domain (Apodaca et al., 1994; Barroso and Sztul, 1994).

IX. INVOLVEMENT OF THE CYTOSKELETON

In view of the location of actin networks at the periphery of the cell, the possibility of a role of an actin-myosin system in endocytosis cannot be overlooked. This chapter frequently suggested a role of actin in most kinds of endocytosis. However, the cited evidence was very indirect. The involvement of actin in endocytosis at the molecular level is just beginning to be understood (e.g., see Riezman et al., 1997; McPherson, 2002. The formation of clathrin coated pits which takes place at certain specific sites in the cell surface involves actin (e.g., Santini et al., 2002)

A major role of actin in endocytosis is well established in *Saccharomyces cerevisiae* (see Geli and Riezman, 1998) and Wendland et al., 1998). Mutations in actin and actin binding proteins inhibit receptor-mediated and fluid endocytosis. (e.g. Kübler and Riezman, 1993; Raths et al., 1993; Munn et al., 1995). In contrast, β-tubulin mutant strains showed no defect in this process indicating no major role for microtubules. Actin and cofilin mutants indicate that the rapid turnover of actin is required for endocytosis (see Lappalainen and Drubin, 1997; Belmont and Drubin, 1998). Cofilin is an actin-depolymerizing factor (see Chapter 24). Among other actin-binding proteins, S1a2p (also known as End4p and Mop2p), has been identified as an actin binding protein (e.g., Wesp et al., 1997) implicated in endocytosis (e.g., Raths et al., 1993).

Experiments using cytochalasin, which destabilize actin filaments and blocks endocytosis, suggest a similar mechanism for endocytosis at the apical membrane (but not the basolateral membrane) of polarized mammalian cells (e.g., Jackman et al., 1994). A role of actin in endocytosis is also confirmed by the observation that many of the proteins involved in endocytosis bind to actin and some of the components of the clathrin coat (see McPherson, 2002). Among these *dynamin* which binds to many endocytotic adaptor proteins as well as proteins capable of binding to actin (see Schmid et al., 1998). Dynamin the GTPase usually thought to be involved in the scission of the the vesicles from the plasma membrane (see Chapter 11), binds to proteins capable of binding actin and has a role in the production of actin tails (Orth et al., 2002; Lee and De Camilli, 2002) suggesting that it is part of the a protein machinery needed for nucleation of actin from membranes.

Search in mammalian data bases (see Chapter 1) for proteins with a similar amino acid sequence to

known yeast proteins already implicated in endocytosis (such as Sla2p), offers a way of extending these studies to the role of actin in mammalian cells. The mouse protein mHip1R was identified in this way (Engqvist-Goldstein et al., 1999) and was found to be very similar to the human *huntingtin interacting protein 1* (Hip1). Huntingtin is the protein responsible for Huntington disease when the polyglutamine section is expanded to more then 35 repeats. Huntington disease is an inherited progressive neurodegenerative deficity (e.g., see Freiman and Tjian, 2002). HIP1 and the related protein, HIP1R/HIP12, have been found in the clathrin coat of clathrin coated pits and vesicles (Engqvist-Goldstein et al., 1999; Mishra et al., 2001). HIP1R/HIP12 colocalizes with clathrin, AP-2, and endocytosed transferrin. Indirectly, it binds to clathrin and stimulate clathrin assembly (e.g., (Legendre-Guillemin et al., 2002). At least in vitro, HIP1 does not bind to actin but does bind to clathrin and AP2, whereas HIP1R/HIP12 co-sediments with F-actin, binds to actin and but does not bind to AP2 or clathrin. However, HIP1R and HIP1 form heterodimers (Legendre-Guillemin et al., 2002) so that the complex could bind to both clathrin and actin. HIP1 and HIP1R/HIP12 contain a phosphatidylinositol 4,5-biphosphate-binding motif (ENTH). Phosphatidylinositol 4,5-biphosphate has also been shown to bind to a variety of other proteins involved in endocytosis (e.g., AP2, epsin and AP180). Furthermore, phosphatidylinositol 4,5-biphosphate is present in the plasma membrane of resting neurons. The level in endocytized membranes increases following stimulation of the presynaptic cells (Micheva et al., 2001). These findings are intriguing and suggest a role of phosphatidylinositol 4,5-biphosphate in endocytosis, perhaps in the localization of the endocytotic machinery and actin in the specialized spots of the plasma membrane.

In view of the involvement of actin comets tails (see Chapter 24) in vesicle movement, actin polymerization alone could result in movement associated with endocytosis (see Taunton, 2000; Taunton, 2001). However, the motor myosin could also be involved in budding and other movements (see Chapter 24). Most myosins move toward the plus-end of actin filaments (see Chapter 24), toward the periphery. The one exception is myosin VI, which is directed toward the minus-end . An experimental examination of this question made use of myosin VI-GFP constructs in polarized cells (Buss et al., 2001). The constructs were found to localize mostly at the apical surface where endocytosis takes place. In addition, they were associated with clathrin-AP2-coated vesicles, since myosin VI co-precipitates in a complex with AP-2 and clathrin. In transfected fibroblasts, the inhibition of the interaction by over-expression of mutants of myosin VI lacking the motor domain, decreased the endocytotic uptake of transferri in fibroblasts, again supporting a role of myosin VI in clathrin-mediated endocytosis.

Not surprisingly clathrin coats have been found to be connected to cytoskeletal elements that play either a functional role or that link actin or microtubules to the plasma membrane . The spectrin-binding protein ankyrin$_R$ facilitates the budding of clathrin-coated vesicles from the plasma membrane (Michaely et al., 1999). The β1 subunit of the AP-1 complex was found to bind to KIF13A, a kinesin that responsible for the transport from the TGN to the plasma membrane (Nakagawa et al., 2000). In addition, the Cdc42-associated tyrosine kinase (ACK) were found to be a clathrin binding proteins (Teo et al., 2001; Yang et al., 2001). Cdc42 is a Rho GTPase involved in actin polymerization at the plasma membrane and the Golgi.

SUGGESTED READING

Bonifacino, J.S. and Weissman, A.M. (1998) Ubiquitin and the control of protein fate in the secretory and endocytic pathways, *Annu. Rev. Cell. Dev Biol.* 14:19-57.(Medline)

Marsh, M. and McMahon, H.T. (1999) The structural era of endocytosis, *Science* 285:215-220. McNiven, M.A., Cao, I., Pitts, K.R. and Yoon, I. (2000) The dynamin family of mechanoenzymes: pinching in new places, *Trends Biochem Sci* 25:115-120. (MedLine)

Parton, R.G. (1996) Caveolae and caveolins, *Curr. Opin. Cell Biol* 8:542-548 (Medline)

Parton, R.G. (2003) Caveolae - from ultrastructure to molecular mechanisms, *Nature Rev. Mol. Cell Biol.* 4:162-167. (MedLine)

Pishvaee, B. and Payne, G.S. (1998) Clathrin coats--threads laid bare, *Cell* 95:443-446. (Medline)

Riezman, H., Woodman, P.G., van Meer, G. and Marsh, M. (1997) Molecular mechanisms of endocytosis, *Cell* 91:731-738. (Medline)

Schmid, S.L. and Damke, H. (1995) Coated vesicles: a diversity of form and function, *FASEB J.* 9:1445-1453. (Medline)

Travis, J. (1993) Cell biologists explore 'tiny caves', *Science* 262: 1208-1209.

WEB RESOURCES

Maniak, M. et al. Dynamic redistribution of the cytoskeleton during phagocytosis. http://www.cellsalive.com/dictyo.htm

REFERENCES

Search the textbook

Back to Chapter 9

REFERENCES

Aderem, A. and Underhill, D.M. (1999) Mechanisms of phagocytosis in macrophages, *Annu. Rev. Immunol.* 17:593-623. (MedLine)

Al Awqati, Q. (1986) Proton-translocating ATPases, *Annu. Rev. Cell Biol.* 2:179-199. (MedLine)

Anderson, R.G.W. (1998) The caveolae membrane system, *Annu. Rev. Biochem.* 67:199-225. (Medline)

Anderson, R.G.W. (1993) Plasmalemma caveolae and GPI-anchored membrane proteins, *Curr. Opin. Cell Biol.* 5:647-652. (Medline)

Anderson, R.G.W., Brown, M.S. and Goldstein, J.L. (1977a) Role of the coated endocytotic vesicle in the uptake of receptor bound low density lipoprotein in human fibroblasts, *Cell* 10:351-364. (Medline)

Anderson, R.G.W., Goldstein, J.L. and Brown, M.S. (1977b) A mutation that impairs the ability of lipoprotein receptors to localize in coated pits in the cell surface of human fibroblasts, *Nature* 270:695-699. (Medline)

Anderson, R.G.W., Vasile, E., Mello, R.J., Brown, M.S. and Goldstein, J.L. (1978) Immunochemical visualization of coated pits and vesicles in human fibroblasts: relation to low density lipoprotein receptor distribution, *Cell* 15:919-933. (Medline)

Anderson, R.G.W., Kamen, B.A., Rothberg, K.G. and Lacey, S.W. (1992) Potocytosis: sequestration and transport of small molecules by caveolae, *Science* 255:410-411. (Medline)

Apodaca, G. (2001) Endocytic traffic in polarized epithelial cells: role of the actin and microtubule cytoskeleton, *Traffic* 2:149-159. (MedLine)

Apodaca, G., Katz, L.A. and Mostov, K.E. (1994) Receptor-mediated transcytosis of IgA in MDCK cells is via apical recycling endosomes, *J. Cell Biol.* 125:67-86. (Medline)

Aroeti, B., Kosen, P.A., Kuntz, I.D., Cohen, F.E. and Mostov K.E. (1993) Mutational and secondary structural analysis of the basolateral sorting signal of the polymeric immunoglobulin receptor, *J, Cell Biol.* 123:1149-1160. (Medline)

Babst, M., Katzmann, D.J., Estepa-Sabal, E.J., Meerloo, T. and Emr, S.D. (2002a) Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting, *Dev. Cell* 3:271-282. (MedLine)

Babst, M., Katzmann, D.J., Snyder, W.B., Wendland, B. and Emr, S.D. (2002b) Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body *Dev Cell* 3:283-289. (MedLine)

Bansal, A. and Gierasch, L.M. (1991) The NPXY internalization signal of the LDL receptor adopts a reverse-turn conformation, *Cell* 67:1195-1201. (Medline)

Barroso, M. and Sztul, E.S. (1994) Basolateral to apical transcytosis in polarized cells is indirect and involves BFA and trimeric G protein sensitive passage through the apical endosome. *J. Cell Biol.* 124:83-100. (Medline)

Beck, K.A. and Keen, J.H. (1991) Interaction of phosphoinositide cycle intermediates with the plasma membrane-associated clathrin assembly protein AP-2, *J. Biol. Chem.* 266:4442-4447. (MedLine)

Belmont, L.D. and Drubin, D.G. (1998) The yeast V159N actin mutant reveals roles for actin dynamics in vivo, *J. Cell Biol.* 142:1289-1299. (Medline)

Benmerah, A., Lamaze, C., Begue, B., Schmid, S.L., Dautry-Varsat, A. and Cerf-Bensussan, N. (1998) AP-2/Eps15 interaction is required for receptor-mediated endocytosis, *J. Cell Biol.* 140:1055-1062. (Medline)

Benmerah, A., Bayrou, M., Cerf-Bensussan, N. and Dautry-Varsat, A. (1999) Inhibition of clathrin-coated pit assembly by an Eps15 mutant, *J. Cell Sci.* 112:1303-1311. (MedLine)

Berkower, C., Loayza, D. and Michaelis, S. (1994) Metabolic instability and constitutive endocytosis of STE6, the a-factor transporter of *Saccharomyces cerevisiae*, *Mol. Biol. Cell* 5:1185-1198. (Medline)

Bleil, J.D. and Bretscher, M.S. (1982) Transferrin receptor and its recycling in HeLa cells, *EMBO J.* 1:351-355. (Medline)

Boll, W., Ohno, H., Songyang, Z., Rapoport,I., Cantley, L.C., Bonifacino, J.S. and Kirchhausen, T. (1996) Sequence requirements for the recognition of tyrosine-based endocytotic signals by clathrin AP-2 complexes, *EMBO J.* 15:5789-5795. (Medline)

Bonifacino, J.S. and Dell'Angelica, E.C. (1999) Molecular bases for the recognition of tyrosine-based sorting signals, *J. Cell Biol.* 145:923-926. (MedLine)

Bradshaw, R.A. (1978) Nerve growth factor, *Annu. Rev. Biochem.* 47:191-217. (Medline)

Braell, W.A., Schlossman, D.M., Schmid, S.L. and Rothman, J.E. (1984) Dissociation of clathrin coats coupled to the hydrolysis of ATP: role of an uncoating ATPase, *J. Cell Biol.* 99:734-741. (MedLine)

Bretscher, M.S. and Pease, B.M.F. (1984) Coated pits in action, *Cell* 38:3-4. (Medline)

Brett, T.J., Traub, L.M. and Fremont, D.H. (2002) Accessory protein recruitment motifs in clathrin-mediated endocytosis, *Structure* (Camb)10:797-809. (MedLine)

Brodin, L., Low, P. and Shupliakov, O. (2000) Sequential steps in clathrin-mediated synaptic vesicle endocytosis, *Curr. Opin. Neurobiol.* 10:312-320. (MedLine)

Brown, M.S., Anderson, R.G., Basu, S.K. and Goldstein, J.L. (1981) Cell surface receptors. Observations from the LDL receptor, *Cold Spring Harbor Symp. Quant. Biol.* 46:713-721. (Medline)

Buss, F., Arden, S.D., Lindsay, M., Luzio, J.P. and Kendrick-Jones, J. (2001) Myosin VI isoform localized to clathrin-coated vesicles with a role in clathrin-mediated endocytosis, *EMBO J.* 20:3676-3684. (MedLine)

Cadena, D.L., Chan, C.L. and Gill, G.N. (1994) The intracellular tyrosine kinase domain of the epidermal growth factor receptor undergoes a conformational change upon autophosphorylation, *J. Biol. Chem.* 269:260-265. (Medline)

Cao, H., Garcia, F. and McNiven, M.A. (1998) Differential distribution of dynamin isoforms in mammalian cells, *Mol. Biol. Cell* 9:2595-2609. (Medline)

Carozzi, A.J., Roy, S., Morrow, I.C., Pol, A., Wyse, B., Clyde-Smith, J., Prior, I.A., Nixon, S.J., Hancock, J.F. and Parton, R.G. (2002) Inhibition of lipid raft-dependent signaling b y a dystrophy-associated mutant of caveolin-3, *J. Biol. Chem.* 277:17944-17949. (MedLine)

Casanova, J.E., Breitfeld, P.P., Ross, S.A. and Mostov, K.E. (1990) Phosphorylation of the polymeric immunoglobulin receptor required for its efficient transcytosis, *Science* 245:743-745. (Medline)

Casanova, J.E., Apodaca, G. and Mostov, K.E. (1991) An autonomous signal for basolateral sorting in the cytoplasmic domain of the polymeric immunoglobulin receptor, *Cell* 66:65-75. (Medline)

Chambliss, K.L., Yuhanna, I.S., Mineo, C., Liu, P., German, Z., Sherman, T.S., Mendelsohn, M.E., Anderson, R.G.and Shaul, P.W. (2000) Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae, *Circ. Res* 87:E44-52. (MedLine)

Chang, W.J., Ying, Y.S., Rothberg, K.G., Hooper, N.M., Turner, A.J., Gambliel, H.A., De Gunzburg, J., Mumby, S.M., Gilman, A.G. and Anderson, R.G. (1994) Purification and characterization of smooth muscle cell caveolae, *J. Cell Biol.* 126:127-138. (Medline)

Chen, H., Fre, S., Slepnev, VI, Capua, M.T., Takei, K., Butler, M.H., Di Fiore, P.P. and De Camilli, P. (1998) Epsin is an EH-domain-binding protein impolicated in clathrin mediated endocytosis, *Nature* 394:793-797. (Medline)

Cohen, C.J., Bacon, R., Clarke, M., Joiner, K. and Mellman I. (1994) *Dictyostelium discoideum* mutants with conditional defects in phagocytosis, *J. Cell Biol.* 126:955-966. (Medline)

Collawn, J.F., Stangel, M., Kuhn, L.A., Esecogwu, V., Jing, S., Trowbridge, I.S. and Tainer, J.A. (1990) Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis, *Cell* 63:1061-1072. (Medline)

Collawn, J.F., Kuhn, L.A., Liu, L.-F.S., Tainer, J.A. and Trowbridge, I.S. (1991) Evidence from lateral mobility studies for dynamic interaction of a mutant influenza hemagglutin with coated pits, *EMBO J.* 10: 3247-3253.

Couet, J., Li. S., Okamoto, T., Ikezu, T. and Lisanti, M.P. Identification of peptide and protein ligands for the caveolin-scaffolding domain. Implications for the interaction of caveolin with caveolae-associated proteins, *J. Biol. Chem.* 272:6525-6533. (Medline)

Cremona,O. and De Camilli, P. (1997) Synaptic vesicle endocytosis, *Curr. Opin. Neurobiol.* 7: 323-330. (Medline)

Cremona, O., Di Paolo, G., Wenk, M.R., Luthi, A., Kim, W.T., Takei, K., Daniell, L., Nemoto, Y., Shears, S.B., Flavell, R.A., McCormick, D.A. and De Camilli, P. (1999) Essential role of phosphoinositide metabolism in synaptic vesicle recycling, *Cell* 99:179-188. (MedLine)

Daro, E., van der Sluijs, P., Galli, T. and Mellman, I. (1996) Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling, *Proc. Natl. Acad. Sci. USA* 93:9559-9964. (Medline)

Das, K., Lewis, R.Y., Scherer, P.E. and Lisanti, M.P. (1999) The membrane-spanning domains of caveolins-1 and -2 mediate the formation of caveolin hetero-oligomers.Implications for the assembly of caveolae membranes in vivo, *J. Biol. Chem.* 274:18721-18728. (Medline)

De Camilli, P. and Takei, K. (1996) Molecular mechanisms in synaptic vesicle endocytosis and recycling, *Neuron* 16:481-486. (Medline)

De Camilli, P., Chen, H., Hyman, J., Panepucci, E., Bateman, A. and Brunger, A.T. (2002) The ENTH domain, *FEBS Lett.* 513:11-18. (MedLine)

de Melker, A.A., van der Horst, G., Calafat, J., Jansen, H. and Borst, J. (2001) c-Cbl ubiquitinates the EGF receptor at the plasma membrane and remains receptor associated t hroughout the endocytic route, *J. Cell Sci.* 114:2167-2178. (MedLine)

Dell'Angelica, E.C., Klumperman, J., Stoorvogel, W. and Bonifacino, J.S. (1998) Association of the AP-3 adaptor complex with clathrin, *Science* 280:431-434. (Medline)

Dell'Angelica, E.C., Mullins, C. and Bonifacino, J.S. (1999) AP-4, a novel protein complex related to clathrin adaptors, *J. Biol. Chem.* 274:7278-7285. (Medline)

Dell'Angelica, E.C. (2001) Clathrin-binding proteins: got a motif? Join the network! *Trends Cell Biol.* 11:315-318. (MedLine)

Desjardins, M., Huber, L.A., Parton, R.G. and Griffiths, G (1994) Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus, *J. Cell Biol.* 124:677-688. (Medline)

Deveraux, Q., Ustrell, V., Pickart, C. and Rechsteiner, M. (1994) a 26S protease subunit that binds ubiquitin conjugates, *J. Biol. Chem.* 269:7059-7061. (Medline)

Doxsey, S.J., Brodsky, F.M., Blank, G. S. and Helenius, A. (1987) Inhibition of endocytosis by anticlathrin antibodies, *Cell* 50:453-463. (Medline)

Drab, M., Verkade, P., Elger, M., Kasper, M., Lohn, M., Lauterbach, B., Menne, J., Lindschau, C., Mende, F., Luft, F.C., Schedl, A., Haller, H. and Kurzchalia, T.V. (2001) Loss of Caveolae, Vascular Dysfunction, and Pulmonary Defects in Caveolin-1 Gene-Disrupted Mice, *Science 2001* Aug 9; [epub ahead of print] (MedLine)

Dunn, W.A. and Hubbard, A.L. (1984) Receptor-mediated endocytosis of epidermal growth factor by hepatocytes in the perfused rat liver: ligand and receptor dynamics, *J. Cell Biol.* 98:2148-2159. (Medline)

Dunn, K.W. and Maxfield, F.R. (1992) Delivery of ligands from sorting endosomes to late endosomes occurs by maturation of sorting endosomes, *J. Cell Biol.* 117:301-310. (MedLine)

Dupré, S., Volland, C. and Haguenauer-Tsapis, R. (2001) Membrane transport: ubiquitylation in endosomal sorting, *Curr. Biol.* 11:R932-934. (MedLine)

Dupree, P., Parton, R.G., Raposo, G., Kurzchalia, T.V. and Simons, K. (1993) Caveolae and sorting in the trans-Golgi network of epithelial cells, *EMBO J.* 12:1597-1605. (Medline)

Eberle, W., Dander, C., Klaus, W., Schmidt, B., Von Figura, K. and Peters, C. (1991) The essential tyrosine of the internalization signal of lysosomal acid phosphatase is part of a turn, *Cell* 67:1203-1209. (Medline)

Engqvist-Goldstein, A.E., Kessels, M.M., Chopra, V.S., Hayden, M.R. and Drubin, D.G. (1999) An actin-binding protein of the Sla2/Huntingtin interacting protein 1 family is a novel component of clathrin-coated pits and vesicles, *J. Cell Biol.* 147:1503-1518. (Medline)

Ezekowitz, R.A., Williams, D.J., Koziel, H., Armstrong, M.Y., Warner, A., Richards, F.F. and Rose R.M. (1991) Uptake of *Pneumocystis carinii* mediated by the macrophage mannose receptor, *Nature* 351:155-158. (Medline)

Ford, M.G.J., Pearse, B.M.F, Higgins, M.K., Vallis, Y., Owen, D.J., Gibson, A., Hopkins, C.R., Evans, P.R. and McMahon, H.T. (2001) Simultaneous binding of PtdIns(4,5)P_2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes, *Science* 291:1051-1055. (MedLine)

Franke, W.W., Lüder, M.R., Kartenbeck, J., Zerban, H. and Deenan, T.W. (1977) Involvement of vesicle coat material in casein secretion and surface regeneration, *J. Cell Biol.* 69:173-195. (Medline)

Freiman, R.N. and Tjian, R. (2002) Neurodegeneration. A glutamine-rich tail leads to transcription factors, *Science* 296:2149-2150. (MedLine)

Friend, D.S. and Farquhar, M.G. (1967) Functions of coated vesicles during protein absorption in the rat vas deferens, *J. Cell Biol.* 35:357-376. (Medline)

Fujimoto, T., Nakade, S., Miyawaki, A., Mikoshiba, K. and Ogawa, K. (1992) Localization of inositol-1,4,5-triphosphate receptor like protein in plasmalemmal caveolae, *J. Cell Biol.* 119:1507-1513. (Medline)

Fujimoto, T. (1993) Calcium pump of the plasma membrane is localized in caveolae, *J. Cell Biol.* 120:1147-1157. (Medline)

Futter, C.E., Connolly, C.N., Cutler, D.F. and Hopkins, C.R. (1995) Newly synthesized transferrin receptors can be detected in the endosome before they appear on the cell surface, *J. Biol. Chem.* 270:10999-11003. (MedLine)

Futter, C.E., Pearse, A., Hewlett, L.J. and Hopkins, C.R. (1996) Multivesicular endosomes containing

internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes, *J. Cell Biol.* 132:1011-1023. (MedLine)

Futter, C.E., Gibson, A., Allchin, E.H., Maxwell, S., Ruddock, L.J., Odorizzi, G., Domingo, D., Trowbridge, I.S. and Hopkins, C.R. (1998) In polarized MDCK cells basolateral vesicles arise from clathrin-γ-adaptin-coated domains on endosomal tubules, *J. Cell Biol.* 141:611-623. (Medline)

Gad, H., Low, P., Zotova, E., Brodin, L. and Shupliakov, O. (1998) Dissociation between Ca^{2+}-triggered synaptic vesicle exocytosis and clathrin-mediated endocytosis at a central synapse, *Neuron* 21:607-616. (MedLine)

Gagescu, R., Demaurex, N., Parton, R.G., Hunziker, W., Huber, L.A and, Gruenberg, J. (2000) The recycling endosome of Madin-Darby canine kidney cells is a mildly acidic compartment rich in raft components, *Mol. Biol. Cell* 11:2775-2791. (MedLine)

Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P.H., Steele-Mortimer, O., Paiement, J., Bergeron, J.J.M. and Desjardins, M. (2002) Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages, *Cell* 110:119-131.

Gaidarov, I. and Keen, J.H. (1999) Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits, *J. Cell Biol.* 146:755-764. (MedLine)

Gaidarov, I., Chen, Q., Falck, J.R., Reddy, K.K. and Keen, J.H (1996) A functional phosphatidylinositol 3,4,5-trisphosphate/phosphoinositide binding domain in the clathrin adaptor AP-2 α subunit. Implications for the endocytic pathway, *J. Biol. Chem.* 271:20922-20929. (Medline)

Gaidarov, I., Santini, F., Warren, R.A. and keen, J.H. (1999) Spatial control of coated-pit dynamics in living cells, *Nature Cell Biol.* 1:1-7. (Medline)

Galan, J.M., Moreau, V., Ander, B., Volland, C. and Haguenauer-Tsapis, R. (1996) Ubiquitination mediated by Np1p/Rsp5p ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease, *J. Biol. Chem.* 271:10946-10952. (Medline)

Galloway, C.J., Dean, G.E., Marsh, M., Rudnick, G. and Mellman, I. (1983) Acidification of macrophage and fibroblast endocytotic vesicles *in vitro, Proc. Natl. Acad. Sci. U.S.A.* 80:3334-3338. (Medline)

Geli, M.I. and Riezman, H. (1998) Endocytic internalization in yeast and animal cells: similar and different, *J. Cell Sci.* 111:1031-1037. (Medline)

Geppert, M., Goda, Y., Hammer, R.E., Li, C., Rosahl, T.W., Stevens, C.F. and Sudhof, T.C. (1994)

Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse, *Cell* 79:717-727. (MedLine)

Geuze, H.J., Slot, J. W., Strous, G., Lodish, H.F. and Schwartz, A.L. (1983) Intracellular sites of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectronmicroscopy during receptor-mediated endocytosis, *Cell* 32:277-287. (Medline)

Geuze, H.J., Slot, J.W., Strous, G.J., Peppard, J., von Figura, K., Hasilik, A. and Schwartz, A. L. (1984) Intracellular receptor sorting during endocytosis: comparative immunoelectronmicroscopy of multiple receptors in rat liver, *Cell* 37:195-204. (Medline)

Ghitescu, L., Fixman, A., Simionescu, M. and Simionescu, N. (1986) Specific binding sites for albumin restricted to plasmalemmal vesicles of continuous capillary endothelium: receptor mediated transcytosis, *J. Cell Biol.* 102:1304-1311. (Medline)

Gibson, A., Futter, C.E., Maxwell, S., Allchin, E.H., Shipman, M., Kraehenbuhl, J., Domingo, D., Odorizzi, G., Trowbridge, I.S. and Hopkins, C.R. (1998) Sorting mechanisms regulating membrane protein traffic in the apical transcytotic pathway of polarized MDCK cells, *J. Cell Biol.* 143:81-94. (Medline)

Gillooly, D.J. and Stenmark, H. (2001) A lipid oils the endocytosis machine, *Science* 291:993-994.

Gillooly, D.J., Morrow, I.C., Lindsay, M., Gould, R., Bryant, N.J., Gaullier, J.M., Parton, R.G. and Stenmark, H. (2000) Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells, *EMBO J.* 19:4577-4588. (MedLine)

Glenney, J.R. Jr, (1989) Tyrosine phophorylation of a 22 kDa protein is correlated with transformation by Rous sarcoma virus, *J. Biol. Chem.* 264:20163-20166. (Medline)

Glenney, J.R. Jr, (1992) The sequence of human caveolin reveals identity with VIP21, a component of transport vesicles, *FEBS Lett.* 314:45-48. (Medline)

Glickman, J.N., Conibear, E. and Pearse, B.M.F. (1989) Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth-factor II, *EMBO J.* 8:1041-1047. (Medline)

Gliemann, J. (1998) Receptors of the low density lipoprotein (LDL) receptor family in man. Multiple functions of the large family members via interaction with complex ligands, *Biol. Chem.* 379:951-964. (MedLine)

Goldstein, J.L. and Brown, M.S. (1977) The low-density lipoprotein pathway and its relation to atherosclerosis, *Annu. Rev. Biochem.* 46:897-930. (Medline)

Goldstein, J.L., Basu, S.K., Brunschede, G.Y. and Brown, M.S. (1976) Release of low density lipoprotein from its cell surface receptor by sulfated glycosaminoglycans, *Cell* 7:85-95. (Medline)

Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1979) Coated pits, coated vesicles, and receptor-mediated endocytosis, *Nature* 279:679-685. (Medline)

Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russel, D.W. and Schneider, W.J. (1985) Receptor-mediated endocytosis: concepts emerging from the LDL receptor system, *Annu. Rev. Cell Biol.* 1:1-39.

Goodman, O.B. Jr., Krupnick, J.G., Gurevich, V.V., Benovic, J.L. and Keen, J.H. (1997) Role of arrestins in G-protein-coupled receptor endocytosis, *Adv. Pharmacol.* 42:429-433. (Medline)

Greenberg, S., Burridge, K. and Silverstein, S.C. (1990) Colocalization of F-actin and talin during Fc receptor-mediated phagocytosis in mouse macrophages, *J. Exp. Med.* 172:1853-1856. (Medline)

Greenberg, S., el Khoury, J., di Virgilio, F., Kaplan, E.M. and Silverstein, S.C. (1991) Ca(2+)-independent F-actin assembly and disassembly during Fc receptor-mediated phagocytosis in mouse macrophages, *J. Cell Biol.* 113:757-767. (Medline)

Griffiths, G. and Gruenberg, J. (1991) The arguments for pre-existing early and late endosomes, *Trends in Cell Biol.* 1:5-9.

Griffiths, G., Hoflack, B., Simons, K., Mellam, I. and Kornfeld, S. (1988) The mannose-6-phosphate receptor and the biogenesis of lysosomes, *Cell* 52:329-341. (Medline)

Griffiths, G., Back, R. and Marsh, M.A. (1989) Quantitative analysis of the endocytic pathway in baby hamster kidney cells, *J. Cell Biol.* 109:2703-2720. (Medline)

Grimes, M.L., Zhou, J., Beattie, E.C., Yuen, E.C., Hall, D.E., Valletta, J.S., Topp, K.S., LaVail, J.H., Bunnett, N.W. and Mobley, W.C. (1996) Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes, *J. Neurosci.* 16:7950-7964. (Medline)

Gruenberg, J. (2001) The endocytic pathway: a mosaic of domains, *Nature Rev. Mol. Cell Biol.* 2:721-730. (MedLine)

Gruenberg, J., Griffiths, G. and Howell, K.E. (1989) Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle fusion in vitro. *J. Cell Biol.* 108:1301-1316. (MedLine)

Hagler, H., Ash, J., Singer, S.J. and Cohen, S. (1978) Visualization by fluorescence of binding and internalization of epidermal growth factor in human carcinoma cells A-431, *Proc. Natl. Acad. Sci. USA* 75:3317-3321.

Hailstones, D., Sleer, L.S., Parton, R.G. and Stanley, K.K. (1998) Regulation of caveolin and caveolae by cholesterol in MDCK cells, *J. Lipid Res.* 39:369-379. (Medline)

Harada, S., Loten, E.G. Smith, R.M. and Jarett, L. (1992) Nonreceptor mediated nuclear accumulation in H35 rat hepatoma cells, *Cell Physiol.* 153: 607-613. (Medline)

Haucke, V. and De Camilli, P. (1999) AP-2 recruitment to synaptotagmin stimulated by tyrosine-based endocytic motifs, *Science* 285:1268-1271. (MedLine)

Heilker, R., Manning-Krieg, U., Zuber, J.-F. and Spiess, M. (1996) *In vitro* binding of clathrin adaptors to sorting signals correlates with endocytosis and basolateral sorting, *EMBO J.* 15:2893-2899. (Medline)

Henley, J.R., Krueger, E.W., Oswald, B.J. and McNiven M.A. (1998) Dynamin-mediated internalization of caveolae, *J. Cell Biol.* 141:85-99. (Medline)

Herz, J. and Strickland, D.K. (2001) LRP: a multifunctional scavenger and signaling receptor, *J. Clin. Invest.* 108:779-784. (MedLine)

Heuser, J.E. and Keen, J. (1988) Deep-etch visualization of proteins involved in clathrin assembly, *J. Cell Biol.* 107:877-886. (Medline)

Heuser, J.E., Keen, J.H., Amende, L.M., Lippoldt, R.E. and Prasad, K. (1987) Deep-etch visualization of 27S clathrin: a tetrahedral tetramer, *J. Cell Biol.* 105:1999-2009. (Medline)

Hewlett, L.J., Prescott, A.R. and Watts, C. (1994) The coated pit and macropinocytic pathways serve distinct endosome populations, *J. Cell Biol.* 124:689-703. (Medline)

Hicke, L. (1997) Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins, *FASEB J.* 11:1215-1226. (Medline)

Hicke, L. (2001) Protein regulation by monoubiquitin, *Nature Rev. Mol. Cell Biol.* 2:195-201. (MedLine)

Hicke, L. and Riezman, H. (1996) Ubiquitination of yeast plasma membrane receptor signals its ligand stimulated endocytosis, *Cell* 84:277-287. (Medline)

Higgins, M.K. and McMahon, H.T. (2002) Snap-shots of clathrin-mediated endocytosis, *Trends Biochem.*

Sci. 27:257-263.

Hirsch, J.G., Fedorko, M.E. and Cohn, Z.A. (1968) Vesicle fusion and formation at the surface of pinocytic vacuoles in macrophages, *J. Cell Biol.* 38:629-632. (MedLine)

Hirst, J. and Robinson, M.S. (1998) Clathrin and adaptors, *Biochim. Biophys. Acta* 1404:173-193. (MedLine)

Holzman, E., Novikoff, A.B. and Villaverde, H. (1967) Lysosomes and GERL in dermal and chromatolytic neurons of the rat ganglion nodosum, *J. Cell Biol.* 33:419-435. (Medline)

Hopkins, C.R. (1985) The appearance and internalization of transferrin receptors at the margins of spreading human tumor cells, *Cell* 40:199-208. (Medline)

Hughson, E.J. and Hopkins, C.R. (1990) Endocytic pathways in polarized Caco-2 cells: identification of an endosomal compartment accessible from both apical and basolateral surfaces, *J. Cell Biol.* 110:337-348. (Medline)

Isberg, R.R. and Tran Van Nhieu, G. (1994) Binding and internalization of microorganisms by integrin receptors, *Trends Microbiol.* 2:10-14. (Medline)

Itin, C., Kappeler, F., Lindstedt, A.D. and Hauri, H.-P. (1995) A novel endocytotic signal related to KKXX ER-retrieval signal, *EMBO J.* 14:2250-2256. (Medline)

Itoh, T., Koshiba, S., Kigawa ,T., Kikuchi, A., Yokoyama, S. and Takenawa, T. (2001) Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis, *Science* 291:1047-1051. (MedLine)

Izumi, T., Shibata, Y. and Yamamoto, T. (1988) Striped structures on the cytoplasmic surface membranes of endothelial vesicles of rat aorta revealed by quick-freeze, deep-etching replicas, *Anat. Rec.* 220:225-232. (Medline)

Jackman, M.R., Shurety, W., Ellis, J.A. and Luzio, J.P. (1994) Inhibition of apical but not basolateral endocytosis of ricin and folate in Caco-2 cells by cytochalasin D, *J. Cell Sci.* 107:2547-2556. (Medline)

Jeffers, M., Taylor, G.A., Weidner, K.M., Omura, S. and Woude, G.F. (1997) Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway, *Mol. Cell. Biol.* 17:799-808. (Medline)

Jones, B.G., Thomas, L., Malloy, S.S., Thulin, C.D., Fry, M.D., Walsh, K.A. and Thomas, G. (1995) Intracellular trafficking of furin is modulated by the phosphorylation state of casein kinase II site in its

cytoplasmic tail, *EMBO J.* 14:5869-5883. (Medline)

Jones, S.M., Howell, K.E., Henley, J.R., Cao, H. and McNiven, M.A. (1998) Role of dynamin in the formation of transport vesicles from the trans-Golgi network, *Science* 279:573-577. (Medline)

Jorgensen, E.M., Hartwieg, E., Schuske, K., Nonet, M.L., Jin. Y. and Horvitz, H.R. (1995) Defective recycling of synaptic vesicles in synaptotagmin mutants of *Caenorhabditis elegans*, *Nature* 378:196-199. (MedLine)

Katzmann, D.J., Odorizzi, G. and Emr, S.D. (2002) Receptor downregulation and multivesicular-body sorting, *Nature Rev. Mol. Cell Biol.* 3:893-905. (MedLine)

Kay, B.K., Yamabhai, M., Wendland, B. and Emr, SD. (1999) Identification of a novel domain shared by putative components of the endocytic and cytoskeletal machinery, *Protein Sci.* 8:435-438. (MedLine)

Keller, G.A., Siegel, M.W., and Caras, I.W. (1992) Endocytosis of glycophospholipid-anchored and transmembrane forms of CD4 by different endocytotic pathways, *EMBO J.* 11:863-874. (Medline)

Kibbey, R.G., Rizo, J., Gierasch, L.M. and Anderson, R.G. (1998) The LDL receptor clustering motif interacts with the clathrin terminal domain in a reverse turn conformation, *J. Cell Biol.* 142:59-67. (MedLine)

Kirchhausen, T.(1993) Coated pits and coated vesicles - sorting it all out, *Curr. Opin. Struct. Biol.* 3:182-188.

Kirchhausen, T. (1999) Adaptors for clathrin-mediated traffic, *Annu. Rev. Cell Dev. Biol.* 15:705-732. (MedLine)

Kirchhausen, T. (2000a) Clathrin, *Annu. Rev. Biochem.*69:699-727. (MedLine)

Kirchhausen, T. (2000b) Three ways to make a vesicle, *Nature Rev. Mol. Cell Biol.* 1:187-198. (MedLine)

Kirchhausen, T., Bonifacino, J.S. and Riezman, H. (1997) Linking cargo to vesicle formation: receptor tail interactions with coat proteins, *Curr. Opin. Cell Biol.* 9:488-495. (Medline)

Knight, A., Hughson, E., Hopkins, C.R. and Cutler, D.F. (1995) Membrane protein trafficking through the common apical endosome compartment of polarized Caco-2 cells, *Mol. Biol. Cell* 6:597-610. (Medline)

Kobayashi, T., Stang E., Fang, K.S., de Moerloose, P., Parton, R.G. and Gruenberg, J. (1998) A lipid associated with the antiphospholipid syndrome regulates endosome structure and function, *Nature* 392:193-197. (Medline)

Kobayashi, T., Beuchat, M.H., Lindsay, M., Frias, S., Palmiter, R.D., Sakuraba, H., Parton, R.G. and Gruenberg, J. (1999) Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport, *Nature Cell Biol.* 1:113-118. (MedLine)

Koenig, J.H. and Ikeda, K. (1989) Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval, *J. Neurosci.* 9:3844-3860. (MedLine)

Kölling, R. and Hollenberg, C.P. (1994) The ABC-transporter Ste6 accumulates in the plasma membrane in a ubiquitinated form in endocytotic mutants, *EMBO J.* 13:3261-3271. (Medline)

Kübler, E. and Riezman, H. (1993) Actin and fimbrin are required for the internalization step of endocytosis in yeast, *EMBO J.* 12:2855-2862. (Medline)

Kurten, R.C., Cadena, D.L. and Gill, G.N. (1996) Enhanced degradation of EGF receptors by a sorting nexin, SNX1, *Science* 272:1008-1010. (MedLine)

Kurzchalia, T.V., Dupree, P., Parton, R.G., Kellner, R., Virta, H., Lehnert, M. and Simons, K. (1992) VIP21, a 21-kD membrane protein is an integral component of *trans*-Golgi-network-derived transport vesicles, *J. Cell Biol.* 118:1003-1014. (Medline)

Lamaze, C. and Schmid, S.L. (1995a) The emergence of clathrin-independent pinocytic pathways, *Curr. Opin. Cell Biol.* 7:573-580. (Medline)

Lamaze, C. and Schmid, S.L. (1995b) Recruitment of epidermal growth factor receptors into coated pits requires their activated tyrosine kinase, *J. Cell Biol.* 129:47-54. (Medline)

Lamaze, C., Chuang, T.-S., Terlecky, L.J., Bokoch, G.M. and Schmid, S.L. (1996) Regulation and receptor-mediated endocytosis by Rho and Rac, *Nature* 382:177-179. (Medline)

Lamaze, C., Dujeancourt, A., Baba, T., Lo, C.G., Benmerah, A. and Dautry-Varsat, A. (2001) Interleukin 2 receptors and detergent-resistant membrane domains define clathrin-independent endocytotic pathway, *Mol. Cell* 7:661-671.

Lappalainen, P,. and Drubin, D.G. (1997) Cofilin promotes rapid actin filament turnover in vivo, *Nature* 388:78-82; (see also 289:211). (Medline)

Lee, E. and De Camilli, P. (2002) Dynamin at actin tails, *Proc. Natl. Acad. Sci. USA* 99:161-166. (MedLine)

Legendre-Guillemin, V., Metzler, M., Charbonneau, M., Gan, L., Chopra, V., Philie, J., Hayden, M.R. and McPherson, P.S. (2002) HIP1 and HIP12 display differential binding to F-actin, AP2, and clathrin. Identification of a novel interaction with clathrin light chain, *J. Biol. Chem.* 277:19897-19904. (MedLine)

Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W.Y., Beguinot, L., Geiger, B. and Yarden, Y. (1998) c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor, *Genes Dev.* 12:3663-3674. (MedLine)

Li, C., Ullrich, B., Zhang, J.Z., Anderson, R.G., Brose, N. and Sudhof, T.C. (1995) Ca^{2+}-dependent and -independent activities of neural and non-neural synaptotagmins, *Nature* 375:594-599. (MedLine)

Li, S., Song, K.S. and Lisanti, M.P. (1996) Expression and characterization of recombinant caveolin. Purification by polyhistidine tagging and cholesterol-dependent incorporation into defined lipid membranes, *J. Biol. Chem.* 271:568-573. (Medline)

Lin, H.C., Südhof, T.C. and Anderson, R.G.W. (1992) Annexin VI is required for budding of clathrin-coated pits, *Cell* 70:283-291. (Medline)

Lisanti, M.P., Tang, Z.L. and Sargiacomo, M. (1993) Caveolin forms a hetero-oligomeric protein complex that interacts with an apical GPI-linked protein: implications for the biogenesis of caveolae, *J. Cell Biol.* 123:595-604. (Medline)

Lisanti, M.P., Tang, Z., Scherer, P.E., Kubler, E., Koleske, A.J. and Sargiacomo, M. (1995) Caveolae, transmembrane signalling and cellular transformation, *Mol. Membr. Biol.* 12:121-124. (Medline)

Littleton, J.T., Stern, M., Perin, M. and Bellen, H.J. Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in *Drosophila* synaptotagmin mutants, *Proc. Natl. Acad. Sci. USA* 91:10888-10892. (MedLine)

Liu, P., Ying, Y., Ko, Y.G. and Anderson, R.G. (1996) Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae, *J. Biol. Chem.* 271:10299-10303. (Medline)

Liu, J., Oh, P., Horner, T., Rogers, R.A. and Schnitzer, J.E. (1997) Organized endothelial cell surface signal transduction in caveolae distinct from glycosylphosphatidylinositol-anchored protein microdomains, *J. Biol. Chem.* 272:7211-7222. (Medline)

Machleidt, T., Li, W.P., Liu, P., Anderson, R.G.W. (2000) Multiple Domains in Caveolin-1 Control its

Intracellular Traffic, *J. Cell Biol.* 148:17-28. (Medline)

Marks, B. and McMahon, H.T. (1998) Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals, *Curr. Biol.* 8:740-749. (Medline)

Marks, M.S., Woodruff, L., Ohno, H. and Bonifacino, J.S. (1996) Protein targeting by tyrosine- and di-leucine-based signals: evidence for distinct saturable components, *J. Cell Biol.* 135:341-354. (Medline)

Marks, M.S., Ohno, H., Kirchhausen, T. and Bonifacino, J.S. (1997) Protein sorting by tyrosine-based signals: adapting to the Ys and wherefores, *Trends in Cell Biol.* 7:124-128.

Marsh, M. and Helenius, A. (1980) Adsorptive endocytosis of Semliki Forest virus, *J. Mol. Biol.* 142:439-454. (Medline)

Marsh, M. and McMahon, H.T. (1999) The structural era of endocytosis, *Science* 285:215-220. (Medline)

Maxfield, F.R., Davies, P.J.A., Klempner, L., Willingham, M.C. and Pastan, I. (1979) Epidermal growth factor stimulation of DNA synthesis is potentiated by compounds that inhibit its clustering in coated pits, *Proc. Natl. Acad. Sci. USA.* 76:5731-5735. (Medline)

May, R.C. and Machesky, L.M. (2001) Phagocytosis and the actin cytoskeleton, *J. Cell Sci.* 114:1061-1077. 9MedLine)

Mayor, S., Rothberg, K.G. and Maxfield F.R. (1994) Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking, *Science* 264:1948-1951. (Medline)

Mayor, S., Sabharanjak, S. and Maxfield, F.R.(1998) Cholesterol-dependent retention of GPI-anchored proteins in endosomes, *EMBO J.* 17:4626-4638. (MedLine)

McMahon, H.T. (1999) An assembly protein for clathrin cages, *Curr. Biol.* 9:R332-335. (Medline)

McPherson, P.S. (2002) The endocytotic machinery at the interface with the actin cytoskeleton: a dynamic, hip version, *Trends in Cell Biol.*12:312-315.

McPherson, P.S., Garcia, E.P., Slepnev, V.I., David, C., Zhang, X., Grabs, D., Sossin, W.S., Bauerfeind, R., Nemoto, Y.and DeCamilli PA (1996) presynaptic inositol-5-phosphatase, *Nature* 379:353-357. (Medline)

Mellman, I. (1996) Endocytosis and molecular sorting, *Ann. Rev. Cell Develop. Biol.* 12:575-625. (Medline)

Mellman, I.S., Plutner, H., Steinman, R.M., Unkeless, J.C. and Cohn, Z.A. (1983) Internalization and degradation of macrophage Fc receptors during receptor-mediated phagocytosis, *J. Cell Biol.* 96:887-895. (Medline)

Michaely, P., Kamal, A., Anderson, R.G. and Bennett, V. (1999) A requirement for ankyrin binding to clathrin during coated pit budding, *J. Biol. Chem.* 274:35908-35913. (MedLine)

Micheva, K. D., Holz, R. W., and Smith, S. J. (2001) Regulation of presynaptic phosphatidylinositol 4,5-biphosphate by neuronal activity , *J. Cell Biol.* 154, 355-368. (MedLine)

Miller, D.S. (1988) Stimulation of RNA and protein synthesis by intracellular insulin, *Science* 240: 506-509. (Medline)

Mineo, C., Gill, G.N. and Anderson, R.G.W (1999)Regulated migration of epidermal growth factor receptor from caveolae, *J. Biol. Chem.* 274:30636-30643. (Medline)

Mishra, S.K., Agostinelli, N.R., Brett, T.J., Mizukami, I., Ross, T.S. and Traub, L.M. (2001) Clathrin- and AP-2-binding sites in HIP1 uncover a general assembly role for endocytic accessory proteins, *J. Biol. Chem.* 276:46230-46236. (MedLine)

Monier, S., Parton, R.G., Vogel, F., Behlke, J., Henske, A. and Kurzchalia, T.V. (1995) VIP21-caveolin, a membrane protein constituent of the caveolar coat, oligomerizes *in vivo* and *in vitro Mol. Biol. Cell* 6:911-927. (Medline)

Montesano, R., Roth, J., Robert, A. and Orci, L. (1982) Non-coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxins, *Nature* 296:651-653. (Medline)

Mori, S., Tanaka, K. Omura, S. and Saito, Y. (1995) Degradation process of ligand-stimulated platelet-derived growth factor, β-receptor involves ubiquitin-proteasome proteolytic pathway, *J. Biol. Chem.* 270:29447-29452. (Medline)

Mostov, K.E. and Simister, N.E. (1985) Transcytosis, *Cell* 43:389-390. (Medline)

Mostov. K., Apodaca, G., Aroeti, B. and Okamoto, C. (1992) Plasma membrane protein sorting in polarized epithelial cells, *J. Cell Biol.* 116:577-583. (Medline)

Mukherjee, S, Ghosh, R.N. and Maxfield, F.R. (1997) Endocytosis, *Physiol. Rev.* 77:759-803. (Medline)

Mukherjee, S. and Maxfield, F.R.(2000) Role of membrane organization and membrane domains in endocytic lipid trafficking, *Traffic* 1:203-211. (MedLine)

Muhlberg, A.B., Warnock, D.E. and Schmid, S.L. (1997) Domain structure and intramolecular regulation of dynamin GTPase, *EMBO J.* 16:6676-6683. (Medline)

Mukherjee, S., Ghosh, R.N. and Maxfield, F.R. (1997) Endocytosis, *Physiol. Rev.* 77:759-803. (Medline)

Munn, A.L., Stevenson, B.J., Geli, M.I. and Riezman, H. (1995) end5, end6, and end7: mutations that cause actin delocalization and block the internalization step of endocytosis in *Saccharomyces cerevisiae*, *Mol. Biol. Cell* 6:1721-1742. (Medline)

Murata, M., Peränen, J., Schreiner, R., Wieland, F., Kurzchalia, T.V. and Simons, K. (1995) VIP21/caveolin is a cholesterol-binding protein, *Proc. Natl. Acad. Sci. USA* 92:10339-10343. (Medline)

Nakagawa, T., Setou, M., Seog, D., Ogasawara, K., Dohmae, N., Takio, K. and Hirokawa, N. (2000) A novel motor, KIF13A, transports mannose-6-phosphate receptor to plasma membrane through direct interaction with AP-1 complex, *Cell* 103:569-581. (MedLine)

Nichols, B.J. and Lippincott-Schwartz, J. (2001) Endocytosis without clathrin coats, *Trends Cell Biol.*11:406-412. (MedLine)

Nichols,B.J., Kenworthy, A.K., Polishchuk, R.S., Lodge, R., Roberts, T.H., Hirschberg, K., Phair, R.D. and Lippincott-Schwartz, J. (2001) Rapid cycling of lipid raft markers between the cell surface and Golgi complex, *J. Cell Biol.* 153(3):529-541. (MedlLine)

Nielsen, M.S., Madsen, P., Christensen, E.I., Nykjaer, A., Gliemann, J., Kasper, D., Pohlmann, R. and Petersen, C.M. (2001) The sortilin cytoplasmic tail conveys Golgi-endosome transport and binds the VHS domain of the GGA2 sorting protein, *EMBO J.* 20:2180-2190. (MedLine)

Nykjaer, A. and Willnow, T.R. (2002) The low-density lipoprotein receptor gene family: a cellular Swiss Army knife? *Trends in Cell Biol.* 12:273-280.

Odorizzi, G., Pearse, A., Domingo, D., Trowbridge, I.S. and Hopkins, C.R. (1996) Apical and basolateral endosomes of MDCK cells are interconnected and contain a polarized sorting mechanism, *J. Cell Biol.* 135:139-152. (Medline)

Oh, P., McIntosh, D.P. and Schnitzer, J.E. (1998) Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium, *J. Cell Biol.* 141:101-114. (Medline)

Ohno, H., Stewart, J., Fournier, M.-C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Galluser, A., Kirchhausen, T. and Bonifacino, J.S. (1995) Interaction of tyrosine-based sorting signals with clathrin-

associated proteins, *Science* 269:1872-1875 (Medline)

Ohno, H., Fournier, M.-C., Poy, G. and Bonifacino, J.S. (1996) Structural determinants of interaction of tyrosine-based sorting signals, *J. Biol. Chem.* 271:29009-29015. (Medline)

Okamoto, T., Schlegel, A., Scherer, P.E. and Lisanti, M.P. (1998) Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane, *J. Biol. Chem.* 273:5419-5422.. (Medline)

Orci, L., Carpentier, I.-L., Perrelet, A., Anderson, R.G.W., Goldstein, J.L. and Brown, M.S. (1978) Occurrence of low density lipoprotein receptors within large pits on the surface of human fibroblasts as demonstrated by freeze etching, *Exp. Cell Res.* 113:1-13.

Orth, J.D., Krueger, E.W., Cao, H. and McNiven, M.A. (2002) The large GTPase dynamin regulates actin comet formation and movement in living cells, *Proc. Natl. Acad. Sci. USA* 99:167-172. (MedLine)

Owen, D.J. and Evans, P.R. (1998) A structural explanation for the recognition of tyrosine-based endocytotic signals, *Science* 282:1327-1332. (Medline)

Palade, G.E. and Bruns, R.R.(1968) Structural modulations of plasmalemmal vesicles, *J. Cell Biol.* 37:633-649.

Parton, R.G. (1996) Caveolae and caveolins, *Curr. Opin. Cell Biol* 8:542-548.

Pearse, B.M.F. (1975) Coated vesicles from pig brain: purification and biochemical characterization, *J. Mol. Biol.* 97:92-98. (Medline)

Pearse, B.M.F. (1988) Receptors compete for adaptors found in plasma membrane coated pits, *EMBO J.* 7:3331-3336. (Medline)

Peiro, S., Comella, J.X., Enrich, C., Martin-Zanca, D. and Rocamora, N. (2000) PC12 cells have caveolae that contain TrkA. Caveolae-disrupting drugs inhibit nerve growth factor-induced, but not epidermal growth factor-induced, MAPK phosphorylation, *J. Biol. Chem.* 275:37846-37852. (MedLine)

Pelkmans, L., Kartenbeck, J. and Helenius, A.(2001) Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER, *Nature Cell Biol.* 3:473-483. (MedLine)

Pelkmans, L., Püntener, D. and Helenius, A. (2002) Local Actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae, *Science* 296:535-539. (MedLine)

Peralta Soler, A., Alemany, J., Smith, R.M., de Pablo, F. and Jarett, L. (1990) The state of differentiation of embryonic chicken lens cells determines the insulin-like growth factor I internalization, *Endocrinology* 127: 595-603. (Medline)

Peters, K.-R., Carley, W.W. and Palade, G.E. (1982) Endothelial plasmalemmal striped bipolar surface structure, *J. Cell Biol.* 101:2233-2238. (Medline)

Pol, A., Calvo, M., Lu, A. and Enrich, C. (1999) The "early-sorting" endocytic compartment of rat hepatocytes is involved in the intracellular pathway of caveolin-1 (VIP-21), *Hepatology* 29:1848-1857. (Medline)

Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M.R., Bossi, G., Chen, H., De Camilli, P. and Di Fiore, P.P. (2002) A single motif responsible for ubiquitin recognition and monoubiquitination i n endocytic proteins, *Nature* 416:451-455. (MedLine)

Pond, L., Kuhn, L.A., Teyton, L., Schutze, M.-P., Tainer, J.A. Jackson, M.R. and Peterson, P.A. (1995) A role of acidic residues in the di-leucine motif-based targeting to the endocytotic pathway, *J. Biol. Chem.* 270:19989-19997. (Medline)

Puertollano, R., Aguilar, R.C., Gorshkova, I., Crouch, R.J. and Bonifacino, J.S. (2001) Sorting of mannose 6-phosphate receptors mediated by the GGAs, *Science* 292:1712-1716. (MedLine)

Puri, V., Watanabe, R., Singh, R.D., Dominguez, M., Brown, J.C., Wheatley, C.L., Marks, D.L. and Pagano, R.E. (2001) Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways, *J. Cell Biol.* 154:535-547. (MedLine)

Qualmann, B., Kessels, M.M. and Kelly, R.B. (2000) Molecular links between endocytosis and the actin cytoskeleton, *J. Cell Biol.* 150:F111-F116.)MedLine)

Radulescu, R.T. (1994) Nuclear localization in insulin-like growth factor-binding protein type 3, *Trends in Biochem. Scie.* 19:278. (Medline)

Raiborg, C., Bache, K.G., Gillooly, D.J., Madshus, I.H., Stang, E. and Stenmark, H. (2002) Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes, *Nature Cell Biol.* 4:394-398. (MedLine)

Rapoport, I., Miyazali, M., Boll, W., Duckworth, B., Cantley, L.C., Shoelson, S. and Kirschhausen, T. (1997) Regulatory interactions in the recognition of endocytotic sorting signals by AP-2 complexes, *EMBO J.* 16:2240-2250. (Medline)

Raposo, G., Dunia, I., Marullo, S., André, C., Guillet, J.-G., Strosberg, A.D., Benedetti, E.L. and

Hoebeke, J.(1987) Redistribution of muscarinic acetylcholine receptors on human fibroblasts induced by regulatory ligands, *Biol. Cell* 60:117-124. (Medline)

Raposo, G., Dunia, I., Delavier-Klutchko, C., Kaveri, S., Strosberg, A,D. and Benedetti, E.L. (1989) Internalization of β-adrenergic receptor in A431 cells involves non-coated vesicles, *Eur. J. Cell Biol.* 50:340-352. (Medline)

Raths, S., Rohrer, J., Crausaz, F. and Riezman, H. (1993) end3 and end4: two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae*, *J. Cell Biol.* 120:55-65. (Medline)

Razani, B., Schlegel, A., and Lisanti, M. P. (2000) Caveolin proteins in signaling, oncogenic transformation and muscular dystrophy, *J. Cell Sci.* 113, 2103-2109. (MedLine)

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekman, D. and Hall, A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling, *Cell* 70:401-410. (Medline)

Riezman, H., Woodman, P.G., van Meer, G. and Marsh, M. (1997) Molecular mechanisms of endocytosis, *Cell* 91:731-738. (Medline)

Rindler, M.J., Ivanov, I.E., Plesken, H., Rodriguez-Boulan, E. and Sabatini, D.D. (1984) Viral glycoproteins destined for apical or basolateral plasma membrane domains traverse the same Golgi apparatus during their intracellular transport in doubly infected Madin-Darby canine kidney cells, *J. Cell Biol.* 98:1304-1319. 9MedLine)

Ringstad, N., Gad, H., Löw, P., Di Paolo, G. Brodin, L., Shupliakov, O. and De Camilli, P. (1999) Endophilin/SH3p4 is required for the transition from early to late stages of clathrin-mediated veisle endocytosis, *Neuron* 24:143-154. (Medline)

Ritter, T.E., Fajardo, O., Matsue, H., Anderson, R.G. and Lacey, S.W. (1995) Folate receptors targeted to clathrin-coated pits cannot regulate vitamin uptake, *Proc. Natl. Acad. Sci. USA* 92:3824-3828. (Medline)

Robbins, S.M., Quintrell, N.A. and Bishop, J.M. (1995) Myristoylation and differential palmitoylation of the HCK protein-tyrosine kinases govern their attachment to membranes and association with caveolae, *Mol. Cell Biol.* 15:3507-3515. (Medline)

Robinson, M.S. (1994) The role of clathrin, adaptors and dynamin in endocytosis, *Curr. Opin. Cell Biol.* 6: 538-544. (Medline)

Rodriguez-Boulan, E. and Powell, S.K. (1992) Polarity of epithelial and neuronal cells, *Annu. Rev. Cell*

Biol. 8:395-427. (Medline)

Roseberry, A.G. and Hosey, M.M. (2001) Internalization of the M2 muscarinic acetylcholine receptor proceeds through an atypical pathway in HEK293 cells that is independent on clathrin and caveolae, *J. Cell Scie.* 114:739-746. (MedLine)

Rothberg, K.G., Ying, Y.-S., Kolhouse, J.F., Kamen, B.A. and Anderson, R.G. (1990) Glycophospholipid-linked folate receptor internalizes folate without entering the coated pit endocytotic pathway, *J. Cell Biol.* 110:637-649. (Medline)

Rothman, J.E. and Schmid, S.L. (1986) Enzymatic recycling of clathrin from coated vesicles, *Cell* 46:5-9. (MedLine)

Rotin, D., Staub, O. and Haguenauer-Tsapis, R. (2000) Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases, *J. Membr. Biol.* 176:1-17. (MedLine)

Roy, S., Luetterforst, R., Harding, A., Apolloni, A., Etheridge, M., Stang, E., Rolls, B., Hancock, J. F., and Parton, R. G. (1999) Dominant-negative caveolin inhibits H-Ras function by disrupting cholesterol-rich plasma membrane domains.*Nature Cell Biol.* 1:98-105. (MedLine)

Sabharanjak, S., Sharma, P., Parton, R.G. and Mayor, S. (2002) GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway, *Dev. Cell* 2:411-423. (MedLine)

Sandvig, K. and van Deurs, B. (1991) Endocytosis without clathrin, *Cell Biol. Int. Rep.* 15:3-8.

Sandvig, K. and van Deurs, B. (1994) Endocytosis without clathrin, *Trends Cell Biol.* 4:275-277. (Medline)

Sandvig, K., Olsens, S., Petersen, O.W. and van Deurs, B. (1988) Inhibition of endocytosis from coated pits by acidification of the cytosol, *J. Cell Biochem.* 36:73-81. (Medline)

Santini, F., Gaidarov, I. and Keen, J.H. (2002) G protein-coupled receptor/arrestin3 modulation of the endocytic machinery, *J. Cell Biol.*156:665-676. (MedLine)

Sargiacomo, M., Sudol, L., Tang, Z.-L. and Lisanti, M.P. (1993) Signal transducing molecules and glycosyl-phoshatidylinositol-linked proteins form caveolin-rich insoluble complex in MDCK cells, *J. Cell Biol.* 122:789-807. (Medline)

Sargiacomo, M., Scherer, P.E., Tang, Z., Kubler, E., Song, K.S., Sanders, M.C. and Lisanti, M.P. (1995) Oligomeric structure of caveolin: implications for caveolae membrane organization, *Proc. Natl. Acad. Sci. USA* 92:9407-9411. (Medline)

Scales, S.J. and Scheller, R.H. (1999) Lipid membranes shape up, *Nature* 401:123-124. (Medline)

Schaerer, E., Neutra, M.R. and Kraehenbuhl, J.-P. (1991) Molecular and cellular mechanisms involved in transepithelial transport, *J. Membr. Biol.* 123:93-103. (Medline)

Scherer, P.E., Lewis, R.Y., Volonte, D., Engelman, J.A., Galbiati, F., Couet, J., Kohtz D.S., van Donselaar, E., Peters, P. and Lisanti. M.P. (1997) Cell-type and tissue-specific expression of caveolin-2. Caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo, *J. Biol. Chem.* 272:29337-29346. (Medline)

Schlessinger, J., Ullrich, A. (1992) Growth factor signaling by receptor tyrosine kinases, *Neuron* 9:383-391. (Medline)

Schlessinger, J., Shechter, Y., Willingham, M.C. and Pastan, I. (1978) Direct visualization of binding, aggregation, and internalization of insulin and epidermal growth factor on living fibroblastic cells, *Proc. Natl. Acad. Sci. USA.* 75:2659-2663. (Medline)

Schmalzing, G., Richer, H.P., Hausen, A., Schwarz, W. and Just, I. (1995) Involvement of GTP binding protein Rho in consituttive endocytosis in *Xenopus laevis* oocytes, *J. Cell Biol.* 130:1319-1332. (Medline)

Schmid, S.L. (1997) Clathrin-coated vesicle formation and protein sorting: an integrated process, *Annu. Rev. Biochem.* 66:511-548. (Medline)

Schmid, S.L., McNiven, M.A. and De Camilli, P. (1998) Dynamin and its partners: a progress report, *Curr. Opin. Cell Biol.* 10:504-512. (MedLine)

Schmid, S.L. and Smythe, E. (1991) Stage-specific assays for coated pit formation and coated vesicle budding *in vitro*, *J.Cell Biol.* 114:860-880

Schmid, S.L. and Damke, H. (1995) Coated vesicles: a diversity of form and function, *FASEB J.* 9:1445-1453. (Medline)

Schmid, S.L., Fuchs, R., Male, P. and Mellman, I. (1988) Two distinct subpopulations of endosomes involved in membrane recycling and transport to lysosomes, *Cell* 52:73-83. (Medline)

Schmid, S.L., McNiven, M.A. and De Camilli, P. (1998) Dynamin and its partners: a progress report,

Curr. Opin. Cell. Biol. 10:504-512. (Medline)

Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A.V., Witke, W., Huttner, W.B.and Soling, H.D. (1999) Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid, *Nature* 401:133-141. (Medline)

Schneider, W.J, Beisiegel, U., Goldstein, J.L. and Brown, M.S. (1982) Purification of the low density lipoprotein receptor, an acidic lipoprotein of 164,000 molecular weight, *J. Biol. Chem.* 257:2664-2673. (Medline)

Schnitzer, J.E. (1997) The endothelial cell surface and caveolae in health and disease, in *Vascular Endothelium. Physiology, Pathology and Therapeutic Opportunities* ed. Born, G.V.R. and Schwartz, C.J., Schattauer, Stuttgard, Germany, pp. 77-95.

Schnitzer, J.E., Oh, P., Pinney, E. and Allard, J. (1994) Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules, *J. Cell Biol.* 127:1217-1232. (Medline)

Schnitzer, J.E., Liu, J., Oh, P. (1995a) Endothelial caveolae have the molecular transport machinery for vesicle budding, docking, and fusion including VAMP, NSF, SNAP, annexins, and GTPases, *J. Biol. Chem.* 270:14399-14404. (Medline)

Schnitzer, J.E., Oh, P., Jacobson, B.S., Dvorak, A.M. (1995b) Caveolae from luminal plasmalemma of rat lung endothelium: microdomains enriched in caveolin, Ca(2+)-ATPase, and inositol trisphosphate receptor, *Proc. Natl. Acad. Sci. USA* 92:1759-1763. (Medline)

Schnitzer, J.E., McIntosh, D.P., Dvorak, A.M., Liu, J. and Oh, P. (1995c) Separation of caveolae from associated microdomains of GPI-anchored proteins, *Science* 269:1435-1439. (Medline)

Schnitzer, J.E., Oh, P. and McIntosh, D.P. (1996) Role of GTP hydrolysis in fission of caveolae directly from plasma membranes, *Science* 274:239-242. (Medline)

Sever, S., Muhlberg, A.B. and Schmid, S.L. (1999) Impairment of dynamin's GAP domain stimulates receptor-mediated endocytosis, *Nature* 398:481-486. (Medline)

Sevinsky. J.R., Rao, L.V. and Ruf, W. (1996) Ligand-induced protease receptor translocation into caveolae: a mechanism for regulating cell surface proteolysis of the tissue factor-dependent coagulation pathway, *J. Cell Biol.* 133:293-304. (Medline)

Shih, S.C., Katzmann, D.J., Schnell, J.D., Sutanto, M., Emr, S.D. and Hicke, L. (2002) Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis, *Nature Cell Biol.*

4:389-393. (MedLine)

Shupliakov, O., Low, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., De Camilli, P. and Brodin, L. (1997) Synaptic vesicle endocytosis impaired by disruption of dynamin-SH3 domain interactions, *Science* 276:259-263. (Medline)

Simionescu, N. (1983) Cellular aspects of transcapillary exchange, *Physiol. Rev.* 63:1536-1560. (Medline)

Simionescu, N., Siminoescu, M. and Palade, G.E. (1975) Permeability of muscle capillaries to small heme-peptides. Evidence for the existence of patent transendothelial channels, *J. Cell Biol.* 64:586-607. (Medline)

Simons, K, and Wandinger-Ness, A. (1990) Polarized sorting in epithelia, *Cell* 62:207-210. (Medline)

Slepnev, V.I. and De Camilli, P. (2000) Accessory factors in clathrin-dependent synaptic vesicle endocytosis, *Nature Rev. Neurosci.* 1:161-172. (MedLine)

Slepnev, V.I., Ochoa, G.C., Butler, M.H., Grabs, D. and Camilli, P.D. (1998) Role of phosphorylation in regulation of the assembly of endocytic coat complexes, *Science* 281:821-824. (Medline)

Smart, E.J., Foster, D.C., Ying, Y.S., Kamen, B.A. and Anderson, R.G. (1994) Protein kinase C activators inhibit receptor-mediated potocytosis by preventing internalization of caveolae, *J. Cell Biol.* 124:307-313. (Medline)

Smart, E.J., Ying, Y.S. and Anderson, R.G. (1995a) Hormonal regulation of caveolae internalization, *J. Cell Biol.* 131:929-938. (Medline)

Smart, E.J., Ying, Y.S., Mineo, C. and Anderson, R.G. (1995b) A detergent-free method for purifying caveolae membrane from tissue culture cells, *Proc. Natl. Acad. Sci. USA* 2:10104-10108. (Medline)

Smart, E.J., Ying, Y.S., Donzell, W.C. and Anderson, R.G. (1996) A role for caveolin in transport of cholesterol from endoplasmic reticulum to plasma membrane, *J. Biol. Chem.* 271:29427-29435. (Medline)

Smith, C.J. and Pearse, B.M. (1999) Clathrin: anatomy of a coat protein, *Trends Cell Biol.* 9:335-338. (Medline)

Smith, C.J., Grigorieff, N. and Pearse, B.M. (1998) Clathrin coats at 21 A resolution: a cellular assembly designed to recycle multiple membrane receptors, *EMBO J.* 17:4943-4953. (Medline)

Smythe, E., Pypaert, M., Lucocq, J. and Warren, G.(1989) Formation of coated pits from coated vesicles in broken A431 cells, *J. Cell Biol.*108:843-853. (Medline)

Snyder, P.M., Price, M.P., McDonald, F.J., Adams, C.M., Volk, K.A., Zeiher, B.G., Stokes, J.B. and Welsh, M.J. (1995) Mechanism by which Liddle's syndrome mutations increase activity of a human epithelial Na^+ channel, *Cell* 83:969-978. (Medline)

Song, S.K., Li, S., Okamoto, T., Quilliam, L.A., Sargiacomo, M. and Lisanti, M.P. (1996) Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains, *J. Biol. Chem.* 271:9690-9697. (Medline)

Sorkin, A., Eriksson, A., Heldin, C.H., Westermark, B. and Claesson-Welsh, L. (1993) Pool of ligand-bound platelet-derived growth factor β-receptors remain activated and tyrosine phosphorylated after internalization, *J. Cell Physiol.* 156:373-382. (MedLine)

Stefanová, I., Hoejí, V., Ansotegui, I.J., Knapp, W. and Stockinger, H. (1991) GPI-anchored cell-surface molecules complexed to tyrosine kinases, *Science* 254:1016-1019. (Medline)

Storrie, B. (1988) Assembly of lysosomes: perspectives from comparative molecular cell biology, *Int. Rev. Cytol.* 111:53-105. (Medline)

Strosberg, A.D. (1991) Structure/function relationship of proteins belonging to the family of receptors coupled to GTP-binding proteins, *Eur. J. Biochem.*196:1-10.

Strous, G.J., Van Kerthof, P., Govers, R., Ciechanover, A. and Schwartz, A.L. (1996) The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor, *EMBO J.* 15:3806-3812. (Medline)

Stowell, M.H.B., Marks, B., Wigge, P. and McMahon, H.T. (1999) Nucleotide-dependent conformational changes in dynamin: evidence for a mechanochemical molecular spring *Nature Cell Biol.* 1:27-32. (Medline)

Sugita, S., Han, W., Butz, S., Liu, X., Fernandez-Chacon, R, Lao, Y. and Sudhof, T.C.(2001) Synaptotagmin VII as a plasma membrane Ca^{2+} sensor in exocytosis, *Neuron* 30:459-473. (MedLine)

Swanson, J.A. and Watts, C. (1995) Macropinocytosis, *Trends in Cell Biol.* 5:424-428.

Sweitzer, S.M. and Hinshaw, J.E. (1998) Dynamin undergoes a GTP-dependent conformational change causing vesiculation, *Cell* 93:1021-1029. (Medline)

Sztul, E., Kaplin, A., Saucan, L. and Palade, G. (1991) Protein traffic between distinct plasma membrane domains: isolation and characterization of vesicular carriers involved in transcytosis, *Cell* 64:81-89. (Medline)

Takei, K., Haucke, V., Slepnev, V., Farsad, K., Salazar, M., Chen, H. and De Camilli, P. (1998) Generation of coated intermediates of clathrin-mediated endocytosis on protein-free liposomes, *Cell* 94:131-141. (Medline)

Takei, K. and Haucke, V. (2001) Clathrin-mediated endocytosis: membrane factors pull the trigger, *Trends Cell Biol.* 11:385-391. (MedLine)

Tang, Z., Scherer, P.E., Okamoto, T., Song, K., Chu, C., Kohtz, D.S., Nishimoto, I., Lodish, H.F. and Lisanti, M.P. (1996) Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle, *J. Biol. Chem.* 271:2255-2261. (Medline)

Taunton J. (2001) Actin filament nucleation by endosomes, lysosomes and secretory vesicles, *Curr. Opin. Cell Biol.* 13:85-91. (MedLine)

Taunton, J., Rowning, B.A., Coughlin, M.L., Wu, M., Moon, R.T., Mitchison, T.J. and Larabell, C.A. (2000) Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP, *J. Cell Biol.* 148:519-530. (MedLine)

Tebar, F., Sorkina, T., Sorkin, A., Ericsson, M. and Kirchhausen, T. (1996) Eps15 is a component of clathrin-coated pits and vesicles and is located at the rim of coated pits, *J. Biol. Chem.* 271:28727-28730. (Medline)

Teo, M., Tan, L., Lim, L. and Manser, E. (2001) The tyrosine kinase ACK1 associates with clathrin-coated vesicles through a binding motif shared by arrestin and other adaptors, *J. Biol. Chem.* 276:18392-18398. (MedLine)

ter Haar, E., Musacchio, A., Harrison, S.C. and Kirchhausen, T. (1998) Atomic structure of clathrin: a β propeller terminal domain joins an α zigzag linker, *Cell* 95:563-573. (Medline)

Thompson, L.P., Ruedi, J.M., Glass, A., Low, M.G. and Lucas, A.H. (1989) Antibodies to 5-nucleotidase (CD73), a glycosyl-phosphatidylinositol-anchored protein, cause human peripheral blood T cells to proliferate, *J. Immunol.* 143:1815-1821. (Medline)

Tjelle, T.E., Lovdal, T. and Berg, T. (2000) Phagosome dynamics and function. *BioEssays* 22:255–263. (MedLine)

Tran, D., Carpentier, J.-L., Sawano, F., Gorden, P., and Orci, L.. (1987) Ligand internalized through coated and noncoated invaginations follow a common intracellular pathway, *Proc. Natl. Acad. Sci. USA*84:7957-7961. (Medline)

Trigatti, B.L., Anderson, R.G.W. and Gerber, G.E. (1999) Identification of caveolin-1 as a fatty acid binding protein, *Biochem. Biophys. Res. Commun.* 255:34-39. (Medline)

Trowbridge, I.S., Collawn, J.F. and Hopkins, C.R. (1993) Signal-dependent membrane protein trafficking in the endocytotic pathway, *Annu. Rev. Cell Biol.* 9:129-161. (Medline)

Tycko, B. and Maxfield, F. R. (1982) Rapid acidification of endocytotic vesicles containing macroglobulin, *Cell* 28:640-651.

Ungewickell, E. and Ungewickell, H. (1991) Bovine brain clathrin light chains impede heavy chain assembly in vitro, *J. Biol. Chem.* 266:12710-12714. (Medline)

Ungewickell, E., Ungewickell, H., Holstein, S.E., Lindner, R., Prasad, K., Barouch, W., Martin, B., Greene, L.E. and Eisenberg, E. (1995) Role of auxilin in uncoating clathrin-coated vesicles, *Nature* 378:632-635. (Medline)

Urrutia, R., Henley, J.R., Cook, T.and McNiven, M.A. (1997) The dynamins: redundant or distinct functions for an expanding family of related GTPases? *Proc. Natl. Acad. Sci. USA* 94:377-384. (Medline)

van der Bliek, A.M. (1999a) Functional diversity in the dynamin family, *Trends Cell Biol.* 9:96-102. (Medline)

van der Bliek, A.M. (1999b) Is dynamin a regular motor or a master regulator? *Trends Cell Biol.* 9:253-254. (Medline)

van der Sluijs, P., Hull, M., Webster, P., Male, P., Goud, B. and Mellman, I. (1992) The small GTP-binding protein rab4 controls an early sorting event on the endocytic pathway, *Cell* 70:729-740. (Medline)

van IJzendoorn, S.C. and Hoekstra, D. (1998) (Glyco)sphingolipids are sorted in sub-apical compartments in HepG2 cells: a role for non-Golgi-related intracellular sites in the polarized distribution of (glyco)sphingolipids, *J. Cell Biol.* 142:683-696. (Medline)

van IJzendoorn, S.C., Zegers, M.M., Kok, J.W. and Hoekstra, D. (1997) Segregation of glucosylceramide and sphingomyelin occurs in the apical to basolateral transcytotic route in HepG2 cells, *J. Cell Biol.* 137:347-357. (Medline)

van IJzendoorn, S.C.D. and Hoekstra, D. (1999) The subapical compartment: novel sorting centre, *Trends Cell Biol.* 9:144-149. (Medline)

van Meer, G., Stelzer, E.H.K., Wijnaendts-van-Resandt, R.W. and Simons, K. (1987) Sorting of sphingolipids in epithelial (Madin-Darbin Canine Kidney) cells, *J. Cell Biol.* 105:1623-1635. (Medline)

Vasile, E., Simionescu, M. and Simionescu, N. (1983) Visualization of the binding, endocytosis and transcytosis of low-density lipoprotein in the arterial endothelium *in situ*, *J. Cell Biol.* 96:1677-1689. (Medline)

Vaux, D. (1992) The structure of an endocytotic signal, *Trends in Cell Biol.* 2: 189-192.

Vickery, R.G. and von Zastrow, M. (1999) Distinct dynamin-dependent and -independent mechanisms target structurally homologous dopamine receptors to different endocytic membranes, *J. Cell Biol.* 144(1):31-43. (MedLine)

Vieira, A.V., Lamaze, C. and Schmid, S.L. (1996) Control of EGF receptor signaling by clathrin-mediated endocytosis, *Science* 274:2086-2089. (Medline)

Vieira, O.V., Botelho, R.J., Rameh, L., Brachmann, S.M., Matsuo, T., Davidson, H.W., Schreiber, A., Backer, J.M., Cantley, L.C. and Grinstein, S. (2001) Distinct roles of class I and class III phosphatidylinositol 3-kinases in phagosome formation and maturation, *J. Cell Biol.* 155:19-25. (MedLine)

Vigers, G.P., Crowther, R.A. and Pearse, B.M. (1986) Three-dimensional structure of clathrin cages in ice, *EMBO J.* 5:529-534. (Medline)

Voorhees, P., Deignan, E., van Donselaer, E., Humphrey, J., Marks, M.S., Peters, P.J. and Bonaficino, J.S.(1995) An acidic sequence within the cytoplasmic domain of furin functions as a determinant of trans-Golgi network localization and internalization from the cell surface, *EMBO J.* 14:4961-4975. (Medline)

Wang, J.Y.J. (1994) Nuclear protein tyrosine kinases, *Trends Biochem. Sci.* 19:373-376. (Medline)

Warnock, D.E., Hinshaw, J.E. and Schmid, S.L. (1996) Dynamin self-assembly stimulates its GTPase activity, *J. Biol. Chem.* 271:22310-22314. (Medline)

Wary, K.K., Mainiero, F., Isakoff, S.J., Marcantonio, E.E. and Giancotti, F.G. (1996)The adaptor protein Shc couples a class of integrins to the control of cell cycle progression, *Cell* 87:733-743. (Medline)

Wells, A., Welsh, J.B., Lazar, C.S., Wiley, H.S., Gill, G.N. and Rosenfeld, M.G. (1990) Ligand-induced

transformation by a noninternalizing epidermal growth factor receptor, *Science* 247:962-964. (Medline)

Wendland, B., Emr, S.D. and Riezman, H. (1998) Protein traffic in the yeast endocytic and vacuolar protein sorting pathways, *Curr. Opin. Cell Biol.* 10:513-522. (Medline)

Wendland, B., Steece, K.E. and Emr, S.D. (1999) Yeast epsins contain an essential N-terminal ENTH domain, bind clathrin and are required for endocytosis, *EMBO J.* 18:4383-4393. (MedLine)

Wesp, A., Hicke, L., Palecek, J., Lombardi, R., Aust, T., Munn, A.L. and Riezman, H. (1997) End4p/Sla2p interacts with actin-associated proteins for endocytosis in *Saccharomyces cerevisiae*, *Mol. Biol. Cell* 8:2291-2306. (Medline)

Wigge, P., Vallis, Y. and McMahon, H.T. (1997) Inhibition of receptor-mediated endocytosis by the amphiphysin SH3 domain, *Curr. Biol.* 7:554-560. (Medline)

Wilde, A. and Brodsky, F.M. (1996) *In vivo* phosphorylation of adaptors regulates their interaction with clathrin, *J. Cell Biol.* 135:635-645. (Medline)

Wilde, A., Beattie, E.C., Lem, L. Riethof, D.A., Liu, S.-H., Mobley, W.C., Soriano, P. and Bordsky, F.M. (1999) EGF receptor signaling stimulated SRC kinase prhopshorylation of clathrin , influencing clathrin redistribution and EGF uptake, *Cell* 96:677-687. (Medline)

Worby, C.A. and Dixon, J.E. (2002) Sorting out the cellular functions of sorting nexins, *Nature Rev. Mol. Cell Biol.* 3:919-931. (MedLine)

Xu, Y., Hortsman, H., Seet, L., Wong, S.H. and Hong, W. (2001) SNX3 regulates endosomal function through its PX-domain-mediated interaction with PtdIns(3)P, *Nature Cell Biol.* 3:658-666. (MedLine)

Yamada, E. (1955) The fine structure of the gall bladder epithelium of the mouse, *J. Cell Biol.* 1:445-458.

Yamashiro, D.J., Tycko, B., Fluss, S.R. and Maxfield, F.R. (1984) Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway, *Cell* 37:789-800. (Medline)

Yang, W., Lo, C.G., Dispenza, T. and Cerione, R.A. (2001) The Cdc42 target ACK2 directly interacts with clathrin and influences clathrin assembly, *J. Biol. Chem.* 276:17468-17473. (MedLine)

Ybe, J.A., Brodsky, F.M., Hofmann, K., Lin, K., Liu, S.H., Chen, L., Earnest, T.N., Fletterick, R.J. and Hwang, P.K (1999) Clathrin self-assembly is mediated by a tandemly repeated superhelix, *Nature* 399:371-375. (Medline)

Yoshimori, T., Keller, P., Roth, M.G. and Simons, K. (1996) Different biosynthetic transport routes to the plasma membrane in BHK and CHO cells, *J. Cell Biol.* 133:247-256. (Medline)

Zhang, F., Crise, B., Su, B., Hou, Y., Rose, J.K., Bothwell, A. and Jacobson, K.. (1991) Lateral diffusion of membrane-spanning and glycosylphosphatidylinositol-linked proteins: towards establishing rules governing the lateral mobility of membrane proteins, *J. Cell Biol.*115:75-84. (Medline)

Zhang, J.Z., Davletov, B.A., Sudhof, T.C. and Anderson, R.G. (1994) Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling, *Cell* 78:751-760. (MedLine)

Zhang, Y., Moheban, D.B., Conway, B.R., Bhattacharyya, A. and Segal, R.A. (2000) Cell surface Trk receptors mediate NGF-induced survival while internalized receptors regulate NGF-induced differentiation, *J. Neurosci.* 20:5671-5678. (MedLine)

10. Biosynthesis and Cytoplasmic Trafficking:

Synthesis, Packaging and the Golgi Complex

Web Resources
References

Back to List of Chapters

All cells are engaged in the synthesis of proteins and other components destined to be transported to various locations. In *regulated secretion*, the destination of proteins is the storage secretory granule that discharges its contents to the extracellular medium on receipt of the appropriate physiological signal, such as the release of a neurotransmitter. In all cases the discharge is by a process known as *exocytosis*. In exocytosis, the vesicular membrane becomes continuous with the plasma membrane and simultaneously discharges its contents (Breckenbridge and Almers, 1987). The secretory granules serve as stores in which the concentration of secretory products is greater than in the Golgi cisternae from which they originated, as much as 10 times greater in exocrine secretion and 200 times greater in endocrine secretion. Other proteins may be destined to be delivered to intracellular organelles such as lysosomes, while still others may be targeted to the cell surface, to form new plasma membrane or discharge the contents to the outside in the process of *constitutive secretion*. Because of the mechanism of exocytosis, the transport of integral plasma membrane proteins and constitutive secretion are facets of the same process. This question was addressed by immunoelectronmicroscopy (Strous et al., 1983). Antibodies were produced against either integral plasma membrane proteins or secreted proteins. Each distinct antibody was labelled with colloidal gold particles of distinct size. The membrane proteins and the products of constitutive secretion were found associated with the same vesicles.

Recycling of components requires transport to occur in the opposite direction. The transport from the ER to the Golgi apparatus and beyond is referred to as *anterograde* transport. When it is in the opposite direction, it is called *retrograde* transport.

The transfer of materials (referred to as *cargo*) from one compartment to another is generally thought to occur in vesicles. However, the translocation of cargo between the Golgi compartments may be by alternative mechanisms (see Section I and Section IIIC). In addition, mRNA itself may be transported and targeted to a specific location and translated there (Section V). However, most of this chapter will address the pathway that transports in vesicles, the topic of Sections I to III. Just as many proteins are transferred to their target in transport vesicles, so are membrane lipids (Section VI).

The regulated secretory system was the first to be examined in detail. Its study has influenced the experiments that examined other intracellular biosynthetic protein transport. For this reason, it is introduced first.

I. PROTEIN SYNTHESIS AND INTRACELLULAR TRANSPORT

What is the general pattern of this synthesis and transport? The fate of a newly synthesized protein can be traced, after incubation in a medium containing a radioactive amino acid, by techniques using electron microscopy (EM) and autoradiography. The radioactivity is recorded by placing a photographic emulsion or film next to a tissue section. Generally, the autoradiograph has to be stored in the dark for weeks. Where a radioactive disintegration has taken place, a silver grain will appear. Sharp localization can be obtained using transmission electron microscopy (TEM) with components labelled with [^{3}H]. The radiation of [^{3}H] is of low energy and therefore cannot travel very far, permitting localizations within 0.1 to 0.2 µm. The amount of

incorporation can be estimated by counting the grains. Alternatively, the radioactivity of various cell fractions can be measured after they are isolated.

In early experiments, [^{14}C]leucine was injected into guinea pigs and then various cell fractions were isolated (Siekevitz and Palade, 1960). The highest specific radioactivity (radioactivity per milligram of enzyme) was detected in the *rough endoplasmic reticulum* (RER, i.e., polysomes attached to the endoplasmic vesicles) at the earliest possible sampling time, and not in the detached polysomes. These observations show that the synthesis takes place in the polysomes associated with the endoplasmic reticulum.

The chronological sequence of events after the initial synthesis can be followed by a *pulse-chase* procedure. In pulse-chase, the incubation of the cells or tissue with a radioactively labelled precursor for a short time period (*pulse*), is followed by the introduction of a very large excess of unlabelled precursor (*chase*). In essence, the chase excludes from observation any incorporation of the radioactive precursor that occurs after the pulse incubation. Sampling the cells or tissue at various times recognizes the migration of the labelled material.

Autoradiographic pulse-chase studies have been carried out with slices of guinea pig pancreas. Experiments with the autoradiographic technique using L-[^{3}H]leucine are illustrated in Fig. 1 (Jamieson and Palade, 1967). The electron micrograph of an acinar cell shows the vesicles of the RER, mitochondria, the nucleus and secretory granules, the dense spherical inclusions on the left side of the figure. The autoradiograph corresponds to an incubation of the pancreatic slice for 3 minutes, and the grains are predominantly on the RER. A summary of the autoradiographic data as a function of time is shown in graphical form in Fig. 2. In this figure, each point represents the percent of label corresponding to the incubation time shown in the abcissa. Curve 1 represents the percentage of grains over the RER. The radioactivity is incorporated into the condensing vacuoles of the Golgi complex (curve 2) and eventually the secretory granules (curve 3) release their contents into the acinar lumen (not shown). A qualitatively similar chronology was found for the incorporation of [^{3}H]leucine in monocytes, in which the predominant proteins synthesized are sequestered in lysosomes.

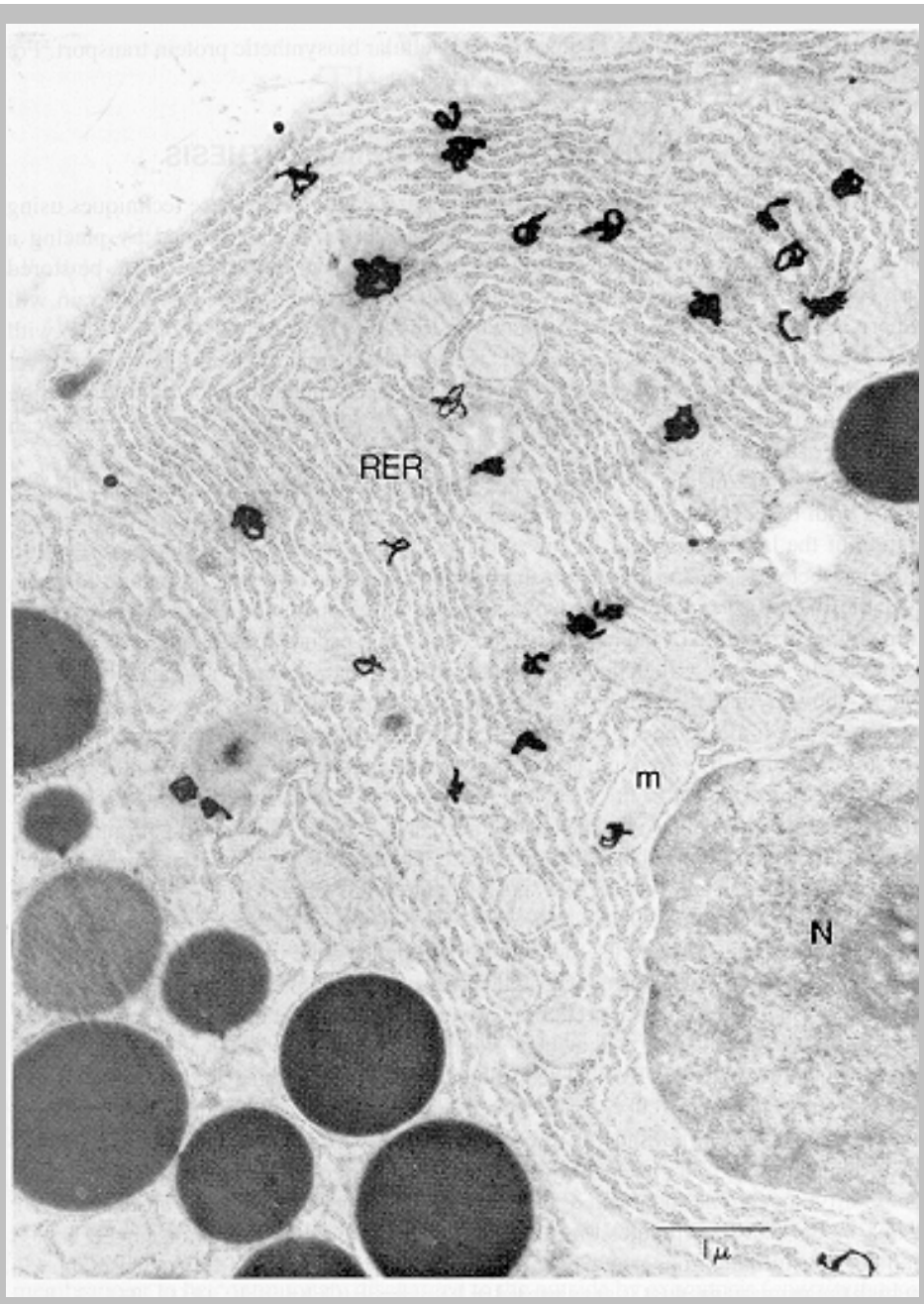


Fig. 1 Electron microscopic autoradiograph of an acinar cell at the end of pulse labeling for 3 min with L-[^{3}H]leucine. The autoradiographic grains are located almost exclusively over elements of the RER. A few grains partly overlie mitochondria. These may be associated with adjacent RER. m, Mitochondrion; N, nucleus. X17,000. Reproduced from *The Journal of Cell Biology* by copyright © permission of The Rockefeller University Press.

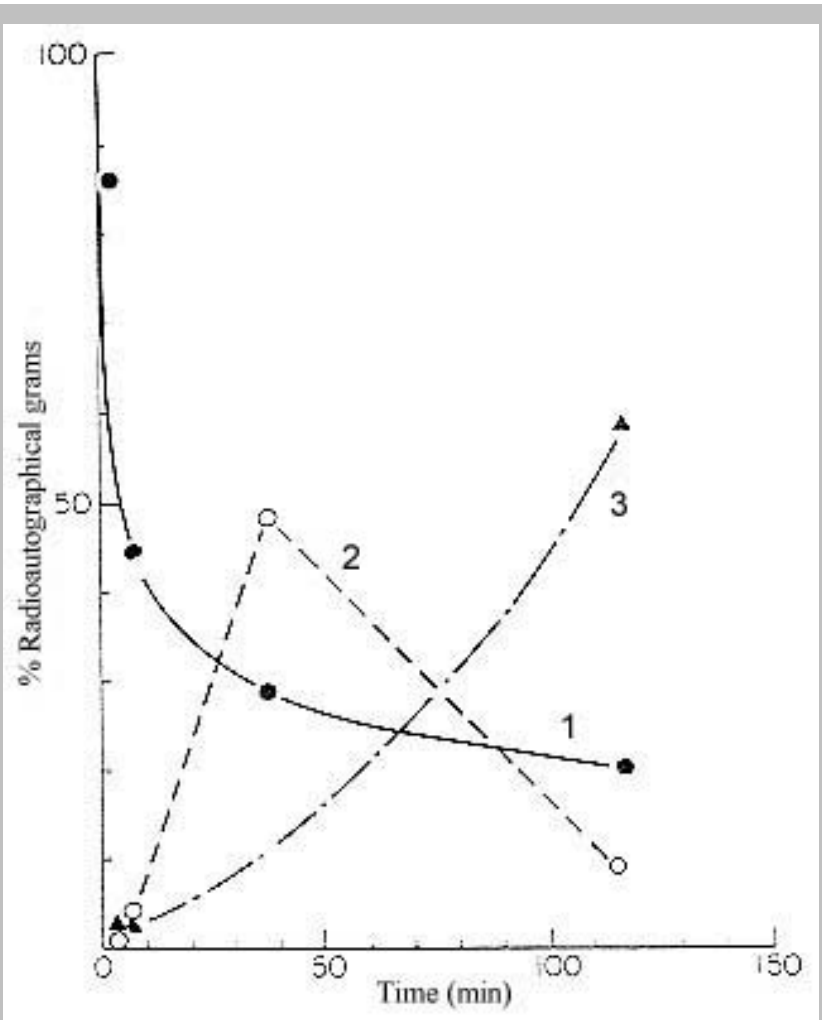


Fig. 2 Pulse-chase of pancreatic acinar cell. See text. Taken from the data of Jamieson and Palade, 1967.

As indicated by the pulse-chase experiments just discussed, newly synthesized proteins are transferred from the RER to the Golgi apparatus. From the Golgi, various vesicles are sorted out to form either storage secretory vesicles, lysosomes, or vesicles involved in constitutive secretion. The divergent fates of the various components suggest the sorting out of the proteins or the vesicles, as already indicated in Chapter 9, for the endocytotic pathway. The Golgi system seems to have a central role in this sorting. Fig. 3A (Farquhar, 1985) summarizes a model that incorporates this and other information. For a more realistic description of the structure of the Golgi apparatus see Section III. The main features of this model are: (1) each cisterna represents a separate subcompartment with a distinctive membrane composition and internal milieu; the stacks closest to the nucleus are referred to as *cis*, the more distant ones are referred to as *trans*; (2) products move vectorially from RER to transitional elements, that include the *vesicular tubular cluster* (VTCs) also referred to as *intermediate compartments* (IC) located on the *cis* side of the Golgi complex, and then unidirectionally across the stacks (*cis* to *trans*), traversing the cisternae one-by-one; (3) transport along the route occurs in vesicles; and (4) the main flow of traffic, that is the reception of vesicles and their budding, is at the rims of the cisternae.

The model in which vesicles are the vehicle for transport of cargo within the Golgi is supported by a wealth of data. However, there are indications that it may be an oversimplification. The *maturation* model of intra-Golgi transport suggests that the various cisternae of the Golgi are formed on the cis side, modified and displaced

without mediation of vesicles (e.g., Bannykh and Balch, 1997; Mironov et al., 1997). In this model, the Golgi proteins that reside in the cisternae would be recovered by retrograde transport (see Allan and Balch, 1999). This model is represented in cartoon form in Fig. 3B. There is considerable evidence supporting alternative models such as this, at least for some special cases. Transport could also take place by yet another mechanism, via the tubules that connect the various Golgi cisternae. In one study, three-dimensional reconstruction of the Golgi apparatus have shown that tubules connect the cisternae (e.g., Rambourg and Clermont, 1990; Weidman et al., 1993) although a more recent study (Ladinsky et al., 1999) has not found tubular connections between successive cisternae. This tubule model of transport is displayed in Fig. 3C. For an evaluation of these models see Section IIIC.

In addition to these different views in relation to the intra-Golgi transport, some experiments indicate that the transfer from post-ER compartments to the Golgi (see Section III) and from the Golgi to the cell surface (see Section IIIC), in at least some special cases, may occur through elongated large structures or large vesicles rather than the small vesicles discussed in most of this Chapter and Chapter 11. These experiments are discussed later, as indicated.

The diagram of Fig. 3 represents reasonably well the process thought to take place in secretory cells (e.g., see Palade, 1975). The VTCs are in close proximity to the cis face of the Golgi stacks. However, studies using other cell lines have revealed that export from the ER can occur at multiple sites, some very far from the Golgi apparatus. A more thorough examination of the geometry of the VTCs has revealed that the ER, in the process of budding, is present in morphological units referred to as *export complexes* (Bannykh et al., 1996, 1998). The ER vesicles are disposed around VTCs with their COPII buds facing the center. The VTCs are separate entities containing COPI proteins (thought to be involved in retrograde transport). The coat proteins, COPI, COPII and clathrin will be discussed in more detail in Chapter 11.

The various processes of sorting are discussed in the rest of this chapter. The mechanisms involving the vesicles, their targeting and fusion with the target membranes is the subject of the next chapter.

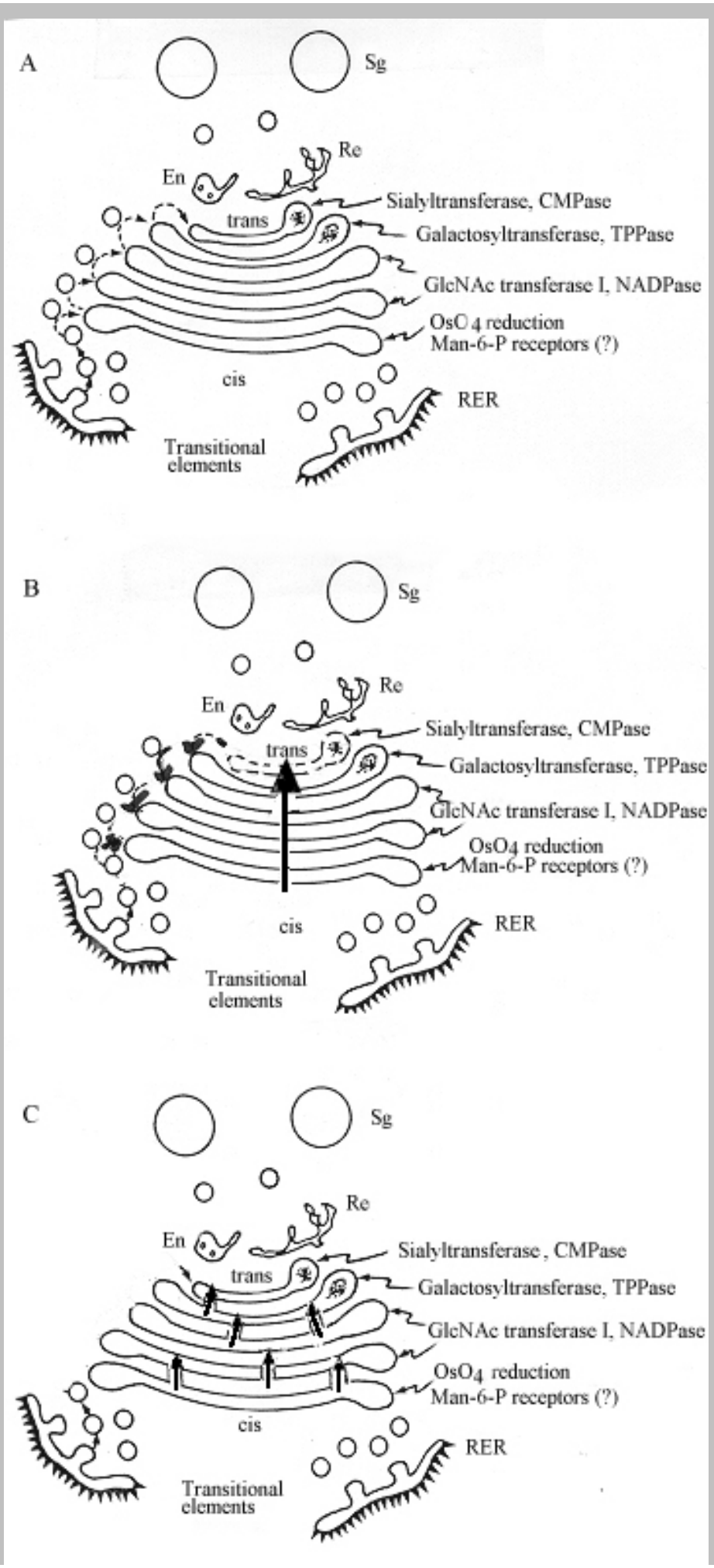

A
Sg
Re
En
trans
Sialyltransferase, CMPase
Galactosyltransferase, TPPase
GlcNAc transferase I, NADPase
OsO 4 reduction
Man-6-P receptors (?)
cis
RER
Transitional elements
B
Sg
Re
En
trans
Sialyltransferase, CMPase
Galactosyltransferase, TPPase
GlcNAc transferase I, NADPase
OsO4 reduction
Man-6-P receptors (?)
cis
RER
Transitional elements
C
Sg
Re
En
trans
Sialyltransferase, CMPase
Galactosyltransferase, TPPase
GlcNAc transferase I, NADPase
OsO4 reduction
Man-6-P receptors (?)
cis
RER
Transitional elements

Fig. 3A Stationary cisternae model of the Golgi complex. The membrane components that have been localized in situ, together with their most frequent localization in either cis, middle, or trans cisternae, are indicated on the right. The flow of biosynthetic products through the Golgi complex is diagrammed on the left. Sg, Secretory granules; En, endosome; Re, reticular element (Farquhar, 1985). Reproduced, with permission, from the *Annual Review of Cell Biology*, Volume 1, copyright &copy1985, by Annual Reviews Inc. **B.** Representation of the maturation model of intra-Golgi transport. The cisternae themselves move from the cis to the trans direction. The appropriate resident enzymes are recovered by retrograde transport involving vesicles. **C.** Representation of the tubular model of intra-Golgi transport. Materials are exchanged through the tubules connecting the cisternae.

II. ROLE OF THE ENDOPLASMIC RETICULUM

The previous section discussed how polysomes of the RER are responsible for the synthesis of proteins destined for secretion, or for packaging in the lysosomes. The synthesis of plasma membrane integral proteins also occurs in these polysomes.

The information for the delivery of newly synthesized or nascent polypeptides into the RER vesicles resides in a discrete segment of the polypeptide, the *signal sequence* or *leader sequence*. The translocation of the polypeptides into the vesicles requires interaction of the signal sequence with receptors in the cytoplasm or in the RER membrane. The receptors have a role in targeting the protein to the RER and may have a role in its translocation into the RER lumen. In addition, a special sequence is required for integral proteins to be located in the bilayer of the RER membrane.

Chapter 5 discussed the presence of certain protein domains needed to transfer proteins into the nucleus, the NLSs. The uptake and targeting of receptor proteins taken up by endocytosis, discussed in Chapter 9, also require the recognition of an amino acids sequence motif. Signal sequences represented by discrete segments of targeted proteins and the corresponding binding domains of receptors on the acceptor membrane, are also thought to play a role in the targeting of proteins synthesized in the free polysomes of the cytoplasm to mitochondria and chloroplasts (see Haucke and Schatz, 1997).

The translocation reactions for secretory, lysosomal and some integral proteins have been studied in isolated systems and are discussed in some detail in the rest of this section.

A. Signal Sequences

As discussed in Chapter 3, protein synthesis generally proceeds one amino acid at a time along the mRNA thread from the initiation triplet in the 5' to the 3' direction of the mRNA. The process of translation is outlined diagrammatically in Fig. 4. The ribosome with attached nascent polypeptide is displaced a step at a time (corresponding to a triplet), to read the mRNA (C to F). The polypeptide attached to the ribosome becomes progressively longer as it advances along the mRNA thread. Eventually, the complex reaches the termination codon, the ribosome's subunits disassemble, and the new polypeptide detaches (G). Many ribosomes are independently and simultaneously involved in the translation of a single mRNA thread to form a native protein, the polypeptide also has to be folded and, in some cases, assembled into a larger protein. The folding and assembly will not be discussed here.

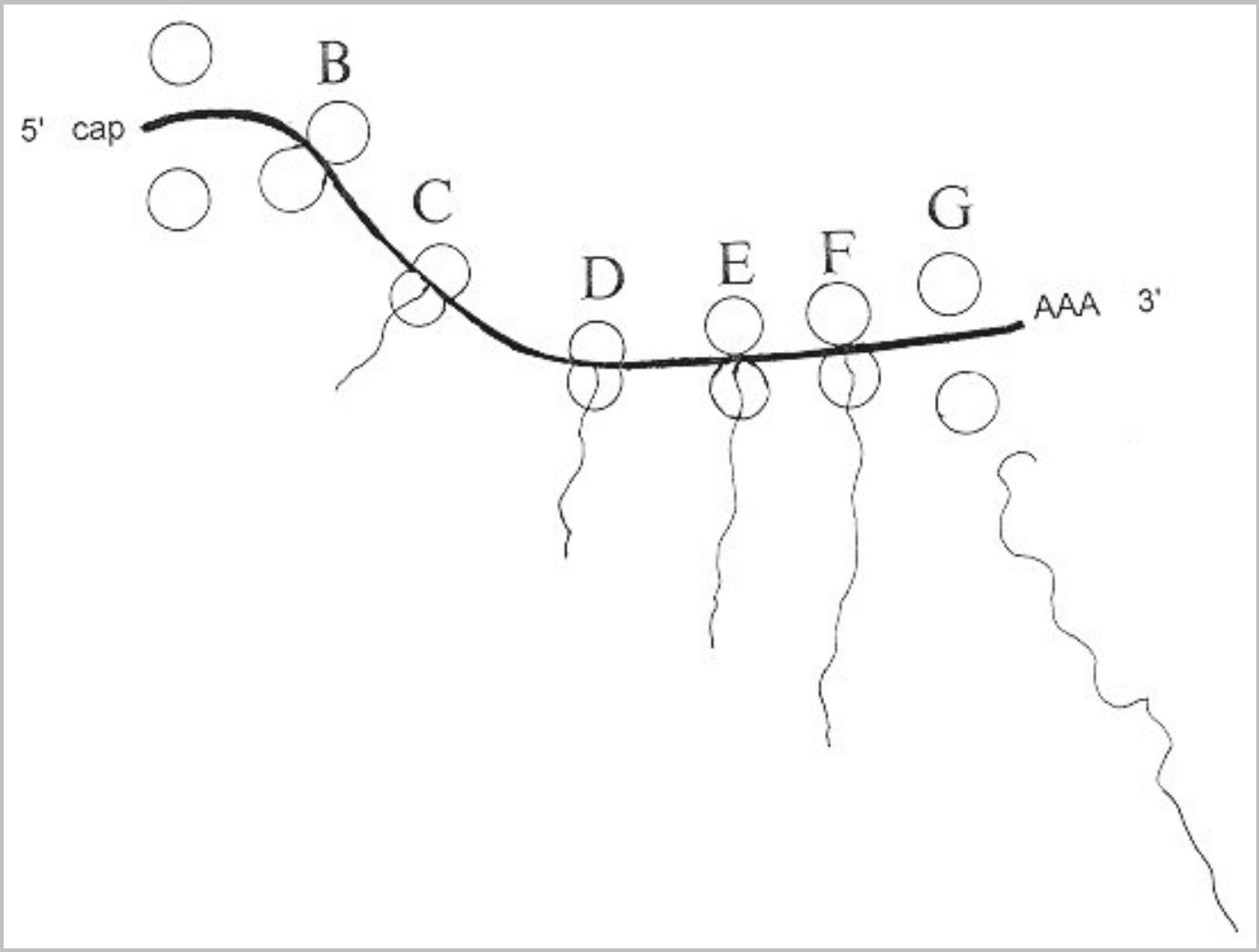


Fig. 4 Diagram summarizing the events involved in the translation of a protein that is free in the cytoplasm.

The interaction between polysomes, RER membranes (forming microsome vesicles in isolated preparations), and the nascent polypeptide, chain has been studied in many cell types. Murine myeloma cells engage in the synthesis and secretion of immunoglobulin (Blobel and Dobberstein, 1975a, 1975b). The mRNA for the light chain of immunoglobulin was found exclusively in membrane-bound polysomes. Surprisingly, when this mRNA was used in a vesicle-free translation system, the product was a protein larger than the secreted light chains (Blobel and Dobberstein, 1975a). In contrast, completion of chains contained by RER vesicles produced only chains of normal length. These experiments suggest that the vesicle components are responsible for the processing needed to produce shorter mature proteins by cleavage of a short segment by a peptidase. Later experiments demonstrated that this short segment corresponds to the signal peptide. Results of experiments using mRNA, the translational system and various concentrations of microsomes stripped of polysomes, are shown in Table 1 (Blobel and Dobberstein, 1975b). The processed and unprocessed proteins were characterized by their size, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The first column indicates the addition of a preparation of stripped microsomes, and the second and third columns show the production of processed (P) and unprocessed (U) light IgG chains, respectively. In the absence of stripped microsomes (row a), there is no synthesis of processed light chains; only the unprocessed proteins are produced. However, processing does take place (rows b-e) when the stripped microsomes are added to the

mixture. The processing is stopped by heating the membranes before the incubation (row f), as expected if the membranes play an active role in the protein processing. Increasing the concentration of microsomes beyond optimal values (rows d and e) decreases the synthesis of either processed or unprocessed protein, possibly because of nonspecific binding of needed components at the higher concentrations of membranes.

Table 1 Synthesis of Processed (P) and Nonprocessed (U) light Chains of IgG in an initiation system containing light chain mRNA and either no added EDTA-Stripped Microsomes (µl RM-EDTA), increasing amounts of RM-EDTA (5, 10, 25, 50 µl), or heat Inactivated RM-EDTA (25µl).

	RM-EDTA (µl)	P	U
(a)	0	0.0	3.4
(b)	5	4.9	0.8
(c)	10	4.8	0.9
(d)	25	2.8	0.4
(e)	50	0.8	0.0
(f)	25 (55º)	0.0	3.2

The experiment of Fig. 5 (Blobel and Dobberstein, 1975b) provides more information. A translation mixture containing intact microsomal vesicles is first incubated in the presence of radioactive amino acids, and then the vesicles are disrupted with detergent. The time of addition of the detergent corresponds to 0 time in the abscissa of the figure. Synthesis of processed protein (curve 1) continues after the disruption, from the pool of polypeptides not yet completed when the vesicles are disrupted. At about the time when the synthesis of processed polypeptides ceases, the unprocessed peptides begin to make their appearance (curve 2) and continue being produced thereafter. These experiments show that the processing by the microsomal membranes is cotranslational and the cleavage of the signal peptide takes place before the polypeptide is completed, because processed nascent chains continue to be produced even after the vesicles are removed. However, when the synthesis is initiated in the absence of membranes, the polypeptides remain unprocessed. Note, however, that posttranslational translocation can occur in mammalian systems and yeast cells exhibit both cotranslational and posttranslational translocation (see Section C, below).

Signal sequences are usually on the amino terminal of nascent peptide chains and they direct the protein to

translocation sites on the ER. Similar sequences fulfill much the same function in transferring proteins to the inner membrane of mitochondria and the thylakoid membrane of chloroplasts. After membrane insertion, the signal sequences are typically cleaved by a membrane bound signal peptidase. In some proteins, the signal sequence is not cleaved. Furthermore, the signal sequence can also be at the carboxy terminal or within the peptide chain (see Simons et al., 1987; Kutay et al., 1995).

Characteristically, the signal sequences have a hydrophobic core (h-) region that is 6 to 15 amino acid residues long in the case of the signal sequences that are cleaved. This sector is the most important for function, as determined using mutants (von Heijne, 1990). The h- region is flanked on the carboxy terminal side by the polar c- region which contains proline and glycine residues that are known to interfere with a helical configuration. This region also has uncharged residues in positions -3 and -1 that determine the cleavage site (see von Heijne, 1990). In the amino terminal side, the signal sequences have a relatively polar n-region generally with a positive charge. The length of this sector is very variable and ranges between 15 to more than 50 amino acids (von Heijne, 1990).

Although in many respects the signal sequences are interchangeable (see Gierasch 1989), many are specialized as to their target and mode of insertion (Zheng and Gierasch, 1996; Hedge and Lingappa, 1997). They appear to have information to carry out a variety of distinct functions, direct the peptide in variety of mechanisms of insertion and even contain instructions for the role of the cleaved sequence after the cleavage. Signal sequences can direct to different targeting pathways (see Ng et al., 1996; Berks, 1996) and mediate the translocation at the amino or the carboxy terminal of the protein across the membrane (Spiess, 1995). In addition, they determine whether the protein remains in the cytoplasm, is inserted in the membrane or is translocated to the lumen (Swameye and Schaller, 1997; Belin et al., 1996). What happens to the signal sequence once it is severed? Apparently, it is cleaved again and transferred to the cytoplasm (Lyko et al., 1995) where it may have other functions (Martoglio, 1997; Long, 1998; Braud et al., 1998).

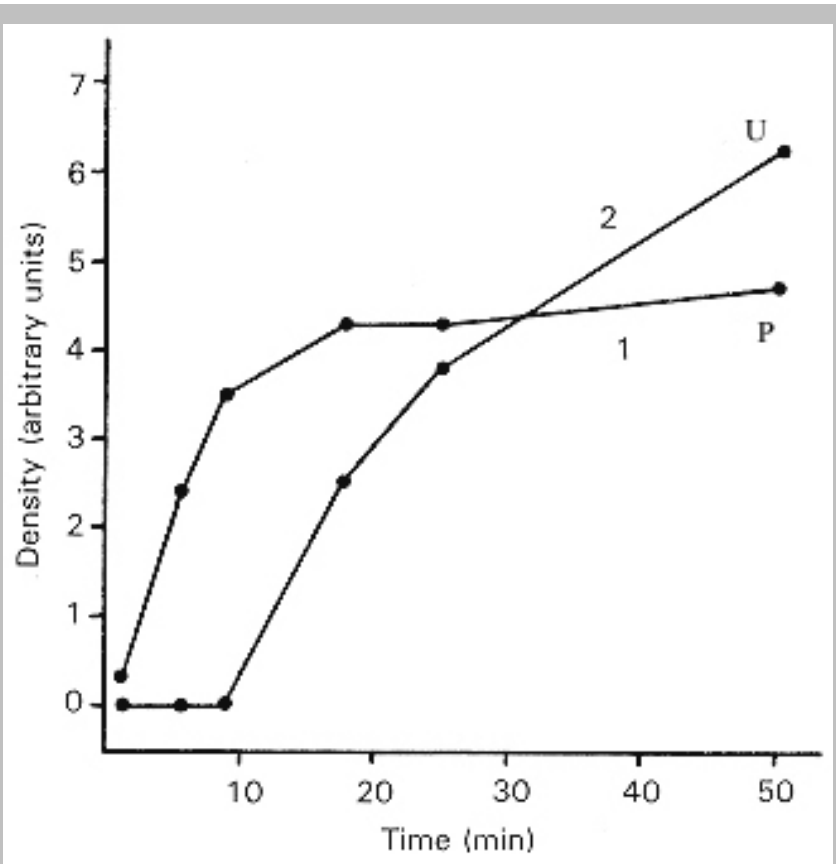


Fig. 5 Relation between processing and the presence of microsomal membranes. Densitometry of an autoradiograph was used to estimate the radioactivity. U and P designate the unprocessed and the processed light chains of IgG, respectively. See text. Reproduced from *Journal of Cell Biology* by copyright © permission of The Rockefeller University Press.

B. Targeting

A model for the complete cycle of the translation in the RER is represented in Fig. 6A (Walter et al., 1984). As indicated in the diagram, two subunits form a ribosome at the initiation codon of the mRNA (steps A and B). As the translation starts, the *signal recognition particle* (SRP) binds to the ribosome and the signal sequence (B and C). In the presence of SRP, but in the absence of RER membranes, the translation is arrested, showing that synthesis of the entire polypeptide requires the complete system. The SRP is involved in the arrest of elongation. When a nascent polypeptide emerges, the SRP-ribosome complex is targeted to the membrane of the RER by an interaction of the SRP with its receptor (*docking protein* or SRP receptor, SR; more recently called the *translocon associated protein*, TRAP). The ribosomes are also attached via the ribosome receptor (step D). The ribosome-nascent polypeptide complex remains attached to the RER membrane, forming a ribosome-membrane junction where the translocation of the nascent chain takes place. The SRP and the docking protein are released to enter a new cycle. Translocation begins as the peptide is synthesized (steps E and F). The signal sequence is cleaved cotranslationally by the signal peptidase. The ribosomal subunits are freed and are ready to start another cycle.

The model is based on several observations (Walter and Blobel, 1980, 1981). In salt-extracted canine pancreatic microsomes, recognition of the nascent peptide by the membrane system requires addition of the SRP. This particle includes a 300-nucleotide 7S RNA and six nonidentical polypeptides (Walter and Blobel,

1982a). The SRP core region has a signal sequence recognition surface composed of both protein and RNA (Batey et al., 2000).

The SRP receptor was first implicated in the translation-translocation system of the RER when it was found that proteolytic digestion of RER membranes blocked translocation and the activity could be reconstituted by addition of an extract solubilized by partial protease treatment (Meyer and Dobberstein, 1980; Walter et al., 1979). The active factor was subsequently shown to be a 52-kDa fragment of a 69-kDa integral membrane protein of the ER, as demonstrated using immunological and peptide mapping techniques (Gilmore et al., 1982a, 1982b). The 69-kDa protein was isolated using affinity chromatography in which SRP was conjugated to the Sepharose beads (Meyer et al., 1982b). The receptor would then remain attached to the column, because it would bind to the immobilized SRP.

The 69-kDa docking protein (referred to as the SR subunit) is thought to be part of a complex with a 30-kDa protein, because the two have been found tightly bound in most preparations (Tajima et al.,1986).

The SRP interacts directly with the signal sequence, as indicated by the binding of SRP to the signal peptide in the isolated translating systems. This has been shown for a signal peptide rich in lysine, where the attachment of SRP is blocked by the lysine analog β-hydroxyleucine (Walter and Blobel, 1981). In addition, in the case of the peptide hormone precursor preprolactin, photoactivated crosslinking reagents were shown to be incorporated into the amino region of the polypeptide and to crosslink to the SRP (Kurzchalia et al.,1986).

One of the subunits of SRP (SRP54) contains a GTPase-domain at the amino-terminal and a carboxy-domain that binds to the signal sequence and the SRP-RNA. The two subunits of the SR both contain GTPase-domains. Current models (Fig. 6B, Bacher et al., 1996) propose that SRP (oval black particle) binds to both the ribosome and signal sequence (SS) (I, II and III in the figure). Then the binding to GTP activates the docking of the complex to the SRP receptor (IV). The SRP receptor stabilizes the binding of GTP to SRP54 and induces its dissociation from the ribosome-signal sequence complex (V). Binding to GTP facilitates the binding of the complex to the *translocon* (the heterotrimeric Sec61p complex discussed below). The ribosome (Bacher et al., 1996) and SRP receptor binding to SRP54 (Miller et al., 1993) increases its affinity to GTP. Then the GTP hydrolysis induces dissociation of the SRP from the membrane. In Fig. 6B, the nascent polypeptide is shown entering as a loop, as discussed below.

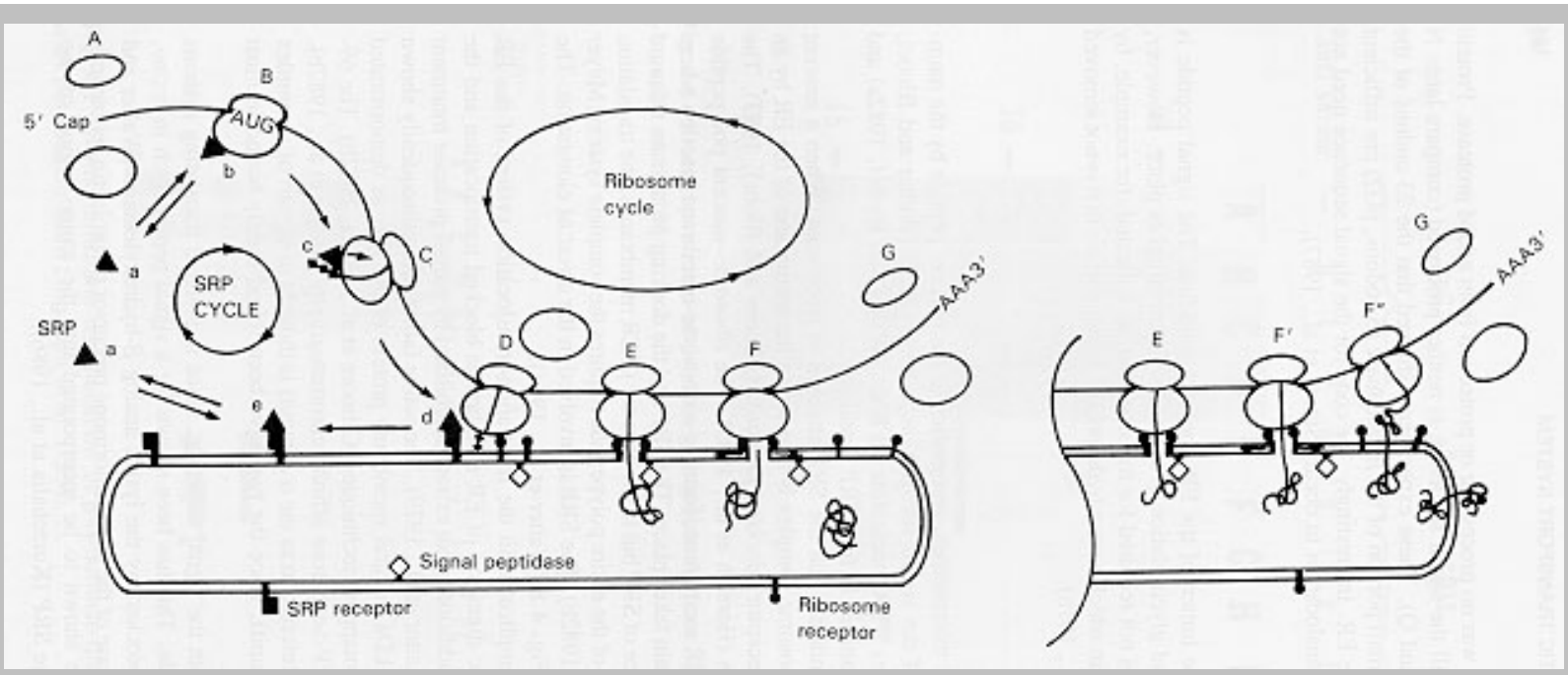


Fig. 6A Protein synthesis, targeting and translocation across or into the membrane of the endoplasmic reticulum. Reproduced with permission from Walter et al. (1984), copyright &copy1984 by Cell Press.

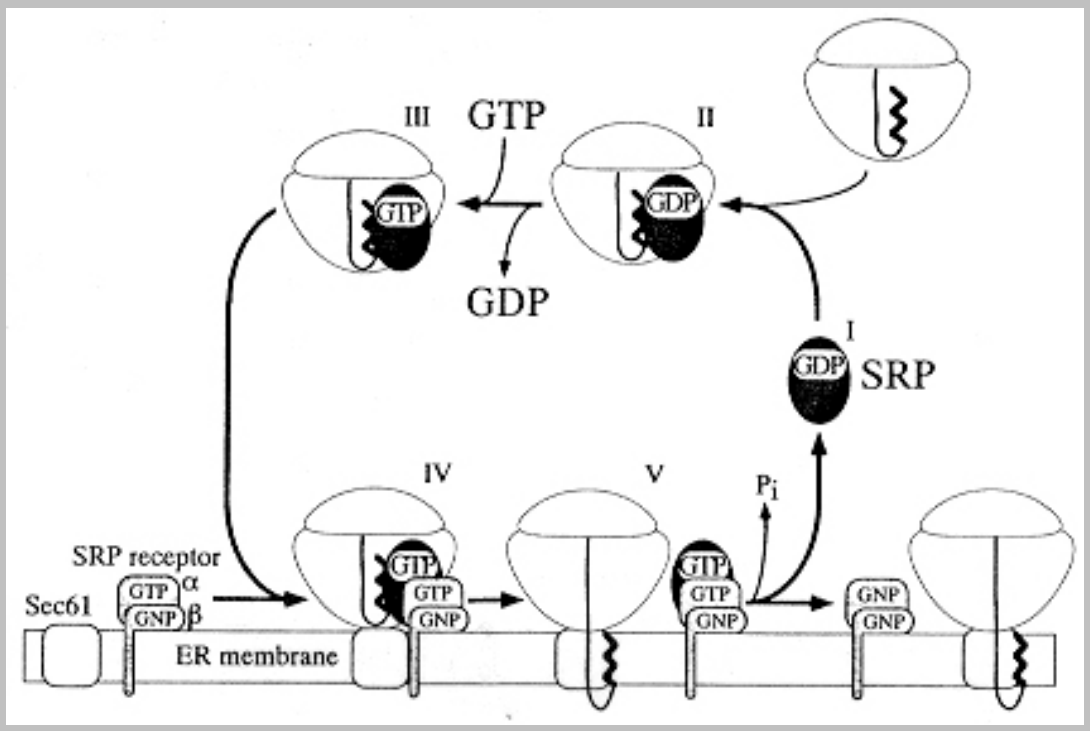


Fig. 6B The role of GTP in the targeting. I. Free SRP binding GDP. II. Complexing of SRP to the ribosome and the SS. III. Displacement of GDP by GTP. IV. binding of SS-ribosome and SRP to the SRP receptor (α and β) and attachment to the translocon, V initiation of translocation and hydrolysis of GTP on the SRP. Reproduced with permission from *Nature*, Bacher et al., 381:248-251, copyright &copy1996, MacMillan Magazines Ltd.

A signal sequence targets a protein for delivery to the ER. The *signal-anchor* sequence that determines the orientation of integral proteins, serves two functions. It targets the protein to the ER and acts as an uncleaved transmembrane sequence (see section C below).

This representation eventually had to be modified, amended and refined. For example, the model of Fig. 6A shows the nascent protein being transferred linearly from the amino to the carboxy terminal. As shown by the model of Fig. 6B, this is not likely to be the case. Some proteins containing a signal sequence are targeted to the ER membrane without the involvement of SRP and are translocated post-transcriptionally (Rapoport et al., 1996a). Another group of proteins is inserted independently of the translocon (Kutay et al, 1995). These proteins are inserted posttranscriptionally and at least some of them are ER proteins.

C. Translocation

The precise events of translocation and the involvement of membrane components are still not entirely clear (see Brodsky, 1998 for a review). In the mammalian in vitro systems, translocation is generally cotranslational, as shown by the results represented in Fig. 5. However, translation seems to be unrelated to the mechanism of translocation. This is indicated by several observations. A potential for posttranslational translocation has been demonstrated in mammalian systems. The glucose transporter protein produced in the absence of vesicles has been shown to be subsequently transferred into added vesicles (Mueckler and Lodish, 1986). Similar experiments have also demonstrated that translocation is an independent phenomenon (Perara et al., 1986). Following transcription and translation from cDNA without termination codons and in the absence of microsomes, newly formed proteins remained attached to the ribosomes. However, subsequent addition of the microsomes stripped of polysomes elicited their transfer into the vesicles. These findings suggest that the translocation machinery is part of the ER membrane and the mechanism is not part of the translational process itself. The posttranslational transfer and, presumably, the cotranslational transfer, were found to require an energy source, in these experiments supplied by ATP, GTP, and phosphocreatine in the presence of creatine phosphokinase (Perara et al., 1986). The role of phosphocreatine and phosphocreatine kinase is to replenish the terminal phosphate of ATP, hydrolyzed during the activation. As we saw, GTP is required for targeting of the ribosome-nascent polypeptide complex (section IIB, above).

Many of the proteins involved in the cotranslational translocation of the nascent polypeptides were identified by a strategy in which the components were isolated after crosslinking. A protein crosslinked to the nascent chain must have been in the proximity of the translocated polypeptide. The crosslinking requires special crosslinking reagents (some of them photoactivated) or UV radiation. The components identified include: the translocon associated protein (TRAP) (previously called the signal sequence receptor, SR; Wiedmann et al., 1987; Hartmann et al., 1993), translocating chain associated protein (TRAM) (Görlich et al., 1992a), and Sec61p, the translocon complex, first discovered in yeast (Görlich et al., 1992b). The minimal complement required for in vitro translocation includes TRAP (SR), the translocon complex or channel (the Sec61p complex), and, for some proteins, TRAM (Görlich and Rapoport, 1993). Although not needed for the translocation itself, other enzymes needed to produce the mature protein may be at the translocation site. These include the enzymes involved in glycosylation, oligosaccharide transferase and the signal peptidase. So far, there is evidence for the involvement of nine or more separate proteins. For example, the oligosaccharide portion is added to a nascent protein by the oligosaccharide-transferase when only 15 residues are exposed to the lumen (Whitley et al., 1996). This indicates that the enzyme is located very close to the channel. The various proteins need not be associated with the translocon all the time. This has led to the concept of the translocon as a dynamic unit, involving any protein that interacts with the polypeptide when still attached to the tRNA (Andrews and Johnson, 1996) and involving time dependent composition, conformation and structure.

The signal peptidase was shown to be predominantly in the RER lumen, suggesting that the excision of the signal sequence is in the lumen. TRAP was also identified as a protein that is in close proximity to the signal sequence during the initial SRP-dependent targeting, by crosslinking. Antibodies to the cytoplasmic carboxy-terminal of the α-subunit of TRAP block translocation and, therefore, also implicate this protein in the process (Hartmann et al., 1989a). Two integral proteins, named *ribophorins*, were found associated with the ribosomes of the rough endoplasmic reticulum, suggesting a role in the process of translocation. Recently, the ribophorins have been shown to be oligosaccharide transferases (e.g., Kelleher et al., 1992).

Reconstitution of the system, including membrane bound elements, has demonstrated the involvement of the TRAP (SR) and TRAM in translocation. In the study of Migliaccio et al., (1992), TRAP (SR) and TRAM were removed from detergent extracts using immunoaffinity columns. Without affecting ribosome binding, depletion of TRAP (SR) resulted in a failure to release the system from elongation and, in addition, arrest, failure in the targeting and failure to translocate the protein.

At least in systems reconstituted using proteoliposomes, the translocation of a few specific secretory proteins does not require the presence of TRAM. The need for TRAM depends on the nature of the signal sequence (Voight et al., 1996). Generally, in vitro, cotranslational translocation can be carried out with only TRAM, the heterotrimeric Sec61p, and the TRAP (SR) (Görlich and Rapoport, 1993).

In *Saccharomyces cerevisiae*, the translocation of proteins into the ER can be either posttranslational or cotranslational and not all preproteins require a functioning SRP for translocation into the ER. Mutants were found that specifically impaired the translocation of SRP-independent preproteins in vivo and in vitro, without affecting the SRP-dependent pathway. Which pathway is followed by the preprotein depends on the amino acids present in the hydrophobic core of the signal sequence (Ng et al., 1996; Zheng and Gierasch, 1996). Some preproteins were found to be able to use either the SRP or the SRP-independent route. Furthermore, some proteins distribute between the cytoplasm and the secretory pathway as a function of the nature of the signal sequence (Belin et al., 1996).

In postranslational translocation in yeast, instead of the SRP related system, the Sec62p-Sec63p complex is involved (e.g., Ng et al., 1996). The translocation system can be reconstituted in proteoliposomes containing the tetrameric Sec62p-Sec63p complex, the trimeric Sec61p (translocon) complex and, in addition, the Sbh1p protein (in yeast, equivalent to BiP) (Panzner et al., 1995). The SRP-pathway is regulated by three GTPases, the 54 kDa SRP protein and the α β subunits of the SRP receptor. These are not necessary for the SRP-less pathway. The two pathways converge at the Sec translocon assembled from Sec61p complex and involving BiP (Rapoport et al., 1996b; Hamman et al., 1998).

The ER lumenal protein BiP, involved in posttranslational translocation, is a chaperone (see Chapters 15 and below) of the Hsp70 family. In yeast, it is required for the efficient import of precursors in vivo and in vitro. Mutations in the gene for BiP (*KAR2*) prevents early translocation before the precursor protein reaches the channel (Sanders et al, 1992; Lyman and Schekman, 1995). Like other chaperones, BiP functions with a partner protein (Sec63p) in *Saccharomyces cerevisiae* (DnaJ in *E. coli*). Homologues of DnaJ have been found in several eukaryotic compartments including the ER (see Cyr et al., 1994). Most DNAJ-like proteins are soluble

or loosely attached to membranes. In contrast, Sec63p is an integral protein (Feldheim et al., 1992) with a sector in the lumen homologous to the J domain. A mutation in the conserved residue of the luminal sector of Sec63p causes a defect in precursor translocation (Rothblatt et al., 1989). The lumenal domain of Sec63p stimulates the ATPase activity of BiP and mediates BiP recruitment to the translocon (Corsi and Schekman, 1997). BiP is also thought to act in closing the translocon channel (see below).

How is the nascent peptide transferred into the RER? The hydrophobicity of the signal peptide suggests that at least the initial insertion occurs through the hydrophobic part of the membrane. The capacity of these peptides to insert in the bilayer is supported by the spontaneous insertion of isolated signal sequences into phospholipid bilayers (e.g., McKnight et al., 1991). However, the signal sequences crosslink to protein components (Robinson et al., 1987), arguing for a more complex mechanism.

The passage of an entire polypeptide through the RER membrane suggests the involvement of a channel. Electrophysiological techniques implicate a channel (Simon and Blobel, 1991). In these studies, RER vesicles were fused to planar bilayers that separated two chambers containing 45 mM potassium glutamate. In this procedure, the cytoplasmic faces of the vesicles were exposed to only one side of the bilayer. The addition of puromycin to the cytoplasmic side, increased the conductance of the bilayer and in some cases induced single channel activity of about 220 pS. Puromycin by itself did not produce channels. Puromycin combines with nascent peptides and releases them to the lumen side; this process presumably would leave open the channel needed for the transfer. Extraction of the ribosomes at high KCl concentrations closed the channels, suggesting that the ribosomes themselves are involved in gating the channels. These experiments are consistent with the presence of a water-filled channel, and the walls of the channels that have been studied so far have invariably been protein in nature.

Experiments using fluorescent probes have provided more decisive evidence for this idea (Crowley et al., 1993; 1994). Nascent chains can be synthesized in the presence of photoreactive probes attached to Lys-tRNA. The fluorescent Lys derivative is incorporated in the position of a lysine codon. The position can be varied by using mRNA of various lengths or by the choice of protein, so that the probes can be positioned at any point in the pathway. Stable conformational intermediates were produced by the use of truncated mRNA for secreted proteins lacking termination codons, so that the selected piece of the nascent chains remained bound to the ribosomes. The probes do not interfere with translation or translocation and can serve as indicators of the polarity of their environment. The probe used was 6-(7-nitrobenz-2-oxa-1,3 diazol-4-yl)amino hexanoic acid (NBD) attached to the side chain of Lysine. NBD derivatives have been shown to differ in fluorescence lifetime depending on whether they are present in an aqueous (1.4 ns) or hydrophobic (7-10 ns) medium. In addition, the fluorescence in an aqueous solution is quenched by the presence of I^-. The fluorescent lifetimes of the NBD-lysines in the ribosome and in the membrane, were those expected from an aqueous medium. Since there was no quenching by iodide from the cytoplasmic side, the channel is sealed at the cytoplasmic end by a tight membrane-ribosome-nascent peptide complex. I^- quenching from the vesicle side (by making them leaky with the addition of streptolysin O) indicated that the channel opens in the ER lumen only after the nascent chain has grown to approximately 70 amino acids. With the ER lumen gate open, I^- from the ER side was able to quench probes located inside the ribosome, showing that the water-filled channel is continuous.

Later image reconstruction using the EM confirmed this conclusion. Cryo-electron microscopy of the ribosome-Sec61 complex and its three-dimensional reconstruction (Beckmann et al. 1997) show that the Sec61 oligomer

is attached to the large ribosomal subunit by a single connection. The pore of the Sec61 oligomer is aligned with a channel traversing the large ribosomal subunit. These reconstructions favor a mechanism in which the nascent peptide is transferred from ribosome to the ER lumen via this continuous composite channel.

A recent study has indicated that in the mammalian system BiP seals the nontranslocating and newly targeted translocons (Hamman et al., 1998)

As already mentioned, the minimal apparatus for cotranslational translocation required in reconstituted vesicles (Görlich and Rapoport, 1993) includes the SRP receptor of two subunits (probably required only for targeting) and the Sec61p complex (the heterotrimeric *translocon*). TRAM is also required only for some proteins and transiently at the beginning of the translocation of the peptides. Cross-linking experiments using single photoreactive groups located at various positions in the peptide showed that, before cleavage, the entire polypeptide segment extending from the ribosome is almost exclusively in contact with Sec61 (Mothes et al., 1994), which is then likely to constitute the walls of the channel. Sec61 probably spans the membrane 10 times, and the spanning segments have several hydrophilic amino acid residues. After passage through the channel, the peptide comes in contact with many other proteins, explaining the cross-linking observed in other experiments.

Sec61p purified from mammalian and yeast cells in the presence of detergent, forms a cylindrical structure of 3 to 4 oligomers approximately 8.5 nm in diameter with a central pore of approximately 2 nm (Hanein et al., 1996). These structures are also present in ER membranes and reconstituted proteoliposomes.

The movement is thought to occur by a Brownian ratchet mechanism, in which the nascent polypeptide chain can only move in one direction because it is restricted in its passage by the walls of the channel and the ribosome that closes one end of the channel. In addition, interaction with BiP, other lumenal RER proteins and glycosylation reactions, makes the process vectorial by trapping the protein on the inside of the vesicle.

The transfer and cleavage of the signal sequence require some special features (see Section IIA). These are beginning to be understood in detail. All signal sequences have in common a variable stretch of hydrophobic amino acids, a short positively charged amino terminal region and a polar carboxy terminal region which contains the site that is generally cleaved. After insertion and before cleavage, the carboxy terminal faces the ER lumen and the amino terminal, attached to the nascent polypeptide, remains on the outside. The various steps in the translocation and eventual cleavage of the signal sequence have been revealed by several studies.

During cotranslational translocation, the signal sequence of the nascent protein first encounters a subunit of the SRP that contains a hydrophobic segment lined with flexible methionine side chains which are capable of binding the highly variable central hydrophobic region of the signal sequence (Keenan et al., 1998). The signal peptide is then inserted in the lipid-exposed area of the Sec61α two transmembrane helices (Plath et al., 1998; Mothes et al., 1998), probably opening the channel for polypeptide transport. This coincides with the formation of a tight seal between the ribosome and the translocation channel (Hamman et al., 1998). As already discussed, the arrangement produces a continuous channel, in this case, traversed by the signal sequence from the ribosome through the large ribosomal subunit, the translocation channel and finally arriving at the ER lumen (Beckmann et al., 1997), as revealed by electron microscopy. The signal peptide has its carboxy terminal

end facing the ER lumen and the hydrophobic portion (now in a helical conformation; Plath et al., 1998) exposed to the lipid phase.

The mechanism for cleavage by the peptidase can be deduced from the structure of an *E. coli* signal peptidase, determined by X-ray crystallography (Paetzel et al., 1998). The peptidase has two transmembrane domains. One of these contains the active site which remains exposed to the lumenal side. Apparently, the active site of the peptidase comes in contact with the carboxy terminal portion of the signal sequence, held in place by the its anchoring hydrophobic region. Cleavage at this site follows.

D. Insertion of Integral Proteins

The translocation of integral proteins differs from proteins destined to be delivered to the ER lumen. Integral proteins have to be inserted in the lipid bilayer during or after translocation. In addition, for proteins with several transmembrane domains (*polytopic proteins*) the protein must be properly folded. Note that because of the mechanism of exocytosis (see below), the portion of the molecules located in the face of the ER lumen will be on the external face of the cell when delivered to the plasma membrane. The eukaryotic and bacterial systems are very similar and will therefore discussed together. However,in bacteria, the incorporation is in the inner membrane. The SecY and Sec E of *E.coli* are homologous to Sec61α and Sec61β (see Rapoport et al., 1996a). Four heterodimers of SecY and SecE from the translocation channel. However, in bacteria the translation and membrane insertion need not be coupled.

For proteins that have a single transmembrane segment, the orientation may differ (see Spiess, 1995) (see Fig. 7). The amino terminal (type I and type III) or alternatively the carboxy terminal (type II) may be in the ER lumen. For type II and III, the signal sequence in the midportion of the molecule (corresponding to the transmembrane (TM) segment), is not cleaved. For type IV integral proteins (not shown in Fig. 7), the amino terminal is in the cytoplasmic phase and most of the carboxy section of the molecule is in the transmembrane segment. For this latter case, the signal sequence is also not cleaved. In addition, some integral proteins have both terminals in the cytoplasmic compartment (diagram 6, Fig. 7). Still others span the membrane repeatedly. How do the transmembrane proteins reach their proper orientation and folding? Some insights have been gained in recent years.

The targeting to the ER membrane first requires a signal-sequence (Blobel and Dobberstein, 1975) and generally, in cotranslational synthesis, the interaction between signal sequence, SRP and SRP receptor (see Section IIA). However, SRPs are not needed for the integration of tail-anchored proteins. At least in yeast, a type II signal-anchor protein can be incorporated independently from SRPs (Ng et al., 1996). In type II proteins, a non-cleaved *signal-anchor* need not be at the amino terminal.

For type I proteins, insertion is initiated by a cleavable *signal sequence* at the amino terminal. The translocation is terminated by a hydrophobic sequence, the *stop-transfer sequence*. Typically, cleaved signals have a positively charged short segment followed by a hydrophobic domain of 7 to 15 residues.

Cross-linking studies show that the transmembrane domain comes in contact with at least three different protein environments, suggesting that the process involves at least three steps (Do et al., 1996), suggesting a complex process rather than a simple partitioning in the lipid bilayer.

The translocation of the integral proteins follows the same path as that of secreted proteins. The channel protein Sec61 and, in some cases, TRAM are involved. However, cross-linking experiments also implicate phospholipids (Martoglio et al., 1995).

The details of the translocation are not known. For peptides that span the membrane only once, models that consider the insertion of the peptide as a loop (e.g., Engelman and Steiz, 1981) can explain the orientation of the proteins. The loop could be inserted in the membrane with one site interacting with a hydrophobic sector of the sequence (the zigzag domain in the diagrams)and another site with the polar sector of the protein (see Fig. 7, middle diagram 1). Release of the polar sector to the lumen of the ER would produce type II orientation (Fig. 7, diagram 4). Interaction with the hydrophobic sector only could produce type III (Fig. 7, diagram 5) orientation, as it does for type I (Fig. 7, diagram 3)

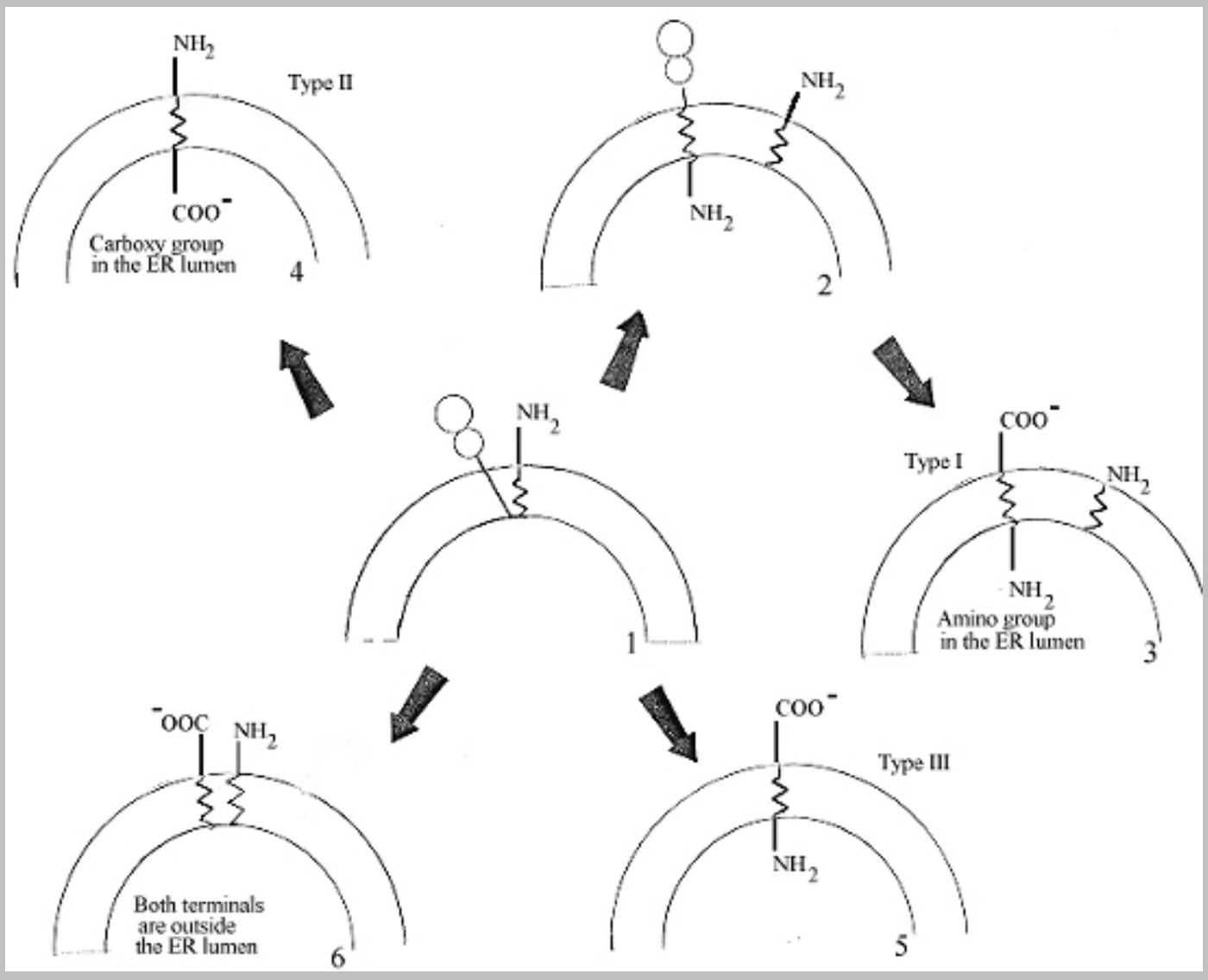


Fig. 7 Diagram showing the insertion of an integral protein so that either the amino (1, 2 and 3) or the carboxy terminal (1 and 4) are in the lumen of the ER (see below) .

In eukaryotes, one of the factors in the orientation of type II (carboxy group in ER) and III (amino group in ER)

proteins appears to be the charge difference between the residues flanking the signal-anchor (more positive on the cytosolic side) (e.g., Hartmann et al., 1989b). Experiments using mutagenesis to alter the sequence support this view. The type III protein cytochrome P-450 was converted to type II by insertion of a positively charged domain at the amino-terminal (e.g., Sato et al., 1990). Similar results were obtained with asialoglycoprotein receptor subunit H1 and paramyxovirus hemagglutinin-neuraminidase, two type II proteins induced to orient in type III orientation by mutation of flanking sequences (e.g. Parks and Lamb, 1993). However, in general the distribution was not unique (e.g., Andrews et al., 1992) suggesting that additional requirements have to be met.

The hydrophobic segment also has an influence in the orientation. The longer sequence favored type III orientation, whereas shorter segments favored type II orientation (e.g., Sakaguchi et al., 1992, Sato et al., 1990). Wahlberg and Spiess (1997) studied the role of hydrophobicity and the length of the apolar domain of a type II signal anchor sequence. Long hydrophobic domains promote amino-terminal translocation (producing type III orientation). Short segments promote carboxy-terminal translocation (producing type II orientation). Wahlberg and Spiess conclude that the topology is determined by the combination of three factors: (a) the charge distribution in the vicinity of the signal sequence, (b) the presence or absence of folded NH_2 segments and (c) the hydrophobicity of the hydrophobic segment. The folding state of the amino-terminal hydrophilic domain has a role, since the translocation is facilitated by the absence of folding (e.g., Denzer et al., 1995).

A nascent polypeptide destined to be located across the membrane must be recognized and moved laterally into the bilayer. This suggests that the channel of the translocon allows access to the phospholipid bilayer. We saw that phospholipids are photo-crosslinked to nascent chains when these contain a photoactive probe in the middle of either the signal or signal-anchor sequence (Martoglio et al., 1995).

For integral proteins with a single transmembrane domain (Type I proteins where the an amino terminal signal sequence is cleaved, see Fig. 7), some studies indicate that the TM domain is integrated into the lipid phase after termination of translation and disassembly of ribosomal-channel assembly (Borel and Simon, 1996; Do et al, 1996). The experiments of Do et al. (1996) indicate that the TM sequence leaves the aqueous pore formed by Sec61α, TRAM, and other proteins during the cotranslational integration of the protein into the membrane. The TM sector traverses three different protein environments (adjacent to Sec61p and TRAM and two adjacent to TRAM). However, the TM sequence is retained by the TRAM site and moves in the bilayer only after translation terminates.

For single-spanning proteins where the signal sequence is the TM domain and is not cleaved (type I or type III), the results are different. The TM domain enters the lipid domain well before translation has ended (e.g., Mothes et al., 1997). For a type III protein, the Sec61p channel in conjunction with the TRAM protein allows the transmembrane domain of the nascent protein to enter the hydrophobic interior of the membrane without coming in contact with the polar head groups of the lipid bilayer (Heinrich et al., 2000). The process depends on the hydrophobicity of the TM domain and the length of the polypeptide segment attached to the ribosome. Initially, SRP targets the TM domain to the Sec61p channel at a chain length of 61 residues. This is similar to secretory proteins where the targeting and membrane insertion takes place between 50 and 60 residues (Jungnickel and Rapaport, 1995; Mothes et al., 1998). As soon as the length of the sector is long enough to span the membrane, the TM enters the lipid phase. (18-23 amino acids of the TM) and immediately leaves the translocation site.

The translocation and insertion of polytopic proteins (with several transmembrane domains) is more complex (see Bibi, 1998; Dalbey, 2000 and Chin et al., 2002). Considerable progress has been made indicating that the translocation and pattern of insertion of these proteins differ significantly. Several studies suggest that in some cases there are interactions between all or some of the transmembrane domains. Folding and the insertion into the bilayer may be two sides of the same coin. However, to facilitate discussion, the two will be approached separately.

In some cases, the transmembrane segments of polytopic membrane proteins are inserted in the membrane sequentially following the amino-terminal. However, there are many variations (see Dalbey, 2000). For the *cystic fibrosis transmembrane conductance regulator* (CFTCR), a cAMP-regulated chloride channel, the first transmembrane segment can act as a non-cleaved signal sequence and the second as a stop-transfer sequence. Alternatively, the role of these two can be reversed. The Shaker-K^+ cannel spans the membrane six times and any of the transmembrane segments can initiate translocation acting as a non-cleavable signal sequence . There are many other factors as well, some indicating interactions between membrane-spanning segments. In the case of the bacterial tetracyclin-export protein, the insertion of the odd numbered membrane spanning segments requires the presence of the even numbered segments, suggesting the formation of hair-pin sectors and interaction between the transmembrane sectors on each side of the hairpin.

The association of helical segments probably depends on van der Waal forces, interhelical polar interaction (see Popot and Engelman, 2000) and hydrogen bonding between Asn, Asp, Gln and Glu residues of the side chains (Zhou et al, 2001; Gratkowski et al., 2001). Cofactor binding is sometimes needed to reach the final folded state (e.g., see Lu and Booth, 2000).

Insertion into the bilayer part of the membrane is a closely related topic. In vitro experiments indicate that hydrophobicity is the main factor determining entry of α-helical segments into the bilayer (von Heijne,1996) The lipids in the membrane have also been found to play an important role. A bilayer structure is probably required. The absence of phosphatidylethanolamine (PE)was shown to produce misfolding of LacY in *E. coli* (see Bogdanov and Dohan, 1999). The role of the lipid is thought to be chaperone-like. Synthesis of LacY in the absence of PE, allowed examining the properties of lipids needed for refolding. Primary amines (either PE or phosphatidylserine, PS) were found to be most effective, whereas phosphatidylcholine (PC) was ineffective. In addition, monoacyl phospholipids were ineffective and diacyl phospholipids had to contain at least one saturated fatty acid with a preference for chain lengths above 14 carbons.

The insertion of several transmembrane sectors of the same protein into the bilayer could occur one at a time. Alternatively, the entire multispan protein could be transferred simultaneously after being packed together in the tranlocon. Although one of the conformations of the translocon is too narrow for allowing more than one peptide at a time (Beckmann et al., 2001), some studies indicate a channel that during translocation can acquire a diameter of 40-60 Å (Hamman et al., 1997). In the case of the *multidrug resistance protein (P-glycoprotein)*, the evidence supports a cooperative release (Borel and Simon, 1996). In these experiments, abbreviated nascent chains containing up to five transmembrane sectors, still attached to ribosomes, were studied. The abbreviated chains were selectively extracted from the membranes with urea, at moderate salt concentrations. This treatment should affect only proteins in an aqueous environment. These findings suggest that all the sectors are not integrated into lipids one at a time, but are maintained in a polar environment, stabilized by electrostatic

interactions (Borel and Simon, 1996). The subsequent release of the nascent chains from the ribosomes made the peptides resistant to urea extraction, suggesting that they have been transferred to the bilayer. The nature of the transmembrane segments seems to make a difference in the transfer to the bilayer. A strongly hydrophobic segment enters the lipid environment almost immediately. A less hydrophobic segment is retained and could be cross-linked to TRAM and Sec61 α under the same conditions, suggesting that the less hydrophobic segments can be retained in the the translocon (Heinrich et al., 2000).

E. Protein Processing and Folding

As the proteins gain access to the lumen of the RER, they are modified. The signal peptide is cleaved, disulfide bonds form, and glycosylation of the amino terminal takes place. However, cleavage by the signal peptidase is not required for translocation, as indicated by secreted proteins such as ovalbumin, which lack a hydrophobic signal removed during processing (Palmiter et al., 1978).

In addition to covalent modification, newly synthesized proteins in the ER go through a series of folding and unfolding reactions and assemble into complexes. These rearrangements are catalyzed by ER-specific chaperones that prevent nonproductive or irreversible folding errors (Rothman, 1989; Gething and Sambrook, 1992). Unfolded or unassembled proteins generally remain in the ER that acts as quality control device for newly synthesized proteins (see Section IIIB and Hurtley and Helenius, 1989). However, they are also degraded (see Chapter 15)

The accumulation of unfolded proteins produces aggregates in the ER. Signals selectively activate transcription of all the genes encoding the chaperones of the *glucose regulated proteins* (GRPs) family (see below) as well as other ER-localized proteins such as PDI (Kozutsumi et al., 1988; Dorner et al. 1989). This response has been termed the *unfolded protein response* (UPR) (also discussed in Chapter 7). Although the longer term regulation is transcriptional, the immediate response to the accumulation of unfolded proteins in the ER occurs at the translational level where translation initiation is inhibited and, therefore, further accumulation of unfolded proteins is prevented. The inhibition results from the phosphorylation of the subunit of eukaryotic translation initiation factor 2 (eIF-2). Another function of UPR is to coordinate the synthesis of lipids and new membrane structures. In *S. cerevisiae*, the UPR is also activated by lipid and sterol deprivation. Sterol deprivation in the ER membrane signals to induce transcription of sterol biosynthetic genes.

Chaperones are present not only in the ER but also in different organelles and in the cytoplasm. They were first discovered as *heat shock proteins* (Hsps) which were induced by heat shock (see Jolly and Morimoto, 2000). In addition to UPR, in mammals they also were found to be induced by environmental stresses including the conditions produced by oxidative stress, exposure to heavy metals, or pathologic states (e.g., inflammation, tissue damage, infection). Some members of the family are constitutively expressed, others are inducible. Hsp genes contain *heat shock elements* (HSE) and in vertebrates heat shock transcription factors are transiently bound to the HSEs. In addition to their induction by UPR and stresses, genes encoding Hsps are transcriptionally regulated during a variety of biological processes such as cell proliferation (e.g., Jerome et al., 1993) or differentiation (Galea-Lauri et al., 1996). Chaperones also have a role in initiating apoptosis, triggered for example, by the inhibition of N-linked glycosylation or disruption of ER calcium stores (see Kaufman, 1999), although in some cases they can block programmed cell death caused by Ca^{2+} depletion (e.g.,

Reddy et al., 1999; Miyake et al., 2000). Chaperones have also been found to have a role in transcriptional regulation by the disassembly of transcriptional regulatory complexes (Freeman et al., 2002) and in particular, those associated with intracellular hormone receptors.

The GRP family of the endoplasmic reticulum are a class of chaperones (see Lee, 1992; Lee, 2001). The *immunoglobulin binding protein* BiP is a GRP. GRPs can be induced in cell in culture by glucose starvation although they are also induced by other stresses (see Lee, 1992; Little et al., 1994). The GRPs, located in the endoplasmic reticulum, are transferred to the nucleus when induced by stress. In the absence of stress they are posttranscriptionally modified into biologically inactive forms (Little et al., 1994). The promoters of two *grp* genes have a high level of redundancy ensuring their expression. The *grp* genes are expressed constitutively, however, the expression is increased under stress conditions (e.g., low glucose or oxygen). As in the case of other Hsps, GRPs are also thought to have a role during development (see Lee, 2001).

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

III. SORTING PROTEINS IN THE ENDOPLASMIC RETICULUM AND THE GOLGI COMPLEX

The passage of proteins through the intracellular vesicular transport system is complex and involves some posttranslational synthetic steps as well as the movement of materials. This section examines the steps that were summarized in Fig. 3 in some detail.

Many proteins are partially glycosylated in the ER, and both glycosylated and unglycosylated proteins are transported out of the ER at variable rates. The Golgi system has a role in the posttranscriptional N-glycosylation of integral membrane proteins and in sorting the various proteins.

The Golgi complex of animal cells generally has three to eight flattened cisternae (see also Section IIIC). In the complex, the displacement of newly synthesized proteins with time, through the various cisternae, is accompanied by stepwise processing by enzymes. These enzymes occupy specific locations in the system in an organization analogous to an assembly line. Depending on their position, the Golgi cisternae are referred to as *cis*, *medial*, or *trans* components, where *cis* corresponds to the elements closest to the RER (see Fig. 3). The tubular-vesicular elements found in the cell's periphery or at the cis-face of the Golgi, have been termed VTCs or intermediate compartments (IC). A network of tubular vesicles, the *trans Golgi network* (TGN), has been observed in the *trans* side of the system. Analogous to the *trans* system, the term *cis Golgi network* (CGN) has been adopted more recently to include *cis* elements.

The transfer from the ER to the VTCs is thought to occur via vesicles. What is responsible for the transport of cargo from VTC to the Golgi stacks? Small vesicles, possibly coated with COPI, could carry the cargo. Alternatively, the VTC units themselves could be transferred.

Presley et al. (1997) tagged the VSV G protein of a temperature sensitive mutant with the green fluorescent protein by introducing the appropriate chimeric cDNA into the cells (see Chapter 1). The mutant is unable to fold the VSV protein at 40ºC, producing an accumulation of fluorescence in the ER of these cells. As we shall see below (Section IIIB), a protein can leave the ER only when folded. The preparation was then maintained at 15ºC, a procedure that enlarges pre-Golgi structures. When the preparation was subsequently shifted to the permissive temperature (32ºC), the fluorescent protein was able to move out of the ER. The fluorescent structures transported to the Golgi were larger than single vesicles (often greater than 1.5 µm in diameter) and frequently acquired tubular shapes. The 15ºC-step used to facilitate the observations was not essential. When the cells were transferred directly to the permissive temperature, the fluorescent vesicular elements were smaller, however, the process was similar. These finding suggest that the transport from pre-Golgi elements to the Golgi takes place in relatively large vesicles.

In the experiments just discussed, the structures were transported toward the Golgi probably using the minus-end motor, *dynein* (see Chapter 24), where *dynactin* serves as an adapter to bind to vesicles (Schroer et al., 1996; Gaglio et al., 1996). In these experiments, an excess of dynactin was found to block the movement suggesting an involvement of dynein. Immunofluorescence showed that the vesicles co-localized with β-COP, a component of COPI coated vesicles (see Chapter 11, Section I and Table 1).

The Golgi cisternae do not separate out when the plasma membrane is disrupted or after micromanipulation, suggesting that they are held together by some adhesive molecules. An *N*-myristoylated Golgi protein of 65 kDa, GRASP65, has been identified in Golgi cisternae (Barr et al., 1997). GRASP65 is likely to be an important player in the formation of stacks. This is indicated, for example, by experiments in which antibodies against GASP65 were shown to block the in vitro assembly of stacks without interfering with the formation of cisternae.

The central position of the Golgi is attributed to its tendency to move along the microtubules in the direction of their minus end, toward the microtubular organizing center (MTOC), which is the centrosome in almost all mammalian cells. The microtubules begin to assemble at this center, generally located to one side of the nucleus.

All proteins processed by the Golgi complex, regardless of eventual destination, can be found throughout the cisternae, as shown, by immunocytochemistry. Sorting must therefore occur when the proteins leave the Golgi at the *trans* end. The TGN has been proposed to play a special role in the sorting (Griffiths and Simon, 1986). Several distinct strategies have been used to examine the mechanisms of these intracellular pathways. These are discussed in the first section (A), followed by a discussion of the information presently available (sections B and C).

A. Different Experimental Approaches

Most of the techniques discussed in previous chapters were used in the study of secretion, including isolation of cell components, EM, and immunoelectronmicroscopy. Some unique strategies were also applied. These are the subjects of this section.

Yeast genetics used as a tool

The yeast *Saccharomyces cerevisiae* functions in many ways like other eukaryotes. In yeast, the central vacuole corresponds functionally to the mammalian lysosome; yeast also secrete proteins into their *periplasmic space*, i.e., the space between the plasma membrane and cell wall. The presence of a structurally distinct Golgi with several compartments is still to be demonstrated. However, the functional evidence for its presence is incontestable (see below).

Yeast have the advantage of permitting the application of genetic techniques so useful in elucidating the molecular biology of cell functions.

The *temperature sensitive* (ts) yeast secretion mutants can be grown at the permissive temperature. They malfunction at the non-permissive temperature and the mutant cells can be readily isolated (Novick et al., 1980). The accumulation of a secretory product progressively increases the mass of the cells, so that they can be isolated by centrifugation techniques. Twenty-three complementation groups were isolated in this fashion. Generally, (but not always, because other strategies and conventions were used) each gene known to be involved in the secretory pathway is referred to as *SECn*, where *n* is an arbitrarily assigned number. Lower case letters indicate a mutant. A protein encoded by an *SECn* gene is referred to as *secnp*.

Mutants defective in steps that take place in the ER required a different strategy, referred to as [^{3}H] mannose suicide (Newman and Ferro-Novick, 1987). The initial glycosylation of proteins takes place in the ER. Therefore, in the presence of excessive concentrations of radioactive mannose at the temperature restrictive for the mutant, wild type yeast will be destroyed. In contrast, the mutants will remain viable because they are unable to incorporate the radioactive mannose. They can be subsequently grown at the permissive temperature and in the absence of radioactive mannose. This approach demonstrates that a minimum of 11 genes contribute to the passage from the ER to the Golgi system.

In addition to the mutants isolated by these direct approaches, several yeast genes involved in vesicular traffic were identified because of their homology to the genes of other organisms. Generally, their products were recognized by their cross-reactivity to antibodies to the homologous protein. The yeast Arf1p and Arf2p were recognized from the mammalian Arf1p, which had been discovered earlier. Arf1p is a Ras-like GTP-binding protein (see Chapter 11). Subsequently, Arf-1p-deficient yeast cells were found to be defective in the transit through the Golgi apparatus.

The genetic approach can also reveal physiological interactions between gene products by the use of double mutants. *sec18*, sec*17* and sec*22* were found to accumulate vesicles produced by the ER (Kaiser and Shekman, 1990). The vesicle accumulation is blocked by four other mutations: *sec12*, *sec13*, sec*16* and *sec23*. This observation indicates that the proteins coded by the last three genes control an earlier step (or steps). Mutations in either one of the two sets are lethal, but only when in combination (e.g., *sec17* and *sec18*). This dependence indicates some sort of cooperation between the various gene products in both vesicle budding and fusion. Suppression of a mutant by another mutant gene is also likely to indicate an interaction of their products at the molecular level. In agreement with this idea, when bacterially produced Sar1p (using recombinant DNA technology) was added to an in vitro membrane system from sec*12* cells, normal function was re-established (Oka et al., 1991). Sar1p is a Ras-like GTP-binding protein (see section IID).

As illustrated by this example, analysis of the mechanisms first suspected from genetic studies requires additional studies involving biochemical techniques. In addition, the location of the proteins in the cell is most readily revealed using antibodies to the proteins (labelled for cytochemistry or electron microscopy), generally produced against the bacterially expressed proteins.

Animal viruses used as tracers

Viruses use the synthetic machinery of infected cells. During completion of the synthesis of new animal virus, the viral envelope containing its own characteristic integral proteins is generated from the plasma membrane of the host cell by a process of budding. The fate of the newly synthesized viral coat proteins therefore can be used as a model for the pathway followed by plasma membrane integral proteins. *Vesicular stomatitis virus* (VSV) and *Semliki Forest virus* (SFV) have been particularly useful. Semliki Forest virus, which infects mosquitoes, is related to yellow fever virus and is named after a forest in Uganda. Vesicular stomatitis virus is a mild pathogen of cattle. Generally, cells in culture such as Chinese hamster ovary (CHO) cells and baby hamster kidney (BHK) fibroblasts, have been used.

Temperature blocks in normal cells

Apart from the localization of components using EM immunocytochemistry, normal cells in culture provide a good deal of useful information because the various steps are differentially affected by temperature. Lowering the temperature to 20ºC slows the exit from the TGN so that many proteins pile up at this step (Griffiths and Simon, 1986). Lowering to 15ºC, blocks at an earlier step, causing an accumulation of proteins in the CGN (Saraste and Kuismanen, 1984).

Reconstitution experiments

In vitro reconstitutions, greatly simplified the molecular dissection of the intracellular transport system. At first, cells with damaged membranes or impure fractions were used. These studies were then followed by others using purer cell fractions (e.g., see Pryer et al., 1992). Blocking a step by a mutation, a chemical inhibitor, or the removal of a key component, should result in an accumulation of vesicles or intermediates. These can be readily recognized. Addition of the missing component, should re-establish function. This strategy would not only identify components of the system, but also reveal the order of the reactions.

Experiments that identified the NEM-sensitive factor (required for vesicle fusion) and recognized the need for ATP and cytosolic components, used this approach. Replacement of a missing component in an extract of cells from one organism with a protein from an unrelated organism, establishes the presence of a functionally equivalent factor for both. Some of these experiments will be discussed in the rest of the chapter.

B. Export from the ER

The ER plays a role in regulation of the intracellular transport by controlling the duration of residence of the various proteins, each having a characteristic half time of residence (e.g., Fries et al., 1984). Presently available evidence indicates that proteins have to be folded and assembled before leaving the ER. For example, the retinol-binding protein cannot be transported unless it binds its ligand (Ronne et al., 1983) and, similarly, the heavy chain of immunoglobulin M (IgM) accumulates in the ER unless it is able to bind to the light chain to form the complete antibody molecule. These observations suggest that the

sorting signal for leaving the ER corresponds to patches in the protein molecule that are conformation dependent. Unfolded or unassembled chains appear to be retained in the ER until they are assembled.

In some cases, unfolded proteins, such as mutant proteins of influenza hemagglutinin, have been found to be associated with a 77-kDa protein (heavy chain-binding protein, BiP; a resident chaperone, involved in protein folding), in a pattern that suggests an association during an intermediate step of folding (Gething et al., 1986). Unfolded and unassembled proteins have a tendency to form aggregates and may be unable to proceed through transport from the ER unless they acquire their native conformation. The disulfide isomerase (PDI) is needed for correct disulfide bond formation, and the BiP protein would facilitate these processes (Pfeiffer and Rothman, 1987). BiP is released from immobilized immunoglobulin heavy chains on addition of ATP (Munro and Pelham, 1986), suggesting a possible energy-requiring step in which proteins are disaggregated so that their transport from the ER can proceed. However, this cannot be the only mechanism for retention in the ER. BiP, PDI, glucose regulating protein (GRP94) and other soluble proteins residing in the ER, have a role in the initial steps of maturation of secretory proteins (see Pelham, 1990). These proteins must be present in a functional form and be properly folded, yet they are retained; therefore, they must be distinguished from other proteins that are transported rapidly through the Golgi complex.

Although retention must play a role, an alternative mechanism, the retrieval of proteins leaving the ER by retrograde transport, has also been shown to be important. As we have seen a number of times, special amino acid motifs in a protein are likely to be involved in targeting. Why not examine whether special motifs have a role in the retention or retrieval of proteins of the ER? This approach can be used by first determining the amino acid sequence of the proteins and then looking for common sequences, perhaps with computers using available data banks (see Chapter 1). The identification of common sequences would also open the way for manipulating protein structure using the bag of tricks supplied by molecular biology, using modified DNA inserted in vectors (Chapter 1). A resident protein from which the suspected sequence is missing should proceed to another compartment. Conversely, a protein generally targeted to another location should acquire residence in the ER when the recognition sequence is attached to the molecule.

The search for common motifs revealed common or similar sequences at the carboxy-terminal of resident proteins corresponding to a tetrapeptide [in mammals, usually Lys-Asp-Glu-Leu (KDEL) and in yeast, Hist-Asp-Glu-Leu (HDEL)]. When expressed in monkey COS cells (transformed kidney cells from the African green monkey), BiP lacking this sequence was secreted (Munro and Pelham, 1987). In contrast, addition of the last 6 amino acids of BiP to secretory, lysosomal, or vacuolar proteins, caused their localization in the ER. Similar results were obtained with other mammalian cells, plants and yeast.

The KDEL or HDEL sequences are not always the ones used for recognition. Although some mammalian liver esterases sometimes use similar tetrapeptides, they frequently have different coding sequences. Residence in the ER is also favored by a double lysine motif in the cytoplasmic tail of a protein (Jackson et al., 1993).

These sequences are retrieval signals, as has been demonstrated ingeniously by adding the KDEL retention sequence to the lysosomal enzyme cathepsin D (Pelham, 1988). Lysosomal enzymes are processed in the Golgi stacks (see sections IIIC and G), in this case by the addition of N-acetylglucosamine-1-phosphate (G1cNAc-1-phosphate). The cathepsin was found in the ER, but with the addition of N-acetylglucosamine (GlcNAc), which must have taken place after cathepsin left the ER. This experiment suggests that a membrane receptor for the retained protein is activated in a post-ER component and the complex is then returned to the ER. Yeast mutants unable to retain the HDEL signal have been isolated, and a putative receptor for post-ER recognition has been found (Semenza et al., 1990).

As mentioned above, many proteins such as enzymes are required in their folded form to perform the tasks of the ER. Is retrieval the only mechanism to retain these resident proteins in the ER? The proteins could be immobilized by binding to receptors excluded from the buds in the process of forming transport vesicles. Removal of either the KDEL sequence or the double lysine motif, permits the secretion of ER resident enzymes. However, the rate of loss is very low (Pelham, 1989) and generally vesicles budding from the ER in an in vitro yeast system do not contain resident proteins (Barlowe et al., 1994, Bednarek et al., 1995, Füllerkrug et al., 1994). These observations suggest that, in these cases, retention is likely to play the predominant role.

In addition to retaining needed or defective proteins in the ER, at least some of the exported proteins are selected. The cargo proteins are loaded by selective binding as demonstrated by the fact that they are concentrated in the anterograde (COPII) vesicles about 10-fold. A concentration of this kind suggests a binding to specific binding sites. The presence of such binding sites is supported by the need of certain domains such as AspXGlu in the carboxy-terminal cytosolic domain of certain transmembrane proteins (e.g., Nishimura and Balch., 1997). In the case of proteins recycling between the ER and the Golgi, the anterograde transport depends on two phenylalanine residues close to the carboxy terminal (e.g., Dominguez et al., 1998) required for binding to coat components of vesicles (the Sec23p/Sec24p complex). The binding between cargo proteins and coat subunits (in this case COPII) should be demonstrable in vitro. As expected, complexes have been found in yeast ER extracts containing COPII subunits and cargo molecules (Kuehn et al., 1998). However, resident proteins were absent. In mammalian ER extracts, components of the COPII vesicles [the GTPase Sar1p (expressed as a glutathione S-transferase fusion protein) and Sec23p/Sec24p] formed a complex in ER vesicles that contained VSV-G cargo glycoprotein (Aridor et al., 1998). Again, a resident protein, ribophorin, was found to be absent.

A variety of known signals for transport and retrieval are displayed in Table 2 (Rothman and Wieland, 1996). These are involved in anterograde and or retrograde transport.

Table 2 Examples of transport signals. Reproduced from Rothman and Wieland, 1996, by permission. Copyright ©1996, American Association for the Advancement of Science.

Signal	Location in protein and with respect to membrane	Fate specified
KDEL	COOH-terminus, luminal	Retrieval of proteins from Golgi to ER
KKXX	COOH-terminus, luminal	Retrieval of membrane proteins from Golgi to ER
XXRR	NH_2-terminus, in cytoplasm	Retrieval of membrane proteins from Golgi to ER
Propeptide	NH_2-terminus, luminal	Transport from Golgi to endosomes or lysosomes
Mannose-6-phosphate	Asn-linked saccharides, luminal	Transport from Golgi to endosomes or lysosomes
Tyrosine-rich dileucine	Cytoplasmic domain	Transport from Golgi to endosomes or lysosomes
YQRL	Cytoplasmic domain	Transport from cell surface to Golgi
NPXY (and similar)	Cytoplasmic domain	Transport from cell surface to endosomes
GPI anchor	COOH-terminus, luminal	Transport from Golgi to apical cell surface in polarized cells

What happens to the proteins that are retained in the ER because they are defective, unassembled or misfolded? The ER possesses what has been called "quality" control (see Kopito, 1997; Ellgaard et al., 1999) and these proteins are degraded (see Bonaficino and Klausner, 1994). In addition, the same degradation system regulates the activity of ER-resident proteins and plasma membrane proteins. The degradation apparently involves a translocation system that removes the soluble (McCracken and Brodsky, 1996) or integral membrane proteins (Wiertz et al., 1996a) from the ER to the cytoplasm. In the cytoplasm, they are degraded by the *proteasome* system (e.g., Wiertz et al., 1996a) (see Chapter 15). At

least in some cases, polyubiquination is required (e.g., Hiller et al., 1996) (see Chapter 15, Section IIB). The proteasomes cover the cytoplasmic face of the ER membrane in secretory cells (see Rivett, 1993) and, at least in yeast, the ER membrane contains ubiquitin conjugating enzymes. (Sommer and Jentsch, 1993).

The translocation from the interior of the ER to the cytoplasm involves Sec61 protein, the translocon component discussed above and, in addition, BiP and Sec63p. The latter two are also involved in posttransciptional import (Plemper et al. 1997) as demonstrated using various yeast mutants. Import into the ER vesicles and reverse translocation may therefore involve, at least in part, the same pathway (Wiertz et al., 1996b). A 97 kDA ATPase, *valosin containing protein* (VCP; also called CDC48 or p97) serves as a link between the export of the proteins from the ER and their degradation in the proteasome. VCP is one of the proteins of the *ATPase with multiple cellular activities* (AAA family). VCP forms a homohexamer that binds to a number of other proteins with a function in the transfer. The VCP complex binds to polyubiquitin chains while these are in the translocon and delivers most of them to the proteasome (e.g., Jarosch et al., 2002; Braun et al., 2002).

The ER-proteasome apparatus may not be the only mechanism used by cells to control the quality of its proteins. A resident protein of the TGN, furin, is degraded by lysosomes. The transfer to the lysosomes is apparently controlled by the state of aggregation of the furin (Wolins et al., 1997).

The production of the misfolded or defective proteins may exceed the capacity of the proteasomes. In these cases, the intracellular deposition of misfolded protein can produce aggregates that form ubiquitin-rich cytoplasmic inclusions, sometimes characteristic of neurodegenerative diseases (see Mayer et al., 1991). *The cystic fibrosis transmembrane conductance regulator* (CFTR), an integral protein, is a transporter molecule that functions as a Cl^--channel in the plasma membrane. Mutations of the corresponding gene produces *cystic fibrosis*, which causes severe chronic bronchopulmonary malfunction and pancreatic insufficiency. Most cystic fibrosis patients carry an allele of CFTR which interferes with the normal folding so that the mutated CFTR is retained in the ER and rapidly degraded. The degradation can be prevented by inhibiting the proteasomes (Ward et al., 1995; Jensen et al., 1995). The mutant CFTR surprisingly can function normally if it were delivered to the cell surface (Cheng et al., 1991). *Presenilin-1* (PS1) is another integral protein predominantly present in the ER. PS1 is present in neurofilament-rich cytoplasmic inclusion bodies in Alzheimer disease (e.g., Busciglio et al., 1997). Both CFTR and PS1 have multiple transmembrane segments. The fate of CFTR and PS1 molecules were studied in transfected human embryonic kidney or Chinese hamster ovary cells where proteasome activity was overexpressed or inhibited (Johnston et al., 1998). The accumulation was in the form of high molecular weight, detergent-insoluble, multiubiquitinated forms in distinct pericentriolar structures that have been called *aggresomes*. The formation of aggresomes redistributes the intermediate filament protein *vimentin* (see Chapter 24) so that it forms an enclosure for the protein. Interference with microtubules blocks the formation of aggresomes.

The retention of mutant misfolded Wilson protein is the basis for *Wilson disease*, an inherited disorder of copper metabolism resulting in neuronal degeneration. The Wilson protein is a Cu transporter localized in the *trans*-Golgi. The mutant form is eliminated by the action of the ER quality control machinery (Payne et al., 1998).

Current thinking suspects inefficient ER quality control to be responsible for prion diseases such as *bovine spongiform encephalopathy* (BSE) or *Creutzfeldt-Jakob disease* (see Dobson and Ellis, 1998). Apparently, the transmission of the diseases depend on the expression and accumulation of abnormal variants of the prion protein PrP (e.g., see Prusiner, 1997; Hegde et al., 1998.

Go to Part 3

REFERENCES

Search the textbook

Back to Part 2

C. Passage Through the Golgi System and Transfer to the Cell Surface

We have seen that the Golgi apparatus is made up of stacks of cisternae. Seen in three-dimensions, the details of the Golgi apparatus differ considerably from cell type to cell type (e.g., see Rambourg and Clermont, 1990, 1997). Some of the early studies have indicated that many of these cisternae are interconnected by tubules as revealed by stereoscopic EM techniques (see Chapter 1) (Rambourg and Clermont, 1997) (however, see below). The imaging of cisternae isolated from mammalian cells in culture using freeze etching, (Weidman et al., 1993) indicates that generally the cisternae have two domains: a central biconcave circular domain bounded by an irregular peripheral domain containing a reticulum of interconnected tubules (referred to as *fenestrations*) and tubules. The peripheral domain has buds with a coating suggesting the presence of coat proteins (see Chapter 11). The central domain is thought to contain oligomers of resident enzymes (Nillson et al., 1993) (see below section on retention). Tubular connections between cisternae are frequent (Weidman et al., 1993).

More recently, a partial three-dimensional reconstruction of the Golgi apparatus from rat kidney cultured cells (Ladinsky et al., 1999) and pancreatic β cells (Marsh et al., 2001) have been carried out and present a picture very similar to that from previous studies. However, some of the details differ significantly. In the study with rat kidney cells, the specimens were subjected to ultrarapid cryofixation and freeze-substitution [where freezing at very low temperatures (e.g., -174ºC) is followed by fixation in an organic solvent also at low temperatures (e.g., -90ºC)]. The observations were carried out with a high-voltage EM using thick sections and then the appropriate tomographic techniques were applied. (see Chapter 1). The study used dual-axis tomography (Mastronarde, 1997). With this technique, the specimen were tilted around two orthogonal axes. The cisternae appeared closely apposed in stacks (the *compact region*) or loosely connected laterally by *bridging tubules* (the *non-compact region*) at equivalent levels, but not at nonequivalent levels. All cisternae were found to be fenestrated and to display buds: the trans-most cisternae displayed clathrin coated buds. Other cisternae displayed non-clathrin coated buds and others were uncoated. Tubules with budding profiles were found at the margins of all *cis* and *trans* cisternae. Vesicles filled holes were found at the *cis* and lateral sides of the stacks and these "wells" may be involved in vesicle transport between the stacks. The stacks of the Golgi were found between *cis*-ER and the *trans*-ER which is very close to the trans-stacks of the Golgi. Between the *cis*-ER and the Golgi, there are many tubular and flattened sac structures. The idea that anterograde transport depends on transient tubular connections between successive cisternae (see Section I, above) is not supported by this study or the study of pancreatic β cells (Marsh et al., 2001), since such connections between successive cisternae were not found. Some tubular projections reaching outside of the Golgi were found and they sometimes bypassed cisternae. The results with cultured pancreatic β cells (Marsh et al., 2001) provides a

reconstructions with a resolution of approximately 6 nm. The observations are similar to those of the previous study. However, in the reconstructed sector the ER is a single continuous compartment in close contact with mitochondria, *trans*-Golgi cisternae, and endosomal and lysosomal elements. The Golgi cisternal openings permit the ER traversing the Golgi ribbon from one side to the other. Microtubules are in close contact with the *cis*-Golgi, the ER, and elements of the endo-lysosomal system.

Sequential events and sequential sites

The Golgi system is the site of synthesis of many sphingolipids, such as sphingomyelin and glucosyl ceramide. Furthermore, the Golgi modifies newly synthesized proteins and lipids. Golgi enzymes glycosylate proteins or trim them by removing carbohydrate components. Still others, add sulfates to tyrosines, attach palmatoyl groups and cleave certain proteins. The reactions are carried out stepwise in various cisternae. The enzymes needed for these reactions are not distributed evenly, but generally in the order of the reactions from the cis to the trans direction. Eventually the Golgi system sorts out the different cargos into vesicles destined to different targets.

The passage of proteins through the Golgi system can be traced by initiating the synthesis of a protein at a specific time and following its travel through the cell's compartments. This can be accomplished by viral infection. The viral glycoproteins enter the Golgi complex at the cis face and exit through the trans face (Bergmann and Singer, 1983, Saraste and Hedman, 1983).

A finished glycoprotein requires several posttranscriptional stepwise enzymatic additions to arrive at its mature structure. The specific locations of the necessary enzymes, mirroring the order of the necessary steps, are evidence of vectorial processing akin to an assembly line. The distribution of enzymes in the ER and the Golgi system is shown in Fig. 8 (Goldberg and Kornfeld, 1983). The evidence for this distribution comes, in part, from the experiments in which the components of CHO cells were fractionated in sucrose gradients and the glycoprotein-processing enzymes were found in different fractions. Enzymes acting earlier in the processing pathway were found in the heavier fractions. This was confirmed in more detail using mouse lymphoma cells, in which the order of fractionation in the sucrose gradients coincided with the order of processing. Although this evidence shows that the location is vectorial, it is somewhat harder to correlate the presence of an enzyme with an actual location inside the cell. Immunocytochemistry has confirmed the location of some of these enzymes in the appropriate Golgi compartment.

Experiments involving a complementation assay that requires cell fusion, confirmed that proteins are transferred from one Golgi stack to another in a unidirectional manner (Rothman et al., 1984a, 1984b). In these experiments, VSV-infected CHO cells were fused to noninfected cells. The Golgi complexes of the two types of cells maintained their integrity. Three clones of cells were used. The fusion of the various types of cells was accomplished by short exposure of the cells to low pH.

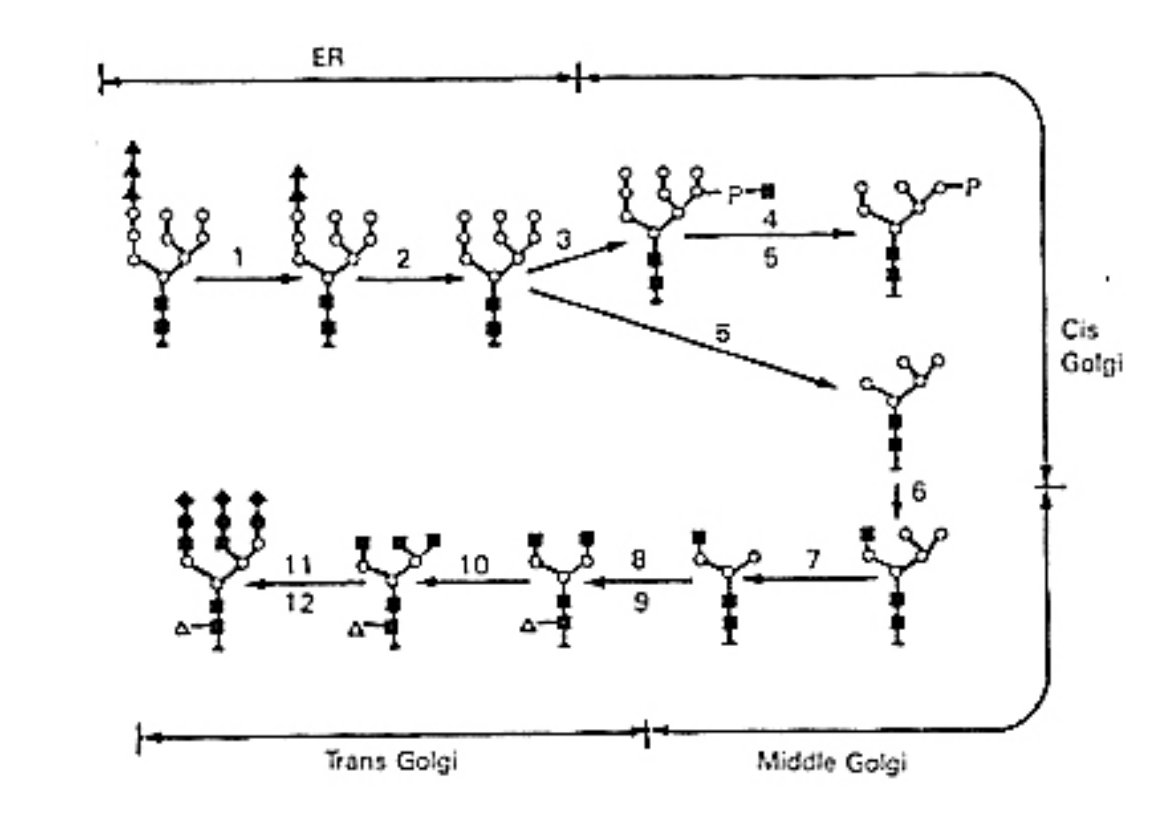


Fig. 8 Steps in the processing of asparagine-linked oligosaccharides and their presumptive intracellular site. Steps for addition of M6P to lysosomal enzymes are indicated in the side branch (3-5). 1; Glucosidase I, 2, glucosidase II; 3, lysosomal enzyme, N-acetylglucosaminylphosphotransferase; 4, lysosomal enzyme, phosphodiester glycosidase; 5, mannosidase I; 6, GlcNAc transferase I; 7, mannosidase II; 8, GlcNAc tranferase II; 9, fucosyltransferase; 10, GlcNAc transferase IV; 11, galactosyltransferase; 12, sialyltransferase.
(▲) Glucose; (■) GlcNAc; (○) mannose; (●) galactose; (△) fucose; (◆) sialic acid; P, phosphate. Note: ER mannosidase is not indicated in the diagram. Reprinted with permission from Goldberg and Kornfeld (1983).

The experiments can be explained most simply by using arbitrary symbols to indicate the direction of the reactions, which occur in separate stacks as follows : A → B → C → D. In the first experiment, infected cells which were able to carry out the first reaction A → B but could not carry out B → C, were fused to non-infected cells which could not carry out the reaction A → B. The virus G-protein was now appropriately modified by reaction B → C. This could only have happened if the viral G protein was transferred from the stacks of one cell to that of the other as shown in Fig. 9.

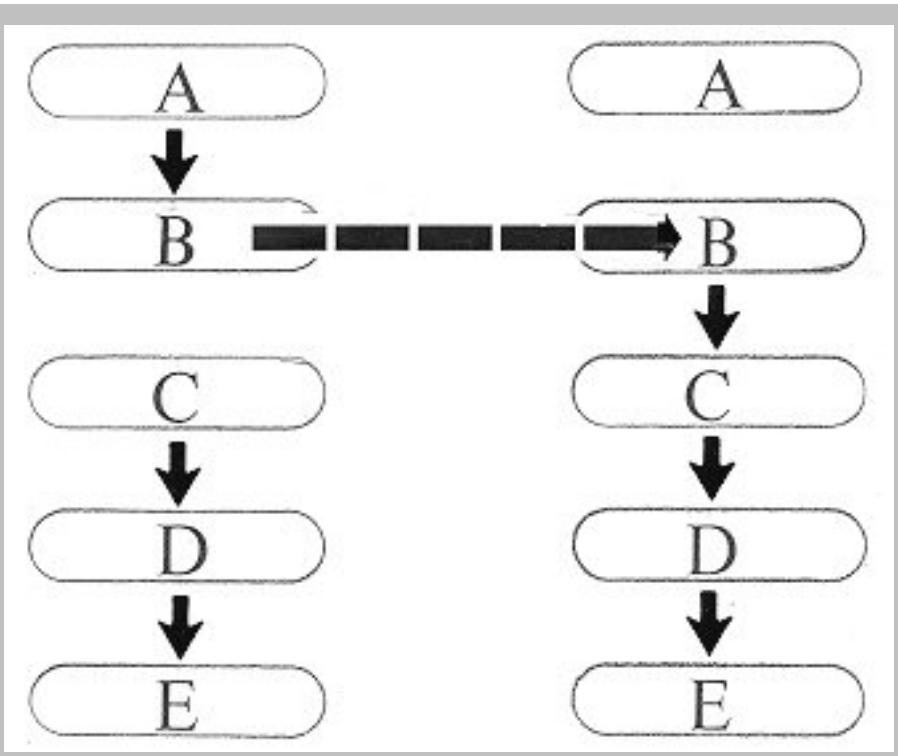


Fig. 9 Cartoon showing the design of an experiment demonstrating the transfer of materials between two different Golgi stacks.

In experiments following the same strategy, the fusion of the two different kinds of cells was carried out either immediately or after delays of different duration. As indicated in Fig. 10, the longer the delay, the lesser the transfer. In this figure, the ordinate shows the extent of the reaction (in this case the incorporation of [^{3}H]GlcNAc into the G viral protein) and the abscissa, the time after the introduction of the labelled material, before fusion was allowed to take place. These results indicate (a) the transfer between Golgi stacks can take place even when separated in space and (b) the passage must be in a single direction (that is vectorial), otherwise it would not follow the pattern shown in Fig. 10.

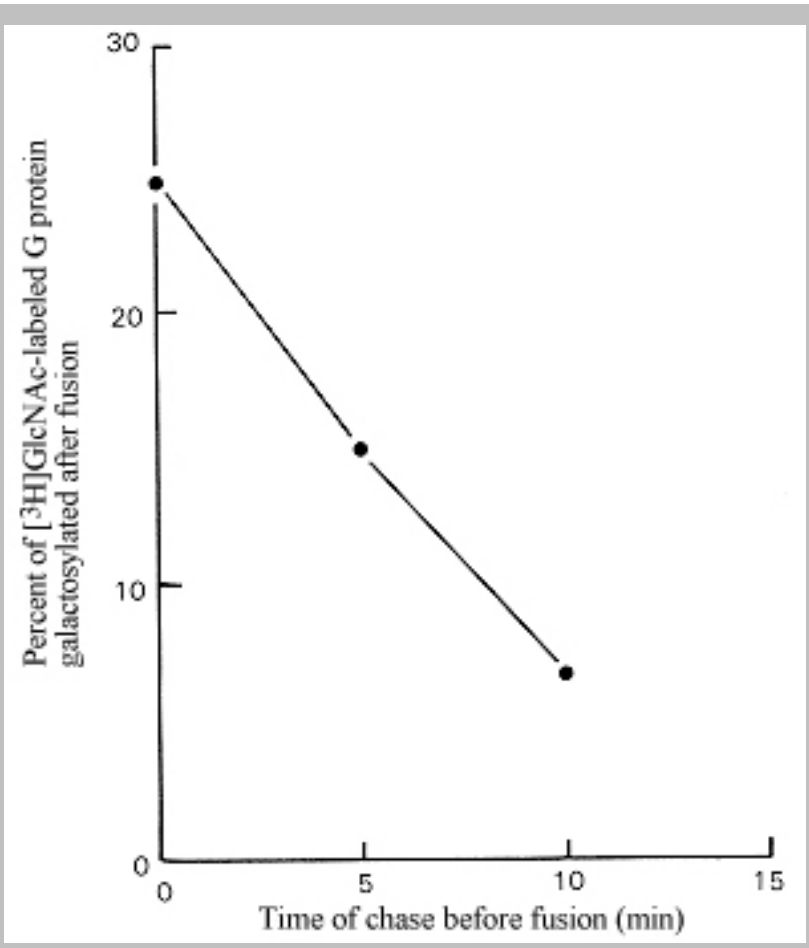


Fig. 10 Kinetics of galactosylation of G-protein labelled with [^{3}H]GlcNAc in VSV-infected clone 13 cells after fusion to uninfected clone 1 15B cells (Rothman et al., 1984a). Reproduced from *The Journal of Cell Biology*, by copyright © permission of the Rockefeller University Press.

Experiments with a somewhat different design have been used to study the vectorial nature of the transfer directly. Infected cells from a clone unable to carry out the reaction C → D but able to carry out the reactions A* → B* and B^o → C^o (where the two suprascripts indicate a different kind of radioactive probe) were fused to infected cells able to carry out reaction B → C and subsequent reactions, but not A → B. The design is shown in Fig. 11. The cells in which the viral G-protein was labelled with *, were able to produce E* to a significant extent. In contrast, the viral G-protein labelled with ^{o} at a later step were unable to incorporate the ^{o} label and produce E^o. This indicates that the G-protein labelled at an earlier step was able to be transferred more readily, as expected for a vectorial transfer. The protein labelled in a later step did not have enough time to proceed to the next step.

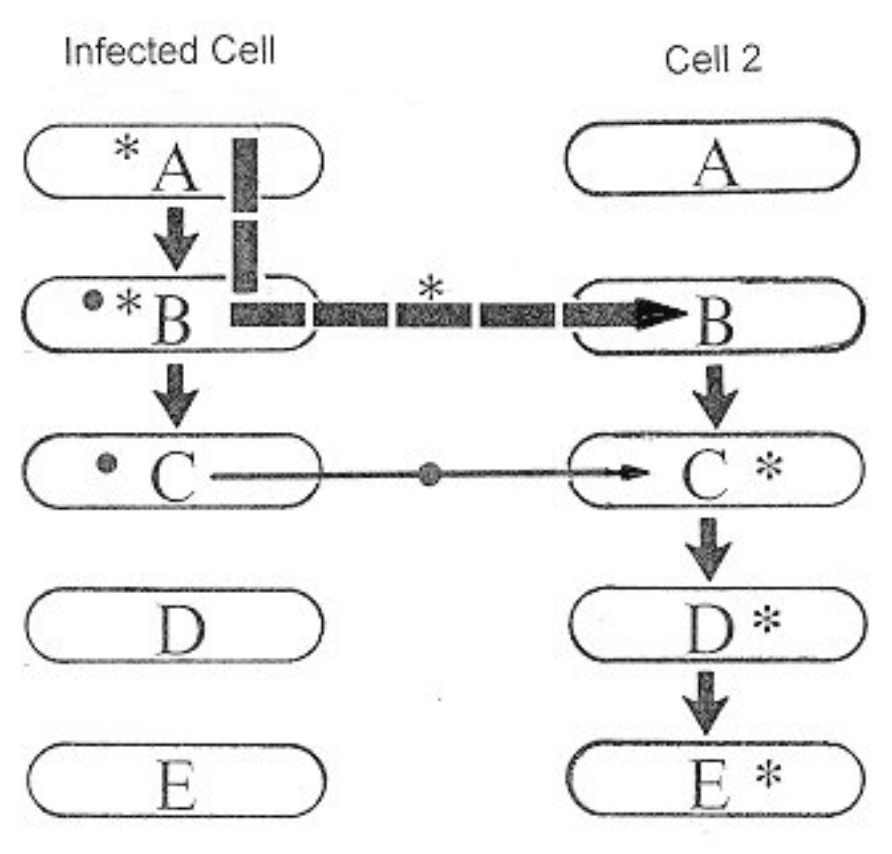


Fig. 11 Cartoon illustrating the experimental design in which the ability to transfer to the same Golgi stack from two different sequential Golgi steps.

Experiments with cell-free systems of CHO cells provide similar evidence (e.g., see Balch 1989). The transport between stacks requires the presence of cytosol extract, ATP, and a protein in the surface of the Golgi. In general, intercompartment transport of G-protein requires sequential steps probably involved in budding and fusion. ATP is required for each step, and cytosol is required for all except the last step.

The experiments just described have been interpreted to mean that in the Golgi, the transport of cargo is vectorial and that the anterograde transport is carried out in packets, such as vesicles. As we shall see in the next section, an involvement of vesicles is contradicted by the results of other studies and this issue is still far from being resolved.

Mechanisms of anterograde transport

Three different mechanisms of transport within the Golgi cisternae have been considered recently: transport mediated by vesicles, the maturation and displacement of cisternae and the transport through the tubules that interconnect the various Golgi elements (for a recent evaluation of the various models, see Pelham, 1998; Allan and Balch, 1999; Pelham, 2001). Present information is insufficient to reach a firm conclusion in relation to the vesicular and maturation mechanisms. In addition, the two models are not mutually exclusive and both may be operating.

Transport via tubules that connect the various cisternae is highly unlikely. We have already indicated that three-dimensional reconstruction of the Golgi apparatus did not find connecting tubules in the *cis*-to-*trans* direction (see above). There is some other evidence that bears on this question. In the tubule-transport model, resident proteins, all of them integral proteins, could be excluded from some Golgi compartments because of oligomerization and possibly binding of their cytoplasmic domains to an underlying matrix (Nilsson et al., 1993) (see discussion of retention that follows). As noted the reactions of the Golgi take place sequentially and the various Golgi compartments differ in biochemical composition as indicated diagrammatically in Fig. 8. There must be some mechanism that keeps the various components from mixing. Studies with chimeric resident integral Golgi proteins linked to the green fluorescent protein (GFP) (see Chapter 1) have been carried out to examine the mobility of proteins residing in the Golgi (Cole et al, 1996) using either photobleaching recovery (FRAP), (see Chapters 1 and Chapter 4) or fluorescence loss in photobleaching (FLIP). In FRAP, the fluorescence of a small area is bleached by a laser flash. The rate of recovery is then measured using an attenuated beam. The return of fluorescence can be interpreted as the exchange of non-fluorescent and fluorescent proteins; under most circumstances bleaching can be considered irreversible. In contrast, FLIP measures the disappearance of the fluorescence from the whole structure after photobleaching a small area repeatedly. Both methods indicate that the proteins are free to move laterally and are not bound. However, the results cannot be interpreted to mean that the membranes of the Golgi system are continuous. Since the Golgi compartments are biochemically specialized, the chimeric proteins used (β-1-4-galactosyltransferase-GFP, mannosidase II-GFP and KDEL-receptor-GFP) are likely to be present in individual compartments of the Golgi (e.g., laterally connected cisternae) and not be distributed throughout the Golgi. In addition, the transport through tubules would require some means of propelling the cargo through the connections. To our knowledge, this aspect has not been addressed so far.

A transport via the small vesicles curently implicated, cannot readily explain the transport of large structures, such as algal scales (Becker et al., 1995), casein micelles in in epithelial mammary cells (Clermont et al., 1993), procollagen in fibroblasts (Bonfanti et al., 1998), and albumin in hepatocyes (Dahan et al., 1994). These cargos progress through the Golgi apparatus but are too large to fit into vesicles (see Mironov et al., 1997, 1998; Glick and Malhotra, 1998). In addition, these components have been found in all Golgi stacks, but not in vesicles.

One of the present maturation models proposes that in anterograde transport the cisternae move from the *cis* to the *trans* direction and simultaneously acquire the appropriate enzymatic complement from the more advanced cisternae by retrograde vesicular transport. In fact, some resident Golgi enzymes are known to move retrogradely through the Golgi stacks (e.g., see below and Harris and Waters, 1996).

That a vectoral movement of Golgi compartments take place is indicated by a study of the flagellate *Triconympha* (Grimstone, 1959) in which the Golgi stacks (forming a so called *parabasal body*) disappear in the absence of food and reappear when the food is reintroduced. The results are in harmony with the interpretation that the cisternae are formed in the *cis* side and disappear on the *trans* side.

Studies of the progression of procollagen in the Golgi apparatus of chick fibroblasts, support the

maturation model. The translocation from *cis*-to-*trans* structures proceeds without showing a presence in COPI vesicles (implicated in intra-Golgi transport). This component, remains within the compartment from which it was transported from the ER (Bonfanti et al., 1998; Mironov et al., 2001). Furthermore, the compartment undergoes a change while moving from a *cis*-to-*trans* position. In the experiments of Bonfanti et al., 1998, the exit from the ER was synchronized first by inhibiting proline hydroxylation (needed for procollagen folding and hence release from the ER) followed by removal of the inhibitor. The polarity of the stacks was determined using clathrin buds as a marker of trans-Golgi, either with serial sections or immunogold using two different sizes of gold particles identifying procollagen and clathrin and, in addition, the visualization of COP coats. Mannosidase II, a medial Golgi enzyme was visualized using an immunological method with peroxidase as a marker (Rabouille et al., 1995). The movement from the ER to the *cis*-Golgi was in tubular-saccular structures greater then 300 nm in length. These were interpreted to correspond to precursors of the cis-Golgi cisternae that then progress through the stacks by a maturation process. The passage from trans-Golgi to the plasma membrane occurs in vesicles.

The cisternal maturation model of anterograde transport across the Golgi apparatus requires a continuous retrograde movement of resident proteins to compensate for their loss. Therefore, retrograde-directed vesicles must contain these proteins and the retrograde transport must equal the anterograde movement. The demonstration of vesicular retrograde transport of Golgi components supports the maturation model. Under steady-state conditions in mammalian cells in culture (Love et al., 1998), a fraction of resident Golgi enzymes was found in vesicles that were depleted of secretory cargo (and therefore not involved in anterograde transport) and could be separated from cisternal membranes. They were capable of binding to and fusing with isolated Golgi. Furthermore, after fusion, their enzymatic complement was able to process newly acquired cargo. Other experiments support the presence of recycling of processing enzymes (Hoe et al., 1995; Harris and Waters, 1996; Wooding and Pelham, 1998). In the experiments of Wooding and Pelham (1998), green fluorescent protein (GFP)-chimeras (see Chapter 1) were used to visualize late and early resident Golgi markers. These are present in distinct sets of scattered moving cisternae. In temperature-sensitive mutants, after shifting to the permissive temperature, late Golgi markers dispersed into vesicle-like structures within minutes. These results agree with the notion that resident Golgi components have to undergo retrograde transport. One of these moved quickly to the ER, suggesting that it cycles between Golgi and the ER.

The results of other experiments are also in harmony with the maturation model of transport between the Golgi stacks. This model of Golgi transport predicts the presence of resident proteins in the peri-Golgi vesicles (i.e., edges of the cisternae and neighboring vesicles) to be recycled to their original stack for the process to continue. In contrast, the vesicular model predicts that anterograde cargo would be contained in these vesicles. Resident proteins were in fact found in the peri-Golgi vesicles. In contrast in the cells expressing VSV-G, a transmembrane protein, used as anterograde marker found this protein mostly absent from these vesicles (Martínez-Menárguez et al., 2001). In the maturation model, large aggregates (such a procollagen) and other cargoes (such as VSV-G) should be found in the cisternae of the Golgi and be transferred at the same rate. Procollagen aggregates were found to be transferred through the Golgi complex without leaving the lumen of the cisternae in agreement with the maturation model (Mironov et al., 2001) and to be transferred at the same rate as the G protein of the VSV. Futhermore, the two were

found to be transported without entering vesicles. In addition, COP-I vesicles were found to contain Golgi resident proteins but minor amounts of anterograde cargo (Lanoix et al., 2001). The early Golgi proteins were in vesicles distinct from those containing proteins of the medial Golgi stacks as might be expected from the retrograde component of the maturation model.

Some in vitro experiments lend strong support to models of transport not dependent on vesicle formation. The production of coated vesicles from Golgi vesicles requires the presence of the small GTPase, ARF (Orci et al., 1993; Taylor et al., 1994). At least in vitro, anterograde transport through the Golgi complex can take place when the formation of coated vesicles is blocked by the absence of ARF (Taylor et al., 1994; Happe and Weidman, 1998). The rate of transport was found to be about the same, with or without endogenous ARF (Happe and Weidman, 1998). However, the density of coated vesicles was reduced fifteen-fold in the ARF-depleted system.

Some observations challenge the maturation model by suggesting that the rate of retrograde transport does not match the rate lf the anterograde transport. Immunoelectronmicroscopy techniques using gold particle markers (see Chapter 1) with human cells in culture were used to localize two proteins, residents of the medial Golgi (Orci et al., 2000). In contrast to the studies just discussed, the resident proteins were found to be excluded from buds and vesicles suggesting that the progression of the Golgi cisternae is much slower than the anterograde protein transport. These observations do not exclude the interpretation that the cisternae do progress in the anterograde direction, but they suggest that this mechanism is not a major player in anterograde transport.

Other recent experiments suggest an alternative explanation for the transfer of large particles, in harmony with the vesicular transport model: the transport seems to occur in very large vesicles (Volchuk et al., 2000). Expression of a construct of a self-aggregating mutant of a binding protein was used (Rollins et al., 2000) in a human cell line. The construct is delivered into the ER and normally would be secreted, if maintained in folded and soluble form. A permeant ligand which allowed the protein to remain in this condition was used. However, the transport was blocked by maintaining the cells at 15º C. When the ligand was removed, protein aggregates ranging up to 400 nm in diameter were formed within the *cis*-cisternae of the Golgi stack. After shifting the cells to 20º C, the huge particles were transported across the Golgi stack within 10 min. An EM study using serial sections showed that during the peak of the transport, about 20% of the aggregates were in megavesicles originating from the rims of the cisternae. These experiments suggest that, at least in part, vesicle size may depend on the size of the cargo.

A strong argument in favor of the vesicular model comes from studies of Orci et al. (1997) which show the presence of anterograde vesicles (proinsulin and VSV G protein) and retrograde vesicles (KDEL receptor, see Chapter 11) budding throughout the Golgi. Segregation of the two sets of proteins can also be demonstrated in vitro. The VSV-G protein and the KDEL proteins are packaged in separate vesicles.

The maturation and tubular transport proposals are unlikely to explain the evidence obtained from experiments using the fusion of cells to demonstrate a vectorial unidirectional anterograde transport (see

previous section) or the demonstration of the presence of both anterograde and retrograde vesicles associated with the Golgi system. As we saw a role of tubules is highly unlikely. However, it is entirely possible that both the maturation and vesicle-transport mechanisms operate, depending on circumstances.

Retention of resident proteins in the Golgi stacks

The individual components of the Golgi system differ in composition, including the lipid elements. This individuality could conceivably result from a steady state where a variety of products would flow through the system, but the composition of each compartment would remain the same at any one moment. A steady state mechanism such as this, is part of any maturation model. There is evidence to support the view that structural elements and the enzymatic machinery of the Golgi, the glycosidases and glycosyl transferases are retained or retrieved to stationary compartments.

The resident proteins of the Golgi, unlike those of the ER, are integral membrane proteins or peripheral proteins on the cytoplasmic face of the cisternae (see Munro, 1998). The characteristics of the proteins are shown in Table 3.

Table 3 Localization of the domains of Golgi proteins

PROTEINS	TRANSMEMBRANE DOMAIN (TMD)	LOCATION IN GOLGI
Glycosylation enzymes	At amino terminal	Lumen
TGN proteases/ TGN 38	Internal sequence	Amino portion lumen, carboxy end in cytoplasm
SNARES	At carboxy terminal	Amino portion in cytoplasm; carboxy terminal in lumen
Peripheral proteins	None	Amino terminal portion bound to cytoplasmic face

If there is a continuous movement of cargo between Golgi compartments and from the Golgi to target sites, what is responsible for the retention of resident proteins? Certain sequences or domains have been

found to be responsible for localization in the Golgi. The localization may be the result of retention or retrieval, and frequently both (e.g., Bryant et al., 1997). Presently available evidence is sometimes insufficient to allow us to generalize.

For glycosylating enzymes, the *transmembrane domain* (TMD) is an important determinant of localization (see Colley, 1997). However, for some of the enzymes, the sequences flanking the TMD and the lumenal portion of the proteins also play a role. The TMD also is important in the localization of the SNAREs, Sed5p and Sft1p (Banfield et al., 1994) to tubules of the cis-Golgi and viral proteins targeted to the Golgi, although cytoplasmic domains are also needed.

Many of the TGN proteins are recycled continuously, from the cell surface and endosomes and are returned to the TGN (e.g., Bos et al., 1993; Molloy et al., 1994). For the proteases of the TGN (e.g., furin in mammalian cells, and in yeast, DPAP-A, Kex1p and Kex2p) as well as TGN38, short sequences in the cytoplasmic tail specify location to the late Golgi probably by retrieval signals (Wilcox et al., 1992; Nothwehr et al., 1993; Bos et al., 1993, Schäfer et al., 1995). In the case of furin and TGN38, the sequences are short motifs containing tyrosine and for DPAP a ten amino acid sequence containing phenylalanine.

What is the mechanism of retention? The TMDs are thought to be resposible for retention by an anchoring mechanism. In contrast to secreted or integral plasma membrane proteins, some of the proteins of the trans-Golgi and TGN are not found at the cell surface (Teasdale et al., 1994; Wong et al., 1992). Therefore, it would seem that they do not enter the vesicles exiting the TGN.

One of the mechanisms proposed is the *kin recognition* model. In this model, like molecules recognize each other and form oligomers. The complexes are then excluded from vesicles. Supporting this view are the observations that two enzymes of the medial Golgi, *N*-acetylglucosaminyltransferase I (NAGT I) and mannosidase II, are tightly associated in vivo (Nilsson et al., 1994). In addition, viral proteins that localize in the Golgi are known to form homo-oligomers (Weisz et al., 1993). However, the association of NAGT I and mannosidase II appears to depend on the lumenal domains of these two proteins and not the TMDs responsible for their retention (Munro, 1995b; Nilsson et al., 1996).

The presence of lipids and sterol in specialized plasma membrane domains, offers another possible explanation for the retention of proteins. The presence of sphingolipids and sterols thicken membrane bilayers (Ren et al., 1997). These domains could exclude the shorter transmembrane domains of resident proteins that are unable to straddle the bilayer. Supporting this view, the TMD of Golgi enzymes are, on the average, five residues shorter than those in the plasma membrane and contain a higher proportion of phenylalanine (Bretscher and Munro, 1993; Munro, 1995a).

The role of the length of the TMD was put to a direct test by adding hydrophobic amino acids to the TMD of certain proteins. The TMD of sialyltransferase or galactosyltransferase were lengthened. These modifications were found to produce a reduction of retention. The experiments found that with a synthetic

TMD of 17 leucines, the proteins were retained. However, when 23 leucines were added, the proteins were no longer retained (Munro, 1991; Masibay et al., 1993: Munro, 1995a).

As already noted, retrieval frequently plays a role for localization in any part of the Golgi. In yeast there is evidence of rapid cycling of an early Golgi enzyme, Och1p (Harris and Waters, 1996) The localization signal for this protein is not known, but for other medial enzymes, TMDs have a role, although lumenal domains and cytoplasmic domains are also involved (Burke et al., 1994; Graham and Krasnov, 1995). Similarly, a protein can achieve a predominant cis-Golgi localization by recycling between the cisternae and the ER, as in the case of the yeast protein Emp47p Schroder et al, 1995) and the KDEL receptors Erd2p and Sec12p (Sato et al., 1996; Füllerkrug, 1997).

An avian coronavirus, infectious bronchitis virus (IBV), has provided information to define the retention signal of the cis compartment. IBV E1 is an integral protein which spans the membrane three times; the retention information is thought to be in the first intramembrane span (Machamer and Rose, 1987). When expressed from cDNA in animal cells, this glycoprotein is trapped in the cis Golgi membranes where the uncharged polar residues Asn, Thr, and Gln appear to be the significant feature of the retention signal (Machamer et al., 1990). This premise can be tested with proteins not normally retained in the cis compartment. When this amino acids motif is added to proteins normally transferred to the plasma membrane, they remain in the cis Golgi (Swift and Machamer, 1991).

Bulk flow and sorting signals

All proteins transported in the anterograde direction share the same pathway until their final packaging in the appropriate vesicle in the TGN. What determines their final cellular destination? The different proteins could be coded by separate sorting signals analogous to the signal domains (or peptides) discussed in the previous sections. Each sorting signal could then mark the destination of the protein. Alternatively, all proteins not containing a sorting signal could be transported through the various compartments, culminating with arrival at the cell surface. These proteins would include those that are constitutively secreted (they do not have to be stored) and the integral proteins of the plasma membrane. This non-specific targeting by default has been termed *bulk flow*. A sorting signal would control a destination to the remaining compartments, such as lysosomes or storage secretory vesicles. Recognition of the signal is likely to require interaction between the signal (i.e., some specific sequence or configuration of the protein molecule) and its corresponding receptor. Therefore, the selective pathways should be saturable, and when the protein is overproduced, it should follow the nonselective (bulk flow) route even when possessing the appropriate signal.

Present evidence suggests that all destinations have a corresponding targeting signal recognized by a corresponding receptor. However, transport via bulk flow is still possible, although it is likely to be much slower (see discussion in Rothman and Wieland, 1996).

Experiments carried out with yeast have been very enlightening in illustrating both the involvement of a receptor in targeting and its saturability by excess of the targeted protein (e.g., Stevens et al., 1986). In

yeast, the vacuolar compartment corresponds to the lysosomes of other cells. The recognition signal in yeast, however, is a polypeptide segment, in contrast to the carbohydrate portion of the glycoprotein, which serves as one of the signals for lysosomal localization (see below). One such protein is carboxypeptidase Y (CPY). Before reaching the vacuole, CPY is present in the cell as proCPY, which contains the sorting signal at its amino terminal. Processing to the mature form of the enzyme takes place in the vacuole. In these cells, overproduction of the vacuolar enzyme results in constitutive secretion to the periplasmic space. In experiments testing the effect of overproduction, the CPY structural gene (PRC 1) was cloned to produce plasmids containing multiple copies, which were used to transfect yeast cells. The results are summarized in Table 4 (Stevens et al., 1986). In these experiments, the increased gene dosage overproduced CPY (in the form of proCPY), presumably saturating the processing system and resulting in constitutive secretion. This interpretation is supported by the observation that deleting the amino-terminal of proCPY leads to constitutive secretion.

Fusion of sequences that code for the amino-terminal portion of CPY to the gene that codes for the secretory enzyme invertase (Inv) has allowed study of the sorting signal (Johnson et al., 1987). Fifty amino acids of the CPY NH_2-terminal appear to be sufficient to code for the transfer to the vacuole. Twenty of these correspond to the signal peptide (which initially targets the protein to the RER), so the 30 remaining are likely to contain the vacuolar sorting signal. In fact, deletion of this segment in the CPY leads to secretion of this protein.

Table 4 Overproduction of CPY Results in Secretion of the Protein

Plasmid	Estimated *PRC1* gene copy number	Relative CPY synthesis	CPY secreted %
2μ	1	1	6
CEN-*PRC1*	2	2	12
2μ-*PRC1*	5	6-8	50-55

In animal cells, as in yeast, when proteins are produced in excess or when the receptor or targeting signal (for lysosomal enzymes, mannose-6-phosphate, M6P) is absent, the proteins spill into the bulk flow pathway. This is the case, for example, in I cells, cultured animal cells which are unable to attach M6P to the lysosomal enzymes (Hasilik and Neufeld, 1980). Similarly, treatment of AtT-20 cells (a line derived

from pituitary cells) with chloroquinone which blocks the storage route, causes constitutive secretion of the ACTH precursor (Moore et al., 1983).

Since in exocytosis the membranes of the secretory vesicles become continuous with the plasma membrane, we would expect integral membrane proteins and constitutively secreted cargo to be part of the same vesicles as in fact found (Strous et al., 1983). Furthermore, in yeast, the two processes are biochemically linked, so that a mutation blocking secretion also blocks membrane growth (Tschopp et al., 1984)

The targeting principles just discussed are summarized and illustrated in Fig. 12 (Rothman and Wieland, 1996). Part A illustrates the packaging of a cargo protein (the open circles) with a transport signal domain (the full rectangle) bound to a receptor, which is shown as part of the coat (indicated by the heavy straight lines). The cargo protein is an integral protein and its signal sequence is in the cytoplasmic domain of the protein. The direct binding of a signal domain to the coat proteins has been shown in a number of cases (e.g., Cosson et al., 1996). The transport signal need not be in the cytoplasmic domain, in which case it may be bound to an auxiliary integral protein acting as a bridge between transport signal and receptor in the coat. Part B shows how retention might take place. The retention signal prevents the protein (full circle) from entering the bud either because it is firmly bound to a special lipid patch (as implied in the figure) or because it is attached to a fixed receptor protein in the membrane (not shown). The transport by bulk flow is shown for proteins (indicated by the open lollipops in Part C) that do not bind to a receptor. The various coats: clathrin, COPI and COPII are discussed in more detail in Chapter 11.

Cargo proteins that span the membrane can interact directly with coat components such as adaptor subunits (see Chapter 11). Proteins that do not span the membrane have to bind to separate cargo-receptors, integral proteins containing a motif capable of binding the cargo-protein and another to bind to a coat component.

In many cases, currently available data strongly suggest that the adaptors have a role in the recognition of signal-motifs. If this were the case, because of the variety of targets yet to be elucidated, we would expect the presence of many more adaptors. New adaptor complexes have been found. One in neuronal cells (β-NAP) is related to β-COP and β-adaptin and is a phosphoprotein associated with transport from the soma to the axon terminals (Newman et al., 1995; Pevsner et al., 1994), (Simpson et al., 1996). This coat protein is involved with TGN and plasma membrane transport. In turn, another complex has been found related to the neuronal complex (Dell'Angelica et al., 1997). These complexes have been referred to as AP-3 and have been localized in the TGN and endosomes (Simpson et al., 1996; Dell'Angelica et al., 1997).

The SDYQRL sequence of TGN38, an integral protein, binds to the μ2 chain of the plasma membrane clathrin-binding AP-2 complex (Ohno et al., 1995) during endocytosis. The same motif also interacts with the AP-1 complex of the Golgi. These observations suggest that the medium chain of the clathrin associated adaptor complexes are the signal recognition molecules for sorting in the TGN or endocytosis.

The proteins of the p24 family of transmembrane proteins have been proposed to act as cargo receptors, selecting the proteins that go into the COPI and COPII vesicles (Schimöller et al., 1995; Stamnes et al., 1995 and Fiedler et al., 1996). Currently, 16 homologous proteins of this family have been recognized. Chop 24a is a component of the Golgi and COPI. A mutation in the analogous yeast component (yp24A) causes a defect in vesicle transport (Stamnes et al., 1995). Similarly, Emp24p is a component of the ER-derived COPII vesicles. In yeast, mutants in Emp24p show a defect in the transport of certain proteins from the ER to the Golgi (periplasmic invertase and a glycosylphosphatidyl inositol-anchored plasma membrane protein) and not others (α-factor, acid phosphatase and two vacuolar proteins), supporting the view the the various cargos need different specific receptors (Schimöller et al., 1995).

Each cargo receptor also has to bind one or more subunits of the coat proteins. The cytoplasmic tail of the p24 protein family binds to the coatomer (COPI) (Fiedler et al., 1996, Sohn et al., 1996). The dilysine motif of hp24d and yp24c binds to the α-, β'- and ε-COP. Three p24 lacking the dilysine motif in their tails but containing a phenylalanine motif, bind preferentially to β-, γ- and ζ-COP. The coatomer can bind two different segments of the p24 tails. One segment binds the dilysine motif, a retrograde transport motif. Another segment binds to a phenylalanine containing motif, which is an anterograde signal. The same p24 could therefore direct a protein in the antero- or retrograde direction depending on the conformation of the coatomer.

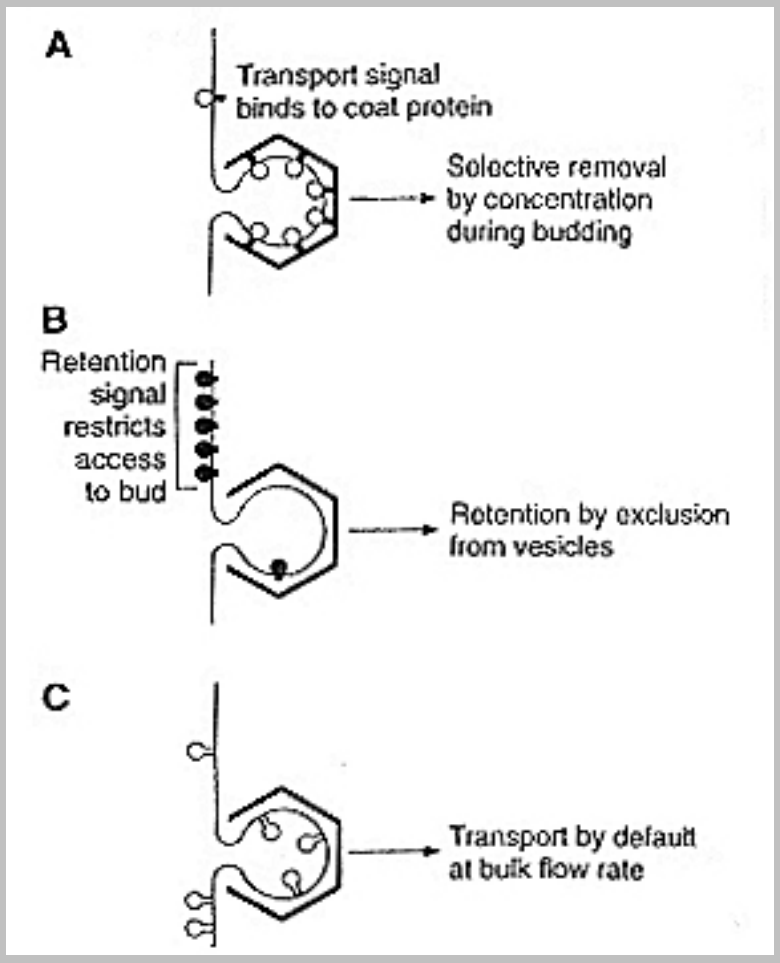


Fig. 12 Models that explain the fate of proteins (see text). A. Selective removal of protein by concentration during budding. B. Retention of protein in donor compartment by exclusion from vesicles, and C. Transport of proteins by default through the bulk phase. Reproduced with permission from Rothman,J.E. and Wieland,F.T., *Science*, Protein sorting by transport vesicles, 272:227-234, Copyright &copy1996, American Association for the Advancement of Science.

Several sorting signals are listed in Table 2 (above).

Certain proteins have to be targeted to more than one target. Furin is an enzyme that catalyzes the proteolytic maturation of many preproteins in the endocytotic and exocytotic pathway. A 56 amino acid cytoplasmic tail is required for TGN concentration and intracellular targeting (Jones et al., 1995). Interestingly enough, different sections of this tail are required for each step in the routing. An 11 amino acid segment containing 2 serines is required for TGN localization. Internalization requires 34 amino acids.

The serine residues of the cytoplasmic tail of furin are phosphorylated by casein kinase II. Mutagenesis of these residues reduces endocytosis of furin from endosomes to the TGN, suggesting that phosphorylation-dephosphorylation regulates furin recycling. Phosphorylation of a 9 amino acid sector localizes furin to the cell surface or early endosomes, whereas removal of the phosphate localizes the furin to the periphery of the TGN. A 280 kDa actin-binding-protein binds tightly to the tail of furin.

The recognition of the sorting signal of a cargo protein may result from a much more subtle mechanism than those already discussed. In the *trans*-Golgi the specific capture into vesicles may depend on the kinetics of the GTP hydrolysis in COPI-ARF1 complexes and not merely the binding to a specific receptor. Vesicle formation in the Golgi requires the assembly of COPI and the GTPase-ARF1 (see also Chapter 11 and Rothman and Wieland, 1996). COPI is released when GTP is hydrolyzed so that COPI becomes available for additional vesicle formation. Therefore ARF1 controls the availability of COPI. The GTP-hydrolysis is coupled to the recognition of the sorting signal (Goldberg, 2000). Different sorting signals have different effects on this reaction, so that the reaction provides specificity to the packaging. hp24a (a p24 protein which are putative cargo receptors discussed above) inhibits coatomer-dependent GTP hydrolysis whereas the dilysine retrieval signal (recognized by COPI and involved in retrograde Golgi to ER transport) has no effect despite the fact that the two compete for the same binding site on COPI (Harter and Wieland, 1998). A cargo sorting signal that inhibits the GTPase reaction increases the probability that the cargo protein will be included in a vesicle. Conversely, a cargo sorting signal that permits the rapid hydrolysis of the GTP bound to ARF1 will not be taken up into a vesicle because the assembly of the coat will be disrupted before a vesicle is formed.

Another small GTPase, Cdc42, may play a role in the dynamics of ARF1-COPI interactions. Cdc42 is present in the Golgi and its GTP-bound form attaches to the γ-COPI subunit through its dilysine motif (Wu et al., 2000). This interaction could well be in competition with the attachment of other cargos containing the dilysine signal. Cdc42 has a prominent role in the assembly of actin filaments and elements involved in the transport of vesicles (see van Aelst and D'Sousa-Schorey, 1997).

The secretion of some proteins is constitutive, that is the proteins are secreted continually. The vesicles originating in the TGN are targeted to the plasma membrane. Alternatively, in regulated secretion, the secretory granules are stored in the cytoplasm and their contents released in response to a signal. Regulated mature secretory granules are formed after the aggregation and sorting out of the proteins in the TGN followed by budding of vesicles. The secretory vesicles fuse to form mature secretory granules. The prohormone or immature protein in these vesicles are then cleaved at dibasic amino acid residues by prohormone convertases (see Zhou et al., 1999.).

In regulated secretion, the targeting and formation of secretory granules probably depend on more than one mechanism and these mechanisms may play different roles in different cell types (see Tooze et al, 2001). In some cases, sorting in the TGN involves a disulfide-bonded amino terminal loop in the protein (Glombik et et al., 1999) supposedly segregated by binding to a membrane receptor as we discussed above for other cargos. Aggregation of the regulated secretory proteins is also thought to magnify the effect by capturing more proteins at the membrane sites (e.g., see Thiele and Huttner, 1998). The amino terminal loop has been found in many proteins which are secreted by a regulated pathway. In addition, in some cases when the loop is disrupted by reducing the disulfide bridge, the protein is constitutively secreted (Chanat et al., 1993). Conversely, inserting the loop in a constitutively secreted protein transforms the protein into one that segregates as a regulated protein (Glombik et al., 1999). The loop structure also has a role in multimerization. Multimerization and aggregation also may act as mechanism independent of the loop structure. Other secretory proteins being transported may also play a role by serving as nuclei for binding proteins at a membrane site. A role for specialized spots in the membrane, the so-called lipid rafts (see Chapter 4), is also suspected. The secretory proteins following the regulated pathway have also been found associated with membranes (e.g., see Pimplikar and Huttner, 1992) possibly by attaching to the lipid rafts (Martin-Belmonte et al., 2000). Several regulated secretory proteins were found associated with membrane fractions thought to correspond to rafts (e.g., Dhanvantari and Loh, 2000). Furthermore, interfering with cholesterol (Wang et al., 2000) or sphingolipids (Blásquez et al., 2000), both components of rafts, inhibited the formation of regulated secretory granules.

Post-Golgi signals

As we saw in Chapter 9, tyrosine signals in the cytoplasmic tails of integral proteins serve as sorting signals in endocytosis and in targeting to post-Golgi compartments. Among these, the NPXY or YXXθ (where θ stands for bulky, hydrophobic amino acids) combinations have been found frequently (see Chapter 9; see Marks et al., 1997). YXXθ sorting signals have a role in the localization to endosomes, lysosomes, basolateral plasma membrane of polarized cells, the TGN and special organelles. Many of these signals utilize pathways that are common, at least part way, as indicated by saturability experiments (Marks et al., 1996). The YXXθ-signals have common capacities. They all can direct proteins from the plasma membrane to the endosomes. However, subsets of these motifs can have very narrow specificity. This specificity may rest in the sequence itself (e.g., the nature of the X-amino acid) or in sequences around the consensus sequence (e.g. Ohno et al., 1996). However, in other cases, the signal's effectiveness

is unaffected by the adjacent amino acids (Collawn et al., 1990). A glycine preceding tyrosine increases the targeting of acid phosphatase to lysosomes (Peters et al., 1990) and the exact position of the motif may determine its recognition (e.g., see Ohno et al., 1996). For example, the displacement of the signal by one amino acid in the lysosomal glycoprotein lamp-1 (Rohrer et al., 1996) eliminates lysosomal targeting. However, this is not always the case. In some instances, the location of the sequence does not matter; the signal may be present in any part of the cytoplasmic domain (e.g., Collawn et al., 1990). Other signals can modify the effect of the targeting signal. For example, a lysosomal avoidance signal has been reported for the M6P receptor (MPR) (e.g., Pond et al., 1995a, Rohrer et al., 1995). In some cases, multiple signals are present. In the case of the cation-dependent MPR, dileucine is required for efficient entry in the TGN. Two signals are needed for endocytosis, one includes Phe 13 and 18. A second involves Tyr 45. In addition, the incorporation of fatty acid chains (e.g., by palmotoylation) was found to be needed, suggesting that anchoring to the lipid bilayer is needed (see Schweizer et al., 1996). In some cases, the dileucine motif is needed for endocytotic targeting. However, dileucine motifs can also mediate internalization and targeting to lysosomes or the basolateral surface of polar cells. Another independent signal is contained in a methionine adjacent to a leucine. However, delivery into a large endosome compartment requires the presence of additional acidic amino acid residues (see Pond et al., 1995b). Apparently, the targeting by the dileucine pathway is through different recognition mechanisms than that of the tyrosine signals. Although the dileucine signals use a saturable pathway (Marks et al., 1996), they do not compete with tyrosine signals. As discussed below, the sorting depends on adaptor complexes. µ1 and µ2 do not bind to the dileucine motif (Ohno et al., 1995), although in at least one case, they can be recognized by AP-1 and AP-2 complexes (Heilker et al., 1996), possibly using other binding regions of the adaptor complex. Other types of signals include a cluster of acid amino acids (Pond et al., 1995b, Voorhees et al., 1995, Jones et al., 1995), the dilysine signal KKFF (Itin et al., 1995) present in the protein VIP36 the ER protein ERGIC-53 that cycle between the plasma membrane and the Golgi. ERGIC-53 cycles in this way only when overexpressed. Ubiquitin added to lysine residues in plasma membrane proteins, also serves as an internalization signal (Hicke and Riezman, 1996; Strous et al., 1996). Coat proteins may be involved in these processes as well. The acidic cluster in the cation-dependent-M6P-receptor, favors recruitment of AP-1 to the TGN in vitro (Mauxion et al., 1996). Many studies suggest the presence of novel coat structures (Narula and Stow, 1995; Stoorvogel et al., 1996). The targeting of lysosomal enzymes requires two distinct signals at the carboxyl terminus of the cytoplasmic domain of the cation-dependent-MPR that are different from tyrosine-based endocytosis motifs (e.g., Voorhees et al., 1995). The first is a casein kinase II phosphorylation site. This site is is required for high affinity binding of AP-1, needed for cation dependent-MPR sorting in the trans-Golgi network (Mauxion et al., 1996). However, an adjacent di-leucine motif not involved on AP-1 binding, is required for a downstream sorting event (Pond et al., 1995b).

Exocytosis and transport to the cell surface

The release of the secretory material occurs after the secretory vesicle and the plasma membrane fuse and the vesicle opens to the outside. This process is known as *exocytosis*. In regulated secretion, exocytosis occurs in response to an external stimulus that often triggers an increase in cytoplasmic Ca^{2+}. Exocytosis

in relation to synaptic transmission is discussed in Chapter 22. A role of lysosomes in exocytosis is discussed below (Section IV). Exocytosis also has a very important function in bringing needed integral proteins to the plasma membrane. The effect of the hormone vasopressin, depends on the insertion in the plasma membrane of the water channel AQP2 (see Chapter 19). One of the effects of insulin requires the recruitment of the glucose transporter GLUT4 from an intracellular pool to the cell surface (see Chapter 19). The specific targeting of secretory vesicles depends intimately on the complexes and structures that establish polarity. These are discussed in Chapter 11.

Current evidence suggests that exocytosis occurs at specialized plasma membrane domains. Specialized domains in the plasma membrane, the so called-lipid rafts (see Chapter 4), have been shown to selectively assemble specific proteins. There is evidence for two different kinds of rafts. The most studied is recognized biochemically by being insoluble in the detergent Triton X-100. An additional kind of raft has been found which is soluble in Triton X-100 but insoluble in Lubrol WX (Roper et al., 2000). A role of these domains in exocytosis is suggested by the finding that many proteins associated with vesicle fusion with the plasma membrane (see Chapter 11) have in fact been found in the Triton-X100 insoluble rafts, [e.g., tSNARE proteins (syntaxin 1A), synaptosomal-associated protein of 25 kDa (SNAP-25) the *SNARE vesicle-associated membrane protein* (VAMP2)]. However, VAMP2 was found associated with the Lubrol insoluble rafts (Chamberlain et al., 2001; Lang et al., 2001). Furthermore, syntaxins and (SNAP)-25 were found to be concentrated in 200 nm cholesterol-dependent clusters where secretory vesicles preferentially dock and fuse (Lang et al., 2001). However, these rafts correspond to the Triton X-100-soluble domains and do not co-localize with Triton X-100 insoluble raft markers. Cholesterol depletion disperses these clusters and sharply reduces the rate of secretion.

Studies of cell surfaces of living cells using atomic force microscopy (see Chapter 1) (e.g., Schneider et al., 1997) and conventional transmission EM (Jena et al., 2003) have revealed structures that have been implicated in exocytosis. A variety of cells such as pancreatic acinar cells (e.g., Schneider et al., 1997), growth hormone secreting pituitary cells (Cho et al., 2002a) and chromaffin cells (Cho et al., 2002b) exhibit circular pits about 0.4-1.2 μm in diameter with depressions 100 to 150 nm in diameter and a depth of 15-30 nm. These were called *fusion pores*. The depressions increase in size with exposure to a secretagogue, however, their numbers remain unchanged (Cho et al., 2002d) suggesting that they are docking sites for secretory vesicles that fuse and release the secretory vesicular contents. An involvement of these structures in secretion was shown using immunogold EM techniques with antibodies to amylase establishing the depressions as sites of fusion in pancreatic acinar cells (Cho et al., 2002c) and growth hormone antibodies establishing the same for somatotrophs of the pituitary (Cho et al., 2002b). Furthermore, immunochemical studies demonstrated that t-SNAREs, NSF, actin, vimentin, α-fodrin and the calcium channels α 1c and β 3 are associated with the fusion complex providing the machinery for docking and release of secretory products from intracellular vesicles (Jena et al., 2003).

Exocytotic vesicles are likely to be present in three distinct pools: those already docked at the plasma membrane, those forming an easily recruitable pool (moved to the surface using myosin as a motor) and a

more slowly recruited pool (delivered to the easily recuitable pool using kinesin as a motor). This distribution in three distinct pools is supported by a study in which local burst of cytoplasmic Ca^{2+} were delivered from the medium to the cytoplasm of sea urchin eggs by reversibly damaging the cell surface using laser beams (Bi et al., 1997). The extracellular phase was made bright using rhodamine dextran. The exocytotic vesicles were identified from confocal fluorescence images (see Chapter 1) as bright disks occurring against a dark intracellular background. These images indicate vesicles that have become continuous with the external medium. Microinjected anti-kinesin antibody targeted to the motor domain of the molecule or kinesin tails (that act as inhibitors of kinesin) were without effect on an early exocytotic burst. However, they inhibited a slow phase exocytotic burst. The myosin inhibitor butanedione monoxime (BDM) inhibited both the slow and the fast release. A peptide derived from the Ca^{2+}-calmodulin dependent protein kinase II was also microinjected. This peptide has the property of blocking the native enzyme (presumably by competing with it). The peptide also inhibited the two phases. This kinase has been implicated in facilitation of transmitter release in the squid giant synapse (Llinas et al., 1991). These findings suggest that kinesin and myosin may mediate two sequential events. The results are consistent with myosin affecting an event downstream from that controlled by kinesin ($t_{1/2}$~10 s). Either block did not affect all of the injury-induced exocytosis. This BDM insensitive pool was found to be fastest and was referred to as *immediate* and would represent docked vesicles and lasted only for a few seconds ($t_{1/2}$~2-4 s). These experiments suggest that regulated secretion involves three separate pools of secretory vesicles as proposed from other studies.

As discussed in Chapter 22 (Section V B), unphosphorylated *synapsin* is attached to neurotransmitter vesicles and probably secretory vesicles of other cell-types so that they cannot participate in exocytosis. Synapsin is a fibrous molecule as long as 33 nm that is associated with the cell surface. Phosphorylated synapsin is unable to bind to the vesicles. Phosphorylation via Ca^{2+}-calmodulin dependent protein kinase II would therefore be the trigger for exocytosis from the "immediate" pool.

Exocytosis has been studied in some detail in mast cells of a mutant mouse (beige mouse) (Spruce et al., 1990). In these cells, exocytosis can be initiated by introducing guanosine-5-O-thiotriphosphate (GTPS) to the cell interior. This observation suggests an involvement of GTP-binding proteins, which have a very important role in vesicle fusion (see Chapter 11). These cells have extremely large secretory vesicles, 1-5 µm in diameter, facilitating electrophysiological studies of the plasma membrane and its fusion with the vesicles. Surface capacitance is proportional to membrane surface area, and, therefore, provides a convenient assay of fusion. Capacitance (C) is the charge (Q) per unit voltage (V), so that (C) = Q/V .

The electrical studies were carried out with a pipette containing a salt solution in contact with the cell's interior. Upon fusion, there was an outward current, indicating the discharge of the vesicle's membrane potential suggesting the opening of a pore. This was followed by a capacitance increase of the mast plasma membrane. The capacitance increase occurred stepwise, representing the fusion of individual vesicles. During this period (within the first 100 µs) the conductance of the membrane also increased, first by a few hundred pS and then by progressively higher conductances, indicating an enlargement of the pore. The approximate pore diameter calculated from the conductance is 2 to 2.5 nm.

A possible connection between a family of proteins, the *annexins*, and exocytosis is intriguing (see Creutz et al., 1992). Annexins form part of a family of proteins that, in the presence of Ca^{2+}, foster the aggregation of secretory vesicles (usually studied in adrenal medullary chromaffin granules). Considering the pore behavior exhibited by cells undergoing exocytosis, a particularly interesting feature of annexin V is its probable tertiary structure, deduced from the amino acid sequence. These reconstructions are compatible with the presence of a channel. In addition, this annexin and annexin VII (*synexin*) have been shown to form voltage sensitive channels when reconstituted in planar lipid bilayers (Rojas et al., 1990; Karshikov et al., 1992). Annexin II (also known as *calpactin*) forms a tetramer consisting of two molecules of annexin and two of another protein (p10) (Glenney et al., 1986). Chromaffin granule-aggregation of this complex requires as little as 1 µM Ca^{2+}. As discussed later (Chapter 22), the local concentration of Ca^{2+} may reach even higher levels. Other molecules similar to p10 may be associated with other annexins. Addition of cis-unsaturated fatty acids increases fusion dramatically (Creutz, 1981). This may be relevant because arachnoic acid is liberated by membranes when secretion is stimulated.

An involvement of annexin II in secretion is shown by immunoelectronmicroscopy using colloidal gold, a technique which shows a localization of annexin in chromaffin cells activated for secretion (Nakata et al., 1990). The annexin molecules were found to be associated with the inner face of the plasma membrane and to be conspicuous between plasma membrane and adjacent chromaffin secretory vesicles. Annexins also have a role in endocytosis, as suggested by the requirement of annexin VI in an in vitro study of the formation of endocytotic vesicles.

Transport to the cell surface has been studied with a cell-free system from cells infected with influenza virus (Woodman and Edwardson, 1986). In this study, the transfer of the viral neuraminidase to the plasma membrane was followed. To detect its arrival, an acceptor fraction was prepared by binding [^{3}H]sialic acid-labelled Semliki Forest virus to cell surfaces before cell rupture. The observed production of free [^{3}H]sialic acid served as an assay of the fusion of exocytotic vesicles containing the enzyme with the labelled acceptor preparation. This reaction requires ATP and a variety of proteins.

Most of the evidence discussed so far, indicates that the transport between the Golgi and the cell surface involves small vesicles. However, there are studies that implicate larger structures perhaps for certain special cases.

The use of VSVG-green fluorescent protein chimeric protein (see Chapter 1) allowed following the protein traffic through the various compartments of the secretory pathway (Hirschberg et al., 1998). This approach, applied to the passage from the ER to the Golgi, is discussed in Section III. A temperature mutant was used where the proteins misfold and are retained in the ER at 40^{o}C. However, when shifted to 32^o, they are moved synchronously to the Golgi complex and from there to the plasma membrane.This study found that the protein molecules move from the Golgi complex in large irregular tubular structures that detach from the Golgi in a cytochalasin B sensitive manner, suggesting an involvement of actin in this process. The movement to the periphery was in rounder structures and was sensitive to nocadazole,

suggesting that it takes place on microtubular tracks. Photobleaching experiments outside the Golgi region indicate that 60% of the traffic is in these large structures. Similar experiments (Toomre et al., 1999; Polishchuk et al., 2000) reach similar conclusions. In the experiments of Polishchuk et al (2000), both light and immunoelectronmicroscopy were used to follow individual vesicles in mammalian cells in culture. The fusion of large vesicles to the plasma membrane was found to be direct.

The kinetics of the discharge of secretory vesicles can also be followed using fluorescent proteins. The cargo proteins can be labelled by the transfection with vectors containing DNA coding for protein-GFP conjugates (see Chapter 1). The lumen of granules can also be labelled with fluorescent weak basic dyes that accumulate inside acid compartments (e.g., acridine orange). Similarly, a membrane component can be visualized when an integral protein is conjugated to GFP (e.g., phogrin-GFP; Tsuboi et al., 2000). Events occurring at the cell surface such as exocytosis have been studied using confocal fluorescence microscopy (see Chapter 1) as well as *evanescent field fluorescence microscopy* (also referred to as total internal reflection microscopy). This latter technique is ideally suited for observing events at the cell surface (see Chapter 1).

The membrane of secretory granule was found to remain intact at the cell surface for several seconds and in some cases it was endocytized after the discharge (Tsuboi et al., 2000). Secretory vesicles from neuroendocrine cells (rat pituitary prolactin secreting cells) and their components have been shown to be recovered in endocytotic vesicles after exocytosis (Angleson et al., 1999).

In constitutive exocytosis of epithelial cells many of the secretory vesicles were found to be tubular as well as spherical (Schmoranzer et al., 2000; Toomre et al., 2000) . In addition, the membrane of the tip of the tubular containers fused only temporarily with the plasma membrane and then closed their fusion pore.

IV. ALTERNATIVE SECRETORY PATHWAY AND ALTERNATIVE MECHANISMS

There are many indications that the translocation and targeting of proteins do not always follow the mechanisms discussed in most of this chapter. Several proteins are secreted from cells although they lack a typical signal sequence required for entry into the ER. Furthermore, pharmacological agents, such as monensin or brefeldin A, which perturb Golgi function, do not interfere with the release of these proteins (e.g., Florkiewicz et al., 1995). The possibility that they are exported via a different mechanism from the Golgi pathway is likely, and is referred to as "nonclassic secretion". It is not known whether all these proteins follow the same pathway. Cleves et al. (1996) explored the alternative pathway by expressing in yeast, the small (14 kDa) galactose binding mammalian lectin, *galectin-1*. The transfected yeast secreted galectin-1 even when the conventional secretory pathway was blocked.

Protein kinases and phosphatases have a central role in the regulation of cell activity, including the activity of enzymes, the processes of transcription and translation, and various events accompanying the cell cycle. Although these enzymes must act in a very specific manner, in vitro they are very unspecific,

reacting even with non-physiological substrates. Apparently, the specificity is determined by the precise localization at special sites (see Hubbard and Cohen, 1993). The localization depends on the presence of targeting domains in the enzyme molecule or, in the case of some heteromeric enzymes, in targeting subunits.

The translocation of polypeptides or proteins through membranes, may involve proteins of the *ATP-binding cassette* (ABC) superfamily (see Higgins, 1992; Kuchler and Thorner, 1992). These transporters are present in many intracellular membranes and participate in the transport of many substrates including drugs, ions, metabolites, peptides and proteins. In *Saccharomyces cerevisiae*, 27 genes have been identified that encode proteins containing the ABC cassette. ABC transport systems are involved in transport of peptides into the ER lumen, part of the immunological system (see Williams et al., 1996).

The ER degradation of defective protein involves cytoplasmic proteasomes, suggesting that the ER membrane can translocate these proteins to the cytoplasm (Hayes and Dice, 1996; Cuervo and Dice, 1996). The uptake is powered by ATP hydrolysis.

Several studies suggest that lysosomes are involved in exocytosis where they function as regulated secretory vesicles. The increase in intracellular Ca^{2+} to 1-5 μM initiates the exocytosis of lysosomes in several mammalian cell lines (Rodríguez et al., 1997) indicating that this might be a general phenomenon (for a general discussion of the role of Ca^{2+}, see Chapter 7 and for a role in exocytosis in the nervous sysem see Chapter 22).

A role of lysosomes in exocytosis is most prominent in hematopoietic cells (see Stinchcombe and Griffiths, 1999). Generally, the targeting of the secreted products follow the usual lysosomal pathway (see Page et al., 1998). However, at least in some cases, in hematopoietic cells, there are distinct pathways and targeting apparently to specialized lysosomes for some of the proteins (Bossi and Griffiths, 1999). In part, the role of lysosomal exocytosis might be to dispose of discarded materials from the cell (e.g., Swank et al., 1998), as long recognized for certain protozoans such as *Paramecium*.

The exocytosis elicited by such low calcium concentration is likely to involve a Ca^{2+}-binding protein, binding with high affinity. Such a protein could be *synaptotagmin* (Syt), a protein that has been implicated in neurotransmitter release (see Chapter 22 and Sudhof and Rizo, 1996). Syt proteins constitute a family of at least twelve isoforms with a unique amino-terminal domain and a conserved carboxy-terminal domain (see Schiavo et al., 1998; Craxton and Goedert, 1999). Although most Syt found so far are in neuronal tissue, the appropriate mRNAs are detected at low level elsewhere (Butz et al., 1999; Craxton and Goedert, 1999). The isoform most likely to play a role in lysosomal secretion is Syt VII whose action (binding to syntaxin and phospholipids) has the appropriate Ca^{2+}-dependence (Li et al., 1995). Syt VII was found in dense lysosomes in rat kidney fibroblasts and GFP-SytVII is targeted to lysosomes after transfection. Antibodies against a Syt VII domain and fragments of the same domain inhibit lysosomal exocytosis (Martinez et al., 2000).

V. TARGETING mRNA

A variety of cytoplasmic proteins are distributed unevenly in the cytoplasm. This uneven distribution is likely to play an important role in development (see Chapter 2) and in the function of a variety of cells, for example, neurons (e.g., Kuhl and Skehel, 1998). We have seen that many proteins are transported in vesicles that are targeted to various locations. Free protein molecules can also be targeted directly. However, the unequal distribution of some proteins is the result of the transport of mRNA and not that of the translated protein. So far, as many as 90 mRNAs have been found to be localized to specific regions in the cell (e.g., see Jansen, 2001). mRNA localization is probably more efficient than the transfer of protein molecules since a single mRNA molecule can give rise to many protein molecules. The protein generated in place will be in very high concentrations. In situ translation has also the advantage of assembling cotranslationally large complexes. In embryonic development, the location of mRNA appears to play an important role in establishing gradients of proteins that determine cell fate. For example, in *Xenopus* and *Drosophila* oocytes (see Gavis, 1997) the mRNA distribution establishes a special protein distribution in the animal and vegetal poles. The unevenly distributed proteins and RNAs are then further fractionated into separate cells by cell divisions. These components could then play a role in differentiation, perhaps by being involved in gene expression. In axons and dendrites, the mRNA of microtubule associated proteins (MAPs) is targeted directly and it has a role in determining the packaging of microtubules (Chen et al., 1992), thereby playing a primary role in the laying down of structure.

The localization of specific mRNAs appears to be a general phenomenon. In polarized rat fetal enterocytes, the mRNAs was shown with light microscopic techniques (by hybridization with the appropriate cDNA; see Chapter 1) to localize in the same region of the cell as the corresponding enzyme (shown immunologically) (Rings et al., 1992). The mRNA of the β-subunit of F_1-ATPase was found by electronmicroscopy [using an hybridization and recognition of the cDNA with an immunogold technique (see Chapter 1)] to localize in clusters, in close proximity to mitochondria whereas the mRNA for the α-subunit was found to be dispersed throughout the cytoplasm. (Egea et al., 1997).

The mRNA is thought to form complexes with certain proteins in the nucleus and then to be transported in the cytoplasm in the form of very large ribonucleoprotein particles. In oligodendrocytes, the transport particles or mRNA granules are as large as 0.7 μm in diameter. Besides containing different mRNA, the granules have been found to contain components the protein synthesizing machinery (e.g., see Barbarese et al., 1995). In neurons, the transport of RNA also occurs in granules which contain various proteins, mRNAs and densely packed clusters of ribosomes. The granules are not active in translation and therefore can be regarded as storage units. Interestingly, when the neuron is depolarized, many mRNAs, including those involved in neuronal plasticity (see Chapter 22), leave the granules and are found in polysomes where translation can take place (Krichevsky and Kosik, 2001). Plants have been shown to transport mRNA over long distances through their vascular system (Xoconosle-Càzares et al., 1999) and the transfer of mRNA from one cell to another may well occur in other systems.

How can mRNA be targeted to special areas? Conceivably, the mRNA or granules containing the mRNA could diffuse randomly and be trapped at a particular location, or they could be targeted through the cell's motor system along cytoskeletal elements. The direct visualization of the transport using fluorescently labelled mRNA injected into cells, could offer important insights. Ainger et al., (1993) injected fluorescently labelled mRNA encoding myelin basic proteins into oligodendrocytes. The mRNA was found in particles that undergo unidirectional transport, suggesting a mechanism similar to that of the transport of vesicles. Furthermore, the particles moved on either microtubules or actin filaments at a rate similar to that of vesicles. The mRNA particles were found in close proximity to microtubules, suggesting that these are responsible for the transport. During *Drosophila* oogenesis, *bcd*-mRNA coding for the bicoid protein (Bcd) becomes localized to the anterior pole of the oocytes (Schnorrer et al., 2000). Bicoid is a protein whose gradient determines the anterior pattern of the *Drosophila* embryo. The transport of the *bcd*-mRNA to that location is mediated by a protein with an RNA binding domain (Swa). The transport is powered by the microtubular motor, dynein (see Chapter 24) which also binds to Swa.

The directed transport of RNA involves both the actin network for short distances and the microtubules for long range transport (see Palacios and St. Johnston 2001). For example, β-actin mRNA and the binding protein *Zipcode* (ZBP1) are found to be transported in granules in a microtubule dependent pathway in axons (Zhang et al., 2001). Actin has also been found to be involved in mRNA transport, for example, in the movement of mRNA to the leading edge of fibroblasts (Hill and Gunning, 1993). ZBP1 proteins have an additional role in determining RNA stability and translational control and are found mostly in the cytoplasm, although they posses a nuclear localization and an export sequence (see Chapter 5, Sections I B and I C), suggesting that they cycle between the two sites. Another protein of 92 kDa (ZBP2) (Gu et al., 2002) has been found to bind to actin mRNA . ZBP2 is predominatly nuclear but has also been found in the leading edge of fibroblasts or neuronal growth cones. The two ZBP proteins (ZBP1 and 2) may function in tandem where ZBP1 receives the mRNA from ZBP2 and transfers it to the appropriate location

These observations can be used to construct a model in which an RNP particle is formed, translocated through the microtubular or actin system of the cell, anchored at a specific location, and translated. Obviously once synthesized, the proteins must also be anchored to maintain their localization.

As might be expected, each mRNA docks by a different mechanism. In some cases the translation of the protein coded by the mRNA is needed for docking. In some cases, short untranslated RNA (UTRs) sequences complementary to the small portions of the mRNA are thought to anchor the mRNA. Certain localization sequences in the 3'UTR or the mRNA are required for targeting and anchoring and they require mRNA binding proteins (see Bashirullah et al., 1998). Presumed targeting signals include several hundred nucleotides in some of the mRNAs, suggesting that there may be multiple signals, each mediating different localization steps, as in the mRNA of the *oskar* gene (see Kim-Ha et al., 1993). The *oskar* gene is required in *Drosophila* for posterior body patterning and germ cell determination.

How could the mRNA be anchored to its target? There are suggestions that the mRNA is attached to

cytoskeletal elements, because it is not solubilized by the detergent Triton X-100 (Yisraeli et al., 1990). Furthermore, actin is likely to be involved because the mRNA is dispersed after cytochalasin treatment. Cytochalasin, a fungal product, is known to interfere with actin polymerization.

In summary, mRNA-containing particles are targeted to structures where they remain anchored. Similarly, the proteins, newly translated at these locations, remain anchored. The microtubular system seems to be involved in the transport of the mRNA particles, whereas its anchoring is likely to involve actin.

VI. THE TRAFFIC OF LIPIDS

In Chapter 4, we discussed lipids in relation to the structure and function of cell membranes. Aside from their insulating and structural roles, recent research has recognized a wide range of lipid functions. Like many glycoproteins, certain glycolipids at the cell surface play an important role in cell recognition. Lipids can also serve as anchors for proteins. Some membrane lipids act as precursors of second messengers. Furthermore, lipid composition is also thought to play a role in the regulation of exocytotic vesicle transport.

We have already examined (section III) how newly synthesized proteins transported in vesicles are targeted to different cell compartments or the plasma membrane. These processes result in a flow of membranes toward the cell surface. The processes culminating in exocytosis produce a flow of membranes, which would augment the surface area of the plasma membrane. Some of this flow must be compensated for by an inward flux of membranes, so that they are primarily recycled in endocytosis and subsequent events. Although we concentrated on the protein components of the membrane, obviously the same problems of targeting and maintaining the characteristics in each compartment applies to the lipid components of membranes as well. In Chapter 4, we saw that the dynamics of the plasma membrane cause it to maintain an asymmetry, so that the external leaflet differs from the inner leaflet of the lipid bilayer. This section will examine what is known about the processes responsible for the targeting of lipid components to the various subcellular compartments (generally in their membranes) and the plasma membrane.

The fate of phospholipids has been followed using several approaches. Arrival at a target organelle can be followed by monitoring the metabolic conversion of the lipid. The use of fluorescent phospholipids offers a more direct and general technique. Phospholipid analogues containing dipyrromethene difluoride (BODIPY) have been used successfully as lipid tracers (Pagano et al., 1991).

The sorting of cholesterol has been difficult to follow because few convenient techniques are available. However, pulse labelling with [^{3}H] acetate in cells cultured in LDL-deficient medium will label newly synthesized cholesterol in minutes. Similarly, [^{3}H]cholesteryl linoleate can be incorporated into LDL. The acetate labelling would reveal the fate of newly synthesized cholesterol, whereas the cholesteryl labelling would reveal the fate of exogenous cholesterol. Generally, in these studies the membranes of the various vesicles or the plasma membranes were isolated and the cholesterol extracted.

A. Glycerophospholipids

PC, PE, PS and PI are synthesized in the cytosolic surface of the ER (Dennis and Vance, 1992). They rapidly distribute laterally in the ER membrane and they can flip to the inner leaflet (Devaux, 1993).

Newly synthesized PC is rapidly transferred to the plasma membrane and the outer mitochondrial membrane, possibly by transfer proteins that would interact with lipids in the membrane, removing them from one location and delivering them to another (Kaplan and Simoni, 1993). PE is transferred to the plasma membrane more slowly (Yaffe and Kennedy, 1983). PI synthesized in the ER, is phosphorylated by PI- and PIP-kinases en route to the plasma membrane and the nucleus (Helms et al., 1991; Banfi et al., 1993).

The asymmetric distribution of glycerolipids in the plasma membrane is thought to be produced by the aminosphingolipid translocase, an ATP-dependent pump that transfers PS and PE to the inner leaflet of the plasma membrane (Berr et al., 1993, Devaux, 1993). However, the asymmetry is not easily explained by models based on the translocase activity alone.

B. Sphingolipids

Ceramide, the precursor of all complex sphingolipids, is synthesized in the ER. After being transported to the Golgi, ceramide is converted into sphingomyelin (SM) (Kallen et al., 1993) and glycosphingolipids (Collins and Warren, 1992; Moreau et al., 1993).

In the previous section we saw that proteins that are constitutively secreted proceed via vesicles from the ER and through the Golgi system, to eventually reach the cell surface. It follows that in this case these proteins and the membrane lipids must move together. The cotransport of glycolipids and glycoproteins has been shown in in vitro systems, and the rate of this transport is consistent with bulk flow (Young et al., 1992). Experiments supporting this view have shown that inhibitors of sphingomyelin biosynthesis slow down intracellular movement (Rosenwald et al., 1992).

Taken together, this information strongly suggests that both glycoproteins and sphingolipids within the Golgi system are transported in vesicles. The vesicles are then delivered to the plasma membrane. Other information indicates that from the cell surface they are recycled by endocytosis (e.g., Kok et al., 1992) and may be degraded in lysosomes (van Eichten and Sanhoff, 1993).

Different sphingolipids are targeted to different cell surfaces (see van Meer, 1993). As already discussed, at the surface they may form special lipid domains, which may play a role in the distribution of proteins anchored by glycosyl-phosphatidyl inositol (GPI).

Although a sharing of the pathway and the targeting by glycoproteins and glycolipids is likely, other

lipids may be transferred by a different pathway, as discussed below for cholesterol and glycerophospholipids.

Sphingolipids can be redistributed in polarized cells (see Chapter 11) by transcytosis. A cell system that has been found useful in the study of polarized cells is that of polarized hepatocytes, HepG2. In these cells, the apical and the basolateral domains are well defined. The fate of lipids can be followed using fluorescently tagged sphingolipids (e.g., Pagano and Sleight, 1985). The localization of glucosylceramide (GC) and SM is distinct. SM is localized in the basolateral surface, whereas GC is localized in the apical surface. When the cells were incubated in the presence of the labelled compounds (van IJzendoorn et al., 1997) both lipids were found in punctate fluorescent bodies presumed to be vesicles and, in addition, at the apical (in this case the bile canicular surface) and the basolateral cell surface. Treatment of the cells with bovine serum albumin in a saline solution removed the fluorescence from the basolateral but not the apical surface. After incubation, the lipids were found in their characteristic localization. SM-was rapidly transferred by transcytosis from the apical to the basolateral surface. The transfer system was found not to involve the Golgi apparatus, but to occur via vesicles seen below the apical surface.

C. Cholesterol

Mammalian cells can acquire cholesterol from extracellular sources in the form of low density lipoprotein (LDL), which is taken up by receptor-mediated endocytosis (Chapter 9). The cholesteryl ester core is hydrolyzed to free cholesterol in lysosomes. [^{3}H] cholesterol reaches the plasma membrane within minutes of the hydrolysis of [^{3}H] cholesteryl linoleate (Brasaemle and Attie, 1990). The Golgi system is thought to be involved in the movement because the cisternae are enriched in cholesterol when normal cells are incubated in LDL for long periods (Blanchette-Mackie et al., 1979). Furthermore, the transport from lysosomes to the plasma membrane is monensin-sensitive, implicating vesicular transport.

There is no direct information on the distribution of cholesterol between the two leaflets of the lipid bilayer of the plasma membrane. However, cholesterol is known to interact primarily with sphingomyelin and, therefore, may be predominantly in the outer leaflet.

In mammals, the enzymes that synthesize cholesterol reside in the ER (see (Reinhart et al., 1987). However, some studies have also implicated peroxisomes (Krisans, 1992). From the the ER, cholesterol is translocated to other cellular destinations, in most cells the plasma membrane being the major recipient (65-90% of the cellular cholesterol, Lange et al., 1989; Warnock et al., 1993). In hepatocytes, cholesterol is needed for lipoprotein and bile acid synthesis, both initiated in the ER. In steroid-producing cells, cholesterol may be transported directly to the mitochondria, the site of synthesis of the hormones.

Transport to the plasma membrane is rapid and involves lipid-rich vesicles (see Liscum and Munn, 1999). The transfer is energy dependent and is blocked when the cells are incubated at 15^{o}C. At this temperature the cholesterol accumulates in vesicles. However, the Golgi apparatus is probably not involved and the vesicles are distinct from those involved in secretion. For example, VSV-G protein and cholesterol are in

different vesicles (e.g., Urbani and Simoni, 1990). Furthermore, monensin or Brefeldin A, which disrupt the Golgi system and block the delivery of VSV-G protein to the surface, are ineffective in blocking the cholesterol movement. Other agents that interfere with the cytoskeletal organization, and therefore block vesicular traffic, also do not interfere with cholesterol transport.

There is some evidence that a sterol carrier is involved in cholesterol transport (*protein-2*, SCP-2 of 13.2-kDa; Puglielli et al., 1995) and furthermore that there are two separate pathways for transport of cholesterol inside the cell and that the SCP-2 pathway is the major normal pathway for the transport of cholesterol. In normal cells, cholesterol transfer was found to be rapid, cytoskeleton-independent, and Golgi-independent in normal cells. However, in SCP-2-deficient cells it was slower, required a functioning cytoskeleton and Golgi. In these experiments, the need for the various mechanisms was tested using brefeldin or mononesin to disrupt Golgi, colchicine to disrupt microtubules and cytochalasin B to disrupts actin filaments. An involvement of caveolin (see Chapter 9) in cholesterol transport is also indicated (Uittenbogaard et al., 1998).

Similar experiments confirm some these findings (Heino et al., 2000). The transport of newly synthesized cholesterol were compared to that of influenza virus hemagglutinin (HA) from the endoplasmic reticulum to the plasma membrane. Brefeldin A which disrupts the Golgi apparatus was found to completely block the passage of HA. In contrast, the cholesterol transport was not affected. However, the exposure to nocodazole which disrupts microtubules was found to block the transport of both cholesterol and HA, contradicting some of the earlier findings. The transport of cholesterol was studied in more detail and found the incorporation into low-density detergent-resistant membranes assumed to be specialized membrane elements or rafts (see Chapter 4). Interference with cholesterol transport (by lowering the temperature) decreased the amount of cholesterol in the presumed rafts. The results suggest that the transport of cholesterol involves membranes containing rafts and possibly caveolae (see Chapter 4 and Chapter 9) as suggested by an involvement of caveolin.

SUGGESTED READING

Bannykh, S.I., Nishimura, N. and Balch, W.E. (1998) Getting into the Golgi, *Trends in Cell Biol.* 8:21-25. (Medline)

Brodsky, J.L. (1998) Translocation of proteins across the endoplasmic reticulum membrane, *Int. Rev. Cytol.* 178:277-328. (Medline)

Ellgaard, L., Molinari, M. and Helenius, A. (1999) Setting the standards: quality control in the secretory pathway, *Science* 286:1882-1888. (Medline)

Farquhar, M.G. and Palade, G. (1998) The Golgi apparatus: 100 years of progress and controversy, *Trends in Cell Biol.* 8:2-10. (Medline)

Higgins, C.F. (1993) Introduction; the ABC transporter channel superfamily-an overview, *Seminar in Cell Biol.* 4:1-5.

Kreis, T.E., Goodson, H.V., Perez, F. and Rönnholm, R. (1997) Golgi apparatus-cytoskeleton interactions, in *The Golgi Apparatus* (ed. Berger, E.G. and Roth, J.), Birkhäuser Verlag, Basel, Boston, pp.179-193.

Marsh, B.J. and Howell, K.E. (2002) The mammalian Golgi--complex debates, *Nature Rev. Mol. Cell Biol.* 3: 789-795.

Martin, T.F.J. (1997) Stages of regulated exocytosis, *Trends in Cell Biol.* 7:271-276.

Plemper, R.K. and Wolf, D.H. (1999) Retrograde protein translocation: ERADication of secretory proteins in health and disease, *Trends Biochem. Sci.* 24:266-270. (Medline)

Rambourg, A. and Clermont, Y. (1997) Three-dimensional structure of the Golgi apparatus in mammalian cells, in *The Golgi Apparatus* Birkhäuser Verlag, Basel, Boston, Berlin, (ed. Berger, E.G. and Roth, J.) p.37-61.

Rapoport, T.A., Rolls, M.M. and Jungnickel, B. (1996) Approaching the mechanism of protein transport across the ER membrane, *Curr. Opin. Cell Biol.* 8:499-504.

Rothman, J.E. and Wieland, F.T. (1996) Protein sorting by transport vesicles, *Science* 272:227-234.(MedLine)

Schatz, G. and Dobberstein (1996) Common principles of protein translocation across membranes, *Science* 271:1519-1526. (Medline)

Valee, R.B. and Sheetz, M.P. (1996) Targeting of motor proteins, *Science* 271:1539-1544.

WEB RESOURCES

ER to Golgi transport: Quick time movie sequence (see Presley et al., 1997)
http://dir.nichd.nih.gov/CBMB/pb1labob.html

REFERENCES

Search the textbook

Back to Chapter 10

REFERENCES

Ainger, K., Avossa, D., Morgan, F., Hill, S.J., Barry, C. and Carson, J.H. (1993) Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes, *J. Cell. Biol.* 123:431-441. (Medline)

Allan, B.B. and Balch, W.E. (1999) Protein sorting by directed maturation of Golgi compartments, *Science* 285:63-66. (Medline)

Andrews, D.W. and Johnson, A.E. (1996) The translocon: more than a hole in the ER membrane? *Trends in Biochem. Scie.* 21:365-369. (Medline)

Andrews, D.W., Young, J.C., Mirles, L.F., and Czarnota, G.J. 4(1992) Role of the N-region in signal sequence and signal-anchor function, *J. Biol. Chem.* 267:7761-7769. (Medline)

Angleson, J.K., Cochilla, A.J., Kilic, G., Nussinovitch, I. and Betz , W.J. (1999) Regulation of dense core release from neuroendocrine cells revealed by imaging single exocytic events, *Nature Neurosci.* 2:440-446. (MedLine)

Aridor, M., Weissman, J., Bannykh, S., Nuoffer, C. and Balch, W.E. (1998) Cargo selection by the COPII budding machinery during export from the ER, *J. Cell Biol.* 141:61-70. (MedLine)

Bacher, G., Lütke, H., Jungnickel, B., Rapoport, T.A. and Dobberstein, B. (1996) Regulation by the ribosome of the GTPase of the signal-recognition particle during protein targeting, *Nature* 381:248-251. (Medline)

Balch, W. E. (1989) Biochemistry of interorganelle transport. A new frontier in enzymology emerges from versatile in vitro model systems, *J. Biol. Chem.* 264:16965-16968. (Medline)

Balch, W.E., McCaffery, J.M., Plutner, H. and Farquhar, M.G. (1994) Vesicular stomatitis virus glucoprotein is sorted and concentrated during export from the endoplasmic reticulum, *Cell* 76:841-852. (Medline)

Banfic, H., Zizak, M., Divecha, N. and Irvine, R.F. (1993) Nuclear diacylglycerol is increased during cell proliferation *in vivo*, *Biochem. J.* 290:633-636. (Medline)

Banfield, D.K., Lewis, M.J., Rabouille, C., Warren, G. and Pelham, H.R.B. (1994) Localization of Sed5, a putative vesicle targeting molecule, to the cis-Golgi network involves both its transmembrane and cytoplasmic domains, *J. Cell Biol.* 127:357-371. (Medline)

Bannykh, S.I., Rowe, T. and Balch, W.E. (1996) The organization of the endoplasmic reticulum export complexes, *J. Cell Biol.* 135:19-35. (Medline)

Bannykh, S.I. and Balch, W.E. (1997) Membrane dynamics at the endoplasmic reticulum-Golgi interface, *J. Cell Biol.*138:1-4. (Medline)

Bannykh, S.I., Nishimura, N. and Balch, W.E. (1998) Getting into the Golgi, *Trends in Cell Biol.* 8:21-25. (Medline)

Barbarese, E., Koppel, D.E., Deutscher, M.P., Smith, C.L., Ainger, K., Morgan, F. and Carson, J.H. (1995) Protein translation components are colocalized in granules in oligodendrocytes, *J. Cell Sci.* 108 :2781-2790. (MedLine)

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Ravazzola, M. Amherdt, M. and Shekman, P. (1994) COPII: a membrane coat formed

by SEC proteins that drive vesicle budding from the endoplasmic reticulum, *Cell* 77: 895-907. (Medline)

Barr, F.A., Puype, M., Vandekerckhove, J. and Warren, G. (1997) GRASP65, a protein involved in the stacking of Golgi cisternae, *Cell* 91:253-262. (Medline)

Bashirullah, A., Cooperstock, R.L. and Lipshitz, H.D. (1998) RNA localization in development, *Annu. Rev. Biochem.* 67:335-394. (MedLine)

Batey, R.T., Rambo, R.P., Lucast, L., Rha, B. and Doudna, J.A. (2000) Crystal structure of the ribonucleoprotein core of the signal recognition particle, *Science* 287:1232-1239. (MedLine)

Becker, B., Bolinger, B. and Melkonian, M. (1995) Anterograde tansport of algal scales through the Golgi complex is not mediated by vesicles, *Trends Cell Biol.* 5:305-307.

Beckmann, R., Bubeck, D., Grassucci,R., Penczek, P., Verschoor,A., Blobel,G. and Frank, J., (1997) Alignment of Conduits for the Nascent Polypeptide Chain in the Ribosome-Sec61 Complex, *Science* 278:2123-2126. (Medline)

Beckmann, R., Spahn, C.M., Eswar, N., Helmers, J., Penczek, P.A., Sali, A., Frank, J. and Blobel, G. (2001) Architecture of the protein-conducting channel associated with the translating 80S ribosome, *Cell* 107:361-372. (MedLine)

Bednarek, S.Y., Ravazzola, M., Hosobuchi, M., Amherdt, M., Perrlet, A., Shekman R. and Orci, L. (1995) COPI and COPII-coated vesicles bud directly from the endoplasmic reticulum in yeast, *Cell* 83:1183-1196. (Medline)

Belin, D., Bost, S., Vassali, J.-D. and Strub, K. (1996) A two-step recognition of signal sequences determines the translocation efficiency of proteins, *EMBO J.* 15:468-478. (Medline)

Bergmann, J. E. and Singer, S. J. (1983) Immunoelectron microscopic studies of the intracellular transport of the membrane glycoprotein (G) of vesicular stomatitis virus in infected Chinese hamster ovary cells, *J. Cell Biol.* 97:1777-1784. (Medline)

Berks, B.C. (1996) A common export pathway for proteins binding complex redox cofactors? *Mol. Microbiol.* 22:393-404. (Medline)

Berr, F., Meier, P.J. and Stieger, B. (1993) Evidence for the presence of a phosphatidylcholine translocator in isolated rat liver canicular plasma membrane vesicles, *J. Biol. Chem.* 268:3976-3979. (Medline)

Bi, G.-Q., Morris, R.L., Liao, G., Alderton, J.M., Scholey, J.M. and Steinhardt, R.A. (1997) Kinesin- and myosin-driven steps of vesicle recruitment for Ca^{2+}-regulated exocytosis, *J. Cell Biol.* 138:999-1008. (Medline)

Bibi, E. (1998) The role of the ribosomes-translocon complex in translation and assembly of polytopic membrane proteins, *Trends in Biochem. Sci.* 23:51-55. (Medline)

Blanchette-Mackie, E.J., Dwyer, N.K., Amende, L.M., Kruth, H.S., Butler, J.D., Sokol, J., Comly, M.E., Vanier, M.T., August, J.T., Brady, R.O. and Pentchev, P.G. (1988) Type-C Niemann-Pick disease: low density lipoprotein uptake is associated with premature cholesterol accumulation in the Golgi complex and excessive cholesterol storage in lysosomes, *Proc. Natl. Acad. Sci. USA* 85:8022-8026. (Medline)

Blásquez, M.., Thiele, C., Huttner, W.B., Docherty, K. and Shennan, K.I.J. (2000) Involvement of the membrane lipid bilayer in sorting prohormone convertase-2 into the regulated secretory pathway, *Biochem. J.* 349: 843-852.

Blobel, G. and Dobberstein, B. (1975a) Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma, *J. Cell Biol.* 67:835-851. (Medline)

Blobel. G. and Dobberstein, B. (1975b) Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from

heterologous components, *J. Cell Biol.* 67:852-862. (Medline)

Bogdanov, M. and Dowhan, W. (1999) Lipid-assisted protein folding, *J. Biol. Chem.* 274:36827-36830. < a href="http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=10601231&dopt=Abstract">(MedLine)

Bonaficino, J.S. and Klausner, R.D. (1994) Degradation of proteins retained in the endoplasmic reticulum, in *Cellular Proteolytic Systems*, ed. Ciechanover, A.J. and Schwartz, A.L., Wiley-Liss, New York p.137-160.

Bonfanti, L., Mironov, A.A. Jr, Martinez-Menarguez, J.A., Martella, O., Fusella, A., Baldassarre, M., Buccione, R., Geuze, H.J., Mironov, A.A. and Luini, A. (1998) Procollagen traverses the Golgi stack without leaving the lumen of cisternae: evidence for cisternal maturation, *Cell* 95:993-1003. (Medline)

Borel, A.C. and Simon, S.M. (1996) Biogenesis of polytopic membrane proteins: membrane segments assemble within translocation channels prior to membrane integration, *Cell* 85:379-389. (MedLine)

Bos, K., Wraight, C. and Stanley, K. (1993) TGN38 is maintained in the *trans* Golgi network by a tyrosine containing motif in the cytoplasmic domain, *EMBO J.* 12:2219-2228. (Medline)

Bossi, G. and Griffiths, G.M. (1999) Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells, *Nature Med.* 5:90-96. (MedLine)

Braell, W.A, Schlossman, D.M., Schmid, S.L. and Rothman, J.E. (1984b) Dissociation of clathrin coats coupled to the hydrolysis of ATP. Role of an uncoating ATPase, *J. Cell Biol.* 99:734-741. (Medline)

Braks, J.A.M. and Martens, G.J.M.. (1994) 7B2 is a neuroendocrine chaperone that transiently interacts with prohormone convertase PC2 in the secretory pathway, *Cell* 78:263-273. (MedLine)

Brasaemle, D.L. and Attie, A.D.(1990) Rapid intracellular transport of LDL-derived cholesterol to the plasma membrane in cultured fibroblasts, *J. Lipid Res.* 31:103-112. (Medline)

Braud, V.M., Allan, D.S., O'Callaghan, C.A., Soderstrom, K., D'Andrea, A., Ogg, G.S., Lazetic, S., Young, N.T., Bell, J.I., Phillips, J.H., Lanier, L.L. and McMichael, A.J. (1998) HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C, *Nature* 391:795-799. (Medline)

Braun, S., Matuschewski, K., Rape, M., Thoms, S. and Jentsch, S. (2002) Role of the ubiquitin-selective CDC48(UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates, *EMBO J.* 21:615-621. (MedLine)

Breckenbridge, L. J. and Almers. W. (1987) Final steps in exocytosis observed in a cell with giant secretory granules, *Proc. Natl. Acad. Sci. USA* 84:1945-1949. (Medline)

Breitfeld, P.P., McKinnon, W.C. and Mostov, K.E. (1990) Effect of nocodazole on vesicular traffic to the apical and basolateral surfaces of polarized MDCK cells, *J. Cell Biol.* 111: 2365-2373. (Medline)

Bretscher M.S.and Munro S. (1993) Cholesterol and the Golgi apparatus, *Science* 261:1280-1281. (Medline)

Brodsky, J.L. (1998) Translocation of proteins across the endoplasmic reticulum membrane, *Int. Rev. Cytol.* 178:277-328. (Medline)

Bryant, N.J. and Stevens, T.H. (1997) Two separate signals act independently to localize yeast late Golgi membrane protein through a combination of retrieval and retention, *J. Cell Biol.* 136:287-297. (Medline)

Burke, J., Pettitt, J.M., Humphris, D. and Gleeson, P.A. (1994) *Medial*-Golgi retention of *N*-acetylglucosominyltransferase I. Contribution from all domains of the enzyme, *J. Biol. Chem.* 269:12049-12059. (Medline)

Busciglio, J., Hartmann, H., Lorenzo, A., Wong, C., Baumann, K., Sommer, B., Staufenbiel, M.amd Yankner, B.A. (1997) Neuronal localization of presenilin-1 and association with amyloid plaques and neurofibrillary tangles in Alzheimer's disease, *J. Neurosci.* 17:5101-5107. (Medline)

Butz, S., Fernandez-Chacon, R., Schmitz, F., Jahn, R. and Sudhof, T.C. (1999) The subcellular localizations of atypical synaptotagmins III and VI. Synaptotagmin III is enriched in synapses and synaptic plasma membranes but not in synaptic vesicles, *J. Biol. Chem.* 274:18290-18296. (MedLine)

Casanova, J.E., Breitfeld, P.P., Ross, S.A., and Mostov, K.E. (1990) Phosphorylation of a polymeric immunoglobulin receptor required for efficient transcytosis, *Science* 248:742-745. (Medline)

Casanova, J.E., Apodaka, G. and Mostov, K.E. (1991) An autonomous signal for basolateral sorting in the cytoplasmic domain of the polymeric immunoglobulin receptor, *Cell* 66:65-75.

Chamberlain, L.H., Burgoyne, R.D. and Gould, G.W. (2001) SNARE proteins are highly enriched in lipid rafts in PC12 cells: implications for the spatial control of exocytosis, *Proc. Natl. Acad. Sci. USA* 98:5619-5624. (MedLine)

Chanat, E., Weiss, U., Huttner, W.B. and Tooze, S.A. (1993) Reduction of the disulfide bond of chromogranin B (secretogranin I) in the trans-Golgi network causes its missorting to the constitutive secretory pathways, *EMBO J.* 12:2159-2168. (MedLine)

Chen, J., Kanai, Y., Cowan, N.J. and Hirokawa, N. (1992) Projection domains of MAP2 and tau determine the spacing between microtubules in dendrites and axons, *Nature* 360:674-677. (Medline)

Cheng, S.H., Rich, D.P., Marshall, J., Gregory, R.J., Welsh, M.J. and Smith, A.E. (1991) Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel, *Cell* 66:1027-1036. (Medline)

Chin, C.-N, von Heijne, G. and de Gier, J.-W. L.(2002) Membrane proteins shaping up, *Trends Biochem. Scie* 27:231-234.

Cho, S.J., Jeftinija, K., Glavaski, A., Jeftinija, S., Jena, B.P. and Anderson, L.L. (2002a) Structure and dynamics of the fusion pores in live GH-secreting cells revealed using atomic force microscopy, *Endocrinology* 143:1144-1148. (MedLine)

Cho, S.J., Wakade, A., Pappas, G.D and Jena, B.P. (2002b) New structure involved in transient membrane fusion and exocytosis, *Ann. N. Y. Acad. Sci.* 971:254-256. (MedLine)

Cho, S.J., Quinn, A.S., Stromer, M.H., Dash, S., Cho, J., Taatjes, D.J. and Jena, B.P. (2002c) Structure and dynamics of the fusion pore in live cells, *Cell Biol. Int.* 26:35-42. (MedLine)

Cho, S.J., Cho. J. and Jena. B.P. (2002) The number of secretory vesicles remains unchanged following exocytosis, *Cell Biol. Int.* 26:29-33. (MedLine)

Clermont, Y., Xia, L., Rambourg, A., Turner, J.D. and Hermo, L.(1993) Transport of casein submicelles and formation of secretion granules in the Golgi apparatus of epithelial cells of the lactating mammary gland of the rat, *Anat. Rec.* 235:363-373. (Medline)

Cleves, A.E., Cooper, D.N.W., Barondes, S.H. and Kelly, R.B. (1996) A new pathway for protein export in *Saccharomyces cerevisiae*, *J. Cell Biol.* 133: 1017-1026. (Medline)

Cole, N.B., Smith, C.L., Sciaky, N., Terasaki, M., Edidin, M., Lippincott-Schwartz, J., (1996b) Diffusional mobility of Golgi proteins in membranes of living cells, *Science* 273:797-801. (Medline)

Colley, K.J. (1997) Golgi localization of glycosyltransferases: more questions than answers, *Glycobiology* 7:1-13. (Medline)

Collins, R.N. and Warren, G. (1992) Sphingolipid transport in mitotic HeLa cells, *J. Biol. Chem.* 267:24906-24911. (Medline)

Collawn, J.F., Stangel, M., Kuhn, L.A., Esecogwu, V, Jing, DS., Trowbridge, I.S. and Tainer, J.A. (1990) Transferrin receptor internalization sequence YXRF implicates a tight turn as a structure motif for endocytosis, *Cell* 63:1061-1072. (Medline)

Corsi, A.K. and Schekman, R. (1997) The lumenal domain of Sec63p stimulates the ATPase activity of BiP and mediates BiP recruitment to the translocon in *Saccharomyces cerevisiae*, *J. Cell Biol.* 137:1483-1493. (Medline)

Cosson, P., Démollière, C., Henecke, S., Duden, R. and Letourneur, F. (1996) and -COP two coatomer subunits homologous to clathrin-associated proteins are involved in ER retrieval, *EMBO J.* 15:1792-1798. (Medline)

Craxton, M. and Goedert M. (1999) Alternative splicing of synaptotagmins involving transmembrane exon skipping, *FEBS Lett.* 460:417-422. (MedLine)

Creutz, C.E. (1981) cis-unsaturated fatty acids induce the fusion of chromaffin granules aggregated by sinexin, *J. Cell Biol.* 91:247-256. (Medline)

Crowley, K.S., Reinhart, G.D. and Johnson, A.E. (1993) The signal sequence moves through a ribosomal tunnel into a non-cytoplasmic aqueous environment at an early stage of translocation, *Cell* 73:1101-1115. (Medline)

Crowley, K.S., Liao, S., Wrell, V.E., Reinhart, G.D. and Johnson, A.E. (1994) Secretory proteins move through the endoplasmic reticulum membrane via an aqueous, gated pore, *Cell* 78:461-471. (Medline)

Cuervo, A.M. and Dice, J.F. (1996) A receptor for selective uptake and degradation by lysosomes, *Science* 273:501-503. (Medline)

Cyr, D.M., Langer, T. and Douglas, M.G. (1994) DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70, *Trends Biochem. Sci.* 19:176-181. (Medline)

Dahan, S., Ahluwalia, J.P., Wong, L., Posner, B.I. and Bergeron, J.J. (1994) Concentration of intracellular hepatic apolipoprotein E in Golgi apparatus saccular distensions and endosomes, *J. Cell Biol.* 127:1859-1869. (Medline)

Dalbey, R.E., Chen, M., Jiang, F. and Samuelson, J.C. (2000) Understanding the insertion of transporters and other membrane proteins, *Curr. Opin. Cell Biol.* 12:435-442. (MedLine)

Dell'Angelica, E.C., Ohno, H., Ooi, C.E., Rabinovich, E., Roche, K.W. and Bonifacino, J.S. (1997) AP-3: an adaptor-like protein complex with ubiquitous expression binds YQRL, *EMBO J.* 16:917-928. (Medline)

Dennis, E.A. and Vance, D.E. (1992) Phospholipid biosynthesis, *Methods in Enzymol.* 209:1-544.

Denzer, A.J., Nabholtz, C.E. and Spiess, M. (1995) Transmembrane orientation of signal-anchor proteins is affected by a folding state but not the size of N-terminal domain, *EMBO J.* 14:6311-6317. (Medline)

Devaux, P.F. (1993) Transmembrane asymmetry and flip-flop in biological membranes and in lipid bilayers, *Curr. Opin. Struct. Biol.* 3:489-494.

Dhanvantari, S. and Loh, Y.P. (2000) Lipid raft association of carboxypeptidase E is necessary for its function as a regulated secretory pathway sorting receptor, *J. Biol. Chem.* 275:29887-29893. (MedLine)

Do, H., Falcone, D., Lin, J., Andrews, D.W. and Johnson, A.E. (1996) The cotranslational integration of membrane proteins into the phospholipid bilayer is a multistep process, *Cell* 85:369-378. (MedLine)

Dobson, C.M. and Ellis, R.J. (1998) Protein folding and misfolding inside and outside the cell, *EMBO J.* 17:5251-5254. (Medline)

Dominguez, M., Dejgaard, K., Fullekrug, J., Dahan, S., Fazel, A., Paccaud, J.P., Thomas, D.Y., Bergeron, J.J. and Nilsson, T. (1998) gp25L/emp24/p24 protein family members of the cis-Golgi network bind both COP I and II coatomer, *J. Cell Biol.* 140:751-765. (MedLine)

Dorner, A.J., L.C. Wasley, D.G. Bole, and R.J. Kaufman. 1989. Increased synthesis of secreted proteins induces expression of glucose regulated proteins in butyrate treated CHO cells, *J. Biol. Chem.* 264: 20602-20607. (MedLine)

Egea, G., Izquierdo, J.M., Ricart, J., San Mart¡n, C. and Cuezva, J.M. (1997) mRNA encoding the β subunit of the mitochondrial F_1-ATPase complex is a localized mRNA in rat hepatocytes, *Biochem. J.* 322:557-565. (Medline)

Ellgaard, L., Molinari, M. and Helenius, A. (1999) Setting the standards: quality control in the secretory pathway, *Science* 286:1882-1888. (Medline)

Engelman, D.M. and Steitz, T.A. (1981) The spontaneous insertion of proteins into and across membranes: the helical hairpin hypothesis, *Cell* 23:411-422. (Medline)

Farquhar, M. G. (1985) Progress in unraveling pathways of Golgi traffic, *Annu. Rev. Cell Biol.* 1:447-488. (Medline)

Feldheim, D., Rothblatt, J. and Schkman, R. (1992) Topology and functional domains of Sec63p, an ER protein required for secretory protein translocation, *Mol. Cell. Biol.* 4:931-939. (Medline)

Fiedler, K., Veit, M. Stamnes, M.A. and Rothman, J.E. (1996) Bimodal interaction of coatomer with the p24 family of putative cargo receptors, *Science* 273:1396-1399 (Medline)

Florkiewicz, R.Z., Majack, R.A., Buechler, R.D. and Florkiewicz, E. (1995) Quantitative export of FGF-2 occurs through an alternative, energy dependent, non-ER/Golgi pathway, *J. Cell Physiol.* 162: 388-399. (Medline)

Freeman, B.C. and Yamamoto, K.R. (2002) Disassembly of transcriptional regulatory complexes by molecular chaperones, *Science* 296:2232-2235. (MedLine)

Fries, E., Gustaffson, L. and Petersen, P. (1984) Four proteins synthesized by hepatocytes are transported from the endoplasmic reticulum to Golgi complex at different rates, *EMBO J.* 3:147-152. (Medline)

Füllerkrug, J., Sönnichsen, B., Wünsch, U., Arseven, K., Van, P.N., Söling, H.-D. and Mieskes, G. (1994) CaBP1, a calcium binding protein of the thioredoxin family, is a resident KDEL protein of the ER and not the intermediate compartment, *J. Cell Scie.* 107:2719-2727. (Medline)

Füllerkrug, J., Boehm, J., R”ttger, S., Nilsson, T., Mieske, G. and Schmitt, H.D. (1997) Human Rer1 is localized in the Golgi apparatus and complements the deletion of the homologus Rer1 protein of Saccharomyces cerevisiae, *Eur. J. Cell Biol.* 74:31-40. (Medline)

Gaglio, T., Saredi, A., Bingham, J.B., Hasbani, M.J., Gill, S.R., Schroer, T.A. and Compton, D.A. (1996) Opposing motor activities are required for the organization of the mammalian spindle pole, *J. Cell Biol.* 135:399-414. (Medline)

Galea-Lauri, J., Latchman, D.S. and Katz, D.R. (1996) The role of the 90-kDa heat shock protein in cell cycle control and differentiation of the monoblastoid cell line U937, *Exp. Cell Res.* 226:243-254. (MedLine)

Gavis, E.R. (1997) Expeditions to the pole: RNA localization in *Xenopus* and *Drosophila*, *Trends in Cell Biol.* 7:485-492.

Gething, M.J. and Sambrook, J. (1992) Protein folding in the cell, *Nature* 355:33-45. (Medline)

Gething, M.-J., McCammon, K. and Sambrook, J. (1986) Expression of wild type and mutant forms of influenza hemagglutinin, the role

of folding in intracellular transport, *Cell* 46:939-950. (Medline)

Gierasch, L.M. (1989) Signal sequences, *Biochemistry* 28:923-930. (MedLine)

Gilmore, R., Blobel, G. and Walter, P. (1982a) Protein translocation across the endoplasmic reticulum. I. Detection in the microsomal membrane of a receptor for the signal recognition particle, *J. Cell Biol*. 95:463-469. (Medline)

Gilmore, T., Walter, P. and Blobel, G. (1982b) Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor, *J. Cell Biol.* 95:470-477. (Medline)

Glenney, J.R., Boudreaux, M., Galyean, R., Hunter, T. and Tack, B. (1986) Association of the S-100 related calpactin I light chain with the NH_2-terminal tail of the 36 kDa heavy chain, *J. Biol. Chem.* 261:10485-10488. (Medline)

Glick, B.S. and Malhotra, V. (1998) The curious status of the Golgi apparatus, *Cell* 95:883-889. (Medline)

Glombik, M.M., Kromer, A., Salm, T., Huttner, W.B. and Gerdes, H.H. (1999) The disulfide-bonded loop of chromogranin B mediates membrane binding and directs sorting from the trans-Golgi network to secretory granules, *EMBO J.* 18:1059-1070. (MedLine)

Goldberg, D. E. and Kornfeld, S. (1983) Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation, *J. Biol. Chem.* 258:3159-3165. (Medline)

Goldberg, J. (2000) Decoding of sorting signals by coatomer through a GTPase switch in the COPI coat complex, *Cell* 100:671-679. (MedLine)

Görlich, D. and Rapoport, T.A. (1993) Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane, *Cell* 75:615-630. (Medline)

Görlich, D., Hartmann, E., Prehn, S. and Rapoport, T.A. (1992a) A protein of the endoplasmic reticulum involved early in polypeptide translocation, *Nature* 357:47-52. (Medline)

Görlich, D., Prehn, S., Hartmann, E., Kalies, K.-U. and Rapoport, T.A. (1992b) A mammalian homolog of Sec61p and DecYp is associated with ribosomes and nascent polypeptides during translocation, *Cell* 71:489-503. (Medline)

Graham, T.R. and Krasnov, V.A. (1995)Sortin of yeast α1,3 mannosyltransferase is mediated by a lumenal domain interaction, and a transmembrane domain signal that can confer clathrin-dependent Golgi localization to a secreted protein, *Mol. Biol. Cell* 6:809-824. (Medline)

Gratkowski, H., Lear, J.D. and DeGrado, W.F. (2001) Polar side chains drive the association of model transmembrane peptides, *Proc. Natl. Acad. Sci. USA* 98:880-885. (MedLine)

Griffiths, G. and Simon, K. (1986) The *trans* Golgi network: sorting at the exit site of the Golgi complex, *Science* 234:438-443.

Grimstone, A. V. (1959) Cytoplasmic membranes and the nuclear membrane in the flagellate *Triconympha*, *J. Cell Biol.* 6:369-377.

Gu, W., Pan, F., Zhang, H., Bassell, G.J. and Singer, R.H. (2002) A predominantly nuclear protein affecting cytoplasmic localization of β-actin mRNA in fibroblasts and neurons, *J. Cell Biol.*156:41-51. (MedLine)

Hamman, B.D., Chen, J.C., Johnson, E.E. and Johnson,. A.E. (1997) The aqueous pore through the translocon has a diameter of 40-60 A during cotranslational protein translocation at the ER membrane, *Cell* 89:535-544. (MedLine)

Hamman, B.D., Hendershot, L.M., Johnson, A.E. (1998) BiP maintains the permeability barrier of the ER membrane by sealing the lumenal end of the translocon pore before and early in translocation, *Cell* 92:747-758. (Medline)

Hanein D. Matlack, K.E.S., Jungnickel, B., Plath, K., Kalies, K.- U., Miller, K.R., Rapoport, T.A. and Akey, K.W.(1996) Oligomeric rings of the Sec61p complex induced by ligands required for protein translocation, *Cell* 87:721-732. (Medline)

Happe, S. and Weidman, P. (1998) Cell-free transport to distinct Golgi cisternae is compartment specific and ARF independent, *J. Cell Biol.* 140:511-523. (Medline)

Harris, S.L. and Waters, M.G. (1996) Localization of yeast early Golgi mannosyltransferase, Och1p, involves retrograde transport, *J. Cell Biol.* 132:985-998. (Medline)

Harter, C. and Wieland, F.T. (1998) A single binding site for dilysine retrieval motifs and p23 within the γ subunit of coatomer, *Proc. Natl. Acad. Sci. USA* 95:11649-11654. (MedLine)

Hartmann, E., Wiedmann, M. and Rapoport, A. (1989a) A membrane component of the endoplasmic reticulum that may be essential for protein translocation, *EMBO J.* 8:2225-2229. (Medline)

Hartmann, E., Rapoport, T.A. and Lodish, H.F. (1989b) Predicting the orientation of eukaryotic membrane spanning proteins, *Proc. Natl. Acad. Sci. USA* 86:5786-5790. (Medline)

Hartmann, E., Görlich, D., Kotska, S., Otto, A., Kraft, R., Knepsel, S., Bürger, E., Rapoport, T.A. and Prehn, S. (1993) A tetrameric complex of membrane proteins in the endoplasmic reticulum, *Eur. J. Biochem.* 214:375-381. (Medline)

Hasilik, A. and Neufeld, E. F. (1980) Biosynthesis of lysosomal enzymes in fibroblasts. Synthesis as precursor of a higher molecular weight, *J. Biol. Chem.* 255:4937-4950. (Medline)

Haucke, V. and Schatz, G. (1997) Import of proteins into mitochondria and chloroplasts, *Trends In Cell Biol.* 7:103-106.

Hayes, S.A. and Dice, J.F. (1996) Roles of molecular chaperones in protein degradation, *J. Cell Biol.* 132:255-258. (Medline)

Hedge, R.S. and Lingappa, V.R. (1997) Membrane protein biogenesis: regulated complexity at the endoplasmic reticulum, *Cell* 91:575-582. (Medline)

Hegde, R.S., Mastrianni, J.A., Scott, M.R., DeFea, K.A., Tremblay, P., Torchia, M., DeArmond, S.J., Prusiner, S.B. and Lingappa, V.R. (1998) A transmembrane form of the prion protein in neurodegenerative disease, *Science* 279:827-834. (Medline)

Heilker, R., Manning,-Krieg, U., Zuber, J.-F. and Spiess, M. (1996) *In vitro* binding of clathrin adaptors to sorting signals correlates with endocytosis and basolateral sorting, *EMBO J.* 15:2893-2899. (Medline)

Heinrich, S.U., Mothes, W., Brunner, J. and Rapoport, T.A. (2000) The Sec61p complex mediates the integration of a membrane protein by allowing lipid partitioning of the transmembrane domain., *Cell.* 102:233-244. (MedLine)

Heino, S., Lusa, S., Somerharju, P., Ehnholm C., Olkkonen, V.M. and Ikonen, E.(2000) Dissecting the role of the Golgi complex and lipid rafts in biosynthetic transport of cholesterol to the cell surface, *Proc. Natl. Acad. Sci. USA* 97:8375-8380. (MedLine)

Helms, J.B., Karrenbauer, A., Wirtz, K.W.A., Rothman, J.E. and Wieland, F.T. (1990) Reconstitution of steps in the constitutive secretory pathway in permeabilized cells. Secretion of glycosylated tripeptide and truncated sphingomyelin, *J. Biol. Chem.* 265:20027-20032. (Medline)

Hicke, L. and Riezman, H. (1996) Ubiquination of yeast plasma membrane receptor signals its ligand stimulated endocytosis, *Cell* 84:277-287. (Medline)

Higgins, C.F., ABC transporters: from microorganisms to man (1992), *Annua. Rev. Cell Biol.* 8:67-113. (Medline)

Hill, M.A. and Gunning, P. (1993) Beta and gamma actin mRNAs are differentially located within myoblasts, *J. Cell Biol.* 122:825-832. (MedLine)

Hiller, M.M., Finger, A., Schweiger, M. and Wolf, D.H. (1996) ER degradation of misfolded luminal protein by a cytosolic ubiquitin-proteasome pathway, *Science* 273:1725-1728. (Medline)

Hirschberg, K. Rodger, J. and Futerman, A.H. (1993) The long- chain sphingolipid base of sphingolipids is acetylated at the cytosolic surface of the endoplasmic reticulum in rat liver, *Biochem. J.* 290:751-757. (Medline)

Hirschberg, K., Miller, C.M., Ellenberg, J., Presley, J.F., Siggia, E.D., Phair, R.D. and Lippincott-Schwartz, J. (1998) Kinetic analysis of secretory protein traffic and characterization of Golgi to plasma membrane transport intermediates in living cells, *J. Cell Biol.* 143:1485-1503. (Medline)

Hoe, M.H., Slusarewicz, P., Misteli, T., Watson, R. and Warren G. (1995) Evidence for recycling of the resident medial/trans Golgi enzyme, N-acetylglucosaminyltransferase I, in ldlD cells, *J. Biol. Chem.* 270:25057-25063. (Medline)

Hubbard, M.J. and Cohen, P. (1993) On target with a new mechanism for the regulation of protein phosphorylation, *Trends in Biochem. Sci.* 18: 172-177. (Medline)

Hurtley, S.M. and Helenius, A. (1989) Protein oligomerization in the endoplasmic reticulum, *Annu. Rev. Cell Biol.* 5:277-307. (Medline)

Itin, C., Kappeler, F., Lindstedt, A.D. and Hauri, H.-P. (1995) A novel endocytotic signal related to KKXX ER-retrieval signal, *EMBO J.* 14:2250-2256. (Medline)

Jackson, M., Nilsson, T. and Peterson, P. (1993) Retrieval of transmembrane proteins in the endoplasmic reticulum, *J. Cell Biol.* 121:317-333. (Medline)

Jamieson, J. D. and Palade, G. E. (1967) Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport to condensing vacuoles and zymogen granules, *J. Cell Biol.* 34:597-615. (Medline)

Jansen, R.P. (2001) mRNA localization: message on the move, *Nature Rev. Mol. Cell Biol.* 2001 2:247-256. (MedLine)

Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D.H. and Sommer, T. (2002) Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48, *Nature Cell Biol.* 4:134-139. (MedLine)

Jena, B.P., Cho, S.J., Jeremic, A., Stromer, M.H. and Abu-Hamdah, R. (2003) Structure and composition of the fusion pore, *Biophys. J.* 84:1337-1343. (MedLine)

Jensen, T.J., Loo, M.A., Pind, S., Williams, D.B., Goldberg, A.L. and Riordan, J.R. (1995) Multiple proteolytic systems, including the proteasome, contribute to CFTR processing, *Cell* 83:129-135. (Medline)

Jerome, V., Vourc'h, C., Baulieu, E.E. and Catelli, M.G. (1993) Cell cycle regulation of the chicken hsp90 alpha expression, *Exp. Cell Res.* 205:44-51. (MedLine)

Johnson, L. M., Bankaitis, V. A. and Emr, S. D. (1987) Distinct sequence determinants direct intracellular sorting and modification of yeast vacuolar protease, *Cell* 48:875-885 (Medline)

Johnston, J.A., Ward, C.L. and Kopito, R.R. (1998) Aggresomes: a cellular response to misfolded proteins, *J. Cell Biol.* 143:1883-1898. (Medline)

Jolly, C. and Morimoto, R.I. (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death, *J. Natl.*

Cancer Inst. 92:1564-1572. (MedLine)

Jones, B.G., Thomas, L., Molloy, S.S., Thulin, C.D., Fry, M.D., Walsh, K.A. and Thomas, G. (1995) Intracellular trafficking of furin is modulated by the phosphorylated state of a casein kinase II site in its cytoplasmic tail, *EMBO J.* 14:5869-5883. (Medline)

Jungnickel, B. and Rapoport, T.A. (1995) A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane, *Cell* 82:261-270. (MedLine)

Kaiser, C.A. and Shekman, R.(1990) Distinct sets of SEC genes govern transport vesicle formation and junction early in the secretory pathway, *Cell* 61:723-733. (Medline)

Kallen, K.-J., Quinn, P. and Allan, D. (1993) Monensin inhibits synthesis of plasma membrane sphingomyelin by blocking transport of ceramide through the Golgi: evidence for two sites of sphingomyelin synthesis in BHK cells, *Biochim. Biophys. Acta* 1166:305-308. (Medline)

Kaplan, M.R. and Simoni, R.D. (1985) Intracellular transport of phosphatidylcholine to the plasma membrane, *J. Cell Biol.* 101:441-445. (Medline)

Karshikov, A., Berendes, R., Burger, A., Cavalié, A., Lux, H.-D. and Huber, R. (1992) Annexin V membrane interaction: an electrostatic potential study, *Eur. Biophys. J.* 20:337-344.

Kaufman, R.J. (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls, *Genes Dev* 13(10):1211-1233. (MedLine)

Keenan, R.J., Freymann, D.M., Walter, P. and Stroud, R.M. (1998) Crystal structure of the signal sequence binding subunit of the signal recognition particle, *Cell* 94:181-191. (Medline)

Kelleher, D.J., Krebich, G. and Gilmore (1992) Oligosaccharyltransferase activity is associated with a protein complex composed of ribophorins I and II and a 48 kd protein, *Cell* 69:55-65. (Medline)

Kim-ha, J., Webster, P.J., Smith, J.L. and MacDonald, P.M. (1993) Multiple RNA regulatory elements mediate distinct steps in the localization of the *oskar* mRNA, *Development* 119:169-178. (Medline)

Kislauskis, E.H. and Singer, R.H. (1992) Determinants of RNA localization, *Curr. Opin. Cell Biol.* 4: 975-978 (Medline)

Kok, J.W., Hoekstra, K., Eskelinen, S. and Hoekstra, D. (1992) Recycling pathways of glucosylceramide in BHK cells: distinct involvement of early and late endosomes, *J. Cell Sci.* 103:1139-1152. (Medline)

Kopito, R.R. (1997) ER quality control: the cytoplasmic connection, *Cell* 88:427-430. (Medline)

Kozutsumi, Y., M. Segal, K. Normington, M.J. Gething, and J. Sambrook. (1988) The presence of malfolded proteins in the endoplasmic reticulum signals, the induction of glucose-regulated proteins, *Nature* 332: 462-464. (MedLine)

Krisans, S.K. (1992) The role of peroxisomes in cholesterol metabolism, *Am. J. Respir. Cell Mol. Biol.* 7:358-364. (Medline)

Kuchler, K. and Thorner, J. (1992) Secretion of peptides and proteins lacking the hydrophobic signal sequences: the role of ATP-driven membrane translocators, *Endocr.Rev.* 13:499-514. (Medline)

Kuehn, M.J., Herrmann, J.M. and Schekman, R. (1998) COPII-cargo interactions direct protein sorting into ER-derived transport vesicles, *Nature* 391:187-190. (MedLine)

Kuhl, D. and Skehel, P. (1998) Dendritic localization of mRNAs *Curr. Opin. Neurobiol.* 8:600-606. (MedLine)

Kuroiwa, T., Sakaguchi, M., Omura, T. and Mihara, K. (1996) The reinitiation of protein translocation across the endoplasmic reticulum membrane for the topogenesis of multispanning membrane proteins, *J. Biol. Chem.* 271: 6423-6428. (Medline)

Kurzchalia, T. V., Wiedmann, M., Girshovich, A. S., Bochkareva, E. S., Bielka, H. and Rapoport, T. A. (1986) The signal sequence of nascent preprolactin interacts with the 54 K polypeptide of the signal recognition particle, *Nature* 320:634-636. (Medline)

Kutay, U., Ahnert-Hilger, G., Hartmann, E., Wiedenmann, B. and Rapoport, T.A. (1995) Transport route for synaptobrevin via a novel pathway of insertion into the endoplasmic reticulum membrane, *EMBO J.* 14:217-223. (Medline)

Ladinsky, M.S., Mastronarde, D.N., McIntosh, J.R., Howell, K.E. and Staehelin, L.A. (1999) Golgi structure in three dimensions: functional insights from the normal rat kidney cell, *J. Cell Biol.* 144:1135-1149. (Medline)

Laird, V. and High, S. (1997) Discrete cross-linking product identified during membrane protein biosynthesis, *J. Biol. Chem.* 272:1983-1989. (Medline)

Lang, T., Bruns, D., Wenzel, D., Riedel, D., Holroyd, P., Thiele, C. and Jahn, R. (2001) SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis, *EMBO J.* 20:2202-2213. (MedLine)

Lange, Y., Swaisgood, M.H., Ramos, B.V. and Steck, T.L. (1989) Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts, *J. Biol. Chem.* 264:3786-3793. (MedLine)

Lanoix, J., Ouwendijk, J., Stark, A., Szafer, E., Cassel, D., Dejgaard, K., Weiss, M. and Nilsson, T. (2001) Sorting of Golgi resident proteins into different subpopulations of COPI vesicles: a role for ArfGAP1, *J. Cell Biol.* 155:1199-1212. 9MedLine)

Lee, A.S. (1992) Mammalian stress response: induction of the glucose-regulated protein family, *Curr. Opin. Cell Biol*:267-273. (MedLine)

Lee, A.S. (2001) The glucose-regulated proteins: stress induction and clinical applications, *Trends Biochem. Sci.* 26:504-510. (MedLine)

Li, C., Ullrich, B., Zhang, J.Z., Anderson, R.G., Brose, N. and Sudhof, T.C. (1995) Ca^{2+}-dependent and -independent activities of neural and non-neural synaptotagmins, *Nature* 375:594-599. (MedLine)

Liscum, L. and Munn, N.J. (1999) Intracellular cholesterol transport, *Biochim. Biophys. Acta.* 1438:19-37. (MedLine)

Little, E., Ramakrishnan, M., Roy, B., Gazit, G. and Lee, A.S. (1994) The glucose-regulated proteins (GRP78 and GRP94): functions, gene regulation, and applications, *Crit. Rev. Eukaryot. Gene. Expr.* 4:1-18. (MedLine)

Llinas, R., Gruner, J.A., Sugimori, M., McGuinness, T.L. and Greengard, P (1991) Regulation by synapsin I and Ca^{2+}-calmodulin dependent protein kinase II on the transmitter release in squid giant synapse, *J. Physiol. (London)* 436:257-282. (Medline)

Long, E.O. (1998) Signal sequences stop killer cells, *Nature* 391:740-741, 743. (Medline)

Love, H.D., Lin, C.C., Short, C.S. and Ostermann, J. (1998) Isolation of functional Golgi-derived vesicles with a possible role in retrograde transport, *J. Cell Biol.* 140:541-551. (Medline)

Lu, H. and Booth, P.J. (2000) The final stages of folding of the membrane protein bacteriorhodopsin occur by kinetically indistinguishable parallel folding paths that are mediated by pH, *J. Mol. Biol.* 299:233-243. (MedLine)

Lyko, F., Martoglio, B., Jungnickel, B., Rapoport, T.A. and Dobberstein, B. (1995) Signal sequence processing in rough microsomes, *J. Biol. Chem.* 270:19873-19878. (Medline)

Lyman, S.K., and Schekman, R. (1995) Interaction between BiP and Sec63p is required for the completion of protein translocation in the ER of *Saccharomyces cervisiae, J. Cell Biol.* 131:1163-1171. (Medline)

Ma, D., Zerangue, N., Lin, Y.F., Collins, A., Yu, M., Jan, Y.N. and Jan, L.Y. (2001) Role of ER export signals in controlling surface potassium channel numbers, *Science* 291:316-319. (MedLine)

Machamer, C. E. and Rose. J. K. (1987) A specific transmembrane domain of coronavirus E1 glycoprotein is required for its retention in the Golgi region, *J. Cell Biol*. 105:1205-1214. (Medline)

Machamer, C. E., Mentone, S. A., Rose, J. K. and Farquhar, M. G. (1990) The El glycoprotein of an avian coronavirus is targeted to the cis Golgi complex, *Proc. Natl. Acad. Sci.* 87:6944-6948. (Medline)

Malhotra, V., Serafini, T., Orci. L., Shepherd, J. C. and Rothman, J. E. (1989) Purification of a novel class of coated vesicles mediating biosynthetic protein transport through Golgi stacks, *Cell* 58:329-336. (Medline)

Marks, M.S., Woodruff, L., Ohno, H. and Bonifacino, J.S. (1996) Protein targeting by tyrosine- and di-leucine-based signals: evidence for distinct saturable components, *J. Cell Biol.* 135:341-354. (Medline)

Marks, M.S., Ohno, H., Kirchhausen, T. and Bonifacino, J.S. (1997) Protein sorting by tyrosine-based signals: adapting to the Ys and wherefores, *Trends in Cell Biol.* 7:124-128.

Marsh, B.J., Mastronarde, D.N., Buttle, K.F., Howell, K.E. and McIntosh, J.R. (2001) Inaugural Article: Organellar relationships in the Golgi region of the pancreatic β cell line, HIT-T15, visualized by high resolution electron tomography, *Proc. Natl. Acad. Sci. USA* 98:2399-2406. (MedLine) movies for this article

Martin-Belmonte, F., Alonso, M.A., Zhang, X. and Arvan, P. (2000) Thyroglobulin is selected as luminal protein cargo for apical transport via detergent-resistant membranes in epithelial cells, *J. Biol. Chem.* 275:41074-41081. (Medline)

Martinez, I., Chakrabarti, S., Hellevik, T., Morehead, J., Fowler, K. and Andrews, N.W. (2000) Synaptotagmin VII regulates Ca^{2+}-dependent exocytosis of lysosomes in fibroblasts, *J. Cell Biol.* 148:1141-1150. (MedLine)

Martínez-Menárguez, J.A., Prekeris, R., Oorschot, V.M., Scheller, R., Slot, J.W., Geuze, H.J. and Klumperman, J. (2001) Peri-Golgi vesicles contain retrograde but not anterograde proteins consistent with the cisternal progression model of intra-Golgi transport, *J. Cell Biol.* 155:1213-1224. (MedLine)

Martoglio, B., Hofmann, M.W., Brunner, J. and Dobberstein, B. (1995) The protein conducting channel in the membrane of the endoplasmic reticulum is open laterally toward the lipid layer, *Cell* 81:207-214. (Medline)

Martoglio, B., Graf, R.and Dobberstein, B. (1997) Signal peptide fragments of preprolactin and HIV-1 p-gp160 interact with calmodulin, *EMBO J.* 16:6636-6645. (Medline)

Masibay A.S., Balaji P.V., Boeggeman E.E. and Qasba P.K. (1993) Mutational analysis of the Golgi retention signal of bovine β-1,4-galactosyltransferase, *J. Biol. Chem.* 268:9908-9916. (Medline)

Mastronarde, D.N. (1997) Dual-axis tomography: an approach with alignment methods that preserve resolution, *J. Struct. Biol.* 120:343-352. (Medline)

Mauxion, F., Le Borgne, R., Munier-Lehman, H. and Hoflack, B. (1996) A casein kinase II phosphorylation site and the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor determines the high affinity interaction of the AP-1 Golgi proteins to membranes, *J. Biol. Chem.* 271:2171-2178. (Medline)

Mayer, R.J., Arnold, J., Laszlo, L., Landon, M. and Lowe, J. (1991) Ubiquitin in health and disease, *Biochim. Biophys. Acta* 1089:141-

157. (Medline)

McCracken, A.A. and Brodsky, J.L. (1996) Assembly of ER-associated protein degradation in vitro: dependence of cytosol, calnexin and ATP, *J. Cell Biol.* 132:291-298. (Medline)

McKnight, C.J., Rafalski, M. and Gierasch, L.M. (1991) Fluorescence analysis of the tryptophan-containing variants of the LamB signal sequence upon insertion into a lipid bilayer, *Biochemistry* 30:6241-6246. (Medline)

Meyer, D. I. and Dobberstein, B. (1980) A membrane component essential for vectorial translocation of nascent proteins across the endoplasmic reticulum: requirements for its extraction and reassociation with the membrane, *J. Cell Biol.* 87:498-502. (Medline)

Meyer, D. I., Krause, E. and Dobberstein, B. (1982a) Secretory protein translocation across membranes. The role of the docking proteins, *Nature* 297:647-650. (Medline)

Meyer, D. I., Louvard, D. and Dobberstein, B. (1982b) Characterization of molecules involved in protein translocation using a specific antibody, *J. Cell Biol.* 92:579-583. (Medline)

Migliaccio, G., Nicchitta, C.V. and Blobel, G. (1992) The signal sequence receptor, unlike the signal recognition particle receptor is not essential for protein translocation, *J.Cell Biol.* 117:15-25. (Medline)

Miller, J.D., Wilhelm, H., Gierasch, L., Gilmore, R. and Walter, P. (1993) GTP binding and hydrolysis by the signal recognition particle during initiation of protein translocation, *Nature* 366:351-354. (Medline)

Mironov, A.A., Weidman, P. and Luini, A. (1997) Variations on the intracellular transport theme: maturing cisternae and trafficking tubules, *J. Cell Biol.*138:481-484. (Medline)

Mironov, A. Jr., Luini, A. and Mironov, A. (1998) A synthetic model of intra-Golgi traffic, *FASEB J.* 12:249-252. (Medline)

Mironov, A.A., Beznoussenko, G.V., Nicoziani, P., Martella, O., Trucco, A., Kweon, H.S., Di Giandomenico, D., Polishchuk, R.S., Fusella, A., Lupetti, P., Berger, E.G., Geerts, W.J., Koster, A.J., Burger, K.N. and Luini, A. (2001) Small cargo proteins and large aggregates can traverse the Golgi by a common mechanism without leaving the lumen of cisternae, *J. Cell Biol.* 155:1225-1238. (MedLine)

Miyake, H., Hara, I., Arakawa, S. and Kamidono, S. (2000) Stress protein GRP78 prevents apoptosis induced by calcium ionophore, ionomycin, but not by glycosylation inhibitor, tunicamycin, in human prostate cancer cells, *J. Cell. Biochem.* 77:396-408. (MedLine)

Molloy, S.S, Thomas, L., VanSlyke, J.K, Stenberg, P.E.and Thomas, G. (1994) Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface, *EMBO J.* 13:18-33. (Medline)

Moore H.-P., Gumbiner, B. and Kelly, R. B. (1983) Chloroquine diverts ACTH from a regulated to a constitutive secretory pathway in AtT-20 cells, *Nature* 302:434-436. (Medline)

Moreau, P., Cassagne, C., Keena, T.W. and Morré, D.J. (1993) Ceramide excluded from cell-free vesicular lipid transfer from endoplasmic reticulum to Golgi apparatus-evidence for lipid sorting, *Biochim. Biophys. Acta* 1146:9-16. (Medline)

Mothes, W., Prehn, S. and Rapoport, T.A. (1994) Systematic probing of the environment of a translocating secretory protein during translocation through the ER membrane, *EMBO J.*13:3973-3982. (Medline)

Mothes, W., Heinrich, S.U., Graf, R., Nilsson, I., von Heijne, G., Brunner, J. and Rapoport, T.A. (1997) Molecular mechanism of membrane protein integration into the endoplasmic reticulum, *Cell* 89:523-533. (MedLine)

Mothes, W., Jungnickel, B., Brunner, .J. and Rapoport, T.A. (1998) Signal sequence recognition in cotranslational translocation by

protein components of the endoplasmic reticulum membrane, *J. Cell Biol.* 142:355-364. (MedLine)

Mueckler, M. and Lodish, H. F. (1986) The human glucose transporter can insert posttranslationally into microsomes,*Cell* 44:629-637. (Medline)

Munro, S. (1991) Sequences within and adjacent to the transmembrane segment of alpha-2,6-sialyltransferase specify Golgi retention, *EMBO J.* 10:3577-3588. (Medline)

Munro, S. (1995a) A comparison of the transmembrane domains of Golgi and plasma membrane proteins, *Biochem. Soc. Trans.* 23:527-530. (Medline)

Munro, S. (1995b) An investigation of the role of transmembrane domains in Golgi protein retention, *EMBO J.* 14:4695-4704. (Medline)

Munro, S. (1998) Localization of proteins to the Golgi apparatus, *Trends in Cell Biol.* 8:11-15. (Medline)

Munro, S. and Pelham, H. R. B. (1986) An H5P70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein, *Cell* 46:291-300. (Medline)

Munro, S. and Pellham, H.R. (1987) A C-terminal signal prevents secretion of luminal ER proteins, *Cell* 48:899-907. (Medline)

Nakata, T., Soboue, K., Hirokawa, N. (1990) Conformational change and localization of calpactin I complex involved in exocytosis is revealed by quick-freeze, deep-etch electron microscopy and immunochemistry, *J. Cell Biol.* 110:13-25. (Medline)

Narula, N. and Stow, J.L. (1995) Distinct coated vesicles labeled for p200 bud from the trans-Golgi network membranes, *Proc. Natl. Acad. Sci. USA* 92:2874-2878. (Medline)

Newman, A. P. and Ferro-Novick, S. (1987) Characterization of new mutants in the early part of the yeast secretory pathway isolated by a ^{3}[H]mannose suicide selection, *J. Cell Biol*, 105:1587-1594. (Medline)

Newman, L.S., McKeever, M.O., Okano, H., and Darnell, R.B. (1995) β-NAP, a cerebellar degeneration antigen, is a neuron specific coast protein, Cell 82:773-783. (Medline)

Ng, D.T.W., Brown, J.D. and Walter, P. (1996) Signal sequences specify the targeting route to the endoplasmic reticulum, *J. Cell Biol.* 134: 269-278. (Medline)

Nilsson, T., Slusarewicz, P., Hoe, M.H. and Warren, G. (1993) Kin recognition. A model for the retention of Golgi enzymes, *FEBS Lett.* 330:1-4. (Medline)

Nilsson, T., Hoe, M.H., Slusarewicz, O., Rabouille, C., Watson, R., Hunte, F., Watzele, G., Berger, E.G. and Warren, G. (1994) Kin recognition between *medial* Golgi enzymes in HeLa cells, *EMBO J.* 13:562-574. (Medline)

Nilsson T., Rabouille C., Hui, N., Watson, R. and Warren, G. (1996) The role of the membrane-spanning domain and stalk region of N-acetylglucosaminyltransferase I in retention, kin recognition and structural maintenance of the Golgi apparatus in HeLa cells, *J. Cell Scie.* 109:1975-1989. (Medline)

Nishimura, N. and Balch, W.E. (1997) A di-acidic signal required for selective export from the endoplasmic reticulum, *Science* 277:556-558. (MedLine)

Nothwehr, S.F., Roberts, C.J. and Stevens, T.H. (1993) Membrane protein retention in the yeast Golgi apparatus: dipeptidyl aminopeptidase A is retained by a cytoplasmic signal containing aromatic residues, *J. Cell Biol.* 121:1197-1209. (Medline)

Novick, P., Field, C. and Shekman, R. (1980) Indentification of 23 complementation groups required for post-translational events in the yeast secretory pathway, *Cell* 21:205-215. (Medline)

Ohno, H., Stewart, J., Fournier, M.-C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Galluser, A., Kirschhausen, T. and Bonifacino, J.S. (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins, *Science* 269:1872-1875. (Medline)

Ohno, H., Fournier, M.-C., Poy, G. and Bonifacino, J.S. (1996) Structural determinants of interaction of tyrosine-based sorting signals, *J. Biol. Chem.* 271:2909-29015.

Oka, T., Nishikawa, S., Nakano, A. (1991) Reconstitution of GTP- binding Sar1 protein function in ER and Golgi transport, *J. Cell Biol.* 114:671-679. (Medline)

Orci, L., Palmer, D.J., Amherdt, M. and Rothman, J.E. (1993) Coated vesicle assembly in the Golgi requires only coatamer and ARF proteins from the cytosol, *Nature* 364:732-734. (Medline)

Orci, L., Stamnes, M., Ravazzola, M., Amherdt, M., Perrelet, A., S”llner, T.H. and Rothman, J.E. (1997) Bidirectional transport by distinct populations of COPI-coated vesicles, *Cell* 90:335-349. (Medline)

Orci, L., Amherdt, M., Ravazzola, M., Perrelet, A. and Rothman, J.E. (2000) Exclusion of Golgi residents from transport vesicles budding from Golgi cisternae in intact cells, *J. Cell Biol.* 150:1263-1270. (MedLine)

Paetzel, M., Dalbey, R.E. and Strynadka, N.C.J. (1998) Crystal structure of a bacterial signal peptidase in complex with a β-lactam inhibitor, *Nature* 396:186-190. (Medline)

Pagano, R.E. and Sleight, R.G. (1985) Defining lipid transport in animal cells, *Science* 229:1051-1057. (Medline)

Pagano, R., Martin, O., Kang, H. and Haugland, R.(1991) A novel fluorescent ceramide analog for studying membrane traffic in animal cells: accumulation in the Golgi apparatus results in altered spectral properties of the sphingolipid precursor, *J. Cell Biol.* 113:1267-1279. (Medline)

Page, L.J., Darmon, A.J., Uellner, R. and Griffiths, G.M. (1998) L is for lytic granules: lysosomes that kill, *Biochim. Biophys. Acta.* 1401:146-156. <(MedLine)

Palacios, I.M. and St. Johnston, D.S. (2001) Getting the message across: the intracellular localization of mRNAs in higher eukaryotes, *Annu. Rev. Cell Dev. Biol.* 17:569-614. (MedLine)

Palade, G.E. (1975) Intracellular aspects of the process of protein transport, *Science* 189:347-354. (Medline)

Palmiter, R. D., Gagnon, J. and Walsh, K. A. (1978) Ovalbumin: a secreted protein without a transient hydrophobic leader sequence, *Proc. Natl. Acad. Sci. USA* 75:94-98. (Medline)

Panzner, S., Dreier, L., Hartmann, E., Kostka, S. and Rapoport, T.A. (1995) Posttranscriptional protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p, *Cell* 81:561-570. (Medline)

Parks, G.D. and Lamb, R.A. (1993) Role of NH2-terminal positively charged residues in establishing membrane protein topology, *J. Biol. Chem.* 268:19101-19109. (Medline)

Payne, A.S., Kelly, E.J. and Gitlin, J.D. (1998) Functional expression of the Wilson disease protein reveals mislocalization and impaired copper-dependent trafficking of the common H1069Q mutation, *Proc. Natl. Acad. Sci. USA* 95:10854-10859. (Medline)

Pelham, H.R.B. (1989) Control of protein exit from the endoplasmic reticulum, *Ann. Rev. Cell Biol.* 5:1-23. (Medline)

Pelham, H. R. B. (1990) The retention signal for soluble proteins of the endoplasmic reticulum, *Trends in Biochem. Sci.* 15:483-486. (Medline)

Pelham, H.R.B. (1998) Getting through the Golgi complex, *Trends in Cell Biol.* 8:45-49. (Medline)

Pelham, H.R.B. (2001) Traffic through the Golgi apparatus, *J. Cell Biol.*155:1099-1101. (MedLine)

Perara, E., Rothman, R. E. and Lingappa, V. R. (1986) Uncoupling translocation from translation: implications for transport of protein across membranes, *Science* 232:348-352. (Medline)

Peters, C., Braun, M., Weber, B., Wendland, M., Schmidt, B., Pohlmann, R., Waheed, A. and von Figura, K. (1990) Targeting of lysosomal proteins: a tyrosine-containing endocytosis signal in the cytoplasmic tail of lysosomal acid phosphatase is necessary and sufficient for targeting to lysosomes, *EMBO J.* 9:3497-3506. (Medline)

Pevsner, J., Voknadnt, W., Wong, B.R. and Scheller, R.H. (1994) Two rat homologs of clathrin associated adaptor proteins, *Gene* 146:279-283. (Medline)

Pfeiffer, S. R. and Rothman, J. E. (1987) Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi, *Annu. Rev. Biochem.*, 56:829-852.

Pimplikar, S.W. and Huttner, W.B. (1992) Chromogranin B (secretogranin I), a secretory protein of the regulated pathway, is also present in a tightly membrane-associated form in PC12 cells, *J. Biol. Chem.* 267:4110-4118. (MedLine)

Plath, K., Mothes, W., Wilkinson, B.M., Stirling, C.J. and Rapoport, T.A. (1998) Signal sequence recognition in posttranslational protein transport across the yeast ER membrane, *Cell* 94:795-807. (Medline)

Plemper, R.K., Böhmler, S., Bordallo, J.Sommer, T. and Wolf, D.H. (1997) Mutant analysis links translocon and BiP to retrograde protein transport for ER degradation, *Nature* 388:891-895. (Medline)

Polishchuk, R.S., Polishchuk, E.V., Marra, P., Alberti, S., Buccione, R., Luini, A. and Mironov, A.A. (2000) Correlative Light-Electron Microscopy Reveals the Tubular-Saccular Ultrastructure of Carriers Operating between Golgi Apparatus and Plasma Membrane, *J. Cell Biol.* 148:45-58. (Medline)

Pond, L., Kuhn, L.A., Teyton,L., Schutze, M.-P., Tainer, J.A., Rohrer, J., Schweizer, A., Johnson, K.F. and Kornfeld, S. (1995a) A determinant in the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor prevents trafficking to lysosomes, *J. Cell Biol.* 130: 1297-1306. (Medline)

Pond, L., Kuhn, L.A., Teyton, L., Schutze, M.-P., Tainer, J.A. Jackson, M.R. and Peterson, P.A. (1995b) A role of acidic residues in the di-leucine motif-based targeting to the endocytotic pathway, *J. Biol. Chem.* 270:19989-19997. (Medline)

Popot, J.L. and Engelman, D.M. (2000) Helical membrane protein folding, stability, and evolution, *Annu. Rev. Biochem* 69:881-922. (Medline)

Presley, J.F., Cole, N.B., Schroer, T.A., Hirschberg, K., Zaal, K.J.M. and Pippincott-Schwartz, J. (1997) ER-to-Golgi transport visualized in living cells, *Nature* 389:81-85. (Medline)

Prusiner, S.B. (1997) Prion diseases and the BSE crisis, *Science* 278:245-251. (Medline)

Pryer, N.K., Wuestehube, L.J. and Schekman, R. (1992) Vesicle-mediated protein sorting, *Annu. Rev. Biochem.* 61:471-516. (MedLine)

Puglielli,L., Rigotti,A., Greco, A.V., Santos, M.J. and Nervi, F. (1995) Sterol carrier protein-2 is involved in cholesterol transfer from the endoplasmic reticulum to the plasma membrane in human fibroblasts, *J. Biol. Chem.* 270: 18723-18726. (MedLine)

Rabouille, C., Hui, N., Hunte, F., Kieckbusch, R., Berger, E.G., Warren, G. and Nilsson, T. (1995) Mapping the distribution of Golgi enzymes involved in the construction of complex oligosaccharides, *J. Cell Sci.* 108:1617-1627. (Medline)

Rambourg, A. and Clermont, Y. (1990) Three-dimensional electron microscopy: structure of the Golgi apparatus, *Eur. J. Cell Biol.* 51:189-200. (Medline)

Rambourg, A. and Clermont, Y. (1997) Three-dimensional structure of the Golgi apparatus in mammalian cells, in *The Golgi Apparatus*, Birkhäuser Verlag, Basel, Boston, Berlin, ed. Berger, E.G. and Roth, J. p.37-61.

Rapoport, T.A., Jungnickel, B. and Kutay, U. (1996a) Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes, *Annu. Rev. Biochem.* 65:271-303. (Medline)

Rapoport, T.A., Rolls, M.M. and Jungnickel, B. (1996b) Approaching the mechanism of protein transport across the ER membrane, *Curr. Opin. Cell Biol.* 8:499-504. (Medline)

Reddy, R.K., Lu, J. and Lee, A.S. (1999) The endoplasmic reticulum chaperone glycoprotein GRP94 with Ca^{2+}-binding and antiapoptotic properties is a novel proteolytic target of calpain during etoposide-induced apoptosis, *J. Biol. Chem.* 274:28476-28483. (MedLine)

Reinhart, M.P., Billheimer, J.T., Faust, J.R. and Gaylor, J.L. (1987) Subcellular localization of the enzymes of cholesterol biosynthesis and metabolism in rat liver, *J. Biol. Chem.* 262:9649-9655. (MedLine)

Ren, J., Lew, S., Wang, Z. and London, E. (1997) Transmembrane orientation of hydrophobic α-helices is regulated both by relationship of helix length to bilayer thickness and the cholesterol concentration, *Biochemistry* 36:10213-10220. (Medline)

Rings, E.H.H.M., B•ller, H.A., de Boer, P.A.J., Grand, R.J., Montgomery, R.K., Lamers, W.H., Charles, R. and Moorman, A.F.M. (1992) Messenger RNA sorting in enterocytes. Colocalization with encoded proteins, *FEBS Lett.* 300:183-187. (Medline)

Rivett, A.J. (1993) Proteasomes: multicatalytic proteinase complexes, *Biochem. J.* 291:1-10 (Medline)

Robinson, A., Kaderbhai, M.A. and Austen, B.M. (1987) Identification of signal sequence binding proteins integrated into the rough endoplasmic reticulum membrane, *Biochem. J.* 242:767-777. (Medline)

Rodríguez, A., Webster, P., Ortego, J. and Andrews, N.W. (1997) Lysosomes behave as Ca^{2+}-regulated exocytic vesicles in fibroblasts and epithelial cells, *J. Cell Biol.* 137:93-104. (MedLine)

Rohrer, J., Schweizer, A., Russell, D. and Kornfeld, S. (1996) The targeting of Lamp1 to lysosomes is dependent on the spacing of its cytoplasmic tail tyrosine sorting motif relative to the membrane, *J. Cell Biol.* 132:565-576. (Medline)

Rojas, E., Pollard, H.B., , Haigler, H.T., Parra C. and Burns, A.L. (1990) Calcium activated endonexin II forms calcium channels across acidic phospholipid bilayer membrane, *J. Biol. Chem.* 265:21207-21215. (Medline)

Rollins, C.T., Rivera, V.M., Woolfson, D.N., Keenan, T., Hatada, M., Adams, S.E., Andrade, L.J., Yaeger, D., van Schravendijk, M.R., Holt, D.A., Gilman, M. and Clackson, T. (2000) A ligand-reversible dimerization system for controlling protein-protein interactions, *Proc. Natl. Acad. Sci. USA.* 97:7096-7101. (MedLine)

Ronne, H., Ocklind, C., Wiman, K., Rask, L., Obring. B. and Peterson, P. A. (1983) Ligand dependent regulation of intracellular protein transport: effect of vitamin A on the secretion of the retinol binding protein, *J. Cell Biol.* 96:907-910. (Medline)

Roper, K. , Corbeil, D. & Huttner, W. B. (2000) Retention of prominin in microvilli reveals distinct cholesterol-based lipid micro-domains in the apical plasma membrane, *Nature Cell Biol.* 2, 582-592. (MedLine)

Rosenwald, A., Machamer,C. and Pagano, R. (1992) Effect of sphingolipid inhibitor in membrane transport through the secretory pathway, *Biochem.* 31:3581-3590. (Medline)

Rossi, G., Jiang, Y., Newman, A. and Ferro-Novick (1991) Dependence of Ypt1 and Sec 4 membrane attachment on Bet2, *Nature* 351:158-161. (Medline)

Rothblatt, J.A., Deshaies, R.J., Sanders, S.L., Daum, G. and Schekman, R. (1989) Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast, *J. Cell Biol.* 109:2641-2652. (Medline)

Rothman, J.E. (1989) Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells, *Cell* 59:591-601. (Medline)

Rothman, J.E. and Wieland, F.T. (1996) Protein sorting by transport vesicles, *Science* 272:227-234. (Medline)

Rothman, J.E., Urbani, L.H. and Brands, R.(1984a) Transport of protein between cytoplasmic membranes of fused cells: correspondence to processes reconstituted in a cell free system, *J. Cell. Bio* 99:248-259. (Medline)

Rothman, J. E., Miller, R. L. and Urbani, L. J. (1984b) Intercompartmental transport in the Golgi complex is a dissociative process: facile transfer of membrane proteins between two Golgi populations, *J. Cell Biol.* 99:260-271. (Medline)

Sakaguchi, M., Tomiyoshi, R., Kuroiwa, T., Mihara, K. an Omura, T. (1992) Functions of signal and signal-anchor sequences is determined by the balance between the hydrophobic segment and the N-terminal charge, *Proc. Natl. Acad.Sci. USA* 88:16-19. (Medline)

Sanders, S.L., Whitfield, K.M., Vogel, J.P., Rose, M.D., and Schekman, R.W. (1992) Sec61p and Bip directly facilitate polypeptide translocation into the ER, *Cell* 69:353-365. (Medline)

Saraste, J. and Hedman, K. (1983) Intracellular vesicles in the transport of Semliki Forest virus membrane proteins to the cell surface, *EMBO J.* 2:2001-2006. (Medline)

Saraste, J. and Kuismanen, E. (1984) Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface, *Cell* 38:535-549. (Medline)

Sato, T., Sakaguchi, M., Mihara, K. and Omura, T. (1990) The amino terminal structures that determine topological orientation of cytochrome P-450 in microsomal membrane, *EMBO J.* 9:2391-2397. (Medline)

Sato, M., Sato, K. and Nakano, A. (1996) Endoplasmic reticulum localization of Sec12p is achieved by two mechanisms: Rer1p-dependent retrieval that requires the transmembrane domain and Rer1p-independent retention that involves the cytoplasmic domain, *J.Cell Biol.* 134:279-293. (Medline)

Schatz, G. and Dobberstein (1996) Common principles of protein translocation across membranes, *Science* 271:1519-1526. (Medline)

Schiavo, G., Osborne, S.L. and Sgouros, J.G. (1998) Synaptotagmins: more isoforms than functions?, *Biochem. Biophys. Res. Commun.* 248:1-8. (MedLine)

Schimöller, F., Singer-Krüger,B., Schröder, S., Krüger, U., Barlowe, C. and Riezman, H. (1995) The absence of Emp14p, a component of the ER-derived COPII-coated vesicles, causes a defect in transport of selected proteins to the Golgi, *EMBO J.* 14:1329-1339. (Medline)

Schmoranzer, J., Goulian, M., Axelrod, D. and Simon, S.M. (2000) Imaging constitutive exocytosis with total internal reflection fluorescence microscopy, *J. Cell Biol.* 149:23-32. (MedLine)

Schneider, S.W., Sritharan, K.C., Geibel, J.P., Oberleithner, H. and Jena, B.P. (1997) Surface dynamics in living acinar cells imaged by atomic force microscopy: identification of plasma membrane structures involved in exocytosis, *Proc. Natl. Acad. Sci. USA* 94:316-321. (MedLine)

Schnorrer, F., Bohmann. K. and Nüsslein-Volhard, C. (2000) The molecular motor dynein is involved in targeting swallow and bicoid RNA to the anterior pole of *Drosophila* oocytes, *Nature Cell Biol.* 2:185-190. (MedLine)

Schroer, T.A., Bingham, J.B. and Gill, S.R. (1996) Actin-related protein 1 and cytoplasmic dynein-based motility, *Trends Cell Biol.* 6:212-215.

Schweizer, A., Kornfeld, S., and Rohrer, J. (1996) Cysteine^{34} of the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor is reversibly palmitoylated and required for normal trafficking and lysosomal enzyme sorting, *J. Cell Biol.* 132:577-584. (Medline)

Semenza, J. C., Hardwick, K. G., Dean. N. and Pelham, H. R. B. (1990) ERD2, a yeast gene required for the receptor mediated retrieval of luminar ER proteins from the secretory pathway, *Cell* 61:1349-1357. (Medline)

Serafini. T., Stenbeck, G., Brecht, A., Lottspeich. F., Orci, L., Rothman. J. E. and Wieland. F. T. (1991) A coat subunit of Golgi-derived non-clathrin vesicles with homology to the clathrin-coated vesicle protein -adaptin, *Nature* 349:215-220. (Medline)

Sevier, C.S., Weisz, O.A., Davis, M. and Machamer, C.E. (2000) Efficient export of the vesicular stomatitis virus G protein from the endoplasmic reticulum requires a signal in the cytoplasmic tail that includes both tyrosine-based and di-acidic motifs, *Mol. Biol. Cell* 11:13-22. (MedLine)

Schäfer, W., Stroh, A., Berghofer, S., Seiler, J., Vey, M., Kruse, M.-L., Kern, H.F., Klenk, H.-D. and Garten, W.(1995) Two independent targeting signals in the cytoplasmic domain determine *trans*-Golgi network localization and endosomal trafficking of the protein convertase furin, *EMBO J.* 14:2424-2435. (MedLine)

Siekevitz, P. and Palade, G. E. (1960) A cytochemical study on the pancreas of guinea pig. V. In vivo incorporation of leucine-1-C_{14} into chymotrypsinogen of various cell fractions,*J. Cell Biol.* 7:619-630.

Simon, S.M and Blobel, G.A. (1991) Protein-conducting channel in the endoplasmic reticulum, *Cell* 65:371-380. (Medline)

Simons, K., Perara, E. and Lingappa, V.R. (1987) Translocation of globin fusion proteins across the endoplasmic reticulum membrane in *Xenopus laevis* oocytes, *J. Cell Biol.* 104:1165-1172. (Medline)

Simpson, F., Bright, N.A., West, M.A., Newman, L.S., Darnell, R.B. and Robinson, M.S. (1996) A novel adaptor protein complex, *J. Cell Biol.* 133:749-760. (Medline)

Sohn, K., Orci, L., Ravazzola, M., Amherdt, M., Bremser, M., Lootspeich, F., Fiedler, K., Helms, J.B. and Wieland, F.T. (1996) A major transmembrane protein in Golgi-derived COP-I coated vesicles involved in coatomer binding, *J. Cell Biol.*135:1239-1248. (Medline)

Sommer, T. and Jentsch, S. (1993) A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum, *Nature* 365:176-179. (Medline)

Spiess, M. (1995) Heads or tails-what determines the orientation of proteins in the membrane, *FEBS Lett.* 369:76-79. (Medline)

Spruce, A.E., Breckenridge, L.J., Lee, A.K. and Almers, W. (1990) Properties of the fusion pore that forms during exocytosis of a mast cell secretory vesicle, *Neuron* 4:643-654. (Medline)

Stamnes, M.A., Craighead, M.W., Hoe, M.W., Lampen, N., Geromanos, S., Tempst, P. and Rothman, J.E (1995) An integral membrane component of coatomer-coated transport vesicles defines a family of proteins involved in budding, *Proc. Natl. Acad. Sci. USA* 92:8011-

8015. (Medline)

Stevens, T. H., Rothman, J. H., Payne, G. S. and Shekman, R. (1986) Gene dependent secretion of yeast vacuolar carboxypeptidase, *J. Cell Biol.* 102:1551-1537. (Medline)

Stinchcombe, J.C. and Griffiths, G.M. (1999) Regulated secretion from hemopoietic cells, *J. Cell Biol.* 147:1-6. (MedLine)

Stoorvogel, W., Oorschot, V. and Geuze, H.J. (1996) A novel class of clathrin-coated vesicles budding from endosomes, *J. Cell Biol.* 132:21-33. (Medline)

Strous, G. J. A. M., Willemsen, R., van Kerkkof, P., Slot, P. W., Geutze, H. J. and Lodish, H. F. (1983) Vesicular stomatitis virus glycoprotein, albumin and transferrin are transported to the cell surface via the same Golgi vesicles, *J. Cell Biol.* 97:1815-1822. (Medline)

Strous, G.J., Van Kerthof, P., Govers, R., Ciechanover, A. and Schwartz, A.L. (1996) The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone, *EMBO J.* 15:3806-3812. (Medline)

Sudhof, T.C. and Rizo, J. (1996) Synaptotagmins: C2-domain proteins that regulate membrane traffic, *Neuron* 17:379-388. (MedLine)

Swameye, I. and Schaller, H. (1997) Dual topology of the large envelope protein of duck hepatitis B virus: determinants preventing pre-S translocation and glycosylation, *J. Virol.* 71:9434-9441. (Medline)

Swank, R.T., Novak, E.K., McGarry, M.P., Rusiniak, M.E. and Feng, L. (1998) Mouse models of Hermansky Pudlak syndrome: a review, *Pigment Cell Res.* 11:60-80. (MedLine)

Swift, A. M. and Machamer, C. E. (1991) A Golgi retention signal in a membrane-spanning domain of coronavirus E1 protein, *J. Cell Biol.* 115:19-30. (Medline)

Tajima, S., Lauffer, L., Rath, V. L. and Walter, P. (1986) The signal recognition particle receptor is a complex that contains two distinct polypeptide chains, *J. Cell Biol.* 103:1167-1178. (Medline)

Taylor, T.C., Kanstein, P., Weidman, P. and Melan‡on, P. (1994) Cytosolic ARFs are required for vesicle formation but not for cell-free intra-Golgi transport; evidence for coated vesicle-independent transport, *Mol. Biol. Cell* 5:237-252. (Medline)

Teasdale, R.D., Matheson, F. and Gleeson, P.A. (1994) Post-translational modifications distinguish cell surface from Golgi-retained beta 1,4 galactosyltransferase molecules. Golgi localization involves active retention, *Glycobiology* 4:917-928. (Medline)

Thiele, C. and Huttner, W.B. (1998) Protein and lipid sorting from the trans-Golgi network to secretory granules-recent developments, *Semin. Cell Dev. Biol.* 9:511-516. (MedLine)

Toomre, D., Keller, P., White, J., Olivo, J.C. and Simons, K. (1999) Dual-color visualization of trans-Golgi network to plasma membrane traffic along microtubules in living cells, *J. Cell Sci.* 112:21-33. (Medline)

Toomre, D., Steyer, J.A., Keller, P., Almers, W. and Simons, K. (2000) Fusion of constitutive membrane traffic with the cell surface observed by evanescent wave microscopy, *J. Cell Biol.* 149:33-40. (MedLine)

Tooze, S.A., Martens G.J.M. and Huttner, W.B. (2001) Secretory granule biogenesis: rafting to the SNARE, *Trends in Cell Biol.* 11:116-122. (MedLine)

Trowbridge, I.S., Collawn, J.F. and Hopkins, C.R. (1993) Signal-dependent membrane protein trafficking in the endocytotic pathway, *Annu. Rev. Cell Biol.* 9:129-161. (Medline)

Tschopp, J., Esmon, P. C. and Shekman, R. (1984) Defective plasma membrane assembly in yeast secretory mutants, *J. Bacteriol.* 160:966-970. (Medline)

Tsuboi, T., Zhao, C., Terakawa, S. and Rutter, G.A. (2000) Simultaneous evanescent wave imaging of insulin vesicle membrane and cargo during a single exocytotic event, *Curr. Biol.* 10:1307-1310. (MedLine)

Uittenbogaard, A., Ying, Y. and Smart, E.J. (1998) Characterization of a cytosolic heat-shock protein-caveolin chaperone complex. Involvement in cholesterol trafficking, *J. Biol. Chem.* 273:6525-6532. (MedLine)

Urbani, L. and Simoni, R. (1990) Cholesterol and vesicular stomatitis virus G protein take separate routes from the endoplasmic reticulum to the plasma membrane, *J. Biol. Chem.* 265:1919-1923. (Medline)

Van Aelst, L. and D'Souza-Schorey C. (1997) Rho GTPases and signaling networks, *Genes Dev.* 11:2295-2322. (MedLine)

van Eichten, G. and Sandhoff, K. (1993) Ganglioside metabolism. Enzymology, topology and regulation, *J. Biol. Chem.* 268:5341-5344. (Medline)

van IJzendoorn, S.C.D., Zegers, M.M.P., Kok, J.W. and Hoekstra, D. (1997) Segregation of the apical and basolateral transcytotic route in HepG2 cells, *J. Cell Biol.* 137:347-357. (Medline)

van Meer, G. (1993) Transport and sorting of membrane lipids, *Current Opin. Cell Biol.* 5:661-673. (Medline)

Voigt, S., Jungnickel, B., Hartmann, E. and Rapoport, T.A. (1996) Signal sequence-dependent function of the TRAM protein during early phase protein transport across the endoplasmic reticulum membrane, *J. Cell Biol.*134:25-35. (Medline)

Volchuk, A., Amherdt, M., Ravazzola, M., Brügger, B., Rivera, V.M., Clackson, T., Perrelet, A., Söllner, T.H., Rothman, J.E. and Orci, L. (2000) Megavesicles implicated in the rapid transport of intracisternal aggregates across the Golgi stack, *Cell* 102:335-348. (MedLine)

von Heijne, G. (1990) The signal peptide *J. Membr. Biol.* 115:195-201. (Medline)

von Heijne, G. (1996) Principles of membrane protein assembly and structure, *Prog. Biophys. Mol. Biol.* 66:113-139. (Medline)

Voorhees, P., Deignan, E., van Donselaer, E., Humphrey, J., Marks, M.S., Peters, P.J. and Bonaficino, J.S.(1995) An acidic sequence within the cytoplasmic domain of furin functions as a determinant of trans-Golgi network localization and internalization from the cell surface, *EMBO J.* 14:4961-4975. (Medline)

Wahlberg, J.M. and Spiess, M. (1997) Multiple determinants direct the orientation of signal-anchor proteins: the topogenic role of hydrophobic signal domain, *J. Cell Biol.* 137:555-562. (Medline)

Walter, P. and Blobel, G. (1980) Purification of a membrane- associated protein complex required for protein translocation across the endoplasmic reticulum, *Proc. Natl. Acad. Sci. USA* 77:7112-7116. (Medline)

Walter, P. and Blobel, G. (1981) Translocation of proteins across the endoplasmic reticulum. II. Signal recognition protein (SRP) mediates selective binding to microsomal membranes of in-vitro assembled polysomes synthesizing secretory protein, *J. Cell Biol.* 91:551-556. (Medline)

Walter, P. and Blobel, G. (1982a) Signal recognition particle contains a 75 RNA essential for protein translocation across the endoplasmic reticulum, *Nature* 299:691-698. (Medline)

Walter, P. and Blobel, G. (1982b) Translocation of proteins across the endoplasmic reticulum. III. Signal recognition protein (SRP) causes signal sequence dependent and site specific arrest of chain elongation which is released by microsomal membranes, *J. Cell Biol.*

91:557-561. (Medline)

Walter, P., Jackson, R. C., Marcus, M. M., Lingappa, V. R. and Blobel, G. (1979) Tryptic dissection and reconstitution of translocation activity for nascent presecretory proteins across microsomal membranes, *Proc. Natl. Acad. Sci. USA* 76:1795-1799. (Medline)

Walter, P., Gilmore, R. and Blobel, G. (1984) Protein translocation across the endoplasmic reticulum, *Cell* 38:5-8. (Medline)

Wang, Y., Thiele, C. and Huttner, W.B. (2000) Cholesterol is required for the formation of regulated and constitutive secretory vesicles from the trans-Golgi network, *Traffic*1:952-962. (MedLine)

Ward, C.L., Omura, S. and Kopito, R.R. (1995) Degradation of CFTR by the ubiquitin-proteasome pathway, *Cell* 83:121-127. (Medline)

Warnock, D.E., Roberts, C., Lutz, M.S., Blackburn, W.A., Young, W.W. Jr. and Baenziger, J.U. (1993) Determination of plasma membrane lipid mass and composition in cultured Chinese hamster ovary cells using high gradient magnetic affinity chromatography, *J. Biol. Chem.* 268:10145-10153. (MedLine)

Weidman, P., Roth, R. and Heuser, J. (1993) Golgi membrane dynamics imaged by freeze-etch electron microscopy: view of different membrane coatings involved in tubulation versus vesiculation, *Cell* 75:123-133. (Medline)

Weiner, J.H., Bilous, P.T., Shaw, G.M., Lubitz, S.P., Frost, L., Thomas, G.H., Cole, J.A., Turner, R.J. (1998) A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins, *Cell* 93:93-101. (Medline)

Weisz, O.A., Swift, A.M. and Machamer, C.E. (1993) Oligomerization of a membrane protein correlates with its retention in the Golgi complex, *J. Cell Biol.* 122:1185-1196. (Medline)

Wessels, H.P. and Spiess, M. (1988) Insertion of a multispanning membrane protein occurs sequentially and requires only one signal sequence, *Cell* 55:61-70 (Medline)

Whitley, P., Nilsson, I. and von Heijne, G. (1996) A nascent secretory protein may traverse the ribosome/endoplasmic reticulum translocase complex as an extended chain, *J. Biol. Chem.* 271:6241-6244. (Medline)

Wiedmann, M., Kurzchalia, T.V., Hartmann, E. and Rapoport, T.A. (1987) A signal sequence receptor in the endoplasmic reticulum membrane, *Nature* 328:830-833. (Medline)

Wieland, F.T., Gleason, M.L., Serafini, T.A. and Rothman, J.E. (1987) The rate of bulk flow from the endoplasmic reticulum to the cell surface, *Cell* 50:289-300. (Medline)

Wiertz, E.J.H.J., Jones, T.R., Sun, L., Bogyo, M., Geuze, H.J. and Ploegh, H.L. (1996a) The human cytomegalovirus US11 gene product dislocates MHC Class I heavy chains from the endoplasmic reticulum to the cytosol, *Cell* 84:769-779. (Medline)

Wiertz, E.J.H.J., Tortella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T.R., Rapoport, T.A. and Ploegh, H.L. (1996b) Sec61-mediated transfer of membrane protein from the endoplasmic reticulum to the proteasome for destruction, *Nature* 384:432-438. (Medline)

Wilcox, C.A., Redding, K., Wright, R. and Fuller, R.S. (1992) Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole, *Mol. Biol. Cell* 3:1353-1371. (Medline)

Wilhelm, J.E. and Vale, R.D. (1993) RNA on the move: the mRNA localization pathway, *J. Cell Biol.*123:269-274. (MedLine)

Williams, D.B., Vassilakos, A. and Suh, W.-K. (1996) Presentation by MHC class I molecules, *Trends in Cell Biol.* 6:267-273.

Wolins, N., Bosshart, H., Kuster, H. and Bonifacino, J.S. (1997) Aggregation as a determinant of protein fate in post-Golgi compartments: role of the luminal domain of furin in lysosomal targeting, *J. Cell. Biol.* 139:1735-1745. (MedLine)

Wong, S.H., Low, S.H. and Hong, W. (1992) The 17-residue transmembrane domain of β-galactoside α2,6-sialyltransferase is sufficient for Golgi retention, *J. Cell Biol.* 117:245-258. (Medline)

Wooding, S. and Pelham, H.R. (1998) The dynamics of Golgi protein traffic visualized in living yeast cells, *Mol. Biol. Cell* 9:2667-2680. (Medline)

Woodman, P. G. and Edwardson, J. M. (1986) A cell free assay for the insertion of viral glycoprotein into the plasma membrane, *J. Cell Biol.* 103:1829-1835. (Medline)

Wu, W.J., Erickson, J.W., Lin, R. and Cerione, R.A. (2000) The γ-subunit of the coatomer complex binds Cdc42 to mediate transformation, *Nature* 405:800-804. (MedLine)

Xoconostle-Cazàres, B., Xiang, Y., Ruiz-Medrano, R., Wang, H.L., Monzer, J., Yoo, B.C., McFarland, K.C., Franceschi, V.R. and Lucas, W.J. (1999) Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem, *Science* 283:94-98. (MedLine)

Yaffe, M.P. and Kennedy, E.P. (1983) Intracellular phospholipid movement and the role of phospholipid transfer proteins in animal cells, *Biochemistry* 22:1497-1507. (Medline)

Young, W.W. Jr., Lutz, M.S. and Blackburn, W.A. (1992) Endogenous glycosphingolipids move at the cell surface at a rate consistent with bulk flow estimates, *J. Biol. Chem.* 267:12011-12015. (Medline)

Yisraeli, J.K., Sokol, S. and Melton, D.A. (1990) A two step model for the localization of maternal mRNA in Xenopus oocytes: involvement of microtubules and microfilaments in the translocation and anchoring of Vg1 mRNA, *Develop.* 108:289-298. (Medline)

Zhang, H.L., Eom, T., Oleynikov, Y., Shenoy, S.M., Liebelt, D.A., Dictenberg, J.B., Singer, R.H. and Bassell, G.J. (2001) Neurotrophin-induced transport of a β-actin mRNP complex increases β-actin levels and stimulates growth cone motility, *Neuron* 31:261-275. (MedLine)

Zheng, N. and Gierasch, L. (1996) Signal sequences: the same yet different, *Cell* 86:849-852. (Medline)

Zhou, A., Webb, G., Zhu, X. and Steiner, D.F. (1999) Proteolytic processing in the secretory pathway, *J. Biol. Chem.* 274:20745-20748. (MedLine)

Zhou, F.X., Merianos, H.J., Brunger, A.T. and Engelman, D.M. (2001) Polar residues drive association of polyleucine transmembrane helices, *Proc. Natl. Acad. Sci. USA.* 98:2250-2255. (MedLine)

11. Biosynthesis and Cytoplasmic Trafficking:

Membranes, Vesicles and Intracellular Transport

Suggested Readings
References
Web Resources

Back to List of Chapters

Understanding the intricate details of the interactions between membranes and membrane bound compartments is a challenging task. Electronmicroscopy using immunological methods together with genetic approaches and molecular studies of reconstituted systems are beginning to provide the needed framework of information. This chapter focuses on some of these processes.

I. TRANSPORT VESICLES

As we saw in Chapter 10, most intracellular transport is thought to be mediated by small vesicles, although an alternative mechanism for the transfer from the VTCs to the *cis*-Golgi (see Chapter 10, Section III) is likely and, in addition, transport between Golgi stacks may involve alternative mechanisms (see Chapter 10, Section IIIC). The transport can occur in either direction: from the ER to the periphery of the cell or in the opposite direction. The transport processes are discussed in relation to endocytosis in Chapter 9, in relation to the motility of cell components in Chapter 23 and the discussion of neuronal transport can be found in that Chapter and in Chapter 24.

Chapter 9 discussed the involvement of clathrin in the formation of endocytotic vesicles. Some other transport vesicles are also clathrin-coated. They carry proteins originating from the *trans*-Golgi system (see Brodsky, 1988). They include transport vesicles destined to the lysosomes (e.g., Schulze-Lohoff et al., 1985) containing acid hydrolases and mannose-6-phosphate receptors, and those destined to storage vesicles containing densely packed secretory products (see Section II F, below). Vesicles coated with proteins other than clathrin (non-clathrin coated) represent another set of transport vesicles. These are involved in the translocations within the Golgi stack, between the ER and the Golgi, from the TGN in the constitutive secretory pathway and in retrograde transport (e.g., from Golgi to ER)

The present conventional view maintains that non-clathrin coated vesicles are involved in the transport between ER and Golgi and between Golgi stacks. The transport from the ER to the *cis*-Golgi is carried out by COPII vesicles (see Barlowe, 1998). In contrast, retrograde transport from Golgi to ER takes place in coatomer protein (COPI) coated vesicles (Cosson and Letourneur, 1997; Gaynor et al., 1998) for proteins either with KKXX, KKXX-like (see below) or the KDEL amino acid motif (see Chapter 10) but not by glycosylated Golgi enzymes or Shiga toxin (Girod et al., 1999). Forward and retrograde transport between Golgi stacks is also thought to be mediated by COPI (Orci et al., 1997). In this latter case, electron microscope immunocytochemistry shows that both anterograde-cargo and retrograde-cargo are present in separate COPI-coated vesicles budding from all stacks of the Golgi. Packaging of anterograde and retrograde cargo into separate vesicles can also be demonstrated in vitro even when budding is driven by highly purified coatomer and a recombinant, small GTPase, ARF.

All in all, at this time four different coat proteins are clearly defined, including two clathrin proteins and COPI and COPII (listed in Table 1, Rothman and Wieland, 1996). In Table 1, the two different clathrin coats are shown to function in conjunction with different adaptor molecules (see below). All these coats are associated with GTP-binding proteins (or GTPases) (see below). Their origin and initial destination are listed in the fourth column. Besides the four coat proteins listed in the Table, more coat proteins have been demonstrated and others are likely to be revealed by further studies. A lace-like coat has been described in the TGN (Ladinsky et al., 1994; Narula et al., 1995), a neuronal variant of COPI has been found (Newman et al., 1995) and another is thought to be associated with endosomal vesicle traffic (Whitney et al., 1995). A distinct coating for vesicles involved in endosome-to-Golgi retrograde transport of sorting receptors is operative in yeast (Seaman et al., 1998). The vesicles are slightly larger than the COPI vesicles. In *Saccharomyces cerevisiae*, a novel vesicle 30-40 nm in diameter transferring fructose-1,6-bisphosphatase to the vacuole for degradation, has been described (Huang and Chiang, 1997) . There is growing evidence for other coat proteins similar to clathrin. A gene (HC22) has been identified with the potential of coding for a protein similar to the clathrin heavy chain. mRNA corresponding to HC22 is expressed predominantly in skeletal muscle and alternative transcripts of H22 are expressed in a tissue specific fashion (see Brodsky, 1997).

Table 1. Coat proteins or vesicle shuttles (from Rothman and Wieland 1996, reproduced by permission)

Type of coated vesicle	Subunits of coat	GTPase	Origin-destination
AP-1 clathrin	Clathrin, AP1 adaptor	ARF	TGN-prelysosomes
AP-2 clathrin	Clathrin, AP2 adaptor	ARF?	Plasma membrane-endosomes
COPI	Coatomer (COPI proteins)	ARF	ER-Golgi; bidirectional within Golgi; Golgi-ER
COPII	COPII proteins	SAR	ER-Golgi

It is now recognized that the process of targeting and interaction between components occurs in many steps starting from the translocation of vesicles to their target and ending with their fusion to their target. The translocation of the vesicles (see Section IID and Chapter 24, section on cytoplasmic dynein and kinesin) has not been elucidated in detail. Upon arrival processes involved in membrane recognition (*tethering*) leaves the two sets of membranes at some distance from each

other. The binding of SNAREs (*docking*) leaves the membranes in close proximity and is followed by fusion. One kind of SNARE, $SNARE_v$,resides on the surface of the vesicle being transported and $SNARE_t$ resides on its membrane target (see Section C). Tethering is thought to be the primary step in determining specificity. Rab GTP-binding proteins have been implicated as well (see Pfeffer, 1999; Novick and Zerial, 1997; Zerial and McBride, 2001). However, a variety of proteins and protein complexes have been found to play essential roles in targeting and to be specific for individual steps. Before fusion, priming events must take place (Klenchin and Martin, 2000) and a fusion trigger, most frequently Ca^{2+}, is needed (e.g., Heidelberger et al., 1994; Peters and Mayer, 1998; Beckers and Bach, 1989; Colombo et al., 1997). The priming includes ATP-dependent steps, such as the NSF-mediated priming of SNARE protein complexes, the ATP-dependent synthesis of phosphoinositides, and protein kinase-mediated protein phosphorylation. The protein munc 13 is also involved in the priming.

The intracellular transport of proteins in vesicles raises a number of issues: (a) how the appropriate proteins are selected in the formation of specific vesicles, (b) the nature of the mechanism of budding, (c) how the vesicles are targeted unidirectionally and in an orderly fashion, and (d) the mechanism of fusion of the targeted vesicles. Unfortunately, only partial answers are known.

The first issue, (a), was discussed in Chapter 10. The formation of vesicles (Section A), their targeting (Section B) and fusion to their target membranes (Section C) and the special role of the GTPases (Section D) will be discussed first, followed by an examination of some of the details of the various pathway segments (Section E).

A. Assembly of Coats, Budding and Formation of Vesicles

This and the following sections will be referring to several components: NSF, SNAP and SNARE. As in many other cases in this book, these acronyms stand for certain appellations that are only of historical importance. NSF stands for *N-ethyl maleimide sensitive factor,* SNAP for *soluble NSF-attachment protein* and SNARE for *SNAP receptors.* The acronysms will be used in the text that follows.

The process of vesicle formation and their delivery to a target are summarized in the diagram of Fig. 1 which will serve as the basis of this discussion. This representation reflects the bare bones, so-called SNARE hypothesis. As discussed below some of the present evidence indicates that SNAREs are responsible for docking and act downstream from tethering. The assembly described in Fig. 1A typified for COPI vesicles, occurs stepwise with (i) the binding of the small GTP-GTPase first (see Section E) (in this case ARF; open spheres: GDP-GTPase; closed spheres: GTP-GTPase) where GDP has been replaced by GTP (i-ii), followed by activation by GTP and formation of coated bud by recruitment from the cytoplasmic pool. The pinching off of the vesicle requires acyl-CoA binding (not shown, Ostermann, et al., 1993) and the GTPase, dynamin (not shown, e.g., Takei et al., 1995; Hinshaw and Schmid, 1995). Dynamin is thought to assemble around the neck of endocytotic invagination and participates in pinching off the vesicles (see below and Chapter 9). Subsequently, the hydrolysis of GTP induces the detachment of the GTP-binding protein, now bound to GDP (Melançon et al., 1987; Orci et al., 1989) (iii-iv). Finally, dissociation of the coat follows (v) . The

steps diagrammed in Fig. 1B and C are discussed in more detail below. Basically, the uncoated vesicles bind to the target protein on the target membrane (part B of the diagram). The binding is mediated by the v-SNARE and its cognate, t-SNARE, in the target membrane (see Section C). The fusion (part C of the diagram) requires ATP, GTP, AcylCoA, the NEM-sensitive-factor (NSF), SNAPs and other factors. NSF catalyzes the hydrolysis of ATP which disrupts the SNARE complex (see Section C), and initiates fusion.

This model is supported by experimental observations: (a) the presence of coated vesicles coincides with biochemically defined transport (Orci et al., 1986); (b) coated vesicles containing cargo proteins, in this case G-VSV-protein, transfer from donor to acceptor stacks of the Golgi and can be trapped at this stage by the presence of GTPγS (Orci et al., 1989); (c) after a GTPγS block is reversed the vesicles disappear; (d) budding requires fatty acylCoA; (e) addition of NEM (or AlF_4^-, which acts as a phosphate analog) causes a buildup of uncoated 75 nm vesicles at the Golgi complex in intact cells; and (f) the accumulation of uncoated vesicles produced by NEM is reversed by the addition of a soluble cytoplasmic factor (NSF) (Malhotra et al., 1988).

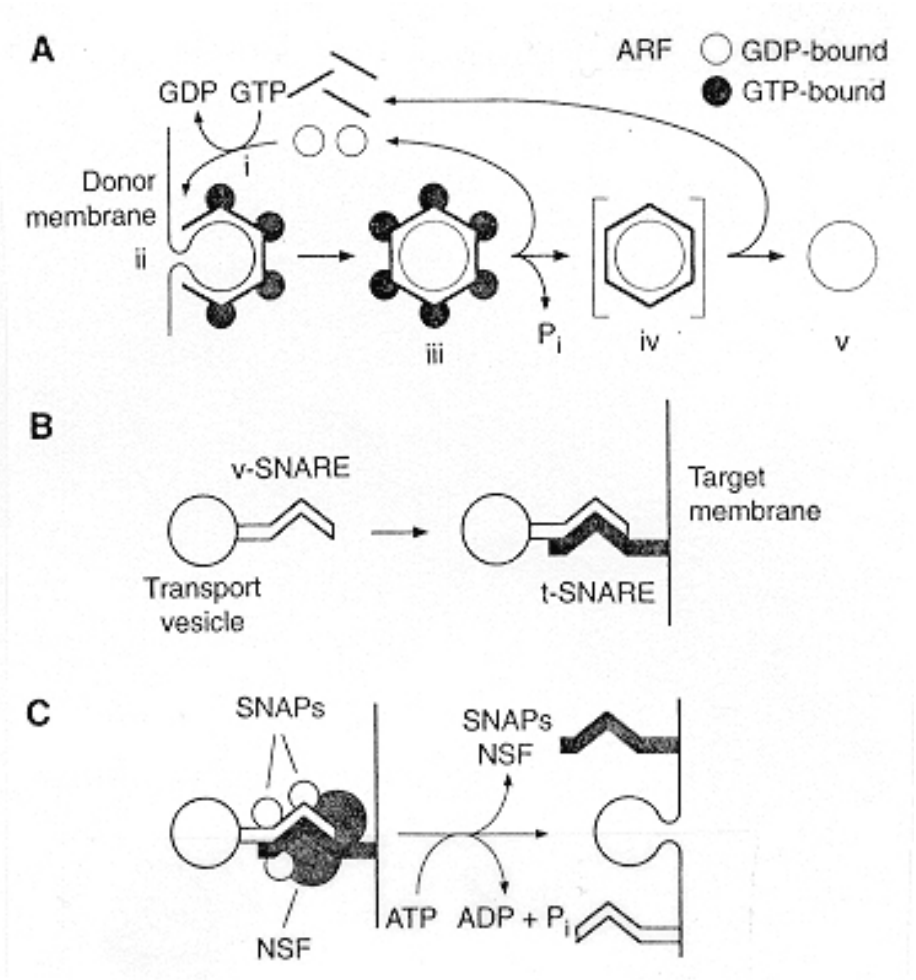


Fig. 1 Model of the vesicle shuttle (see text) (A) Vesicle budding. (B) Targeting of vesicles. (C) Fusion. Reproduced with permission from Rothman, J.E. and Wieland, F.T. (1996) Protein sorting by transport vesicles, *Science* 272:227-234, copyright &copy1996, American Association for the Advancement of Science.

As implied by Fig. 1A, several features in the assembly of coated vesicles are common to all three systems that have been studied. They all first require the recruitment of small GTP-GTPases. Later binding of coat proteins induces a deformation of the membrane to form a bud. Transmembrane proteins of the donor membranes recruit the proteins that will form the coat (see Fig. 12A of Chapter 10). In addition, they can function in the selection of cargo. However, there are significant differences. The assembly of COPI-coated vesicles recruits a pre-assembled coat present as a soluble complex (e.g., Zhao et al., 1997, 1999), whereas COPII proceeds stepwise. The recruitment of clathrin is also nucleotide dependent and requires ARF1. However, unlike COPI vesicles, in clathrin vesicle assembly, ARF1 only recruits AP-1 (an adaptor protein, see below) (e.g. Zhu et al, 1998) followed by formation of the coat. In addition, clathrin disassembly in neurons requires the heat shock protein Hsc70 together with the cofactor auxilin and proceeds with the hydrolysis of ATP. In other cells cyclin G-associated protein kinase (GAK) acts similarly to auxilin. The disassembly of the clathrin coat (e.g., Ungewickell et al., 1995; Umeda et al., 2000) differs from the dissociation of the other two coats.

The ability of clathrin to self assemble into polyhedral cages (Woodward and Roth, 1978) has suggested that budding occurs by a stepwise assembly of coat structure from subunits in the cytoplasm. In this model, each addition progressively deforms the membrane to eventually form the bud and then a detached vesicle (see Le Borgne and Hoflack, 1998).

However, the process is best understood for the cases of budding from the ER (Barlowe et al., 1994) and the Golgi cisternae that do not involve clathrin. As already mentioned, the budding process is regulated by GTP-binding proteins (see Table 1, Fig. 1A), which initiate the process (Donaldson et al., 1992; Helms and Rothman, 1992). Then the coat proteins can begin to be assembled. In contrast, the hydrolysis of the bound GTP to form bound GDP, initiates the release of the coat (Tanigawa et al., 1993). An enzyme in the donor compartment catalyzes the exchange of GTP for GDP (Barlowe and Shekman, 1993).

The components of the COPII coat needed for vesicle assembly can be shown by genetic approaches in yeast. In addition, ER membranes can be used in an in vitro assay after extraction of peripheral proteins. The stripped membranes produce vesicles by budding after the addition of cytosol extracts and GTP. The active ingredients of the cytosol extracts correspond to a 700 kDa complex of Sec31p/Sec13p, a 400 kDa complex of Sec23p/Sec24p and the small GTPase, Sarp1 (Barlowe and Sheckman, 1993). Sar1p-GDP is normally in the cytoplasm. It is recruited to the ER membrane by Sec12p, an integral membrane protein of the ER. Sec12p also functions as a *guanine exchange factor* (GEF) (Barlowe and Sheckman, 1993), thereby facilitating the exchage of GTP for GDP. In contrast, the disassembly, required for fusion, involves GTP hydrolysis activated by Sec23p (see Kaiser and Ferro-Novick, 1998).

Another protein of 240 kDa, Sec16p, is tightly bound to the ER and acts as a scaffold for the assembly. Sec23p, Sec24p, Sec31p and Sed4p (a homolog of Sec12p) are bound to different sites in the Sec16p molecule (e.g., Shaywitz et al., 1997).

The minimum system to function in the formation of COPII vesicles and buds has also been

determined by reconstituting purified proteins in entirely synthetic liposomes (Matsuoka et al., 1998). By adding 5'guanylyl imidodiphosphate (GMP-PNP), a non-hydrolyzable analog of GTP, Sec12p (which functions as a nucleotide exchange protein) is not required. After Sar1p-GMP-PNP is bound to the membrane, the Sec23p/Sec24p and Sec31p/Sec13p complexes are sequentially attached in that order. The process differs somewhat from the native process suggesting that other factors (e.g., the presence of cargo) may facilitate the process. Apparently a minimum of 10% acidic phospholipids is required (Matsuoka et al., 1998).

Sorting of integral membrane proteins is thought to require binding of the cytoplasmic domain of the protein to the Sec23p-Sec24p complex (Aridor, et al., 1998). Sorting of soluble proteins requires transmembrane receptors. These receptors would require at least one transmembrane domain, a lumenal domain that can bind to the cargo and a cytoplasmic exposed domain that would bind to coat subunits. One of these is the KDEL receptor that binds to a carboxy-terminal KDEL peptide and retrieves the proteins that have escaped from the ER (Lewis and Pelham, 1992). Two proteins of the p24 family are receptors that have been found in COPII-coated vesicles (Schimoller et al., 1995) and are needed for the secretion of certain proteins. Their cytoplasmic domain contains a diphenyl and a dibasic motif at the carboxy terminals.

In order to fuse to their target the vesicles must contain v-SNAREs (see Section B). vSNAREs have been demonstrated in COPI and COPII coats. Two ER to Golgi v-SNAREs, Bet1p and Bos1p, interact specifically with Sar1p, Sec23p, and Sec24p in a guanine nucleotide-dependent fashion (Springer and Sheckman, 1998).

The assembly of COPI-coated vesicles proceeds as follows. First the GTPase ARF1 (ADP-ribosylation factor 1) binds to the membrane. This requires binding to its GEF. ARF1 is myristoylated. The myristoyl moiety is exposed when the GTPase binds GTP, allowing ARF1 to bind to lipids. The hydrolysis of GTP leads to the retraction of the meristoyl-moiety into a pocket of the ARF1 molecule, so that the GTPase is no longer able to attach to lipids. The assembly of the coat takes place by recruitment of a preassembled coat via its β and γ-COP subunits, resulting into a deformation of the membrane to form the bud (Zhao et al., 1997; 1999).

Adaptors and receptors

The assembly of coated vesicles probably involves many more interactions than those considered so far. The adaptor molecules AP-1 (present in the clathrin coated vesicles that originated from the TGN) and AP-2 (from the clathrin coated vesicles originating during endocytosis) appear to have a key role in the processes involved in vesicle formation. Their structure and function have been recently reviewed (Traub, 1997; Robinson, 1997). In addition to AP-1 and AP-2, a third adaptor protein has been identified, AP-3 (Dell'Angelica et al., 1997). AP-3 is likely to function in transport to the lysosomes (in yeast the vacuole). Deletion of any of the subunits, leads to mistargeting of some of the vacuolar proteins but not others (Cowles et al., 1997; Stepp et al., 1997; Vowels and Payne, 1998). Similarly, in mammalian cells, antisense oligonucleotides for the AP-3 gene (see Chapter 1) also send lysosomal glycoproteins to the cell surface without affecting AP-1 mediated transport, such as that of the mannose 6-phosphate receptors (Le Borgne et al., 1998). An additional

function is suggested by the observation that in vitro synaptic vesicle can be formed from endosomes in the presence of AP-3 (Faundez et al., 1998).

AP-1, AP-2 and AP3 are heterotetramers [two large δ subunits of 160 kDa, and two β3A (120 kDa) subunits associated with μ3 (47 kDa) and σ3 (22 kDa)].The β-subunits of these adaptors promote clathrin-cage formation (e.g., Gallusser and Kirchhausen, 1993). AP-1 and -2 also bind in vitro to the cytoplasmic domains of membrane receptors (e.g., Pearse, 1988, Glickman et al., 1989) and clathrin (e.g., Ahle and Ungewickell, 1989; Shih et al., 1995; Traub et al., 1995, Schröder and Ungewickell, 1995). In addition, they bind the tyrosine or di-leucine sorting motifs (Heilker et al., 1996) important for endocytosis and lysosomal targeting (see Sandoval and Bakke, 1994). The μ-1 and μ-2 subunits of AP-1 and AP-2, bind to the tyrosine endocytotic motif (Ohno et al., 1995). The adaptors are therefore responsible for the selectivity of the vesicle as well as their assembly.

The receptor molecules, such as the mannose 6-phosphate receptors (MPRs) sorted out in the TGN, were found to be needed for the recruitment of AP-1 to the clathrin vesicles in an in vitro system (Le Borgne et al., 1996). The amount of AP-1 recruited by clathrin coated vesicles of the TGN was found to depend on the presence of the MPRs and the integrity of their cytoplasmic domains (Le Borgne and Hoflack, 1997).

Many homologues of adaptor subunits AP-1, AP-2 and AP-3 have been found. In one case, clathrin coated vesicles associated with endosomes have been shown to have neither AP-1 nor AP-2, suggesting the presence of another adaptor complex (see Stoorvogel et al., 1996). Some of the adaptors function independently of clathrin (e.g., Stepp et al., 1995; Simpson et al., 1996). These adaptors could function in conjunction with clathrin-like molecules or other unknown coat proteins, possibly interacting with COPI.

In summary, adaptors, receptors and clathrin appear to act in concert to produce coated vesicles.

The subunits of COPI, coatomer, (β, δ and ζ-COP) have some sequence homology with the β, μ and σ-adaptor subunits of AP-1 and AP-2. Interestingly, the B subcomplex of COPI binds the di-lysine motif (e.g., Fiedler et al., 1996). Therefore, in contrast to clathrin, coatomer subunits have a role similar to the adaptor molecules. Coatomer coat subunits have been shown to interact with KKXX motif (Letourneur et al., 1994) needed for retrieval to the ER. Therefore they do function as receptors for retrograde transport.

The interaction of coatomer with a domain of the peptide p23 (a p24 protein thought to act as a receptor for cargo) has been studied in vitro (Reinhard et al., 1999). The binding of the two kinds of protein results in a conformational change and polymerization of the complex in vitro with a stoichiometry of 1:4, COPI:peptide. This conformation is also seen on the surface of isolated COPI vesicles. These results suggest a mechanism by which the induced conformational change of coatomer accompanying its polymerization is responsible for the formation of the bud on the Golgi membrane during biogenesis of a COPI vesicle. In vitro experiments using liposomes reveal the formation of coatomer vesicles requires ARF, GTP and the cytoplasmic tails of the p24 proteins receptors, or cargo proteins with the KKXX retrieval signal (Bremser et al., 1999).

Knowledge of the possible role of adaptor-like molecules in COPII is still lacking. However, COPII-coated vesicles are selective, in some cases, as shown by the fact that they concentrate proteins exported from the ER (Balch et al., 1994; Bednarek et al., 1995). We saw that only the COPII proteins are required for vesicle formation (see above) (Matsuoka et al, 1998). However, phosphatidylinositol 4-phosphate or phosphatidylinositol 4,5-bisphosphate are needed to bind them to liposomes and the GTP-bound form of Sar1p is needed to recruit the proteins to either liposomes or the ER membranes.

Heteromeric complexes of proteins of the p24 family are found in both COPI and COPII vesicles as well as ER and Golgi membranes (e.g., Füllekrug et al., 1999; Marzioch et al., 1999) suggesting that p24 cycles between ER and Golgi as would be expected for receptor proteins. In mammals the p24 proteins are thought to be involved in exit from the ER (Lavoie et al., 1999). In yeast there are eight genes encoding these family members. Mutations of several of these genes exhibit selective protein transport defects or secretion of the ER lumenal protein Kar2p (e.g., Marzioch et al., 1999). For example, deletion of one p24 gene slows down the transport of Gas1p from ER to Golgi (e.g., Marzioch et al., 1999). Gas1p is a GPI-anchored protein.

Mutations of the genes coding for p24 proteins do not inhibit anterograde passage from the ER completely and the export of some of the proteins from the ER do not require p24-proteins at all. This suggests that only some proteins require this receptor or any receptor at the ER exit step. In agreement with this notion, many secreted proteins have been shown not to be concentrated in COPII-coated vesicles (Martínez-Menárguez et al., 1999). The incomplete inhibition by some of the mutants that seem to be involved in the p24 system may be explained by the occurrence of bulk flow which would be of significance in the absence of receptors. However, in some cases, the role of p24-proteins is clearly that of receptors. The Emp24 complex is needed for for packing Gas1p into vesicles of the ER (Muñiz et al., 2000). In agreement with the notion that Emp23 acts a receptor, Gap1p was shown to become chemically cross-linked to two of the Emp24 proteins in experiments using cross-linking reagents.

Cargo proteins have been found to have a role in the assembly of coat proteins (see Springer et al., 1999; Bremser et al., 1999). In addition, in view of the central role of the GTPases in coat assembly, it would seem likely that the GTPases have a role in regulating cargo recognition. A special role of GTPases is suggested by experiments using a cell-free system. During COPI vesicle formation, competing sorting signals were shown to act through a GTPase switch thereby providing specificity for the process (Goldberg, 2000). The signal sequence of hp24a (a p24 protein, supposedly a receptor) inhibits coatomer-dependent GTP hydrolysis. In contrast, the di-lysine retrieval signal (see above) that binds the same coatomer site has no effect on the GTPase. The results suggest that the activity of the GTPase can select or discard a cargo protein.

Involvement of phosphatides

Several phospholipid modifying enzymes are involved in the formation of vesicles from buds (e.g., endophilin, synaptojanin and phospholipase D). Endophilin is an acyltransferase that binds to the small GTPase, dynamin (see below), and transfers fatty-acyl from arachidonic and palmitic acid to

lysophosphatidic acid to produce phosphatidic acid (Schmidt et al., 1999). This reaction may induce negative membrane curvature by converting an inverted-cone-shaped lipid into a cone-shaped lipid in the cytoplasmic layer of the bilayer. Phospholipase D produces phosphatidic acid from phospholipids, possibly affecting membrane curvature (Zimmerberg, 2000). Synaptojanin is a polyphosphoinositide phosphatase. See also Chapter 4 for a discussion of membrane curvature.

Although their role is not well understood, the phosphoinositides (PIs) have been shown to be part of the machinery responsible for the formation of vesicles, for example, in the the TGN, the plasma membrane and the endosomes (see Martin, 1997; De Camilli, 1996; Ohashi et al., 1995). The phosphoinositides are thought to be part of the discrete membrane sites (see Chapter 4) required for the recruitment of cytoplasmic proteins needed for vesicle formation and budding.

An involvement of the lipid system is shown by the dependence of secretion on *phosphatitylinositol transfer proteins* (PITPs). PITPs are enzymes reponsible for the transfer of phospholipids between membrane structures or serum lipoproteins (see Wirtz et al., 1991). These enzymes have a distinct preference for phosphatidylinositol over phosphatidylcholine. The β isoform, present in the Golgi system, has a high transfer activity in relation to sphingomyelin (see deVries et al., 1995). In yeast, mutations in the *SEC14* gene which codes for a PITP, block post-Golgi secretory traffic (e.g., Bankaitis et al., 1990). In agreement with these results, experiments carried out on a cell free system from a neuroendocrine cell line (Ohashi et al., 1995) found that the α and β isoforms of PITPs stimulate formation of vesicles from the TGN.

Phosphatidylinositol kinases (PIKs) which phosphorylate PIs have also been implicated. In yeast, the Vps15 protein kinase and the Vps34 PI-3-kinase have been shown to function as a membrane-associated complex which facilitates the delivery of proteins to the yeast vacuole (e.g., Stack and Emr, 1994) which has a similar function than lysosomes in a mammal. Subsequent work has indicated that polyphosphoinosides phosphorylated at the 4' and 5' or alternatively the 3' positions of the inositol ring determine the location of events involved in membrane traffic. It is now generally recognized that phosphorylation-dephosphorylation of the polar heads of phosphoinositides in specific locations coincides with the recruitment or the activation of proteins essential for vesicular transport (see De Camilli et al., 1996).

The compartmentation of PPIs is probably the result of synthesis at specific locations. The targeting of PI 4-kinase would permit a segregated synthesis. PI 4-kinase catalyzes the phosphorylation of phosphatidylinositol to form PI 4-phosphate. PI 4-kinases have been found in plasma membranes and intracellular organelles including Golgi, lysosomes, ER, nuclear envelope, coated vesicles, exocytotic vesicles and secretory granules (see De Camilli et al., 1996; Carpenter and Cantley, 1996). The immunoreactivity for this PI 4-kinase molecule was found mostly in close association with the membranes of the Golgi vesicles and vacuoles (Nakagawa et al., 1996).

The association between PI-kinases and membranes occurs despite the absence of transmembrane sectors. However, these proteins could bind directly to lipids or indirectly by anchoring to integral proteins. Some of the mammalian kinases have a *pleckstrin-homology* (PH) domain (Nakagawa et al., 1996) that would allow them to interact with lipids. Alternatively, the kinase could be anchored

by binding to membrane receptors, as is the case of PI 4-kinase that links to transmembrane proteins and integrins (Berditchevski et al., 1997). A mammalian adaptor protein p150 (homolog of the yeast VPS15) that recruits the PI 3-kinase to the Golgi membrane, has been identified (Panaretou et al., 1997). Recombinant p150, PI 3-kinase and PITP form a complex also present in the cytoplasm of human cells.

Not surprisingly, a polyphosphoinositide phosphatase (*synatpojanin 1*) has been found to be involved in presynaptic endocytotic vesicle recycling in neurons. Synaptojanin 1 was found at high levels at nerve terminals (McPherson et al., 1996), which are involved in the exocytosis of synaptic vesicles. Synaptojanin 1-deficient mice exhibit neurological defects and die shortly after birth, phosphatidylinositol 4,5-biphosphate (PIP_2) levels are increased and clathrin-coated vesicles accumulate at the nerve endings (Cremona et al., 1999)

Undoubtedly, the binding of phosphoinositides to coat components has a role in trafficking. They bind AP-2 (e.g., Gaidarov et al., 1996), AP-3 (AP180) (e.g., Hao et al., 1997) and COPI coatomer (e.g., Chaudhary et al., 1998), dynamin, synaptojanin (Cremona et al., 1999) and in addition arrestins (Gaidarov et al., 1999). Dynamin is involved in vesicle formation (see Chapter 9). *Arrestins* are involved in the inhibition of G_s proteins (e.g., see Chapter 7) and also act as clathrin adaptors (see Goodman et al., 1996). Mutants of arrestin3 expressed in cells in culture fail to participate in β2-adrenergic receptor internalization and fail to be recruited to the coated pits although they are recruited to the plasma membrane (Gudrov et al., 1999). Similarly, mutation of the α subunit of the AP-2 adaptor protein at high expression levels results in failure to localize at clathrin coated pits (Gaidarov and Keen, 1999).

In addition to their separate roles in vesicle formation and secretion, the phosphoinositide system and the GTP-binding proteins are functionally and intimately linked. ARF activates phospholipase D (PLD) (Brown et al., 1993). PLD catalyzes the conversion of phosphatidyl choline to phosphatidic acid. The latter activates PI-4 kinase to produce PIP_2 (see Martin, 1997; DeCamilli et al., 1996) which has been shown to be involved in vesicle formation (Tüscher et al., 1997). In addition, ARF has a direct effect in increasing the level of PIP_2 (Godi et al., 1998) and PI-4 kinase-β and, by recruitment, another unidentified kinase in the Golgi (Godi et al., 1999). This increases the synthesis of PI-4-phosphate and PI_2 levels independently of PLD activation. The PI-4 kinase is required to maintain the integrity of the Golgi. Mutants that lack the kinase exhibit a disorganized Golgi (Godi et al., 1999).

Other effects of ARF on the Golgi and vesicle pathway are less clearly understood. The actin binding proteins ankyrin and spectrin, are at the cytoplasmic surface of the Golgi (see Beck et al., 1998; De Matteis et al., 1998). ARF recruits a specific spectrin to Golgi membranes (Godi et al., 1998) in a mechanism involving the PIP_2 binding domain of spectrin [the *pleckstrin-homology (PH) domain*]. Spectrin is needed for maintaining the structural integrity of the Golgi. Agents that block the binding of spectrin inhibit the transport of vesicular stomatitis virus G protein from the ER to the medial compartment of the Golgi complex.

B. Targeting of Proteins and Vesicles; SNAREs

As we saw in Chapter 10, the sorting signals of cargo proteins are responsible for the targeting of proteins. Similarly, retention signals are likely to be required for retention by the appropriate compartment. The sorting signal is recognized directly or indirectly by the proteins that form the vesicle coat to insure packaging of the cargo protein in the corresponding vesicle. Once assembled, specific tethering and docking of the transport vesicle are needed to deliver the vesicle to the acceptor compartment. At the target site, the docking depends on the complementary SNAREs.

SNAREs are integral proteins of intracellular membranes with a large domain on the cytoplasmic phase. SNAREs present in a transport vesicle (v-SNARE; e.g. *synaptobrevin* in neurons) (Söllner et al., 1993a) are recognized by another similar protein on the surface of the target compartment (t-SNARE; e.g. *syntaxin* in neurons) (Rothman, 1994; Søgaard et al., 1994) (see Fig. 1B). The two usually correspond to R-and Q-SNAREs respectively (i.e., named after the glutamine and arginine residues of the their cytoplasmic domains) (see Jahn and Südhof, 1999).The specific interaction is apparently mediated by the extended cytoplasmic domain of the two different kinds of SNAREs (Chapman et al., 1994). The role of SNAREs has been delineated most completely for the shuttles between the ER and the Golgi (see Rothman; 1994, Paek et al., 1997) and from the Golgi to the plasma membrane (Brennwald et al, 1994). A SNARE protein that functions within the Golgi system has been isolated from mammalian cells in culture (Hay et al., 1997; Nagahama et al., 1997). This protein of 28 kDa is associated with Golgi membranes. The transport from the ER to the *trans*-Golgi and TGN is blocked by the cytoplasmic domain of this protein or an antibody to it. These agents are thought to block transport between the *medial*-Golgi and the *trans*-Golgi and TGN (Hay et al., 1997). A yeast v-SNARE protein has been implicated in retrograde transport to the *cis*-Golgi (Lupashin et al., 1997) and probably the anterograde transport from late Golgi and a prevacuolar compartment (von Mollard, et al., 1997).

The two kinds of SNARES, v-SNARES and t-SNARES, must be capable of binding each other specifically. Complementary pairs have been identified in yeast for the ER-Golgi step (Lian and Ferro-Novick, 1993; Søgaard et al., 1994) and the Golgi-plasma membrane step (Aalto et al., 1993; Propopov et al., 1993; Brennwald et al., 1994). In addition, SNARE-pairs have been identified in regulated exocytosis of neuronal synapses (see Südhof, 1995). A study of Nichols et al. (1997) has shown, in yeast vacuoles, the need for t-SNARE in one membrane and v-SNARE in another for fusion to occur. In a more systematic approach, McNew et al. (2000) tested all of the potential v-SNAREs encoded in the yeast genome to examine whether they can partner t-SNAREs of the Golgi, the vacuole and the plasma membrane. Vesicle and target SNAREs were reconstituted into two separate sets of liposomes and tested for fusion (see Weber et al., 1998). In these experiments, the phospholipids in the vesicles reconstiuted with v-SNAREs were labeled with a mixture of probes whose fluorescence is quenched. When fusion to the vesicles containing t-SNAREs occurs, the fluorescent probes are diluted and become fluorescent as the quenching decreases. The SNARE proteins reconstituted in liposomes were found to function as predicted by the SNARE hypothesis by exhibiting the appropriate specificity. However, other factors seem to play an important role as well (see below). These are proteins involved in the localization of vesicles, such as tethering factors, or those involved in the activation of the SNARE complexes (see Waters et al., 1999;

Mellman and Warren, 2000).

The assembled v-SNARE/t-SNARE complex consists of a bundle of four helices (Parlati et al., 2000; Fukuda et al., 2000). For the transition between ER and Golgi, one SNARE contains three of the helices (t-SNARE) and the other one (v-SNARE). Fusion does not take place with any other combination. For t-SNAREs on the plasma membrane, the protein *syntaxin*, supplies one helix and a SNAP-25 protein contributes the other two. The assembly of SNARE complexes involves Rab (see Rothman, 1994; Søgaard et al., 1994) and Sec1. Rab proteins are GTP-binding proteins (Simons and Zerial, 1993, see below). Sec1 proteins (Aalto et al., 1992) bind specifically to t-SNARE subunits (Pevner et al., 1994).

The need for separate and complementary v-SNAREs and t-SNARES, one in the cargo carrying vesicle and the other in the target membrane, explains many of the available data. However, in COPII vesicles the situation is more complex. The vesicles originating from the ER must first cluster to form VTCs (see Chapter 10). The large vesicles of the VTC are then targeted to the *cis*-Golgi with which they fuse. Consistent with this view, yeast vesicles can be isolated in clusters (Lian and Ferro-Novick, 1993). In addition, the t-SNARE syntaxin 5 is present in the vesicles, as we might expect if they fuse and not in the Golgi membranes (Rowe et al., 1998) and is essential for the assembly of vesicular-tubular-preGolgi intermediates as well as for the delivery of the cargo to Golgi.

We saw that the SNARE hypothesis postulates that the specificity of membrane fusion events resides on the SNARE receptors, $SNARE_v$ and $SNARE_t$ joining with each other. A good deal of data just reviewed supports this view (e.g., see Weber et al., 1998). However, recent studies suggest that the process is more complex. It has been argued that the specificity of targeting may not depend on SNAREs (e.g., Kaiser and Ferro-Novick, 1998). t-SNAREs are not localized at specific sites of the target membrane (e.g., Garcia et al., 1995) and v-SNARE can bind to more than one t-SNARE (von Mollard, et al., 1997; Holthuis et al., 1998). In some cases the system may be able to bypass SNAREs entirely. The disruption of SNAREs [e.g., caused by microinjection of the cytoplasmic domain of synaptobrevin (Hunt et al., 1994) or cell mutants lacking synaptobrevin or syntaxin (Broadie et al., 1995)] does not prevent vesicle docking. A v-SNARE can reside in both anterograde and retrograde-directed vesicles and a single v-SNARE can bind to several t-SNARES and a single t-SNARE can bind to several different v-SNAREs (see Götte and Fischer von Mollard, 1998; Pfeffer et al., 1999). In the case of the ER-to-Golgi transport, another entity, the 800 kDa complex of 8 subunits, the *transport protein particle* (TRAPP) may account for the specificity. TRAPP, located in the *cis*-Golgi, has a role in targeting and fusion (Sacher et al., 1998) and may have a role in providing specificity since it is unique to this step, whereas all SNARE molecules are very similar.

As reviewed above, t-SNAREs and v-SNAREs present in separate vesicles interact. However, other components are active in this process (Ungermann et al., 1998; Peters and Mayer, 1998). The small GTP-binding protein Ypt7p holds together the vacuoles in a reversible reaction (Ungermann et al., 1998). This stage requires the presence of various factors (see next section) but not SNAREs. A similar SNARE independent attachment was first described in the attachment of the ER vesicles to the Golgi (Cao et al., 1998a).

In *Saccharomyces cerevisiae*, Vam7 (a t-SNARE) is targeted to vacuoles in a manner dependent on the presence of phosphatidylinositol 3-phosphate in the vacuolar membrane. This interaction involves the *phox homology* (PX) domain of Vam7 (Cheever et al., 2001). The PX domain is an 80-125 amino acid residue region of proteins involved in binding phosphoinositides (Kanai et al., 2001).

C. Fusion

The machinery responsible for fusion is composed of three distinct proteins (Söllner et al., 1993): NSF (*Sec18p* in yeast), SNAP *(Sec17p* in yeast) family members, and the SNAREs. Once assembled, the complex forms a 20S particle (where 20S refers to the sedimentation coefficient). Models of the structures of the proteins in the complex derived from crystallography give us some understanding of the assembly of the 20S fusion particle (May et al. 1999; Yu et al., 1999; Rice and Brunger, 1999). These studies provide us with a detailed view of the very similar structures seen with the EM of the 20S particles (Hohl et al., 1998). The SNARE complex is rod shaped (2.5 x 15 nm). SNAP binds laterally, whereas NSF binds to one end of the complex to form a particle 22 nm in length.

The association of SNAP and NSF with SNARE to form the 20S particle is sequential. The v-SNARE-t-SNARE complex binds 3 to 6 SNAP proteins and subsequently NSF (Söllner et al., 1993b; Hayashi et al, 1995). The release of SNAP, which requires NSF, is accomplished before vesicle docking (Mayer et al., 1996). Subsequently, v-SNARE from t-SNARE dissociate (Söllner et al., 1993) and the fusion takes place. The SNAP and NSF proteins are common to most, if not all, different transport steps. It might be expected the SNAREs are specialized since their interaction determines the final fate of the cargo (Fig. 1B) (see Ferro-Novick and Jahn, 1994). However, this was found not to be the case. In vitro testing showed very promiscuous binding of SNAREs derived from different sources (Yang et al., 1999).

The SNAP proteins (Clary et al., 1990) and an ATPase, the NSF (Block et al., 1988; Malhotra et al, 1988; Wilson et al, 1989) assemble after docking. The v-SNARE-t-SNARE complex binds 3 to 6 SNAP proteins and subsequently NSF (Söllner et al., 1993b; Hayashi et al, 1995). The hydrolysis of ATP dissociates v-SNARE from t-SNARE and releases the SNAP molecules (Söllner et al., 1993).The fusion process is still not well understood.

In one of the current models of fusion, SNAREs have a role in bringing the two bilayers together in a zipper like process (see Chen and Scheller, 2001). When the two bilayers become fused a pore opens and then pore expands producing one continuous bilayer. At least in exocytosis, freeze fracture EM (Chandler and Heuser, 1980) and patch clamping (Breckenridge and Almers, 1987) confirm the formation of a pore. Present thinking suggests that the pore is in the lipid components with protein scaffolding, possibly SNAREs (see Monck and Fernandez, 1994, 1996;Lee and Lentz, 1997)

In mammals, the fusion of early endosomal vesicles and more mature endosomal vesicles requires

the *early endosome-associated protein* (EEA1) and Rabaptin-5. EEA1 binds to phosphatidylinositol 3-phosphate in the endosomal membrane through its FYVE domain (e.g., Simonsen et al., 1998)

NSF

NSF was discovered in isolated systems. In low concentrations, the sulfhydryl alkylating agent NEM, inactivated the acceptor membrane fraction of the intra-Golgi transport system so that it would no longer be able to fuse to the incoming vesicles (Glick and Rothman, 1987). Untreated peripheral proteins of the Golgi membranes restored function. A 76 kDa polypeptide, forming a homotetramer in its native form, NSF, was found to be responsible for the activity (Block et al., 1988). EM examination of the Golgi membranes of blocked cells (Diaz et al., 1989) indicated an accumulation of uncoated intermediate transport vesicles associated with membranes. This observation suggested that NSF acts as part of the complex needed to fuse the vesicle membrane with the acceptor membrane. NSF was subsequently found to be needed for the in vitro fusion of vesicles involved in the transport from ER to Golgi and for the fusion of endocytotic vesicles.

NSF shows 48% sequence identity to *Sec18p* of yeast. Furthermore, *Sec18p* can replace NSF in the mammalian cell free system. NSF is a hydrophilic molecule not likely to interact with hydrophobic domains such as the hydrocarbon leaflets of the bilayer membrane. However, it has two ATP-binding domains and displays ATPase activity, which determines its attachment to the membrane. In addition to NSF, five other proteins have been found to be necessary in vesicle mediated transport. Many others might also be required. The in vivo requirement for *Sec18p* was demonstrated using a temperature sensitive mutant (Graham and Emr, 1991). The proteins of the cells were labelled by exposure for a short period to radioactive amino acids at the permissive temperature (20ºC), followed by a chase at the nonpermissive temperature (37ºC). The fate of two proteins, F and CPY, was examined. These two were selected because the proteins undergo stepwise modifications in the Golgi complex that can be easily recognized using SDS gel electrophoresis and immunoprecipitation. In the case of the wild type cells, the F precursor proteins were rapidly chased to the mature form of the protein (mF). In contrast, in the mutant cells the inactivation of the *sec18* protein left the F protein in all intermediate compartments. These results indicate that each sequential step must occur in a separate compartment, as we saw for other systems, and *Sec18p* is involved in each step.

NSF is a member of the ATPases responsible for varied cellular activities, the AAA (*ATPases associated with a variety of cellular activities*) superfamily (see Patel and Latterich, 1998; Confalonieri and Duguet, 1995, see Frölich on the Web) which also includes proteasomal components (see also Chapter 15). Some members of this family perform chaperone tasks (i.e., folding tasks) (see Chapter 15), others are associated with assembly, remodeling and disassembly. They act in a variety of cellular functions (see Neuwald et al., 1999), including cell-cycle regulation, protein degradation, organelle biogenesis and vesicle-mediated protein transport, and the initiation of transcription. The AAA motif corresponds to a 230-amino-acid domain that contains Walker ATP-binding homology sequences and imparts ATPase activity.

NSF is involved in eukaryotic fusion events which also require SNAREs. The SNAREs are associated at their cytoplasmic domains forming stable complexes (Hay and Scheller, 1997; Weber

et al., 1998). SNAREs (v and t) constitute the minimum requirement for fusion of membranes for one round of fusion. NSF and SNAPs being required for separation of the two SNAREs to allow for the next round of fusions (see Weber et al., 1998). This activity depends on ATP hydrolysis. The dissociation events require the *soluble NSF attachment proteins* (SNAPs) to bind to SNAREs providing a binding site for NSF (Whiteheart et al., 1992; Wilson et al., 1992). The binding to SNARE and SNAP increases the ATPase activity of NSF and at the same time the complex is disassembled (Morgan et al., 1994, Barnard et al., 1997). NSF is made up of three domains: the amino-terminal domain which is capable of interacting with SNAPs and SNAREs, and two similar ATPase domains (D1 and D2). D1 is thought to be involved in remodeling the 20S particle; D2 is thought to be responsible for the formation of NSF-hexamers (Neuwald, 1999). The complex is thought to function similarly to chaperones in driving the remodeling of effector molecules using the free energy generated by the hydrolysis of ATP (see Patel and Latterich, 1998; Neuwald, 1999)

SNAPs

SNAPs constitute a family of soluble proteins required for NSF to bind to Golgi membranes (Weidman et al., 1989, Clary et al., 1990). α, β and γ SNAPs are 35, 36 and 39 kDa in molecular weight, respectively. α-SNAP has also been shown to be required in vivo. In yeast, α-SNAP is encoded by the *SEC17* gene. In the absence of this protein, vesicles are accumulated (Kaiser and Shekman, 1990). SNAPs bind to specific sites on the membranes the SNAREs) and this binding is required for interaction with NSF. The binding site can be identified by crosslinking with crosslinking reagents (Whiteheart et al., 1992). These show that α-SNAP attaches to a 30-40 kDa protein. The various SNAPs bind at different sites of a receptor complex.

D. The GTPases

The GTP-binding proteins or GTPases discussed above (also referred to as G-proteins, not to be confused with the VSV-G glycoproteins) have been shown to have a key role in intracellular transport. Mutations in the genes known to code for GTP-binding proteins block several steps in secretion. Furthermore, in mammalian cells, the Rab or Ras proteins which correspond to GTPases are localized in specific membranes associated with the secretory pathway and intracellular transport is blocked by treatments that inhibit small GTP-binding proteins.

There are seven major groups of GTPases. They share domains needed for guanine binding and GTP hydrolysis. These domains are so similar that the three-dimensional crystal structure of the proteins are superimposable. They diverge significantly in amino acid sequences in other regions of the molecules. Presumably, these are the regions which determine functional specificity.

The role of heterotrimeric GTP-binding proteins in signal transduction was discussed in Chapter 7. In this complex, the α-subunit of 40-50 kDa binds to the guanidine nucleotide. There are also two additional subunits (β and γ of 35-36 and 8 kDa respectively). The present section will discuss both the heterotrimeric and the lower molecular weight GTPases in relation to intracellular trafficking.

In contrast to the heterotrimeric GTPase, those of the Ras superfamily (also called Rab when

derived from cDNA libraries from rat brain) are monomers (Sar1, ARF, Rab/*YPT*, Rac/*CDC42* and Rho families) 20 to 30 kDa in molecular weight. Those of the dynamin family are 60-80 kDa. With the exception of ARF, all form complexes with cytoplasmic or membrane proteins which may have a function similar to the β and γ subunits of the trimeric GTPase. Fatty acid residues are added posttranscriptionally to several GTPases. In some cases, these have been shown to permit direct interaction with membranes (Rossi et al., 1991). Rab, Rho, Rac and the γ-subunit of the heterotrimeric GTP-binding protein have one or two prenyl moieties at the carboxyl terminal or at cysteine residues. Members of the ARF and G_α family are myristylated at an amino-terminal glycine residue. Palmitylation of cysteine residues may occur in the amino or carboxyl terminal of Ras and G.

GTPases were originally shown to have a role in all the steps of intracellular transport through the effects of guanosine-5-O-thiotriphosphate (GTPγS) and AlF_4^-. GTPγS, a non-hydrolyzable analog of GTP blocks all GTPases. It inhibits almost all of the steps in the transport.

The heterotrimeric GTPase appears to function as an inhibitor of transport. Two specific α-subunits are associated with the Golgi. Overexpression (by transfection, see Chapter 1) of one of these G_αs leads to slowing of the transport of proteoglycan through the Golgi (Stow et al., 1991). Conversely, inhibition of G_α speeds up transport. A similar role is suspected for ER to Golgi transport at the budding stage (Schwaninger et al., 1992).

The well-defined morphology of the mammalian system provides a clear picture of the role of the small GTP-binding proteins. A minimum of 12 mammalian *rab* genes have been isolated from cDNA libraries using probes recognizing *ras* sequences. The proteins of six *rab* genes (*rab1* to *rab6*) have been shown to bind and hydrolyze GTP. Immunofluorescence, immunoeletronmicroscopy and the immunological reactions after cell fractionation have provided information on the localization of these proteins, as summarized in Table 2 (Rothman and Orci, 1992). This association of each Rab protein with a specific structure in the intracellular transport pathway suggests that each GTP protein acts at a different step. However, direct evidence has been provided by genetic approaches in yeast. These findings were extended to other systems using cDNA technology and in vitro reconstitution systems. Numerous additional rab[110,111] and ARF[65] genes have been cloned and sequenced but the proteins have not yet been localized. In most cases, there is a substantial pool of soluble GTPases in addition to the membrane-bound forms.

The transport of VSV G-protein was followed in vitro by measuring the incorporation of [^{3}H]-N-acetylglucosamine (GlcNAc) from donor membranes (from a cell line lacking GlcNAc-transferase) to acceptor membranes isolated from wild type cells (containing GlcNAc transferase) (Balch et al., 1984a,b). The use of synthetic polypeptides representing partial amino acid sequences of specific GTPases, or the use of antibodies to the individual GTPases, identified the individual steps in which they are involved. A polypeptide will block the action of a GTPase when it competes with it, for example, for a receptor site. Similarly, a specific antibody can block individual steps by depleting the cells of the Rab-protein.

Table 2 Partial list of GTPases and their location.

	Small GTP-binding protein	Location(s) when membrane bound
Rab family	rab1Ap/rab1Bp/ypt	ER and Golgi
	rab2p	GCN
	rab3Ap	Neurosecretory vesicles
	rab4p	Early endosomes, plasma membrane
	rab5p	Early endosomes, plasma membrane
		Golgi stack
	rab6p	Late endosomes
	rab7b	Post-Golgi vesicles, plasma membrane
	sec4p	
ARF family	ARF1p	Golgi stack
	SAR1p	ER

Reproduced with permission from Nature (1992) Rothman, J.E. and Orci, L. 355:409-415.

Research efforts are now directed toward understanding how the small GTP-binding proteins interact with specific membranes. This is likely to require the identification and study of multiple binding proteins in the various membranes.

A summary of some of the functions of GTPases is presented in Table 3. In this table, an important function of the GTPase is listed in the first column, however, some of them have multiple functions. The GTPase responsible is listed in the second column and the evidence available is listed in the third.

The precise mechanism of the action of small GTPases is not known. However, their properties provide hints that they can function as switches, initiating or terminating biological processes. We have seen this kind of role in the docking of ribosomes and nascent polypeptide chains in the ER.

The GTPases change in conformation in response to the phosphorylative state of the bound nucleotide. The active conformation occurs when the bound GDP exchanges with GTP. Hydrolysis of the GTP returns the protein to its inactive conformation. The reactions of the GTPases require the presence of other proteins. Two proteins regulate the conformational changes. The exchange of GDP for GTP is facilitated by the *guanine nucleotide dissociation proteins* (GEPs) and inhibited by *guanine nucleotide dissociation inhibitors* (GDIs). In addition, the GTPase activity of the Ras superfamily is slow in the absence of *GTPase activating proteins* (GAPs).

Table 3 Role of GTPases in Intracellular Transport

FUNCTION	GTPase	EVIDENCE
vesicle budding from ER	Sar 1	overcoming mutant block (1)
		location in ER, trnaslational elements and Golgi (2)
formation of COP vesicles from Golgi	ARF (ADP ribosylation factor)	in vitro assay (3)
endocytotic role	dynamins	accumulation of coated pits in defective mutants (4)
transport from ER and cis to trans Golgi	Rab1	in vitro assay (5)
early endosome fjunction	Rab2, 5 and 7	Rab domains target to endosomes (6)
TGN vesicle budding	Rab6	antibodies to Rab6 block TGN budding (7)
exocytosis: synaptic vesicle	Rab3A	required for transfer from vesicle to cell surface (8)
exocytosis: mast cells		discharge of granules when Rab delivered into cells (9)

Ref. (1) Nakano, A. and Maramatsu, M. (1969), *J. Cell Biol.* 109:2677-2691.
(2) Kuge, O., Dascher, C., Orid, L., Amherdt, M., Plutner, H., Ravazzola, M., Tanigawa, G. Rothman, J.E. and Balch, W.E. (1994) *J. Cell Biol.*. 125:51-65

(3) Orci, L., Palmer, D.J.,, Perrelet, A., Amerdt, M.., Palmer, D.J. and Rothman, J.E.. *Nature* 362:648-652; 364:732-734.
(4) Kosaka, T. and Ikeda, L. (1983) *J. Neurobiol*.. 14:207-225.
(5) Plutner, H., Cox, A.D., Pind, S., Khosravi-Far, R., Bourne, J.R. et al., (1991) *J. Cell Biol*. 115:31-43.
(6) Chavier, P., Gorvel, J.P., Steizer, E., Simons, K., Greenberg, J. and Zerila, M. (1991) *Nature* 353:769-772.
(7) Jones, S.M., Crosby, J.R., Salamero, J. and Howell, K.E. (1993) *J. Cell Biol* . 122:775-788.
(8) Matteoli, M., Takel, K., Cameron. R., Hurbult, P., Johnston, P.A. et al. (1991) *J. Cell Biol* . 115:625-633.
(9) Oberhauser, A.F., Monck, J.R., Balch, W.E. and Fernandez, J.M.. (1992) *Nature* 360:270- 273.

Dynamins (see McNiven et al., 2000) constitute a family of large (100-kDa) GTPases that seem to fulfill several roles in membrane trafficking in eukaryotic organisms. Different dynamin isoforms are present in different tissues and even in the same tissue. At least 25 different mRNAs are produced by the 3 dynamin genes by alternative splicing (Cao et al., 1998b).

Dynamins have been shown to play important roles in endocytosis and vesicle formation (see Chapter 9 and above). Dynamin is needed for clathrin-mediated endocytosis (e.g., Herskovits et al., 1993; van der Bliek, 1993) and the formation of vesicles from caveolae (see Chapter 9) (Henley et al, 1998; Oh et al., 1998). Inhibition of dynein function, inhibits the scission of caveolae both in vivo and in vitro. Dynamin has also been implicated in other kinds of endocytosis (see Sandvig and van Deurs, 1996), including the intake of fluid in cultured mammalian cells (Henley et al., 1999) and phagocytosis in macrophages (Gold et al., 1999).

Dynamins have a role in vesicle formation in steps of the endocytotic pathway other than vesicle formation from the plasma membrane. In HeLa cells, the expression of a mutant of dynamin does not affect clathrin-independent endocytosis. However, the pathway from endosomes to Golgi is blocked (Llorente et al., 1998). Similarly, disruption of a dynamin-family proteins in *Dictyostelium discoideum* has a very broad effect (including a defective fluid-phase uptake) (Wienke et al., 1999). In addition, dynamin was found to colocalize with vacuolin, a marker of a postlysosomal compartment. In mammalian cells, confocal imaging (see Chapter 1) showed that dynamin is associated not only with the plasma membrane but also the *trans*-Golgi network, and a perinuclear cluster of structures containing cation-independent mannose 6-phosphate receptor. Electron microscopy showed that the structures correspond to late endosomes with a localization of dynamin preferentially in tubulo-vesicular processes of these endosomes (Nicoziani et al., 2000). In other studies, dynamin was found associated with the TGN (Henley et al., 1996; Maier et al., 1996). GFP-dynamin chimeras (see Chapter 1) expressed in cultured rat hepatocytes appeared in the clathrin coated vesicles at the cell surface and the TGN (Jones et al., 1998). An in vitro system produced dynamin in clathrin coated and non-clathrin coated vesicles. Cells expressing a mutant of dynamin were found to accumulate GFP-protein chimeras in the TGN (Kreizer et al., 2000). In *Saccharomyces cerevisiae*, a dynamin, dnm1p, has been shown to be involved in transport to the vacuole from the TGN (Gammie et al., 1995).

The dynamins are capable of interacting with several components. Their *pleckstrin homology* (PH) domain allows them to bind to phosphoinositides and their proline-rich domain (PRD) allows to bind to the SH3-domains of variety of effector molecules. A coiled-coil region (CC domain) f dynamins is a GTPase effector domain (Sever et al., 1999).

In vivo, dynamin interacts with other proteins that may alter the geometry of lipid structures to produce vesicles. An interaction with endophilin 1 (Schmidt et al., 1999) (that transfers arachidonate to lysophosphatidic acid) has been demonstrated. This reaction is likely to favor the formation of highly curved lipid structures. There is at least one example of this mechanism: BARS (Weigert et al, 1999) (that acylates lysophosphatidic acid) was shown to induce the formation of vesicles in Golgi membranes of rat brain. However, in this case the system seems to operate independently of dynamin.

Several studies have indicated the dynamin is associated with actin or actin-binding or actin-depolymerizing proteins, suggesting a role of these interactions in the formation of vesicles (e.g., Witke et al., 1998; Qualmann et al., 1999)

E. ER and Golgi Transport

ER to Golgi Transport

Present information indicates that COPII vesicles mediate the transfer of cargo from the ER to the Golgi. However, the process may be more complex. We saw that larger vesicular components, formed by vesicle clustering, carry cargo to the *cis*-Golgi (see Chapter 10, Section III). Therefore, the *vesicular tubular clusters* (VTC) and not the COPII vesicles are targeted to the *cis*-Golgi. Consistent with this view, the t-SNARE, *syntaxin* 5, is present in the vesicles as well as in the *cis*-Golgi (Rowe et al., 1998) and is essential for the assembly of VTCs. What this suggests is that the VTCs are formed by an aggregation of these vesicles. Unfortunately, since retrograde transport also takes place, it is difficult to consider this argument decisive.

The role of COPII in anterograde transport from the ER is well established. However, much of the present evidence also supports a role of COPI for anterograde transport between ER and Golgi and between Golgi stacks (Chapter 10, Section 3C; see also Kreis et al., 1995). However, studies with yeast COPI mutants demonstrate ER to Golgi transport in COPI-impaired cells for some proteins but a complete block for others (Gaynor and Emr, 1997). In contrast, the remaining ER to Golgi transport required COPII.

A possible explanation for the complex role of COPI in anterograde transport from the ER, might rest on a sequential role of the two coat complexes. In in vitro experiments using isolated ER fragments, showed that vesicles released by the ER-derived system were COPII coated and contained VSV-G protein and p58. p58 is an endogenous recycling protein. However, preparations from ARF1 mutants that prevent COPI recruitment blocked subsequent movement to the isolated Golgi membranes (Rowe et al., 1996). These observations suggest that COPII drives the export from the ER and then COPI replaces COPII in the vesicles.

In yeast, the transfer of cargo from the ER to the *cis*-Golgi involves *the transport protein particle* (TRAPP), a 1100 kDa complex (Sacher et al., 1998). TRAPP mediates vesicle docking and fusion. Proteins analogous to the subunits of TRAPP have been found in mammalian species (see Guo et al., 2000). Subunits of TRAPP have been found in early Golgi membranes, in X-100 insoluble membrane components suggesting a presence in rafts.The protein p115 (also known as TAP) is needed for the transport from the VTC to the *cis*-Golgi (Nelson et al., 1998). It plays a more general role in intra-Golgi transport (see next section). In yeast, two other factors have been implicated in ER-to-Golgi transfer of cargo. Uso1p acts before SNAREs and is required for tethering (Cao et al., 1998). Sec34p and Sec35p also function in tethering (VanRheenen et al., 1998).

Transport from the Golgi stacks

Isolated Golgi stacks incubated with cytosol and ATP generate 75 nm vesicles from the cisternae (Balch et al., 1984a) (now recognized as COPI-coated vesicles). They correspond to transport vesicles because they can be shown to contain transported proteins (in these experiments the G-protein of vesicular stomatatis virus, VSV). The transport between cisternae, also mediated by vesicles, can be followed biochemically through the progression in the glycosylation of proteins. Like other transfers studied so far in vitro, these also require cytosol and ATP (e.g., Braell et al., 1984a).

The 75 nm vesicles can be either coated or uncoated (Orci et al., 1986). The relationship between these two kinds of vesicles is revealed by the effect of two inhibitors. GTPγS, a non-hydrolyzable analog of GTP, produces an accumulation of buds and coated vesicles (Melançon et al., 1987). GTP hydrolysis is needed for the fusion of vesicles onto their target compartment and GTPγS blocks this process. N-ethylmaleimide (NEM) block, on the other hand, produces an accumulation of uncoated vesicles (Melançon et al., 1987). As discussed later, NEM blocks the docking of vesicles to the target membrane (see below). Simultaneous treatment with GTPγS and NEM produces an accumulation of coated vesicles (Orci et al., 1989); therefore, the coated vesicles are the precursors of the uncoated vesicles. Both kinds of vesicles should accumulate if the two were produced independently. Alternatively, if the uncoated vesicles were the precursor, only the uncoated vesicles would accumulate.

The accumulation of vesicles derived from the Golgi stacks in a cell free system in the presence of GTPγS, permitted the isolation and direct biochemical study of the COPI (coatomer) coated vesicles (Malhotra et al., 1989; Serafini and Rothman et al., 1992).

The COPI coatomer is composed of seven proteins (α, β, β', γ, ε and ζ) ranging in molecular weight between 60 and 160 kDa. β-COP has been localized with immunofluorescence and immunoelectron microscopy in the CGN and TGN and has also been found in soluble cytoplasmic complexes (Duden et al., 1991b, Waters et al., 1991). Presumably, the cytosolic β-COP is the source of this subunit of the vesicle's coat.

Members of the p24 protein family bind to COPI coatomeres and may have a role in the recruitment

of COPI to the Golgi membranes (Dominguez et al., 1998). p24 and p23 are cargo receptors found in both COPI and COPII coated vesicles. In COPI coated vesicles, they are present in stoichiometric amounts relative to coatomer and the GTPase, ARF. The cytoplasmic domains of these proteins bind to coatomer (e.g., Sohn et al., 1996) and are needed for cycling in the early secretory pathway (e.g., Nickel et al., 1997). COPI coatomer vesicles also bind to KKXX (di-lysine motif) and KXKXX containing proteins. The KDEL receptor that binds to proteins containing the KDEL motif is also transported in these vesicles. The γ subunit recognized the lysine motifs (Harter et al., 1996). These motifs are retrieval signals (see Table 2, Chapter 10) in line with the role of COPI coated vesicles in retrograde transport.

COPI is required for intra-Golgi retrograde transport in vitro (see Lin et al., 1999). COPI vesicles or transport intermediates can be isolated or produced in vitro and have a high level of Golgi-resident enzymes (Lanoix et al., 1999; Love et al., 1998) and KDEL receptors (Sönnichsen et al., 1996) suggesting that they are recycling intermediates.

Although there is evidence for the involvement of COPI in anterograde transport (see previous section), the evidence for a role of COPI in retrograde transport is less ambiguous. Subunit of the coatomer bind directly to proteins with the di-lysine retrieval motif (Cosson and Letourneur, 1994). Furthermore, mutation in some of these subunits prevents recovery to the ER of proteins with the di-lysine retrieval motif (Cosson et al., 1996). In view of these findings, some investigators are postulating that the role of COPI in anterograde transport is indirect, recycling components needed for COPII transport by retrograde transport. However, results of several experiments continue to support a direct role of COPI in both anterograde and retrograde transport. Immunocytochemistry with the electron microscope using colloidal gold and antibodies (to COPI subunits, KDEL receptors and proinsulin) have demonstrated that both anterograde transport of proinsulin and VSV G protein and retrograde transport of a KDEL receptor occur in COPI vesicles. These vesicles constitute two distinct populations that together account for at least 80% of the vesicles present. The COPI vesicles bud from every level of Golgi cisternae. Similar results were obtained in in vitro experiments (Orci et al., 1997).

In addition to a retrograde pathway which involves COPI coat proteins, a transport pathway from Golgi to ER has been found which functions independently from COPI coat proteins (see Storrie et al., 2000). This pathway returns Golgi resident proteins (as well as protein toxins) to the ER and may have a primary role in the recycling of lipids. Microinjection of antibodies to coatomer, block recycling of KDEL receptor and a lectin-like molecule, ERGIC-53, from Golgi to ER. Proteins containing sequences recognized by the KDEL receptor are also inhibited (Girod et al., 1999). In contrast, microinjection of anti-COPI antibodies or an Arf-1 mutant (Arf is required for COPI-coated vesicle assembly) does not interfere with the transport to the ER of Golgi-resident glycosylation enzymes or Shiga toxin/Shiga-like toxin-1. However, overexpression of a Rab6 (a small GTPase, see discussion below) mutant blocks retrieval of Golgi-resident glycosylation enzymes and Shiga toxin/Shiga-like toxin-1. However, it has no effect on KDEL receptor, KDEL containing proteins or ERGIC-53. These observations indicate that there are two pathways for the retrieval of proteins from the Golgi to the ER.

The protein p115 (TAP), which has a role in the transport from the VTC to the *cis*-Golgi (see

previous section) is required for intra-Golgi transport (Waters et al., 1992) and is likely to function in tethering several steps. It has been implicated in the binding to the plasma membrane during transcytosis (Barroso et al., 1995).

II. RECOGNITION OF TARGETS

A. Cell Polarity

In an organized tissue, many cells have regions of the cytoplasm and the cell surface that differ in composition and function. They are said to be polar. This polarity can be preserved when the cells are cultured on a solid medium. Epithelial and endothelial cells form sheets in which they are held together by junctional complexes which prevent exchange between the two domains and prevent exchanges between compartments. A diagrammatic representation of polar cells in Fig. 2 shows apical and basolateral surfaces. The *adherens junction* is responsible for adhesion between the cells. In vertebrates the *tight junction* and in other animals the *septate junction*, prevent the exchanges.

Vertebrate epithelial and endothelial cells, are held together by the tight junctions at contact points, the *desmosomes* which serve as anchoring points for intermediate filaments. In the polarized cell, the apical and the basolateral surfaces have distinct lipid and protein domains (Simons and Fuller, 1985). This distinct composition could not be maintained unless the two were prevented from exchanging materials and the various components were specifically targeted when newly synthesized.

Essentially, tight junctions play a dual role as *barriers* and *fences* (see Chapter 4) (e.g., see Gumbiner, 1993; Anderson and Van Itallie, 1995). The barrier function refers to the tight seal that prevents diffusional exchanges between separate compartments The fence function refers to the prevention of exchanges between the basolateral and apical domains of the plasma membrane to maintain specialized functions, such as unidirectional secretion or active transport across the sheets.

In addition to their structural role, cell junctions can also provide signals that initiate cascades involved in cell growth and differentiation (e.g., see Clark and Brugge, 1995; Takahashi et al., (1998); Reichert et al., 2000). For example, note that components of the signaling systems are present at junctions (see below). In some cases, the role of the cell junction component may more direct, for example, the adherens junction protein β-catenin (see below) is translocated into the nucleus and binds to a transcription factor (see Nusse, 1997; Eger et al., 2000). Similarly, the junctional component CASK (see Hata et al., 1996) is translocated into the nucleus (Hsueh et al., 2000) and is required for EGF receptor localization and signalling in the nematode *Caenorhabditis elegans*. CASK is membrane-associated guanylate kinase that is bound to the adhesion protein, *syndecan*, at epithelial cell junctions (Cohen et al., 1998). In the nucleus, a complex of CASK and Tbr-1 binds to a specific DNA sequence. Tbr-1 is a T-box transcription factor that is involved in forebrain development. The complex of the CASK and Tbr-1 activates several genes containing the T-box. T-box gene family have a binding domain, the T-domain which is important for development.

The structure and formation of tight junctions as well as the factor that underlie the formation and maintenance of distinct membrane domains have been explored.

Maintenance of polarity

Some progess has been made in the study of the maintenance of polarity in *Drosophila* epithelium. Proteins which localize to one of three surfaces and suspected to have a role in organizing the corresponding domain have been called *epithelial cell surface organizers* (ECSOs) (see Tepass, 1997). These include *cadherins* (see below) that localize laterally (see Drubin and Nelson, 1996), Crumbs proteins of *Drosophila* that localize to the apical surface in (e.g., Wodarz et al., 1995) and β_1, α integrins (see Chapter 6) located in the basal surface of kidney epithelium (Sorokin et al., 1990) and *Madin-Derby canine kidney* (MDCK) cells (e.g., Schoenenberger et al., 1994). ECSOs are thought to be recruited and retained to their domain by external signals such as homophilic adhesion in the case of cadherin. The interaction of ECSDOs with cytoplasmic factors permit interactions with the cytoskeletal system.

In *Drosophila*, the mutation in four genes (*stardust, sdt, bazooka, baz, crumbs, crb,* and *discs lost, dlt*) has been shown to be lethal by eliminating polarity in epithelial cells (see Tepass, 1997). CRB and DE-cadherin are considered key regulators of polarity. CRB is an integral membrane protein with a single transmembrane domain. It contains thirty EGF-like (for other examples see Chapter 6, Fig. 10 and 11) and four laminin AG domain-like repeats in its extracellular segment and a short cytoplasmic tail of thirty seven amino acids. CRBs is expressed apically in ectodermally derived epithelia. Interestingly the mRNA for CRBs is found in the apical cytoplasm (Tepass et al., 1990), suggesting that it is the mRNA and not the protein which is targeted to this region. Several examples of proteins targeted via their mRNA are known (see Chapter 10). Overexpression of CRB expands the apical plasma membrane and reduces the basolateral domain. In contrast, BAZ and DLT are cytoplasmic proteins with protein-protein interacting domains such as the PDZ domain. At least in vitro, one of the PDZ domains of DLT binds to the cytoplasmic domains of CRB (Bhat et al., 1999, Klebes and Knust, 2000) and the laterally localized *Neurexin IV* (NRXIV). Interference with DLT (mutations or introduction of double-stranded RNA) lead to mistargeted CRB and NRX IV and disruption of epithelial polarity. The apical distribution of DLT depends on the presence of CRB. These and other observations suggest that CRB and DLT together with other proteins define the localization of the zonula adherens (Klebes and Knust, 2000) and independently the loss of cell polarity (Grawe et al., 1996). An additional protein, *Scribble* was found to be necessary to maintain the polarization of cells (Bilder and Perrimon, 2000), as development proceeds in *Drosophila* embryogenesis. Scribble is localized to the epithelial septate junction. Without Scribble, adherens proteins no longer assemble in the apical-basolateral region interface and apical proteins are no longer localized at the apical region. Scribble, a protein of 195 kDa (calculated from the cDNA), was found to contain many protein-protein binding domains and is likely to correspond to a scaffold needed for other proteins to assemble.. The carboxy-end has four PDZ domains (the initials of the first three proteins that were discovered with the motif). The amino-end contains sixteen leucine-rich repeats. Similar repeats are known to bind RhoA and Rac-GTPases, known to be associated with polarity and junctions (Jou and Nelson, 1998; see Kaibuchi et al., 1999). Many PDZ proteins, including Scribble localize at septate junctions, although others localize elsewhere. The actual molecular role of Scribble is still unknown. It may have a a role in targeting or it might even be part

of the fence that prevents exchanges between the two regions.

Components of tight junctions

EM studies of thin sections (Farquhar and Palade, 1963) revealed discrete sites at which tight junctions produced close contacts between cells ("kissing points"). Freeze-fracture EM showed anastomosing strands in the cytoplasmic leaflet of the plasma membrane with complementary grooves in the external face of the bilayer (see Staehelin, 1974).

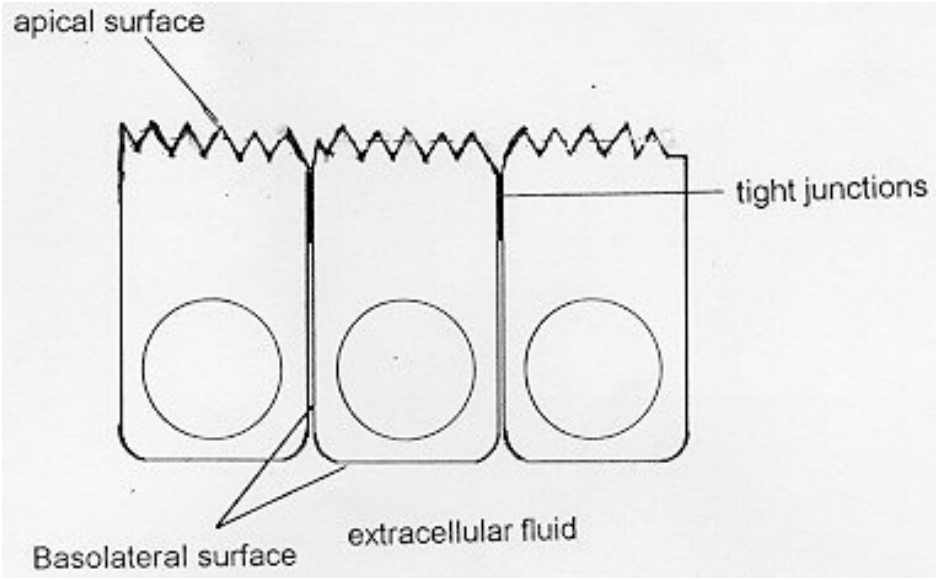


Fig. 2. Arrangement of epithelial cells forming a sheet.

Occludin, a 60 kDa integral protein was identified in tight junction strands (Furuse et al., 1993; Ando-Akatsuka et al., 1996). The cloning of the corresponding cDNA (see Chapter 1) and hydrophobicity plots (see Chapter 4) showed that occludin had four possible transmembrane domains and three cytoplasmic domains including the amino- and carboxy-terminals (see Ando-Akatsuka et al., 1996). Occludin is involved in forming barriers and fences. The barrier function is shown, for example, by the increase electrical resistance across the epithelium when chicken occludin is overexpressed in under conditions in which they formed tight junctions (McCarthy et al., 1996). Expression of the carboxy-terminally truncated occludin, rather than wild-type occludin (Balda et al., 1996), was found to render MDCK cells incapable of maintaining a fluorescent lipid in a specifically labeled cell surface domain, indicating that occludin is also involved in providing an apical/basolateral intramembrane diffusion barrier.

More recently, *claudin-1* and *claudin-2*, two 23 kDa integral membrane proteins, were found in tight junctions of chick liver or transfected MDCK cells (Furuse et al., 1998a). Hydrophobicity analysis (see Chapter 4) indicated four possible transmembrane domains and cDNA analysis showed no sequence similarity to occludin. Immunofluorescence and immunoelectron microscopy (see Chapter 1) revealed that both claudins were present in the tight junction strands. When introduced in fibroblasts lacking tight junctions, these proteins induced the formation of a network of strands and

grooves at contact sites (Furuse et al., 1998b). The strands more closely resemble those in native tight junctions, suggesting that the occludins are major components of the junctions. Extensive studies indicate that the claudin family contains many members, as many as fifteen have been found in data-base searches of cDNA (Morita et al., 1999a,b,c; Tsukita and Furuse, 1999).

Certain claudins appear to be associated with specific tissues. Claudin-5 and -6 have been found in tight-junctional strands of endothelial, but not of epithelial cells (Morita et al., 1999b). *Clostridium perfringes* enterotoxin, which binds specifically to claudin-3 and -4, were shown to disrupt tight junction strands in transfected L fibroblast cells and MDCK cells (Sonoda et al., 1999) implicating their presence in those junctions. The possibility of other molecular components forming tight junctions cannot be excluded at this time (see Tsukita and Faruse, 1999)

Aside from the proteins forming tight junctions, there must be some structural basis for establishing polarity. In addition to targeting sequences and corresponding receptors there must be an assembly that includes cytoskeletal elements and permits the transfer of cargo to specific locations. The generation of polarity is of fundamental importance in the physiology of a variety of cells. A general model of the development of cell polarity has been presented (Drubin and Nelson, 1996). In this model, clues at the surface lead to localized assemblies of submembrane elements, including the cytoskeleton responsible for the organization of a pathway along an axis of polarity.

In the case of epithelial cells, the position of adhesion receptor proteins is determined by adhesion to other cells or the *extracellular matrix* (ECM) (for cell-cell adhesion, E-cadherin; for adhesion to the ECM, the integrins) (see also Fig. 7C, Chapter 4). This interaction generates localized assembly of cytoskeletal elements. Integrins are bound by α-actinin and talin which recruit actin and actin-associated proteins. In turn, these serve as a scaffold for the assembly of signaling components, such as adhesion kinase and components of the Ras pathway (such as SOS and Grb2 and GTP-binding proteins), and may even lead to regulating gene expression as already discussed. Similarly, cadherin recruits cytoplasmic proteins such as β-catenin, plakiglobin and P120. The binding of these proteins to α-catenin (that has some homology with vinculin) may connect the complex to actin. Cadherin-catenin complexes recruit kinases (Src and Yes) and protein-tyrosine phosphatase, components associated with signaling (see Chapter 7). GTP-binding proteins and Ras may associate with the complex. In addition, the region that remains free, the apical region, also seems to respond and assemble a distinct actin network cross-linked with villin, fimbrin and myosin I. Talin, α-actinin and vinculin are involved in the formation of fibers containing actin at focal contacts (see Chapter 23). Catenin, vinculin, α-actinin and plakoglobin have a similar mission but are part of an actin containing belt connecting epithelial cells and are attached to cadherins. Fimbrin and villin are actin bundling proteins (villin is present only in microvilli) (see Chapter 24). Talin, α-actinin and vinculin are involved in the formation of fibers containing actin at focal contacts.

The events accompanying cell adhesion also redistribute the microtubules with consequences on the targeting of secretory products. Depletion of kinesin disrupts the basolateral delivery, and depletion of kinesin and dynein disrupts apical delivery (Lafont et al., 1994). Kinesin and dynein are motor molecules that move along microtubules (see Chapter 24). The difference between the microtubules transport to the two faces suggest that the organization of the microtubules is distinct in the two

systems.

The determination of specific targeting of secretory vesicles is clearly closely dependent on these events. In yeast, this is shown by mutations of the genes coding for sec6/sec8 which lead to the accumulation of secretory vesicles in the bud of a daughter cell (Novick et al., 1990; TerBush, 1996). The proteins are found only at the tip of the bud, hence they determine polarity.

The *sec6/sec8* complex, also known as *exocyst*, has been implicated in exocytosis and is specifically located at sites of vesicle fusion. In yeast, the complex contains seven subunits of 70 to 155 kDa, whereas in the rat, the complex has eight components ranging from 71 to 110 kDa (see Hsu et al., 1996, 1998). In rat brain, sec6 and sec8 are two components of a 17S complex of 743 kDa homologous to the yeast Sec6/8/15 complex of 834 kDa, which is required for exocytosis. The rat brain complex associated with the plasma membrane has been implicated in exocytosis by its immunoprecipitation with *syntaxin*, a plasma membrane protein critical for neurotransmission (see above and Chapter 22).

In the rat brain, the complex is present at sites of neurosecretion such as the hippocampal synapses (Hsu et al., 1996) and is essential for survival. Mice with a mutation in Sec8 die early during embryogenesis at the primitive streak stage (Friedrich et al., 1997). In the MDCK cell line, the complex is required for calcium dependent cell adhesion (Grindstaff et al., 1998). When the cells are rendered permeable by streptolysin, Sec8 antibodies inhibit delivery of LDL receptor to the basal-lateral membrane, but not the delivery of the receptor for the nerve growth factor $p75^{NTR}$ to the apical membrane. These findings suggest that the complex is needed to recruit vesicles to specific domains. Similar conclusions were reached in neuronal tissues where the sec6/8 complex seems to specify sites for targeting vesicles at domains of neurite outgrowth and potential active zones during synaptogenesis (Hazuka et al., 1999).

B. Targeting of Plasma Membrane Proteins

Viral coat proteins with different plasma membrane targets share the transport pathway through the Golgi system (Rindler et al., 1984), as shown by immunological EM methods using colloidal gold markers of different sizes in doubly infected cells. What routes do these glycoproteins follow after leaving the TGN? Are they targeted directly to their surface of residence or do they make a stopover at the other surface?

The proteins are sorted out in the TGN (Rodriguez-Boulan and Nelson, 1989). Whether the delivery is direct or indirect can be determined by growing the cells in sheets so that either the apical or basolateral surface is separately accessible to antibodies or proteases. A block in the transport would implicate a stopover in the alternative surface. The answers are not simple. Some of the glycoproteins are sorted out in the TGN so that they are targeted directly to either the apical or the basolateral surface. The delivery of the G-protein of VSV to the basolateral surface (Pfeiffer et al., 1985) and of the hemagglutinin of influenza virus to the apical surface is direct (Matlin et al., 1983; Matlin and Simons, 1984). However, other patterns are possible. In rat hepatocytes, all membrane proteins appear to be delivered to the basolateral surface and eventually, the proteins destined to the

apical surface are then rerouted to their final destination (Bartles et al., 1987; Schell et al., 1992). In other cells, such as a polarized intestinal epithelium cell line (Caco-2), apical proteins can either proceed directly to the apical surface or arrive first to the basolateral surface (e.g., Matter et al., 1990a, Le Bevic et al., 1990).

How are the membrane proteins targeted? The targeting need not differ in principle from other targeting processes. The transported protein may have a targeting domain. Targeting could also have an entirely different mechanism, possibly involving the lipid components of the membrane and acyl chains attached to the targeted protein. As discussed below, there is evidence in some cases, for a targeting mechanism involving glycosphingolipids. The sorting machinery (such as the TGN) must recognize the signal. In addition, the protein must then be targeted to the appropriate membrane site, possibly by the presence of another targeting domain. A separate but related problem is the preservation of the makeup of the target membrane itself.

Originally, most investigators assumed that plasma membrane proteins were targeted by a signal to the apical surface, a basolateral targeting occurring by default. However, sorting signals have been demonstrated in the cytoplasmic domain of proteins destined to the basolateral surface (Hunzinker et al., 1991, Casanova et al., 1991, Mostov et al, 1992). One set of signals appears to be contained in the Tyr-containing signals for endocytosis via clathrin-coated pits (Mostov et al., 1992). Mutations of the cytoplasmic tail block basolateral targeting and the proteins are delivered to the apical surface. Internalization signals, required for endocytosis, can substitute for basolateral signals (Collawn et al., 1991). It has been suggested that the signal consists of the presence of a reverse β-turn in the protein. Some signals are distinct from the endocytotic signal (e.g., Hunziker et al., 1991, Aroeti et al., 1993) and, in some cases, mutation of the cytoplasmic Tyr that blocks endocytosis has no effect on basolateral sorting (Hunziker et al., 1991).

A short amino acid sequence (approximately 14 residues) serves as a signal for basolateral localization of some proteins (Yokode et al., 1992; Mostov et al., 1992). When expressed in livers of transgenic mice, the LDL receptor containing this sequence is targeted to the basolateral surface. Mutant receptors lacking the sequence are delivered to the apical side. The probable sequence of a cytoplasmic domain is Arg Asn X Asp XX Ser/Thr XX Ser, perhaps recognized by an adaptor molecule of the Golgi.

In polarized MDCK cells the transferrin receptor (TR) is localized in the basolateral surface. After binding transferrin, ligand and receptor are taken up in coated pits by endocytosis and then returned to the basolateral surface. TR, whether synthesized de novo or recycled from vesicles, depends on its cytoplasmic tail for targeting. The targeting signal for both pathways is contained in residues 19 to 41. However, within this region the targeting sequence for the biosynthetic pathway is distinct from that for the endocytotic pathway (Odorizzi and Trowbridge, 1997)

A variety of observations suggest more complexity than that presented in this discussion. For example, integrins and laminin have been shown to be transported from the TGN to the basolateral surface in separate vesicles (Boll et al., 1991), demonstrating the possibility that more than one pathway may be responsible for the same localization.

The glycosyl phosphatidylinositol (GPI) anchor of certain proteins (see Chapter 4) could serve as an apical signal (see Simons and Ikonen, 1997) by clustering with glycosphingolipids, forming "rafts" that may correspond, at least in part, to caveolin containing elements (see Chapter 4 and Chapter 9). After the separation of lipid-linked proteins in the Golgi, vesicle budding could segregate them from other components. A role of GPI in apical targeting has been demonstrated in Madin-Darby canine kidney (MDCK) cells and intestinal cells by attaching a GPI anchor to proteins not originally destined to the apical surface (e.g., see Soole et al., 1985; Brown et al., 1989; Lisanti et al., 1989). Conversely, replacement of the GPI anchor of placental alkaline phosphatase with the transmembrane and cytoplasmic domains of VSV G, switched its targeting from the apical to the basolateral surface (Brown et al., 1989).

The recognition of GPI-anchored proteins could occur in an early step of the *cis*-Golgi (see Brown and Rose, 1992) because these proteins were found associated with the glycosphingolipid microdomains at stages requiring *cis*-Golgi reactions.

Although many observations are compatible with the raft -GPI model, other significant factors are likely to come into play. Fisher rat thyroid cells (FRT) (Zurzolo et al., 1993) and MDCK Concanavalin A-resistant cells (MDCK-ConAr) (Zurzolo et al., 1994) behave differently from other polarized epithelial cell lines. FRT cells target glycosphingolipids and six out of nine detectable endogenous GPI-anchored proteins to the basolateral surface. In contrast, two other GPI-anchored proteins are apical and one is present at either surface. Transfection (see Chapter 1) of several model GPI proteins, previously shown to be apically targeted in MDCK cells, also led to unexpected results. The GPI anchored form of *Decay accelerating factor* (DAF) was targeted to the basolateral domain. Similarly, the *Herpes simplex* gD-1 protein attached to GPI in the form of fusion protein, gD1-DAF, was targeted basolaterally, where gD1-DAF was delivered directly from the Golgi apparatus to the basolateral surface.

Similar discrepancies are exhibited by MDCK-ConAr cells. In most polarized epithelial cell lines (e.g., MDCK), both gD1-DAF and glucosylceramide (GlcCer) are sorted to the apical membrane. In contrast, in MDCK-ConAr cells, gD1-DAF was sorted to both surfaces, but GlcCer was still targeted to the apical surface (Zurzolo et al., 1994). In both MDCK and MDCK-ConAr cells, gD1-DAF became associated with TX-100-insoluble GSL clusters during transport to the cell surface. In the FRT cell line gD1-DAF and GlcCer were both targeted basolaterally. Although gD1-DAF and glucosyl ceramide distributed to the basolateral surface, gDI-DAF did not associate with membrane clusters. Among several possible alternatives, this surprising finding could be explained by the presence of a small subset of specialized clusters available for basolateral targeting.

In addition, in some cases, the role of the GPI anchor is in doubt. In MDCK cells, Thy-1 (a glycoprotein of 25 kDa and unkownn function present in mouse thymocytes, T-cells and neurons) anchored to GPI was delivered apically. However, a truncated form of Thy-1, lacking 22 out of 31 hydrophobic amino acids at the carboxy-terminal, still resulted in apical secretion of Thy-1 despite the fact that the GPI anchor was not attached (Powell et al., 1991). It would therefore seem that Thy-1 contains apical targeting information in its protein sector, as well as in the GPI anchor.

GPI anchors are synthesized in the ER and added to primary translation products while they are

being translocated across the ER membrane (see Thomas et al, 1990). In the plasma membrane, the GPI anchors are attached to the external leaflet of the plasma membrane. The motif in the protein directing attachment to the GPI resides in the amino acid sequence. The signal at the carboxy-end of the proteins differ with the protein (see Medof et al., 1996). Characteristically, GPI anchored proteins lack charged amino acids at the carboxy-end of the protein. An additional signal, 15 to 30 amino acids upstream of the terminal hydrophobic stretch of the protein, is needed for GPI anchoring (see Medof et al., 1996).

In at least some cases, targeting may be a function of the lipid composition of the membrane. The outer leaflet of the apical membrane of the epithelial Madine-Darby canine kidney (MDCK) cells contains mainly glycosphingolipids held together by H-bonds. These lipids exclude glycerol-based phospholipids (Thompson and Tillack, 1985). Similarly, glycosphingolipid clusters are present in other membranes (see Chapter 4, Section VI).

Signals other than GPIs have also been found (e.g. see Weimbs et al., 1997). Saturated acylated proteins (e.g., Src family kinases) appear to be targeted to detergent resistant membrane domains which are thought to correspond to rafts (see Chapter 4). In contrast prenylated proteins (e.g., Ras) are usually not found in these domains (Melkonian et al., 1999). This is likely to be the consequence of the ordered environment of lipid rafts or caveolae (see Brown and London, 1998) which is more likely to favor the incorporation of acyl chains. These chains tend to be present in an extended configuration, whereas the prenyl moieties are branched and bulky. Studies in intact cells confirm these observations (Zacharias et al., 2002). This study used FRET (see Chapter 1) a technique which allows the study the interaction between two chromatophores separated by 10 nm or less and therefore the proximity of the protein pairs. GFP variant pairs (donor-acceptor) were used. They were combined to peptides with consensus sequences for either acylation or prenylation or with caveolin, a protein components of rafts. The fluorescent GFP variants had to be modified to prevent their dimerization. Again, the acyl proteins but not the prenylated proteins were found in rafts. Furthermore, in contrast to the acylated proteins, the prenylated proteins were found insensitive to cholesterol depletion.

The finding of the location of proteins in microdomains or caveolae is significant in relation to regulatory cascades. The lipid microdomains contain signaling kinases so that when receptors are bound to their ligand they move to these microdomains initiating a signaling cascade (see Pierce, 2002).

N-glycans of secretory (Scheiffele et al., 1995) or in N- and O-glycans of membrane proteins act as apical signals (Yeaman et al., 1997; Gut et al., 1998). The hypothesis that N-linked carbohydrates are responsible for apical targeting was tested with three membrane proteins (Gut et al., 1998). Two are normally not glycosylated and another is a glycoprotein. In all three cases, N-linked carbohydrates were clearly able to mediate apical targeting. However, the presence of cytoplasmic basolateral targeting motifs remained basolateral even when attached to N-linked sugars. To compare the role of GPI and N-glycans, GPI was attached to the rat growth hormone (rGH) which is normally secreted in non-polarized manner (Benting et al., 1999). This modification did not lead to an apical delivery. However, the addition of N-glycans to the GPI-anchored rGH did target mostly to the apical surface. A transmembrane form of rGH accumulated intracellularly unless attached to

N-glycans that delivered them to the apical surface. The N-or O-linked oligosaccharide could possibly attach to lectins present in the rafts (such as VIP36) (see Fiedler and Simons, 1995).

As with other transport systems, it has been possible to partially reconstitute the basolateral targeting system (Gravotta et al., 1990). The fusion of the vesicles to the surface requires energy and cytoplasmic extracts. A GTP-binding protein is presumed to be involved because the fusion is inhibited by GTPγS. As already discussed, GTP-binding proteins of the Rab family are thought to be involved in fusion of vesicles in the intracellular transport system. There are some indications that Rab8 is localized in the basolateral transport vesicles in MDCK cells.

Once incorporated into one of the cell membrane faces, the diffusibility of the proteins in the plane of the membrane appears to be constrained by binding to the cortical cytoskeleton (Vega-Salas, 1987). Na^+-K^+, ATPase, the Na^+-channel, and the anion exchange protein bind to ankyrin/fodrin complexes (see Nelson and Hammerton, 1989).

The probable interactions involved in establishing and maintaining cell polarity are summarized in Fig. 3 (Nelson, 1992).

C. Targeting in Secretion and Transcytosis

Secretion may also require targeting to a specific cell surface. This may be a consequence of the common mechanism accounting for plasma membrane transport and secretion. Although some cells release secretory products around their perimeter, many polar cells release them only in specific regions of the membranes. Thus, the same kind of targeting has to be considered in secretion. In MDCK cells, endogenous constitutively secreted proteins are released in the apical domain (Kondor-Koch et al., 1985; Gottlieb et al., 1986), whereas exogenous proteins (produced after transfection) release at both the apical and basolateral cell surfaces.

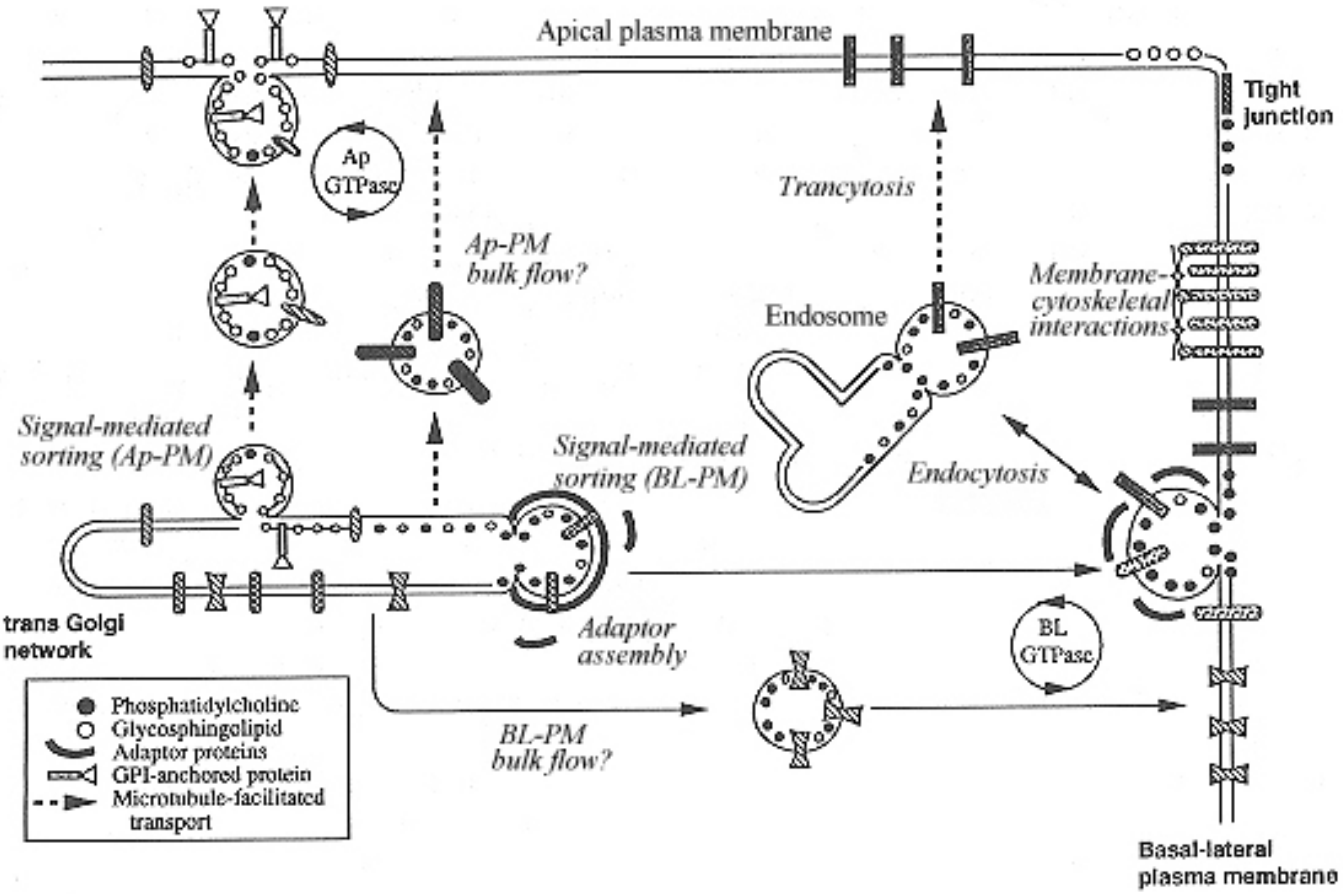


Fig. 3 Mammalian polarized epithelial cell organization of protein trafficking pathways. Protein sorting and transport between the trans Golgi network (TGN) and different cell surface domains are regulated. In the TGN, proteins can be sorted by signal-mediated or bulk flow pathways into different vesicles. Signal-mediated sorting of proteins to the apical plasma membrane (Ap-PM) is regulated by glycosphingolipid patching, whereas signal-mediated sorting of protein to the basolateral plasma membrane (BL-PM) is regulated by protein clustering of adaptor proteins; other signal-mediated pathways may also exist. Docking with targeting patches in each membrane is regulated by domain-specific GTPase cycles (Ap-GTPase, apical membrane GTPase; BL-GTPase, basal-lateral membrane GTPase) (from Nelson 1992). Reproduced by permission.

Most cells carry out receptor-mediated endocytosis (Chapter 9), in which the ligand is generally degraded in the lysosomes and the receptor is either degraded or recycled to the surface. However, in *transcytosis*, which is ubiquitous in epithelial cells, the endosomes transfer receptor and ligand to the surface of opposite polarity, so that the material traverses the cell.

The transcytosis of polymeric immunoglobulin (poly-Ig) is probably the best understood of the various known cases. Poly-Ig is produced by plasma cells and transported through epithelial cells by transcytosis. In this process, the epithelial cell adds a polypeptide, the secretory component (SC) or secretory piece, part of the receptor molecule, to the poly-Ig. The poly-Ig receptor is originally incorporated in the basolateral surface, where it binds poly-Ig. After transcytosis, the endocytotic vesicle discharges its contents at the apical surface by exocytosis and the receptor is cleaved. So far, only one signal has been identified in transcytosis, the phosphorylation of a Ser in the cytoplasmic domain of the receptor (Casanova et al., 1990).

Despite the flow of membranes from one pole of the cell to the other in transcytosis, the two surface domains remain distinct. This is illustrated by VSV G-protein, which is normally in the basolateral surface of the infected MDCK cells. When artificially inserted into the apical surface by fusing it to viral coats at low pH, the VSV-G protein is taken up by endocytosis and delivered to the basolateral surface (Matlin et al., 1983). Therefore, it would seem possible that portions of the membranes involved in transcytosis can be recycled to their original location by a process akin to transcytosis in reverse.

D. Transport of the Vesicles

What is responsible for the movement of vesicles? The information presently available suggests a varied pattern (see Bloom and Goldstein, 1998).

The diffusion coefficient of granules in cells is approximately 2.5 x 10^{-10} cm^2/s (Felder and Kam, 1994). It has been calculated that a vesicle 160 nm in diameter can diffuse for a distance of 10 μm in 10 minutes (Bloom and Goldstein, 1998). Therefore, no special mechanism is required in small cells for a variety of vesicular transport events. For a neuron, the distances from cell body to periphery, however, is prohibitive (the vesicles may have to travel 1 m or more from the cell body to the neuron terminal) and microtubular transport is essential.

Normally, the transport from the compartments intermediate between ER and Golgi (ICs) to the Golgi, occurs on microtubules (Presley et al., 1997; Scales et al., 1997) as shown by direct observation using conjugates of VSV-G glycoprotein and green fluorescent protein (see Chapter 1, Chapter 10, Section III). However, in some cases, the absence of MTs does not preclude secretion at close to normal rates (e.g., van de Moortele et al., 1993). Apparently, in these cases, the Golgi cisternae segment in ministacks. These ministacks distribute throughout the cytoplasm (Rogalski and Singer, 1984) adjacent to IC sites and the ER (Cole et al., 1996). Presumably in these cases the transport can proceed efficiently by diffusion.

These considerations and other presently available information suggest that in anterograde transport microtubules have no role in transport from ER to IC or in intra-Golgi transport. However, they are needed for transport from IC to Golgi or from TGN to the cell surface (see Lippincott-Schwartz, 1998).

In the retrograde pathway, microtubules are also involved in many steps. Golgi to ER transport is driven by kinesin (Lippincott-Schwartz et al., 1995), a microtubular motor, and microtubules are involved in the transport of endosomes and lysosomes toward the centrosome, as shown by direct observation and the use of microtubular inhibitors (Matteoni and Kreis, 1987).

In polarized epithelial cells, microtubules seem to be involved in the movement of vesicles to the apical surface (Nelson, 1991, 1992). Depolymerization of microtubules interferes with trafficking between TGN and the apical membrane (e.g., Rindler et al.,1987; Van Zeijl and Matlin, 1990) and decreases transcytosis from the basolateral to the apical surface (e.g., Matter et al., 1990b, Breitfeld et al., 1990). The presence of colchicine, vinblastine or nocodazole (all drugs which interfere with

the microtubules) redirects the vesicles from the apical to the basolateral surface. In contrast, the basolateral traffic is not affected. However, in budding yeast, microtubules do not have a role in polarized secretion (e.g., Huffaker et al., 1988; Jacobs et al., 1988). In contrast, in these cells, actin (e.g., see Novick and Botstein, 1985) has been found to be involved in conjunction with Myo2p, a myosin V (see Chapter 24) (e.g., Santos and Snyder, 1997; Catlett and Weisman, 1998; Schott et al., 2000).

The experiments carried out with mammalian cells indicate a primary role of microtubules in apical vesicular transport. The presence of myosin I in Golgi derived vesicles (Fath and Burgess, 1993) and in apical membranes (Mooseker and Coleman, 1989) also suggests a role of the actomyosin system in at least some of the processes of intracellular transport. Myosin I is found in vesicles in intestinal brush border cells where they are linked to actin filaments (Drenckhahn and Dermietzel, 1988). Similarly, myosin I was localized by immunoblotting and immunolabel negative staining of the isolated vesicles during the assembly of these cells (Fath and Burgess, 1993). This protein was found at the outer surface of Golgi associated vesicles during the assembly of these cells. The vesicles contained galactosyl transferase, a *trans*-Golgi enzyme, as well as alkaline phosphatase, an apical membrane targeted enzyme. The vesicles were also shown to bundle actin, suggesting that the actomyosin system functions in the peripheral translocation of vesicles. The results suggest an involvement of both microtubules and the actomyosin system, the latter perhaps only in the final step of the delivery in the apical pathway. However, the details are still not clear.

How are the vesicles connected to the transporting systems? Present evidence implicates *dynactin.* Dynactin is a 1.2 MDa complex of ten peptides (and includes actin) that is required for cytoplasmic dynein motility and in vitro vesicle movement (Schroer and Sheetz, 1991).

Electron microscopy of this complex shows an actin-like filament, 37 nm long, with laterally projecting sidearms (Schafer et al., 1994). A model of dynactin is shown in Fig. 4 (Schroer et al., 1996). The filamentous part of the molecule is made up of the a*ctin related protein* (Arp1) and probably one molecule of actin. The interaction between dynactin and the MT-dynein system probably involves the sidearms that contain a microtubule binding site (Waterman-Storer et al., 1995) and also bind dynein (Collins and Vallee, 1989). The most likely role of the Arp1 portion of the complex is to provide a connection to the cargo vesicle.

Many myosins of the myosin I family bind directly to phospholipid. Myosin I isoforms associate with specific membranes (Baines et al., 1995). It is entirely possible then that dynactin binding to the vesicles is mediated by myosin, although other possibilities are also likely (e.g., *ponticulin* or an *annexin*, see Schroer et al., 1996).

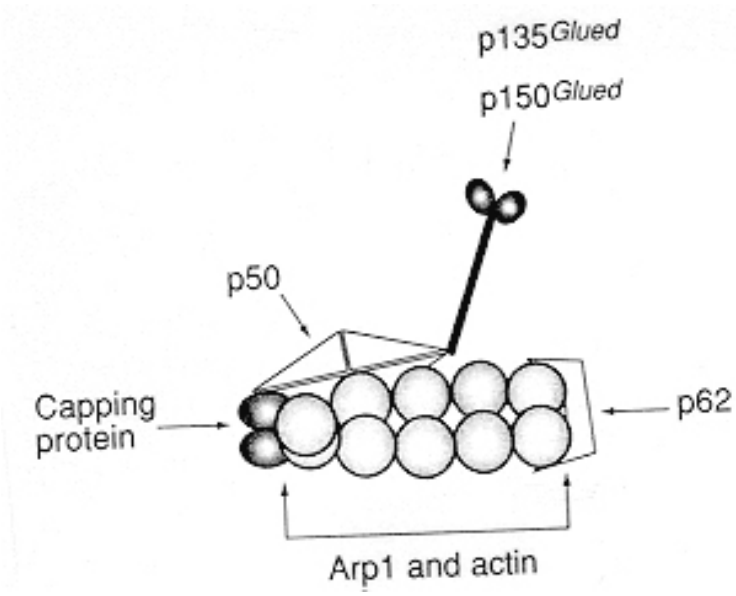


Fig. 4 Current model of dynactin structure. The localization of Arp1, actin-capping protein, p62 and $p150^{Glued}$/$P135^{Glued}$ are based on ultrastructural analysis of antibody decorated molecules. The location of actin, p50, p24 and p27 is uncertain. Because of their similar properties, actin is likely to be in the Arp1 filament. The overexpression of p50 causes $p150^{Glued}$ to dissociate suggesting the location in the diagram. From Schroer et al., 1996, reproduced by permission.

E. Recycling of the Plasma Membrane

The process of exocytosis adds a considerable amount of material to the plasma membrane. Much of this material is recycled through endocytosis. Because of this continuous recycling, the turnover time of the membranes of granules is relatively long compared to that of ordinary cytoplasmic proteins (Meldolesi, 1974).

The availability of antibodies to membrane proteins of the luminal side of the secretory vesicle membrane has permitted following the fate of the secretory vesicles in the regulated secretion of catecholamine granules (Patzak and Winkler, 1986). Glycoprotein III (gpIII) is exposed to its fluorescently labelled antibody at exocytosis. The protein appears in coated pits and vesicles in the first 5 min after exocytosis. Then it passes through the smooth ER and reappears in the trans Golgi network and in dense-core secretory granules within 30 to 45 min. The protein was never found in the cisternal lumen, indicating that the membrane itself is being recycled. Similar results were found for the transferrin receptors (Woods et al. 1986). Their presence was demonstrated in several Golgi cisternae; therefore, recycling must involve some of the same steps followed by the transport of newly synthesized protein.

These observations imply that some steps in the recycling process must involve transport in the opposite direction from that discussed in most of this chapter, that is retrograde transport. Retrograde transport is beginning to be studied by taking advantage of the effect of the antibiotic Brefeldin A (Tan et al., 1992). Brefeldin, a macrocyclic lactone synthesized by fungi, prevents the assembly of nonclathrin coated vesicles and blocks the transport from ER to Golgi (e.g., Klausner et al., 1992). The retrograde transport, however, is not inhibited, redistributing material such as enzymes from the Golgi back to the ER. This transport is also blocked by GTPγS, suggesting that

GTP-binding proteins are involved in both forward and backward transport.

F. Formation of Lysosomes and Secretory Storage Vesicles

Lysosomes

The study of the transport system in the formation of lysosomes received great impetus from the recognition of at least 30 human lysosomal storage disorders. I-cell disease, a deficiency in lysosomal enzymes, results from a failure in the recognition marker needed for targeting. Study of I-cell mutants has permitted the identification of the recognition marker, the mannose-6-phosphate (M6P) residue, and the *M6P receptor*, MPR (Sahagian et al., 1981). The receptor spans the membrane and 10 kDa of its carboxy-terminal protrudes into the cytosol (Sahagian and Steer, 1985). Two distinct but related MPRs are known (see Kornfeld, 1992). One is a type I (amino group external to the cell) transmembrane glycoprotein of 275 kDa which does not require divalent cations. The other receptor is also a type I glycoprotein of 46 kDa. The bovine and murine forms of the latter, but not the human or porcine forms, require divalent cations for optimal binding.

M6PRs bind to lysosomal hydrolases while they are transported from the *trans*-Golgi to the lysosomes. In turn, targeting signals in the M6PRs are needed to arrive at their final destination. The *Golgi-localized* γ*-ear containing, ARF-binding* (GGAs) adaptors have been found to bind to the lysosomal targeting signals of the cation-independent M6PRs, the acidic cluster-dileucine motif of their cytoplasmic tails. The GGAs, already implicated in protein trafficking between the Golgi and the endosomes, have all the properties expected for an adaptor mediating the binding of the receptors to the components needed for transport. They contain a Vps27p/Hrs/STAM (VHS) domain, binding sites for clathrin, a GTP-ARF binding domain and a domain that binds to proteins involved in coat assembly such as γ-synergin. The VHS domain of 153 residues is present in various proteins involved in endocytic trafficking (Lohi and Lehto, 1998). The M6PRs bind to the VHS domain of the GGAs. The GGAs were found to be present in the TGN, tubules and vesicles which bud from the TGN and the cell surface as expected from its presumed function (Zhu et al., 2001; Puertollano et al., 2001).

Lysosomal enzyme precursors are transported through the common pathway, as indicated by immunocytochemical experiments (Geutze et al., 1984). However, processing continues on arrival in the *cis*-Golgi (Fig. 5, Kornfeld, 1987), where lysosomal hydrolases are recognized by GlcNAc-phosphotransferase, which adds GlcNAc-phosphate to α-1,2-mannose residues of the hydrolases. The phosphotransferase probably recognizes a signal patch, because recognition is very sensitive to conformation changes. After this modification, M6P residues are exposed by removal of the N-acetylglucosamine and are recognized by MPR. The interaction between receptors and M6P residues is responsible for the lysosomal enzyme segregation (see von Figura and Hasilik, 1986; Kornfeld, 1987). The two can be detected together in buds and coated vesicles in the TGN, which eventually form lysosomes (Geutze et al., 1985, Griffiths et al., 1985).

In addition to the processing of the oligosaccharides, lysosomal enzymes are proteolytically cleaved to their mature form (Gieselman et al., 1983).

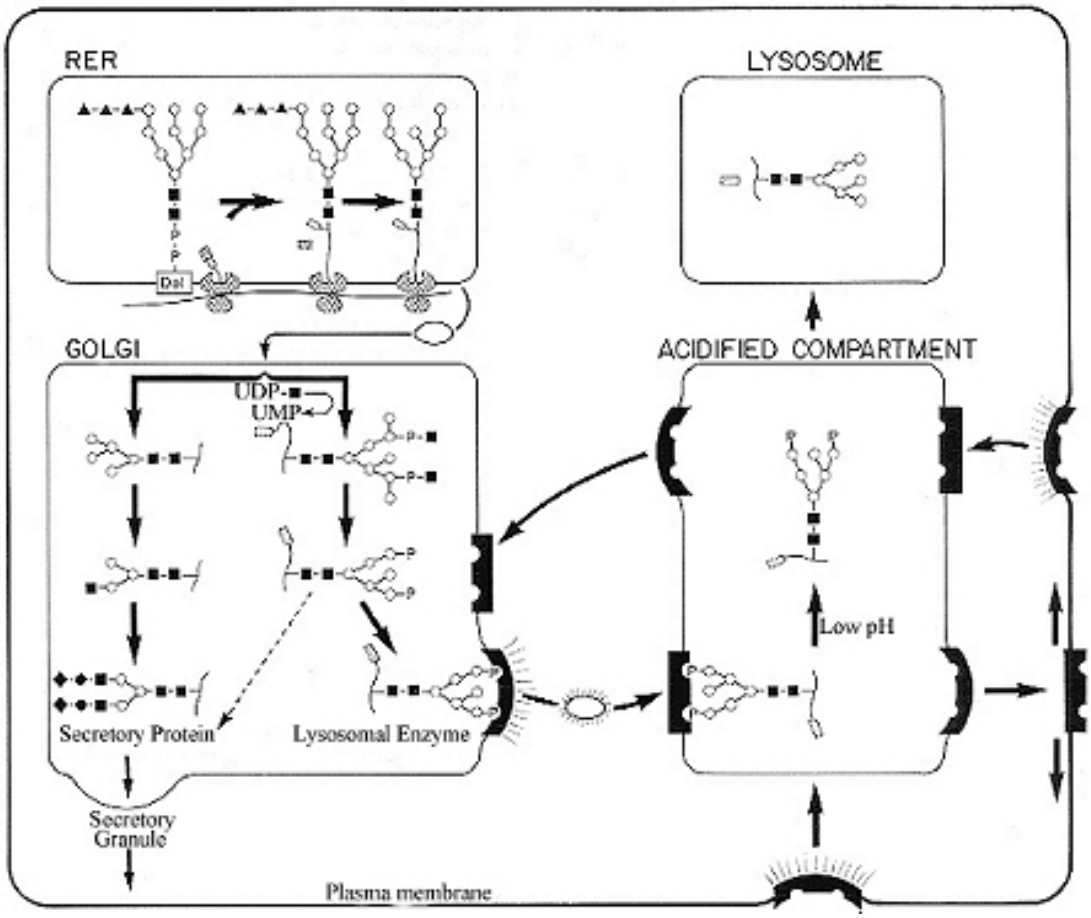


Fig. 5 Schematic pathway of lysosomal enzyme targeting to lysosomes. Lysosomal enzymes and secretory proteins are synthesized in the rough endoplasmic reticulum (RER) and glycosylated by the transfer of a performed oligosaccharide from dolichol-P-P-oligosaccharide (DOL). In the RER, the signal peptides (hatching) are excised. The proteins are translocated to the Golgi, where the oligosaccharides of secretory proteins are processedto complex-type units and the oligosaccharides of lysosomal enzymes are phosphorylated. Most of the lysosomal enzymes bind to mannose6-phosphate receptors (MPRs) (▬) and are translocated to an acidified prelysosomal compartment where the ligand dissociates. The receptors recycle back to the Golgi or to the cell surface, and the enzymes are packaged into lysosomes where cleavage of their propieces is completed (▭). The P_i may also be cleaved from the mannose residues. A small number of the lysosomal enzymes fail to bind to the receptors and are secreted along with secretory proteins (⇢). These enzymes may bind to surface MPRs in coated pits (◠) and be internalized into the prelysosomal compartment. (■) N-Acetylglucosamine; (○) mannose; (▲) glucose; (●) galactose; (◆) sialic acid. Reprinted with permission from S. Kornfield, *Federation of American Societies for Experimental Biology Journal*, Vol.1, No.6: 463, 1987.

The MPRs are recycled. Before the primary lysosomes are formed, the MPRs are sent back to the trans Golgi. Recovery of receptor probably follows its dissociation from the ligands brought about by lowering the pH, as in the case of endocytotic vesicles discussed in Chapter 9. Although most lysosomal enzymes remain segregated in vesicles, a small portion of the lysosomal enzymes are secreted and are thought to be recovered by endocytotic uptake after binding MPRs present at the surface (Willingham et al., 1981).

Secretory storage granules

Regulated secretion differs from constitutive secretion in the need to store the secreted products in the secretory vesicles until a physiological signal permits their discharge. Therefore, they must be separated from the other products destined to the cell surface. This separation occurs in the *trans*-Golgi, but the selection mechanism is still unknown. The process can be very selective. However, it also allows for packaging very different proteins in the same vesicle.

The possibility that a receptor is involved in targeting storage secretory products is also supported by the observation that the precursor of insulin, proinsulin, is bound to Golgi membranes (Munro and Pelham, 1986), indicating the likelihood that a receptor is present.

G. Synaptic Vesicles

Presynaptic cells discharge neurotransmitters by exocytosis of their synaptic vesicles. Postsynaptic membrane receptors bind the neurotransmitters. These receptors are channels that open more frequently after binding the neurotransmitters. The increased conductance of the membrane initiates the depolarization that can culminate in an action potential (see Chapter 22). The presynaptic neurotransmitter vesicles are continuously discharged and continuously reformed. Most of the recycling involves the recovery of synaptic vesicles by endocytosis and their reloading with neurotransmitter (See Chapter 22). However, there is also a turnover of the synaptic vesicles themselves; their components are degraded and resynthesized in the cell body. Eventually, the newly formed vesicles must be targeted to the nerve terminals through axonal transport. The integral proteins follow the usual path from RER to TGN. These proteins obviously can be found at synaptic sites, but also (with few exceptions) throughout the Golgi system.

All indications are that the forward or anterograde transport of newly synthesized synaptic vesicle components is microtubular and is powered by the motor protein of the *kinesin* family (Chapter 24). Kinesin is implicated by its association with vesicles (e.g., Morin et al., 1993) and by its accumulation when the axonal flow is blocked by ligation (Hirokawa et al., 1991). The vesicles and the kinesin (indentified by immunocytochemistry) accumulate on the cell body side of the ligature. Evidence from genetic studies also implicates a kinesin-like molecule. The *unc-104* gene codes for a kinesin-like motor, thought to be neuron specific in the nematode *Caenorhabditis elegans*. This protein has a kinesin-like motor domain at the amino terminal, but otherwise it has little homology to other kinesins. Mutant alleles of this gene block the accumulation of synaptic vesicles in axons (Hall and Hedgecock, 1991). The movement of other vesicles is not affected, suggesting a unique targeting role for the protein coded by the *unc-104* gene.

The study of the biogenesis of synaptic vesicle proteins can be carried out most readily in neuroendocrine cells and neurons in culture. PC12 cells, derived from pheochromocytoma of the rat adrenal medulla, have characteristics of both neural and endocrine cells. They synthesize, store and release the neurotransmitter acetylcholine (Greene and Rein, 1977) and have both regulated and constitutive secretory pathways. In these cells, regulated secretion involves large dense-core granules related to chromaffin granules. Small electron-translucent vesicles are also present and are thought to be related to cholinergic synaptic vesicles. Dense granules were purified and shown to

contain the regulated secretory protein *secretogranin II*. The synaptic protein *synaptophysin* was used as a marker for the smaller vesicles (Cutler and Cramer, 1990). Synaptophysin is a major integral protein of synaptic vesicle membranes. Pulse-chase experiments using immunoprecipitation, demonstrated that synaptophysin is associated with the smaller vesicles and does not occupy the dense granules at any time. These findings indicate two separately regulated secretion routes. In another study, synaptophysin was also traced by pulse-chase (Régnier-Vigouroux et al., 1991). It was found to follow a route involving the trans Golgi network (TGN). The protein was found to reach the cell surface from the TGN with a half time of 10 min and was found to cycle between cell surface and vesicles. The endosomal fraction was identified by exposing the terminals to peroxidases and tracing this enzyme. Peroxidase is taken up by endocytosis and represents the material dissolved in the external medium.

The molecular mechanism by which the synaptic vesicle proteins are sorted out is not clear. A common sequence motif that could be recognized by receptors used in targeting has not been found in examining the various proteins of the secretory vesicles, and signals involving secondary or tertiary folding are suspected. A role of specific complexes of synaptic proteins in targeting is also possible because multimeric complexes can be recovered from synaptic vesicles after detergent treatment (Bennett et al., 1992).

SUGGESTED READING

Bloom, G.S. and Goldstein, L.S.B. (1998) Cruising along microtubule highways: how membranes move through the secretory pathway, *J. Cell Biol.* 140:1277-1280. (MedLine)

Kirchhausen, T. (2000) Clathrin, *Annu. Rev. Biochem.* 69:699-727. (MedLine)

Lippincott-Schwartz, J. (1998) Cytoskeletal proteins and Golgi dynamics, *Curr. Opin. Cell Biol.* 10:52-59.

McNiven, M.A., Cao, I., Pitts, K.R. and Yoon, I. (2000) The dynamin family of mechanoenzymes: pinching in new places, *Trends Biochem Sci* 25:115-120.(MedLine)

Mellman, I. and Warren, G. (2000) The road taken: past and future foundations of membrane traffic, *Cell* 100:99-112. (MedLine)

Morgan, A. (1995) Exocytosis, *Essays in Biochemistry* 30:77-95.

Nelson, W. J. (1992) Regulation of cell surface polarity from bacteria to mammals, *Science* 258:948-955. (MedLine)

Robinson, M.S. (1997) Coats and vesicle budding, *Trends in Cell Biol.* 7:99-102.

Schekman, R. and Orci, L. (1996) Coat proteins and vesicle budding, *Science* 271:1526-1533. (MedLine)

Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes, *Nature* 387:569-572. (MedLine)

Springer, S., Spang, A. and Schekman, R. (1999) A primer on vesicle budding, *Cell* 97:145-148. (MedLine)

Weimbs, T., Low, S.H., Chapin, S.J. and Mostov, K.E. (1997) Apical targeting in polarized epithelial cells: there's more afloat than rafts, *Trends in Cell Biol.* 7:393-399.

Wieland, F. and Harter, C. (1999) Mechanisms of vesicle formation: insights from the COP system, *Curr. Opin. Cell Biol.* 11:440-446. (MedLine)

WEB RESOURCES

Kirchhausen, T. and Bruce, A. Clathrin-coat formation in time and space: modelling. http://www.hms.harvard.edu/news/clathrin

REFERENCES

Search the textbook

Back to Chapter 11

REFERENCES

Aalto, M.K., Keränen, S. and Ronne, H. (1992) A family of proteins involved in intracellular transport, *Cell* 68:181-182. (Medline)

Aalto, M.K., Ronnel, H. and Carragheenin, S. (1993) Yeast syntaxis Sso1p and Ssop2 belong to a family of related membrane proteins that function in vesicular transport, *EMBO J.* 12:4095-4104. (Medline)

Ahle, S. and Ungewickell, E. (1989) Identification of clathrin binding subunit in the HA-2 adaptor protein complex, *J. Biol. Chem.* 264:20089-20093. (Medline)

Anderson, J.M. and Van Itallie, C.M. (1995) Tight junctions and the molecular basis for regulation of paracellular permeability, *Am. J. Physiol.* 269:G467-475. (Medline)

Ando-Akatsuka, Y., Saitou, M., Hirase, T., Kishi, M., Sakakibara, A., Itoh, M., Yonemura, S., Furuse, M. and Tsukita, S. (1996) Interspecies diversity of the occludin sequence: cDNA cloning of human, mouse, dog, and rat-kangaroo homologues, *J. Cell Biol.* 133:43-47. (Medline)

Aridor, M., Weissman, J., Bannykh, S., Nuoffer, C. and Balch, W.E. (1998) Cargo selection by the COPII budding machinery during export from the ER, *J. Cell Biol.* 141:61-70. (MedLine)

Aroeti, B., Kosen, P.A., Kuntz, I.D., Cohen F.E. and Mostov, K.E. (1993) Mutational and secondary structural analysis of the basolateral sorting signal of the polymeric immunoglobulin receptor, *J. Cell. Biol.* 123:1149-1160. (Medline)

Baines, I.C., Corigliano-Murphy, A. and Korn, E.D. (1995) Quantification and localization of phosphorylated myosin I isoforms in *Acanthamoeba castellanii*, *J. Cell Biol.* 130:591-603. (Medline)

Bhat, M.A., Izaddoost, S., Lu, Y., Cho, K.O., Choi, K.W. and Bellen, H.J. (1999) Discs Lost, a novel multi-PDZ domain protein, establishes and maintains epithelial polarity, *Cell* 96:833-845. (MedLine)

Balch, W. E., Dunphy, W. G., Braell, W. A. and Rothman, J. E. (1984a) Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine, *Cell* 39:405-416. (Medline)

Balch, W. E., Glick, B. S. and Rothman, J. E. (1984b) Sequential intermediates in the pathway of intercompartmental transport in a cell free system, *Cell* 39:525-536. (Medline)

Balch, W. E., Wagner, R. R. and Keller, D. S. (1987) Reconstitution of transport vesicular stomatitis virus G protein from the endoplasmic reticulum to the Golgi complex using a cell free system, *J. Cell Biol.* 104:749-760. (Medline)

Balch, W.E., McCaffery, J.M., Pluttner, H. and Farquhar, M.G. (1994) Vesicular stomatitis virus glycoprotein is sorted and concentrated during export from the endoplasmic reticulum, *Cell* 76:841-852. (Medline)

Balda, M.S., Whitney, J.A., Flores, C., Gonzalez, S., Cereijido, M. and Matter, K. (1996) Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein, *J. Cell Biol.* 134:1031-1049. (Medline)

Bankaitis, V.A., Aitken, J.R., Cleves, A.E. and Dowhan, W. (1990) An essential role for a phospholipid transfer protein in yeast Golgi function, *Nature* 347:561-562. (Medline)

Bannykh, S.I. and Balch, E.E. (1997) Membrane dynamics at the endoplasmic reticulum-Golgi interface, *J. Cell Biol.*138:1-4. (Medline)

Barlowe, C. (1998) COPII and selective export from the endoplasmic reticulum, *Biochim. Biophys. Acta* 1404:67-76. (MedLine)

Barlowe, C. and Shekman, R. (1993) SEC12 encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER, *Nature* 365:347-349. (Medline)

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Ravazzola, M. Amherdt, M. and Shekman, P. (1994) COPII: a membrane coat formed by SEC proteins that drive vesicle budding from the endoplasmic reticulum, *Cell* 77: 895-907. (Medline)

Barnard, R.J., Morgan, A. and Burgoyne, R.D. (1997) Stimulation of NSF ATPase activity by α-SNAP is required for SNARE complex disassembly and exocytosis, *J. Cell Biol.* 139:875-883. (Medline)

Barroso, M., Nelson, D.S. and Sztul, E.(1995) Transcytosis-associated protein (TAP)/p115 is a general fusion factor required for binding of vesicles to acceptor membranes, *Proc. Natl. Acad. Sci. USA* 92:527-531. (MedLine)

Bartles, J. R., Feracci, H. M., Stieger, B. and Hubbard, A. L. (1987) Biogenesis of the rat hepatocyte

plasma membrane in vivo: comparison of the pathways taken by apical and basolateral proteins using subcellular fractionation. *J. Cell Biol.* 105:1241-1251. (Medline)

Beck, K.A. and Nelson, W.J. (1998) A spectrin membrane skeleton of the Golgi complex, *Biochim. Biophys. Acta.* 1404:153-160. (Medline)

Beckers, C.J. and Balch, W.E. (1989) Calcium and GTP: essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus, *J. Cell Biol.* 108:1245-1256. (MedLine)

Bednarek, S.Y., Ravazzola, M., Hosobuchi, M., Amherdt, M., Perrelet, A., Shekman, R. and Orci, L. (1995) COPI and CopII-coated vesicles bud directly from the endoplasmic reticulum in yeast, *Cell* 83:1183-1196. (Medline)

Bennett, M.K., Calakos, N., Kreiner, T. and Scheller, R.H. (1992) Synaptic vesicle membrane proteins interact to form a multimeric complex, *J. Cell Biol.* 116:761-775. (Medline)

Benting, J.H., Rietveld, A.G. and Simons, K. (1999) N-Glycans mediate the apical sorting of a GPI-anchored, raft-associated protein in Madin-Darby canine kidney cells, *J. Cell Biol.* 146:313-320. (Medline)

Berditchevski, F., Tolias, K.F., Wong, K., Carpenter, C.L. and Hemler, M.E. (1997) A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase, *J. Biol. Chem.* 272:2595-2598. (Medline)

Bilder, D. and Perriman, N. (2000) Localization of apical epithelial determinants by the basolateral PDZ protein Scribble, *Nature* 403:676-680. (Medline)

Block, M.R., Glick, B.S., Wilcox, C.A., Wieland, F.T. and Rothman, J.E. (1988) Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport, *Proc. Natl. Acad. Sci. USA* 85:7852-7856. (Medline)

Bloom, G.S. and Goldstein, L.S.B. (1998) Cruising along microtubule highways: how membranes move through the secretory pathway, *J. Cell Biol.* 140:1277-1280. (Medline)

Boll, W., Partin, J.S.,Katz, A.I., Caplan, M.J.. and Jamieson, J.D. (1991) Distinct pathways for basolateral targeting of embrane and secretory proteins in polarized epithelial cells, *Proc. Natl. Acad. Sci. USA* 88:8592-8596. (Medline)

Braell, W. A., Balch, W. E., Dobbertin, D. C. and Rothman, J. E. (1984a) The glycoprotein that is transported between successive compartments of the Golgi in a cell free system resides in stacks of cisternae, *Cell* 39:511-524. (Medline)

Breckenridge, L.J. and Almers, W. (1987) Currents through the fusion pore that forms during exocytosis of a secretory vesicle, *Nature* 328:814-817. (MedLine)

Breitfeld, P.P., McKinnon, W.C. and Mostov, K.E. (1990) Effect of nocodazole on vesicular traffic to the apical and basolateral surfaces of polarized MDCK cells, *J. Cell Biol*. 111:2365-2373. (Medline)

Bremser, M., Nickel, W., Schweikert, M., Ravazzola, M., Amherdt, M., Hughes, C.A., Sollner, T.H., Rothman, J.E. and Wieland, F.T. (1999) Coupling of coat assembly and vesicle budding to packaging of putative cargo receptors, *Cell* 96:495-506. (MedLine)

Broadie, K., Prokop, A., Bellen, H.J., O'Kane, C.J., Schulze, K.L. and Sweeney, S.T. (1995) Syntaxin and synaptobrevin function downstream of vesicle docking in *Drosophila*, *Neuron* 1 5:663-673. (MedLine)

Brodsky, F.M. (1988) Living with clathrin: its role in intracellular membrane traffic, *Science* 242:1396-1402. (Medline)

Brodsky, F.M. (1997) New fashions in vesicle coats, *Trends in Cell Biol.* 7:175-179.

Brown, D.A. and Rose, J.K. (1992) Sorting of GPI-anchored proteins to the glycolipid-enriched membrane subdomains during transport to the apical cell surface, *Cell* 68:533-544. (Medline)

Brown, D.A. and London, E. (1998) Structure and origin of ordered lipid domains in biological membranes, *J. Membr. Biol.* 164:103-114. (MedLine)

Brown, D.A., Crise, B., and Rose, J.K. (1989) Mechanism of membrane anchoring affects polarized expression of two proteins in MDCK cells, *Science* 245:1499-1501. (Medline)

Brown, H.A., Gutowski, S., Moomaw, C.R., Slaughter, C. and Sternweis, P.C. (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity, *Cell* 75:1137-1144. (Medline)

Cao, X., Ballew, N., and Barlowe, C. (1998a) Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins, *EMBO J.* 17:2156-2165. (Medline)

Cao, H., Garcia, F. and McNiven, M.A (1998b) Differential distribution of dynamin isoforms in mammalian cells, *Mol. Biol. Cell* 9:2595-2609. (MedLine)

Carpenter, C.L. and Cantley, L.C. (1996) Phosphoinositide kinases, *Curr. Opin. Cell Biol.* 8:153-158. (Medline)

Casanova, J.E., Breitfeld, P.P., Ross, S.A., and Mostov, K.E. (1990) Phosphorylation of a polymeric immunoglobulin receptor required for efficient transcytosis, *Science* 248:742-745. (Medline)

Casanova, J.E., Apodaka, G. and Mostov, K.E. (1991) An autonomous signal for basolateral sorting in the cytoplasmic domain of the polymeric immunoglobulin receptor, *Cell* 66:65-75. (Medline)

Catlett, N.L and Weisman, L.S. (1998) The terminal tail region of a yeast myosin-V mediates its attachment to vacuole membranes and sites of polarized growth, *Proc. Natl. Acad. Sci. USA* 95:14799-14804.

Chandler, D.E. and Heuser, J.E. (1980) Arrest of membrane fusion events in mast cells by quick-freezing, *J. Cell Biol.* 86:666-674. (MedLine)

Chapman, E.R., Au, S., Barton, N. and Jahn, R. (1994) SNAP-25, a t-SNARE which binds to syntaxin and synaptobrevin via domains that may form coiled coils, *J. Biol. Chem.* 269:27427-27432. (Medline)

Chaudhary, A., Gu, Q.M., Thum, O., Profit, A.A., Qi, Y., Jeyakumar, L., Fleischer, S. and Prestwich, G.D. (1998) Specific interaction of Golgi coatomer protein α-COP with phosphatidylinositol 3,4,5-trisphosphate, *J. Biol. Chem.* 273:8344-8350. (Medline)

Cheever, M.L., Sato, T.K., de Beer, T., Kutateladze, T.G., Emr, S.D. and Overduin, M. (2001) Phox domain interaction with PtdIns(3)P targets the Vam7 t-SNARE to vacuole membranes, *Nature Cell Biol.* 3:613-618. (MedLine)

Chen, Y.A. and Scheller, R.H. (2001) SNARE-mediated membrane fusion, *Nature Rev. Mol. Cell Biol.* 2:98-106. (MedLine)

Clark, E.A. and Brugge, J.S. (1995) Integrins and signal transduction pathways: the road taken, *Science* 268:233-239. (MedLine)

Clary, D.O., Griff, I.C., Rothman, J.E. (1990) SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast, *Cell* 61:709-721. (Medline)

Cohen, A.R., Woods, D.F., Marfatia, S.M., Walther, Z., Chishti, A.H., Anderson, J.M. and Wood, D.F. (1998) Human CASK/LIN-2 binds syndecan-2 and protein 4.1 and localizes to the basolateral membrane of epithelial cells, *J. Cell Biol.* 142:129-138. (MedLine)

Cole, N.B., Sciaky, N., Marotta, A., Song, J. and Lippincott-Schwartz, J. (1996) Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites, *Mol. Biol. Cell.* 7: 631-650. (Medline)

Collawn, J.F., Kuhn, L.A., Liu, L.-F. C., Tainer, J.A. and Trowbridge, I.S. (1991) Transplanted LDL and mannose-6-phosphate receptor internalization signals promote high-efficiency endocytosis of transferrin receptor, *EMBO J.* 10:3247-3254. (Medline)

Collins, C.A. and Vallee, R.B. (1989)Preparation of microtubules from rat liver and testis: cytoplasmic dynein is a major microtubule associated protein, *Cell Motility and Cytosk.* 14:491-500. (Medline)

Colombo, M.I., Beron, W. and Stahl, P.D. (1997) Calmodulin regulates endosome fusion, *J. Biol. Chem.* 272:7707-7712. (MedLine)

Confalonieri, F. and Duguet, M. (1995) A 200-amino acid ATPase module in search of a basic function, *BioEssays* 17:639-650. (Medline)

Cosson, P. and Letourneur, F. (1994) Coatomer interaction with di-lysine endoplasmic reticulum retention motifs, *Science* 263:1629-1631. (MedLine)

Cosson, P. and Letourneur, F. (1997) Coatomer (COPI)-coated vesicles: role in intracellular transport and protein sorting, *Curr. Opin. Cell Biol.* 9:484-487. (MedLine)

Cosson, P., Demolliere, C., Henneke, S., Duden, R. and Letourneur, F. (1996)δ and ζ-COP, two coatomer subunits homologous to clathrin-associated proteins are involved in ER retrieval, *EMBO J.* 15:1792-1798. (Medline)

Cowles, C.R., Odorizzi, G., Payne, G.S., Emr, S.D. (1997) The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole, *Cell* 91:109-118. (Medline)

Cremona, O., Di Paolo, G., Wenk, M.R., Luthi, A., Kim, W.T., Takei, K., Daniell, L., Nemoto, Y., Shears, S.B., Flavell, R.A., McCormick, D.A. and De Camilli, P (1999) Essential role of phosphoinositide metabolism in synaptic vesicle recycling, *Cell* 99:179-188. (MedLine

Cutler, D.F. and Cramer, L.P. (1990) Sorting during transport to the surface of PC12 cells: divergence of synaptic vesicle and secretory granule proteins, *J. Cell Biol.* 110:721-730. (Medline)

De Camilli, P., Emr, S.D., McPherson, P.S. and Novick, P. (1996) Phosphoinositides as regulators in membrane traffic, *Science* 271:1533-1539. (Medline)

Dell'Angelica, E.C., Ohno, H., Ooi, C.E., Rabinovich, E., Roche, K.W. and Bonifacino J.S. (1997) AP-3: an adaptor-like protein complex with ubiquitous expression, *EMBO J.* 16:917-928. (Medline)

De Matteis, M.A. and Morrow, J.S. (1998) The role of ankyrin and spectrin in membrane transport and

domain formation, *Curr. Opin. Cell Biol.* 10:542-549. (Medline)

de Vries, K.J., Heinrichs, A.A., Cunningham, E., Brunink, F., Westerman, J., Somerharju, P.J., Cockcroft, S., Wirtz, K.W. and Snoek, G.T. (1995) An isoform of the phosphatidylinositol-transfer protein transfers sphingomyelin and is associated with the Golgi system, *Biochem. J.* 310:643-649. (Medline)

Diaz, R., Mayorga, L.S., , Weidman, P.J., Rothman, J.E. and Stahl, P.D.(1989) Vesicle fusion following receptor-mediated endocytosis requires a protein active in Golgi transport, *Nature* 339:398-400. (Medline)

Dominguez, M., Dejgaard, K., Fullekrug, J., Dahan, S., Fazel, A., Paccaud, J.P., Thomas, D.Y., Bergeron, J.J. and Nilsson, T. (1998) gp25L/emp24/p24 protein family members of the cis-Golgi network bind both COP I and II coatomer, *J. Cell Biol.* 140:751-765. (MedLine)

Donaldson, J.G., Finazzi, D. and Klausner, R.D. (1992) Brefeldin A inhibits Golgi-membrane catalyzed exchange of guanine nucleotide into ARF protein, *Nature* 360:350-352. (Medline)

Drenckhahn, D. and Dermietzel. R. (1988) Organization of the actin filament cytoskeleton in the intestinal brush border: a quantitative and qualitative immunoelectron microscope study, *J. Cell Biol*. 107:1037-1048. (Medline)

Drubin, D.G. and Nelson, W.J. (1996) Origins of cell polarity, *Cell* 84:335-344. (MedLine)

Duden, R., Allan, V. and Kreis, T. (1991a) Involvement of p-COP in membrane traffic through the Golgi complex, *Trends Cell Biol.* 1:14-19.

Duden, R., Griffiths, G., Frank, R., Argos, P. and Kreis, T. E. (1991b) p-COP, a 110 kd protein associated with non-clathrin-coated vesicles and Golgi complex, shows homology to ξβ-adaptin, *Cell* 64:649-665. (Medline)

Eger, A., Stockinger, A., Schaffhauser, B., Beug, H. and Foisner, R. (2000) Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of β-catenin and upregulation of β-catenin/lymphoid enhancer binding factor-1 transcriptional activity, *J. Cell Biol.* 148:173-188. (MedLine)

Farquhar, M.G. and Palade, G.E. (1963) Junctional complexes in various epithelia, *J. Cell Biol.* 17: 375-412.

Ferro-Novick, S. and Jahn, R. (1994) Vesicle fusion from yeast to man, *Nature* 370:191-193. (Medline)

Fiedler, K. and Simons, K. (1995) The role of N-glycans in the secretory pathway, *Cell* 81:309-312. (Medline)

Frölich K.U. http://yeamob.pci.chemie.uni-tuebingen. de/AAA/Tree.html

Fukuda, R., McNew, J.A., Weber, T., Parlati, F., Engel, T., Nickel, W., Rothman, J.E. and Söllner, T.H. (2000) Functional architecture of an intracellular membrane t-SNARE, *Nature* 407:198-202. (MedLine)

Füllekrug, J., Suganuma, T., Tang, B.L., Hong, W., Storrie, B. and Nilsson, T. (1999) Localization and recycling of gp27 (hp24gamma3): complex formation with other p24 family members, *Mol. Biol. Cell* 10:1939-1955. (MedLine)

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita, S. and Tsukita, S. (1993) Occludin: a novel integral membrane protein localizing at tight junctions, *J. Cell Biol.* 123:1777-1788. (Medline)

Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K. and Tsukita, S.(1998a) Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin, *J. Cell Biol.* 141:1539-1550. (Medline)

Furuse, M., Sasaki, H., Fujimoto, K. and Tsukita, S. (1998b) A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts, *J. Cell Biol.* 143:391-401. (Medline)

Faundez, V., Horng, J.T. and Kelly, R.B. (1998) A function for the AP3 coat complex in synaptic vesicle formation from endosomes, *Cell* 93:423-432. (Medline)

Fath, K.R. and Burgess, D.R. (1993) Golgi derived vesicles from developing epithelial cells bind actin filaments and possess myosin-I as a cytoplasmically oriented peripheral membrane protein, *J. Cell Biol.* 120:117-27. (Medline)

Felder, S. and Kam, Z. (1994) Human neutrophil motility: time-dependent three-dimensional shape and granule diffusion, *Cell. Motil. Cytoskel.* 28: 285-302. (Medline)

Fiedler, K., Veit, M., Stamens, M.A. and Rothman, J.E. (1996) Bimodal interaction of coatamer with p24 family of putative cargo receptors, *Science* 273:1396-1399. (Medline)

Friedrich, G.A., Hildebrand, J.D. and Soriano, P. (1997) The secretory protein Sec8 is required for paraxial mesoderm formation in the mouse, *Dev. Biol.* 192:364-374. (Medline)

Gaidarov, I. and Keen, J.H. (1999) Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits, *J. Cell Biol.* 146:755-764. (Medline)

Gaidarov, I., Chen, Q., Falck, J.R., Reddy, K.K. and Keen, J.H. (1996) A functional phosphatidylinositol 3,4,5-trisphosphate/phosphoinositide binding domain in the clathrin adaptor AP-2 α subunit. Implications for the endocytic pathway, *J. Biol. Chem.* 271:20922-20929. (Medline)

Gaidarov, I., Krupnick, J.G., Falck, J.R., Benovic, J.L. and Keen, J.H. (1999) Arrestin function in G protein-coupled receptor endocytosis requires phosphoinositide binding, *EMBO J.* 18:871-881. (Medline)

Gallusser, A. and Kirchhausen (1993) The 1 and 2 subunits of the AP complexes are the clathrin coat assembly components, *EMBO J.* 12:5237-5244. (Medline)

Gammie, A.E., Kurihara, L.J., Vallee, R.B. and Rose, M.D. (1995) DNM1, a dynamin-related gene, participates in endosomal trafficking in yeast, *J. Cell Biol.* 130:553-566. (Medline)

Gaynor, E. C. and Emr, S.D. (1997) COPI-independent anterograde transport: cargo-selective ER to Golgi protein transport in yeast COPI mutants, *J. Cell Biol.* 136:789-802. (Medline)

Gaynor, E.C., Graham, T.R., Emr, S.D. (1998) COPI in ER/Golgi and intra-Golgi transport: do yeast COPI mutants point the way? *Biochim. Biophys. Acta* 1404:33-51. (MedLine)

Geutze, H. J., Slot, J. W., Strous, J. A. M., Hasilik, A. and von Figura, K. (1984) The ultrastructural localization of the mannose 6-phosphate receptor in rat liver, *J. Cell Biol*. 98:2047-2054. (Medline)

Geutze, H. J., Slot, J. W., Strous, J. G., Hasilik, A. and von Figura, K. (1985) Possible pathway for lysosomal enzyme delivery. *J. Cell Biol.* 101:2253-2263. (Medline)

Gieselman, V., Pohlmann, R., Hasilik, A. and van Figura, K. (1983) Biosynthesis and transport of cathepsin D in cultured human fibroblasts, *J. Cell Biol.* 97:1-5. (Medline)

Girod, A., Storrie, B., Simpson, J.C., Johannes, L., Goud, B., Roberts, L.M., Lord, J.M., Nilsson, T. and Pepperkok, R. (1999) Evidence for a COP-I-independent transport route from the Golgi complex to the endoplasmic reticulum, *Nature Cell Biol.* 1:423-430. (MedLine)

Glick, B.S. and Rothman J.E. (1987) Possible role for fatty acyl-coenzyme A in intracellular protein transport, *Nature* 326:309-312. (Medline)

Glickman, J.N., Conibear, E., and Pearse, B.M.F. (1989) Specificity of binding of clathrin adaptors to signals on the mannose 6-phosphate / insulin-like growth factor II receptor, *EMBO J.* 4:1041-1047.

(Medline)

Godi, A., Santone, I., Pertile, P., Devarajan, P., Stabach, P.R., Morrow, J.S., Di Tullio, G., Polishchuk, R., Petrucci, T.C., Luini, A.and De Matteis, M.A. (1998) ADP ribosylation factor regulates spectrin binding to the Golgi complex, *Proc. Natl. Acad. Sci. USA* 95:8607-8612. (Medline)

Godi, A., Pertile, P., Meyers, R., Marra, P., Di Tullio, G., Iurisci,C., Luini, A., Corda, D. and De Matteis, G. (1999) ARF mediates recrutiment of PtdIns-4-OH kinase-β and stimulates synthesis of PtdIns(4,5)P_2 on the Golgi complex *Nature Cell Biol.* 1:280-287. (Medline)

Gold, E.S., Underhill, D.M., Morrissette, N.S., Guo, J., McNiven, M.A. and Aderem, A. (1999) Dynamin 2 is required for phagocytosis in macrophages, *J. Exp. Med.* 190:1849-1856. (MedLine)

Goldberg, J. (2000) Decoding of sorting signals by coatamer through GTPase switch in COPI coat complex, *Cell* 100:671-679. (Medline)

Goodman, O.B., Jr., Krupnick, J.G., Santini, F., Gurevich, V.V., Penn, R.B., Gagnon, A.W., Keen, J.H. and Benovic, J.L. (1996) β-arrestin acts as a clathrin adaptor in endocytosis of the β2-adrenergic receptor, *Nature* 383:447-450. (Medline)

Götte, M. and Fischer von Mollard, G. (1998) A new beat for the SNARE drum, *Trends Cell Biol.* 8:215-218. (MedLine)

Gottlieb, T. A., Beaudry, G., Rizzolo, L., Colman, A., Rindler, M., Adesnik, M. and Sabatini, D. D. (1986) Secretion of endogenous and exogenous proteins from polarized MDCK cell monolayers, *Proc. Natl. Acad. Sci. USA.* 83:2100-2104. (Medline)

Graham, T.R. and Emr, S. (1991) Compartmental organization of Golgi-specific modification and vacuolar protein sorting events defined in yeast *sec18* (NSF) mutant, *J. Cell Biol.* 114:207-218. (Medline)

Gravotta, D., Adesnik, M. and Sabatini, D.D. (1990) Transport of influenza HA from the *trans*-Golgi network to the apical surface of MDCK cells permeabilized in their basolateral plasma membranes: energy dependence and involvement of GTP-binding proteins, *J. Cell Biol*. 111:2893-2908. (Medline)

Grawe, F., Wodarz, A., Lee, B., Knust, E. and Skaer, H. (1996) The *Drosophila* genes *crumbs* and *stardust* are involved in the biogenesis of adherens junctions, *Development* 122:951-959. (MedLine)

Greene, L.A. and Rein, G. (1977) Synthesis, storage and release of acetylcholine by a noradrenergic phaeochromocytoma cell line, *Nature* 268:349-351. (Medline)

Griffiths, G., Pfeiffer, S., Simon, K. and Matlin, K. (1985) Exit of newly synthesized membrane proteins from the trans cisternae of the Golgi complex to the plasma membrane, *J. Cell Biol.* 101:949-964. (Medline)

Grindstaff, K.K., Yeaman, C., Anandasabapathy, N., Hsu, S.C., Rodriguez-Boulan, E., Scheller, R.H. and Nelson, W.J. (1998) Sec6/8 complex is recruited to cell-cell contacts and specifies transport vesicle delivery to the basal-lateral membrane in epithelial cells, *Cell* 93:731-740. (Medline)

Gumbiner, B.M. (1993) Breaking through the tight junction barrier, *J. Cell Biol.* 123:1631-1633. (Medline)

Guo, W., Roth, D., Walch-Solimena, C. and Novick, P. (1999) The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis, *EMBO J.*18:1071-1080. (MedLine)

Guo, W., Sacher, M., Barrowman, J., Ferro-Novick, S. and Novick, P. (2000) Protein complexes in transport vesicle targeting, *Trends Cell Biol.* 10:251-255. (MedLine)

Gut, A., Kappeler, F., Hyka, N., Balda, M.S., Hauri, H.P. and Matter, K. (1998) Carbohydrate-mediated Golgi to cell surface transport and apical targeting of membrane proteins, *EMBO J.* 17:1919-1929. (Medline)

Hall, D.H.and Hedgecock, E.M. (1991) Kinesin related gene unc-104 is required for axonal transport of synaptic vesicles, *Cell* 65:837-847. (Medline)

Hao, W., Tan, Z., Prasad, K., Reddy, K.K., Chen, J., Prestwich, G.D., Falck, J.R., Shears, S.B. and Lafer, E.M. (1997) Regulation of AP-3 function by inositides. Identification of phosphatidylinositol 3,4,5-trisphosphate as a potent ligand, *J. Biol. Chem.* 272:6393-6398. (Medline)

Harter, C., Pavel, J., Coccia, F., Draken, E., Wegehingel, S., Tschochner, H. and Wieland, F. (1996) Nonclathrin coat protein γ, a subunit of coatomer, binds to the cytoplasmic dilysine motif of membrane proteins of the early secretory pathway, *Proc. Natl. Acad. Sci. USA* 93:1902-1906. (MedLine)

Hata, Y., Butz, S. and Sudhof, T.C. (1996) CASK: a novel dlg/PSD95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins, *J. Neurosci.* 16:2488-2494. (MedLine)

Hay, J.C. and Scheller, R.H. (1997) SNAREs and NSF in targeted membrane fusion, *Curr. Opin. Cell Biol.*9:505-512. (Medline)

Hayashi, T., Yamasaki, S., Nauenburg, S., Binz, T. and Niemann, H.(1995) Disassembly of the reconstituted synaptic vesicle membrane fusion complex *in vitro*, *EMBO J.* 14:2317-2325. (Medline)

Hazuka, C.D., Foletti, D.L., Hsu, S.C., Kee, Y., Hopf, F.W., and Scheller, R.H. (1999) The sec6/8 complex is located at neurite outgrowth and axonal synapse-assembly domains, *J. Neurosci.* 19:1324-1334. (Medline)

Heidelberger, R., Heinemann, C., Neher, E. and Matthews, G. (1994) Calcium dependence of the rate of exocytosis in a synaptic terminal, *Nature* 371:513-515. (MedLine)

Heilker, R., Manning-Krieg, U., Zuber, J.-F., and Spiess, M. (1996) In vitro binding of clathrin adaptors to sorting signals correlates with endocytosis and basolateral sorting, *EMBO J.* 15:2893-2899. (Medline)

Helms, J.B. and Rothman, J.E. (1992) Inhibition by brefeldin A of a Golgi enzyme that catalyzes exchange of guanine nucleotide bound to ARF, *Nature* 360:352-354. (Medline)

Henley, J.R. and McNiven, M.A. (1996) Association of a dynamin-like protein with the Golgi apparatus in mammalian cells, *J. Cell Biol* 133:761-775. (MedLine)

Henley, J.R., Krueger, E.W., Oswald, B.J. and McNiven, M.A. (1998) Dynamin-mediated internalization of caveolae, *J. Cell Biol.* 141:85-99. (MedLine)

Henley, J.R., Cao, H. and McNiven, M.A. (1999) Participation of dynamin in the biogenesis of cytoplasmic vesicles, *FASEB J.* 13, Suppl 2:S243-S247. (MedLine)

Herskovits, J.S., Burgess, C.C., Obar, R.A., Vallee, R.B. (1993) Effects of mutant rat dynamin on endocytosis, *J. Cell Biol.* 122:565-578. (Medline)

Hinshaw, J. E. and Schmid, S.L. Dynamin self-assembles into rings suggeting a mechanism for coated vesicle budding, (1995) *Nature*374:190-192. (Medline)

Hirokawa, N., Sato-Yoshitake, R., Kobayashi, N., Pfister, K.K., Bloom, G.S. and Brady, S.T. (1991) Kinesin associates with anterogradely transported membranous organelles in vivo, *J. Cell Biol.* 114:295-302. (Medline)

Hohl, T.M., Parlati, F., Wimmer, C., Rothman, J.E., Söllner, T.H. and Engelhardt, H. (1998) Arrangement of subunits in 20 S particles consisting of NSF, SNAPs, and SNARE complexes, *Mol Cell* 2:539-548. (Medline)

Hope, H.R. and Pike, L.J. (1996) Phosphoinositides and phosphoinositide-utilizing enzymes in detergent-insoluble lipid domains, *Mol. Biol. Cell* 7:843-881. (Medline)

Hsu, S.C., Ting, A.E., Hazuka, C.D., Davanger, S., Kenny, J.W., Kee, Y. and Scheller, R.H. (1996) The mammalian brain rsec6/8 complex, *Neuron* 17:1209-1219. (Medline)

Hsu, S.C., Hazuka, C.D., Roth, R., Foletti, D.L., Heuser, J. and Scheller, R.H. (1998) Subunit composition, protein interactions, and structures of the mammalian brain sec6/8 complex and septin filaments, *Neuron* 20:1111-1122. (Medline)

Hsueh, Y.P., Wang, T.F., Yang, F.C. and Sheng, M. (2000) Nuclear translocation and transcription regulation by the membrane-associated guanylate kinase CASK/LIN-2, *Nature* 404:298-302. (MedLine)

Huang, P.-H. and Chiang, H.-L. (1997) Identification of novel vesicles in the cytosol to vacuole protein degradation pathway, *J. Cell Biol.* 136:803-810. (Medline)

Huffaker, T.C., Thomas, J.H. and Botstein, D. (1988) Diverse effects of β-tubulin mutations on microtubule formation and function, *J. Cell Biol.* 106:1997-2010. (MedLine)

Hunt, J.M., Bommert, K., Charlton, M.P., Kistner, A., Habermann, E., Augustine, G.J. and Betz, H. (1994) A post-docking role for synaptobrevin in synaptic vesicle fusion, *Neuron* 12:1269-1279. (MedLine)

Hunziker, W., Harter, C., Matter, K. and Mellman, I. (1991) Basolateral sorting in MDCK cells requires a distinct cytoplasmic domain determinant, *Cell* 66:907-920. (Medline)

Jacobs, C.W., Adams, A.E., Szaniszlo, P.J. and Pringle, J.R. (1988) Functions of microtubules in the *Saccharomyces cerevisiae* cell cycle, *J. Cell Biol.* 1071409-1426. (MedLine)

Jahn, R. and Südhof, T.C. (1999) Membrane fusion and exocytosis, *Annu. Rev. Biochem.* 68:863-911. (MedLine)

Jones, S.M., Howell, K.E., Henley, J.R., Cao, H. and McNiven, M.A. (1998) Role of dynamin in the formation of transport vesicles from the trans-Golgi network, *Science* 279:573-577. (MedLine)

Jou, T.S. and Nelson, W.J. (1998) Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity, *J. Cell Biol.* 142:85-100. (Medline)

Kaibuchi, K., Kuroda, S., Fukata, M. and Nakagawa, M. (1999) Regulation of cadherin-mediated cell-cell adhesion by the Rho family GTPases, *Curr. Opin. Cell Biol.* 11:591-596. (Medline)

Kaiser, C.A. and Shekman, R.(1990) Distinct sets of SEC genes govern transport vesicle formation and junction early in the secretory pathway, *Cell* 61:723-733. (Medline)

Kanai,F., Liu, H., Field, S. J., Akbary, H., Matsuo, T., Brown, G. E.,Cantley, L.C. and Yaffe, M.B. (2001) The PX domains of p47phox and p40phox bind to lipid products of PI(3)K, *Nature Cell Biol.* 3:675-678 . (MedLine)

Klausner, R.D., Donaldson, J.G. and Lippincott-Schwartz, J. (1992) Brefeldin A: insights into the control of membrane traffic and organelle structure, *J. Cell Biol*. 116:1071-1080. (Medline)

Klebes, A. and Knust, E. (2000) A conserved motif in Crumbs is required for E-cadherin localisation and zonula adherens formation in *Drosophila*, *Curr. Biol.* 10:76-85. (MedLine)

Klenchin, V.A. and Martin, T.F. (2000) Priming in exocytosis: attaining fusion-competence after vesicle docking, *Biochimie* 82:399-407. (MedLine)

Kondor-Koch, C., Bravo, R., Fuller, S. D., Cutler, D. and Garoff, H. (1985) Exocytotic pathways exist to both apical and basolateral cell surface of the polarized epithelial cell MDCK, *Cell* 43:297-306. (Medline)

Kornfeld, S. (1987) Trafficking of lysosomal enzymes, *FASEB J.* 1:462-468. (Medline)

Kornfeld, S. (1992) Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors, *Annu. Rev. Biochem.* 61:307-330. (MedLine)

Kreis, T.E., Lowe, M. and Pepperkok, R. (1995) COPs regulating membrane traffic, *Ann. Rev. Cell Biol.* 11:677-706. (Medline)

Kreitzer, G., Marmorstein, A., Okamoto, P., Vallee, R. and Rodriguez-Boulan, E. Kinesin and dynamin are required for post-Golgi transport of a plasma-membrane protein, *Nature Cell Biol.* 2:125-127. (MedLine)

Ladinsky, M.S, Kremer, J.R, Furcinitti, P. S., McIntosh, J.R. and Howell, K.E. (1994), HVEM tomography of the *trans*-Golgi network: structural insights and identification of a lace-like vesicle coat, *J. Cell Biol.* 127:29-38. (Medline)

Lafont, F., Burkhardt, J.K. and Simons, K. (1994) Involvement of microtubule motors in basolateral and apical transport in kidney cells, *Nature* 372:801-803. (Medline)

Lanoix, J., Ouwendijk, J., Lin, C.C., Stark, A., Love, H.D., Ostermann, J. and Nilsson T. (1999) GTP hydrolysis by arf-1 mediates sorting and concentration of Golgi resident enzymes into functional COP I vesicles, *EMBO J.* 18:4935-4948. (MedLine)

Lavoie, C., Paiement, J., Dominguez, M., Roy, L., Dahan, S., Gushue, J.N. and Bergeron, J.J. (1999)

Roles for alpha(2)p24 and COPI in endoplasmic reticulum cargo exit site formation, *J. Cell Biol.*146:285-299. (MedLine)

Le Bevic, A., Quaroni, A., Nichols, B. and Rodriguez-Boulan, E. (1990) Biogenetic pathways of plasma membrane proteins in Caco-2, a human intestinal epithelial cell line, *J. Cell Biol*. 111:1351-1361. (Medline)

Le Borgne, R., Griffiths, G. and Hoflack, B. (1996) Mannose 6-phosphate receptors and ADP-ribosylation factors cooperate for high affinity interaction of the AP-1 Golgi assembly with membranes, *J. Biol. Chem.* 271:2162-2170. (Medline)

Le Borgne, R. and Hoflack, B. (1997) Mannose 6-phosphate receptors regulated the formation of clathrin-coated vesicles in the TGN, *J. Cell Biol.* 137:335-345. (Medline)

Le Borgne, R. and Hoflack, B. (1998) Mechanisms of protein sorting and coat assembly: insights from clathrin coated vesicle pathway, *Curr. Opin. Cell Biol.* 10:499-503. (Medline)

Le Borgne, R., Alconada, A., Bauer, U. and Hoflack, B. (1998) The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins, *J. Biol. Chem.* 273:29451-29461. (Medline)

Lee, J. and Lentz, B.R. (1997) Evolution of lipidic structures during model membrane fusion and the relation of this process to cell membrane fusion, *Biochemistry* 36:6251-6259. (MedLine)

Letourneur, F., Gaynor, E.C., Hennecke, S., Démollière, C., Duden, R., Emr, S.D., Riezman, H. and Cosson, P. (1994) Coatamer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum, *Cell* 79:1199-1207. (Medline)

Lewis, M.J. and Pelham, H.R. (1992) Ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum, *Cell* 68:353-364. (MedLine)

Lin, C.-C., Love, H.D., Gushue, J.N., Bergeron, J.J. and Ostermann, J. (1999) ER/Golgi intermediates acquire Golgi enzymes by brefeldin A-sensitive retrograde transport in vitro, *J. Cell Biol.* 147:1457-1472. (MedLine)

Lippincott-Schwartz, J. (1998) Cytoskeletal proteins and Golgi dynamics, *Curr. Opin. Cell Biol.*10:52-59. (MedLine)

Lippincott-Schwartz, J., Cole, N.B., Marotta, A., Conrad, P.A and Bloom, G.S. (1995) Kinesin is the motor for microtubule-mediated Golgi-to-ER membrane traffic, *J. Cell. Biol.* 128: 293-306. (Medline)

Lisanti, M.P., Caras, I.W., Davitz, M.A. and Rodriguez-Boulan, E. (1989) A glycophospholipid membrane anchor acts as an apical targeting signal in polarized epithelial cells, *J. Cell Biol.* 109:2145-2156. (Medline)

Llorente, A., Rapak, A., Schmid, S.L., van Deurs, B. and Sandvig, K. (1998) Expression of mutant dynamin inhibits toxicity and transport of endocytosed ricin to the Golgi apparatus, *J. Cell Biol.* 140:553-563. (MedLine)

Lohi, O., and Lehto, V.-P. (1998) VHS domain marks a group of proteins involved in endocytosis and vesicular trafficking, *FEBS Lett.* 440: 255-257. (MedLine)

Love, H.D., Lin, C.C., Short, C.S. and Ostermann, J. (1998) Isolation of functional Golgi-derived vesicles with a possible role in retrograde transport, *J. Cell Biol.* 140:541-551. (Medline)

Maier, O., Knoblich, M. and Westermann, P. (1996) Dynamin II binds to the trans-Golgi network, *Biochem. Biophys. Res. Commun.* 223:229-233. (MedLine)

Malhotra V., Orci, L., Glick, B.S.. Block, M.R. and Rothman, J.E. (1988) Role of an N-ethylmaleimide-sensitive transport component in promoting fusion of transport vesicles with cisternae of the Golgi stack, *Cell* 54:221-227. (Medline)

Malhotra, V., Serafini, T., Orci, L., Shepherd, J.G. and Rothman, J.E. (1989) Purification of a novel class of coated vesicles mediating the biosynthetic protein transport through the Golgi stacks, *Cell* 58:329-336. (Medline)

Martin, T.F. (1997) Phosphoinositides as spatial regulators of membrane traffic, *Curr. Opin. Neurobiol.* 7:331-338. (Medline)

Martínez-Menárguez, J.A., Geuze, H.J., Slot, J.W. and Klumperman, J. (1999) Vesicular tubular clusters between the ER and Golgi mediate concentration of soluble secretory proteins by exclusion from COPI-coated vesicles, *Cell* 98:81-89. (MedLine)

Marzioch, M., Henthorn, D.C., Herrmann, J.M., Wilson, R., Thomas, D.Y., Bergeron, J.J. and Solari, R.C. and Rowley, A. (1999) Erp1p and Erp2p, partners for Emp24p and Erv25p in a yeast p24 complex, *Mol. Biol. Cell* 10:1923-1938. (MedLine)

Matlin, K. and Simons, K. (1984) Sorting of a plasma membrane glycoprotein occurs before it reaches the cell surface in cultured epithelial cells, *J. Cell Biol.* 99:2131-2139. (Medline)

Matlin, K., Bainton, D. F., Pesonen, M., Louvard, D., Genty, N. and Simons, K. (1983) Transepithelial transport of viral membrane glycoprotein implanted into the apical plasma membrane of Madin-Darby

canine kidney cells. I. Morphological evidence, *J. Cell Biol.* 97:627-637. (Medline)

Matsuoka, K., Orci, L., Amherdt, M., Bednarek, S.Y., Hamamoto, S., Schekman, R. and Yeung, T. (1998) COPII-coated vesicle formation reconstituted with purified coat proteins and chemically defined liposomes, *Cell* 93:263-275. (MedLine)

Matteoni, R. and Kreis, T.E. (1987) Translocation and clustering of endosomes and lysosomes depends on microtubules, *J. Cell. Biol.* 105:1253-1265 (Medline)

Matter, K., Brauchbar, M. and Hauri, H.-P. (1990a) Sorting of endogenous plasma membrane proteins occurs from two sites in cultured human intestinal epithelial cells (Caco-2), *Cell* 60:429-437. (Medline)

Matter, K., Bucher, K. and Hauri, H.-P.(1990b) Microtubule perturbation retards both the direct and the indirect pathway but does not affect sorting of plasma membrane proteins in intestinal cells (Caco-2) *EMBO J.* 9:3163-3170. (Medline)

May, A.P., Misura, K.M.S., Whiteheart, S.W. and Weiss, W.I. (1999) Crystal structure of the amino-terminal domain of *N*-ethylmaleimide-sensitive fusion protein, *Nature Cell Biol.* 1:175-182. (Medline)

Mayer, A., Wickner, W. and Haas, A. (1996) Sec18p (NSF)-driven release of Sec17p (α-SNAP) can precede docking and fusion of yeast vacuoles, *Cell* 85:83-94. (Medline)

McCarthy, K.M., Skare, I.B., Stankewich, M.C., Furuse, M., Tsukita, S., Rogers, R.A., Lynch, R.D. and Schneeberger, E.E. (1996) Occludin is a functional component of the tight junction, *J. Cell Sci.* 109:2287-2298. (Medline)

McNew, J.A., Parlati, F., Fukuda, R., Johnston, R.J., Paz, K., Paumet, F., Söllner, T.H. and Rothman, J.E. (2000) Compartmental specificity of cellular membrane fusion encoded in SNARE proteins, *Nature* 407:153-159. (MedLine)

McNiven, M.A., Cao, I., Pitts, K.R. and Yoon, I. (2000) The dynamin family of mechanoenzymes: pinching in new places, *Trends Biochem Sci* 25:115-120. MedLine

McPherson, P.S., Garcia, E.P., Slepnev, V.I., David, C., Zhang, X., Grabs, D., Sossin, W.S., Bauerfeind, R., Nemoto, Y. and De Camilli, P. (1996) A presynaptic inositol-5-phosphatase, *Nature* 379:353-357. (Medline)

Medof, M.E., Nagarajan, S. and Tykocinski, M.L. (1996) Cell-surface engineering with GPI-anchored proteins, *FASEB J.* 10:574-586. (Medline)

Melançon, P., Glick, B.S., Malhotra, V., Weidman, P.J., Serafini, T., Gleason, M.L., Orci, L. and Rothman, J.E. (1987) Involvement of GTP-binding "G" proteins in transport through the Golgi stack, *Cell* 51:1053-1062. (Medline)

Meldolesi, J. (1974) Dynamics of cytoplasmic membranes in guinea pig pancreatic acinar cells. I. Synthesis and turnover of membrane proteins, *J. Cell Biol.* 61:1-13.

Melkonian, K.A., Ostermeyer, A.G., Chen, J.Z., Roth, M.G. and Brown, D.A. (1999) Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts. Many raft proteins are acylated, while few are prenylated, *J. Biol. Chem.* 274:3910-3917. (MedLine)

Mellman, I. and Warren, G. (2000) The road taken: past and future foundations of membrane traffic, *Cell* 100:99-112. (MedLine)

Monck, J.R. and Fernandez, J.M. (1994) The exocytotic fusion pore and neurotransmitter release, *Neuron* 12:707-716. (MedLine)

Monck, J.R. and Fernandez, J.M. (1996) The fusion pore and mechanisms of biological membrane fusion, *Curr. Opin. Cell Biol.* 8:524-533. (MedLine)

Mooseker, M.S. and Coleman, T.R. (1989) The 110-kD protein-calmodulin complex of the intestinal microvillus (brush border myosin I) is a mechanoenzyme, *J. Cell Biol.* 108:2395-2400. (Medline)

Morgan, A., Dimaline, R. and Burgoyne, R.D. (1994) The ATPase activity of N-ethylmaleimide-sensitive fusion protein (NSF) is regulated by soluble NSF attachment proteins, *J. Biol. Chem.* 269:29347-29350. (Medline)

Morin, P.J., Johnson, R.J. and Fine, R.E. (1993) Kinesin is rapidly transported in the optic nerve as a membrane associated protein, *Biochim. Biophys. Acta* 1146:275-281. (Medline)

Morita, K., Furuse, M., Fujimoto, K. and Tsukita, S. (1999a) Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands, *Proc. Natl. Acad. Sci. USA* 96:511-516. (Medline)

Morita, K., Sasaki, H., Furuse, M. and Tsukita, S. (1999b) Endothelial claudin. claudin-5/TMVCF constitutes tight junction strands in endothelial cells, *J. Cell Biol.* 147:185-194. (Medline)

Morita, K., Sasaki, H., Fujimoto, K., Furuse, M. and Tsukita, S. (1999c) Claudin-11/OSP-based tight junctions of myelin sheaths in brain and Sertoli cells in testis, *J. Cell Biol.* 145:579-588. (Medline)

Mostov, K., Apodaca, G., Aroeti, B. and Okamoto, C. (1992) Plasma membrane protein sorting in

polarized epithelial cells, *J. Cell Biol.* 116:577-583. (Medline)

Muñiz, M., Nuoffer. C., Hauri, H.P. and Riezman, H. (2000) The Emp24 complex recruits a specific cargo molecule into endoplasmic reticulum-derived vesicles, *J. Cell Biol.* 148:925-930. (MedLine)

Munro, S. and Pelham, H. R. B. (1986) An H5P70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein, *Cell* 46:291-300. (Medline)

Nakagawa, T., Goto, K. and Kondo, H. (1996) Cloning, expression, and localization of 230-kDa phosphatidylinositol 4-kinase, *J. Biol. Chem.* 271:12088-12094. (Medline)

Narula, N. and Snow, J.C.(1995) Distinct coat vesicles labeled for p200 bud from trans-Golgi neet work membranes, *Proc. Natl. Acad. Sci. USA* 92:2874-2878. (Medline)

Nelson, W.J. (1991) Cytoskeleton functions in membrane traffic in polarized cells, *Seminars in Cell Biol.* 2:375-385. (Medline)

Nelson, W. J. (1992) Regulation of cell surface polarity from bacteria to mammals, *Science* 258:948-955. (Medline)

Nelson, W. J. and Hammerton, R. W. (1989) A membrane-cytoskeleton complex containing Na^+, K^+ ATPase, ankyrin and fodrin in Madin-Darby canine kidney (MDCK) cells: implications from the biogenesis of epithelial cell polarity. *J. Cell Biol.* 108:893-902. (Medline)

Nelson, D.S., Alvarez, C., Gao, Y.S., Garcia-Mata, R., Fialkowski, E. and Sztul, E. (1998) The membrane transport factor TAP/p115 cycles between the Golgi and earlier secretory compartments and contains distinct domains required for its localization and function, *J. Cell Biol.* 143:319-331. (MedLine)

Neuwald, A.F. (1999) The hexamerization domain of N-ethylmaleimide-sensitive factor: structural clues to chaperone function, *Structure Fold. Des.* 7:R19-23. (Medline)

Neuwald, A.F., Aravind, L., Spouge, J.L. and Koonin, E.V. (1999) AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes, *Genome Res.* 9:27-43. (Medline)

Newman, L.S., McKeever, M.O., Okano, H.J. and Darnell, R.B. (1995) β-NAP, a cerebellar degeneration antigen, is a neuron-specific vescile coat protein, *Cell* 82:773-783. (Medline)

Nichols, B.J., Ungermann, C., Pehham, H.R.B., Wickner, W.T. and Haas, A. (1997) Homotypic vacuolar fusion mediated by t- and v-SNAREs, *Nature* 386:199-902. (Medline)

Nickel, W., Sohn, K., Bunning, C. and Wieland, F.T. (1997) p23, a major COPI-vesicle membrane protein, constitutively cycles through the early secretory pathway, *Proc. Natl. Acad. Sci. USA* 94:11393-11398. (MedLine)

Nicoziani, P., Vilhardt, F., Llorente, A., Hilout, L., Courtoy, P.J., Sandvig, K. and van Deurs, B. (2000) Role for dynamin in late endosome dynamics and trafficking of the cation-independent *Mol. Biol. Cell* 11:481-495. (MedLine)

Novick, P. and Zerial, M. (1997) The diversity of Rab proteins in vesicle transport, *Curr. Opin. Cell Biol.* 9:496-504. (MedLine)

Novick, P., Field, C. and Schekman, R. (1980) Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway, *Cell* 21:205-215. (Medline)

Novick, P. and Botstein, D. (1985) Phenotypic analysis of temperature-sensitive yeast actin mutants, *Cell* 40:405-416. (MedLine)

Nusse, R. (1997) A versatile transcriptional effector of Wingless signaling, *Cell* 89:321-323. (MedLine)

Odorizzi, G. and Trowbridge, I.S. (1997) Structural requirements for basolateral sorting of human transferrin receptor in biosynthetic and endocytotic pathways in Madin-Darby canine kidney cells, *J. Cell Biol.* 137:1255-1264. (Medline)

Oh, P., McIntosh, D.P. and Schnitzer, J.E. (1998) Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium, *J. Cell Biol.* 141:101-114. (MedLine)

Ohashi, M., Jan de Vries, K., Frank, R., Snoek, G., Bankaitis, V., Wirtz, K. and Huttner, W.B. (1995) A role for phosphatidylinositol transfer protein in secretory vesicle formation, *Nature* 377:544-547. (Medline)

Ohno, H., Stewart, J.,m Fournier, M.C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kichhausen, T. Banifacion, J.S. (1995) Interactions of tyrosine-based sorting signals with clathrin-associated proteins, *Science* 269:1872-1875. (Medline)

Orci, L., Glick, B. S. and Rothman, J. E. (1986) A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stacks, *Cell* 46:171-184. (Medline)

Orci, L., Malhotra, V., Amherdt, M., Serafini, T. and Rothman, J.E. (1989) Dissection of a single round of vesicular transport: sequential intermediates for cisternal movement in Golgi stack, *Cell* 56:357-368.

(MedLine)

Orci, L., Stamnes, M., Ravazzola, M., Amherdt, M., Perrelet, A., Söllner, T.H. and Rothman, J.E. (1997) Bidirectional transport by distinct populations of COPI-vesicles, *Cell* 90:335-349. (Medline)

Ostermann, J., Orci, L., Tani, K. Amherdt. M., Ravazzola, M., Elazar, Z. and Rothman, J.E. (1993) Stepwise assembly of functionally active transport vesicles, *Cell* 75: 1015-1025. (MedLine)

Panaretou, C., Domin, J., Cockcroft, S. and Waterfield, M.D. (1997) Characterization of p150, an adaptor protein for the human phosphatidylinositol (PtdIns) 3-kinase. Substrate presentation by phosphatidylinositol transfer protein to the p150.Ptdins 3-kinase complex, *J. Biol. Chem.* 272:2477-2485. (Medline)

Parlati, F., McNew, J.A., Fukuda, R., Miller, R., Sollner, T.H. and Rothman, J.E. (2000) Topological restriction of SNARE-dependent membrane fusion, *Nature* 407:194-198. (MedLine)

Patel, S. and Latterich, M. (1998) The AAA team: related ATPases with diverse functions, *Trends Cell Biol.* 8:65-71. (Medline)

Patzak, A. and Winkler, H. (1986) Exocytotic exposure and recycling of membrane antigens of chromaffin granules: ultrastructural evaluation after immunolabeling, *J. Cell Biol*. 102:510-515. (Medline)

Pearse, B.M. (1988) Receptors compete for adaptors found in plasma membrane coated pits, *EMBO J.* 11:3331-3336. (Medline)

Pelham, H.R.B. (1999) SNAREs and the secretory pathway-lessons from yeast, *Exp. Cell Res.* 247:1-8. (MedLine)

Pelham, H.R.B. (2001) SNAREs and the specificity of membrane fusion, *Trends Cell Biol.* 99-101.

Peter, F., Plutner, H., Zhu, H., Kreis, T.E. and Balch, W.E. (1993) β-COP is essential for transport of protein from the endoplasmic reticulum to the Golgi in vitro, *J. Cell Biol.* 122:1155-1167. (Medline)

Peters, C. and Mayer, A (1998) Ca2+/calmodulin signals the completion of docking and triggers a late step of vacuole fusion, *Nature* 396:575-580. (MedLine)

Pevner, J., Hsu, S.-C. and Scheller, R.H. (1994) n-Sec1: a neural-specific syntaxin-binding protein, *Proc. Natl. Acad. Sci. USA* 91:1445-1449. (Medline)

Pfeffer, S.R. (1999) Transport-vesicle targeting: tethers before SNAREs, *Nature Cell Biol.* 1:E17-22. (MedLine)

Pfeiffer, S., Fuller, S. D. and Simons, K. (1985) Intracellular sorting and basolateral appearance of the G protein of vesicular stomatitis virus in Madin-Darby canine kidney cells. *J. Cell Biol.* 101:470-476. (Medline)

Pierce, S.K. (2002) Lipid rafts and B-cell activation, *Nature Rev. Immunol.* 2:96-105. (MedLine)

Pike, L.J. and Casey, L. (1996) Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains, *J. Biol. Chem.* 271:26453-26456. (Medline)

Powell, S.K., Lisanti, M.P. and Rodriguez-Boulan, E.J. (1991) Thy-1 expresses two signals for apical localization in epithelial cells, *Am. J. Physiol. Cell Physiol.* 29:C715-720. (Medline)

Presley, J.F, Cole, N.B., Schroer, T.A., Hirschberg K., Zaal K.J., Lippincott-Schwartz J. (1997) ER-to-Golgi transport visualized in living cells, *Nature* 389:81-85. (Medline)

Propopov, V., Govindan, B., Novick, P., and Gerst, J.E. (1993) Homologs of the syaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway of *S. cerevisiae*, *Cell* 74:855-861. (Medline)

Puertollano, R., Aguilar, R.C., Gorshkova, I., Crouch, R.J and Bonifacino, J.S. Sorting of mannose 6-phosphate receptors mediated by the GGAs, *Science* 292:1712-1716. (MedLine)

Qualmann, B., Roos, J., DiGregorio, P.J. and Kelly, R.B. (1999) Syndapin I, a synaptic dynamin-binding protein that associates with the neural Wiskott-Aldrich syndrome protein, *Mol. Biol. Cell* 10:501-513. (MedLine)

Régnier-Vigouroux,A., Tooze, S.A., and Huttner, W.B.(1991) Newly synthesized synaptophysin is transported to synaptic-like microvesicles via constitutive secretory vesicles and the plasma membrane, *EMBO J.* 10:3589-35601. (Medline)

Reichert M, Muller T, Hunziker W (2000) The PDZ domains of zonula occludens-1 induce an epithelial to mesenchymal transition of madin-darby canine kidney I cells. Evidence for a role of β-catenin/tcf/lef signaling, *J. Biol. Chem.* 275:9492-9500. (MedLine)

Reinhard, C., Harter, C., Bremser, M., Br gger, B., Sohn, K., Helms, J.B. and Wieland, F. (1999) Receptor-induced polymerization of coatomer, *Proc. Natl. Acad. Sci. USA* 96:1224-1228. (Medline)

Rice, L.M. and Brunger, A.T. (1999) Crystal structure of the vesicular transport protein Sec17: implications for SNAP function in SNARE complex disassembly, *Mol. Cell.* 4:85-95. (Medline)

Rindler, M. J.. Ivanov, I. E., Plesken, H., Rodriguez-Boulan, E. and Sabatini, D. D. (1984) Viral glycoproteins destined for apical or basolateral plasma membrane domains traverse the Golgi apparatus during the intracellular transport in doubly infected Madine-Darby canine kidney cells (MDCK), *J. Cell Biol.* 98:1304-1319. (Medline)

Rindler, M.J., Ivanov, I.E. and Sabatini, D.D. (1987) Microtubule -acting drugs lead to the non-polarized delivery of influenza hemagglutinin to the cell surface of polarized Madin-Darby canine idney cells, *J. Cell Biol.* 104:231-241. (Medline)

Robinson, M.S. (1997) Coats and vesicle budding, *Trends in Cell Biol.* 7:99-102

Rodriguez-Boulan, E. and Nelson, W.J. (1989) Morphogenesis of the polarized epithelial cell phenotype, *Science* 245:718-724. (Medline)

Rogalski, A.A. and Singer, S.J. (1984) Associations of elements of the Golgi apparatus with microtubules, *J. Cell. Biol.* 99: 1092-1100 (Medline)

Rossi, G., Jiang, Y., Newman, A. and Ferro-Novick (1991) Dependence of Ypt1 and Sec 4 membrane attachement on Bet2, *Nature* 351:158-161. (Medline)

Rothman, J.E. (1994) Mechanisms of intracellular protein transport (1994) *Nature* 372:55-62. (Medline)

Rothman, J. E. and Orci, L. (1992) Molecular dissection of the secretory pathway *Nature* 355:409-415. (Medline)

Rothman, J.E. and Wieland, F.T. (1996) Protein sorting by transport vesicles, *Science* 272:227-234. (Medline)

Rowe, T., Aridor, M., McCaffery, J.M., Plutner, H. and Nuoffer, C. and Balch, W.E. (1996) COPII vesicles derived from mammalian endoplasmic reticulum microsomes recruit COPI, *J. Cell Biol.* 135:895-911. (Medline)

Sacher, M., Jiang, Y., Barrowman, J., Scarpa, A., Burston ,J., Zhang, L., Schieltz, D., Yates, J.R. 3rd, Abeliovich, H. and Ferro-Novick, S. (1998) TRAPP, a highly conserved novel complex on the cis-Golgi that mediates vesicle docking and fusion, *EMBO J.* 17:2494-2503. (Medline)

Sahagian, G. G. and Steer, C. J. (1985) Transmembrane orientation of a mannose-6-phosphate receptor in

isolated clathrin coated vesicles, *J. Biol. Chem*. 260:9838-9842. (Medline)

Sahagian, G. G., Distler, J. and Jourdian, G. W. (1981) Characterization of a membrane-associated receptor from bovine liver that binds phosphomannosyl residues of bovine testicular -galactosidase, *Proc. Natl. Acad. Sci. USA.* 78:4289-4293. (Medline)

Salama, N.R., Yeung, T. and Shekman, R.W. (1993) The sec 13p complex and reconstitution of vesicle budding from ER with purified cytosolic proteins, *EMBO J.* 12:4073-4082. (Medline)

Sandoval , I.V. and Bakke, O. (1994) Targeting of membrane proteins to endosomes and lysosomes, *Trends in Cell Biol.* 4:292-297.

Sandvig, K. and van Deurs, B. (1996) Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin, *Physiol. Rev.* 76:949-966. (MedLine)

Santos, B. and Snyder, M. (1997) Targeting of chitin synthase 3 to polarized growth sites in yeast requires Chs5p and Myo2p, *J. Cell Biol.* 136:95-110. (MedLine)

Scales, S.J., Pepperkok, R. and Kreis, T.E. (1997) Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI, *Cell* 90:1137-1148. (Medline)

Schimmoller, F., Singer-Kruger, B., Schroder, S., Kruger, U., Barlowe, C. and Riezman, H. (1995) The absence of Emp24p, a component of ER-derived COPII-coated vesicles, causes a defect in transport of selected proteins to the Golgi, *EMBO J.* 14:1329-1339. (MedLine)

Schoenenberger, C.A., Zuk, A., Zinkl, G.M., Kendall, D. and Matlin, K.S. (1994) Integrin expression and localization in normal MDCK cells and transformed MDCK cells lacking apical polarity, *J. Cell Sci.* 107:527-541. (MedLine)

Schafer, D.A., Gill, S.R., Cooper, J.A., Heuser, J.E. and Schroer, T.A. (1994) Ultrastructural analysis of the dynactin complex: an actin-related protein is a component of a filament that resembles F-actin, *J. Cell Biol.*126:403-412. (Medline)

Scheiffele, P., Peranen, J. and Simons, K. (1995) N-glycans as apical sorting signals in epithelial cells, *Nature* 378:96-98. (Medline)

Schell, M.J., Maurice, M., Stieger, B. and Hubbard. A.L. (1992) 5' nucleotidase is sorted to the apical domain of hepatocytes via an indirect route, *J. Cell Biol.*, 119:1173-1182. (Medline)

Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A.V., Witke, W., Huttner, W.B. and Soling, H.D. (1999) Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to

lysophosphatidic acid, *Nature* 401:133-141. (MedLine)

Schott, D., Ho, J., Pruyne, D. and, Bretscher, A. (1999) The COOH-terminal domain of Myo2p, a yeast myosin V, has a direct role in secretory vesicle targeting, *J. Cell Biol.* 147:791-808. (MedLine)

Schröder, S. and Ungewickell, E. (1991) Subunit interaction and function of clathrin-coated vesicle adaptors for the Golgi and the plasma membrane, *J. Biol. Chem.* 266:7910-7918. (Medline)

Schroer, T.A. and Sheetz, M.P. (1991) Two activators of microtubule-based vesicle transport, *J. Cell Biol.* 115:1309-1318. (Medline)

Schroer, T.A., Bingham, J.B. and Gill S.R. (1996) Actin-related protein 1 and cytoplasmic dynein based motility-what's the connection, *Trends Cell Biol.* 6:212-215.

Schulze-Lohoff. E., Hasilik, A. and von Figura, K. (1985) Cathepsin D precursors in clathrin coated organelles from human fibroblasts, *J. Cell Biol.* 101:824-829. (Medline)

Schwaninger, R., Plutner, H., Bokoch, G.M. and Balch, W.E. (1992) Multiple GTP-binding proteins regulate vesicular transport from the ER to Golgi membranes, *J. Cell Biol.* 119:1077-1096. (Medline)

Seaman, M.N., McCaffery, J.M., Emr, S.D. (1998) A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast, *J. Cell. Biol.* 142:665-681. (Medline)

Serafini, T. and Rothman, J.E. (1992) Purification of Golgi cisternae-derived non-clathrin-coated vesicles, *Methods Enzymol.* 219:286-299. (MedLine)

Sever, S., Muhlberg, A.B. and Schmid, S.L.(1999) Impairment of dynamin's GAP domain stimulates receptor-mediated endocytosis, *Nature* 398:481-486. (MedLine)

Shaywitz, D.A., Espenshade, P.J., Gimeno, R.E. anf Kaiser, C.A. (1997) COPII subunit interactions in the assembly of the vesicle coat, *J. Biol. Chem.* 272:25413-25416. (Medline)

Shih, W., Galluser, A. and Kirchhausen, T. (1995) A clathrin binding site in the hinge of the β2 chain of mammalian AP-2 complexes, *J. Biol. Chem.* 270:31083-31090. (Medline)

Simons, K. and Fuller, S. D. (1985) Cell surface polarity in epithelium, *Annu. Rev. Cell Biol.* 1:243-288. (Medline)

Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes, *Nature* 387:569-572. (Medline)

Simons, K. and Zerial, M. (1993) Rab proteins and the road map for intracellular transport, *Neuron* 11:789-799. (Medline)

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J.M., Brech, A., Callaghan, J., Toh, B.H., Murphy, C., Zerial, M. and Stenmark, H. (1998) EEA1 links PI(3)K function to Rab5 regulation of endosome fusion, *Nature* 394:494-498. (MedLine)

Simpson, F., Bright, N.A., West, M.A., Newman, L.S., Darnell, R.B. and Robinson, M.S. (1996) A novel adaptor-related protein complex, *J. Cell Biol.* 133:749-760. (Medline)

Sohn, K., Orci, L., Ravazzola, M., Amherdt, M., Bremser, M., Lottspeich, F., Fiedler, K., Helms, J.B. and Wieland, F.T. (1996) A major transmembrane protein of Golgi-derived COPI-coated vesicles involved in coatomer binding, *J. Cell Biol.* 135:1239-1348. (MedLine)

Sönnichsen, B., Watson, R., Clausen, H., Misteli, T. and Warren, G. (1996) Sorting by COP I-coated vesicles under interphase and mitotic conditions, *J. Cell Biol.* 134:1411-1425. (MedLine)

Soole, K.L., Jepson, M.A., Hazlewood, G.P., Gilbert, H.J., and Hirst, B.H. (1985) Epithelial sorting of a glycosylphosphatidylinositol-anchored bacterial protein expressed in polarized renal MDCK and intestinal Caco-2 cells, *J. Cell Sci.* 108:369-377. (Medline)

Sorokin, L., Sonnenberg, A., Aumailley, M., Timpl, R. and Ekblom, P. (1990) Recognition of the laminin E8 cell-binding site by an integrin possessing the α_6 subunit is essential for epithelial polarization in developing kidney tubules, *J. Cell Biol.* 111:1265-1273. (MedLine)

Søgaard, M., Tani, K., Ye, R.R., Geromanos, S., Tempst, P., Kirchhausen, T. Rothman, J.E. and Söllner, T. (1994) A rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles, *Cell* 78:937-948. (Medline)

Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S. Tempst, P. and Rothman, J.E. (1993a) SNAP receptors implicated in vesicle targeting and fusion, *Nature* 362:318-324. (Medline)

Söllner,T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H. and Rothman, J.E. (1993b) A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation and fusion. *Cell* 75:409-418. (Medline)

Sonoda, N., Furuse, M., Sasaki, H., Yonemura, S., Katahira, J., Horiguchi, Y. and Tsukita, S. (1999) *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands. Evidence for direct involvement of claudins in tight junction barrier, *J. Cell Biol.* 147:195-204. (Medline)

Springer, S. and Schekman, R. (1998) Nucleation of COPII vesicular coat complex by endoplasmic reticulum to Golgi vesicle SNAREs, *Science* 281:698-700. (MedLine)

Springer, S., Spang, A. and Schekman, R. (1999) A primer on vesicle budding, *Cell* 97:145-148. (MedLine)

Stack, J.H. and Emr, S.D. (1994) Vps34p required for yeast vacuolar protein sorting is a multiple specificity kinase that exhibits both protein kinase and phosphatidylinositol-specific PI 3-kinase activities, *J. Biol. Chem.* 269:31552-31562. (Medline)

Staehelin, L.A. (1974) Structure and function of intercellular junctions, *Int. Rev. Cytol.* 39:191-283. (Medline)

Stepp, J.D., Pellicena-Palle, A., Hamilton, S., Kirchhausen, T. and Lemmon, S. K. (1995) A late Golgi sorting function for *Saccharomyces cerevisiae* Apm1p, but not Apm2p, a second yeast clathrin AP medium chain-related protein, *Mol. Biol. Cell* 6:41-58. (Medline)

Stepp, J.D., Huang, K. and Lemmon, S.K. (1997)The yeast adaptor protein complex, AP-3, is essential for the efficient delivery of alkaline phosphatase by the alternate pathway to the vacuole, *J. Cell Biol.* 139:1761-1774. (Medline)

Stone, S., Sacher, M., Mao, Y., Carr, C., Lyons, P., Quinn, A.M. and Ferro-Novick, S. (1997) Bet1p activates the v-SNARE Bos1p, *Mol. Biol. Cell* 8:1175-1181 (Medline)

Stoorvogel, W., Oorschot, V. and Geutze, H.J. (1996) A novel class of clathrin-coated vesicles budding from endosomes, *J. Cell Biol.* 132:21-33. (Medline)

Storrie, B., Pepperkok, R. and Nilsson, T. (2000) Breaking the COPI monopoly on Golgi recycling, *Trends Cell Biol.* 10:385-390. (MedLine)

Stow, J.L. de Almeida, J.B., Narula, N., Holzman, E.J., Ercolani, L. and Ausiello, D.A. (1991) A heterotrimeric G protein $G_{1\text{-}3}$, on Golgi membranes regulates the secretion of a heparan sulfate proteoglycan in LLC-PK_1 epithelial cells, *J. Cell Biol.* 114:1113-11124. (Medline)

Takahashi, K., Matsuo, T., Katsube, T., Ueda, R. and Yamamoto, D. (1998) Direct binding between two PDZ domain proteins Canoe and ZO-1 and their roles in regulation of the jun N-terminal kinase pathway in *Drosophila* morphogenesis, *Mech. Dev.* 78:97-111. (MedLine)

Takei, K., McPherson, P.S., Schmid, S.L. and De Camilli, P. (1995) Tubular membrane invaginations coated by dynamin rings are induced by GTP-S in nerve terminals, *Nature* 374:186. (Medline)

Tan, A., Bolscher, J., Feltkamp, C. and Ploegh, H. (1992), Retrograde transport from the Golgi region to the endoplasmic reticulum is sensitive to GTPγS, J. Cell Biol. 116, 1357-1367. (Medline)

Tanigawa, G., Orci, L., Amherdt, M., Ravazzola, M., Helms, J.B. and Rothman, J.E. (1993) Hydrolysis of bound GTP by ARF protein triggers uncoating of Golgi-derived COP-coated vesicles, *J. Cell Biol.* 123:1365-1371. (Medline)

Tepass, U. (1997) Epithelial differentiation in *Drosophila*, *BioEssays* 19:673-682. (MedLine)

Tepass, U., Theres, C. and Knust, E. (1990) crumbs encodes an EGF-like protein expressed on apical membranes of *Drosophila* epithelial cells and required for organization of epithelia, *Cell* 61:787-799. (MedLine)

TerBush, D.R., Maurice, T., Roth, D. and Novick, P. (1996) The Exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*, *EMBO J.* 15:6483-6494. (Medline)

Thomas, J.R., Dwek, R.A. and Rademacher, T.W. (1990) Structure, biosynthesis and function of glycosylphosphatidylinositols, *Biochemistry* 29:5413-5422. (Medline)

Thompson, T.E. and Tillack, T.W. (1985) Organization of glycosphingolipids in bilayers and plasma membranes of mammalian cells, *Ann. Rev. Biophys. Biophys. Chem.* 14:361-386. (Medline)

Traub, L.M. (1997) Clathrin-associated adaptor proteins-putting it all together, *Trends in Cell Biol.* 7:43-46.

Traub, L.M., Kornfeld, S. and Ungewickell, E. (1995) Different domains of the AP-1 adaptor complex are required for Golgi membrane binding and clathrin recruitment, *J.Biol. Chem.* 270:4933-4942. (Medline)

Tsukita, S. and Furuse, M. (1999) Occludin and claudins in tight-junction strands: leading or supporting players? *Trends Cell Biol.* 9:268-273. (Medline)

Umeda, A., Meyerholz, A. and Ungewickell, E. (2000) Identification of the universal cofactor (auxilin 2) in clathrin coat dissociation, *Eur. J. Cell Biol.* 79:336-342. (MedLine)

Ungewickell, E., Ungewickell, H., Holstein, S.E., Lindner, R., Prasad, K., Barouch, W., Martin, B., Greene, L.E. and Eisenberg E. (1995) Role of auxilin in uncoating clathrin-coated vesicles, *Nature* 378:632-635. (MedLine)

van de Moortele, S., Picart, R., Tixier-Vital, A., and Tougard, C. (1993) Nocodazole and taxol affect subcellular compartments but not secretory activity of GH3B6 prolactin cells, *Eur. J. Cell Biol.* 60:217-

227. (Medline)

van der Bliek, A.M., Redelmeier, T.E., Damke, H., Tisdale, E.J., Meyerowitz, E.M. and Schmid, S.L. (1993) Mutations in human dynamin block an intermediate stage in coated vesicle formation, *J. Cell Biol.* 122:553-563. (MedLine)

VanRheenen, S.M., Cao, X., Sapperstein, S.K., Chiang, E.C., Lupashin, V.V., Barlowe, C. and Waters, M.G. (1999) Sec34p, a protein required for vesicle tethering to the yeast Golgi apparatus, is in a complex with Sec35p, *J. Cell Biol.* 147:729-742. (MedLine)

Van Zeijl, M.J.A.H. and Matlin, K.S (1990) Microtubule perturbation inhibits intracellular transport of an apical membrane glycoprotein in a substrate dependent manner in polarized Madin-Darby canine kidney cells, *Cell Reg.* 1:921-936. (Medline)

Vega-Salas, D.E., Salas, P.J.I., Gundersen, D. and Rodriguez-Boulan, E. (1987) Formation of the apical pole of the epithelial (Madin-Darby canine kidney) cells:polarity of an apical protein is independent of tight junctions while segregation of a basolateral marker requires cell-cell interactions, *J. Cell Biol.* 104:905-916.

von Figura. K. and Hasilik, A. (1986) Lysosomal enzymes and their receptors. *Annu. Rev. Biochem.* 55:167-193. (Medline)

von Mollard, F.G.and Stevens, T.H. (1999) The *Saccharomyces cerevisiae* v-SNARE Vti1p is required for multiple membrane transport pathways to the vacuole, *Mol. Biol. Cell.* 10:1719-1732. (MedLine)

von Mollard, G.F., Nothwehr, S.F. and Stevens, T.H. (1997) The yeast v-SNARE Vti1p mediates two vesicle transport pathways through interactions with the t-SNAREs Sed5p and Pep12p, *J. Cell Biol.* 137:1511-1524. (MedLine)

Vowels, J.J. and Payne, G.S. (1998) A dileucine-like sorting signal directs transport into an AP-3-dependent, clathrin-independent pathway to the yeast vacuole, *EMBO J.* 17:2482-2493. (Medline)

Waterman-Storer, C.M., Karki, S. and Holzbauer, E.L.F. (1995) The p150^{Glued} component of the dynactin complex binds to both microtubules and the actin related protein centractin (Arp1) *Proc. Natl. Acad. Sci. USA* 92:1634-1638. (Medline)

Waters, M.G. and Pfeffer, S.R. (1999) Membrane tethering in intracellular transport, *Curr. Opin. Cell Biol.* 11:453-459. (MedLine)

Waters, M. G., Serafini, T. and Rothman. J. E. (1991) 'Coatamer': a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles, *Nature* 349:248-251. (Medline)

Waters, M.G., Clary, D.O. and Rothman, J.E. (1992) A novel 115-kD peripheral membrane protein is required for intercisternal transport in the Golgi stack, *J. Cell Biol.* 118:1015-1026. (MedLine).

Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Söllner, T.H. and Rothman, J.E. (1998) SNAREpins: minimal machinery for membrane fusion, *Cell* 92:759-972. (MedLine)

Weidman, P.J., Melançon, P., Block, M.R. and Rothman, J.E. (1989) Binding of an N-ethylmaleimide-sensitive fusion protein to Golgi membranes requires both soluble protein(s) and an integral membrane receptor, *J. Cell Biol.* 108:1589-1596. (Medline)

Weigert, R., Silletta, M.G., Spano, S., Turacchio, G., Cericola, C., Colanzi, A., Senatore, S., Mancini, R., Polishchuk, E.V., Salmona, M., Facchiano, F., Burger, K.N., Mironov, A., Luini, A. and Corda, D. (1999) CtBP/BARS induces fission of Golgi membranes by acylating lysophosphatidic acid, *Nature* 402:429-433. (MedLine)

Weimbs, T., Low, S.H., Chapin, S.J. and Mostov, K.E. (1997a) Apical targeting in polarized epithelial cells: there's more afloat than rafts, *Trends in Cell Biol.* 7:393-399.

Weimbs, T., Low, S.H., Chapin, S.J., Mostov, K.E., Bucher, P. and Hofmann K.(1997b) A conserved domain is present in different families of vesicular fusion proteins: a new superfamily, *Proc. Natl. Acad. Sci. USA* 94:3046-3051. (MedLine)

Whiteheart, S.W., Brunner, M., Wilson, D.W., Wiedmann, M. and Rothman, J.E. (1992) Soluble N-ethylmaleimide-sensitive fusion attachment proteins (SNAPs) bind to a multi-SNAP receptor complex in Golgi membranes, *J. Biol. Chem.* 267:1239-12243.

Whitney, J.A., Gomez, M., Sheff, D., Kresi, T.E. and Mellman, I. (1995) Cytoplasmic coat proteins involved in endosome function, *Cell* 83:703-713. (Medline)

Wickner, W. and Haas, A. (2000) Yeast homotypic vacuole fusion: a window on organelle trafficking mechanisms, *Annu. Rev. Biochem.* 69:247-275. (MedLine)

Wienke, D.C., Knetsch, M.L., Neuhaus, E.M., Reedy, M.C. and Manstein, D.J. (1999) Disruption of a dynamin homologue affects endocytosis, organelle morphology, and cytokinesis in *Dictyostelium discoideum Mol. Biol. Cell* 10:225-243. (MedLine)

Willingham, M. C., Pastan, I. H., Sahagian, G. G., Jourdian, G. W. and Neufeld, E. F. (1981) Morphologic study of the internalization of a lysosomal enzyme by mannose-6-phosphate receptor in cultured Chinese hamster ovary cells, *Proc. Natl. Acad. Sci. USA* 78:6967-6971. (Medline)

Wilson, D.W., Wilcox, C.A., Flynn, G.C., Chen, E., Kuang, W.-J., Henzel, W.J., Block, M.R., Ulrich, A. and Rothman, J.E. (1989) A fusion protein required for vesciel-mediated transport in both mammalian cells and yeast, *Nature* 339:355-359. (Medline)

Wilson, D.W., Whiteheart, S.W., Wiedmann, M., Brunner, M. and Rothman, J.E. (1992) A multisubunit particle implicated in membrane fusion, *J. Cell Biol.* 117:531-538. (Medline)

Wirtz, K.W. (1991) Phospholipid transfer proteins, *Annu. Rev. Biochem.* 60:73-99. (Medline)

Witke, W., Podtelejnikov, A.V., Di Nardo, A., Sutherland, J.D., Gurniak, C.B., Dotti, C. and Mann, M. (1998) In mouse brain profilin I and profilin II associate with regulators of the endocytic pathway and actin assembly, *EMBO J.* 17:967-976. (MedLine)

Wodarz, A., Hinz, U., Engelbert, M. and Knust, E. (1995) Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*, *Cell* 82:67-76. (MedLine)

Woods, J.W., Doriaux, M. and Falquhar, M.G. (1986) Transferrin receptors recycle to the *cis* and middle as well as *trans* Golgi cisternae in Ig-secreting myeloma cells, *J. Cell Biol*. 103, 277-286. (Medline)

Woodward, M.P. and Roth, T.F. (1978) Coated vesicles: characterization, selective dissociation, and reassembly, *Proc. Natl. Acad. Sci. USA* 75:4394-4398. (Medline)

Yang, B., Gonzalez, L. Jr., Prekeris, R., Steegmaier, M., Advani, R.J. and Scheller, R.H. (1999) SNARE interactions are not selective. Implications for membrane fusion specificity, *J. Biol. Chem.* 274:5649-5653. (Medline)

Yeaman, C., Le Gall, A.H., Baldwin, A.N., Monlauzeur, L., Le Bivic, A. and Rodriguez-Boulan, E. (1997) The O-glycosylated stalk domain is required for apical sorting of neurotrophin receptors in polarized MDCK cells, *J. Cell Biol.* 139:929-940. (Medline)

Yokode, M., Pathak, R.K., Hammer, R.E., Brown, M.S.. Goldstein, J.L. and Anderson, R.G.W. (1992) Cytoplasmic sequence required for basolateral targeting of LDL receptor in liver of transgenic mice, *J. Cell Biol.* 117:39-46. (Medline)

Yu, R.C., Hanson, P.I., Jahn, R. and Brunger, A.T. (1998) Structure of the ATP-dependent oligomerization domain of N-ethylmaleimide sensitive factor complexed with ATP, *Nature Struct. Biol.* 5:803-8011. (Medline)

Yu, R.C., Jahn, R. and Brunger, A.T. (1999) NSF N-terminal domain crystal structure: models of NSF function, *Mol. Cell* 4:97-107. (Medline)

Zacharias, D.A., Violin, J.D., Newton, A.C. and Tsien, R.Y. (2002) Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells, *Science* 296:913-916. 9MedLine)

Zerial, M. and McBride, H. (2001) Rab proteins as membrane organizers, *Nature Rev. Mol. Cell Biol.* 2:107-117. (MedLine)

Zhao, L., Helms, J.B., Brugger, B., Harter, C., Martoglio, B., Graf, R., Brunner, J. and Wieland, F.T. (1997) Direct and GTP-dependent interaction of ADP ribosylation factor 1 with coatomer subunit β, *Proc. Natl. Acad. Sci. USA* 94:418-423. (MedLine)

Zhao, L., Helms, J.B., Brunner, J. and Wieland, F.T. (1999) GTP-dependent binding of ADP-ribosylation factor to coatomer in close proximity to the binding site for dilysine retrieval motifs and p23, *J. Biol. Chem.* 274:14198-14203. (MedLine)

Zhu, Y., Traub, L.M. and Kornfeld, S. (1998) ADP-ribosylation factor 1 transiently activates high-affinity adaptor protein complex AP-1 binding sites on Golgi membranes, *Mol. Biol. Cell* 9:1323-1337. (MedLine)

Zhu, Y., Doray, B., Poussu, A., Lehto, V.P. and Kornfeld, S. (2001) Binding of GGA2 to the lysosomal enzyme sorting motif of the mannose 6-phosphate receptor, *Science* 292:1716-1718. (MedLine)

Zimmerberg, J. (2000) Are the curves in the right places? *Traffic* 1:366-368.

Zurzolo, C., Lisantu, M.P., Caras, I.W., Nitsch, L. and Rodriguez-Boulan, E. (1993) Glycosylphosphatidylinositol-anchored proteins are preferentially targeted to the basolateral surface in Fischer rat thyroid epithelial cells, *J. Cell. Biol.* 121:1031-1039. (Medline)

Zurzolo, C., van't Hof, W., van Meer, G. and Rodriguez-Boulan, E. (1994) VIP21/caveolin, glycosphingolipid clusters and the sorting of glycosylphosphatidylinositol-anchored proteins in epithelial cells, *EMBO J.* 13:42-53. (Medline)

12. Energy and Biological Systems.

Energy is central to cell function. Energy is needed for carrying out the tasks of the cell, the displacement of mass in biological movement, the fluxes of solutes through membranes against electrochemical gradients (concentration and electrical potential gradients), the emission of light (bioluminescence), and the synthesis of macromolecules to replace cell components that are broken down. Ultimately, the energy must come from the environment: from the light absorbed by photosynthetic pigments and from the substances that serve as substrates in the cell's metabolism. In a sense, cells or cell organelles may be regarded as transducer systems, i.e., devices that convert one form of energy into another. The present chapter reviews very briefly some of the principles of bioenergetics and is intended to serve as a framework for the discussion of later chapters.

I. FREE ENERGY

The Gibbs free energy change, ΔG, expresses the maximal amount of work that can be performed by a system or, conversely, the minimal amount of energy input required for work.

The definition of ΔG is such that net work can be performed by a reaction when ΔG is less than zero; i.e., the reaction is exergonic. Unless coupled to exergonic reactions, endergonic reactions ($\Delta G > 0$) take place to a very limited extent. When $\Delta G = 0$, the system is at equilibrium. For reactions taking place at constant temperature, ΔG can be expressed as shown in Eq.(1). ΔH is the heat transferred between the system under discussion and the surrounding at constant temperature. In an exothermic reaction, heat is released by the system ($\Delta H < 0$), whereas in an endothermic reaction, heat is absorbed by the system ($\Delta H > 0$). The entropy of the system, ΔS, is defined as shown in Eq. (2), where dQ is the chance in the heat of the system.

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

$$dS = -dQ/T \quad (2)$$

In practical terms, the entropy is related to the organization of the system. In an isolated system (i.e., one that does not exchange heat or matter with its environment) ΔS must be positive.

Two examples of exergonic reactions are shown below in Eqs. (3) and (4). The superscript^o (e.g., in ΔG^o, ΔH^o) means that the quantity has been determined under standard temperature (usually 298 K), pressure (1 atmosphere), and concentration (1 molal). Since biological reactions do not take place under these standard conditions, we should be concerned with ΔG, but for simplicity the system will be assumed to be at standard conditions. The difference between ΔG and ΔG^o and the meaning of these two parameters are discussed in Sections IV and V. The oversimplification of equating ΔG to ΔG^o has been vigorously challenged (Banks and Vernon, 1970). The ΔG value changes as a function of concentration and, in the case of active transport of ions, ΔG is also a function of membrane potential. This aspect will be taken up in Sections IV and VI.

$$ATP \rightarrow ADP + P_i \quad (3)$$

ΔH^o = -7.4 kcal;

$-T\Delta S^o$ = -2.6 kcal

ΔG^o = -10.0 kcal

Equation (3) represents the hydrolysis of the terminal phosphate of adenosine triphosphate (ATP) to form adenosine diphosphate (ADP) and inorganic orthophosphate (P_i). In this reaction ΔH^o and ΔG^o are both less than zero. However, ΔH need not always be less than zero for a reaction to take place, as shown for the activation of NADH dehydrogenase, represented by E in Eq. (4).

$$E + AMP \rightarrow E\text{-}AMP \quad (4)$$

ΔH^o = 12.5 kcal

$-T\Delta S^o$ = -17.3 kcal

ΔG^o = -4.8 kcal

The units used for energy vary in part for convenience, in part for historical reasons. The calorie (or kilocalorie = 1000 cal) has been frequently used, and kilocalories can easily be converted to electrical

potential units by dividing by the Faraday constant, *F* (23 kcal/V). For a direct comparison of ΔG values to the experimentally obtained redox potentials, see Section III. In recent years, the joule (1 X 10^7 ergs, 0.24 cal) or kjoule have been frequently used.

II. COUPLED REACTIONS

Chemical reactions with $\Delta G > 0$ can take place to a significant extent, only if coupled to another reaction in which $\Delta G < 0$. At constant pressure and temperature, the coupling requires the product of the first reaction to be the reactant in the subsequent reaction. If the reactions were not linked in this manner, they would occur independently of each other and therefore no energy could be transferred between these molecules by ordinary means. An hypothetical set of coupled reactions is shown in Eq. (5).

$$A + B \rightleftharpoons C + D \quad \Delta G_1^\circ = 5\,kcal \quad (5a)$$

$$C \rightleftharpoons X + Y \quad \Delta G_2^\circ = -7\ kcal \quad (5b)$$

$$A + B \rightleftharpoons D + X + Y \quad \Delta G^\circ = -2\ kcal \quad (5c)$$

The hypothetical product of reaction (5a), C, is a reactant in the second reaction (5b). Consequently, in the overall reaction (5c), C does not appear at all, since no net change in C occurs. The *G* of the overall reaction (5c) is less than zero since the *G* of the coupled reactions is additive. Equation (6a) gives the formal description of an actual biochemical reaction that requires an energy input in order to proceed to a significant extent. The energy-yielding reaction is represented by Eq. (6b). Equations (6a) and (6b) ignore the reaction needed to link the two. In fact, the molecular details need not be known; only knowledge of the overall reaction is necessary.

$$glutamate + NH_4^+ \rightarrow glutamine \quad \Delta G^\circ = 3.7kcal \quad (6a)$$

$$ATP \rightarrow ADP + P_i \quad \Delta G^\circ = -7.4kcal \quad (6b)$$

$$NH_4 + Glutamate + ATP \rightleftharpoons Glutamine + P_i + ADP \quad \Delta G^\circ = -3.7kcal \quad (6c)$$

The reaction actually takes place by the mechanism shown in Eqs. (6d) and (6e). An intermediate glutamyl-

P-E in the first reaction is a participant in the second reaction.

$$Glutamate + ATP + E \rightleftharpoons Glutamyl\text{-}P\text{-}E + ADP \quad (6d)$$

$$Glutamyl\text{-}P\text{-}E + NH_4^+ \rightleftharpoons Glutamine + P_i + E \quad (6e)$$

In Eqs. (6d) and (6e), E represents an enzyme molecule. The energy for reaction (6a) is said to be coupled to the hydrolysis of ATP, (reaction 6b), and the actual common intermediate (glutamyl-P-E) need not be known to carry out calculations involving the energy balance of the reactions, i.e., ΔG.

The synthesis of ATP from ADP and P_i in so-called substrate-level phosphorylation, takes place by a coupling similar to that discussed in these reactions. They involve as an intermediate a phosphorylated substrate. In contrast, in most cells, the reactions responsible for most of the synthesis of ATP involve oxidation-reduction reactions of the cytochrome system in a process referred to collectively as oxidative phosphorylation. These reactions are likely to occur by a distinct mechanism (see later discussion). In eukaryotic cells, the cytochrome system is in the mitochondria.

An example of substrate-level phosphorylation is the oxidation of glyceraldehyde 3-phosphate to form 3-phosphoglycerate in one of the reactions of glycolysis. In this step, ADP is concomitantly phosphorylated to form ATP. Equations (7a) and (7b) represent the process schematically.

$$Glyceraldehyde\ 3\text{-}phosphate + NAD^+ \rightleftharpoons$$

$$\rightleftharpoons 1,3\text{-}Phosphoglycerate + NADH + H^+ \quad (7a)$$

$$ADP + P_i \rightleftharpoons ATP \quad (7b)$$

The oxidation of glyceraldehyde 3-phosphate has a ΔG of less than -10 kcal, whereas the phosphorylation of ADP is endergonic and has a ΔG of about 10 kcal. In effect, the energy yielded by one reaction is trapped by the synthesis of ATP. A later event, hydrolysis of the ATP, can yield the energy necessary for other processes.

The details of the reactions show that in one reaction, Eq. (8a), a product, 1,3-phosphoglycerate, is formed that is used in the subsequent reaction depicted by Eq. (8b). The coupling scheme shown by Eqs. (8a) and (8b) is somewhat oversimplified but sufficiently detailed to illustrate this mechanism.

$$Glyceraldehyde\ 3\text{-}phosphate + NAD^+ + H^+ \rightleftharpoons$$

$$\rightleftharpoons 1,3\text{-}Phosphoglycerate + NADH + H^+ \quad (8a)$$

$$1,3\text{-}Phosphoglycerate + ADP \rightleftharpoons 3\text{-}Phosphoglycerate + ATP \quad (8b)$$

In the past, couplings similar to those represented in Eqs. (8a) and (8b) have also been postulated for the phosphorylation of ADP in the oxidative phosphorylation reactions of the cytochrome chain. The mechanism of phosphorylation involving the cytochrome chain is still not completely understood; however, it does not involve a phosphorylated intermediate.

Not all the energy derived from a reaction can be used for the synthesis of ATP. Some of the energy is released as heat (Poe and Estabrook, 1969). This release of heat is of great physiological importance in mammals. The nonshivering thermogenesis that occurs in brown fat apparently results from an increase in the energy dissipated as heat in the oxidative reactions in mitochondria (Nicholls and Locke, 1984). This form of thermogenesis plays a fundamental role in cold adaptation and arousal from hibernation.

The idea of energy coupling and in particular the concept that ATP can be used to power in vivo reactions, has been questioned at various times (e.g., see Banks and Vernon, 1970). Although the concepts are sometimes misunderstood and should be used with caution (e.g., see McClare, 1972), they are nevertheless valid.

III. REDOX POTENTIALS

In some reactions, a reactant serves as an electron donor and another as an electron acceptor. In these cases, the ability to exchange electrons can be expressed as an oxidation-reduction potential (*redox potential*). Removal of electrons from the donor and acceptance of electrons by the acceptor can be formulated as separate reactions, as shown in Eqs. (9a) and (9b). In these equations, e represents an electron that is being exchanged. Equation (9c) represents the overall reaction. "Red" and "ox" indicate, respectively, the reduced and oxidized species of the compound. Oxidation-reduction reactions must be coupled to each other in order to take place, but they can be separated out, as done in Eqs. (9a) and (9b), for calculations involving the energetics of the system.

$$red_1 \rightleftharpoons ox_1^{n+} + ne^- \quad (9a)$$

$$ox_2 + ne^- \rightleftharpoons red_2 \qquad (9b)$$

$$red_1 + ox_2^{n+} \rightleftharpoons ox_1^{n+} + red_2 \qquad (9c)$$

If an electrical connection is made between two containers, these reactions can actually take place separately. Each container is called a "half-cell." The system made up of two half-cells is represented in Fig. 1, in which the electrode of half-cell A receives an electron that serves to reduce the component of half-cell B.

$$2H^+ + 2e^- \rightleftharpoons H_2 \qquad (10)$$

The potential of a half-cell is conventionally defined by comparison to that of an H_2 half-cell that operates as shown in Eq. (10). When electrons are given to the H electrode, the potential is considered to be less than zero (i.e., E_h is negative). When the hydrogen half-cell is the electron donor, the potential is considered greater than zero (E_h is positive). In comparing the tendency of two reactions to take place, we are actually concerned with the difference in the redox potential between the two reactions (E_{h1} - E_{h2}) which is expressed as the ΔE of the two half-cells, and the ΔG of the overall reaction is represented by Eq. (11).

$$\Delta G = -nF\,\Delta E \quad (11)$$

Here, ΔE is the redox potential difference (in volts), n refers to the number of electrons and F is the Faraday constant (23 kcal/V). The use of E and its interconvertibility with ΔG can best be illustrated by an example. A hypothetical redox scheme is shown in Eq. (12a). The two half-reactions (i.e., the portions of the reactions occurring in the two half-cells) are shown in Eqs. (l2b) and (l2c).

$$AH_2 + B \rightleftharpoons BH_2 + A \qquad (12a)$$

$$AH_2 \rightleftharpoons A + 2H^+ + 2e^- \quad E_h^\circ = 0.30\ V \qquad (12b)$$

$$B + 2H^+ + 2e^- \rightleftharpoons BH_2 \quad E_h^\circ = -0.25\ V \qquad (12c)$$

Since reaction (12c) is driven by reaction (6.12b),

ΔE^o = 0.30 V - (-0.25 V) = 0.55 V

or

ΔG^o = -2 X 0.23 kcal (V mol)$^{-1}$ x 0.55 V = -25.3 kcal

The reaction depicted in Eq. (l2c) involves H^+ and, therefore, ΔE will depend on the pH of the mixture (pH = -log[H^+]). Usually the redox potentials have been evaluated from values determined at some standard pH (e.g., pH 7).

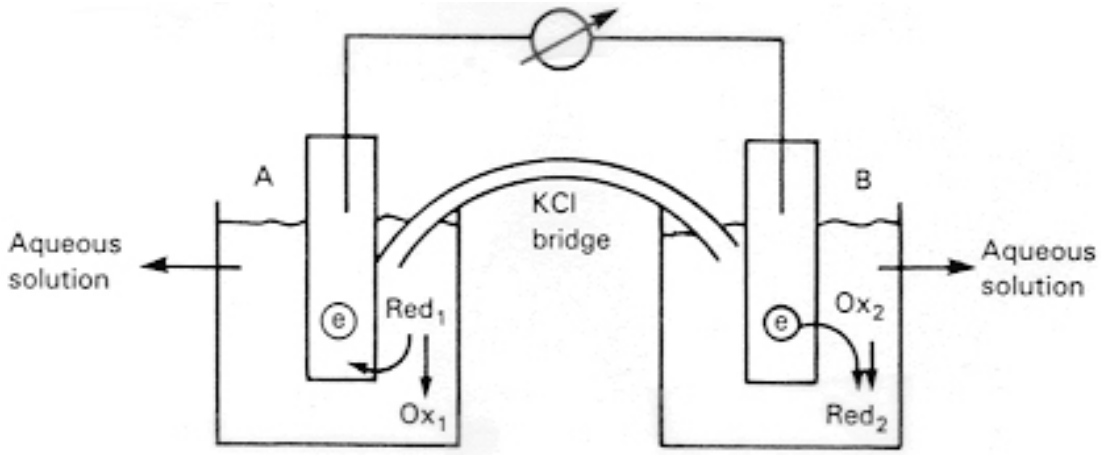


Fig. 1 Two half-cells.

IV. ΔG AS A FUNCTION OF THE CONCENTRATION OF REACTANTS

As already mentioned, the actual parameter that is pertinent to the energy available to perform work is ΔG rather than ΔG^o and in this section, we will focus on this parameter.

The total capacity of a substance to perform useful work actually depends on its chemical potential, μ. The change in μis as a function of concentration as shown in Eq. (13a), which follows directly from the gas laws and is integrated in Eq. (13b)

$$d\mu = RT\, dC / C \quad (13a)$$

$$\mu = \mu^o + RT \log_e C \quad (13b)$$

In these equations, C is the concentration of the substance in question, and μ is the chemical potential under standard conditions. In a chemical reaction such as AB, the ΔG will correspond to the difference in chemical potential between the two components as shown in (Eq. 14).

$$\Delta G = \mu_B - \mu_A = \mu_B^o - \mu_A^o + RT \ln C_B - RT \ln C_A \quad (14)$$

$$= \Delta G^o + RT \ln C_B - RT \ln C_A$$

$$= \Delta G^o + RT \ln(C_B/C_A)$$

In Eq. (14), $\Delta G^o = \mu_B^o - \mu_A^o$. Following Eq. (14), the ΔG for the synthesis of 1 mol of ATP would be

$$\Delta G = \Delta G^\circ + RT \ln \frac{[ATP]}{[ADP]\ [P_i]} \qquad (15a)$$

To convert $\log_e$ (ln) to $\log_{10}$ and to simplify the calculation, $2.3RT$ can be considered to be 1.4 kcal/mol (i.e., 2.3 x 2 cal mol^{-1} deg^{-1} X 300 K). Equation (15a) can be represented by Eq. (15b) if ΔG is assumed to be 10 kcal/mol.

$$\Delta G = 10 \text{ kcal/mol} - 1.4 \text{ kcal/mol } (\log[P_i]) + 1.4 \text{ kcal/mol } (\log_{10}[ATP]/[ADP]) \quad (15b)$$

Equations (15a) and (15b) show that the ΔG for the synthesis or, inversely, the ΔG available from the hydrolysis of ATP, depend on the concentrations of the components. This fact has important consequences. For example, in muscle the ATP concentration is maintained maximally by the transfer of the phosphate from phosphocreatine. Creatine phosphokinase replenishes any ATP hydrolyzed by muscle contraction and other energy-requiring reactions. It is only when 90% of the phosphocreatine is used up that ATP begins to fall significantly, to about 10% of its resting state. The high [ATP]/[ADP] ratio permits a ΔG of -12.5 kcal/mol, a much greater magnitude than the ΔG, which may be as low as -7.6 kcal/mol.

Generally, the hydrolysis of ATP or the reverse reaction, the synthesis of ATP, is written in a simplified form that involves only ATP, ADP, and P_i, as in (Eq. 3). In actuality, the reaction involves H^+, as shown in (Eq. 16).

$$ATP \text{ ---> } ADP + P_i + H^+ \quad (16)$$

The calculation of the ΔG for ATP synthesis therefore would follow Eq. (17a).

$$\Delta G = \Delta G^\circ + RT \ln \frac{[ATP]}{[ADP]\ [P_i]\ [H^+]} \qquad (17a)$$

Since pH = $-\log_{10}[H^+]$, Eq. (a) can be represented as shown in Eq. (17b).

$$\Delta G = \Delta G^\circ + 1.4\ kcal/mol\ pH + 1.4\ kcal/mol\ \log_{10}\frac{[ATP]}{[ADP]\ [P_i]} \quad (17b)$$

When the simpler Eq. (15a) is used, the ΔG is assumed to be at a standard pH (e.g., 7.0 or 7.4). As noted previously, ΔG is significantly sensitive to pH, and a difference of one pH unit lowers the ΔG for the formation of ATP by 1.4 kcal/mol. Since the synthesis of ATP during oxidative phosphorylation (Chapters 16 and 18) or photophosphorylation (Chapters 17 and 18) is associated with membranes, the actual pH values are not known. There have been proposals that during oxidative phosphorylation or photosynthetic phosphorylation the pH of the phosphorylation sites is changed. A decrease of pH from 7 to 5, for example, would lower the ΔG as much as 2.8 kcal/ mo, driving the reaction of Eq. (6.16) toward ATP synthesis.

Actually, the ΔG depends on many other factors, although in the present discussion, we have assumed that the ΔG for the synthesis of ATP (i.e., $-\Delta G$ of its hydrolysis) is in the neighborhood of 10 kcal/mol. Published estimates of ΔG for the hydrolysis of ATP (pH range 6-9, at several Mg^{2+} concentrations and at 25º-35ºC) was calculated to be between -6.1 to -10.9 kcal/mol (see Bridger and Henderson, 1983).

The energy required for the synthesis of 1 mol of ATP is frequently referred to as the *phosphate potential* and is expressed in either kilocalories or units of electrical potential (millivolts). Electrical potential units make it possible to compare the energy needed for ATP synthesis directly with the redox potentials for the cytochromes. Calculations of phosphate potentials for metabolizing isolated mitochondria have been published (Slater et al., 1973).

V. ΔG^o

As mentioned in Section 1, ΔG^o is the ΔG under standard conditions of temperature, pressure, and concentration. ΔG^o is related to the equilibrium constant (K) of the reaction. In Eq. (14), ΔG has been shown to correspond to $\Delta G^o + RT \ln(C_A/C_B)$. Since $\Delta G = 0$ at equilibrium, ΔG^o can be expressed as shown in Eq. (18).

$$\Delta G^\circ = RT \ln \frac{[C_B]}{[C_A]} = -RT \ln K_{eq} \quad (18)$$

VI. ENERGY COST OF TRANSPORT

Biological systems are made up of many compartments, such as kidney tubules, cells, or subcellular organelles, enclosed by selective membranes of varying complexity. The movement of molecules from one biological compartment to another is known as transport. Transport requires an expenditure of energy whenever it occurs against an electrochemical gradient (i.e., the transport is in an uphill direction). In these cases, it is referred to as *active transport*.

For a nonelectrolyte, the chemical potential μ of a compartment in relation to a single solute is represented by Eq. (19a), where n represents the number of moles and μ_1, the chemical potential per mole of the solute.

$$\mu = n\mu_1 \quad (19\ a)$$

The total chemical potential is the summation of μ's for all solutes as in Eq. (19b). Each subscript of μ or *n* indicates a different solute. Here the discussion will be restricted to a single solute.

$$\mu = n_1\mu_1 + n_2\mu_2 + n_3\mu_3 + ... + n_i\mu_i \quad (19b)$$

In a system made up of two compartments separated by a biological membrane, the ΔG for the transfer of 1 mole of solute from one compartment to another will correspond to the difference in chemical potential of the solute between the two compartments. If μ_A represents the chemical potential of the solute in compartment A and μ_B the chemical potential in compartment B, the ΔG can be shown to correspond to Eq. (20) by subtracting the individual chemical potentials as expressed by Eq. (13). Since the standard chemical potential is the same in both compartments, $\mu_B - \mu_A = 0$.

$$\Delta G = \mu_B - \mu_A = \mu_B^o - \mu_A^o + RT \ln C_B - RT \ln C_A = RT \ln (C_B/C_A) \quad (20)$$

In the case of ions, the electrical potential across the membrane and the charges of the solute molecules also have to be taken into account. We are then concerned with the electrochemical potential of the solute. The electrochemical potential (μ) for a given solute corresponds to Eq. (21).

$$\mu = \mu_1 + z\Psi\ F \quad (21)$$

Here Ψ is the electrical potential, *z* is the valence, and *F* is the Faraday constant. Consequently, the ΔG for the transfer of 1 mole of the ion is represented by Eq. (22), where $\Delta\Psi_m$ is the membrane potential.

$$\Delta G = RT \ln(C_B/C_A) + z\Delta\Psi_m F \quad (22)$$

Eq. (22) was obtained by subtracting the μ of phase B from that of phase A.

One of the compartments may contain a nondiffusible charged component such a macromolecule or a colloid. This can give rise to a special kind of equilibrium, called a Donnan equilibrium, in which the diffusible ions distribute unequally between the two phases. Ions opposite in charge to the nondiffusible component will have a higher concentration in its compartment. The reverse is true for ions with charges of the same sign as that of the nondiffusible component. The exact proportion can be calculated from Eq. (22). When $\Delta G = 0$, $RT \ln(C_B/C_A) = -zF\Delta\Psi_m$ or $C_B/C_A = [\exp(-z\Delta\Psi_m F)]/RT$. For this special case, the electrical potential is known as the *Donnan potential*. The $\Delta\Psi_m$ is related to the charge of the nondiffusible component. A Donnan distribution does not require the presence of a membrane. The nondiffusible component may be fixed to a structure rather than restrained by a limiting membrane.

The energy required for the transport of ions can be calculated from Eq. (22). For instance, consider the transport of Na^+ and K^+. In most cells, the two transports are coupled and they take place in opposite directions. The Na^+ is transported outward, whereas K^+ is transported inward. The ion concentrations used in the calculation, which are shown in Table 1, approximately correspond to those of the squid giant axon. Columns 2 and 3 give the internal and external concentrations of Na^+ and K^+. The potential across the membrane is shown in column 4. The inside of the axon is negative in relation to the outside. The electrical potential across the cell membrane would therefore favor the entry of K^+ and oppose the exit of Na^+, as shown in column 5, which represents the electrical potential component of ΔG. The chemical potential component of ΔG is represented in column 6, and column 7 shows the ΔG for each transfer. The energy expenditure is due primarily to the transport of the Na^+. If we assume that the transport is coupled to ATP hydrolysis, the breakdown of 1 mol of ATP would suffice for more than the simultaneous transfer of 1 mol of Na^+ and K^+, since the ΔG of hydrolysis of ATP is approximately -10 kcal/mol. More realistic calculations (Tedeschi and Kinnally, 1992) indicate the cost for 3 Na^+ and 2 K^+ transported per ATP hydrolyzed to be approximately 10 kcal under conditions in which ATP hydrolysis provides -13.2 kcal/mole.

So far, the discussion has concerned the expenditure of energy necessary for active transport and the minimal energy cost (i.e., assuming 100% efficiency) has been calculated for one example. We may well want to ask the opposite question. Can the energy available from the passage of solute in the direction of the electrochemical gradient be harnessed and used for some other process? As shown by substituting the appropriate values in Eq. (20) or (22), the transfer in the direction of the gradient provides a $\Delta G < 0$, for example, in the transfer of a nonelectrolyte from phase A to phase B where C_A/C_B. Such coupling should be feasible at least as far as the energy available for the process is concerned. The flow of one solute in the direction of its gradient can be coupled to the flow of another solute against the electrochemical gradient and in the opposite direction. This is shown, for example, in the experiment depicted in Fig. 2 (Rosenberg and Wilbrandt, 1958). In this experiment, a suspension of human red blood cells has been equilibrated with [^{14}C]glucose. The radioactivity of the external medium is represented on the ordinate and time is shown on the abscissa. Although it has been recognized that a special mechanism is needed for the transfer of the sugars across the plasma membrane, these cells normally do not transport against a concentration gradient. However, on addition of mannose or unlabeled glucose to the medium, the cells begin to transport labeled glucose outward against the concentration gradient, as shown in curves 1 and 2. Curve 3 represents a

control in which only a salt solution was used; the radioactivity of the external medium corresponds to the appropriate dilution. The passage against the concentration gradient is maintained only for a limited period, and eventually the system equilibrates again. This equilibration is to be expected, since the gradient for the mannose or the unlabeled glucose is dissipated. This experiment demonstrates that the energy from the concentration gradient of the solute added to the medium can be harnessed to provide an outward flow of another solute against a concentration gradient. Outflow of one solute in response to the inflow of another has been called counterflow, and it will be discussed later.

Table 1 Energy Requirement for the Transport of Na^+ and K^+ in an axon of a Marine Invertebrate[a]

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Ions	Internal Concentration (M)	External Concentration (M)	m (V)	zF_m kcal/mole	2.3RT log (C_2/C_1) kcal/mole	ΔG kcal/mole	ΔG kcal/mol
K^+	0.40	0.010	0.06	-1.4	2.2	0.8	3.5
Na^+	0.05	0.460	0.06	+1.4	1.3	2.7	

[a]2.3 RT was assumed to be 1.4 kcal/mole

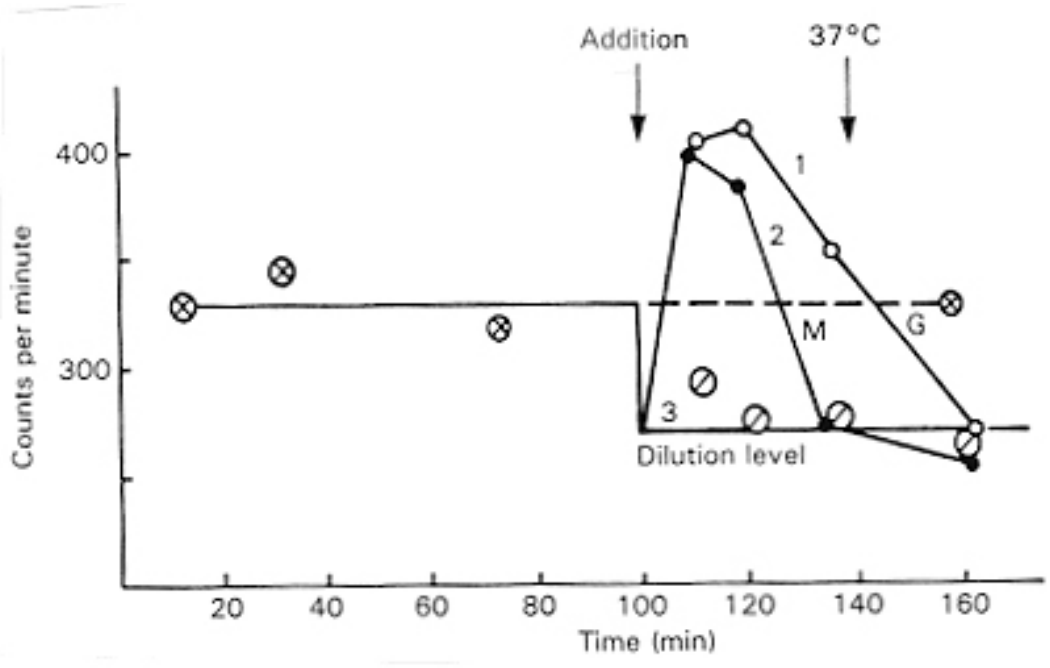


Fig. 2 Experiment showing uphill transport of labeled glucose across the human red cell membrane induced by counterflow of (●) mannose or (○) unlabeled glucose. Ordinate, activity in 10 µl of the external medium. Circle with x in center, activity before addition of 0.16 volume of unlabeled sugar (0.72 M in

saline). Circle with bar, activity after addition of 0.16 volume of saline. Temperature 0ºC until second arrow, then 37ºC. (The temperature was raised to accelerate the penetration, which, however, proved to be unnecessary.) The calculated maximal concentration ratio for labeled glucose is approximately 4 (Rosenberg and Wilbrandt, 1958). Reproduced from *Journal of Gen. Physiol*., ©1958, vol. 41, pp. 289-296 by copyright permission of The Rockefeller University Press.

The transport of one solute may also proceed against its concentration gradient when it is coupled to the flow of another solute in the direction of its own concentration gradient, providing the necessary energy. This type of translocation has been called *cotransport*. A study of pigeon red cells (Vidaver, 1964) has shown inward cotranspont of Na^+ and glycine, with two Na^+ transferred per glycine molecule. The dependence of the glycine transport on the Na^+ gradient has been shown in experiments where the internal concentration of ions has been varied. Red blood cells can be made leaky, exposed to a medium of the desired composition, and then resealed. Table 2 shows how the internal composition of human red blood cells can be varied over a wide range of concentrations. In this example, both Na^+ and K^+ levels were varied. A similar procedure was used with pigeon red cells to vary the internal concentration of Na^+. The flow of glycine in response to changes in the Na^+ gradient is shown in Table 3. The initial internal concentration of Na^+ is represented in column 4; that in the medium, in column 5. The initial concentration ratios of glycine (internal/external) are shown in column 6. Column 7 represents the final ratio after a period of incubation. A comparison of these ratios shows that a higher external concentration of Na^+ results in accumulation of internal glycine. When the internal concentration of Na^+ is higher than that outside, the glycine is actively transported outward (results marked with arrows). In the intact cell, since the Na^+ concentration inside the cell is low, the flow of glycine is invariably inward. In the intact cell, the internal Na^+ concentration is kept low by the net outward transport of Na^+, the so-called *sodium pump*, which expends energy from the hydrolysis of ATP. Table 1 shows the calculation of the energy requirement for the inward transport of 1 mole of Na^+ in the squid axon.

In Chapters 21 and 20, we will examine evidence for the coupled outward transport of Na^+ and inward transport of K^+ powered by the hydrolysis of ATP, and the possible mechanism of this pump.

The converse process, transfer of ions in the direction of the electrochemical gradient coupled to the synthesis of ATP from ADP and P_i, is also possible. An experiment to test this was carried out with human red blood cells in a medium with a high external concentration of Na^+ and low external concentration of K^+ (Glynn and Lew, 1970). The synthesis of ATP from metabolic sources was inhibited with iodoacetate (which inhibits glycolysis, the major metabolic pathway in these cells). Results of the experiment are shown in Fig. 3. Curve 1 represents the incorporation of ^{32}P into ATP as a function of external Na^+ concentration. The concentration of Na^+ was varied without changing the osmotic pressure or the ionic strength of the medium by replacing the Na^+ with choline whenever necessary. Placing the red blood cells in a medium with a higher Na^+ concentration

Table 2 Variations of Erythrocyte Cation Composition after Special Treatment

Concentration of cation in loading medium (mM)		**Final cation content of cells (μEq/ml of cells)**		
Na^+	**K^+**	**Na^+**	**K^+**	**Na^++K^+**
40	260	12	108	120
50	250	16	101	117
75	225	24	89	113
100	200	31	78	109
150	150	50	65	115
300	0	101	12	113

Whittam and Ager (1965) Reproduced by permission from *Biochemistry Journal* , 97:214-227, copyrights ©1965 The Biochemical Society, London.

increases the gradient for Na^+, since the low initial internal Na^+ level remained the same. Increased external Na^+ led to increased ATP synthesis. Curve 2 represents the effects of ouabain, a drug that inhibits the Na^+ pump. Since the incorporation into ATP was prevented by the inhibitor, it is most likely that the reaction does correspond to the reverse of transport against the electrochemical gradient.

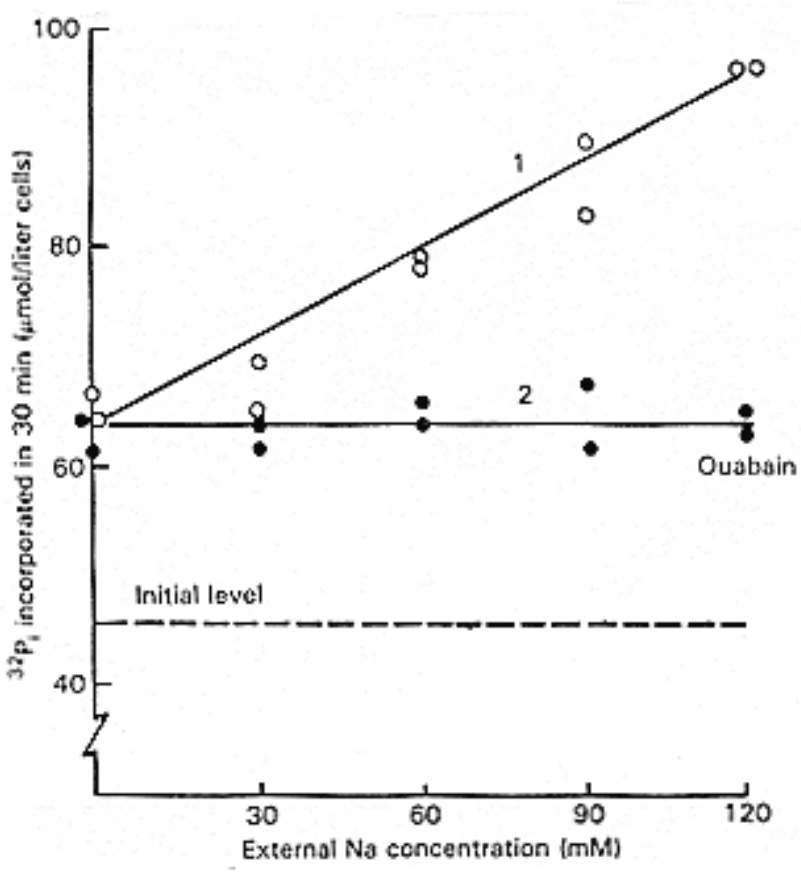


Fig. 3 ATP synthesis as a function of external sodium concentration. Choline was used to replace Na^+ to maintain constant osmotic pressure and ionic strength. Reproduced with permission from I.M. Glynn and V.L. Lew, *Journal of Physiology*, 207:393-402. Copyright ©1970 The Physiological Society, Oxford, England.

The incorporation of $^{32}P_i$ into ATP can be related to the flow in the direction of the gradient by following either the penetration of Na^+ or the exit of K^+, since the transport of the two in opposite directions is coupled. In these experiments, the exit of K^+ was estimated by the appearance of ^{42}K in the medium after suitable loading of the cells with the labeled ion. A correction for the hydrolysis of $[^{32}P_i]$ATP, which takes place presumably by independent processes, makes it possible to relate the K^+ exit with the ATP synthesis. The exit of 2 to 3 moles of K^+ (accompanied by the Na^+ influx) produces 1 mole of ATP.

Similar results have been obtained in experiments with other membrane-bound systems that can carry out active transport of ions. In isolated *sarcoplasmic vesicles*, the release of Ca^{2+} in the direction of the electrochemical gradient results in phosphorylation of ADP (Makinose and Hasselbach, 1971a, 1971b). The *sarcoplasmic reticulum* is a system of tubes and vesicles of striated muscle. Elements of the reticulum liberate Ca^{2+} as a signal for muscle contraction and subsequently sequester it during relaxation by an active transport. The active transport depends on the hydrolysis of ATP and is thought to resemble the Na^+ pump.

In isolated mitochondria, the antibiotic valinomycin induces active transport of K^+ inward. The energy for

this transport can be

Table 3 Glycine Accumulation and Expulsion by Intact or Lysed and Restored Cells

(1)	(2)	(3)	(4)	(5)	(6)	(7)
Experiment	Sample	Preparation	Initial cell Na^+(mM)	Na^+ in medium (mM)	Initial ratio $glycine_{in}/glycine_o$	Final ratio $glycine_{in}/glycine_o$
1	a b	lysed and restored	24 24	140 140	1.45 1.45	2.76 3.02
2	c d	lysed and restored Intact	24 17.5	140 140	1.17 6.06	2.31 8.22
3	e f g h	lysed and restored " "	24 24 115 126	140 0 0 0	1.59 1.57 1.43 1.24	2.39 1/1.08 1/2.06 1/2.34
4	i j	lysed and restored	24 126	140 0	1.12 1.07	1.95 1/1.94
5	k l m	lysed and restored "	115 84 24	140 134 125	1.63 1.67 1.68	1.73 1.91 2.57

From: Vidaver (1964). Reproduced with permission from *Biochemistry* 3:795-799. Copyright ©1964 American Chemical Society.

supplied by either hydrolysis of ATP or respiratory reactions. Conversely, the efflux of K^+ in response to the addition of valinomycin results in net synthesis of ATP from P_i and ADP (Cockrell et al., 1967). Valinomycin is one of the compounds, referred to as ionophores, that are capable of ligating ions. Because of their high solubility in the membrane lipid, they are involved in the transport of ions. The mechanism by which valinomycin affects mitochondrial transport is still a matter of debate.

These experiments show that the transport mechanism can operate in either direction. In one direction, an

exergonic chemical reaction powers the transport against an electrochemical gradient. In the other direction, the passage of ions along the electrochemical gradient can be harnessed to drive the synthesis of a chemical bond.

The minimal energy expenditure necessary to transport solute is given by Eq. (20) or (22). According to these relationships, the minimal expenditure necessary to transport 1 mole of solute (i.e., the ΔG per mole transported) increases with the steepness of the gradient. Experimental examination of transport at different gradients leads to a number of conclusions that bear on the mechanism of transport.

As already discussed, it is possible to vary either the internal or external ionic composition of red blood cells. The internal composition of the red blood cell can be changed by several experimental manipulations. The result of one such procedure has been shown in Table 2. In other work discussed above (see Table 1), the transport of glycine was studied as a function of the Na^+ gradient. Other studies concerned the influx of K^+ as a function of external K^+ concentration and the efflux of Na^+ as a function of internal Na^+ concentration. The ATP hydrolyzed can be calculated from the P_i liberated by the reaction. It is necessary to correct this value by subtracting the amount of ATP produced by the red blood cell's metabolism. The latter can be calculated from the lactate produced by the glycolytic reactions that represent the major metabolic pathway of the red blood cell. In Fig. 4, the influx of K^+ is shown on the ordinate (Whittam and Ager, 1965). Each point represents an experimental determination at a different external K^+ concentration. The hydrolysis of ATP inhibited by ouabain is shown on the abscissa. Ouabain has little or no effect on reactions other than the active transport. The slope of the line indicates the K^+ transported per ATP hydrolyzed and remains constant (mean of 2.4 ± 0.1 K ATP) over a wide range of K^+ concentrations in many independent experiments. In experiments in which the Na^+ efflux was estimated at various internal Na^+ concentrations, the Na^+ transported per ATP hydrolyzed was found to correspond to 1.1 ± 0.2. The results indicate that the energy cost of transporting one Na^+ or one K^+ by the Na^+/K^+ pump is the same regardless of the magnitude of the gradient. This suggests that the transport mechanism functions in precise stoichiometry, in a manner analogous to any other biochemical reaction. The implication of this observation can best be seen by calculating transport in specific cases.

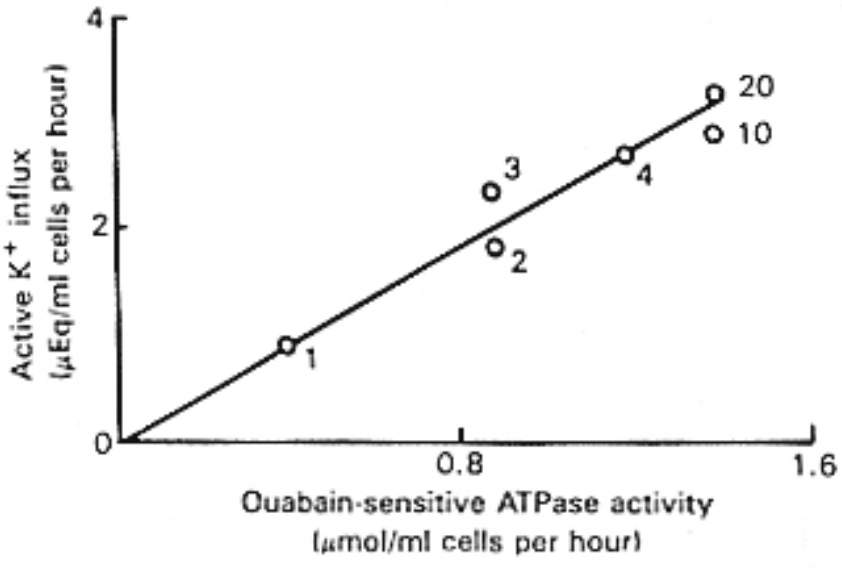


Fig. 4 Relationship between active K^+ influx and ouabain sensitive ATPase activity in media with different K^+ concentrations (mM, shown by the numbers next to the data points). From Whittam and Ager (1965). Reproduced by permission from *Biochemistry Journal*, 97:214-227, copyright ©1965 The Biochemical Society, London.

Table 4 Calculation for ΔG of Transport Corresponding to the hydrolysis of 1 ATP

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Experiment	**Na_o**	**Na_i**	**K_o**	**K_i**	**Na_o/Na_i**	**K_i/K_o**	**n2.3RT x $\log_{10}$ Na_0/Na_i (kcal)**	**m2.3RT x$\log_{10}$ K_i/K_o (kcal)**
1	150	10	10	96	15	9.6	5.3	2.8
2	150	101	10	12	1.48	1.2	0.8	0.3

Table 4 shows some of the concentrations reported by Whittam and Ager (1965) and gives the calculation of *G* for these two cases (as in Table 1). The external concentrations of Na^+ and K^+ are shown in columns 2 and 4 and the internal concentrations in columns 3 and 5. Columns 8 and 9 show the calculations of *G* for the transport of Na^+ and K^+. In these calculations, the membrane potential of the red blood cell has been neglected because its contribution to the energy is small. The values in columns 8 and 9 were obtained by multiplying the appropriate equation by *n* or *m*, the equivalents of Na^+ or K^+ transported per ATP hydrolyzed. The total energy required for transport under the conditions of experiment 1 is about 8.1 kcal (columns 8 and 9), and under the conditions of experiment 2 it is only 1.1 kcal. Nevertheless, as in Fig. 4, the ATP hydrolyzed per Na^+ or K^+ transported remains invariant. Under these conditions, the ΔG for the hydrolysis of one mole of ATP is about -13 kcal, sufficient for the transport under either of the conditions. Therefore, a constant stoichiometry is maintained by varying the efficiency. When the gradient becomes smaller the efficiency drops, even if theoretically there is sufficient energy in each ATP hydrolyzed to support a much larger cation/ATP ratio.

VII. MUSCLE CONTRACTION

Biological systems expend energy in a number of processes. We examined in some detail the transport of ions. Cells also expend energy in moving by processes involved in contraction. The contraction of striated vertebrate muscle has been studied intensively. The work performed by muscle can be calculated readily, since it corresponds to the mass of the object lifted (or, alternatively, the tension τ exerted) times the displacement (ΔL). The energy expended that is not used to perform work is liberated as heat. The heat liberated can be calculated from the change in temperature of an insulated system whose heat capacity (ΔH

= $\mu C_H \Delta T$) is known. Note that if no work has been performed (the muscle has been held stationary in a so-called *isometric* contraction) or a contraction-relaxation cycle has been completed, all energy expended must appear as heat. The heat released by muscle is of great physiological importance in animals (even in some fish, which are considered ectothermic, or cold blooded) and in the physiological control of body temperature. In mammals, the heat released by contraction-relaxation cycles is expended in the basic function of shivering.

Fig. 5 shows the work performed (lower curve) and the heat liberated (upper curve) as a function of tension (τ) relative to peak tension τ_0. An apparatus kept tension constant during the contractile event. The amount of work is expressed solely by the shortening. The results show that the work performed and heat liberated undergo parallel changes. This is expressed most simply in Fig. 6, where the heat liberated is presented as a function of work. It follows from these results that the energy expenditure is proportional to the work performed. However, some energy is expended whether work is performed or not. In Fig. 5, at zero work, 2.95 mcal/g was expended; this minimal expenditure is known as the *activation energy*. The chemical events underlying contraction would, therefore, be graded with the amount of work performed. The efficiency of the system does not vary with increases in the amount of work performed. It has been shown (discussed in Chapter 23) that the amount of ATP (or phosphocreatine) hydrolyzed is proportional to the work, as we would expect from these considerations (Cain et al., 1962). Phosphocreatine is one of the energy reservoirs of muscle and can transfer its terminal phosphate to ADP to regenerate ATP.

We saw that in the transport of Na^+ and K^+, the energetic cost of transferring 1 mol of Na^+ is the same, regardless of the steepness of the gradient. Therefore, the case of ion active transport seems to be quite different in principle from that of contraction of striated muscle. The results suggest that muscle contraction corresponds to a graded process, as if contraction were the result of small finite steps. The molecular mechanisms of contraction are likely to involve such small finite steps (see Chapter 22 and 23).

As noted above, some heat is evolved by muscle whether work is performed or not. This heat evolution seems inherent to the active state. When contracting muscle is slowly stretched, the total heat evolved is less than the sum of the heat of activation and the heat evolved from the work performed on the muscle by the stretching process (Hill, 1960). The difference corresponds rather closely to the work being performed on the muscle.

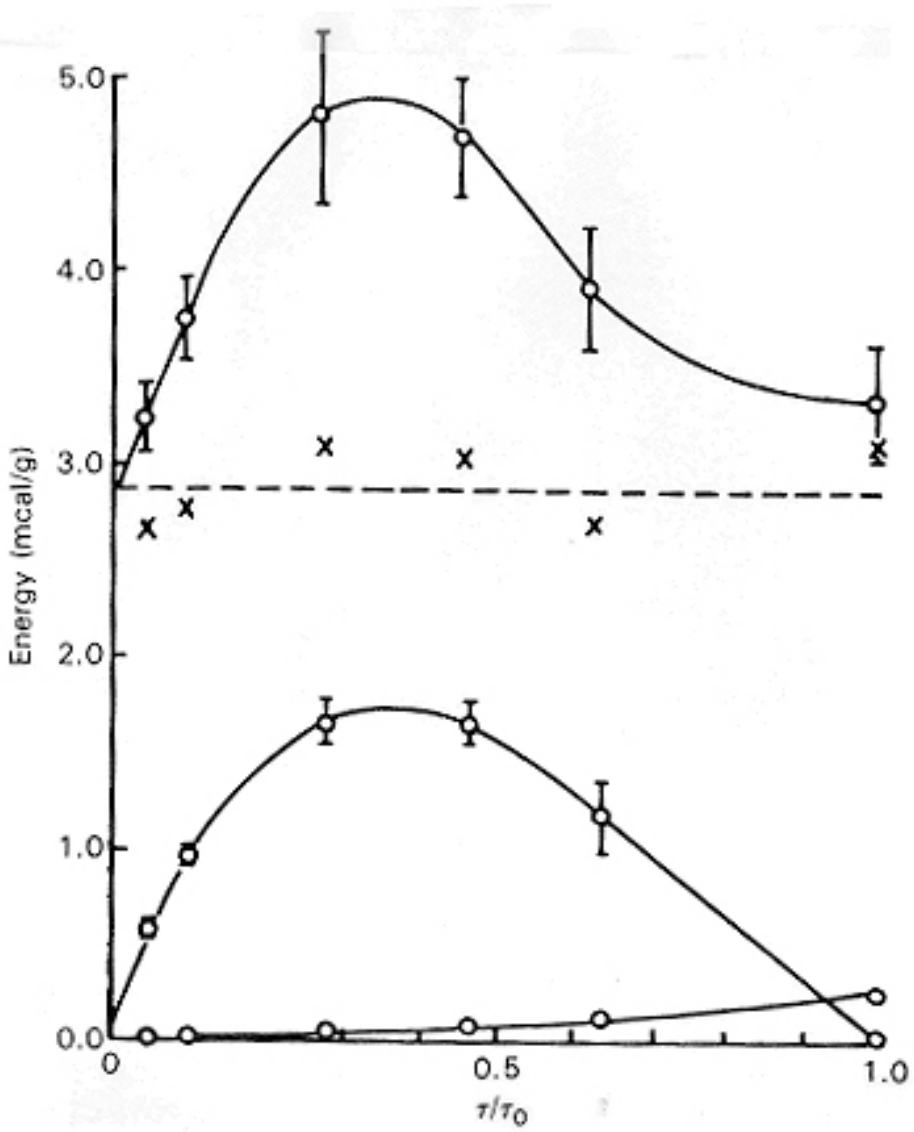


Fig. 5 Variation of energy output with load in after load isotonic twitches; the load was allowed to fall during relaxation. Abscissa, load as a fraction of the peak isometric twitch tension $_0$. Ordinate, energy output in millicalories per gram and twitch (mean of 100 twitches, 20 by each of five muscles, ± 1 standard error plotted as a vertical bar). Upper curve, total heat; lower curves, external and internal work. Crosses, total heat minus total work. Reproduced with permission from F.D. Carlson, et al., *Journal of General Physiology*, 40:851-882. Copyright ©1963 Rockefeller University Press.

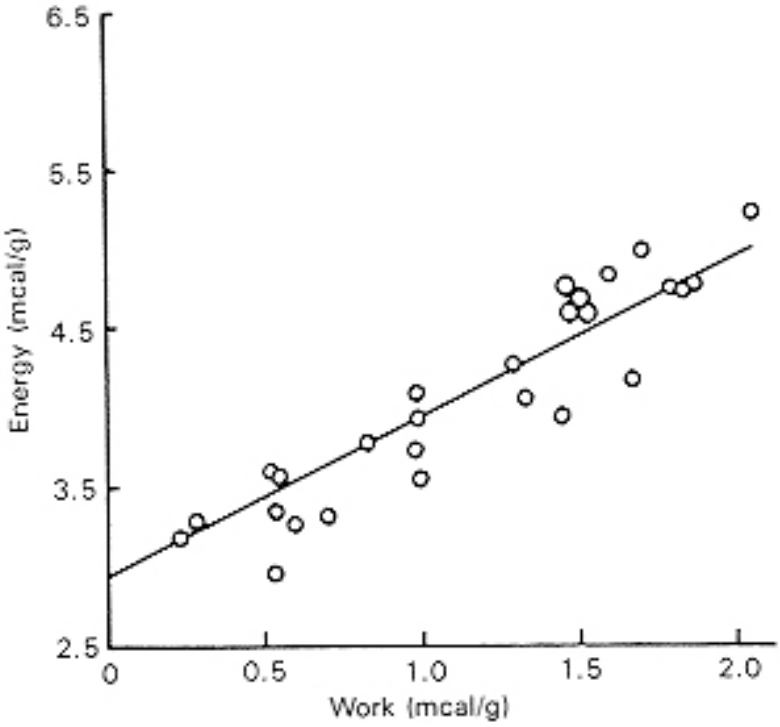


Fig. 6 Ordinate, total heat, millicalories per gram and twitch. Abscissa, total work, millicalories per gram and twitch. Reproduced with permission from F.D. Carlson, et al., *Journal of General Physiology*, 40:851-882. Copyright ©1963 Rockefeller University Press.

The results lend themselves to two possible interpretations: either the stretching has arrested the active state, or the energy input (so-called negative work) has been absorbed by reversing the contractility process. The latter alternative, which is supported by some investigators, would have rather far-reaching repercussions. The energy of contraction originates from the hydrolysis of high-energy phosphates. It has been found that the stretching of striated vertebrate muscle does not result in the synthesis of the main high-energy compound, phosphocreatine or ATP. These experiments have been interpreted as being consistent with the idea that the primary event in contraction does not directly involve the hydrolysis of ATP or phosphocreatine. However, in insect flight muscle preparations, stretching does increase the incorporation of $^{32}P_i$ into ATP (Ulbrich and Ruegg, 1971). In addition, the interpretation involving the "disappearance" of work may well be incorrect. A more likely explanation is that the events of contraction are arrested and that the heat evolved is simply a quantitative conversion of the work performed on the system into heat.

In Chapters 13 to 18 we will examine primarily the processes involved in capturing energy in a biologically utilizable form. The mechanisms involved in dissipating this energy will be taken up primarily in Chapters 19 to 24.

SUGGESTED READING

Becker, W.M. (1977) *Energy and the Living Cell*, Lippincott, Philadelphia.

Christensen, H.N. (1975) Thermodynamic aspects of transport. In *Biological Transport*, 2d ed. Benjamin,

Reading, Mass.

Cramer, W.A. and Knaff, D.B. (1990) *Energy Transduction in Biological Membranes*, Chapters 1 and 2. Springer-Verlag, New York.

Dutton, P.L. (1978) Redox potentiality: determination of midpoint potential of oxidation reduction components of biological electron transfer systems, *Methods Enzymol*. 54:411-425. (Medline)

Morris, J.G. (1968) *The Biologist's Physical Chemistry*, Addison-Wesley, Reading Mass.

Woledge, R.C., Curtin, N.A. and Homsher, E. (1985) *Energetic Aspects of Muscle Contraction*, Academic Press, New York (see Chapters 1, 2, and 4). (Medline)

Alternative References

Harold, F.M. (1986) *The Vital Force: A Study of Bioenergetics*, Chapter 1 to 3. W.H. Freeman, New York.

Tedeschi, H. and Kinnally, K.W. (1992) *Bioenergetics* in *Fundamentals of Medical Cell Biology*, Bittar, E.E. ed., JAI Press Inc., Greenwich, CT., vol. 3B pp. 609-642.

WEB RESOURCES

Diwan, J.J., Bioenergetics, http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/bioener.htm

REFERENCES

Search the textbook

Back to Chapter 12

REFERENCES

Banks, E.C. and Vernon, C.A. (1970) Reassessment of the role of ATP in vivo, *J. Theor. Biol.* 29:301-306. (Medline)

Bridger, W.A. and Henderson J.H. (1983) *Cell ATP*, Chapter 2. Wiley, New York.

Cain, D.F., Infante, A.A. and Davies, R.E. (1962) Chemistry of muscle contraction. Adenosine triphosphate and phophoryl creatine as energy supplies for single contractions of working muscle, *Nature* 196:214-217. Carlson, F.D., Hardy, D.J. and Wilkie, D.R. (1963) Total energy and phosphocreatine hydrolysis in the isotonic twitch, *J. Gen. Physiol.* 46:851-882.

Cockrell, R.S., Harris, E.J. and Pressman, B.C. (1967) Synthesis of ATP driven by potassium gradient in mitochondria, *Nature* 215:1487-1488. (Medline)

Glynn, I.M. and Lew, V.L. (1970) Synthesis of adenosine triphosphate at the expense of downhill cation movements in intact red cell, *J. Physiol. (London)* 207:393-402. (Medline)

Hill, A.V. (1960) Production and absorption of work by muscle, *Science* 131:897-903.

McClare, C.W.F. (1972) In defense of the high energy phosphate bond, *J. Theor. Biol.* 35:233-246. (Medline)

Makinose, M. and Hasselbach, W. (1971a) Calcium efflux dependent formation of ATP from ADP and orthophosphate by membranes of the sarcoplasmic vesicles, *FEBS Lett.* 12:269-270.

Makinose, M. and Hasselbach, W. (1971b) ATP synthesis by the reverse of the sarcoplasmic pump, *FEBS Lett.* 12:271-272.

Nicholls, D.G. and Locke, R.M. (1984) Thermogenic mechanism in brown fat, *Physiol. Rev.* 64:1-64. (Medline)

Poe, M. and Estabrook, R.W. (1969) Kinetic studies of temperature changes and oxygen uptake concomitant with substrate oxidation by mitochondria: the enthalpy of succinate oxidation during ATP formation in Mitochondria, *Arch. Biochem. Biophys.* 126:320-330. (Medline)

Rosenberg, T. and Wilbrandt, W. (1958) Uphill transport induced by counterflow, *J. Gen. Physiol.* 41:289-296.

Rosing, J. and Slater, E.C. (1972) The value of ΔG for the hydrolysis of ATP, *Biochim. Biophys. Acta* 267:275-290. (Medline)

Slater, E.C., Rosing, J. and Mol, A. (1973) The phosphorylation potential generated by respiring mitochondria, *Biochim. Biophys. Acta* 292:534-553. (Medline)

Tedeschi, H. and Kinnally, K.W. (1992) Bioenergetics, in *Fundamentals of Medical Cell Biology*, JAI Press Inc., Greenwich, CT, vol 3B, pp. 609-642.

Ulbrich, M. and Ruegg, J.C. (1971) Stretch induced formation of [P]ATP in glycerinated fibers of insect flight muscle, *Experientia* 27:45-46. (Medline)

Vidaver, G.A. (1964) Glycine Transport by hemolyzed and restored pigeon red cells, *Biochemistry* 3:795-799. (Medline)

Whittam, R. and Ager, M.E. (1965) The connexion between active cation transport and metabolism in erythrocytes, *Biochem. J.* 97:214-227.

13. Enzymes and Enzyme Complexes

The cell is a dynamic system, in continuous change and in continuous motion. The underlying events must necessarily reflect molecular changes, namely chemical reactions. Most chemical reactions occurring in living systems are catalyzed by enzymes. As a result, the study of enzyme reactions of cells goes far in explaining the basis of their functional behavior. The genetic makeup of the cells and the processes involved in the expression of the genetic information determine the kinds of enzymes or structural proteins that are present. These enzymes and structural proteins are the ultimate functional units of the cell.

This chapter addresses first chemical reactions, and enzyme-catalyzed reactions in particular, and then proceeds to a discussion of the properties of the enzymes, their regulation, and the organization in enzyme complexes.

I. CHEMICAL REACTIONS

In a population of molecules, the kinetic energy varies with each molecule. The distribution should be random and follow a pattern such as that represented in Fig. 1. In this plot, the fraction of the molecules, $(dn/dv)n_T^{-1}$, having velocities between v and $v + dv$, is represented as a function of the velocity v. A simple model describing the energy distribution in two dimensions is given by Eq. (1).

$$\frac{dn}{n_T dv} = \frac{mv}{kT} e^{-mv^2/2kt} \qquad (1)$$

In Eq. (1), m corresponds to mass, $\frac{1}{2}mv^2$ is the kinetic energy, k is the Boltzmann constant ($k = R/N$, where R is the gas constant and N is Avogadro's number), and T is temperature. This relationship is represented graphically in Fig. 1.

Representing the kinetic energy by E and $dE = mv\,dv$, Eq. (1) becomes

$$\frac{dn}{n_T} = \frac{1}{kT} e^{-E/kt} dE \qquad (2)$$

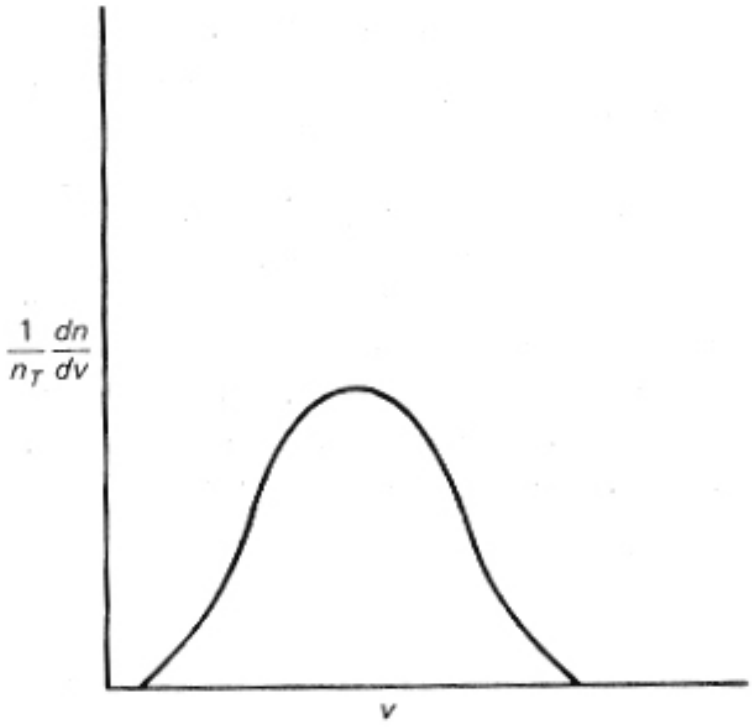


Fig. 1 Distribution of velocities of gas molecules or of molecules in an ideal solution. dn/n_T is the fraction of molecules with velocity in the range of v to $v + dv$. The area under the curve has a value of unity.

If we were to ask what fraction of the molecules has energy greater than a particular value of *E*, it becomes necessary to integrate under the curve [Eqs. (3) and (4)].

It would seem reasonable to consider that molecules need a critical minimal energy, termed the activation energy (*E*), in order to react. Equation (4) represents the proportion of molecules that surpass the activation energy at some specified temperature and hence the proportion of molecules that can react.

$$\frac{dn}{n_T} = \frac{1}{kT} e^{-\frac{E}{kt}} \, dE \qquad (2)$$

$$\int_o^n \frac{dn}{n_T} = \frac{1}{kT} \int_E^\infty e^{E/kT} dE \qquad (3)$$

This idea is illustrated in Fig. 2a and b. Fig. 2a represents the energy of a molecule through the course of the reaction. The reaction is assumed to involve a single reactant. The molecules that can react are represented in Fig. 2b by the portion of the curve that is crosshatched. An increase in temperature increases the kinetic energy of the system. Accordingly, more molecules reach the critical energy level as represented in a diagram in which the bell-shaped distribution is shifted to the right (Fig. 2c). As implied by Eq.(4), the rate of the reaction should be proportional to n/n_T. When the logarithm of the reaction rate constant (represented in this chapter as k_r) is plotted as a function of $1/T$, the slope of the line is $-E/k$.

Generally, the relationship of Eq.(4) is not followed precisely. A number of other parameters are likely to come into play. For example, where more than one molecular species is involved, the two molecules must meet and, furthermore, they must meet in such a fashion that the reactive groups are specifically apposed. Therefore, we would expect the reaction rate to depend not just on temperature but on other factors as well. We would expect that the shape of the molecule or the nature or location of the reactive groups plays a significant role. Accordingly, the reaction rate constant is more closely predicted by a more complex equation. Here *K* is a constant, mostly empirical, and *h* is Planck's constant.

$$\frac{n}{n_T} = e^{-E/kT} \qquad \log_e\left(\frac{n}{n_T}\right) = -\frac{E}{kt} \qquad (4)$$

II. THE ROLE OF ENZYMES

Since the metabolic activity of the cell is to a large extent the sum of its enzymatic activities, it is very much to the point to examine some of the properties of enzymes. Some insights into the control of metabolism can be gained by examining the biochemical properties of purified, soluble enzymes. However, this approach, while providing very useful information, is not likely to reveal a realistic pattern of control in the living cells because of the complexity of the intact system. The level of our ignorance has been illustrated in yeast by varying the concentrations of glycolytic enzymes through the introduction of the appropriate genes in multicopy vectors (Schaaff et al., 1989). The specific activity of individual enzymes was increased approximately 4 to 14 times over that of the wild-type. However, the rate of ethanol production by the yeast remained unchanged even with increases in enzymes catalyzing irreversible steps which have been presumed to play a regulatory role. Part of the reason for the lack of

understanding results from (a) the complexity of the system, (b) the fact that many and perhaps all enzymes are present in multienzyme complexes and (c) they are present in specialized compartments or attached to specific structures which alter their properties and their environment (see Section VI).

Almost all enzymes studied are proteins, generally ranging between 10 and 500 kDa in molecular weight. The reactants that interact with the enzymes are called substrates. Enzymes sometimes require small organic molecules (coenzymes) for activity. Carboxylase, for example, requires thiamine pyrophosphate and enzymes involved in acetylation require coenzyme A. Phosphorylase, which is discussed later in this chapter, requires pyridoxal 5-phosphate. In addition, many enzymes require certain metal ions for their activity. For example, Mg^{2+} is necessary for many enzyme-catalyzed reactions.

The RNA portion of ribonuclease P (a ribonucleoprotein) has been found to have catalytic activity (Guerrier-Takada and Altman, 1984). In addition, a portion of an RNA *intron* of 395 nucleotides released by the self-splicing of a ribosomal RNA precursor (Zaug and Cech, 1986) acts as a ribonuclease and an RNA polymerase. Introns are the noncoding sequences of either a gene or the corresponding primary RNA transcript that are excised while the coding sequences are simultaneously linked during RNA processing to form mature mRNA (see Chapter 3). The large subunit of the ribosome catalyzes peptide bond formation. Although 30 proteins are present, the site at which the peptide bonds are formed is an RNA domain (see Ban et al., 2000). The RNA enzymes are sometimes referred to as *ribozymes*.

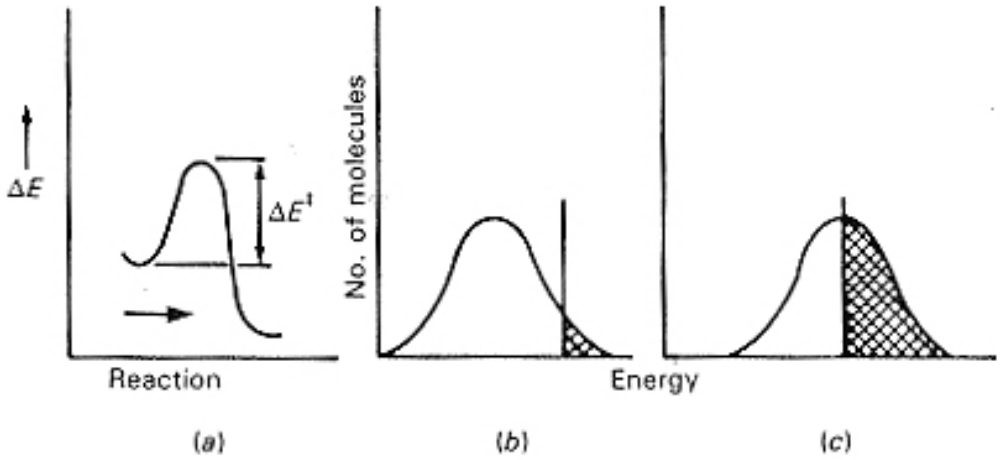


Fig. 2 Illustration of how an increase in temperature allows more molecules to reach the energy of activation, *E*·

The site primarily involved in catalysis, the *active site*, complexes with substrate and may interact with cofactors such as coenzymes and metal ions. It is but a very small portion of the enzyme molecule, as suggested for example, by the low molecular weight of many substrates. The specificity of the interaction between enzyme and substrate implies that a finite number of specific groups in the enzyme molecule are involved. The restricted nature of the active site has also been demonstrated directly. For example, low molecular weight analogs of the substrate can be covalently bonded to a single site in an enzyme molecule and thereby block its activity (Schoellmann and Shaw, 1963).

The technique of X-ray crystallography has permitted the deduction of the structure of many

macromolecules, including enzymes. In some cases, when the crystals of the enzyme are exposed to the substrate, electron density maps reconstructed from the diffraction data show an increased density at a discrete small site (e.g., see Ludwig et al. 1967). The location of this site generally implicates the same groups which were previously suggested by direct chemical studies. Why a large molecule is needed for biological catalysis is still a subject of debate. Some of the conformational rearrangements thought to occur during enzyme catalysis or during the regulation of enzyme activity may require the involvement of a macromolecule.

Many proteins, such as enzymes, contain domains. Domains are portions of proteins that carry out specific functions (see Rashin, 1981). They are identified by computer algorithms that search in proteins data bases for segments (50 to 500 residues) with sequence similarity (see Khosla and Harbury, 2001). There are three kinds of modular enzymes: enzymes in which the substrate specificity and catalytic activity are separable (e.g., see Fig. 10), multisubstrate enzymes where the binding sites for each substrates are modular and multienzyme systems that catalyze metabolic pathways (see Section IIIF).

The role of an enzyme consists of accelerating the rate of a given reaction. The tendency of a reaction to occur and its final equilibrium position are entirely expressed by the free energy, ΔG, of the reaction and remain unchanged in the presence of the enzyme. The thermodynamic considerations discussed in Chapter 12 apply whether a reaction is enzyme catalyzed or not. The enzyme may be regarded as increasing the rate of a reaction entirely by lowering the energy of activation. The principle can best be illustrated by comparing Fig. 2 and Fig. 3.

Fig. 2, as we have seen, represents a hypothetical chemical reaction. Fig. 2a shows the energy as a function of the extent of the reaction, and Fig. 2b shows the proportion of molecules at any given energy. The proportion of molecules having sufficient energy to react is indicated by the crosshatched area under the curve. The energy of the molecules can be increased by heating (Fig. 2c). In contrast, introduction of an enzyme lowers the activation energy to the level represented in Fig. 3a. As a consequence, a larger proportion of the molecules now has sufficient energy to react, as indicated by the increase in the crosshatched area under the curve in Fig. 3b (compared to that in Fig. 2b) without a change in their energy. Catalysis of a chemical reaction is illustrated by the data in Table 1 (Koshland, 1956). In this table, V_o represents the rate or velocity of the reaction in the absence of enzyme and V_E represents the velocity in the presence of enzyme. It is apparent that enzymes can speed up reactions as much as 10^{12} times the rate of the uncaltalyzed reaction.

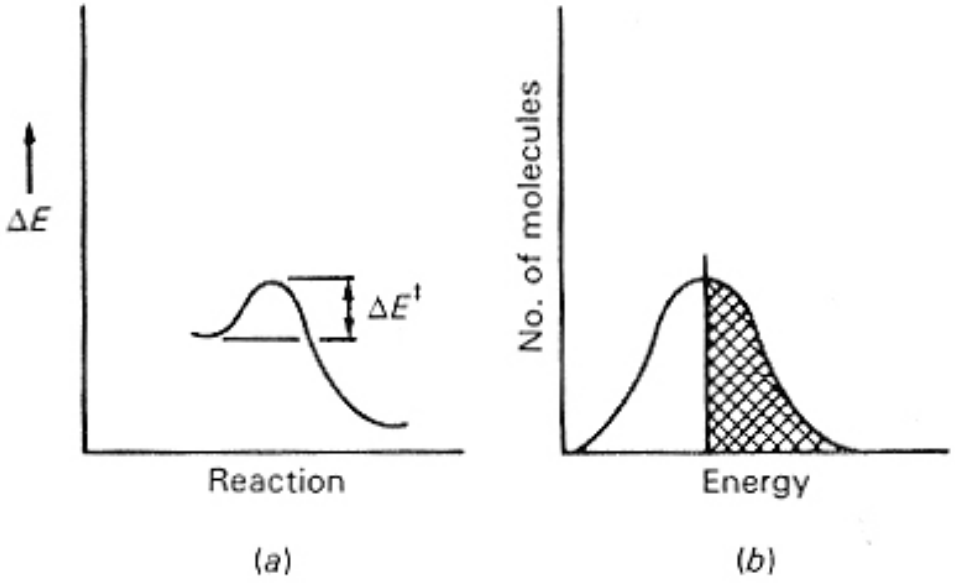


Fig. 3 Illustration of how an enzyme acts. The energy level of the molecules remains unchanged but the *E* is decreased.

Table 1 Rate of a Chemical Reaction in the Presence (V_E) or Absence (V_0) of the Appropriate Enzyme

			Observerd (mol min^{-1} $liter^{-1}$)	
Enzyme	**Substrate & Concentration**	**[E_T] (Eq/liter)[a]**	**V_O**	**V_E**
Hexokinase	0.0003 M Glucose 0.002 M ATP	10^{-7}	1 x 10^{-13}	1.3 x 10^{-3}
Phosphorylase	0.016 M Glucose 1-phosphate 10^{-5} M Glycogen	6 x 10^{-7}	5 x 10^{-15}	1.6 x 10^{-3}
Alcohol dehydrogenase	10^{-4} M NAD 0.04 M Ethanol	4 x 10^{-7}	6 x 10^{-12}	2.7 x 10^{-3}

Creatine kinase	0.024 M Creatine 0.004 M ATP	3×10^{-9}	3×10^{-9}	4×10^{-5}

From D.E. Koshland, *Journal of Comparative Physiology* 47:217-234. Copyright &copy1956 Alan R. Liss, Inc.

[a](Number of moles/ liter) X (number of active sites per molecule)

It is possible to develop some insights into the mechanisms of enzyme catalysis by studying model compounds. These compounds are simple organic molecules that have been found to catalyze certain reactions, although with an efficiency much lower than that of enzyme catalysis. Studies with simpler molecules involve fewer complications and are generally easier to interpret. The possibility of synthesizing molecules that differ from each other in specific ways, permits the evaluation of various factors in the mechanisms of catalysis. In addition, from model reactions the contribution of various mechanisms that are suspected to play a role can be approximated.

Cloning of DNA that codes specific proteins and site-directed mutagenesis (see Appendix) have made it possible to change systematically single amino acids in selected enzymes. An example of this approach as applied to the study of tyrosyl-tRNA synthetase (Wilkinson et al., 1984) is shown in Table 2. The substituted site, occupied by threonine in position 51 in the wild type, is shown in the first column, and the effects of the indicated substitutions on the kinetic constants k_{cat} and K_m are shown in the rest of the table. The constant k_{cat} is the rate constant of the reaction and K_m is the apparent Michaelis-Menten constant, discussed in Section III, A. The ratio of the two (k_{cat}/K_m) relates the reaction rate to the concentration of free enzyme and corresponds to an index of specificity in relation to alternative or competing substrates.

The study of specifically altered enzymes is a very powerful method for elucidating enzyme mechanisms. However, model compounds still provide an important alternative. Alteration of a single amino acid in an enzyme may provide results that are difficult to interpret because it may produce alterations in the overall conformation of the enzyme, with dramatic effects on enzyme activity but only an indirect effect on the catalytic site.

In a reaction involving two or more reactants, the lowering of the activation energy by the enzyme is in part the result of an increase in the number of potentially fruitful collisions between the substrate molecules. In this role, the enzyme orients the active groups of the substrates relative to each other in a manner most conducive for a reaction to occur (*proximity effect*). In contrast, the chances of the active groups free in solution coming into fruitful apposition is much lower.

During enzyme catalysis, the active site, or some component held at the active site, may act as a *nucleophilic* (electron-donating) or *electrophilic* (electron-attracting) agent. The active site of the enzyme will make the reactive group more reactive by making it more electrophilic or nucleophilic. In many cases, this takes place by addition or removal of a proton.

Residues at the active site act as acids (H^+ donors) or bases (H^+ acceptors) in general *acid-base* catalysis. The reactivity of the substrate may be altered by a nonpolar microenvironment at the active site. By virtue of its lower dielectric constant, the hydrophobic spot would alter the ionization of the substrate. The presence of oppositely charged residues (i.e., *ion pairs*) may also have an effect on the rate of the enzyme-catalyzed reaction.

The enzyme may contribute to the orientation of the substrates much more precisely than by the proximity effects already discussed. Perhaps the interaction of the active site substrate introduces a *strain* on the molecule by producing a distortion. The distortion could be produced by a straightforward interaction. Alternatively, a change in the conformation at the active site may distort the substrates and facilitate the reaction (the *rack and strain theory*).

Generally, when an enzyme interacts with a substrate, the conformation of the enzyme changes (see Olson and Allgyer, 1973). In addition, changes in the shape of the enzyme molecule are frequently produced by substances involved in the regulation of enzyme activity, and these changes in conformation affect the ability of the enzyme to interact with the substrate (see Section III C).

Table 2 Pyrophosphate Exchange Activity of Tyrosyl-tRNA Synthetases[a]

Enzyme	k_{cat} (s^{-1})	K_m (ATP) (mM)	k_{cat}/K_m ($s^{-1}M^{-1}$)
TyrTS	7.6	0.9	8,400
TyrTS (Ala 51)	8.6	0.54	15,900
TyrTS (Pro 51)	12.0	0.058	208,000

From; Wilkinson et al (1984) Reproduced by permission from *Nature* 307:187-188. Copyright &copy1984 Macmillan Magazines Ltd.

[a] Synthetase exchange activity was measured at 25ºC, in 144 mM Tris-Cl, pH 7.8, 10 mM $MgCl_2$, 0.1 mM phenylmethanesulfonyl chloride, 10 nM 2-mercaptoethanol, 2mM pyrophosphate, 50 µM tyrosine, and 100-200 nM enzyme.

A number of facts that are known about enzymes can be summarized as follows.

1. The active site of an enzyme binds the substrate and interacts with the substrate to catalyze the reaction.

2. The active site is but a small portion of the total enzyme molecule.

3. The enzyme itself is regenerated after the reaction.

4. The nature of the active group is such that the enzyme can interact only with specific substrates that have certain characteristic groupings.

5. The interaction brings about a lowering of the activation energy and thereby increases the proportion of the substrate molecules that can react at any one time. The result is an increase in the rate of the reaction.

III. KINETICS OF ENZYME REACTIONS

A. Michaelis-Menten Kinetics

We have discussed how the rate of reaction depends on the number of molecules that have reached a critical energy level, permitting the reaction to take place. The number of molecules reacting will, therefore, be directly proportional to the concentration of molecules present. Representing the reactant as A and the product as P, the reaction can be represented as in Eq. (6a):

$$k_r = e^{-E/kt} K\frac{(kt)}{h} \qquad (5)$$

where k_1 represents the rate constant of the forward reaction and k_2 that of the reverse reaction. The ratio k_1/k_2 corresponds to the equilibrium constant. At the beginning of the reaction, with only A present, the backward reaction from P to A need not be considered, as shown in Eq. (6b). Equation (6c) represents the analogous relationship when the back reaction becomes significant.

$$d\frac{(P)}{dt} = k_1(A) \qquad (6b)$$

$$d\frac{(P)}{dt} = k_1(A) - k_2(P) \qquad (6c)$$

Where more than one reactant is involved, the same reasoning applies. In addition, the different reactant molecules must meet before the reaction takes place. Again, the probability of their meeting will be proportional to their concentration. A reaction involving two reactants, A and B, can be represented by Eq. (7a). Accordingly, Eq. (7b) represents the rate of the reaction when the concentration of P is negligible.

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} P \qquad (7a)$$

$$d\frac{(P)}{dt} = k_1 (A)\ (B) \qquad (7b)$$

When an enzyme is involved in the reaction, the same considerations apply, except that the enzyme itself is regenerated as a product. In a reaction involving a single substrate, either the enzyme or the substrate could represent *A* or *B*. Generally, the enzyme is present only in very low concentrations. For this reason, its concentration becomes limiting in determining the rate of the reaction, and an increase in substrate concentration beyond a particular level will not increase the rate of the reaction. There is no free enzyme left to bind *S*. The equations describing the rate of an enzyme-catalyzed reaction can be modified to take this fact into consideration. An enzyme reaction involving a single substrate is represented by

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P + E \qquad (8a)$$

Here, *E* and *S* represent enzyme and substrate, *ES* is the enzyme-substrate complex, and *P* is the product of the reaction.

As we have seen, the rate of a reaction is proportional to the concentration of reactants and the formation of product (*P*) is proportional to $k_3(ES)$. Therefore, the rate of the reaction, conventionally called the velocity of the reaction (*V*), can be represented as

$$V = \frac{dP}{dt} = k_3 (ES) \qquad (8b)$$

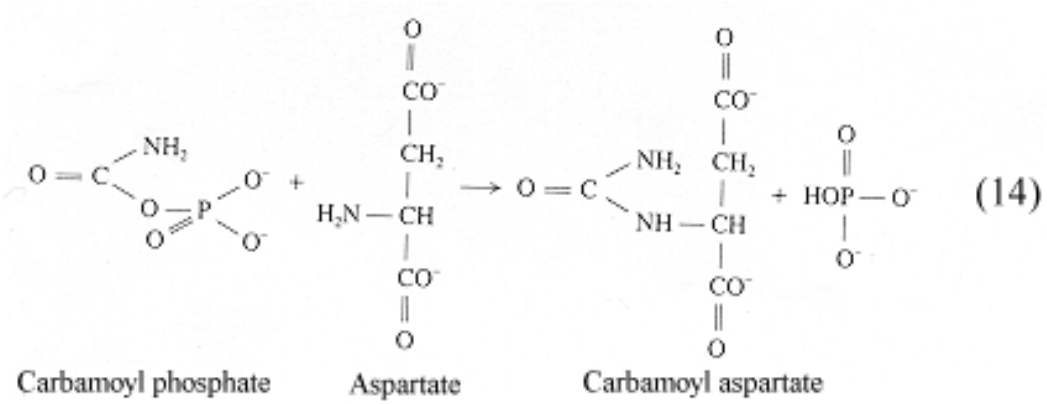
Under ordinary circumstances, the total amount of enzyme (E_T) does not change (the enzyme is neither synthesized nor activated). Therefore, as in Eq. (9):

$$E_T = E + ES \qquad (9)$$

In addition, under steady-state conditions, which are established rapidly, the concentration of *ES* does not change significantly, i.e., $d(ES)/dt = 0$ or

$$d\frac{(ES)}{dt} = 0 = k_1 (E)\ (S) - k_2 (ES) - k_3 (ES) \qquad (10a)$$

On rearrangement, this becomes



(14)

Substituting $(k_2+k_3)/k_1 = K_m$:

$$(E)\,(S) = K_m\,(ES) \qquad (10c)$$

Since $(E) = (E_T) - (ES)$ [Eq. (9)], Eq. (l0c) can be rewritten in the form

$$(ES) = \frac{(E_T)\,(S)}{K_m + S} \qquad (10d)$$

The rate (V) of appearance of the product corresponds to $k_3(ES)$, as shown by Eq. (8b). Therefore, Eq. (10d) can be modified to give

$$V = k_3\,(ES) = \frac{k_3\,(E_T)(S)}{K_m + (S)} \qquad (11)$$

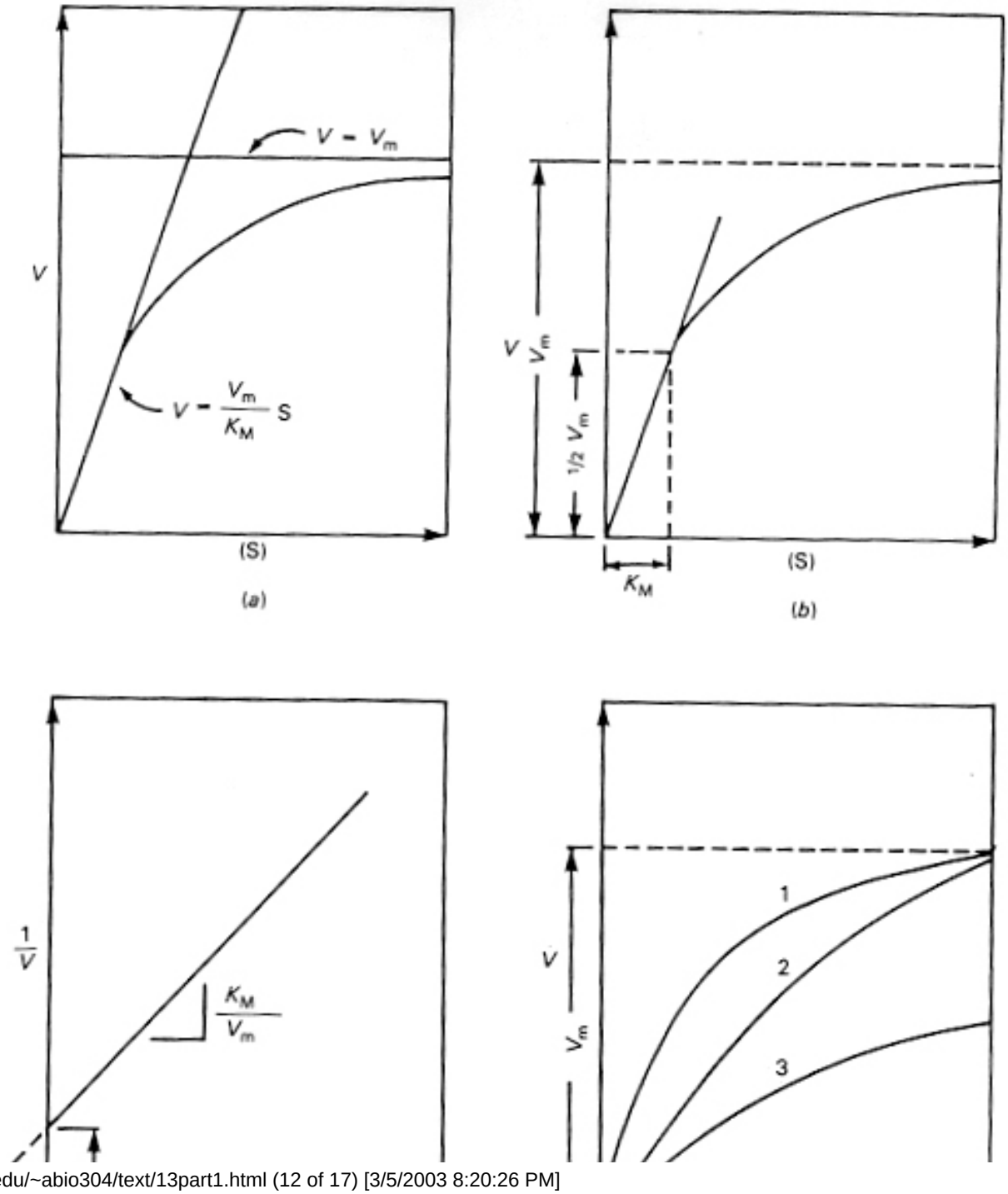
$k_3(E_T)$ represents the maximum possible rate [when $(ES) = (E_T)$]; it is a constant that can be represented by V_m. Equation (11) now assumes the following form, known as the *Michaelis-Menten equation*:

$$V = \frac{V_m\,(S)}{K_m + (S)} \qquad (12)$$

A close examination of Eq. (12) is very revealing. When the amount of substrate is low, (S) becomes negligible in the denominator and the velocity approximates $V = (V_m/K_m)(S)$ (see Fig. 4a). In other words, the velocity varies linearly with substrate concentration. On the other hand, when (S) is very large compared to K_m, K_m can be dropped from the equation. Consequently, (S) is a factor in both numerator and denominator and cancels out, and V now equals the constant V_m: the velocity of the reaction is independent

of the substrate concentration (horizontal line in Fig. 4a). The enzyme is entirely complexed to the substrate (it is saturated); *(ES)* approaches (E_T). The velocity of the enzyme reaction as a function of increasing substrate concentration, expressed in Eq. (12), is represented in Fig. 4a and b. Enzyme reactions following this pattern are said to exhibit Michaelis-Menten kinetics.

The constants V_m and K_m are characteristic of the enzyme reaction and describe the kinetic properties of the system: V_m is directly proportional to the amount of enzyme present, and K_m is characteristic of the enzyme in question. These two constants can be readily evaluated. The maximum number of substrate molecules transformed per unit time by a single enzyme molecule, $V_m/(E)$, is the *turnover number*, a frequently used parameter of enzyme activity. The *specific activity* is the number of enzyme units (1 unit transforms 1 µmol of substrate per minute at 25ºC) per milligram of protein.



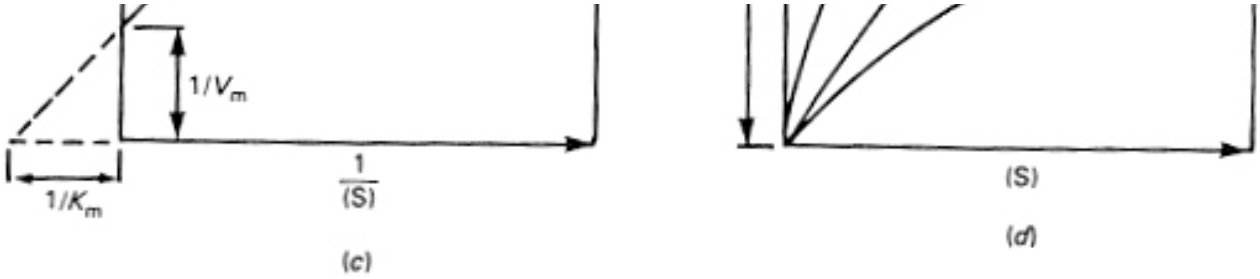


Fig. 4 Relationship between the various kinetic parameters in an enzyme-catalyzed reaction. (a) Rate of the reaction (*V*) as a function of substrate concentration (S). Initial slope and asymptote. (b) Rate of the reaction (*V*) as a function of substrate concentration *(S)*. Determination of K_m from *(S)* when $V = V_m/2$. (c) Lineweaver-Burk plot. (d) Rate of a reaction (*V*) as a function of substrate concentration (S). Curve 1, control; curve 2, competitive inhibition; curve 3, noncompetitive inhibition.

The constant V_m can be estimated by evaluating the asymptote from a graphical representation, such as Fig. 4b, and K_m can be readily evaluated when $V = \frac{1}{2}V_m$, where K_m corresponds to the value of *(S)*. It is common practice to evaluate the constant of the Michaelis-Menten relationship from a plot of the reciprocal of *V* against the reciprocal of *(S)* (known as a *Lineweaver-Burk plot*). The reciprocal of Eq. (12) takes the form

$$\frac{1}{V} = \frac{K_m}{V_m (S)} + \frac{1}{V_m} \qquad (13)$$

In a plot of $1/V$ as a function of $1/(S)$ the slope is K_m/V_m. The $1/V$ intercept is $1/V_m$ and the $1/(S)$ intercept is $1/K_m$.

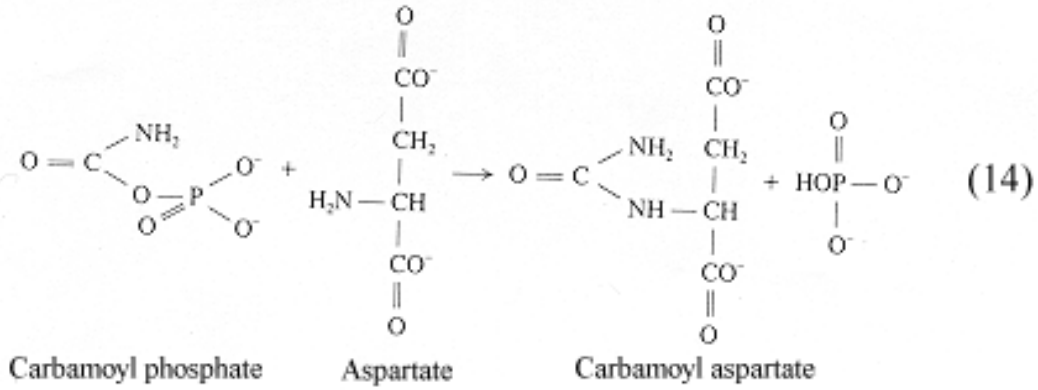
None of these methods are entirely satisfactory. Software is now readily available to calculate the constants for standard treatments or for more complex equations. These programs generally include statistical error analysis (e.g., Software for Science, http://www.scitechint.com/scitech/).

The presence of inhibitors could alter the kinetics represented in Fig. 4a. For example, the *V* may be lowered (e.g., Fig. 4d; compare curves 2 and 3 to curve 1) if the inhibitor combines with the enzyme to make less enzyme available for the reaction. This would result in a decrease in rate as well as V_m (noncompetitive inhibition, curve 3). On the other hand, the inhibitor could compete directly or indirectly for the active site. In this case, the substrate at a high enough concentration should be able to compete successfully, so the V_m would remain unaffected by the presence of inhibitor (curve 2).

B. Sigmoidal Kinetics

Equations (12) and (13) and Fig. 4a represent reasonably well the kinetics of many enzyme reactions. However, many other reactions do not follow these kinetics but, instead, a sigmoidal pattern, where the curve expressing rate as a function of substrate concentration is S shaped. The aspartate

carbamoyltransferase reaction, which is one of these, takes place as shown in Eq. (14).



(14)

This reaction is the first in the pathway that converts carbamoyl phosphate and aspartate into cytidine triphosphate (CTP). The kinetics of the reaction, represented in Fig. 5 (Gerhart and Pardee, 1962), clearly do not follow the predictions of Eq. (12). In this figure, the rates of the reaction at various concentrations of aspartate are shown. The carbamoyl phosphate level is kept constant.

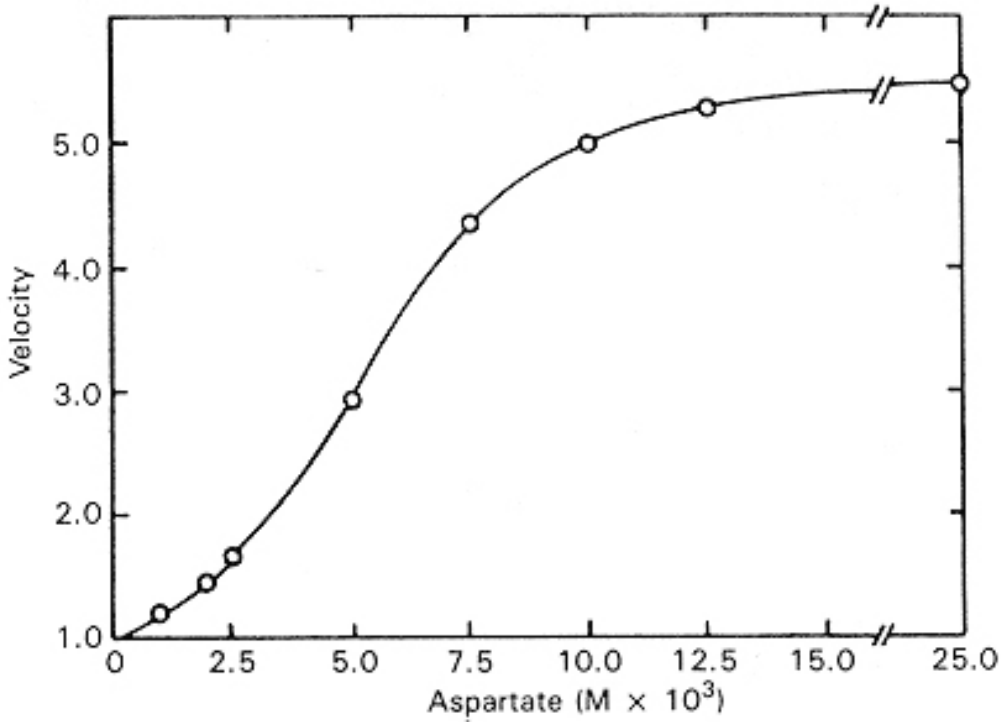


Fig. 5 Kinetics of aspartate carbamoyltransferase reaction. Velocity, units of activity (in this case micromoles of carbamoyl aspartate per hour) per milligram of protein x 10^{-3}. Reaction mixture: 3.6 x 10^{-3} M carbamoyl phosphate and 9.0 x 10^{-3} µg of enzyme per ml. The mixture was in 0.04 M potassium phosphate buffer at pH 7.0. Reproduced with permission from J. C. Gerhart and A. B. Pardee, *Journal of Biological Chemistry*, 237:891-896. Copyright &copy1962 The American Society of Biological Chemists, Inc.

At low concentrations of aspartate, the rate of the reaction does not vary linearly with substrate concentration. Rather, it increases more sharply, as if the effectiveness of the enzyme were increased at the higher concentration of substrate. A number of explanations could account for this phenomenon. The most

likely one is that the reactivity of the enzyme does, in fact, change with increasing concentrations of substrate. It is difficult to visualize an effect of this kind unless we postulate that a combination of the substrate with the enzyme alters the structure of the enzyme so that it reacts more effectively with another molecule of substrate. This implies that each enzyme molecule has more than one functional unit and active site. A substrate at the active site can facilitate the reaction at another active site only if the two active sites are part of the same enzyme molecule. Many enzymes have been found to be made up of subunits that are held together by noncovalent bonds. In some enzymes these subunits are identical, whereas in others the subunits are dissimilar. Generally, but not always, the subunits alone have little or no activity. Reconstitution of the enzyme from its subunits restores the activity.

The idea that these particular kinetics are the consequence of changes in reactivity of the carbamoyltransferase at different substrate concentrations, is supported by experiments in which the enzyme is manipulated chemically. The kinetic pattern is altered when the enzyme is treated with compounds that react with sulfhydryl groups or when it is heated to 60ºC for short periods. This alteration is shown in Fig. 6 (Gerhart and Pardee, 1962) and consists of a conversion to a Michaelis-Menten curve from the original sigmoidal curve. The lower curve in Fig. 6 indicates results obtained with the untreated enzyme. The upper curve indicates results obtained after treatment with $Hg(NO_3)_2$. The rates in the upper curve are increased and the *V* is higher, evidence that the treatment has increased the activity of the enzyme. The lack of a sigmoidal shape for the kinetics of the treated enzyme can be explained by postulating that the altered enzyme can now react only maximally, and that the conformation of the enzyme has been changed by the treatment.

There is considerable evidence that the conformation of some enzymes, changes when the enzymes interact with their substrates. In the case of aspartate carbamoyltransferase, the rate at which the enzyme sediments when centrifuged changes with the addition of succinate, a substrate analog (Gerhart and Schachman, 1968). The rate of sedimentation of macromolecules is a function of their size and shape (i.e., conformation). Presumably, the same changes would occur in the presence of aspartate. The presence of CTP has the opposite effect on the sedimentation of the enzyme. The metabolic significance of the regulation of aspartate carbamoyltransferase by CTP is examined in more detail in the next section (III, C). Changes produced by interactions with the substrate have also been detected by observing changes in the reactivity of some of the residues of the enzyme (e.g., -SH groups). These changes imply a change in the shielding of the residues and hence in the conformation of the enzyme. Similarly, conformational changes of some enzymes have been detected with X-ray diffraction and nuclear magnetic resonance (NMR) techniques.

Recent crystallographic studies have demonstrated substrate induced changes in conformation in enzymes such as phosphoglycerate kinase (PGK). PGK plays a pivotal role in photosynthesis and glycolysis. This enzyme catalyzes a transfer of phosphate from 1,3 bisphosphoglycerate to ADP, producing ATP and 3-phosphoglycerate (3PGA). The two substrates are bound to widely separated domains: one in the amino half of the molecule and the other in the carboxy-half of the molecule. However, the transfer of phosphate is direct since it takes place only when both substrates are present. The transfer could only take place with a substantial hinge-bending conformational change to bring the two substrates together in the catalytic process. Bernstein et al. (1997) have shown by crystallography that complexes of Mg, ADP, PGK and 3PGA are shifted as much as 27 Å compared to the unliganded enzyme with a domain rotation of 32º. Fig.7 shows the changes in conformation deduced from a variety of studies .

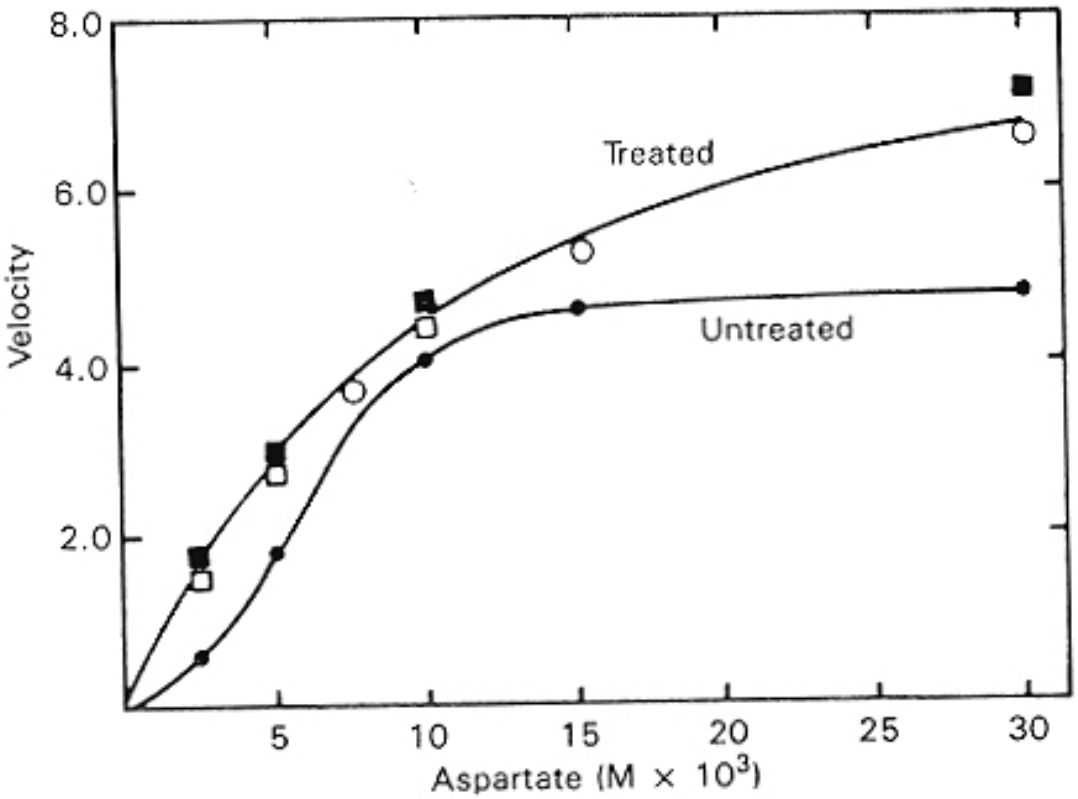


Fig. 6 Dependence of the velocity of the aspartate carbamoyltransferase reaction on aspartate concentration after loss of feedback inhibition. Velocity units are activity per milligram of protein x $l0^{-3}$ (●) Native (untreated) enzyme; (■) 10^{-6} M $Hg(NO_3)_2$ present during assay (heavy metal-treated aspartate carbamoyltransferase); (○) enzyme heated for 4 min at 60C and cooled before assay; (□) heated enzyme assayed in presence of 2 x 10^{-1} M CTP. The reaction mixture contained 3.6 x 10^{-3} M carbamoyl phosphate; aspartate varied as indicated; 2 x 10^{-1} M CTP when used; 0.04 M potassium phosphate, pH 7.0; and 9.0 x 10^{-1} µg of enzyme protein/ml. Reproduced with permission from, J. C. Gerhart and A. B. Pardee, *Journal of Biological Chemistry*, 237:891-896. Copyright &copy1962 The American Society of Biological Chemists, Inc.

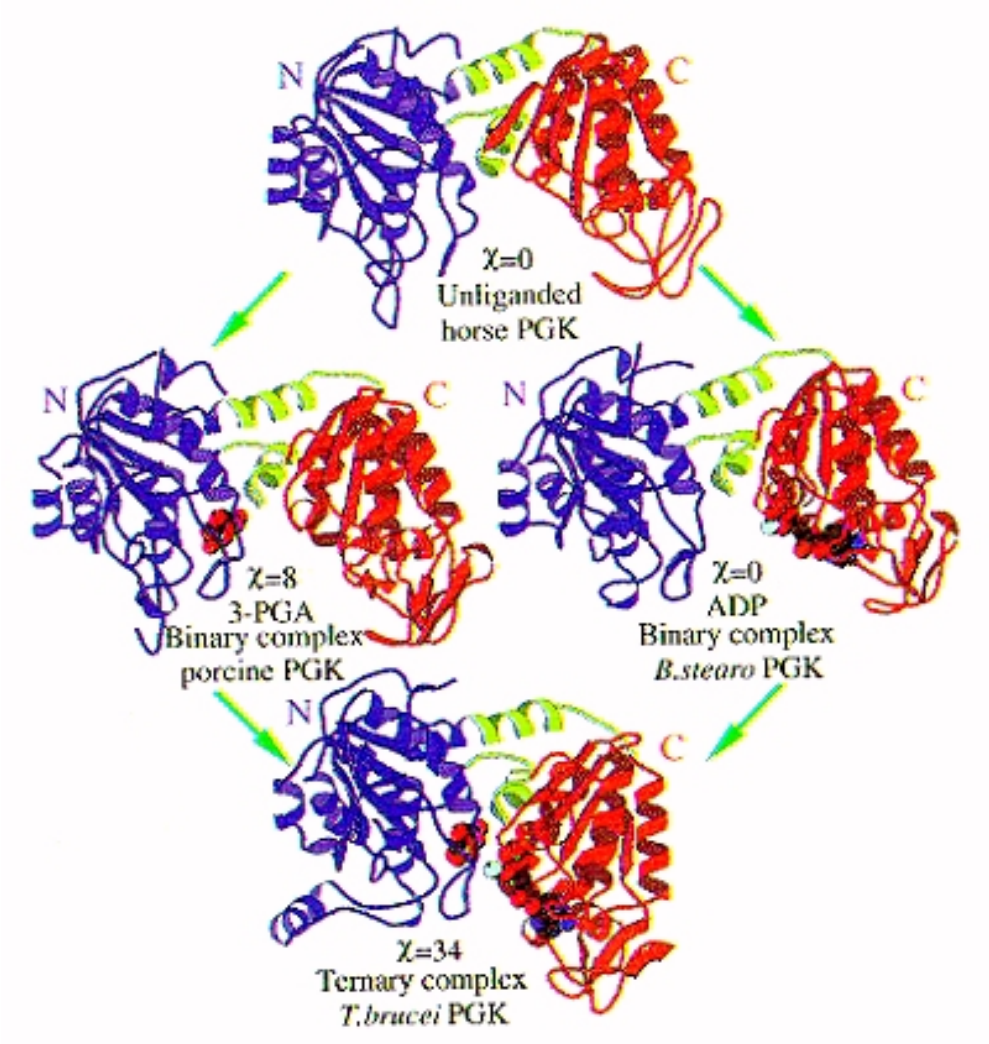


Fig. 7 Substrate-induced conformational change in PGK catalysis. Enzymes from various sources are oriented by superimposing the amino-terminal domains. Reproduced with permission from Bernstein, B.E., Michels, P.A.M. and Hol, W.G.J. Synergistic effects of substrate-induced conformational changes in phosphoglycerate kinase activation, *Nature* 385:275-278. Copyright &copy1997 MacMillan Magazines Ltd.

X-ray diffraction at very low temperatures (e.g., 100 º K will probably permit the study of various steps of enzyme catalyzed reactions (e.g., see Edman et al., 1999).

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

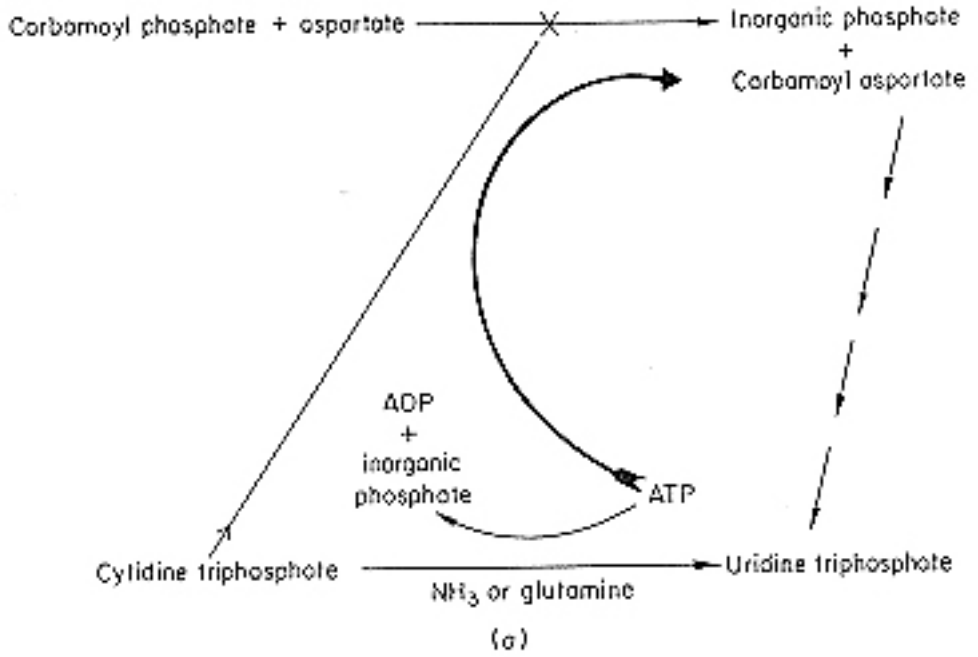
C. Regulatory Mechanisms

Allosteric interactions

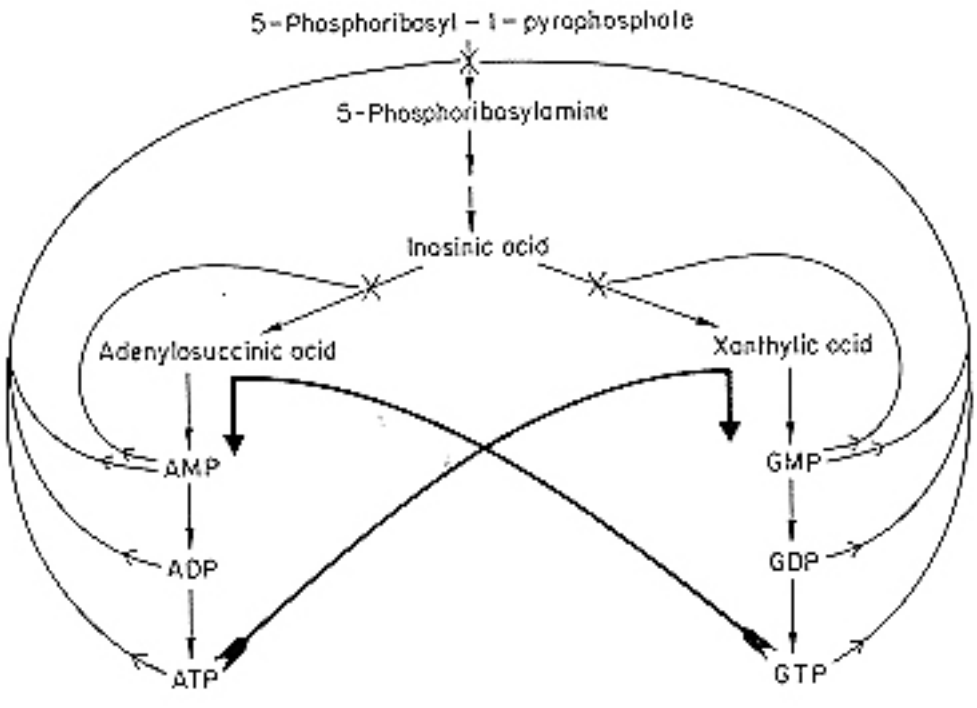
As previously mentioned, the fact that the presence of substrate facilitates the enzyme reaction argues for the presence of more than one functional unit or binding site per molecule of enzyme. In fact, treatment with mercurials (e.g., *p*-mercuribenzoate) separates the aspartate carbamoyltransferase enzyme into two types of subunits. One type is composed of two units containing the catalytic activity, whereas the other contains four units carrying the sites that react with the substances regulating the activity of the enzyme.

A great number of enzymatic reactions can be inhibited or stimulated by compounds that are chemically quite distinct from the natural substrates. These substances interact at sites other than the active site. Such interactions are referred to as *allosteric interactions*. Allosteric inhibitions are frequently exerted by products of metabolic pathways and may be of great physiological significance by permitting precise control of the reaction by *feedback inhibition* (end product inhibition). In this manner, overproduction of end product leads to a slowing down of the pathway that produces it. The functional effect of such an inhibition is illustrated in Fig. 8a. In Fig. 8a and b, the hatched line and heavy arrow indicate stimulation and the black line and cross indicate inhibition. The pathway represented in Fig. 8a synthesizes the cell's uridine triphosphate (UTP) and CTP. Feedback inhibition by the end product (CTP) can adjust the rate of production, depending on the needs of the cell. Regulation can be much more complex, particularly where the pathways are complex. The diagram of Fig. 8b represents the biosynthetic pathway of the purine nucleotides: adenosine triphosphate (ATP) and guanosine triphosphate (GTP). The pathway is branched and its regulation includes feedback inhibition of the initial reaction before the branching point, as well as inhibition of the two separate branches independently. Furthermore, accumulation of the end product of one branch of the pathway stimulates the other branch. In this pathway, inosinic acid is converted into either ATP or GTP, depending on which branch of the reaction sequence is traversed. One of the early steps in the pathway, the first shown in the figure, involves transfer of an amino group to 5-phosphoribosyl-1-pyrophosphate, forming 5-phosphoribosylamine (Fig. 8b). The aminotransferase catalyzing this reaction is inhibited by ATP, ADP, and AMP or, alternatively, GTP, GDP, and GMP, with the two groups of nucleotides acting at two separate regulative sites in the enzyme. The two divergent pathways are subject to separate feedback control at the point of divergence. The branch producing the G-containing nucleotides is controlled by GMP, whereas that producing the A-containing nucleotides is controlled by AMP. In addition, the end products of the two divergent pathways reciprocally facilitate each other: ATP facilitates the GTP pathway and GTP facilitates the ATP pathway. This mode of control ensures smooth integration of the two systems synthesizing the two purine nucleotides.

The data available for the allosteric inhibition of aspartate carbamoyltransferase are shown in Fig. 9a (Gerhart and Pardee, 1962). In this experiment, the velocity of the reaction is measured at various concentrations of aspartate while the concentration of the other substrate, carbamoyl phosphate, is kept constant. The upper curve represents a control in the absence of CTP. The lower curve shows the same experiment carried out in the presence of CTP, the product of the metabolic pathway. Inhibitory compounds of this kind have been called *negative effectors* by some investigators. Other metabolites act as *positive effectors*; ATP acts as a positive effector in this reaction (Fig. 9b). Such a role may be of fundamental physiological importance. When the energy supply is plentiful (reflected in a high level of ATP), nucleic acid synthesis and, consequently growth, are favored by increasing the availability of the nucleic acid precursors. On the other hand, a decrease in ATP slows down the biosynthetic pathway.



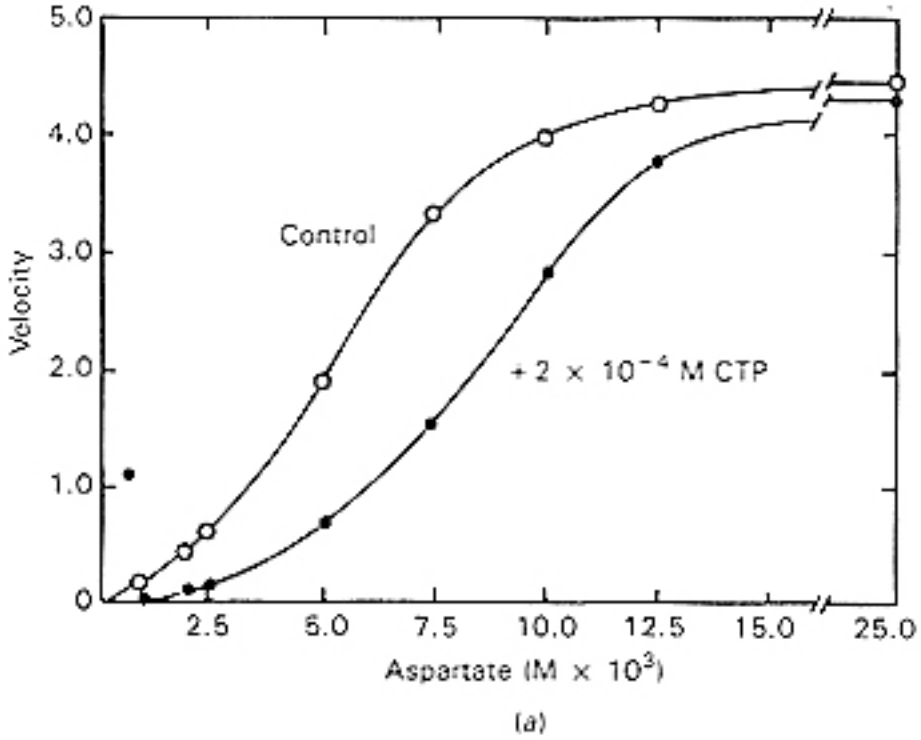
(a)



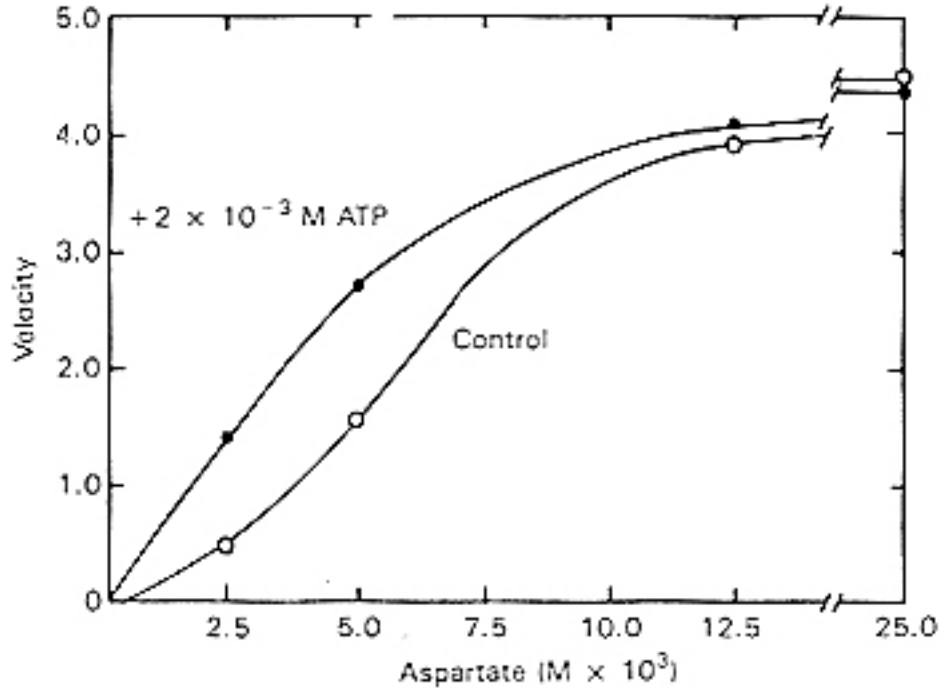
(b)

(b)

Fig. 8 (a) Feedback control of the aspartate carbamoyltransferase reaction by CAP and its stimulation by ATP. (b) Schematic diagram of the regulation of purine nucleotide metabolism.



(a)



(b)

Fig. 9 (a) Reversal of CTP inhibition of aspartate carbamoyltransferase by aspartate. (b) Effect of ATP on reaction velocity. Velocity, units of activity per milligram protein x 10^{-3}. The reaction mixture contained

3.6 x 10^{-3} M carbamoyl phosphate, 0.04 M potassium phosphate, pH 7.0, and 9.0 x 10^{-2} g of enzyme per ml. Reproduced with permission from J. C. Gerhart and A. B. Pardee, *Journal of Biological Chemistry*, 237:891-896. Copyright &copy1962 The American Society of Biological Chemists, Inc.

The increase in enzymatic activity that occurs when the aspartate carbamoyltransferase is treated with mercurials (Fig. 9) is accompanied by loss of regulative ability. The inhibition by CTP is lost completely, as shown in Table 3, column 1. Columns 2 and 3 correspond to the apparent V_m and K_m, respectively.

Table 3 Selective Destruction of Feedback Inhibition

Treatment	(1) Inhibition by 2 x 10^{-4}M CTP (%)	(2) Maximal velocity (units/mg protein x 10^{-3})	(3) Aspartate for half-saturation (molarity x 10^{-2})
1. None	70	4.5	6
2. 10^{-6} M Hg $(NO_3)_2$	0	10.0	12
3. 5 x 10^{-3} M p-hydroxymercuribenzoate	0		
4. 5 x 10^{-3} M mersalyl[b]	0		
5. 10^{-3} M $AgNO_3$	0		
6. Preheat 4 min at 60°C	0	9	12
7. 0.8 M urea	0	>4.5	

Reproduced with permission from J.C. Gerhardt and A.B. Pardee, *J. Biol. Chem.* 237:891-896. Copyright &copy1962 The American Society of Biological Chemists, Inc.

The fact that loss of regulative ability and loss of enzymatic activity do not go hand in hand speaks for two different reactive groups being responsible for the two effects. As mentioned, two different kinds of subunits are separated out from aspartate carbamoyltransferase upon treatment with *p*-mercuribenzoate. One kind of subunit involves enzymatic activity only, whereas the other, when complexed with the active portion, permits control by end product or end product analog inhibition (Fig. 10). Alternatively, the activity of the enzyme can be enhanced by ATP. The recoupling after removal or dilution of the mercurial compound is spontaneous.

Fig. 10 (Gerhart and Schachman, 1965) represents a demonstration of the separation of the subunits of carbamoyltransferase by *p*-mercuribenzoate. It is possible to separate protein molecules on the basis of their size and density differences by means of centrifugation techniques. One such technique is centrifugation through a sucrose solution, forming a density gradient. The preparation, in this case the enzyme treated with *p*-mercuribenzoate, is layered on a tube containing the sucrose gradient. Centrifugation of the tube at very high speed causes the molecules to descend through the gradient, with the heaviest and largest molecules moving most rapidly. After centrifugation, the various layers of the gradient can be separated and sampled for enzymatic activity. The assay is carried out by mixing the layer with a substrate mixture of aspartate and carbamoyl phosphate. The appearance of the product of the reaction, carbamoyl aspartate, is measured by the appropriate chemical method. In Fig. 10, the distribution of the enzyme in the various layers is shown by the closed circles and dashed curve. The capacity of the fractions to inhibit the aspartate carbamoyltransferase (after dilution to decrease the concentration of *p*-mercuribenzoate) is shown by the filled triangles and line. The enzyme activities were assayed as follows: one of the fractions that is unable to respond to the negative effector (fraction 6) was mixed in the presence of CTP with the fraction to be sampled for inhibitory activity. Clearly, the enzymatic activity (filled circles) and the ability to respond to the inhibition (triangles) are in two separate molecules. The open circles represent the ultraviolet light absorption of each fraction (at a wavelength of 280 nm) and indicate protein concentration.

In contrast to aspartate carbamoyltransferase, many of the enzymes studied so far do not have separate subunits responsible for regulation. One of these enzymes is glutamine synthetase from *Escherichia coli*. The enzyme synthesizes glutamine from glutamate, ATP, and ammonia. Glutamine is thought to serve as an important reserve of available nitrogen and is required by several metabolic pathways. Glutamine synthetase is made up of 12 identical subunits, each with a molecular weight of about 50 kDa (Shapiro and Stadtman, 1967). The activity of this enzyme is inhibited by at least eight separate feedback inhibitors which apparently attach to eight independent allosteric sites.

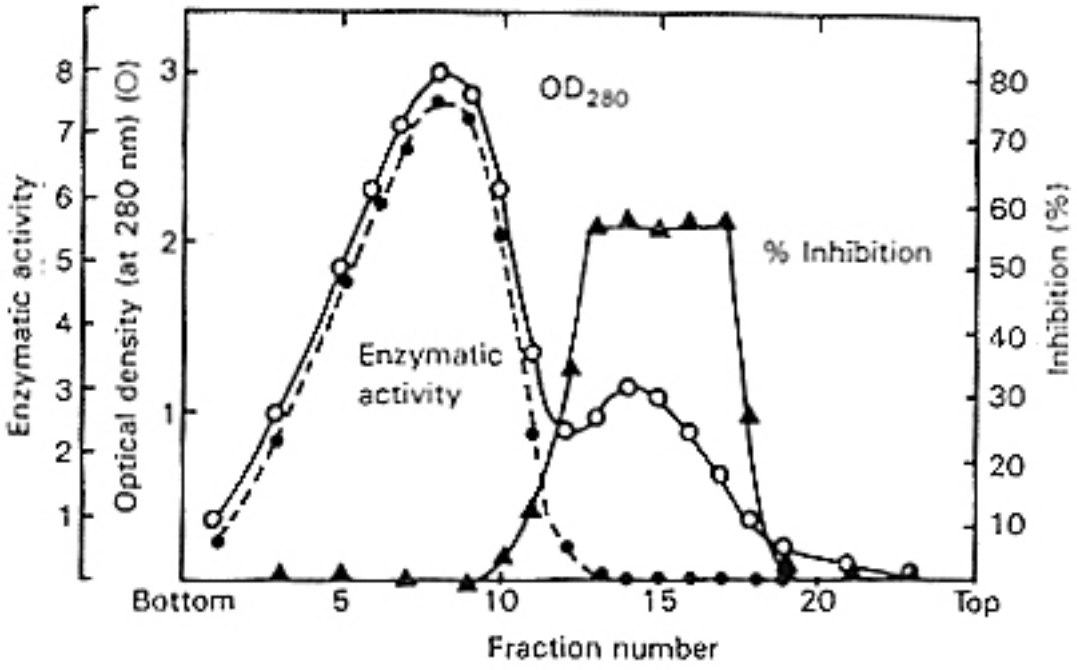


Fig. 10 Subunits of aspartate carbamoyltransferase separated by sucrose gradient centrifugation. The layers contained 6-25% sucrose, 0.04 M potassium phosphate, pH 7.0, and 10 M *p*-mercuribenzoate. Samples were centrifuged for 20 h at 10ºC at 38,000 rpm in a W-39 rotor of a Spinco model L centrifuge. Reproduced with permission from Gerhart and Schachman (1965) Biochemistry 4:1054-1062, copyright &copy1965 by the American Chemical Society.

A model representing an enzyme made up of two identical subunits is shown in Fig. 11 (Koshland and Neet, 1968). Allosteric effectors, R_2, interact with special regulator sites of the molecule (part B) as in the case of glutamine synthetase. In some cases, such as aspartate carbamoyltransferase, part B is a separate regulatory subunit. The shaded sites in part A of the molecule correspond to the active site bindings substrate, S, with two residues, X_1 and X_2. The effector molecule R_1 can interact directly with the active site. The outside portion of the active site represents a flexible arm that is adjusted into position by the substrate itself and the effector R_2 (the so-called *induced fit*). There is considerable evidence that induced fit plays a significant role in enzyme catalysis.

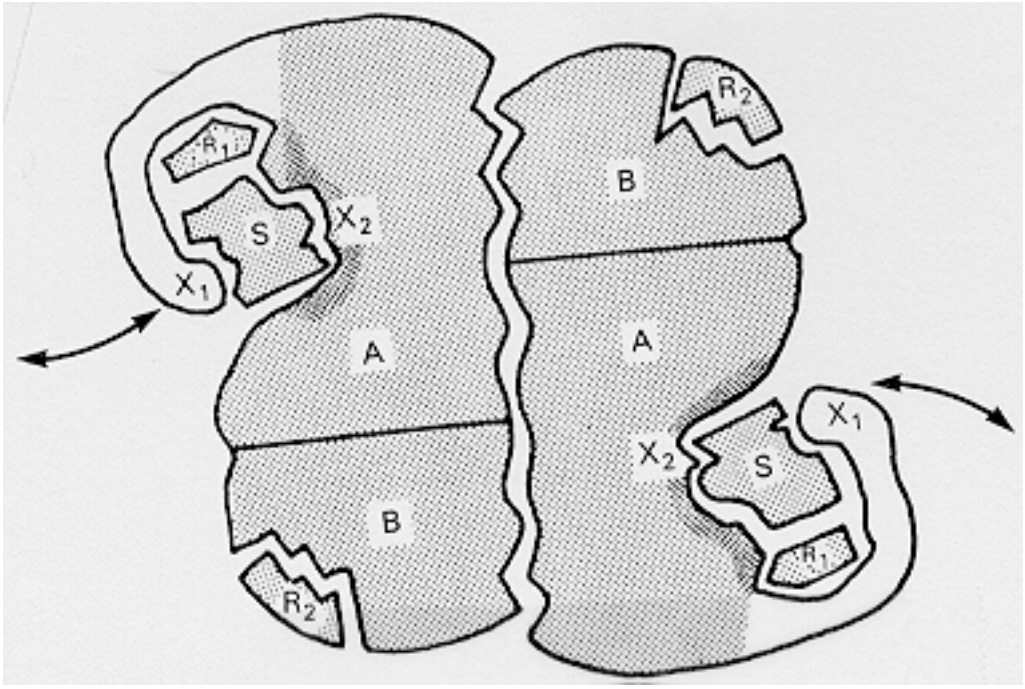


Fig. 11 Hypothetical structure of an enzyme containing two identical subunits. The enzyme has two residues in the active site (X_1 and X_2). The substrate is denoted by S, and R_1 and R_2 are regulator molecules, functioning at the active site and at allosteric site, respectively. Modified from Koshland and Neet (1968). Reproduced, with permission, from the *Annual Review of Biochemistry*, Volume 37, 1968 by Annual Reviews Inc.

The model represented in this figure depicts neither a conformational change imposed by the allosteric effector nor a conformational change induced in one subunit by the interaction of the other with the substrate. The nature of the conformational changes in enzymes are still not entirely clear.

As shown in Fig. 12 (Atkinson, 1965) the effects of positive and negative effectors are well suited for the regulation of enzyme activity. The presence of an effector can produce a major change in the reactivity of the enzyme. The small shifts in the curve, depicting the rate of the reaction as a function of substrate concentration, represent major changes in the rate of enzyme activity at certain fixed substrate concentrations (e.g., S') (compare 3, the control curve to 2 or 4 in Fig. 12). The bar diagrams (left side of the figure) show the rates at these critical substrate concentrations in the presence or in the absence of positive and negative effectors.

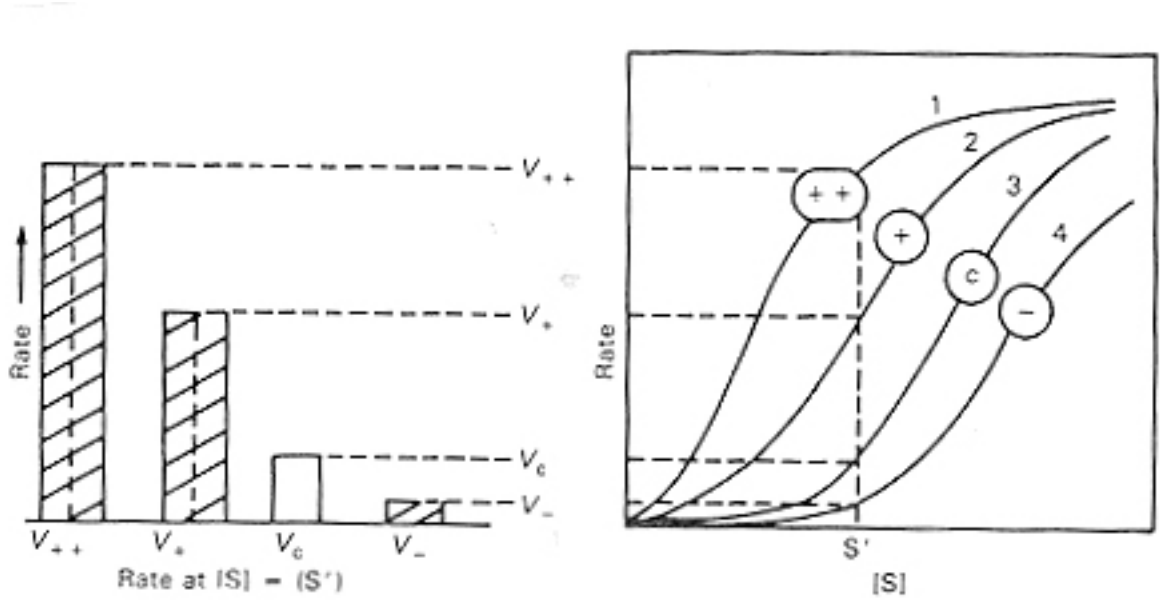


Fig. 12 Generalized substrate response curve for an enzyme that is regulated by positive effectors (+) or negative effectors (-). The control (no effectors) is indicated by the letter c. Reproduced with permission from D. E. Atkinson, *Science*, 150:851-857. Copyright &copy1965 by the AAAS.

Generally, allosteric effects can vary the activity of an enzyme over a 10-fold range. However, because in some cases the forward and backward reactions of a step are catalyzed by separate enzymes which are regulated independently, the actual regulation can result in a 100-fold change (see Chapter 14, Section IIC).

Intrasteric interactions

Regulation may involve internal sequences resembling the substrate and binding to the active site. This kind of regulation has been called *intrasteric regulation* (see Kobe and Kemp, 1999). Intrasteric regulation may result from allosteric interactions. The regulatory domain is referred to as a *pseudosubstrate* or *intrasteric autoregulatory sequence* (IARS). The pseudosubstrate domain may be in the protein itself or in another subunit of a complex and may even be in a separate protein molecule entirely (e.g., protein proteinase inhibitors, see Kobe and Kemp, 1999). Originally, intrasteric regulation had been observed only in protein kinases and phosphatases (Kemp and Pearson, 1991). However, intrasteric regulation occurs in a variety of enzymes and also in receptors and protein-targeting domains.

Evidence for intrasteric regulation includes: (a) the inhibition of the enzyme by synthetic peptides corresponding to the pseudosubstrates, (b) absence of regulation with the removal of the pseudosubstrate domain, (c) or in the presence of antibodies against the regulatory domain, and (d) structural crystallographic studies that show interactions of a pseudosubtrate domain with the active site.

IV. TRANSITIONS FROM INACTIVE TO ACTIVE FORMS

Some of the regulative events of a cell's biochemical reactions seem to involve allosteric interactions, such as the end product inhibitions or simulations just discussed. However, enzyme activity may also be regulated by covalent modifications in which the enzyme is converted from an inactive form to an active form. Furthermore, at least in the case of phosphorylation, the modification may determine whether the enzyme can be controlled allosterically. A number of covalent modifications have been implicated, such as conversions from S-S to SH groups, acetylation or deacetylation, methylation or demethylation, adenylation or deadenylation. Activation of enzymes by proteolytic cleavage of the inactive form of the enzyme, the *proenzyme*, is involved in many physiological (Hall et al., 1979) and developmental processes (Hasilik and Tanner, 1978; Neurath and Walsh, 1976). At least in eukaryotic cells, many enzymes have been shown to undergo phosphorylative and dephosphorylative transitions. Table 4 (Krebs and Beavo, 1979) gives a relatively short list, and more are likely to be involved. Generally ATP, and in sometimes GTP, is implicated in phosphorylation.

In some cases, the phosphorylated form is the more active form (as in the case of phosphorylase b), and in others it may be a less active form. Generally, the enzymes involved in a breakdown pathway are activated by phosphorylation, whereas enzymes involved in synthetic pathways are inactivated by phosphorylation. Some examples of these effects are shown in Table 5 (Cohen, 1980).

Table 4 Initial Reports on Enzymes Undergoing Phosphorylation-Dephosphorylation

Enzyme	Year reported
Glycogen phosphorylase	1955
Phosphorylase kinase	1959
Glycogen synthase	1963
Hormone-sensitive lipase	1964,1970
Fructose-1,6-bisphosphatase	1966,1977
Pyruvate dehydrogenase	1969
Hydroxymethylglutaryl-CoA reductase	1973
Acetyl-CoA carboxylase	1973
DNA-dependent RNA polymerase	1973

Pyruvate kinase (liver)	1974
Cholesterol ester hydrolase	1974
R subunit of type II cAMP-dependent protein kinase	1974
Reverse transcriptase	1975
Phosphofructokinase (liver)	1975
Tyrosine hydroxylase	1975
Phosphorylase phosphatase inhibitor	1975
Phenylalanine hydroxylase	1976
elF2-kinase	1977
cGMP-dependent protein kinase	1977
Tryptophan hydroxylase	1977
NAD-dependent glutamate dehydrogenase (yeast)	1978
Glycerophosphate acyltransferase	1978

From: Krebs and Beavo (1979) Reproduced with permission, from the *Annual Review of Biochemistry*, vol. 48 © 1979 by Annual Reviews Inc.

Phosphorylations are catalyzed by protein kinases; dephosphorylation by phosphatases (see Section V)

These systems are under the control of hormonal or neural signals. Cyclic AMP, cyclic GMP, Ca^{2+} - calmodulin complex, and other second messengers (see Chapter 6) have been implicated in some of these regulative events.

Table 5 Enzymes Found in the Cytoplasm of Mammalian Cells That Are Regulated by Phosphorylation

	Types of protein kinase involved			
	cAMP	**Ca^{2+}-calmodulin**	**Other**	
ACTIVATION BY PHOSPHORYLATION:				**Biodegradative pathway:**
Glycogen phosphorylase	-	+	-	Glycogenolysis
Phosphorylase kinase	+	+	-	Glycogenolysis
Myosin	-	+	-	ATP hydrolysis
Triglyceride lipase	+	-	-	Triglyceride breakdown
Cholesterol esterase	+	-	?	Cholesterol ester hydrolysis
INACTIVATION BY PHOSPHORYLATION:				**Biosynthetic pathway:**
Glycogen synthase	+	+	+	Glycogen synthesis
Acetyl-CoA carboxylase	+	-	+	Fatty acid synthesis
Glycerol phosphate acyltransferase	+	-	-	Triglyceride synthesis
HMG-CoA reductase	-	-	+	Cholesterol synthesis
eIF	-	-	+	Protein synthesis

From: P. Cohen, *Molecular Aspects of Cell Regulation*, vol 1, Copyright &copy1980 Elsevier Science Publishers, Amsterdam.

A general role of the phosphorylation-dephosphorylation of proteins in cellular physiology has been proposed (Greengard, 1978); the process is likely to affect the functions of chromosomes, ribosomes, microtubules, and cell or intracellular membranes.

A diverse enzyme regulatory role by ubiquitin conjugation, analogous to the phosphorylative mechanism, has also been proposed. Ubiquitin has been found in all cells examined (hence the name, which is derived from ubiquitous). Ubiquitin is an intracellular peptide which is ligated to proteins by a series of reactions involving several enzymes (Chapter 15, II, B). A regulatory role of ubiquitin conjugation is suggested by the presence of ubiquitin hydrolases, which remove ubiquitin from conjugates. The conjugation between ubiquitin and various proteins is involved in the pathway of protein degradation that takes place in all cells (Chapter 15, II, B). However, the role of ubiquitin is likely to touch a broader spectrum of the cell's activities (Jentsch et al., 1990). Metabolically stable ubiquitin-protein conjugates are found in eukaryotes. Furthermore, a large part of chromosomal histones are conjugated to ubiquitin, suggesting a role in gene expression. Several integral membrane proteins as well as actin, have also been found to be conjugated to ubiquitin. The conjugation to ribosomal enzymes enhances protein synthesis (e.g., see Spence et al., 2000). In addition, ubiquitin-conjugating enzymes appear to be involved in DNA repair and the control of the cell cycle (e.g., Goebl et al., 1988) (see Chapter 8).

A general role of acetylation in regulation similar to the phosphorylation-dephosphorylation system has been envisioned (see Kouzarides, 2000). The role of acetylation of histones in gene expression is well understood (see Chapter 2, Section B and C). Many proteins have been modified by acetylation (such as transcription factors, nuclear import factors and α-tubulin). Acetylation has been found to regulate many functions including DNA recognition, protein-protein interaction and protein stability. Bromodomains recognize acetylated residues and may serve as signalling domains. In DNA-binding transcription factors, acetylation increases DNA binding. Acetylation inhibits protein-protein interactions and increases the half life of some proteins including α-tubulin and microtubules. Acetylated α-tubulin is present mostly stable microtubules and absent from dynamic cellular structures such as neuronal growth cones and the leading edges of fibroblasts. So far only one enzyme has been identified in the acetylation-deacetylation regulatory process, a tubulin deacetylase (Hubbert et al., 2002). The enzyme is cytoplasmic and associates with the microtubule motor complex containing $p150^{glued}$ (see Chapter 24, Section IV B and Section IV C).

V. REGULATION OF PHOSPHORYLATION AND DEPHOSPHORYLATION

We saw that phosphorylation and dephosphorylation have a key role in the regulation of enzymatic activity. Furthermore, the transcriptional and translational machinery of the cell is regulated by phosphorylation-dephosphorylation. Phosphorylation appears to function in both repression, positive

regulation, stability, localization and the ability to interact with other proteins or DNA (see Holmberg et al., 2002). The phosphorylation of proteins at multisite sites permits the precise tuning of activity. For example, p53 which mediates cell-growth block and apoptosis has 16 phosphorylation sites (see Holmberg et al., 2002) leading to different effects (e.g., see Vousden, 2002). The concerted activity of protein kinases (PKs) and phosphatases (PPs) has a central role in this mechanism of regulation. About 200 PKs and 100 PPs have been identified. Approximately 30 of the PPs are protein tyrosine phosphatases (Mauro and Dixon, 1994).

PKs and PPs are relatively nonspecific, at least in vitro, and can even catalyze reactions with artificial substrates. Their specificity may rest in a specific localization. Present information suggests that these enzymes, in addition to a catalytic domain, contain a targeting domain which directs them to specific targets in the cell (see also Chapter 10, Section VI). An additional role of this domain may be regulatory, via an allosteric interaction. The sections that follow address these question in more detail.

The regulation of protein kinases and phosphatases is also discussed in some detail in relation to intracellular signals in Chapter 7.

A. Protein Kinases

PKs mediate the interaction between extracellular ligands and biological activity (Chapter 6 and 7). At least two classes of protein kinases have been shown to be regulated by targeting subunits: cAMP-dependent protein kinases (PKAs), which are activated by the presence of the second messenger cAMP (see Chapter 7) and cyclin-dependent kinases. Cyclins are protein molecules involved in the regulation of cell division (Chapter 8). Protein kinases have been found to be profoundly affected by domains that allow them to interact docking sites on their substrate.

Interactions with substrate docking sites

Protein kinases have been found to complex tightly to their substrates even when inactive. The enzyme and the complex may interact directly. The small domain in the substrate molecule that binds to the enzyme is known as a *docking site* (see Table in Holland and Cooper, 1999). Alternatively, the interaction may be through a third protein. This binding increases the efficiency of the kinase (see e.g., Jacobs et al., 1999; Gavin and Nebreda, 1999; Smith et al., 1999). Several docking sites can be present and are modular: when experimentally attached to different substrates they change their specificity for the kinase.

The presence of substrate docking sites has several consequences. It may determine the location of the kinase within the cell. For example, the MSAP kinase p38 is localized in the nucleus when not active because of its binding to its substrate, the MAPKAP kinase 3 (Ben-Levy et al., 1998). Docking sites increase the specificity of the kinases which normally would be capable of reacting with a variety of substrates. Furthermore, the preassembly of kinase and substrate allows for very rapid enzymatic action

after activation (see Holland and Cooper, 1999).

The tightness of the interaction between substrate and enzyme would slow down the reaction if it were not for the fact that the complex of the two is destabilized by the phosphorylation of the substrate (Zhao et al., 1996; Waskiewicz et al., 1997) so that there is an increase in V_{max} accompanying the increase in affinity (see Jacobs et al., 1999)

cAMP-dependent protein kinases

PKAs are present in the cytoplasm as an inactive tetramer of two catalytic and two regulatory subunits. In this form, the enzymes are excluded from the nucleus. Two cAMPs bind to each regulatory subunit; this binding induces the dissociation of the complex into a regulatory dimer and two catalytic monomers. The free catalytic monomers can enter the nucleus (Fantozzi et al., 1992). In the nucleus, they regulate transcription factors such as the *cAMP response element binding protein* (CREB)(see Chapter 7, Fig. 18). The regulatory subunit can also serve as a targeting device. Regulatory subunits of type II PKAs keep the enzyme at specific locations. For example, the regulatory subunit of PKA type II sequesters most of the cAMP of the brain and binds to the *microtubular associated protein 2* (MAP-2).

Cyclin-dependent protein kinases

The cyclin dependent PKs are activated at different stages of the cell cycle (see Chapter 8). These enzymes are heterodimers. The catalytic subunit attaches to a cyclin, its regulatory subunit, which activates the enzyme and targets it to particular sites.

B. Protein Phosphatases

The PPs have been studied less than the PKs. Obviously, they must be as significant in regulation as the better understood PKs. A general discussion of PPs will be found in Chapter 7. Several of the PPs involved in the regulation of metabolism have been studied in detail. PP1 associated with glycogen particles is a protein serine/threonine phosphatase. In vitro it is capable of dephosphorylating proteins that have been phosphorylated by different PKs. However, in vivo its specificity is assured by its association with glycogen. The next section will discuss this kind of PP1 ($PP1_G$).

$PP1_G$ is present as a heterodimer containing a catalytic subunit of 31 kDa and a glycogen targeting subunit of 124 kDa (Strålfors et al., 1985, Tang et al., 1991). The regulatory subunit also contains a sarcoplasmic reticulum targeting domain and several phosphorylated sites (see Hubbard and Cohen, 1993). $PP1_G$ binds glycogen with high affinity and is present exclusively in its bound form. Phosphorylase kinase and glycogen synthase are also bound to glycogen.

VI. MULTIENZYME COMPLEXES

The organization of functionally related enzymes in complexes concentrates catalytic activity and allows for a high steady-state concentration of intermediates so that substrates are transferred from one active site to another with a minimum of diffusional effects or dilution. Furthermore, the presence of enzymes in a complex makes possible coordinated allosteric control of several activities. α-ketoacid dehydrogenase and fatty acid synthetase are soluble multienzyme complexes that have been studied in some detail. In the next section, the pyruvate dehydrogenase complex of *E. coli* will serve as an example of a complex of several distinct enzymes. The combination of enzymes into complexes involving membranes is discussed primarily in relation to oxidative phosphorylation (Chapter 16), photosynthesis (Chapter 17), and the reactions involved in the translocation of solutes across the cell membrane (Chapters 19, 20, and 21). Many other enzymes are present in multimolecular assemblies. As our knowledge gains in detail, the number of known complexes increases. The splicing of mRNA is carried out by spliceosome, and transcription takes place in transcriptional complexes. Protein synthesis involves a variety of enzymes, mRNA and ribosomes, forming translational complexes. The degradation of proteins involve proteosomes. In addition, there is evidence for multienzyme systems in other pathways (referred to as *metabolons*), such as glycolysis, nucleotide synthesis (e.g., Jones, 1980), urea biosynthesis, tricarboxylic acid cycle, fatty acid oxidation and amino acid metabolism. The sections that follow will first discuss pyruvate dehydrogenase. Other cases that have been studied less will be discussed in later sections.

A. The Pyruvate Dehydrogenase Complex

In *E. coli*, the pyruvate dehydrogenase reaction is carried out by enzyme aggregates made up of three types of enzymes with a total molecular weight of about 4,000 kDa. Similar complexes are thought to cata1yze these reactions in other organisms, including mammals.

The reactions catalyzed by the pyruvate dehydrogenase complex are represented in Fig. 13, in which part (a) represents the overall reaction and part (b) some of the details. As shown in this figure, pyruvate in the presence of coenzyme A and NAD^+ is oxidized and decarboxylated to form acetyl coenzyme A, reduced NAD, and CO_2. This pyruvate dehydrogenation involves several separate reactions and the three enzymes represented in Fig. 13b. These enzymes are held together in the pyruvate dehydrogenase complex. In reaction 1, pyruvate reacts with pyruvate dehydrogenase (E_1). The reaction involves the coenzyme thiamine pyrophosphate (TPP). Decarboxylation of the pyruvate forms the α-hydroxyethyl-TPP-E_1 complex. Reaction 2 involves lipoyl reductase-transacetylase (E_2). The lipoyl moiety of E_2 oxidizes the hydroxyethyl residue that is transferred from E_1-TPP as the acetyl moiety. In reaction 3, the acetyl-reduced lipoyl-E_2 transfers the acetyl group to coenzyme A, and, in reaction 4, the lipoyl-E_2 molecule reduced in reaction 2 is oxidized by the flavoprotein dihydrolipoyl dehydrogenase (E_3-FAD). The reducing equivalents from this reaction are transferred to NAD^+ (reaction 5) and eventually oxidized by the cytochrome chain. In these reactions, the lipoyl moiety of E_2 (abbreviated Lip) interacts with both E_1-TPP (reaction 1) and E_3 (reactions 3 and 4). It serves to transfer reducing equivalents as well as acetyl groups (see Fig 13).

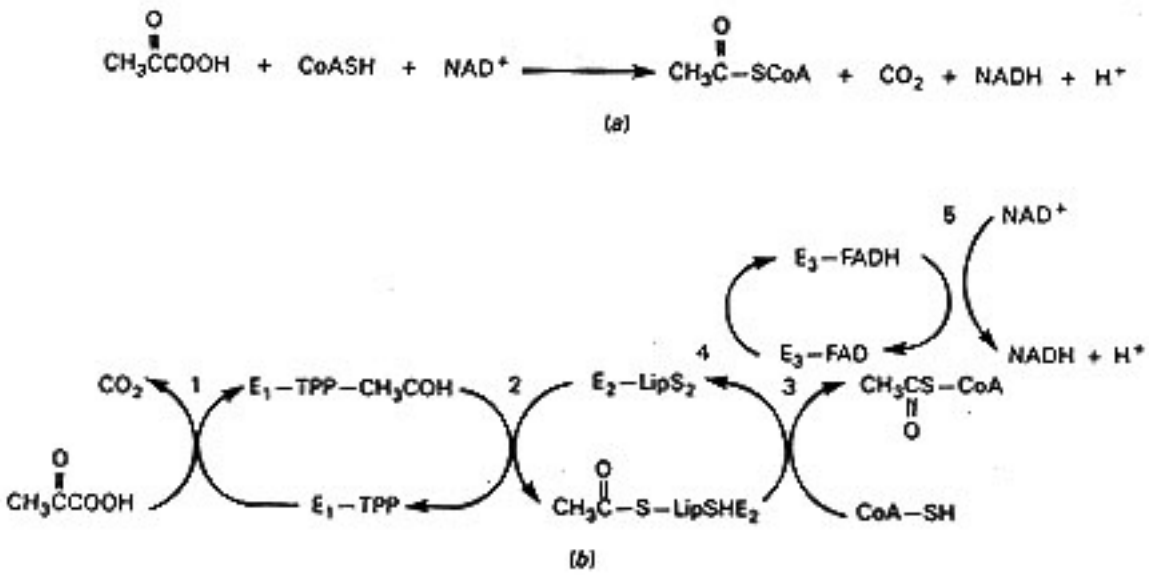
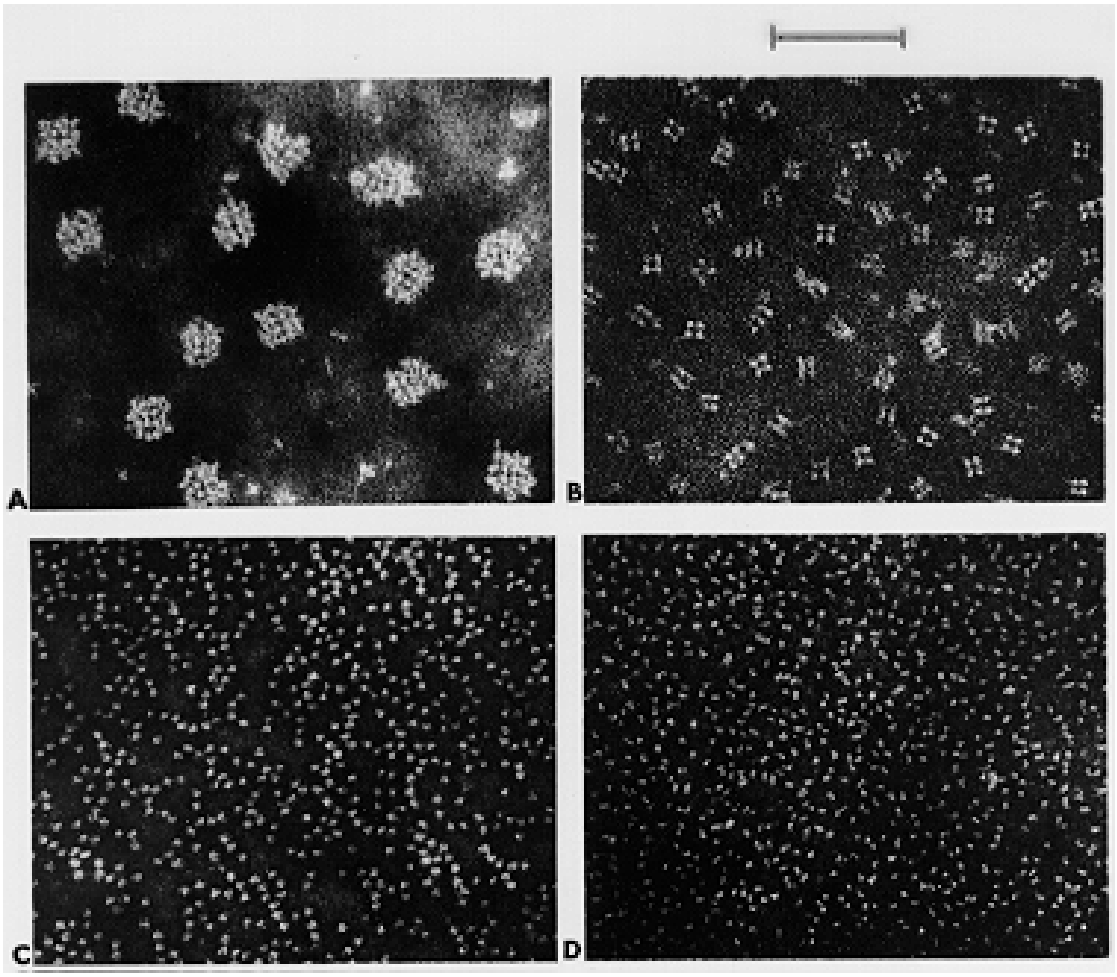


Fig. 13 Diagrammatic representation of the pyruvate dehydrogenase reaction. E_1, Pyruvate dehydrogenase; E_2, dihydrolipoyl transacetylase; E_3, dihydrolipoyl dehydrogenase.

The three enzymes can be separated out from the pyruvate dehydrogenase complex by relatively gentle means since they are held together by noncovalent bonds. The separated molecules reassemble spontaneously when mixed together at neutral pH. Alone, each kind of enzyme molecule forms an active complex made up of several subunits; these complexes have a specific morphology. Complexes are also formed between the molecules of pyruvate dehydrogenase (E_1) or dihydrolipoyl dehydrogenase (E_3) and the transacetylase (E_2). The characteristic appearance of these complexes and that of the entire dehydrogenase complex have permitted their reconstruction. A reconstruction of the entire pyruvate dehydrogenase complex is shown in Fig. 14. Figure 14a shows an electron micrograph of the complex of the three enzymes after negative staining with phosphotungstate. In this procedure, the particles, suspended in a solution of phosphotungstate, are placed on a grid in a thin layer by a suitable procedure and dried. When viewed with the electron microscope, the molecules appear white against the dense background of the phosphotungstate (Fig. 14a). A model based on various observations is shown in Fig. 14b.


A
B
C
D

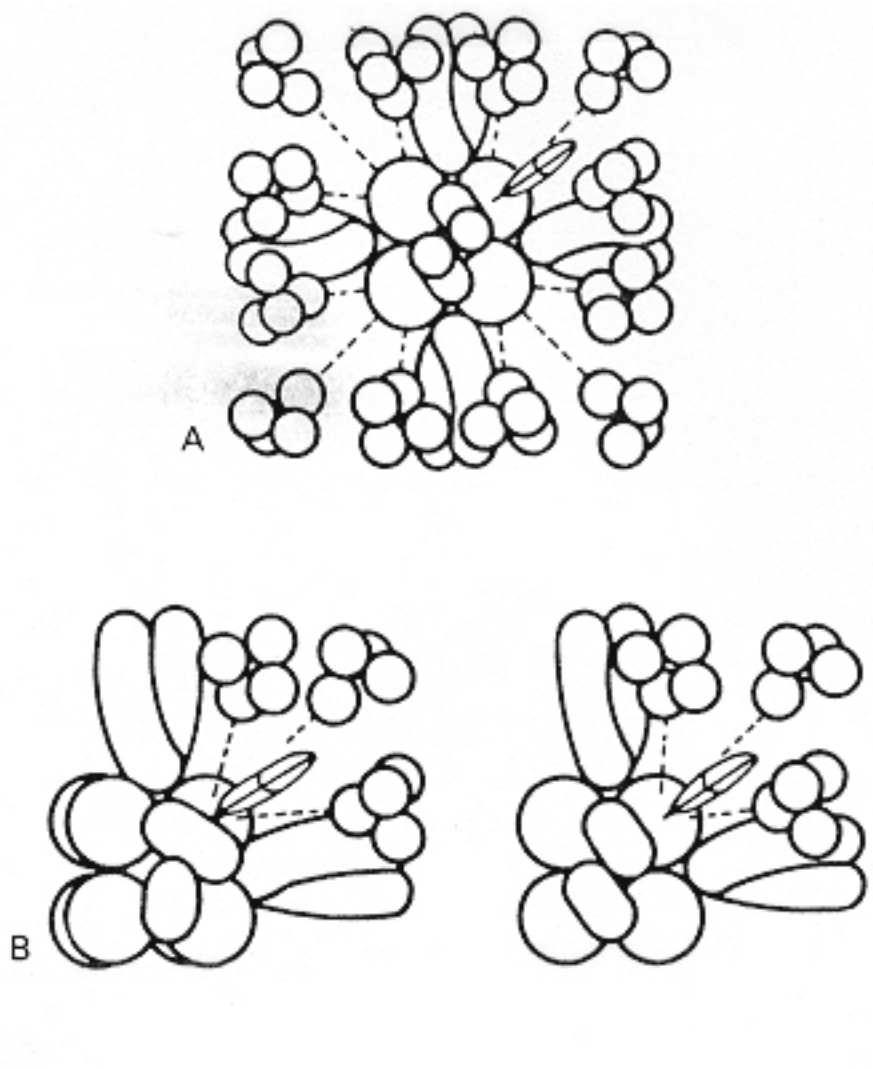


Fig. 14 (a) Electron micrographs of the *E. coli* pyruvate dehydrogenase complex and its componenent enzymes (bar corresponds to 75 nm), *A*, Pyruvate dehydrogenase complex; *B*, dihydrolipoyl transacetylase (E_2); *C*, pyruvate dehydrogenase (E_1); *D*, dihydrolipoyl dehydrogenase (E_3). (b) Interpretative model of *E. coli* pyruvate dehydrogenase complex: *A*, Model viewed along the fourfold axis of the dihydrolipoyl transacetylase (E_2) core, illustrating the proposed architectural organization of the complex; *B*, stereoscopic drawings of a portion of the model showing the spatial relationships of the components. The eight trimers of E_2 binding domains are represented by large spheres, and the single E_2 lipoyl domain shown is represented by an ellipsoid. Pyruvate dehydrogenase (E_1) chains are represented by tetrahedra consisting of four lobes (small spheres) bound along the edges of the cubelike "inner" core of E_2 binding domains. In this projection of the model, the 12 E_1 chains shown are superimposed over 12 other similarly positioned chains of E_1 (not shown). The dimers of dihydrolipoyl dehydrogenase (E_3) are located on the faces of the cubelike E_2 "inner" core and are represented by pairs of cylinders. From R. M. Oliver and L. S. Reed, Electron Microscopy, 2:1-48, 1982, with permission of Academic Press.

The molecules of each of the three enzymes occupy fixed positions in the complex. Because the active groups correspond to a very small portion of the enzyme, it is difficult to see how an interaction can take place between the dihydrolipoyl transacetylase active site and the other two enzymes unless parts or all of the molecule move in relation to the others, first to react with one enzyme (pyruvate dehydrogenase)

and then with the other (dihydrolipoyl dehydrogenase). The dihydrolipoyl transacetylase portion is composed of 24 polypeptide chains each bearing two lipoyl moieties, which function in both acetylation and redox reactions (see Fig. 15). The interaction could result from the swinging of the lipoyl-lysyl 1.4 nm arm of the dihydrolipoyl transacetylase as shown in Fig. 15a. Alternatively, the polypeptides to which the lipoyl moiety is attached could be mobile (Collins and Lester, 1977; Perham and Duckworth, 1981). These two models (A and B) are shown in Fig. 15b.

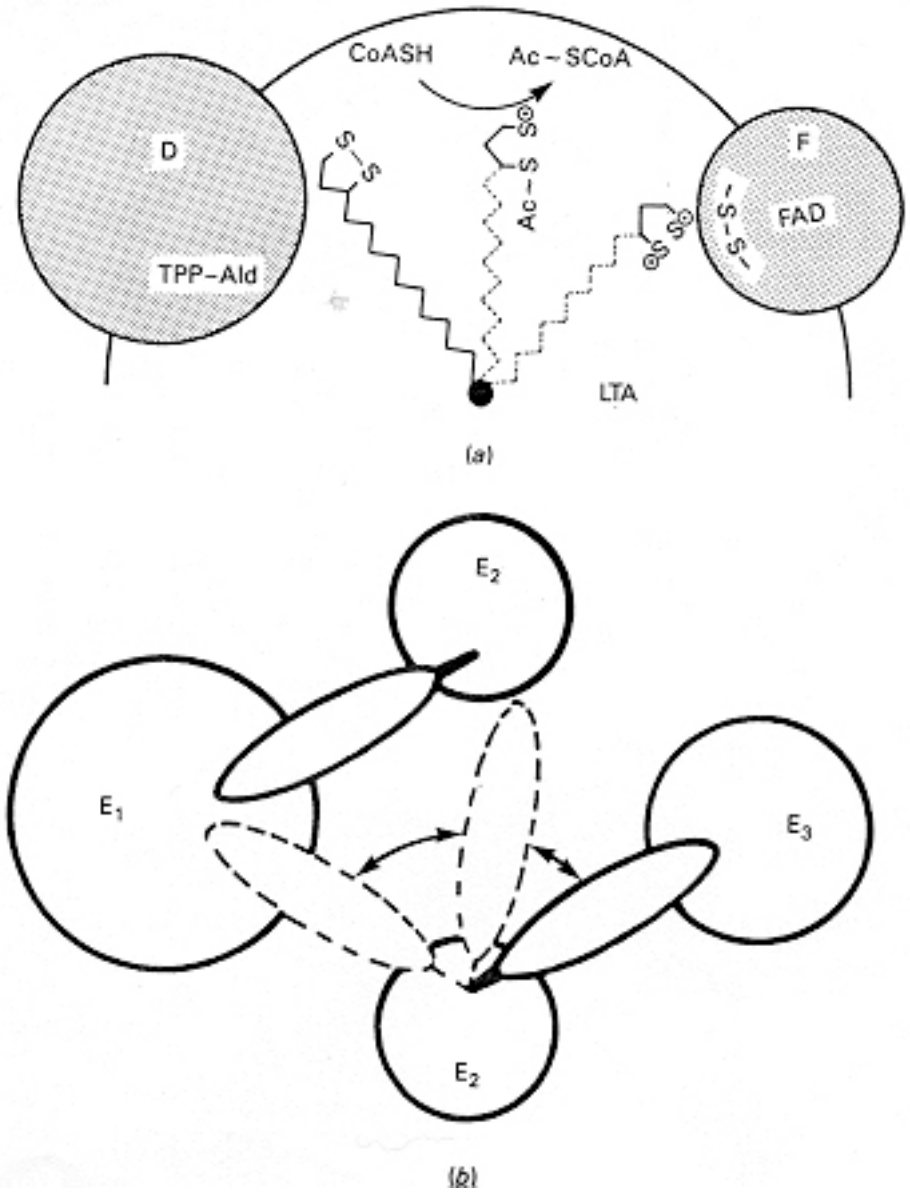


Fig. 15 (a) Schematic representation of the possible rotation of a lipoyl-lysyl moiety between α-hydroxyethylthiamine pyrophosphate (TPP-Ald) bound to pyruvate dehydrogenase (D) (the site for acetyl transfer to CoASH) and the reactive disulfide of the flavo-protein (F). From Oliver and Reed (1982), with permission. (b) Model illustrating movement of lipoyl domains (and not simply rotation of lipoyl moieties) to span the physical gaps between catalytic sites on the complex. A dihydrolipoyl transacetylase (E_2) subunit is represented by a sphere (subunit binding domain) and its attached ellipsoid (lipoyl domain). The catalytic site for transacetylation resides on the subunit binding domain, whereas the two lipoyl moieties are on the lipoyl domain. It is visualized that movement of lipoyl domains permits their covalently attached lipoyl moieties (not shown) to service the catalytic sites on the complex. Each E_1, E_2,

and E_3 subunit is serviced by at least two lipoyl moieties, which apparently reside on two separate lipoyl domains. The E1, E2, and E3 subunits shown need not be adjacent to each other in the complex. From Stepp et al. (1981). Reproduced with permission from *Biochemistry* 20:4555-4560. Copyright &copy1981 American Chemical Society.

In the previous section, we saw that enzyme complexes made up of identical subunits undergo conformational changes during enzyme-catalyzed reactions. In this section, we saw how the larger multienzyme complexes discussed must also involve some movement, either of the molecules of the complex in relation to each other, or of a portion of one of the molecules. These movements, or conformational rearrangements, appear to be a fundamental property of many enzyme-catalyzed systems and may be involved in a wide range of biological mechanisms. The molecular mechanisms of muscular contraction or other events linked with cellular motility, the changes in shape of cells, and the mechanisms of solute transport across biological membranes, may well involve specialized modifications of these conformational rearrangements.

B. Other Multienzyme Complexes

Some of the evidence for the presence of metabolic enzymes associated in complexes comes from indirect information. For example, the centrifugation of intact cells has been shown to sediment most cytosolic proteins (Zalokar, 1960; Kempner and Miller, 1968); this observation suggests that a large portion of the proteins of the cell must be present as aggregates. It has also been argued that the high concentration of proteins in the mitochondrial matrix (calculated to be 56% by weight) (Hackenbrock, 1968) corresponds to the densest possible packing for proteins, i.e., that of the maximum packing of identical spheres. This suggests that these proteins are present in a semi-crystalline state and therefore must be in the form of interacting complexes. Similar arguments can be made for the glycolytic enzymes in muscle, where most of the space not occupied by the contractile apparatus is likely to be occupied by glycolytic enzymes (Pette and Brandau, 1962; Sigel and Pette, 1969). Pette and Brandau estimate that in the muscle of *Locusta migratoria*, the concentration of these enzymes by themselves corresponds to 0.2 g per ml. Other evidence is more direct and is discussed below.

A strong argument for the idea that glycolytic enzymes are organized in assemblies comes from experiments with permeabilized fibroblasts, where the efficiency of the conversion of [^{14}C]-glucose to [^{14}C] lactate (Clegg and Jackson, 1990) suggests that the endogenously produced intermediates are channeled as if they were in complexes.

Mitochondrial enzymes

The complexes involved in electron transport and phosphorylation in mitochondria (Chapters 16 and 18) and chloroplasts (Chapters 17 and 18) will be discussed separately. Evidence exists for the organization of mitochondrial matrix enzymes in complexes and that in some cases the localization of certain enzymes play an important role in metabolism.

Each mitochondrial enzyme of the citric acid cycle is able to bind the next enzyme in the sequence. Furthermore, they do not bind to isoenzymes catalyzing the same reaction in other locations (e.g., fumarase binds only the mitochondrial malate dehydrogenase and not the cytoplasmic enzyme, Beekmans and Kanarek, 1981). The interactions are summarized in Table 6. The enzymes are listed in sequential order, beginning with citrate synthase. All enzymes are present either in the first or second column, with the exception of succinate dehydrogenase. Together with the fact that the enzymes are present in very high concentrations, as discussed above, the results suggest very strongly that the enzymes are present in a complex.

In addition to the ability to form complexes, the tricarboxylic acid cycle enzymes also bind to the inner mitochondrial membrane (D'Souza and Srere, 1983). These experiments took advantage of the inner mitochondrial vesicle preparations that are inside-out. Isoenzymes that catalyze the same reactions but correspond to other organelles (e.g.,+ in yeast citrate synthase from peroxisomes) (Kispal and Srere, 1991) do not bind to these vesicles. Although there was no binding of the enzymes to liposomes free of protein components, mitochondrial malic dehydrogenase did bind to liposomes in which the mitochondrial Complex I was incorporated (Sumegi and Srere, 1984). Complex I is capable of accepting electrons from dehydrogenases (see Chapter 16). Mitochondria disrupted by ultrasound (as demonstrated by the accessibility of citrate synthase to its antibody or of malic dehydrogenase to high molecular weight blue dextran) contain tricarboxylic acid cycle enzymes (Robinson et al., 1985). This association supports the view that they are bound to the inner mitochondrial membrane. In these complexes, the rate of metabolism is much higher than in the solubilized state (Robinson et al., 1987; Sumegi et al., 1991), suggesting that the enzyme properties are quite distinct when bound.

The localization of mitochondrial components is also important. Apparently, the respiratory rate of cardiac mitochondria is under the control of creatine kinase, which catalyzes the phosphate exchange between creatine and ATP. This enzyme is present between the mitochondrial inner and outer membranes (Jacobus et al., 1983). Furthermore, the mitochondrial outer membrane has binding sites for hexokinase (Felgner et al., 1979) probably at sites at which the inner and outer membrane make contact (the so-called *contact sites*), so the ATP synthesized can be used without delay to phosphorylate hexoses.

Table 6 Interactions of TCA Enzymes

Enzyme	Binds to	Reference
citrate synthase	malate dehydrogenase, aconitase, pyruvate dehydrogenase citrate transporter	Halper and Srere, 1977; Tyiska et al., 1986; Sumegi and Alkonyi, 1983; Persson and Srere, 1992

aconitase	NADH-isocitrate dehydrogenase	Tyiska et al., 1986
succinyl-CoA synthase	α-ketoglutarate dehydrogenase complex	Porpaczy et al., 1983
fumarase	malate dehydrogenase	Beekmans and Kanarek, 1981

Binding of glycolytic enzymes to components of the contractile systems

The location of enzymes at specific sites is shown in muscle, where most glycolytic enzymes (but not hexokinase, which is localized primarily in mitochondria) are located within the I-band, a site shared with F-actin (Dolken et al., 1975; Sigel and Pette, 1969). A number of glycolytic enzymes have been shown to bind to F-actin (Arnold and Pette, 1968, 1970; Arnold et al., 1971). The binding is most pronounced in the presence of the regulatory proteins tropomyosin and troponin (Clarke and Morton, 1976; Clarke and Masters, 1975, 1976; Stewart et al., 1980). Binding to actin was observed for fructose-1,6-biphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase.

In *Drosophila* flight muscle, glycerophosphate dehydrogenase (GPDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and aldolase can be localized at Z discs and M lines using immunofluorescence (Wojtas et al., 1997). The Z lines were identified using anti-α-actinin, a Z-disc protein. The position of the M-lines can be deduced from the fact that they are equidistant between Z discs.

GPDH is not a glycolytic enzyme. Its substrate, glycerophosphate, is generated from the breakdown of lipids. However, it is oxidized to dihydroacetone phosphate and the latter, after an isomerization reaction, produces glyceraldehyde 3-phosphate, an intermediate in glycolysis.

Transgenic flies able to synthesize only one of the GPDH isoforms (GPDH-3) lacked the characteristic distribution of GPDH, GADPH and aldolase, and were unable to fly although they contained the normal complement of glycolytic enzymes (Wojtas, 1997). GPDH-3 differs from the normal GPDH-1 only in lacking a tripeptide at the carboxy terminal. These observations show that the organization of enzymes in relation to cytoplasmic structures is of fundamental importance in function and that the distribution of related enzymes is codependent.

The glycolytic reaction sequence catalyzed by aldolase, triosephosphate isomerase, glyceraldehyde-phosphate dehydrogenase and phosphoglycerate kinase have been found in isolated muscle triads (see Chapter 24). In the triad, these enzymes synthesize ATP in the presence of glyceraldehyde 3-phosphate

or fructose 1,6-bisphosphate (Han et al., 1992). The synthesized ATP appears associated with the triad and is not in equilibrium with the bulk ATP.

Phosphofructokinase (Vertessy et al., 1997; Volker and Knull, 1997) and aldolase (Vertessy et al., 1997) have been found associated with microtubules.

Glycogen particles

Glycogen particles isolated from skeletal muscle contain the enzymes of glycogen metabolism. These include: phosphorylase b and its kinase and phosphatase; glycogen synthase and its respective kinase and phosphatase (Meyer et al, 1970); as well as the debranching enzyme, glycogen synthase kinase 2, protein phosphatase 2, and inhibitors 1 and 2 (Nimmo et al., 1976). The glycogen particles themselves are closely associated to the sarcoplasmic reticulum near the thin filaments (Wanson and Drochmans, 1968). The kinetic behavior of the enzymes in the particles is distinct from those in solution (Fischer, et al., 1971).

Binding of enzymes to the plasma membrane

Certain enzymes are located in the cell's periphery. At least some of this binding is to the cytoplasmic domain of integral membrane proteins. In the red blood cell, Glyceraldehyde-3-phosphate dehydrogenase and fructose-1,6-bisphosphate aldolase bind to Band 3 protein, the anion transporter (see Yu and Steck, 1975; Tsai et al., 1982). In the case of glyceraldehyde-3-phosphate dehydrogenase, the binding exhibits 1:1 stoichiometry.

The bound enzymes have different kinetic properties from the free enzymes. For example, phosphofructokinase, upon binding, loses its allosteric inhibition by ATP (Karadsheh and Uyeda, 1977) and glyceraldehyde-3-phosphate dehydrogenase is inhibited by binding (Tsai et al., 1982).

The presence of organized multienzyme complexes or clusters offers significant opportunities for integrated activity and regulation that are just beginning to be appreciated (Welch, 1977; Paul et al., 1989). There are indications, for example, that at least portions of the enzymes glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase function at the inner surface of the red cell membrane to provide ATP to Na^+,K^+ -ATPase but not to the rest of the cell (Mercer and Dunham, 1981).

SUGGESTED READING

Introductory

Creighton, T.E. (1993) *Proteins, Structures and Molecular Properties*, W.H. Freeman, New York, Chapter 9.

Kraut, J. (1988) How do enzymes work? *Science* 242:533-540.(Medline)

Saier, M. H., Jr. (1987) *Enzymes in metabolic pathways*. Harper Row, New York, Chapters 3 and 4.

Stryer, L. (1994) In *Biochemistry*. 4th ed. Freeman, New York Chapters 8 and 9.

More Advanced

Fersht, A. (1985) *Enzyme Structure and Mechanism* 2nd Ed., Freeman, New York Chapters 1-3, 8, 10 and 15.

Schachman, H. K. (1988) Can a simple model account for the allosteric transitions of aspartate transcarbamoylase? *J. Biol. Chem.* 263:18583-18586.(Medline)

Special aspects

James, H.A. and Turner, P.C. (1995) Ribozymes, *Essays in Biochemistry*, 29:175-192.(Medline)

REFERENCES

Search the textbook

Back to Chapter 13

REFERENCES

Arnold, H. and Pette, D. (1968) Binding of glycolytic enzymes to structure proteins of the muscle, *Eur. J. Biochem.* 6:163-171.(Medline)

Arnold, H. and Pette, D. (1970) Binding of aldolase and triosephosphate dehydrogenase to F-actin and modification of catalytic properties of aldolase, *Eur. J. Biochem.* 15:360-366.(Medline)

Arnold, H., Henning, R. and Pette, D. (1971) Quantitative comparisons of the binding of glycolytic enzymes to F-actin and the interaction of aldolase with G-actin, *Eur. J. Biochem.* 22:121-126.(Medline)

Atkinson, D. E. (1965) Biological feedback control at the molecular level, *Science* 150:851-857.(Medline)

Ban, N., Nissen, P., Hansen, J., Moore, P.B. and Steitz, T.A.. (2000) The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution, *Science* 2890:905-920. (MedLine)

Beekmans, S. and Kanarek, L. (1981) Demonstration of physical interactions between consecutive enzymes of the citric acid cyclic and of the aspartate-malate shuttle, *Eur. J. Biochem.* 117:527-535.(Medline)

Ben-Levy, R., Hooper, S., Wilson, R., Paterson, H.F. and Marshall, C.J. (1998) Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2, *Curr. Biol.* 8:1049-1057.(Medline)

Bernstein, B.E., Michels, P.A.M. and Hol, W.G.J. (1997) Synergistic effects of substrate-induced conformational changes in phosphoglycerate kinase activation, *Nature* 385:275-278.(Medline)

Clarke, F.M. and Masters, C.J. (1975) On the association of glycolytic enzymes with structural proteins in skeletal muscle, *Biochim. Biophys. Acta* 381: 37-46.(Medline)

Clarke, F.M. and Masters, C.J. (1976) Interactions between muscle proteins and glycolytic enzymes, *Inter. J. Biochem.* 7:359-365.

Clarke, F.M. and Morton, C.J. (1976) Aldolase binding to actin-containing filaments, *Biochem. J.* 159: 797-798.(Medline)

Clegg, J.S. and Jackson, S.A. (1990) Glucose metabolism and the channeling of glycolytic intermediates in permeabilized L-929 cells, *Arch. Biochem. Biophys.* 278:452-460.(Medline)

Cohen, P. (1980) Protein phosphorylation and the coordinate control of intermediate metabolism, *Mol. Aspects Cell. Regul.*1:255-268.

Collins, J.H., and Lester, L.J. (1977) Acyl group and electron pair relay system: a network of interacting lipoyl moieties in pyruvate and α-ketog1utarate dehydrogenase complexes from *Echerichia coli*, *Proc. Natl. Acad. Sci. USA.* 74:4223-4227.(Medline)

Dölken, G., Leisner, E. and Pette, D. (1975) Immunofluorescent localization of glycogenolytic and glycolytic enzyme proteins and of malate isozymes in cross-striated skeletal muscle and heart of rabbit, *Histochemistry* 43:113-121.(Medline)

D'Souza, S.F. and Srere, P.A. (1983) Binding of citrate synthase to mitochondrial inner membranes, *J. Biol. Chem.* 258:4706-4709.(Medline)

Edman, K., Nollert, P., Royant, A., Belrhali, H., Pebay-Peyroula, E., Hajdu, J., Neutze, R. and Landau, E.M. (1999) High-resolution X-ray structure of an early intermediate in the bacteriorhodopsin photocycle, *Nature* 401:822-826.(Medline)

Fantozzi, D.A., Taylor, S.S., Howard, P.W., Maurer, R.A., Feramisco, J.R. and Meinkoth, J.L. (1992) Effect of the thermostable protein kinase inhibitor on intracellular localization of the catalytic subunit of cAMP-dependent protein kinase, *J. Biol. Chem.* 267:16824-16828. (MedLine)

Felgner, P. L., Messer, J. and Wilson, J. E. (1979) Purification of a hexokinase-binding protein from the outer mitochondrial membrane, *J. Biol. Chem.* 254:4946-4949.(Medline)

Fischer, E.H., Heileyer, L.M.G. Jr. and Hasche, R.H. (1971) Phosphorylase and the control of glycogen degradation, *Curr. Top. Cell. Reg.* 4:211-251.

Gavin, A.C. and Nebreda, A.R. (1999) A MAP kinase docking site is required for phosphorylation and activation of $p90^{rsk}$/MAPKAP kinase-1, *Curr. Biol.* 9:281-284.(Medline)

Gerhart, J.C. and Pardee, A.B. (1962) The enzymology of control by feedback inhibition, *J. Biol. Chem.*237: 891-896.

Gerhart, J.C. and Schachman, H.K. (1965) Distinct subunits for the regulation and catalytic activity of aspartate transcarbamoylase, *Biochemistry* 4:1054-1062.(Medline)

Gerhart, J.C. and Schachman, H.K. (1968) Allosteric interactions in aspartate transcarbamoylase. II. Evidence for different conformational states of the protein in the presence and absence of specific ligands, *Biochemistry* 7:538-552.(Medline)

Goebl, M.G., Yochem, J., Jentsch, 5., McGrath, J. P., Vashavsky, A. and Byers, B. (1988) The yeast cell cycle gene CDC34 encodes a ubiquitin-conjugating enzyme, *Science* 241:1331-1335.(Medline)

Greengard, P. (1978) Phosphorylated proteins as physiological effectors, *Science* 199:146-152.(Medline)

Guerrier-Takada, C. and Altman, S. (1984) Catalytic activity of an RNA molecule prepared by transcription *in vitro*, *Science* 223:285-286.(Medline)

Hackenbrock, C.R. (1968) Chemical and physical fixation of isolated mitochondria in low and high energy states, *Proc. Natl. Acad. Sci. USA* 61:598-605.(Medline)

Hall. E.R., McCully, V., and Cottam, G.L. (1979) Evidence for proteolytic modification of pyruvate kinase in fasted rats, *Arch. Biochem. Biophys.* 195:315-324.(Medline)

Halper, L.A. and Srere, P.A. (1977) Interaction between citrate synthase and malate dehydrogenase in the presence of polyethylene glycol, *Arch. Biochem. Biophys.* 184:529-534.(Medline)

Han, J.W., Thieleczek, R., Varsanyi, M. and Heilmeyer, L.M. Jr. (1992) Compartmentalized ATP synthesis in skeletal muscle triads, *Biochemistry* 31:377-384.(Medline)

Hasilik, A. and Tanner, W. (1978) Biosynthesis of the vacuolar yeast glycoprotein carboxypeptidase Y. Conversion of precursor into enzyme, *Eur. J. Biochem.* 85:599-608.(Medline)

Holland, P.M. and Cooper, J.A. (1999) Docking sites for kinases, *Curr Biol.* 9:R329-331. (MedLine)

Holmberg, C.I., Tran, S.E., Eriksson, J.E. and Sistonen, L.(2002) Multisite phosphorylation provides sophisticated regulation of transcription factors, *Trends Biochem. Sci.* 27:619-627. (MedLine)

Hubbard, M.J. and Cohen, P. (1993) On target with a new mechanism for the regulation of protein phosphorylation, *Trends Biochem. Sci.* 18:172-177.(Medline)

Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito. A., Nixon, A., Yoshida, M., Wang, X.F. and Yao, T.P. (2002) HDAC6 is a microtubule-associated deacetylase. *Nature*417:455-458. (MedLine)

Jacobs, D., Glossip, D., Xing, H., Muslin, A.J. and Kornfeld, K. (1999) Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase, *Genes Dev.* 13:163-175.(Medline)

Jacobus, W.E., Moreadith, R.W., and Vandegaer, K.M. (1983) Control of heart oxidative phosphorylaton by creatine kinase in mitochondrial membranes, *Ann. N. Y. Acad. Sci.* 414:73-89.(Medline)

Jentsch, S., Seufert, W., Soomer, T. and Reins, H.-A. (1990) Ubiquitin-conjugating enzymes: novel regulators of eukaryotic cells, *Trends Biochem. Sci.* 15:195-198.(Medline)

Jones, M.E. (1980) Pyridine nucleotide biosynthesis in animals: genes, enzymes and regulation of UMP biosynthesis, *Ann. Rev. Biochem.* 49:253-279.(Medline)

Karadsheh, N.S. and Uyeda, K. (1977) Changes in the allosteric properties of phosphofructokinase bound to erythrocyte membranes, *J. Biol. Chem.* 252:7418-7420.(Medline)

Kemp, B.E. and Pearson, R.B. (1991) Intrasteric regulation of protein kinases and phosphatases, *Biochim. Biophys. Acta.* 1094:67-76.(Medline)

Kempner, E.S. and Miller, J.H. (1968) The molecular biology of *Euglena gracilis*. IV. Cellular stratification by centrifuging, *Exp. Cell Res.* 51:141-149.(Medline)

Khosla, C. and Harbury, P.B. (2001) Modular enzymes, *Nature* 409:247-252.

Kispal, G., and Srere, P.A. (1991) Studies of yeast peroxisomal citrate synthase, *Arch. Biochem. Biophys.* 286:132-137.(Medline)

Kobe, B. and Kemp, B.E. (1999) Active site-directed protein regulation, *Nature* 402:373-376.(Medline)

Koshland, D.E., Jr. (1956) Molecular geometry in enzyme action, *J. Cell. Comp. Physiol.* 47 (Suppl. 1):217-234.

Koshland, D.E., Jr. and Neet, K.E. (1968) The catalytic and regulatory properties of enzymes, *Annu. Rev. Biochem*. 37:349-410.

Kouzarides T. (2000) Acetylation: a regulatory modification to rival phosphorylation? *EMBO J.* 19:1176-1179. (MedLine)

Krebs, E.G. and Beavo, J.A. (1979) Phosphorylation-dephosphorylation of enzymes, *Annu. Rev.*

Biochem. 48:923-959.(Medline)

Ludwig, M.L., Hartsuck, J.A., Steitz, T.A., Muirhead, H., Coppola, J.C., Reeke, G.N. and Lipscombe, W.N. (1967) The structure of carboxypeptidase A. IV. Preliminary results at 2.8 Å resolution and a substrate complex at 6 Å resolution, *Proc. Natl. Acad. Sci. U.S.A.* 57:511-514.

Mauro, L.J. and Dixon, J.E. (1994) 'Zip codes' direct intracellular protein tyrosine phosphatases to correct cellular 'address', *Trends in Biochem. Sci.* 19:151-155.(Medline)

Mercer, R.W. and Dunham, P.B. (1981) Membrane bound ATP fuels the Na/K pump. Studies on membrane-bound glycolytic enzymes on inside-out vesiclcs from human red cell membranes, *J. Gen. Physiol.* 78:547-568. (MedLine)

Meyer, F., Heilmeyer, L.M.G., Jr., Haschke, R.H. and Fischer, E.H. (1970) Control of phosphorylase activity in a muscle glycogen particle. I. Isolation and characterization, *J. Biol. Chem.* 245:6642-6648.(Medline)

Neurath, H. and Walsh, K.A. (1976) Role of proteolytic enzymes in biological regulation, *Proc. Natl. Acad. Sci. U.S.A.* 73:3825-3832.(Medline)

Nimmo, H.G., Proud, C.G. and Cohen, P. (1976) The phosphorylation of rabbit skeletal muscle glycogen synthase by glucose synthase kinase 2 and adenosine-3,5-monophosphate-dependent protein kinase, *Eur. J. Biochem.* 68:31-44.(Medline)

Oliver, R.M. and Reed. L.J. (1982) Multienzyme complexes. In *Electron Microscopy of Proteins*, Vol. 2 (Harris, J.R., ed.) Academic Press, New York, pp.1-48.

Olson, M.S. and Allgyer, T.T. (1973) The regulation of nicotinamide adenine dinucleotide-linked substrate oxidation in mitochondria, *J. Biol. Chem.* 248:1582-1597.(Medline)

Paul, R.J., Hardin, C.D., Raeymaekers, L., Wuytack, F. and Casteels, R. (1989) Preferential support of Ca^{2+} uptake in smooth muscle plasma membrane vesicles by an endogenous glycolytic cascade, *FASEB J.* 3:2298-2301.(Medline)

Perham, R.N. and Duckworth, H.W. (1981) Mobility of polypeptide chain in the pyruvate dehydrogenase complex revealed by proton NMR, *Nature* 292:474-477.(Medline)

Persson, L.-O. and Srere, P.A. (1991) Purification of mitochondrial citrate transporter in yeast, *Biochem. Biophys. Res. Comm.* 183:70-76. (MedLine)

Pette, D. and Brandau, H. (1962) Intracellular localization of glycolytic enzymes in cross-straited muscle of *Locusta migratoria, Biochem. Biophys. Res. Comm.* 9:367-370.

Porpáczy, Z., Sümegi, B. and Alkonyi, I. (1983) Association between the α-ketoglutarate dehydrogenase complex and succinate thiokinase, *Biochim. Biophys. Acta* 749: 172-179.(Medline)

Rashin, A.A. (1981) Location of domains in globular proteins, *Nature* 291:85-87. (MedLine)

Reed, L. J., and Cox, D. J. (1966) Macromolecular organization of enzyme systems, *Annu. Rev. Biochem.* 35:57.

Robinson, J.B. Jr. and Robinson, J.B., Jr. and Srere, P.A., (1985) Organization of Krebs tricarboxylic acid cycle enzymes in mitochondria, *J.Biol. Chem.* 260:10800-10805.(Medline)

Robinson, J.B. Jr., Inman, L., Sumegi, B. and Srere, P.A. (1987) Further characterization of the Krebs tricarboxylic acid cycle-metabolon, *J. Biol. Chem.* 262:1786-1790.(Medline)

Smith, J.A., Poteet-Smith, C.E., Malarkey, K. and Sturgill, T.W. (1999) Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK *in vivo, J. Biol. Chem.* 274:2893-2898.(Medline)

Srere, P.A. (1985) Organization of Krebs tricarboxylic acid cycle enzymes in mitochondria, *J. Biol.Chem.*260:10800-10805.(Medline)

Schaaff, I., Heinisch, J. and Zimmermann, F.K. (1989) Overproduction of glycolytic enzymes in yeast, *Yeast* 5:285-290.(Medline)

Schoellmann, G. and Shaw, E. (1963) Direct evidence for the presence of histidine in the active center of chymotrypsin, *Biochemistry* 2:252-255.

Shapiro, B.M., and Stadtman, E.R. (1967) Regulation of glutamine synthetase. IX. Reactivity of the sulfhydryl groups of the enzyme from *Escherichia coli, J. Biol. Chem.* 242:5069-5079.(Medline)

Sigel, P. and Pette, D. (1969) Intracellular localization of the glycogenolytic and glycolytic enzymes in white and red rabbit skeletal muscle. A gel film method for coupled enzyme reactions in histochemistry, *J. Histochem. Cytochem.*17:225-237.(Medline)

Spence, J., Gali, R.R., Dittmar, G., Sherman, F., Karin, M. and Finley, D. (2000) Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain, *Cell* 102:67-76. (MedLine)

Srere, P.A. (1993) Wanderings (wonderings) in metabolism, *Hoppe-Seyler* 374:833-842.(Medline)

Stepp, L.R., Bleile, D.M., McRorie, D.K., Pettit, F.H. and Reed, L.J. (1981) Use of trypsin and lipoamidase to study the role of lipoic acid moieties in the pyruvate and α-ketoglutarate dehydrogenase complexes, *Biochemistry* 20:4555-4560.(Medline)

Stewart, M., Morton, D.J. and Clarke, F.M. (1980) Interaction of aldolase with actin-containing filaments. Structural studies, *Biochem. J.* 186:99-104.(Medline)

Strålfors, P., Hiraga, A. and Cohen, P. (1985) The protein phosphatases involved in cellular regulation. Purification and characterization of glycogen-bound form of protein phosphatase-1, *Eur. J. Biochem.* 149:295-303.(Medline)

Sumegi, B. and Alkonyi, I. (1983) A study of the physical interaction between the pyruvate dehydrogenase complex and citrate synthase, *Biochem. Biophys. Acta* 749:163-171.(Medline)

Sumegi, B. and Srere, P.A. (1984) Complex I binds several mitochondrial NAD-coupled dehydrogenases, *J. Biol. Chem.* 259:15040-15045.(Medline)

Sumegi, B., Propaczy, Z. and Alkonyi, I. (1991) Kinetic advantage of the interaction between the fatty acid β-oxidation enzymes and complexes of the respiratory chain, *Biochim. Biophys. Acta* 1081: 122-128.

Tang, P.M., Bondor, J.A., Swiderek, K.M. and DePaoli-Roach, A.A.(1991) Molecular cloning and expression of the regulatory (R_{G1}) subunit of the glycogen-associated protein phosphatase, *J. Biol. Chem.* 266: 15782-15789.(Medline)

Tsai, I.-H., Murthy, S.N.P. and Steck, T.L. (1982) Effect of red cell membrane binding on the catalytic activity of glyceraldehyde-3-phosphate dehydrogenase, *J. Biol. Chem.* 257: 1438-1442.(Medline)

Tyiska, R.L., Williams, J.S., Brent, L.G., Hudson, A.P., Clark, B.J., Robinson, J.B. Jr. and Srere, P.A. (1986) in *Organization of Cell Metabolism* (Welch, G.R. and Clegg, J.S., eds), *NATO Series A: Life Sciences* 127:177-189.

Vertessy, B.G., Orosz, F., Kovacs, J. and Ovádi, J. (1997) Alternative binding of two sequential glycolytic enzymes to microtubules. Molecular studies in the phosphofructokinase/aldolase/microtubule system, *J. Biol. Chem.* 272:25542-25546.(Medline)

Volker, K.W. and Knull, Hr. (1997) A glycolytic enzyme binding domain on tubulin, *Arch. Biochem. Biophys.* 338:237-243.(Medline)

Vousden, K.H. (2002) Activation of the p53 tumor suppressor protein, *Biochim. Biophys. Acta* 1602:47-59. (MedLine)

Wanson, J.-C. and Drochmans, P. (1968) Rabbit skeletal muscle glycogen, A morphological and biochemical study of glycogen -particles isolated by the precipitation-centrifugation method, *J. Cell Biol.* 38:130-150.(Medline)

Waskiewicz, A.J., Flynn, A., Proud, C.G. and Cooper, J.A. (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2, *EMBO J.*16:1909-1920.(Medline)

Welch, G.R. (1977) On the role of organized multienzyme systems in cellular metabolism: a general synthesis, *Prog. Biophys. Mol. Biol.* 32:103-191.

Wilkinson, A.J., Fersht, A.R., Blow, D.M.. Carter, P. and Winter, G. (1984) A large increase in enzyme substrate affinity by protein engineering, *Nature* 307:187-188.(Medline)

Wojtas, K., Slepecky, N., von Kalm, L. and Sullivan, D. (1997) Flight muscle function in *Drosophila* requires colocalization of glycolytic enzymes, *Mol. Biol. Cell* 8:1665-1675.(Medline)

Yu, J. and Steck, T.L. (1975) Association of band 3, the predominant polypetide of the human erythrocyte membrane, *J. Biol. Chem.* 250: 9176-9184.

Zaug, A.J. and Cech, T.R. (1986) The intervening sequence RNA of *Tetrahymena* is an enzyme, *Science* 231:470-475.(Medline)

Zhao, Y., Bjorbaek, C. and Moller, D.E. (1996) Regulation and interaction of pp90(rsk) isoforms with mitogen-activated protein kinases, *J. Biol. Chem.* 271:29773-29779.(Medline)

Zalokar, M. (1960) Cytochemistry of centrifuged hyphae from *Neurospora*, *Exp. Cell Res*, 19:114-132.

14. Regulation of Metabolism

Chapter 13 discussed how enzyme activity is regulated allosterically by positive and negative effectors. An additional mechanism is provided by the cycling of the enzyme molecules between active and inactive states, frequently involving phosphorylation and dephosphorylation of the enzyme. The present chapter will concentrate on the control of energy yielding reactions of cell metabolism on moment-to-moment adaptive changes.

I. PROBLEMS IN APPLYING IN VITRO OBSERVATIONS TO INTACT CELL

The metabolism of cells and its regulation are so complex that it is difficult to arrive at a quantitative evaluation of the effect of control mechanisms on the overall economy of the cell. Much is known about the properties of the purified soluble enzymes involved in the cell's metabolic pathways. Although some inferences can be drawn from this information, the control is complex and involves factors that have not been studied in detail. The enzymes of the glycolytic pathway have been studied in detail and their regulation might be thought to involve few surprises. However, some experiments suggest that our understanding is very limited. One experiment illustrates this point effectively. In yeast, the concentration of glycolytic enzymes can be increased by introducing the appropriate genes in multicopy vectors (Schaaff et al., 1989). This process increases the activity of individual enzymes approximately 4 to 14-fold compared to the wild-type enzymes. However, the rate of ethanol production by the yeast remains unchanged, even with increases in enzymes catalyzing irreversible steps that are thought to have a key regulative role (see below). This observation may be explained by the presence of enzymes in "assemblies" where the enzymes are held in precise stoichiometry. The free excess enzyme is simply left out of the pathway.

Many biochemical pathways interact in ways which are unpredictable with our present knowledge. Most are branched at least to some extent. The kinetics of even the simplest hypothetical pathway have unsuspected complexities. For example, a linear pathway with no branching has kinetics that depend on the constants of each step, except for the reactions subsequent to an irreversible step. Similarly, when the concentration of one substrate in a sequence is very large (for example, provided by a side feeder reaction) the rate of the pathway depends not only on the rate of that one step but also on the rates of all the proceeding steps (Hearon, 1948, 1949). Furthermore, the information obtained in vitro is not likely to be very realistic. The organization of the cytoplasm, both structurally and functionally, is very complex. Isolated enzymes in solution are not likely to duplicate the in situ conditions.

A. Organization of Enzymes

Enzymes are not free in solution; they are frequently attached to structures or are present in multienzyme complexes. In intact cells, the properties of enzymes are much different from those of isolated, purified forms. When attached to structures they acquire very different properties. When bound to actin, the maximal enzymatic rate *(V_m)* of aldolase doubles and the K_m for fructose 1,6-bisphosphate (Arnold and Pette, 1970) increases. Upon binding to structures, phosphofructokinase loses its allosteric inhibition by ATP (Karadsheh and Uyeda, 1977) and glyceraldehyde-3-phosphate dehydrogenase is inhibited (Tsai et al., 1982).

Organization in structures and specific sites have other unexpected results. The effective local concentration of substrates or regulatory molecules may attain unsuspected levels. The Ca^{2+} concentration at axon terminals may reach concentrations as high as 0.1 mM, whereas it is well below the µM level in most of the cell (see Chapter 22). At the synapse, Ca^{2+} triggers neurotransmitter release. In most cells, Ca^{2+} initiates many other functions as a second messenger (see Chapter 7 and 24).

The organization of enzymes in assemblies may well be the rule rather than the exception. Their behavior is distinct from that of their individual components and not readily predictable from conventional models. As our knowledge gains in detail, the number of known enzyme assemblies increases. *Spliceosomes* carry out the splicing of mRNA; transcriptional complexes carry out transcription; and translational complexes which contain a variety of enzymes, mRNA and ribosomes, are involved in protein synthesis. Similarly, *proteasomes* containing several enzymes, are responsible for the degradation of proteins. Evidence abounds for multienzyme complexes (referred to as *metabolons*) playing a role in metabolic pathways. These pathways include glycolysis, nucleotide synthesis (e.g., Jones, 1980), urea biosynthesis, tricarboxylic acid (TCA) cycle, fatty acid oxidation (e.g., Gillevet and Dakshinamurti, 1982) and amino acid metabolism.

Some of the evidence for the presence of metabolic enzymes associated in complexes comes from indirect information. For example, the centrifugation of intact cells has been shown to sediment most cytosolic proteins (Zolokar, 1960; Kempner and Miller, 1968). This observation suggests that a large portion of the proteins of the cell must be present as aggregates. The very high concentration of proteins suggests that they are not in solution and may well be present in a semi-crystalline state. In the mitochondrial matrix, the protein concentration has been calculated to be 56% by weight (Hackenbrock, 1968), a proportion which corresponds to the densest possible packing of proteins, assuming them to be identical spheres. Similar arguments can be made for the glycolytic enzymes in muscle, where most of the space not occupied by the contractile apparatus is likely to be occupied by glycolytic enzymes (Pette and Brandau, 1962; Sigel and Pette, 1969). Pette and Brandau estimate that in the muscle of *Locusta migratoria* the concentration of these enzymes by themselves corresponds to 0.2 g per ml. Other evidence is more direct. The discussion

below will address the question for metabolic enzymes.

B. Mitochondrial Matrix Enzymes

Each enzyme of the citric acid cycle is able to bind the next enzyme in the sequence, indicating that they are likely to be present in a complex. Citrate synthase associates specifically with mitochondrial malate dehydrogenase, but not cytoplasmic malate dehydrogenase (Halper and Srere, 1977). Similarly, citrate synthase binds to mitochondrial, but not cytoplasmic aconitase (Tyiska et al., 1986), the pyruvate dehydrogenase complex (Sumegi and Alkonyi, 1983), and the citrate transporter (Persson and Srere, 1991). Similar results are obtained with pure enzymes. Enzymes which are sequential in the cycle bind to each other: mitochondrial aconitase binds to NADH-isocitrate dehydrogenase (Tyiska et al., 1986); fumarase binds to mitochondrial malate dehydrogenase (but not the cytoplasmic enzyme) (Beekmans and Kanarek, 1981); and the α-ketoglutarate dehydrogenase complex binds succinyl-CoA synthase (succinate thiokinase) (Porpaczy et al., 1983).

An additional level of organization is revealed by the observation that the tricarboxylic acid cycle enzymes bind to inside-out inner mitochondrial membrane vesicles (D'Souza and Srere, 1983). The binding is specific; isoenzymes that catalyze the same reactions in other organelles (such as citrate synthase from peroxisomes) do not bind (Kispal and Srere, 1991). In addition, mitochondrial malic dehydrogenase binds to liposomes containing the mitochondrial Complex I (Sumegi and Srere, 1984), with which it must interact for the oxidation of the NADH generated by this enzyme. Binding is also demonstrated by the observation that mitochondria disrupted by ultrasound do not lose the citric acid cycle enzymes, even though citrate synthase has become accessible to an anti-synthase antibody and malic dehydrogenase to high molecular weight blue dextran (Robinson and Srere, 1985). The properties of these bound enzymes suggest that the organization in the mitochondrial matrix has an important role in determining the metabolic rate. The bound enzymes have a much higher rate than those in soluble form (Robinson et al., 1987; Sumegi et al., 1991).

C. Binding of Glycolytic Enzymes to Actin

In muscle, F-actin and most glycolytic enzymes are located within the I-band (Dolken et al., 1975; Sigel and Pette, 1969). The exception is hexokinase, which is localized primarily in mitochondria. A number of glycolytic enzymes have been shown to bind to F-actin (Arnold and Pette, 1968, 1970; Arnold et al., 1971). The binding is most pronounced in the presence of the regulatory proteins tropomyosin and troponin (Clarke and Morton, 1976; Clarke and Masters, 1975; Stewart et al., 1980). Binding to actin has been observed for fructose-1,6-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase. The implications of these findings are not easyly assessed.

D. Glycogen Particles

Glycogen particles isolated from skeletal muscle contain the enzymes of glycogen metabolism. These include: phosphorylase b and its kinase and phosphatase, glycogen synthase and its respective kinase and phosphatase (Meyer et al., 1970), as well as its debranching enzyme, glycogen synthase kinase 2, protein phosphatase 2, and inhibitors 1 and 2 (Nimmo and Cohen, 1976). The glycogen particles themselves are closely associated with the sarcoplasmic reticulum near the thin filaments (Wanson and Drochmans, 1968). The kinetics of the enzymes in the particles are distinct from that in solution (Fischer, et al. 1971).

E. Binding of Enzymes to the Plasma Membrane

Certain enzymes are located in the cell's periphery, and some of them have been shown to bind to the cytoplasmic domain of integral membrane proteins. In the red blood cell, glyceraldehyde-3-phosphate dehydrogenase and fructose-1,6-bisphosphate aldolase bind to the Band 3 protein, the anion transporter (see Yu and Steck, 1975; Tsai et al., 1982). In the case of glyceraldehyde-3-phosphate dehydrogenase, the binding exhibits 1 to 1 stoichiometry. The functional significance of these associations is not clear.

II. REGULATORY ELEMENTS

The regulation of enzyme specific activity (in contrast to regulating the number of enzyme molecules present) can take place by different mechanisms. Regulation may occur by the phosphorylation-dephosphorylation of enzymes by specific kinases (Chapter 13). The kinases respond to the level of a metabolite or, when responding to hormonal signals, to second messengers. This kind of regulation generally affects key entry reactions. In this chapter we will discuss the regulation of glycogen phosphorylase, which is activated by phosphorylation, and that of pyruvate dehydrogenase, which is inhibited by phosphorylation. These two enzymes control the entry into the glycolytic pathway (when glycogen is the substrate) and the TCA cycle, respectively.

The control of enzyme activity can also be through allosteric regulation. Sometimes the allosteric regulator is a component of the pathway. At other times the regulator is synthesized in a side reaction of the pathway. The function of this side reaction is probably solely to provide regulation.

The presence of regulatory cycles (originally called futile cycles) is thought to offer a very effective regulatory mechanism, for example in glycolysis. Substrate availability and feedback inhibition are also significant.

Some of these regulatory mechanisms require more thorough examination in light of the information considered in Section I.

The general features of metabolic regulation will be discussed first (A to D) followed by a more specific discussion of the regulation of metabolic reactions (Sections III-VI).

A.Cascades

The activity of enzymes or enzymatic pathways are controlled by complex mechanisms. A signal can be amplified by a series of biochemical reactions (a *cascade* mechanism). This kind of effect was examined in Chapter 7 in relation to intracellular signals. The initial signal may be a hormone or a regulative metabolite. The activity of an enzyme may be through phosphorylation-dephosphorylation reactions as also discussed in Chapter 7. We will examine two cascades that reflect these two distinct triggers: the AMP-activated protein kinase and the mobilization of glucose by the hormone epinephrine.

AMP-activated protein kinase.

AMP-activated protein kinase (AMPK) occupies an important position in the regulation of metabolism because AMP is a sensitive sensor of metabolic states as discussed in Section IIE. The precise mechanisms are not always clear at this time but the basic role of AMPK is well recognized. AMPK inhibits anabolic pathways to limit the use of ATP (Hardie and Carling, 1997) because it inhibits enzymes involved in glycogen, fatty acid and cholesterol synthesis. In contrast, AMPK accelerates reactions generating ATP, such as those of fatty acid oxidation in striated muscle and heart. In the heart, 60-70% of metabolism originates from fatty acid oxidation. AMPK, a heterotrimer, is activated by high AMP and low ATP in a cascade which involves allosteric regulation, facilitation of phosphorylation by an upstream protein kinase (AMPK kinase), and inhibition of dephosphorylation. The activity of the AMPK kinase and two phosphatases depends on the concentration of AMP (Davies et al., 1995). This protein-kinase cascade represents a sensitive system, which responds to the depletion of ATP. AMPK is equivalent to the yeast SNF1 protein-kinase complex. SNF1 is activated by glucose starvation which in yeast leads to ATP depletion. Apparently, it is involved in derepression of glucose-repressed genes.

AMPK phosphorylates and activates hydroxymethylglutaryl-CoA reductase and acetyl-CoA carboxylase, key regulatory enzymes of sterol synthesis and fatty acid synthesis, respectively. It probably phosphorylates many other target enzymes as well. Inhibition of acetyl-CoA carboxylase by AMPK reduces malonyl-CoA. In this way, it relieves the allosteric inhibition of mitochondrial carnitine palmitoyl transferase (CTP1) (e.g., see Kudo et al., 1995). CTP1 is the rate-limiting enzyme involved in mitochondrial uptake of fatty acids transport into mitochondria (McGarry et al., 1989). In mammals, AMPK also induces an increase in glucose transport following exercise by a mechanism independent of the action of insulin (Hayashi et al., 1998). In muscle, phosphocreatine has a role in maintaining the ATP concentration constant. AMPK phosphorylates and inhibits creatine kinase (Ponticos et al., 1998) thereby blocking the exchange of high energy phosphate between creatine and ATP, possibly shutting off a high energy phosphate sink. AMPK

is itself regulated by a mechanism involving phosphocreatine, creatine and pH. The creatine kinase-creatine phosphate system is clearly of great significance in the energy balance of the cell (see Wallimann et al., 1992). However, the significance of the AMPK regulation is not obvious particularly since the phosphocreatine system is compartmentalized and the physiological effect will depend on the location of the enzyme.

The hormone leptin, secreted by adipocytes, has been found to increase the oxidation of fatty acids, the uptake of glucose and to prevent the accumulation of lipids in nonadipose tissues. The effect of leptin on fatty acid oxidation rests on its activation of AMPK in muscle which inhibits the synthesis of malonyl-CoA , thereby favoring fatty acid oxidation (Minokoshi et al., 2002). Leptin is a hormone secreted by adipocytes. It has a role in food intake and in controlling energy expenditure and neuroendocrine function (see Friedman and Halaas, 1998).

Hormonal control: the case of epinephrine.

Glycogen phosphorylase of skeletal muscle is controlled by the cascade shown in Fig. 1 (Fisher et al., 1970). The less active form of phosphorylase, phosphorylase *b*, is converted to the active phosphorylated species, phosphorylase *a* (Fig. 1). In turn, phosphorylase *a* can be inactivated by the appropriate phosphatase. Phosphorylase *a* releases glucose-1-phosphate from glycogen. Glucose-1-phosphate can then be metabolized by the muscle cell's glycolytic machinery.

The phosphorylase cascade is very significant in the mobilization of the carbohydrate stores of muscle. The conversion of phosphorylase *b* to phosphorylase *a* is indirectly regulated by the hormone *epinephrine* (also called *adrenaline*), secreted by the adrenal medulla and other components of the sympathetic nervous system. In muscle, epinephrine activates adenyl cyclase (see Fig. 1), which catalyzes the conversion of ATP to cyclic AMP (cAMP). cAMP, in turn, activates the kinase that phosphorylates phosphorylase kinase (i.e., a kinase kinase). In the presence of Ca^{2+}, the phosphorylated active form of phosphorylase kinase, along with ATP, phosphorylate the phosphorylase *b*, thereby producing phosphorylase *a*. The binding of a small amount of epinephrine has triggered the production of many molecules of glucose-1-phosphate and thereby stimulated the major metabolic pathway of muscle through a cascade of reactions. The activity of phosphorylase is also under the control of several effectors, such as ATP, glucose-6-phosphate, glucose, and uridine diphosphoglucose.

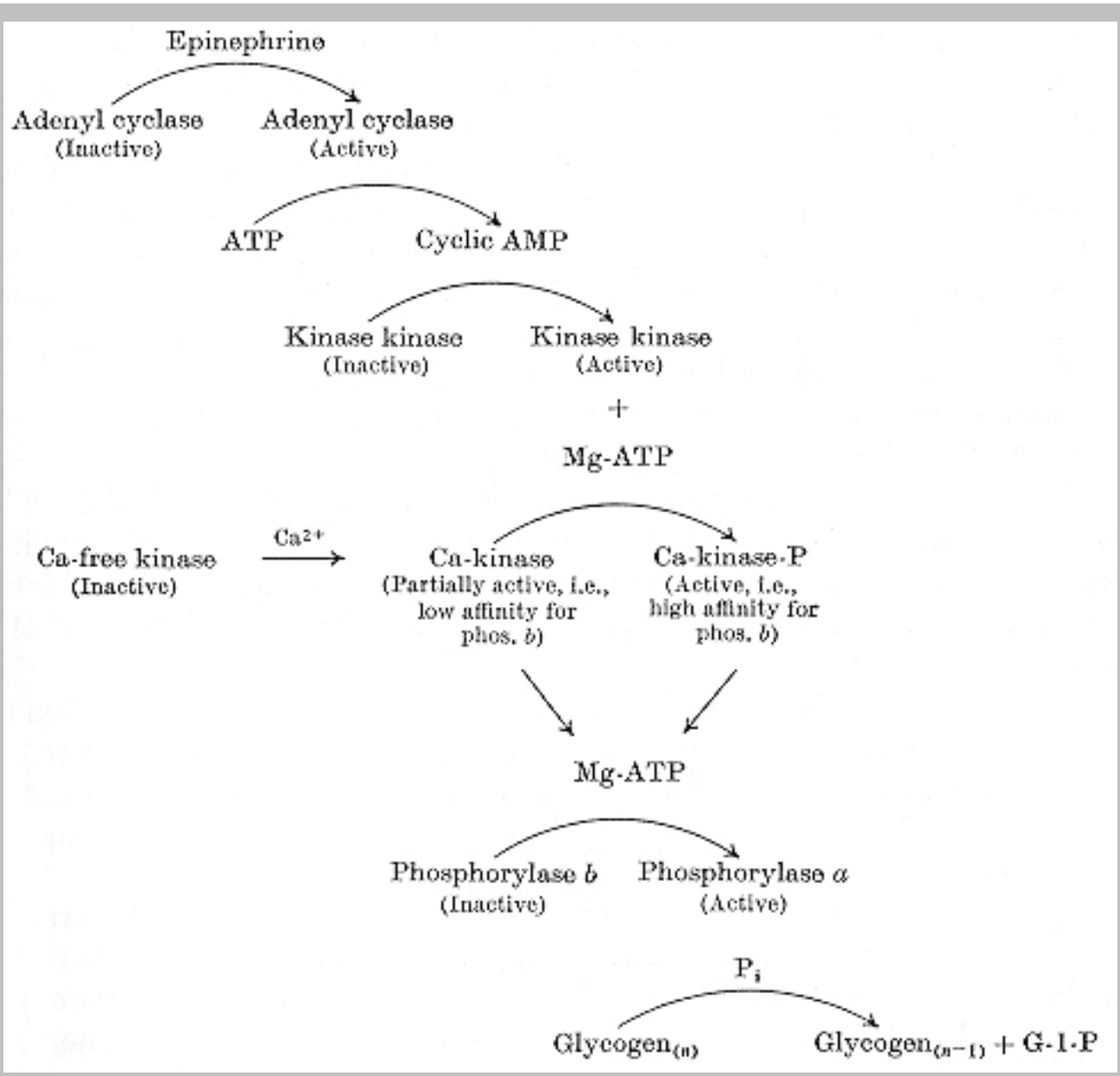


Fig. 1 Control of glycogen phosphorylase (Fisher et al., 1970). Reproduced by permission from *Essays in Biochemistry* 6:23-68, copyright &copy1970 The Biochemical Society, London.

The mechanism involving muscle phosphorylase increases the glucose available for glycolysis in muscle. In liver, phosphorylase is also involved in the regulation of blood glucose and hence in the metabolism of the organism as a whole. The control of the release of glucose by epinephrine in muscle is but one of the reactions involved in mobilizing glucose in mammals (Exton et al., 1971). cAMP inhibits phosphorylase phosphatase, thereby prolonging the presence of phosphorylase in its active form. cAMP also mediates the transformation of glycogen synthetase to a less active form, thereby blocking the storage of glucose favoring its release.

Epinephrine is not the only hormone involved in the release of cAMP. *Glucagon* also tends to increase the concentration of cAMP, while *insulin* decreases the concentration. The interactions between the effects of the three hormones are responsible for the final effect on glucose metabolism. Both glucagon and insulin are pancreatic endocrine hormones.

A number of key enzymes controlled by a variety of signals are also regulated through a phosphorylation-dephosphorylation mechanism; an example is pyruvate dehydrogenase. However, in contrast to the phosphorylase, the dephosphorylated pyruvate dehydrogenase is the active form.

B. Role of Exergonic Steps

Most of the component reactions in a pathway proceed rapidly and are close to equilibrium (i.e., $\Delta G = 0$). Therefore, they are not subject to regulation. The metabolic pathways themselves are highly exergonic. They are virtually irreversible because at least one step highly exergonic. This step has been called the *first committed step* and is frequently at the beginning of a metabolic pathway. Since it is far from equilibrium, it can be regulated. The regulation, through the control of a rate-limiting exergonic step, is analogous to the way in which a dam controls the flow of a whole river by adjusting the flow of the water passing through the dam itself. Besides the requirement that the step be exergonic, other conditions must also be met. The enzyme must be capable of responding to the appropriate regulator. Furthermore, the substrate concentration should be in a range allowing regulation; the rate of enzyme activity cannot be increased when the enzyme is saturated.

In the regulated steps, generally forward and backward reactions are catalyzed by separate enzymes, as discussed in the next section.

C. Separate Enzymes for Forward and Backward Reactions

In key metabolic steps, frequently one enzyme catalyzes the forward reaction and another the backward reaction. This feature allows irreversible steps in either direction and the independent regulation of the forward and backward pathways. In addition, the continuous formation and degradation of substrate (*substrate cycling*) has an important regulatory role. Its purpose was not originally understood and the process is undoubtedly wasteful in terms of energy. For these reasons, the cycling was originally called *futile cycling*. Whereas allosteric regulation allows variation of the rate of a step over a ten-fold range, substrate cycling permits regulation over a hundred-fold range.

Let us examine this process in more detail (Newsholme et al., 1984). The flow of metabolites through a pathway, that is, the flux (J_{net}) in moles per unit time through a rate-limiting step, can be represented as the difference between the forward (J_f) and the backward flux (J_b). The two fluxes are catalyzed different enzymes. This can be represented as:

$$J_{net} = J_f - J_b \quad (1)$$

Selecting reasonable values in arbitrary units [based on the regulation of the phosphorylation of

fructose-6-phosphate (F6P) to form fructose-1,6-bisphosphate (F1,6P)], J_f may be 10 and J_b, 9. J_{net} will be 1. If J_f is increased tenfold and J_b decreased tenfold, J_{net} will be 99, i.e., approximately a one hundredfold increase. In the case of this metabolic step, the forward reaction is catalyzed by phosphofructokinase (PFK), whereas the reverse reaction is catalyzed by fructose-1,6-bisphosphate phosphatase (FBPase). The two enzymes are regulated by AMP, which stimulates the kinase and inhibits the phosphatase. The stimulation of PFK by AMP actually results from blocking an inhibition by ATP (Mansour and Ahlfors, 1968).

This metabolic step is also sensitive to a variety of other metabolites, illustrating how complex a regulative pattern can be. One of the allosteric activators of PFK is cAMP, a second messenger under hormonal control.

The fact that, in the case discussed, the regulating metabolite is primarily AMP leads us to another basic principle of regulation. The effector must be a chemical which changes in concentration significantly with physiological conditions. This question will be discussed in the next section.

D. Effectors Sensitive to Small Changes

Effectors are produced in two distinct ways. The pathway itself may provide a metabolite that can serve as a sensitive indicator of metabolic conditions. Basically this mechanism corresponds to feedback inhibition, discussed in Chapter 13. Alternatively, the enzymatic machinery of the cell can produce an effector from side reactions, sometimes under the control of a hormone.

The first reaction of the glycolysis illustrates feedback control. The phosphorylation of glucose to form glucose-6-phosphate (G-6-P), is catalyzed by hexokinase (Hk). Hk is inhibited allosterically by G-6-P (McDonald et al., 1979). In some cells, this effect is very important, and the reaction, the gateway to glycolysis, regulates the whole pathway.

A side reaction may also provide a sensitive indicator of metabolic state. The concentration of AMP, which we discussed above, serves as an indicator of the energy balance. Fructose-2,6-bisphosphate (F2,6P), one of the most powerful allosteric regulators now known, is also generated in a step that is not in the main metabolic pathway. The regulative role of F2,6P differs with the function of the tissue. Glucose-1,6-phosphate (G1,6P), which has an important role as a cofactor, is also a significant effector in glycolysis. The regulation by AMP, F2,6P, and G1,6P are discussed in more detail below.

E. AMP as a Metabolic Effector

In muscle, as we saw for PFK, it is AMP, not ATP, that is the major controlling effector. Why not ATP, which is directly involved in supplying the power to cells? Many enzymes (including PFK) are, in fact, regulated by ATP. However, in this case, and particularly in heart muscle, the effect of AMP is preeminent. The answer lies in the fact that the ATP and ADP concentrations remain

relatively constant (Helmreich and Cori, 1965). In vertebrate muscle and nerve, ATP is rapidly regenerated by two reactions: (a) the creatine kinase reaction where ATP is formed from ADP and phosphocreatine as shown in Eq. (2) and (b) the adenylate kinase reaction that generates ATP and AMP from 2 ADPs, as shown in Eq. (3).

$$\text{creatine-P} + \text{ADP} \rightarrow \text{ATP} + \text{creatine} \quad (2)$$

$$2\ \text{ADP} \rightarrow \text{ATP} + \text{AMP} \quad (3)$$

The adenylate kinase reaction rate is rapid and can be considered at equilibrium. In turn, AMP concentration plays a very important physiological role. The equilibrium constant

$K_{eq} = (\text{ATP})\ (\text{AMP}) / (\text{ADP})^2 \sim 1$. The concentrations of ADP and ATP are rather large compared to AMP. A small increment in (ATP) will produce a very significant decrease in AMP. Actual calculations assuming a decrease in ATP of 10% will produce a large decrease in AMP (approximately 4-fold), so that AMP can serve as a sensitive indicator of the energy stores of the cell.

F. F2,6P

Fructose 2,6-bisphosphate (F2,6P) is an important regulator of glycolysis in several mammalian tissues (Hers et al., 1982). In liver, it acts to block glycolysis, thereby favoring the release of glucose produced by the concurrent degradation of glycogen. The liver is the key organ responsible for regulating the concentration of blood glucose. The regulation of the glucose level via F2,6P is part of this mission. In heart muscle, which degrades glycogen for its own glycolysis, F2,6P instead, stimulates glycolysis.

The level of F2,6P depends on the balance between the enzyme activity responsible for the phosphorylation of F6P (phosphofructokinase 2, PFK-2) and the enzyme activity responsible for its hydrolysis (fructose-2,6-bisphosphatase, FBPase-2). The same protein is responsible for the two activities. In liver, the cAMP-dependent phosphorylation of the enzyme by a protein kinase inhibits PFK-2 and activates the phosphatase. In heart, the opposite is true: phosphorylation activates PFK-2 and blocks FBPase-2.

G. G1,6P

Glucose 1,6-bisphosphate (G1,6P) is a cofactor of the phosphoglucomutase reaction which converts glucose-1-phosphate (G1P) to glucose-6-phosphate (G6P). It is generated by the phosphorylation of G1P and it is degraded by a specific phosphatase. G1,6P is an activator of PFK, pyruvate kinase (PK), and is an inhibitor of HK and FBPase. G1,6P also releases phosphoglucomutase from ATP and citrate inhibition. Because its level is subject to a variety of physiological controls, including the presence of several second messengers (summarized in Table

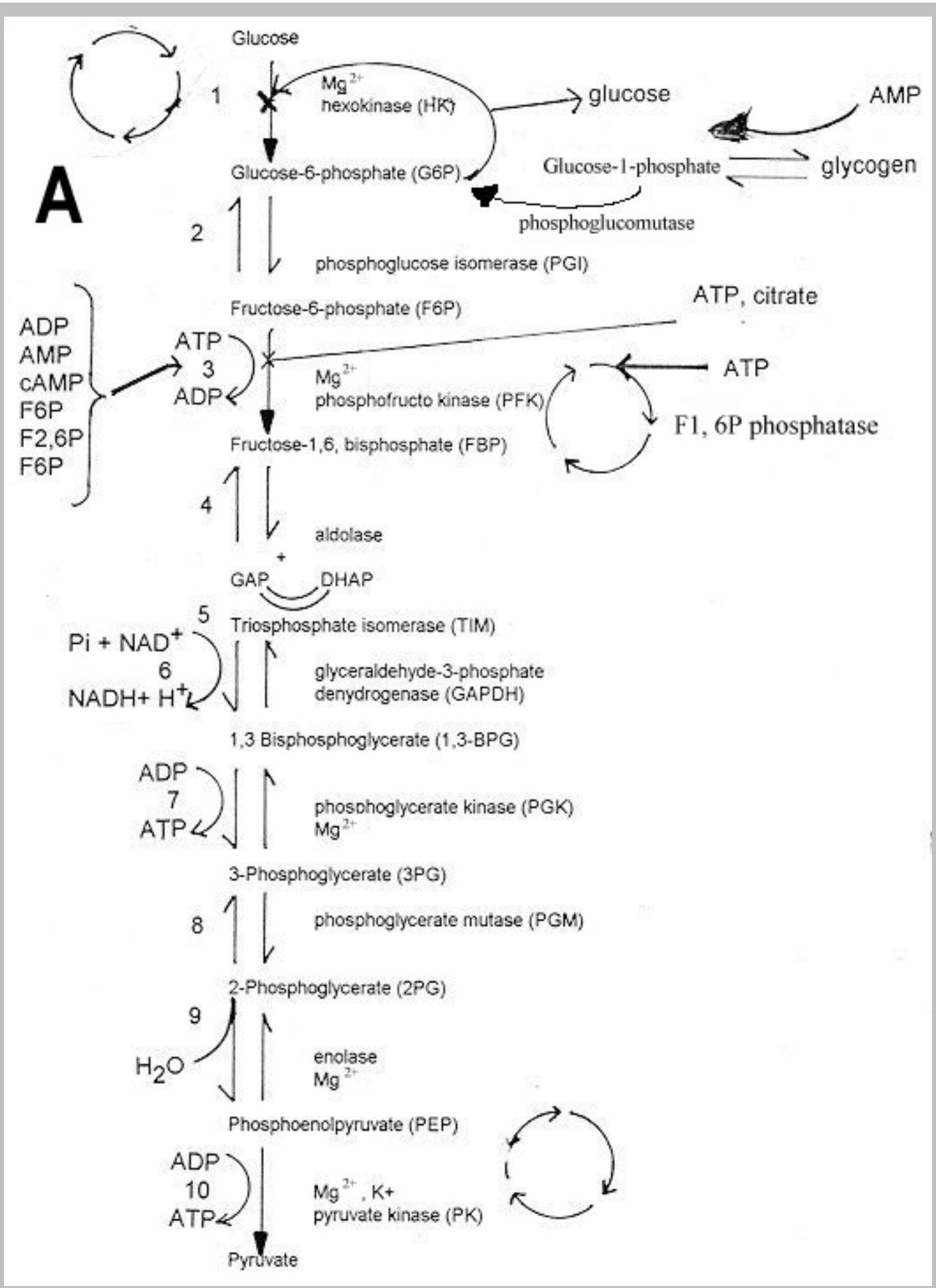
1), it must act as a significant physiological signal (Beitner, 1984).

III. CONTROL OF GLYCOLYSIS

The regulation of glycolysis is fairly complex, involving most of the mechanisms discussed. Many of these regulatory effects are summarized in Fig. 2, part A.

A. ΔG and Regulation

Insights into the regulation of a metabolic pathway require information that is not always available. As discussed above, regulated steps must be exergonic, i.e., far from equilibrium. Therefore, ΔG of the various reactions can predict in what steps the regulation may occur inside cells. However, ΔG alone merely indicates feasibility of regulation. As already mentioned, the enzyme catalyzing the step must be capable of responding to the appropriate effectors. As we saw in Chapter 12, calculation of ΔG requires knowing not only the ΔG^o of the reactions (and this can be calculated, see Chapter 12), but also the actual substrate concentrations. Fortunately, data is available for some tissues, for example, heart muscle cells (Newsholme and Start, 1973). The calculated ΔGs show that regulation is likely to occur only at three glycolytic steps: the HK (ΔG = - 6.5 kcal), PFK (ΔG = - 6.2 kcal), and PK (ΔG = -3.3 kcal) reactions. The major negative effectors of these enzymes are G6P for HK and ATP for PK. ATP and citrate are inhibitors of PFK. Only PFK has positive effectors, among which is AMP. As we saw in heart muscle, AMP is very sensitive to the energy balance of the cell. Furthermore, the regulatory effect is enhanced by the mechanism of substrate cycling, permitting as much as a one hundred-fold activation. For these reasons, in most mammalian tissues, PFK is thought to be the most important glycolytic control site (e.g., see Bosca and Corredor, 1984). These regulatory steps are indicated in Fig. 2, part A.


A
Glucose
1
Mg^{2+}
hexokinase (HK)
glucose
AMP
Glucose-6-phosphate (G6P)
Glucose-1-phosphate
glycogen
phosphoglucomutase
2
phosphoglucose isomerase (PGI)
Fructose-6-phosphate (F6P)
ATP, citrate
ADP
AMP
cAMP
F6P
F2,6P
F6P
ATP
3
ADP
Mg^{2+}
phosphofructo kinase (PFK)
ATP
F1, 6P phosphatase
Fructose-1,6, bisphosphate (FBP)
4
aldolase
GAP
DHAP
5
Triosphosphate isomerase (TIM)
Pi + NAD^+
6
$NADH+ H^+$
glyceraldehyde-3-phosphate
denydrogenase (GAPDH)
1,3 Bisphosphoglycerate (1,3-BPG)
ADP
7
ATP
phosphoglycerate kinase (PGK)
Mg^{2+}
3-Phosphoglycerate (3PG)
8
phosphoglycerate mutase (PGM)
2-Phosphoglycerate (2PG)
9
H_2O
enolase
Mg^{2+}
Phosphoenolpyruvate (PEP)
ADP
10
ATP
Mg^{2+}, K+
pyruvate kinase (PK)
Pyruvate

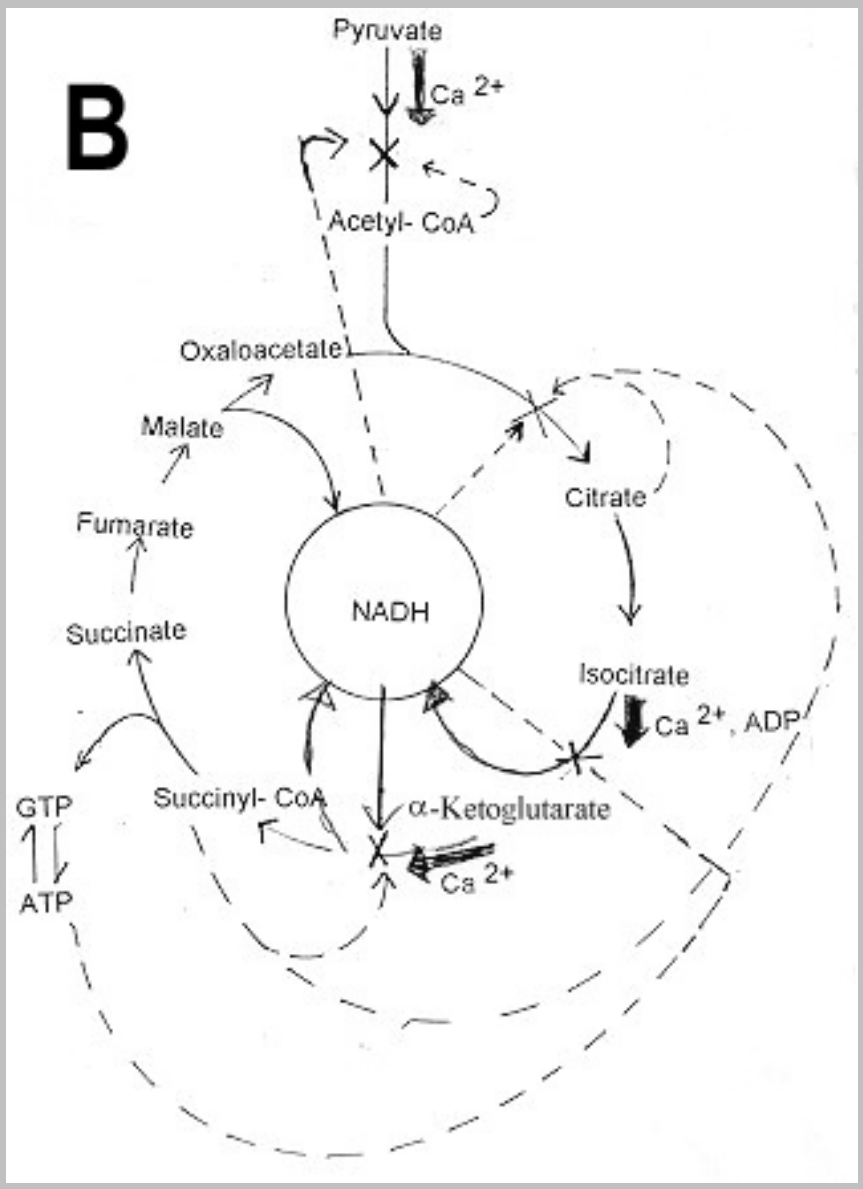


Fig. 2 Representation of the control of metabolism. The oxidation-phosphorylation steps of mitochondria were left out to simplify the diagram.

B. Substrate Cycling

Substrate cycling occurs not only in the F6P to F1,6P step (e.g., Clark, D.G. et al., 1973; Clark, M.G. et al., 1973; Rognstad and Katz, 1980; Challis et al., 1984), but also in glucose to G6P (Surholt and Newshome, 1983) and the PK step (Freidmann et al., 1971), suggesting that in at least some of the systems studied, these steps can be regulated through this mechanism. The substrate cycling steps are indicated in Fig. 2 on the left of part A of the diagram.

Table 1 Factors and Conditions That Control Cellular G1,6P Levels.

Effectors	Tissues	Changes in G1,6P levels
Cyclic AMP	Diaphragm Cultured muscle	↑↑
Cyclic GMP	Diaphragm Cultured muscle	↓↓
Ca^{2+}	Diaphragm Cultured muscle	↓↓
Hormones:		
Epinephrine	Diaphragm Cultured muscle	↑↑
Epinephrine + propranolol	Cultured muscle	↓
Vasopressin	Cultured muscle	↓
Serotonin	Skeletel muscle Skin	↓↓
Bradykinin	Skeletal muscle Skin	↓↓
Phospholipase A_2	Diaphragm	↓
Lysolecithin	Diaprhagm	↓
Muscular dystrophy	Skeletal muscle	↓
Fasting	Skeletal muscle Normal Dystrophic	↓↓

Refeeding	Skeletal muscle Normal Dystrophic	↑↑
Anoxia	Diaphragm	↓
Ischemia	Brain	↓
Aerobiosis	Diaphragm	↑
Differentiation (fusion)	Cultured muscle	↑
Growth	Skeletal muscle Heart Brain Skin	↑↔↓↓
Old age	Skeletal muscle	↓
Glucose	Pancreatic islets	↑
Pharmacological agents Local anesthetics Lithium **Trifluoroperazine**	Diaphragm Skeletal muscle Normal; Dystrophic Diaphragm Brain Liver Skeletal muscle	↓ ↓↓↓↓↔↑

↑ = increase ; ↓=decrease; ↔ = no change

From Beitner (1984). Reproduced with permission from *International Journal of Biochemistry* 16. Beitner, R., Control levels of glucose 1,6-bisphosphate, copyright &copy1984, Pergamon Journals, Ltd.

IV. CONTROL OF THE TRICARBOXYLIC ACID CYCLE

The tricarboxylic acid (TCA) cycle acts in concert with NADH oxidation and ATP synthesis and

is, therefore, inexorably linked to these processes. Glycolysis and other sources of acetyl-CoA provide one of the needed substrates. Because it provides NADH to be oxidized by the electron transport chain, the cycle depends on the rate of oxidation by the mitochondrial complexes. Therefore, for many reasons it would be more appropriate to examine the regulation of the tricarboxylic acid cycle in relation to the whole metabolic system, as done in the next section (V). However, some of the details presented in this section facilitate our understanding of the whole.

Regulation of the TCA cycle primarily involves the regulation of the availability of the entry substrate and feedback inhibition (Hansford, 1980) (see Fig. 2, part B). Because the entry substrates, acetyl-CoA and oxaloacetate, are present at less than saturation level, their influx into the system can control the flow through the cycle. A major product of the cycle is NADH, and as might be expected, acts as a feedback inhibitor.

A. Substrate Availability

Pyruvate dehydrogenase (PDH), a multienzyme complex, is responsible for the production of one molecule of acetyl-CoA and one molecule of NADH from the oxidation of each pyruvate. This reaction controls the entry into the TCA cycle of substrate from the glycolytic pathway (see Randle et al., 1978). PDH is inactivated by phosphorylation catalyzed by a specific kinase. The kinase, in turn, is activated by NADH and acetyl-CoA (see Reed et al., 1985). NADH and acetyl-CoA, the products of the PDH reaction, also act directly on PDH as feedback inhibitors. Both mechanisms then inhibit the production of acetyl-CoA in response to excess products. PDH is also regulated by nucleotides (GTP inhibiting and AMP activating), and its inhibition by phosphorylation is increased by high ATP/ADP ratios.

Acetyl-CoA and oxaloacetate are the substrates of citrate synthase, the enzyme which catalyzes the first reaction of the TCA cycle. Oxaloacetate is produced by malate dehydrogenase. The reaction is in equilibrium so that:

K_{eq} = (oxaloacetate) (NADH) / (NAD^+) (malate) (4)

As shown in Eq. (4), the oxaloacetate concentration varies inversely with that of NADH. Conversely, the increase in NAD^+ (and consequent decrease in NADH) will increase the level of oxaloacetate. This reaction serves as a delicate sensor of the redox state of NAD^+ and responds by adjusting the amount of oxaloacetate fed into the TCA cycle.

The two substrates of the entry reaction to the TCA cycle are therefore under tight regulation by at least two separate mechanisms. The citrate synthase reaction itself is also inhbited by a high $NADH/NAD^+$ ratio (see below).

B. Inhibition by Products and Intermediates

As already discussed, regulation is likely to occur at exergonic steps in the TCA cycle, such as the citrate synthase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase steps. In fact, these appear to be control steps (see Fig. 2, part B). NADH produced by the cycle inhibits all three. In addition, citrate inhibits citrate synthase, while succinyl-CoA inhibits α-ketoglutarate dehydrogenase and citrate synthase. As might be expected, ADP and ATP have opposite roles. ADP stimulates isocitrate dehydrogenase, whereas ATP inhibits it.

An interesting role is played by Ca^{2+}, a second messenger (see Chapter 7), which stimulates pyruvate, isocitrate and α-ketoglutarate dehydrogenase, thereby tending to increase the metabolic rate. The mechanism controlled by Ca^{2+} can override all other factors. The regulation of metabolism by Ca^{2+} is also discussed in Chapter 7 (section IA).

V. CONTROL OF OXIDATIVE PHOSPHORYLATION AND GLYCOLYSIS IN TANDEM

The interactions of the various components of the metabolic reactions cannot be appreciated without examining them together. As we saw in the discussion of glycolysis and the TCA cycle, the availability of substrates and regulation by indicators of energy metabolism are important components. This section will discuss these factors in some detail.

A. Regeneration of NAD^+

One of the products of glycolysis is NADH, which is formed from NAD^+ by the glyceraldehyde-3-phosphate dehydrogenase reaction as shown schematically in Fig. 3. The total amount of NAD, reduced or oxidized, is finite, and the NAD^+ has to be regenerated if glycolysis is to proceed. During oxidative metabolism, NADH can be oxidized by the electron transport chain (see Chapter 16 and discussion below). However, when the oxidative metabolism cannot keep up with the recycling of NADH, NAD^+ is regenerated by an anaerobic reaction. In mammalian tissues, lactate is produced from pyruvate by lactate dehydrogenase, as shown in Fig. 3 and Eq. (5). In yeast, the decarboxylation of pyruvate by the pyruvate decarboxylase (Eq. 6) is followed by the reduction of acetaldehyde to produce ethanol (Eq. 7).

$$CH_3COCOO^- + NADH + H^+ \rightarrow CH_3CHOH + NAD^+ \quad (5)$$

$$CH_3COCOO^- \rightarrow CH_3CHO + CO_2 \quad (6)$$

$$CH_3CHO + NADH + H^+ \rightarrow CH_3CH_2OH \quad (7)$$

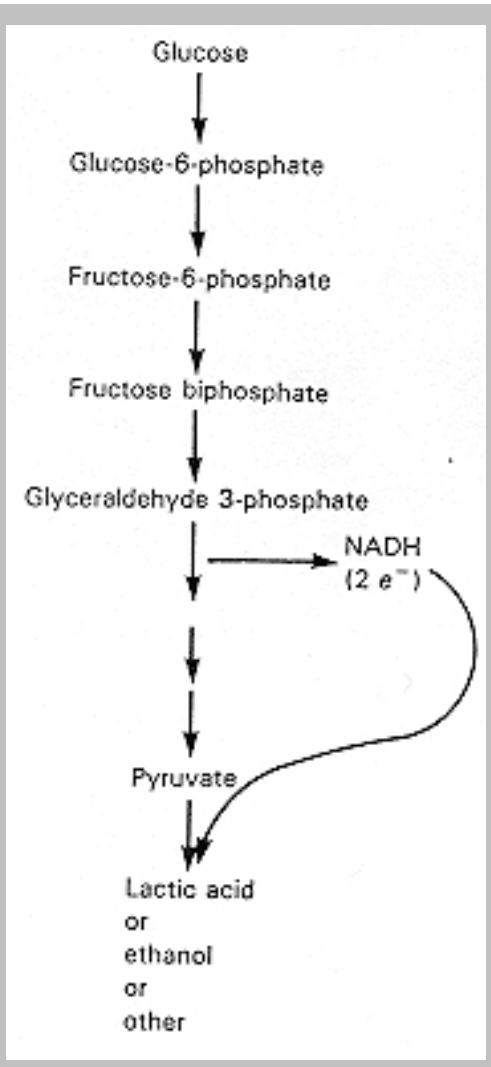


Fig. 3 Involvement of NADH in anaerobic glycolysis.

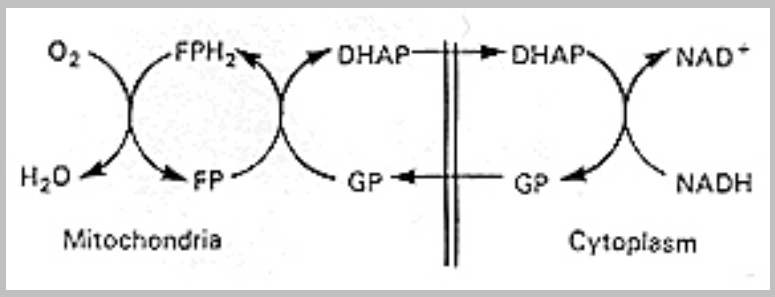


Fig. 4 Representation of shuttle carrying reducing equivalents from cytoplasmic NADH to intramitochondrial NAD^+.

Mitochondrial NADH can be oxidized readily by the electron transport system (Chapter 16). However, glycolysis occurs in the cytoplasm. How does the NADH reach the NADH-coenzyme Q reductase (complex I) of mitochondria? This multienzyme complex reacts with the NADH only on the matrix side of the mitochondrial inner membrane. Apparently, the oxidation of the cytoplasmic NADH is mediated by shuttles. Two possible shuttles are likely to take place. Dihydroxyacetone phosphate (DHAP), one of the intermediates of glycolysis, can be reduced to form

glycerophosphate (GP), a reaction catalyzed by glycerol phosphate dehydrogenase (Eq. 8).

$$CH_2COCH_2OPO_3H_2 + NADH + H^+ \rightarrow CH_2OHCHOHCH_2OPO_3H_2 + NAD^+ \quad (8)$$

The mitochondria from muscle can oxidize GP to regenerate DHAP, which can then be transferred to the cytoplasm (Fig. 4). A similar shuttle may occur in liver, where β-hydroxybutyrate can be formed from acetoacetate in the cytoplasm, which can then be oxidized in the mitochondria, regenerating the β-hydroxybutyrate.

Shuttles such as these may play an important role in the regulation of metabolism. For example, inhibition of the GP shuttle would favor the formation of lactic acid. Lactic acid is favored in some cells, for example, tumor cells. This effect has been referred to as the *Crabtree effect*. The actual mechanism for the Crabtree effect is not known, and a number of other possibilities have been proposed. In other systems, under aerobic conditions, no lactic acid is accumulated, a phenomenon referred to as the *Pasteur effect*. Again, the mechanism is not well understood, and it has been suggested that a competition between the glycolytic and the electron transport systems for NADH, ADP, and P_i may be responsible. P_i may be involved in the expression of both the Crabtree and the Pasteur effect in some cells (Krebs, 1972). In some tissues, the oxidation of NADH by peroxisomes may have an effect on the regulation of metabolism.

B. Energy Balance

In Chapter 13 we saw the importance of the adenosine phosphates acting as allosteric regulators in directing metabolites in the purine nucleotide biosynthetic pathway. In this role, ATP is a positive effector of reactions favoring the synthesis of macromolecules. The role of ATP, ADP, and AMP in metabolic regulation was discussed above from the limited perspective of the individual pathway, for example, glycolysis and the TCA cycle. These compounds, by indicating the energy balance of the cell, may serve as ideal general regulators of metabolism. Therefore, a more global perspective is very useful. The role of these compounds in regulating metabolism has long been recognized and referred to as the *energy charge* (Atkinson, 1977). A high energy charge (high ATP concentration) inhibits ATP-generating reactions and facilitates energy-yielding reactions. Generally, ATP acts in the opposite direction of AMP or ADP, although different reactions may be the target of the individual adenine nucleotide.

The examination of the regulation of the individual steps clearly indicates that ATP, ADP and AMP play a role. For example, in the PK reaction, ATP present in high concentrations favors the formation of the inactive form of the enzyme, whereas ADP has the opposite effect. This, in effect, can regulate the amount of pyruvate that can enter the TCA cycle.

AMP or ADP (depending on the organism) favor the hydrolysis of polysaccharide by the polysaccharide phosphorylase reaction, thereby permitting the mobilization of glucose. They speed up the machinery of glycolysis through the activation of PFK and decrease the formation of

polysaccharide stores by inhibiting FBPase. Through the activation of isocitrate dehydrogenase, AMP or ADP stimulate the turnover of the TCA cycle. In the same way, P_i stimulates succinate dehydrogenase and fumarase. ATP, in contrast, has the opposite effect by activating FBPase, which favors the synthesis of polysaccharide. Likewise, ATP inhibits isocitrate dehydrogenase and tends to slow down the TCA cycle.

We have already seen that the ATP concentration does not vary significantly in muscle, because it can be regenerated from creatine phosphate, with AMP being a much more sensitive indicator of the energy level. However, phosphocreatine (PC), which decreases during muscular activity, can also act as a regulative signal. An increased concentration of PC activates FDPase (Fu and Kemp, 1973) and inhibits PFK (Uyeda and Racker, 1965) and PK (Moyed, 1961). Molecules that are interconvertible with ATP may also have an effect; for example, GTP inhibits α-ketoglutarate dehydrogenase (Olson and Algyer, 1973).

There are other controls superimposed on this basic pattern. G6P is a positive effector for the polysaccharide synthase reaction: favoring the laying down of carbohydrate stores while discouraging glycolysis through its inhibitory effect on hexokinase. Citrate, which inhibits citrate synthase, stimulates fatty acid synthesis from acetyl-CoA, thereby favoring the formation of fat deposits. Succinate dehydrogenase is inhibited by oxaloacetate (Pardee and Potter, 1948) and activated by succinate, ATP, reduced CoQ, and succinylCoA (Pardee and Potter, 1948; Kearney et al., 1972). Some of these reactions, other reactions previously discussed, and their regulation are shown in Fig. 2. The heavy arrows indicate positive effects, whereas lighter lines with crosses indicate negative effects. For simplicity, the figure leaves out the regulation by F2,6P and G1,P.

C. Control of Oxidative Phosphorylation in Mitochondria

In eukaryotic cells, mitochondrial oxidative phosphorylation provides 95% of the total ATP required. Furthermore, any event affecting oxidative phosphorylation will have repercussions in the pathways that precedes it by oxidizing their metabolic products. Therefore, understanding how the mitochondrial system is regulated is of paramount importance to understand the regulation of metabolism.

The mitochondrial electron transport chain is responsible for accepting reducing equivalents from NADH and reduced flavoprotein (FPH_2) and oxidizing them to ultimately produce water and ATP. The chain is a multienzyme system consisting of four complexes embedded in the mitochondrial inner membrane (see Chapter 16). The appropriate dehydrogenases and the ATP synthase complexes are located on the inner face of the mitochondrial inner membrane. The transfer of ATP to the cytoplasm, where it is mostly used, requires the adenine nucleotide translocator, an integral protein of the inner membrane which mediates the exchange between the mitochondrial ATP^{4-} and the cytoplasmic ADP^{3-}.

Near equilibrium and translocase hypotheses

Two major hypotheses have been advanced to explain the regulation of mitochondrial oxidative phosphorylation. One hypothesis proposes that the first two sites of oxidative phosphorylation (in complexes I and III) are near equilibrium. The regulation of the system results from the virtually irreversible reactions in which reduced cytochrome c (2 cyt c^{2+}) is oxidized by O_2 in complex IV. This proposal has been referred to as the *near equilibrium hypothesis* (see Erecinska and Wilson, 1982).

An alternative hypothesis the *translocase hypothesis* (Klingenberg, 1980a) proposes a key role of the adenine nucleotide translocase. The transport mediated by the adenine nucleotide translocase (the ADP/ATP transporter, seeChapter 5) is rate limiting and is responsible for setting the metabolic rate.

The concept of the near equilibrium hypothesis can be illustrated most simply by representing the events of the first two phosphorylative sites as follows:

$$NADH + 2\ cyt\ c^{3+} + 2\ ADP + P_i \rightarrow NAD^+ + 2\ cyt\ c^{2+} + 2\ ATP \quad (9)$$

In this representation, cyt c^{3+} and cyt c^{2+} represent the oxidized and the reduced species of cytochrome c. If we now assume that the reactions are at equilibrium:

$$K_{eq} = [(NAD+)/(NADH)]^{½}\ (ATP)/(ADP)(P_i)\ (cyt\ c^{2+})/(cyt^{3+}) \quad (10)$$

then at equilibrium,

$$(cyt\ c^{2+})/(cyt\ c^{3+}) = (NADH)^{½}/(NAD^+)^{½}\ (ADP)\ (P_i)/(ATP)\ (1/K_{eq}) \quad (11)$$

As shown in Eq. (11), any increase in the NADH/NAD^+ or (ADP) (P_i)/ATP ratios will be reflected in an increase in cyt c^{2+}. Therefore, this increase in cyt c^{2+} will lead to an increase in oxidation, because the cyt c-oxidase is not saturated and the reaction is irreversible.

The key and contrasting points of the two models have to do with the equilibrium position. The near equilibrium hypothesis assumes equilibrium for the first two phosphorylating steps, which include adenylate translocase. The translocase hypothesis assumes that the adenylate translocase is not at equilibrium.

Can we support one model over the other at this time? Examination of the available data shown in Table 2 indicates that, at steady state, in many biological systems these reactions appear to be close to equilibrium (Erecinska and Wilson, 1978). The table calculates the redox potential for the

reactions between NADH and cyt c by subtraction. The values were calculated from the experimentally determined concentrations for the system listed in the first column. The corresponding ΔG values are listed in the fourth column. When the ΔG for the synthesis of ATP, also calculated from the experimentally determined concentration (column 5), is subtracted from this value (displayed in column 6 and represented as ΔΔG), the results show little deviations from equilibrium (i.e., ΔG = 0). ΔG (expressed as ΔΔG) for the whole system is close to zero.

Unfortunately, arguments for or against either model depend crucially on the accuracy of the rather complex measurements needed to calculate concentrations. Furthermore, some of the components may be bound and not free in solution. Therefore, at this time it is difficult to reach a firm conclusion (see Erecinska and Wilson, 1982 for a discussion).

Regulation of mitochondrial permeability

Mitochondrial metabolism may also be regulated by mechanisms affecting the accessibility of the mitochondrial internal compartment to substrates. NADH and NADPH reduce the permeability of the outer mitochondrial membrane to ADP (Lee et al., 1994) apparently by closing the outer mitochondrial membrane channel VDAC (voltage dependent anion channel). The permeability to other substrates may also be affected.

Uncoupling proteins

Uncoupling proteins stimulate heat production in mitochondria by uncoupling respiration from ATP synthesis (e.g., see Klingenberg, 1990). Presumably, the proteins act as channels that discharge the proton-electrochemical gradient in mitochondria. The uncoupling protein-1 (UCP1), the first to be discovered, is a mitochondrial protein of brown adipose tissue (BAT) and is essential for nonshivering thermogenesis (see Himms-Hagen, 1990). Shivering is the immediate mechanism for thermogenesis on exposure to cold, whereas nonshivering thermogenesis increases metabolism for long periods by affecting the metabolic machinery. UCP-1 plays an important role in arousal from hibernation, in cold adaptation in rodents and in neonatal animals. A role in humans is still in question.

Two other uncoupling proteins similar to UCP1 have been discovered. UCP-2 has a 59% amino acid correspondence to UCP1. In contrast to UCP1, UCP2 is widely distributed in animal and human tissues. It is present in white fat, skeletal muscle, heart and tissues rich in macrophages. The expression of UCP2 mRNA is strongly influenced by dietary factors, for example, it is sharply increased by fasting (Boss et al., 1997) or a fat diet (Fleury et al., 1997). It is presently thought to have a role in body weight regulation with implications for obesity and hyperinsulinemia. The brain distribution of UCP2-mRNA in mouse brain (Richard et al., 1998) suggests that UCP2 is present in neurons, perhaps having a role in neuroendocrine functions (e.g., it is abundant in the hypothalamus), autonomic responses and arousal.

UCP3 is highly expressed in skeletal muscle of rodents and humans (Boss et al., 1998a). In a mouse myoblast cell line, overexpression of UCP3 by transfection dissipated part of the mitochondrial proton motive force. A similar uncoupling was shown when incorporated into yeast (Gong et al., 1997). In contrast to UCP2, which showed little hormonal regulation, UCP3-RNA levels are regulated hormonally. Muscle UCP3 levels were decreased 3-fold in hypothyroid rats and increased 6-fold in hyperthyroid rats, suggesting that UCP3 may be responsible for the the effect of thyroid hormone on thermogenesis. In the skeletal muscle of rodents, UCP3-mRNA expression did not change in response to cold exposure. However, it decreased by 81% with food restriction and increased 5.6-fold with fasting. Skeletal muscle is an important site of catecholamine and diet-induced thermogenesis in rats and in humans and, therefore, might play an important role in whole body thermogenesis. In rats, both UCP3 and UCP2 mRNAs in muscle are downregulated by excercise, presumably to direct the energy expenditure to the motor activity (Boss et al., 1998b).

A role of UCP-3 in lipid metabolism has been suspected for some time (see Muzzin et al., 1999; Samec et al., 1998). A role in regulation of metabolism is supported by experiments in which transgenic mice (see Chapter 1) overproduced UCP-3 in skeletal muscle (Clapham et al., 2000). The mice ate excessively but maintained a low body weight and a reduced adipose tissue mass.

It has been proposed that the UCPs could function in reducing the concentration of reactive oxygen species which produce oxidative damage (see Chapter 2). In agreement with this thesis, superoxide has been found to increase mitochondrial proton conductance through effects on UCP1, UCP2 and UCP3 (Echtay et al., 2002). This activation required the presence of fatty acids and was inhibited by purine nucleotides.

Table 2 Free-Energy Relationships Between the Oxidation-Reduction Reactions of the Respiratory Chain and ATP Synthesis. From Erecinska and Wilson, 1978.

Material	E_h(V) cyt c	E_h(V) NAD	ΔE_h	$\Delta G_{ox\text{-}red}$ (kcal/2e)	ΔG_{ATP} (kcal/2ATP)	$\Delta\Delta G$ (kcal)

Liver cells (no substrate)	0.272	-0.242	0.514	23.7	23.8	-0.1
	0.269	-0.260	0.529	24.4	24.2	0.2
Liver cells (lactate + ethanol)						
	0.253	-0.263	0.516	23.8	22.6	1.2
Perfused liver (no substrate)						
	0.260	-0.270	0.530	24.4	23.6	0.8
Ascites tumor cells						
	0.271	-0.252	0.523	24.1	24.2	-0.1
Cultured kindey cells						
	0.251	-0.236	0.487	22.5	22.4	0.1
Tetrahymena pyriformis	0.276	-0.244	0.520	24.0	24.1	-0.1
Paracoccus dentrificans	0.253	-0.313	0.566	26.1	26.3	-0.2
Perfused heart (80 cm H_2O)	0.270	-0.343	0.613	28.4	29.8	-1.4
Pigeon heart mitochondria (succinate)						

E = $E_{h\ NAD}$-$E_{h\ cyt\ c}$ see Chapter 12. Reproduced from *Trends in Biochemical Sciences* vol. 3, M. Erecinska and D.F. Wilson, pp.219-223, copyright &copy1978 with permission from Elsevier Science Publishers, Amsterdam.

VI. CONTROL OF CARBOHYDRATE METABOLISM IN PLANTS

In plants, the energy captured from light is trapped and converted into ATP (synthesized from ADP and P_i) and NADPH (reduced from $NADP^+$) (see Chapter 17). Eventually, after a series of reactions that fix CO_2, the energy is stored in the synthesis of starch or sucrose. Starch is formed in the chloroplasts, whereas sucrose is formed in the cytoplasm. The pathways generate dihydroxyacetone phosphate (DHAP) or triose phosphates derived from DHAP. In the production of starch, DHAP is converted first to hexose phosphate. In the production of sucrose, triose phosphate is first transported across the chloroplast envelope to the cytoplasm. In the absence of light, the plant cells can draw on energy reserves by breaking down starch to form triose phosphates, which can be metabolized further. These two alternatives are summarized in Fig. 5

(Buchanan, 1984). As shown, the enzymes that synthesize starch and those that catalyze its breakdown, coexist in the chloroplast. For this reason, the system is tightly regulated. The enzymes involved in synthesis are activated by light (when energy is available from light) and the degradative enzymes are deactivated by light, so that in plants, carbon assimilation predominates during the day and carbohydrate degradation during the night. Light regulates the enzymes indirectly, functioning of the photosynthetic machinery of the chloroplast. Ferredoxin, a component of the electron transport chain of the thylakoid vesicles, is reduced during photosynthesis (see Chapter 17). Light controls the enzymes through the redox changes of ferredoxin as discussed in the next section.

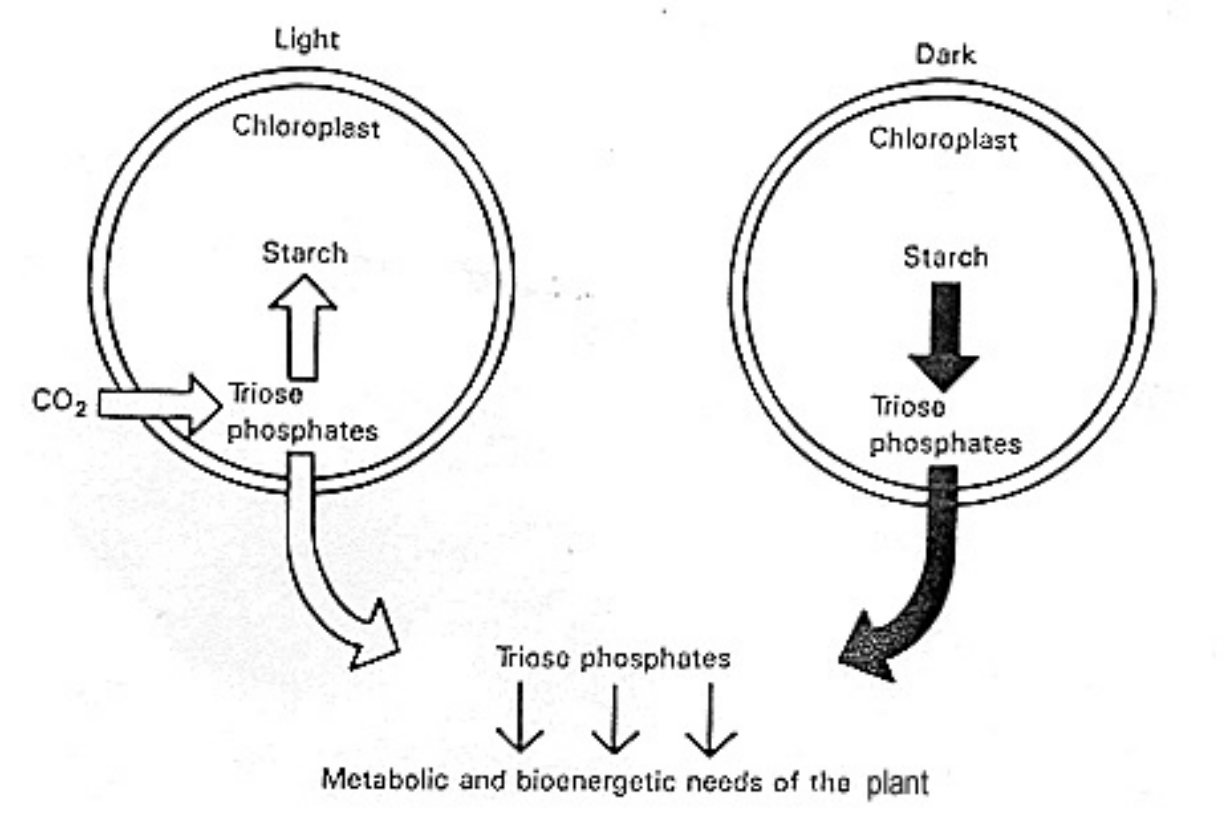


Fig. 5 Role of chloroplasts in producing and storing energy-rich compounds in the plant. From B.B. Buchanan, *BioScience*, 34:378-383 Copyright &copy1984 by the American Institute of Biological Sciences.

VII. REGULATION VIA THE REDOX STATE OF CELLS

The redox state of cells is emerging as an important regulatory factor. The redox state of any cellular compartment depends on the redox state of certain molecular species such as protein thiols, glutathione, pyridine nucleotides (e.g., Gilbert 1990; Chance et al., 1962). This state is regulated by enzymes that sense and respond to the redox state of cells. In animal cells, redox signaling has been shown to have effects on metabolism as well as in the regulation of transcription, translation and apoptosis (see e.g., Xanthoudakis et al., 1994; Hampton et al., 1998; Sen, 1998).

A. Control of metabolism in plants.

In plants, regulation by the redox state of the system is responsible for the activation of the chloroplast's Calvin cycle enzymes and inhibition degradative reactions by light (e.g., see Buchanan, 1994; Jacquot et al., 1997). The enzyme ferredoxin-thioredoxin reductase (FTR), an iron sulfur protein, is key to this system. During photosynthesis, PSI reduces ferredoxins which produces NADPH catalyzed by $NADP^+$-ferredoxin reductase. In addition, the reduced ferredoxin is used by FTR to reduce thioredoxins. Thioredoxins (Trxs) are responsible for the activation of the enzymes of the Calvin cycle and other enzymes (see Jacquot et al., 1997). Trxs correspond to a group of small proteins about 12 kDa in size, probably present in all organisms. The thiol groups of Trxs undergo redox changes so that 2SH are oxidized to S-S.

The FTR of plants is distinct from either mammalian or bacterial FTR. The plant FTR is an iron-sulfur enzyme heterodimer of a catalytic subunit (β) of 13 kDa and a redox active disulfide and a 4FeS-4S center. The α subunit is of similar size but differs from organims to organism. FTRs are disulfide reductases which use as an electron donor, $[Fe2S2]^{2+/+}$ ferredoxin and an FeS cluster as a prosthetic group (e.g., see Staples, 1996, 1998). The FTR molecule is thin and flat, with the iron sulfur cluster in close contact with the iron-sulfur center (Dai et al., 2000).

The functioning of the ferredoxin-thioredoxin system in plants is summarized in Fig. 6. Reduced thioredoxin activates the enzymes of carbohydrate biosynthesis and those of the reductive pentose phosphate cycle, and deactivates glucose-6-phosphate dehydrogenase, which is needed for degradation through the pentose phosphate cycle. The reductive pentose phosphate and the regulation by light are shown in Fig. 7 (Buchanan, 1984). Table 3 summarizes the enzymes which are activated by light or dark (Anderson et al., 1982). Thioredoxin acts by reducing or oxidizing the enzymes that can also undergo thiol-disulfide transitions. The action of two different varieties of Trx is summarized in Fig. 8. As shown, the ferredoxin-thioredoxin system also activates NADP-malate dehydrogenase (NADP-MDH) and ATP synthase (CF_1ATPase). In non-photosynthesizing tissues, Trx can be reduced by NADPH.

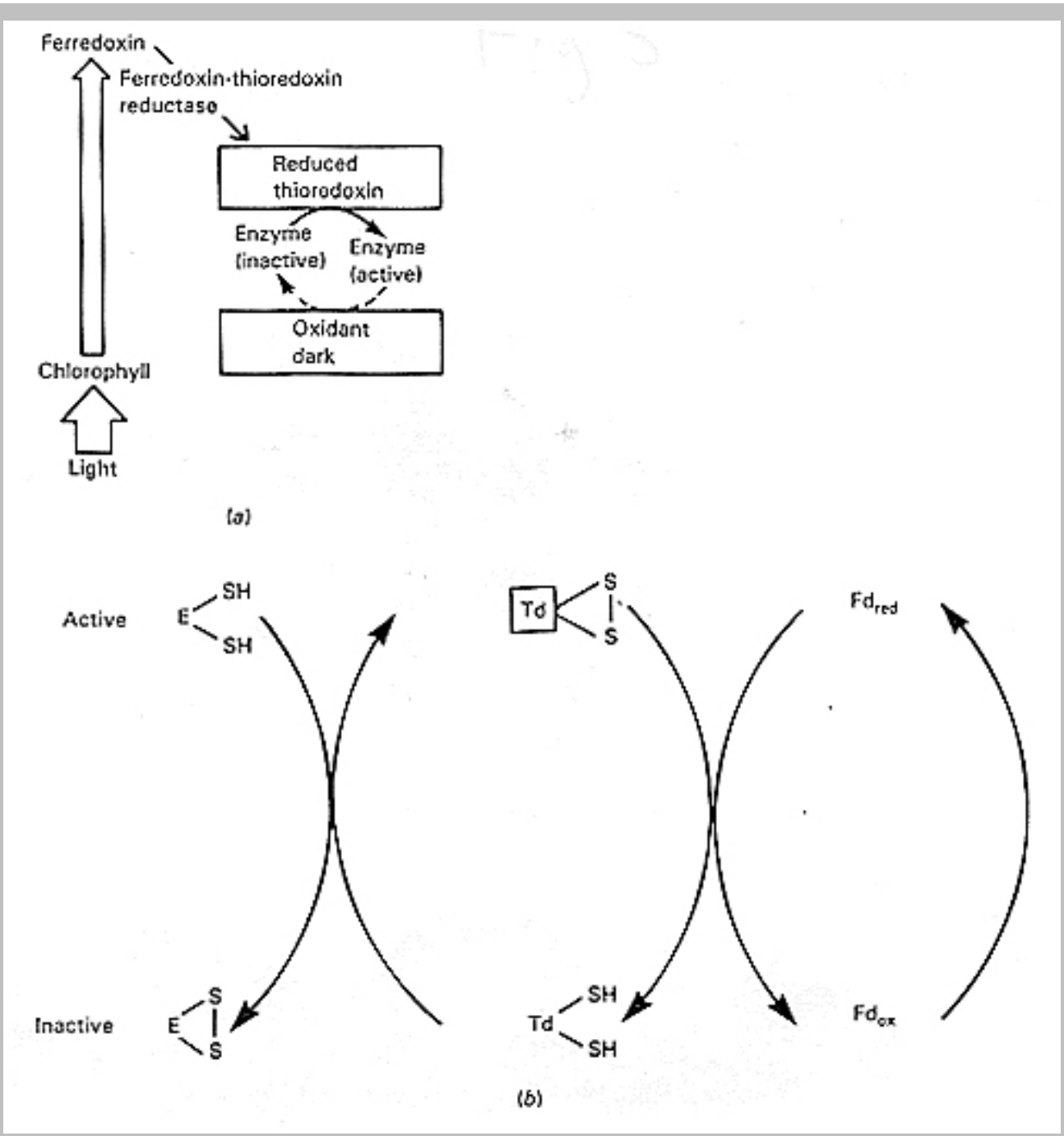


Fig. 6 (a) Ferredoxin-thioredoxin system of enzyme photoregulation (light activation/ dark deactivation). From B. B. Buchanan, *BioScience*, 34:378-383. Copyright &copy1984 by the American Institute of Biological Sciences. (b) Outline of the mechanism of activation and inactivation of NADP-MDH. E, Enzyme; Td, thioredoxin; Fd, ferredoxin.

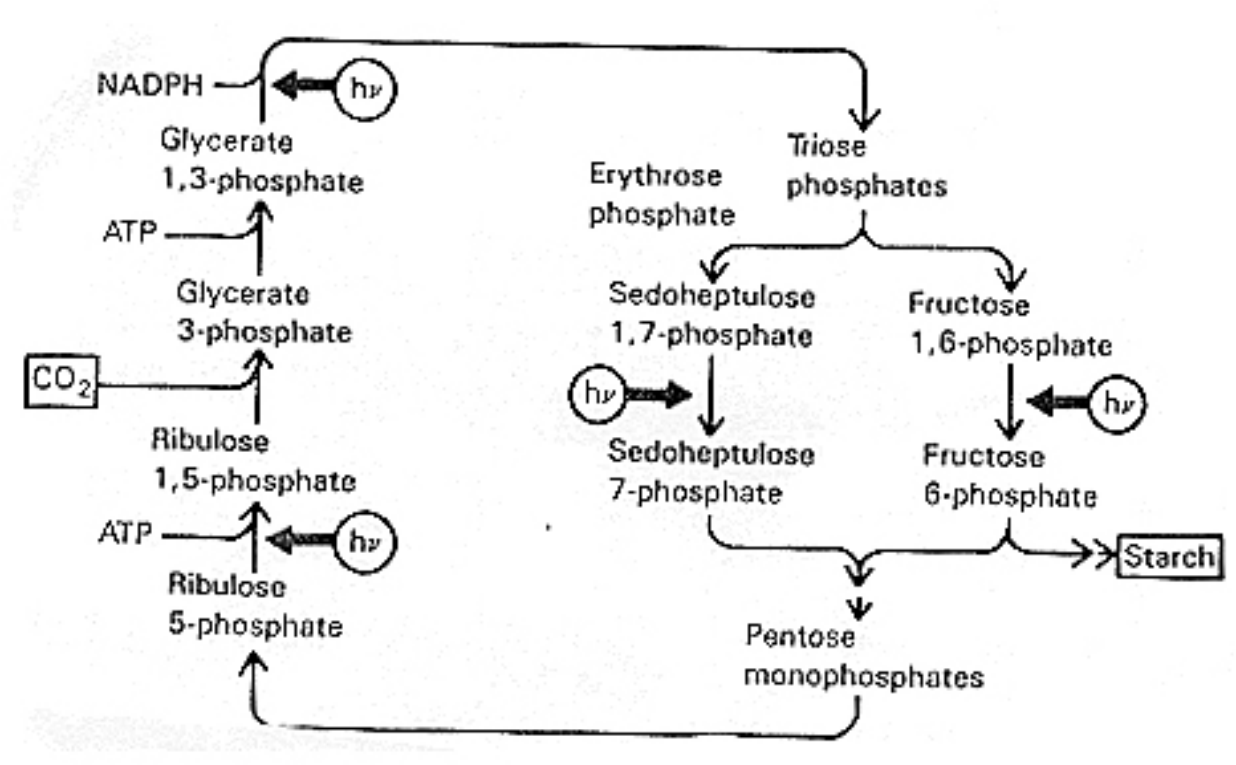


Fig. 7 Steps of the reductive pentose phosphate cycle regulated by light through the Trx system. The light dependent reactions are indicated by h and the heavy arrows. From B. B. Buchanan, *BioScience*, 34:378-383. Copyright &copy1984 by the American Institute of Biological Sciences.

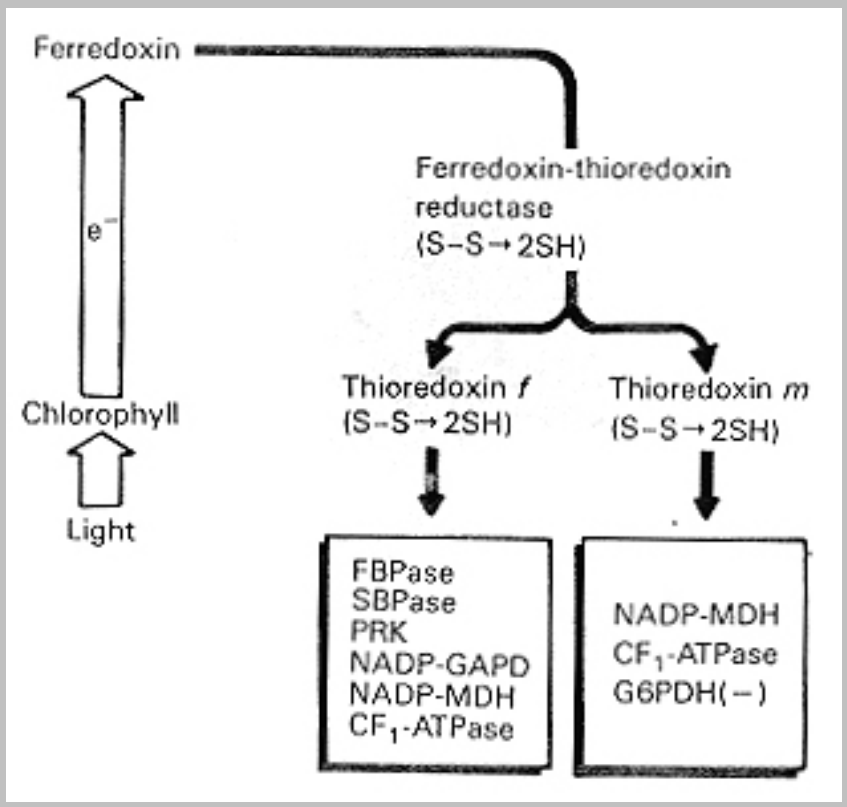


Fig. 8 Enzymes regulated by the ferredoxin-thioredoxin system. Reproduced from *Biochimica et Biophysica Acta*, vol.853, Cseke and Buchanan, pp.43-63. Copyright &copy1986 with permission

from Elsevier Science Publishers, Amsterdam.

NADP-MDH, which catalyzes the reduction of oxalate by NADPH, can serve as an example for the scheme discussed and summarized in Fig. 6. The reaction proceeds as shown in Eq. 12.

$$\text{Oxaloacetate} + \text{NADPH} + H^+ \rightarrow \text{malate} + NADP^+ \quad (12)$$

The enzyme is rapidly inactivated when leaves are kept in the dark and is reactivated by light (Johnson, 1971; Johnson and Hatch, 1970; Scheibe and Anderson, 1981). Added thiols keep the enzyme active in the presence of oxygen and reactivate the enzyme when it has been inactivated. When the activation is produced by light, it is blocked by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Hatch, 1977) that blocks the electron transport chain of the chloroplast. Activation of the inactive enzyme requires the presence of thioredoxin (Kagawa and Hatch, 1977), that also undergoes dithiol-disulfide transitions.

Table 3 Light Modulation in Different Plants Reproduced with permission from Anderson et al., 1982.

	Prokaryote	**Eukaryotes**			
	cyanobacterium	**Algae**	**C_3 plants**	**C_4 plants**	**CAM**
	Anacystis nidulans	**A.Green B.Brown**	***A.Pisum B.Spinacia***	***A.Zea B.Tidestromia***	***Kalanchoe***
Light-activated enzymes			Light stimulatioin x-fold		
NADP-linked malic dehydrogenase			14 x	50 3.3	1.7
NAPD-linked glyceraldehyde-3-P dehydrogenase	Nil	~2 Nil	2.4 5	2 3.1	2
Ribulose-5-P kinase	2		7.7 3.2	1.6 4	4.4
Fructose-1,6-P_2 phosphatase	Nil		1.7 2.2	Nil	Nil

Sedoheptulose-1,7-P_2 phosphatase	Nil	1.8 1.7		1.7
Pyruvate, orthophosphate dikinase			~12	
NAD-linked malic dehydrogenase		1.7		
Dark-activated enzymes		Dark stimulation x-fold		
Glucose-6-P dehydrogenase	4.8	2 3		3
		1.4		
Phosphofructokinase		2		
		11		
Phosphorylase		1.6		
Phosphoglucomutase		3.7		
Phosphoglucoisomerase		3.6		

Apart from the ferredoxin-thioredoxin regulation in plants, there is evidence that the redox state of cells plays an important role in a variety of systems (Ziegler, 1985). Sulfides and sulfhydryl groups are pervasive in enzymes. Similarly, there are a variety of enzymes which catalyze transitions between the two. In addition, many cells are rich in gluthione. Trx is also thought to play a regulatory role in mammalian cells, including the control of growth (Holmgren, 1989).

B. Role in animal cells.

The precise role of redox state in animal cells, frequently through thiol-reactive proteins, such as Trx, is far reaching. However, the physiological implications of this regulation are still far from clear. The role of Trxs in animal cells is undeniable. In mammals, mutant redox-inactive forms of Trx are unable to stimulate cell growth or inhibit apoptosis (Oblong et al., 1994; Freemerman et al. 1999). Increases in gene expression for thioredoxin reductases (TrxRs) and Trx, are thought to play a protective role against oxidative damage (see Mustacich and Powis, 2000). In addition to protecting the cell from oxidative stress by maintaining Trx in its reduced state, TrxR is likely to

be responsible for the recycling of ascorbate. In humans, this role is very important since humans lack the ability to synthesize ascorbic acid.

The mammalian TrxRs are a family of flavoproteins containing selenium that are pyridine nucleotide-disulfide oxidoreductases with mechanistic and sequence identity (see Mustacich and Powis, 2000). They include lipoamide dehydrogenase, glutathione reductase and mercuric ion reductase. The redox activity of this catalytic site is necessary for their biological activity. In mammals, TrxRs are the only enzymes known to reduce oxidized Trx. TrxRs are homodimers where each monomer includes an FAD prosthetic group, an NADPH binding site and an active site containing a redox-active disulphide. Electrons are transferred from NADPH via FAD to the active-site disulphide of TrxR, which then reduces the substrate (see Williams, 1995). Substrates other than Trx are reduced by TrxRs, including lipoic acid, lipid hydroperoxides, vitamin K3, dehydroascorbic acid and the tumor-suppressor p53. However, the physiological role of these reactions is not known.

Trx proteins supply reducing equivalents to enzymes such as ribonucleotide reductase (Laurent et al., 1964) and thioredoxin peroxidase (Chae et al., 1994). They also reduce cysteine residues in certain transcription factors, increasing their binding to DNA, thereby altering gene transcription.

In mammalian cells low oxygen increases the transcription of genes that favor the delivery of oxygen (e.g., angiogenesis, erythropoiesis and vasomotor function) or that permit metabolic adjustments to low oxygen (e.g., regulation of glycolytic enzymes and glucose transport). The responses to low oxygen are through the transcription factor know as the *hypoxia inducible factor* (HIF) (see Semenza, 1999). HIF-1 is also needed for normal development. HIF-1 has two subunits, one of 91-94 kDa and the other of 120-130 kDa.. HIF-1 is stable at low oxygen but is degraded at higher oxygen concentration via the E3 ubiquitin-ligase system (see Chapter 15),which contains the von Hippel-Lindau (pVHL) tumor suppressor protein. pVHL binds to a domain of HIF-1 when the proline of this domain is hydroxylated (Ivan, et al., 2001; Jaakkola et al., 2001). The hydroxylation requires Fe^{2+} and molecular oxygen.

The ubiquitination system, responsible for various physiological functions including protein degradation (see Chapter 15, Section IIB) is regulated by the cellular ratio of oxidized to reduced glutathione (GSSG:GSH ratio) (Jahngen-Hodge et al., 1997; Obin et al., 1998).

In eukaryotes several transcription factors such a AP-1 and NF-κ B respond to oxidative stress (see Dalton et al. 2000). In addition, kinases and phosphatases (see Section V, Chapter 13; Section IV, Chapter 7) are thought to have a significant role in signal regulation by the redox state. The activity of some serine/threonine and tyrosine phosphatases (e.g., calcineurin, PP1 and PP2A) depend on the redox state of their active sites (see Rusnak and Reiter, 2000) such as that of the Fe ion (e.g., Yu et al., 1997; Merkx and Averill, 1998) for the former and cysteine residues for the latter (e.g., Denu and Tanner, 1998). These phosphatases could therefore play a central role in the

regulation of signal transduction and responses to oxidative stress caused by the presence of ROSs (Huie et al., 1999).

Several systems involved in transcription of certain genes have been found to be regulated by nicotinamide adenine nucleotides. The *carboxyl-terminal binding protein* (CtBP) is a corepressor with an important role in development in *Drosophila* and vertebrates. It has been shown to have a role in growth and differentiation in *Drosophila* and vertebrates. The binding of CtBP transcriptional repressors is facilitated by NAD^+ and NADH, the reduced form being two to three orders of magnitude more effective (Zhang et al., 2002). The clock mechanism of the mammalian forebrain has been found to be regulated by the redox state of nicotinamide adenine dinucleotide cofactors, supposedly in response to metabolic conditions. The DNA-binding activity of the Clock:BMAL1 and NPAS2:BMAL1 is increased by the presence of NADH and NADPH and inhibited by the oxidized forms. (see see Chapter 15). Sir2 proteins in mice and yeast have been found to be nicotinamide adenine dinucleotide-dependent histone deacetylases (Imai et al., 2000). The acetylation state of the histones have been shown to have a role in gene expression (see Chapter 2). This finding suggests that Sir2 proteins might be sensors of the redox-state of the cell. The deacetylase activity of Sir2 proteins has a role in the mechanism by which Sir2 silences transcription of several genes, suppresses recombination of rDNA and promotes longevity in yeast.

SUGGESTED READING

Beitner, R. (1984) Control of levels of glucose 1,6-bisphosphate, *Int. J. Biochem.* 16:579-585.(Medline)

Boss, O., Muzzin, P., Giacobino, J.P. (1998) The uncoupling proteins, *Eur. J. Endocrinol.* 139:1-9.(Medline)

Cséke, C. and Buchanan, B. B. (1986) Regulation of the formation and utilization of photosynrthate in leaves, *Biochim. Biophys. Acta* 853:43-63.

Erecinska, M., and Wilson, D. F. (1982) Regulation of cellular energy metabolism, *J. Membr. Biol.* 70: 1-14.(Medline)

Goodwin, T. W. and Mercer, E. I. (1983) *Introduction to Plant Biochemistry*, 2nd ed. Pergamon. New York. See pp. 139-159.

Jacquot, J.-P., Lancelin, J.-M., Meyer, Y. (1997) Thioredoxins: structure and function in plant cells, *New Phytol.* 136:543-570.

Klingenberg, M. (1979) ATP shuttle of the mitochondrion, *Trends in Biochem. Sci.* 4:249-252.

Mustacich, D. and Powis, G. (2000) Thioredoxin reductase, *Biochem. J.* 346:1-8. (Medline)

Newsholme, E. A., Challis, R. A., and Crabtree, B. (1984) Substrate cycles: their role in improving sensitivity in metabolic control, *Trends in Biochem. Sci.* 9:227-280.

Ricquier, D. (1998) Neonatal brown adipose tissue, UCP1 and the novel uncoupling proteins, *Biochem. Soc. Trans.* 26:120-123.(Medline)

Saier, M. H., Jr. (1987) *Enzymes in Metabolic Pathways.*, Chapters 5 and 6. Harper and Row, New York.

WEB RESOURCES

Diwan, J.J., Molecular Biochemistry

REFERENCES

Search the textbook

Back to Chapter 14

REFERENCES

Akamatsu, Y., Ohno, T., Hirota, K., Kagoshima, H., Yodoi, J. and Shigesada, K. (1997) Redox regulation of the DNA binding activity in transcription factor PEBP2. The roles of two conserved cysteine residues, *J. Biol. Chem.* 272:14497-14500.(Medline)

Anderson, L. E., Ashton, A. R., Mohamed, A. H., and Sheibe, R.(1982) Light/dark modulation of enzyme activity in photosynthesis, *BioScience* 32:103-107.

Arnold, H. and Pette, D. (1968) Binding of glycolytic enzymes to structure proteins of the muscle, *Eur. J. Biochem.* 6:163-171.(Medline)

Arnold, H. and Pette, D. (1970) Binding of aldolase and triosephosphate dehydrogenase to F-actin and modification of catalytic properties of aldolase, *Eur. J. Biochem.* 15:360-366.(Medline)

Arnold, H., Henning, R. and Pette, D. (1971) Quantitative comparisons of the binding of glycolytic enzymes to F-actinand the interaction of aldolase with G-actin, *Eur. J. Biochem.* 22:121-126.(Medline)

Atkinson, D. E. (1977) Cellular energy metabolism and its regulation. Academic Press, New York.

Beitner, R. (1984) Control of levels of glucose 1,6- bisphosphate, *Int.J. Biochem.*16:579-585.(Medline)

Beekmans, S., and Kanarek, L. (1981) Demonstration of physical interactions between consecutive enzymes of the citricacid cyclic and of the aspartate-malate shuttle, *Eur.J. Biochem.* 117:527-535.(Medline)

Bosca, L., and Corredor, C. (1984) Is phosphofructokinase the rate-limiting step of glycolysis?, *Trends in Biochem.Sci.* 9:372-373.

Boss, O., Samec, S., Dulloo, A., Seydoux, J., Muzzin, P. and Giacobino, J.P. (1997) Tissue-dependent upregulation of rat uncoupling protein-2 expression in response to fasting or cold, *FEBS Lett.* 412:111-114.(Medline)

Boss, O., Samec, S., Kuhne, F., Bijlenga, P., Assimacopoulos-Jeannet, F., Seydoux, J., Giacobino, J.P. and Muzzin, P. (1998a) Expression in rodent skeletal muscle is modulated by food intake but not by changes in environmental temperature, *J. Biol. Chem.* 273:5-8.(Medline)

Boss, O., Samec, S., Desplanches, D., Mayet, M.H., Seydoux, J., Muzzin, P. and Giacobino, J.P. (1998b) Effect of endurance training on mRNA expression of uncoupling proteins 1, 2, and 3 inthe rat, *FASEB J.* 12:335-339.(Medline)

Buchanan, B. B. (1984) The ferredoxin/thioredoxin system: a key element in the regulatory function of light in photosynthesis, *BioScience* 34:378-383.

Buchanan, B.B., Schurmann, P. and Jacquot, J.P. (1994) Thioredoxin and metabolic regulation, *Semin. Cell Biol.* 5:285-293.(Medline)

Chae, H.Z., Chung, S.J. and Rhee, S.G. (1994) Thioredoxin-dependent peroxide reductase from yeast, *J. Biol. Chem.* 269:27670-27678.(Medline)

Challiss, R. A. J., Arch, J. R. S., and Newsholme, E. A. (1984) The rate of substrate cycling between fructose 6-phosphate and fructose-1,6-bisphosphate in skeletalmuscle, *Biochem. J.* 221:153-161.(Medline)

Chance, B.. Cohen, P., Jobsis, F. and Schoener, B. (1962) Intracellular oxidation-reduction states *in vivo*, *Science* 137:499-508.

Clapham, J.C., Arch J.R.S., Chapman, H., Hayners, A., Lister, C., Moore, G.B.T., Piercy, V., Carter, S.A., Lehner, I., Smith, S.A., Beely, L.J., Godden R.J., Herrity, N., Skehel, M., Kumar Changani, K., Hockings, P.D., Reid, D.G., Squires, S.M., Hatcher, J., Trail, B., Latcham, J., Rastan, S., Harper, A.J., Cadenas, S., Buckingham, J.A. Brand, M.D. and Abuin, A. (2000) Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean, *Nature* 406:415-418.

Clark, D. G., Rognstad, R., and Katz, J. (1973) Isotopic evidence for futile cycles in liver cells, *Biochem. Biophys. Res. Comm.* 54:1141-1148.(Medline)

Clark, M. G., Bloxham, D. P., Holland, P. C., and Lardy, H. A. (1973) Estimation of fructose diphosphatase-phosphokinase substrate cycle in the flight muscle of*Bombus affinis*, *Biochem. J.* 134:589-597.

Clarke, F. M. and Masters, C. J. (1975) On the association of glycolytic enzymes with structural proteins in skeletalmuscle, *Biochim. Biophys. Acta* 381: 37-46.(Medline)

Clarke, F. M. and Morton, C. J. (1976) Aldolase binding to actin-containing filaments, *Biochem. J.* 159: 797-798.(Medline)

Cseke, C. and Buchanan, B. B. (1986) Regulation of the formation and utilization of photosynthate in

leaves, *Biochim. Biophys. Acta* 853:43-63.

Dai, S., Schwendtmayer, C., Schurmann, P., Ramaswamy, S. and Eklund, H. (2000) Redox signaling in chloroplasts: cleavage of disulfides by an iron-sulfur cluster, *Science* 287:655-658.(Medline)

Dalton, T.P., Shertzer, H.G. and Puga, A. (1999) Regulation of gene expression by reactive oxygen, *Annu. Rev. Pharmacol. Toxicol.* 39:67-101. (MedLine)

Davies, S.P., Helps, N.R., Cohen, P.T. and Hardie, D.G. (1995) 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C α and native bovine protein phosphatase-2AC, *FEBS Lett.* 377:421-425.(Medline)

Denu, J.M. and Tanner, K.G. (1998) Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation, *Biochemistry* 37:5633-5642. (MedLine)

Dölken, G., Leisner, E. and Pette, D. (1975) Immunofluorescent localization of glycogenolytic and glycolytic enzymeproteins and of malate isozymes in cross-striated skeletalmuscle and heart of rabbit, *Histochemistry* 43:113-121.(Medline)

D'Souza, S.F. and Srere, P.A. (1983) Binding of citrate synthase to mitochondrial inner membranes, *J. Biol. Chem.* 258:4706-4709.(Medline)

Echtay, K.S., Roussel, D., St-Pierre, J., Jekabsons, M.B., Cadenas, S., Stuart, J.A., Harper, J.A., Roebuck, S.J., Morrison, A., Pickering, S., Clapham, J.C. and Brand, M.D. (2002) Superoxide activates mitochondrial uncoupling proteins, *Nature* 415:96-99. (MedLine)

Erecinska, M. and Wilson, D. F. (1978) Homeostatic regulation of cellular energy metabolism, *Trends Biochem. Sci.*3:219-223.

Erecinska, M. and Wilson, D. F. (1982) Regulation of cellular energy metabolism, *J. Membr. Biol.* 70:1-l4.(Medline)

Exton, J. H., Lewis, S. B., Ho, R. J., Robinson, G. A., and Park, C. R. (1971) The role of cyclic AMP in theinteraction of glucagon and insulin in the control of the liver, *Ann. N.Y. Acad. Sci.* 185:85-100.(Medline)

Fisher, E. H., Packer, A., and Saari, J. C. (1970) The structure, function and control of glycogen phosphorylase, *Essays Biochem.* 6:23-68.

Fischer, E. H., Heileyer, L. M. G. Jr., and Hasche, R. H. (1971) Phosphorylase and the control of glycogen degradation, *Curr. Top. Cell. Reg.* 4:211-251.

Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Levi-Meyrueis, C., Bouillaud, F., Seldin, M.F., Surwit, R.S., Ricquier, D. and Warden, C.H. (1997) Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia, *Nature Genet.* 15:269-272.(Medline)

Freemerman, A.J., Gallegos, A. and Powis, G. (1999) Nuclear factor κB transactivation is increased but is not involved in the proliferative effects of thioredoxin overexpression in MCF-7 breast cancer cells, *Cancer. Res.* 59:4090-4094.(Medline)

Freidmann, B., Goodman, E. H. Jr., Saunders, H. L., Kostos, V., and Weinhouse, S. (1971) An estimation of pyruvate recyclingduring gluconeogenesis in the perfused rat liver, *Arch.Biochem. Biophys.* 143:566-578.(Medline)

Friedman, J.M. and Halaas, J.L. (1998) Leptin and the regulation of body weight in mammals, *Nature* 395:763-770. (MedLine)

Fu, J. Y., and Kemp, R. G. (1973) Activation of muscle fructose-1,6-diphosphatase by creatine phosphate and citrate, *J.Biol. Chem.* 248:1124-1125.(Medline)

Gilbert, H.F. (1990) Molecular and cellular aspects of thiol-disulfide exchange, *Adv. Enzymol. Relat. Areas Mol. Biol.* 63:69-172. (MedLine)

Gillevet, P.M. and Dakshinamurti, K. (1982) Rat-liver fatty-acid-synthesizing complex, *Biosci. Rep.* 2:841-848. (MedLine)

Gong, D.W., He, Y., Karas, M. and Reitman, M. (1997) Uncoupling protein-3 is a mediator of thermogenesis regulated by thyroid hormone, β3-adrenergic agonists, and leptin, *J. Biol. Chem.* 272:24129-24132.(Medline)

Gulbis, J.M., Mann, S. and MacKinnon, R. (1999) Structure of a voltage-dependent K^+ channel β subunit, *Cell* 97:943-952.(Medline)

Hackenbrock, C. R. (1968) Chemical and physical fixation of isolatedmitochondria in low and high energy states, *Proc. Natl. Acad. Sci. USA*61:598-605.(Medline)

Halper, L. A. and Srere, P. A. (1977) Interaction between citrate synthase and malate dehydrogenase in the Presence ofpolyethylene glycol, *Arch. Biochem. Biophys.* 184:529-534.(Medline)

Hampton, M.B., Fadeel, B. and Orrenius, S. (1998) Redox regulation of the caspases during apoptosis, *Ann. N. Y. Acad. Sci.* 854:328-335.(Medline)

Hansford, R. G. (1980) Control of mitochondrial substrate oxidation, *Curr. Topics Bioenerg.* 10:217-278.

Hardie, D.G. and Carling, D. (1997) The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur. J. Biochem.* 246:259-273.(Medline)

Hatch, M. D. (1977) Light/dark mediated activation and inactivation of NADP-malate dehydrogenase in isolated chloroplasts from *Zea mays*. In *Photosynthetic Organelles* (Miyachi, S., Katoh, S., Fujita, Y., and Shibata, J., eds.),pp. 311-314, *Plant Cell Physiology*, special issue.

Hayashi, T., Hirshman, M.F., Kurth, E.J., Winder, W.W. and Goodyear, L.J. (1998) Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport, *Diabetes* 47:1369-1373.(Medline)

Hearon, J. Z. (1949a) The steady state kinetics of some biologicalsystems: I, *Bull. Math. Biophys.* 11: 29-50.

Hearon, J. Z. (1949b) The steady state kinetics of some biologicalsystems: II, *Bull. Math. Biophys.* 11: 83-95.

Helmreich, E. and Cori, C. F. (1965) Regulation of glycolysis in muscle, *Adv. Enzyme Regul.* 3:91-107.

Hers, H.-G., Hue, L., and Van Scaftingen, E. (1982) Fructose 2,6 bisphosphate, *Trends in Biochem. Sci.*7:329-333 (1982).

Himms-Hagen, J. (1990) Brown adipose tissue thermogenesis: interdisciplinary studies, *FASEB J.* 4:2890-2898.(Medline)

Holmgren, A. (1989) Thioredoxin and glutaredoxin systems, *J. Biol. Chem.* 264:13963-13965. (MedLine)

Huie et al. (1999) Chemistry of reactive oxygen species, in *Reactive oxygen species in biological systems: an interdisciplinary approach* (Gilbert, D.L. and Colton, C.A., ed.) pp 33-73, Kluwer, Academic/Plenum Publishers, New York.

Imai, S., Armstrong, C.M., Kaeberlein, M. and Guarente, L. (2000) Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase, *Nature* 403:795-800. (MedLine)

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S. and Kaelin, W.G. Jr. (2001) HIF α targeted for VHL-mediated destruction by proline hydroxylation:

implications for O_2 sensing, *Science* 292:464-468. (MedLine)

Jacquot, J.-P., Lancelin, J.-M., Meyer, Y. (1997) Thioredoxins: structure and function in plant cells *New Phytol.* 136:543-570.

Jahngen-Hodge, J., Obin, M.S, Gong, X, Shang, F., Nowell, T.R. Jr, Gong, J., Abasi, H., Blumberg, J. and Taylor, A. (1997) Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress, *J. Biol, Chem.* 272:28218-28226.(Medline)

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim Av, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (2001) Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation, *Science* 292:468-472. (MedLine)

Johnson, H. S. (1971) NADP-malate dehydrogenase: photoactivation in leaves of plants with Calvin cycle photosynthesis, *Biochem. Biophys. Res. Commun.* 43:703-709.(Medline)

Johnson, H. S. and Hatch, M. D. (1970) Properties and regulation of leaf nicotinamide-adenine dinucleotide phosphate-malate dehydrogenase and 'malic' enzyme in plants with C_4-dicarboxylic acid pathway in photosynthesis, *Biochem. J.* 119:273-280.(Medline)

Jones, M. E. (1980) Pyridine nucleotide biosynthesis in animals: genes, enzymes and regulation of UMP biosynthesis, *Ann. Rev. Biochem.* 49:253-279.(Medline)

Kagawa, T., and Hatch, M. D. (1977) Regulation of C_4 photosynthesis: characterization of a protein factor mediatingthe activation and inactivation of NADP-malate dehydrogenase,*Arch. Biochem. Biophys.* 84:290-297.(Medline)

Karadsheh, N. S. and Uyeda, K. (1977) Changes in the allosteric properties of phosphofructokinase bound to erythrocyte membranes, *J. Biol. Chem.* 252:7418-7420.(Medline)

Kearney, E. B., Mayer, M., and Singer, T. P. (1972) Regulatory properties of succinate dehydrogenase: activation by succinyl CoA, pH and anions, *Biochem. Biophys. Res. Commun.* 46:531-537.(Medline)

Kempner, E. S. and Miller, J. H. (1968) The molecular biology of *Euglena gracilis*. IV. Cellular stratification by centrifuging, *Exp. Cell Res.* 51:141-149.(Medline)

Kispal, G, and Srere, P. A. (1991) Studies of yeast peroxisomal citrate synthase, *Arch. Biochem. Biophys.* 286:132-137.(Medline)

Klingenberg, M. (1980a) The ADP-ATP translocation in mitochondria, a membrane potential controlled transport, *J. Membr. Biol.* 56:97-105.(Medline)

Klingenberg, M. (1990b) Mechanism and evolution of the uncoupling protein of brown adipose tissue, *Trends Biochem. Sci.* 15 :108-112.(Medline)

Laurent, T. C., Moore, E. C. and Reichard, P. (1964) *J. Biol. Chem.* 239, 3436 3444.

Lee, A.-C., Zizi, M. and Colombini, M. (1994) NADH decreases the permeability of the mitochondrial outer membrane by ADPby a factor of 6, *J. Biol. Chem.* 269:30974-30980.(Medline)

Liu, H., Lightfoot, R. and Stevens, J.L. (1996) Activation of heat shock factor by alkylating agents is triggered by glutathione depletion and oxidation of protein thiols, *J. Biol. Chem.* 271:4805-4812.(Medline)

Krebs, H. A. (1972) The Pasteur effect and the relation between respiration and fermentation, *Essays Biochem.* 8:1-34.(Medline)

Kudo, N., Barr, A.J., Barr, R.L., Desai, S. and Lopaschuk, G.D. (1995) High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP-activated protein kinaseinhibition of acetyl-CoA carboxylase, *J. Biol. Chem.* 270:17513-17520.(Medline)

Mansour, T. E. and Ahlfors, C. E. (1968) Studies in heart phosphofructokinase. Some kinetic and physical properties of the crystalline enzyme, *J. Biol. Chem*. 243:2523-2533.(Medline)

McDonald, R. C., Steitz, T. A., and Engleman, D. M. (1979) Yeast hexokinase in solution exhibits a large conformational change upon binding glucose or glucose 6-phosphate, *Biochemistry* 18:338-342.(Medline)

McGarry, J.D., Woeltje, K.F., Kuwajima, M. and Foster, D.W. (1989) Regulation of ketogenesis and the renaissance of carnitine palmitoyltransferase, *Diabetes Metab. Rev.* 5:271-584.(Medline)

Merkx, M. and Averill, B.A.. (1998) The activity of oxidized bovine spleen purple acid phosphatase is due to an Fe(III)Zn(II) 'impurity', *Biochemistry*37:11223-11231. (MedLine)

Meyer, F., Heilmeyer, L. M. G., Jr., Haschke, R. H. and Fischer, E.H. (1970) Control of phosphorylase activity in a muscle glycogen particle. I. Isolation and characterization, *J. Biol. Chem.* 245:6642-6648.(Medline)

Minokoshi, Y., Kim, Y.B., Peroni, O.D., Fryer, L.G., Muller, C., Carling, D. and Kahn, B.B. (2002) Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase, *Nature* 415:339-343.

(MedLine)

Moyed, H. S. (1961) Interference with feedback control of enzyme activity, *Cold Spring Harbor Symp. Quant. Biol.* 26:323-329.

Mustacich, D. and Powis, G. (2000) Thioredoxin reductase, *Biochem. J.* 346:1-8.(Medline)

Muzzin, P., Boss, O. and Giacobino, J.P. (1999) Uncoupling protein 3: its possible biological role and mode of regulation in rodents and humans, *J. Bioenerg. Biomembr.* 31:467-473. (MedLine)

Newsholme, E. A., Challis, R. A., and Crabtree, B. (1984) Substrate cycles: their role in improving sensitivity in metabolic control, *Trends Biochem. Sci.* 9:227-280.

Newsholme, E. A. and Start, C. (1973) *Regulation in metabolism*, p. 97. Wiley, New York.

Nimmo, H. G., Proud, C. G. and Cohen, P. (1976) The phosphorylation of rabbit skeletal muscle glycogen synthase by glucose synthase kinase 2 and adenosine-3,5-monophosphate-dependent protein kinase, *Eur. J. Biochem.* 68:31-44.(Medline)

Obin, M., Shang, F., Gong, X., Handelman, G., Blumberg, J. and Taylor, A. (1998) Redox regulation of ubiquitin-conjugating enzymes: mechanistic insights using the thiol-specific oxidant diamide, *FASEB J.* 12:561-569.(Medline)

Oblong, J.E., Berggren, M., Gasdaska, P.Y. and Powis, G. (1994) Site-directed mutagenesis of active site cysteines inhuman thioredoxin produces competitive inhibitors of human thioredoxin reductase and elimination of mitogenic properties of thioredoxin, *J. Biol. Chem.* 269:11714-11720.(Medline)

Olson, M. S. and Allgyer, T. T. (1973) The regulation of nicotinamide adenine dinucleotide-linked substrate oxidation in mitochondria, *J. Biol. Chem.* 248:1582-1597.(Medline)

Pardee, A. B. and Potter, V. R. (1948) Inhibition of succinic dehydrogenase by oxaloacetate, *J. Biol. Chem.* 176:1085-1094.

Persson, L.-O. and Srere, P. A. (1991) Purification of mitochondrial citrate transporter in yeast, *Biochem. Biophys. Res. Comm.* 183:70-76.

Pette, D. and Brandau, H. (1962) Intracellular localization of glycolytic enzymes in cross-striated muscle of locusta migratoria, *Biochem. Biophys. Res. Comm.* 9:367-370.

Ponticos, M., Lu, Q.L., Morgan, J.E., Hardie, D.G., Partridge, T.A. and Carling, D. (1998) Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine

kinase in skeletal muscle, *EMBO J.* 17:1688-1699.(Medline)

Porpáczy, Z., Sümegi, B. and Alkonyi, I. (1983) Association between the α-ketoglutarate dehydrogenase complex and succinate thiokinase, *Biochim. Biophys. Acta* 749: 172-179.(Medline)

Randle, P. J. (1978) Pyruvate dehydrogenase complex-meticulous regulator of glucose disposal in animals, *Trends Biochem. Sci.* 31:2217-2219.

Reed, L. J., Damuni, Z., and Merryfield, M. L. (1985) Regulation of mammalian pyruvate and branched-chain α-keto-aciddehydrogenase complexes by phosphorylation and dephosphorylation, *Curr. Topics Cell Reg.* 27:41-49.(Medline)

Richard, D., Rivest, R., Huang, Q., Bouillaud, F., Sanchis, D., Champigny, O. and Ricquier, D. (1998) Distribution of the uncoupling protein 2 mRNA in the mouse brain, *J. Comp. Neurol.* 397:549-60.(Medline)

Robinson, J. B. Jr. and Srere, P. A. (1985) Organization of Krebs tricarboxylic acid cycle enzymes in mitochondria, *J. Biol. Chem.* 260:10800-10805.(Medline)

Robinson, J. B. Jr., Inman, L., Sumegi, B. and Srere, P. A. (1987) Further characterization of the Krebs tricarboxylic acid cycle-metabolon, *J. Biol. Chem.* 262:1786-1790.(Medline)

Rognstad, R. and Katz, J. (1980) Control of glycolysis in the liver by glucagon at the phosphofructokinase-fructose-1,6-diphosphatase site, *Arch. Biochem. Biophys.* 203:642-646.(Medline)

Rusnak, F. and Reiter, T. (2000) Sensing electrons: protein phosphatase redox regulation, *Trends Biochem. Scie.* 25:527-529. (MedLine)

Samec, S., Seydoux, J. and Dulloo, A.G. (1998) Role of UCP homologues in skeletal muscles and brown adipose tissue: mediators of thermogenesis or regulators of lipids as fuel substrate? *FASEB J.* 12:715-724. (MedLine)

Schaaff, I., Heinisch, J. and Zimmermann, F. K. (1989) Overproduction of glycolytic enzymes in yeast, *Yeast* 5:285-290.(Medline)

Scheibe, R. and Anderson, L.E. (1981) Dark modulation of NADP-dependent malate dehydrogenase and glucose-6-phosphatedehydrogenase in the chloroplast, *Biochim. Biophys. Acta.* 636:58-64. (MedLine)

Semenza, G.L. (1999) Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1, *Annu. Rev. Cell Dev. Biol.* 15:551-578. (MedLine)

Sen, C.K. (1998) Redox signaling and the emerging therapeutic potential of thiol antioxidants, *Biochem. Pharmacol.* 55:1747-1758. (MedLine)

Shrimpton, C.N., Glucksman, M.J., Lew, R.A., Tullai, J.W, Margulies, E.H., Roberts J.L. and Smith, A.I. (1997) Thiol activation of endopeptidase EC 3.4.24.15. A novel mechanism for the regulation of catalytic activity, *J. Biol. Chem.* 272:17395-17399.(Medline)

Sigel, P. and Pette, D. (1969) Intracellular localization of the glycogenolytic and glycolytic enzymes in white and red rabbit skeletal muscle. A gel film method for coupledenzyme reactions in histochemistry, *J. Histochem. Cytochem*,17:225-237.(Medline)

Staples, C.R., Ameyibor, E., Fu, W., Gardet-Salvi, L., Stritt-Etter, A.L., Schurmann, P., Knaff, D.B. and Johnson, M.K. (1996) The function and properties of the iron-sulfur center in spinach ferredoxin: thioredoxin reductase: a new biological role for iron-sulfur clusters, *Biochemistry* 35:11425-11434.(Medline)

Staples, C.R., Gaymard, E., Stritt-Etter, A.L., Telser, J., Hoffman, B.M., Schurmann, P., Knaff, D.B. and Johnson, .M.K. (1998) Role of the [Fe4S4] cluster in mediating disulfide reduction in spinach ferredoxin:thioredoxin reductase, *Biochemistry* 37:4612-4620.(Medline)

Stewart, M., Morton, D. J. and Clarke, F. M. (1980) Interaction of aldolase with actin-containing filaments. Structural studies, *Biochem. J.* 186:99-104.(Medline)

Sumegi, B. and Alkonyi, I. (1983) A study of the physical interaction between the pyruvate dehydrogenase complex and citrate synthase, *Biochem. Biophys. Acta* 749:163-171.(Medline)

Sumegi, B. and Srere, P. A. (1984) Complex I binds several mitochondrial NAD-coupled dehydrogenases, *J. Biol. Chem.* 259:15040-15045.(Medline)

Sumegi, B., Propaczy, Z. and Alkonyi, I. (1991) Kinetic advantage of the interaction between the fatty acid - oxidationenzymes and complexes of the respiratory chain, *Biochim.Biophys. Acta* 1081: 122-128.

Surholt, B. and Newsholme, E. A. (1983) The rate of substrate cycling between glucose and glucose 6-phosphate in muscle and fat body of the hawk moth (*Acherontia atropos*) at rest and during flight, *Biochem. J.* 210:49-54.(Medline)

Tsai, I.-H., Murthy, S. N. P. and Steck, T. L. (1982) Effect of red cell membrane binding on the catalytic activity of glyceraldehyde-3-phosphate dehydrogenase, *J. Biol. Chem.* 257:1438-1442.(Medline)

Tyiska, R. L., Williams, J. S., Brent, L. G., Hudson, A. P., Clark, B. J., Robinson, J. B. Jr. and Srere, P. A.

(1986) in *Organization of Cell Metabolism* (Welch, G. R. and Clegg, J. S.,eds) *NATO Series A: Life Sciences* 127:177-189.

Uyeda, K. and Racker, E. (1965) Regulatory mechanisms in carbohydrate metabolism. VII. Hexokinase and phosphofructokinase, *J. Biol. Chem.* 240:4682-4688.(Medline)

Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K., Eppenberger, H.M. (1992) Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissueswith high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis, *Biochem. J.* 281:21-40.(Medline)

Wanson, J.-C. and Drochmans, P. (1968) Rabbit skeletal muscle glycogen, A morphological and biochemical study of glycogen-particles isolated by the precipitation-centrifugation method, *J. Cell Biol.* 38:130-150.(Medline)

Williams, Jr., C. H. (1995) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., ed.), CRC Press, Boca Raton, pp. 121 211.

Xanthoudakis, S., Miao, G.G. and Curran, T. (1994) The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains, *Proc. Natl. Acad. Sci. USA*91:23-27.(Medline)

Yu, J. and Steck, T. L. (1975) Association of band 3, the predominant polypeptide of the human erythrocyte membrane, *J.Biol. Chem.* 250: 9176-9184.

Yu, L., Golbeck, J., Yao, J. and Rusnak, F. (1997) Spectroscopic and enzymatic characterization of the active site dinuclear metal center of calcineurin: implications for a mechanistic role, *Biochemistry* 36:10727-10734. (MedLine)

Ziegler, D. M. (1985) Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation, *Annu. Rev. Biochem.* 54:305-329.(Medline)

Zolokar, M. (1960) Cytochemistry of centrifuged hyphae from, *Neurospora*, *Exp. Cell Res*, 19:114-132.

15. Regulation by Synthesis and Degradation of Macromolecules

As discussed in Chapters 13 and 14, metabolic activity can be regulated by adjusting the rates of the cell's biochemical reactions either by activating previously inactive enzymes or, more subtly, by adjusting the activity of the appropriate enzymes. Alternatively, cells may alter the concentration of enzymes and hence the rate of certain metabolic reactions. In eukaryotes, the concentration of any one enzyme is controlled by the balance between the ability of cells to synthesize the enzyme and their ability to degrade it. In bacteria, enzymes are generally stable. However, because of their much shorter generation time, in exponentially growing bacterial cells, unneeded enzymes are rapidly diluted by cell division.

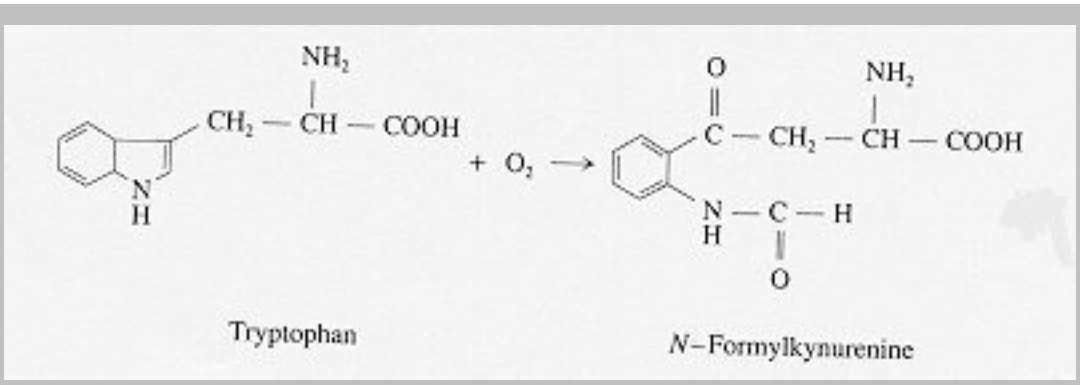
The synthesis of specific enzymes can be regulated at several biochemical levels in the various processes occurring between transcription of the gene and the actual synthesis of the enzyme. The present chapter will concentrate on exploring the regulation of these events in eukaryotes. In bacteria, the adjustment of the enzymatic composition to face an environmental challenge, such as the presence of a new nutrient in the medium, is a major function of the regulatory mechanisms. Regulation is primarily at the transcriptional and translational levels. Lower eukaryotes, such as yeast, may also be subject to the same nutritional challenges. To some extent, the problem is different for multicellular organisms. Since their tissues are specialized, only some (for example, the liver) have to adjust to nutritional changes. The environment and nutrient composition of other tissues are maintained relatively constant. However, protein and enzyme synthesis is regulated by hormones or growth factors. Furthermore, the protein composition of a cell depends on its developmental stage. Each stage reflects differential gene expression that eventually results in differentiation. In the differentiated state, only some of the proteins encoded by the genome are fully expressed, as discussed in Chapter 2.

In multicellular eukaryotes, the control of protein or enzyme levels is very complex. As in prokaryotes, protein and enzyme synthesis is controlled by transcriptional and posttranscriptional mechanisms. In addition, however, specific enzymes and their corresponding mRNAs are differentially degraded, and the transfer of mRNA from the nucleus may be regulated. This is the topic of this chapter. Some of the molecular events of transcriptional and posttrascriptional regulation are discussed in Chapter 3. One of the *heat shock proteins*, Hsp90, has been found to have a role in the folding of certain key proteins so that it regulates the formation and localization of nuclear hormonal receptors, transcription factors, protein kinases involved in signaling, and translational events (see Chapter 7). Hsp is also needed for the maturation and maintenance of the chloride channel CFTR (Loo et al., 1998). The proteins requiring Hsp90 are often labile, have very complex folding patterns, or have to be maintained in a particular conformation to insert a cofactor or other ligand (see Mayer and Bukau, 1999). Hsp90 and co-chaperones remain associated with the multiprotein complexes after maturation and may be involved in facilitating ligand induced conformational changes (see Mayer and Bukau, 1999).

I. PRODUCTION AND DEGRADATION OF SPECIFIC ENZYMES

The balance between production and breakdown of enzymes plays an important role in the regulation of the cell's metabolic machinery in eukaryotes.

The concentration of an enzyme can be increased in response to the presence of its substrate, to the secretion of a hormone, or to general nutritional conditions. In rat liver, the level of the enzyme tryptophan pyrrolase (also called tryptophan 2,3-dioxygenase) is increased sharply by administering hydrocortisone or a large concentration of tryptophan. The enzyme catalyzes the reaction shown in Eq. (1). (1)



This reaction is the first in one of the two major biochemical pathways responsible for the breakdown of tryptophan. The formation of the product of the reaction, N-formylkynurenine with time, can be followed spectrophotometrically.

The adrenal cortex secretes a number of steroid hormones, some of which increase the breakdown of proteins to produce glucose (*gluconeogenesis*) in the intact animal. Steroid induction of enzymes such as tryptophan pyrrolase, tyrosine transaminase, serine dehydratase, serine deaminase, and phosphoenolpyruvate carboxykinase, is likely to be part of this catabolic effect. The hormonal control of tyrosine transaminase and serine dehydratase is discussed in more detail later in this chapter.

In the experiment shown in Table 1 (Schimke et al., 1965), *adrenalectomized* rats (i.e., rats whose adrenal gland was surgically removed) were used to avoid the complication of endogenous release of the steroid hormone by the adrenal cortex. The tryptophan pyrrolase activity in a liver extract increased with the addition of tryptophan and hydrocortisone (column 3 of Table 1). Other enzyme activities also increased (columns 4 and 5), although more moderately. Compared to an untreated control (in which a NaCl solution was injected), both hydrocortisone- and tryptophan-treated animals had large increases in the activity of tryptophan pyrrolase. The two treatments administered together had an even greater effect.

Since a latent form of tryptophan pyrrolase is known, the increase in enzyme activity could result from activation of the enzyme rather than de novo synthesis. An immunological assay can resolve which of the two possible mechanisms is responsible for the effect.

Immunological techniques depend on the interaction between antibodies and their corresponding antigens. Serum antibodies specific for regions (i.e., *epitopes*) of an enzyme molecule can be produced by injecting the pure protein into animals, generally rabbits. The amount of antibody-antigen complex formed after the addition of excess antibody to an extract, quantitatively measures the amount of enzyme present. In the commonly used *radioimmunoassay* technique, radioactive iodine is introduced into the antibody molecule and the presence of antibody-antigen complex is assayed in the precipitate by simply measuring its radioactivity. Similarly, if the proteins of an animal are made radioactive by injection of radiolabeled amino acids, an enzyme can be recognized with an antibody in the same way. Experiments using this latter approach will be discussed later.

Table 1 Effect of Repeated Doses of Tryptophan and Hydrocortisone on Liver Weight and

Tryptophan Pyrrolase, Formylkynurenine Formylase, and Arginase of Rat Liver

		Enzyme activity (μmol product/h per g liver weight)		
(1) Treatment	**(2) Total liver weight (g)[a]**	**(3) Tryptophan pyrrollase**	**(4) Formylase**	**(5) Arginase**
NaCl	25.4	3.0	1800	13,800
Hydrocortisone (HC)	27.4	38	1810	19,740
Tryptophan (Try)	28.7	28	2060	15,200
HC + Try	25.0	116	2180	20,160

[a] Combined weight of 4 animals

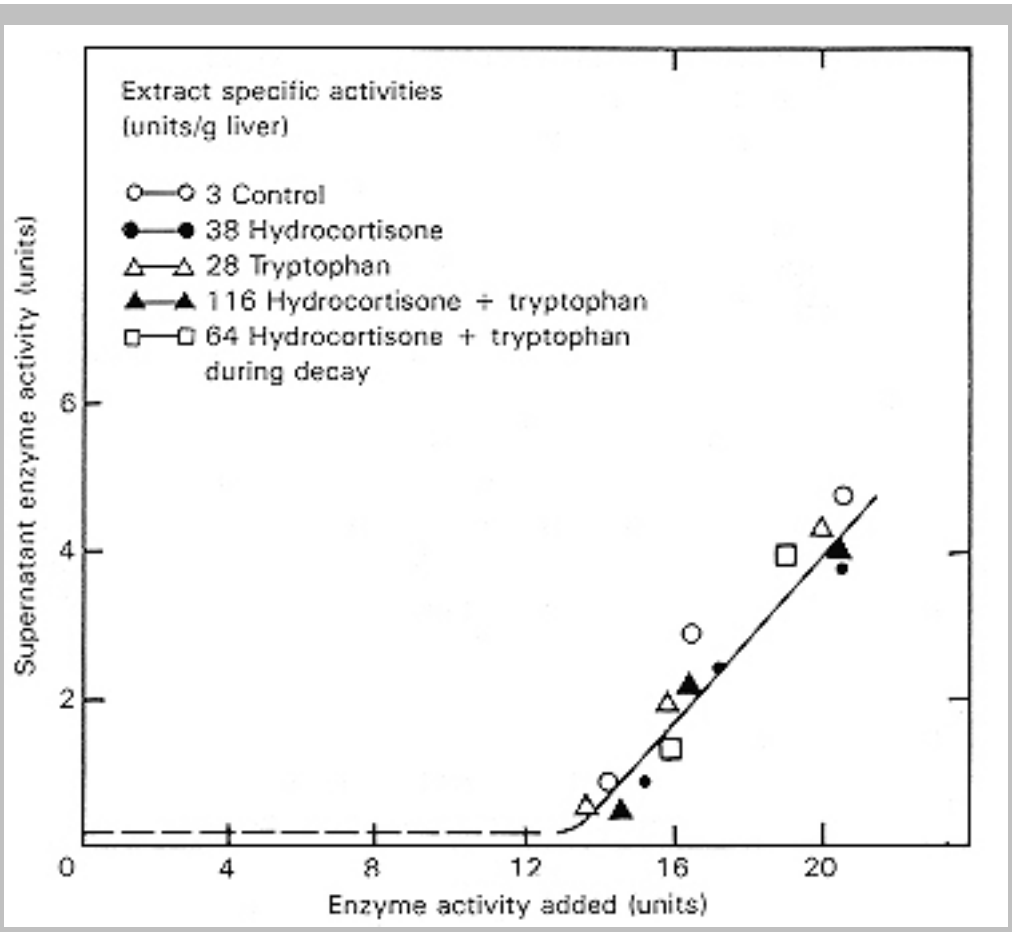


Fig. 1 Immunological analysis of tryptophan pyrrolase activity of rat liver extracts. Adrenalectomized rats weighing 150-170 g were given repeated administrations of 0.85% NaCl (control) or hydrocortisone 2l-phosphate, L-tryptophan, or both. At the end of 16 h, the livers were removed. Two rats that had received both hydrocortisone and tryptophan were killed 3 h later, at a time when the enzyme level was falling. The dashed line indicates that no enzyme activity was detectable in the supernatant fluid. Reproduced with permission from R. T. Schimke, et al., *Journal of Biological Chemistry* 240:322-331, Copyright © 1965 American Society of Biological Chemists, Inc.

In the experiment of Fig. 1 (Schimke et al., 1965), aliquots of liver homogenate were added sequentially to a fixed amount of antibody. The supernatant was sampled for the enzyme activity that remains after precipitation of the antibody-enzyme complex. This manipulation is analogous to an analytical procedure in which aliquots of a reagent are added to a sample until an end point is reached-in this case, the appearance of the enzymatic activity in the supernatant. The enzyme activity of the aliquots, before addition to the antibody, is shown on the abscissa and the enzyme activity remaining in solution after precipitation is shown on the ordinate. As soon as the added enzyme is in excess of the antibody present, the activity appears in the supernatant. Presumably, the antibody will interact with the enzyme whether the latter is active or inactive. Inactive enzyme should remove antibody but will not be detected in the assay of the activity of the homogenate. Any masked enzyme would therefore show up in a graph, such as that of Fig. 1, as an earlier appearance of activity (at a lower homogenate activity) because there will be less antibody to bind the active enzyme. In this experiment, regardless of whether the animal receives only weak salt solution (the control) or is treated with hydrocortisone, tryptophan, or both, the results are the same. The appearance of enzymatic activity in the supernatant occurs at precisely the same level of

added enzyme activity in all experimental preparations, indicating that the active enzyme and the antigen bound by the antibody are precisely equivalent. Therefore, there is no inactive enzyme before induction. This indicates that the increase in activity corresponds to an increase in the enzyme present. Other criteria (see below) are in agreement with the conclusions of this study.

Since the amount of enzyme has increased, either more enzyme is being made or less is being broken down. After [^{14}C]-labeled amino acid (in these experiments [^{14}C]-leucine) is injected into rats, immunological precipitation of the enzyme from homogenates serves as a measure of its synthesis, assuming that [^{14}C] leucine inside the cells remains the same under the different experimental conditions. The results of an experiment depicted in Fig. 2 (Schimke et al., 1965) show that this assumption is correct. Curve 2 in the figure represents the amount of radioactive trichloroacetic acid-soluble material (TCA extract) that corresponds primarily to free amino acid. The radioactivity in the protein corresponds to the material precipitated with TCA (curve 1). Each point corresponds to a different group of rats treated in the same way. In Fig. 2, the squares and the triangles represent the radioactivity present in liver extracts of hydrocortisone- and tryptophan-treated animals, respectively; the circles represent controls. The radioactivity is equivalent in all three cases. Therefore, the concentration of [^{14}C] leucine incorporated represents the same degree of protein synthesis in all three cases.

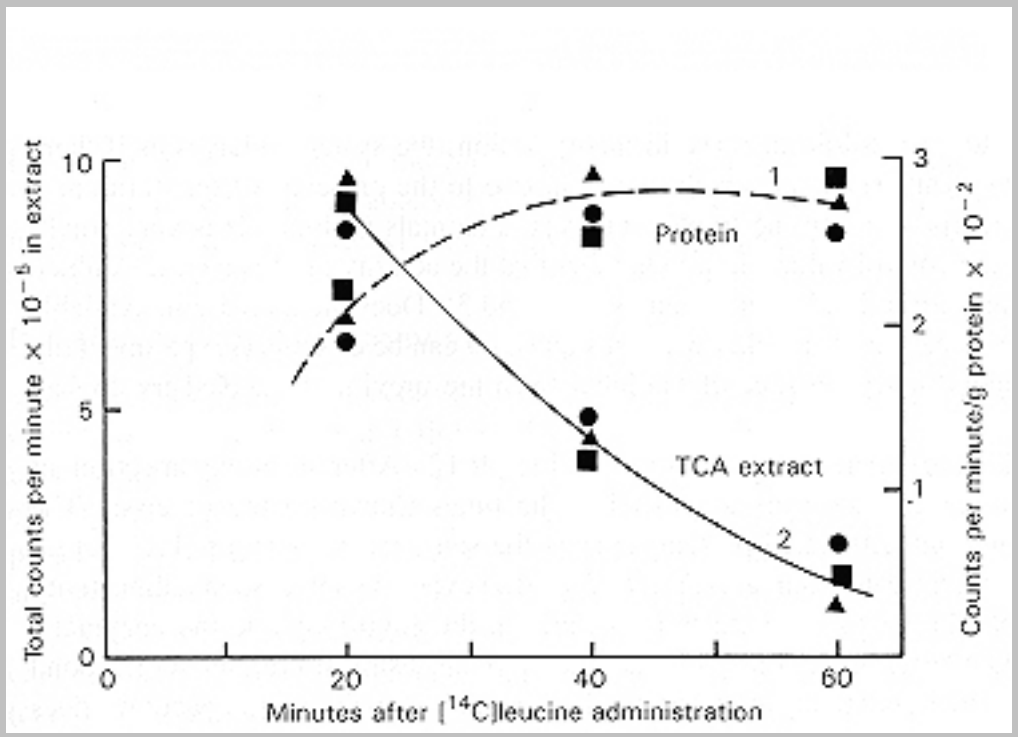


Fig. 2 Effect of hydrocortisone 21-phosphate and tryptophan administration on free pool of administered L-[^{14}C]leucine and its incorporation into liver protein. Reproduced with permission from R. T. Schimke, et al., *Journal of Biological Chemistry*, 240:322-331. Copyright © 1965 American Society of Biological Chemists, Inc.

The incorporation of a label into immunologically precipitated tryptophan pyrrolase is shown in Table 2 (Schimke et al., 1965). The label incorporated in the enzymes of the saline-treated control animals

corresponds to about 1400 counts/min (column 4). The incorporation in the hydrocortisone-treated animals (column 4) is considerably higher, about 9500 counts/minute. Since this corresponds approximately to a sevenfold increase in incorporation, the system behaves as if there were an increase in the synthesis of the enzyme in response to the presence of the hormone.

Table 2 Immunological Precipitation of Tryptophan Pyrrolase from Liver Extracts of Rats that Received [^{14}C]L-lysine[a]

	Total enzyme activity in (units/hour)			
	(1)	(2)	(3)	(4)
Treatment	**Homogenate**	**Supernatant**	**DEAE-cellulose eluate**	**Corrected incorporation (total counts/min)**
NaCl	41.1	26.0	23.1	1406
Hydrocortisone	189	118	107	9466
Tryptophan	80.1	56.7	51.3	1954

[a] Adrenalectomized rats weighing 150 to 160 g each were given single intraperitoneal injections of component in 0.85% NaCl. After 3 h and 20 min each animal was given intraperitoneally injection of 20 µCi of L-[^{14}C]lysine (specific activity 80 mCi/mmol) in 1 ml od 0.85 NaCl. After 40 min the animals were killed, and extracts prepared by immunological precipitation.

In contrast, the incorporation in the tryptophan-treated animals (column 4) is very small, about 40% more than the control value, despite the fact that the activity of the enzyme doubled (compare tryptophan and control values in columns 1, 2, and 3). Does the increase in available enzyme result from a decrease in its breakdown? This question can be examined experimentally by observing the temporal disappearance of the label from the previously labeled tryptophan pyrrolase molecule.

The results of these experiments are shown in Fig. 3 (Schimke et al., 1965). After a single injection of [^{14}C]leucine, some of the animals were sacrificed at the times shown on the abscissa. The radioactivity of the total unfractionated protein remains the same in the controls (Fig. 3a, squares) and the tryptophan-

treated animals (Fig. 3b). However, the decrease in radioactivity of the enzyme with time (Fig. 3a, triangles) is rapid in the controls, while the enzymatic activity remains constant (Fig. 3a, circles). The rate of synthesis must correspond precisely to the rate of breakdown. In contrast, in the tryptophan-treated animals, the radioactivity does not decrease with time (Fig. 3b, triangles), while enzyme activity increases (Fig. 3b, circles). The increase in activity must result from inhibition of the degradation of the pyrrolase.

Rat liver serine dehydratase exhibits a similar response to a dietary intake and the presence of the hydrocortisone (Jost et al., 1968). This enzyme catalyzes the dehydration and deamination of serine to produce pyruvate. The pyruvate is subsequently oxidized or used in the production of glucose. Administration of a mixture of amino acids to rats, results in introduction of the enzyme unaccompanied by changes in the total protein content of the liver. Glucagon, a polypeptide hormone that is secreted by the pancreas and favors gluconeogenesis, has a similar effect. Administration of glucagon or the amino acids produces an increase in the incorporation of [^{14}C]-valine into serine dehydratase molecule, as shown by an immunological precipitation similar to that in the experiment of Table 1. In contrast, glucose inhibits production of the enzyme and increases its rate of breakdown (shown by experiments analogous to those of Fig. 3).

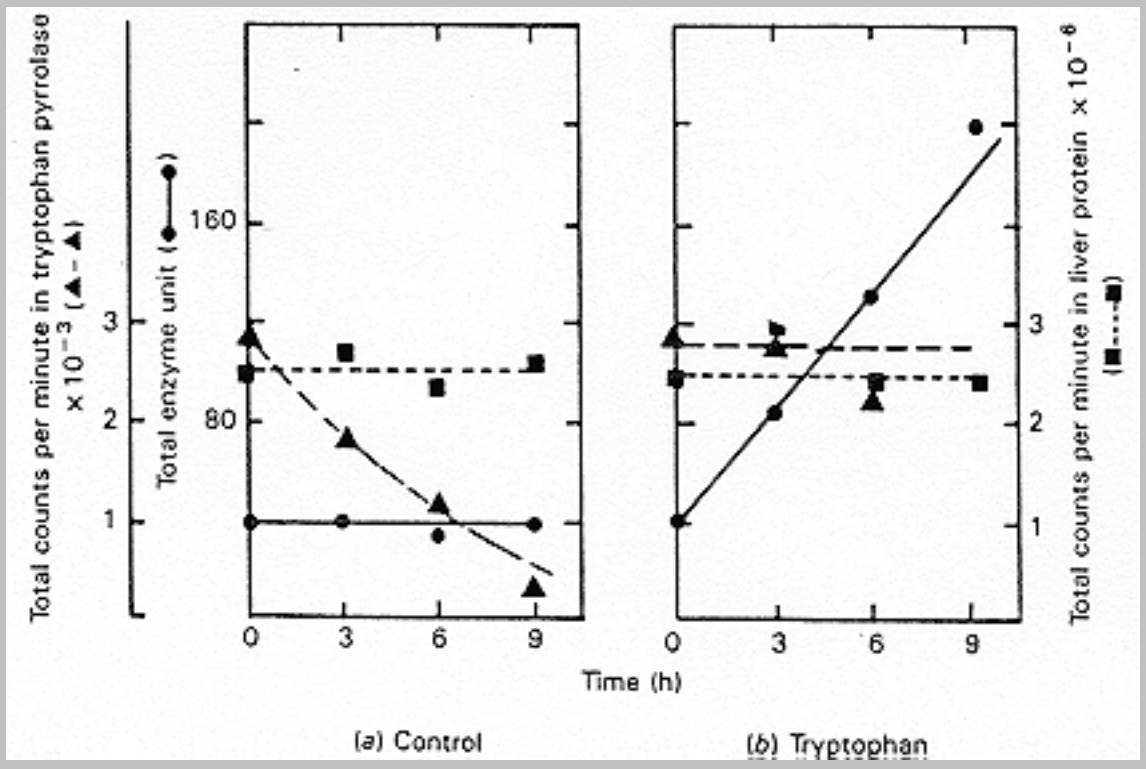


Fig. 3 Effect of L-tryptophan administration on loss of tryptophan pyrrolase prelabeled with L-[^{14}C]leucine. The values represent total enzyme activity present in the combined extracts from two animals ● . Counts per minute represent total radioactivity present in protein precipitated by the tryptophan pyrrolase antiserum ▲ or in total cellular protein ■ from two livers. Reproduced with permission from R. T. Schimke, et al., *Journal of Biological Chemistry*, 240:322-331 Copyright © 1965 American Society of Biological Chemists, Inc.

II. THE DEGRADATIVE PATHWAYS

The previous section showed how synthesis and degradation play an important role in the regulation of the concentration of the enzyme. This section will concentrate on the degradative processes. The degradation of proteins contributes to the turnover of the cellular components. Under basal conditions, i.e., at rest and with a sufficient food supply, protein turnover has been estimated to account for 15% of the energy expenditure of humans. In addition to its role in the regulation of enzyme concentration, proteolysis is also thought to function in removing abnormal proteins produced by mistakes in translation (Goldberg, 1972). Such proteins would fulfill no function, and their breakdown allows their use as a metabolic fuel.

We have seen how degradation plays a role in the regulation of enzyme activity. In yeast, changes in the supply of nutrients, such as addition of glucose or removal of carbon sources (carbon starvation), irreversibly inactivate certain enzymes, and at least glutamine synthetase (Ferguson and Sims, 1974) is degraded. The enzyme activity returns after a return to the original conditions, but only if protein synthesis is not blocked by inhibitors such as cycloheximide.

A. Degradation as a General Regulatory Mechanism

In eukaryotes, the rate of proteolysis is as significant as the rate of synthesis. This conclusion is drawn from the demonstration that most enzymes have a characteristic lifetime.

At steady state, the balance between synthesis and degradation is generally expressed as a half-time ($t_{1/2}$). The $t_{1/2}$, can be estimated from data such as those shown in Fig. 3a representing the decay of the radioactivity in the enzyme molecule (E). This rate, dE/dt, can be expressed as a function of the rate constant for synthesis k_s (the rate of synthesis will not change if short synthetic periods are considered) and a degradation rate constant k_d as shown in Eq. (2).

$$dE/dt = k_s - k_sE \quad (2)$$

If the original radioactivity has been rapidly diluted by adding unlabeled amino acid (i.e., by a chase) $k_s = 0$. Equation (9.2) can be integrated to the form

$$\log_e E/E_0 = -k_dt \quad (3)$$

where E_o represents the total amount of radioactivity present at time zero. When half of the enzyme has decayed, the half-time can be expressed as shown by Eq. (4); k_d can be readily calculated from a logarithmic plot of E/E_0 as a function of time, where it would correspond to the slope.

$$t_{1/2} = \log_e 2 / k_d \quad (4)$$

The regulation of an enzyme through its turnover rate must be reflected in a characteristic $t_{1/2}$ for the enzyme. Table 3 (Ballard, 1977) shows that enzymes indeed have characteristic half-times, in agreement with this idea. Therefore, proteolytic degradation seems to choose specific targets. What mechanism or mechanisms provide such precise specificity? It is possible to clarify these processes by examining the properties of known cellular proteinases (Barrett, 1980; Matern and Holzer, 1979). These enzymes differ significantly from each other, but many have a common characteristic: they must be activated or they need special conditions for their action. However, aside from the fact that they are frequently located at specific sites (e.g., microsomal or mitochondrial membranes, the Z band of the sarcomere), nothing suggests that they can act on certain proteins and not on others. An initial inactivation has frequently been found to precede the actual hydrolysis. There are indications that some enzymes are marked for proteolysis by phosphorylation. In other cases, other forms of modification, such as the inactivation by oxidation, may mark an enzyme for proteolysis (e.g., Fucci et al., 1983; Rivett, 1985). Most of the degradation of proteins follows complex and specialized pathways, which are discussed in some detail in the next section.

Table 3 Characteristic Half-Times of Enzymes

	$t_{1/2}$ in vivo (days)
Lactate dehydrogenase	6.0
Fructose bisphosphate aldolase	4.9
Glucose-6-phosphate dehydrogenase	1
Glucokinase	1
Phosphoenolpyruvate carboxykinase	0.3
Thymidine kinase	0.1
Ornithine decarboxylase	0.008
RNA polymerase I	0.05
Tyrosine aminotransferase	0.06

Tryptophan oxygenase	0.08
Phosphoenolpyruvate carboxykinase	0.25
Acetyl-CoA carboxylase	2
Glyceraldehyde phosphate dehydrogenase	3.4
Arginase	4.5

From Ballard (1977 and 1978), Reproduced with permission from *Essays in Biochemistry* 13:1-37 copyright © 1977 The Biochemical Society, London.

B. Biochemical Pathways of Proteolysis

A good deal is known about the biochemical pathways of protein degradation. Present evidence indicates that there are two distinct processes, one relatively unspecific, the other exquisitely specific.

Multiple proteolytic pathways

Perfusion of rat liver or incubation of cell cultures with a medium devoid of amino acids, induces an increase in the breakdown of proteins. This enhancement of proteolysis can be blocked by supplementing the medium with amino acids (Woodside and Mortimore, 1972) or by introducing insulin (Mortimore et al., 1973) or some other growth factor.

The breakdown of a number of components after endocytosis has been shown to occur in lysosomes (Chapter 9). Therefore, a general role of lysosomes in proteolysis would not be surprising. In fact, proteolysis, which accompanies amino acid deprivation, correlates well with the enlargement of lysosomes and the presence of *autophagic vacuoles* (Mortimore and Schworer, 1977), i.e., vacuoles that enclose cellular structures and function in their digestion. Conversely, these events are prevented by the presence of amino acid supplements.

How can this problem be studied in more detail? As many cell organelles continue to function after isolation, it would seem possible to examine lysosomal activity after isolation of the vesicle. Under a variety of conditions, including amino acid deprivation and amino acid supplementation (Mortimore and Ward, 1981), lysosomes isolated from amino acid-deprived animals release amino acids or acid-soluble material at a rate compatible with the overall protein breakdown (Mortimore et al., 1973). Despite this observation, it is difficult to imagine that lysosomes are responsible for the very specific breakdown of the rapidly turning over enzymes we discussed. In contrast, it would be easy to imagine the involvement of lysosomes in the nonspecific hydrolysis of protein at a lower rate, in response to a generalized signal

(however, see exceptions discussed below).

The fate of these two kinds of proteins could be followed after a short pulse of a radioactive amino acid. Such a pulse should predominantly label the rapidly turning over proteins. A more prolonged pulse would label both kinds of protein. The view that there are two different processes is supported by the finding that inhibitors of lysosomal proteases, which have little or no effect on the proteolysis of short-lived or abnormal proteins (Neff et al., 1979), do block the proteolysis of the longer-lived proteins. Furthermore, a temperature-sensitive mutant has been found for one of the enzymes of the ubiquitin conjugation system (Finley et al., 1984). Reactions involving ubiquitin correspond to a second proteolytic pathway and, as we shall see later in this section, are responsible for the rapid turnover of proteins. In the mutant, at a permissive temperature, 70% of the rapidly turning over proteins are degraded in 4 h (Ciechanover et al., 1984). However, only 15% of this fraction is degraded at the nonpermissive temperature.

The effect of protease inhibitors, which inhibit lysosomal proteolysis, is shown in Table 4 (Neff et al., 1979). The experiment was carried out with cultured hepatocytes. Part A of the table shows the appearance of [^{3}H]-labeled material in the medium of cells that have either been labeled for long periods (column 1), pulse-labeled with [^{14}C]-leucine (column 2), or pulse-labeled with an analog that will be incorporated to form an abnormal protein (column 3). The inhibitors have no effect on the pulse-labeled proteins or abnormal proteins, but inhibit the proteins that have been labeled for a long period. Although the effect is small, it is significant. Table 4B shows a much more marked effect for some of the inhibitors - as much as 54% inhibition. In addition, the effect is not additive, suggesting that the inhibitors are acting on the same mechanism. The amount of inhibition is not complete, possibly because of permeability barriers to the inhibitors. More significantly, the inhibitors failed to inhibit the breakdown of defective protein, even when its concentration was sharply increased by prolonged incubation. The results are therefore consistent with the presence of two independent pathways: one for the slowly turning over proteins broken down by lysosomes and the other for the breakdown of rapidly turning over proteins including abnormal proteins (however, see Gronostajski et al., 1985).

Three pathways of lysosomal proteolysis have been recognized. Microautophagy takes place in well-nourished cells and is non-selective. The lysosomal membrane invaginates at multiple locations (Marzella and Glaumann, 1987) and internalizes proteins which are then digested by lysosomal proteases. Macroautophagy (Seglen et al., 1990) which is induced in cell cultures, for example, by removal of growth factors, is similarly nonspecific. Autophagic vacuoles are formed in the cytoplasm in which newly formed membranes sequester cytoplasmic components. These are then exposed to the lysosomal hydrolytic enzymes. An additional pathway, induced by serum deprivation in confluent cultured cells in which growth is arrested, is selective. In this mechanism, proteins with a specific sequence of five amino acids are imported into the lysosomes (Dice et al., 1990) and subsequently digested.

Table 4A Effects of Protease Inhibitors on Degradation of Different Classes of Cellular Proteins

	Normal		Anaolg containing
	(1)	(2)	(3)
Inhibitor	**Chronically labeled**	**Pulse labeled**	**Pulse labeled**
Protein degraded/ (% of total)			
None	1.65±0.13	21.2±0.7	26.7±2.6
Percent inhibition			
Leupeptin	21	0	0
Chymostatin	27	0	0
Antipain	18	0	0
Pepstatin	0	-----	-----

Table 4B Lack of Additivity of Inhibitors on Protein Degradation

Inhibitor	Protein degraded/h (%of total)	Inhibition (%)
None	3.32±0.11	-----
Leupeptin	2.62±0.17	21
Chymostatin	1.72±0.09	48
Antipain	2.97±0.16	11
Leupeptin + chymostatin	1.54±0.08	54
Leupeptin + chymostatin + antipain	1.72±0.05	48

The ubiquitin pathway

The nonlysosomal pathway has been partially elucidated by use of a special strategy (Gronostajski et al., 1985). All protein breakdown in the cell requires energy. Therefore, proteolysis can be arrested in extracts by the simple expedient of leaving ATP out of the incubation medium. This would allow the fractionation and subsequent reconstitution of the system, which can then be activated at will by the

addition of ATP. Reticulocyte extracts have proved particularly useful because they synthesize hemoglobin almost exclusively and also possess a very active proteolytic system to dispose of abnormal globin.

This approach can best be illustrated by showing the results of the original experiments which defined the ubiquitin proteolytic pathway and were carried out with the reticulocyte extract (Table 5) (Ciechanover et al., 1978). The degradation of [^{3}H]-globin, previously labeled by incubation of the intact cells with [^{3}H]-labeled amino acids, provides a measure of proteolytic activity. As shown in line 1, ATP is required for proteolysis. The two fractions derived from the extract, fractions I and II, have little activity when alone, as shown by lines 2 and 3. However, the two together reconstitute the proteolytic system as long as ATP is present, as shown in line 4. Further extraction of fraction I demonstrates the presence of a heat-stable factor with a molecular weight of 10 kDa (Ciechanover et al., l980a) and containing 76 amino acids. In the complete system, this low molecular weight component is found to bind to protein when the system was incubated in the presence of ATP and fraction II (Ciechanover et al., 1980b). The binding is covalent, since the association is resistant to severe treatment such as heat denaturation, exposure to acid and alkali, or exposure to a chemical reducing agent. The heat-resistant protein corresponds to a form of ubiquitin containing two extra glycine residues. Several molecules of this factor bind a higher molecular weight protein. The ubiquitin attaches through its carboxyl terminal to the amino groups of the lysines in the higher molecular weight proteins.

Table 5 Resolution of the ATP-Dependent Cell-Free Proteolytic System into Complementing Activities

	Degradation of [^{3}H] globin (%/h)	
Enzyme fraction	**-ATP**	**+ATP**
1. Extract	1.5	10.0
2. Fraction I	0	0
3.Fraction II	1.5	2.7
4. Fractions I and II	1.6	10.6

Reproduced by permission from Ciechanover et al., 1978.

When ATP is removed from the extract, covalent binding of diglycine ubiquitin to proteins ceases. However, the extract continues to break down the protein portion of the protein-ubiquitin complex (Hershko et al., 1980). In this fashion, ubiquitin is regenerated and, in the presence of ATP, can be used again (Ciechanover et al., 1984). A version of this cycle is illustrated in Fig. 4 (Hershko, 1988), where E_1, E_2, and E_3 correspond to a family of enzymes required for ubiquitin ligation and protein breakdown. In order to be efficiently degraded, the proteins targeted for destruction must be polyubiquinated. An

additional factor, E_4 (UFD2 in yeast) (Koegl et al., 1999) is required for polyubiquination.

Conjugation of proteins to ubiquitin has been observed in many mammalian tissues (e.g., Ciechanover et al., 1984), so the ubiquitin-proteolytic system seems to be a general mechanism not limited to reticulocytes. Furthermore, mutations in two of the three yeast genes coding for E_2-proteins greatly reduce protein degradation (Seufert and Jentsch, 1990). Deletion of all three is lethal (Seufert et al., 1990). It follows that the ubiquitin-linked proteolysis system plays a vital role.

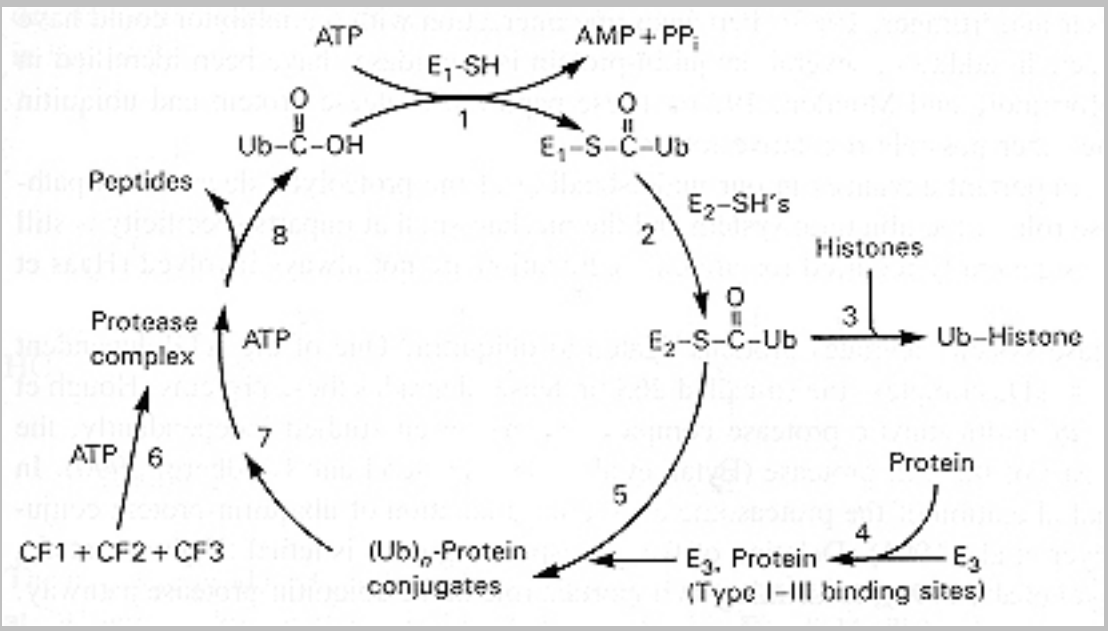


Fig. 4 Proposed sequence of events in ubiquitin-mediated protein breakdown. Ub, Ubiquitin; CF1, CF2, and CF3, conjugate-degrading factors 1-3, respectively; E_1, E_2, and E_3 are enzymes. Reproduced with permission from A. Hershko, *Journal of Biological Chemistry*, 263:15237-15240. Copyright © 1988 American Society of Biological Chemists, Inc.

Despite the important advances in our understanding of the proteolytic degradation pathways, the precise role of the ubiquitin system and the mechanism that imparts specificity is still not entirely clear. The multiplicity of E_2s and E_3s may have a role in providing specificity to the protein degradation system. In *Saccharomyces cerevisiae* there are thirteen different E_2s. In some cases, the E_2s can transfer the ubiquitin directly to the substrate. More generally the transfer is carried out by E_3s, the *ubiquitin-protein ligases*. There are several kinds of E_3s that differ significantly in sequence and mechanism, and may be the ones capable of most substrate recognition. Some E_3s facilitate transfer form E_2s to substrates. Others provide thioester intermediates. At least some of the E_3 are in large multicomponent complexes (see Hochstrasser, 1996). Specificity of the E_3 ligases is provided by the presence of many substrate specific-adapters that recruit the proteins to the core ubiquitination complexes. There are several reports of E_3 ubiquitin ligase complexes containing adapters (e.g., see Peters, 1998): the SCF, APC and VCB families of complexes (Kamura et al., 1999; Skowyra et al., 1999 and Stebbins et al., 1999).

The SCF family complexes contain adapter subunits called F-boxes. They recognize different substrates through specific protein-protein binding domains (Bai et al., 1996). The presence of the F-box containing proteins in protein data bases, suggests that many proteins may be targeted for degradation by the SCF pathway. In fact, cyclins, CDK inhibitors and transcriptional regulators have been found to be substrates of SCF complexes (see Patton et al., 1998; Maniatis, 1999).

APC is a second kind of E_3 ligase complex that uses different adapters for different substrates. Their substrates include cyclins and other proteins involved in the cell cycle (see Peters, 1998).

The VCB complex contains the *von Hippel-Lindau tumor suppressor protein* (VHL) and associated proteins. At this time, a role of this complex in E_3 ligase is suspected but not yet established. The VCB complex recognizes proteins that contain a SOCS-box (see Starr and Hilton, 1999).

A new aspect of the functioning of the ubiquitination system is provided by recent developments involving the Nedd4 protein family. *Nedd4* is a gene expressed in various tissues (e.g., Kumar et al., 1997). At this time, members of the Nedd4 family have been found in yeast, mouse, rat and human. The Nedd4 proteins regulate a variety of functions. A group of proteins of the Nedd4 family (see Harvey and Kumar, 1999) are ubiquitin ligases with a Ca^{2+}-lipid-binding domain (C2 domain) and three repeats of approximately 40 amino acids, the *WW* domains known to be protein-protein binding modules (see Sudol, 1996; Staub and Rotin, 1996). In addition, the carboxy-terminal region of Nedd4 was found to be similar to the oncoprotein *E6-associated protein* (E6-AP) which is an E_3 ubiquitin ligase involved in the ubiquitination of the protein p53 (Scheffner et al., 1993; Scheffner et al., 1995).

The epithelial Na^+ channel is down regulated by Nedd4 probably by ubiquination (Goulet et al., 1998). In humans, malfunction in this regulation leads to hypertension (*Liddle's syndrome*). The equivalent protein in *Saccharomyces cerevisiae* (Rsp5p/Npi1p) has varied functions. It is involved in the ubiquitin mediated regulated turnover of various permeases (see Harvey and Kumar, 1999). Rsp5p/Npi1p ubiquitinates RNA polymerase II (Huibregtse et al., 1997). Human nEDD4 and Rsp5p are needed for the hormonal activation of transcription by progesterone and glucocorticoid receptors independently from the ubiquitin-ligase function (Imhof and McDonnell, 1996).

Specificity must also be determined by some characterstic of the protein to be degraded, such as a destruction signal or domain recognized by the ubiquitin-protease system. In some cases, the stability of the protein seems to be determined by the amino acid present at the amino terminal (this has been termed the N-end rule, see Varshasky, 1997). In contrast, the signal for degradation of the large subunit of RNA polymerase II of yeast, the repeat sequence SPTSPSY, resides at the carboxy terminal (Huibregtse, et al., 1997). These are probably recognized by the WW domains an ubiquitin ligase (e.g., Wang et al., 1999). Some proteins have domains similar to ubiquitin and they have a short half life (e.g., Watkins et al., 1993). More frequently, *destruction boxes* (e.g., RIALGSLTD in yeast uracil permease, Galan et al.,

1994) appear to be responsible. These boxes are short stretches that are strongly conserved in proteins degraded by the system. One of these boxes corresponds to a stretch of 27 amino acids (Treier et al., 1994). Another sequence recognized by the system contains proline, aspartate, glutamate, serine and threonine (the *PEST* region; Roger et al., 1986). Removal of these sequences leads to partial stabilization. In contrast to these short recognition sequences, the signal for the mating type transcription factor α2 is the surface of the folded protein corresponding to a region of about 60 amino acids predicted to form an amphipatic helix and containing a hydrophobic face (Johnson et al., 1998).

Some of the proteins degraded by the 26S proteasome and the degradation signals are listed in the first column of Table 6.

What protease system degrades proteins ligated to ubiquitin? One of the ATP-dependent proteases, a 1,300 kDa complex, the so-called 26S proteasome, degrades these proteins (Hough et al., 1987). A 20S multicatalytic protease complex that has been studied independently, the *proteasome*, is part of the 26S proteasome. In yeast, mutational alteration of the proteasome causes accumulation of ubiquitin-protein conjugates (Heinemeyer et al., 1991). Deletion of the corresponding genes is lethal (Fujiwara et al., 1990; Heinemeyer et al., 1991), confirming its important role in the ubiquitin-protease pathway.

In many cases, phosphorylation of cytoplasmic proteins has been shown to be needed for ubiquitination and degradation via the proteasomes, for example, in the case of cyclins and transcriptional regulators (e.g., Yaglom et al., 1995; Chen et al., 1995). In yeast, the degradation of integral membrane proteins also requires phosphorylation (e.g., Medintz et al., 1996). Although not demonstrated as clearly, phosphorylation also seems to be required for ubiquitination of integral membrane proteins in mammalian systems (e.g. Cenciarelli et al., 1996).

In summary, it seems that two different pathways operate in proteolysis. A generalized pathway involving all proteins operates through the action of lysosomes. This pathway can be stimulated by major switches, such as nutritional step-down or insulin. The proteins broken down in this pathway are those with a long half-life. Proteins with a short half-life, including defective proteins, are broken down in an alternative pathway that involves primarily covalent binding to ubiquitin. There may be additional ubiquitin-independent pathways of degradation (McGuire et al., 1988).

The ubiquitin-conjugating system has actually been implicated in many important and diverse regulatory functions (see Hershko and Ciechanover, 1998) only some of which are related to protein degradation (see Chapter 7), with possible roles in gene expression, regulation of enzyme activity, the assembly of ribosomal proteins, DNA repair, yeast sporulation, regulation of the cell cycle (see Chapter 8) and response to stress. In the initial steps of the immune response, the antigen is partially cleaved. This cleavage involves proteasomes (Michalek et al., 1993; Niedermann et al., 1995). In some cases, ubiquitination is not a signal for degradation. Certain ribosomal proteins are attached to ubiquitin at their amino terminal. In this case, ubiquitin is thought to function in the folding of the proteins (Finley et al.,

1989). In addition, in higher eukaryotes, a portion of the histones (see Chapter 2) are monoubiquitinated and the ubiquitin, in this case, is not a degradation signal. Monoubiquitination marks a protein at the cell surface for endocytosis in both *Saccharomyces cervisiae* and in mammals (see Hicke, 2001). It also has a role in regulating the endocytotic machinery (see Chapter 9). In a number of cases, a special role of the ubiquitin system is indicated by indirect evidence, e.g., the synaptic development at the *Drosophila* neuromuscular junction was found to be a ubiquitin-dependent mechanism (DiAntonio et al., 2001). Overexpression of the deubiquitination enzyme *fat facets* in *Drosophila* leads to an increase in synaptic boutons and branching and a breakdown in synaptic function. The results suggest a balance between positive and negative regulators of ubiquitination in the development of synapses.

Ubiquitin binding domains have been identified in many proteins, although the function of these is not always clear (see Buchberger, 2002). The UBA domain of about 40 amino acid residues has been found in some proteins, some of which are thought to act as inhibitors of polyubiquitination. The *ubiquitin interacting motif* (UIM or LALAL-motif) of 20 residues has been found in the 20S subunit of proteasome, as well as in proteins involved in ubiquitin metabolism and receptor mediated endocytosis.

Several proteins, the *ubiquitin-like* proteins (ULPs), have sequence similarity to ubiquitin and can also covalently attach to proteins (see Yeh et al., 2000). They require enzyme complexes equivalent to E_1, E_2 and E_3 for their binding to target proteins. The ULPs include UCRP/ISG15, Sentrin (also called *small ubiquitin-like modifier*, SUMO), NEDD8 (in yeast the equivalent protein is the *related to ubiquitin 1*, Rub1) and Apg12. Their function is not always clear since this is a relatively new focus of research.

The amino acid sequences of all the Sentrins (Sentrin-1, -2, and -3). are the same in all mammals studied and Sentrin homologues have been found in many other organisms. Sentrinization does not serve as a degradation signal but rather targets proteins to different cellular compartments (e.g., nuclear body, NPC). Sentrinization and desentrinization play a role in cell cycle progression. Sentrins conjugate mostly with nuclear or nuclear envelope proteins among these homeodomain-interacting protein kinase 2 (HIPK2), p53 and RanGAP1, a major regulator of the Ras-like GTPase Ran. Sentrinization is required for RanGAP1 localization in nucleopore complex. Ran is the small GTPase that plays an important role in both nuclear import and export (see Chapter 5). Sentrins are also conjugated to non-nuclear proteins. among these, I κ B α, the inhibitor of the transcription factor NF-κB (Desterro et al., 1998) and in addition, the glucose transporters, GLUT1 and GLUT4 (see Chapter 19). Sentrinization decreases GLUT1 and increases GLUT4. The latter is translocated from the cytoplasm to the plasma membrane following the action of insulin.

The binding of SUMO-1 to transcriptional factors is thought to have a role enhancing or decreasing transcriptional activity of factors and in subcellular localization (e.g., Ross et al., 2002)

The *neural precursor cell-expressed developmentally regulated* (Nedd8) is a ULP of 81-amino acids which conjugates to a large number of nuclear proteins, the cullins. In yeast Rub1 binds to Cdc53, a 94 kDa protein required for the G_1-S progression. Two cullins conjugated to Nedd8 are components of the

ubiquitin E3 ligase. Nedd8 can form polymers which are thought to target proteins to proteasomes. The protein NUB1 is a strong down-regulator of Nedd8 by recruiting Nedd8 containing complexes for proteasomal degradation (Kamitani et al., 2001). Nedd8 modification of the Cul-1 component of the a ubiquitin-ligase enzyme complex, SCF, is important for function of SCF in the ubiquitination of I κ B α.

The *Ubiquitin cross-reactive protein* (UCRP) targets substrates to the cytoskeleton (Loeb and Haas, 1994). The role of the ubiquitin-like proteins has just begun to be explored and many more may be found.

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

Proteasomes

We have seen that protein degradation is an important mechanism in regulating enzyme activity. Furthermore, the degradation of proteins is required for vital cell functions such as cell division and changes in transcriptional, translational and developmental programs, and elimination of defective proteins (see Patton et al., 1998).

The primary process for protein degradation is through the proteasome pathway (e.g., see Hochstrasser, 1996). In view of the very important role of proteasome in the functioning of cells, it is not surprising to find that drugs that inhibit proteasomes induce cell cycle arrest and apoptosis in cancer cell lines (Orlowski et al., 1998; Adams et al., 1999) by unknown mechanisms. The sparing of inhibitors of cell division (see Chapter 8, Section IIID) may play a role in this effect.

As discussed below (Section IIIB), the degradation of mRNA is another important regulatory mechanism. The process by which degradation of RNA takes place is beginning to be understood. In part, it involves a complex, the exosome. The degradation of cytokine mRNAs containing AU-rich elements in the 3' untranslated region, appears to involve, in some way, proteasomes (Laroia et al., 1999).

Some of the roles of proteasome mediated proteolysis are summarized in the second column of Table 6 (Hilt and Wolf, 1996), which lists a variety of known substrates of the 26S proteasome (column 1), their function (column 2) and their degradation signal (column 3).

Table 6 Substrates and physiological role of the 26S proteasome (modified from Hilt and Wolf, 1996). Reproduced by permission.

Substrate protein	Cellular function	Degradation signal

Metabolic adaptation		
Ornithine decarboxylase	Metabolic enzyme; gluconeogenesis	?
Fructose-1,6-bisphosphatase	Metabolic enzyme; gluconeogenesis	?
Gcn4	Transcriptional activator; amino acid and purine synthesis	PEST sequences
Cell differentiation		
MAT α2 repressor	Repression of mating type specific genes	Degradation box
Gα	GTP-protein; signal transduction	N-end rule
Cell cycle control/cell growth		
Cln2	G1 cyclin; control of cdc28 kinase	PEST sequences
Cln3	G1 cyclin; control of cdc28 kinase	PEST sequences
Clb5	S-phase cyclin; control of cdc28 kinase	Destruction box
Clb2	Mitotic cyclin; control of cdc28 kinase	Destruction box
Sic1	CDK inhibitor; inhibition of Clb5-cdc28 complex	?
	Protein kinase; meiotic arrest in oocytes	Amino terminal, 2nd amino acid

c-Mos c-Jun p53	Transcriptional activator; signal transduction Tumor suppressor; cell cycle pause	δ-domain ?
Stress response NFκ-B I-κB	Transcriptional regulator; immune and inflammatory response Inhibitor of NF-κB	? ?
Removal of waste Canavanyl proteins Fas2 CTFR	Undefined α-subunit of fatty acid synthase Ion pump; Cl^- transport across plasma membrane	?

The proteasomes are located in the cytoplasm and the nucleus (Hügle et al., 1983, Peters et al., 1994), as shown by immunofluorescence localization (see Chapter 1) in diverse organisms and cell types. They are found in the form of 20S and 26S particles [most accurately the larger unit is 30.3S (Yoshimura et al., 1993) but not currently used in the nomenclature]. S refers to the sedimentation coefficient in the ultracentrifuge. The 20S particle is also part of the 26S proteasome. However, the 20S particle may function independently as well, although this is not established. The 20S particle is also present in complexes other than the 26S proteasome (Peters, 1994), arguing for a separate and independent role. The

26S proteasomes degrade ubiquitinated proteins only, whereas the 20S proteasomes do not degrade ubiquitinated proteins but rather unfolded proteins (Wenzel and Baumeister, 1995). In vitro, the 20S proteasome has been shown to degrade only certain denatured or oxidized proteins (see Grune et al., 1998). The 20S proteasome recognizes amino acid residues that are exposed during the conformational rearrangement accompanying the oxidative denaturation, in particular, hydrophobic residues that are usually shielded.

The 20S proteasome has a molecular mass of 700 kDa and is constituted of many low molecular weight components arranged in a stack of four rings, each of seven subunits. A reconstruction is shown in Fig. 5 (Hilt and Wolf, 1996). Many recent studies have been carried out with the 20S proteasome of the archaebacterium *Thermoplasma acidophilum*, that resembles the eukaryotic proteasome and is thought to be its ancestor. This structure has been studied with X-ray crystallography with a resolution of 3.4 Å. The complex is a hollow cylinder, 11.3 nm in diameter and 14.8 nm in length (Löwe et al., 1995). The 20S proteasome is made up of two kinds of subunits. The 7 α-subunits are probably not catalytic and can assemble into a seven membered ring. The 7 β-subunits cannot assemble by themselves but have catalytic activity. The α and β components arrange themselves in a stack of 4 rings, α on the outside and β on the inside. A similar structure was shown by X-ray diffraction of the bovine (Morimoto et al., 1995) and yeast (Groll et al., 1997) 20S proteasomes.

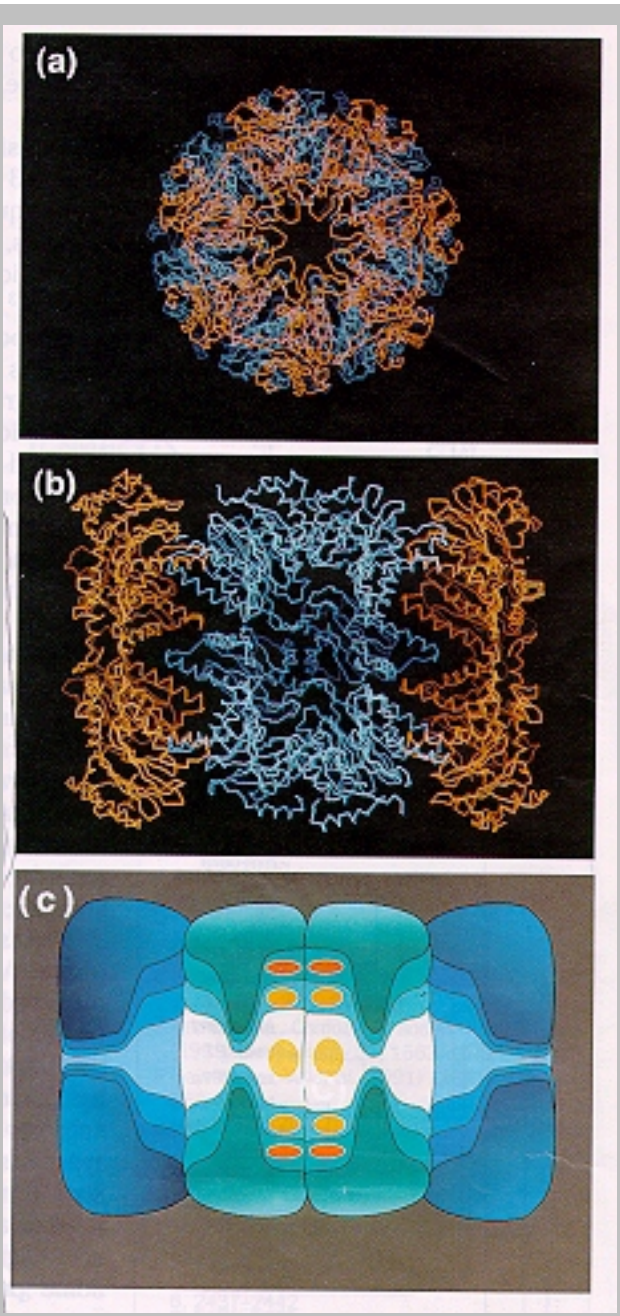


Fig. 5 3D structure of the 20S proteasome derived from X-ray, (a) top view (C_α atoms only) showing the 13 A entrance to the channel, (b) view of proteasome when cut along sevenfold axis, and (c) schematic representation of cut proteasome showing the three cavities and the proteolytic sites .Reproduced from *Trends in Biochemical Science*, vol. 21, Hilt, W. and Wolf, D.H., Proteasomes: destruction as a programme, pp. 96-102, copyright © 1996 with permission from Elsevier Science.

The 26S proteasome of eukaryotes is formed by 20S proteolytic core, capped by one or two 19S regulatory complexes (see Tanaka, 1998; Glickman et al., 1998a,b) and has a molecular mass of 1,700 kDa. The 20S and the 19S complexes separate out when ATP is depleted, and ATP is required for their reassembly into the 26S particle. The regulatory complex, only found in eukaryotic cells, is formed by at least 17 polypeptides. The regulatory particle selects ubiquitinated substrates for translocation into the core particle. It can be dissociated into an 8 subunits subcomplex or lid, and a base that contains 6 ATPases of the AAA-ATPase (see below) family and connects the regulatory complex to the core particle

(e.g., Glickman et al., 1998a,b). The eight subunit base activates the core particle to degrade peptides in a ubiquitin dependent manner. In order to degrade a protein (see Baumeister et al., 1998; Larsen and Finley, 1997; Gottesman et al., 1998), the 26S proteasome must be able to translocate it through a narrow channel for the protein to come in contact with the proteolytic chamber. In contrast to the bacterial proteasome, the interior of the yeast 20 S proteasome is accessible only through very narrow side entrances (Groll et al., 1997). The passage of the substrate can only take place if the protein is unfolded, a function that is chaperone-like (see below). In accordance with this view, the base alone or the whole 26S proteasome (in human and yeast 26S proteasome) were found to bind and refold a denatured protein (non-ubiquitinated citrate synthase) in an ATP dependent manner (Braun et al., 1999). The actual cleavage of the protein takes place by a N-terminal nucleophilic mechanism (see Brannigan et al., 1995). The polyubiquitin chain must be removed to allow the translocation of the unfolded substrate polypeptide into the 20S portion. The POH1 (also known as Rpn11 in yeast) subunit of the 19S complex, a Zn^{2+}-dependent protease, is responsible for substrate deubiquitination during proteasomal degradation (other deubiquitinating enzymes (DUBs) are cysteine proteases) (Yao and Cohen, 2002).

Surprisingly the 19 S regulatory unit has been implicated in transcription. For example, two-hybrid assays and in vitro experiments have shown the binding of one of its components to a protein involved in the activation of transcription of the galactose pathway (Gal4p) (Chang et al., 2001). In addition, yeast strains carrying alleles of two of the 19 S components exhibit elongation defects and in vitro transcription is inhibited by antibodies against one of these proteins. An elongation factor, immunoprecipitates with the 19S complex (Ferdous et al., 2001). Furthermore, the 19S complex has been shown to be recruited by the GAL promoter by the Gal4 activator upon induction by galactose (Gonzalez et al., 2002).

We saw that covalent binding of ubiquitin to a protein substrate is generally needed for degradation by the 26S proteasome. Generally, the targeting requires the attachment of polyubiquitin so that the molecular weight of the ubiquitinated protein is much greater than that of the non-ubiquitinated variety. In addition, ubiquitin can also serve as a targeting signal for alternative degradation pathways. Ubiquitin targets certain proteins to lysosomes (Hicke and Riezman, 1996). Other mechanisms for targeting to the 26S proteasome must also exist because some proteins, such as ornithine decarboxylase, are degraded by the proteasome without ubiquitination (Murakami et al., 1992). Ornithine decarboxylase is targeted to the proteasome by non-covalent binding to a protein factor, the *antizyme*.

The presence of polyubiquitin chains (Chau et al., 1989) and their targeting function was revealed relatively recently (Gregori et al., 1990). The ubiquitins are linked by an isopeptide bond (attached to the ε rather than the α amino group) between Lys48 of ubiquitin *n* and Gly76 of ubiquitin *n+1*. Mutation of Lys48 to Arg (K48R) is lethal in *S. cerevisiae* (Finley et al., 1994) and blocks β-galactosidase degradation in vitro (Chau et al., 1989). Furthermore, conditional expression of K48R inhibits the turnover of short-lived proteins and causes cell cycle arrest at G2/M.

Although polyubiquitination seems to be the most important signal, single ubiquitin molecules can still

serve as signals, but of lesser significance. Polyubiquitin may be the favored signal because it is bound more tightly to the proteasome, for example, octaubiquitin binds 90 times more tightly than diubiquitin (Piotrowski et al., 1997). One of the possible mechanisms of binding may involve hydrophobic patches from different ubiquitin moieties. Such a mechanism would permit regulating the degree of binding by adding or removing ubiquitin and, hence, changing the rate of degradation of a protein (see Pickart, 1997).

Polyubiquitin can attach at other Lys sites. However, only Lys48 is essential (Spence et al., 1995). The polyubiquitins linked to proteins at other Lys sites have been shown to function in targeting. However, mutants (Lys to Arg) at these sites (Spence et al., 1995) do not show a major proteolytic defect, so that the ubiquitination of the Lys48 site must be considered the major targeting signal.

Although proteasomes are the major agents of extralysosomal protein degradation, there have been some indications that another pathway is also present. For example, mouse lymphoma cells in culture, can become adapted to the presence of a proteasomal inhibitor (Glas et al., 1998). In fact, an additional large protease, a tripeptidyl peptidase (TPII) (Geier et al., 1999), has been found to co-purify with proteasomes. TPII is larger than the proteasome and may be able to substitute for some of the proteasome functions. A large protease complex distinct from proteasomes has also been demonstrated in the archaebacterium, *Thermoplasma*, the *tricorn protease* (Tamura et al., 1996). The tricorn protease is a complex of 720 kDa, involving six identical subunits that form a channel 8 nm in diameter.

Polypeptides that are not properly folded, bind to chaperones or are ubiquitinated and degraded by proteasomes (see Wickner et al., 1999). When the two systems are insufficient to eliminate unfolded proteins, they form aggregates. A variety of prion (see Harris, 1999; Prusiner, 1998) and amyloid diseases (Martin, 1999) are associated with abnormal aggregation of proteins. Prion diseases are fatal neurodegenerative disorders of humans and animals caused by conformational conversion of a normal host glycoprotein into an infectious isoform without the intervention of nucleic acid. The amyloid diseases include a variety of disorders, such as Alzheimer's, Huntigton's, Parkinson's disease and others (see Martin, 1999).

The chaperones are complexes involved in the proper folding of proteins (also discussed in Chapter 10 and in Section III below). They are represented by several families of ATP-dependent proteins that interact with a variety of proteins. Chaperonins are barrel shaped complexes organized as two stacked rings, each of seven to nine subunits (see Kessel et al., 1995). The internal chamber serves for recognition and sequestration of unfolded proteins. Other chaperones act as monomers in conjunction with a co-chaperones (Bukau and Horwich, 1998). The alternative fate of misfolded proteins is to be degraded by ATP-dependent proteases, mostly the 26S proteasome complex. Chaperones recognize hydrophobic residues not usually exposed in native folded protein. As already discussed, proteasomes depend on the ubiquitin conjugating system for their selectivity.

A perinuclear structure containing 20S proteasome machinery has been identified in mammalian cells in

culture (Johnston et al., 1998; Wigley et al., 1999) and is particularly evident when unfolded proteins are produced in excess. (Johnston et al.), coined the term *aggresome* for this aggregate. Aggresome formation is accompanied by redistribution of the intermediate filament protein vimentin to form a cage surrounding a pericentriolar core of aggregated, ubiquitinated protein. Disruption of microtubules blocks the formation of aggresomes (Johnston et al., 1998). The structure is surrounded by endoplasmic reticulum and is adjacent to the Golgi (Wigley et al., 1999). It colocalizes with γ-tubulin, considered to be a centrosomal marker, and it is therefore thought to be associated with the centrosomes which may be acting as a scaffold (Wigley et al., 1999). Density gradient fractions containing purified centrosomes are enriched in proteasomal components and cell stress chaperones. The structure enlarges in response to inhibition of proteasome activity and the level of misfolded proteins [misfolded protein is produced by transfection using vectors containing mutants of the *cystic fibrosis transmembrane conductance regulator* (CFTR)]. When the level of misfolded protein is high, the structure recruits the cytosolic pools of ubiquitin and proteasomal components.

Degradation of membrane proteins

Cell surface receptors and other integral proteins (e.g., growth factor receptors, channel proteins or transporters) that have been internalized by endocytosis can be recycled to the plasma membrane or degraded by the lysosomes (or vacuole in the case of *Saccharomyces cerevisiae*) (see Gruenberg and Maxfield, 1995). The choice of target, either the plasma membrane or the degradation pathway, offers a way by which the activity of the protein can be regulated. In the degradation pathway, late endosomes produce multivesicular bodies (MVB) by invagination of the surface membrane into its interior. Some of the receptors are sorted into these vesicles. Fusion of the MVB with the lysosomes delivers these receptors into the lysosomal lumen for degradation (Futter et al., 1996). The degradation of receptors is also discussed below (Section C)

Ubiquitination has been found to act as a sorting signal in both the endosomal and the biosynthetic pathway of certain membrane proteins (see Hicke, 2001). In *Saccharomyces cerevisiae* and other eukaryotes, integral proteins have to be ubiquitinated, most generally monoubiquitinated, on specific residues in order to be taken up by endocyosis. The ubiquitin offers a 3-D internalization signal. Ubiquitination of proteins not normally internalized by this mechanism has been shown to lead to their internalization (e.g., Roth and Davis, 2000). The proteins taken up by this mechanism are degraded in lysosomes (or the vacuole in yeast) and not proteasomes (e.g., see Hicke 1999).

Ubiquitination also has a role in controlling the machinery of endocytosis. For example, a *growth hormone receptor* (GHR) truncation mutant (which lacks ubiquitination sites) internalizes via a di-leucine motif and does not require ubiquitination to be taken up by endocytosis. However, ubiquitinating enzymes have to be present presumably to act on other proteins needed for the machinery of endocytosis (Govers et al., 1999) which may act on components of the clathrin machinery (van Delft et al., 1997). Similarly, in *Saccharomyces cerevisiae*, a receptor chimera containing ubiquitin does not require ubiquitination for its internalization but requires ubiquitin-conjugating enzymes (Dunn and Hicke, 2001).

Generally, the choice between recycling or the degradation pathway after internalization, depends on the ubiquitination state of the protein. The degradation requires ubiquitination (Lee et al., 1999b). In the absence of ubiquitination the fate of the integral protein is to be recycled to the plasma membrane. In the case of the *colony-stimulating factor-1* (CSF-1 receptor), a polyubiquitination is required for both endocytotic uptake and targeting to the lysosomes for degradation. In mammals, CSF-1 is involved in the survival, proliferation and differentiation of mononuclear phagocytic cells.

Ubiquitination also plays a role in the fate of integral proteins packaged in the TGN. In *Saccharomyces cerevisiae*, the presence of the permeases at the cell surface are adjusted depending on nutrient availability. The tryptophan permease Tat2 is present constitutively and is regulated by degradation in the absence of sufficient nitrogen sources when it is transferred to the vacuole, a targeting that requires ubiquitination (Beck et al., 1999). An internal pool of Tat2 appears to be directed to the vacuole independently of the plasma membrane. The general amino acid permease Gap is induced when grown on a relatively poor nitrogen source and is targeted to the plasma membrane, where it is active in transport. When grown on a relatively rich nitrogen source, Gap is directed to the vacuole and is degraded. The transport to the cell surface requires monoubiquitination or no ubiquitination, whereas polyubiquitination targets the protein to the vacuole (Helliwell et al., 2001).

In contrast to the degradation of integral membrane proteins of the plasma membrane, membrane proteins of the ER are degraded by the ubiquitin-proteasome system (see Plemper and Wolf, 1999). A member of the ubiquitin-conjugating enzyme Ubc6p of yeast has been found in the cytoplasmic face of the ER (Sommer and Jentsch, 1993). In contrast, mitochondria, chloroplasts and bacteria possess ATP-dependent proteases: the AAA-proteases which are embedded in the membranes. The AAA ATPases are also discussed in Chapter 11.

AAA-proteases (see Langer, 2000) are present as large complexes of 850 kDa, made up of identical or at least very similar subunits of 70 to 80 kDa (e.g., Arlt et al., 1996; Leonhard et al., 1996). The AAA-proteases have one or two transmembrane segments. In *Saccharomyces cerevisiae*, absence of AAA-protease function produces respiratory defects (Tzagoloff et al., 1994; Arlt et al., 1998). Apparently, the proteolytic activity is required to maintain a functional respiratory chain (e.g., Arlt et al., 1998). In humans, mutations of a mitochondrial AAA-protease, *paraplegin*, is responsible for a hereditary spastic paraplegia (Casari et al., 1998).

The AAA-proteases degrade unfolded proteins as well as regulatory proteins (see Langer, 2000). In order to recognize proteins, AAA-proteases must have chaperone-like properties which apparently are present at the ATPase domain. The hydrolysis of ATP is essential for the degradation of substrates (e.g., Arlt et al., 1996). The possibility of a role of the AAA-proteases other than protein-degradation is also suggested by mutations in the FtsH *E. coli* protease which cause abnormal orientation of membrane proteins, overcome by the overproduction of chaperone proteins (Shirai et al., 1996).

The action of the AAA-proteases appears to be regulated by an additionmal large complex of 2000 kDa, the *prohibitin* complex with assembles with the proteases and inhibits degradation of unassembled protein (Steglich et al., 1999)

Caspases

In addition to the proteolytic systems already discussed, the cysteine proteases (*cysteinyl aspartate specific proteinases*, caspases) play a major role in *apoptosis* (see Chapter 2). Apoptosis is the process of programmed death involved in development and the maintenance of multicellular organisms (see Jacobson et al., 1997, Thornberry and Lazebnik, 1998). The importance of caspases is shown by experiments with the nematode, *Caenorhabditis elegans*. Deletion or mutation of a single gene coding for a caspase abolishes the programmed death of 131 cells. Similarly, mice deficient in caspase-3 have defects in the apoptosis that normally accompanies the early development of mouse brain. Caspases have been found to selectively cleave proteins involved in the early stages of apoptosis (see Thornberry et al., 1997; Thornberry and Lazebnik).

C. Degradation of Receptors

As discussed in Chapters 6 and 7, a variety of receptors are responsible for the binding of certain hormones and growth factors. Their activation produces a cascade of events that have important results in the cell's function. The removal of receptors and ligands from the cell surface by endocytosis is part of the mechanism by which cells return to the unstimulated condition. The degradation of the protein is carried out either by lysosomes or proteasomes (see Chapter 9). One of the mechanisms for the internalization of receptors and other plasma membrane proteins depends on ubiquitination and has been discussed above.. We saw that membrane proteins of the ER are ubiquitinated and degraded by the proteasome pathway. A variety of cell membrane proteins are ubiquitinated (e.g., see Bonifacino and Weissman, 1998; Hicke, 1999), a signal for internalization into the endocytotic pathway and degradation in the lysosome (vacuole in yeast). In mammals, the lysosomal pathway is primarily responsible for the degradation, although at least one case involving proteasomes is known (see Bonifacino and Weissman, 1998, pp. 42 and 43).

A typical case, the regulation of the epithelial growth factor receptors (EGFRs) can serve as an example of the targeting process. EGFRs are downregulated by the degradation mechanism. The EGFRs are sorted out by the MVB pathway where they are degraded by lysosomes or recycled to the plasma membrane (Carter and Sorkin, 1998) . After binding the ligand, the EGFRs are internalized quickly. A portion is degraded after delivery to the lysosome lumen. The marking for degradation depends on EGFR kinase, sorting signals in the EGFR tail and the ubiquitin ligase c-Cbl which supposedly polyubiquinates the EGFR (e.g., Felder et al., 1990, Kornilova et al., 1996; Yokouchi et al., 1999). Ubiquitination was found to be required for endosomal sorting and for later sorting for lysosomal degradation (Katzmann et al., 2001). A 350 kDa complex (ESCRT-I) recognizes ubiquinated cargo and is involved on sorting it into the MVB vesicles.

What domains recognize the ubiquitin signals? A domain of approximately 20 amino acids (the ubiquitin interacting motif, UIM) has been identified in several proteins and is suspected to act as a site for ubiquitin binding during the endocytotic process (Hofmann and Falquest, 2001).

How do ubiquitinated proteins avoid degradation by the proteasomes? In yeast, this is probably because a polyubiquitin chain at least four units long (Deveraux et al., 1994) is required for targeting to the proteasomes. In contrast, a single ubiquitin residue is sufficient to initiate endocytosis (Terrell et al., 1998). In animal cells [e.g., *growth hormone* (GH) and *platelet-derived growth factor* (PDGF) receptors], polyubiquitination of receptors does take place (Mori et al., 1992; Strous et al., 1996). However, it is not clear at this time whether the ubiquitin is attached to multiple or single lysine sites.

Plasma membrane ubiquitination is positively regulated by phosphorylation. In yeast, in response to ligand binding, the phosphorylation activates both the ubiquitination and the internalization (e.g., Reneke et al., 1988; Hicke et al., 1998).

In yeast, G-protein coupled receptors undergo ubiquitination dependent internalization. In mammals, a variety of tyrosine kinase or kinase linked receptors undergo ligand stimulated ubiquitination and internalization. Mammalian cells deficient in the enzyme of the ubiquitin-activating enzymes are impaired in internalization (Strous et al., 1996). Furthermore, the degradation of several receptors requiring ubiquitination is blocked by inhibitors of proteasome and lysosomal activity (Mori et al., 1995; Jeffers et al., 1997).

Several observations suggest that the ubiquitination of proteins other then those being internalized play an important role. For example, the ubiquitination of the growth hormone (GH) receptor is not required for internalization. However, blocking internalization of the receptor also seems to prevent ubiquitination events (Govers et al., 1997).

III. FOLDING AND QUALITY CONTROL

Native proteins are in a folded configuration. Cells possess mechanisms of quality control which prevent the accumulation of defective and misfolded polypeptides (see Ma and Hendershot 2001; Ellgaard and Helenius, 2001). Inside cells, the *chaperones* mediate the folding of nascent proteins. The folding is coupled to ATP hydrolysis. In addition, chaperones have a role in the folding and degradation of misfolded or defective proteins. Protein misfolding and consequent aggregation can have dire consequences because misfolded polypeptides have exposed hydrophobic surfaces which interact with each other to cause aggregation. Chaperones are also discussed above in relation to the proteasomes and in Chapter 10 in relation to events occurring in the ER.

Chaperones occupy a central position in the folding of proteins and in the process of degradation. Chaperones function cotranslationally on nascent chains as they emerge from ribosomes so that they are stabilized in a nonaggregated state. They can also act in the cytoplasmic or organelle environment (see

Frydman, 2001). Chaperones recognize hydrophobic residues and unstructured backbone regions which are normally buried inside the native folded protein. Chaperones bind to substrates to prevent defective folding and either mediate folding or transfer the substrates to other chaperones, the chaperonins. The chaperonins (see Saibil and Ranson, 2002) are large, double ringed cylindrical complexes of about 800 kDa forming a central cavity in which a single protein chain is folded (e.g., see Frydman, 2001; Hartl and Hayer-Hartl, 2002). The multiple subunits of the chaperonins first bind to the hydrophobic regions of their substrate. Then the substrate is transferred to the central cavity where the folding takes place. Eukaryotes have group II chaperonins which contain eight orthologous subunits per ring.

The chaperones Hsp70 and Hsp90 together with co-chaperones function to fold proteins in the cytoplasm (see Cyr et al., 2002, Table 1 for listing). Co-chaperones mediate the binding of denatured proteins to the chaperones. Sometimes Hsp70 and Hsp90 function sequentially to fold the same protein. The co-chaperone Hop connects the two chaperones. When proteins are destined for degradation, the two chaperones bind to a set of co-chaperones that have a degradation function (see Hohfeld et al., 2001; Connell et al., 2001). Supposedly these co-chaperones bridge chaperones and components of the ubiquitin-proteasome system.

Short nascent polypeptides may fold without the intervention of chaperones. When acting cotranslationally, the chaperones mediate substrate binding cycles as the nascent polypeptide emerges from the ribosome. These cycles are coupled to the chaperone's ATPase activity and involve a number of cofactor proteins. Some chaperones such as *trigger factor* or Hsp70 bind to the ribosome so that they can interact with the nascent chain. Longer polypeptides require the action of other chaperones. In some cases, the peptide is passed from Hsp70 to the Hsp90.

Misfolded polypeptides are recognized and degraded by the proteasome system. As already indicated, the folding and the degrading pathways are thought to have some steps in common. Current thought assigns the role of partitioning between the folding and proteases to the kinetics of folding (Wickner, 1999). However, the finding that the co-chaperone CHIP functions as an E_3 complex , a ubiquitin ligase, indicates a possible regulation between these two pathways. E_3 proteins are enzyme complexes responsible for ubiquitination (see above) and hence degradation via the proteasome pathway.

Ubiquitination requires E_1, E_2 and E_3 (see above) and sometimes E4 (Koegl et al., 1999). After four or more ubiquitins are added, the protein is taken up by proteasomes and degraded. Many E_3 enzymes contain the HECT domain of 350 residues. A cysteine residue accepts ubiquitin from E_2. The HECT domain selects the protein to which ubiquitin is attached. Many E_3 enzymes possess *really interesting new gene* (RING) domains or U-box domains which bind to E_2 and the substrate. The RING family of E_3 ubiquitin ligases are involved in the selection of misfolded proteins for degradation. Some of these have a role in quality control involving the ER. Some of the E_3s recognize hydrophobic groups (e.g., doa10). Others recognize polar residues exposed in the lipid bilayer (e.g., Tul1). Another (Parkin) appears to recognize unfolded proteins. These regions serve as sorting signals for the endocytotic pathway and in

yeast the proteins are degraded by the vacuole rather than the proteasomes.

At the sites of protein synthesis such as the ER or the cytoplasm there are multiple quality control mechanisms. In the ER, the oligosaccharide chains are trimmed by glucosidases which monitor the folded state together with glycoprotein-specific chaperones such as calnexin, calreticulin and glucose transferase (Ellgaard et al., 1999). When the folding is slow or the glyprotein is misfolded, the chain is trimmed to provide a signal for entry into the cytoplasm, followed by conjugation with ubiquitin and degradation by proteasomes (*ER-associated degradation*, ERAD) (see Fewell et al., 2001; Plemper and Wolf , 1999; Hohfeld et al., 2001).

IV. REGULATION OF ENZYME SYNTHESIS

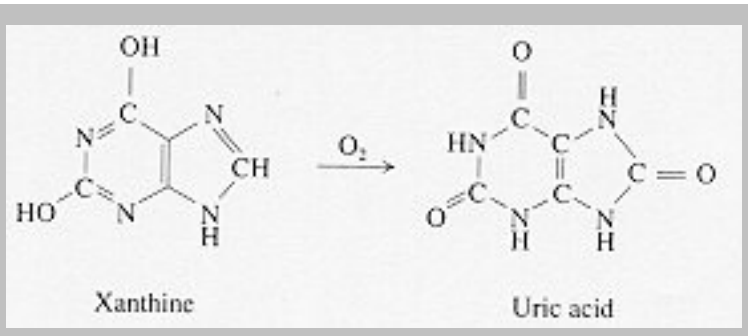
In eukaryotes, the control of the synthesis of specific proteins may depend on a variety of regulatory mechanisms that are much more complex than those occurring in bacteria. Animal cells are thought to contain at least 1000 times more genetic information than bacteria. This genetic information is contained in the chromosomes, where DNA is combined with proteins, such as histones that may have a role in controlling transcription. The structure of the chromosome itself is likely to have an effect on its availability for transcription. The nuclear envelope may play a role in the release of mRNA and its modification after transcription. Furthermore, the mRNA used in translation is constantly being broken down, although this process takes place much more slowly in animal cells than in prokaryotes. The possible mechanisms of control in eukaryotic cells are depicted in Fig. 6 (Walker, 1977).

One of the emerging threads in the control of metabolic reactions is the role of thiol-reactive proteins, such as thioredoxin. These proteins act through a variety of mechanisms including direct effects on enzyme activity, transcription (e.g., Akamatsy et al., 1997) and ubiquination (see Chapter 14).

There are two special cases of regulation. Regulation in mammalian striated muscle is discussed below (see Section IIID). Neuronal plasticity is discussed in Chapter 22.

A. Transcriptional Control

Transcription of the appropriate genes may be repressed or activated, depending on the signal received. There are many indications that the level of certain enzymes, such as xanthine oxidase, is regulated by a transcriptional mechanism. Xanthine oxidase is an enzyme with a molecular weight of about 300 kDa and contains two flavin nucleotides. The enzyme acts in the pathway responsible for the degradation of purine rings and catalyzes the oxidation of xanthine to form uric acid, as shown in Eq. (5).

(5) 

Using a spectrophotometer, the uric acid produced can be estimated by measuring the light absorbed at 292 nm, as done in the experiments represented in Fig. 7, Table 7, and Fig. 8. The optical density represented in the figures can be considered to be directly proportional to the amount of uric acid produced and is expressed per milligram of liver protein. In contrast, the results of Table 7 (Rowe and Wyngaarden, 1966) are expressed as specific activity of the enzyme, i.e., activity per milligram of purified enzyme.

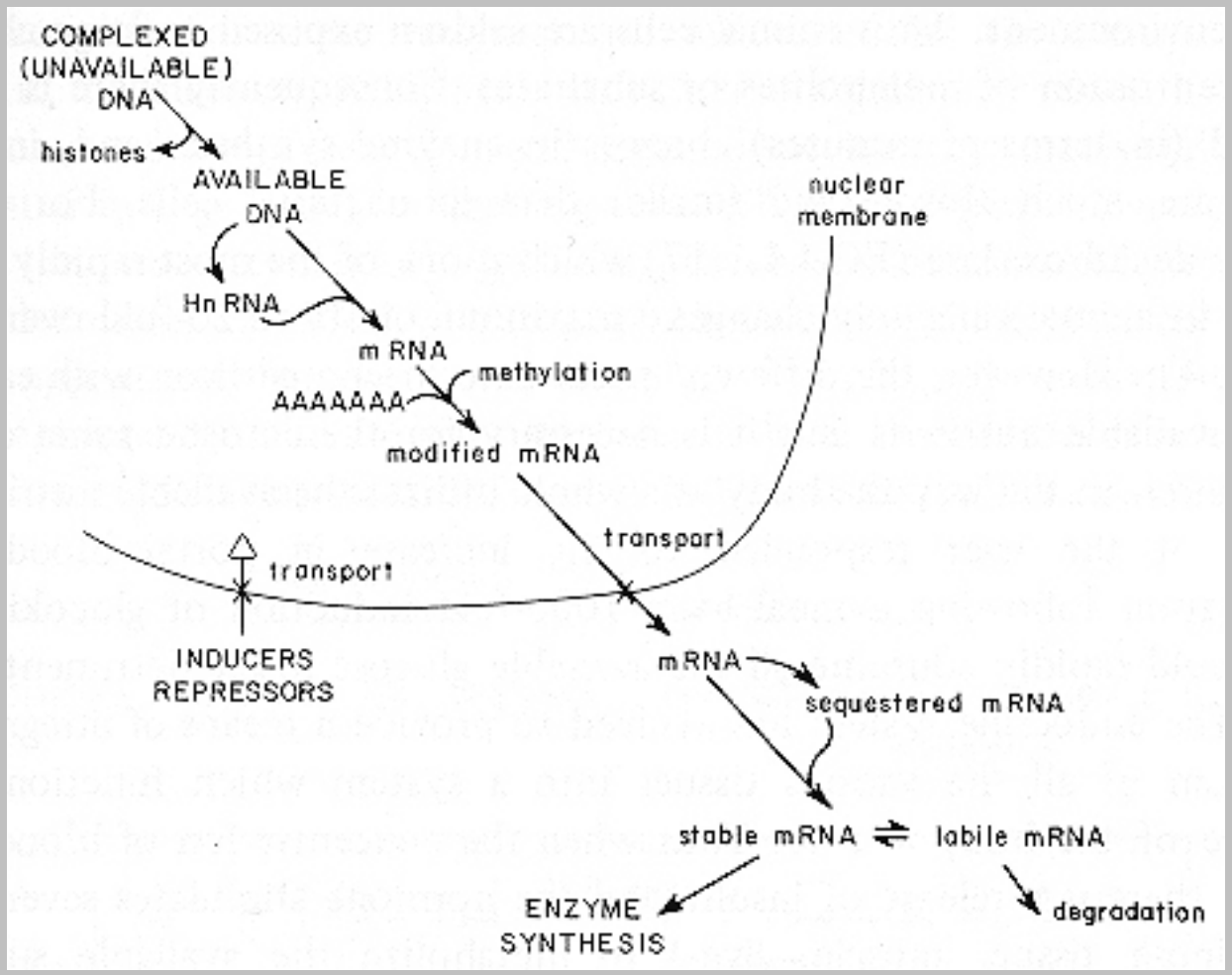


Fig. 6 Transcriptional and posttranscriptional stages in the production of translatable mRNA in animal cells (Walker, 1977). Reproduced with permission from *Essays in Biochemistry* 13:39-69, copyright © 1977 by The Biochemical Society, London.

In some species, uric acid is the final breakdown product of the pathway, and this product is excreted

directly. In in most mammals, uric acid is further degraded to produce urea. In the rat, the xanthine oxidase activity of the liver is increased by a high-protein diet (e.g., 23% protein) and decreased by a low-protein diet (e.g., 8%). The effect of diet is shown in Fig. 7. Rats normally fed a 23% protein diet are switched at time zero to an 8% diet (first arrow). After 14 days on the low-protein diet, the animals are again placed on the high-protein diet (second arrow), and a subsequent increase in xanthine oxidase is observed. In Figs. 7 and 8, the xanthine oxidase activity is expressed in terms of optical density change (in relative units). The values represent the amount of uric acid produced in the test tube assay in relative units. As shown in Fig. 7 (first part of the curve), switching to a low-protein diet causes a 10-fold drop in activity. The return to a high-protein diet dramatically increases the level of the enzyme after a short lag. The enzyme activity after reintroduction of the high-protein diet is also shown in Fig. 8 (Rowe and Wyngaarden, 1966), curve 1.

The results of Table 7 (Rowe and Wyngaarden, 1966) show that the increase in enzyme activity does not correspond to activation of preexisting molecules. Rats were injected with [^{14}C]leucine 6 h before being sacrificed. Items 1 and 2 represent control animals that were maintained on 23% protein for 14 days, and items 3 and 4, represent results obtained with rats on an 8% protein diet. Items 5 and 6 represent animals treated as in items 1 and 4 but subsequently fed the high-protein diet for 12 h. The activity per milligram of enzyme (specific activity of the enzyme, column a) is the same regardless of physiological condition. Activation would be expected to increase the enzyme activity per milligram of enzyme. The incorporation of label into the enzyme molecule (column b) is the same for the control animals and the animals maintained on an 8% protein diet, although the xanthine oxidase activity is low. Hence, the decrease in the activity of xanthine oxidase probably corresponds to a higher rate of breakdown. In animals for which the low-protein diet was replaced after 14 days by 23% protein, there is an increase in radioactivity that roughly corresponds to the five-fold increase in enzymatic activity shown in Fig. 7. Therefore, the increase in enzyme activity appears to be the result of increased synthesis of the enzyme.

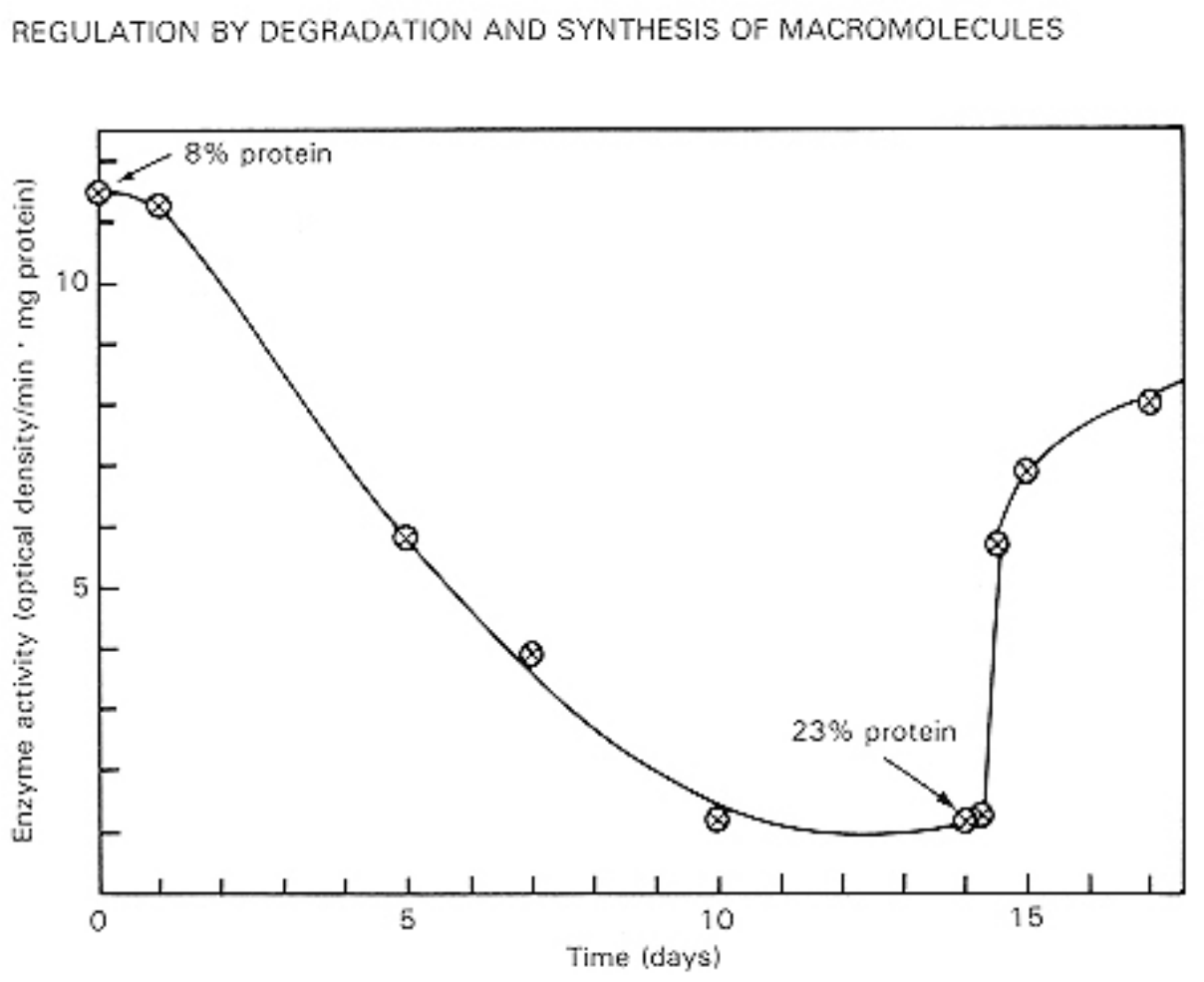


Fig. 7 Induction of xanthine oxidase in rat liver. Data from Rowe and Wyngaarden (1966), expressed per milligram of liver protein. Reproduced by permission.

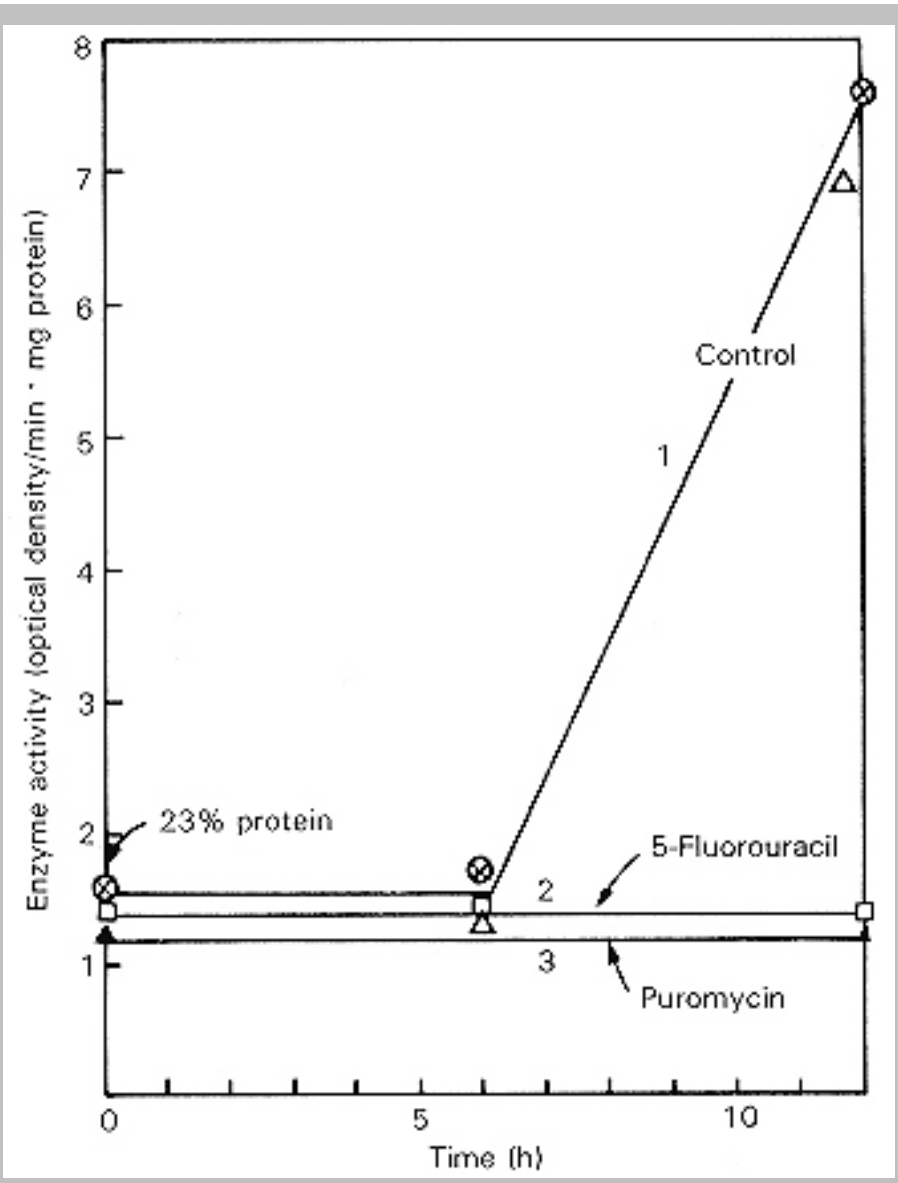


Fig. 8 Effects of inhibitors of protein synthesis or RNA in the induction of xanthine oxidase. Data from Rowe and Wyngaarden (1966), expressed per milligram of liver protein. Reproduced by permission.

The results shown in Fig. 8 confirm that the increase in enzyme activity is the result of an increased enzyme synthesis. The controls, originally maintained on a diet containing 8% protein, are transferred to a 23% protein diet at 0 time. They exhibit an increase in xanthine oxidase activity as previously shown (curve 1). Puromycin (curve 3), 5-fluorouracil (curve 2), or actinomycin D (not shown) block the increase. Puromycin blocks protein synthesis, whereas 5-fluorouracil and actinomycin D block RNA synthesis. The control is therefore transcriptional. Similar results are obtained for the induction of rat liver serine dehydratase (Jost et al., 1968).

Many other types of controls are likely to involve a mechanism at the level of transcription. For example, the synthesis by guinea pig peritoneal cells of one of the serum proteins associated with the inflammatory response (protein C4) seems to depend on a factor that switches on the production of C4. The response can be inhibited by actinomycin D (Cahoun and Hatfield, 1975). Similarly, purine biosynthesis in hepatoma cells in tissue culture is repressed by adenine (Martin and Owen, 1972). The derepression is

sensitive to both actinomycin D and cycloheximide (the latter also blocks protein synthesis). Since the amount of mRNA coding for tryptophan pyrrolase parallels the amount of enzyme induced, the control is probably transcriptional (Schutz et al., 1975). A number of hormonal controls are thought to be exerted at the transcriptional level and some examples are shown in Table 8. Enzyme production is not always affected by the mechanisms controlling transcription. In some cases, the control is probably at a posttranscriptional level, as also shown in this table.

An increase in specific mRNA in response to a hormone has been clearly demonstrated in the induction of yolk proteins by estrogen in rooster liver. DNA synthesized by reverse transcriptase (cDNA) from purified specific mRNA can recognize the mRNA in hybridization tests, where nucleic acid strands combine by virtue of their complementarity (see Chapter 1). The mRNA used as a template is not difficult to isolate by standard procedures, provided that it is present in the tissue in sufficient amounts. In vitro, translation systems can confirm the nature of the mRNA isolated. After production of the cDNA, the template RNA is removed by degradation in alkali, and DNA polymerase is used to replicate the missing complementary strand of the DNA duplex. The duplex inserted into a vector, either a bacterial plasmid or a phage, can reproduce multiple copies or clones in a bacterial host. Hybridization of the cDNA bound to a filter can then be used to recognize the appropriate mRNA.

Table 7 Effect of Diet of Specific Enzyme activity and ^{14}C-Labelled Amino Acid Incorporation in Xanthine Oxidase

Group	Specific activity [optical density min^{-1}(mg enzyme)$^{-1}$]	Enzyme specific radioactivity [counts min^{-1} (mg protein)$^{-1}$]
Control (23% protein diet)		
1	2000	170
2	3260	195
8% Protein diet		
3	2000	158
4	3180	200

8% Protein diet for 14 days-shifted to 23% protein diet for 12 h	2100	778
5	3245	900
6		

Administration of estrogen induces the production of egg yolk protein. Figure 9 (Wiskocil et al., 1980) shows various specific mRNAs as a function of time in the liver of roosters after a single injection of estrogen. The mRNA for serum albumin remains relatively unchanged (curve 1). However, the mRNAs for the yolk proteins apoVLDLII (curve 2) and vitellogenin (curve 3) are induced. Interestingly, after the initial synthesis they are also rapidly degraded suggesting that both transcription and degradation of the mRNA play a regulatory role.

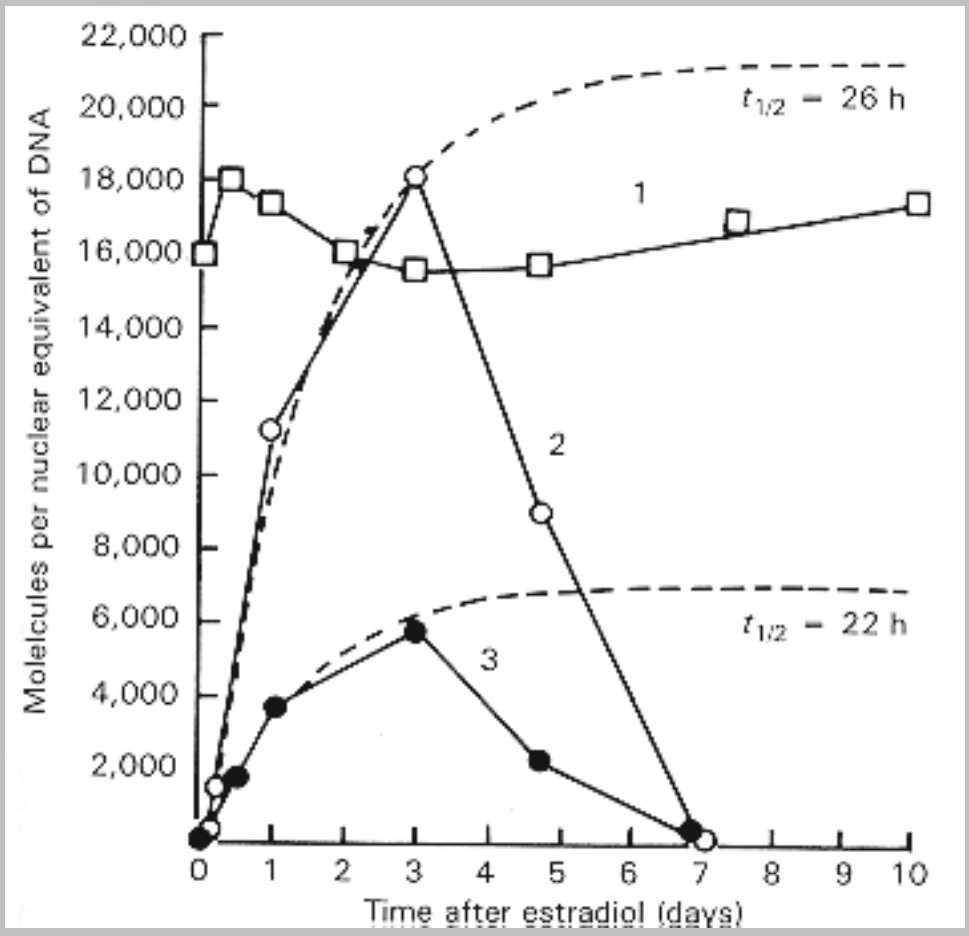


Fig. 9 Levels of apoVLDLII mRNA, vitellogenin mRNA,and serum albumin mRNA during 10 days after

primary stimulation with 17-estradiol. Absolute levels of serum albumin mRNA (□), apoVLDLII mRNA (◌), and vitellogenin mRNA (●) after injection of hormone. The data are expressed as molecules of mRNA per nuclear equivalent of DNA. The theoretical accumulation curves (--) were calculated from data of Wiskocil et al., with permission, *Proceedings of the National Academy of Sciences*, 77:4474-4478, 1980.

Table 8 Hormonal Control of Macromolecular Synthesis[a]

		Evidence for level of control	
Hormone	**Experimental system or target organ**	**Transcriptional**	**Translational**
Protein Hormones			
Growth	Rat tissue (liver muscle and others)	RNA ↑(including mRNA), RNA polymerase ↑	In vitro stimulation of protein synthesis [b]
Insulin	Muscle	RNA ↑, RNA polymerase ↑	Actinomysin D inability to block some actions, RNA and ribosome interaction aided[b]
Peptide hormones			
ACTH	Adrenals	Not clear	b
TSH	Thyroid	RNA ↑	b
LH	Ovaries	Not clear	b
Thyroxine	Rat and amphibian liver	RNA ↑, including mRNA	Amino acid incorporation before increased transcription and with administration to cell-free system[b]
Steroid			
Androgen	Accessory sex tissue	RNA ↑, RNA polymerase ↑, ribosomes ↑	b
Estrogen	Uterus	RNA ↑, actinomycin D inhibition, increased template activity of chromatin, appropriate RNA mimics the affect of hormone, RNA polymerase ↑	b

		New class of RNA (hybridization) RNA polymerase ↑	
		Inhibitors of RNA or protein synthesis block effects, new mRNA appears for one induced enzyme	b
	Chick oviduct	Appearance of new mRNA	New mRNA appears for one induced enzyme[c,d,e,]
		RNA ↑	
Corticosteroids		Specific RNA ↑	
	Embryonic chick retina	Increased template activity, hormone blocks mRNA synthesis	f
		Actinomycin D inhibition of hemoglobin synthesis	f
		RNA ↑ in chromosomal loci	b
	Rat kidney cortex	Actinomysin D inhibition of avidin synthesis, RNA polymerase ↑, new RNA made (hybridization)	b
	Kidney cortex		g
	Chick embryo		In vitro simulation of cytoplasmic extract
	Retina, liver		b
Ecdysone	Thymus nuclei		b
Progesterone	Erythoid cells		
	*Calliphora*larvae		
	Chick oviduct		

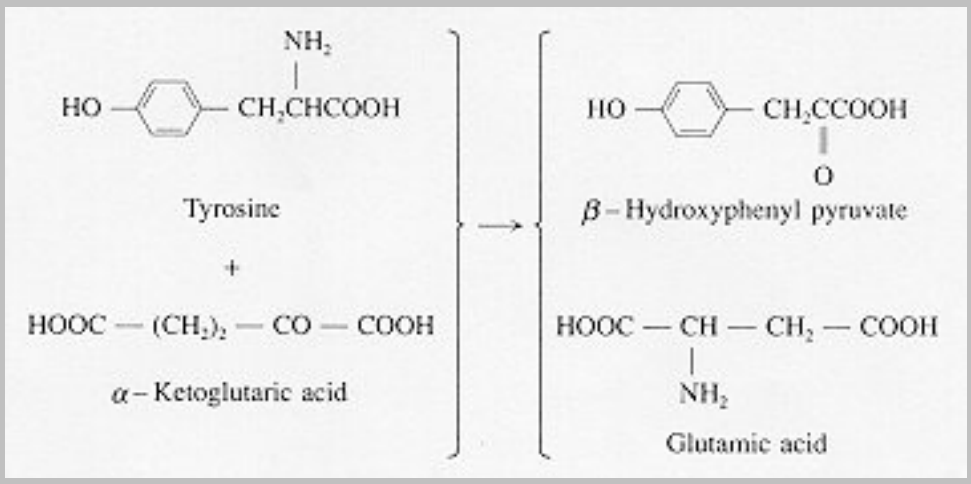
[a] Upward arrow indicates increases

[b]O'Malley, B.W., *Trans. N.Y. Acad. Scie.* 31:478-503 (1969)
[c]Moscona, A.A. et al., *Proc. Natl. Acad. Sci. USA* 61: 160-167 (1968)
[d]Reif-Lehrer, L. and Amos, H., *Biochem. J.* 106: 425-430 (1968)
[e]Schwartz, R.J., *Nature, New Biol.* 237: 121-125 (1972)
[f]Congote, L.F. and Trachewsky, D., *Biochem. Biophys. Res. Comm.*. 46: 957-971 (1972)
[g]Abraham, A.D. and Sekeris, C.E., *Biochem. Biophys. Res. Comm.* 47: 562 (1971)

B. Posttranscriptional Control

Transcriptional control mechanisms seem to play a prominent role in the regulation of enzyme synthesis. As outlined in Fig. 6, a number of regulative events may occur after transcription. Several experiments indicate that the specific degradation of mRNA is significantly involved in the regulation (Styles et al., 1976). Conceivably, the rate of synthesis of an enzyme could also be regulated by changing the rate at which the translational events take place. The molecular mechanics of translation are discussed in Chapter 3, section III and some examples at the molecular level of posttranscriptional regulation in Chapter 3, Section IVB).

The induction of tyrosine transaminase in rat liver or rat liver tumor cells (HTC cells) by adrenal steroid hormones or their analogs (e.g., dexamethasone phosphate), suggests that the control is exerted after the formation of the appropriate mRNA.

(6) 

The transamination of tyrosine is represented in Eq.(6). In this reaction, the amino group of the amino acid is transferred to α-ketoglutaric acid with the formation of β-hydroxyphenyl pyruvate and glutamic acid. The β-hydroxyphenyl pyruvate can be broken down by several oxidative steps that eventually lead to the formation of acetoacetate, which is metabolized by the mitochondria after the appropriate activation reaction. The regulation of tyrosine transaminase may shed some light on the physiological mode of action of the adrenal steroid hormones, as mentioned for tryptophan pyrrolase. The experiment illustrated in Fig. 10 (Thompson et al., 1966) shows that induction of the enzyme in HTC cells is triggered by the presence of dexamethasone phosphate. The inducer brings about a 20-fold increase in enzyme activity after a lag period of 1.5 to 2h. The induction occurs in the presence of actinomycin D (Tomkins et al., 1969). Immunological precipitation (as described in Fig. 1 for the tryptophan pyrrolase assay) demonstrates that the induction corresponds to actual formation of the enzyme and not to activation of a preexisting molecule (Granner et al., 1968). The degradation of the enzyme, which normally takes 3 to 7 h, seems to be unchanged by administration of the drug (Auricchio et al., 1969; Martin et al., 1969).

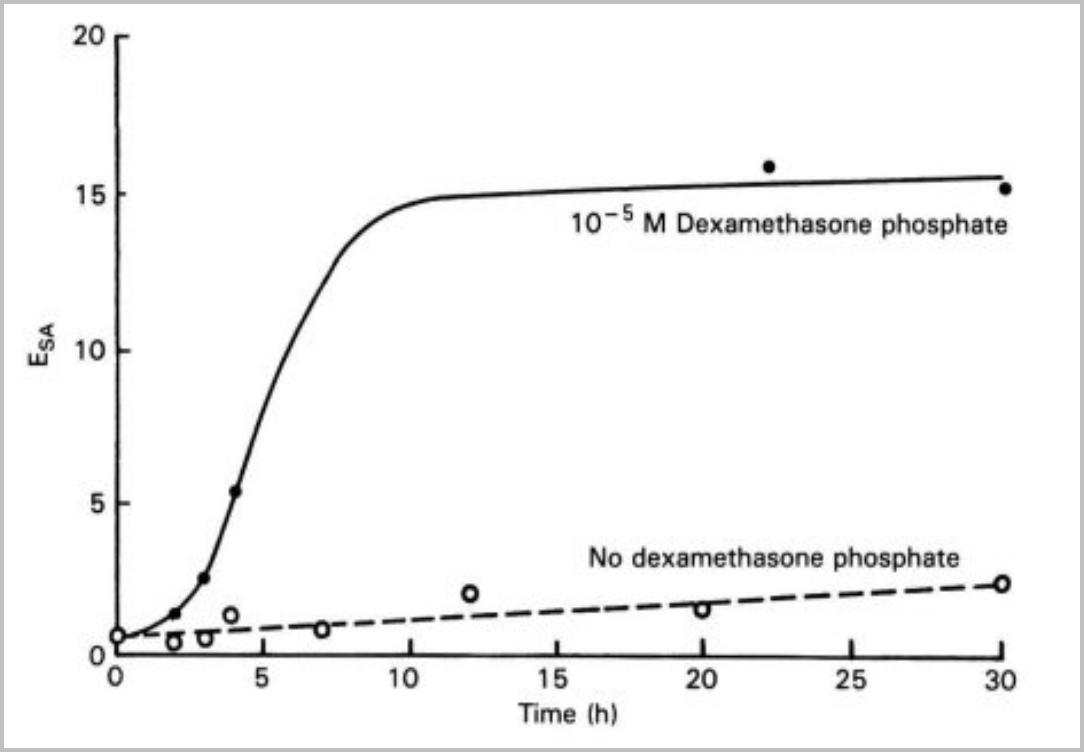


Fig. 10 Kinetics of induction of tyrosine transaminase activity in HTC cells at 37C. E_{SA} refers to tyrosine transaminase specific activity. Reproduced with permission from E. B. Thompson, et al., *Proceedings of the National Academy of Sciences*, 56:296-303, 1966.

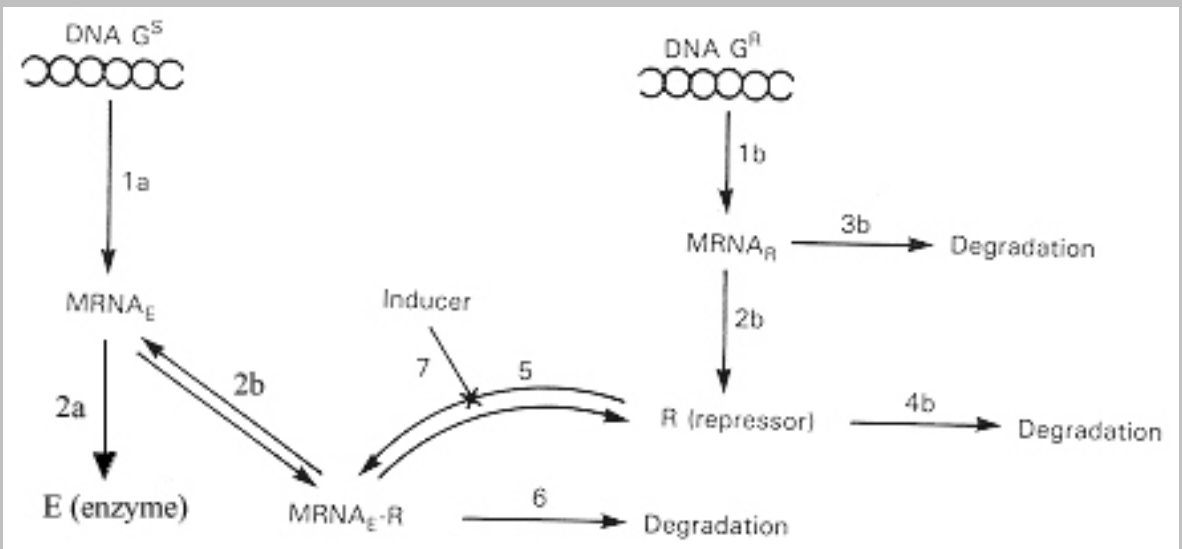


Fig. 11 Model to explain the observed results in the regulation of tyrosine transaminase production (see text).

The data presently available permit the formulation of a model, shown in Fig. 11, which has some similarities to that presented for the *lac* operon of *E. coli*. However, it also has a number of significant differences. The enzyme is coded by gene G^S and the repressor by G^R (Fig. 11). Both genes are transcribed to produce the corresponding mRNAs ($mRNA_E$ and $mRNA_R$, respectively) in steps 1a and 1b of Fig. 11. The two mRNAs can be translated to produce enzyme (E) or repressor (R) (steps 2a and 2b).

The repressor acts by binding to the mRNA (reaction 5), thereby making it unavailable for production of the enzyme. In this model, the inducer (adrenal steroid) can complex with a protein repressor, thus removing its inhibitory ability to bind to the mRNA coding for the tyrosine transaminase (step 7). Normally, the mRNA repressor combination is degraded more rapidly than the free mRNA. The model assumes that the repressor and the mRNA corresponding to the repressor are labile (reactions 3b and 4b in Fig. 11).

These features have been proposed on the basis of several experiments. The addition of cycloheximide to the HTC cells inhibits protein synthesis by about 97%. Naturally, this technique blocks induction, since the enzyme cannot be formed (mutt and Kipnis, 1972). However, on washing the cells and adding the inducer (dexamethasone phosphate), the system is again capable of responding. If the inducer is added after washing, without pretreatment with cycloheximide, induction occurs after a lag period of 1.5 to 2 h (Fig. 12a curve 2) (Peterkofsky and Tomkins, 1968). The washing itself has no effect on the induction, as shown in curve 1. After the cycloheximide is washed off in the presence of inducer, the cells synthesize the enzyme (Fig. 12b). When the inducer is added after washing, the customary lag period occurs (curve 2). However, when the inducer and cycloheximide are present from the beginning, the synthesis of the enzyme no longer involves a lag period; it begins without delay (curve 4). In fact, synthesis occurs when the inducer, present in the original incubation medium together with cycloheximide, is washed away and is not reintroduced (curve 1). The cycloheximide alone has no significant effect on induction (curve 3). The accumulation of the capacity to produce the enzyme in the absence of protein synthesis suggests that it is the result of the accumulation of mRNA.

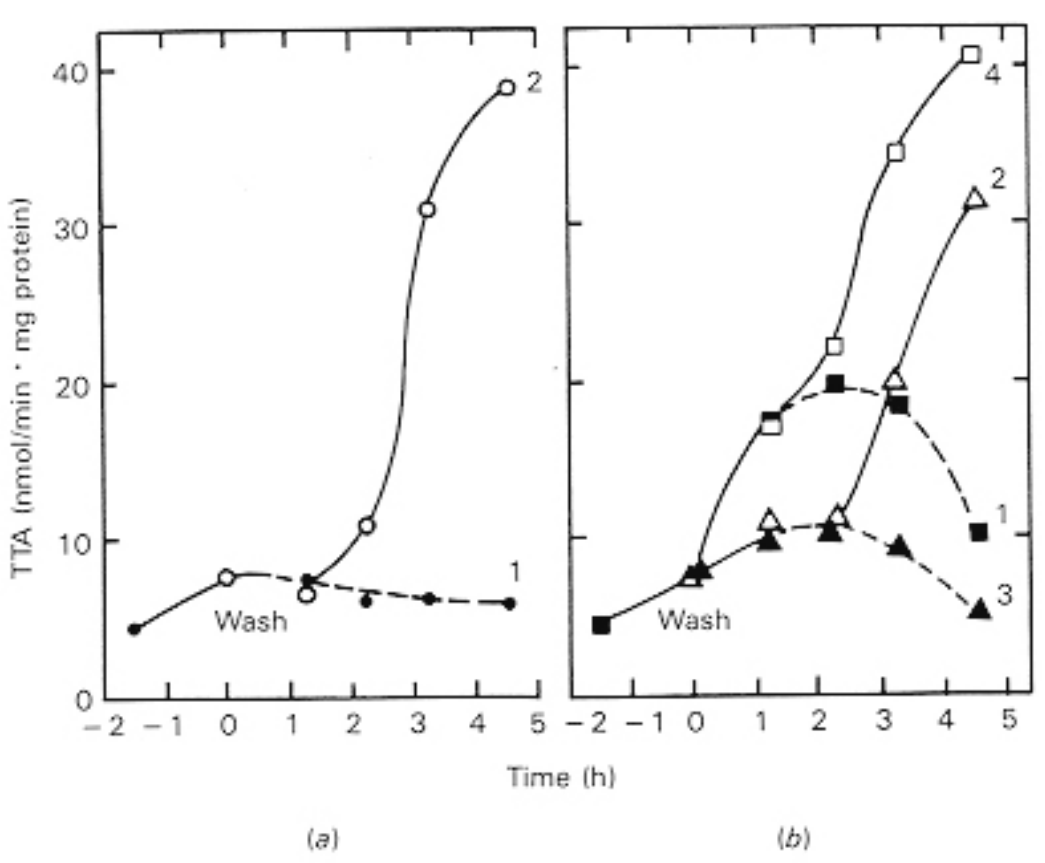


Fig. 12 Level of tyrosine transaminase (TTA) activity after preincubation of HTC cells with or without various additions. Cells were preincubated for 1.5 h, washed with medium, and reincubated. (a) Curve 1 (●), no preincubation addition, no reincubation addition; curve 2 (◌), no preincubation addition, Dex addition at reincubation. (b) Curve 1 (■), preincubation CH + Dex addition, no reincubation addition; curve 2 (▲), preincubation CH addition, Dex addition reincubation; curve 3 (▲), preincubation CH addition, no reincubation addition, curve 4 (❐), preincubation CH + Dex addition, Dex addition reincubation. Dex, dexamethasone phosphate; CH, cycloheximide. Reproduced with permission from B. Peterkofsky and G. M. Tomkins, *Proceedings of the National Academy of Sciences*, 60:222-228, 1968.

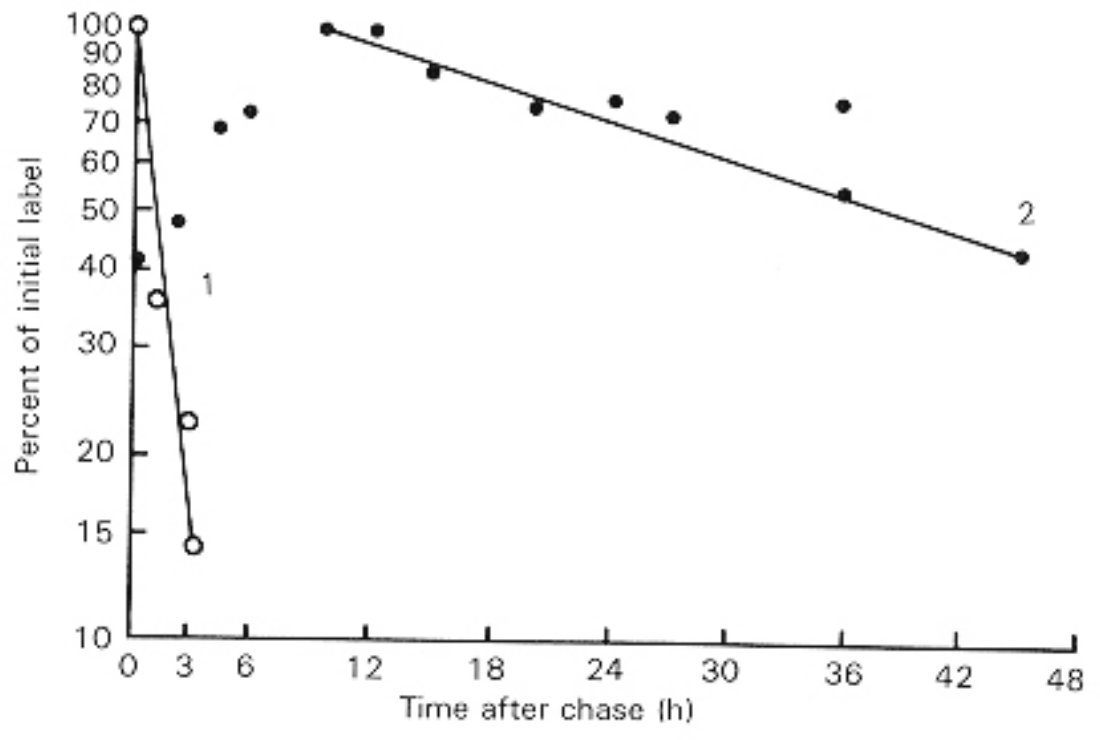


Fig. 13 Degradation of casein mRNA. Both control (curve 1) and experimental (in the presence of prolactin, curve 2) runs were in the presence of insulin and hydrocortisone. In the absence of prolactin, the half-life corresponds to 1.1 h, and in its presence to 28.5 h. Reproduced with permission from Guyette et al. (1979); copyright © 1979 by Cell Press.

A role of mRNA degradation in posttranscriptional regulation has been shown directly in a number of cases. Chapter 3 discusses the control of tubulin production by the level of tubulin itself. This mechanism operates through the degradation of its mRNA. The mRNA of breast cells is also regulated primarily by controlling the rate of its degradation. Casein is the major protein in milk secreted by breast cells. The secretion is hormonally controlled in part by prolactin. Cultured breast cells respond to the presence of prolactin by increasing the casein mRNA 100-fold. However, the nuclei of breast cells can increase the synthesis of casein mRNA only three-fold in response to prolactin -- apparently, the major effect is reduction of the degradation of mRNA. Results of an experiment showing this are presented in Fig. 13 (Guyette et al., 1979).

In this experiment, cultured breast cells were pulse-chased with [^{3}H]-labeled uridine in the absence (curve 1) or presence of prolactin (curve 2). The casein mRNA (ordinate) was estimated from the radioactivity hybridized to the corresponding cDNA probes (seeChapter 1). The points correspond to cell samples withdrawn at different times. The results show that prolactin blocks the degradation of casein mRNA.

Various hormones alter the half-life of specific mRNAs, indicating that this control mechanism is common. Some of these hormones and the corresponding mRNA half-lives are shown in Table 9 (Shapiro and Brock, 1985).

Table 9 Biological Systems That Exhibit Regulation of mRNA Stability

mRNA	Tissue	Regulatory signal	mRNA Half-life[a]	
			+Effector	-Effector
Vitellogenin	*Xenopus* liver	Estrogen	+E: 500 h	-E: 16 h
Vitellogenin	Rooster liver	Estrogen	+E: ~24 h	-E: &lt3 h
Ovalbumin, conalbumin	Hen oviduct	Estrogen, progesterone	+E: ~24 h	-E: 2-5 h
Casein	Rat mammary gland	Prolactin	+Pro: 92h	-Pro: 5h
Prostatic steroid-binding protein	Rat ventral prostate	Androgen	$\Delta t_{1/2}$ ~30x	
Lactate dehydrogenase, A subunit	Rat C6 glioma cells	cAMP, isoprotenerol, dibutyl cAMP	+Ipt: 2.5 h	-Ipt: 45 min
β-Interferon	Human fibroblasts	Poly(I.C)+cycloheximide or Newcastle virus	(IC)+ChX or (IC)+N.V., $t_{1/2}$&gt12 h	(IC) $t_{1/2}$&lt30 min
Histones	HeLa cells	DNA replication	During replication 11 h after 13 min	

Histones	Yeast	DNA replication	During replication ~15 min after ~5 min
Adenovirus 1A (9S), 1B (14S)	HeLa cells	Early/late infection	Late, 60-100 min; early, 6-10 min
L3 (ribosomal protein)	Yeast	mRNA overproduction	$\Delta t_{1/2}$~2x
γ integrase (int)	γ infected *E. coli*	Early/late infection (terminator read through)	$\Delta t_{1/2}$~10x

[a]Abbreviations: E: estrogen; pro: prolactin; Ipt: isoprotenerol; IC: poly(IC); ChX: cycloheximide; NV: Newcastle virus.

Reproduced by permission from: D.J. Shapiro and M.l. Brock, *Biochem. Action of Hormones* 12 :139-172 (1985).

Obviously, the regulation of mRNA degradation must have a prominent role in gene expression. Some insight into this aspect is provided by studies carried out with *Saccharomyces cerevisiae* (see Herrick et al., 1990; Wang et al., 2002) which reveal that mRNA of housekeeping genes (i.e., those involved in the day to day tasks for survival of cells) have fairly invariant and prolonged life-spans. In contrast, the mRNAs whose half-lives differ in response to external signals have much shorter life-spans, as we have already seen (e.g., the case of casein mRNA shown in Fig.23). In the study of Wang et al., the rate of decay of mRNA was studied in a massive survey after blocking mRNA production by thermal inactivation of a temperature-sensitive RNA polymerase II. The individual mRNAs were recognized with DNA microarrays representing over four thousand genes (Wang et al., 2002). The half-lives were found to be variable, in the range of 3 more than 90 minutes. The decay rates of mRNAs of proteins present in stoichiometric complexes were very close. Physiological function was found to be most closely related to mRNA decay rates. Not surprisingly, malfunctions in the mechanisms that regulate mRNA stability have been implicated in disease (see Chen and Shuyu, 1995; Conne et al., 2000).

What is responsible for the degradation of the RNAs? Two major pathways have been found. However, the rate of degradation of mRNA is determined by the mRNA molecules themselves and proceeds at the same rate irrespective of pathway.

Specific sequences which affect the decay rates of mRNA have been identified in yeast by deletions and point mutations. Instability elements occur throughout the length of the transcripts. There is also evidence that the degradation may depend on the translational process.

The major pathway of mRNA degradation in *Saccharomyces cerevisiae* first shortens the 3' poly(A) tail. This is followed by removal of the cap at the 5' end, followed by 5' to 3' degradation (see Caponigro and Parker, 1996). Sm-like (Lsm) proteins related to the Sm proteins, play a major role in decapping, whereas the Sm-proteins are active in splicing (e.g., Stevens and Abelson, 1999). Lsm and Sm proteins contain a common sequence (the Sm sequence). Mutations in seven Lsm proteins in yeast, block mRNA decapping. These proteins also co-precipitate with the mRNA decapping enzyme (Dcp1), a decapping activator and mRNA. The Lsm complex that functions in mRNA decapping is different from the U6-associated Lsm complex suggesting that Lsm proteins form distinct complexes affecting different mRNA-reactions (Tharun et al., 2000).

A complex of 3'-to-5' exonucleases, the *exosome*, has been found to have a role in either processing RNAs to shorter forms or degrading them (e.g., see van Hoof and Parker, 1999). The exosomes are needed for maturation of 5.8S rRNA (Mitchell et al., 1996, Mitchell et al., 1997) that requires the removal of 140 nucleotides from the 3' end of an intermediate. In addition, the complex is active in the degradation of mRNA (Jacobs et al., 1998). Exosomes are present in the nucleus and in the cytoplasm (e.g., Allmang et al., 1999a; Zanchin and Goldfarb, 1999). In *Saccharomyces cerevisiae*, the exosome is formed by a minimum of 10 proteins (Allmang et al., 1999a). The nuclear exosome has an extra subunit. The molecular weight of the complex suggests that each subunit is represented once in the 300-400 kDa complex (Mitchell et al., 1997). Each subunit is thought to have 3'-to-5' exoribonuclease activity. Six of these are phosphorylytic enzymes: they attack phosphate during RNA hydrolysis, resulting in the production of nucleotides 5' diphosphates. Each of the subunits are essential for function, possibly because of a failure to assemble if one of them is missing: the inactivation of any one of them produces a failure of exosome function (e.g., Jacobs et al., 1998; Allmang et al., 1999a and b). It is also possible that each subunit carries out a specific step in the degradation (see van Hoof and Parker, 1999). Similar complexes are present in other eukaryotes, including humans. The system has been shown to operate under conditions in which the deadenylation-decapping mechanism is blocked. In addition, the 3'-to-5' mechanism has been shown to be present while 5'-to-3' degradation is proceeding. In yeast, the exosome pathway also requires the product of three other genes: *SKI2, SKI3* and *SKI8* (Jacobs et al., 1998). These may be involved in the assembly of the exosome or may modify the mRNA to facilitate interaction between the RNA molecules and the exosomes.

Since *Saccharomyces cerevisiae* strains lacking both the 3'-to-5' and the 5'-to-3' RNA-degradation pathways cannot survive, it is likely that the two are the major RNA-degradation pathways of the cells.

In yeast, unspliced pre-mRNAs are degraded in the nucleus in the 3'-to-5' direction by the exosome and in the 5' to 3' direction by an exonuclease (Rat1p), with the exosome pathway predominating (Bousquet-Antonelli et al., 2000). Defective pre-mRNAs are degraded. In addition, the degradation of normal pre-mRNA takes place in competition with splicing and is physiologically regulated, for example, it is increased by glucose. Inhibition of pre-mRNA degradation increases pre-mRNAs and consequently spliced mRNAs. Therefore this pathway appears to be part of the machinery regulating transcription. Similar mechanisms may be present in mammalian cells since it has been observed that defective pre-

mRNA are degraded mostly in the nucleus (see Hentze and Kulozik, 1999)

A close regulatory interaction between transcription and degradation is also suggested by the finding that in yeast the mRNA deadenylases Crc4p and Caf1p bind to transcription factors (e.g., Chang et al., 1999; Tucker et al., 2001). In fact, the corresponding genes (CCR4 and CAF1) have been shown to affect the initiation of transcription of some genes .

In vertebrates, similar mechanisms are thought to be operating. Deadenylation is one of the processes involved in mRNA degradation. A poly(A)-specific deadenylating nuclease (poly(A) ribonuclease) is involved in mammals and *Xenopus* (e.g., Martinez et al., 2000). Decapping is likely to be the next step since decapped mRNA intermediates have been isolated from mouse liver cells (Couttet et al., 1997). It is not clear however whether the rest of the mRNA is degraded by a 5'-to-3' or a 3'-to-5' exoribonuclease.

In general, the poly(A) tails play a major role in mRNA degradation. The tails block mRNA decay by binding to the *poly(A)-binding protein* (PABP) (Bernstein et al., 1989). In addition, PABP binds to the initiation translation factor eIF4G which complexes to the cap-binding protein eIF4E which promotes translation (Gingras et al., 1998). These complexes block access to the deadenylation and decapping enzymes. The involvement of proteins active in the process of translation also suggest a role of translation on mRNA stability. In fact, inhibition of translation initiation destabilizes mRNA (Schwartz and Parker, 1999), whereas inhibiting translational elongation favors mRNA stabilization (Beelman and Parker, 1994)

Cytokines, proto-oncogene and transcription factors mRNAs are rapidly degraded (see Chen and Shyu, 1995), a process requiring *AU-rich elements* (AREs) in the 3' untranslated region (3'UTR). Exactly how this happens is a matter of conjecture. However, the degradation depends on the binding of the AU-rich region by a AU-binding protein, AUF1 (also known as *heterogeneous ribonucleoprotein D* (hnRNP D) (Laroia et al., 1999). AUF1 complexes with heat shock proteins hsc70-hsp70, translation initiation factor eIF4G and poly(A) binding protein. eIF4G is displaced from AUF1 and then AUF1 is ubiquitinated. The AUF1 is degraded by proteasomes. Interference with any of these processes interferes with the degradation. The ARE-binding proteins also determine their subcellular localization and translation of the mRNA. Other sequence elements with similar functions are also known and some of these are located in the 5'UTRs (see Wilusz et al., 2001).

Some proteins that shuttle between the nucleus and the cytoplasm have been found to have a role in the localization of the mRNA (which in many cases is translated at a specific cytoplasmic target site), its translation and its turnover (see Shyu and Wilkinson, 2000). Possibly the best understood role is that of the hnRNPs, which are also involved in a variety of functions such as transcriptional regulation, maintenance of telomere length, alternative pre-RNA splicing and processing of the 3' end (see Krecic and Swanson, 1999) (see also Chapter 5).

hnRNPs have been found to accompany mRNAs as they exit the nucleus and to shuttle between the

nucleus and the cytoplasm. They control the localization of the mRNA in the cytoplasm, its translation and its turnover (see Shyu and Wilkison, 2000). For example, the localization of the mRNA for myelin basic protein (MBP) depends of hnRNP A2 as shown, for example, when antisense oligonucleotides (see Chapter 1) complementary to hnRNP A2 mRNA, inhibited the efflux of MBP mRNA and a reporter RNA with an A2 binding site (Munro et al., 1999). Since many proteins are translated only after their mRNA has arrived at the intended target, it is not surprising that hnRNP A2 also has a role in the regulation of translation (Kwon et al., 1999). In one case, hnRNP was found to be involved in translational silencing. hnRNP K and E1 were found to block the translation of 15-lipoxygenase (an enzyme involved in mitochondrial membrane breakdown during erythroid cell differentiation) by inhibiting the assembly of 80S ribosomes on the mRNA coding for the oxygenase (Ostareck et al., 1997).

Nonsense-mediated decay (NMD) eliminates faulty transcripts with premature termination codons (see Hentze and Kulozik, 1999). An involvement of an hnRNP-like protein (HRP1) is shown by the fact that a mutation in HRP1 stabilizes nonsense-containing mRNAs and abolishes its binding to the defective mRNA (Gonzalez et al., 2000). At least in yeast, NMD depends on a *downstream sequence element* (DSE), and an *hnRNP-like factor*, Hrp1. Apparently, when DSE is located within normal coding sequences the ribosome blocks the interaction between Hrp1 and DSE. However, with a prematurely terminated translation the DSE is in the untranslated region and the binding of Hrp1 triggers the degradation events (Gonzalez et al., 2000) (see Wilusz et al., 2001).

C. Regulation in Skeletal Muscle

Mammalian skeletal muscle has to adapt to changing conditions, such as exercise, by undergoing changes in the kind of myosin present in its fibers and in the metabolic pathway favored.

Skeletal muscle fibers differ in their speed of contraction from very slow (type 1) to very fast (type 2B) depending on the type of myosin heavy chain present (see Schiaffino et al., 1989; Bottinelli et al., 1994). There are four major kinds of myosin heavy chains. In contrast, endurance of a muscle fiber does not depend on the myosin component but on the availability of ATP. Type 2B fibers have a high level of glycolytic enzymes and can provide ATP rapidly from glycogen. However, the supply of glycogen is limited. Type 1 fibers depend mostly on oxidative-phosphorylation, which produces ATP more slowly. With increased stimulation, muscle fibers can go from one phenotype to another, for example, from type 2B to type 1 (see Gorza et al., 1988; Windisch et al., 1998).

What mediates these changes? Several myogenic transcription factors are known to regulate muscle gene expression (see Molkentin and Olson, 1996) and may respond to electrical activity. One of these factors is *myogenin*. The overexpression of myogenin in transgenic mice (see Chapter 1) (Hughes et al., 1999) produced as much as three fold elevation of the oxidative-phosphorylation levels of fast fibers, accompanied by a decrease in glycolytic enzymes. In this particular study, there was no alteration in the myosin types.

V. Circadian Rhythms

Daily rhythms, the *circadian* rhythms, are characteristic of all living matter (see Pittendrigh, 1993; Takahashi, 1995). They have three basic properties that include a self-sustained oscillation with a period of approximately 24 hours in an organism's activity. The activity can be expressed in an animal's behavior or an organism's physiology or biochemistry. The oscillations are intrinsic and persist even under constant conditions. The cycle is synchronized (*entrained*) (see below) in both period and phase by environmental cycles of light or temperature. The circadian rhythm compensates the period length of the rhythms for temperature changes. The role of circadian rhythms in human physiology, disease and the efficacy of drugs is well recognized (see Kraft and Martin, 1995).

A. Clock mechanisms

The general components of circadian systems that have been shown to be universal, center on a circadian pacemaker or clock that generates the oscillations. Input pathways convey environmental information for *entraining* (i.e. setting) the pacemaker (see below), while efferent pathways communicate the rhythms to the rest of the organism. Although the pacemakers may have common elements in different organisms, the input and the efferent pathways differ significantly from organism to organism.

A circadian oscillator is a structure which expresses self-sustained oscillation. A pacemaker is a circadian oscillator that drives a cyclic process. The circadian oscillators are cell based. This is not only clear from unicellular organisms but also has been shown in primary cell cultures of a single cell type. Furthermore, in the case of the marine mollusc, *Bulla*, cultured, isolated single cells express circadian rhythms (Michel et al., 1993). The oscillatory system of the *suprachiasmatic nucleus* (SCN) of mammals has entire clock elements in individual neurons (Welsh et al., 1995; Liu et al., 1997c).

In mammals, the pacemaker has been shown to be localized in the brain in the SCN of the hypothalamus. For example, surgical removal of the SCN in hamsters eliminates rhythmicity. Neuronal grafts from other animals reestablish the rhythm that corresponds to that of the donor (Ralph et al., 1990). Although the SCN is recognized as the main center responsible for rhythmicity, there are other independent centers. It has become increasingly apparent that clock mechanisms function independently in a variety of tissues and can be independently regulated (that is entrained, see below) by different signals. In the golden hamster, a circadian oscillator that operates independently from the SCN has been found in the retina (Tosini and Menaker, 1996). The mouse oscillator gene, *Clock*, is expressed in a variety of tissues examined (King et al., 1997; Stockan et al., 2001). Similarly, the *per1* gene, also involved in rhythmicity (see below), is expressed in a variety of tissues in the rat . In the latter experiments, the *per1* promoter was linked to a luciferase reporter. The activation of of *per1* could be monitored by recording light emission of the luciferase system in vitro (Yamazaki et al., 2000). When the feeding was restricted to a limited time each day, the rhythmicity in the SCN remained entrained by the light-dark cycle. However, liver cells were entrained rapidly to the feeding schedule (Stokkan et al., 2001). In addition, in the vascular system, the clock mechanism is regulated by retinoic acid via the appropriate nuclear receptors

(see Chapter 7) (McNamara et al., 2001) and a clock mechanism in the mammalian forebrain (Reick et al., 2001) is regulated by the redox state of nicotinamide adenine dinucleotide (NAD) cofactors, supposedly in response to metabolic conditions and may constitute a system of intracellular redox sensors. (Rutter et al., 2001). These results indicate that a peripheral clock can behave quite independently of the SCN. The presence of several oscillators may be a general biological phenomenon in multicellular organisms. In *Drosophila melanogaster*, independent oscillators are present throughout the fly's tissues (Plautz et al., 1997).

In mammals, the neurons of the SCN secrete neuropeptides with circadian rhythmicity. The SCN also signals the pineal gland to secrete melatonin (see Foulkes et al., 1997). Several inputs impinge on the SCN, including visual clues from the retina which in mammals entrain circadian rhythms to the light-dark cycle (see below).

Molecular components of circadian oscillators (see Dunlap, 1996) must: (a) show a rhythm in activity or amount corresponding to the overall rhythm, (b) be affected by mutational changes such that some basic property of the clock is also changed, (c) have some sort of feed-back regulation at the molecular level, (d) respond to abrupt changes in the component by resetting the clock, (e) stop the clock when clamped at any level, and (f) reset the phase of the component within one cycle with changes in light-dark cycle (reflecting the entrainment properties of the clock).

Macromolecular synthesis is inextricably involved in circadian rhythms and is required for the functioning of the circadian pacemaker, as well as for the input and the efferent pathways. The dependence of the oscillator on protein synthesis is shown by the fact that single-gene mutations affect the circadian period. In addition, pulses of inhibitors of protein synthesis applied at critical periods cause phase shifts. The effects of inhibitors of RNA synthesis also cause similar phase shifts. The application of inhibitors for long periods stops the pacemaker entirely. Similarly, entrainment depends on transcriptionally controlled protein synthesis. This is shown by the fact that light stimuli induce the expression of transcription factors at the site of the oscillator (such as the SCN in mammals). The need for expression of certain genes for entrainment has been shown using antisense oligonucleotides (Wollnick et al., 1994). These presumably block gene expression by hybridizing to mRNA (see Chapter 1). In mammals, the DNA-arrays technique (see Chapter 1) has allowed recognizing the expression of 650 genes involved in circadian rhythm in the SCN and the liver. Approximately 650 transcripts were implicated. Only 28 were found to be common to both tissues (Panda et al., 2002), indicating that the two are regulated independently

Many of the proteins involved in the oscillations are similar in various organisms, although their role may be quite different. In order to distinguish the genes or proteins, those of *Drosophila* are preceded by the letter *d*, those of mice by *m* and *h*denotes the human variety.

In *Drosophila* (see Hall, 1995), the circadian rhythm depends on two genes, the *period*, *per* gene (Konopka and Benzer, 1971) and the *timeless*, *tim* gene. Nonsense mutations in *per*, result in loss of

locomotor rhythm and single amino acid replacements alter the length of the cycle (see Takashi, 1995). The PER protein is controlled transcriptionally and by degradation, as witnessed by the short half-life of its mRNA (Hardin et al., 1992). Mutations of the *tim* gene block the oscillations in *per* expression. Similarly, the cyclic expression of *tim* is affected by *per* mutants (see Reppert and Sauman, 1995). As suggested by the mutual dependence of their expression, the TIM protein binds the PER protein to form a dimer, which is active in cyclicity. PER contains a domain referred to as the PAS domain needed for interaction between two proteins (Huang et al., 1993) that is characteristic of clock proteins. However, the PAS domain is not present in TIM (see Reppert and Sauman, 1995).

In *Drosophila* present evidence agrees with the diagram represented in Fig. 14. The positive transcriptional regulation is from dCLOCK (dCLK) and dCYCLE (dCYC also called dBMAL1) (coded by the genes *dclk* and *dcyc*). dCLK and BMAL1 have basic helix-loop-helix (bHLH)and PAS domains. The bHLH domains bind to a DNA sequence (referred to as an E box). The PAS domain is needed for dimerization (see Chapter 6). The two proteins heterodimerize and the complex binds to E box enhancers in the promoter region of the genes [(1)in Fig. 14](Hao et al., 1997; Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998)and activate the transcription of genes coding for proteins required for function (e.g., in eclosion behavior and locomotion) (2) and the clock genes *per* and *tim* (3). The RNA and protein of these genes oscillate and the cycling depends on dimerization and entry of the PER and TIM proteins(4) into the nucleus (5). PER and TIM function as negative regulators of their own transcription, forming a feedback loop (6). In the nucleus, these proteins inhibit transcription of the corresponding genes by interfering with CLK-BMAL1-mediated transcription (Darlington et al., 1998; Lee, 1999)). Thus, dCLK drives the expression of *per* and *tim*, and in turn PER and TIM inhibit dCLK's activity and close the feedback loop. The return to dCLK's activity requires the decrease in concentration of PER. Therefore, the degradation of PER is as necessary as its initial expression. The proteasome inhibitors lactacystin, a microbial metabolite (Fenteany et al., 1995) and MG115, a synthetic peptide aldehyde inhibitor (Rock et al., 1994), were applied to the central nervous system of *Drosophila* larvae and found to inhibit the degradation of TIM accompanying light pulses (used to modify the timing of the clock, see below) (Naidoo et al., 1999). These results implicate the proteasome in clock function.

Other findings have added a complexity to this simple picture. The transcription of another gene, *vri*, cycles in phase with *per* and *tim* (Blau and Young, 1999) and is directly regulated by the transcription factors dCLK and dCYC. *vri*-less mutants block *per* and *tim* expression. When the *vri* cycle is interfered with, *per* and *tim* expression is suppressed and long-period behavioral rhythms and arrhythmicity take place, suggesting the *vri* is an essential part of the *Drosophila* clock. Oscillations in the absence of light require the kinase DOUBLE-TIME (DBT) which controls the phosphorylation and stability of PER. Without DBT, PER proteins accumulate and the clock is stopped (Price et al., 1998; Kloss et al., 1998).

Genetics have been applied to mammalian circadian systems only recently (see Dunlap, 1999). In the SCN, the basic elements determining the circadian rhythm are conserved in mammals [PER, TIM, CLK and CYC (BMAL)]. The NPAS2 gene encodes an analog of Clock. which expressed differently in various tissues (e.g., Reick et al., 2001). However, there are three *per* genes expressed in the SCN of

mammals that are differentially regulated by light (e.g., Shearman et al, 1997; Takumi et al., 1998) (*per3* is expressed independently of light). As in *Drosophila*, in mammals, CLK/BMAL1 also drive transcription of *mper* (Jin et al., 1999). The mouse PER proteins have a PAS domain and a bHLH domain (Albrecht et al., 1997; Antoch et al., 1997). The bHLH domain is absent in the *Drosophila* PER. *mper3* knockout mice have a normal clock (Shearman et al., 2000) whereas *a mper2* mutants have been found to have a shortened circadian period with loss of rhythmicity in the dark (Zheng et al., 1999). Null mutations in the *mper1* gene produces a shorter circadian period with less precision. Mice deficient in both *mper1* and *mper2* display no circadian rhythms. mPER2 acts at the transcriptional level. In contrast mPER1 regulates mPER2 at the posttrascriptional level. mPER1 and mPER2 regulate clock-controlled genes independently (Zheng et al., 2001). In contrast to the behavior in *Drosophila* (Section B, below), the *cryptochrome* genes (*mcry1* and *mcry2*) are essential for circadian behavioral rhythms (van der Horst et al., 1999; Vitaterna et al., 1999) (see below). They are also transcriptionally controlled by CLOCK-BMAL1 heterodimers (e.g., Jin et al., 1999). mPER and mCRY feed-back inhibit the transcription mediated by CLOCK/BAML1 (e.g., Kume et al., 1999). The role of *mtim* is unknown (see Field et al., 2000) but does not seem to be involved in the oscillations, although mTIM inhibits the CLK.BMAL1 transcriptional activation (e.g., Jin et al., 1999; Kume et al. 1999). In mammals, PER-TIM complexes are not detected (Kume et al., 1999).

In mammals, the *casein kinase I* ε (*cki* ε) gene corresponds to the *dbt* gene of *Drosophila* (Lowrey et al., 2000). *tau* is a mutant of *ckiε* with reduced phosphorylating ability. The casein kinase lε enzyme (CKI), a serine/threonine kinase, binds and phosphorylates mammalian PER (Vielhaber et al., 2000). Supposedly, when the kinase phosphorylates PER1, it masks the nuclear localization sequence (NLS) and PER1 remains in the cytoplasm. Furthermore, the phosphorylation of PER decreases its stability (Price et al, 1998). With a defective kinase (e.g., in *tau* mutants), the PER moves to the nucleus more rapidly with the consequent early repression of the transcription of *per* genes. A mutation in human *per2* (*hper2*) has ben shown to speed up the circadian clock in people suffering from *familial advanced sleep phase syndrome* (Toh et al., 2001). In individuals with this syndrome, a serine was replaced by glycine in the casein kinase I ε (CKI ε) binding region of hPER2, which causes hypophosphorylation of the protein.

In the fungus *Neurospora*, the *frequency*, *frq* gene is a major part of the oscillator (see Luo et al., 1998). Expression of *frq* at the wrong time interrupts the rhythm. The mRNA from *frq* produces two distinct proteins by alternative initiation of mRNA translation (Liu et al., 1997b). The choice of protein depends on temperature. In addition, several phosphorylated versions of these two proteins may act as distinct entities (Garceau et al., 1997). The FRQ protein has properties of transcription factors, such as a nuclear localization sequence (NLS) (see Chapter 5) and a DNA binding domain. A negative loop is also present in *Neurospora*: FRQ negatively regulates the level of its own transcription. The proteins encoded by the *white collar 1* and *white collar 2* genes favor the transcription of *frq* (see Crosthwaite et al., 1997). The FRQ protein depresses the level of *frq* transcription, probably by interfering with the activation of the transcription by *White collar 1* and *White collar 2* protein.

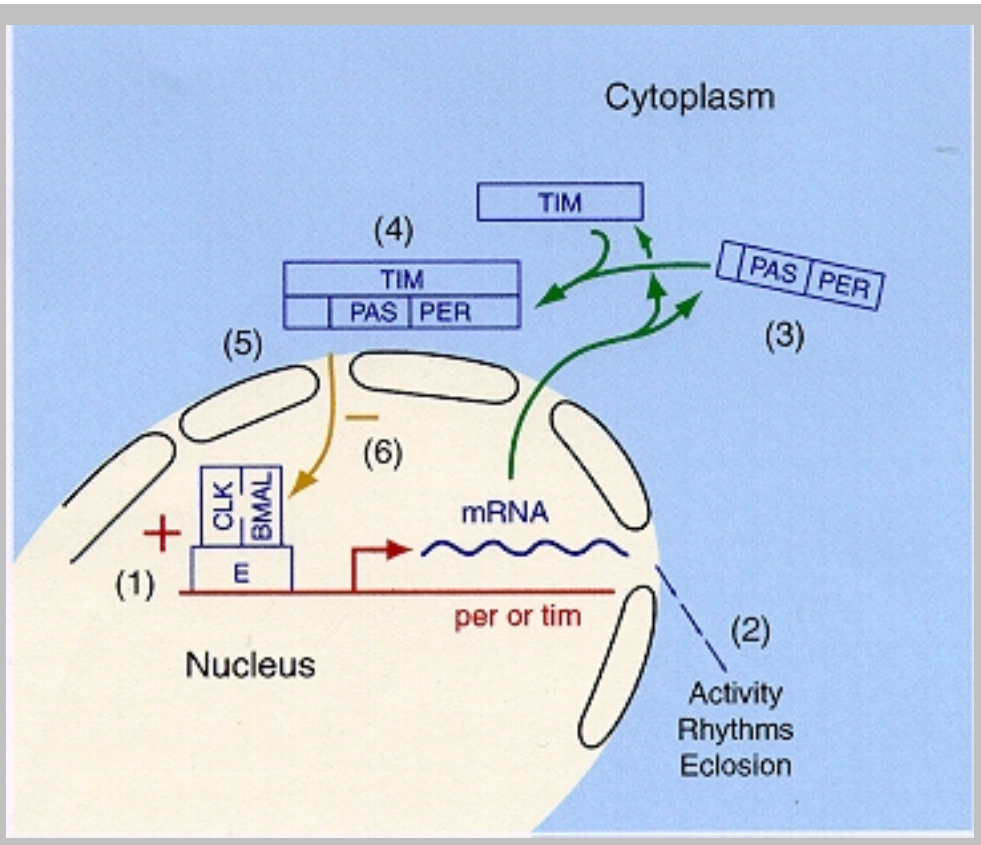


Fig. 14 Diagram representing the feedback control of circadian rhythmicity in *Drosophila*. The proteins coded by *clock* and *bmal* continuously activate the transcription of *per* or *tim* (1). The mRNA from these genes code for the proteins responsible for activity and eclosion (2) and in addition for proteins (3) that upon phosphorylation dimerize (4) are translocated into the nucleus (5) and block the action of the activators (6).

B. Entrainment

Entrainment sets the clock in response to external clues. The major factor determining entrainment in plants and animals is light, in particular blue light (of a wavelength of 400-500 nm). Therefore, pigments capable of absorbing this wave-length range have been looked for some time. Some of these are *cryptochromes* (CRYs), pterin/flavin containing proteins (e.g., see Cashmore et al., 1999). CRY receives the light information and transmits it to the core oscillator in plants (Somers et al., 1998) and *Drosophila* (Stanewsky et al., 1998; Emery et al., 1998; Egan et al., 1999). However, in mammals the nature of photoreceptor molecules in entrainment is still obscure (see below).

The plant, *Arabidopsis*, has two cryptochrome genes and five genes for *phytochrome* photoreceptors (see Quail et al., 1995). Phytochrome protein is a protein dimer of 120 kDa each. The prosthetic group of phytochrome is an open chain tetrapyrrole attached to the protein as a cysteine thioester. It absorbs light mostly in the red and also to some extent in the blue range. Entrainment in *Arabidopsis* has an input from both kinds of photoreceptors (Somers et al., 1998). Similarly in *Drosophila*, CRY and *rhodopsin* also

function in entrainment (see Stanewsky et al., 1998). Redundancy in photoreceptors is also found in non-mammalian vertebrates (see Yoshikawa and Oishi, 1998) and, as discussed below, is likely to be the case in rodents.

In *Drosophila* embryonic cells in culture (Ceriani et al., 1999), CRY was shown to block the function of PER-TIM heteromeric complexes in a light dependent fashion and to block the degradation of TIM. CRY and TIM are part of the same complex. After absorption of light, PER-TIM and CRY are found in the nucleus. The CRYs bind to the corresponding PER proteins and translocate each from cytoplasm to nucleus. The interaction between the light-activated CRY and TIM, blocks the negative feedback effect of the PER-TIM complex (shown diagramatically in Fig. 14). In the peripheral tissues of *Drosophila*, CRY has also been implicated in the clock mechanism independently from photoreceptor function. A CRY mutation has been found that does not affect the circadian oscillator function of the central circadian pacemaker neurons but makes the peripheral circadian oscillators arrhythmic (Krishnan et al., 2001). Note that in mice, a role of CRY1 and CRY 2 in the clock is also likely (see below).

As in other systems, entrainment of the SCN clock in mammals depends mostly on light, with the primary effect exerted by light exposure during the "night" phase of the spontaneous rhythm. When the animal is maintained in continuous darkness, light exposure during the early "night" (that is the portion of the spontaneous oscillation corresponding to early night) causes phase delays, whereas late "night" exposure causes advancements (De Coursey, 1960). The information is conveyed to the clock from the retina by the retinal projections of the retinohypothalamic tract (e.g., Johnson et al., 1988) and is mediated by the neurotransmitter glutamate (e.g., see Ding et al., 1994).

The study of entrainment has provided a number of insights on the process (see Ding et al., 1994). The direct application of glutamate to the SCN brain slices induces light-like clock resetting (Ding et al., 1994). The data indicate that resetting of the clock, following light stimulation, first activates glutamate receptors by glutamate originating from the retinal projections. This is then followed by Ca^{2+} influx in the cytoplasm and subsequent activation of nitric oxide synthase. NO may stimulate guanylyl cyclase (see Chapter 7). The increase in cGMP activates cGMP-dependent protein kinase (PKG) (see Chapter 7). Activation of the cGMP pathways advances the clock at night, but not during the day (e.g., Liu et al, 1997a). Injection of a PKG specific inhibitor was found to block light induced advances in glutamate-induced effect at late night (e.g., Weber et al., 1995). The early "night" effect is not blocked by the PKG inhibitor, but the effect apparently depends on intracellular Ca^{2+} stores (see Chapter 7) and the Ca^{2+} release mediated by the ryanodine receptors (Ding, 1998). The results indicate that there are two divergent pathways for the early and late "night" light exposure. The light-induced resetting at late "night" involves a pathway where cGMP advances the clock. The early "night" light effect follows a distinct pathway where Ca^{2+} release delays the clock. In mammals, the entrainment of the SCN must transmit some sort of signal to the peripheral tissues under its control (see Rusak and Zucker, 1979).

Since light signals entrain the clock, in mammals the stimulus is likely to come through the eyes as might

be expected. With some exceptions, rodents and humans without eyes are unable to set the clock (see Foster, 1998). However, rods and cones are not involved. A gene that destroys retinal rod and cone cells in mice was unable to stop the clock, as seen from running behavior (Freedman et al., 1999) or the secretion of melatonin (Lucas et al., 1999). Therefore, under these conditions, other photoreceptors are suspected as responsible for entrainment, and the cryptochromes are natural candidates in view of their role in other organisms. CRY1 and CRY2 were found to be expressed in the ganglion cells of the retina (Miyamoto and Sancar, 1998; Thresher et al., 1998). In addition, CRY1 is expressed at a high level in the SCN and oscillates in this tissue in a circadian manner. In mutants lacking either CRY1 or CRY2 (van der Host et al., 1999), the free-running periodicity of locomotor function was accelerated or delayed respectively. These data indicate antagonistic clock-adjusting functions, which are essential for circadian rhythmicity. When both proteins were absent, under dark-dark conditions the, free running rhythmicity was entirely lost indicating that CRY1 and CRY2 must have an important function in the clock mechanism itself. However, under light-dark conditions, the double mutants exhibited a 24 hours rhythm, demonstrating that a light input is still present and therefore a pigment other than CRY1 or CRY2 must be involved. In mice (Kume et al., 1999), CRY1 and CRY2 bind to the PER proteins and translocate them from cytoplasm to nucleus. The PER and CRY proteins appear to inhibit the transcriptional complex differentially. Either CRY1 or CRY2 alone block CLK.BMAL mediated transcription. Present data indicates that in mice, CRYs play a role in the negative feed-back loop of the clock mechanism. However, a role in light-entrainment is also possible as shown by some of the studies already discussed. In mammals, molecular clues to a possible role of photoreceptor molecules in light-entrainment would require a study of events taking place in the ganglion cells of the retina. To our knowledge, these have not been carried out.

As already discussed, in mammals peripheral tissues can express circadian rhythms independently of the SCN. Not surprisingly, entrainment can also occur in a tissue specific manner. Liver cells can be entrained to a feeding schedule (Stockan et al., 2001). In addition, in vascular cells, the binding to ligand activated retinoic acid receptors (RAR α and RXR α) bind to the protein NPAS2 or CLOCK and thereby negatively regulate the CLOCK/MOP4:BMAL1 induced transcription and act as a negative-feedback loop (McNamara et al., 2001) (see Fig. 14). In addition, at least in neural tissues, the DNA-binding activity of the Clock/BMAL1 and NPAS2/BMAL1 is regulated by the redox state of nicotinamide adenine dinucleotide (NAD) cofactors in a purified system. The reduced forms of the redox cofactors, NAD(H) and NADP(H), strongly enhance DNA binding of the Clock/BMAL1 and NPAS2/BMAL1, whereas this binding is inhibited by the oxidized (Rutter et al., 2001) (see Fig. 14).

The situation is quite different in zebrafish. Zebrafish tissues in vivo or culture show endogenous oscillations (Whitmore et al., 1998). The peripheral organ clocks have been found to be set by light-dark cycles either in culture or in derived cell lines in culture (Whitmore et al., 2000).

The resetting of circadian clocks due to temperature changes is also an important factor in entrainment (e.g., see Pittendrigh, 1960). High temperatures elicit responses similar to light and low temperatures elicit those elicited by darkness (e.g., Zimmerman et al., 1968). Levels of FRQ protein were measured in

Neurospora at various temperatures (Liu et al., 1998). The amount of FRQ oscillates at higher concentrations when the temperature is higher. However, the level of *frq*-mRNA remains unchanged, indicating that the effect is posttranscriptional. The same concentration of FRQ corresponds to different times at different temperatures. Therefore, temperature shifts result in shifts in clock time without immediate synthesis of FRQ.

SUGGESTED READING

Protein and RNA Degradation

Baumeister, W., Walz, J., Zuhl, F. and Seemuller, E. (1998) The proteasome: paradigm of a self-compartmentalizing protease, *Cell* 92:367-380 (Medline)

Caponigro, G. and Parker, R. (1996) Mechanisms and control of mRNA turnover in *Saccharomyces cerevisiae*, *Microbiol. Rev.* 60: 233-249. (Medline)

Ciechanover, A., Orian, A., and Schwartz, A.L. (2000) Ubiquitin-mediated proteolysis: biological regulation via destruction, *BioEssays* 22:442-451. (MedLine)

Hargrove, J. L. and Schmidt, F. H. (1989) The role of mRNA and protein stability in gene expression, *FASEB J.* 3:2360-2370.(Medline)

Olson, T. C. and Dice, J. F. (1989) Regulation of protein degradation rates in eukaryotes, *Curr. Opin. Cell Biol.* 1:1194-1200.(Medline)

Control of Protein Synthesis and Folding

Bukau, B. and Horwich, A.L. (1998) The Hsp70 and Hsp60 chaperone machines, *Cell* 92:351-366. (MedLine)

Frydman, J. (2001) Folding of newly translated proteins in vivo: the role of molecular chaperones, *Annu. Rev. Biochem.* 70:603-647. (MedLine)

Merrick, W.C. (1992) Mechanism and regulation of eukaryotic protein synthesis, *Microbiol. Rev.* 56:291-315.(Medline)

Circadian Rhythms

Cashmore, A.R., Jarillo, J.A., Wu, Y.J. and Liu, D. (1999) Cryptochromes: blue light receptors for plants and animals, *Science* 284:760-765. (Medline)

Dunlap, J.C. (1999) Molecular bases of circadian clocks, *Cell* 96:271-290. (Medline)

King, D.P. and Takahashi, J.S. (2000) Molecular genetics of circadian rhythms in mammals, *Annu. Rev. Neurosci.* 23:713-742. (MedLine)

Pittendrigh, C.S. (1993) Temporal organization: reflections of a Darwinian clock-watcher, *Annu. Rev. Physiol.* 55:16-54. (Medline)

Reppert, S. (1998) A clockwork explosion! *Neuron* 21:1-4. (Medline)

Takahashi, J.S. (1995) Molecular neurobiology and genetics of circadian rhythms in mammals, *Annu. Rev. Neurosci.* 18:531-553. (Medline)

REFERENCES

Search the textbook

Back to Chapter 15

REFERENCES

Adams, J., Palombella, V.J., Sausville, E.A., Johnson, J., Destree, A., Lazarus, D.D., Maas, J., Pien, C.S., Prakash, S. and Elliott, P.J. (1999) Proteasome inhibitors: a novel class of potent and effective antitumor agents, *Cancer Res.* 59:2615-2622.(Medline)

Albrecht, U., Sun, Z.S., Eichele, G. and Lee, C.C. (1997) A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light, *Cell* 91:1055-1064.(Medline)

Allada, R., White, N.E., So, W.V., Hall, J.C. and Rosbash, M. (1998) A mutant *Drosophila* homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless, *Cell* 93:791-804.(Medline)

Allmang, C., Petfalski, E., Podtelejnikov, A., Mann, M., Tollervey, D. and Mitchell, P. (1999a) The yeast exosome and human PM-Scl are related complexes of 3' --> 5' exonucleases, *Genes Dev.* 13:2148-2158.(Medline)

Allmang, C., Kufel, J., Chanfreau, G., Mitchell, P., Petfalski, E. and Tollervey, D. (1999b) Functions of the exosome in rRNA, snoRNA and snRNA synthesis, *EMBO J.* 18:5399-5410.(Medline)

Antoch, M.P., Song, E.J., Chang, A.M., Vitaterna, M.H., Zhao, Y., Wilsbacher, L.D., Sangoram, A.M., King, D.P., Pinto, L.H. and Takahashi, J.S. (1997) Functional identification of the mouse circadian Clock gene by transgenic BAC rescue, *Cell* 89:655-667.(Medline)

Arlt, H., Tauer, R., Feldmann, H., Neupert, W. and Langer, T. (1996) The YTA10-12 complex, an AAA protease with chaperone-like activity in the inner membrane of mitochondria, *Cell* 85:875-885. (MedLine)

Arlt, H., Steglich, G., Perryman, R., Guiard, B., Neupert, W. and Langer, T. (1998) The formation of respiratory chain complexes in mitochondria is under the proteolytic control of the m-AAA protease, *EMBO J.* 17:4837-4847. (MedLine)

Auricchio, F., Martin, D., Jr. and Tomkins, G. (1969) Control of degradation and synthesis of induced tyrosine aminotransferase studied in hepatoma cells in culture, *Nature* 224:806-808.(Medline)

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W. and Elledge, S.J. (1996) SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box, *Cell* 86:263-274.(Medline)

Bukau, B. and Horwich, A.L. (1998) The Hsp70 and Hsp60 chaperone machines, *Cell* 92:351-366. (MedLine)

Ballard, F. J. (1977) Intracellular protein degradation, *Essays Biochem.* 13:1-37.(Medline)

Barrett, A. J. (1980) The many forms and functions of cellular proteinases, *Fed.Proc.* 39:9-l4.(Medline)

Baumeister, W., Walz, J., Zuhl, F. and Seemuller, E. (1998) The proteasome: paradigm of a self-compartmentalizing protease, *Cell* 92:367-380.(Medline)

Beck, T., Schmidt, A. and Hall, M.N. (1999) Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast, *J. Cell Biol.* 146:1227-1238. (MedLine)

Beelman, C.A. and Parker, R. (1994) Differential effects of translational inhibition in cis and in trans on the decay of the unstable yeast MFA2 mRNA, *J. Biol. Chem.* 269:9687-9692. (MedLine)

Bernstein, P., Peltz, S.W. and Ross J (1989) The poly(A)-poly(A)-binding protein complex is a major determinant of mRNA stability in vitro, *Mol .Cell. Biol.* 9:659-670. (MedLine)

Blau, J. and Young, M.W. (1999) Cycling *vrille* expression is required for a functional *Drosophila* clock, *Cell* 99:661-671.(Medline)

Bonifacino, J.S. and Klausner, R.D. (1994) Degradation of proteins retained in the endoplasmic reticulum in, *Cellular Proteolytic Systems* (Ciechanover, A.J. and Schwartz, A.L., eds.) p. 137-160, Wiley-Lis, New York.

Bonifacino, J.S. and Weissman, A.M. (1998) Ubiquitin and the control of protein fate in the secretory and endocytic pathways, *Annu. Rev. Cell. Dev Biol.* 1998;14:19-57.(Medline)

Bottinelli, R., Canepari, M., Reggiani, C. and Stienen, G.J. (1994) Myofibrillar ATPase activity during isometric contraction and isomyosin composition in rat single skinned muscle fibres, *J. Physiol. (London)* 481:663-675.(Medline)

Bousquet-Antonelli, C., Presutti, C. and Tollervey, D. (2000) Identification of a regulated pathway for nuclear pre-mRNA turnover, *Cell* 102:765-775. (MedLine)

Brannigan, J.A., Dodson, G., Duggleby, H.J., Moody, P.C.E., Smith, J.L., Tomchick, D.R. and Murzin, A.G. (1995) A protein catalytic framework with an N-terminal nucleophile is capable of self-activation, *Nature* 378:416-419.(Medline)

Braun, B.C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P.M., Finley, D. and Schmidt, M. (1999) The base of the proteasome regulatory particle exhibits chaperone-like activity, *Nature Cell Biol.* 1:221-226.(Medline)

Brodsky, J.L. and McCracken, A. A. (1997) ER-associated and proteasome-mediated protein degradation: how two topologically restricted events came together, *Trends in Cell Biol.* 7:151-156.

Buchberger, A. (2002) From UBA to UBX: new words in the ubiquitin vocabulary, *Trends in Cell Biol.* 12:216-221.

Bukau, B. and Horwich, A.L. (1998) The Hsp70 and Hsp60 chaperone machines, *Cell* 92:351-366.(Medline)

Busch, H. and Goldknopf, I.L. (1981) Ubiquitin-protein conjugates, *Mol. Cell. Biochem.* 40:173-187.(Medline)

Cahoun, D. H. and Hatfield, G. W. (1975) Autoregulation of gene expression, *Annu. Rev. Microbiol.* 29:275-299.

Caponigro, G. and Parker, R. (1996) Mechanisms and control of mRNA turnover in *Saccharomyces cerevisiae*, *Microbiol. Rev.* 60: 233-249.(Medline)

Carter, R.E. and Sorkin, A. (1998) Endocytosis of functional epidermal growth factor receptor-green fluorescent protein chimera, *J. Biol. Chem.* 273:35000-35007.

Casari, G., De Fusco, M., Ciarmatori, S., Zeviani, M., Mora, M., Fernandez, P., De Michele, G., Filla, A., Cocozza, S., Marconi, R., Durr, A., Fontaine, B. and Ballabio A. (1998) Spastic paraplegia and OXPHOS impairment caused by mutations in paraplegin, a nuclear-encoded mitochondrial metalloprotease, *Cell* 93:973-983. (MedLine)

Cashmore, A.R., Jarillo, J.A., Wu, Y.J. and Liu, D. (1999) Cryptochromes: blue light receptors for plants and animals, *Science* 284:760-765.(Medline)

Cenciarelli, C., Wilhelm, J.K.G., Guo, A. and Weissman, A.M. (1996) T cell antigen receptor ubiquitination is consequence of receptor-mediated tyrosine kinase activity, *J. Biol. Chem.* 271:87090-87130.(Medline)

Ceriani, M.F., Darlington, T.K., Staknis, D., Mas, P., Petti, A.A., Weitz, C.J. and Kay, S.A. (1999) Light-dependent sequestration of TIMELESS by CRYPTOCHROME, *Science* 285:553-556.(Medline)

Chang, M., French-Cornay, D., Fan, H.Y., Klein, H., Denis, C.L. and Jaehning, J.A. (1999) A complex containing RNA polymerase II, Paf1p, Cdc73p, Hpr1p, and Ccr4p plays a role in protein kinase C signaling, *Mol. Cell Biol.* 19:1056-1067. (MedLine)

Chang, C., Gonzalez, F., Rothermel, B., Sun, L., Johnston, S.A. and Kodadek, T. (2001) The Gal4 activation domain binds Sug2 protein, a proteasome component, in vivo and in vitro, *J. Biol. Chem.* 276:30956-30963. (MedLine)

Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K., and Varshavsky, A. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein, *Science* 243:1576-1583.(Medline)

Chen, A.C.-Y. and Shyu, A.-B. (1995) AU-rich elements: characterization and importance in mRNA degradation, *Trends Biochem. Sci.* 20:465-470. (MedLine)

Chen, Z., Hagler, J., Palombella, V.J., Melandrini, F., Scherer, D., Ballard, D. and Maniatis, T. (1995) Signal-induced site-specific phosphorylation targets IkBα to the ubiquitin-proteasome pathway, *Genes Dev.* 9:1586-1597.(Medline)

Ciechanover, A., Hod, Y. and Hershko, A. (1978) A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes, *Biochem. Biophys. Res. Commun.* 81:1100-1105. (MedLine)

Ciechanover, A., Elias, S., Heller, H., Ferber, S. and Hershko, A. (l980a) Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes, *J. Biol. Chem.* 255:7525-7528.(Medline)

Ciechanover, A., Heller, H., Elias, S., Haas, A. L. and Hershko, A. (l980b) ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation, *Proc. Natl. Acad. Sci. USA* 77:1365-1368.(Medline)

Ciechanover, A., Finley, D. and Varshavsky, A. (1984) Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85, *Cell* 37:57-66.(Medline)

Conne, B., Stutz, A. and Vassalli, J.D. (2000) The 3' untranslated region of messenger RNA: A molecular 'hotspot' for pathology? *Nature Med.* 6:637-641. (MedLine)

Connell, P., Ballinger, C.A., Jiang, J., Wu, Y., Thompson, L.J., Hohfeld, J. and Patterson, C. (2001) The

co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins, *Nature Cell Biol.* 3(1):93-96. (MedLine)

Couttet, P., Fromont-Racine, M., Steel, D., Pictet, R. and Grange T. (1997) Messenger RNA deadenylylation precedes decapping in mammalian cells, *Proc. Natl. Acad. Sci. USA* 94:5628-5633. (MedLine)

Crosthwaite, S.K., Dunlap, J.C. and Loros, J.J. (1997) Neurospora wc-1 and wc-2: transcription, photoresponses, and the origins of circadian rhythmicity, *Science* 276:763-769.(Medline)

Cyr, D.M., Hohfeld, J. and Patterson, C. (2002) Protein quality control: U-box-containing E3 ubiquitin ligases join the fold, *Trends Biochem. Sci.* 27:368-375. (MedLine)

Darlington, T.K., Wager-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steeves, T.D.L., Weitz, C.J., Takahashi, J.S. and Kay, S.A. (1998) Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*, *Science* 280:1599-1603.(Medline)

De Coursey, P.J. (1960) Daily light sensitivity rhythm in a rodent, *Science* 131:33-35.

Deveraux, Q., Ustrell, V., Pickart, C. and Rechsteiner, M. (1994) A 26 S protease subunit that binds ubiquitin conjugates, *J. Biol. Chem.* 269:7059-7061.(Medline)

DiAntonio, A., Haghighi, A.P., Portman, S.L., Lee, J.D., Amaranto, A.M. and Goodman, C.S. (2001) Ubiquitination-dependent mechanisms regulate synaptic growth and function, *Nature* 412:449-452. (MedLine)

Dice, J. F., Terlecky, S. R., Chiang, H.-L., Olson, T. S. Isenman, L. D., Short-Russell, S. R., Freundlieb, S. and Terlecky, L. J. (1990) A selective pathway for degradation of cytosolic proteins by lysosomes, *Seminars in Cell Biol.* 1:449-455.(Medline)

Ding, J.M, Chen, D., Weber, E.T., Faiman, L.E., Rea, M.A. and Gillette, M.U. (1994) Resetting the biological clock: mediation of nocturnal circadian shifts by glutamate and NO, *Science* 266:1713-1717. (MedLine)

Ding, J.M., Faiman, L.E., Hurst, W.J., Kuriashkina, L.R. and Gillette, M.U. (1997) Resetting the biological clock: mediation of nocturnal CREB phosphorylation via light, glutamate, and nitric oxide, *J. Neurosci.* 17:667-675.(Medline)

Ding, J.M., Buchanan, G.F., Tsichkau, S.A., Chen, D., Kuriashkina, L., Faiman, L.E,, Alster, J.M., McPherson, P.S., Campbell, K.P. and Gillette, M.U. (1998) A neuronal ryanodine receptor mediates light-

induced phase delays in the circadian clock, *Nature* 394:381-384.(Medline)

Dunlap, J.C. (1996) Genetics and molecular analysis of circadian rhythms, *Annu. Rev. Genet.* 30:579-601.(Medline)

Dunlap, J.C. (1999) Molecular bases for circadian clocks, *Cell* 96:271-290.(Medline)

Dunn, R. and Hicke, L. (2001) Multiple roles for Rsp5p-dependent ubiquitination at the internalization step of endocytosis, *J. Biol. Chem.* 276:25974-25981. (MedLine)

Egan, E.S., Franklin, T.M., Hilderbrand-Chae, M.J., McNeil, G.P., Roberts, M.A., Schroeder, A.J., Zhang, X. and Jackson, F.R. (1999) An extraretinally expressed insect cryptochrome with similarity to the blue light photoreceptors of mammals and plants, *J. Neurosci.* 19:3665-3673.(Medline)

Ellgaard, L., Molinari, M. and Helenius, A. (1999) Setting the standards: quality control in the secretory pathway, *Science*286:1882-1888. (MedLine)

Ellgaard, L. and Helenius, A. (2001) ER quality control: towards an understanding at the molecular level, *Curr. Opin. Cell Biol.* 13:431-437. (MedLine)

Emery, P., So, W.V., Kaneko, M., Hall, J.C. and Rosbash, M. (1998) CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity, *Cell* 95:669-679.(Medline)

Felder, S., Miller, K., Moehren, G., Ullrich, A., Schlessinger, J. and Hopkins, C.R. (1990) Kinase activity controls the sorting of the epidermal growth factor receptor within the multivesicular body, *Cell* 61:623-634. (MedLine)

Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J. and Schreiber, S.L. (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin, *Science* 268:726-731.(Medline)

Ferdous, A., Gonzalez, F., Sun, L., Kodadek, T. and Johnston, S.A. (2001) The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II, *Mol. Cell.* 7:981-991. (MedLine)

Ferguson, A. R. and Sims, A. P. (1974) The regulation of glutamine metabolism in *Candida utilis*: the inactivation of glutamine synthetase, *J. Gen. Microbiol.* 80:173-185.(Medline)

Fewell, S.W., Travers, K.J., Weissman, J.S. and Brodsky, J.L. (2001) The action of molecular chaperones

in the early secretory pathway, *Annu. Rev. Genet.* 35:149-191. (MedLine)

Finley, D., Ciechanover, A. and Varshavsky, A. (1984) Thermolability of ubiquitin-activating enzyme from the mammalian cell cycle mutant ts85, *Cell* 37:43-55.(Medline)

Finley, D., Bartel, B. and Varshaky, A. (1989) The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis, *Nature* 338:394-401.(Medline)

Finley, D., Sadis, S., Monia, B.P., Boucher, P., Ecker, D.J., Crooke, S.T.and Chau, V. (1994) Inhibition of proteolysis and cell cycle progression in multiubiquitination-deficient yeast mutants, *Mol. Cell. Biol.* 14:5501-5509.(Medline)

Foster, R.G. (1998) Shedding light on the biological clock, *Neuron* 20:829-832.(Medline)

Foulkes, N.S., Borjigin, J., Snyder, S.H. and Sassone-Corsi, P. (1997) Rhythmic trascription: the molecular basis of circadian melatonin synthesis, *Trends in Neurosci.* 20:487-492.(Medline)

Freedman, M.S., Lucas, R.J., Soni, B., von Schantz, M., Muñoz, M., David-Gray, Z. and Foster, R. (1999) Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors, *Science* 284:502-504.(Medline)

Frydman, J. Folding of newly translated proteins in vivo: the role of molecular chaperones, *Annu. Rev. Biochem.* 70:603-647. (MedLine)

Fucci, L., Oliver, C. N., Coon, N. J. and Stadtman, E. R. (1983) Inactivation of key metabolic enzymes by mixed-function oxidation reactions: possible implications in protein turnover and aging, *Proc. Natl. Acad. Sci. USA* 80:1521-1525.(Medline)

Fujiwara, T., Tanaka, K., Orino, E., Yoshimura, T., Kumatori, A., Tamura, T., Hung, C. H., Nakai, T., Yamaguchi, K., Sin, S., Kakizuka, A., Nakanishi, A. and Ichihara, A. (1990) Proteasomes are essential for yeast proliferation. cDNA cloning and gene disruption of two major subunits, *J. Biol. Chem.* 265:16604-l3.(Medline)

Futter, C.E., Pearse, A., Hewlett, L.J. and Hopkins, C.R. (1996) Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes, *J. Cell Biol.* 132:1011-1023. (MedLine)

Galan, J. and Haguenauer-Tsapis, R. (1997) Ubiquitin lys63 is involved in ubiquitination and endocytosis of a yeast plasma membrane protein, *EMBO J.* 16:5847-5854. (MedLine)

Galan, J.M., Volland, C., Urban-Grimal, D. and Haguenauer-Tsapis, R. (1994) The yeast plasma membrane uracil permease is stabilized against stress induced degradation by a point mutation in a cyclin-like "destruction box", *Biochem. Biophys. Res. Comm.* 201:769-775.(Medline)

Garceau, N.Y., Liu, Y., Loros, J.J. and Dunlap, J.C. (1997) Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY, *Cell* 89:469-476.(Medline)

Geier, E., Pfeifer, G., Wilm, M., Lucchiari-Hartz, M., Baumeister, W., Eichmann, K. and Niedermann, G. (1999) A giant protease with potential to substitute for some functions of the proteasome, *Science* 283:978-981.(Medline)

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S., Weitz, C.J. (1998) Role of the CLOCK protein in the mammalian circadian mechanism, *Science* 280:1564-1569.(Medline)

Gingras, A.C., Raught, B. and Sonenberg, N. (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation, *Annu. Rev. Biochem.* 68:913-963. (MedLine)

Glas, Bogyo, M., McMaster, J.S. Gaczynska, M. and Ploegh, H.L. (1998) A proteolytic system that compensates for loss of proteasome function, *Nature* 392:618-622.(Medline)

Glickman, M.H., Rubin, D.M., Fried, V.A. and Finley, D. (1998a) The regulatory particle of the *Saccharomyces cerevisiae* proteasome, *Mol. Cell. Biol* 18:3149-3162.(Medline)

Glickman, M.H., Rubin, D.M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V.A. and Finley, D. (1998b) A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3, *Cell* 94:615-623.(Medline)

Goldberg, A. L. (1972) Degradation of abnormal proteins in Escherichia coli, *Proc. Natl. Acad. Sci. USA* 69:422-426.(Medline)

Gonzalez, C.I., Ruiz-Echevarria, M.J., Vasudevan, S., Henry, M.F. and Peltz, S.W. (2000) The yeast hnRNP-like protein Hrp1/Nab4 marks a transcript for nonsense-mediated mRNA decay, *Mol. Cell* 5:489-499. (MedLine)

Gonzalez, F., Delahodde, A., Kodadek, T. and Johnston, S.A. (2002) Recruitment of a 19S proteasome subcomplex to an activated promoter, *Science* 296:548-550. (MedLine)

Gorza, L., Gundersen, K., Lomo, T., Schiaffino, S. and Westgaard, R.H. (1988) Slow-to-fast transformation of denervated soleus muscles by chronic high-frequency stimulation in the rat, *J.*

Physiol.(London) 402:627-649.(Medline)

Gottesman, S., Maurizi, M.R. and Wickner, S. (1997) Regulatory subunits of energy-dependent proteases, *Cell* 91:435-438.(Medline)

Goulet, C.C., Volk, K.A., Adams, C.M., Prince, L.S., Stokes, J.B. and Snyder, P.M (1998) Inhibition of the epithelial Na^+ channel by interaction of Nedd4 with a PY motif deleted in Liddle's syndrome, *J. Biol. Chem.* 273:30012-30017.(Medline)

Govers, R., van Kerkhof, P., Schwartz, A.L. and Strous, G.J. (1997) Linkage of the ubiquitin-conjugating system and the endocytic pathway in ligand-induced internalization of the growth hormone receptor, *EMBO J.* 16:4851-4858. (MedLine)

Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A.L. and Strous, G.J. (1999) Identification of a novel ubiquitin conjugation motif, required for ligand-induced internalization of the growth hormone receptor, *EMBO J.* 18:28-36. (MedLine)

Granner, D. K., Hayashi, S.-I., Thompson, E. B. and Tomkins, G. M. (1968) Stimulation of tyrosine aminotransferase synthesis by dexamethasone phosphate in cell culture, *J. Mol. Biol.* 35:291-301.(Medline)

Gregori, L., Poosch, M.S., Cousins, G. and Chau, V. (1990) A uniform isopeptide-linked multiubiquitin chain is sufficient to target substrate for degradation in ubiquitin-mediated proteolysis, *J. Biol. Chem.* 265:8354-8357.(Medline)

Groll, M., Ditzel, L., Löwe, J., Stock, D., Bochtler, M., Bartunik, H.D. and Huber, R. (1997) Structure of the 20S proteasome from yeast at 2.4 Å resolution, *Nature* 386:463-474.(Medline)

Gronostajski, R. M., Pardee, A. B. and Goldberg, A. L. (1985) The ATP dependence of the degradation of short- and long-lived proteins in growing fibroblasts., *J. Biol. Chem.* 260:3344-3349.(Medline)

Gruenberg, J. and Maxfield, F.R. (1995) Membrane transport in the endocytic pathway, *Curr. Opin. Cell Biol.* 7:552-563.

Grune, T., Reinheckel, T. and Davies, K.J.A. (1997) Degradation of oxidized proteins in mammalian cells, *FASEB J.* 11:526-534.(Medline)

Guarante, L. (1984) Yeast promoters; positive and negative elements, *Cell* 36:799-800.

Guyette, W. A., Matusik, R. J. and Rosen, J. M. (1979) Prolactin-mediated transcriptional and

posttranscriptional control of casein gene expression, *Cell* 17:1013-1023.(Medline)

Hall, J.C. (1995) Tripping along the trail to the molecular mechanisms of biological clocks, *Trends Neurosci.* 18:230-240.(Medline)

Hardin, P.E., Hall, J.C. and Rosbash, M. (1992) Circadian oscillations in period gene mRNA levels are transcriptionally regulated, *Proc. Natl. Acad. Sci. USA* 89:11711-11715.(Medline)

Harris, D.A. (1999) Cellular biology of prion diseases, *Clin. Microbiol. Rev.* 12:429-444.(Medline)

Hartl, F.U. (1996) Molecular chaperones in cellular protein folding, *Nature* 381:571-580.(Medline)

Hartl, F.U. and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein, *Science* 295:1852-1858. (MedLine)

Harvey, K.F. and Kumar, S. (1999) Nedd-4-like protein: an emerging family of ubiquitin protein ligases implicated in diverse cellular functions, *Trends Cell Biol.* 9:166-169.(Medline)

Heinemeyer, W., Kleinschmidt, J. A., Saidowsly, C. E. and Wolf. D. H. (1991) Proteinase YScE, the yeast proteasome/multicatalytic - multifunctional proteinase: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival., *EMBO J.* 10:555-562.(Medline)

Helliwell, S.B., Losko, S. and Kaiser, C.A. (2001) Components of a ubiquitin ligase complex specify polyubiquitination and intracellular trafficking of the general amino acid permease, *J. Cell Biol.* 153:649-662. (MedLine)

Hentze, M.W. and Kulozik, A.E. (1999) A perfect message: RNA surveillance and nonsense-mediated decay, *Cell* 96:307-310. (MedLine)

Herrick, D., Parker, R. and Jacobson, A. (1990) Identification and comparison of stable and unstable mRNAs in *Saccharomyces cerevisiae Mol. Cell. Biol.* 10:2269-2284. (MedLine)

Hershey, J. W. (1991) Translational control in mammalian cells, *Ann. Rev. Biochem.* 60:717-755.(Medline)

Hershko, A. (1988) Ubiquitin-mediated protein degradation, *J. Biol. Chem.* 263:15237-15240.(Medline)

Hershko, A. and Ciechanover, A. (1998) The ubiquitin system, *Annu. Rev. Biochem.* 67:425-479. (MedLine)

Hershko, A., Ciechanover, A., Heller, H., Haas, A. L. and Rose, I. A. (1980) Proposed role of ATP in protein breakdown: conjugation of proteins with multiple chains of the polypeptide of ATP-dependent proteolysis, *Proc. Natl. Acad. Sci. USA* 77:1783-1786.(Medline)

Hicke, L. (1999) Gettin'down with ubiquitin: turning off cell-surface receptors, transporters and channels, *Trends Cell Biol.* 9:107-111.(Medline)

Hicke, L. and Riezman, H. (1996) Ubiquitination of yeast plasma membrane receptor signals its ligand-stimulated endocytosis, *Cell* 84:277-287.(Medline)

Hicke, L., Zanolari, B. and Riezman, H. (1998) Cytoplasmic tail phosphorylation of the α-factor receptor is required for its ubiquitination and internalization, *J. Cell Biol.* 141:349-358.(Medline)

Hicke, L. (2001) A new ticket for entry into budding vesicles-ubiquitin, *Cell* 106:527-530. (MedLine)

Hilt, W. and Wolf, D.H. (1992) Stress induced proteolysis in yeast, *Mol. Microbiol.* 6:2437-2442.(Medline)

Hilt, W. and Wolf, D.H. (1996) Proteasomes: destruction as a programme, *Trends in Biochem.. Scie.* 21:96-102.(Medline)

Hochstrasser, M. (1996), Ubiquitin-dependent protein degradation, *Annu. Rev. Genet.* 30:405-439.(Medline)

Hofmann K. and Falquet, L. (2001) A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems, *Trends Biochem. Sci.* 26:347-350. (MedLine)

Hohfeld, J., Cyr, D.M. and Patterson, C. (2001) From the cradle to the grave: molecular chaperones that may choose between folding and degradation, *EMBO Rep.* 2:885-890. (MedLine)

Hough, R., Pratt, G. and Rechsteiner, M. (1987) Purification of two high molecular weight proteases from rabbit reticulocyte lysate, *J. Biol. Chem.* 262:8303-8313.(Medline)

Hsu, C.L. and Stevens, A. (1993) Yeast cells lacking 5'-->3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure, *Mol. Cell. Biol.* 13:4826-4835. (MedLine)

Huang, Z.J., Edery, I. and Rosbash, M. (1993) PAS is a dimerization domain common to Drosophila period and several transcription factors, *Nature* 364:259-262.(Medline)

Hughes, S.M., Chi, M.M., Lowry, O.H. and Gundersen, K. (1999) Myogenin induces a shift of enzyme

activity from glycolytic to oxidative metabolism in muscles of transgenic mice, *J. Cell Biol.* 145:633-642.(Medline)

Huibregtse, J.M., Yang, J.C. and Beaudenon, S.L. (1997) The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase, *Proc. Natl. Acad. Sci. USA* 94:3656-3661.(Medline)

Hügle, B., Kleinschmidt, J.A. and Franke, W.W. (1983) The 22S cylinder particles from *Xenopus laevis* II. Immunological characterization and localization of their proteins in tissues and cultured cells, *Eur. J. Cell Biol.* 32:157-163.(Medline)

Imhof, M.O. and McDonnell, D.P. (1996) Yeast RSP5 and its human homolog hRPF1 potentiate hormone-dependent activation of transcription by human progesterone and glucocorticoid receptors, *Mol. Cell Biol.* 16:2594-2605.(Medline)

Jacobs, J.S., Anderson, A.R. and Parker, R.P. (1998) The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex, *EMBO J.* 17:1497-1506.(Medline)

Jacobson, M.D., Weil, M. and Raff, M.C. (1997) Programmed cell death in animal development, *Cell* 88:347-354.(Medline)

Jeffers, M., Taylor, G.A., Weidner, K.M., Omura, S., and Vande Woude, G.F. (1997) Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway, *Mol. Cell. Biol.*17:799-808.(Medline)

Jin, X., Shearman, L.P., Weaver, D.R., Zylka, M.J., de Vries, G.J., Reppert, S.M. (1999) A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock, *Cell* 96:57-68.(Medline)

Johnson, R.F., Moore, R.Y. and Morin. L.P. (1988) Loss of entrainment and anatomical plasticity after lesions of the hamster retinohypothalamic tract, *Brain Res.* 460:297-313.(Medline)

Johnson, P.R., Swanson, R., Rakhilina, L. and Hochstrasser, M. (1998) Degradation signal masking by heterodimerization of MATα2 and MATa1 blocks their mutual destruction by the ubiquitin-proteasome pathway, *Cell* 94:217-227.(Medline)

Johnston, J.A., Ward, C.L. and Kopito, R.R. (1998) Aggresomes: a cellular response to misfolded proteins, *J. Cell Biol.* 143:1883-1898.(Medline)

Jost, J., Khairallah, E. A. and Pitot, H. C. (1968) Studies on the induction and repression of enzymes in rat liver. V. Regulation of the rate of synthesis and degradation of serine dehydratase by dietary amino acids and glucose, *J. Biol. Chem.* 243:3057-3066.(Medline)

Kamitani, T., Kito, K., Fukuda-Kamitani, T. and Yeh, E.T. (2001) Targeting of NEDD8 and its conjugates for proteasomal degradation by NUB1, *J. Biol. Chem.* 276:46655-46660. (MedLine)

Kamura, T., Koepp, D.M., Conrad, M.N., Skowyra, D., Moreland, R.J., Iliopoulos, O., Lane, W.S., Kaelin, W.G. Jr., Elledge, S.J., Conaway, R.C., Harper, J.W. and Conaway, J.W. (1999) Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase, *Science* 284:657-661.(Medline)

Kandror, O., Busconi, L., Sherman, M. and Goldberg, A.L. (1994) Rapid degradation of an abnormal protein in *Escherichia coli* involves the chaperones GroEL and GroES, *J. Biol. Chem.* 269:23575-23582.(Medline)

Katzmann, D.J., Babst, M. and Emr, S.D. (2001) Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I, *Cell* 106:145-155. (MedLine)

Kessel, M., Maurizi, M.R., Kim, B., Kocsis, E., Trus, B.L., Singh, S.K. and Steven, A.C. (1995) Homology in structural organization between *E. coli* ClpAP protease and the eukaryotic 26S proteasome, *J. Mol. Biol.* 250:587-594.(Medline)

King, D.P, Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D., Vitaterna, M.H., Kornhauser, J.M., Lowrey, P.L., Turek, F.W. and Takahashi, J.S. (1997) Positional cloning of the mouse circadian clock gene, *Cell* 89:641-653.(Medline)

Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S. and Young, M.W. (1998) The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase IE, *Cell* 94:97-107.(Medline)

Koegl, M., Hoppe, T., Schenkler, S., Ultich, H.D., Mayer, T.U. and Jentsch, S. (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly *Cell* 96:635-644.(Medline)

Konopka, R. and Benzer, S. (1971) Clock mutants of *Drosophila melanogaster*, *Proc. Natl. Acad. Sci. USA* 68:2112-2116.(Medline)

Kornilova, E., Sorkina, T., Beguinot, L. and Sorkin, A. (1996) Lysosomal targeting of epidermal growth factor receptors via a kinase-dependent pathway is mediated by the receptor carboxyl-terminal residues 1022-1123. *J. Biol. Chem.* 271:30340-30346. (MedLine)

Kraft, M. and Martin, R.J. (1995) Chronobiology and chronotherapy in medicine, *Dis. Mon.* 41:501-575.(Medline)

Krecic, A.M. and Swanson, M.S. (1999) hnRNP complexes: composition, structure, and function, *Curr. Opin. Cell Biol.* 11:363-371. (MedLine)

Krishnan, B., Levine, J.D., Lynch, M.K., Dowse, H.B., Funes, P., Hall, J.C., Hardin, P.E. and Dryer, S.E. (2001) A new role for cryptochrome in a *Drosophila* circadian oscillator, *Nature* 411:313-317. (MedLine)

Kumar, S., Harvey, K.F., Kinoshita, M., Copeland, N.G., Noda, M. and Jenkins, N.A. (1997) cDNA cloning, expression analysis, and mapping of the mouse Nedd4 gene, *Genomics* 40:435-443.(Medline)

Kume, K., Zylka, M.J., Sriram, S., Shearman, L.P., Weaver, D.R., Jin, X., Maywood, E.S., Hastings, M.H. and Reppert SM (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop, *Cell* 98:193-205.(Medline)

Kwon, S., Barbarese, E. and Carson, J.H. (1999) The cis-acting RNA trafficking signal from myelin basic protein mRNA and its cognate trans-acting ligand hnRNP A2 enhance cap-dependent translation, *J. Cell Biol.* 147:247-256. (MedLine)

Langer, T. (2000) AAA proteases: cellular machines for degrading membrane proteins, *Trends Biochem. Sci.* 25:247-251. (MedLine)

Laroia, G., Cuesta, R., Brewer, G. and Schneider, R.J. (1999) Control of mRNA decay by heat shock-ubiquitin-proteasome pathway, *Science* 284:499-502.(Medline)

Larsen, C.N. and Finley, D. (1997) Protein translocation channels in the proteasome and other proteases, *Cell* 91:431-434.(Medline)

Lee, D.L., Sherman, M.Y. and Goldberg, A.L. (1996) Involvement of molecular chaperone Ydj1 in the ubiquitin-dependent degradation of short-lived and abnormal proteins in *Saccharomyces cerevisiae* , *Mol. Cell. Biol.* 16:4773-4781.(Medline)

Lee, C., Bae, K. and Edery, I. (1999) PER and TIM inhibit the DNA binding activity of a *Drosophila* CLOCK-CYC/dBMAL1 heterodimer without disrupting formation of the heterodimer: a basis for circadian transcription, *Mol. Cell Biol.* 19:5316-5325. (MedLine)

Lee, P.S., Wang, Y., Dominguez, M.G., Yeung, Y.G., Murphy, M.A., Bowtell, D.D. and Stanley, E.R. (1999b) The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation, *EMBO J.* 18:3616-3628. (MedLine)

Leonhard, K., Herrmann, J.M., Stuart, R.A., Mannhaupt, G., Neupert, W. and Langer, T. (1996) AAA proteases with catalytic sites on opposite membrane surfaces comprise a proteolytic system for the ATP-

dependent degradation of inner membrane proteins in mitochondria, *EMBO J.* 15:4218-4229. (MedLine)

Liu, C., Ding, J.M., Faiman, L.E. and Gillette, M.U. (1997a) Coupling of muscarinic cholinergic receptors and cGMP in nocturnal regulation of the suprachiasmatic circadian clock, *J. Neurosci.* 17:659-666.(Medline)

Liu, Y., Garceau, N.Y., Loros, J.J. and Dunlap J.C. (1997b) Thermally regulated translational control of FRQ mediates aspects of temperature responses in the Neurospora circadian clock, *Cell* 89:477-486.(Medline)

Liu, C., Weaver, D.R., Strogatz, S.H. and Reppert, S.M. (1997c) Cellular construction of a circadian clock: period determination in the suprachiasmatic nuclei, *Cell* 91:855-860.(Medline)

Liu, Y., Merrow, M., Loros, J.J. and Dunlap, J.C. (1998) How temperature changes reset the circadian oscillator, *Science* 281:825-829.(Medline)

Loeb, K.R. and Haas, A.L. (1994) Conjugates of ubiquitin cross-reactive protein distribute in a cytoskeletal pattern, *Mol. Cell. Biol.* 14:8408-8419.(Medline)

Loo, M.A., Jensen, T.J., Cui, L., Hou, Y., Chang, X.B. and Riordan, J.R. (1998) Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome, *EMBO J.* 17:6879-6887.(Medline)

Löwe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W. and Huber, R. (1995) Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution, *Science* 368:533-539.(Medline)

Lowrey, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., Ralph, M.R., Menaker, M., Takahashi, J.S. (2000) Positional syntenic cloning and functional characterization of the mammalian circadian mutation *tau*, *Science* 288:483-492. (MedLine)

Lucas, R.J., Freedman, M.S., Muñoz M, Garcia-Fernández, J.M. and Foster, R.G. (1999) Regulation of the mammalian pineal by non-rod, non-cone, ocular photoreceptors, *Science* 284:505-507.(Medline)

Luo, C., Loros, J.J. and Dunlap, J.C. (1998) Nuclear localization is required for function of the essential clock protein FRQ, *EMBO J.* 17:1228-1235.(Medline)

Ma, Y. and Hendershot, L.M. (2001) The unfolding tale of the unfolded protein response, *Cell* 107:827-830. (MedLine)

Mahajan, R., Delphin, C., Guan, T., Gerace, L., Melchior, F. (1997) A small ubiquitin-related polypeptide

involved in targeting RanGAP1 to nuclear pore complex protein RanBP2, *Cell* 88:97-107.(Medline)

Maniatis, T. (1999) A ubiquitin ligase complex essential for the NF-kappaB, Wnt/Wingless, and hedgehog signaling pathways, *Genes Dev.* 13:505-510.(Medline)

Martin, J.B. (1999) Molecular basis of the neurodegenerative disorders, N. Engl. J. Med. 340:1970-1980.(Medline)

Martin, D. W., Jr. and Owen, N. T. (1972) Repression and derepression of purine biosynthesis in mammalian hepatoma cells in culture, *J. Biol. Chem.* 247:5477-5485.(Medline)

Martin, D. W., Jr., Tomkins, G. M. and Bresler, M. A. (1969) Control of specific gene expression examined in synchronized mammalian cells, *Proc. Natl. Acad. Sci. USA* 63:842-849.(Medline)

Martinez, J., Ren, Y.G., Thuresson, A.C., Hellman, U., Astrom, J., and Virtanen, A. (2000) A 54-kDa fragment of the Poly(A)-specific ribonuclease is an oligomeric, processive, and cap-interacting Poly(A)-specific 3' exonuclease, *J. Biol. Chem.* 275:24222-24230. (MedLine)

Marzella, L. and Glaumann, H. (1987) Autophagy, microautophagy, and crinophagy as mechanisms for protein degradation. In *Lysosomes: their role in protein breakdown* (Glaumann, H. and Ballard. F. J., eds.) pp. 319-367. Academic Press, New York.

Matern, H. and Holzer, H. (1979) Endogenous proteolytic modulation of yeast enzymes. *In Modulation of Protein Function* (Atkinson, D. E. and Fox, C. F., eds.) pp. 81-92. Academic Press, New York.

Matunis, M.J., Coutavas, E. and Blobel, G. (1996) A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex, *J. Cell Biol.* 135:1457-1470.(Medline)

Mayer, M.P. and Bukau, B. (1999) The busy life of Hsp90, *Curr. Biol.* 9:R322-325.(Medline)

McCracken, A.A. and Brodsky, J.L. (1996) Assembly of ER-associated protein degradation in vitro: dependence on cytosol, calnexin, and ATP, *J. Cell Biol.* 132:291-298.(Medline)

McGuire, M. J., Croall, D. E. and DeMartino, G. N. (1988) ATP-stimulated proteolysis in soluble extracts of BHK 21/CB cells. Evidence for multiple pathways and a role of an enzyme related to the high-molecular weight protease macropain, *Arch. Biochem. Biophys.* 262:273-285.(Medline)

McNamara, P., Seo, S.P., Rudic, R.D., Sehgal, A., Chakravarti, D. and FitzGerald, G.A. (2001) Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral

mechanism to reset a peripheral clock, *Cell* 105:877-889. (MedLine)

Medintz, I., Jiang. H., Han, E., Cui, W. and Michels, C. (1996) Characterization of the glucose-induced inactivation of maltose permease in *Saccharomyces cerevisiae., J. Bacteriol* 178:2245-2254.(Medline)

Michalek, M.T., Grnat, E.P., Gramm, C., Goldberg, A.L. and Rock, K.L. (1993) A role for the ubiquitin-dependent proteolytic pathway in MHC class I, restricted antigen presentation, *Nature* 363:552-554.(Medline)

Michel, S., Geusz, M.E., Zaritsky J.J., Block, G.D. (1993) Circadian rhythm in membrane conductance expressed in isolated neurons, *Science* 259:239-241.(Medline)

Mitchell, P., Petflaski, E. and Tollervey, D. (1996) The 3' end of yeast 5.8S rRNA is generated by an exonuclease processing mechanism, *Genes Dev.* 10:502-513.(Medline)

Mitchell, P. Petfalski, E., Sevchenko, A., Mann, A. and Tollervey, D. (1997) The exosome: a conserved eukaryotic RNA processing complex containing multiple 3'→5' exonucleases, *Cell* 91:457-466.(Medline)

Miyamoto, Y. and Sancar, A. (1998) Vitamin B2-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals, *Proc. Natl. Acad. Sci. USA.* 95:6097-60102.(Medline)

Molkentin, J.D. and Olson, E.N. (1996) Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors, *Proc. Natl. Acad. Sci. USA* 93:9366-9373.(Medline)

Mori, S., Heldin, C.H. and Claesson-Welsh, L. (1992) Ligand-induced polyubiquitination of the platelet-derived growth factor β-receptor, *J. Biol. Chem.* 267:6429-6434.(Medline)

Mori, S., Tanaka, K., Omura, S. and Saito, Y. (1995) Degradation process of ligand-stimulated platelet-derived growth factor β-receptor involves ubiquitin-proteasome proteolytic pathway, *J. Biol. Chem.* 270:29447-29452.(Medline)

Morimoto, Y., Mizushima, T., Yagi, A., Tanahashi, N., Tanaka, K., Ichihara, A. and Tsukihara, T. (1995) Ordered structure of the crystallized bovine 20S proteasome, *J. Biochem.* 117:471-474.(Medline)

Morris, M.E., Viswanathan, N., Kuhlman, S., Davis, F.C. and Weitz C.J. (1998) A screen for genes induced in the suprachiasmatic nucleus by light, *Science* 279:1544-1547.(Medline)

Mortimore, G. E. and Schworer, C. M. (1977) Induction of autophagy by amino-acid deprivation in perfused rat liver, *Nature* 270:174-176.(Medline)

Mortimore, G. E. and Ward, W. F. (1981) Internalization of cytoplastic protein by hepatic lysosomes in basal and deprivation-induced proteolytic states, *J. Biol. Chem.* 256:7659-7665.(Medline)

Mortimore, G. E., Neely, A. N., Cox, J. R. and Guinivan, R. A. (1973) Proteolysis in homogenates of perfused rat liver: responses to insulin, glucagon and amino acids, *Biochem. Biophys. Res. Commun.* 54:89-95.(Medline)

Munro, T.P., Magee, R.J., Kidd, G.J., Carson, J.H., Barbarese, E., Smith, L.M. and Smith, R. (1999) Mutational analysis of a heterogeneous nuclear ribonucleoprotein A2 response element for RNA trafficking, *J. Biol. Chem.* 274:34389-34395. (MedLine)

Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K. and Ichihara, A. (1992) Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination, *Nature* 360:597-599.(Medline)

Naidoo, N., Song, W., Hunter-Ensor, M. and Sehgal, A. (1999) A role for the proteasome in the light response of the timeless clock protein, *Science* 285:1737-1741.(Medline)

Neff, N., DeMartino, G. N. and Goldberg, A. L. (1979) The effect of protease inhibitors and decreased temperature on the degradation of different classes of proteins in cultured hepatocytes, *J. Cell Pysiol.* 101:439-458.(Medline)

Niedermann, G., Butz, S., Ihlenfeldt, H.G., Grimm, R., Lucchiari, M., Hoschutzy, H., Jung, G., Maier, B. and Eichmann, K. (1995) Contribution of proteasome-mediated proteolysis to the hierarchy of epitopes presented by major histocompatibility complex class I molecules, *Immunity* 2:289-299.(Medline)

Orlowski, R.Z., Eswara, J.R., Lafond-Walker, A., Grever, M.R., Orlowski, M., Dang, C.V. (1998) Tumor growth inhibition induced in a murine model of human Burkitt's lymphoma by a proteasome inhibitor, *Cancer Res.* 58:4342-4348.(Medline)

Ostareck, D.H., Ostareck-Lederer, A., Wilm, M., Thiele, B.J., Mann, M. and Hentze, M.W. (1997) mRNA silencing in erythroid differentiation: hnRNP K and hnRNP E1 regulate 15-lipoxygenase translation from the 3' end, *Cell* 89:597-606. (MedLine)

Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S. and Hogenesch, J.B. (2002) Coordinated transcription of key pathways in the mouse by circadian clock, *Cell* 109: 307-320.

Patton, E.E., Willems, A.R. and Tyers, M. (1998) Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis, *Trends Genet.* 14:236-243.(Medline)

Permutt, M. A. and Kipnis, D. M. (1972) Insulin biosynthesis. 1. On the mechanism of glucose stimulation, *J. Biol. Chem.* 247:1194-1199.(Medline)

Peterkofsky, B. and Tomkins, G. M. (1968) Evidence for the steroid induced accumulation of tyrosine aminotransferase messenger RNA in the absence of protein synthesis, *Proc. Natl. Acad. Sci. USA* 60:222-228.(Medline)

Peters, J.-M. (1994) Proteasomes: protein degradation machines of the cell, *Trends Biochem. Sci.* 19:377-382.(Medline)

Peters, J.M. (1998) SCF and APC: the Yin and Yang of cell cycle regulated proteolysis, *Curr. Opin. Cell Biol.* 10:759-768.(Medline)

Peters, J.-M., Franke, W.W. and Kleinschmidt, J.A. (1994) Distinct 19S and 20S subcomplexes of the 26S proteasome and their distribution in the nucleus and the cytoplasm, *J. Biol. Chem.* 269: 7709-7718.(Medline)

Pickart, C.M. (1997) Targeting of substrates to the 26S proteasome, *FASEB J.* 11:1055-1066.(Medline)

Piotrowski, J., Beal ,R., Hoffman, L., Wilkinson, K.D., Cohen, R.E. and Pickart, C.M. (1997) Inhibition of the 26 S Proteasome by polyubiquitin chains synthesized to have defined lengths, *J. Biol. Chem.* 1997 272: 23712-23721.(Medline)

Pittendrigh, C.S. (1960) Circadian rhythms and the circadian organization of living systems, *Cold Spring Harbor Symp.Quant. Biol.* 25:159-182.

Pittendrigh, C.S. (1993) Temporal organization: reflections of a Darwinian clock-watcher, *Annu. Rev. Pysiol.* 55:16-54.(Medline)

Plautz, J.D., Kaneko, M., Hall, J.C. and Kay, S.A. (1997) Independent photoreceptive circadian clocks throughout *Drosophila, Science* 278:1632-1635.(Medline)

Plemper, R.K. and Wolf, D.H. (1999) Retrograde protein translocation: ERADication of secretory proteins in health and disease, *Trends Biochem. Sci.* 24:266-270. (MedLine)

Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B. and Young, M.W. (1998) *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation, *Cell* 94:83-95.(Medline)

Prusiner, S.B. (1998) Prions, *Proc. Natl. Acad. Sci. USA* 95:13363-13383. (MedLine)

Quail, P.H., Boylan, M.T., Parks, B.M., Short, T.W., Xu, Y. and Wagner, D. (1995) Phytochromes: photosensory perception and signal transduction, *Science* 268:675-580. (MedLine)

Ralph, M.R., Foster, R.G., Davis, F.C. and Menaker, M. (1990) Transplanted suprachiasmatic nucleus determines circadian period, *Science* 247:975-978.(Medline)

Reick, M., Garcia, J.A., Dudley, C. and McKnight, S.L. (2001) NPAS2: an analog of Clock operative in the mammalian forebrain, *Science* 293:506-509. (MedLine)

Reneke, J.E., Blumer, K.J., Courchesne, W.E. and Thorner, J. (1988) The carboxy-terminal segment of the yeast α-factor receptor is a regulatory domain, *Cell* 55:221-234.(Medline)

Reppert, S.M. and Sauman, I. (1995) Period and timeless tango: a dance of two clock genes, *Neuron* 15:983-986.(Medline)

Reppert, S.M. and Weaver, D.R. (1997) Forward genetic approach strikes gold: cloning of a mammalian clock gene, *Cell* 89:487-490.(Medline)

Rivett, A. J. (1985) Preferential degradation of the oxidatively modified form of glutamine synthetase by intracellular mammalian proteases, *J. Biol. Chem.* 260:300-305.(Medline)

Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D. and Goldberg, A.L. (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules, *Cell* 78:761-771.(Medline)

Roger, S., Wells, R. and Rechsteiner, M. (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis, *Science* 234:364-368.(Medline)

Ross, S., Best, J.L., Zon, L.I. and Gill, G. (2002) SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization, *Mol. Cell* 10:831-842. (MedLiner)

Roth, A.F. and Davis, N.G. (2000) Ubiquitination of the PEST-like endocytosis signal of the yeast a-factor receptor, *J. Biol. Chem.* 275:8143-8153. (MedLine)

Rowe, P. B. and Wyngaarden, J. B. (1966) The mechanism of dietary alterations in rat hepatic xanthine oxidase levels, *J. Biol. Chem.* 241:5571-5576.(Medline)

Rusak B. and Zucker, I. (1979) Neural regulation of circadian rhythms, *Physiol. Rev.* 59:449-526. (MedLine)

Rutila, J.E, Suri, V., Le, M., So, W.V., Rosbash, M. and Hall, J.C. (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*, *Cell* 93:805-814.(Medline)

Rutter, J., Reick, M., Wu, L.C. and McKnight, S.L. (2001) Regulation of Clock and NPAS2 DNA binding by the redox state of NAD cofactors, *Science* 293:510-514. (MedLine)

Saibil, H.R. and Ranson, N.A. (2002) The chaperonin folding machine, *Trends Biochem. Sci.*27:627-632. (MedLine)

Sassone-Corsi, P. (1994) Rhythmic transcription and autoregulatory loops: winding up the biological clock, *Cell* 78:361-364.(Medline)

Sassone-Corsi, P. (1998) Molecular clocks: mastering time by gene regulation, *Nature* 392:871-874.(Medline)

Scheffner, M., Huibregtse, J.M., Vierstra, R.D. and Howley, P.M. (1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53, *Cell* 75:495-505.(Medline)

Scheffner, M., Nuber, U. and Huibregtse, J.M. (1995) Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade, *Nature* 373:81-83.(Medline)

Schiaffino, S., Gorza. L., Sartore, S., Saggin, L., Ausoni, S., Vianello, M., Gundersen, K. and Lømo, T. (1989) Three myosin heavy chain isoforms in type 2 skeletal muscle fibres, *J. Muscle Res. Cell Motil.* 10:197-205.(Medline)

Schimke, R. T., Sweeney, E. W. and Berlin, C. M. (1965) The roles of synthesis and degradation in the control of rat liver tryptophan pyrrolase, *J. Biol. Chem.* 240:322-331.

Schutz, G., Killewich, L., Chen, G. and Feigelson, P. (1975) Control of the mRNA or hepatic tryptophan oxygenase during hormonal and substrate induction, *Proc. Natl. Acad. Sci. USA* 72:1017-1020.(Medline)

Schwartz, D.C. and Parker, R. (1999) Mutations in translation initiation factors lead to increased rates of deadenylation and decapping of mRNAs in *Saccharomyces cerevisiae*, *Mol. Cell Biol.* 19:5247-5256. (MedLine)

Seglen, P. O., Gordon, P. B. and Holen, I. (1990) Non-selective autophagy, *Seminars In Cell Biol.* 1:441-448.(Medline)

Seufert, W. and Jentsch, S. (1990) Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective

degradation of short-lived and abnormal proteins, *EMBO J.* 9:543-550.(Medline)

Seufert, W., McGrath, J. P. and Jentsch, S. (1990) UBC1 encodes a novel member of an essential subfamily of yeast ubiquitin-conjugating enzymes involved in protein degradation, *EMBO J.* 9:4535-4541.(Medline)

Shapiro, D. J. and Brock, M. L. (1985) Messenger RNA stabilization and gene transcription in the estrogen induction of vitellogenin mRNA, *Biochem. Action Hormones* 12:139-172.

Shatkin, A. J. (1976) Capping of eukaryotic mRNA, *Cell* 9:645-653.(Medline)

Shearman, L.P., Zylka, M.J., Weaver, D.R., Kolakowski, L.F. Jr. and Reppert, S.M. (1997) Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei, *Neuron* 19: 1261-9.(Medline)

Shearman, L.P., Jin, X., Lee, C., Reppert, S.M. and Weaver, D.R. (2000) Targeted disruption of the mPer3 gene: subtle effects on circadian clock function, *Mol. Cell. Biol.* 20:6269-6275. (Medline)

Shirai, Y., Akiyama, Y. and Ito K. (1996) Suppression of ftsH mutant phenotypes by overproduction of molecular chaperones, *J. Bacteriol.* 178:1141-1145. (MedLine)

Shyu, A.-B. and Wilkinson, M.F. (2000) The double lives of shuttling mRNA binding proteins, *Cell* 102:135-138.

Skowyra, D., Koepp, D.M., Kamura, T., Conrad, M.N., Conaway, R.C., Conaway, J.W., Elledge, S.J. and Harper, J.W. (1999) Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and Rbx1, *Science* 284:662-665.(Medline)

Somers, D.E., Devlin, P.F. and Kay, S.A. (1998) Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock, *Science* 282:1488-1490.(Medline)

Sommer, T. and Jentsch, S. (1993) A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum, *Nature* 365:176-179. (MedLine)

Spence, J., Sadis, S., Haas, A.L. and Finley, D. (1995) A ubiquitin mutant with specific defects in DNA repair and multiubiquitination, *Mol. Cell. Biol.* 15:1265-1273.(Medline)

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S.A., Rosbash, M. and Hall JC (1998) The cryb mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*, *Cell* 95:681-692.(Medline)

Starr, R. and Hilton, D.J. (1999) Negative regulation of the JAK/STAT pathway, *BioEssays* 21:47-52.(Medline)

Staub, O. and Rotin, D. (1996) WW domains, *Structure* 4:495-499.(Medline)

Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L. and Rotin, D. (1997) Regulation of stability and function of the epithelial Na^+ channel (ENaC) by ubiquitination, *EMBO J.* 16:6325-6336. (MedLine)

Stebbins, C.E., Kaelin, W.G. Jr. and Pavletich, N.P. (1999) Structure of the VHL-ElonginC-elonginB complex: implications for VHL tumor suppressor function, *Science* 284:455-461.(Medline)

Steglich, G., Neupert, W. and Langer, T. (1999) Prohibitins regulate membrane protein degradation by the m-AAA protease in mitochondria, *Mol. Cell. Biol.* 19:3435-3442. (MedLine)

Stevens, S.W. and Abelson, J. (1999) Purification of the yeast U4/U6.U5 small nuclear ribonucleoprotein particle and identification of its proteins, *Proc. Natl. Acad. Sci. USA* 96:7226-7231. (MedLine)

Stokkan, K.-A., Yamazaki, S., Tei, H., Sakaki, Y. and Menaker, M. (2001) Entrainment of the circadian clock in the liver by feeding, *Science* 291:490-493. (MedLine)

Strous, G.J., van Kerkhof, P., Govers, R., Ciechanover, A. and Schwartz, A.L. (1996) The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor, *EMBO J.* 15:3806-3812.(Medline)

Strous G.J., van Kerkhof, P., Govers, R., Rotwein, P. and Schwartz, A.L. (1997) Growth hormone-induced signal tranduction depends on an intact ubiquitin system, *J. Biol. Chem.* 1997 Jan 3;272(1):40-43. (MedLine)

Styles, C. D., Lee, K. L. and Kenney, F. T. (1976) Differential degradation of messenger RNAs in mammalian cells, *Proc. Natl. Acad. Sci. USA* 73:2634-2638.(Medline)

Sudol, M. (1996) Structure and function of the WW domain, *Prog. Biophys. Mol. Biol.* 65:113-132.(Medline)

Takahashi, J.S. (1995) Molecular neurobiology and genetics of circadian rhythms in mammals, *Annu. Rev. Neurosci.* 18:531-553.(Medline)

Takumi, T., Taguchi, K., Miyake, S., Sakakida, Y., Takashima, N., Matsubara, C., Maebayashi, Y., Okumura, K., Takekida, S., Yamamoto, S., Yagita, K., Yan, L., Young, M.W. and Okamura, H. (1998) A

light-independent oscillatory gene mPer3 in mouse SCN and OVLT *EMBO J.* 17:4753-4759. (MedLine)

Tamura, T., Tamura, N., Cejka, Z., Hegerl, R., Lottspeich, F. and Baumeister, W. (1996) Tricorn protease --the core of a modular proteolytic system, *Science* 274:1385-1389.(Medline)

Tanaka, K. (1998) Molecular biology of the proteasome, *Biochem. Biophys. Res. Comm.* 247:537-541.(Medline)

Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M. and Sakaki, Y. (1997) Circadian oscillation of a mammalian homologue of the *Drosophila* period gene, *Nature* 389:512-516.(Medline)

Terrell, J., Shih, S., Dunn, R. and Hicke, L. (1998) A function for monoubiquitination in the internalization of a G protein-coupled receptor, *Mol Cell* 1:193-202.(Medline)

Tharun, S., He, W., Mayes, A.E., Lennertz, P., Beggs, J.D. and Parker, R. (2000) Yeast Sm-like proteins function in mRNA decapping and decay, *Nature* 404:515-518. (MedLine)

Thompson, E., Tomkins, G. M., and Curran, F. (1966) Induction of tyrosine α-keto-glutarate transaminase by steroid hormones in a newly established tissue culture cell line, *Proc. Natl. Acad. Sci. USA* 56:296-303.(Medline)

Thornberry, N.A., Rosen, A. and Nicholson, D.W. (1997) Control of apoptosis by proteases, *Adv. in Pharmacol.* 41:155-177.(Medline)

Thornberry, N.A. and Lazebnik, Y. (1998) Caspases: enemies within, *Science* 281:1312-1316. (Medline)

Thresher. R.J., Vitaterna, M.H., Miyamoto, Y., Kazantsev, A., Hsu, D.S., Petit, C., Selby, C.P., Dawut, L., Smithies, O., Takahashi, J.S. and Sancar, A. (1998) Role of mouse cryptochrome blue-light photoreceptor in circadian photoresponses, *Science* 282:1490-1494.(Medline)

Toh, K.L., Jones, C.R., He, Y., Eide, E.J., Hinz, W.A., Virshup, D.M., Ptacek, L.J. and Fu, Y.H. (2001) An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome, *Science* 291:1040-1043. (MedLine)

Tomkins, G. M., Gelehrter, T. D., Granner, D., Martin, D., Jr., Samuels, H. and Thompson, E. B. (1969) Control of specific gene expression in higher organisms, *Science* 166:1474-1480.

Tosini, G. and Menaker, M. (1996) Circadian rhythms in cultured mammalian retina, *Science* 272:419-421.(Medline)

Treier, M., Straszewski, L.M. and Bohmann, B. (1994) Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain, *Cell* 78:787-798.(Medline)

Tucker, M., Valencia-Sanchez, M.A., Staples, R.R., Chen, J., Denis, C.L. and Parker, R. (2001) The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in *Sacchromyces cerevisiae*, *Cell* 104:377-386.

Tzagoloff, A., Yue, J., Jang, J. and Paul, M.F. (1994) A new member of a family of ATPases is essential for assembly of mitochondrial respiratory chain and ATP synthetase complexes in *Saccharomyces cerevisiae*, *J. Biol. Chem.* 269:26144-26151. (MedLine)

van Delft, S., Govers, R., Strous, G.J., Verkleij, A.J. and van Bergen en Henegouwen, P.M. (1997) Epidermal growth factor induces ubiquitination of Eps15, *J. Biol. Chem.* 272:14013-14016. (MedLine)

van der Horst, G.T.J., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S-i, Takao, M., de Wit, J., Verkek, A., Eker, A.P.M., van Leene, D., Buijs, R., Bootsma, D., Heojmakers, J.H.J. and Yasui, A. (1999) Mammalian Cry1 and cry2 are essential for maintenance of circadian rhythms, *Nature* 398:627-630.(Medline)

van Hoof, A. and Parker, R. (1999) The exosome: a proteasome for RNA? *Cell* 99:347-350.(Medline)

Varshavsky, A. (1997) The N-end rule pathway of protein degradation, *Genes Cells* 2:13-28.(Medline)

Vielhaber, E., Eide, E., Rivers, A., Gao, Z.H. and Virshup, D.M. (2000) Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase I ε *Mol. Cell Biol.* 20:4888-4899. (MedLine)

Vitaterna, M.H., Selby, C.P., Todo, T., Niwa, H., Thompson, C., Fruechte, E.M., Hitomi, K., Thresher, R.J., Ishikawa, T., Miyazaki, J., Takahashi, J.S. and Sancar, A. (1999) Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2, *Proc. Natl. Acad. Sci. USA.* 96:12114-12119. (MedLine)

Wagner, I., Arlt, H. Van Dyck, L., Langer, T. and Neupert, W. (1994) Molecular chaperones cooperate with P1M1 protease in the degradation of misfolded proteins in mitochondria, *EMBO J.* 13:5135-5145.(Medline)

Walker, P. R. (1977) The regulation of enzyme synthesis in animal cells, *Essays Biochem.* 13:39-69.

Wang, G., Yang, J. and Huibregtse, J.M. (1999) Functional domain of the Rsp5 ubiquitin-protein ligase, *Mol. Cell. Biol.* 19:342-352.(Medline)

Wang, Y., Liu, C.L., Storey, J.D., Tibshirani, R.J., Herschlag, D. and Brown, P.O. (2002) Precision and functional specificity in mRNA decay, *Proc. Natl. Acad. Sci. USA.* 99:5860-5865. (MedLine)

Watkins, J.F., Sung, P., Prakash, L. and Prakash, S. (1993) The *Saccharomyces cerevisiae* DNA repair gene RAD23 encodes a nuclear protein containing a ubiquitin-like domain required for biological function, *Mol. Cell. Biol.* 13:7757-7765.(Medline)

Weber, E.T., Gannon, R.L. and Rea, M. (1995) cGMP dependent protein kinase inhibitor blocks light-induced phase advances in circadian rhythms *in vivo*, *Neuroscie. Lett.* 197:227-230.(Medline)

Welsh, D.K., Logothetis, D.E., Meister, M. and Reppert, S.M. (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms, *Neuron* 14:697-706.(Medline)

Wenzel, T. and Baumeister, W. (1995) Conformational constraints in protein degradation by the 20S proteasome, *Nat. Struct. Biol.* 2:199-204.(Medline)

Whitmore, D., Foulkes, N.S., Strahle, U. and Sassone-Corsi P. (1998) Zebrafish Clock rhythmic expression reveals independent peripheral circadian oscillators, *Nature Neurosci.* 1:701-707.(Medline)

Whitmore, D., Foulkes, N.S. and Sassone-Corsi, P. (2000) Light acts directly on organs and cells in culture to set vertebrate clock, *Nature* 404:87-91.(MedLine)

Wickner, S., Maurizi, M.R. and Gottesman, S. (1999) Posttranslational quality control: folding, refolding, and degrading proteins, *Science* 286:1888-1893.(Medline)

Wigley, W., Fabunmi, R.P., Lee, M.G., Marino, C.R., Muallem, S. and DeMartino, G.N. and Thomas, P.J. (1999) Dynamic association of proteasomal machinery with the centrosome, *J. Cell Biol.* 145:481-490.(Medline)

Wilusz, C.J., Wormington, M. and Peltz, S.W. (2001) The cap-to-tail guide to mRNA turnover, *Nature Rev. Mol. Cell Biol.* 2:237-246. (MedLine)

Windisch, A., Gundersen, K., Szabolcs, M.J., Gruber, H. and Lømo, T. (1998) Fast to slow transformation of denervated and electrically stimulated rat muscle, *J. Physiol. (London)* 510:623-632.(Medline)

Wiskocil, R., Bensky, P., Dower, W., Goldberger, R. F., Gordon, J. I. and Deely, R. G. (1980) Coordinate regulation of two estrogen dependent genes in avian liver, *Proc. Natl. Acad. Sci. USA* 77:4474-4478.(Medline)

Wollnik, F., Brysch, W., Uhlmann, E., Gillardon, F., Bravo, R. and Zimmermann, M., Schlingensiepen, K.H. and Herdegen, T.(1995) Block of c-Fos and JunB expression by antisense oligonucleotides inhibits light-induced phase shifts of the mammalian circadian clock, *Eur. J. Neurosci.* 7:388-393.(Medline)

Woodside, K. H. and Mortimore, G. E. (1972) Suppression of protein turnover by amino acids in the perfused rat liver, *J. Biol. Chem.* 247:6474-6481.(Medline)

Yaglom, J., Linskens, M.M.H.K., Sadis, S., Rubin, D.M. Fuchter, B. and Finley, D. (1995) $p34^{cdr28}$-mediated control of Cln3 cyclin degradation, *Mol. Cell. Biol.* 15:731-741.(Medline)

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G.D., Sakaki, Y., Menaker, M. and Tei, H. (2000) Resetting central and peripheral circadian oscillators in transgenic rats, *Science* 288:682-685. (MedLine)

Yao, T. and Cohen, R.E. (2002) A cryptic protease couples deubiquitination and degradation by the proteasome, *Nature* 419:403-407. (MedLine)

Yeh, E. T. H., Gong, L., and Kamitani, T. (2000) Ubiquitin-like proteins: new wines in new bottles *Gene* 248, 1-14. (MedLine)

Yokouchi, M., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W.C., Zhang, H., Yoshimura, A. and Baron, R. (1999) Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7, *J. Biol. Chem.*274:31707-31712. (MedLine)

Yoshimura, T., Kameyama, K., Takagi, T., Ikai, A., Tokunaga, F., Koide, T., Tanahashi, N., Tamura, T., Cejka, Z., Baumeister, W., et al (1993) Molecular characterization of the "26S" proteasome complex from rat liver *J. Struct. Biol.* 111:200-211.(Medline)

Zanchin, N.I. and Goldfarb, D.S. (1999) The exosome subunit Rrp43p is required for the efficient maturation of 5.8S, 18S and 25S rRNA, *Nucleic Acids Res.* 27:1283-1288.(Medline)

Zheng, B., Larkin, D.W., Albrecht, U., Sun, Z.S., Sage, M., Eichele, G., Lee, C.C. and Bradley, A. (1999) The mPer2 gene encodes a functional component of the mammalian circadian clock., *Nature* 400:169-173. (MedLine)

Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., Li, Q., Sun, Z.S., Eichele, G., Bradley, A. and Lee, C.C. (2001) Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock, *Cell* 105:683-694. (MedLine>

Zimmerman, W.F., Pittendrigh, C.S. and Pavlidis, T. (1968) Temperature compensation of the circadian

oscillation in *Drosophila pseudoobscura* and its entrainment by temperature cycles, *J. Insect Physiol.* 14:669-684.(Medline)

Zylka, M.J., Shearman, L.P., Weaver, D.R. and Reppert, S.M. (1998) Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain, *Neuron* 20:1103-1110.(Medline)

16. Oxidative Phosphorylation and Mitochondrial Organization

I. GENERAL CONSIDERATIONS

A. Photosynthesis and Oxidative Phosphorylation

In green plants and in photosynthetic bacteria, energy is generated by photosynthetic reactions. In most other cells, the energy is generated primarily by oxidative phosphorylation. The products of photosynthesis in plants, fuel oxidative phosphorylation in animals. The role of these two groups of reactions is so fundamental and their mechanisms are so intriguing that a good deal of effort has gone into their study. Although photosynthesis and oxidative phosphorylation are formally very different, they share many features. Differences and similarities are summarized in Table 1. Another metabolic pattern that occurs in some bacteria is *chemolithotrophy*, i.e., the oxidation of reduced inorganic substrates, which occupies an important biological niche (see Wood, 1988; Ehrlich, 1995) but is not discussed further in this book.

In oxidative phosphorylation, the oxidation of substrates takes place by a process significantly different from that of nonbiological systems. The latter involves oxygen or other oxidants directly and results in the release of energy in the form of heat. In contrast, the oxidative reactions of the cell proceed in steps, each progressively dissipating part of the energy as heat and, in some of these steps, part of the energy is used to phosphorylate ADP. Oxygen is involved only in the terminal reaction. The discrete steps of

oxidative phosphorylation may be regarded as the passage of electrons through the *electron transport* chain until finally oxygen itself accepts the electrons and water is formed. In photosynthesis, light is the source of energy. The photosynthetic pigments, chlorophyll and the accessory pigments, absorb the radiant energy. The excited chlorophyll of the reaction centers transfers an electron to the primary acceptor and then to the electron transport system. In both oxidative phosphorylation and photosynthetic reactions, the reactions involved in the passage of electrons - i.e., the reactions of the electron transport chain - are associated with the internal membranes of specialized organelles or structures. Oxidative phosphorylation takes place in mitochondrial or in bacterial membranes. Similarly, photosynthesis takes place in the *choloroplasts* of green plants and in the membranes of bacteria.

Table 1 Energy Capturing Systems of Cells

Characteristics	**Photosynthesizing systems**	**Oxidative phosphorylation systems**
Source of energy	Entirely or in part energy from sunlight	Oxidizable substrates
Location in cells	Membrane structure: chloroplasts or chromatophores	Membrane structure: mitochondria or mesosomes
Biochemical organization	Electron transport systems involving cytochromes	Electron transport systems involving cytochromes
Primary form of chemical energy trapping	Synthesis of ATP, reduction of NADP	Synthesis of ATP
Overall results	a. Release of o_2 or other oxidized component (S or H_2SO_4)	O_2 uptake CO_2release
	b. Fixation of CO_2 or some other C source	

General pattern of system	Light →photosynthetic reaction involving chlorophyll ↓ e^- ↑ electron transport steps ↑e^- $H_2O \rightarrow O_2$ photosynthetic reaction involving chlorophyll	Substrate → oxidized substrate ↓ e^- electron transport stepd ↓ e^- $O_2 \rightarrow H_2O$

B. Oxidation Without Phosphorylation

In brown fat, a number of substrates can be oxidized in mitochondria without phosphorylation (Nedergard and Cannon, 1984). In nonshivering thermogenesis, the mitochondria of brown adipose tissue oxidize substrates, but the energy is dissipated as heat to warm the animal. This takes place by the uncoupling of oxidation from phosphorylation by a special mechanism (Flatmark and Pederson, 1975). Three *uncoupling proteins* have been found (UCPs) and these are the subject of discussion in Chapter 14. Other oxidative reactions that do not produce ATP take place elsewhere in the cell.

The P450 cytochromes, a family of hemoproteins, (see Guengerich, 1992; Coon et al., 1992), oxidize a variety of important chemicals including drugs, carcinogens, steroids, pesticides, hydrocarbon and endogenous compounds, such as fatty acids, steroids, acetone, vitamins, and many others. Accordingly, they are significant in understanding pharmacology and human disease. In mammals, the enzymes are present in the endoplasmic reticulum membranes and to a lesser extent in mitochondria. Their ubiquitous presence in other organisms, including prokaryotes such as bacteria, is not only of general interest but has facilitated research on characterizing the system. The P450 enzymes are coded by a gene superfamily; 150 of these enzymes have been identified so far. The compounds oxidized by P450 induce the expression of the P450 genes. The activation of the genes is mediated by nuclear hormone receptors (see Chapter 7), some of them the so-called orphan receptors (receptor for a yet unknown ligand) (see Waxman, 1999). Mice without a nuclear hormone receptor cannot induce the P450 genes (Lee, 1995; Wei et al., 2000, Xie et al., 2000). Some of the isoforms of P450 may be fairly specific, but others, such as those in the hepatic endoplasmic reticulum, have been estimated to catalyze about 250,000 different reactions involving foreign substances. Newly created chemicals synthesized in the laboratory are likely to become substrates.

P450 enzymes are generally but not always monoxygenases; that is, the steps involved in the oxidation of a substrate (e.g., RH, where R represents an organic compound) involve one oxygen atom (to form ROH).

Studies of the P450 system should make it possible to screen drugs with liver microsomal preparations or reconstituted P450 systems which would permit predictions of metabolic stability and toxicity. Similarly,

it may be possible to place the P450 systems in the appropriate microorganisms using recombinant DNA techniques (see Chapter 1,IIA) to dispose of toxic compounds in the environment. The desaturation of fatty acids, which requires O_2 and NADPH, and their β-oxidation (i.e., the oxidation at the β-carbon atom) is carried out in the endoplasmic reticulum by the P450 system. The function of the β-oxidation is unknown. Production of the endoplasmic system and P450 can be induced by exposure of the animal to foreign substances.

The *peroxisomal* oxidative system does not involve cytochromes (Huang et al., 1981). The enzymes of this system are contained in vesicular cellular elements, the *peroxisomes*, which are enclosed by a single membrane. Peroxisomes (see Titorenko and Rachubinski, 2001) are very heterogeneous organelles which originate from a common precursor. They differ in composition, morphology and enzymatic activity depending on cell type as well as metabolic and environmental condition. Peroxisomes range in size from 0.1 μm to 1 μm. They are related to the *glyoxysomes* of plants (see below) and *glycosomes* of trypanasomes. Together these organelles form a family of *microbodies*. The peroxisomal proteins are coded by nuclear genes and are generally synthesized in the cytoplasm.

23 proteins are required for peroxisome assembly. These proteins are encoded by the *PEX* genes (see Subramani et al., 2000). Mutations in 11 of these genes cause lethal disorders in humans (see Gould and Valle, 2000). The proteins are generated by free polysomes and then posttranslationally transferred to peroxisomes (see Tabak et al., 1999). The various components are imported in steps to form the mature peroxisome (see Titorenko and Rachubinski, 2001). In addition, peroxisomes undergo division when cells divide or when there is an increase in enzymes induced by external signals (Marshall et al., 1996). For example, hypolipidemic drugs and plasticizers produce a proliferation of these organelles (see Lock et al., 1989). The nuclear hormone receptor *peroxisome proliferator activated receptors* (PPARs) (see Schoonjans et al., 1996 and Chapter 7) are involved in peroxisomal proliferation in response to a variety of ligands.

In mammals, the peroxisomal matrix is granular and, at least in rat liver, contains a crystalline or polytubular structure made of urate oxidase. In mammalian cells, peroxisomes contain enzymes capable of carrying out the β-oxidation of fatty acids (see below). Shortened fatty acids are exported from peroxisomes to mitochondria (see van den Bosch et al., 1992). In *S. cervisiae*, fatty acids are exclusively oxidized in peroxisomes (see Erdmann et al., 1997) indicating that acetylCoA and NADH are produced there. The peroxisome system can oxidize a variety of substances in two steps. In one step, H_2O_2 is formed from oxygen using reducing equivalents from the substrate, and substances such as urate, D- and L-amino acids, L--hydroxy acids and glyoxylate are oxidized. In the second step, the H_2O_2 is used as an oxidant; phenols, nitrites, ethanol, methanol and formate can be oxidized. The system can also operate to oxidize NADH indirectly. The glycolate oxidized by the peroxisomes to form glyoxylate can be regenerated in a reaction that uses NADH as a source of reducing equivalents. Catalytic amounts of these two metabolites operating in oxidation-reduction cycles can oxidize NADH indefinitely. In plants, a similar cycle is thought to be responsible for *photorespiration* (see below).

The purpose of these oxidative reactions is not entirely clear. In mammalian liver or intestine, where peroxisomes are also present, they may be important in the regulation of the concentration of metabolites. The oxidation of NADH by the peroxisomes may be significant in the regulation of glycolysis that requires the regeneration of NAD^+ for its continuous operation. NADH oxidation may also play a role in the prevention of high levels of O_2 which might be damaging.

The enzyme system capable of β-oxidation is likely to function in the breakdown of the fatty acids, which are poorly processed by mitochondria, and may play a significant role when the need for fatty acid breakdown is high (Hashimoto, 1982; Mannaerts and Debeer, 1982; Osmundsen, 1982). However, the peroxisomes are most likely to act in concert with mitochondria, since they are not capable of processing short-chain fatty acids.

In higher plants, glyoxysomes are found in the oil-rich tissues of seeds (Huang et al., 1983). In these tissues, they are exclusively responsible for β-oxidation of fatty acids and they contain glyoxylate cycle enzymes. The glyoxylate cycle can convert 2 mol of acetyl-CoA to 1 mol of succinate, thereby permitting the synthesis of sugars in conjunction with reactions occurring in the cytoplasm and in mitochondria.

Leaf peroxisomes (Huang et al., 1983) are involved in photorespiration, in which they oxidize reducing equivalents formed by photosynthesis in conjunction with reactions taking place in the cytoplasm and the mitochondria. The complex reactions can function in the carbon reduction pathway of the chloroplasts or in the oxidative pathway, depending on the competition between oxygen and CO_2. Oxygen favors the oxidation of ribulose-1,5-biphosphate in the oxidative pathway, whereas CO_2 favors the carboxylation of ribulose-1,5-biphosphate with the formation of phosphoglycerate.

The precise physiological role of photorespiration is not known, but the process appears to be essential. Mutants lacking the system (Sommerville and Ogren, 1980) do not survive in the presence of oxygen. Without the photorespiratory system, oxygen and light cause a loss of photosynthetic capacity, which suggests that photorespiration is involved in maintaining a low oxygen concentration. Photorespiration may also be required to consume excess photosynthetically produced reducing equivalents.

This chapter focuses primarily on the electron transport system of mitochondria. Sections II and III describe the structural and biochemical organization of mitochondria. Chapter 18 presents in more detail electron transport and its coupling to ATP synthesis and ion transport.

C. Anaerobic Mitochondria

Several kinds of mitochondria exist in of eukaryotes, including protists and metazoans (e.g., gold fish!), that deal with electrons resulting from carbohydrate oxidation in the absence of sufficient oxygen. The electron pathways produce ATP (see Tielens et al., 2002). These mitochondria use as terminal electron

acceptors nitrite, nitric oxide, succinate and other metabolites. The anaerobic mitochondria may use electron acceptors present in the medium (e.g., NO_3^-) or some generated by metabolism (e.g., fumarate).

The *hydrogenosome* is another organelle related to mitochondria which is present in anaerobic protists and produces H_2 without intervention of electron carriers (e.g., see Hackstein et al., 1999; Van der Giezen et al., 2002).

II. STRUCTURAL ORGANIZATION OF MITOCHONDRIA

The protein complexes which constitute the machinery of oxidative phosphorylation are organized in the mitochondria in the inner mitochondrial membrane. In bacteria, they are present in infoldings of the plasma membrane. Mitochondria, seen with the electron microscope, are distinctive. Their appearance varies with the tissue and the preparative technique. The mitochondria appear either spherical or tubular, depending on the tissue. The mitochondria from fibroblasts are long (as long as 46 µm). Two membranes enclose the mitochondrial matrix: an outer membrane and an inner membrane. The inner mitochondrial membrane lining the mitochondrial lumen forms deep folds called *cristae*. These folds have been thought to be lamellar, however, more recent technology shows them to be pleiomorphic.

EM tomographic reconstruction of the mitochondrial structure has yielded a wealth of detail (see e.g., Frey and Mannella, 2000) and has shown that the morphology of the cristae depends on the tissue and physiological state. In liver and fibroblasts, three dimensional reconstructions from high resolution scanning electron microscopy (HRESEM) (see Chapter 1) show them to be predominantly tubular and approximately 30 nm in diameter (Lea et al., 1994). In some cristae the tubular elements merge, some forming flattened lamellae (e.g., in neurons). Those of cold adapted brown fat adipocytes were lamellar. In muscle, they were found to be both lamellar and tubular. Fig. 1 shows a electron micrograph of a mitochondrion from an hepatocyte (Lea at al., 1994). The view also illustrates the close association of some mitochondria to the endoplasmic reticulum (see below).

The cristae connect to the intramembrane space by a tubular structure that have been referred to as *peculi crista* (Daems and Wisse, 1966) or *crista junctions* (Perkins and Frey, 2000), generally 30 to 40 µm in diameter. Despite the narrowness of the crista junctions (and the presence of an outer membrane), the surface of the inner membrane appears to be available to external solutes of low molecular weight as shown by permeabilty studies of isolated mitochondria (Tedeschi, 1959 as corrected by Garlid and Beavis, 1985).Where inner and outer membrane make contact (the so-called *contact sites*) show a structure 14 nm in length and 14 nm in diameter (see Frey and Mannella, 2000). It has been suggested that these sites represent macromolecule assemblies involving channels.

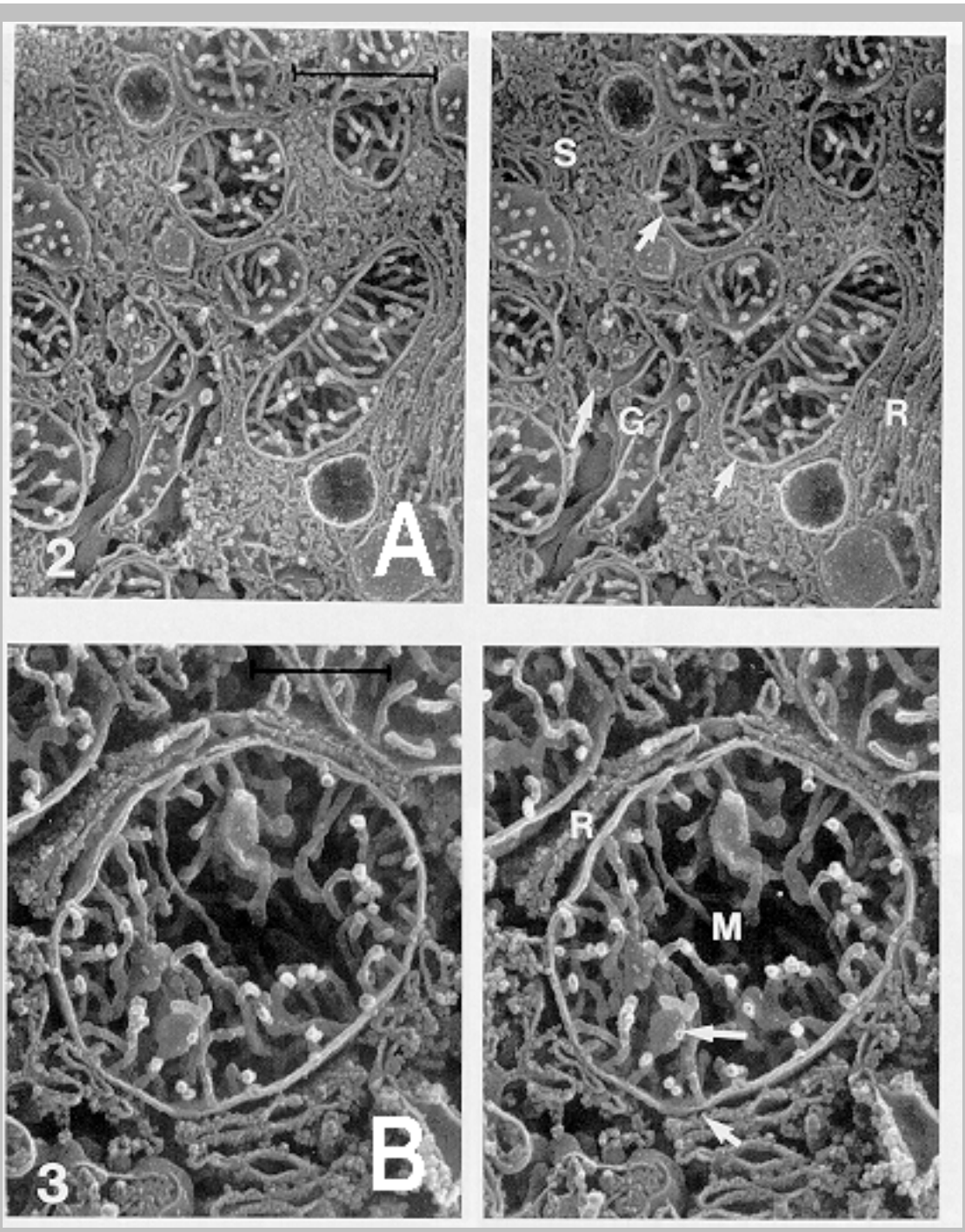


Fig. 1 Stereo pair scanning electronmicrograph of (A) a portion of a human liver cell showing several mitochondria (bar corresponds to 880 nm) and (B) a mitochondrion (bar corresponds to 494 nm). S stands for smooth endoplasmic reticulum, R for rough enoplasmic reticulum and M for mitochondrion. From Lea et al., 1994, reproduced by permission.

Variations in the volume of the internal mitochondrial lumen would increase or decrease the separation between the internal and external membranes. There is evidence that these structural alterations actually take place during changes in metabolic state (Hackenbrock, 1966). The matrix is expanded in the absence of phosphorylation (the *orthodox* configuration) and condensed when phosphorylating (the *condensed* configuration). It has been suggested that these changes in the volume of the lumen are osmotic in nature (Anagnosti and Tedeschi, 1970; Izzard and Tedeschi, 1970).

3-D reconstructions (Mannella et al., 1994) show that the spaces between cristae, tubular when in the orthodox configuration, become sac-like in the condensed configuration. The cristae are connected to the outside medium and to each other by tubes 20 nm in diameter.

In negatively stained preparations (Fig. 2) (Parson, 1963), the mitochondrial membranes have the appearance of tubes. The inner surfaces of the internal membranes are studded with particles 8 nm in diameter, which are attached to the membranes by a stalk. At times, this arrangement has been observed with conventional electron microscopy (Ashhurst, 1965; Schneider et al., 1972). These particles are not always visible after the usual preparatory procedures, perhaps due to their lability. They do not appear with negative staining after conventional fixation. In submitochondrial preparations or reconstituted systems, these granular structures are correlated with the presence of F_1, the ATP synthase.

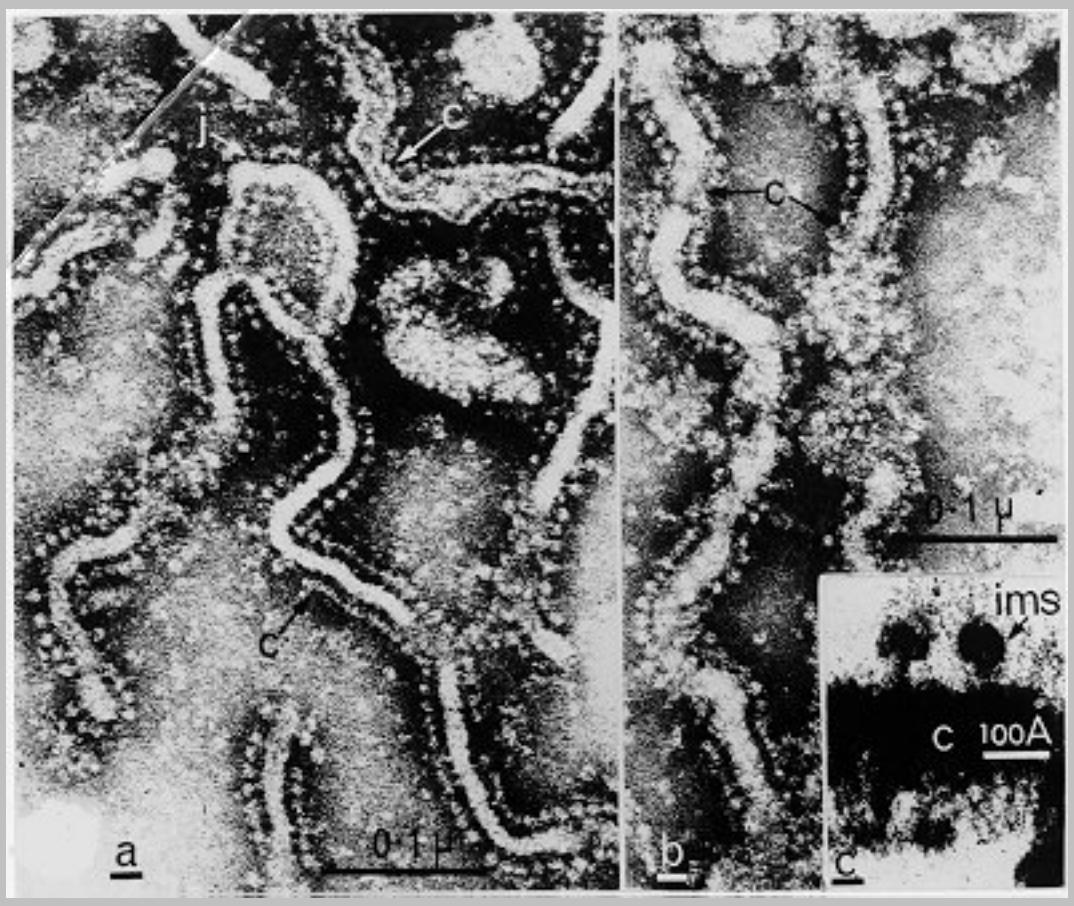


Fig. 2 (a) Negatively stained mitochondria spread on potassium phosphotungstate. (b and c) Subunits associated with the inner membrane or cristae. The particles are shown in more detail in the inset (in which the contrast is reversed). Abbreviations: om, outer membrane; c, a crista; g, an intramitochondrial granule; ims, a particle; p, projection of outer membrane; j, branching of crista. Reproduced with permission from Parson, D.F. (1963) Mitochondrial structure: two types of subunits on negatively stained mitochondrial membranes. *Science*, 140:985-988, copyright &copy1963 American Association for the Advancement of Science.

Freeze-fracture, also used in the study of membranes, is entirely different from the two electron microscopic methods already discussed. In this method, a very small piece of tissue is rapidly frozen at

very low temperature. The preparation is then fractured by using a chilled microhammer. The fractured preparation is etched by sublimating the water to a depth of a few tens of nanometers. A carbon or a platinum and carbon layer is then condensed onto the exposed surface to produce a replica, which is then viewed with the electron microscope. The fracture is thought to expose the inner hydrophobic central surface of the unit membrane. Observations suggest that the inner hydrophobic face of the inner membrane has many particles ranging in diameter between 10 and 15 nm which probably correspond to intrinsic proteins, i.e., proteins embedded in the lipid framework (Wrigglesworth et al., 1970).

Anatomically, ER and mitochondria are frequently in close contact (e.g., Perkins et al., 1997) and are likely to interact. Electron microscopic tomography of mitochondria (see Chapter 1) in situ, show mitochondria in clusters with stacks of the ER forming extended structures (Mannella et al., 1998). The structural relationship of the two has been studied in living HeLa cells (Rizzuto et al., 1998) using specifically targeted green fluorescent proteins with different spectral characteristics (see Chapter 1) one to identify mitochondria and the other ER. Many close contacts were observed between the ER and mitochondria. Furthermore, in situ, mitochondria formed a closely associated dynamic network. Not surprisingly, mitochondria and ER interact in Ca^{2+} exchanges (see Chapter 7) and in biochemical reactions. Glycosylphosphatidylinositols (GPIs) biosynthetic reactions involve the sequential addition of monosaccharides, fatty acid, and phosphoethanolamine(s) to phosphatidylinositol (PI) that take place in the ER. The subcellular fractions of homogenates of mammalian cells (Vidugiriene et al., 1999) revealed that some of the reactions take place in a sub-compartment of the ER associated with mitochondria.

The relationship between mitochondria and ER membranes is likely to be regulated. In digitonin-permeabilized *Madin-Derby canine kidney* (MDCK) cells, rat liver cytosol stimulates the dissociation of the two at low Ca^{2+}, whereas high concentrations (higher than 1 μM) favor association (Wang et al., 2000).

III. BIOCHEMICAL ORGANIZATION OF MITOCHONDRIA

Although the major function of mitochondria is in oxidative phosphorylation other roles are also known. For example, mitochondria have been found to have a significant role in programmed cell death (see Chapter 2).

Substrate oxidation coupled to phosphorylation involves three kinds of reaction sequences: reactions in which the substrates themselves are oxidized (e.g., in the tricarboxylic acid cycle), reactions in which the electrons are transported through the cytochrome chain, and reactions involved in the coupling of the electron transport to ATP synthesis or ion transport.

The three kinds of reactions can actually be dissociated by isolating functional complexes or by lysing the mitochondria. Some of the enzymes concerned directly with the oxidation of substrates can be isolated by relatively simple procedures. They are thought to be either loosely bound to the membrane or dissolved in the mitochondrial lumen. The isolation of succinate dehydrogenase, α-glycerophosphate

dehydrogenase, and the complexes involved in the electron transport requires disruption of the membranes, since these enzymes are either firmly attached or part of the lipoprotein framework of the inner mitochondrial membrane. Part of the ATP synthase complex (the F_o portion) requires disruption of the lipoprotein framework, and part (the F_1, portion) can be removed by simpler disruptive procedures.

Interactions between the matrix enzymes suggest that they may be associated in physiologically significant complexes. The measured rates in a pathway are in excess of the rate calculated from the known intramitochondrial concentrations of substrates and enzymes free in solution (e.g., Halper and Srere, 1977). Furthermore, enzymes that catalyze consecutive reactions in a metabolic pathway tend to associate with each other (Beckman and Kanarek, 1981), and at least some of them are capable of being bound to the inner mitochondrial membrane (Sumegi and Srere, 1984).

In intact mitochondria or submitochondrial vesicles (i.e., vesicles derived from mitochondria), electron transport may be coupled to or uncoupled from phosphorylation. Except for the evolution of heat in mitochondria of brown fat cells, mitochondria are involved in oxidative phosphorylation. Mitochondria capable of phosphorylation may be uncoupled by the addition of chemicals, so-called *uncouplers*, such as 2,4-dinitrophenol. In this condition, they continue to oxidize substrates, frequently at an increased rate, but without producing ATP. Other chemicals have other distinct effects. Some of them block the flow of electrons at specific sites without substantially affecting the coupling. In contrast, oligomycin and aurovertin block ATP synthesis and hydrolysis without directly interfering with coupling or electron transport. Uncouplers and inhibitors have been used extensively in studies of the mechanisms of electron transport and phosphorylation. The points at which some of the inhibitors block the respiratory chain are discussed later.

A. The Electron Transport Chain

The metabolic breakdown of carbohydrates and fatty acids eventually results in the production of reduced equivalents and acetyl-CoA. The formation of acetyl-CoA, further oxidation of the acetyl moiety through the enzymes of the tricarboxylic acid cycle and the oxidation of reducing equivalents through the electron transport chain, take place in the mitochondria. Some of the amino acids are also metabolized in reactions in which they form acetyl-CoA, although the carbon skeleton of other amino acids may enter the catabolic pathway through the tricarboxylic acid cycle or the glycolytic pathway (see Fig. 3). This chapter primarily addresses the reactions of the electron transport chain: a series of oxidative-reductive steps in which each reduced component is in turn oxidized by the one that follows. In the last oxidative step, the reducing equivalents are directly oxidized by oxygen. In essence, the electrons are passed from one component of the electron transport chain to the one that follows. The reactions can be represented as shown below.

$$NADH + FP + H^+ \rightarrow NAD^+ + FPH_2 \quad (1)$$

$$FPH_2 + 2\ \text{cyt}\ b^{3+} \rightarrow FP + 2H^+ + 2\ \text{cyt}\ b^{2+} \quad (2)$$

$$2\ \text{cyt}\ b^{2+} + 2\text{cyt}\ c^{3+} \rightarrow 2\ \text{cyt}\ c^{2+} + 2\ \text{cyt}\ b^{3+}\ (3)$$

Here FP is flavoprotein and *cyt* represents a is cytochrome. The final reaction of the series involves oxygen:

$$2\ \text{cyt}\ a_3^{2+} + \tfrac{1}{2}O_2 \rightarrow 2\ \text{cyt}\ a_3^{3+} + 2\ \text{cyt}\ b^{3+}\ (4)$$

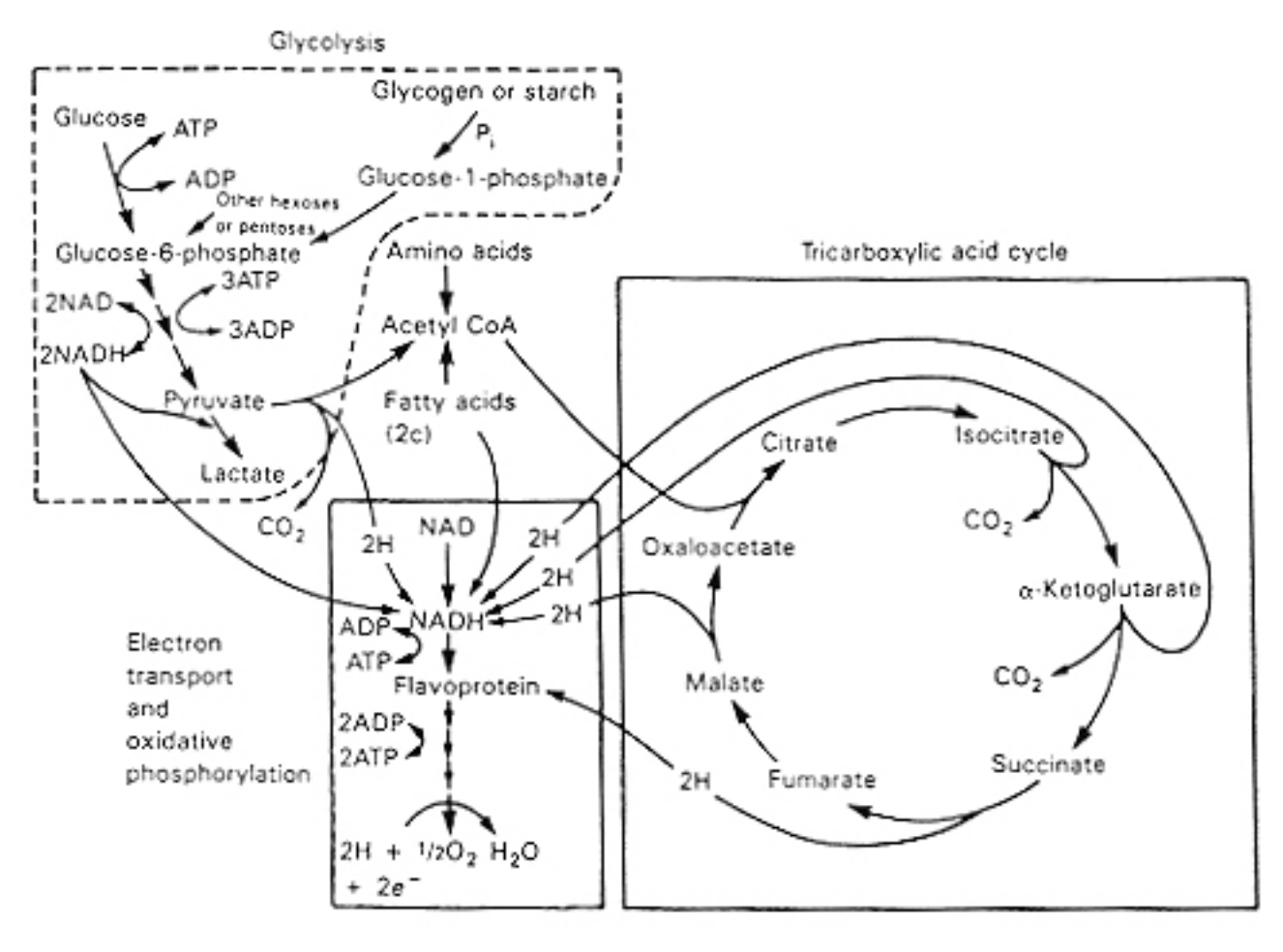


Fig. 3 Diagram of metabolic reactions show the role of reactions taking place in mitochondria (in the solid line boxes).

More schematically, the reactions of Eqs. (1) to (4) can be represented as a single equation:

$$\text{NAD} \rightarrow \text{FP} \rightarrow 2\ \text{cyt}\ b \rightarrow 2\ \text{cyt}\ c \ldots \rightarrow \text{cyt}\ a_3 \rightarrow O_2\ (5)$$

In this representation, the arrows indicate the direction of the electron flow.

Many of the components of the electron transport chain have been known and intensely studied for a number of years. To evaluate the role of each of these components in oxidative phosphorylation, it is first necessary to know whether they are actually functioning in the redox reaction chain, and then to

determine their order in the chain. The electron transport chain includes NAD, the cytochromes, flavoproteins, coenzyme Q (CoQ), and nonheme iron. Copper is involved in oxidase activity (e.g., via cytochrome-c oxidase) in a way that is still unclear. There is some evidence that one of the coppers in cytochrome-c oxidase is an electron carrier and the other is involved in ligand binding. The flavoproteins are specific proteins containing flavin groups (riboflavin-5'-phosphate or flavin-adenine dinucleotide). The cytochromes contain as a redox component Fe^{3+} (or Fe^{2+}) in the form of iron porphyrins. CoQ (ubiquinone) is a lipid soluble quinone. Some of the known components of the cytochrome chain and their probable position in the electron transport chain, are discussed in more detail below (see Figs. 6, 7, and 10).

Oxidation and reduction of the cytochrome system are reflected in changes in the light absorbed, as shown in Fig. 4 (Chance and Williams, 1956). The solid line in Fig. 4 represents the difference in absorption spectra between mitochondria in the presence of substrate under anaerobic conditions (more reduced) and under aerobic conditions (more oxidized). The various peaks correspond to the components indicated. Curve 1 pertains to the present discussion; curve 2 is discussed later.

The redox changes of some of the components of the respiratory chain have also been studied with *electron paramagnetic resonance* (EPR), also called electron spin resonance (ESR). This technique has been very useful in the study of the electron transport, particularly at low temperatures (e.g., 13K). Electrons usually occur in pairs, and the paired electrons have opposed spins. Unpaired electrons can be present either in free radicals or in compounds such as the electron transport components. The unpaired electrons respond to changes in the magnetic field, and the interaction can be detected by changes in the absorption of microwaves. In EPR, generally the magnetic field is varied and the transmitted radiation is amplified, recorded, and displayed, most commonly as the derivative of the absorbed radiation in relative units. The relationship between the magnetic field and the absorption is dependent on the constant g or g factor, following the relationship of Eq. (6). In this equation, h is Planck's constant; β is the Bohr magneton, also a constant; and H and ν are the strength of the magnetic field and the frequency of the microwave radiation, respectively. For an electron without any disturbances, g = 2.00232. However, other charged groups may disturb this pattern so that the g value is different, the usual case in biological samples.

$$h\nu = g\beta H \quad (6)$$

Since the frequency is generally maintained constant, the position of resonance in the spectrum (i.e., the absorption of the radiation) can be indicated by the magnitude of either H or g. In biological studies it is frequently indicated by the g value. Figure 5 shows the parameters of the EPR spectrum of complex I from beef heart mitochondria (see Section III,B) at 11°K (obtained with cold helium gas).

The proportion of the components reduced under various conditions can provide information about the organization of the electron transport chain. The order in which the cytochromes function should be deducible from the order in which they are oxidized when O_2 is suddenly introduced into the system. If the mitochondria are first exposed to a nitrogen atmosphere, the cytochromes become entirely reduced;

the electrons originating from the substrate no longer have a terminal acceptor. When O_2 is introduced, the components closer to O_2 should be oxidized first. The results indicate that cytochrome a is oxidized first, followed by cyt c, cyt b and flavoprotein.

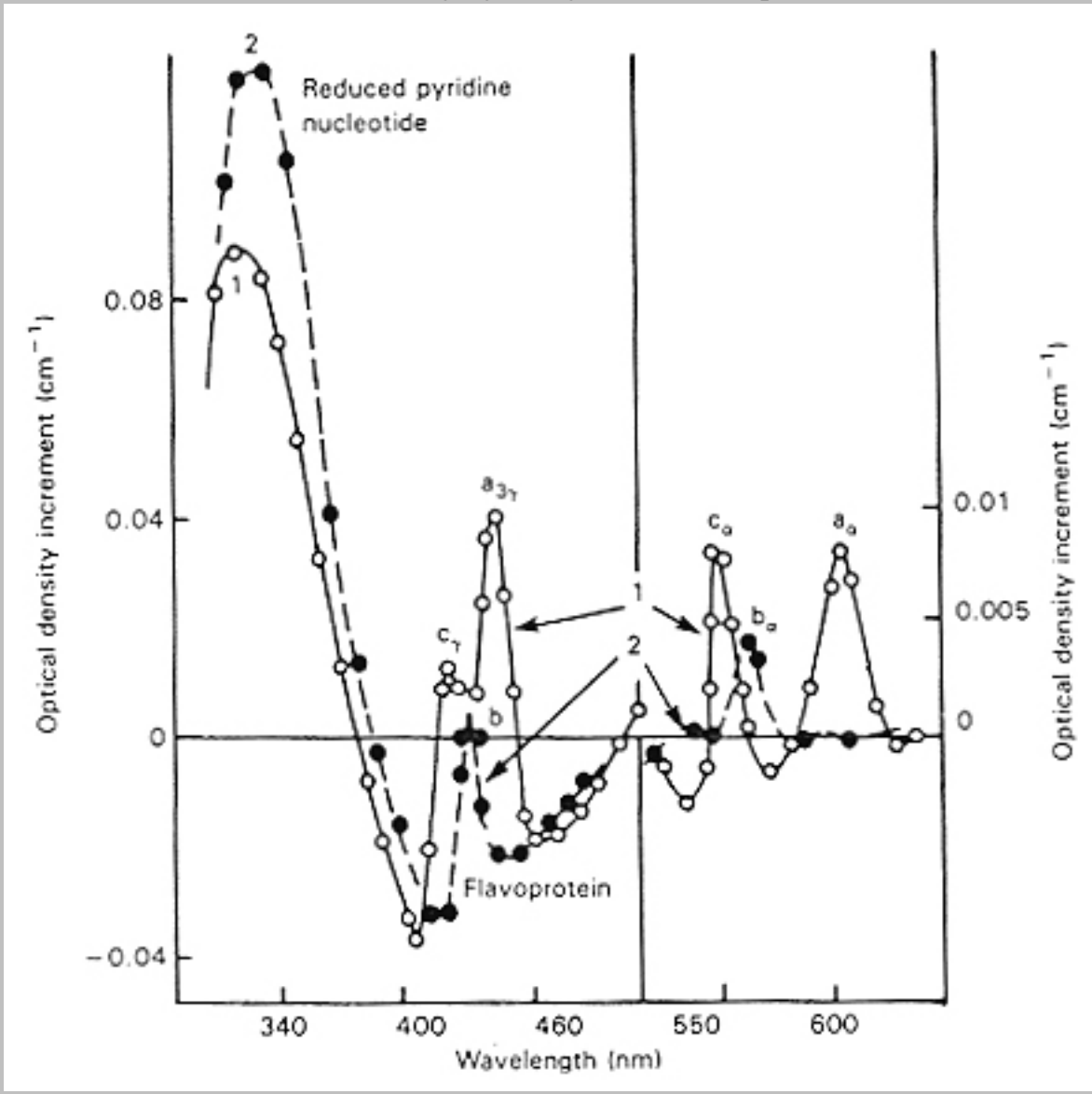


Fig. 4 Difference spectra of the respiratory carriers in rat liver mitochondria. The solid line represents absorbancy changes brought about by the presence of substrate under anaerobic conditions. The dashed line represents the change produced by substrate in the presence of antimycin A. The results are measured as differences from the absorption under aerobic conditions. Reproduced by permission from B. Chance and G. R. Williams (1956), *Advances in Enzymology*, vol. 17, p. 74, Copyright &copy1956 by John Wiley & Sons, Inc. Data from *The Journal of Biological Chemistry*.

Selective blocks can be introduced at various points in the chain. The components on the NADH side of the block, no longer connected to an electron acceptor, will become more reduced. The components on the oxygen side of the block will be unable to receive electrons and will become more oxidized. The location of a block of this kind, where the redox state of two neighboring components sharply differs, is known as a *crossover point*. Specific reactions in the sequence can be blocked by means of inhibitors. Addition of the inhibitor antimycin to a metabolizing suspension blocks the oxidation. Changes in the

absorption spectrum induced by the presence of antimycin are shown by the dashed line (curve 2) of Fig. 4, where it is clear that cytochromes *c* and a are fully oxidized, whereas cytochrome b, flavoprotein and pyridine nucleotides remain reduced. Thus, cytochromes *c* and a are situated on the oxygen side of the block. Similar experiments can be carried out with other inhibitors, such as amytal, which blocks between the pyridine nucleotide-linked flavoproteins and CoQ. A number of others, including British antilewisite (BAL), leave only cytochrome b, flavoprotein and pyridine nucleotide in reduced forms. In contrast, in the presence of substrate and cyanide, all carriers remain reduced. Cyanide is known to react with cyt a_3; thus cytochrome a_3 can be considered the terminal component of the chain, in line with its well-known reactivity with CO and the competition for binding between CO and O_2. We can conclude that, as far as this experimental approach can be carried, the findings are entirely in agreement with the order shown in Figs. 6 and 7. Figure 6 summarizes the steps in the cytochrome chain. Figure 7 incorporates the iron sulfur protein detected with EPR techniques (Ohnishi and Salerno, 1982).

Any functional component of the electron transport chain must respond rapidly to account for the rates observed under limiting conditions, such as sudden admission of O_2 or ADP, withdrawal of ADP, or inhibition of electron transport with an appropriate inhibitor (e.g., see Klingenberg and Kroger, 1967).

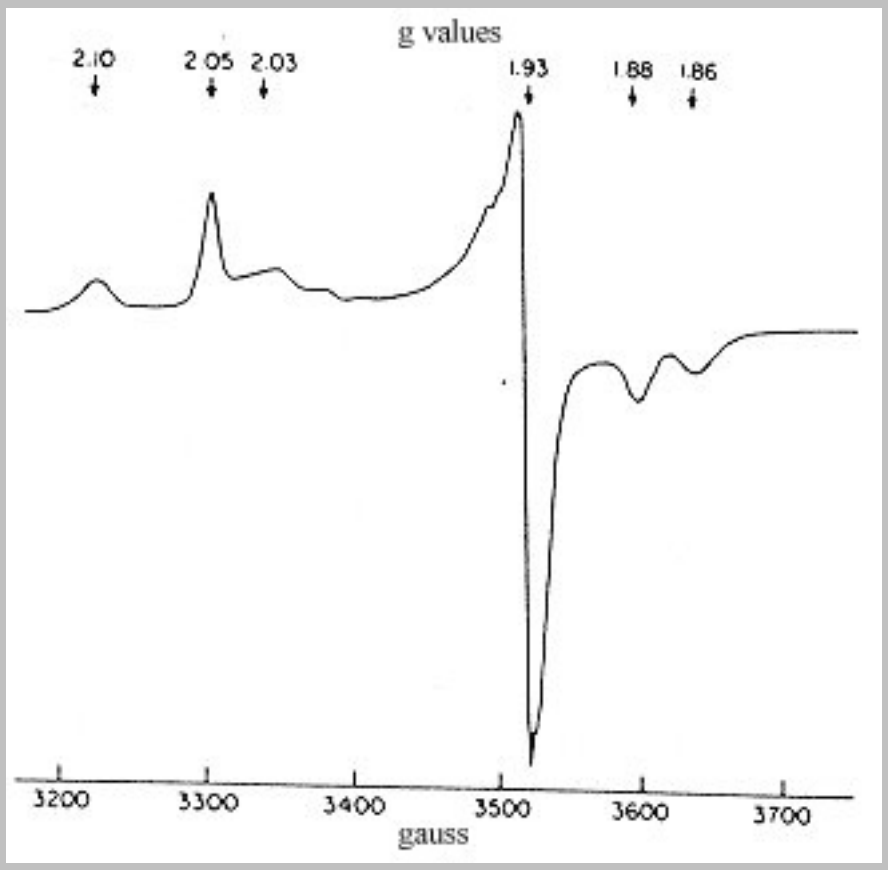


Fig. 5 Parameters of the electron paramagnetic resonance spectrum of beef heart complex I at 12 mW, 9.49 MHz, at 11°K. (Courtesy of John Salerno.) The centers, g values, and redox potentials are as follows:

	Center	g	g	g	E_h(mV)

(2Fe, 2S)	N-la	2.03	1.94	1.91	-400
	b	2.03	1.94	1.91	-250
(4Fe, 4S)	N-2	2.05	1.93	1.93	-20
	N-3	2.04	1.93	1.86	-250
	N-4	2.10	1.93	1.88	-250

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

B. The Complexes

From the very first studies of mitochondria, biochemists have attempted to break down the system into simpler fractions more amenable to elucidating the mechanisms of the reactions. The close association of the electron transport components with the mitochondrial membrane structure has made this type of analysis difficult.

The citric acid cycle enzymes (e.g., isocitrate dehydrogenase, malate dehydrogenase, fumarase, condensing enzyme, and aconitase) and the enzymes for fatty acid oxidations (fatty acid synthase and thiokinases) can be brought into solution by relatively mild treatments of rat liver or beef heart mitochondria. The so-called electron transport particles (ETPs), vesicles prepared from mitochondria by sonication or mechanical disruption, can carry out all electron transport reactions from either NADH or succinate. Some lipoprotein complexes extracted from mitochondria or ETPs can carry out electron transport, but they are unable to phosphorylate. Four such complexes corresponding to portions of the electron transport chain have been isolated (see Hatefi, 1985). The reconstitution of such a system is represented in Fig. 8. The complexes do not function together without the addition of CoQ and cyt c, which are therefore presumed to have been lost during the preparation and to operate normally in the spans shown in the figure. Further fractionation of this preparation is possible with a number of disruptive procedures.

As seen previously, the inner mitochondrial membranes are encrusted with particles attached to the membranes by stalks. Stalks, particles and the corresponding piece of membrane (the so-called base piece or F_o) constitute the basic unit of phosphorylation. The particles (termed F_1) have ATPase activity and by all indications normally function as an ATP synthase.

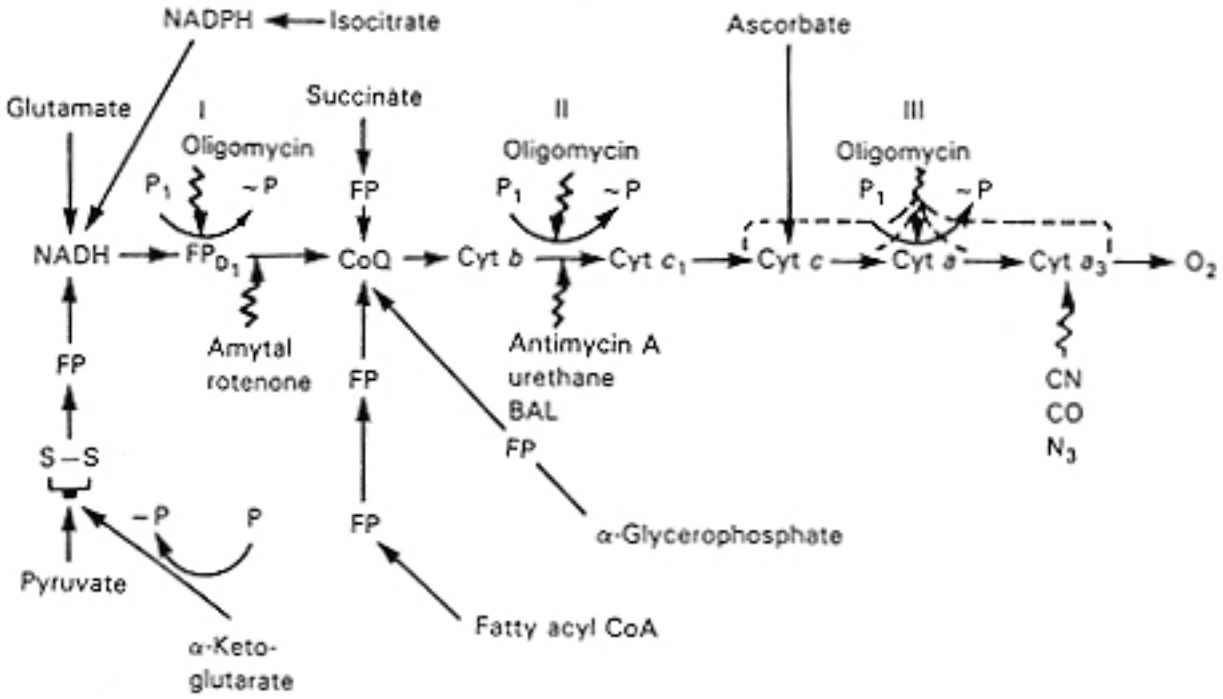


Fig. 6 Diagrammatic presentation of the respiratory chain. Roman numerals indicate probable sites of phosphorylation. Wavy arrows indicate probable sites of action of inhibitors.

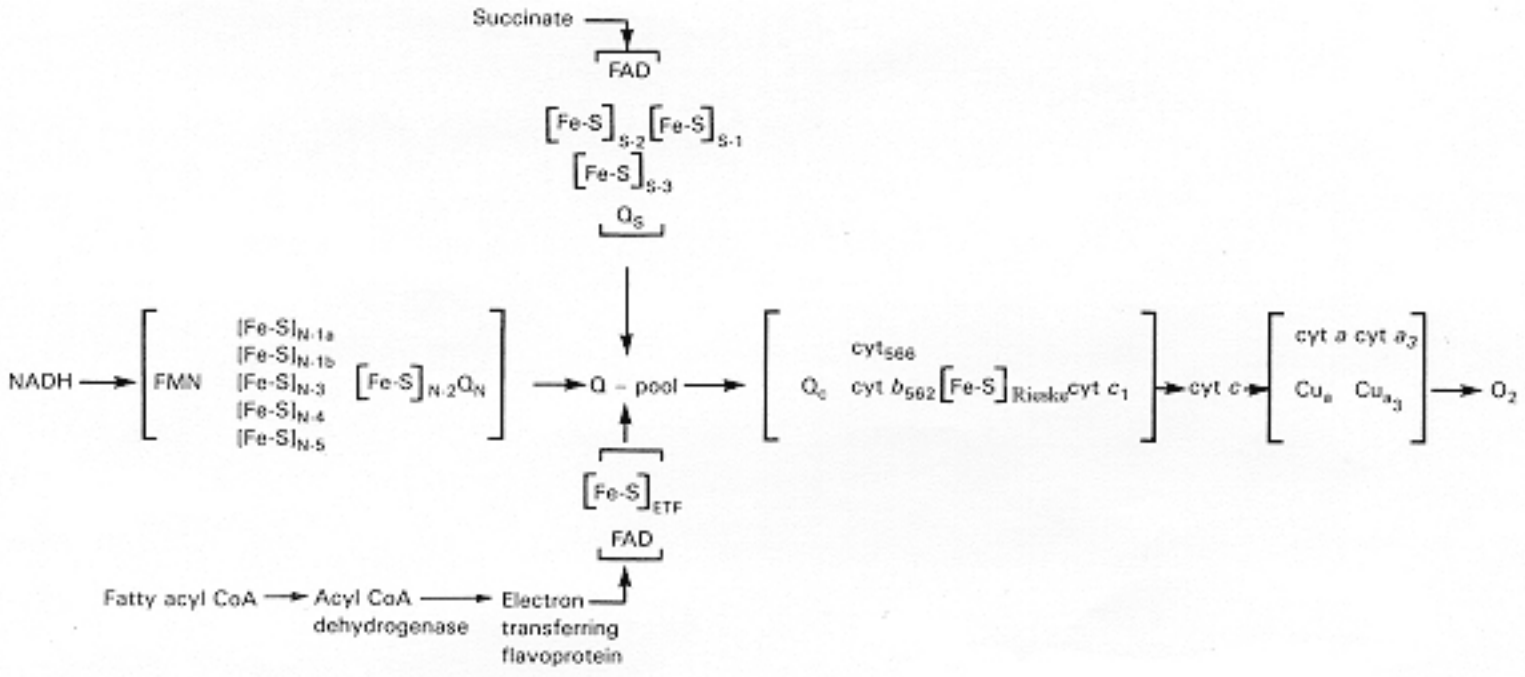


Fig. 7 Respiratory chain redox components present in the inner mitochondrial membrane. Fe-S clusters associated with NADH-UQ and succinate-UQ reductase segments are designated with suffixes N-x and S-x, respectively. Q_s, Q_n, and Q_c are protein-associated pools of ubiquinone in succinate-UQ NADH-UQ, and ubiquinol-cytochrome c reductase segments, respectively, which can be distinguished from the bulk ubiquinone pool. Reproduced by permission from Ohnishi and Salerno (1982). Iron-sulfur clusters in the mitochondria electron transport chain, in *Iron Sulfur Proteins* ed. T.G. Spiro, p.288, Copyright &copy1982 by John Wiley and & Sons, Inc.

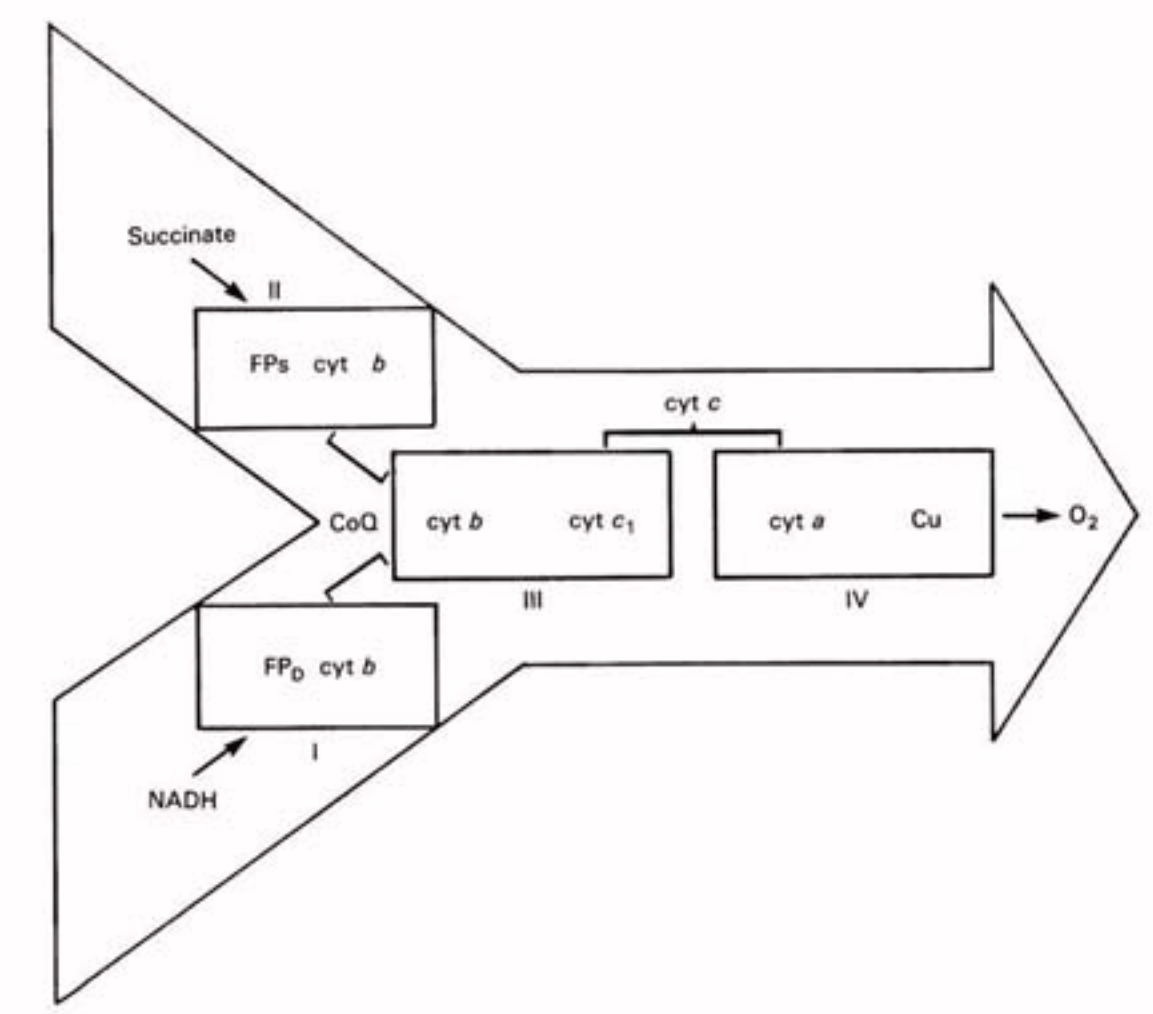


Fig. 8 Diagrammatic representation of the sections of the electron transport chain that can be isolated.

The stalk piece is necessary for the attachment to the membrane (MacLennan and Asai, 1968).

The presence of enzymes and enzyme complexes fixed to a membrane may also have other implications. Since the position of each enzyme molecule is critical, components with a structural role may be significant in the functioning of the assembly. This may be one of the reasons why the functioning of the cytochrome chain depends on the presence of lipid components.

This dependence is illustrated by the experiment represented in Table 2 (Fleischer et al., 1967). As shown by sample a in the table, mitochondria can readily oxidize succinate with the concomitant reduction of cytochrome c (columns 3-5). These reactions are essentially abolished by the removal of lipids or CoQ (ubiquinone) from the system by acetone extractions (column 3, samples b-d). The reactions are readily reestablished by adding the missing components (samples b, c, and d, column 5; or sample d, column 4). This reactivation can be accounted for by the addition of CoQ to the systems containing some phospholipid (samples b and d), but where the amount of phospholipid is low, addition of CoQ by itself is insufficient to reestablish activity (samples c and e, column 4). Thus, phospholipid components are

essential for the maintenance of the enzymatic activity. Apparently, phospholipid is required by the electron transport chain in all three of the spans tested (from succinate to CoQ, from reduced CoQ to cytochrome c, and from reduced cytochrome c to oxygen) (Green and Fleischer, 1963). Although the lipid components of the membrane may play a role by holding the various enzymes and electron carriers in appropriate positions, it is also conceivable that they help to maintain them in their appropriate conformation.

In Chapter 4, we saw that many domains of integral proteins are accessible from only one phase. Similar information is available for mitochondrial complexes. Fig. 9 (Capaldi et al., 1987; Ohnishi, 1987; Ragan, 1987; Weiss, 1987) summarizes the orientation of the four electron transport complexes of mitochondria in relation to the mitochondrial inner membrane. The information comes from a variety of experimental approaches including: electron microscopy of two-dimensional crystals (see Chapter 1), the use of photoactive hydrophobic probes to label the portions in contact with the lipid components, chemical cross-linking, which provides information about the spatial relationships between the polypeptides of a complex, and the predictions of hydropathy profiles.

The complexes that make up the electron transport chain and the ATP synthase complex can be separated (Hatefi, 1985) by appropriate techniques (see Figs. 8 and 10) and can be recombined with reconstitution of activity. These complexes are thought to represent enzyme assemblies present as independent units in the native membrane. Studies of the mobility of the four complexes,and in addition cytochrome c and ubiquinone by a photobleaching and recovery technique similar to the one described in Chapter 4, suggest that all these components are free to move in the inner mitochondrial membrane (Hackenbrock et al., 1986). The kinetics of the appropriate redox reactions are consistent with diffusional control of electron transport (Hackenbrock et al., 1986). We would expect ubiquinone (lipid soluble) and cytochrome *c* (water soluble), which have high diffusion coefficients and link the electron transport between the various complexes, to play an important role as shuttles in these exchanges. In Chapter 17, we will see that plastoquinone (lipid soluble) and plastocyanin (water soluble) play a similar role in chloroplast electron transport. The idea that the components of the electron transport chain are free to move in the plane of the membrane is supported by other observations. For example, when some of the electron transport chains are partially blocked with CO, many of them are nevertheless oxidized, but at a lower rate (Chance et al., 1970). From this observation it may be postulated that the electron transport chains are interconnected in some way. However, since the interaction is relatively slow, the possibility of diffusional movement in the plane of the membrane deserves attention. A summary of the complexes, associated components. and their stoichiometry is represented in Fig. 10 (Hatefi, 1985).

Table 2 Phospholipid Content and Enzymic Activity of Beef Heart Mitochondria

			Succinate cytochrome reductase[a] activity after addition of		
	(1)	**(2)**	**(3)**	**(4)**	**(5)**
Sample	**Treatment**	**P (μg/mg protien)**	**Nothing**	**CoQ**	**CoQ + MPL**
a. Mitochondria	None	16.8	0.59	0.52	0.53
b."Neutral lipid-depleted" mitochondria	4% water in acetone	14.5	0.02	0.84	1.00
c. "Lipid-deficient" mitochondria	10% water in acetone	3.7	0.01	0.08	0.97
c + MPL		10.5	0.07	0.77	1.03
e. "Lipid-free" mitochondria	10% water in acetone + NH_3	2.2	0.01	0.01	0.39

From Fleischer et al., (&copy1967) reproduced from *The Journal of General Physiology*, 1967, vol 32:193-208, by copyright permission of the Rockefeller University Press.

[a] micromoles cytochrome reduced per min per mg of protein at 30ºC; CoQ, coenzyme Q; MPL, mitochondrial phospholipid.

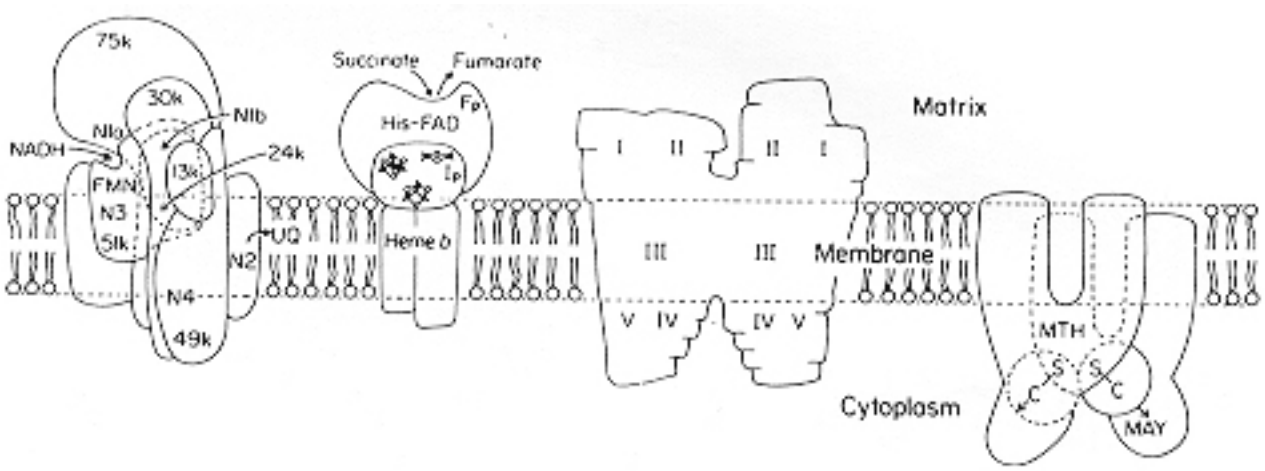


Fig. 9 Representation of the structure of the various electron transport complexes, their components and their arrangement in relation to the mitochondrial inner membrane. From left to right: NADH-ubiquinone reductase (complex I) (Ragan, 1987), succinate-ubiquinone oxidoreductase (complex II) (Ohnishi, 1987), ubiquinone-cytochrome-c reductase (complex III) (Weiss, et al., 1987) and cytochrome-c oxidase (Complex IV) (Capaldi et al., 1987). Reproduced by permission.

The transfer of electrons within a complex may involve adjacent amino acids, such as tryptophan, that are capable of transferring electrons, or it may take place through a distance by the phenomenon of electron tunneling. Electron tunneling is thought to be involved in a variety of electron transfers in mitochondria. In part, this is suggested by the evidence for short distances between the active centers (Salerno and Ohnishi, 1979). Its likelihood is indicated by the events of photosynthetic electron transport. Cytochrome oxidation in photosynthetic bacteria is independent of temperature at very low temperatures (4-lOO ^{o}K), and this is also the case for the transfer of electrons from the primary electron acceptor back to the oxidized reaction center (DeVault, 1979).

A summary of our present understanding of the biochemical organization of the mitochondrial complexes is shown in Fig. 10 (Hatefi, 1985). In this diagram, the top line represents the redox potentials of the components. The corresponding mitochondrial electron transport complexes are indicated below this line. The numbers in parentheses indicate the stoichiometry of the complexes in the intact mitochondrion compared to complex I (which is taken as unity). The wavy lines indicate the site at which the listed inhibitors act. As shown, the electron transport reactions of each complex are thought to produce a translocation of protons from the mitochondrial matrix (H^{+}_{m}) to the cytoplasmic phase (H^{+}_{c}) resulting in an electrochemical proton gradient (indicated by μ^{+}_{H}) The translocation of protons in complex V (the F_oF_1, complex) in the opposite direction generates ATP from ADP and P_i. This process is discussed in more detail in Chapter 18.

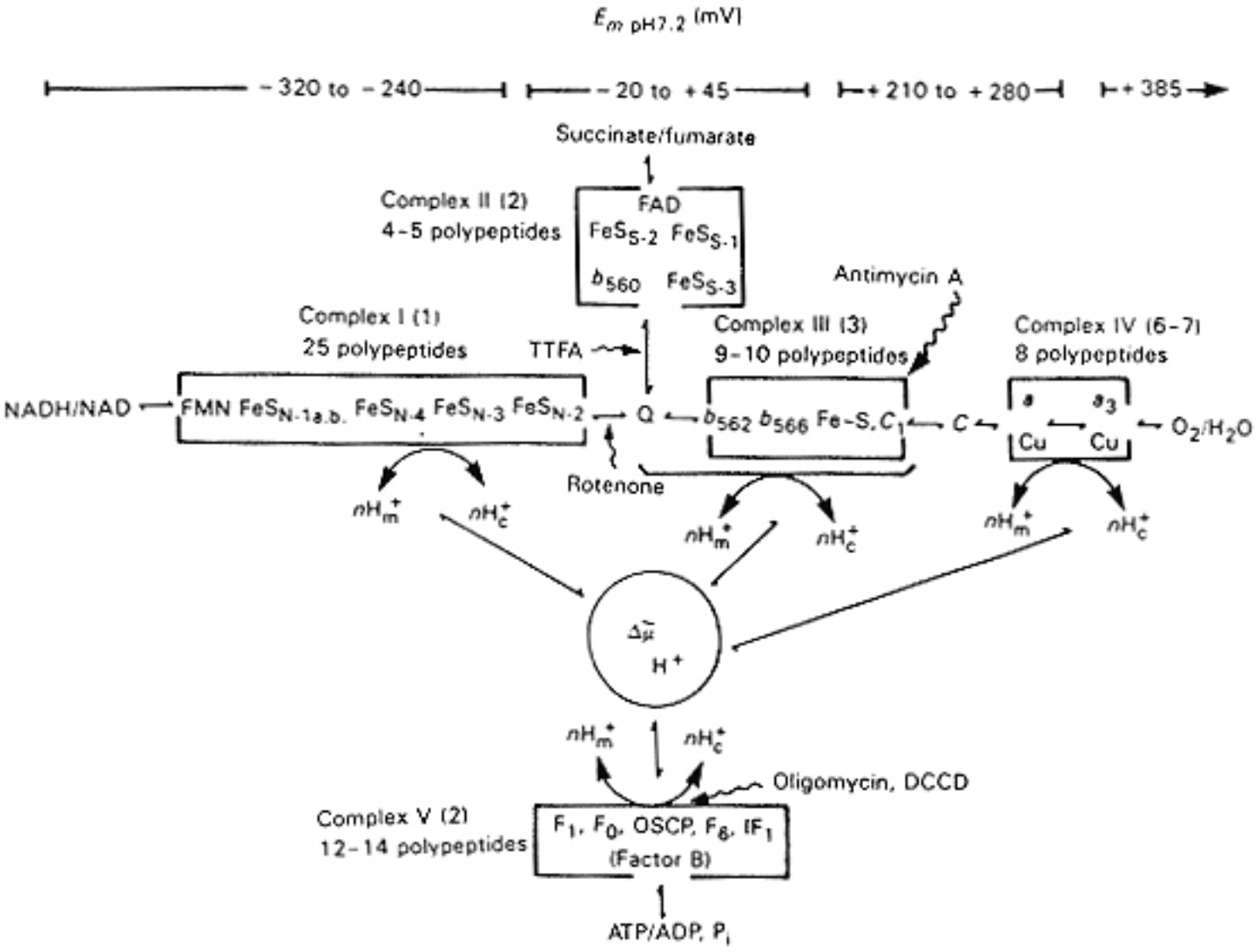


Fig. 10 Profile of the mitochondrial electron transport-oxidative phosphorylation system showing the well-characterized components of complexes I, II, III, IV, and V. DCCD stands for N,N'-dicyclohexylcarbodiimidole and TTFA for thenoyltrifluoroacetone. The abbreviations in complex V represent various peptides or peptide assemblies (Hatefi, 1985). Reproduced, with permission, from the *Annual Review of Biochemistry*, vol. 54, Copyright &copy1985 by Annual Reviews Inc.

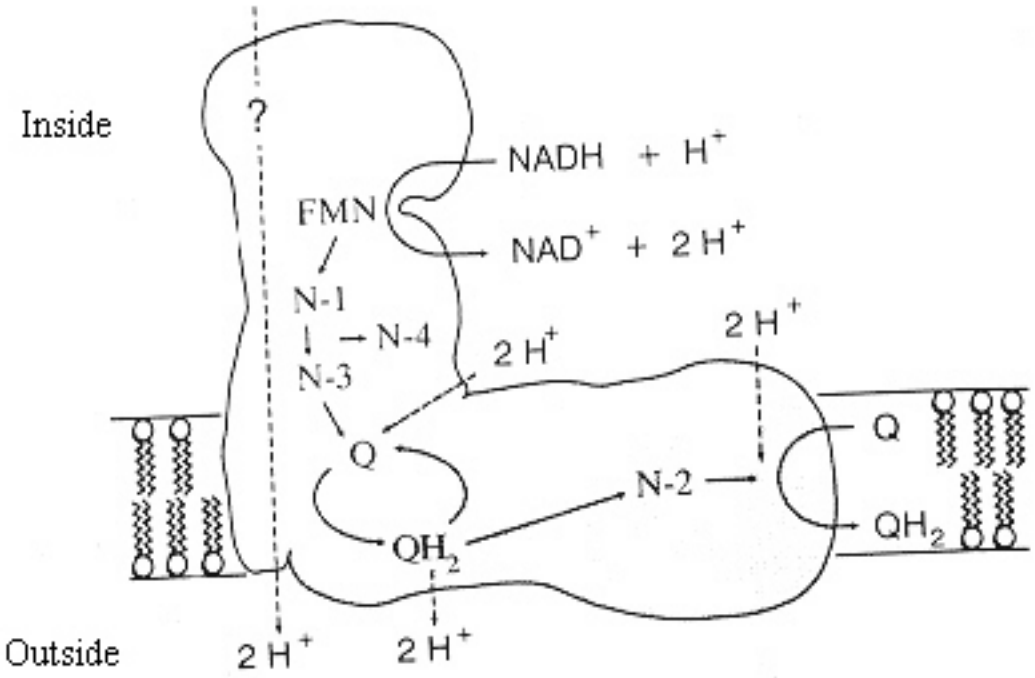
C. Proton Pumping

The link between the electron transport reactions and phosphorylation presumably is the production of an electrochemical gradient for H^+. In mitochondria, the electron transport chain pumps H^+s out of the mitochondrial matrix; in thylakoid vesicle, it pumps into the vesicle's interior. The passage of H^+ in the direction of the gradient and involving the ATP synthase is coupled to the synthesis of ATP. The question of how protons are pumped by the electron transport chain is therefore of prime importance.

The previous section has shown that electrons are transferred from NADH to O_2 through three large

complexes: complex I, NADH-ubiquinone oxidoreductase; complex III, ubiquinol-ferricytochrome c oxidoreductase; and complex IV, ferrocytochrome c-oxygen oxidoreductase. The most probable stoichiometry is a minimum of 4 H^+ per electron pair at each coupling site (e.g., Reynafarje et al., 1976). How do the three complexes of the electron transport chain function as H^+ pumps? The mechanisms are not clear, however some of the details are beginning to emerge.

The NADH-ubiquinone oxidoreductase has been shown to produce at least 4 H^+ per electron pair transferred through the complex (see Weiss et al., 1991). The redox potential gaps predict that the energy transduction would occur between FMN and the isopotential clusters N-1, N-3 and N-4, and between these and the N-2 cluster. Weiss et al. (1991) proposed that the reoxidation of $FMNH_2$ is linked to the translocation of 2 H^+ to the external face of the inner mitochondrial membrane. From the isopotential FeS cluster, the electrons might be transferred to an hypothesized internal quinone near the outer face of the inner mitochondrial membrane. Quinone reduction would produce an uptake of $2H^+$ on the matrix side and a release of $2H^+$ at the external face. Cluster N-2 would then transfer the two electrons across the membrane to reduce ubiquinone. Two additional H^+ are taken up in the matrix side. This model would account for the $4H^+$ per electron pair found experimentally. This model is represented in Fig.11



(Weiss et al., 1991).

Fig. 11 Model of the operation of NADH-ubiquinone oxidoreductase. From Weiss et al., 1991. Reproduced by permission.

Much of our knowledge of complex III, the ubiquinol-cytochrome c oxidoreductase (the bc_1 complex) comes from the bacterial bc_1 systems which catalyze the same reactions as the eukaryotic equivalents, but with less subunits. Use of the bacterial systems also permits the use of genetic techniques. An additional advantage of the use of bacteria rests on the presence of alternative pathways that allow

metabolism despite a malfunctioning bc_1 complex. Furthermore, in bacteria the components of the complex are coded by the conventional genetic apparatus, whereas in eukaryotes cytochrome b is coded by mitochondrial DNA (see Gennis et al., 1993).

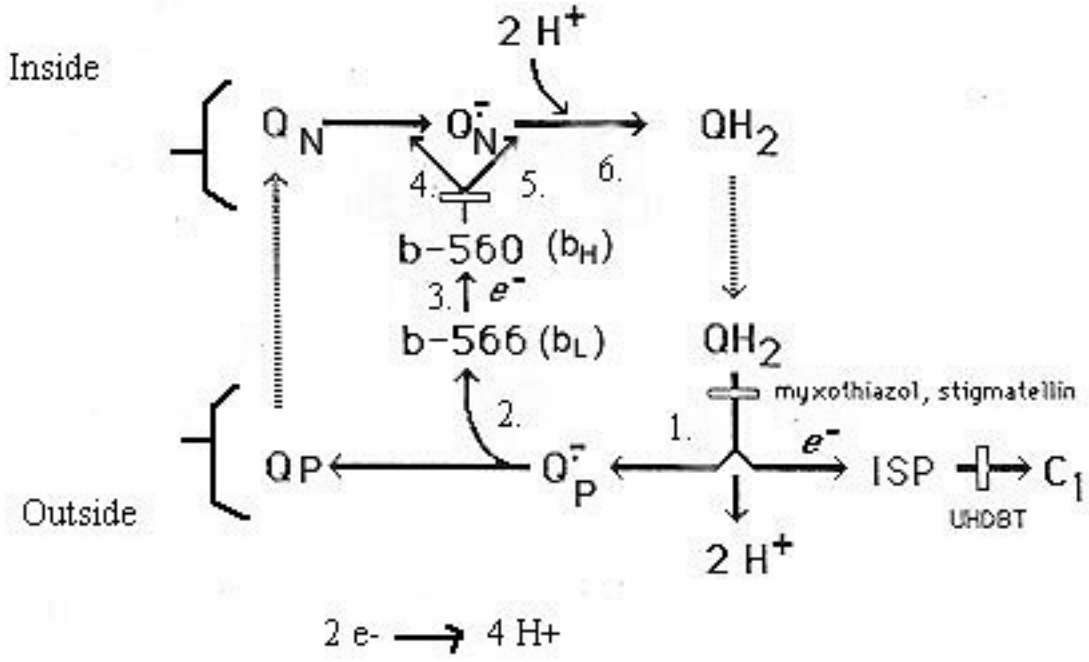


Fig. 12 Protonmotive Q-cycle of the mitochondrial bc_1 complex. See text. The open bars indicate a block by inhibitors of electron transport, ISP stands for FeS protein. The broken lines indicate the movement. From Trumpower (1990a) reproduced by permission.

In the so-called protonmotive Q-cycle pathway, electrons are transferred from ubiquinol to cytochrome c in a branched pathway known as the protonmotive Q cycle (Fig.12, Trumpower, 1990a) (see Trumpower, 1990b). In this process, one molecule of ubiquinol is oxidized and two molecules of cytochrome c_1 are reduced. This generates four protons on the outer side of the mitochondrial inner membrane and consumes two protons on the inside. The process occurs in two phases. In the first, one electron is removed from ubiquinol via FeS protein (ISP) and cytochrome c_1. The resulting ubisemiquinone dissociates to the anion and one H^+ (step 1). This step generates two H^+ at the outer surface (one from the reduction and the other from the ionization). The second electron from the ubisemiquinone, reduces cytochrome b-566 (b_L)(step 2) and is transferred to b-560 (b_H)(step 3). Ubiquinone at the inner surface is then reduced to the ubisemiquinone anion by accepting an electron from b-560 (step 4) at the inner mitochondrial surface. In the second phase, one additional ubiquinol molecule is oxidized in the same way (step 1) with the production of $2H^+$ at the outer surface and one electron following the cytochrome c pathway. Through step 2 and 3, Cytochrome b-560 transfers one electron to the ubisemiquinone anion at the inner surface (formed in the previous phase). The reduction of the ubisemiquinone anion to ubiquinol consumes 2 H^+ (step 5 and 6).

For one complete cycle (involving both phases) two molecules of ubiquinol are oxidized to ubiquinone,

one molecule of ubiquinol is formed by reduction of one of these two quinones, two molecules of cytochrome c are reduced, and 4 H^+ are formed at the outer surface. 4 H^+ are produced per electron pair. The FeS protein, cyt c_1 and the b cytochromes go through two redox turnovers. Alternatively, b-560 reduces ubiquinone to ubisemiquinone anion and ubisemiquinone to ubiquinol. As represented in the diagram, cyt b must traverse the membrane, FeS protein and cyt c_1 must be at the outer portion of the membrane. All of the presently available observations on electron transport and proton translocation are consistent with this model.

As already suggested, the functioning of complex III is very dependent on the geometry of the system. The complex is a dimer. Each monomer is a complex of 11 polypeptides and 250 kDa in molecular weight which contains b heme, c_1 heme and an iron sulfur protein (ISP) (the Rieske protein) in a 2:1:1 stoichiometry. The arrangement of the subunits in relation to the inner mitochondrial membrane were defined by biochemical studies (e.g., Gonzalez-Halphen et al., 1988) that included proteinase digestion, the use of lipid soluble probes and crosslinking studies. The latter allowed detecting the location of the various subunits in relation to each. The position of the various players is that suggested in Fig. 13 (Smith, 1998). The transmembrane region spans 38 Å and contains most of cytochrome b. The largest portion of the complex (about 75 Å) extends into the mitochondrial matrix. A large part of cytochrome c_1 and ISP represent a projection of 38 Å into the intermembrane space (that is in the cytoplasmic side).

Crystallographic studies provided more information. The initial study (Xia et al., 1997) of 80% of the complex with a resolution of 2.9 Å, gave the general features of the complex, including the positions of the metal centers. Zhang et al. (1998) reported a 3 Å resolution structure of 9 of the subunits with complete tracing of the extrinsic domains of cytochrome c_1 and the Rieske subunits. Iwata et al. (1998) report two new crystal forms of the bovine cytochrome bc_1 complex. The position of the hemes b_L and b_H and c_1 are invariant. The external domain of the Rieske Fe_2S_2 center moves. In one position, it is close to Q_P, allowing for the reduction of the ISP. In another, it is close to the heme of cytochrome c_1, permitting its oxidation (Zhang et al., 1998; Iwata et al., 1998; Kim et al., 1998). In one conformation, the histidines that protrude from the ISP bind the cytochrome b in close proximity to the Q_P. Release from this binding is controlled by the redox state of Q_P. Oxidation releases the histidines from the cytochrome b binding (Zhang et al., 1998; Kim et al., 1998) and allows them to move close to the heme

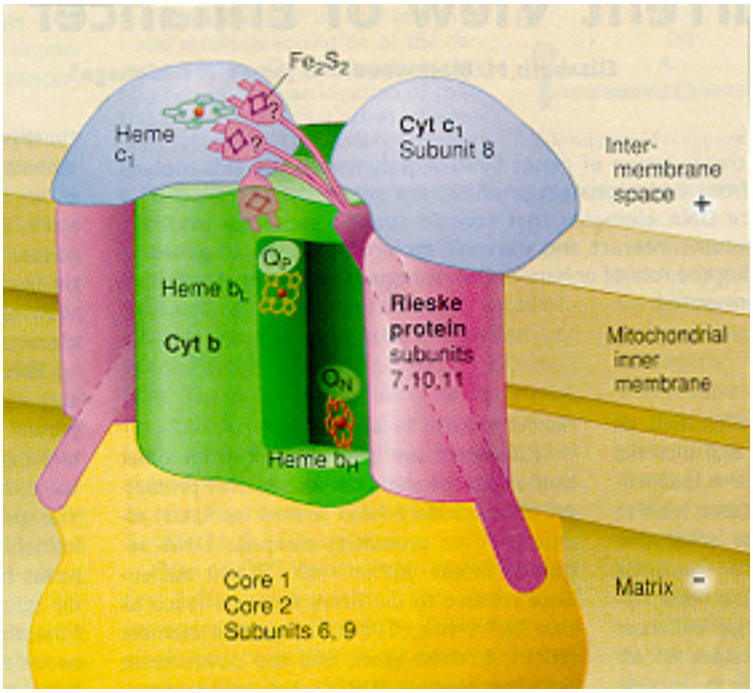
of cytochrome c_1. 

Fig. 13 Structure of cytochrome bc_1. The hollow between its two monomers allow the shuttling of ubiquinol/ubiquinone to and from the complex and betwen Q_p and Q_N. The Rieske extrinsic domain (represented by the plug) also shuttles between a site near Q_p (the socket) and cytochrome c_1. The passage of each electron from Q_p through Rieske and cytochrome c_1 to cytochrome c displaces 2 H^+. Reproduced with permission from Smith J.L., *Science* 281:58-59. Copyright &copy1998 American Association for the Advancement of Science. http://www.sciencemag.org

The presence as a dimer is required for the bc_1 complex to carry out its biochemical reactions. This is indicated by the location of the ISP intrinsic and extrinsic domains in opposite monomers and the significance of this arrangement in relation to the Q cycle, which explains how the transfer of two electrons through the complex can give rise to 4 H^+ (shown in Fig. 13). The major role in the cycle is played by hemes b_L and b_H and the adjacent ubiquinol/ubiquinone sites (Q_P and Q_N), all attached to the transmembrane domain of cytochrome b. In the Q cycle, Q_P is at the site of bifurcation in the pathway of electron transport. One electron supposedly goes to the ISP extrinsic domain and the other is cycled through the b hemes to return to Q_N.

These processes require a specific geometry. The ubiquinone species must be free to move in and out of the bc_1 complex. In addition there must be a rapid exchange between Q_P and Q_N. These needs are taken care of by the arrangement of the bc_1 dimer. Together, two cytochrome b proteins form a space accessible from the bilayers with an opening of 25 Å. The opening allows rapid exchanges of the very lipid soluble Qs. The transmembrane helices of the cytochrome bc_1 form the walls and its external domains form the floor and ceiling. Q_P and Q_N are present in the walls, each in different monomers of the composite complex.

The structure of cytochrome *c*-oxidase has been elucidated at high resolution on both bacteria (Iwata et al., 1995) and mammals (Tsukihara et al., 1995). $4H^+$ are produced per electron pair transferred through the complex. At least when reconstituted in liposomes, cytochrome *c* oxidase translocates two protons during its oxidation and two during its re-reduction (Verkhovsky et al., 1999).

The electrons are thought to follow a linear path: *c* Cu_A a_3-Cu_B. The bacterial complex is formed by only four subunits, whereas the eukaryotic complex has thirteen. This discussion will generally refer to the simpler system. The bacterial subunits I, II and III are common to all members of the heme-copper oxidase family. The arrangement is represented in Fig.14 (Gennis and Ferguson-Miller, 1996). Cytochrome c oxidase has four metal redox centers which are contained in subunits I and II. One redox center (Cu_A) is in subunit II. Heme a and the heme a_3-Cu_B bimetallic center are in subunit I and they are at approximately the same level: 15 Å below the outer surface of bacteria. Heme a is ligated to two histidines, heme a_3 to a single histidine and Cu_B to three.

The interaction with oxygen occurs at the heme a_3-Cu_B site which is deep in the protein. It follows that there must be pathways (referred to as channels) for the passage of the protons from or to the appropriate surface and the redox centers of the molecule. Water molecules and amino acid side chains act as proton wires (see Nagle et al., 1980; Rammelsberg et al., 1998). These routes that could function as conduits for the protons have been implicated by site-directed mutagenesis (e.g., Thomas et al., 1993; Fetter et al., 1995) . One of the channels involved in the uptake of protons from the mitochondrial interior (the D channel) is lined with polar residues (e.g., Ostermeier et al., 1997) and a chain of hydrogen-bonded water (e.g., see Riistama et al., 1997; Hofacker and Schulten, 1998). The D channel ends at a hydrophobic cavity containing water molecules. Other channels are less certain.

The actual pumping mechanism is still a mystery. Some models propose a displacement of the histidines which ligate the heme a_3-Cu_B center and undergo cyclic protonation-deprotonation to produce a unidirectional proton flow (Morgan et al., 1994).

A possible mechanism for pumping protons by cytochrome *c* oxidase, might involve movement of an acidic group of the protein which would be exposed alternatively to the two faces of the inner mitochondrial membrane depending on the redox state of the heme a_3-Cu_B sites. To function as a pump, the movement would have to be accompanied by a change in pK. This mechanism has been proposed based on crystallographic data from bovine heart cytochrome c oxidase (Yoshikawa et al., 1998). The results of this study are consistent with a movement of an aspartate residue (Asp51). With the reduction of the enzyme the aspartate becomes accessible to the external phase, a change accompanied by a decrease in pK . The results also suggest an acidified tyrosine residue as a source of protons for the oxygen reduction. These events require only minor changes in structure that can be deduced by comparing the crystal structure in various states.

In this model, in the oxidized state, Asp51 is connected to the inner surface by a network that includes a

peptide unit, hydrogen bonds, a cavity and a water path. The network interacts with heme a_3. Upon reduction, Asp51 loses its accessibily to the matrix phase and is accessible from the intramembrane space.

Studies of another redox system, that of ferredoxin I of *Azotobacter vinelandii* lend support to this kind of model. Ferredoxin is a small protein with a buried 3Fe-3S cluster. A single long-range electron-coupled proton transfer occurs in ferredoxin I. Reduction of the Fe-S cluster brings in a proton from the water. In contrast, re-oxidation requires a proton release to the water. The proton is directed through the protein matrix by movement of the side-chain of an aspartate (Asp 15) close to the surface. The pK of the aspartate changes in response to the electrostatic charge of Fe-S cluster (Chen et al., 2000).

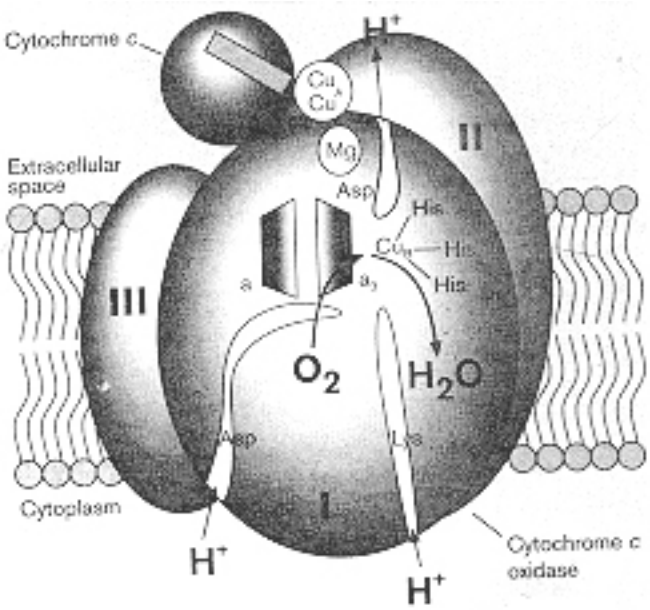


Fig. 14 Model of bacterial cytochrome c oxidase based on the crystal structure and site directed mutagenesis, showing predicted pathways and key residues. Reproduced from Gennis and Ferguson-Miller (1996) by permission. (Available in the BioMedNet library at: http://biomednet.com/cbiology/cub)

Presumably, the main function of mitochondrial electron transport is the production of ATP from ADP and P_i. One of the functions of photosynthesis is to support electron transport coupled to the phosphorylation of ADP. Therefore, the similarity between the two systems is considerable, so the oxidative and photophosphorylative reactions are best discussed together. For this reason, various aspects of photosynthesis are presented in Chapter 17, whereas the phosphorylative reactions of the mitochondrial and chloroplast electron transport chains are treated together in Chapter 18.

SUGGESTED READING

Overall view

Capaldi, R.A. (2000) The changing face of mitochondrial research, *Trends Biochem. Sci.* 25:212-214.

(MedLine)

Saraste, M.(1999) Oxidative phosphorylation at the *fin de siècle*, *Science* 283:1488-1493. (Medline)

Wallace, D.C. (1999) Mitochondrial diseases in man and mice, *Science* 283:1482-1488. (Medline)

Other references

Cramer, A.W. and Knaff, D.B. (1990) *Energy Transduction in Biological Membranes*, Chapters 3,4 and 5. Springer-Verlag, New York.

Gennis, R.B. and Ferguson-Miller, S. (1996) Protein structure; Proton-pumping oxidases, *Curr. Biol.* 6:36-38. (Medline)

Nicholls, D. (1984) Mechanisms of energy transduction. In *Bioenergetics* (Ernster, L., ed.), pp. 29-48. Elsevier, New York.

Tzagoloff, A. (1982) *Mitochondria*, Chapters 2-5. Plenum, New York.

Wikstrom, M. and Saraste, M. (1984) The mitochondrial respiratory chain. In *Bioenergetics* (Ernster, L., ed.), pp. 49-94. Elsevier, New York.

Alternative References

Harold, F.M. (1986) *The Vital Force: A Study Bioenergetics*, Chapter 7, pp. 197-250. W.H. Freeman, New York.

Nicholls, D.G. and Ferguson, S.J. (1992) *Bioenergetics 2*, Academic Press, Chapter 1-8.

Structure of mitochondria

Frey, T.G. and Mannella, C.A. (2000) The internal structure of mitochondria, *Trends Biochem. Sci.* 25:319-324. (MedLine)

Special Aspects

Capaldi, R.A., Takamiya, S., Zhang, Y. -Z., Gonzalez-Halphen, D. and Yanamura, W. (1987) Structure of cytochrome-c oxidase, *Curr. Top. Bioenerg.* 15:91-108.

Ehrlich, H. (1995) *Geomicrobiology*, 3rd ed., Dekker, New York.

Friedrich, T., Steinmüller, K. and Weiss, H. (1995) The proton-pumping respiratory complex I of bacteria and mitochondria and its homologue in chloroplasts, *FEBS Lett.* 367:107. (Medline)

Hackenbrock, C.R., Chazotte, B. and Gupte, S.S. (1986) The random collision model and a critical assessment of diffusion and collision in mitochondrial electron transport. *J. Bioenerg. Biomembr*. 18:331-368. (Medline)

Hatefi, Y., Ragan, I. and Galante, Y.M. (1985) The enzymes and the enzyme complexes of the mitochondrial oxidative phosphorylation system. In *The Enzymes of Biological Membranes*, 2d ed., Vol. 4, pp. 1-70 (Martonosi, A.N., ed.). Plenum, New York.

Jones, C.W. (1988) Membrane associated energy conservation in bacteria: a general introduction. In *Bacterial Energy Transduction* (Anthony, C., ed.), pp. 1-82. Academic Press, New York.

Kadenbach, B., Kuhn-Nentwig, I. and Buge, U. (1987) Evolution of a regulatory enzyme: cytochromec oxidase (complex IV). *Curr. Top. Bioenerg.* 15:114-151.

Mitchell, P. (1987) Respiratory chain systems in theory and practice, in *Advances in Membrane Biochemistry and Bioenergetics* (Kim, C.H., Tedeschi, H., Diwan, J.J. and Salerno, J.C., eds.), pp. 25-52. Plenum, New York.

Moynagh, P.N. (1995) Contact sites and transport in mitochondria, *Essays in Biochemistry* 30: 1-14. (Medline)

Ohnishi, T. (1987) Structure of the succinate-ubiquinone oxido-reductase (complex II), *Curr. Top. Bioenerg.* 15:37-66.

Ragan, C.I. (1987) Structure of NADH-ubiquinone reductase (complex I), *Curr. Top. Bioenerg.* 15:11-36.

Rutter, G.A. and Rizzuto, R. (2000) Regulation of mitochondrial metabolism by ER Ca^{2+} release: an intimate connection, *Trends Biochem. Sci.* 25:215-221. (MedLine)

Senior, A.E. (1988) ATP synthesis by oxidative phosphorylation, *Physiol. Rev.* 68:177-231.

Senior, A.E. (1990) The proton translocating ATPase of *Escherichia coli*, *Annu. Rev. Biophys. Chem.* 19:7-41.(Medline)

Tolbert, N.E. (1981) Metabolic pathways in peroxisomes and glyoxysomes, *Annu. Rev. Biopys. Chem.* 50:133-158.(Medline)

Weiss, H. (1987) Structure of mitochondrial ubiquinol-cytochrome c reductase (complex III), *Curr. Top. Bioenerg.* 15:67-90.

Wikström, M. (1998) Proton translocation by bacteriorhodopsin and heme-copper oxidases, *Curr. Opin. Struct. Biol.* 8:480-4888. (MedLine)

Yoshida, M., Muneyuki, E. and Hisabori, T. (2001) ATP synthase - a marvellous rotary engine of the cell, *Nature. Rev. Mol. Cell Biol.* 2:669-677. (MedLine)

WEB RESOURCES

Diwan, J.J., Electron transfer chain, http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/redox.htm

REFERENCES

Search the textbook

Back to Chapter 16

REFERENCES

Anagnosti, E. and Tedeschi, H. (1970) Mechanism of low-amplitude orthophosphate-induced swelling in isolated mitochondria, *J. Cell Biol.* 47:520-525.

Ashhurst, D.E. (1965) Mitochondrial particles seen in sections, *J. Cell Biol.* 244:497-499.

Beckman, S. and Kanarek, L. (1981) Demonstration of physical interaction between consecutive enzymes of the citric acid cycle and the aspartate-malate shuttle, *Eur. J. Biochem.* 117:527-535.

Capaldi, R.A., Takamiya, S., Zhang, Y.-Z., Gonzalez-Halphen, D. and Yanamura, W. (1987) Structure of cytochrome-c oxidase,*Curr. Top. Binenerg.* 15:91-113.

Chance, B. and Williams, G.R. (1956) The respiratory chain and oxidative phosphorylation, *Adv. Enzymol.* 17:65-134.

Chance, B., Erecinska, M. and Wagner, M. (1970) Mitochondrial responses to carbon monoxide toxicity, *Ann.N.Y. Acad. Sci.* 174:193-204.(Medline)

Chen, K., Hirst, J., Camba, R., Bonagura, C.A., Stout, C.D., Burgess, B.K. and Armstrong, F.A. (2000) Atomically defined mechanism for proton transfer to a buried redox centre in a protein, *Nature* 405:814-817.

Coon, M.J., Ding, X., Pernecky, S.J. and Vaz, A.D.N. (1992) Cytochrome P450: progress and predictions, *FASEB J.* 6:669-673.(Medline)

Daems, W.T. and Wisse, E. (1966) Shape and attachment of the cristae mitochondriales in mouse hepatic cell mitochondria, *J. Ultrastruct. Res.* 16:123-140. (MedLine)

DeVault, D. (1979) Introduction to biological aspects. In *Tunneling in Biological Systems* (Chance, B., DeVault,D.C., Fraunfelder, H., Marcus, R.A., Schrieffer, J.R. and Sutin, N., eds.), pp. 303-316. Academic Press, New York.

Ehrlich, H. (1995) *Geomicrobiology*, 3rd ed., Dekker, New York.

Erdmann, R., Veenhuis, M. and Kunau, W.-H. (1997) Peroxysomes: organelles at the crossroads, *Trends Cell. Biol.* 7:400-407.

Fetter, J.R., Qian, J., Shapleigh, J., Thoams , J.W., Garcia-Horsman, A., Schmidt, E., Hosler, J.P., Babcock, G., Gennis, R.B. and Ferguson-Miller, S, (1995) Possible proton relay pathway in cytochrome c oxidase, *Proc. Natl. Acad. Sci. USA* 92:1604-1608.(Medline)

Flatmark, T. and Pederson, J.I. (1975) Brown adipose tissue mitochondria, *Biochim. Biophys. Acta* 416:53-103.(Medline)

Fleischer, S., Fleischer, B. and Stoekenius, W. (1967) Fine structure of lipid depleted mitochondria, *J. Cell Biol.* 32:193-208.

Frey, T.G. and Mannella, C.A. (2000) The internal structure of mitochondria, *Trends Biochem. Sci.* 25:319-324. (MedLine)

Friedrich, T., Steinmüller, K. and Weiss, H. (1995) The proton-pumping respiratory complex I of bacteria and mitochondria and its homologue in chloroplasts, *FEBS Lett.* 367:107.(Medline) Garlid, K.D. and Beavis, A.D. (1985) Swelling and contraction of the mitochondrial matrix. II. Quantitative application of the light scattering technique to solute transport across the inner membrane, *J.Biol.Chem.* 260:13434-13441. (MedLine)

Gennis, R.B. and Ferguson-Miller, S. (1996) Protein structure; Proton-pumping oxidases, *Curr. Biol.* 6:36-38.(Medline)

Gennis, R.B., Barquera, B., Hacker, B., Van Doren, S.R., Arnaud, SE., Crofts, A.R., Davidson, E., Gray, K.A. and Daldal, F. (1993) The bc_1 complexes of *Rhodobacter sphaeroides* and *Rhodobacter* capsulatus, *J. Bioenerg. Biomembr.* 25:195-209.(Medline)

Goldenberg, H. (1982) Plasma membrane redox activities, *Bichim. Biophys. Acta* 694:203-223.(Medline)

Gonzalez-Halphen, D., Lindorfer, M.A. and Capaldi, R.A. (1988) Subunit arrangement in beef heart complex III, *Biochemistry* 27:7021-7031.(Medline)

Gould, S.J. and Valle, D. (2000) Peroxisome biogenesis disorders: genetics and cell biology, *Trends Genet.* 16:340-345. (MedLine)

Green, D.E. and Fleischer, S. (1963) The role of lipids in mitochondrial electron transfer and oxidative phosphorylation, *Biochim. Biophys. Acta* 70:554-581.

Green, D.E., Allman, D.W., Bachmann, E., Baum, H., Kopaczyk, K., Korman, E.F., Lipton, S., MacLennan,D.H., McConnell, D.G., Perdue, J.F., Rieske, J.S. and Tzagoloff, A. (1967) Formation of membranes by repeating units, *Arch. Biochem. Biophys.* 119:312-325.(Medline)

Guengerich, F. P. (1992) Cytochrome P450: advances and prospects, *FASEB J.* 6, 667-668.(Medline)

Hackenbrock, C.R. (1966) Ultrastructural bases for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria, *J. Cell Biol.* 30:269-297.(Medline)

Hackenbrock, C.R., Chazotte, B. and Gupte, S.S. (1986) The random collision model and a critical assessment of diffusion and collision in mitochondrial electron transport, *J. Bioenerg. Biomembr.* 18:331-368.(Medline)

Hackstein, J.H., Akhmanova, A., Boxma, B., Harhangi, H.R. and Voncken, F.G. (1999) Hydrogenosomes: eukaryotic adaptations to anaerobic environments, *Trends Microbiol.* 7:441-447. (MedLine)

Hall, J.D. and Crane, F.L. (1970) An intracristal structure in beef heart mitochondria, *Exp. Cell Res.* 62:480-483.(Medline)

Halper L.A. and Srere, P.A. (1977) Interaction of citrate synthase and mitochondrial malate dehydrogenase in the presence of polyethelene glycol, *Arch. Biochem. Biophys.* 184:529-534.(Medline)

Hashimoto, T. (1982) Individual peroxisomal β-oxidation enzymes, *Ann. N.Y. Acad. Sci.* 386:5-12.(Medline)

Hatefi, Y. (1985) The mitochondrial electron transport and oxidative phosphorylation system, *Annu. Rev. Biochem.* 54:1015-1069.(Medline)

Hofacker, I. and Schulten, K. (1998) Oxygen and proton pathways in cytochrome *c* oxidase, *Proteins* 30:100-107. (MedLine)

Huang, A.H.C., Trelease, R.N. and Moore, T.S., Jr. (1983) *Plant Peroxisomes*, Chapter 4, pp. 87-155. Academic Press, New York.

Iwata, S., Ostermeieer, C., Ludwig, B. and Michel, H. (1995) Structure at 2.8 resolution of cytochorme c oxidase from *Paracoccus denitrificans, Nature* 376:660-669.(Medline)

Iwata, S., Lee, J.W., Okada, K., Lee, J.K. Iwata, M., Rasmussen, N., Link, T.A., Ramaswamy, S. and

Jap, B.K. (1998) Complete structure of the 11-subunit bovine mitochondrial cyrochrome b_1 complex, *Science* 281:64-71.(Medline)

Izzard, S. and Tedeschi, H. (1970) Ion transport underlying metabolically controlled volume changes of isolated mitochondria, *Proc. Natl. Acad. Sci. U.S.A.* 67:702-709.(Medline)

Kim, H., Xia, D., Yu, C.A., Xia, J.Z., Kachurin, A.M., Zhang, L., Yu, L. and Deisenhofer, J. (1998) Inhibitor binding changes domain mobility in the iron-sulfur protein of the mitochondrial bc1 complex from bovine heart, *Proc. Natl. Acad. Sci. USA* 95:8026-8033.(Medline)

Kimura, Y., Vassylyev, D.G., Miyazawa, A., Kidera, A., Matsushima, M., Mitsuoka, K., Murata, K., Hirai, T. and Fujiyoshi, Y. (1997) Surface of bacteriorhodopsin revealed by high-resolution electron crystallography, *Nature* 389:206-211. (MedLine)

Klingenberg, M. and Kroger, A. (1967) On the role of ubiquinone in the respiratory chain. In *Biochemistry of Mitochondria* (Slater, E.C., Kaniuga, Z. and Wojtczak, L., eds.), pp. 11-27. Academic Press, New York.

Lea, P.J., Temkin, R.J., Freeman, K.B., Mitchell, G.A. and Robinson, B.H. (1994) Variations in mitochondrial ultrastructure and dynamics observed by high resolution scanning electron microscopy (HRSEM), *Micr. Res. and Tech.* 27:269-277.(Medline)

Lee, S.S.T., Pineau, T., Drago, J., Lee, E.J., Owens, J.W., Kroetz, D.L., Fernandez-Salguero, P.M., Westphal, H. and Gonzalez, F.J. (1995) Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators, *Mol. Cell. Biol.* 15:3012-3022. (MedLine)

Lock, E.A., Mitchell, A.M. and Elcombe, C.R. (1989) Biochemical mechanisms of induction of hepatic peroxisome proliferation, *Annu. Rev. Pharmacol. Toxicol.* 29:145-163. (MedLine)

MacLennan, D.H. and Asai, J. (1968) Studies on the mitochondrial adenosine triphospbatase system. V. Localizationof the oligomycin-sensitivity conferring protein, *Biochem. Biophys. Res. Commun.* 33:441-447.(Medline)

Mannaerts, G.P. and Debeer, L.J. (1982) Mitochondrial and peroxisomal beta-oxidation of fatty acids in rat liver, *Ann. N.Y. Acad. Sci.* 386:30-38.(Medline)

Mannella, C.A., Marko, M., Penczek, P., Barnard, D. and Frank, J. (1994) The internal compartmentation of rat-liver mitochondria : tomographic study using the high-voltage transmission electronmiscocope, *Micr. Res. and Tech.* 27:278-283.(Medline)

Mannella, C.A., Buttle, K., Rath, B.K. and Marko, M. (1998) Electron microscopic tomography of rat-liver mitochondria and their interaction with the endoplasmic reticulum, *Biofactors* 8:225-228.(Medline)

Marshall, P.A., Dyer, J.M., Quick, M.E. and Goodman, J.M. (1996) Redox-sensitive homodimerization of Pex11p: a proposed mechanism to regulate peroxisomal division, *J. Cell Biol.* 135:123-137. (MedLine)

Morgan, J.E., Verkhovsky. M. I. and Wikström, M. (1994) The histidine cycle; a new model for proton translocation in the respiratory heme-copper oxidases, *J. Bioenerg, Biomembr.* 26:599-608.(Medline)

Nagle, J.F., Mille, M. and Morowitz, H.J. (1980) Theory of hydrogen bonded chains in bioenergetics, *J. Chem. Phys.* 72:3959-3971.

Nedergard, J. and Cannon, B. (1984) *Thermogenic mitochondria*. In Bioenergetics (Ernster, L., ed.), pp. 291-314. Elsevier, New York.

Ohnishi, T. (1987) Structure of the succinate-ubiquinone oxidoreductase (complex II), *Curr. Top. Bioenerg.* 15:37-66.

Ohnishi, T. and Salerno, J.C. (1982) Iron-sulfur clusters in the mitochondrial electron-transport chain. In *Iron Sulphur Proteins* (Spiro, T.G., ed.), pp. 285-327. Wiley, New York.

Osmundsen, H. (1982) Peroxysomal β-oxidation of long fatty acids: effects of high fat diet, *Ann. N.Y. Acad. Sci.* 386:12-27.(Medline)

Ostermeier, C., Harrenga, A., Ermler, U. and Michel, H. (1997) Structure at 2.7 Å resolution of the Paracoccus denitrificans two-subunit cytochrome *c* oxidase complexed with an antibody FV fragment, *Proc. Natl. Acad. Sci. USA* 94:10547-10553. (MedLine)

Parson, D.F. (1963) Mitochondrial structure: two types of subunits on negatively stained mitochondrial membranes, *Science* 140:985-988.

Perkins, G.A. and Frey ,T.G. (2000) Recent structural insight into mitochondria gained by microscopy, *Micron* 31:97-111. (MedLine)

Perkins, G., Renken, C., Martone, M.E., Young, S.J., Ellisman, M. and Frey, T. (1997) Electron tomography of neuronal mitochondria: three-dimensional structure and organization of cristae and membrane contacts, *J. Struct. Biol.* 119:260-272. (MedLine>

Ragan, C.I. (1987) Structure of NADH-ubiquinone reductase (complex I), *Curr. Top. Bioenerg.* 15:1-36.

Rammelsberg, R., Huhn, G., Lubben, M. and Gerwert, K. (1998) Bacteriorhodopsin's intramolecular proton-release pathway consists of a hydrogen-bonded network, *Biochemistry* 37:5001-5009. (MedLine)

Reynafarje, B., Brand, M.D. and Lehninger, A.L. (1976) Evaluation of the H^+/site ratio of mitochondrial electron transport from rate measurements, *J. Biol. Chem.* 251:7442-7451.(Medline)

Riistama, S., Hummer, G., Puustinen, A., Dyer, R.B., Woodruff, W.H. and Wikström M. (1997) Bound water in the proton translocation mechanism of the haem-copper oxidases, *FEBS Lett.* 414:275-280. (MedLine)

Rizzuto, R., Pinton, P., Carrington, W., Fay, F.S., Fogarty, K.E., Lifshitz, L.M., Tuft, R.A. and Pozzan, T. (1998) Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses, *Science* 280:1763-1776.(Medline)

Roux, B., Nina, M., Pomes, R. and Smith, J.C. (1996) Thermodynamic stability of water molecules in the bacteriorhodopsin proton channel: a molecular dynamics free energy perturbation study, *Biophys. J.* 71:670-681. (MedLine)

Salerno, J.C., and Ohnishi, T. (1979) Electron transport in the succinate ubiquinone segment of the respiratory chain. In *Tunneling in Biological Systems* (Chance, B., DeVault, D.C., Fraunfelder, H., Marcus, B.A., Schrieffer, J.R. and Sutin, N., eds.), pp. 473-482. Academic Press, New York.

Schneider, D.L., Kagawa, Y. and Racker, E. (1972) Chemical modification of the inner mitochondrial membrane, *J. Biol. Chem.* 247:4074-4079.(Medline)

Schoonjans, K., Staels, B. and Auwerx, J. (1996) The peroxisome proliferator activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation, *Biochim. Biophys. Acta.* 1302:93-109. (Medline)

Smith, J.L. (1998) Secret life of cytochrome bc_1, *Science* 281:58-9. (MedLine)

Sommerville, C.R. and Ogren, W.L. (1980) Photorespiration mutants in Arabidopsis thaliana deficient in serine-glyoxylate aminotransferase, *Proc. Natl. Acad. Sci.* U.S.A. 77:2684-2687.

Subramani, S., Koller, A. and Snyder, W.B. (2000) Import of peroxisomal matrix and membrane proteins, *Annu. Rev. Biochem.* 69:399-418. (MedLine)

Sumegi, B. and Srere, A. (1984) Complex I binds several mitochondria NAD-coupled dehydrogenases, *J. Biol. Chem.* 259:15040-15045.(Medline)

Sun, I.L., Crane, F.L., Grebing. C. and Low, H. (1984) Properties of a transplasma membrane electron transport system in HeLa cells, *J. Bioenerg. Biomembr.* 16:583-595.(Medline)

Tabak, H.F., Braakman, I. and Distel, B. (1999) Peroxisomes: simple in function but complex in maintenance, *Trends Cell Biol.* 9:447-453. (Medline)

Tedeschi, H. (1959) The structure of the mitochondrial membrane: inferences from permeability properties, *J. Cell Biol.* 6:241-252.

Thomas, J.W., Puustinen, A., Alben, J.O., Gennis, R.B. and Wikström, M. (1993) Substitution of asparagine for aspartate-135 in subunit I of the cytochrome bo ubiquinol oxidase of *Escherichia coli* eliminates proton-pumping activity, *Biochemistry* 32:10923-10928.(Medline)

Tielens, A.G.M., Rotte, C., van Hellemond, J.J. and Martin, W. (2002) Mitochondria as we don't know them, *Trends Biochem. Sci.* 27:564-572. (MedLine)

Titorenko, V.I. and Rachubinski, R.A. (2001) The life cycle of the peroxisome, *Nature Rev. Mol. Cell Biol.* 2:357-368. (MedLine)

Trumpower, B.L. (1990a) Cytochrome bc_1 complexes of microorganisms, *Micr. Rev.* 54:101-129.(Medline)

Trumpower, B.L.(1990b) The protonmotive Q cycle. Energy transduction by coupling of proton translocation to electron transfer by the cytochrome bc_1 complex, *J. Biol. Chem.* 265:11409-11412.(Medline)

Tsukihara, T., Aoyama, H., Yamashita, E., Yamaguchim H., Sinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995) Structure of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 , *Science* 269:1069-1074.(Medline)

Tzagaloff, A., MacLennan, D.H., McConnell, D.G. and Green, D.G. (1967) Studies on the electron transfer system. LXVIII. Formation of membranes as basis of the reconstitution of the mitochondrial electron transfer system, *J. Biol. Chem.* 242:2051-2061.

van den Bosch, H., Schutgens, R.B., Wanders, R.J. and Tager, J.M. (1992) Biochemistry of peroxisomes, *Annu. Rev. Biochem.* 61:157-197. (Medline)

Van der Giezen, M., Slotboom, D.J., Horner, D.S., Dyal, P.L., Harding, M., Xue, G.P., Embley, T.M. and Kunji E.R. (2002) Conserved properties of hydrogenosomal and mitochondrial ADP/ATP carriers: a common origin for both organelles, *EMBO J.* 21:572-579. (MedLine)

Verkhovsky, M.I., Jasaitis, A., Verkhovskaya, M.L., Morgan, J.E. and Wikström, M. (1999) Proton translocation by cytochrome c oxidase, *Nature* 400:480-483. (Medline)

Vidugiriene,J., Sharma, D.K., Smith, T.K., Baumann,N.A. and Menon A.K. (1999) Segregation of glycosylphosphatidylinositol biosynthetic reactions in a subcompartment of the endoplasmic reticulum, *J. Biol. Chem.* 274:15203-15212. (Medline)

Wang, H.-J., Guay, G., Pogan, L., Sauve, R. and Nabi, I.R. (2000) Calcium regulates the association between mitochondria and a smooth subdomain of the endoplasmic reticulum, *J. Cell Biol.* 150:1489-1498. (MedLine)

Waxman, D.J. (1999) P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR, *Arch. Biochem. Biophys.* 369:11-23. (MedLine)

Wei, P., Zhang, J., Egan-Hafley, M., Liang, S. and Moore, D.D. (2000) The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism, *Nature* 407:920-923. (MedLine)

Weiss, H. (1987) Structure of ubiquinol-cytochrome c reductase (complex III), *Curr. Top. Bioenerg.* 15:67-90.

Weiss, H., Friedrich, T. Hofhaus, G. and Preis, D. (1991) The respiratory-chain NADH dehydrogenase (complex I) of mitochondria, *Eur. J. Biochem.* 197:563-576(Medline)

Wood, P.M. (1988) Chemolithotrophy. In *Bacterial Energy Transduction* (Anthony, C., ed.), pp. 183-230. Academic Press, New York.

Wrigglesworth, J.M., Packer, L. and Branton, D. (1970) Organization of mitochondrial structure as revealed by freeze-etching, *Biochim. Biophys. Acta* 205:125-135.(Medline)

Xie, W., Barwick, J.L., Downes, M., Blumberg, B., Simon, C.M., Nelson, M.C., Neuschwander-Tetri, B.A., Brunt, E.M., Guzelian, P.S. and Evans, R.M. (2000) Humanized xenobiotic response in mice expressing nuclear receptor SXR, *Nature* 406:435-439. (MedLine)

Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M.J., Libeu, C.P., Mizushima, T., Yamaguchi, H., Tomizaki, T. and Tsukihara, T. (1998) Redox-coupled crystal structural changes in bovine heart cytochrome c oxidase, *Science* 280:1723-1729.(Medline)

Zhang, Z., Huang, L., Shulmeister, V.M. Chi, Y.-I., Kim, K.K., Hung, L.-W., Crofts, A.R., Berry, E.A. and Kim, S.-H. (1998) Electron transfer by domain movement in cytochrome bc_1, *Nature* 392:677-684.(Medline)

17. Photosynthesis

With few exceptions, such as the chemolithotrophic bacteria, the ultimate source of most of the energy used by biological systems is sunlight. Consequently, almost all organisms depend directly or indirectly on the conversion of radiant energy to chemical free energy. The *heterotrophs*, which do not carry out photosynthesis, degrade complex molecules provided by other organisms.

The overall process of photosynthesis is frequently represented as shown in Eq. (1).

$$CO_2 + 2H_2A + \text{light} \rightarrow [CHOH] + H_2O + 2A \quad (1)$$

In green plants, the A of equation (1) corresponds to oxygen, which originates from water as shown by the equation. This has been demonstrated with ^{18}O-labeled water (Stemler and Radmer, 1975). In some bacteria, the A of equation (1) corresponds to S [Eq. (2)] and even S and H_2O [Eq. (3)].

$$CO_2 + 2H_2S + light \rightarrow (CHOH) + H_2O + 2S \quad (2)$$

$$3CO_2 + 2S + 5H_2O + light \rightarrow 3(CHOH) + 2H_2SO_4 \quad (3)$$

In bacteria, the source of carbon may be not CO_2 but rather an organic molecule, and the final product may be not a carbohydrate but some other reduced organic compound. Several of the known schemes are summarized in Table 1 (Stanier, 1961).

Table 1 Characteristics of Photosynthetic Organisms

	Green plants	**Cyanobacteria**	**Green sulfur bacteria**	**Purple bacteria**
Source of reducing power	H_2O	H_2O	H_2S, reduced organic compounds	H_2S, reduced inorganic & organic compounds
Photosynthetic O_2 evolution	Yes	Yes	No	No
Principle source of carbon	CO_2	CO_2	CO_2	CO_2 or organic compounds
Relation to oxygen	Aerobic	Aerobic	Strictly anaerobic	Strictly or faculatively anaerobic
Site	Chloroplast	Membrane-like structures	Chlorosomes	Internal membranes (chromato-phores)

The absorption of light quanta and the subsequent reactions that convert radiant energy to chemical energy are included in what we call photosynthesis. The *chlorophylls* (sometimes abbreviated in this discussion as Chl) are the most abundant pigments, but many others (such as carotenoids and, in some algae, phycobilins) are also present. Many of these pigment molecules absorb light and funnel the energy

to the reaction centers, which are involved more directly in energy transduction. The characteristics of some of the pigments are shown in Fig. 1. Chlorophylls *a* and *b* are the predominant types of Chl in plants and cyanobacteria (Fig. 1a). Purple photosynthetic bacteria contain bacteriochlorophyll (BChl), either *a* or *b*, whereas green bacteria have a third type of BChl. Chlorophyll and pheophytins, shown in Fig. 1a, resemble protoporphyrin IX, the prosthetic group of hemoglobin and the *c*-type cytochromes. In contrast to those proteins, in Chl, Fe is replaced by Mg^{2+} and, in pheophytins by $2H^{+}$. Fig. 1b shows β-carotene, a major carotene in many plants, and spheroidine, a bacterial carotenoid. The phycobilins, many of which are poorly characterized, are not shown; they are proteins containing tetrapyrroles similar to the bile pigments in animals. Phycobilins are present in large complexes, the phycobilisomes of cyanobacteria (blue-green algae) and red algae. The phycobilisomes absorb light over a wide spectral region.

All the reactions involved in light absorption, transfer of energy, electron transport, and phosphorylation are associated with membranes. Not surprisingly, the latter two resemble the processes in oxidative phosphorylation discussed in Chapter 16.

I. THE PHOTOSYNTHETIC MEMBRANES

The chloroplasts present in eukaryotes are discrete structures enclosed by membranes. They may be spiral-shaped, cup-shaped, star-shaped, or irregular. In higher plants and bryophytes, they are generally shaped like saucers and are approximately 5 to 10 μm in diameter. The light-driven reactions, as well as the biochemical pathways involved in the fixation of CO_2, occur in chloroplasts. Fig. 2 (Staehelin, 1986) shows a chloroplast of a red alga. A thin section of chloroplast of a higher plant as seen with the electron microscope is shown in Fig. 3 (Park, 1966). Fig. 4 illustrates the probable arrangement of a chloroplast similar to that of Fig. 3. The membrane enclosing the organelles is double. The internal membrane system of flattened vesicles or sacs, the *thylakoids*, is embedded in the chloroplast interior, the *stroma*. These vesicles may be closely apposed in a combination of shorter and longer lamellae. These composite structures form stacks, the *grana*. The grana stacks are interconnected by lamellae (the *stroma lamellae*), so at least some of the thylakoid inner spaces are continuous with those in other stacks. Parts of the thylakoid surface are apposed to other thylakoid surfaces and other parts, such as the stroma lamellae, are exposed to the chloroplast interior. Although the general features remain the same, the details may vary considerably, even within the same cell. The thylakoids have been shown to contain the chlorophyll as well as the *light harvesting complexes* (LHCs), the *reaction centers* (RCs), and the components of the electron transport chain, which will be discussed later. The soluble material contains the enzymes responsible for carbon dioxide fixation and the biochemical synthetic pathways. The significance of structural details, such as the stacking, is not clear, since mutants lacking grana are functional (Goodenough et al., 1969; Goodenough and Staelin, 1971).

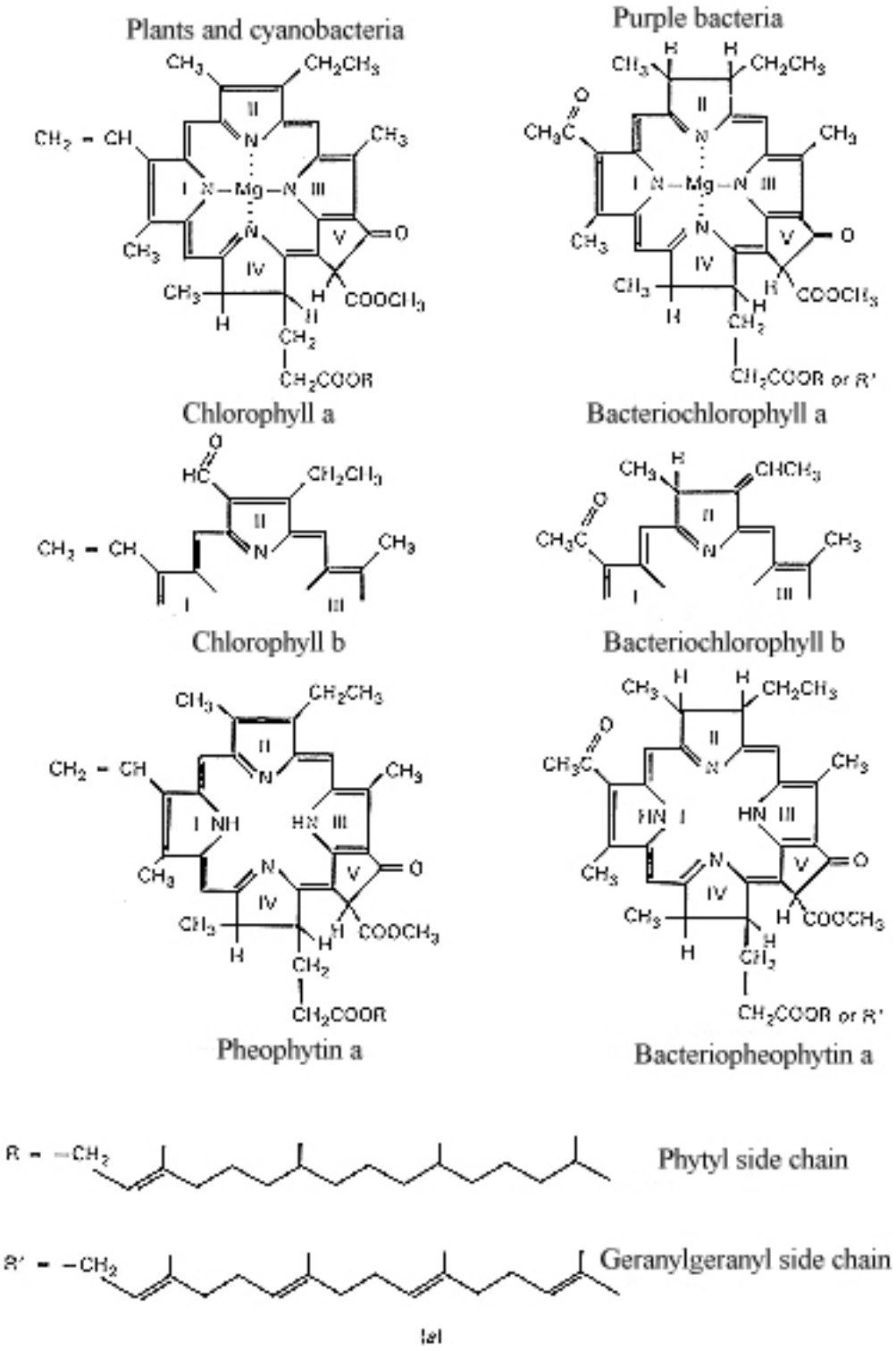


Fig. 1 Structures of photosynthetic pigments. (a) Chlorophylls & pheophytins.

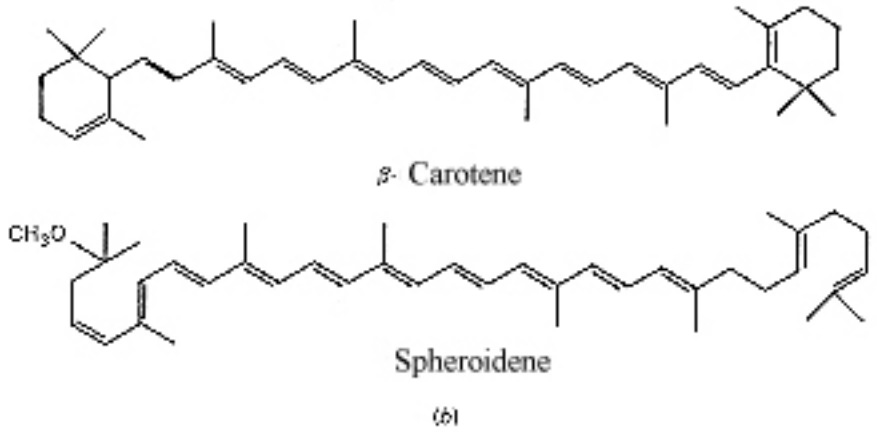


Fig. 1 Structures of Photosynthetic pigments (Continued). (b) Carotenoids.

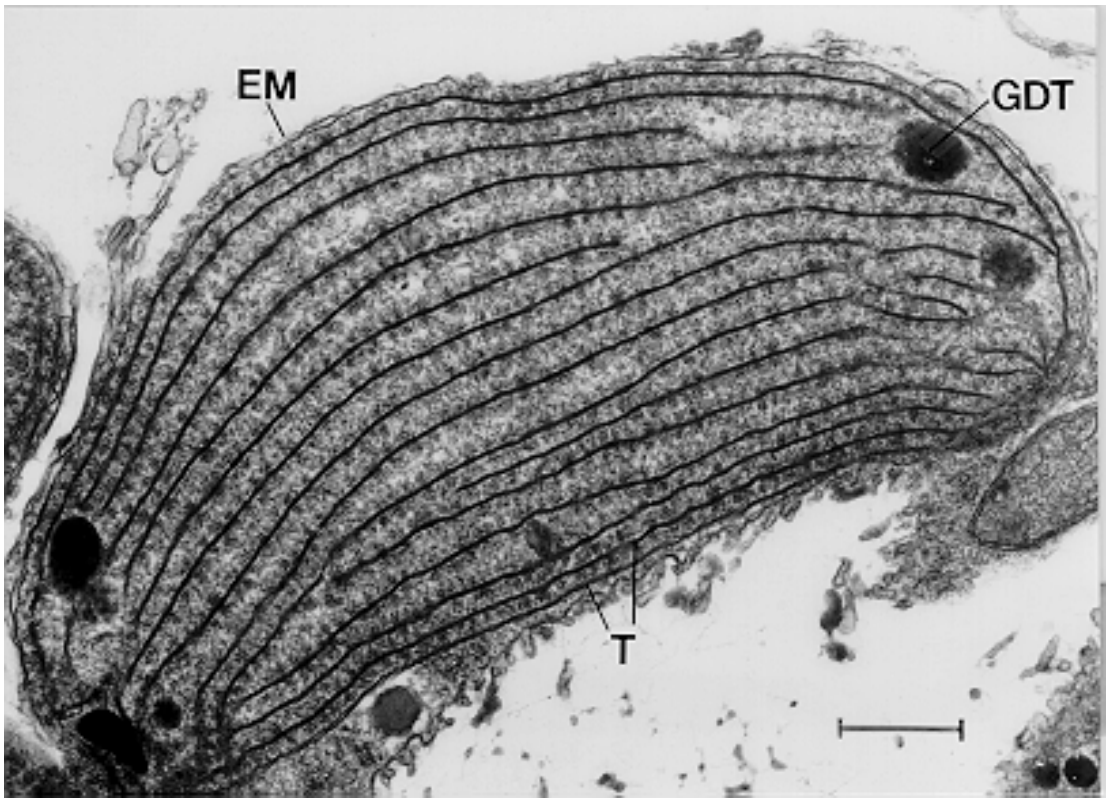


Fig. 2 Section through a chloroplast of the red alga *Spermothamnion tuneri*. The phycobilosomes are attached to the thylakoids (T). EM, Envelope membrane; GDT, girdle thylakoid. Bar corresponds to 769 nm. Reproduced by permission from Staehelin (1986).

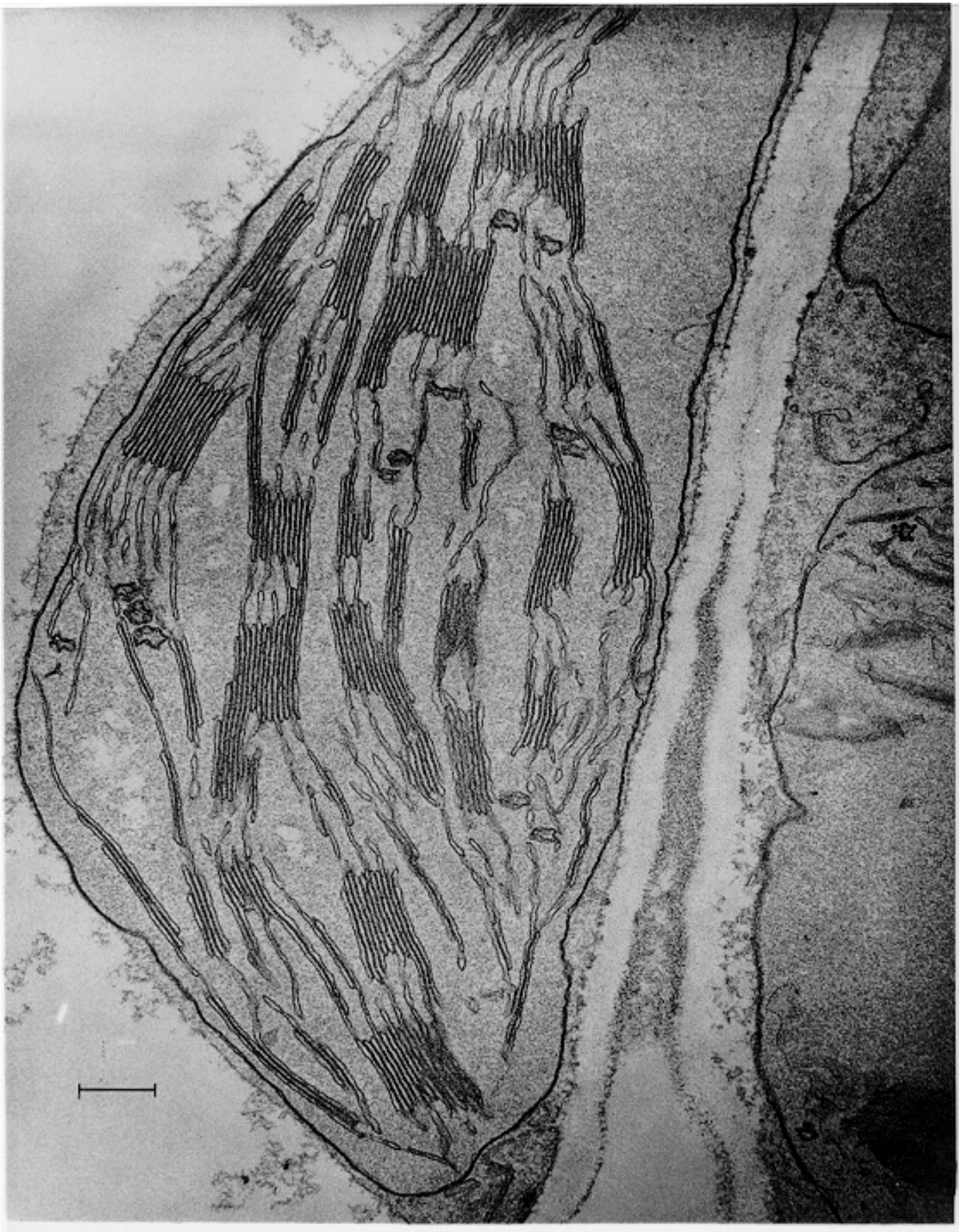


Fig. 3 Thin section of $KMnO_4$-fixed *Spinacea oleracea* chloroplast, bar corresponds to 769 nm.

Reproduced by permission from Park (1966).

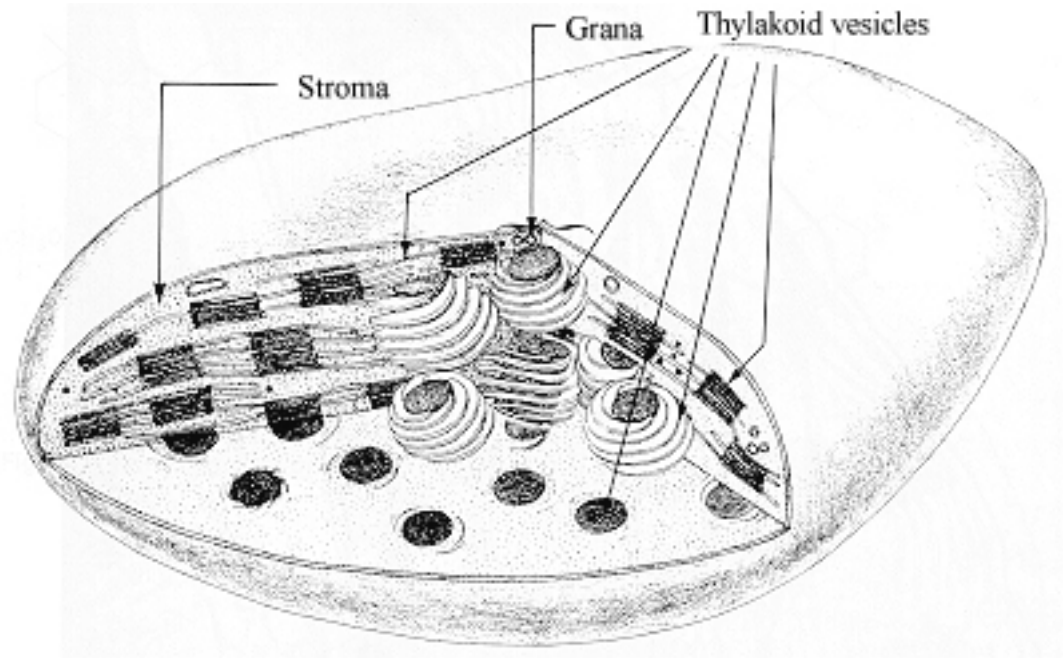


Fig. 4 Chloroplast thylakoid membrane architecture viewed in two and in three dimensions. This drawing of a sectioned chloroplast is intended to aid in visualizing the relationship between the appearance of thylakoid membranes in two dimensions with that in three dimensions. From D. R. Ort (1986), *Encyclopedia of Plant Physiology*, with permission. Copyright ©1986 Springer-Verlag.

In bacteria (Fig. 5) (Sprague and Varga, 1986), the morphology of the photosynthetic membranes varies. The cell membrane may be directly involved, or complex invaginations of the cell membrane or special vesicles, such as the *chlorosomes*, may be present. Vesicles prepared from photosynthetic bacteria, the *chromatophores*, have been found to be active in photosynthesis.

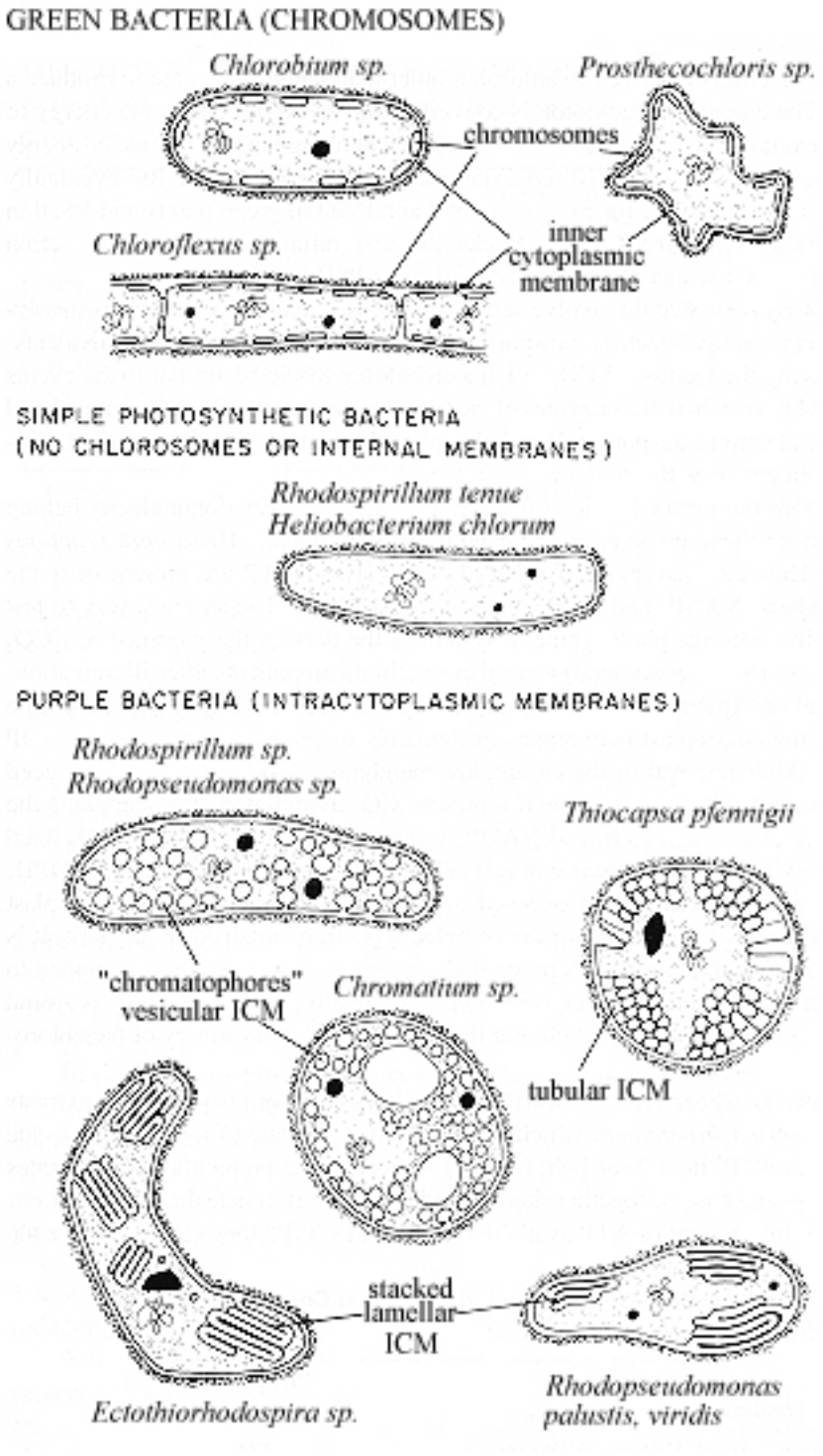


Fig. 5 Morphologies observed in the anoxygenic photosynthetic bacteria for housing the photosynthetic apparatus. The green bacteria contain chlorosomes attached to the inner side of the cytoplasmic membrane (CM). Most purple bacteria elaborate an extensive membrane system within the cytoplasm for the photosynthetic components. A few "simple" bacteria contain only a single CM, which contains the pigments and proteins necessary for photosynthetic growth. From S. G. Sprague and A. R. Varga (1986), *Encyclopedia of Plant Physiology*, with permission. Copyright ©1986 Springer-Verlag.

II. THE EVENTS OF PHOTOSYNTHESIS: LIGHT AND DARK REACTIONS

In photosynthesis, pigments present in specialized complexes, the *antennae*, or light harvesting complexes (LHCs) absorb most of the light. Absorption of photons of incident visible or ultraviolet light produces a change in the electron orbitals of the absorbing molecules (Sauer, 1986). The electrons generally occupy molecular orbitals in the lowest possible energy state, where they are present in one pair per orbital. In the excited state produced by the photon absorption, one of the electrons is moved to an orbital other than the ground state to produce a singlet excited state. The energy of the photon is converted into an orbital electronic energy of the excited state. The excitation is then transferred from pigment to pigment by resonance energy transfer over distances as long as 8 to 10 nm. Energy can also be transferred in this way from PSII to PSI. The energy transferred to the RC eventually results in the oxidation of a pigment. P680 and P700 in green plants and P870 in some bacteria (see below) lose one electron to initiate the events of electron transport (Sauer, 1986)

Conceivably, photosynthesis could involve separate processes: one set of processes involving the trapping of radiant energy, electron transport and the production of reducing equivalents, and the other set involving the fixation of CO_2. Such a dichotomy would be similar to the events of oxidative phosphorylation, where the enzymes of the tricarboxylic acid cycle can be considered separate from those of electron transport and oxidative phosphorylation. The information presently available is in agreement with this view.

The reactions responsible for the fixation of CO_2 or some other carbon donor clearly belong to a separate system from those involved in the capture of light quanta. These so-called *dark reactions* do not require light. They can take place if reduced NADPH and ATP are present or if the system containing oxidized $NADP^+$ and ADP is exposed to the light. The simplest way to test this proposition is to incubate the photosynthetic system in the dark in the presence of $^{14}CO_2$ (or, for bacteria, some other ^{14}C compound) and other required components after illumination. The data from this kind of experiment are shown in Table 2 (Trebst et al., 1958). In sample 1, the mixture containing chloroplast membranes (presumably thylakoids), $NADP^+$ and ADP was exposed to light. After removal of the chloroplast membranes, the $^{14}CO_2$ was introduced into the system in the dark. The counts shown represent CO_2 assimilated. For sample 2, the procedure was identical except that ADP and $NADP^+$ were left out. The amount of CO_2 fixed is very low and probably reflects the presence of a small residual amount of ATP and NADPH. Sample 3 represents the complete system exposed to light with both $^{14}CO_2$ and chloroplast membranes present. Sample 4 corresponds to the complete system maintained in the dark. It is clear that considerable $^{14}CO_2$ fixation occurs in the dark as long as energy has been supplied to the system in the form of ATP and reducing equivalents. Evidently, once the energy is stored in a biochemically utilizable form, neither light nor the photosynthetic machinery of the chloroplast is needed.

Table 2 CO_2 Fixation by a Chlorophyll-Free Extract and a Complete Chloroplast System from Spinach[a]

Treatment	$^{14}CO_2$ fixed (counts/min x 10^3)
1. Chlorophyll-free extract, dark (NADP and ADP present)	134
2. Chlorophyll-free extract, dark (no NADP and no ADP)	9
3. Complete chloroplast system, light	200
4. Complete chloroplast system, dark	20

Trebst et al. (1958). Reproduced with permission from *Nature* 182: 352-355, copyright ©1958 Macmillan Magazines Limited.

Similar experiments have been carried out with bacterial systems, for instance, with extracts of the purple sulfur bacteria *Chromatium*, which incorporate [^{14}C]acetate (Table 3) (Losada et al., 1960). As long as ATP (item 2) or light (item 5) is present, the preparation incorporates [^{14}C]acetate. Hexokinase and glucose together (items 4 and 7) largely eliminate the incorporation, probably by decreasing the amount of ATP available. In this case ATP alone suffices, since the reducing equivalents can be produced from ATP hydrolysis by running electron transport in reverse (see Section III,C).

Table 3 Equivalence of ATP and Light in the Assimilation of [^{14}C]Acetate by Cell-Free Preparations of Chromatium

Treatment	^{14}C fixed (counts/min) x 10^3
1. Dark, Control	27
2. Dark. ATP	180

3. Dark, ATP, hexokinase	186
4. Dark, ATP, hexokinase and glucose	6
5. Light, control	414
6. Light, hexokinase	348
7. Light, hexokinase, glucose	20

Losada et al. (1960). Reproduced by permission from *Nature* 186:753-760 copyright ©1960 by Macmillan Magazines Limited.

Conceivably, the biochemical pathway followed may depend on whether light is present. However, the radioautographic paper chromatographs of Fig. 6a and b (Trebst et al., 1958), obtained with the reaction products of experiments such as those of Table 2, show that this is not the case. The results show that the intermediates accumulating under the two sets of conditions (i.e., in the dark, after illumination or under continuous illumination) are the same. The principles of paper chromatography used to separate these molecules are discussed in the legend of the figure.

The results just discussed show that the light-requiring reactions per se are not involved in carbon fixation, except to supply chemical free energy in the form of NADPH and ATP. Seen from this point of view, photosynthesis is concerned directly only with trapping radiant energy and converting it into biologically utilizable forms: reducing equivalents and a phosphate of high group-transfer potential.

In different organisms, photosynthesis differs in detail. However, the light-requiring processes correspond to the production of ATP and reducing equivalents. The subsequent biochemical reactions can take place in the dark.

III. PHOTOOXIDATlON

The sections that follow are concerned with the events that result in the production of ATP and reducing equivalents during photosynthesis.

After the primary photochemical event -- the light-energized oxidation of Chl in the RCs -- the return of the excited electrons through electron transport carriers could provide enough energy for the synthesis of ATP from ADP and P_i by a process analogous to that taking place in mitochondria. Similarly, these electrons could be used to reduce electron carriers with the eventual production of NADPH.

Let us begin with the photooxidation of chlorophyll. When Chl or BChl are oxidized, one electron is removed, not from the metal as in the case of cytochromes (in this case Mg^{2+}), but from the aromatic electron system of the molecule. The spin of the remaining electron is delocalized over the whole electron system.

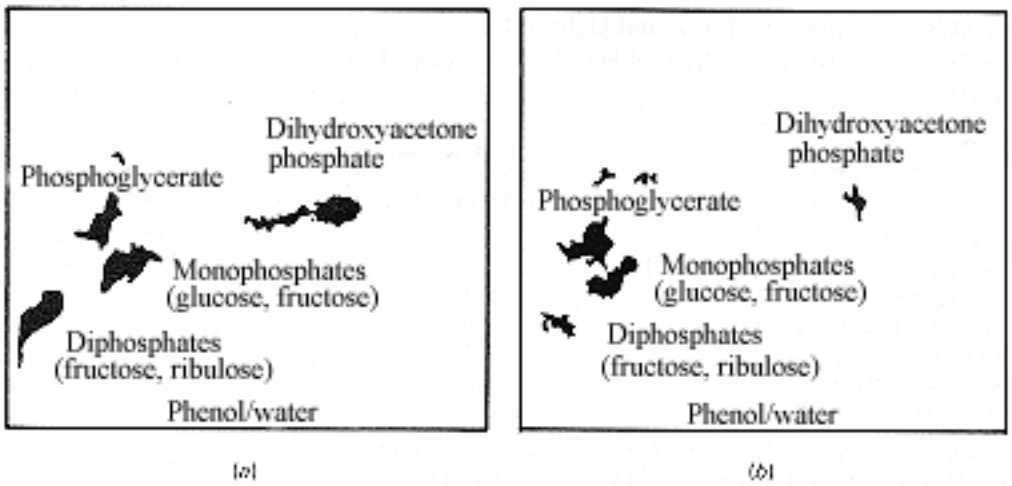


Fig. 6 Tracings of radioautographs of chromatograms showing (a) products of photosynthetic $^{14}CO2$ assimilation by illuminated spinach chloroplasts and (b) products of dark $^{14}CO_2$ assimilation by chlorophyll-free extract of chloroplasts. In paper chromatography, a mixture is placed at one end of the chromatographic paper. The solution moves by capillary action and it is submitted to minute partition steps between the solvent and the wet cellulose fibers of the paper. The migration of various compounds on the paper and in a particular medium will depend on their individual solubility in the solvent. The location of the individual compounds can be seen by means of reactions generating colored compounds or, in the case of radioactive compounds, by autoradiography. Ideally, each spot is unique and corresponds to a single compound. In the chromatographs, the compounds have migrated in one direction in one particular solvent system (phenol:water) and in the other direction, at right angles from the first, in another solvent system (butanol:acetic acid). Separation with such a technique depends on the solubility of the compounds in the different solvents, resulting in increased separation. The position of the compounds was recorded by placing the paper on photographic paper. From Trebst et al. (1958). Reproduced with permission from *Nature*, 182:351-355, copyright ©1958 Macmillan Magazines Limited.

The photooxidation can be studied and detected by electron paramagnetic resonance (EPR) and also by light absorption measurements. For the latter, the experimental design and the apparatus used for spectrophotometric measurements in photosynthetic systems are illustrated in Fig. 7 (Clayton, 1980). In this representation, the monitoring beam provides the light needed to measure changes in absorption. The beam is weak and ideally has no effect on the pigments and therefore none on the absorption spectrum. The detector, which measures the light transmitted through the sample, is shielded in some way from stimulation by the excitatory beam. Since excitatory and monitoring beams are generally at different wavelengths, this can be done with light filters.

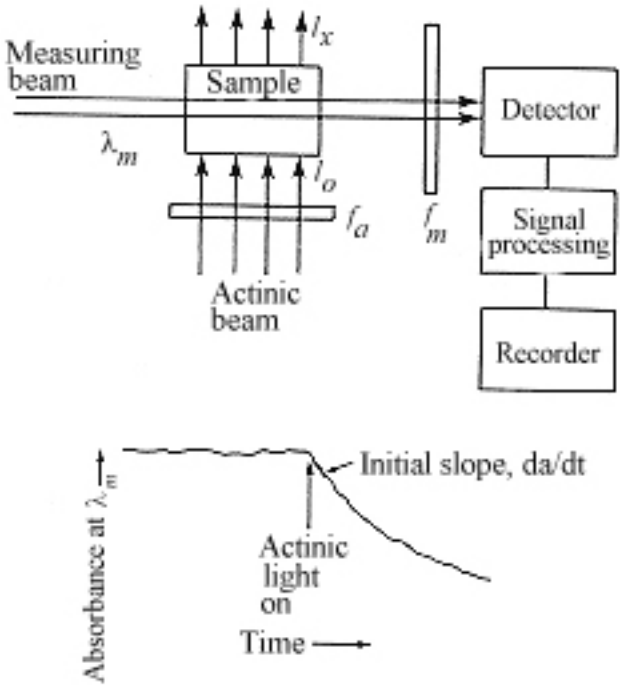


Fig. 7 System for measuring light-induced absorbance changes and the quantum efficiency of a photochemical process. From Clayton (1980). Copyright ©1980 by Cambridge University Press, reproduced with the permission of Cambridge University Press.

Various experiments have demonstrated that the primary photosynthetic event is the oxidation of chlorophyll by light. A mutant of the purple photosynthetic bacteria, *Rhodobacterium sphaeroides*, that lacks carotenoids, was used in the experiment of Fig. 8 (Clayton, 1980). Fig. 8a represents the absorption spectrum of the isolated RCs. The pigments responsible for the peaks are indicated. Fig. 8b represents the change in optical density brought about by illumination, which occurs primarily in the region of absorption of BChl. The effect is an oxidation, as shown by the inhibition of the effect with a reducing compound (Fig. 8c).

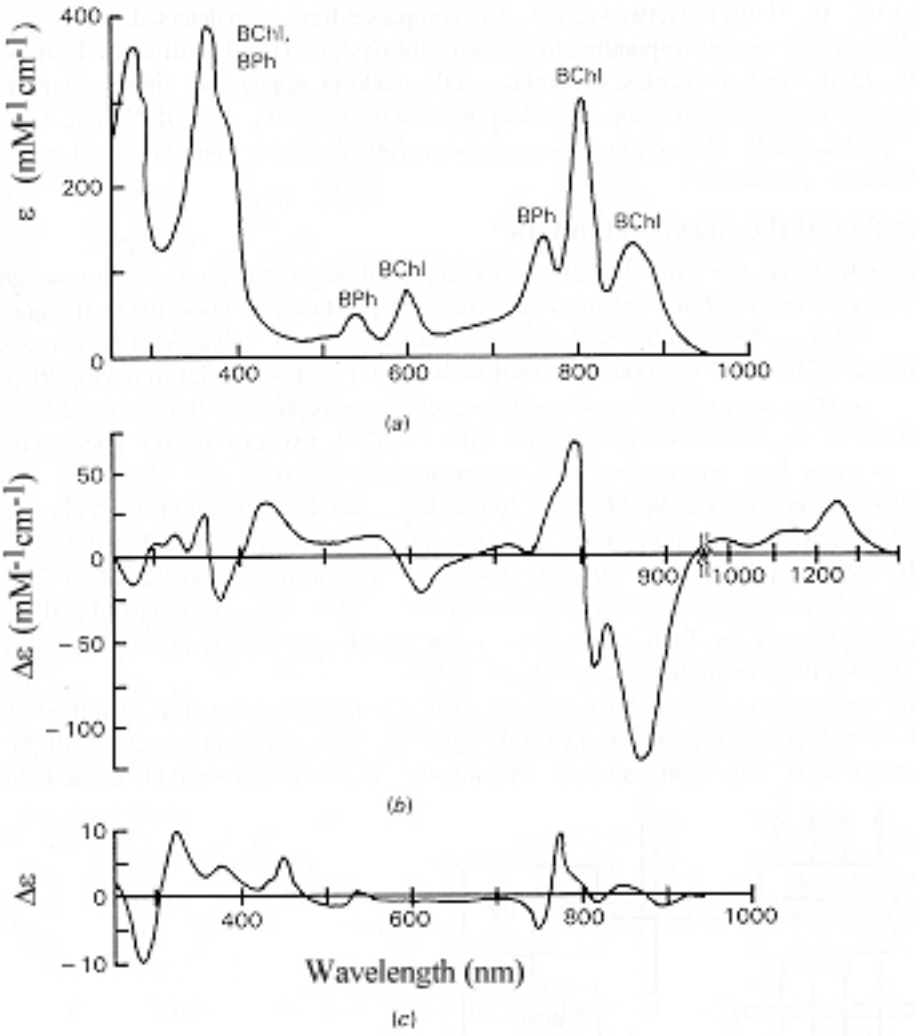


Fig. 8 (a) Absorption spectrum and (b and c) difference spectra of light-induced absorbance changes of reaction centers isolated from carotenoidless mutant *R. sphaeroides*. (b) Reaction centers alone. (c) Reaction centers plus an electron donor to prevent the accumulation of oxidized bacteriochlorophyll during illumination. Changes due to oxidation of the donor have been discounted. From Clayton (1980).

The chlorophylls that are photooxidized have been named after the wavelength, in nanometers, of the major absorbance decrease produced by the photooxidation, in this case, P870 (P stands for pigment). In chloroplasts, two such reactive complexes have been detected spectrophotometrically: P700 and P680, corresponding to the two photosystems (I and II) discussed below. In bacteria, two BChl *a* or *b* molecules, depending on the bacteria, appear to be involved in the photooxidation, since the remaining unpaired electron is delocalized over two BChl molecules. Two Chl *a* molecules are also involved in the chloroplasts.

A. Organization of the Reaction Centers

As indicated in Section II in the native photosynthetic systems, light is absorbed by LHCs (see below), constituted of Chls, carotenoids and bilins, to produce excited electronic states. The antennae then

transfer the excitation to the reaction center (RC) (see Allen and Williams, 1998; Heathcote et al., 2002). The chlorophylls that undergo photooxidation are complexed to the RC. Type I RCs have 4 FeS clusters as electron acceptors, whereas Type II have pheophytin and quinones. Photosystems I and II (PSI and PSII) (see below) include Type I and Type II RCs respectively.

The RCs from two purple photosynthetic bacteria have provided detailed information of the complexes. These features are common to all RCs (e.g., see Fyfe and Jones 2000) and include Chl or BChl dimers as the primary electron donors, the arrangement of the cofactors carrying electrons across the membrane in two branches traversing the bilayer oriented by an axis of symmetry perpendicular to the plane of the membrane (Fig. 9) (see Allen et al., 1987). The purple bacteria RCs contain 4 molecules of BChl *a*, 2 of bacteriopheophythin *a*, 2 of ubiquinone (Q_A and Q_B), 1 non-heme Fe and 1 carotenoid. The Fe is thought to play a structural role, and the carotenoid is thought to have a photoprotective role. These redox cofactors are held at the interface between L and M polypeptides forming a hydropohobic environment. Each of the polyptides have 5 transmembrane α helices. An additional polypeptide (H) is on top of the cytoplasmic faces of the L and M polypeptides. L, M, and H are present in 1:1:1 stoichiometry in most bacteria studied, and their molecular masses are 32, 34, and 29 kDa respectively. The H polypeptide is present only in some bacteria and can be removed without affecting function. Two of the Bchl molecules have been termed B_A and B_B. The two other BChl molecules are packed close together; and their optical absorption changes with illumination identify them as P870.

Fig. 9 shows the arrangements of the prosthetic groups of the center as identified by the three-dimensional reconstruction of x-ray crystallography (Deisenhofer et al., 1984, 1985). The four hemes are in the cytochrome subunit; the other components shown are in the L-M complex.

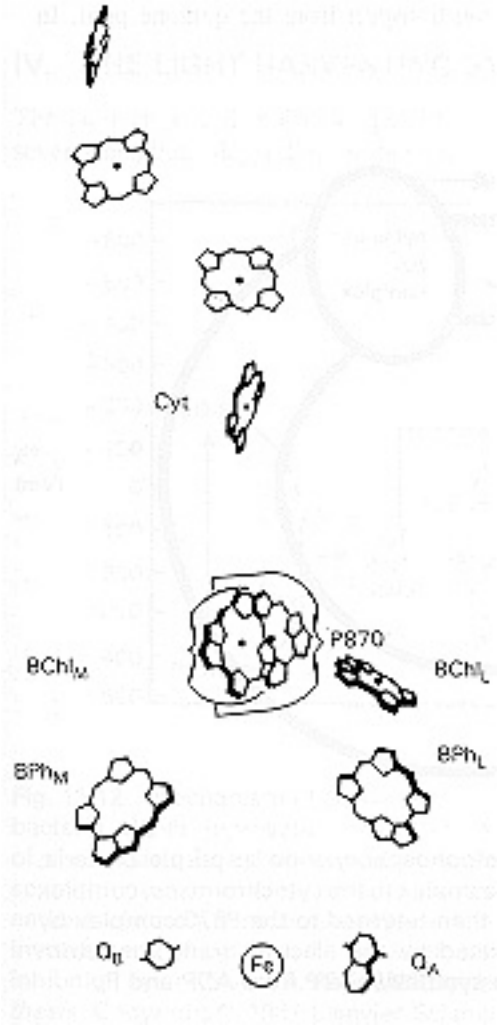


Fig. 9 Arrangement of the prosthetic groups in the *Rhodopseundomonas viridis* reaction center. Q_B is shown at the site identified by Deisenhofer et al. (1984) but the orientation of the quinone in this site is drawn arbitrarily; the exact orientation of Q_B in the crystal structure has not been described. The four hemes at the top are in the cytochrome subunit; the other components are in the L-M complex as indicated by the subscripts. The plane normal to the chromatophore membrane is approximately vertical and the periplasmic side of the complex is at the top. The L and M proteins traverse the membrane, with the periplasmic side represented at the top and the cytoplasmic side at the bottom. The orientation of the quinone in this diagram is arbitrary. Reproduced by permission from W.W. Parson, *Photosynthesis*, pp.43-62. Copyright ©1987 Elsevier Science Publishers, Amsterdam.

The arrangement of plant, cyanobacterial and algal Type II RCs appear to be similar to that of the purple bacteria. In these cases the D1 and D2 polypeptides, each with 5 transmembrane helices, are in positions equivalent to those of the L and M polypeptides. Similarly, the redox cofactors are also similar. In this case, they are constituted by 4 Chl *a*, 2 pheophytins and 2 plastoquinones. However, unlike the RC of purple bacteria, the D1 and D2 polypeptides bind 1 Chl α each. This Chl might act to transmit energy from the antenna to the RC. As in purple bacteria, the cofactors also are arranged as two transmembrane sections. Two light antennae constituted of proteins CP43 and CP47 are present in close proximity. They correspond to 6 transmembrane α helices containing 12 -14 Chl α molecules.

The acceptor side of the purple bacteria and the PSII reaction center are very similar. The electron

transfer across the membrane is carried out from the primary donor to Q_A quinone followed by the formation of quinol Q_B. However, the redox events on the donor side are quite different. In purple bacteria the electron missing in the photooxidized $P870^+$ is replaced from an electron from a cytochrome. In contrast, in PSII RC, $P680^+$ receives an electron from the *oxygen evolving complex* (OEC) (see below) with the production of O_2. The OEC is thought to involve a cluster of 4 Mn as well as Ca^{2+} and Cl ions (see Nugent, 2001; Rutherford and Faller, 2001).

Oxygenic photosynthetic organisms have two photosystems, PSI and PSII and these function in tandem (see Section IV). The PSI RC are larger than those of PSII. The RC of PS1 is thought to be present as a trimer; each monomer consisting of 12 polypeptides (see Jordan et al., 2001). Each PSI RC monomer contains 96 Chls *a*, 22 carotenoid, 4 lipids, 3 FeS clusters and 2 phylloquinones. The redox cofactors are enclosed in 5 carboxy-terminal portion of each PsaA and PsaB polypeptides which traverse the bilayer. The amino-terminal portion contribute 6 α transmembrane helices and connect to antenna domains present along the core domain. The transmembrane sector contains 6 Chl *a*, and 2 phylloquinones (Q_K) present in two branches similar to Type II RCs. The P700 primary donor is a heterodimer of Chl and an epimer Chl a (e.g., see Maeda et al., 1992; Webber and Lubitz, 2001). The acceptors are A, A_0 and A_1. The two branches end at the F_x Fe-S center. The electrons are then tranferred to ferredoxin. Epimerization changes the conformation of the group attached to C13 of the Chl and allows the incorporation of Chl a' to the PsA side. In contrast to Type II complexes the phylloquinones of the PSI RC, transfer electrons to the single redox acceptors of the FeS centers. The question remains of which branch of the redox carriers is responsible for the transmembrane transfer of electrons from P700 and the FeS centers and the phylloquinones. Present evidence suggests that the transfer is over both branches (see Heathcote et al., 2002).

Heliobacteria and green sulfur bacteria have an arrangement similar to the Type I components. However, the RCs have a homodimeric core (similar to PsaC) and a small peptide, not the PsaA-PsaB heterodimer. The polypeptide composition of the Type I complexes are simpler than those of PSI. Similarly to the PSI RC, in green sulfur bacteria the homodimeric RCs possess epimers of Bchl *a* and in heliobacteria Bchl *g*. It is likely that electrons move in both branches of the RC and the primary electron acceptor A_o is a Chl (not a Bchl).

Electron transfers within the RC are likely to occur through tunneling pathways (Balabin and Onuchic, 2000) where constructive and destructive interference can take place (see Betts et al., 1992). The pathways were previously defined from X-ray diffraction data collected at cryogenic temperature from crystals of the RC in the dark or under illumination (Stowell et al., 1997). The electron transfer is coupled through interactions with the surrounding medium which is affected by the thermal motion. The electron transfer from donor to acceptor can take place only if the two are energetically matched. This match will occur occasionally due to the thermal motion of the surrounding protein matrix and will depend on constructive interference.

B. Cyclic Photophosphorylation of Bacteria

When RCs of isolated bacteria are excited with a very short flash, a transient P870 excited single state is formed. This state decays in picoseconds, forming a $P870^{+}BPh^{-}$ radical pair. Accordingly, the absorption bands of P870 and BPh disappear and new bands that have been attributed to the radicals are formed. This is followed by the reduction of one quinone to produce a semiquinone radical, QA. The reactions occur extremely rapidly, essentially with a quantum efficiency of 1 and independent of temperature. This supports the idea that these components are held together tightly with little motion (Dutton, 1986).

The electron of QA^{-} is transferred to a second quinone, Q_b This electron displacement is a much slower process and is temperature dependent. These reactions and the redox potentials are outlined in Fig. 10.

The electron displaced from P870 is replaced from the c-type cytochrome, so the RC can respond again to light. After a second electron is removed from P870, the Q_b has become fully reduced and $Q^{2-}{}_b$ picks up two H^{+} from the cytoplasmic side of the membrane. The cytochrome bc_1 complex oxidizes Q_BH_2. These reactions are thought to proceed with a net proton efflux of as many as four H^{+} per two electrons (Dutton, 1986). The passage of these protons through a channel in the F_0 portion of the ATP synthase is thought to be responsible for ATP synthesis (see Chapter 18). These details are summarized in Fig. 11.

The series of reactions just discussed is entirely cyclic. The electron displaced from the photooxidation of P870 is replaced by the cytochrome bc_1 complex, which in turn recovers it from the oxidation of quinone. Thus, the energy from the light absorption is converted into ATP with no net change in electrons. Most photosynthetic eubacteria carry out cyclic electron transport (Pierson and Olson, 1987).

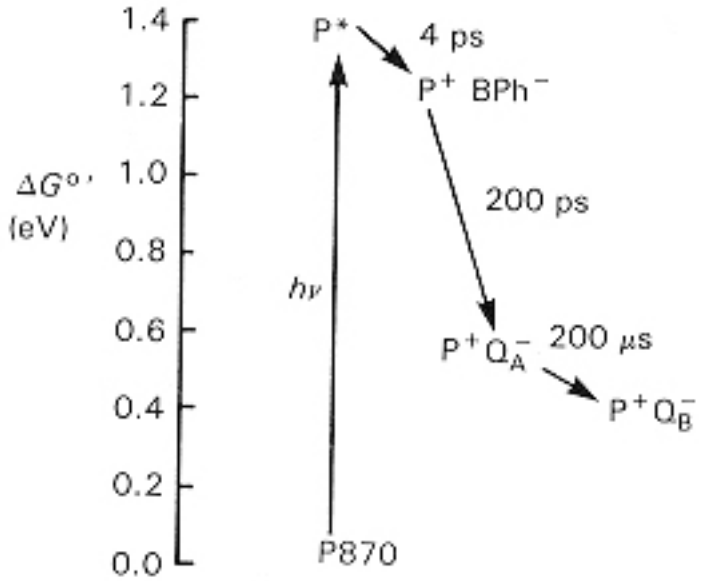


Fig. 10 Kinetics and standard free energy changes of electron transfer steps in reaction centers isolated from *Rb. sphaeroides*. The rates of the transfers are indicated next to the arrows.

C. Production of Reducing Equivalents in Bacteria

What accounts for the production of reducing equivalents? In the RC-2 bacteria, NAD^+ is probably reduced by reverse (energy-requiring) electron transport from the quinone pool. In contrast, green sulfur bacteria reduce ferredoxin directly from the secondary acceptor. Both cases require an external reductant such as succinate or H_2S.

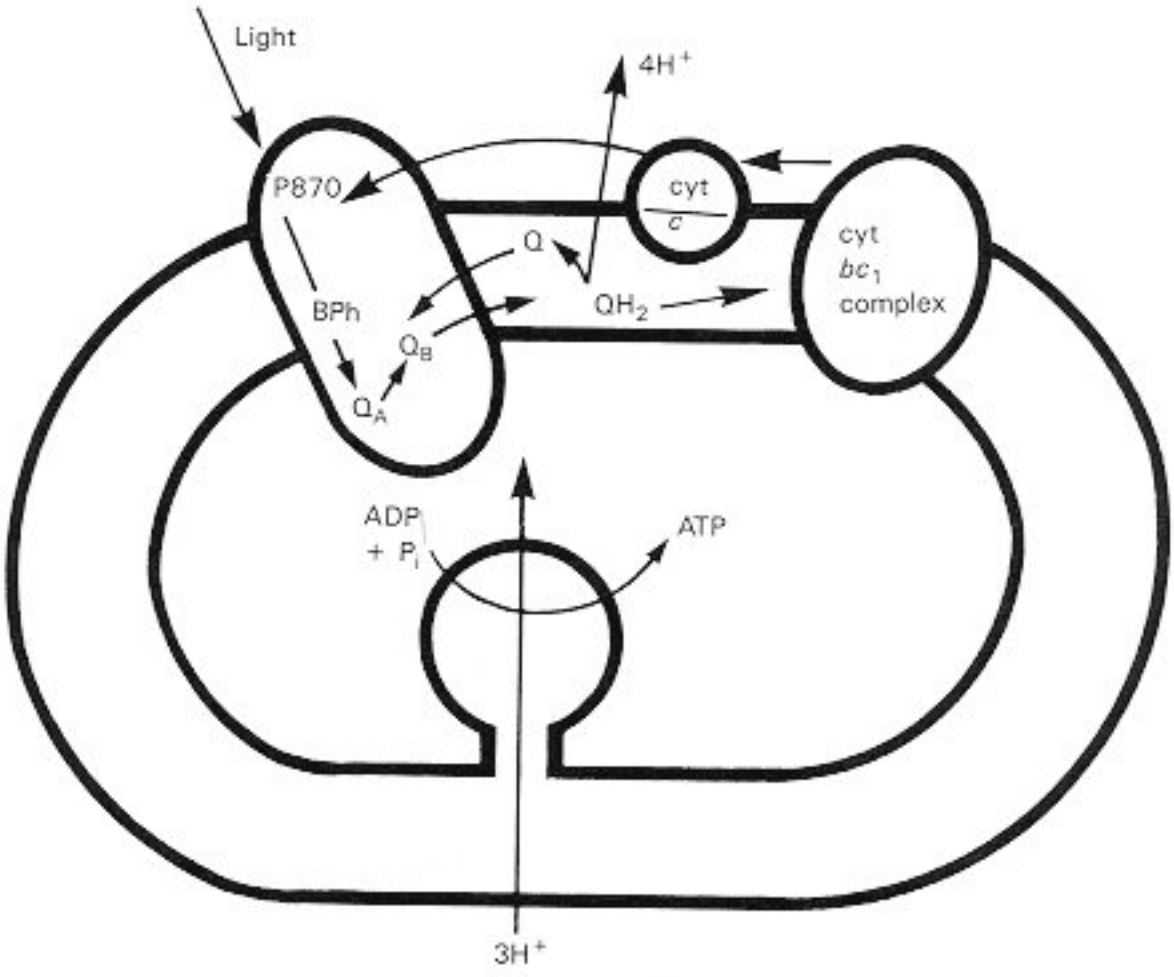


Fig. 11 Schematic representation of cyclic photophosphorylation in purple bacteria. Reducing equivalents are transferred from the P870 complex to the cytochrome bc_1 complex by quinone (dissolved in the membrane), which is then returned to the P870 complex by cytochrome c (water soluble). The protons generated by the electron transport return through the ATP synthase, providing the energy to synthesize ATP from ADP and P_i.

Experiments with chromatophores isolated from *Rhodospirillum rubrum* and other nonsulfur purple bacteria show that electrons can be transferred from succinate to NAD^+ in the dark, provided that ATP or pyrophosphate hydrolysis (presumably through the action of ATP synthase, proceeding in reverse) is available to supply energy (Jones and Vernon, 1969). In the absence of ATP, the reduction requires cyclic electron flow to pump electrons uphill from organic reductants, such as succinate. A block of the cyclic electron flow blocked NAD^+ photoreduction but not the ATP-driven reduction. In addition,

uncouplers of oxidative phosphorylation or photophosphorylation blocked the photoreduction and the ATP-dependent reduction (Hauska et al., 1983; Malkin, 1987). Uncouplers are thought to collapse the energy-dependent H^+ electrochemical gradient, i.e., the proton motive force. Therefore, these experiments suggest that the energy is provided indirectly through the proton motive gradient. The reduction also requires the presence of a functional NADH dehydrogenase. The mechanism suggested by these experiments is summarized in Fig. 12a (Knaff and Kampf, 1987).

In green sulfur bacteria, however, the energy is thought to be directly available, since there is no inhibition by uncouplers (Knaff and Kampf, 1987). The scheme consistent with the information available for these bacteria is summarized in Fig. 12b.

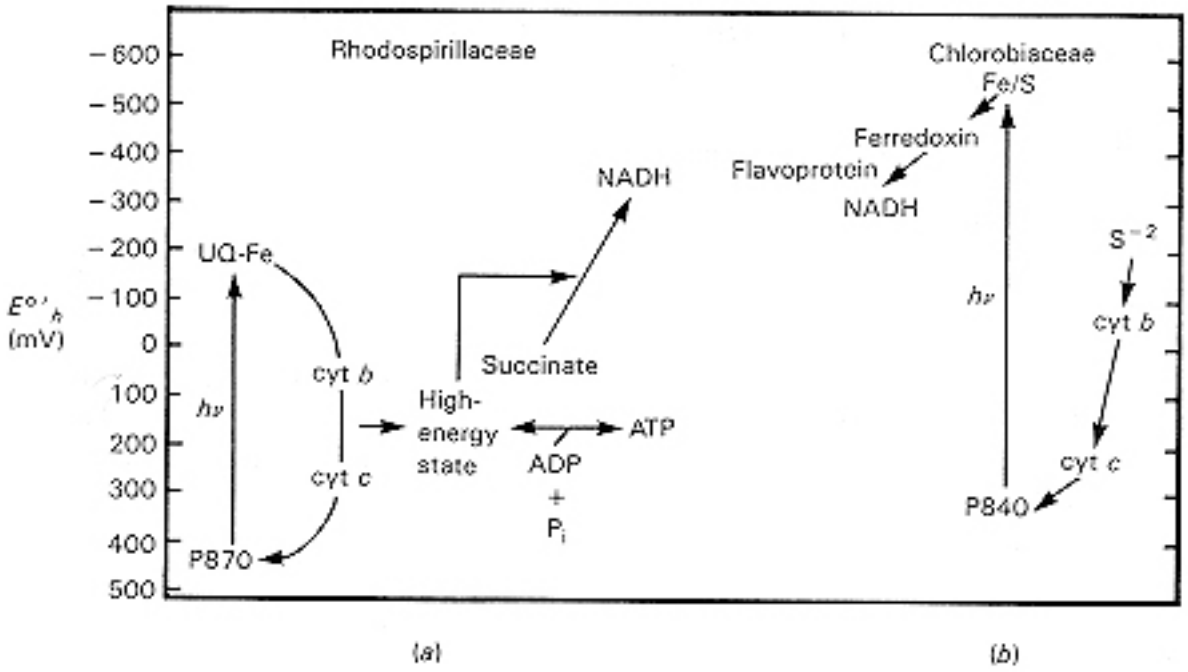


Fig. 12 Mechanism of NAD^+ photoreduction in (a) purple bacteria and (b) green sulfur bacteria. UQ-Fe represents the Fe^{2+}-quinone complex present at the primary quinone site of purple bacteria, although in some species menaquinone replaces ubiquinone. Fe/S represents the iron-sulfur center that functions as an early acceptor in green sulfur bacteria. The earliest electron acceptors have been omitted for both green and purple bacteria. The involvement of cyt b in S^{2-} oxidation in green bacteria is speculative but is based on inhibition by antimycin A. Reproduced by permission from B. Knaff and C. Kampf, *Photosynthesis*, pp.199-212. Copyright ©1987 Elsevier Science Publishers, Amsterdam.

D. The Special Case of Bacteriorhodopsin

Halobacterium halobium is halophilic (salt loving), red colored bacterium of tidal salt flats. Under conditions of oxygen deprivation, purple patches, referred to as purple membranes, appear in the carotenoid containing plasma membrane of *Halobacterium*. The purple membranes contain mostly protein (75% by weight) which corresponds almost entirely of *bacteriorhodopsin* (Brh) (see Blaurock and Stoeckenius, 1971; Oesterhelt and Stoeckenius, 1971). Brh acts as a transducer by coupling the

absorption of light to the translocation of protons from the cytoplasm to the medium. The return of the protons to the cytoplasm is used to synthesize ATP via the ATP-synthase or the protons are exchanged for Na^+ to transport it against a steep gradient, in the face of external NaCl concentrations as high as 4 M.

The structure of Brh (see Khorana, 1988) has been deduced by electron microscopy and crystallographic techniques since this protein forms a highly organized lattice in the purple membrane. The molecule contains seven transmembrane α-helices (A-G) linked by loops. The purple color is the result of its binding to one molecule of all-*trans* retinal which absorbs with a maximum at a wavelength of 570 nm (see Khorana, 1988).

Retinal changes from the all-*trans* form to the 13-*cis*-form, when it absorbs light and the bacteriorhodopsin pushes the proton through components of the seven helices to the outside (see below). The passage of the proton is through several intermediate states of the Brh (K-O), some accompanied by conformational changes in the transmembrane helices. Some of these conformations have been elucidated (see Kühlbrandt, 2000; selected articles are available through http://www.nature.com/nature/fow).

One molecule of Brh contains one retinal molecule with one end of the retinal covalently bound to a lysine of helix G and the other end deep in the transmembrane segments of the Brh. For each photon absorbed by retinal, one H^+ is transferred across the cell membrane against an electrochemical gradient (e.g., see Gennis and Ebrey, 1999). Very small movements (about 1 Å) of components of the Brh accompny the pumping. These movements are induced by the bending and unbending of retinal accompanying its light dependent isomerization (in the 13-*cis*-form one of the retinal terminals is twisted around a double bond and during the transition the pigment moves in relation to the protein). The movements of the Brh change the affinity to protons of neighboring side chains by changing their local chemical environment. The passage of the proton is through several intermediate states in the transmembrane helices in conduits involving several amino acid residues (e.g., aspartate, tyrosine, arginine and water molecules). In essence, the retinal acts as a valve in a conduit permitting the exchange in a single direction (see Kühlbrandt, 2000; selected articles are available through http://www.nature.com/nature/fow).

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

IV. THE TWO PHOTOCHEMICAL SYSTEMS OF CHLOROPLASTS

As already discussed, chloroplasts have two distinct chlorophyll complexes in the RCs: P700 and P680. With their respective electron acceptors and donors, these complexes constitute two distinct assemblies, which are called photosystem I (PSI) and photosystem II (PSII), respectively.

A summary of our present understanding of their organization is shown in Fig. 13 (Blakenship and Prince, 1986). The scale on the left represents the redox potentials of the electron carriers. The large arrows represent the excitation of PSI or PSII by light. As in the figures in Chapter 16, the small arrows represent electron transport steps. As shown in Fig. 13, PSI and PSII are connected in series, i.e., the electron transport chain that receives electrons from PSII replaces the electrons displaced by the light reaction in PSI. This arrangement also imposes two distinct features. One corresponds to a mechanism that replaces the electron lost by the photooxidation of PSII, carried out by the oxygen-evolving complex (OEC) (Dunahay et al., 1984), and the other corresponds to a series of reactions that accept the electrons from PSI to eventually reduce $NADP^+$.

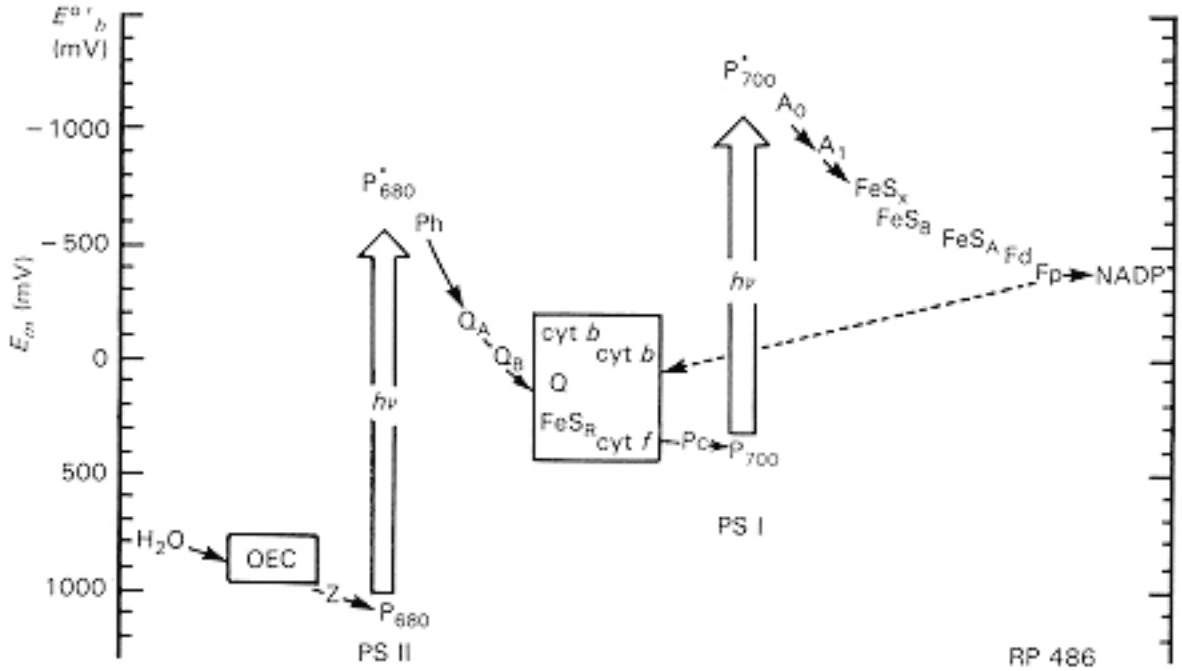


Fig. 13 The Z scheme for oxygenic photosynthesis constructed using excited-state redox potentials. The carriers are placed at the accepted midpoint redox potentials (at pH 7) where they have been measured directly; other potentials are estimated. OEC, oxygen-evolving complex; Z, donor to photosystem II

(PSII); P_{680}, reaction center chlorophyll of PSII; Ph, pheophytin acceptor of PSII; Q, quinone; cyt, cytochrome; FeS_R, Rieske iron-sulfur protein; Pc, plastocyanin; P_{700}, reaction center chlorophyll of PSI; A_0 and A_1, early acceptors of PSI (possibly chlorophyll and quinone species); FeS_X FeS_B, and FeS_A, bound iron-sulfur protein acceptors of PSI; Fd, soluble ferredoxin; Fp, flavoprotein (ferredoxin-NADP reductase). FeS_B and FeS_A may operate in parallel. The dashed line indicates cyclic electron flow around PSI. The pathway of electron flow through the cyctochrome $b_6f$complex is outlined by a box. Reproduced from *Trends in Biochemical Sciences*, vol. 10, R. Blakenship and R. C. Prince, pp. 382-383, copyright &copy1985 Elsevier Science.

Biochemical fractionation of the chloroplast photosynthetic system has shown that the complete system consists of three separate transmembrane complexes: PSI, PSII, and cytochrome b_6f complex. The electron transport between PSII and the cytochrome b_6f complex is mediated by *plastoquinone* (PQ). *Plastocyanin* (PC) mediates the electron transport between the cytochrome b_6f complex and PSI. The three complexes are represented in Fig. 14 in diagrammatic form (Cramer et al., 1985).

Plastoquinone closely resembles ubiquinone (CoQ), a component of the electron transport chain of mitochondria. Plastocyanin is a water-soluble 1l-kDa protein containing copper ion coordinated to the side chains of cystein, methionine and two histidines. Reduced PC and oxidized PC contain Cu^+ and Cu^{2+}, respectively.

The arrangement of Fig. 14 is reminiscent of the four complexes of mitochondria in which electron transport is carried out between complexes by either CoQ or cytochrome *c* (Chapter 16).

The stoichiometry of the photosystems and the Chl distribution in higher plant chloroplasts are summarized in Table 4 (Glazer and Melis, 1987; Whitmarsh and Ort, 1984).

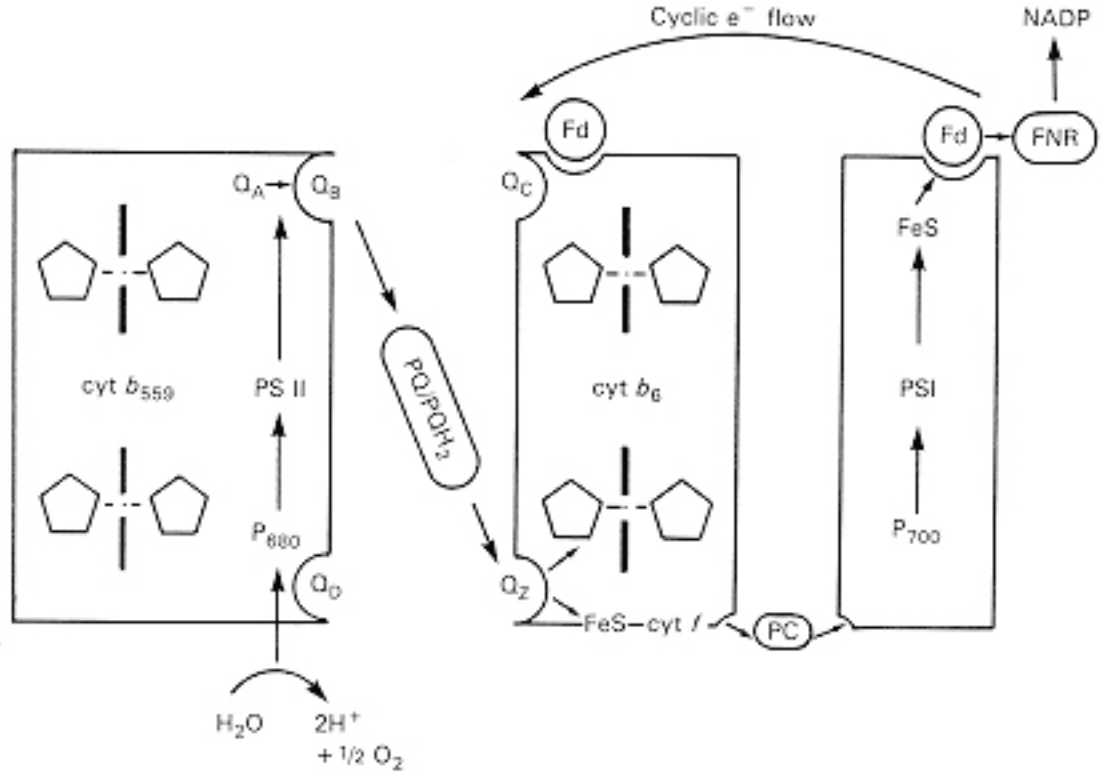


Fig. 14 Arrangement of the three major transmembrane protein electron transport complexes in the thylakoid membrane. The photosystem II complex is involved in water splitting, in electron transfer to noncyclic electron transport chain and proton deposition to the lumen. It includes the 51-and 44-k Da chlorophyll-binding polypeptides that also bind the Q_A acceptor and (perhaps) the Q_D donor plastoquinone; two 32-kDa putative plastoquinone binding proteins to which herbicides and probably the Q_D plastoquinone also bind; and cytochrome b_{559} containing two hemes per P680 reaction center that are hypothesized to span the membrane as shown. Electrons from the PSII complex are transferred by a plastoquinone pool to the cytochrome b_6f complex through a plastoquinone binding site, Q_z. The b_6 *f* complex consists of four subunits: 34-kDa cytochrome *f*, 23-kDa cytochrome b_6 (with two heme groups), a 20-kDa polypeptide FeS redox center, and a fourth 17-kba polypeptide (not shown). The cytochrome *bf* complex is connected to photosystem I by plastocyanin (PC). The transmembrane PSI P700 reaction center complex possibly contains two large (M_r 70,000) P700 reaction center chlorophyll polypeptides and at least three smaller peptides that may contain the FeS electron receptor complex used to reduce ferredoxin (Fd) and NADP. The involvement of Fd in PSI cyclic phosphorylation, possibly through a plastoquinone binding site, Q_c, is shown. The fourth membrane-spanning ATP synthase complex is not shown. Reproduced from *Trends in Biochemical Sciences*, vol.10, W.A. Cramer et al., pp.125. Copyright &copy1985 Elsevier Science.

Table 4 Photosystem Stoichiometry and Chlorophyll Distribution in Wild-Type Higher Plant Chloroplasts

	PSII[a]	PSI[a]	Cyt *b/f*[b]

Stoichiometry	1.7	1.0	1.10
Antenna size	330	200	
Chl *a*	235	180	
Chl *b*	95	20	
Total Chl,%	63	37	

[a] Data from Glazer and Melis (1987). Reproduced, with permission, from the *Annual Review of Plant Physiology*, vol. 38 &copy1987 Annual Reviews, Inc.

[b] Data from Whitmarsh and Ort (1984). Reproduced by permission.

The complexes are not randomly distributed in the membrane; rather, PSII is primarily located in the appressed grana and PSI primarily in the stroma lamellae (Anderson and Anderson, 1982). The interaction between the three complexes is thought to occur by the diffusion of plastoquinone in the membrane from PSII to the cytochrome b_6f complex and by the location of the cytochrome complex close to PSI. The low molecular weight and high lipid solubility of plastoquinone would permit fast diffusion in the plane of the membrane, sufficient to account for the electron transport rate (Haehnel, 1984). This arrangement is shown in Fig. 15 (Haehnel, 1984).

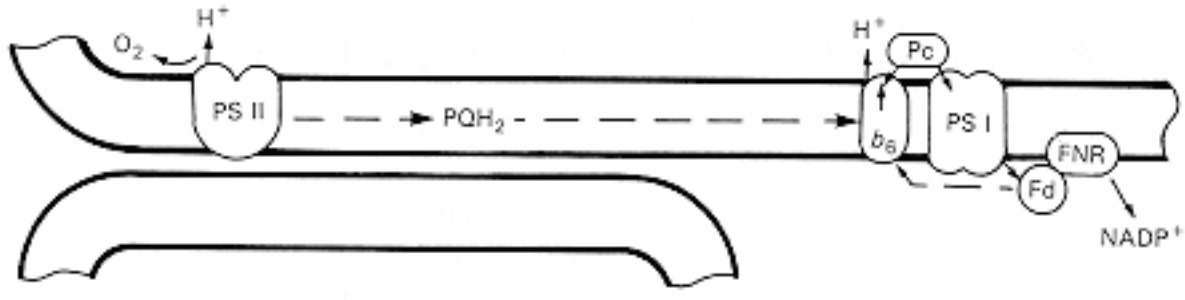


Fig. 15 Schematic representation of photosynthetic electron transport distributions of the integral complexes in chloroplasts with grana stacks. PQH_2, plastoquinol; b_6f cytochrome b_6f complex; Pc, plastocyanin; Fd, ferredoxin; and FNR, ferredoxin-$NADP^+$ reductase. Not shown is the H^+ uptake from outside. The long dashed arrows indicate assumed long-range diffusion of mobile electron carriers. From Haehnel (1984). Reproduced, with permission, from the *Annual Review of Plant Physiology*, volume 35, 1984 by Annual Reviews Inc.

What is the evidence for two photosynthetic events? Part of the story includes results of experiments on

the effectiveness of various wavelengths. The effectiveness of light in photosynthesis can be expressed as the yield of O_2 evolved per einstein of light. The einstein, the energy equivalent of photons, corresponds to *Nhv*, where *N* is Avogadro's number (6×10^{23}), *v* is the frequency of the light (vibrations per second), and *h* is Planck's constant (1.6×10^{-34}). Results of an experiment measuring the yield as a function of wavelength for the grana alga, *Chlorella*, are shown in Fig. 16 (Emerson et al., 1957). Curve 1 represents the yield with a single beam of light. Curve 2 represents the yield with two beams of light, the second beam of constant but of lower intensity and shorter wavelength. The yield is greater for curve 2, particularly at longer wavelengths. The effect must involve separate light-activated units, since it occurs even when the two beams are alternated (Fig. 17) (Myers and French, 1960). In these experiments, the oxygen release is measured with a Pt electrode and the actual current in µA recorded (ordinate). The excitatory beam is alternated between wavelenghts of 700 and 650 nm (as indicated in the upper tier of the figure). The O_2 released is increased whenever the shorter wavelength is turned on (upper trace). However, when the alternating is done between 690 and 700 nm, there is no significant effect (lower curve and numbers in lower tier).

The excitation of PSI and PSII is necessary for optimal functioning of chloroplast photosynthesis, but the two also have separate functional roles. The photochemical O_2-generating system can be dissociated from other events by providing the system with an electron acceptor (e.g., indophenol blue). Such a reaction could be visualized as follows:

$$2OH^- \rightarrow 2e^- = 2H^+ + \tfrac{1}{2}O_2$$

The electrons would be picked up by the electron acceptor (not shown in the equation). On the other hand, the production of reducing equivalents in the form of NADPH would require an electron donor (e.g., ascorbate):

$$NADP^+ + H^+ + 2e^- \rightarrow NADPH$$

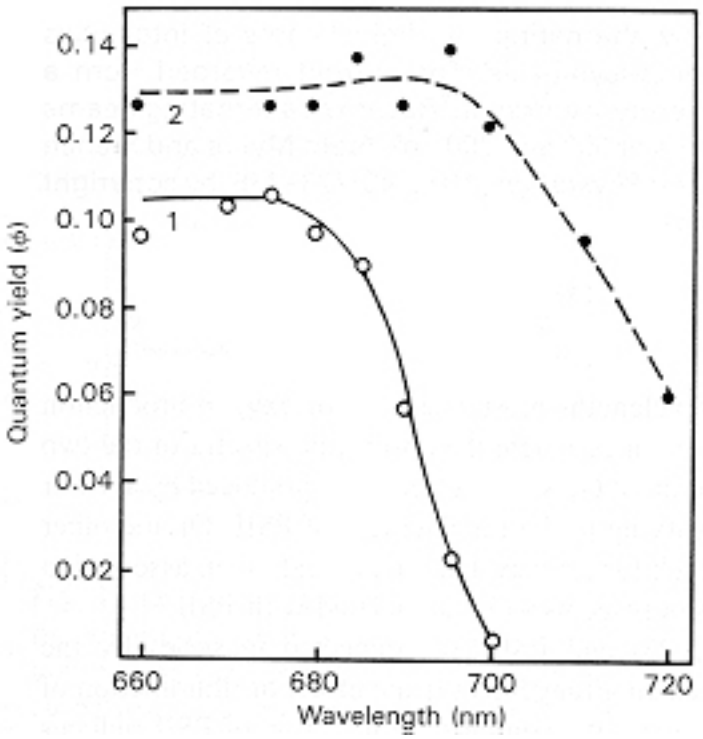


Fig. 16 Effect of supplementary light on quantum yield. Curve 1, control; curve 2, same conditions as used for curve 1 but with supplementary light. Reproduced with permission from R. Emerson, *Science*, 127:1059-1060. Copyright &copy1958 by the American Association for the Advancemnet of Science.

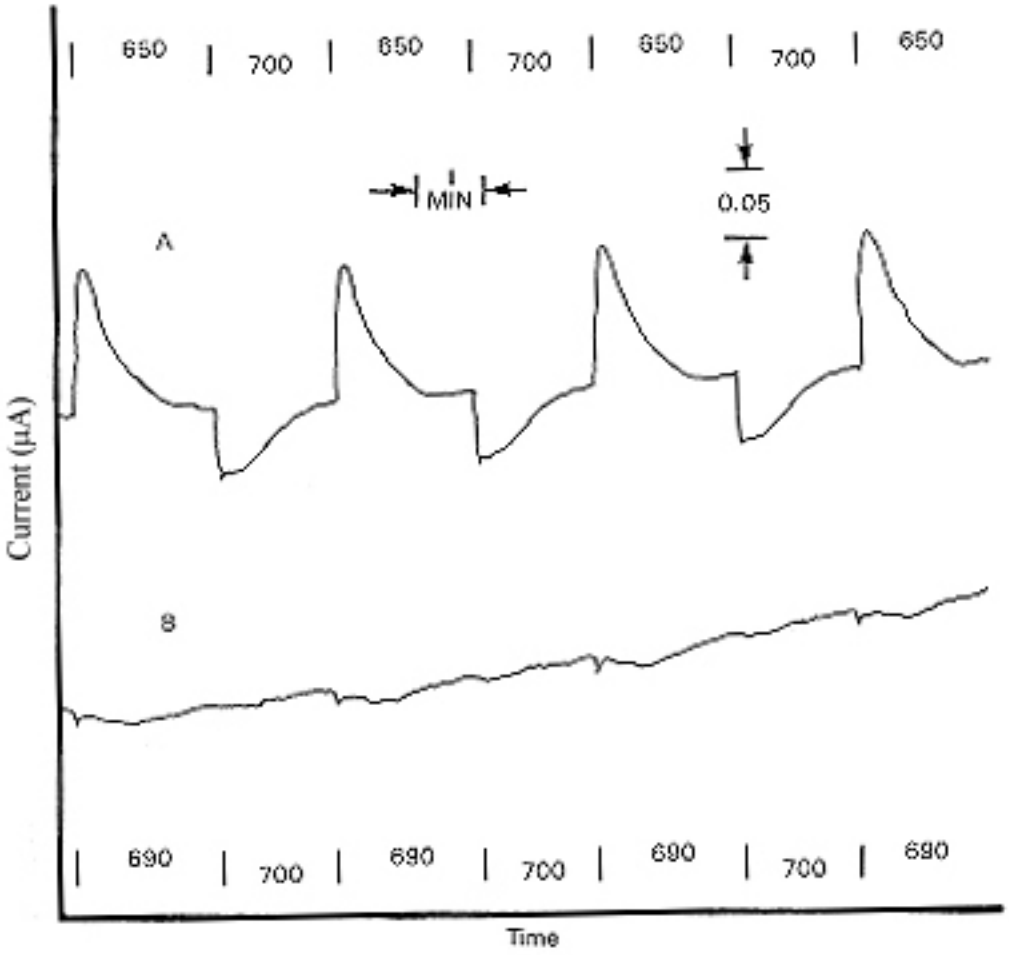


Fig. 17 Chromatic transients observed by alternating two light beams of intensities adjusted to sustain equal steady rates of photosynthesis. The current recorded from a platinum electrode is proportional to the oxygen concentration. Record A, alternating beams of 650 and 700 nm. Record B, alternating beams of 690 and 700 nm. From Myers and French (1960). Reproduced from *The Journal of General Physiology*, 1960, by copyright permission of the Rockefeller University Press.

Fig. 18 (Arnon, 1961) shows the wavelengths most effective for oxygen production or NADP reduction over the narrow wavelength range where the absorption spectra of the two chlorophylls do not overlap. Clearly, the evolution of O_2 is most effectively produced by a lower wavelength than the reduction of $NADP^+$, testifying to the involvement of PSII. On the other hand, $NADP^+$ reduction has action peaks at much longer wavelengths, revealing an association primarily with PSI. Interestingly, ATP production seems to be associated with PSII.

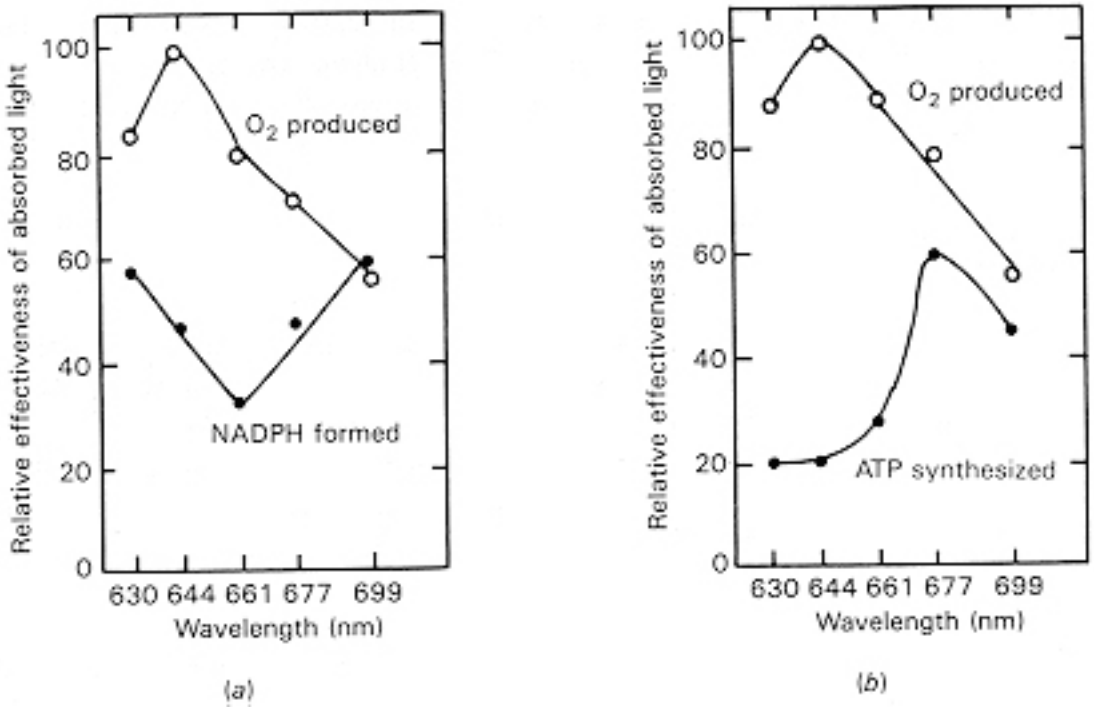


Fig. 18 (a) Effectiveness of monochromatic light, in the red region of the spectrum, in oxygen evolution and NADP reduction by isolated chloroplasts. Ascorbate was used as an electron donor for the photoreduction of NADP. (b) Effectiveness of monochromatic light, in the red region of the spectrum, in oxygen evolution and cyclic photophosphorylation by isolated chloroplasts. The 100 on the ordinate scale is equivalent to 0.16 atom oxygen evolved per micromole of light quanta absorbed; 60 on the ordinate scale is equivalent to 0.10 µmoles ATP formed per micromole of light quanta absorbed. Reproduced from D. Arnon, *Bulletin of Torrey Botany Club*, 88:215-259, with permission of Johns Hopkins Press, 1961.

As represented in Fig. 13 or 14, PSI and PSII are connected in series by the cytochrome b_6f complex. The evidence for such an arrangement is the effect of illumination of either PSI or PSII on the redox state of the cytochrome complex. Stimulation of PSII reduces the cytochrome complex, whereas illumination of PSI oxidizes it. The experiment represented in Fig. 19 (Duysens et al., 1961), carried out with the red alga *Porphyridium*, shows the absorption of cytochromes. In this record, the upward deflections reflect oxidation and the downward arrows the turning off of light. Illumination at 680 nm corresponds to excitation of PSI and that at 562 nm corresponds to excitation of PSII. As predicted by the model in which the two systems are in series, light excitation of PSI oxidizes the cytochromes and excitation of PSII reduces them.

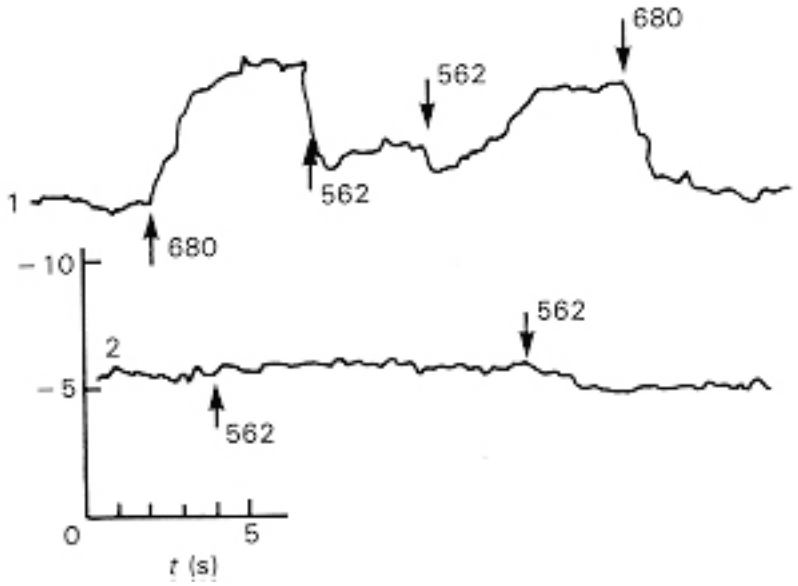


Fig. 19 Time course of cytochrome oxidation in *Porphyridium* in light of 680 and 562 nm of intensities 4.4 x 10^{-10} and 5.3 x 10^{-10} einstein cm^{-2} s^{-1}. Upward and downward arrows indicate that the light is switched on and off, respectively. Tracing 1 shows that cytochrome is oxidized by light of 680 nm but reduced by light of 562 nm. Curve 2 shows no effect by light of 562 nm. From Duysens et al. (1961). Reproduced with permission from *Nature*, 190:510-514, copyright &copy1961 Macmillan Magazines Limited.

V. COMPONENTS OF THE CHLOROPLAST PHOTOSYNTHETIC SYSTEMS

Knowledge of the topology and function of the various components of the thylakoid membrane has advanced rapidly thanks to the application of spectroscopic techniques, fractionation procedures, conventional reagents or antibodies, and the application of X-ray diffraction and cryoelectron microscopy (Chapter 1) accompanied by image reconstuction techniques. Sequencing of the appropriate cDNA (Chapter 1) and computer predictions of structure based on the hydrophobicity of the amino acid side chains of the polypeptides have also provided insights. This section presents some of the information that has been gained by the use of several of these techniques.

A. PSII

The LHC complex of PSII, referred to as LHC II, contains Chl a and b in approximately equal amounts. Part of the complex is tightly bound to PSII. A mobile or peripheral portion, not closely associated with PSII, accounts for 120 Chl molecules. The total corresponds to approximately 250 Chl molecules (Staehelin, 1986).

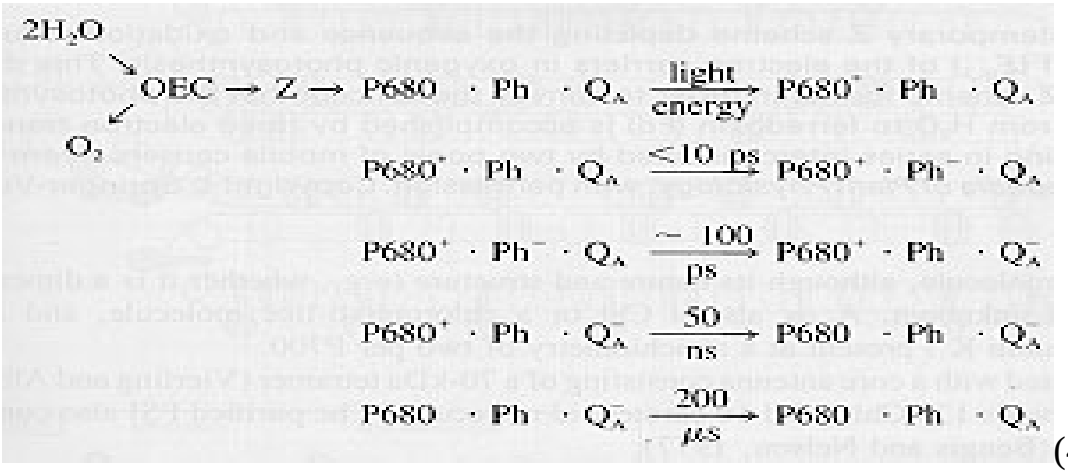
The three dimensional structure of the reaction center of PSII has been analyzed using cryoelectron microscopy and image reconstruction techniques (Rhee et al, 1998) and more recently that of the entire PS II has been elucidated by X-ray diffraction (at a resolution of 3.8 Å) from crystals fully active in water oxidation (Zouni et al., 2001). . The arrangement is very similar to that of PSI (Krauss et al., 1996;

Schubert et al., 1997) or that of purple bacteria (Deisenhofer et al., 1984, 1995). As discussed, the bacterial photochemical activity depends on two polypeptides, L and M, which span the membrane. The PSII reaction center contains a 32 kDa polypeptide, D_1, and a 34 kDa polypeptide, D_2. These two polypeptides have important homologies to L and M (Trebst, 1986) and are therefore thought to correspond in function to the bacterial polypeptides. In addition to D_1 and D_2, the isolated RC of PSII (Namba and Satoh, 1987) contains one molecule of cytochrome *b*559, five Chl, pheophytin and two β-carotenes.

P680 is photooxidized on illumination (Doring et al., 1967). The primary electron acceptor is pheophytin (Klimov et al., 1980), a porphyrin identical to Chl *a*, but lacking Mg. A specialized plastoquinone, Q_A, is a secondary acceptor. These transfers produce an endergonic charge separation between P680 and pheophytin, followed by electron transfer to Q_A.

An electron from Z replaces the electron removed by the photooxidation of P680 b. A tyrosine residue of D_1 is thought to be the electron donor Z (Debus et al., 1988).

Both Z^+ and $P680^+$ can be detected with EPR and the kinetics of the ESR changes implicate Z as the immediate donor (Boska et al., 1983). The electron lost by Z is replaced by the oxidation of water and the evolution of O_2 carried out by the oxygen-evolving complex (OEC) (see Murata and Miyao, 1985), which is tightly bound to PSII. Each individual OEC undergoes a succession of increasing oxidation states from S_0 to S_4. Oxygen is liberated at S_4 (Kok et al., 1970). The reactions of PSII are represented by Eq. (4) and the S-scheme is summarized by Fig. 20.



(4)

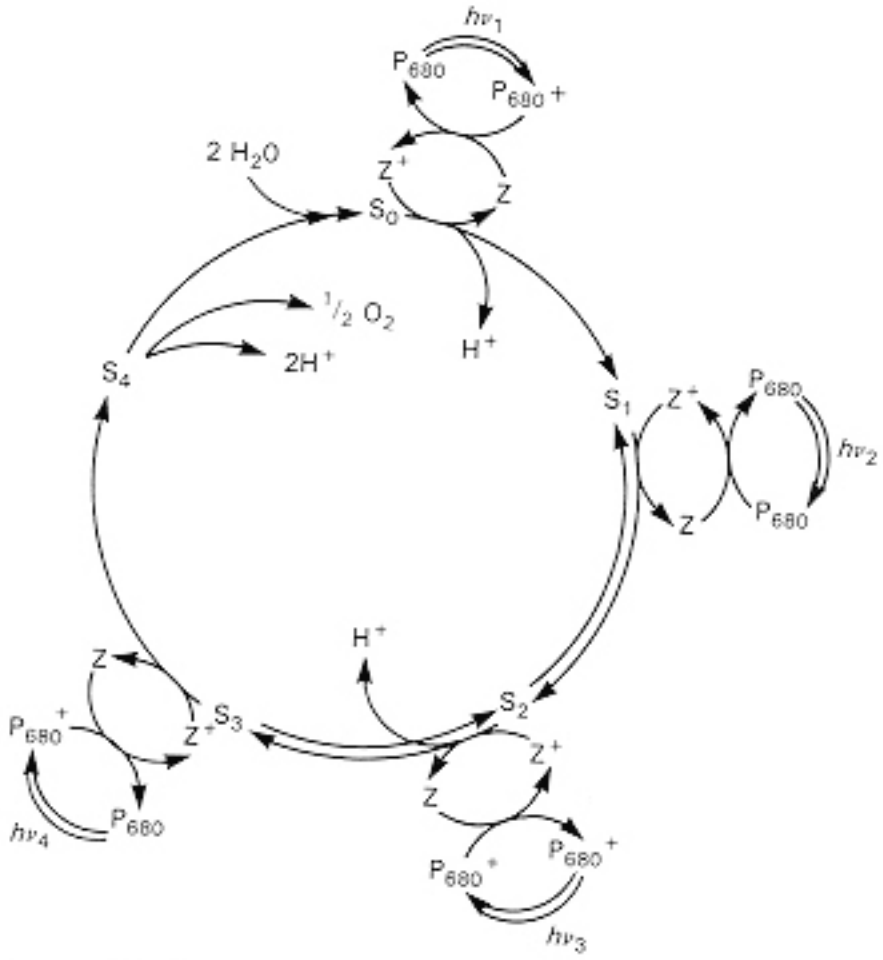


Fig. 20 Scheme in which one electron is removed in each transition from S_0 to S_3. State S_4 decays spontaneously, releasing O_2.

B. PSI

PSI of the thermophilic cyanobacterium *Synechococcus elongatus* contains 12 protein subunits and 127 cofactors comprising 96 Chls, 2 phylloquinones, 3 Fe4S4 clusters, 22 carotenoids and 4 lipids. 90 of the 96 Chls function as antennae; only 6 are involved in electron transfer.

The structure of PSI has been studied in chloroplasts (see Fromme et al., 1996) and in cyanobacteria (Krauss et al., 1996; Schubert et al., 1997; Jordan, et al. 2001). These have many common features with that of purple bacteria (see Deisenhofer et al., 1995) and the RC of PSII (Rhee et al., 1998).

As already discussed, P700 is the electron donor. The primary electron acceptor is A_0 and the intermediate acceptor is A_1. Three iron-sulfur centers, F_X, F_B and F_A, have been identified. The sequence and kinetic constants are shown in Eq. (5).

$$P700\ A_0\ A_1\ XBA \xrightarrow[\text{energy}]{\text{light}} P700^*\ A_0\ A_1\ XBA \xrightarrow{<10\ ps} P700^+\ A_0^-\ A_1\ XBA$$

$$P700^+\ A_0^-\ A_1\ XBA \xrightarrow[ps]{\sim 100} P700^+\ A_0\ A_1^-\ XBA \longrightarrow P700^+\ A_0\ A_1\ X^-BA \quad (5)$$

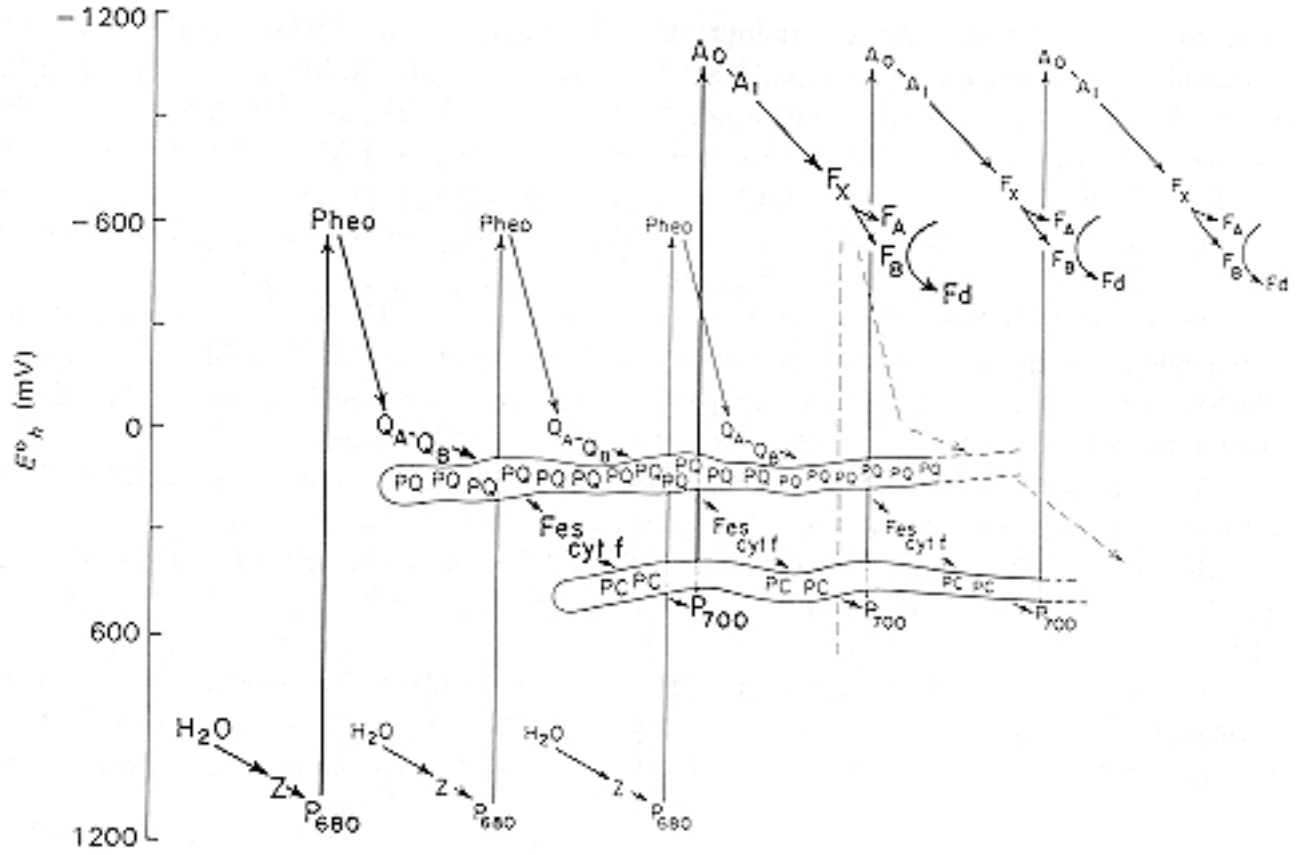


Fig. 21 A contemporary Z scheme depicting the sequence and oxidation-reduction midpoint potential ($E_{m,7}$) of the electron carriers in oxygenic photosynthesis. This differs from the familiar Z scheme format in order to convey the concept that the photosynthetic electron transfer from H_2O to ferredoxin (Fd) is accomplished by three electron transport complexes operating in series, interconnected by two pools of mobile carriers. Reproduced from D. R. Ort (1986), *Encyclopedia of Plant Physiology*, with permission. Copyright &copy1986 Springer-Verlag, Germany.

P700 is a Chl molecule, although its nature and structure (e.g., whether it is a dimer or a monomer) are still unknown. A_0 is also a Chl or a chlorophyll-like molecule and A_1 is phylloquinone (vitamin K_1) present at a stoichiometry of two per P700.

P700 is associated with a core antenna (see Section VI) consisting of a 70-kDa tetramer (Vierling and Alberte, 1983), which also binds 130 Chl a and 16 carotenoid molecules. The purified PSI also contains other polypeptides (Bengis and Nelson, 1977).

The structure of PSI has been studied in chloroplasts (see Fromme et al., 1996) and in cyanobacteria (Krauss et al., 1996; Schubert et al., 1997; Jordan, et al. 2001). It has many common features with that of purple bacteria (see Deisenhofer et al., 1995) and the RC of PSII (Rhee et al., 1998).

The accessory light-harvesting system of PSI, LHC I (see Section VI) contains an additional 60 to 80 Chl a and b molecules, with approximately three times more Chl a. The LHC I polypeptides are in the range of 20 to 25 kDa and are immunologically distinct from those of PSII (Lam et al., 1984). Since LHC I can be separated into two fractions, each could have a distinct function as in the case of LHC II, where one is considered laterally mobile and the other is closely associated with PSII.

C. The Cytochrome b_6f Complex

The cytochrome b_6f complex (Cramer et al., 1987) containing plastoquinone and plastocyanin oxidoreductase, has been isolated (Hauska et al., 1983). In addition, the complex was found to include the 34-kDa polypeptide of cytochrome *f*, a 23-kDa polypeptide with two cytochrome b_6 hemes, a 20-kDa FeS-Rieske protein, two other smaller polypeptides and bound plastoquinol.

The aspects of the organization of the components of the thylakoid membranes discussed in Sections IV and V are summarized in Fig. 21 (Ort et al., 1986) and Fig. 22 (Anderson, 1981, 1987). Fig. 22a shows the organization of the complexes; Fig. 22b shows their organization in relation to the thylakoid structural arrangement.

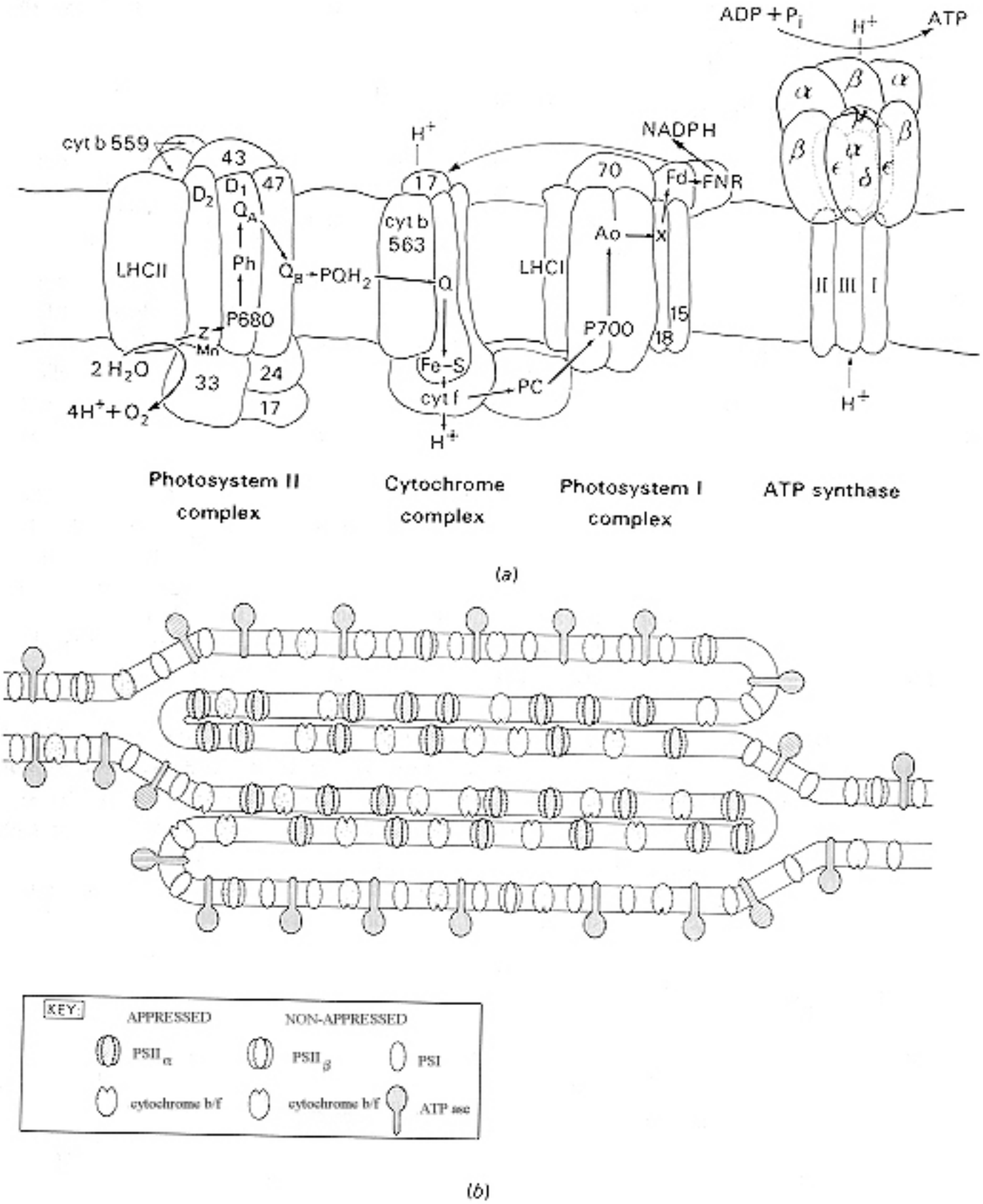


Fig. 22 (a) Arrangement of the supramolecular protein complexes and mobile electron transport carrier in thylakoid membranes. From J.M. Anderson, *Photosynthesis*, p.275, with permission of Elsevier Science Publishers, Amsterdam, &copy1987. (b) Possible static representation of the lateral herterogeneity in the distribution of the supramolecular thylakoid complexes between appressed and nonappressed thylakoids.

Reproduced from J.M. Anderson, *FEBS* Letters, 124(1), 1981, with permisssion.

VI. THE LIGHT-HARVESTING SYSTEM

All photosynthetic processes depend on the efficient capture of energy to drive the biosynthetic processes. The problem is dealt with by the capture of light by light-harvesting complexes (LHCs) and the proximity between LHC and the RC which allows for the rapid transfer of energy.

The number of Chl molecules per RC is very large. It has been estimated to be from 25 to several hundred, depending on the bacteria, to about 300 in the chloroplast. The function of these molecules is to act as an antenna, i.e., absorb light and transfer the energy to neighboring molecules by resonance transfer until it is trapped in the reactions of the RC. Part of the evidence for this mechanism stems from the fact that in oxygen-producing systems, the amount of oxygen evolved increases with intensity of the flash until a maximal saturation rate is obtained. The amount of O_2 produced is very small in relation to the Chl content because the RCs are finite in number and must be reduced again before participating in another photooxidative event. Approximate calculations yield about 2500 Chl molecules per O_2 evolved under saturating conditions or 300 per RC (8 photons are required per O_2 see Fig. 20).

The light-harvesting complexes (LHCs) contain Chl or BChl and a variety of other pigments, such as carotenoids (Zuber et al., 1987). Thus they can absorb light from a very broad region of the spectrum. The pigment molecules are noncovalently attached to integral proteins. A few pigment molecules are associated with each protein. The energy is transferred from short wavelength-absorbing to longer wavelength-absorbing pigments by inductive resonance and the formation of exciton states.

In bacteria, the dominant LHC is LHCII which donates the energy to a second complex LHCI. How this takes place with high efficiency and high speed has been shown in studies of the atomic structure of the bacterial LHCII system with electron crystallography (McDermott et al., 1995). 18 bacteriochlorophyll *a* molecules absorbing at 850 nm (B850) are present in an overlapping ring closely associated with their neighbor and vertical to the plane of the membrane. Nine other chlorophyll *a* molecules (B800) are close to the cytoplasmic surface and are parallel to the plane of the membrane. The phytol chains of the bacteriochlorophyll intertwine. Nine carotenoids molecules span the membrane and make contact with both B850 and B800. The closeness between the B800 and B850 and the carotenoid molecules allows for the rapid and efficient transfer of energy. LHCI, which has a similar composition, is thought to have a similar arrangement. A model is shown in Fig. 23 (McDermott et al., 1995).

The structure of LHCII from green plants which contains both Chl a and b has also been studied by electron crystallography. Each polypeptide has 232 amino acids and contains a minimum of 12 Chl molecules and 2 carotenoids. A representation is shown in Fig 24 (Kühlbrandt et al., 1994). Again all the components are close to each other allowing rapid transfer of energy. In this case, the role of carotenoid is mostly to protect the Chl from photodamage (Kühlbrandt et al., 1994). Apparently there are 6 trimers per RC and two additional polypeptides (CP43 and CP47) with 20 to 30 Chl, accounting for the total

amount of pigment of the LHCII.

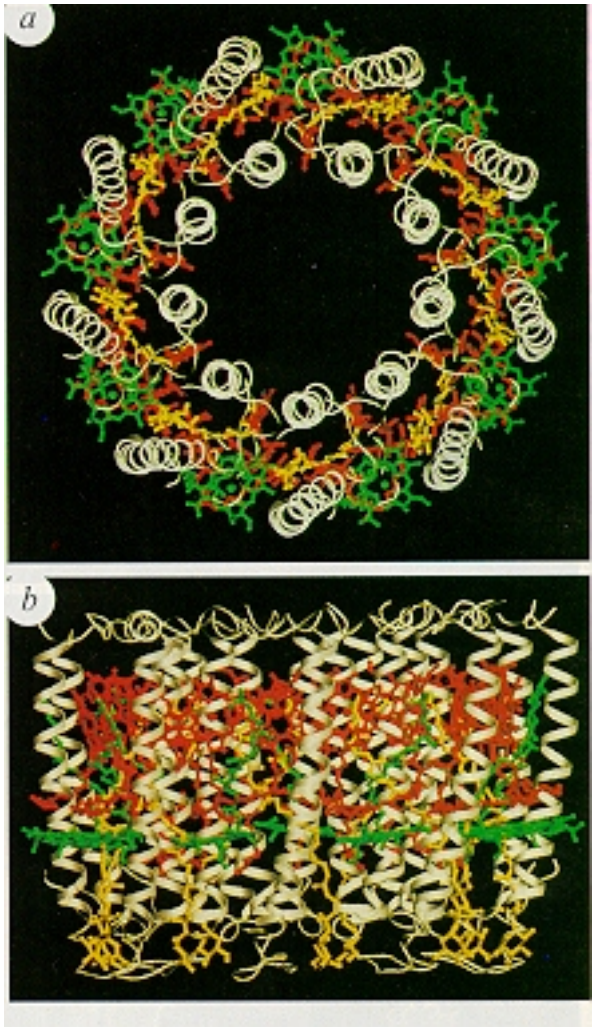
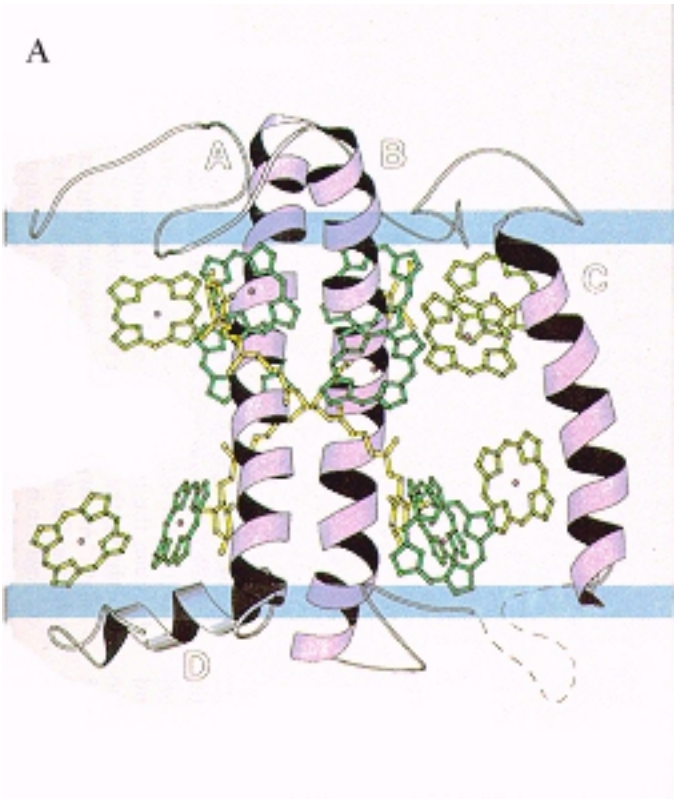


Fig. 23 a. The nanomeric complex viewed from the cytoplasmix side of the membrane. Protein units are shown in white, B800 BChl *a* in green, B850 BChl *a* in red and carotenoid in yellow. b. The complex viewed perpendicular to the symmetry axix. Broad ribbons are in the presumed hydrophobic region of the membrane. Reproduced with permission from *Nature*, McDermott, G., Prince, S.M., Freer, A.A., Hawthornwaite-Lawless, A.M, Papiz, M.Z., Cogdell, R.J. and Isaacs, N.W. (1995) Crystal structure of an integral membrane light-harvesting complex from photosynthetic bacteria, 374:517-521, copyright &copy1995 MacMillan Magazines Ltd.


A
A
B
C
D

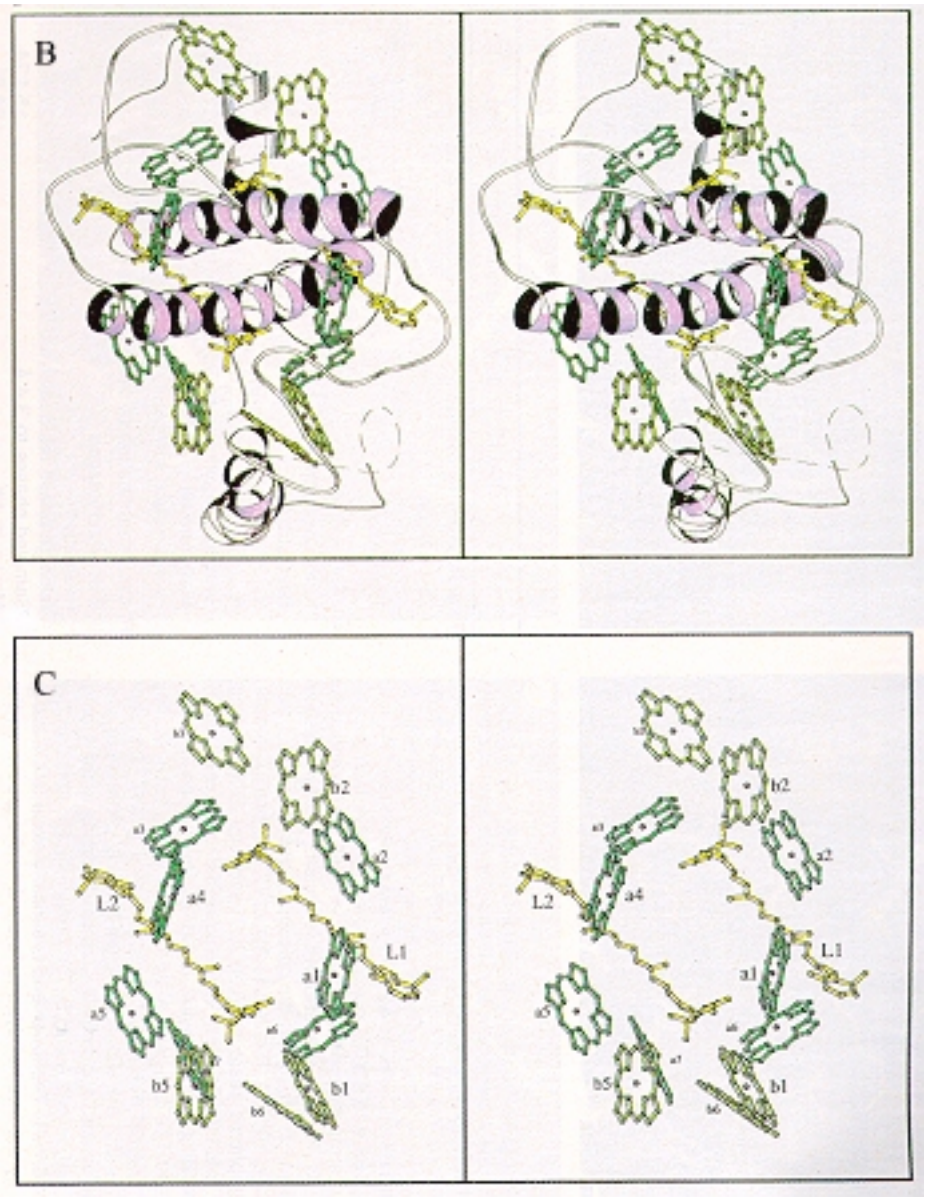


Fig. 24 Overall views of LCII of chloroplasts. a. Side view. Blue bands indicate the approximate location of the bilayer. Helices are labelled A to D. b. Stereodiagram showing the top view of the complex from the stromal side of the membrane, c. Stereodiagram of Chl tetrapyrroles and luteins. Residues 26 to 224 of the polypeptide are shown. The fit indicated by the dashed line for residues 100 to 116 is tentative. Dark green corresponds to Chl *a*, light green to Chl *b*, Mg atoms are pink, luteins are yellow and the membrane spanning helices are violet. Reproduced with permission from *Nature*, Kühlbrandt, W., Wang, D.N. and Fujiyoshi, Y. (1994) Atomic model of plant light-harvetsing complex by electron crystallography, 367:614-621, copyright &copy1994 MacMillan Magazines Ltd.

VII. COORDINATION BETWEEN PSI AND PSII

The efficient operation of the two systems, PSI and PSII, requires smooth coordination. Although PSI and PSII are likely to be excited equally by white light, shade favors PSI. Excess energy absorbed by either system would be dissipated as heat and lost to the system.

The presence of components of the LHCs can be adjusted to decrease light absorption and the

photosynthetic capacity can be adjusted to increase the utilization of the light energy. These mechanisms require synthesis and degradation of the photosyntiesis machinery. For more rapid adjustments, state transitions may take place. Unbalanced excitation of PSI is known as *state* I and that of PSII as *state* II. Apparently, these imbalances are corrected by what have been called state transitions. These are regulatory effects by which the flow of energy between the two systems is altered. The mechanism or mechanisms are still not entirely understood. Conceivably, the amount of energy normally transferred from PSII to PSI could be changed. Some role of Mg^{2+} or Na^{+} in the amount of energy transferred from PSII to PSI is suspected (Wang and Govinjee, 1979). Alternatively, the proportion of the light absorbed by the two photosystems could be altered, an effect referred to as a change in *optical cross section*.

There is some evidence that the optical cross section changes. Barley mutants lacking LHC II fail to show state transitions (Canaani and Malkin, 1984), which suggests that LHC II is responsible for these transitions. Since PSI and PSII are in separate locations in the membrane, a change in the position of the movable portion of LHC II could favor the transfer of energy to either PSI or PSII. The phosphorylation and dephosphorylation of LHC II affect its distribution; phosphorylation favors a position closer to PSI (Bennett, 1983; Kyle et al., 1983). The phosphorylation is catalyzed by a membrane-bound Mg^{2+}-dependent kinase, whereas dephosphorylation is catalyzed by a phosphatase. Presumably, the phosphorylated LHC II moves laterally to approach PSI in the unstacked region, thereby favoring the transfer of energy from LHC II to PSI (Larsson et al., 1983). This could be the result of electrostatic repulsion. In contrast, a phosphatase dephosphorylates LHC II, favoring its displacement toward PSII. The phosphorylation cycle is thought to be regulated by the redox state of plastoquinone acting as a sensor of the energy distribution (Allen et al., 1981). Reduction of plastoquinone, resulting from the electron transport following the photoactivation of PSII, would favor the kinase, whereas the phosphatase would be stimulated by oxidation of plastoquinone. The redox state of plastoquinone also regulates the transcription of the reaction-center apoproteins of PSI and PSII, both coded by chloroplast genes (Pfannschmidt et al., 1999). The rate of transcription is much more rapid than that of nuclear genes. Reduction of plastoquinone induces apoproteins of PSI and oxidation represses them. PSII transcription is controlled in the opposite manner.

VIII. PROTECTION FROM PHOTO-OXIDATION

Oxygenic photosynthesis produces highly reactive oxidizing intermediates and byproducts that can damage the photosynthetic apparatus (see Niyogi, 1999). Photo-oxidative damage decreases the efficiency of photosynthesis and has been called *photoinhibition* (Kok, 1956). The reactions of photo-oxidation take place all the time during photosynthesis. However, they become particularly damaging when the absorption exceeds the capacity of the energy utilization system.

Oxygenic photosynthetic organisms have developed a variety of mechanisms to prevent photo-oxidative damage. These mechanisms range from moving the leaves or moving chloroplasts within cells (to avoid exposure to light) to mechanisms involving molecular interactions. As we saw in Section VII, overexcitation of PSII relative to PSI, is thought to reduce the plastoquinone pool and activate a kinase

that phosphorylates the peripheral LHC associated with PSII. Detachment of the phosphorylated LHC from PSII transfers it to PSI. This mechanism is not likely to play a role at high light intensity since the kinase is inactivated by intense light (e.g., Rintamäki et al., 1997). However, alternative electron transport pathways and thermal dissipation help to remove the energy form excess absorbed light. In addition, many antioxidant molecules and scavenging enzymes are present to deal with reactive oxygen species.

The danger of photo-oxidation is greatest for the LHC associated with PSII, the PSII reaction center and the PSI acceptor side. Absorption of light causes Chl to enter the singlet excited state (^{1}Chl). As already discussed, the energy is transferred to neighboring Chls in the LHC by resonance transfer. Before transferring to the RC, the triplet state (^{3}Chl) can be formed from ^{1}Chl. ^{3}Chl is relatively long-lived and can produce singlet oxygen ($^1O_2$) from O_2. The generation of $^1O_2$ is much greater at PSII-LHC, because the average life time of ^{1}Chl is longest in PSII-LHC (Foote, 1976).

In addition to these reactions, $P680^+$ and Z^+ (see above) of PSII are capable of oxidizing nearby proteins and pigments. The acceptor side of PS1 can reduce O_2 to the superoxide anion (O^-_2) radical and eventually hydroxyl radical.

In excessive light, an increase in the thylakoid ΔpH regulates PSII by dissipating as much as 75% of the excess energy as heat (Demmig-Adams et al., 1996a). The pH dependent thermal dissipation occurs in the PSII- LHC and involves the de-epoxidation of xanthophyll pigments (e.g., Demmig-Adams, 1996b). Presumably, the interaction of the pigments with excited states of oxygen or Chl lead to a de-excitation state.

The thermal energy dissipation can be estimated from the chlorophyll fluorescence emission. Although this emission involves a small portion of the chlorophyll, the fluorescent emission decreases with increases in thermal dissipation. This has been referred to as *non-photochemical quenching* (NPQ). Carotenoids facilitate thermal energy dissipation, as demonstrated in mutants that lack lutein an isomer of zeaxanthin, where dissipation is largely absent (see Niyogi, 1999) but where oxidative damage in the form of lipid peroxidation is evident (Havaux and Niyogi, 1999). A mutant of *Arabidopsis thaliana* with normal level of zeaxanthin, but deficient in energy dissipation, was isolated (Li et al, 2000). The mutant lacks a protein, *chlorophyll binding protein* of 22 kDa (CP22) [the product of the PSII gene (*psbS*), Psb)]. CP22 is part of the LHC, one of the 30 components of LHC that have been identified (see Jansson, 1999). CP22 was found to be required for the thermal energy dissipation which also requires zeaxanthin and a pH gradient across the thylakoid membrane.

In addition to the machinery described, chloroplasts contain many antioxidants that act as scavengers of reactive oxygen species. The scavengers include carotenoids, tocopherols, ascorbate and glutathione (see Niyogi, 1999). In addition, some enzymes are involved in scavenging, including superoxide dismutase (SOD) and ascorbate peroxidase (APX). SOD catalyzes the conversion of O_2^- to H_2O_2. APX reduces the

H_2O_2 and produces monodehydroascorbate radicals which are reduced by PSI.

IX. PHOTOSIGNALING SYSTEMS

Light is essential for the survival of plants, and plants have evolved a remarkable ability to respond to light using sensory receptors (see Quail, 2002). Phytochrome (phy) is one family of these receptor molecules. Each phytochrome has specific physiological functions. The subunits form two main domains. Phys are a soluble homodimeric chromoproteins composed of two polypeptides approximately 125 kDa in size. Phys are encoded by multigene families (see Mathews and Sharrock, 1995). At the amino terminal of phy, a photosensitive domain is covalently attached to tetrapyrrole chromatophore (phytochromobilin). A domain at the carboxy terminal is responsible for dimerization. Light produces a switch from the inactive form (*red-light-absorbing*, Pr) to the biologically active form (*far-red-light absorbing*, Pfr). Phy molecules present in the cytoplasm in the Pr form are transferred to the nucleus following photoactivation (e.g., Kircher et al., 1999). Two hybrid screens (see Chapter 1) have identified some of the proteins interacting with phy. Among these the Phytochrome-interacting factor 3 (PIF3) a basic helix-loop-helix (bHLH) transcriptional regulator (Ni et al., 1998). PIF3 is present in the nucleus (Ni et al., 1998) bound to its DNA binding site, the G-box DNA sequence (CACGTG) which is also present in various other light activated promoters (Martinez-García et al., 2000). Analysis of the RNA shows the PIF3-phyB are responsible for activating two genes: *circadian clock-associated protein 1* (CCA1) and *late elongated hypocytyl* (LHY) genes. These encode transcription-factor-related proteins which regulate the expression of chloroplast components and the circadian clock. Experiments using DNA-microarray technology showed that a large portion of the genes activated by far red light code for transcriptional regulators (Tepperman et al., 2001). These findings suggests direct targeting of light signals to the promoters of genes encoding key transcriptional regulators. These regulators orchestrate the expression of multiple genes.

Another slower pathway also regulated by light involves the depletion in the nucleus of an E3 ubiquitin ligase initiated by ligt. The ubiquitin ligase is involved in the degradation pathway of the proteasome. This results in the accumulation of nuclear HYP5 protein, a transcription factor (see Osterlund et al., 2000; Schwechheimer and Deng, 2001).

SUGGESTED READING

General Reading

Cramer, W.A., and Knaff, D.B. (1990) *Energy Transduction in Biological Membranes*, Chapters 5 and 6. Springer-Verlag, New York.

Hall, D.O. and Rao, K.K., (1994) *Photosynthesis*, Cabridge University Press.

Lawlor, D.W. (1987) Photosynthesis: Metabolism, Control and Physiology. Longman Scientific,

London, and Wiley, New York.

Other

Allen, J.P. and Williams, J.C. (1998) Photosynthetic reaction centers, *FEBS Lett.* 438(1-2):5-9. (MedLine)

Andersson, B. and Franzén, L.G. (1992) The two photosystems of oxygenic photosynthesis in *Molecular Mechanisms in Bioenergetics*, Ernster, L. ed. (*New Comprehensive Biochemistry*, vol.23) pp.121-143, Elsevier, Amsterdam, New York.

Demmig-Adams, B., Gilmore, A.M. and Adams, W.W. III (1996) In vivo function of carotenoids in higher plants, *FASEB J.* 10:403-412. (Medline)

Hunter, C.N. (1995) Rings of light, *Curr. Biol.* 5:826-828. (Medline)

Knaff, D.B., and Kämpf, C. (1987) Substrate oxidation and NAD^+ reduction by phototrophic bacteria. In *Photosynthesis* (Amesz, J., ed.), pp. 199-211. Elsevier, New York.

Ort, D.R. (1985) Energy transduction in oxygenic photosynthesis: an overview of structure and mechanisms. In *Encyclopedia of Plant Physiology*, New Series, Vol. 19, pp. 165-196. Springer-Verlag. Berlin.

Rutherford, A.W. (1989) Photosystym II, the water splitting enzyme. *Trends Biochem. Sci.* 14:227-232. (Medline)

Sauer. R (1986) Photosynthetic light reactions-physical aspects. In *Encyclopedia of Plant Physiology*, New Series, Vol.19, pp. 85-97. Sprinder-Verlag, Berlin.

Staehelin, L.A. (1986) Chloroplast structure and supramolecular organization of photosynthetic membranes. In *Encyclopedia of Plant Physiology*, New Series, Vol. 19, pp. 1-84. Springer-Verlag, Berlin.

WEB RESOURCES

Selected articles on the pump mechanism of bacteriorhodopsin are available through http://www.nature.com/nature/fow).

REFERENCES

Search the textbook

Back to Chapter 17

REFERENCES

Allen, J.P. and Williams, J.C. (1998) Photosynthetic reaction centers, *FEBS Lett.* 438(1-2):5-9. (MedLine)

Allen, J. F., Steinbach, K. E., and Arntzen, K. E. (1981)Chloroplast protein phosphorylation couples plastoquinone redoxstate to distribution of excitation energy between photosystems, *Nature* 291:25-29.

Allen, J.P., Feher, G., Yeates, T.O., Komiya, H. and Rees DC. (1987) Structure of the reaction center from *Rhodobacter sphaeroides* R-26: the cofactors, *Proc. Natl. Acad. Sci. USA* 84:5730-5734. (MedLine)

Anderson, J. M. (1981) Consequences of spatial separation of photosystem 1 and 2 in thylakoid membranes, *FEBS Lett.* 124:1-10.

Anderson, J. M. (1987) Molecular organization of thylakoid membranes. In *Photosynthesis* (Amesz, J., ed.), pp. 273-297, Elsevier. New York.

Anderson, J. M., and Anderson, B. (1982) The architecture of the photosynthetic membrane: lateral and transverse organization, *Trends Biochem. Sci.* 7:288-292.

Arnon, D. I. (1961) Changing concepts of photosynthesis, *BullTorrey Bot. Club* 88:215-259.

Balabin, I.A. and Onuchic, J.N. (2000) Dynamically controlled protein tunneling paths in photosynthetic reaction centers, *Science* 290:114-117. (MedLine)

Bengis, C., and Nelson, N. (1977) Subunit structure of the chloroplast PSI reaction center, *J. Biol. Chem.* 252:4564-4569.(Medline)

Bennett, J. (1983) Regulation of photosynthesis by reversible phosphorylation of the light-harvesting chlorophyll *a/b* protein,*Biochem. J.* 212:1-13.(Medline)

Betts, J.N., Beratan, D.N. and Onuchic, J.N. (1992) Mapping electron tunneling pathways: an algorithm that finds the "minimum length"/maximum coupling pathway between electron donors and acceptor in proteins *J. Am. Chem. Soc.* 114:4043-4046.

Blakenship, R. E., and Prince, R. C. (1986) State redox potentials andZ scheme of photosynthesis, *Trends Biochem. Sci.* 10:382-383.

Blaurock, A.E. and Stoeckenius, W. (1971) Structure of the purple membrane, *Nature New Biol.* 233:152-155.(Medline)

Boska, M., Sauer. K., Buttner, W., and Babcock, G. T. (1983) Similarity of EPR signal II rise and $P680^+$ decay kinetics in Tris-washed chloroplast II preparations as a function of pH, *Biochim.Biophys. Acta* 722:327-330.

Canaani, O., and Malkin, S. (1984) Distribution of light excitationin an intact leaf between the two photosystems of photosynthesis.Changes in absorption cross-section following state 1-state 2transitions, *Biochim. Biophys. Acta* 766:513-524.

Clayton, R. K. (1980) *Photosynthesis: Physical Mechanisms and Chemical Patterns.* Cambridge Univ. Press, London.

Cramer, W. A., Widger, W. R., Herrmann, R. G., and Trebst. A.(1985) Topography and function of thylakoid membrane proteins,*Trends Biochem. Sci.* 10:125-129.

Cramer, W. A.. Black, M. T.. Widger, W. R.. and Girivin. M. E.(1987) Structure and function of photosynthetic cytochrome *b*-$c_1$and b_6-*f* complexes. In *The Light Reaction* (Barber, J., ed.). pp.447-494. Elsevier, New York.

Debus, R.J., Barry, B.A., Sithole, I., Babcock, G.T. and McIntosh, L. (1988) Directed mutagenesis indicates that the donor to P+680 in photosystem II is tyrosine-161 of the D1 polypeptide, *Biochemistry* 27:9071-9074. (Medline)

Deisenhofer, J., Epp, O., Miki. K., Huber, R., and Michel, H.(1984) X-ray structure analysis of a membrane-protein complex, *J.Mol. Biol.* 180:385-398.(Medline)

Deisenhofer, J., Michel, H., and Huber, R. (1985) Structural basis of photosynthetic light reactions in bacteria, *Trends Biochem.Sci.* 10:243-248.

Deisenhofer, J., Epp, O., Sinning, I. and Michel, H. (1995) Crystallographic refinement at 2.3 Å resolution and refined model of the photosynthetic reaction centre from *Rhodopseudomonas viridis, J. Mol. Biol.* 246:429-457. (Medline)

Demmig-Adams, B., Adams, W.W. III, Barker, D.H., Logan, B.A., Bowling D.R. and Verhoeven, A.S. (1996a) Using chrlorophyll, fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of escess excitation, *Physiol. Plant* 98:253-264.

Demmig-Adams, B., Gilmore, A.M. and Adams, W.W. III (1996b) In vivo function of carotenoids in higher plants, *FASEB J.* 10:403-412. (Medline)

Doring, G., Renger, G., Vater, J., and Witt, H. T. (1967) Properties of the photoactive chlorophyll-$_{aII}$ in photosynthesis, *Z.Natyrforsch Teil B* 1139-1143.

Dunahay, T. G., Staehelin, L. A., Siebert, M., Ogilvie. P. D., and Berg, S. P. (1984) Structural, biochemical and biophysical characterization of four oxygen-evolving photosystem II preparations from spinach, *Biochim. Biophys. Acta* 764:179-193.

Dutton, P. L. (1986) Energy transduction in an oxygenic photosynthesis. In *Encyclopedia of Plant Physiology*, New Series, Vol. 19, pp. 197-237.Springer-Verlag, Berlin.

Duysens, L. N. M., Amesz, J., and Kamp, B. M. (1961) Two photochemicalsystems in photosynthesis, *Nature* 190:510-514.

Emerson, R., Chambers, R., and Cederstrand, C. (1957) Some factors influencing the long-wave limit of photosynthesis, *Proc. Natl.Acad. Sci. U.S.A.* 43: 133-143.

Foote, C.S. (1976) Photosensitized oxidation of singlet oxygen: consequences in biological systems, in *Free Radicals in Biology*, ed. Pryor, W.A., Academic Press, New York, 2:85-133.

Fromme, P., Witt, H.T., Schubert, W.D., Klukas, O. Saenger, W. and Krauss, N. (1996) Structure of photosystem I at 4.5 Å resolution. A short review including evolutionary aspects *Biochim. Biophys. Acta* 1275:76-83.

Fyfe, P.K. and Jones, M.R. (2000) Re-emerging structures: continuing crystallography of the bacterial reaction centre, *Biochim. Biophys. Acta* 1459:413-421. (MedLine)

Gennis, R.B. and Ebrey, T.G. (1999) Proton pump caught in the act, *Science* 286:252-253.(Medline)

Glazer. A. N. and Melis, A. (1987) Photochemical reaction centers, structure, organization, and function, *Annu. Rev. Plant Physiol.*38:11-45.

Goodenough, U. W. and Staehelin, L. A. (1971) Structural differentiation of stacked and unstacked chloroplast membranes. Freeze etch electron microscopy of wild-type and mutant strains of *Chlamydomonas, J. Cell Biol.* 48:594-6l9.(Medline)

Goodenough, U. W., Armstrong, J.J., and Levine, R. P. (1969) Photosynthetic properties of ac-31, a mutant strain of *Chlamydomanas reinhardi* devoid of chloroplast membrane stacking, *Plant Physiol.*

44:1001-1012.

Haehnel, W. (1984) Photosynthetic electron transport in higherplants, *Annu. Rev. Plant Physiol.* 35:659-693.

Hauska, G. A., Hurt. E., Gabellini, N.. and Lockau, W. (1983) Comparative aspects of quinol-cytochrome *c*/plastocyaninoxidoreductases, *Biochim. Biophys. Acta.* 726:97-133.(Medline)

Hauska, G., Schoedl, T., Remigy, H. and Tsiotis, G. (2001) The reaction center of green sulfur bacteria *Biochim. Biophys Acta*1507:260-277. (MedLine)

Havaux, M. and Niyogi, K.K. (1999) The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism, *Proc. Natl. Acad. Sci. USA* 96:8762-8767.(Medline)

Heathcote, P., Fyfe, P.K. and Jones, M.R. (2002) Reaction centres: the structure and evolution of biological solar power, *Trends Biochem. Sci.* 27:79-87. (MedLine)

Jansson, S. (1999) A guide to the Lhc genes and their relatives in Arabidopsis/IT>, *Trends Plant Sci.* 4:236-240. (MedLine

Jones C. W. and Vernon, L. P. (1969) Nicotinamide photoreductionin *Rhodospirillum rubrum* chromatophores, *Biochim. Biophys. Acta.*180:144-164.(Medline)

Jordan, P., Fromme, P., Witt, H.T., Klukas, O., Saenger, W. and Krauss, N. (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution, *Nature. 2001* 411:909-917. (MedLine)

Khorana, H.G. (1988) Bacteriorhodopsin, a membrane protein that uses light to translocate protons, *J. Biol. Chem.* 263:7439-7442.(Medline)

Kircher, S., Kozma-Bognar, L., Kim, L., Adam, E., Harter, K., Schafer, E. and Nagy, F. (1999) Light quality-dependent nuclear import of the plant photoreceptors phytochrome A and B, *Plant Cell*11:1445-1456. (MedLine),/a>

Klimov, V. V., Dolan, E., Shaw, E. R., and Ke, B. (1980) Interactionbetween the intermediary electron acceptor (pheophytin) and a possible plastoquinone-iron complex in photosystem II reaction centers, *Proc.Natl. Acad. Sci. U.S.A.* 77:7227-7231.

Knaff, D. B., and Kampf, C. (1987) Substrate oxidation and NAD^+reduction by prototrophic bacteria. In Photosynthesis (Amesz, J.,ed.), pp. 199-212. Elsevier, New York.

Kok, B. (1956) On the inhibition of photosynthesis by intense light, 21:234-244.

Kok, B. Forbush, B., and McGloin, M. (1970). Cooperation of charges in photosynthetic 0_2 evolution. I. A linear four step mechanism, *Photochem. Photobiol.* 11:457-475.(Medline)

Krauss, N., Schubert, W.D., Klukas, O., Fromme, P., Witt, H.T. and Saenger, W, (1996) Photosystem I at 4 Å resolution represents the first structural model of a joint photosynthetic reaction centre and core antenna system, *Nature Struct. Biol.* 3:965-973. (Medline)

Kühlbrandt, W., Wang, D.N. and Fujiyoshi, Y. (1994) Atomic model of plant light-harvesting complex by electron crystallography, *Nature* 367:614-621.(Medline)

Kühlbrandt, W. (2000) Bacteriorhodopsin--the movie, *Nature* 406:569-570. (MedLine)

Kyle, D., Staehelin, L. A., and Amtzen, C. J. (1983) Lateral mobility of the light harvesting complex in chloroplast membranes controls excitation energy distribution in plants, *Arch. Biochem.Biophys.* 222:527-541.(Medline)

Lam, E., Ortiz, W., Mayfield, S., and Malkin, R. (1984) Isolation and characterization of a light harvesting chlorophyll *a/b* proteincomplex associated with PSI, *Plant Physiol.* 74:650-655.

Lanyi, J.K. (1998) Understanding structure and function in the light-driven proton pump bacteriorhodopsin, *J. Struct. Biol.* 124:164-178.(Medline)

Larsson, U. K., Jergil, B., and Anderson, B. (1983) Changes in lateral distribution of the light-harvesting chlorophyll *a/b*-protein complex induced by its phosphorylation, *Eur. J. Biochem.*136:25-29.(Medline)

Li, X.-P., Björkman, O., Shih, C, Gross, A.R., Rosenquist, M., Jansoon, S. and Niyogi, K. (2000) A pigment-binding protein essential for regulation of photosynthetic light harvesting, *Nature* 403:391-395.

Losada, M., Trebst, A. V., Ogata, S., and Amon, D. I. (1960) Equivalence of light and adenosine triphosphate in bacterial photosynthesis, *Nature* 186:753-760.

Luecke, H., Schobert, B., Richter, H.T., Cartailler, J.P. and Lanyi, J.K. (1999) Structural changes in bacteriorhodopsin during ion transport at 2 Angstrom resolution, *Science* 286:255-261.(Medline)

Maeda, H., Watanabe, T., Kobayashi, M. and Ikegami, I. (1992) Presence of two chlorophyll-a' molecules at the core of photosystem-I, *Biochim. Biophys. Acta* 1099:74-80.

Malkin, R. (1987) Photosystem I. In The Light Reaction (Barber, J., ed.). pp. 495-560. Elsevier, New York.

Malkin, R., Chain, R. K., Kraichoke, S., and Knaff, D. B. (1981) Studies of the function of the membrane bound iron-sulfur center of the photosynthetic bacterium *Cheomatium vinosum*, *Biochim.Biophys. Acta.* 637:88-91.

Martinez-García, J.F., Huq, E. and Quail, P.H. (2000) Direct targeting of light signals to a promoter element-bound transcription factor, *Science* 288:859-863. (MedLine)

Mathews, S. and Sharrock, R.A. (1997) Phytochrome gene diversity, *Plant Cell Environ.* 20:666-671.

McDermott, G., Prince, S.M., Freer, A.A., Hawthornwaite-Lawless,A.M, Papiz, M.Z., Cogdell, R.J. and Isaacs, N.W. (1995) Crystal structure of an integral membrane light-harvesting complex from photosynthetic bacteria, *Nature* 374:517-521.

Murata, N., and Miyao, M. (1985) Extrinsic membrane proteins in photosynthetic oxygen-evolving complex, *Trends Biochem.Sci.*10:122-124.

Myers, J., and French, C. S. (1960) Evidence from action spectrafor a specific participation of chlorophyll *b* in photosynthesis,*J. Gen. Physiol.* 43:723-736.

Namba, O., and Satoh, K. (1987) Isolation of photosystem II reaction center consisting of D1 and D2 polypeptides an cytochrome*b*-559, *Proc. Natl. Acad. Sci. U.S.A.* 84:109-112.

Ni, M., Tepperman, J.M. and Quail, P.H. (1998) PIF3, a phytochrome-interacting factor necessary for normal photoinduced signal transduction, is a novel basic helix-loop-helix protein, *Cell* 95:657-667. (MedLine)

Niyogi, K.K. (1999) Photoprotection revisited: genetic and molecular approaches, *Annu. Rev. Plant Physiol. Mol. Biol.* 50:333-359.

Nugent, J. (2001) Photosynthetic water oxidation, *Biochim. Biophys. Acta.* 1503:1. (MedLine)

Ort, D. R. (1986) Energy transduction in oxygenic photosynthesis: an overview of structure and mechanisms. In *Encyclopedia of Plant Physiology*, New Series, Vol. 19, pp. 165-196. Springer-Verlag,Berlin.

Oesterhelt, D. and Stoeckenius, W. (1971) Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*, *Nature New Biol.* 233:149-152.(Medline)

Osterlund, M.T., Hardtke, C.S., Wei, N. and Deng, X.W. (2000) Targeted destabilization of HY5 during light-regulated development of *Arabidopsis Nature* 405:462-466. (MedLine)

Park, R. B. (1966) Thin section of $KMn0_4$ fixed *Spinacea oleracea*chloroplast, x19,500. In *The Chlorophylls* (Vernon, L. P., and Seely, G. R., eds.), pp. 283-311. Academic Press, New York.

Parson, W.W. (1987) The bacterial reaction center. In *Photosynthesis*(Amesz, J., ed.), pp. 43-62. EIsevier, New York.

Pfannschmidt, T., Anders, N. and Allen, J.F. (1999) Photosynthetic control of chloroplast gene expression, *Nature* 397:625-628.

Pierson, B. K., and Olson, J. M. (1987) Photosynthetic bacteria.In *Photosynthesis* (Amesz, J., ed.), pp. 21-42. Elsevier, New York.

Quail, P.H. (2002) Phytochrome photosensory signalling networks, *Nature Rev. Mol. Cell Biol.* 3:85-93. (MedLine)

Rhee, K.H., Morris, E.P., Barber, J. and Kühlbrandt, W. (1998) Three-dimensional structure of the plant photosystem II reaction centre at 8 Å resolution, *Nature* 396:283-286. (Medline)

Rintamäki, E., Salonen, M., Suoranta, U.M., Carlberg, I., Andersson, B. and Aro, E.M. (1997) Phosphorylation of light-harvesting complex II and photosystem II core proteins shows different irradiance-dependent regulation in vivo. Application of phosphothreonine antibodies to analysis of thylakoid phosphoproteins, *J. Biol. Chem.* 272:30476-30482.(Medline)

Rutherford, A.W. and Faller, P. (2001) The heart of photosynthesis in glorious 3D, *Trends Biochem. Sci.* 26:341-344. (MedLine)

Sauer, K. (1986) Photosynthetic light reactions. In *Encyclopedia of Plant Physiology*, New Series, Vol. 19, pp. 603-631. Springer-Verlag, Berlin.

Schubert, W.D., Klukas, O., Krauss, N., Saenger, W., Fromme, P. and Witt, H.T. (1997) Photosystem I of *Synechococcus elongatus* at 4 Å resolution: comprehensive structure analysis, *J. Mol. Biol.* 272:741-769. (Medline)

Schwechheimer, C. and Deng, X.W. (2001) COP9 signalosome revisited: a novel mediator of protein degradation, *Trends Cell Biol.* 11:420-426. (MedLine)

Sprague, S. G., and Varga, A. R. (1986) Topography, composition and assembly of photosynthetic membranes. In *Encyclopedia of PlantPhysiology*, New Series, Vol. 19, pp. 603-631. Springer-Verlag, Berlin.

Staehelin, L. A. (1986) Chloroplast structure and supramolecular organization of photosynthetic membranes. In *Encyclopedia of Plant Physiology*, New Series, Vol. 19, pp. 11-83. Springer-Verlag, Berlin.

Stanier, R. Y. (1961) Photosynthetic mechanisms in bacteria and plants: development of a unitary concept, *Bacterial. Rev.* 25:1-17.

Stemler, A., and Radmer, R. (1975) Source of photosynthetic oxygenin bicarbonate-stimulated Hill reaction, *Science* 190:457-458.

Stowell, M.H., McPhillips, T.M., Rees, D.C., Soltis, S.M., Abresch, E. and Feher, G. (1997) Light-induced structural changes in photosynthetic reaction center: implications for mechanism of electron-proton transfer, *Science* 276:812-816. (MedLine)

Tepperman, J.M., Zhu, T., Chang, H.S., Wang, X. and Quail, P.H. (2001) Multiple transcription-factor genes are early targets of phytochrome A signaling, *Proc. Natl. Acad. Sci. USA* 98:9437-9442. (MedLine)

Trebst, A. (1986) The topology of the plastoquinone and herbicide peptides in photosystem II in the thylakoid membrane, *Z.Naturforsch. Teil C*5 41:240-245.

Trebst, A. V., Tsujimoto, H. Y., and Arnon, D. I. (1958) Separation of light and dark phases in photosynthesis of isolated chloroplasts,*Nature* 182:351-355.

Vierling, E., and Alberte, R. S. (1983) P700 chlorophyll *a*-protein. Purification, characterization and antibody preparation, *Plant Physiol.* 72:625-633.

Webber, A.N. and Lubitz, W. (2001) P700: the primary electron donor of photosystem I, *Biochim. Biophys. Acta* 1507:61-79. (Medline)

Whitmarsh, J., and Ort, D. R. (1984) Stoichiometries of electron transport complexes in spinach chloroplasts, *Arch. Biochem. Biophys.* 23:378-389.(Medline)

Wang, D. and Govinjee (1979) Antagonistic effects of mono- and divalent cations on polarization of chlorophyll fluorescence inthylakoids and changes in excitation energy transfer, *FEBS Lett.*97:373-377.

Zouni, A., Witt, H.T., Kern, J., Fromme, P., Krauss, N., Saenger, W. and Orth, P. (2001) Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution *Nature* 409:739-743. (MedLine)

Zuber, H., Brunischolz, R., and Sidler, W. (1987) Structure and function of light-harvesting pigment-protein complexes. In *Photosynthesis* (Amesz, J., ed.), pp. 233-272. Elsevier, New York.

18. Energy Transduction:

Oxidative Phosphorylation and Photophosphorylation

The major function of the electron transport chains is to provide energy for the synthesis of ATP from ADP and P_i. ATP can be synthesized by either the oxidative reactions in mitochondria or bacteria (i.e., oxidative-phosphorylation) or the reactions of photosynthetic systems (i.e., photophosphorylation). As we saw in Chapters 15 and 16, the electron transport chain functions in either oxidative or photosynthesizing systems as a series of redox reactions. There is every indication that the mechanism of the coupling between electron transport and phosphorylation is also very similar in both systems. Section I deals with the reactions in mitochondria and Section II with photosynthetic systems. Finally, the mechanism of coupling is discussed in more detail in Sections III and IV.

I. ENERGY TRANSDUCTION IN MITOCHONDRIA

The involvement of mitochondria in both phosphorylation and ion transport is illustrated in the experiment of Fig. 1 (Chance, 1965). In this experiment, the respiration of isolated guinea pig kidney mitochondria is monitored by an electrode sensitive to O_2 in the medium. The trace in the figure corresponds to a plot of the oxygen concentration (ordinate) with time (abscissa). Some respiration occurs without the addition of substrate, as indicated by the slight downward trace at the left-hand part of the graph; the mitochondria are presumably oxidizing an endogenous substrate. Addition of succinate (arrow 1) accelerates the respiration. Subsequent addition of ADP (arrow 2) to the suspension that already contains inorganic phosphate (P_i), produces a burst of respiration that coincides with oxidative phosphorylation. When all the ADP is used up, the respiration returns to a lower rate. Similar bursts of respiration occur after additions of Ca^{2+} (arrows 3 and 4), which is translocated into the mitochondrial lumen and precipitated as calcium phosphate. The rate of respiration before the addition of ADP, divided by the rate of respiration during phosphorylation, the *respiratory control ratio*, has been adopted as an index of the degree of coupling in mitochondrial preparations.

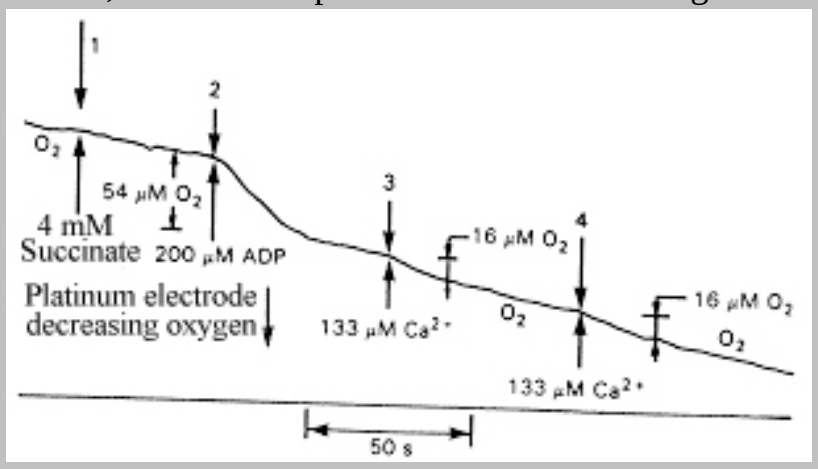


Fig. 1 Consumption of oxygen as a function of time, showing the increment in oxidation in the presence of ADP or Ca^{2+}. Guinea pig kidney mitochondria and an assay medium containing 4 mM phosphate were used. Reproduced from B. Chance, *Journal of Biological Chemistry*, 240:2729-2748, 1965, with permission of the American Society of Biological Chemists, Inc.

Part A of this section concerns oxidative phosphorylation primarily; part B is concerned with the coupling to active transport of ions in mitochondria.

A. Oxidative Phosphorylation

The maximal phosphorylative yields of electron transport can be predicted from thermodynamic considerations (see below). However, these arguments cannot establish the actual yield, which can be determined directly in well-coupled mitochondria. The amount of ATP formed from ADP and P_i is carefully measured and the oxygen taken up by the system is estimated. Each oxygen g-atom represents an electron pair traversing the cytochrome chain. It has become customary to express the phosphorylative yield as a P/O ratio: the moles of inorganic phosphate esterified per oxygen atom taken up. Oxygen uptake can be conveniently assessed with an oxygen electrode (as done in the experiment depicted in Fig. 1). Phosphorylation can be evaluated by measuring the disappearance of P_i or ADP or the appearance of ATP. The incorporation into ATP of the radioactive $^{32}P_i$ gives a reliable and convenient estimate of ATP

synthesized. ATP can also be assayed by using biochemical reactions that are coupled to the hydrolysis of ATP. One of the most sensitive is the luciferin-luciferase reaction using enzymes purified from the firefly tail. The amount of light generated from the system is a function of the ATP concentration.

The amount of ATP synthesized can also be estimated indirectly. When small amounts of ADP are added, phosphorylation proceeds until virtually all the ADP has been used up to synthesize ATP. Therefore, the ATP formed corresponds to the ADP added. This is the method used in the interpretation of Fig. 1.

Phosphorylation sites

The actual yield of ATP per electron pair transported will depend on the substrate used because some of the substrates enter at different sites in the electron transport chain. Substrates that are oxidized by NAD^+ dehydrogenases, such as isocitrate, α-ketoglutarate and malate, make use of the entire electron transport chain and involve all the redox couples of respiration. The oxidation of α-ketoglutarate has one additional substrate-level phosphorylation not involving the cytochrome chain. In practice, the fatty acid β-hydroxybutyric acid is commonly used. In liver mitochondria, this substrate is converted quantitatively into acetoacetate in a reaction that reduces one NAD^+. The P/O ratio of β-hydroxybutyrate oxidation is listed in Table 1 (Copenhaver and Lardy, 1952); the value is between 2 and 3. The mitochondria are thought to be imperfectly coupled. Therefore, the higher figures are generally assumed to be correct and the synthesis of three ATP is thought to occur per electron pair traversing the entire chain (see Lee et al., 1996; for a discussion, Hinkle et al., 1991 and for alternative views Nicholls and Ferguson, 1992).

Table 1 P/O Ratios in Isolated Mitochondria

Substrate	P/O	Reference
β-Hydroxybutyrate	2.4-2.5	a
Pyruvate+ malate	2.9	b
Succinate +(rotenone)	1.7	a
	1.7-1.9	b
ascorbate	0.88	c
cytochrome c	0.61-0.68	c

[a]Copenhaver and Lardy (1952)
[b]Lee et al. (1996)
[c]Lehninger (1955)

The contribution of the various portions of the chain can be examined in more detail using other substrates (e.g., Copenhaver and Lardy, 1952; Lee et al., 1996). Succinic dehydrogenase is a flavoprotein, and the oxidation of succinate does not involve NAD^+; it skips the NAD^+-ubiquinone segment of the electron transport chain (see Fig. 6, Chapter 16). Interestingly, with succinate as the substrate, electrons enter the chain below those for an NAD-linked substrate and the P/O ratio is approximately 2 (Table 1). The results in Fig. 1 show that the phosphorylation of 200 µM ADP results in the uptake of 108 µg-atoms of oxygen. In this case, the P/O ratio is 1.8. These results suggest that one phosphorylative step occurs in the span of the chain preceding CoQ (i.e., NAD-FP-CoQ) and the other two in the span below CoQ (i.e., CoQ-cyt *b*-cyt c_1-cyt *c*-cyt *a*-cyt a_3-O_2).

Although no natural substrate interacts with the chain span below CoQ, it has been found that ascorbate reduces cytochrome *c* nonenzymatically. Frequently, the experiments hare carried out in the presence of catalytic amounts of the dye N,N,N',N'-tetramethyl-*p*-phenylene diamine (TMPD), which mediates the electron transfers between ascorbate and cytochrome c. The resulting P/O ratios approach 1 (Table 1) (Lehninger, 1955). Added reduced cytochrome *c* can also be oxidized by the system, also yielding a P/O of about 1. Therefore, the second phosphorylation must occur in the span between CoQ and cytochrome *c*, and the third between cytochrome c and oxygen, as shown in Fig. 2.

For convenience, Chance and Williams (1956) numbered the various possible conditions of mitochondria. In the presence of substrate and O_2, the mitochondria are said to be in state 4. With the addition of ADP, the phosphorylating mitochondria are said to be in state 3. When all the ADP is used up, the mitochondria undergo a transition from state 3 to state 4. Three other states, not discussed in this chapter, have been described: oxygenated mitochondria without added ADP or substrate (state 1), state 1 conditions with the addition of ADP (state 2), and the complete system in the absence of oxygen (state 5).

In very tightly coupled mitochondria, little oxidation takes place in the absence of ADP. ADP is involved only at the phosphorylative sites; its absence should block electron flow at these sites. Accordingly, the components preceding the block should be more reduced and those following the block more oxidized. The electron transport chain should exhibit crossover points similar to those caused by a block in the chain by a specific inhibitor. In addition, since the oxidative phosphorylation reactions are reversible, the hydrolysis of ATP can reverse the flow of electrons. In this latter case, the components that precede the phosphorylative site in the course of normal electron transport should become more reduced and those that follow should become more oxidized. Therefore, the addition of ADP or ATP under the appropriate conditions aids in pinpointing the location of the phosphorylative sites.

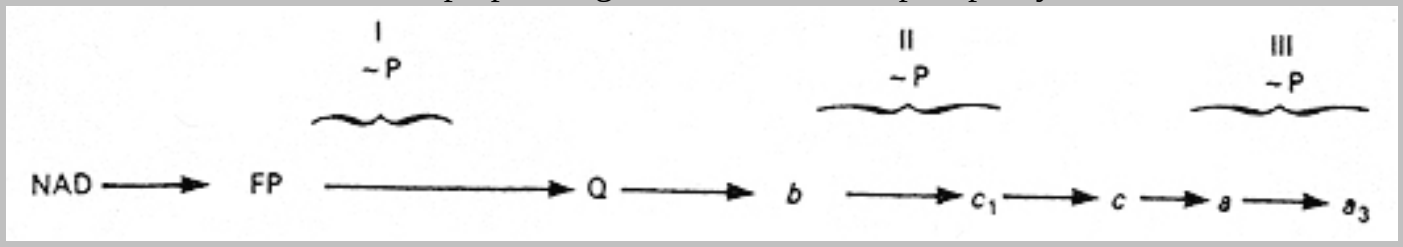


Fig. 2 Diagram summarizing (a) the sequence of electron transport components and (b) the location of the

phosphorylative sites.

The results of an experiment providing data to determine cross-over points is shown in Fig. 3. In this experiment, the hydrolysis of ATP reduces components at the phosphorylation site in the direction opposite to normal electron transport. The addition of ATP induces the reduction of pyridine nucleotide (Fig. 3a) at the expense of the oxidation of the cytochromes and flavoproteins. Na_3S is used to block the cytochrome chain at the cytochrome a_3 site. The two curves correspond to the changes in optical density at wavelengths that allow monitoring of the redox state of NAD (curve 1) and cytochrome *c* (curve 2) with time. The optical density changes have been calibrated in terms of NADH formed (upper ordinate) or expressed in terms of cytochrome oxidation (lower ordinate). In this kind of experiment, several respiratory carriers, including flavoprotein, are oxidized as shown in Fig. 3b.

Similarly, other crossover points can be studied after isolating the relevant part of the chain by means of inhibitors. A block between cytochromes *b* and *c* with antimycin permits the demonstration of an ATP-induced oxidation of cytochrome $a+a_3$ and reduction of cytochrome *c*. A slow reduction of cytochrome *b*, while components closer to the oxygen are oxidized, suggests a possible third point of interaction.

The conclusions reached in experiments in which the reactions are driven backward agree with those obtained for the transition from state 3 to state 4.

These two approaches, i.e., deductions from crossover points, can give insight into the sites responsible for the phosphorylation of ADP. However, it is obvious that phosphorylation can be coupled to the redox couples of the electron transport chain only when enough energy (i.e., ΔG) is available. Therefore the energetics of the system are pertinent as well.

Thermodynamic considerations

The energy available from the redox reactions (i.e., the ΔG) can be estimated directly by measuring the redox potential for the appropriate redox reactions. The redox potentials depend on the physical environment, therefore, redox potentials have a meaning only in a native system. The redox state of the system can be varied chemically by adding precise amounts of oxidants or reductants, and the redox potential of the system as a whole can be measured. As long as the system is anaerobic (i.e., reducing equivalents cannot be oxidized by oxygen), the system is *closed*. Equilibration of the electron carriers should result in the entire system being poised at the same potential (E_h). As discussed in Chapter 12, the ΔG and ΔE of a half-cell can be represented as shown in Eqs. (1) and (2), respectively.

$$\Delta G = \Delta G^{\circ} + RT \ln \frac{[red]}{[ox]} \qquad (1)$$

$$\Delta E = \Delta E^{\circ} + RT \ln \frac{[ox]}{[red]} \qquad (2)$$

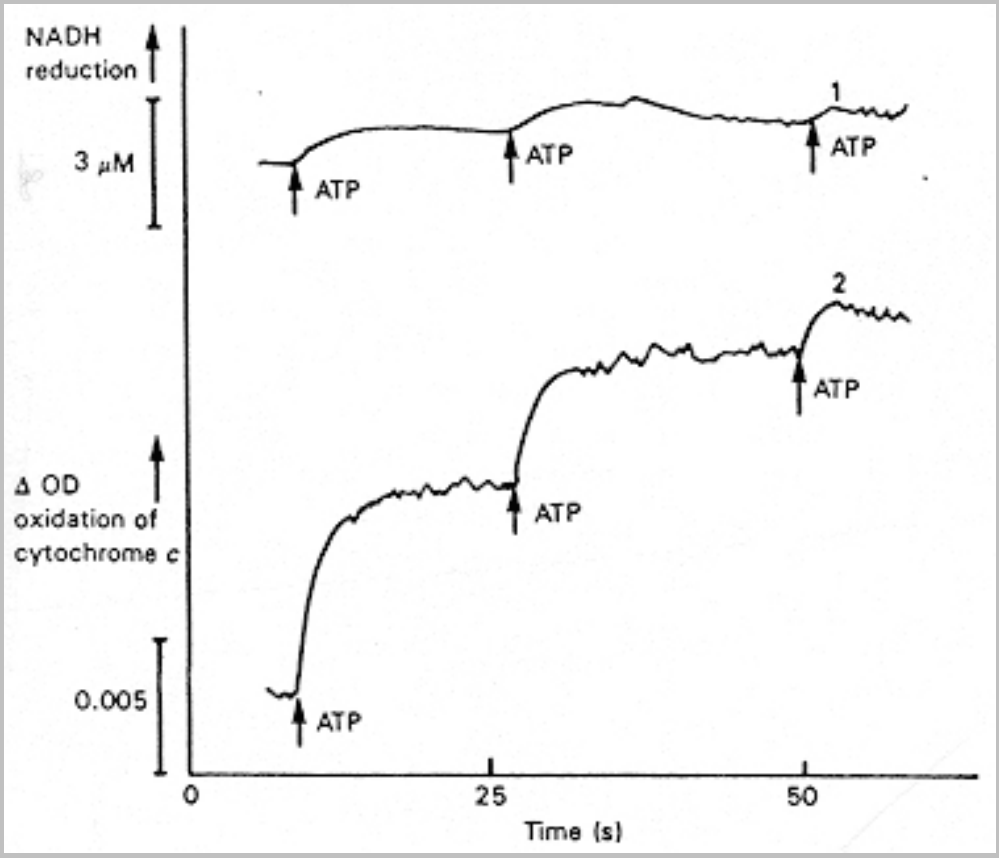


Fig. 3 Reversal of electron transport on addition of ATP. The cytochrome *c* is oxidized and the pyridine nucleotide is reduced in sulfide-inhibited pigeon heart mitochondria, in the absence and presence of ATP. The mitochondria were in a mannitol-sucrose medium, buffered with Tris at pH 7.4 and a temperature of 26C. Reproduced from B. Chance and C. Hollunger, *Journal of Biological Chemistry*, 236:1577-1584, 1961, with permission of the American Society of Biological Chemists, Inc.

In practice, the redox potential can be measured in the presence of low molecular weight mediators (see below) with two electrodes: a *reference electrode* (e.g., calomel electrode) and an *indicator electrode* (e.g., platinum electrode). The indicator electrode responds to the redox components of the system. Basically, each electrode acts as a half-cell. For the calomel electrode the reaction of the half-cell is

$$Hg_2Cl_2 \text{ (solid)} + 2e^- \leftrightarrow 2Hg \text{ (solid)} + 2Cl^- \quad (3)$$

For the platinum electrode the reaction corresponds to

$$Pt^{2+} + 2e^- \leftrightarrow Pt \quad (4)$$

The electron transport chain in the mitochondrial inner membrane cannot interact directly with the platinum electrode. Catalytic amounts of low molecular weight synthetic carriers (electron *donors-acceptors*) are added in order to shuttle electrons from the mitochondria to the indicator electrode.

For each level of oxidation-reduction of each component there will be a corresponding ratio [ox]/[red]. As

shown by Eq. (2), when this ratio is unity, ΔE becomes ΔE^o, the half reduction potential (ΔE_m).

The concentrations of oxidized or reduced components are determined either from their light absorption or, in the case of the iron-sulfur proteins, from the electron spin resonance of the oxidized form. By convention (see Chapter 10) the redox potentials are those that would have been obtained using a H_2-electrode half-cell. Those obtained with the more practical calomel half-cell can be readily converted to the appropriate values for the H_2-electrode (usually indicated as E_h).

The reaction at a H_2-electrode would be:

$$ox + H_2 \leftrightarrow red + 2H^+ \quad (5)$$

In Fig. 4 (Wilson et al., 1983), components of the electron transport chain are displayed as a function of their redox potential (ΔE_h and ΔE^o_h). The various redox potentials seem to fall into three groups of approximately the same potentials. However, between the equipotential groups there is a change of about 250 mV corresponding to a ΔG of -5.7 kcal/mol. Considering that two electrons are needed to go through the cytochrome chain per phosphorylation site, with -7 to -10 kcal needed for the synthesis of ATP, the calculated -11.4 kcal would be sufficient. The redox potentials of three components (cyt a_3, cyt b_1, and a flavoprotein) appear to depend on whether the mitochondria are uncoupled (lower value) or coupled and in the presence of ATP (higher value). In the original studies, this finding was attributed to a direct involvement of these reactions in the synthesis of ATP. However, other interpretations are possible. For example, the ox/red ratio could have been altered by reversed passage of electrons at the expense of energy from ATP hydrolysis, as discussed above.

The order of the electron transport carriers shown previously and the data summarized in Fig. 4 (Wilson et al., 1971) support the location of the three phosphorylation sites shown by the jumps in potentials in the figure, schematically expressed in Fig. 2.

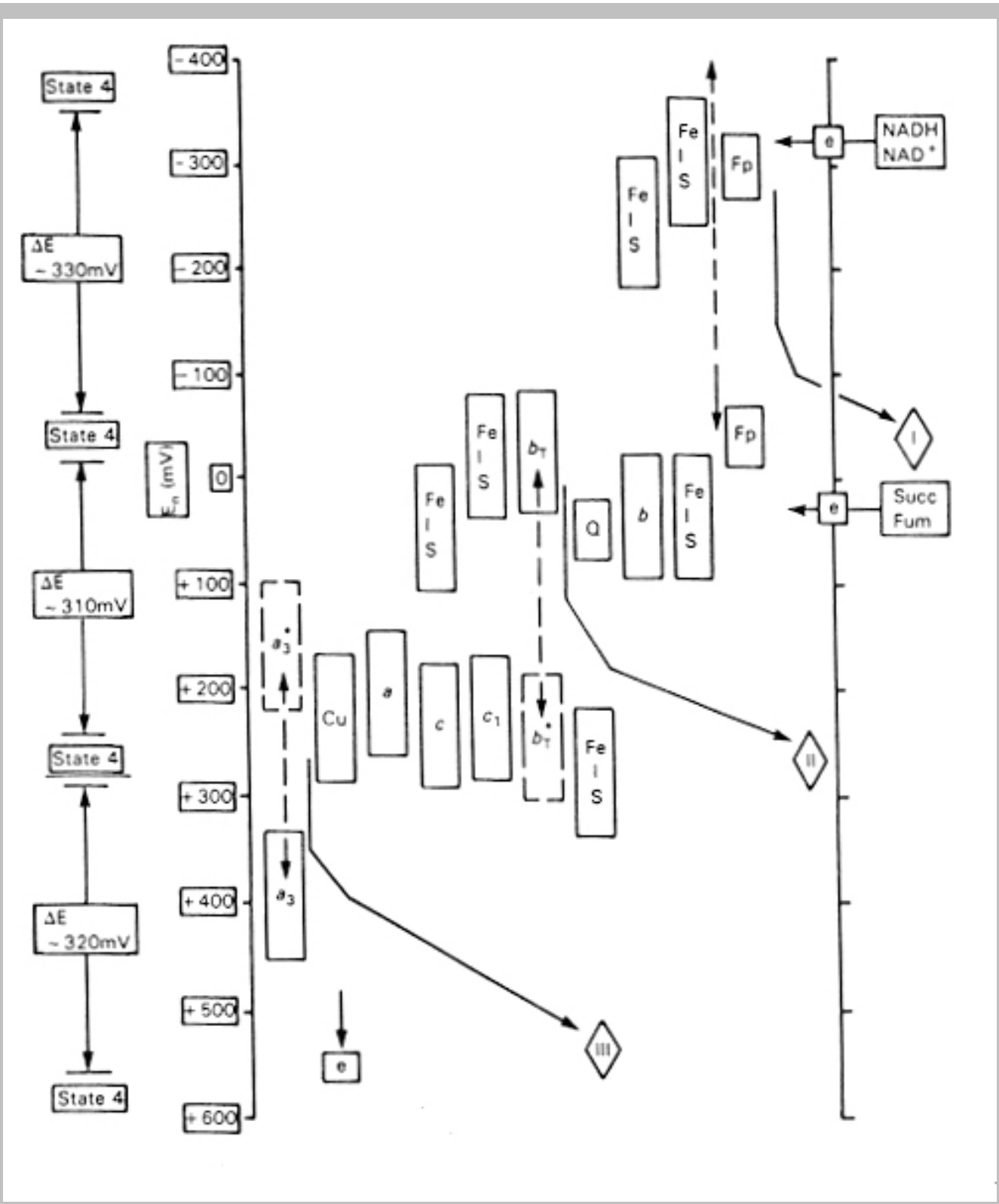


Fig. 4 Thermodynamic profile of the oxidation-reduction components of the respiratory chain of pigeon heart mitochondria. Each component is represented by a rectangle, which is centered on its half-reduction potential at pH 7.2 and extends from the potential at which the component is 9% reduced to the potential at which it is 91% reduced. The positions of the rectangles are not intended to indicate the sequence of electron transfer. On the left side are indicated the E_h values of the isopotential groups in pigeon heart mitochondria under state 4 conditions, with succinate and glutamate as substrate. Two values are indicated for cytochromes b_t and a_3, one measured for uncoupled mitochondria and the other measured for coupled mitochondia in the presence of excess ATP. Reproduced from D. P. Wilson, et al., *Current Topics in*

Bioenergetics, 5:234-265, 1973, with permission of Academic Press.

Proton production

The chemiosmotic model proposes that a flux of H^+ generated by electron transport produces a proton electrochemical gradient, the *protonmotive force*. A flow of protons in the direction of this gradient is coupled to the phosphorylation of ADP. Some of the details of the proposal can be questioned (see section III). However, a link between H^+-flux and the synthesis of ATP is, undeniable. Therefore, sites of proton production must be equivalent to the phosphorylative sites. The reactions translocating protons have been shown in Chapter 16 (Section IIIC). They correspond to the electron transport sites deduced from the experiment just discussed. $4H^+$ are generated per site involving the NADH-ubiquinone oxidoreductase, the ubiquinol-cyt *c* oxidoreductase and the cytochrome c oxidase. Currently, $3H^+$ are thought to be needed per ATP synthesized (see summary of Cramer and Knaff, 1990, p.118).

B. Transport of Ions

As shown in Fig. 1, the addition of certain cations to mitochondrial suspensions results in a burst of respiration. This is the result of the active transport of the ion from the medium to the mitochondrial matrix. Energy-dependent uptakes have been demonstrated for a number of divalent cations (e.g., Mg^{2+}, Mn^{2+}, and Sr^{2+}) and monovalent cations (e.g., K^+, Na^+, or Li^+). The translocations can be supported by oxidative reactions or by the hydrolysis of ATP. As might be expected, the active transport is blocked by uncouplers. However, there are indications that the transport of ions differs significantly from the processes leading to phosphorylation, as indicated by the effect of the antibiotic oligomycin. Oligomycin binds to the ATP-synthase complex (F_0F_1) and blocks both the phosphorylation of ADP and the ATPase activity of the enzyme. Oligomycin also blocks the transport of cations powered by ATP hydrolysis. However, oligomycin does not block the transport of cations powered by respiration. The results of the experiment of Table 2 (Brierley et al., 1962) show the uptake of Mg^{2+} by beef heart mitochondria. The uptake in the complete system is shown in line 2. The uptake of Mg^{2+} requires energy, as indicated by the need for a substrate (line 5) and by the inability to transport in the presence of an uncoupler (line 8) or the inhibitor of electron transport, antimycin (line 9). Transport and oxidative phosphorylation compete as the uptake is reduced in the presence of ADP (line 6) or ADP plus hexokinase and glucose (line 7) (the latter to regenerate ADP by phosphorylating glucose). However, oligomycin, which blocks phosphorylation, fails to affect the transport (line 10), as discussed in more detail later. In the experiments of Table 2, Mg^{2+} is accompanied by P_i. In fact, accumulation of a cation is generally accompanied by the passage of an anion, maintaining electric neutrality; alternatively, an internal cation must leave. The uptake of a divalent cation in the presence of P_i results in precipitation of the salt. The anion is, therefore, functioning in a dual capacity, accompanying the cation and acting as a trapping agent. Under these conditions, massive amounts of the salt can be accumulated. However, the anion need not be phosphate; arsenate, acetate, and many others will do. Without removal of the accumulated ions by precipitation (as in the case of acetate), the uptake is not as marked.

The uptake of cations is sometimes accompanied by a countermovement of H^+. The precise magnitude of the exchange depends on the conditions. For example, in the absence of an excess of penetrating anion, two H^+ appear in the medium per Ca^{2+}. This ratio can be lowered considerably by the presence of acetate or some other anion to which the membranes are permeable, and in some cases no protons are ejected.

Do the same steps in the electron transport chain provide the energy for ion transport and for the phosphorylation of ADP? Several experiments suggest that this the case. The effectiveness of the substrates linked to the various coupling sites (e.g., Brierley and Murer, 1964, Penniston et al., 1966) and the cross-overs in the presence and absence of Ca^{2+} (e.g., Chance and Hollunger, 1961) suggest that the transport of ions involves the same sites in the electron transport chain as oxidative phosphorylation.

Table 2 P_i and Mg^{2+} Accumulation by Isolated Heart Mitochondria

Conditions	P_i[a]	Mg^{2+}[a]
1. Mitochondria alone	30	50
2. Complete system	1010	1800
3. Mg^{2+} omitted	10	35
4. P_i omitted	10	95
5. Substrate omitted	10	75
6. ADP added (10 µmoles)	130	206
7. ADP + hexokinase system	30	85
8. Complete + dinitrophenol (0.3 µmoles)	8	75
9. Complete + antimycin (6 µg)	40	75
10. Complete + oligomycin (12 µg)	980	1800

Reproduced with permission from G.P. Brierley, et al., *Proc. Natl. Acad. Sci. USA* 48:1928-1935, 1962.

II. PHOTOPHOSPHORYLATION

As we saw in Chapter 17, the electron transport chain and the phosphorylative reactions in photosynthetic systems are very similar to those of the mitochondrial system. Analogous diagrammatic schemes can be

designed, as shown in Fig. 5 (Hall, 1976). Figure 5 shows not only the various electron transport components and their position in the photosynthetic electron transport chain, but also inhibitors, electron acceptors and donors. Electron acceptors and donors can functionally isolate part of the chain, as previously shown for the study of PSI or PSII (Chapter 17). For example, with methylviologen (MV) or ferricyanide (FeCy), the electron flow that normally reduces NADP can be bypassed. Similarly, benzidine and ascorbate can bypass the reactions that normally release oxygen.

The relationship between electron transport and phosphorylative reactions can be studied in the chloroplasts by methodology analogous to that used in the study of mitochondria. Figure 6 (Hall, 1976) shows a record of oxygen release in an experiment in which ferricyanide is used as an electron acceptor. It is seen that light in the presence of ferricyanide increases the amount of O_2 released. When ADP is added (state 3), the amount of O_2 released increases. When the ADP is used up, the O_2 released returns to the state 4 value (no ADP). The P/O ratios calculated from these results approach 2. However, such high ratios can be calculated only after correcting for the basal electron transport (see Horton and Hall, 1968; Winget et al., 1965). In addition to determining phosphorylative yield, it is possible to calculate photosynthetic control (PC) ratios analogous to respiratory control (RC) ratios in mitochondria, by dividing the oxygen released in state 3 by that released in state 4.

If the components associated with PSI or PSII are isolated, the portions of the electron transport chain appropriately marked in Fig. 5 can be shown to be involved in the synthesis of ATP. These results suggest one such site per span.

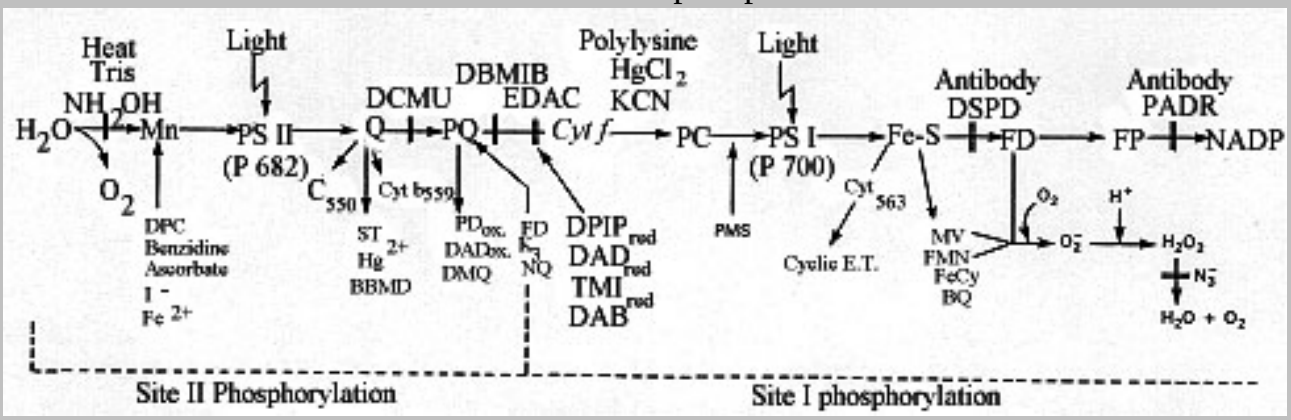


Fig. 5 Scheme for noncyclic electron transport and phosphorylation. From Hall (1976), with permission. Abbreviations are as follows. Electron donors: DPC, diphenyl carbazide; FD, ferrodoxin; K_3, vitamin K_3 (menadione); NQ, napthoquinone; DAD, diaminodurene; TMI, tetramethylindamine; DAB, diaminobenzidine; PMS, phenazine methosulphate. Electron acceptors: ST, silicotungstate; BBMD, benzl-a-bromomalodinitrile; DAD_{ox}, diaminodurene; DMQ, dimethyl-*p*-benzoquinone; MV, methyl viologen; FeCy, ferricyanide; BQ, benzoquinine. Inhibitors: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tris, tris(hydroxy- methlaminomethane); DBMIB, 2,5-dibromo-3-methyl-6-isopopyl-p-benzoquinone;EDAC,1-ethyl-3-(3-dimethylaminopropyl)cardodiimide; DSPD, disalicyclidene propane diamine; PADR, phosphoadenosine diphosphate ribose. Reproduced with permission from D.O. Hall, *The Intact Chloroplast*. Copyright &copy1976 Elsvier Sceince Publishers, Amsterdam.

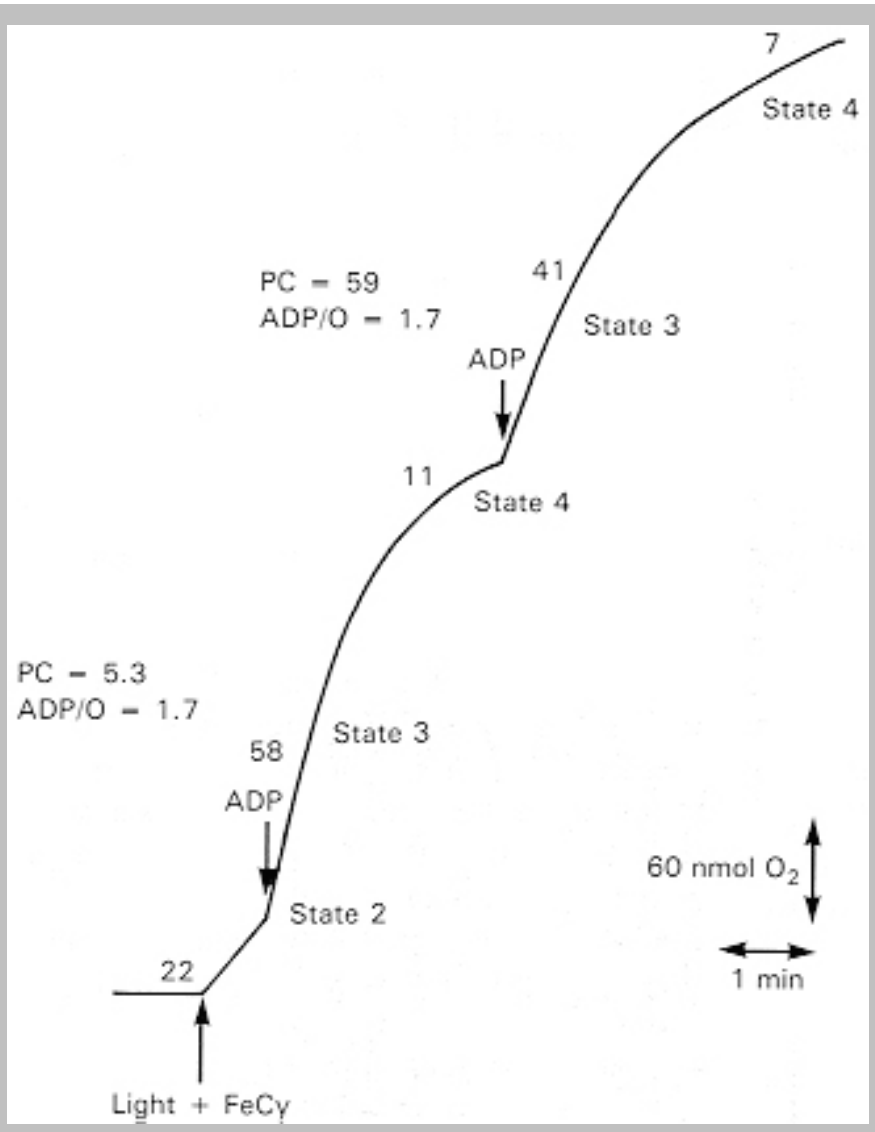


Fig. 6 Record of oxygen release over time. The electron acceptor is ferricyanide (FeCy). Reproduced with permission from D.O. Hall, *The Intact Chloroplast*. Copyright &copy1976 Elsevier Science Publishers, Amsterdam.

The results for photophosphorylation appear analogous to those for oxidative phosphorylation. For this reason the two are thought to involve the same mechanisms, as discussed in Section III.

III. MECHANISMS OF COUPLING

The *chemiosmotic hypothesis* of Mitchell (1966, 1967) proposes the formation of a proton electrochemical gradient by the coupling of electron transport to the flux of H^+, an efflux in the case of mitochondria and an influx in the case of the thylakoid vesicles. The return of H^+ in the direction of its electrochemical gradient involving the ATP-synthase is coupled to the phosphorylation of ADP. The transducing membranes are assumed to be extremely impermeable to ions so that the electrochemical gradient is not passively dissipated. Translocations of ions are assumed to occur mostly by electrically neutral exchanges in which external ions are exchanged for internal ions of the same charge (*antiport*), or oppositely charged ions are simultaneously transferred in the same direction (*symport*).

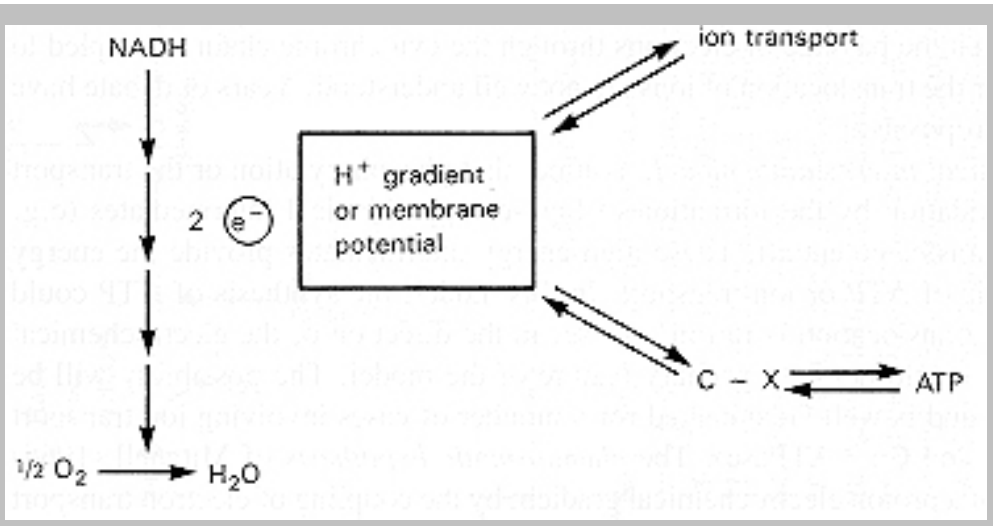


Fig. 7 Diagram summarizing the possible interpretation of the data. A chemiosmotic model.

The system can be operated as an ATPase or as a phosphorylative mechanism, as shown for mitochondria in Fig. 8. The operation of the cytochrome chain to produce an H^+ ion gradient or a membrane potential is represented in Fig. 8a. The operation of the ATP synthase either in phosphorylation (heavy lines) or as an ATPase (dashed lines) is shown in Fig. 8b. The pH differential or an internal negative potential resulting from the expulsion of H^+ during oxidation would drive the H^+ inward for phosphorylation. The functioning of the system in the transport of cations, in this example Ca^{2+}, is shown in Fig. 8c. Fig. 8 represents the chemiosmotic hypothesis as proposed in mitochondria. In the case of the thylakoid vesicles, all the events would have the opposite polarity, since the H^+ transport occurs inwardly.

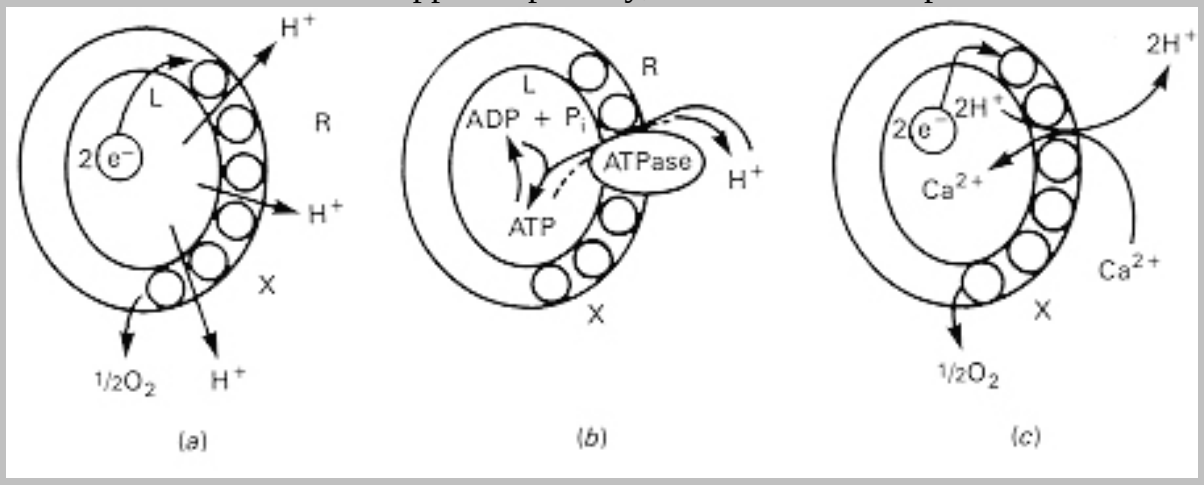


Fig. 8 Diagram representing the chemiosmotic hypothesis. (a) Formation of a membrane potential or a pH gradient by passage of the electrons through the cytochrome chain. (b) ATPase functioning either to hydrolyze ATP (dashed line) or to phosphorylate ADP (solid line). (c) Exchange of cations for H^+.

The possible role of the chemiosmotic model can be evaluated through two basic questions: (1) Can the flux of H^+ in the direction of its electrochemical gradient produce ATP from ADP and P_i? (2) During normal electron transport, is there an H^+ electrochemical gradient sufficient to play a role in the various transducing systems (mitochondria, thylakoid vesicles and bacteria)?

The answer to the first question is yes for thylakoid vesicles, as shown by the classic experiment of Jagendorf and Uribe (1966), summarized in Table 3. In this experiment, leaky chloroplasts, probably equivalent to thylakoid vesicles, were maintained in the dark or in the presence of the appropriate inhibitor. The chloroplasts were first equilibrated with organic acid and then shifted to an alkaline medium. Presumably, the inside would initially be acidic, as it would be with illumination; the proton flow is in the direction opposite to that in mitochondria. The external alkalinization would then provide the appropriate gradient for an H^+ efflux. The first item (*a*) corresponds to the experimental determination with the complete system. The amount of ATP formed was measured either with the luciferin-luciferase reaction (column 4) or by measuring the amount of phosphate incorporated into ATP (column 6); the two methods show that ATP was formed by this procedure. On the other hand, ATP was not synthesized when the initial incubation was at pH 7.0 (item *b*), or when any of the necessary components were left out (such as PO_4, item *c*; ADP, item *d*; Mg^{2+}, item *e*; or chloroplasts, item *f*). Analogous demonstrations are available for submitochondrial particles or reconstituted vesicular preparations containing only ATP synthase, FF, (e.g., Sone et al., 1977). The latter experiments also establish that F_0F_1 (and no other protein) is necessary for the synthesis of ATP.

Table 3 Formation of ATP by Acid-Base Transitions[a]

		ATP (estimated by luciferase assay)		ATP (estimated by phosphomolybdate extraction)	
Reaction mixture	**pH of acid**	**Total**	**Net synthesis**	**Total**	**Net Synthesis**
(1)	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**
a. Complete	3.8	141	129	166	163
b. Complete	7.0	12	--	3	--
c. P_i omitted	3.8	12	--	--	--
d. ADP omitted	3.8	4	--	3	--
e.. Mg^{2+} omitted	3.8	60	48	48	45
f.. Chloroplasts omitted	3.8	7	--	3	--

Reproduced with permission from A.T. Jagendorf and E. Uribe, *Proc. Natl. Acad. Sci. USA* 55:170-177, 1966.

[a]The chloroplast fragments were first exposed to acid then to an alkaline medium (pH 8). The results are given in micromoles/mg protein.

Even though the experiments just described support the chemiosmotic hypothesis, experiments with intact mitochondria do not. In mitochondria, because the H^+ pumping is outward, the matrix will become alkaline. In isolated mitochondria, a small alkaline shift in the medium induces ATP synthesis (Malenkova et al., 1982). Experiments with base followed by acid shifts do not produce ATP. Since in mitochondria a H^+ influx should be coupled to ATP synthesis, these experiments are at variance with the present chemiosmotic model.

The answer to the second question is also not clear. The electrochemical potential gradient (the *proton-motive force* of Mitchell) contains two terms: one referring to the H^+ concentration gradient and the other to the electrical potential across the transducing membrane, Eq. (6):

$$\Delta G = nRT \ln \frac{(H^+)_i}{[H^+]_o} + nF\,\Delta\Psi \qquad (6a)$$

$$\Delta G = -2.3\, nRT\, \Delta pH + nF\Delta\psi \qquad (6b)$$

In these equations, the subscripts *i* and *o* refer to location inside or outside the lumen, *F* is the Faraday and corresponds to the membrane potential. The stoichiometry was thought to correspond to n=2. However, more recently, n=3 has been favored.

The two terms have been evaluated in a number of studies. The mitochondrion will be used for the discussion of the technique used to calculate the pH. Analogous reasoning can be applied to the thylakoid vesicles.

In metabolizing mitochondria in which the interior is alkaline in relation to the outside, the pH has generally been evaluated by measuring the distribution of a weak acid (HA). The acid is assumed to permeate the inner mitochondrial space rapidly and primarily in its undissociated form. With these assumptions, at equilibrium,

$[HA]_i = [HA]_o$ (7) The dissociation constant would be the same for the two phases:

$$\frac{[H^+]_i\,[A^-]_i}{[HA]_i} = \frac{[H^+]_o\,[A^-]_o}{[HA]_o} = K_d \qquad (8)$$

$$[H^+]_i[A^-]_i = [H^+]_o[A^-]_o \quad (9)$$

so that

$$\Delta pH = Log_{10}\,\frac{[A^-]_i}{[A^-]_o} \qquad (10)$$

Therefore, in mitochondria, the pH can be readily calculated from the distribution of the weak acid. In practice, only HA + A^- can be determined. Equation (10) can be used readily for these cases (which requires the appropriate pK_A). A more complex equation permits the calculation when the pK_A is too low for Equation (15) to be applicable. For thylakoid vesicles, the inside is acid in relation to the medium, and the analogous technique uses weak bases. For illuminated chloroplasts at steady state, the values calculated by a similar technique suggest a pH as high as 3 to 3.5 (Pick et al., 1974). In fact, little phosphorylation occurs below a pH of 2.5. In effect, this would allow for 8.4 kcal (at pH=3), which would barely suffice to synthesize one ATP if two H+ were transferred. However, it is generally thought that 3 H+ are transferred per ATP synthesized. In contrast, with mitochondria, the pH under physiological conditions depends on the tissue. In rat liver, it is generally approximately 0.5 (Addanki et al., 1968), not enough to play a significant role (although sometimes values as high as 1 have been reported for mitochondria from other tissues).

Calculations of membrane potentials follow a different rationale. In a number of cells, it is possible to estimate the electric potential across the plasma membrane by indirect techniques, using the distribution (i.e., the ratio of concentrations) of a cation completely dissociated at biological pH values. Commonly, these techniques have been applied to mitochondria or chloroplasts. Equation (11) shows the free energy available from the distribution of a monovalent cation per mole transferred.

$$\Delta G = RT \ln \frac{[C^+]_o}{[C^+]_i} + nF\Delta\psi \qquad (11)$$

At equilibrium $\Delta G = 0$, so Eq. (12), the Nernst equation, is applicable.

$$\Delta\psi = RT \ln \frac{[C^+]_i}{[C^+]_o} \qquad (12)$$

Where it is possible to calculate the potential from the distribution of a cation, the procedure assumes that (1) the system is in equilibrium in relation to the cation used as a probe, (2) the ion distribution has not disturbed the system, and (3) the cation has distributed solely as the result of the potential across the membrane. The third item tacitly makes the a priori assumption that no other mechanism is responsible for the distribution.

Presumably, the same reasoning should apply for the thylakoid vesicles. However, in this case the potential across the membrane would be positive inside and an anion would have to be used as a probe. In the case of the thylakoid vesicles, the distribution shows that the potential is negligible in magnitude, at least at steady state.

The predominantly accepted view at this time is that there is a significant membrane potential across the inner mitochondrial membrane. This $\Delta\Psi$ is the major portion of the driving force for the phosphorylation of ADP or the transport of ions. In support of this view, the rate of influx of Ca^{2+} in isolated mitochondria conforms to that predicted from the calculated $\Delta\Psi$ (Gunter and Pfeiffer, 1990). However, this may result from a common transport mechanism for the various cations, sharing the driving force and the energy requirements.

In the case of mitochondria, there are preparations that phosphorylate in the absence of a significant potential (as calculated by the same method). In other preparations, however, it is possible to calculate a substantial membrane potential, as high as -200 mV. However, there is evidence that a process other than the potential itself is responsible for the distribution, that is, a transport system involving an H^+/C^+ stoichiometric exchange (for a review, see Tedeschi, 1981). Microelectrode impalement of giant mitochondria also shows that phosphorylation takes place in these mitochondria in the absence of a significant membrane potential (e.g., Campo et al., 1984). In addition, the protons that have to be pumped out in the absence of ion transport to produce a potential across the membrane of -200 mV can be easily calculated (see Eq. (6) of Chapter 21) to be approximately 1 x 10^{-6} moles per g protein, as done by Mitchell (1966). When checked experimentally, a significant H^+ efflux has never been observed in the absence of ion transport (e.g., Archbold et al., 1979).

Other findings also suggest that the calculated from cation-probe distributions does not have a role in energy coupling. For example, the presence or absence of respiration has no effect on the rate constant of the efflux of cationic probe molecules; it affects only the rate constant of the influx (Skulskii et al., 1983). Both should be affected (in opposite ways) if a membrane potential were responsible for distribution. Furthermore, in at least some experiments, the membrane potential and the protonmotive force measured by conventional means remain essentially the same when both respiration and phosphorylation are decreased in parallel by the use of inhibitors (e.g., Mandolino et al., 1983).

If we discarded the chemiosmotic model, we would be left with a major dilemma: how could we explain the synthesis of ATP in the experiments in which an H^+ flux has been imposed? Several models have now been proposed that depend on localized effects (e.g., see Dilley and Schreiber, 1984; Hong and Junge, 1983; Kagawa, 1984; Westerhoff et al., 1984). In additon, current evidence indicates that protons generated at the surface of a bilayer membrane diffuse laterally (Heberle et al., 1994; Scherrer et al., 1994; Alexiev et al., 1995), suggesting that these localized protons can be directly coupled to the oxidative- or photo-phosphorylation of ADP (see Ferguson, 1995).

Ferguson (1995) suggests that despite a localization of protons, at steady state there would be "an equilibrium with the bulk aqueous phase." This conclusion is highly unlikely under phosphorylative conditions because the proton flux generated by electron transport in one direction is likely to be be matched by the flux of the protons responsible for the phosphorylation in the opposite direction. In contrast, we would expect an equilibration in the absence of oxidative phosphorylation. This dichotomy would provide a switch between oxidative-phosphorylation (localized mechanism) and cation transport (supported by a membrane potential). Although this latter possibility would seem extremely attractive, it should be noted that we have also demonstrated in giant mitochondria, the accumulation of Ca^{2+} in the absence of a membrane potential (Maloff et al. 1978); there is also evidence that the transport of cations occurs electroneutrally by a stoichiometric exchange with protons (see Tedeschi, 1981).

Fortunately, the same molecular mechanisms can be invoked as long as a role of H^+ is postulated. The electron transport chain could deliver H^+ to the ATP synthetase either in the presence or absence of a conventional protomotive force. Fig. 9 (Senior, 1979) illustrates a model that can operate either with or without a proton gradient. The proton directly delivered to the ATP synthase (step I) produces the release of ATP (by changing the conformation and hence the dissociation constant) in step II. This apparently corresponds to the energy-requiring step. The simultaneous attachment of ADP and P_i (step II) results in the release of H^+ (step III), presumably by decreasing the binding constant for H^+. The synthesis of bound ATP is shown in step IV.

There is considerable evidence that the formation of bound ATP does not require a significant amount of energy (Boyer et al., 1973; Feldman and Sigman, 1982, 1983). Bound ATP, (ATP_b), can be present in the presence of an uncoupler (Boyer et al., 1973; Feldman and Sigman, 1983) and furthermore, in preparations of F_1 alone, at very low concentrations, the equilibrium constant for $[(ATP)_b]/[ADP][P_i]$ is approximately 0.6, corresponding to a ΔG of 0.3 kcal. Similarly, there is evidence for conformational changes of ATP synthase dependent on energy input (e.g., Gogol et al., 1989a, 1989b, 1990) or the presence of ATP (Dilley and Schreiber, 1984), and these changes result in different binding constants of nucleotides. The synthesis of ATP by isolated ATP synthase induced by a shift to an alkaline medium, supports a conformational model (Blumenfeld et al., 1987), although not the details of Fig. 9.

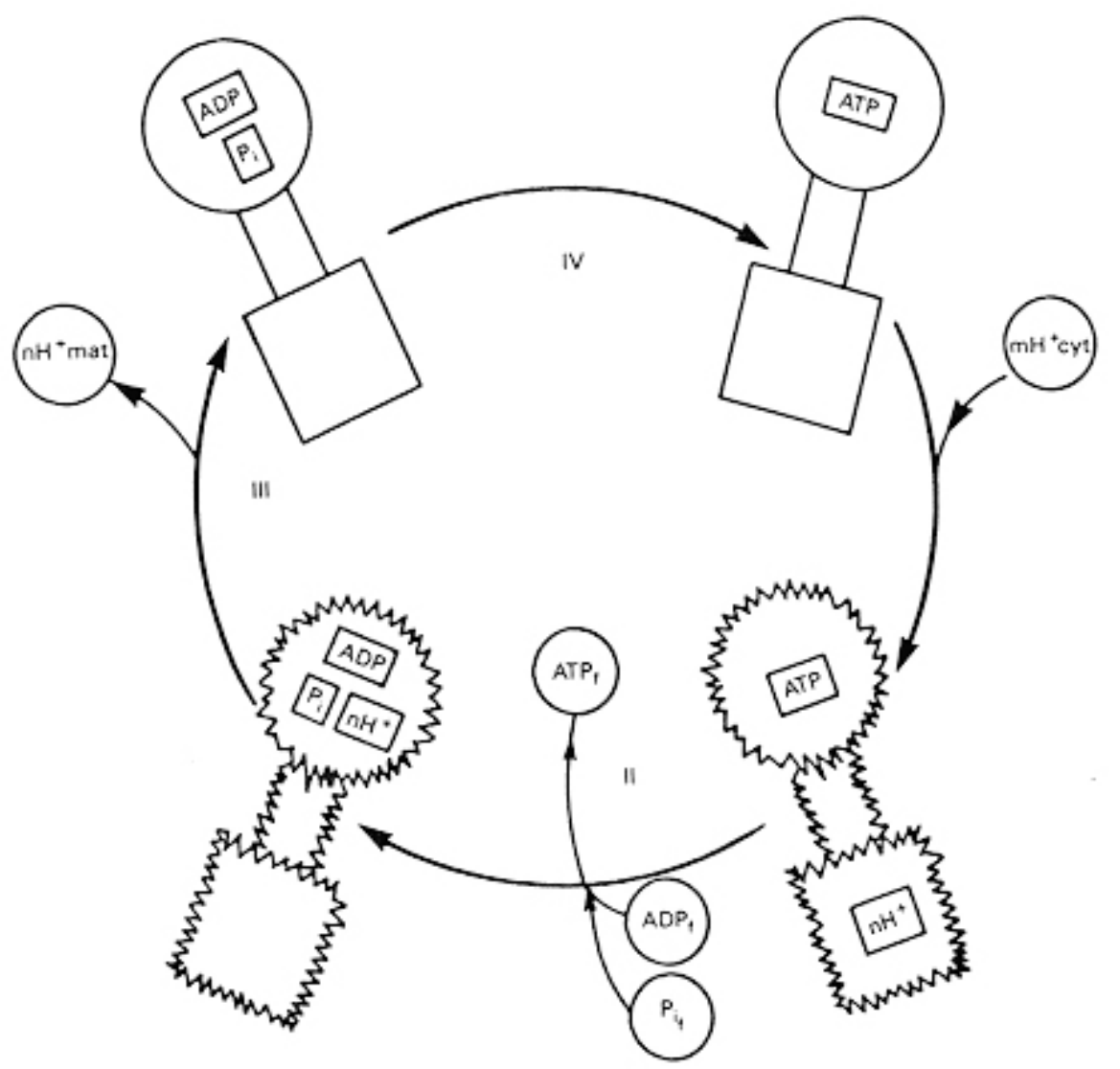


Fig. 9 The conformational model: *Cyt*, cytoplasm; *mat*, mitochondrial matrix. Reproduced from Senior (1979), by courtesy of Marcel Dekker, Inc.

IV. THE ATP SYNTHASE

The ATP synthase or proton-ATPase, F_0F_1, is a complex of many polypeptides and can function either forward in oxidative phosphorylation or photophosphorylation or backward, i.e., to hydrolyze ATP.

A. Structure of F_0F_1

Present understanding of the structure of ATP synthase of *Escherichia coli* is shown in the model depicted in Fig. 10 (Junge et al., 1997). The figure incorporates data from many biophysical approaches. In order to show the arrangement in some detail, the front α and β subunits are not shown in the figure (the αβ complex is actually a hexamer). It includes the water-soluble portion (F_1) and the components associated with the inner mitochondrial membrane (F_o). The ATP synthase subunits are highly conserved in organisms ranging from *E. coli* and thermophilic bacteria to the so-called higher eukaryotes including the

synthase of chloroplasts. In addition, there is a high degree of homology between subunits α and β that contain the catalytic site. The mechanism of action of the ATP synthases of the various organisms is probably identical, since recombination of the various components from different sources produces a hybrid complex that is functional.

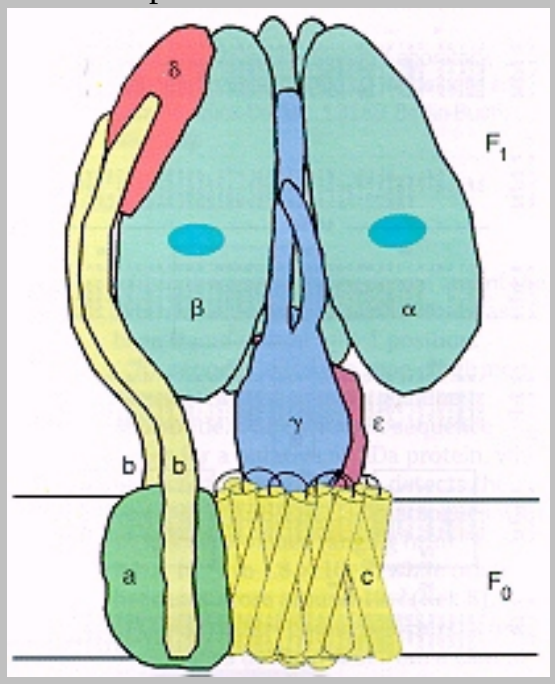


Fig. 10 Model of the general structure of ATP synthase from E. Coli. The F_o portion represents integral and proton-conducting proteins. The components of F_1 contain the nucleotide binding sites. The stator portion (see text) includes *a* , *b*, δ and $(\alpha\ \beta)_3$. The rotor portion includes c_n, ε and γ. From Junge et al., 1997 by permission.

The representation differs from previous versions by the presence of a double stalk, one including γ and ε, components of F_1, and the other including b_2, a component of F_o, in close contact with δ (a subunit of F_1). The γ portion has been shown to rotate in relation to the $(\alpha\beta)_3$ complex. As discussed later, the assembly of a, b, δ and the complex of $(\alpha\beta)_3$ acts as a *stator*, that is the stationary part of a machine. In contrast, *c*, γ and ε correspond to the *rotor* of the motor (e.g. see Junge et al., 1997; Elston et al., 1998).

The polypeptide composition of F_1 is listed in Table 4 for *E. coli* and beef heart mitochondria (Senior, 1988). With the exception of ε from beef heart mitochondria, the subunits from the two different systems appear to be very similar. However, the nomenclature, i.e., the Greek letter denoting each polypeptide, differs. The components are present with the stoichiometry $(\alpha\beta)_3\ \gamma\delta\ \varepsilon$. The composition of the F_o portion is shown in Table 5 (Senior, 1988).

The *E. coli* F_o proteins *a*, *b* and *c* are present in the stoichiometry $a_1b_2c_n$, where n has been estimated to be between 10 and 12. Many other components have been shown in mitochondria: factor B, 11-15 kDa; F_6, 8-9 kDa; subunit 8,6-8 kDa; an uncoupler binding protein, 30 kDa; and the inhibitor polypeptide, 9.6 kDa. The inhibitor peptide probably functions in blocking the ATPase activity in favor of the synthase activity. Subunit 6 of mitochondrial F_o of 25 to 29 kDa is homologous to subunit *a* of *E. coli*, whereas subunit 9, an

8-kDa proteolipid, is homologous to subunit *c*.

Table 4 Subunit Composition of *E coli* and Beef Heart Mitochondrial F_1

	E. coli	Beef Heart mitochondria
Subunit composition	$\alpha_3\beta_3\gamma\delta\varepsilon$	$\alpha_3\beta_3\gamma\delta\varepsilon$
Total molecular size	381 kDa	371 kDa
α subunit		
No. of residues	513	509
molecular size	55.2 kDa	55.2 kDa
β subunit		
No. of residues	459	480
molecular size	50.15 kDa	51.6 kDa
γ subunit		
No. of residues	286	272
molecular size	31.43 kDa	30.14 kDa
δ subunit		
No. of residues	177	190
molecular size	19.33 kDa	21 kDa
ε subunit/ δ subunit[a]		
No. of residues	138	146
molecular size	14.92 kDa	15.1 kDa
ε subunit		
No. of residues		50
molecular size		5.65 kDa

Reproduced by permission from A.E. Senior, *Physiological Reviews* 68:177-231. Copyright &copy1988, American Physiological Society.

[a]Note that in *E.. coli* is analogous to oligomycin sensitive conferral protein, OSCP, *E. coli* is analogous to mitochondrial and mitochondrial has not counterpart in *E. coli*

Table 5 Subunits of F_o

	E. coli	Beef heart mitochondria
Subunit ***a***		
No. of residues	271	226
Molecular size	30.3 kDa	24.8 kDa
Subunit ***b***		
No. of residues	156	
Molecular size	17.2 kDa	
Subunit ***c***		
No. of residues	79	75
Molecular size	8.3 kDa	7.4 kDa
A6L/aapl		
No. of residues	Not present	66
Molecular size		8.0kDa
Other subunits	Not present	Likely
Stoichiometry	$a_1b_2c_n$	Unknown (c_n)

Reproduced by permission from A.E. Senior, *Physiological Reviews*, 68:177-231. Copyright &copy1988 the American Physiological Society.

B. Catalytic Activity of F_1

Each F_1 complex contains six nucleotide binding sites. Three of these exchange rapidly when ATP is added to the medium, and are presumed to be catalytic sites. The other three exchange only slowly, and might be regulatory sites. In fact, occupancy of the presumed noncatalytic sites by ATP is not required for ATPase-catalysis (Weber et al., 1994). The three catalytic sites of the complex function in a highly cooperative fashion. Functioning as an ATPase, the rate of hydrolysis at any one of the three sites is slow, but it is accelerated by several orders of magnitude by binding to a second site (see Senior, 1988; Boyer, 1993).

Binding studies with isolated F_1 show that each α or β subunit binds ATP or ADP. The nucleotides of each subunit exchange rapidly with those in the medium. Affinity labeling of F_1 with nucleotide analogs labels mostly β, although there is a suggestion that α may also be involved, perhaps at the interface between the two.

Binding affinity for substrates and products at one catalytic site was found to be profoundly affected by binding at a second site by ADP aid P_i, which appeared to be needed for net ATP synthesis or release at the first site.

The model presently favored proposes that ADP and P_i tightly bound at one catalytic site can form ATP. However, binding of ADP and P_i to a second catalytic site is obligatory for release of ATP. As discussed previously, the energy for the release would come from the proton gradient. The model involves *alternating* catalytic sites; each β subunit acts in the binding of ADP and P_i, in ATP synthesis and in its release, alternately. Fig. 11 (Senior, 1988) incorporates these features in a model in which three alternating sites are present (see Cross, 1981). In this respect, it is of considerable interest that hybrid F_1 containing one inactive β subunit along with two normal β subunits is inactive.

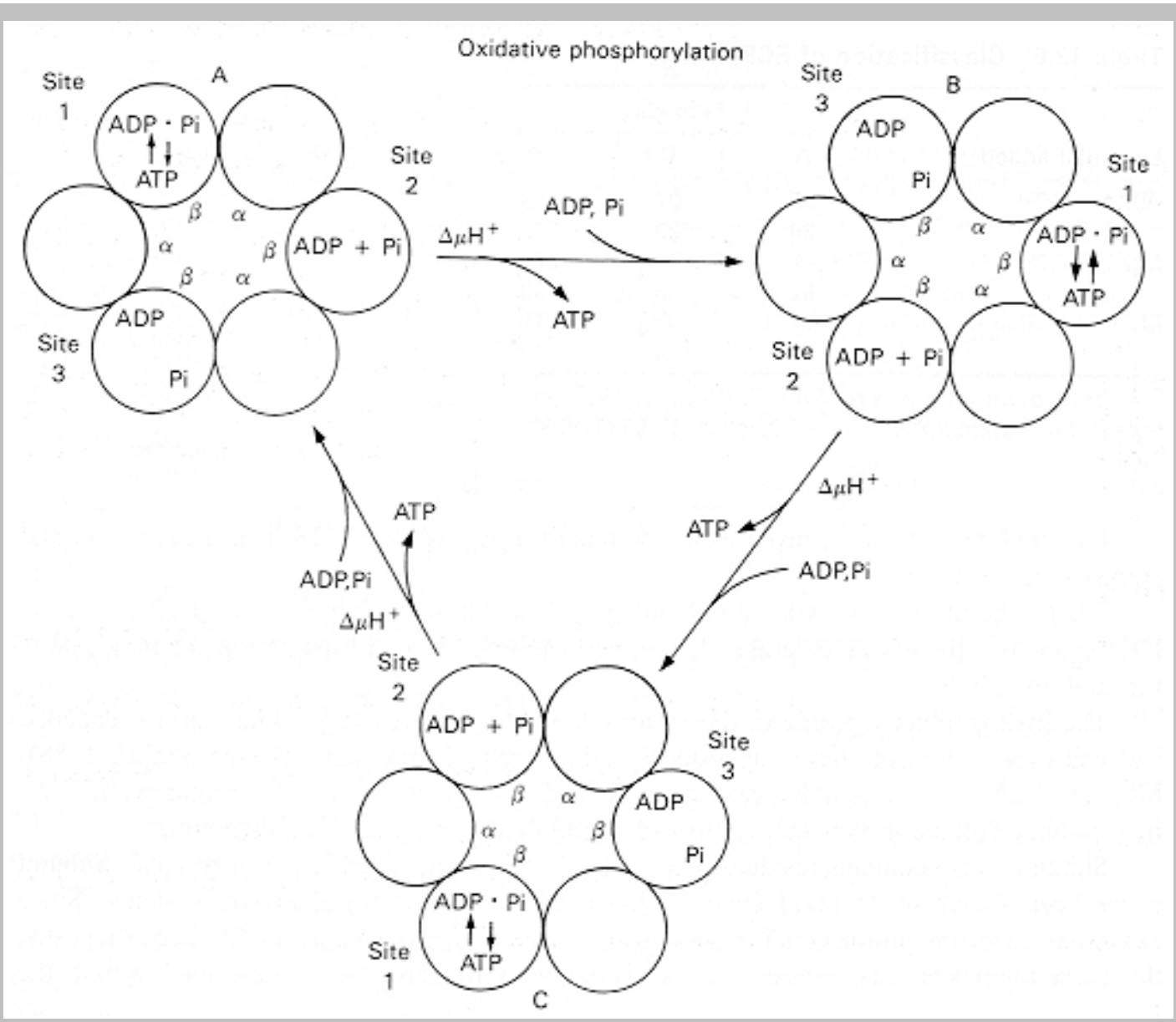


Fig. 11 ATP synthesis: proposed cyclical mechanism using all three catalytic sites of F_1. (A) catalytic site 1 is a high-affinity site, undergoing reversible synthesis of ATP from tightly bound ADP + P_i Site 2 is a catalytic site of intermediate affinity, which must be occupied by ADP + P_i, and site 3 is a loose catalytic site. Energy from the proton gradient causes synchronous binding affinity changes at each site to give state B. (B) Site 1 has become lowest-affinity site, number 3, and ATP has been released and replaced by ADP and P_i from the medium. Site 2 has become high-affinity site, number 1, and reversible ATP synthesis has commenced. Site 3 has become an intermediate-affinity site, now number 2. Another proton gradient-induced binding-affinity change will release ATP and switch sites again to give the situation in state C. Reproduced from Senior (1988), with permission.

NMR, crosslinking data (see Engelbrecht and Junge, 1997) and EM studies (Wilkens et al., 1997; Wilkens and Capaldi, 1998) together with the crystallographic information discussed below, suggest the structure for F_oF_1 shown in Fig.10.

The structure of F_1 has been revealed by X-ray crystallography in three distinct configurations (Abrahams

et al., 1994) at a 2.8 resolution. One corresponded to the empty site, the other was filled by AMP-PNP, a non-hydrolyzable ATP analog, and one with ADP. These three configurations represent snapshots of the rotary mechanism of catalysis and suggest that the γ-subunit moves like a crankshaft, coupled to the opening and closing of the catalytic-binding sites of F_1.

The rotational hypothesis was supported by several experiments. Reversible disulfide cross-links between engineered β and γ subunits showed that ATP hydrolysis switched the β-γ partners (see Duncan et al., 1995; Zhou et al., 1996; Zhou et al., 1997). The rotation was also demonstrated with eosin-linked to γ in CF_1 immobilized in the $(\alpha\beta)_3$ portion. The polarization anisotropy relaxed with ATP hydrolysis, indicating a rotation (Sabbert et al., 1996; 1997).

The occurrence of a rotation in individual molecular assemblies was demonstrated dramatically in recombinant F_1 of thermophilic bacteria (Noji et al., 1997). Noji et al., observed the rotation induced by Mg-ATP after attaching fluorescently labelled actin fibers to the γ-subunit. The $(\alpha\beta)_2$ of F_1 was immobilized. The rotation was counter-clockwise (viewed from the membrane side). The three lines of evidence are summarized in Fig. 12.

More details on the rotational movement of the ATP-synthase were provided by experiments following the same experimental design as that of Noji et al. (1997). In this case (Sambongi et al., 1999), the fluorescently labelled actin filament was attached to a subunit of *c* bound to the components of F_1. The *c* oligomer was found to rotate using ATP-hydrolysis as the source of energy and generating approximately the same amount of force as shown for the γ subunit in the earlier experiments. Considering the structural data that show a close association of the *c* oligomer to the γ and δ subunits of F_1 (e.g., Stock et al., 1999), the results indicate that the c-oligomer and the γ subunit of F_1 are attached and rotate together.

Studies of the rotation of the γ subunit of ATP-synthase at lower ATP concentrations, provided some significant information (Yasuda et al., 1998). This study found that the actin tag attached to the γ subunit moves in discrete 120º steps. Yasuda et al., calculate that the work carried out in each step is very close to the energy available from the hydrolysis of 1 ATP molecule (implying close to 100% efficiency). The energy expended by the work was calculated from the equation of Hunt et al. (1994) to estimate frictional drag. The energy available from the hydrolysis of 1 ATP was assumed to be approximately 80 pN.nm (corresponding to approximately -11.5 kcal; 1 N = 10^5 dynes) and approximately the same as the energy required for one step of the rotation. The actual numbers can only be considered approximate. However there is no doubt that the efficiency of the ATP-synthase operating as a motor is extremely high. This is not too surprising for a reversible pump. In fact, rough calculations for another ion transport pump, the Na^+, K^+-ATPase, while operating in transport, suggest an efficiency as high as 80% (see Chapter 12 and Tedeschi and Kinnally, 1992). When a small gold bead is attached to the γ as a marker (rather than actin, to reduce frictional drag) and high speed photography is used, the 120º steps can be subdivided into substeps of approximately 90º and 30 º (Yasuda et al., 2001). The beads were observed either with transmission light microscopy or with laser dark-field microscopy (i.e., from the light scattered by the

beads) . The first phase is likely to correspond to the binding of ATP, and the second to the hydrolysis step. It is interesting to note that the movement is induced by ATP binding (see discussion in Schnitzer, 2001). Other movements that use ATP binding are the stepping motion of kinesin (see Vale and Milligan, 2000) and the chaperone GroEl . For the latter, binding to one ring structure induces the opening of the other ring structure releasing the folded protein (see Sigler et al., 1998).

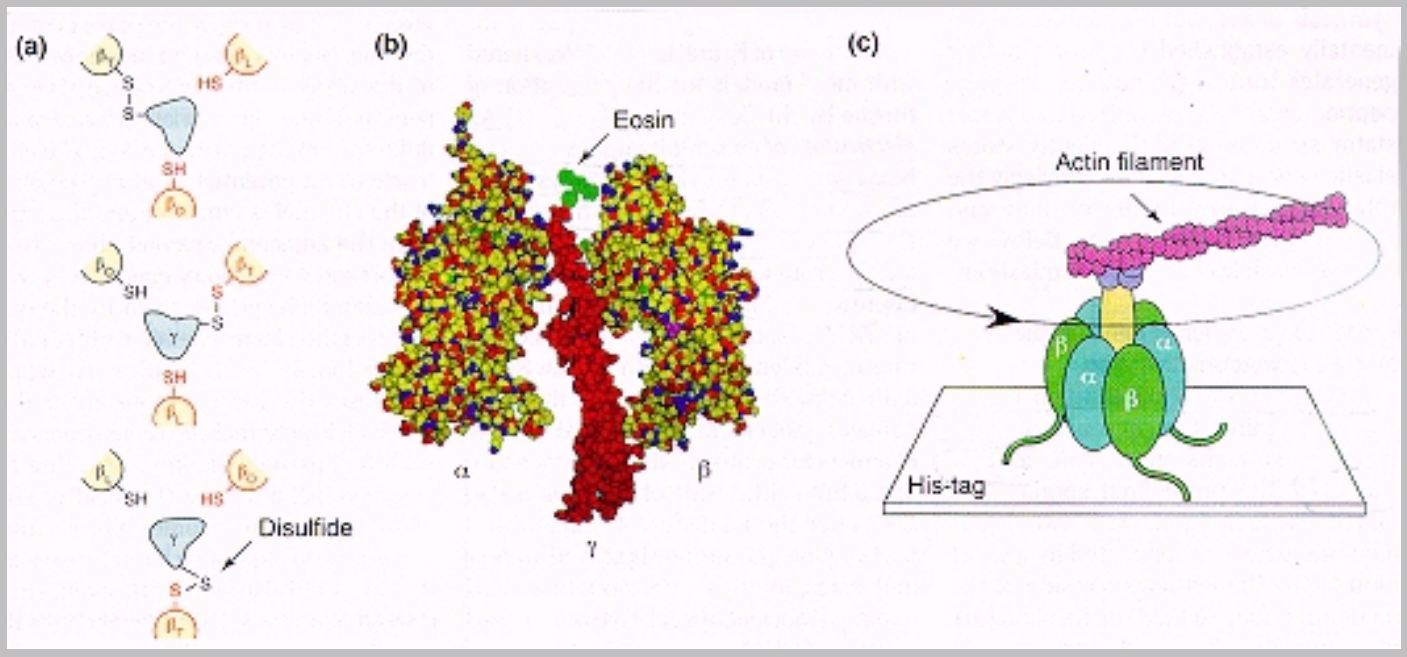


Fig. 12. Illustration of the techniques used to demonstrate rotation of γ in relation to $(\alpha\beta)_3$ during the hydrolysis of ATP by F_1. (a) Schematic diagram showing cleavage and reformation of disulfide bridges between engineered γ and β during ATP hydrolysis, producing new γ-β pairing that could occur only with rotation. (b) Polarized absorption recovery of eosin after photobleaching on immobilized $(\alpha\beta)_3$ showing large scale rotation. (c)Fluorescence microscopy showing movement of a fluorescent actin filament attached γ recorded by video. From Junge et al., 1997. Reproduced by permission.

The question remains of how to couple proton movement and rotation. In *Escherichia coli*, the proteolipid subunit *c* is folded like a hairpin where two transmembrane α-helices, separated by a short loop, face the cytoplasmic side of the membrane (Girvin and Fillingame, 1994; Girvin et al., 1998). These subunits are present in multiple copies probably forming a ring as suggested by images on the EM (Birkenhäger et al., 1995) and atomic force microscope (Singh et al., 1996). Two monomers of *c* are thought to associate to form a dimer where the flattened front surface of one unit interacts with the flattened back surface of the other. Part of the evidence was derived from cross-linking with doubly Cys-substituted variant of the *c* subunit (Jones et al., 1998). Data from extensive cross-linking indicate 12 subunits per *c* oligomer(Jones and Fillingame, 1998). However, X-ray crystallography suggests 10 (Stock et al., 1999). In the oligomer, the α helices form two concentric rings with the amino- and carboxy-terminals located in the inner or outer ring, respectively (Dmitriev et al., 1999). In a model of 12 subunits, the oligomer forms a hollow cylinder with an outer diameter of 55-60 Å and an internal diameter of 11 Å. The Asp-61 residue, corresponding to the H^+-transport residue, is in center of four transmembrane helices of two interacting subunits. Subunits *a*

and *b* are outside the *c* oligomer. Subunit *a* consists of as many as five transmembrane helices and subunit *b* has a single transmembrane stretch and a long hydrophilic head (Fillingame, 1996). A model for the *a* and *c* of F_o is shown in Fig. 13.

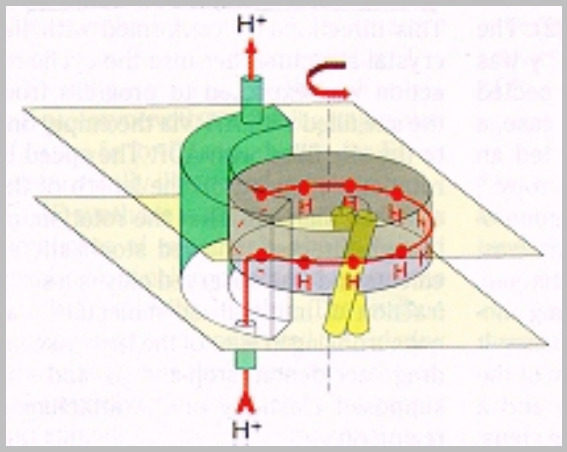


Fig. 13. Model for the generation of torque in F_o driven by protonmotive force. Subunit *c* proteolipid subunits form a ring. Subunit *a* has two proteon access channels for protons on either side of the membrane. From Junge et al., 1997. Reproduced by permission.

A possible mechanism would require that: (a) the carboxyl groups in the *c*-ring (corresponding to Asp 61 of subunit *c*) must be protonated and electroneutral when they face the lipid core, (b) when facing subunit *a*, they can be deprotonated and charged and (c) random Brownian motions become directed when constrained by requirements (a) and (b) (Junge et al., 1997; see also Junge, 1999; Dimroth et al., 1999). As shown in Fig. 13, the subunits of the ring can move only if a proton is bound. When a charged group of *c* facing *a* hits the boundary with the lipid, it is reflected back. When the lower channel of *a* is more acidic than the upper channel, the ring will turn clockwise (from the bottom). It will turn counter-clockwise when the gradient is reversed. The model proposed by Elston et al. (1998) is very similar. It proposes an important role for Arg 210 in the *a* subunit by contributing to the the deprotonation of the carboxyl group when the movement proceeds in the unfavorable direction. Both Asp 61 (Dmitriev et al., 1995) in each *c* subunit and Arg 210 in the single *a* subunit (Vik and Antonio, 1994; Valiyaveetil and Fillingame, 1997) are essential for proton translocation as revealed by mutagenesis.

However, there are indications that the deprotonation of Arg 61 induces a conformational change in the *c* subunit, a 140º rotation of the carboxy-terminal helix with respect to the amino-terminal helix (Rastogi and Girvin, 1999). This rotation drives the movement of the γ-portion. Two possible mechanisms are suggested by Rastogi and Girvin (1999). The *a* subunit may move with the carboxy terminal helix of the *c* subunit. This would produce a 30º rotation of the ring with respect to the *a* subunit. An alternative model proposes the movement of a negatively charged "proton hole" which traverses the ring until it arrives at a ε subunit, which moves to an adjacent *c* subunit, providing a 30º rotation of the ε-γ stalk.

SUGGESTED READING

Overall view

Saraste, M.(1999) Oxidative phosphorylation at the *fin de siècle*, *Science* 283:1488-1493. (Medline)

Boyer, P.D. (1999) What makes ATP synthase spin? *Nature* 402:247-249. (Medline)

Capaldi, R.A. and Aggeler, R. (2002) Mechanism of the F_1F_0-type ATP synthase, a biological rotary motor, *Trends Biochem. Sci.* 27:154-160. (MedLine)

Others

Boyer, P. D. (1989) A perspective of the binding change mechanism for ATP synthesis, *FASEB J.* 3:2l64-2178. (Medline)

Cross, R.L. (1994) Our primary source of ATP, *Nature* 370:594. (Medline)

Cramer, W. A., and Knaff, D. B. (1990) *Energy Transduction in Biological Membranes*, Chapter 8. Springer-Verlag, New York.

Nakamoto, R.K. (1999) Molecular features of energy coupling in the F_oF_1 ATP synthase, *News Physiol. Sci.* 14:40-46.

Nicholls, D.G. and Ferguson, S.J. (1992) *Bioenergetics 2*, Academic Press, New York.

Penefsky, H. S., and Cross, R. L. (1991) Structure and mechanisms of F_0F_1 type ATP synthases and ATPases. *Adv. Enzymology*, 64:173-214. (Medline)

Senior, A. E. (1988) ATP synthesis by oxidative phosphorylation, *Physiol. Rev.* 68:177-231. (Medline)

Senior, A. E. (1990) The Proton-translocating ATPase of *Escherichia coli*, *Annu. Rev. Biophys. Biophys. Chem*. 19:7-41. (Medline)

Tzagaloff, A. (1982) *Mitochondria*, Chapters 6-9. Plenum Press, New York.

Special Aspects

Ferguson, S.J. (1995) Protons fast and slow, *Current Biol.* 5:25-27. (Medline)

Kagawa, Y. (1984) Proton motive ATP synthesis. In *Bioenergetics* (Ernster. L., ed.), pp. 149-186. Elsevier. New York.

Tedeschi. H. (1981) The transport of cations in mitochondria, *Biochim. Biophys. Acta* 639:157-196; see

pp. 162-170. (Medline)

Weber, G. (1972) Addition of chemical and osmotic energies by ligand protein interactions, *Proc. Natl. Acad. Sci. U.S.A.* 69:3000-3003. (Medline)

WEB RESOURCES

Noji, H., Yasuda, R., Yoshida, M. and Kinosita Jr, K. Direct observation of the rotation of F_1-ATPase
Movie 1
Movie 2

Diwan, J.J. Oxidative phosphorylation: chemiosmotic coupling,
http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/oxphos.htm

Oxidative phosphorylation: F1Fo ATPase,
http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/f1fo.htm

REFERENCES

Search the textbook

Search the textbook

Back to Chapter 18

REFERENCES

Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Structure at 2.8 resolution of F_1-ATPase from bovine heart mitochondria, *Nature* 370:621-628.(Medline)

Addanki, S., Dallas, F., Cahill, F. S., and Sotos, J. F. (1968) Determinations of intramitochondrial pH and intramitochondrial extramitochondrial pH gradient of isolated heart mitochondria by the use of 5,5-dimethyl 1-2,4 oxazolinedione, *J. Biol. Chem.* 243:2337-2348.

Alexiev, U., Mollaaghaba, R., Scherrer, P., Khorana, H.G. and Heyn, M.P. (1995) Rapid long-range proton diffusion along the surface of the purple membrane and delayed proton transfer into the bulk, *Proc. Natl. Acad, Sci. USA* 92:372-376.(Medline)

Archbold, G. P. R., Farrington, C. L. Lapping S. A., McKay, A. M., and Malpress, F. H. (1979) Oxygen pulse curves in rat liver mitochondrial suspensions: Some observations and deductions, *Biochem. J.* 180:161-174.(Medline)

Birkenhäger, R., Hoopert, M. Deckers-Hebestreit, G., Mayer, I., and Altendorf, K.(1995) The F_o complex of *Escherichia coli* ATP synthase. Investigation by electron spectrocopic imaging and immunoelectron microscopy, *Eur. J. Biochem.* 230:58-67.(Medline)

Blumenfeld, M., Goldfeld, G., Mikoyan, V. D., and Soloyev, I. S. (1987) ATP synthesis by isolated coupling factors from chloroplasts during acidic and alkaline pH shifts. [translated in *Mol. Biol.* 21:268-274 (1987)] *Molekulyarnaya Biologiya* 21:323-329.

Boyer, P.D. (1993) The binding change mechanism for ATP-synthase - some probabilities and possibilities, *Biochim. Biophys. Acta* 11440:215-250.(Medline)

Boyer, P.D., Cross, R.L., and Momsen, W. (1973) A new concept in energy coupling in oxidative phosphorylation based on a molecular explanation of the oxygen exchange reactions, *Proc. Natl. Acad. Sci. USA* 70:2837-2839.(Medline)

Brierley, G. P., and Murer, E. (1964) Ion accumulation in heart mitochondria supported by reduced cytochrome *c., Biochem. Biophs. Res. Commun.* 14:437-442.(Medline)

Brierley, G. P., Bachman, E., and Green, D. E. (1962) Active transport of inorganic phosphate and magnesium ions by beef heart mitochondria, *Proc. Natl. Acad. Sci. U.S.A.* 48:1928-1935.

Campo, M. L., Bowman, C. L., and Tedeschi, H. (1984) Assays of ATP synthesis using single giant mitochondria, *Eur. J. Biochem.* 141:1-4.(Medline)

Capaldi, R.A., Ageler, R., Turina, P. and Wilkens, S. (1994) Coupling between catalytic sites and the proton channel in the F_1F_o-ATPases, *Trends in Biochem Scie.* 19:284-289.

Chance, B. (1965) The energy-linked reaction of calcium with mitochondria, *J. Biol. Chem.* 240:2729-2748.

Chance, B., and Hollunger, G. (1961) The interaction of energy and electron transfer reactions in mitochondria. VI. The efficiency of the reaction, *J. Biol. Chem.* 236:1577-1584.

Chance, B., and Williams, G. R. (1956) The respiratory chain and oxidative phosphorylation, *Adv. Enzymol.* 17:65-134.

Copenhaver, J. H., Jr., and Lardy, H. A. (1952) Oxidative phosphorylation: pathways and yield in mitochondrial preparations, *J. Biol. Chem.* 195:225-238.

Cramer, W. A., and Knaff, D. B. (1990), *Energy Transduction in Biological Membranes,* Springer-Verlag, New York.

Cross, R. L. (1981) The mechanism and regulation of ATP synthesis by F_1F_0-ATPases, *Annu. Rev. Biochem.* 50:681-714.(Medline)

Dilley, R. A., and Schreiber, U. (1984) Correlation between membrane-localized protons and flash-driven ATP formation in chloroplast thylakoids, *J. Bioenerg. Biomembr.* 16:173-193.(Medline)

Dimroth, P., Wang, H., Grabe, M. and Oster, G. (1999) Energy transduction in the sodium F-ATPase of *Propionigenium modestum*, *Proc. Natl. Acad. Sci. USA* 96:4924-4929.(Medline)

Dmitriev, O.Y., Altendorf, K. and Fillingame, R.H. (1995) Reconstitution of the F_o complex of *Escherichia coli* ATP synthase from isolated subunits: varying the number of essential carboxylates by co-incorporation of wild-type and mutant subunit *c* after purificationon organic solvents, *Eur. J. Biochem.* 233:478-483.(Medline)

Dmitriev, O.Y., Jones, P.C. and Fillingame, R.H. (1999) Structure of the subunit c oligomer in the F_1F_o ATP synthase: model derived from solution structure of the monomer and cross-linking in the native

enzyme, *Proc. Natl. Acad. Sci. USA* 96:7785-7790.(Medline)

Duncan, T.M., Bulygin, V.V., Zhou, Y., Hutcheon, M.L. and Cross, R.L. (1995) Rotation of subunits during catalysis by *Escherichia coli* F_o1-ATPase, *Proc. Natl. Acad. Sci. USA* 92:10964-10968.(Medline)

Engelbrecht, S., and Junge, W. (1997) ATP synthase: a tentative structural model, *FEBS Lett.* 414:485-491.(Medline)

Elston, T., Wang, H. and Oster, G. (1998) Energy transduction in ATP synthase, Nature 391:510-513.(Medline)

Feldman, R.I., and Sigman, D.S. (1982) The synthesis of enzyme bound ATP by soluble chloroplast factor 1, *J. Biol. Chem.* 257:1676-1683.(Medline)

Feldman, R.I., and Sigman, D.S. (1983) The synthesis of ATP by membrane bound ATP synthase complex from medium P under completely uncoupled conditions, *J. Biol. Chem.* 258:12178-12183.(Medline)

Ferguson, S.J. (1995) Protons fast and slow, *Current Biol.* 5:25-27.(Medline)

Fillingame, R.H. (1996) Membrane sectors of F- and V-type H+-transporting ATPases, *Curr. Opin. Struct. Biol.* 6:491-498.

Girvin, M.E. and Fillingame, R.H. (1994) Hairpin folding of subunit c of F_1F_o ATP synthase: ^1H distance measurements to nitroxide-derivatize aspartyl-61, *Biochemistry* 33:665-674.(Medline)

Girvin, M.E., Rastogi, V.K., Abildgaard, F., Markley, J.L. and Fillingame, R.H. (1998) Solution structure of the transmembrane H^+-transporting subunit c of the F_1F_0 ATP synthase, *Biochemistry* 37:8817-8824.(Medline)

Gogol, E. P., Aggeler, R., Sagermann M., and Capaldi, R. A. (1989a) Cryoelectron microscopy of *Escherichi coli* F_1 Adenosinetriphosphatase decorated with monoclonal antibodies to individual subunits of the complex, *Biochem.* 28:4717-4724.(Medline)

Gogol, E. P., Lücken, U., Bork, T., and Capaldi, R. A. (1989b) Molecular architecture of *Escherichia coli* F_1 Adenosinetriphosphatase, *Biochem.* 28:4709-4716.(Medline)

Gogol, E. P., Johnston, E., Aggeler, R., and Capaldi, R. A. (1990) Ligand-dependent structural variation in *Eschierichia coli* F_1 ATPase revealed by cryoelectron microscopy, *Proc. Natl. Acad. Sci.* 87:9585-

9589.(Medline)

Gunter, T.E. and Pfeiffer, D.R.(1990) Mechanisms by which mitochondria transport calcium, *Am. J. Physiol.* 258 *(Cell Physiol.* 27): C755-786.(Medline)

Hall, D. O. (1976) The coupling of photophosphorylation to electron transport in isolated chloroplasts. In *The Intact Chloroplast* (Barber, J., ed.), pp. 135-170. Elsevier, New York.

Heberle, J. Riesle, J. Thiedemann, G., Oesterhelt, D. and Dencher, N.A., (1994) Proton migration along the membrane surface and retarded surface to bulk transfer, *Nature* **370**, 379-382.(Medline)

Hinkle, P.C., Kumar, M.A., Resetar, A. and Harris, D.L. (1991) Mechanistic stoichiometry of mitochondrial oxidative phosphorylation, *Biochemistry* 30:3576-3582.(Medline)

Hong, Y. Q., and Junge, W. (1983) Localized or delocalized protons in photophosphorylation. On the accessibility of the thylakoid lumen for ions and buffers, *Biochim. Biophys. Acta* 722:197-208.

Horton, A. A., and Hall, D. O. (1968) Determining stoichiometry of photosynthetic phosphorylation, *Nature* 218:386-388.

Hunt, A.J., Gittes, F., Howard, J. (1994) The force exerted by a single kinesin molecule against a viscous load, *Biophys. J.* 67:766-781.(Medline)

Jagendorf, A. T., and Uribe, E. (1966) ATP formation caused by acid-base transition of spinach chloroplasts, *Proc. Natl. Acad. Sci. U.S.A.* 55:170-177.(Medline)

Jones, P.C. and Fillingame, R.H. (1998) Genetic fusions of subunit c in the F_0 sector of H^+ transporting ATP synthase. Functional dimers and trimers and determination of stoichiometry by cross-linking analysis, *J. Biol. Chem.* 273:29701-29705.(Medline)

Jones, P.C., Jiang, W. and Fillingame, R.H. (1998) Arrangement of the multicopy H^+ -translocating subunit c in the membrane sector of the *Escherichia coli* F_1F_0 ATP synthase, *J. Biol. Chem.* 273:17178-17185.(Medline)

Junge, W. (1999) ATP synthase and other motor proteins, *Proc. Natl. Acad. Sci. USA* 96:4735-4737.(Medline)

Junge, W., Lill, H. and Engelbrecht, S. (1997) ATP synthase: an electrochemical transducer with rotary mechanics, *Trends in Biochem. Scie.* 22:420-423.(Medline)

Kagawa, Y. (1984) A new model of proton motive ATP synthesis: acid-base cluster hypothesis, *J. Biochem.*95:295-298.(Medline)

Lee, C.P., Gu, Q., Xiong, Y., Mitchell, R.A. and Ernster, L. (1996) P/O ratio reassesed: mitochondrial P/O ratios consistently exceed 1.5 with succinate and 2.5 with NAD-linked substrates, *FASEB J.*10:345-350.(Medline)

Lehninger, A. L. (1955) Oxidative phosphorylation, *Harvey Lect.* 49:176-215.

Malenkova, I. V., Kuprin, S. P., Davydov, R. M. and Blumenfeld, L. A. (1982) pH-jump-induced ADP phosphorylation in mitochondria, *Biochim. Biophys. Acta* 682:179-183.(Medline)

Maloff, B.L., Scordilis, S.P. and Tedeschi, H. (1978) Assays of the viability of single giant mitochondria: experiments with intact and impaled mitochondria, *J. Cell Biol.* 78:214-226.(Medline)

Mandolino, G., De Santis, A., and Melandri, B. A. (1983) Localized coupling in oxidative phosphorylation by mitochondria from Jerusalem artichoke (*Helianthus tuberosus*), *Biochim. Biophys. Acta* 723:428-439.

Mitchell, P. (1966) Chemiosmotic coupling in oxidative and photosynthetic phosphorylation, *Biol. Rev.* 41:445-502.(Medline)

Mitchell, P. (1967) Proton-translocation phophorylation in mitochondria, chloroplasts and bacteria-natural fuel cells and solar cells, *Fed. Proc.* 26:1370-1379.(Medline)

Nicholls, D.G. and Ferguson, S.J. (1992) *Bioenergetics 2*, Academic Press, New York.

Noji, H., Yasuda, R., Yosida, M. and Kinosita, K., Jr. (1997) Direct observation of the rotation of F_1-ATPase, *Nature* 386:299-302.(Medline)

Penniston, J. T., Zande, H. V., and Green, D. E. (1966) Mitochondrial particles resolved for ion translocation. I. Preparation and properties of a particle coupled only at the phosphorylation site III of the electron transfer chain, *Arch. Biochem. Biophys.* 113:507-511.(Medline)

Pick, U., Rottenberg, H., and Avron, M. (1974) The dependence of photophosphorylation in chloroplast on pH and external pH, *FEBS Lett.* 48:32-36.(Medline)

Rastogi, V.K. and Girvin, M.E. (1999) Structural changes linked to proton translocation by subunit c of the ATP synthase, *Nature* 402:263-268.(Medline)

Sabbert, D., Engelbrecht, S. and Junge, W. (1996) Intersubunit rotation in active F-ATPase, *Nature* 381:623-625.(Medline)

Sabbert, D., Englebrecht, S. and Junge, W. (1997) Functional and idling rotatory motion within F_1 - ATPase, *Proc. Natl. Acad. Sci.* (1997) 94:4401-4405.(Medline)

Sambongi, Y., Iko, Y., Tanabe, M., Omote, H., Iwamoto-Kihara, A., Ueda, I., Yanagida, T., Wada, Y. and Futai, M. (1999) Mechanical rotation of the c subunit oligomer in ATP synthase (F_0F_1): direct observation, *Science* 286:1722-1724.(Medline)

Scherrer, P, Alexiev, U., Marti, T., Khorana, H.G. and Heyn, M.P. (1994) Covalently bound pH-indicator *dyes* at selected extracellular or cytoplasmic sites in bacteriorhodopsin 1. Proton migration along the surface of Bacteriorhodopsin micelles and delayed transfer from surface to bulk, *Biochem.* 33:13684-13692.(Medline)

Schnitzer, M.J. (2001) Molecular motors: Doing a rotary two-step, *Nature* 410:878-881. (MedLine)

Senior, A. E. (1979) The mitochondrial ATPase. In *Membrane Proteins in Energy Transduction* (Capaldi, R. A., ed.), pp. 233-278. Marcel Dekker, New York.

Senior, A. E. (1988) ATP synthesis by oxidative phosphorylation, *Physiol. Rev.* 68:177-231.(Medline)

Sigler, P.B., Xu, Z., Rye, H.S., Burston, S.G., Fenton, W.A. and Horwich, A.L. (1998) Structure and function in GroEL-mediated protein folding, *Annu. Rev. Biochem.* 67:581-608. (MedLine)

Singh, S., Turina, P., Bustamante, C.J., Keller, D.J. and Capaldi R. (1996) Topographical structure of membrane-bound *Escherichia coli* F_1F_0 ATP synthase in aqueous buffer, *FEBS Lett.* 397:30-34.(Medline)

Skulskii, I. A., Saris, N. E. L., and Glusunov, V. V. (1983) The effect of the energy state of mitochondria on the kinetics of unidirectional cation fluxes, *Arch. Biochem. Biophys.* 226:337-346.(Medline)

Sone, N., Yoshida, M., Hirata, H., and Kagawa, Y. (1977) Adenosine triphosphate synthesis by electrochemical proton gradient in vesicles reconstituted from purified adenosine triphosphatase and phospholipids of thermophilic bacterium, *J. Biol. Chem.* 252:2956-2960.(Medline)

Stock, D., Leslie, A.G. and Walker, J.E. (1999) Molecular architecture of the rotary motor in ATP synthase, *Science* 286:1700-1705.(Medline)

Tedeschi, H. (1981) The transport of cations in mitochondria, *Biochem. Biophys. Acta* 639:157-196; see

pp. 162-170.(Medline)

Tedeschi, H. and Kinnally, K.W. (1992) Bionergetics, in *Fundamentals of Medical Cell Biology*, JAI Press Inc., Greenwich, CT, vol 3B, pp. 609-642.

Vale, R.D. and Milligan, R.A. (2000) The way things move: looking under the hood of molecular motor proteins, *Science* 288:88-95. (MedLine)

Valiyaveetil, F.I. and Fillingame, R.H. (1997) On the Role of Arg-210 and Glu-219 of Subunit a in Proton Translocation by the *Escherichia coli* F_oF_1-ATP Synthase, *J. Biol. Chem.* 272:32635-32641.(Medline)

Vik, S.B. and Antonio, B.J. (1994) A mechanims of proton translocation by F_oF_1 ATP synthases suggested by double mutants of the a subunit, *J. Biol. Chem.* 269:30364-30369.(Medline)

Weber, J., Wilke-Mounts, S., Grell, E. and Senior, A.E. (1994) Tryptophan fluorescence preovides a direct probe on nucleotide binding in the noncatalytic sites of *Escherichia coli* F_1-ATPase, *J. Biol. Chem.* 269: 11261-11268.(Medline)

Westerhoff, H. V., Melandri, B. A., Venturoli, G., Azzone, G. F., and Kell, D. B. (1984) Mosaic protonic coupling hypothesis for free energy transduction, *FEBS Lett.* 165:1-5.(Medline)

Wilkens, S., Rodgers, A., Ogelvie, I. and Capaldi, R.A. (1997) Structure and arrangement of the δ subunit in the *E. coli* ATP synthase, *Biophys. Chem.* 68:95-102.(Medline)

Wilkens, S. and Capaldi, R.A. (1998) ATP synthase's second stalk comes into focus, *Science* 393:29.(Medline)

Wilson, D. F., Dutton, P. L., and Wagner, M. (1973) Energy transducing components in mitochondrial respiration, *Curr. Top. Bioenerg.* 5:234-265.

Winget, G. D., Izawa, S., and Good, N. E. (1965) The stoichiometry of photophosphorylation, *Biochem. Biophys. Res. Commun.* 21:438-443.(Medline)

Yasuda, R., Noji, H., Kinosita, K. Jr. and Yoshida, M. (1998) F_1-ATPase is a highly efficient molecular motor that rotates with discrete 120º, *Cell* 93:1117-1124.(Medline)

Yasuda, R., Noji, H., Yoshida, M., Kinosita, K. Jr. and Itoh, H. (2001) Resolution of distinct rotational substeps by submillisecond kinetic analysis of F1-ATPase, *Nature* 410:898-904. (MedLine)

Zhou, Y., Duncan, T.M. and Cross, R.L. (1997) Subunit rotation in Escherichia coli F_oF_1-ATP synthase during oxidative phosphorylation, *Proc. Natl. Acad. Sci. USA* 94:10583-10587.(Medline)

Zhou, Y., Duncan, T.M., Bulygin, V.V., Hutcheon, M.L. and Cross, R.L. (1996) ATP hydrolysis by membrane-bound *Escherichia coli* F_oF_1 causes rotation of the γ subunit relative to the β subunits, *Biochim. Biophys. Acta* 1275:96-100.(Medline)

19. The Cell Membrane: Transport and Permeability

The integrity of the cell depends on the presence of the plasma membrane. The membrane prevents the loss of internal components and metabolites. Its ability to exclude some solutes and to permit the passage of others allows the maintenance of an internal environment that differs from the medium external to the cell. The low concentration of Ca^{2+} in the cytoplasm permits its use as a sensitive signal for a variety of physiological regulatory effects. Similarly, the creation of an Na^{+} electrochemical gradient provides a reservoir of energy that can be used for the transport of other solutes, such as amino acids and sugars. Furthermore, the low internal Na^{+} and high K^{+} and the channels that allow their passage, are indirectly responsible for the electrical potential of cells, excitation and conduction (Chapter 22). The membrane controls the entrance of substrates used in either energy metabolism (e.g., glucose) or the synthesis of cell components (e.g., amino acids). The present chapter is directed primarily to general aspects of transport through the plasma membrane. Chapters 20 and 21 discuss the transport of ions. As we shall see the translocation through the plasma membrane involves in many cases the mediation of channels or transporters. Interestingly, although translocations through channels and transporters represent distinct mechanisms, transporter molecules can act as channels when reconstituted into bilayers (see Chapter 21). This indicates that part of the translocation mediated by transporters is through channel like structures (as also suggested by present models of transport). In addition, at least the glutamate transporter of the mammalian nervous system also can act as a channel in vivo (see Slotboom et al., 2001).

Enzymes act to increase the rates of reactions (Chapter 13) and are subject to a variety of regulatory mechanisms affecting either their activity (Chapters 13 and 14) or their turnover (Chapters 15). No less important is the accessibility of the cell's interior to substrates and cofactors which could also control the rate of metabolism.

I. CHARACTERIZATION OF TRANSPORT

Passage of a solute from one biological compartment to another may involve passage of the molecule through a biological membrane. This process need not differ from ordinary diffusion. The passage from, say, compartment 1 to compartment 2, is proportional to the concentration of solute in the first

compartment [S_1], as shown in Eq. (1).

$$J_{12} = k[S_1] \quad (1)$$

In this equation, *k* is a constant related to the diffusion coefficient (diffusion coefficient/thickness of the membrane). Equation (1) is a common sense equation, expressing Fick's law. The probability of the molecules going through a barrier must obviously increase with the number of molecules present. The flux (*J*) can be measured experimentally, simply by measuring the passage from 1 to 2 (J_{12}) of a radioactive isotope before the concentration in the second compartment [S_2] is sufficiently high to produce a significant flux in the opposite direction. The passage in the opposite direction (J_{21}) is given by an identical relationship. The net passage is given by the differences between the two fluxes.

$$J_{21} = k[S_2] \quad (2)$$

$$J_{12} - J_{21} = k([S_2] - [S_1]) = dS_1/dt \quad (3)$$

Equation (3) predicts an equilibrium ($dS_1/dt = 0$) when [S_1] = [S_2]. It also predicts that the net passage, due to diffusion alone, will be in the direction of the concentration gradient.

The chemical potentials (see Chapter 12) for the solute in compartments 1 and 2 are, respectively,

$$\mu_1 = \mu_o + RT \ln[S_1] \quad (4)$$

$$\mu_2 = \mu_o + RT \ln[S_2] \quad (5)$$

$\mu_1 - \mu_2 = \Delta G$ and at equilibrium $\Delta G = 0$ and [S_1] = [S_2].

When μ_1 is not equal to μ_2 and the difference or ΔG is positive, energy would have to be expended to transfer the solute against the concentration gradient. Conversely, when negative, the chemical potential could be used to drive another system to perform work. Either event would require a special mechanism to couple the energy providing reaction to the energy expenditure. Some of these topics were addressed in Chapter 12. For certain cases, the mechanism of coupling is the topic of Chapters 20 and 21.

The constant *k* of Eq. 3 will depend on the nature of the solute and the properties of the biological membrane in question. The rate constant will also depend directly on the surface area exposed to the exchanges. The constant *k*, corrected for surface area (*A*), is the permeability constant (*P*).

$$k/A = P \quad (6)$$

Solute molecules could pass through the phospholipid bilayer of the plasma membrane. First they must break away from the water phase of one

compartment, then they must dissolve into the bilayer to be released to another aqueous phase after diffusing through the hydrocarbon environment. However, evidence has accumulated for the involvement of protein channels in many of these translocations. Channels are responsible for the passage of ions during excitation and conduction of electrical impulses (see Chapter 22) or other cellular events. In addition, specialized channels or pores have been implicated in the passive passage of water and certain non-electrolytes.

A. Diffusion and Channels: the Aquaporins

The cell membrane has a much higher permeability to water than phospholipid bilayers. Furthermore, passage of water in response to an osmotic gradient is much more rapid than diffusion of water, as would be expected if channels are present. In addition, mercurial sulfhydryl reagents block water transport in the cell membrane so that it becomes as impermeable to water as phospholipid bilayers. A dependence on sulfhydryl groups suggests an involvement of proteins, most likely forming channels. Further studies showed that these expectations were correct. An integral membrane protein of approximately 28 kDa has been identified in red blood cells and renal tubules: the *aquaporin channel forming integral protein* (CHIP or aquaporin-1). Other water channel proteins associated with other tissues were found later. All these channels are generally referred to as *aquaporins* (AQPs). The AQPs (Deen and van Os, 1998) are members of a family of channel forming proteins called the major intrinsic proteins (MIP). Members of this family have been partially or fully sequenced. 150 MIPs are known including 10 mammalian AQPs. The MIP proteins constitute a family of proteins widely present in plants, animals and bacteria (Park and Saier, 1996; Deen and van Os, 1998).

AQP1-mediated water channel activity was first demonstrated in *Xenopus laevis* oocytes. These oocytes have a low water permeability to survive in fresh water ponds. The mRNA corresponding to AQP1 was transcribed in vitro using the appropriate cDNA as a template. The newly synthesized mRNA was then microinjected into the cells (Preston et al., 1992; Preston et al., 1993). The increased water permeability closely followed the characteristics of the red blood cells that contain AQP1.

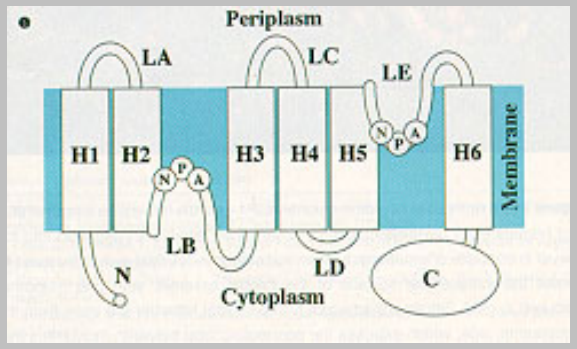
The transport of several nonelectrolytes is also sensitive to reagents that are likely to react with proteins. Therefore, several studies proposed a transfer of these substances through protein channels. AQP0, AQP3, AQP7 and AQP9 are also permeable to glycerol, and AQP3, AQP7 and AQP8 to urea.

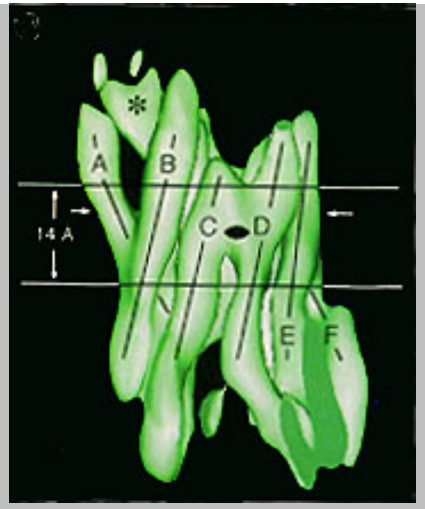
AQP1 has been found to be a homotetramer of the 28 kDa subunit (Denker et al., 1988; Smith and Agre, 1991). AQP1 has been sequenced (Preston and Agre, 1991) and has been found to be an integral protein with 6 hydrophobic domains capable of spanning the membrane, connected by loops A to E. The monomer is thought to have a long polygalactoseaminoglycan. One molecule has two repeats of three α helices which are 180^{o} mirror images. Each repeat consists of an asparagine-proline-alanine (i.e., NPA) sequence in loops B and E (the hourglass model). In this model, the highly conserved loops B and E are essential for the formation of the water pore. The three dimensional structure has been reported (see Fig. 1) from studies using cryo-electron microscopy and electron diffraction (Li and Jap, 1997; Walz et al., 1997; Cheng et al., 1997; Murata et al., 2000) and more recently X-ray crystallography (Sui et al., 2001). The unit cell contains two tetramers oriented in the bilayer in opposite direction (e.g., Walz et al., 1997). The helices form a barrel that encloses a vestibular region leading to the water-selective channel, which is outlined by densities attributed to the functionally important NPA boxes and their connections to the surrounding helices. A model representing an AQP1 monomer is shown in Fig. 1, top (from Walz et al., 1997). A side representation of the six-helix barrel is shown in Fig. 1, middle (Cheng et al., 1997), and Fig. 1, bottom, shows the arrangement of the helices in the tetramer as seen from above (Cheng et al., 1997). Despite its presence as a tetramer, a single subunit can act as a channel, as shown by radiation inactivation, which reveals a target size of about 30 kDa (Van Hoek et al., 1991, 1992). This method works on the principle that the larger the

molecule, the easier it is to hit by radiation (e.g., X-rays) and thereby be inactivated.

In mammals, the expression of AQP1 is regulated by several factors. In rat fetal lung cells (King et al., 1996), corticosteroids induce AQP1. In addition, this protein increases with hypertonic stress (Jenq et al., 1999) in several kinds of cells. Proteasome inhibitors increase AQP1 expression, indicating that the this protein is degraded via the ubiquitination- proteasome pathway (see Chapter 15). In addition, the half-life of the AQP1 protein of cells exposed to hypertonic medium is significantly longer than under isotonic conditions (Leitch et al., 2001), indicating an osmotic regulation of the degradation.

The specificity of AQP1 provides a challenging mystery. This channel protein allows the rapid flow of water but not H^+ (e.g., Zeidel et al., 1992). Recent electron crystallographic data (Murata et al., 2000) with a resolution of 3.8 Å, obtained from AQP1, allows the construction of an atomic model that may have resolved this question. Hydrophobic residues line the water channel. They allow rapid water transport in single file (based on studies carried out with gramicidin A; gramicidin is an antibiotic which forms well defined channels in bilayers) (see Finkelstein and Andersen, 1981). The water molecules can serve as proton wires (e.g., see Pomès and Roux, 1996). Supposedly, the protons interact with the oxygen of the water and are passed along the water column in a succession of hops. In gramicidin A channels, protons move fifteen times faster than K^+ (which have the same mobility in bulk water) and eight times faster than the water molecules themselves (see Pomès and Roux, 1996)! In the AQP1 molecule, selectivity is probably provided by a constriction of the channel with a diameter of about 3 Å over a span of one residue. At the point of constriction, water can form hydrogen bonds via its oxygen with the free amino residues and the consequent rearrangement of the water molecules in the column interrupts the continuous chain of hydrogen bonds needed for the proton transfer. Consequently, H^+ is unable to get past this point. A similar mechanism has been proposed to explain the H^+ impermeability of the *E.coli* glycerol channel and it has been suggested that the same mechanism may operate for all AQPs (Tajkhorshid et al., 2002)





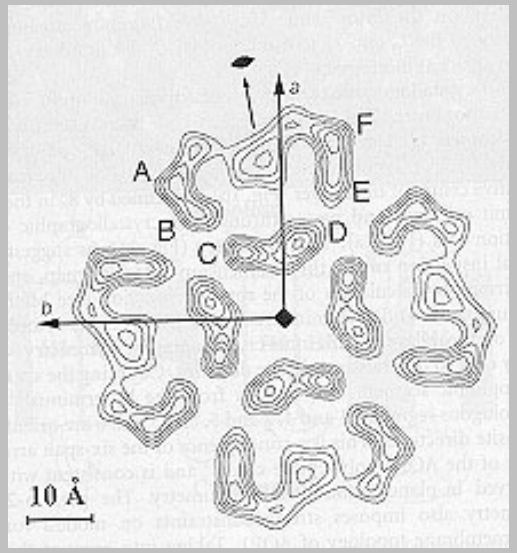


Fig. 1 Representation of aquaporin. Top: model showing the predicted position of the helices and loops. From Walz et al., 1997. Reproduced by permission. Middle: representation of the six-helix barrel parallel to the bilayer. From Cheng et al., 1997. Reproduced by permission. Bottom: top view of the arrangment of the helices in a tetramer. From Cheng et al., 1997. Reproduced by permission. Copyright &copy1997 MacMillan Magazines Ltd.

.

The structure of the glycerol conducting channel from *Escherichia coli*, containing 3 glycerol molecules also has been studied crystallographically (Fu et al., 2000). A region (termed the selectivity filter) is large enough to allow the passage of only 1 glycerol. In addition, the 3 glycerols are present in single file. The pathway is amphipatic and matches successive CH-OH groups. The polar interactions take place only on one side of the pathway. The alkyl portion of glycerol is held against a hydrophobic environment. Successive hydroxyl groups form hydrogen bonds with a pair of acceptor and donor

atoms. The passage of solutes is relatively specific allowing the entry of polyhydroxy-alcohols provided that the carbon backbone is lined along the channel axis and the molecule is sufficiently small.

The distribution and role of AQPs in mammalian organisms have been recently reviewed (King and Agre, 1996; Deen and van Os, 1998) and some of these are summarized in the table.

AQP	Tissue	Function	Malfunction
AQP0	lens fiber cells (1)	water/glycerol transport	cataracts (1)
AQP1	Kidney, red blood cells, lungs, eye, choroid plexus, bile duct, Non-fenestrated epithelia (2)	water/water reabsorption in kidney	impaired water reabsorption in mice (3)
AQP2	renal collecting duct cells (apical side) (e.g. 11)		nephrogenic diabetes insipidus (12)
AQP3	renal collecting duct cells (basolateral side) GI tract airway epithelia, eye conjuctive meningeal cells (4) renal collecting duct cells (basolateral side)		
AQP4	glial and ependymal cells of brain and others (4)	reabsorption of cerebrospinal fluid?	
AQP5	apical plasma membrane of pneumocytes, secretory epithelium and in upper airway and salivary glands (5)	secretion of fluid	
AQP6	Kidney (6)		
AQP7	Late spermatids and sperms Kidney and heart (7)		
AQP8	Hepatocytes, pancreas, heart and testes (8 and 9)		
AQP9	Adipocytes (also heart, kidney and small intestine) (10)		

(1) Shiels and Bassnet, 1996
(2) King and Agre, 1996
(3) Ma et al., 1998
(4) Lee et al., 1997
(5) Nielsen et al., 1997
(6) Ma et al., 1996
(7) Ishibashi et al., 1997
(8) Koyama et al., 1997
(9) Ma et al., 1997
(10) Kuriyama et al., 1997
(11) Saito et al., 1997
(12) Deen and Knoers, 1998

AQPs in mammals have a central role in the reabsorption of water in the kidney. Most of the reabsorption occurs in the proximal tubules and the descending loop of Henley. The remaining 10% takes place in the collecting duct principal cells and is regulated by antidiuretic hormone (ADH) also know as vasopressin. AQP3 and AQP4 are present in the basolateral membrane and AQP2 in the apical membrane of these cells. The release of ADH from the pituitary (when the blood's level of Na^+ is too high or the blood volume is too low) favors the increase in channels. The vasopressin binds to its receptor of the basolateral membrane of the principal cells. This is followed by the formation of cAMP in the cytoplasm and activation of PKA. The PKA phosphorylates several proteins, including AQP2 in intracellular vesicles (Fushimi et al., 1997; Katsura et al., 1997). AQP3 and 4 are not affected in this manner. The vesicles containing the phosphorylated AQP2 fuse to the apical membranes and are responsible for the osmotically driven reabsorption. When the level of vasopressin decreases, the vesicles are removed from the plasma membrane by endocytosis. In addition, the AQP2 and AQP3 levels are increased as a result of increased transcription. The cAMP-responsive elements in the AQP2 promoter region are involved in ther case of AQ2 (Matsumura et al., 1997).

Exocytotic delivery of AQPs is likely to be a general mechanism for increasing water transport. For example, the hormone *secretin* is known to stimulate ductal bile secretion by cholangiocytes. These cells transport water through AQP1. Secretin stimulates exocytosis in cholangiocytes and causes up to a 3-fold increase in the amount of AQP1 in plasma membranes and a proportional decrease in the amount of the water channel in microsomes. These findings suggest that secretin induced redistribution of AQP1 from intracellular to plasma membranes. The redistribution was found to be dependent on vesicular translocation and microtubular function (Marinelli et al., 1997). Similar mechanisms involve the glucose transporter GLUT4. Stimulation of glucose transport by insulin, is linked to translocation of the GLUT4 glucose transporter from an intracellular pool to the cell surface (e.g., see Kanai et al., 1993).

B. Transporter Mediated Translocations

Equation (3) expresses the net flux of a solute transferred by a diffusional process and indicates that the net passage of solute should be directly proportional to the concentration gradient. As we saw, a steady state should be reached eventually in the form of a true equilibrium at which $[S_1] = [S_2]$. Although not explicitly stated by the equation, the passage should be largely independent of the presence of other substances. Furthermore, we would expect a similar rate transfer for isomers. For example, transfer of the D form of a compound should generally be about the same as transfer of the L form.

It must have been very exciting for the early investigators to find glaring exceptions to these predictions. In many cases, the rate of entry was not found not to be a linear function of the concentration of the penetrant. In addition, some of the translocations were found to be surprisingly specific and against the concentration gradient. Fig. 2 (Christensen et al., 1963) shows some of the characteristics of the transport of leucine by Ehrlich ascites tumor cells (a line of mouse peritoneal cells). Fig. 2a shows the rate of transport of L-*tert*-leucine as a function of concentration of the amino acid in the medium. Fig. 2b is the corresponding Lineweaver-Burk plot and Fig. 2c shows the ratio of internal over external concentration of leucine with time of incubation showing that the uptake corresponds to an active transport. In addition, L-*tert*-leucine is taken up more rapidly than the D-isomer, as shown in Fig. 2c. Consequently, the steady-state level reached is greater for the L isomer. The ability to distinguish between isomers is known as *stereospecificity*.

Other apparent anomalies have also been observed. Where many amino acids are used simultaneously, they interfere with each other as if they were

competing for entry.

Some of the findings may be explained without changing the diffusion model significantly. The saturation effect shown in Fig. 2(a) could be the result of passage of the amino acid through a restrictive channel that could admit only a certain number of molecules at a time. However, most of the other observations cannot be explained by such a simple concept. We would not expect, for example, that a simple channel would be able to distinguish between an L and a D isomer. Neither would we expect accumulation against a chemical or electrochemical gradient.

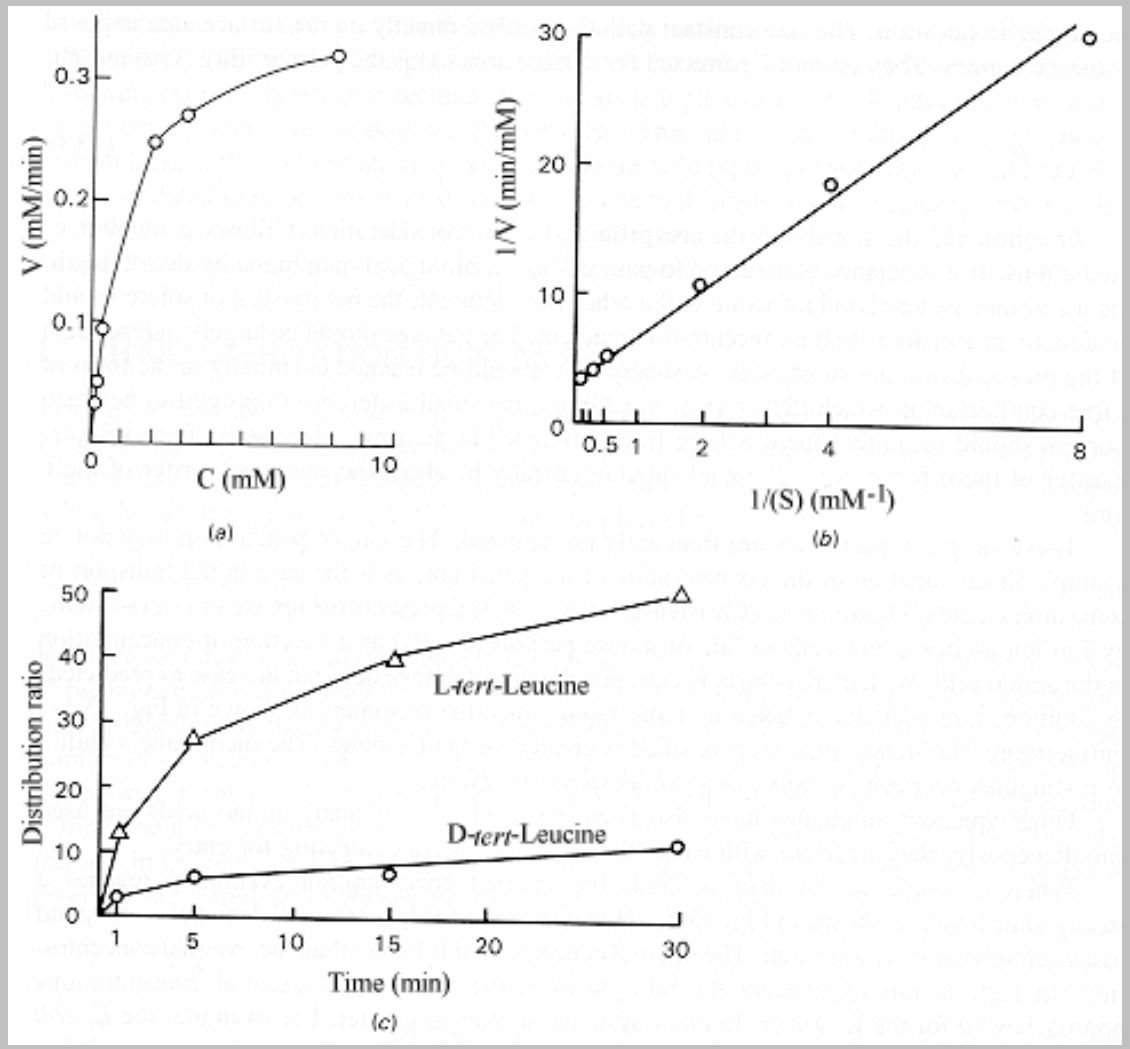


Fig. 2 (a) Kinetics of the transport of L-*tert*-leucine. (b) Lineweaver-Burk plot of L-*tert*-leucine concentrations against its rate of uptake. See text for details. V_m, 3.0 µmol ml^{-1} min^{-1}; K_m 1.0 x 10^{-3} M. (c) Time course of the uptake of the isomers of *tert*-leucine by Ehrlich ascites tumor cells. The cells were incubated as 4% suspensions in 1 mM L- or D-*tert*[1 - ^{14}C]leucine. The distribution ratio represents the ratio of radioactivity per kilogram of cellular water to the radioactivity of the suspending solution. Reproduced from *Biochimica et Biophysica Acta*,vol. 78, H.N. Christensen, et al.,pp. 206-213, copyright &copy1963 Elsevier Science.

In order to explain these anomalies, scientists postulated that the transport is *mediated* by a *transporter* or *carrier* molecule. The present discussion will favor the term "transporter" because the term "carrier" has been used in the past to imply a movement of the whole transporter molecule from one membrane interface to the other. The translocation through a transporter mechanism requires reversible binding of the transported molecule to specific sites in the transporter molecule, followed by transfer of the sites to the opposite interface of the membrane and release of the ligand. These properties are very similar to that of the enzymes discussed in Chapter 13. The model reactions describing the general patterns of enzyme substrate interactions could also be used here, as shown in Eq. (7). In this equation, T = transporter which is restricted to the membrane phase (indicated by the brackets); S is the substance being transported; the subscripts *o* and *i* indicate the location in the inner and the outer phase. The brackets indicate that the reaction occurs in the membrane.

$$S_o + [T \leftrightarrow TS_o] + S_i \quad (7)$$

Where the accumulation is against a concentration gradient, a coupled reaction supplying the needed energy expenditure has to be added to this simple scheme. The transporter itself, or other molecular elements involved in the reaction between substrate and transporter, could have the requisite stereospecificity. As in the case of enzymes, the number of transporter molecules would necessarily be limited and the kinetics of the transfer should therefore exhibit saturation. This saturation could explain the constancy of the rate of transfer with large increases in substrate concentration (Fig. 2a).

Except for the localization of the system in a membrane and the fact that the reaction corresponds to a vectorial displacement rather than catalysis, the model formally corresponds to that discussed for enzyme reactions (Chapter 13). In fact, the equations that are applicable are the same, such as Eq. (8).

$$V = \frac{dS}{dt} = \frac{V_m\ (S_o]}{K_m + (S_o)} \quad \text{V} \frac{1}{V} = \frac{K_m}{V_m\ (S_o)} + \frac{1}{V_m} \qquad (8)$$

As shown in Fig. 2a, when the substrate concentration increases, the rate (or velocity) of the reaction approaches a limiting value (V_m). As for the action of enzymes, and as shown in Eq. (8), a plot of $1/V$ as a function of $1/S_o$ (a Lineweaver-Burk plot), should yield the constants K_m and V_m. In this case, the V_m is 3 mol ml^{-1} min^{-1} and the K_m is 1 x 10^{-3} M.

The K_m of Eq. (8) depends on the various rate constants of the reactions represented in Eq. (7) (see Christensen 1975, pp. 115-119).

Obviously, other features of the transport system will also be the same as those of enzyme reactions, such as competitive inhibition by analogs or a block by certain chemicals that have been shown to react with proteins.

The representation of Eq. (7) does not include the coupling to a reaction supplying energy as required in active transport. The coupling may be through the phosphorylation of the transporter (see Chapters 20 and 21) or involve the cotransport of another ion in the direction of its electrochemical gradient. Eq. (8) does not always predict the rate of net transport accurately, because independent processes such as a diffusional component may also be involved.

Many transport processes have the characteristics just discussed. When the transport is not against an electrochemical gradient, the process is referred to as *facilitated diffusion*. Transport against a gradient is referred to as *active transport*.

II. MOLECULAR MECHANISMS OF TRANSPORT: THE GLUCOSE TRANSPORTERS

What is the molecular mechanism for the transport of solutes? The information amassed over the years is beginning to provide answers (see Chapter 21). The transporters have been isolated and sequenced. These and other data have permitted eliminating many alternative models. However, examination of several models, for example those shown in Fig. 3, helps us define more precisely the characteristics of the transport systems and the mechanisms of transport. The transporter-substrate complex could be transferred from one interface of the membrane to the other by diffusion of the complex through the membrane (model 1). A larger molecule spanning the membrane could also transfer the solute, perhaps by rotating or moving in some other way (model 2). However, the movement need not involve a large portion of the molecule, but simply a small portion (not shown) or a conformational movement of the molecule or molecules constituting the pore structure (model 3). These three models involve movement of either the whole transporter or a portion of the transporter molecule (Fig. 3a). In contrast, fixed groups capable of binding the substrate arranged through a membrane pore (Fig. 3b) could also transfer the solute.

The mechanisms represented by the two sets of general models - (a) or fixed (b) - can be distinguished experimentally (Fig. 4). Part *a* of the figure focuses for simplicity on model 1. However, the reasoning would be identical if we were to consider any of the mobile models. Let us examine the situation in which the substrate does not normally accumulate against a concentration gradient. On reaching a steady state (here, a true equilibrium), the inside and outside concentrations are the same. The inward flux is precisely the same as the outward flux. A competitor should decrease the flux by making the transporter less available to the substrate. As shown in Fig. 4a, if the transporter is mobile, the competitor, present on only one side, would naturally block the passage of substrate in a single direction (i.e., if the competitor is outside, it should affect only the inward flux). The flux in the opposite direction should remain largely unaffected. The inhibition would, therefore, be asymmetric. This situation could result in temporary accumulation against a gradient. The counterflow of a substrate against a concentration gradient involves the dissipation of chemical potential due to the flow of a second substance in the direction of its own concentration gradient. Counterflow is also known as *trans facilitation* (trans indicating that the competitor is on the other side of the membrane from the substrate). This phenomenon is apparently also aided by the fact that the rate of movement of the transporter is considerably higher when it is loaded than when it is empty (Gorga and Lienhard, 1981) (see below).

For the case of a *fixed* carrier (Fig. 4b), both the inward and outward fluxes should be equally inhibited and no trans facilitation should take place.

Many experiments clearly establish that the transporter is mobile (see also below). We already examined the case of the monosaccharide transport of red blood cells (Rosenberg and Wilbrandt, 1958) discussed in Chapter 12.

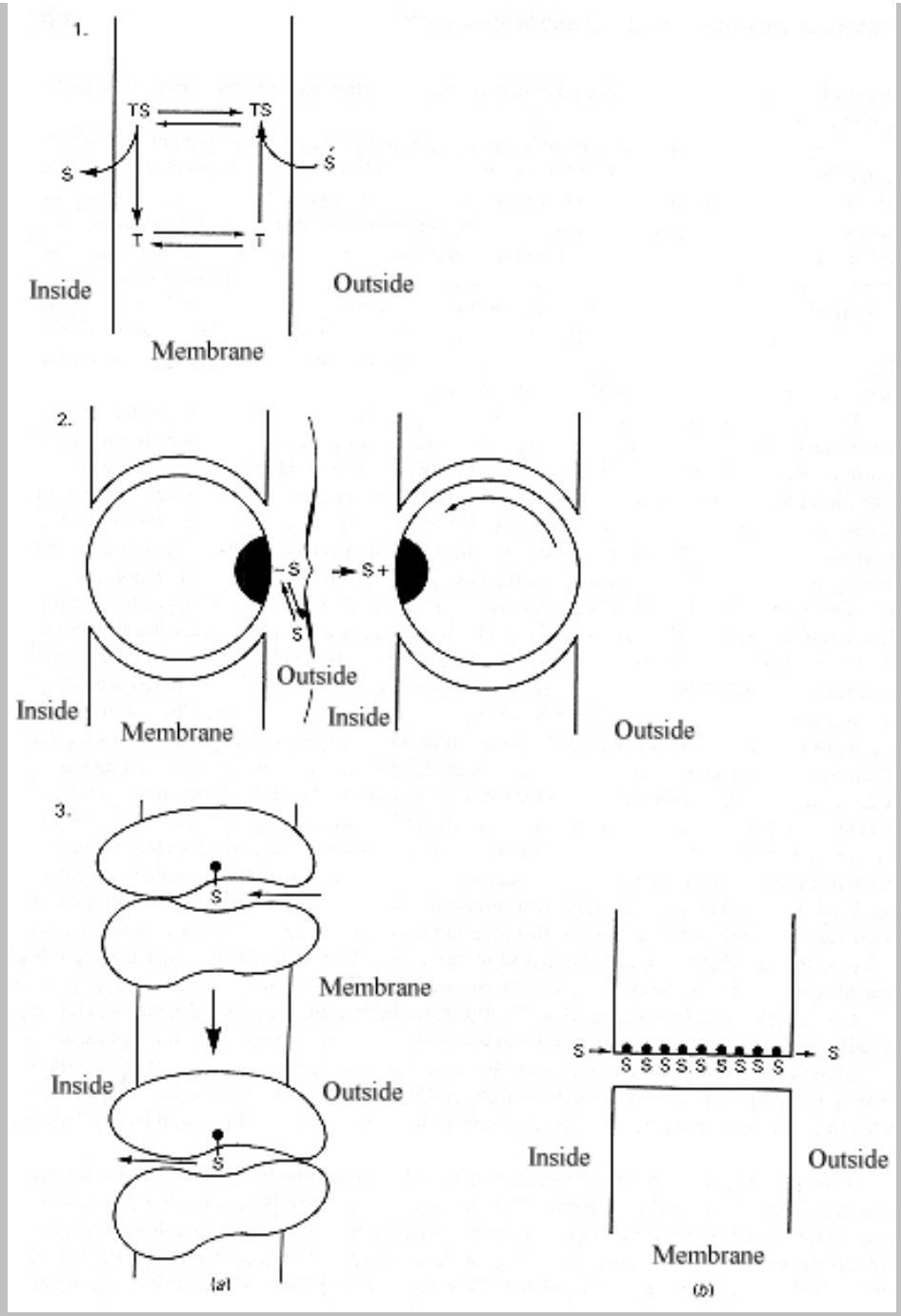


Fig. 3 Diagrammatic representation of carrier models. Circle, carrier; S, substrate. (a) Mobile transporters, (b) Fixed transporters.

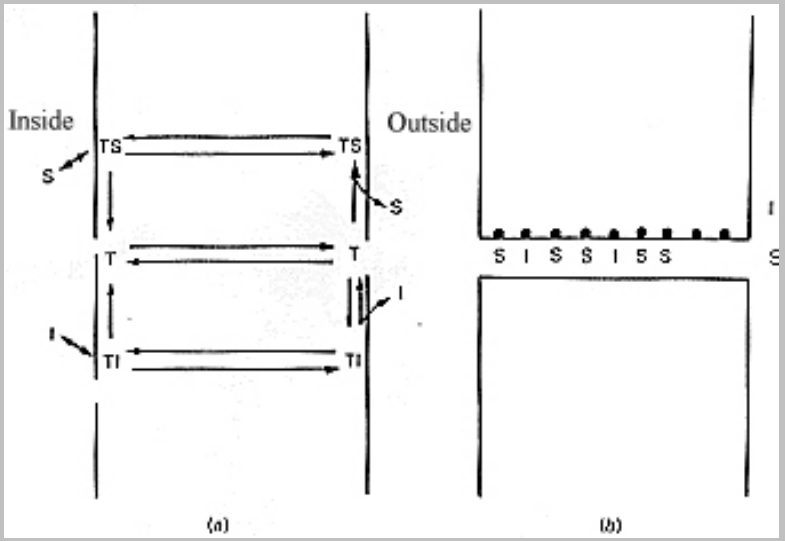


Fig. 4 Diagrammatic representation of transporter models and their behavior in relation to competitive inhibitors. T, Transporter; S, substrate; I, inhibitor; filled circles indicate binding sites. (a) Mobile transporter; (b) fixed transporter.

The movement of the transporter probably corresponds to a relatively small conformational change. As we saw in Chapter 4, integral membrane proteins clearly neither move from one surface to the other (model 1), nor rotate (model 2). In the case of the glucose transporter, the intrinsic fluorescence of the protein changes with binding to the substrate (Gorga and Lienhard, 1981), an indication that the components responsible for the fluorescence have been moved to a different molecular microenvironment. A conformational change is also demonstrated by changes in its sensitivity to chemicals. Similarly, the galactose-binding protein of *E. coli* shows an increase in intrinsic fluorescence and electrophoretic mobility when attached to its substrate (Boos et al., 1972).

Model 3 is the most likely. It shows two different parts of the transporter forming a channel which exposes the binding groups alternatingly to the two phases separated by the membrane. A version of this model will be discussed in more detail in Chapter 21 for ion transport.

The glucose transporters have been studied in detail. These transport systems are of obvious importance in the physiology of cells since the use of glucose as a major food is widespread. Furthermore, homeostatic mechanisms required in mammals for maintenance of glucose concentration are extensive and the diseases resulting from malfunction of this homeostatic control, such as in *diabetes mellitus*, are of obvious significance.

Initially, the study of glucose transport was facilitated by the availability of red blood cells and the abundance of the glucose transporter in these cells, estimated to correspond to 5% of the total membrane proteins (e.g., Allard and Lienhard, 1985). The later DNA sequencing and cloning (see Chapter 1) provided considerable information on the widespread existence of this family of transporters, referred to as *major facilitators* (MFs). Until 1998, 133 have been sequenced, where sugar transporters form a subgroup of 18 members (see Walmsley et al., 1998). Six glucose transporters have been identified in mammals, these are referred to as GLUT1 to GLUT5 and GLUT7 (GLUT6 is non-functional as a transporter). Similar sugar transporters are present in

bacterial systems. The transport in bacteria, however, is active via proton *symporters* (see Henderson, 1993), where glucose and H^+entry are linked. The Na^+-linked sugar transporters present, for example, in the kidney and small intestine, appear to be unrelated (see Hediger et al., 1987).

In mammals, each GLUT has a characteristic tissue distribution (e.g., see tabulation of Gould and Holman, 1993). GLUT1 is one of the major proteins of the red blood cell. This transporter is present mostly in brain, adipose tissue and the blood brain barrier. GLUT2 is present in liver, pancreas β cells, kidney and small intestine, GLUT4 in muscle, heart and adipocytes and GLUT5 primarily in the small intestine. GLUT7 has a different function since it is located in the ER membrane. Some mammalian cells, such as brain have GLUT protein which are constitutive and mostly present in the plasma membranes. In others such as muscle and adipose tissue most of the transporters are recruited to the surface in response to biological signals (e.g., insulin or exercise)

In part, the regulation of GLUT1 and GLUT4 uses an exocytotic mechanism similar to the one discussed for the aquaporins. Functional GLUT1 increases following stresses (such as viral infection, heat shock or alkaline pH) because the transporter is transferred from intracellular sites in the perinuclear region to the cell surface (Pasternak et al., 1991; Widnell et al., 1990). However, prolongued stress, particularly glucose deprivation, increases the total amounts of GLUT-1-mRNA and GLUT1 in the cell (Shetty et al., 1992; Yamada et al., 1983). White and brown fat, skeletal muscle and heart, transfer GLUT4 from the cell's interior to the cell surface in response to insulin (see Pessin et al., 1999). The transfer is rapid and produces as much as a 20-fold increase (Holman et al., 1990). In heart and brown adipocytes, immunological techniques have determined that, in the basal state, 99% of the GLUT4 is in the cell's interior in the *trans*-Golgi and tubular-vesicular structures (Slot et al., 1991a, b). The GLUT4 delivered to the plasma membrane in response to insulin appears to be present in part in small vesicles, 50 nm in diameter, which contain the proteins thought to be necessary for the exocytosis to the plasma membrane and these proteins are depleted with insulin treatment (e.g., Ramm et al., 2000). The activity of GLUT4, but not its distribution in the cell, is also regulated by other hormones such as *adrenocoticotropic hormone* (ACTH) and glucagon (see Simpson and Cushman, 1986). The regulation of GLU4 has been found to be central to the control. of glucose utilization and in diabetes in mammals (e.g., see addendum).

GLUT1, a glycoprotein with an apparent molecular weight of 55 kDa (on SDS PAGE, this is band 4.5), has been extensively studied because of its presence in readily available red blood cells (Allard and Lienhard, 1985). The protein is heavily glycosylated and hydrophobicity plots (see Chapter 4) suggest 12 helices spanning the membrane (e.g., see Mueckler et al., 1985). Hydrogen exchange studies (by dialyzing reconstituted vesicles containing GLUT1 in a $[^2H]_2O$ medium) indicate that a large fraction of the helices are accessible to water, evidence that they may be arranged to form a water filled channel (e.g., Alvarez et al., 1987). Supporting this view, the amino acid sequences of segments III, V, VII, VIII and XI were found to suggest amphipathic α-helices (Mueckler et al., 1985) since they contain several serine, threonine, glutamine and asparagine residues that would be localized on the same face of the α-helix. Both the carboxy and the amino terminals are in the highly charged cytoplasmic surface (Baldwin, 1993; Hresko et al. 1994). The carbohydrate moiety is external, attached to Asp45. Experiments with proteolytic digestion and antibody binding (see Chapter 4) confirm this general arrangement (e.g., Shanahan and D'Artel-Ellis, 1984; Cairns et al., 1984, 1987; Davies et al., 1987).

The mechanism of transport currently favored is that shown in Model 3 of Fig. 3, where the transporter alternates between inward and outward-facing conformations (e.g., see Baldwin, 1993). In this model, the reorientation of the transporter is thought to be the rate-limiting step in the translocation of the glucose. The evidence supporting this model is as follows. Fluorescence spectroscopy shows that the tryptophan fluorescence changes during transport indicating that there is a change in the conformation of the transporter (see Appleman and Lienhard, 1985, 1989). In addition, essentially unidirectional

conversion of one conformation to the other was accomplished by rapidly mixing purified transporter with either 4,6-ethylidene-D-glucose, which preferentially binds to the external phase of GLUT1 or phenyl beta-D-glucoside, which preferentially binds to the internal phase. Other evidence also supports the view that during transport there is a movement of binding sites from one surface to the other. One approach shows that cleavage of the transporter by the protease thermolysin is favored by the antibiotic cytochalasin B (CB) but retarded by the azidosalicoyl derivative of bis(D-mannose) (ASA-BMPA) (Holman and Rees, 1987). This finding suggests that cytochalasin B recruits the transporter site to the inner surface, whereas ASA-BMPA does the opposite. Cytochalasin B binds to the inward facing conformation since it is a competitive inhibitor of glucose efflux but a non-competitive inhibitor of influx. Similarly, ASA-MPA must bind to the outer surface because it is an impermeable competitive inhibitor of glucose transport. Thermolysin, a proteolytic enzyme isolated from bacteria, hydrolyzes internal peptide bonds adjacent to hydrophobic residues.

Two other strategies have demonstrated movement of binding sites from one surface to the other by showing recruitment to the side that binds the competitor. In one case the binding of ligands (e.g., ethylidene glucose) at the external surface of the transporter was shown to interfere with cytochalasin B binding on the inner surface (Deves and Kupka, 1978; Gorga and Lienhard, 1981). In the other approach, the transport sites were shown to be recruited to the external surface by using impermeant, reversible inhibitors. The initial availability of transporter sites was assayed by measuring the initial rate of glucose influx. After treatment with the inhibitor, the subsequent addition of glucose and dilution of inhibitor produced an initial rate of transport considerably faster than the steady state transport in the absence of pretreatment (Lowe and Walmsey, 1987; Critchley and Lowe, 1991).

However, the transport sites that are available to two sets of inhibitors - CB at the inner surface and sugar derivatives at the outer surface - are distinct. UV light cross-links CB to GLUT1 between helices X and XII, and D-glucose inhibits the binding. Sugar derivatives that bind to the external site attach between helices IX and X (see Walmsley, 1988). These sites may be on different transmembrane segments, however, they may be very close together in the tertiary structure. For example, a hydrophilic cleft is predicted between spans VII and VIII and XI and XII (see Baldwin, 1993). The two inhibitor binding sites are very close together since the two labels are separated by a small span of protein and can be separated by some chemical fragmentation procedures (see Holman and Rees, 1987). The two labels can be isolated together down to fragments as small as 7 kDa. A tryptophan cleavage at residue 388, divides the cytochalasin B site and the ASA-BMPA site.

Mutagenesis studies have determined the amino acids residues that are important for the transport (Walmsley et al., 1994). In addition, the orientation of the glucose during transport by GLUT1 has been determined (Barnett et al., 1975). It enters the external face C-1 first and C-4 and C-6 enter last. Other GLUTs bind glucose with a similar polarity (Colville et al., 1993).

Transmembrane chain V of GLUT1 is one of the segments of GLUT1 that has been postulated to form an amphipathic transmembrane helix (Mueckler et al., 1985) that might line the translocation pathway. This notion was put to test (Mueckler and Makepeace, 1999). Each residue supposed to be in segment V were mutated to cysteine and expressed in *Xenopus* oocytes (see e.g., Chapter 1). The mutants had transport activity. At least six of these cysteine residues were found accessible to p-chloromercuribenzoatesulfonate (pCMBS) which blocks transport. pCMBS is presumed to enter only a hydrated space because of its polarity. However, the six residues were clustered at the external surface of the plasma membrane and, therefore, represent only a small portion of segment V.

Many ATP powered transports (e.g., see Chapter 21) and secondary active transports (i.e., powered by a gradient for another solute and not by ATP) have been shown to alternate binding groups between the inner and the outer face (e.g., see Hirayama et al., 1997). The case of the Na^+-dependent

glucose transport has been studied in detail (Panayotova-Heiermann et al., 1995; Hirayama et al., 1994). Binding of Na^+, Li^+ or H^+ induce a change in conformation and increases the affinity for the sugar. The rate of transport is also determined by the cation.

III. VECTORIAL ENZYMES

In many ways, transport resembles an enzyme catalyzed process in which a protein molecule mediates translocation of the substrate rather than a chemical change. However, there is no reason why a protein could not pick up a substrate on one side of the membrane and simultaneously catalyze a chemical change with the discharge of the product on the other side of the membrane. In this case, the reaction would differ from one catalyzed by an enzyme in solution in that it has directionality. The reaction is said to be *vectorial*. A vectorial reaction is thought to be involved in the simultaneous translocation and phosphorylation of glucose and related monosaccharides in *E. coli*, *Salmonella typhimurium* and *Bacillus subtilis*. The sugars are translocated from the external medium into the cell, where they are released in a phosphorylated form. A similar process is thought to take place in yeast (e.g., Van Steveninck, 1972) and in *Aerobacter aerogenes* (Kelker and Anderson, 1972). Simultaneous translocation and enzyme action are by no means restricted to microorganisms. They are thought to take place, for example, in the intestinal transport of sugars, where sucrose is hydrolyzed and the products of the reaction, fructose and glucose, are released into cells.

The most direct evidence for a role of vectorial reactions in translocation comes from studies of the phosphoenolpyruvate phosphotransferase reaction in vesicles obtained from *E. coli* membranes. The enzyme is responsible for the transfer of phosphate from phosphoenolpyruvate to sugar molecules. The experiment is represented in Fig. 5 (Kaback, 1968), where the ordinate represents the amount of sugar taken up as a function of time. The interior of the vesicles was preloaded with [^{14}C]glucose, and [^{3}H] glucose and phosphoenolpyruvate were added to the outside. The phosphorylation and the transfer of the phosphate showed a preference for the outside [^{3}H]glucose. The phosphorylation did not precede the transfer, since the vesicles were unable to take up external glucose-6-phosphate.

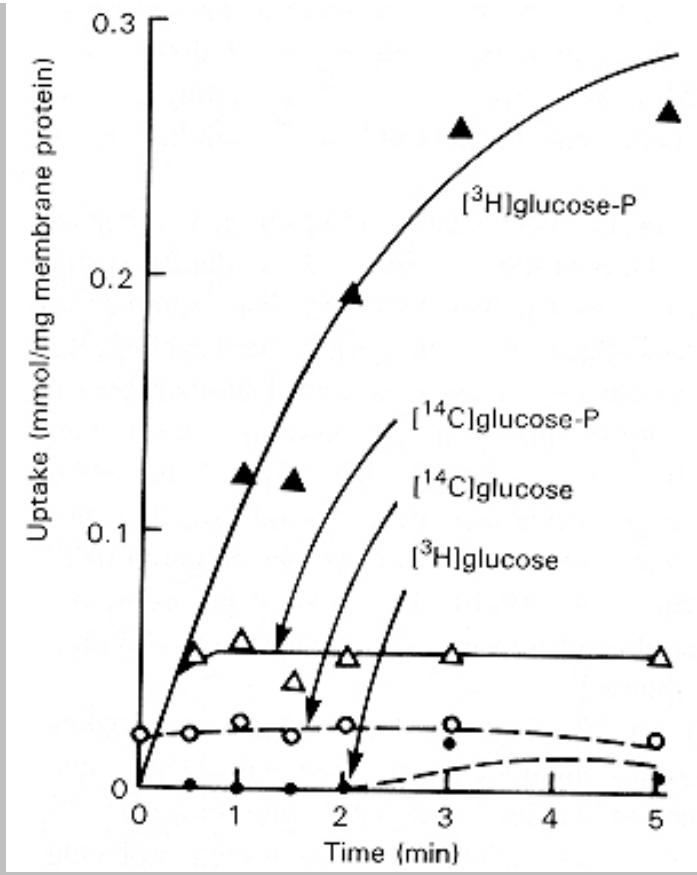


Fig. 5 Uptake and phosphorylation of[^{3}H]glucose by *E. coli* membranes previously loaded with [^{14}C]glucose. Reproduced with permission from H. R. Kaback, *Journal of Biological Chemistry* 243:3711-3724. Copyright &copy1968 American Society of Biological Chemists, Inc.

In some bacteria, the phosphorylation translocation of sugars involves the phosphoenolpyruvate phosphotransferase reaction, as in the example of Fig. 5. In this reaction, sugars are phosphorylated by the transfer of phosphate from phosphoenolpyruvate (PEP). The reaction occurs in two steps: one in which the phosphate is transferred from PEP to a protein (HPr) and another in which it is transferred from the protein to the sugar. This can be represented as:

$$PEP + HPr \xrightarrow{Enzyme\,I,\,Mg^{2+}} Pyruvate + PHPr \qquad (9a)$$

$$PHPr + Sugar \xrightarrow{Enzyme\,II} Sugar\text{-}6\text{-}phosphate + HPr \qquad (9b)$$

HPr is small protein (9.4 kDa) that is phosphorylated at the histidine residue.

The phosphotransferase system of bacteria may well account for the first reaction of many metabolized sugars for which no active kinase has been found. The apparent absence of an appropriate kinase has been one of the most intriguing questions in the physiology of some microorganisms (Wood, 1966). There are many indications that the phosphotransferase system plays a role in the translocation of sugars and that they are accumulated in the phosphorylated form. Mutants of *S. typhimurium* lacking any of the components of the system, fail to accumulate sugars (Simoni et al., 1967). However, it should be recognized that the mutants may be pleiotrophic (Kennedy and Scarborough, 1967) and have more than one deficiency. In some cases, the substrate is accumulated as a phosphorylated intermediate, as in the case of β-glucosides (Fox and Wilson, 1968; Wang and Morse, 1968). It has been proposed that the PEP system is involved in the transport of β-glycosides in *E. coli*. The protein HPr is deficient in treated *E. coli* cells that have impaired β-galactoside transport, and the addition of HPr reestablishes function. On the other hand, the finding that *E. coli* mutants deficient in enzyme I can transport β-galactosides makes this interpretation unlikely (Asensio et al., 1963).

IV. STRATEGIES FOR THE ISOLATION OF TRANSPORT PROTEINS

Our insights into a biological system cannot be considered sufficient until all molecular components and their characteristics are recognized. The ultimate test of our understanding consists of reestablishing biological activities in the test tube after putting the component molecules back together. A number of transport proteins have been isolated, and with many of them it has been possible to reconstitute transport by recombining them with artificial membranes.

Isolation of a transport protein requires disruption of the membrane and some means of recognizing the presence of the molecules through the various fractionation procedures. Detergents must be used because the transporters are integral proteins.

Recognition is not always a simple matter. Transport proteins may be recognized by their ability to bind either substrates or inhibitors. The task is simplest when a specific labelled inhibitor strongly binds to the transporter molecule. The anion transporter of the red blood cell has been recognized in this fashion. The usefulness of this approach can be much extended with the use of modified substrates that can be cross-linked at the sites of attachment, perhaps in a light-dependent fashion (*photoaffinity labeling*).

Some active transports are coupled to a reaction which hydrolyzes ATP. These transporters are called P-ATPases. Their ATPase activity depends on the presence of the appropriate ions (Ca^{2+} for the Ca^{2+}-ATPase, Na^+ and K^+ for the Na^+, K^+-ATPase). Therefore, the appropriate ATPase activity can serve to identify the transporter in the various protein fractions during the fractionation procedure.

The ability of binding either substrates or inhibitors can also be directly exploited. These substances (e.g., glucose, ATP) can be covalently attached to a column and will bind the transporter, but allow other proteins through (this is known as *affinity* chromatography). An excess of the free substrate or inhibitor can then be used to set free the transporter by competing for binding with the substances attached to the column in a process known as *elution*.

A group of related transport proteins with ATPase activity has been identified. The members of this super-family of *traffic ATPases* are also referred to as the *ATP-binding casette proteins* (ABC) (Hyde et al., 1990). They include the multidrug resistance P-glycoprotein (MDR), the cystic fibrosis gene

product (CPTR), and a variety of prokaryotic permeases. CPTR probably functions as a Cl^--conducting channel (Anderson et al., 1991; Kartner et al., 1991).

Many years of work by many people have brought us close to unraveling one of the most intriguing problems of cell physiology: how a cell can control its integrity by controlling the flow of material in and out of its internal compartments. In this chapter, we have dealt primarily with the translocation of nonionic components. The next two chapters (Chapters 20 and 21) discuss the systems responsible for the transfer of ions in the cell.

SUGGESTED READING

Christensen, H.N. (1975) *Biological Transport*, Chapters 1, 4-8. Benjamin, Reading, Mass.

Gould, G.W. and Holman, G.D. (1993) The glucose transporter family: structure, function and tissue-specific expression, *Biochem. J.* 295:329-341. (Medline)

Macey, R. I. (1987) Mathematical models of membrane transport processes. In *Membrane Physiology*, 2d ed. (Andreoli, T. E., Hoffman, J. F., Fanestil, D. D., and Schulz, S. G., eds.), pp. 111-132, Plenum Medical Book Company, New York.

Schafer, J. A. and Andreoli, T. E. (1987) Principles of water and nonelectrolyte transport across membranes. In *Membrane Physiology*, 2d ed. (Andreoli, T. E., Hoffman, J. F., Fanestil, D. D., and Schulz, S. G., eds.), pp. 177-190. Plenum Medical Book Company, New York.

Schulz, S. G. (1987) Ion-coupled transport of organic solutes across biological membranes. In *Membrane Physiology*, 2d ed. (Andreoli, T. E., Hoffman, J. F., Fanestil, D. D., and Schulz, S. G., eds.), pp. 283-294. Plenum Medical Book Company New York.

Stein, W. D. (1986) *Transport and Diffusion Across Cell Membranes*, Chapters 1, 2, 4, and 5. Academic Press, New York.

Zeuthen T. (2001) How water molecules pass through aquaporins, *Trends Biochem. Sci.* 26:77-79. (MedLine)

WEB RESOURCES

Diwan, J.J., Membrane Transport,
http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/carriers.htm">http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/carriers.htm

REFERENCES

Search the textbook

Back to Chapter 19

REFERENCES

Abel, E.D., Peroni, O., Kim, J.K., Kim, Y.B., Boss, O., Hadro, E., Minnemann, T., Shulman, G.I. and Kahn, B.B. (2001) Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver, *Nature* 409:729-733. (MedLine)

Allard, W.J. and Lienhard, G.E. (1985) Monoclonal antibodies to the glucose transporter from human erythrocytes, Identification of the transporter as a Mr = 55,000 protein, *J. Biol. Chem.* 260:8668-8675.(Medline)

Alvarez, J., Lee, D.C., Baldwin, S.A. and Chapman, D. (1987) Fourier transform infrared spectroscopic study of the structure and conformational changes of the human erythrocyte glucose transporter, *J. Biol. Chem.* 262:3502-3509.(Medline)

Anderson, M.P., Berger, H.A., Rich, D.P, Gregory, R.J., Smith, A.E. and Welsh, M.J. (1991) Nucleoside triphosphates are required to open the CFTR chloride channel, *Cell* 67:775-784. (MedLine)

Appleman, J.R. and Lienhard, G.E. (1985) Rapid kinetics of the glucose transporter from human erythrocytes. Detection and measurement of a half-turnover of the purified transporter, *J Biol. Chem.* 260:4575-4578.(Medline)

Appleman, J.R. and Lienhard, G.E. (1989) Kinetics of the purified glucose transporter. Direct measurement of the rates of interconversion of transporter conformers, *Biochemistry* 28:8221-8227.(Medline)

Asensio, C., Avigad, G., and Harecker, B. L. (1963) Preferential galactose utilization in a mutant strain of *E. coli. Arch. Biochim. Biophys.* 103:299-309.

Baldwin, S.A. (1993) Mammalian passive glucose transporters: members of an ubiquitous family of active and passive transport proteins, *Biochim. Biophys. Acta.* 1154:17-49.(Medline)

Barnett, J.E., Holman, G.D., Chalkley, R.A. and Munday, K.A. (1975) Evidence for two asymmetric conformational states in the human erythrocyte sugar-transport system, *Biochem. J.* 145:417-429.(Medline)

Boos, W., Gordon, A. S., Hall, R. E., and Price, H. D. (1972) Transport properties of the galactose-binding protein of *Escherichia coli*. Substrate induced conformational change. *J. Biol. Chem.* 247:917-924.(Medline)

Cairns, M.T., Elliot, D.A., Scudder, P.R. and Baldwin, S.A. (1984) Proteolytic and chemical dissection of the human erythrocyte glucose transporter, *Biochem. J.* 221:179-188.(Medline)

Cairns, M.T., Alvarez, J., Panico, M., Gibbs, A.F., Morris, H.R., Chapman, D. and Baldwin, S.A. (1987) Investigation of the structure and function of the human erythrocyte glucose transporter by proteolytic dissection, *Biochim. Biophys. Acta* 905:295-310.(Medline)

Cheng, A., van Hoek, A.N., Yaeger, M., Verkman, A.S., and Mitra, A.K. (1997) Three-dimensional organization of a human water channel, *Nature* 387:627-630.(Medline)

Christensen, H. N. (1975) *Biological Transport*. Benjamin, Reading, Mass.

Christensen, H. N., Clifford, J. B., and Oxender, D. L. (1963) Stereospecificity of the transport of *tert*-leucine. *Biochim. Biophys. Acta* 78:206-213.

Colville, C.A., Seatter, M.J. and Gould, G.W. (1993) Analysis of the structural requirements of sugar binding to the liver, brain and insulin-responsive glucose transporters expressed in oocytes, *Biochem. J.* 294:753-760.(Medline)

Critchley, A.J. and Lowe, A.G. (1991) Single half-turnovers of the glucose transporter of the human erythrocyte, *Biochem. Soc. Trans.* 19:417S.(Medline)

Davies, A., Meeran, K., Cairns, M.T. and Baldwin, S.A. (1987) Peptide-specific antibodies as probes of the orientation of the glucose transporter in the human erythrocyte membrane, *J. Biol. Chem.* 262:9347-9352.(Medline)

Deen, P.M. and Knoers, N.V.A.M. (1998) Physiology and pathophysiology of the aquaporin-2 channel, *Curr. Opin. Nephrol. Hypertens.* 7:37-42.(Medline)

Deen, P.M. and van Os, C.H. (1998) Epithelial aquaporins, *Curr. Opin. Cell Biol.* 10:435-442.(Medline)

DeFronzo, R.A. (1997) Pathogenesis of type-2 diabetes - metabolic and molecular implications for identifying diabetes genes, *Diabetes Rev.* 5:177-269.

Denker, B.M., Smith, B.L., Kuhajda, F.P. and Agre, P. (1988) Identification, purification, and characterization of a novel M_r 28,000 integral membrane protein from erythrocytes and renal tubules, *J.*

*Biol. Chem.*263:15634-15642.(Medline)

Deves, R. and Krupka, R.M. (1978) Cytochalasin B and the kinetics of inhibition of biological transport: a case of asymmetric binding to the glucose carrier, *Biochim. Biophys. Acta.* 510:339-348.(Medline)

Echevarria, M., Windhager, E.E. and Frindt, G. (1996) Selectivity of the renal collecting duct water channel aquaporin-3, *J. Biol. Chem.* 271:25079-25082.(Medline)

Finkelstein, A. and Andersen, O.S. (1981) The gramicidin A channel: a review of its permeability characteristics with special reference to the single-file aspect of transport, *J. Membr. Biol.* 59:155-171. (MedLine)

Fox, C. F., and Wilson, G. (1968) The role of a phosphoenolpyruvate-dependent kinase in β-glucoside catabolism in *Escherichia coli. Proc. Natl. Acad. Sci. USA* 59:988-995.(Medline)

Fruhbeck, G. and Salvador, J. (2000) Relation between leptin and the regulation of glucose metabolism, *Diabetologia* 43:3-12. (MedLine)

Fu, D., Libson, A., Miercke, L.J., Weitzman, C., Nollert, P., Krucinski, J. and Stroud, R.M. (2000) Structure of a glycerol- conducting channel and the basis for its selectivity, *Science* 290:481-486. (MedLine)

Fushimi, K., Sasaki, S. and Marumo, F. (1997) Phosphorylation of serine 256 is required for cAMP-dependent regulatory exocytosis of the aquaporin-2 water channel, *J. Biol. Chem.* 272:14800-14804.(Medline)

Gorga, F. R., and Lienhard, G. E. (1981) Equilibria and kinetics of ligand binding to the human erythrocyte glucose transporter. Evident for an alternative conformational model, *Biochemistry* 20:5108-5113.(Medline)

Gould, G.W. and Holman, G.D. (1993) The glucose transporter family: structure, function and tissue-specific expression, *Biochem. J.* 295:329-341. (MedLine)

Hediger, M.A., Coady, M.J., Ikeda, T.S. and Wright, E.M. (1987) Expression cloning and cDNA sequencing of the Na+/glucose co-transporter, *Nature* 330:379-381.(Medline)

Henderson, P.J. (1993) The 12-transmembrane helix transporters, Curr. Opin. Cell Biol. 5:708-721.(Medline)

Hirayama, B.A., Loo, D.D. and Wright, E.M. (1994) Protons drive sugar transport through the

Na+/glucose cotransporter (SGLT1), *J. Biol. Chem.* 269:21407-21410.(Medline)

Hirayama, B.A., Loo, D.D. and Wright, E.M. (1997) Cation effects on protein conformation and transport in the Na^+/glucose cotransporter, *J. Biol. Chem.* 272:2110-2115.(Medline)

Holman, G.D. and Rees, W.D. (1987) Photolabelling of the hexose transporter at external and internal sites: fragmentation patterns and evidence for a conformational change, *Biochim. Biophys. Acta* 897:395-405.(Medline)

Holman, G.D., Kozka, I.J., Clark, A.E., Flower, C.J., Saltis, J., Habberfield, A.D., Simpson, I.A. and Cushman, S.W. (1990) Cell surface labeling of glucose transporter isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester, *J. Biol. Chem.* 265:18172-9.(Medline)

Hotamisligil, G.S. and Spiegelman, B.M. (1994) Tumor-necrosis-factor- α - a key component of the obesity-diabetes link, *Diabetes*43:1271-1278. (MedLine)

Hresko, R.C., Kruse, M., Strube, M. and Mueckler, M. (1994) Topology of the Glut 1 glucose transporter deduced from glycosylation scanning mutagenesis, *J. Biol. Chem.* 269:20482-20488.(Medline)

Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E., and Higgins, C. (1990) Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* 346:362-365.(Medline)

Ishibashi, K., Sazaki, S., Fishimi, K., Uchida, S., Kuwahara, M., Saito, H., Furukawa, T., Nakajima, K., Yamaguchi, Y., Gojobori, T. and Marumo, F.(1994) Molecular cloning and expression of a member of the *aquaporin* family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells, *Proc. Natl. Acad. Sci.* 91:6269-6273.(Medline)

Ishibashi, K., Kuwahara, M., Gu, Y., Kageyama, Y., Tohsaka, A., Suzuki, F., Marumo, F. and Sasaki, S. (1997) Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol, and urea, *J. Biol. Chem.* 272:20782-20786.(Medline)

Jenq, W., Cooper, D.R., Bittle, P. and Ramirez, G. (1999) Aquaporin-1 expression in proximal tubule epithelial cells of human kidney is regulated by hyperosmolarity and contrast agents, *Biochem. Biophys. Res. Commun.* 256:240-248. (MedLine)

Kaback, H. R. (1968) The role of the phosphoenolpyruvate- phosphotransferase system in the transport of sugars by isolated membrane preparations of *Escherichia coli. J. Biol. Chem*. 243:3711-3724.(Medline)

Kanai, F., Nishioka, Y., Hayashi, H., Kamohara, S., Todaka, M. and Ebina, Y. (1993) Direct demonstration of insulin-induced GLUT4 translocation to the surface of intact cells by insertion of a c-myc epitope into an exofacial GLUT4 domain, *J. Biol. Chem.* 268:14523-14526.(Medline)

Kartner, N., Hanrahan, J. W., Iensen, T. J., Naismith, A. L. Sun, S., Ackerley, C. A., Reyes, E. F., Tsui, L. C., Rommens, J. M., Bear, C. E., and Riordan, J. R. (1991) Expression of the cystic fibrosis gene in nonepithelial invertebrate cells produce a regulated anion conductance. *Cell* 64:681-691.(Medline)

Katsura, T., Gustafson, C.E., Ausiello, D.A. and Brown, D. (1997) Protein kinase A phosphorylation is involved in regulated exocytosis of aquaporin-2 in transfected LLC-PK1 cells, *Am. J. Physiol.: Renal, Fluid and Electrolyte* 41:F817-F822.(Medline)

Kelker, N. E., and Anderson, R. L. (1972) Evidence for vectorial phosphorylation of *n*-fructose by intact cells of *Aerobacter aerogenes. J. Bacteriol.* 112:1441-1443.(Medline)

Kennedy, E. P., and Scarborough, G. A. (1967) Mechanism of hydrolysis of *o*-nitrophenyl--galactoside in *Staphylococcus aureus* and its significance for theories of sugar transport. *Proc. Natl. Acad. Sci. USA.* 58:225-228.(Medline)

King, L.S. and Agre, P. (1996) Pathophysiology of the aquaporin channels, *Annu. Rev. Physiol.* 58:619-648.(Medline)

King, L. S. , Nielsen, S. & Agre, P. (1996) Aquaporin-1 water channel protein in lung: ontogeny, steroid-induced expression, and distribution in rat, *J. Clin. Invest.* 97: 2183-2191. (MedLine)

Koyama, Y., Yamamoto, T., Kondo, D., Funaki, H., Yaoita, E., Kawasaki, K., Sato, N., Hatakeyama, K. and Kihara, I. (1997) Molecular cloning of a new aquaporin from rat pancreas and liver, *J. Biol. Chem.* 272:30329-30333.(Medline)

Kuriyama, H., Kawamoto, S., Ishida, N., Ohno, I., Mita, S., Matsuzawa, Y., Matsubara, K. and Okubo, K. (1997) Molecular cloning and expression of a novel human aquaporin from adipose tissue with glycerol permeability, *Biochem. Biophys. Res. Commun.* 241:53-58.(Medline)

Lee, M.D., King, L.S. and Agre, P. (1997) The aquaporin family of water channel proteins in clinical medicine, *Medicine* (Baltimore). 76:141-156.(Medline)

Leitch, V. V., Agre, P. and King, L.S. (2001) Altered ubiquitination and stability of aquaporin-1 in hypertonic stress, *Proc. Natl. Acad. Sci. USA* 98:2894-2898. (MedLine)

Li, H., Lee, S. and Jap, B.K. (1997) Molecular design of aquaporin-1 water channel as revealed by

electron crystallography, *Nature Struct Biol.* 4:263-265.(Medline)

Lowe, A.G. and Walmsley, A.R. (1987) A single half-turnover of the glucose carrier of the human erythrocyte, *Biochim. Biophys. Acta.* 903:547-550.(Medline)

Ma, T., Yang, B. and Verkman, A.S. (1996) Gene structure, cDNA cloning, and expression of a mouse mercurial-insensitive water channel, *Genomics* 33:382-388.(Medline)

Ma, T., Yang, B. and Verkman, A.S. (1997) Cloning of a novel water and urea-permeable aquaporin from mouse expressed strongly in colon, placenta, liver, and heart, *Biochem. Biophys. Res. Commun.* 240:324-328.(Medline)

Ma, T., Yang, B., Gillespie, A., Carlson, E.J., Epstein, C.J. and Verkman, A.S. (1998) Severely impaired urinary concentrating ability in transgenic mice lacking aquaporin-1 water channels, *J. Biol. Chem.* 273:4296-4299.(Medline)

Marinelli, R.A., Pham, L., Agre, P. and LaRusso, N.F. (1997) Secretin promotes osmotic water transport in rat cholangiocytes by increasing aquaporin-1 water channels in plasma membrane. Evidence for a secretin-induced vesicular translocation of aquaporin-1, *J. Biol. Chem.* 272:12984-12988.(Medline)

Marples, D., Knepper, M.A., Christensen, E.I. and Nielsen, S. (1997) Redistribution of aquaporin-2 water channels induced by vasopressin in rat kidney inner medullary collecting duct, *Am. J. Physiol.: Cell Physiol.* 42:C655-C664.

Matsumura, Y., Uchida, S., Rai, T., Sasaki, S. and Marumo, F. (1997) Transcriptional regulation of aquaporin-2 water channel gene by cAMP, *J. Am. Soc. Nephrol.* 8:861-867.(Medline)

Mueckler, M., Caruso, C., Baldwin, S.A., Panico, M., Blench, I., Morris, H.R., Allard, W.J., Lienhard, G.E. and Lodish, H.F. (1985) Sequence and structure of a human glucose transporter, *Science* 229:941-945.(Medline)

Mueckler, M. and Makepeace, C. (1999) Transmembrane segment 5 of the Glut1 glucose transporter is an amphipathic helix that forms part of the sugar permeation pathway, *J. Biol. Chem.* 274:10923-10926.(Medline)

Murata, K., Mitsuoka, K., Hirai, T., Walz, T., Agre, P., Heymann, J.B., Engel, A. and Fujiyoshi, Y. (2000) Structural determinants of water permeation through aquaporin-1, *Nature* 407:599-605. (MedLine)

Nielsen, S., King, L.S., Christensen, B.M. and Agre, P., (1997) Aquaporins in complex tissues. II. Subcellular distribution in respiratory and glandular tissues of rat, *Am. J. Physiol.: Cell Physiology*

42:C1549-1561.(Medline)

Panayotova-Heiermann, M., Loo, D.D. and Wright, E.M. (1995) Kinetics of steady-state currents and charge movements associated with the rat Na^+/glucose cotransporter, *J. Biol. Chem.* 270:27099-27105.(Medline)

Park, J.H., Saier, M.H., Jr. (1996) Phylogenetic characterization of the MIP family of transmembrane channel proteins, *J. Membr. Biol.* 153:171-180.(Medline)

Pasternak, C.A., Aiyathurai, J.E., Makinde, V., Davies, A., Baldwin, S.A., Konieczko, E.M. and Widnell, C.C. (1991) Regulation of glucose uptake by stressed cells, *J. Cell Physiol.* 149:324-331. (Medline)

Pessin, J.E., Thurmond, D.C., Elmendorf, J.S., Coker, K.J. and Okada, S. (1999) Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Location! Location! *J. Biol. Chem.* 274:2593-2596.(Medline)

Pomès, R. and Roux, B. (1996) Structure and dynamics of a proton wire: a theoretical study of H^+ translocation along the single-file water chain in the gramicidin A channel, *Biophys. J.* 71:19-39. (MedLine)

Preston, G.M. and Agre, P. (1991) Molecular cloning of red cell integral membrane protein of M_r28,000; a member of an ancient channel family, *Proc. Natl. Acad. Sci. USA* 88: 11110-11114.(Medline)

Preston , G.M., Carroll, T.P., Guggino, W.B. and Agre, P. (1992) Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein, *Science* 256:385-387.(Medline)

Preston, G.M., Jung, J.S., Guggiono, W.B. and Agre, P. (1993) The mercury sensitive residue of cysteine-189 in the CHIP28 water channel, *J. Biol.Chem.* 268:17-10.(Medline)

Ramm, G., Slot, J.W., James, D.E. and Stoorvogel, W. (2000) Insulin recruits GLUT4 from specialized VAMP2-carrying vesicles as well as from the dynamic endosomal/trans-Golgi network in rat adipocytes, *Mol. Biol. Cell* 11:4079-4091. (MedLine)

Rosenberg, T., and Wilbrandt, W. (1958) Uphill transport induced by counterflow. *J. Gen. Physiol.* 41:289-296.

Saito, T., Ishikawa, S.E., Sasaki, S., Fujita, N., Fushimi, K., Okada, K., Takeuchi, K., Sakamoto, A., Ookawara, S., Kaneko, T., Marumo, F. and Saito, T. (1997) Alteration in water channel AQP-2 by removal of AVP stimulation in collecting duct cells of dehydrated rats, *Am. J. Physiol.* 272:F183-191.(Medline)

Shanahan, M.F. and D'Artel-Ellis, J. (1984) Orientation of the glucose transporter in the human erythrocyte membrane. Investigation by *in situ* proteolytic dissection, *J. Biol. Chem.* 259:13878-13884.(Medline)

Shetty, M., Loeb, J.N. and Ismail-Beigi, F. (1992) Enhancement of glucose transport in response to inhibition of oxidative metabolism: pre- and posttranslational mechanisms, *Am. J. Physiol.* 262:C527-532.(Medline)

Shiels, A. and Bassnett, S. (1996) Mutations in the founder of the MIP gene family underlie cataract development in the mouse, *Nature Genet.* 12:212-215.(Medline)

Shulman, G.I. (2000) Cellular mechanisms of insulin resistance, *J. Clin. Invest.* 106:171-176. (MedLine)

Simoni, R. D., Levinthal, L. M., Kundig, F. D., Kunding, W., Anderson, B., Hartman, P. E., and Roseman, S. (1967) Genetic evidence for the role of a bacterial phosphotransferase system in sugar transport. *Proc. Natl. Acad. Sci. USA* 58:1963-1970.(Medline)

Simpson, I.A. and Cushman, S.W. (1986) Hormonal regulation of mammalian glucose transport, *Annu. Rev. Biochem.* 55:1059-1089.(Medline)

Slot, J.W., Geuze, H.J., Gigengack, S., James, D.E. and Lienhard, G.E. (1991a) Translocation of the glucose transporter GLUT4 in cardiac myocytes of the rat, *Proc. Natl. Acad. Sci. USA* 88:7815-7819.(Medline)

Slot, J.W., Geuze, H.J., Gigengack, S., Lienhard, G.E. and James, D.E. (1991b) Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat, *J. Cell Biol.* 113:123-135.(Medline)

Slotboom, D.J., Konings, W.N. and Lolkema, J.S. (2001) Glutamate transporters combine transporter- and channel-like features, *Trends Biochem. Sci.* 26:534-539. (MedLine)

Smith, B. L., and Agre, P. (1991) Erythrocyte M_r exists as a multi-subunit oligomer similar to channel proteins, *J. Biol. Chem.* 266:6407-6415.(Medline)

Steppan, C.M., Bailey, S.T., Bhat, S., Brown, E.J., Banerjee, R.R., Wright, C.M., Patel, H.R., Ahima, R.S. and Lazar, M.A. (2001) The hormone resistin links obesity to diabetes, *Nature* 409:307-312. (MedLine)

Sui, H., Han, B.G., Lee, J.K., Walian, P. and Jap, B.K. (2001) Structural basis of water-specific transport through the AQP1 water channel, *Nature* 414:872-878. (MedLine)

Tajkhorshid, E., Nollert, P., Jensen, M.O., Miercke, L.J., O'Connell, J., Stroud, R.M. and Schulten, K. (2002) Control of the selectivity of the aquaporin water channel family by global orientational tuning, *Science* 296:525-530. (MedLine)

Thorens, B., Charron, M.J. and Lodish, H.F. (1990) Molecular physiology of glucose transporters, *Diabetes Care* 13:209-218.(Medline)

Van Hoek, A.N., Hom, M.L., Luthjens, L.H., de Jong, M.D., Dempster, J.A. and van Os, C.H. (1991) Functional unit of 30 kDa for proximal tubule water channels as revealed by radiation inactivation, *J. Biol. Chem.* 266:16633-16635.(Medline)

Van Hoek, A.N., Luthjens, L.H., Hom, M.L., Van Os, C.H. and Dempster, J.A. (1992) a 30 kDa functional size for the erythrocyte water channel determined in situ by radiation inactivation, *Biochem. Biophys. Res. Comm.* 184:1331-1338.(Medline)

Van Steveninck, J. (1972) Transport and transport associated phosphorylation of galactose in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* 274:575-583.

Walmsley, A.R. (1988) The dynamics of the glucose transporter, *Trends Biochem. Sci.* 13:226-231.(Medline)

Walmsley, A.R., Martin, G.E. and Henderson, P.J. (1994) 8-Anilino-1-naphthalenesulfonate is a fluorescent probe of conformational changes in the D-galactose-H^+ symport protein of *Escherichia coli* , *J. Biol. Chem.* 269:17009-17019.(Medline)

Walmsley, A.R., Barrett, M.P., Bringaud, F. and Gould, G.W. (1998) Sugar transporters from bacteria, parasites and mammals: structure-activity relationships, *Trends Biochem. Sci.* 23:476-481.(Medline)

Walz, T., Typke, D., Smith, B.L., Agre, P. and Engel, A. (1995) Projection map of of aquaporin-1 determined by electron crystallography, *Nature Struct. Biol.* 2:730-732.(Medline)

Walz, T., Hirai, T., Murate, K., Heymann, J.B., Mitsuoka, K., Fujiyoshi, Y., Smith, B.L., Agre, P. and Engel, A. (1997) The three dimensional structure of aquaporin-1, , *Nature* 387:624-627.(Medline)

Wang, R. J., and Morse, M. L. (1968) Carbohydrate accumulation metabolism in *Escherichia coli*. I. Description of pleiotrophic mutants. *J. Mol. Biol.* 32:59-66.(Medline)

Widnell, C.C., Baldwin, S.A., Davies, A., Martin, S. and Pasternak, C.A. (1990) Cellular stress induces a redistribution of the glucose transporter, *FASEB J.* 4:1634-1637.(Medline)

Wood, W. A. (1966) Carbohydrate metabolism. *Annu. Rev. Biochem.* 35:521-558.(Medline)

Yamada, K., Tillotson, L.G. and Isselbacher, K.J. (1983) Regulation of hexose carriers in chicken embryo fibroblasts. Effect of glucose starvation and role of protein synthesis, *J. Biol. Chem.* 258:9786-9792.(Medline)

Zeidel, M.L., Ambudkar, S.V., Smith, B.L. and Agre, P. (1992) Reconstitution of functional water channels in liposomes containing purified red cell CHIP28 protein, *Biochemistry* 31:7436-7440. (MedLine)

20. The Cell Membrane:

Transport of Ions

The active transport of ions has many facets and variations. The translocation of a solute may be coupled to the translocation of another. These have been called secondary transports and are discussed in Section IV, below. When the two are translocated in the same direction, the transporter has been referred to as a *symport*; when in the opposite direction, as an *antiport*. The translocation of a solute can be powered by different mechanisms. In either a symport or an antiport, one of the solutes may be transferred in the direction of its electrochemical gradient and this transfer can pay for the active translocation of the accompanying solute. The active influx of sugars and amino acids in animal cells is powered by a symport in which Na^+ is translocated in the direction of its electrochemical gradient. One of the mechanisms for pumping Ca^{2+} out of cells depends on an antiport which transfers Na^+ in the direction of its gradient.

Other transporters are powered by the coupled hydrolysis of ATP and they function as ion-dependent ATPases.

I. THE P-ATPASES

The P-type ATPases are self phosphorylating enymes which use ATP to phosphorylate a conserved aspartic residue. These include the Ca^{2+}-ATPase of the plasma membrane or that of the sarcoplasmic reticulum (SR), the Na^+,K^+-ATPase of the plasma membrane, the gastric mucosa ATPase which transports H^+ outwards in exchange for K^+ and the H^+-ATPase of the plasma membrane of plants and yeasts maintains the intracellular pH and membrane potential (see Møller et al., 1996). The translocation

reactions of these ATPases are outlined in Eq. (1) to (3). In these equations, the location of the component, inside or outside, is indicated by the subscript *i* or *o*, respectively. These four are probably the only active transport systems coupled to ATP hydrolysis in the plasma membrane of animal cells.

$$2K_o^+ + 3Na_i^+ + MgATP_i \leftrightarrow MgADP_i + P_i + 3Na_o^+ + 2K_i^+ \quad (1)$$

$$H_o^+ + 2Ca_i^{2+} + MgATP_i \leftrightarrow MgADP_i + P_i + 2Ca_o^{2+} + H_i^+ \quad (2)$$

$$nK_o^+ + nH_i^+ + MgADP_i \leftrightarrow MgADP_i + P_i + nH_o^+ + nK_i^+ \quad (3)$$

Each of the four proteins responsible for the ATP-powered active transport of these ions has been isolated (Kyte, 1971; McLennan, 1969; Sachs et al., 1976; see Nakamoto and Slayman, 1989). The Ca^{2+} and the Na^+-K^+ transport systems have been reconstituted in artificial lipid membranes from the purified ATPases and phospholipids. The various transport ATPases resemble each other strikingly. All are polypeptides of approximately 900 to 1200 amino acids and their activity involves phosphorylation in an aspartate residue. The Ca^{2+} and Na^+,K^+-ATPases have been studied in more detail. They appear to share many similarities, in their amino acid sequence and in exhibiting similar hydrophobic segments and adenine nucleotide binding sites. All four involve the transport of one cation in one direction, and another in the opposite direction. Because of the stoichiometry of the exchange, at least the Ca^{2+} pumps and the Na^+, K^+-ATPase are electrogenic.

In its native form, the Na^+,K^+- ATPase has two polypeptides: a larger polypeptide (α of 94 to 106 kDa and a smaller sialoglycoprotein (β) of 41 to 52 kDa (Kyte, 1974), which may have a role in the transport and membrane assembly of the α-subunit (McDonough et al., 1990). The two polypeptides are present in equimolar amounts, but it is generally agreed that the α-component corresponds to the transporter; the β-subunit can be removed without changing the ATPase activity (Freytag, 1983). Furthermore, the very similar Ca^{2+}-ATPase of the SR lacks the smaller polypeptide.

The Na^+-K^+ transport system is associated with almost all cells that have been studied, including those of specialized tissues which carry out Na^+ transport, such as the kidney and the rectal gland of elasmobranchs which function to excrete Na^+. The Ca^{2+}-ATPase functions to maintain a low cytoplasmic Ca^{2+} concentration. One Ca^{2+}-ATPase is associated with the plasma membrane, while another distinct ATPase functions to sequesters Ca^{2+} in the sarcoplasmic reticulum of muscle cells (referred to as CERCA)(seeChapter 23). Similar Ca^{2+} sequestering vesicles are likely to be present in the cytoplasm of other cells. In contrast, the H^+,K^+-ATPase is the means by which acid is accumulated by the gastric mucosa (Sachs et al., 1976). A similar ATPase in the fungus, *Neurospora*, (Scarborough, 1980) transports H^+ outward and is responsible for the high membrane potential of its cells; in this case, the transport is strongly *electrogenic*.

This chapter primarily treats the functional significance and characteristics of the Na^+, K^+-ATPase transport system. The next chapter will discuss the characterization of the proteins responsible for the transport of cations, together with possible models of transport. The Na^+, K^+-ATPase and the Ca^{2+}-ATPases are so similar that the two are frequently discussed interchangeably in that chapter.

II. FUNCTIONAL SIGNIFICANCE OF THE Na^+-K^+ TRANSPORT SYSTEM

In all cells, there seems to be some mechanism for controlling the concentration of ions in the internal medium. A high internal concentration of K^+ and a low concentration of Na^+ is generally the rule. The range of internal K^+ concentration in vertebrates is narrow: 100 to 200 mM. In freshwater organisms, the internal level of K^+ is frequently much smaller, 15 to 30 mM, but this is remarkably high compared to the external environment, in which K^+ may be present only in trace amounts. The capacity to control the internal concentration is such that certain organisms can grow at extreme conditions of salinity and still concentrate K^+ selectively, despite the high Na^+ concentration of the medium and the relative absence of K^+. *Halobacterium salinarium* concentrates K^+ in the face of an external NaCl concentration of 4 M and can attain the remarkable internal K^+ concentration of 4 M (Christian and Waltho, 1962). Examples of the concentrations of Na^+ and K^+ in various organisms are listed in Table 1 (Steinbach, 1963).

The reasons for the universality of these ion distributions are still a subject for speculation. As we shall see in Chapter 22, the plasma membrane's resting and action potentials depend on this ion concentration imbalance between the inside and the outside of the cell. These potentials play a role not only in the signal conduction of nerves and muscle in higher organisms, but in other functions. In protists, they play a role in their responses to environmental stimuli. Furthermore, the internal concentration of cations has to be controlled (in this case by pumping out Na^+) to maintain cell volume within physiological limits. The Na^+ tends to accumulate inside (Tosteson and Hoffman, 1960) to neutralize the negative charges of the macromolecules trapped in the cytoplasm. Inhibition of the Na^+ pumping activity therefore leads to osmotic swelling. There is no doubt that the exclusion of Na^+ plays a role in cell function and integrity in at least animal cells. However, it is difficult to argue that these needs are universal. Some organisms may not use electrical signals and the rigid walls of plant cells and microorganisms are effective in limiting swelling. Other reasons may therefore exist for the universality of the control of the cell's internal environment. Evidence accumulated over the years indicates that K^+ is generally required for growth (Steinbach, 1963). Potassium ions are required for protein synthesis in a number of unrelated organisms or preparations (Table 2). This requirement probably explains the effect of K^+ on growth. In addition, K^+ is required for maximal activity of a number of enzymes concerned primarily with other functions, such as 6-phosphofructokinase and pyruvate kinase (Lubin, 1964 lists a total of 23 important enzymes).

Table 1 Selected Values for Na^+ and K^+

	Erthyrocytes (mmol/kg wet wt.)		Plasma or Serum (mmol/kg wet wt.)	
Animal	**Na**	**K**	**Na**	**K**
Human	11	91	138	4.2
Sheep (average)	82	11	160	4.8
Dog	106	5	150	4.4
Rabbit	16	99	158	4.1
Elephant seal	95	7	142	4.5
Rat	12	100	151	5.9
Duck	7	112	141	6.0
Chicken	18	119	154	6.0
Dolphin (mammal)	13	99	153	4.3
Fish (mackerel)	-	-	183	10.0
Frog	-	-	105	4.8
Reptile (turtle)	-	-	140	4.6

Plant	**Na**	**K**
Asparagus	1	50
Beet, leaves	60	130
Beet, roots	30	75

Lettuce	6	60
String beans	1	60
Broccoli	7	75
Celery	50	90
Yucca, leaves	30	120
Yucca, stem	19	70
Vetch	40	156
Salt grass (sea shore)	70	45
Salt bush	23	63
Rye, tops	4	120
Clover, tops	20	150

The K^+ requirement for protein synthesis is elegantly shown in an experiment with a mutant strain of *E. coli* that is incapable of transporting K^+. The cells can be easily depleted of K^+ and the internal concentration of K^+ mirrors that in the medium. The cells are incubated in a medium containing leucine labeled with [^{14}C]. The ordinate in Fig. 1 (Lubin and Ennis, 1964) represents the labeled leucine incorporated by the bacteria, the abscissa, the intracellular K^+ concentration. In this experiment, leucine incorporation serves as an indicator of protein synthesis. Clearly, the K^+ inside the cells is needed for the incorporation of amino acid into protein. Similar results were obtained in experiments carried out with *B. subtilis* mutants and mammalian tumor cells in culture in which the K^+ transport has been inhibited by the drug amphotericin B.

From these experiments, it is clear that K^+ accumulation has a fundamental role in protein synthesis. In addition, it activates a number of metabolic enzymes.

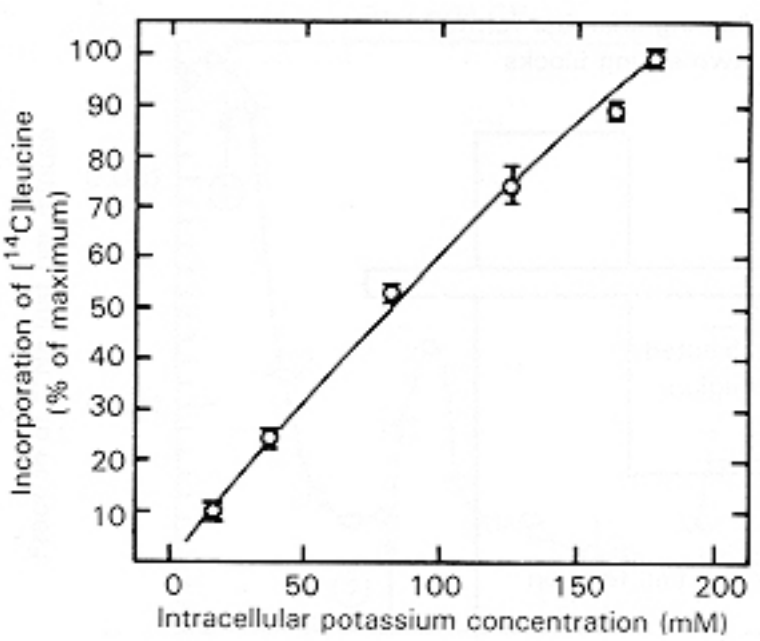


Fig. 1 Incorporation of [^{14}C]leucine into protein at various concentrations of intracellular K^+ at 37C. Reproduced from *Biochimica et Biophysica Acta*, vol.80, M. Lubin and H. Ennis, pp.614-631, copyright ©1964 with permission from Elsevier Science.

III. COUPLING OF ATP HYDROLYSIS TO THE TRANSPORT OF Na^+

The active transport of Na^+, K^+, Ca^{2+}, or H^+ in the plasma membrane is coupled to the hydrolysis of ATP. For practical reasons, the Na^+, K^+-ATPases studied most intensely have been those of giant nerve cell axons and red blood cells. As we shall see, they have obvious advantages. The high internal K^+ and the low Na^+ concentration of these cells, as in many others, are maintained in the presence of high Na^+ and low K^+ concentrations in the external medium. Here we will consider the evidence for an active transport mechanism that functions as an ATPase.

Table 2 Dependence of Protein Synthesis on K^+

System	Reference
Liver extract	Sachs, H. *J. Biol. Chem.* 228:23 (1957)
Pancreas extract	Gazzinelli, G., and Dickman, S.R. *Biochim. Biophys. Acta* 61:980 (1962)
Sea urchin egg	Hultin, T. *Exp. Cell Res.* 25:405 (1961)

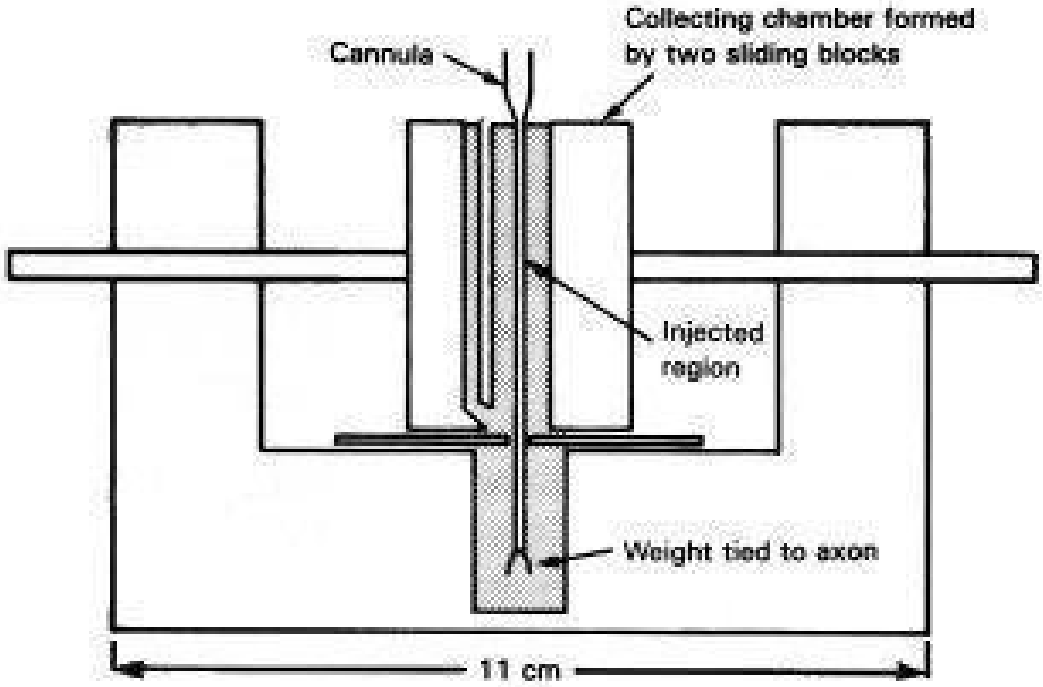
E. coli intact mutant or extract	Lubin, M. *Nature* 213:415 (1967)
Bacillus subtilis intact mutant	Lubin, M. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 23:994 (1964)
Sarcoma 180, intact, pharmacological manipulation	Lubin, M. *Nature* 213:451 (1967)

Giant axons of certain invertebrates are ideal experimental material for the study of ionic transfers and electrical phenomena. Their size, as much as 0.5 mm in diameter, greatly facilitated manipulation, for example, in the internal injection of compounds with micropipettes, even in the days when the fabrication of pipettes was fairly primitive. In addition, considerable data can be collected from a single experiment, since these axons are sturdy enough to allow prolonged observations. With the squid axon, a number of incisive experiments have explored a whole spectrum of problems related to ion transfer and excitability, including that of the coupling of Na^+ transport to energy expenditure.

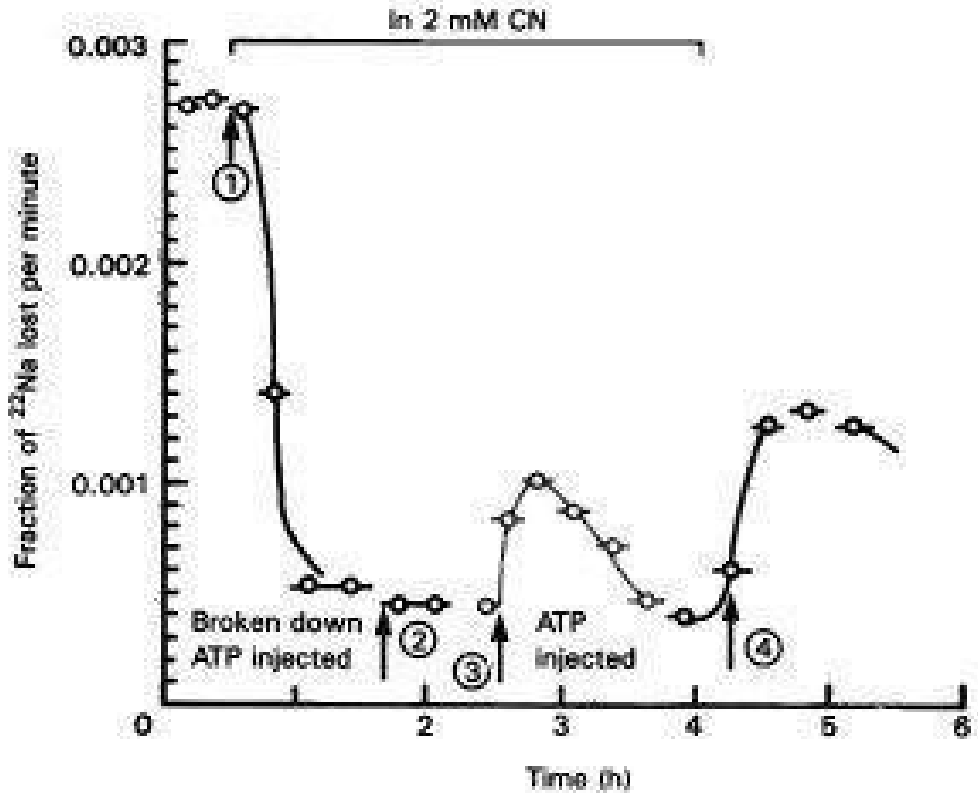
When the radioactive isotope [$^{22}Na^+$] is injected into the nerve fiber, the exit of Na^+ can be followed by placing the axon in unlabeled medium and simply measuring the level of [$^{22}Na^+$] in the external medium. Oxidative metabolism can be blocked with certain inhibitors (e.g., CN^-). The role of high-energy phosphates in Na^+ transport can be tested directly by injecting these compounds into the axon after blocking the metabolic reactions that would regenerate them (see Fig. 2a). Results of such an experiment are shown in Fig. 2b and c. The addition of 2 mM cyanide to the system considerably reduces the Na^+ efflux from the axon (arrow 1, Fig. 2b or c). However, injections of ATP (arrow 4) increase the efflux of [$^{22}Na^+$] significantly, whereas the injection of breakdown products of ATP is ineffective (arrow 2). The results with arginine phosphate, another phosphate ester with a high phosphate group-transfer potential, are similar (Fig. 2c). Arginine phosphate is thought to transfer its terminal phosphate to ADP to regenerate ATP. In the experiments shown, the effects of the ATP or arginine phosphate are transient, but they can be prolonged for several hours if higher concentrations are used.

Although injections of ATP or arginine phosphate into the axon are effective, their addition to the medium is not. Thus the system is asymmetric, as might be expected in a transport ATPase which pumps Na^+ outward. In both experiments, after about 4 h, the cyanide is washed off and the high rate of Na^+ efflux (arrow 4) returns.

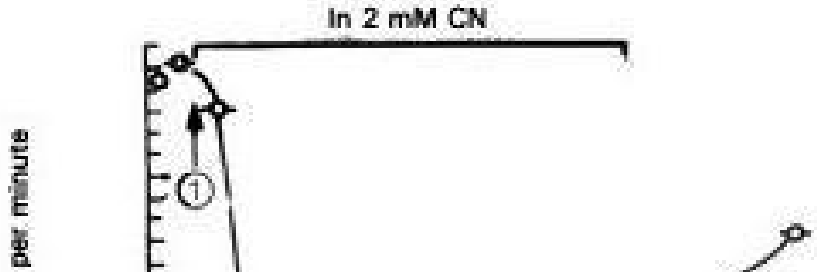
From these experiments, it is relatively simple to calculate the Na/P ratio in much the same way as done for the mitochondrial system. The ratio was found to be about 0.7 using either arginine phosphate or ATP. More recent results show that the value is more likely to be close to 3. The lower value is probably the result of the activity of other cytoplasmic ATPases or phosphatases. Regardless of stoichiometry, these results agree with the notion that an ATP-powered transport is taking place in these axons.


Collecting chamber formed by two sliding blocks
Cannula
Injected region
Weight tied to axon
11 cm

(a)


In 2 mM CN
0.003
0.002
0.001
Fraction of ^{22}Na lost per minute
Broken down ATP injected
ATP injected
0
2
3
4
5
6
Time (h)

(b)


In 2 mM CN
per minute

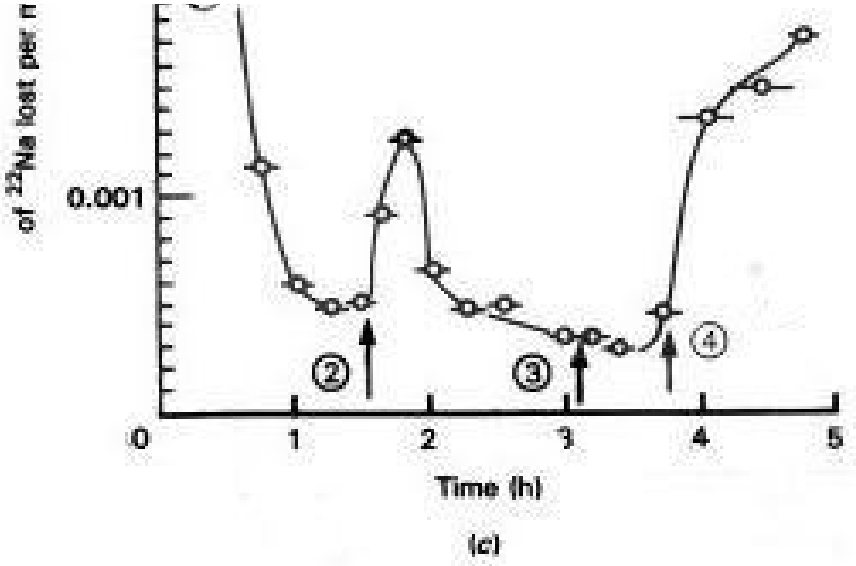


Fig. 2 (a) System for measuring [^{22}Na] efflux in giant axon. (b)[^{22}Na] efflux; the effect of CN^- and ATP and arginine phosphate. Reproduced with permission from P.C. Caldwell, et al. , *Journal of Physiology*, 152:561-590. Copyright ©1960 The Physiological Society, Oxford, England.

Similar experiments have been carried out with intact red blood cells or isolated membranes. Erythrocytes are generally sturdy and, of course, are available in large quantities. Analysis of the internal medium or the suspending solution, can be carried out readily after separating the cells or other vesicles from the medium by centrifugation or filtration. The erythrocytes can be lysed (*hemolyzed*) by hypotonic conditions or the use of detergents. The intact cells are shaped like slightly concave plates. When suspended in a hypoosmotic medium, they swell to a spherical shape so that the surface membrane is stretched. At this point, they become extremely permeable and lose their internal contents, including hemoglobin; what is left are the *erythrocyte ghosts*. Because of their high permeability, the ghosts tend to equilibrate with the external medium. Interestingly, the empty sacs can at least partially regain their low permeability under the appropriate conditions, as if their membranes were capable of "resealing." Because of this property, it is possible to vary the internal contents of the cells not by injection but by hemolysis in media containing the desired components and then resealing them. This procedure is illustrated in Fig. 3 (Whittam, 1962), which shows the good correlation between the Na^+ composition inside the resealed ghosts (ordinate) and that of the suspension medium used for hemolysis (abscissa). Comparable results have been shown for other solutes contained in the hemolysis medium.

Ghost fragments are more amenable to biochemical studies, since the absence of permeability barriers avoids unnecessary complications in experimental design (for example, changes in concentration of ions due to the presence of a permeability barrier). A number of other cell membrane preparations, such as leaky brain and kidney microsomes, are similar to these ghost fragments.

Incorporation of ATP into the ghosts gives results very similar to those obtained with the giant axon. Such an experiment is represented in Fig. 4. Curve 1 shows the results obtained with ATP, and curve 2

shows the inhibition of the ATP effect by the use of an inhibitor of the Na^+ transport system, ouabain (G-strophanthin, a glycoside used as a cardiac drug). Table 3 (Hoffman, 1962) compares the Na^+ exit in the presence of ATP (row 1) with that occurring in its absence (row 5). The effects of several other compounds of high phosphate group transfer potential (rows 2-4) are also shown. From the results, it can be concluded that ATP serves as the source of energy for the translocation of Na^+ in the two systems discussed.

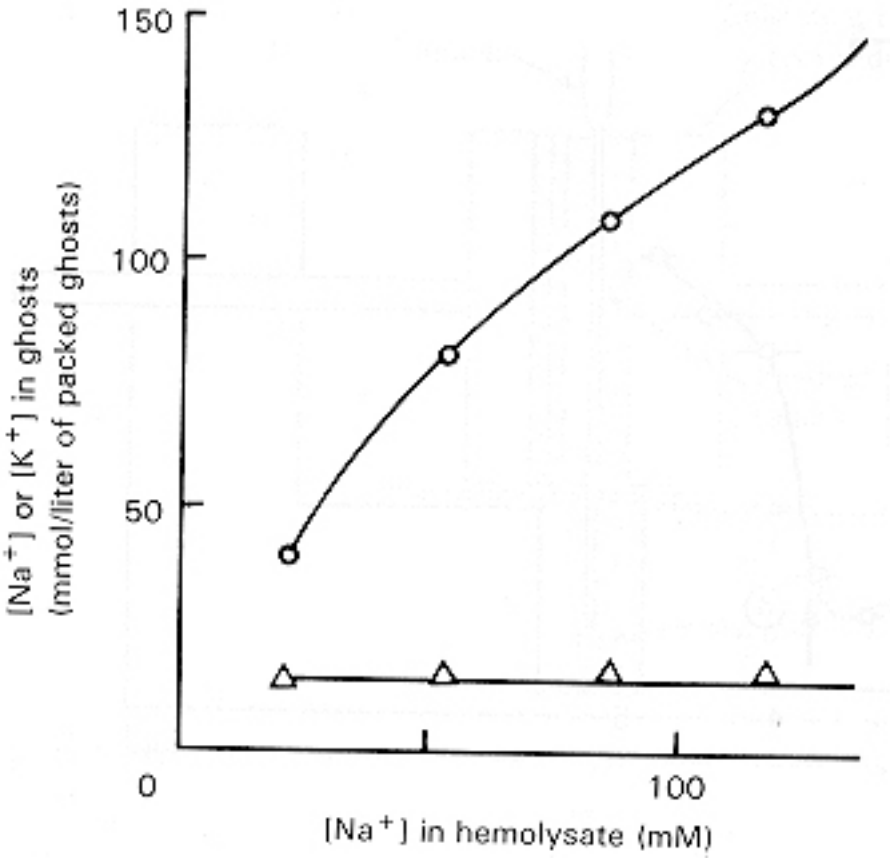


Fig. 3 Ionic composition of resealed ghosts as a function of the composition of the hemolysate. (○) Na^+, (Δ) K^+. From *Biochemistry Journal*, 84:110-118, copyright ©1962 The Biochemical Society, London.

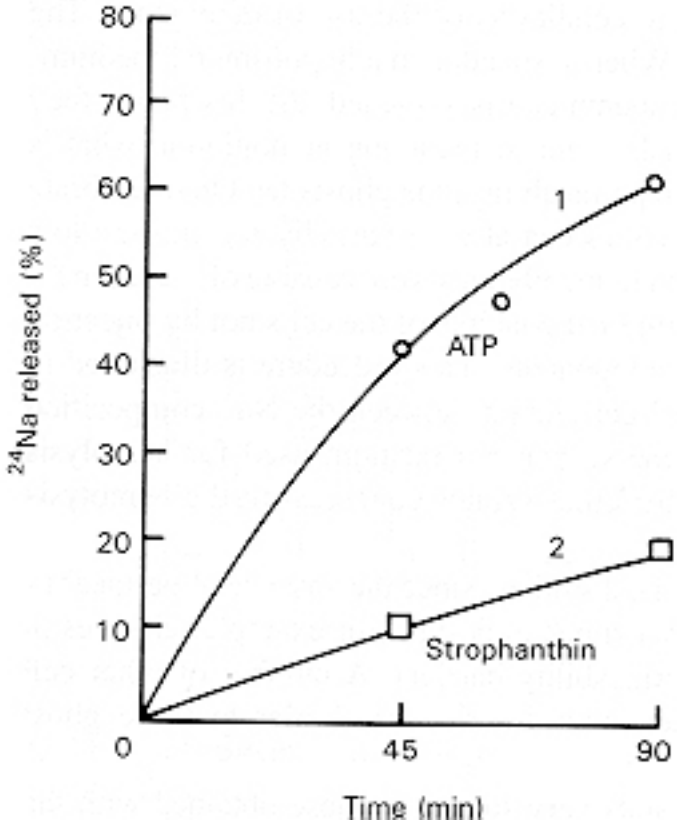


Fig. 4 Release of [^{24}Na] induced by ATP (curve 1) and its inhibition by ouabain (curve 2). Reproduced from J. Hoffman, *Circulation* 26:1201-1213, 1962, by permission of the American Heart Association, Inc.

Table 3 Influence of Different Incorporated Nucleotides on the Activation of Transport in Depleted Ghosts

	^{24}Na released in 80 min (%)	
Incorporated substrate	**Alone**	**+Ouabain**
1. ATP	50.6	22.8
2. ITP	27.6	22.2
3. GTP	25.9	25.5
4. UTP	33.4	33.2
5. Control	29.1	24.2

Reproduced from J. Hoffman, *Circulation* 26:1201-1213, 1962, by permission of the American Heart Association, Inc.

The effect of ouabain is a useful one: this drug inhibits the active transport of Na^+ in many different kinds of cells and organisms. With it, the active transport of Na^+ can easily be distinguished from nonspecific events, such as those brought about by a change in permeability. A parallel change would be expected for an ATPase involved in transport; other unrelated ATPases are not likely to be affected. The ATPases of both nerve and erythrocyte ghosts appear to be sensitive to the inhibitor. At least in squid axon, which has been studied in this respect, cardiac glycosides are effective only when added to the outside; their injection into the axon does not inhibit Na^+ transport. This is a polarity opposite to that of ATP, which is effective only on the inside.

IV. THE Na^+ K^+-TRANSPORT ATPase SYSTEM

The experiments just discussed show that the energy available from the hydrolysis of ATP can be used for the transport of Na^+ (and K^+). In order to function as an ion pump powered by ATP hydrolysis, the ATPase must reside in the plasma membrane. Erythrocyte ghosts are virtually plasma membranes. The results depicted in Fig. 4 were obtained using ghosts and they show considerable oubain-sensitive ATPase. Similar evidence has also been obtained in neurons, where the individual nerve cell sheaths can be mechanically isolated by microdissection, as shown in Fig. 5 (Cummins and Hyden, 1962). The activity of the material isolated in this way is shown in Table 4 (Cummins and Hyden, 1962). It is interesting to see that, in this case, ouabain is capable of inhibiting the membrane ATPase entirely. These results leave little doubt that the Na^+, K^+-transport ATPase is located in the plasma membrane. Many other membrane preparations from widely different tissues, including plants (Lai and Thomson, 1971), have been shown to contain Na^+ and K^+-dependent ATPases.

These experiments opened the way for a more detailed examination of this transport. The maintenance of a high internal concentration of K^+ and a low internal concentration of Na^+ may be explained most simply by a model, such as that represented in Fig. 6 (Glynn, 1957). Here, a high phosphate group transfer potential form of a carrier, Y, in the inner phase (step 1) permits the translocation of Na^+ to the external phase (steps 2-4) against an electrochemical gradient. In this process, Na^+ complexes with the carrier Y (step 2) and, after movement of the complex NaY to the external surface (step 3), the NaY complex dissociates (step 4). The transition from a high-energy form of the carrier (Y) to a low-energy form (X) (step 5) fulfills the thermodynamic requirement of an energy expenditure. It also presents K^+ with a carrier for its translocation to the internal phase (steps 6-8).

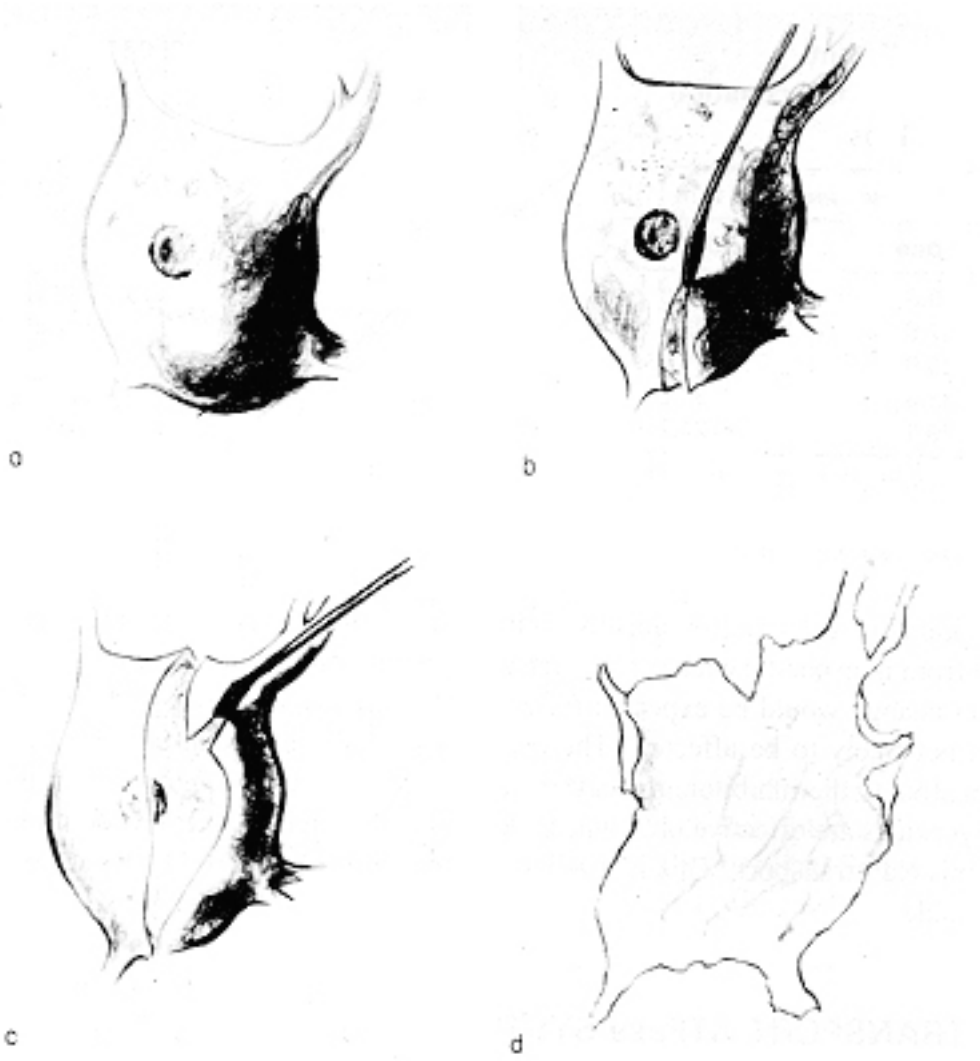


Fig. 5 Microsurgical procedure to obtain nerve cell membrane. (a) Whole nerve cells; (b) initial incision; (c) the incision; (d) final membrane preparation. Reproduced from *Biochimica et Biophysica Acta*, vol. 60, J. Cummins and H. Hyden, pp.271-283. Copyright ©1962 with permission from Elsevier Science.

The cyclic operation of this model could account for the Na^+ efflux and K^+ influx coupled to ATP hydrolysis. NaY and KX could be moved inside the membrane from one membrane interface to the other. However, a mechanism in which the binding groups of the transporter are alternatively exposed to the two membrane interfaces through a conformational rearrangement is more likely (see Chapter 21).

The form of the energy expended is not specified in Fig. 6; however, the evidence discussed for the Na^+-K^+ transport shows that ATP hydrolysis is coupled to the transport, so that the model can be modified to include this detail, as represented in Fig. 7.

Table 4 Effect of Ouabain on the ATPase of Nerve Cell Membrane[a]

Concentration of ouabain (M)	ATPase activity (pmoles ATP hydrolyzed per membrane/h)
0	0.4
2×10^{-6}	0.2
2×10^{-5}	0

Reproduced with permission from J. Cummins and H. Hyden, *Biochim. Biophys. Acta* 60: 271-283. Copyright ©1962 Elsevier Publishing, Amsterdam.

[a] Composition of saline was NaCl, 66 mM,; KCl, 33 mM; $MgCl_2$, 5 mM, Tris, 25 mM (pH 8.0); final volume 6 µl. 15 pmol of radioactive ATP added (250 counts/min). Total of 10 experiments consisting of 8-10 membranes each.

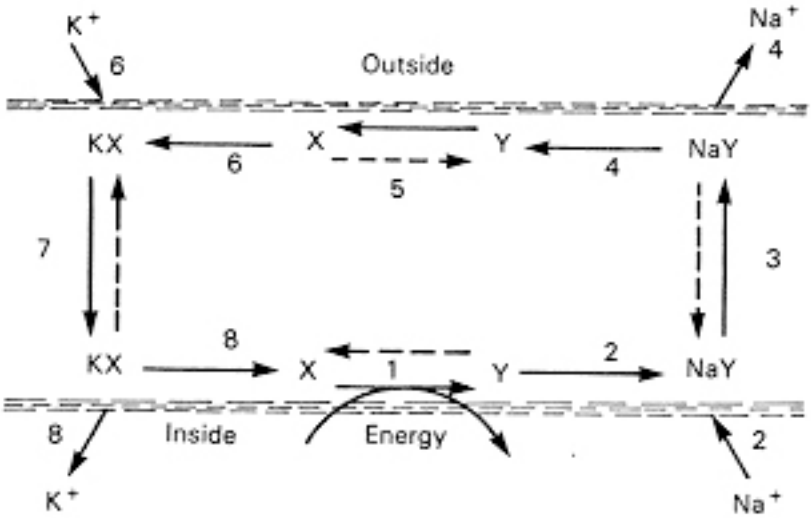


Fig. 6 Shaw's hypothesis of a K^+ carrier that is converted to an Na^+ carrier by the expenditure of energy. Reproduced with permission from *Progress in Biophysics and Molecular Biology*, 8:241-307, I. M. Glynn, copyright ©1957 Pergamon Journals, Ltd.

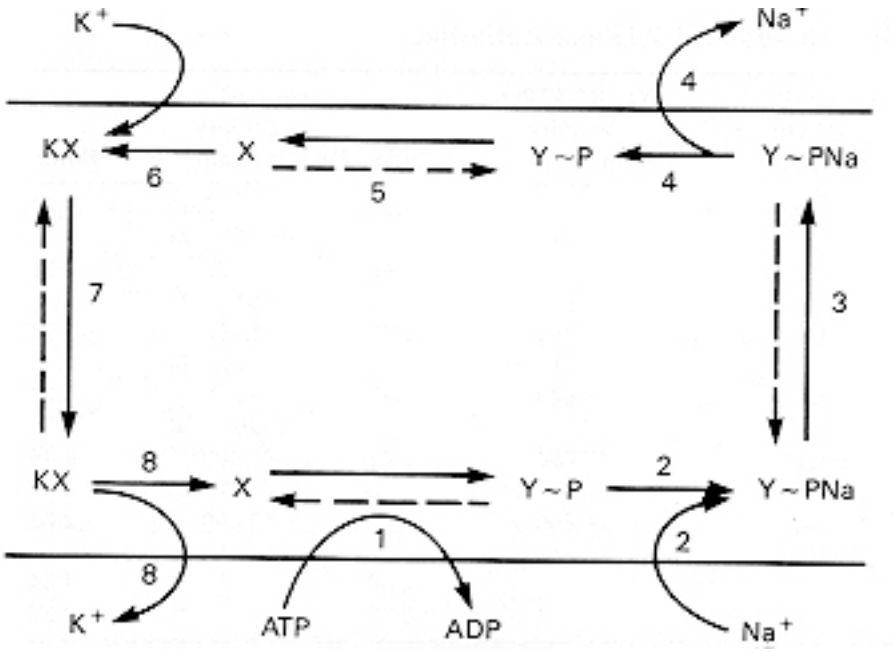


Fig. 7 Shaw's hypothesis modified to specify the high-energy intermediate as a phosphorylated form.

Figure 7 specifies a phosphorylated form of the carrier as the transporter of Na^+ (step 1) and another phosphorylated form (formed in step 5) as the transporter of K^+. Otherwise, it does not differ from the model of Fig. 6. As shown in Fig. 7, the transport system, and hence the energy-expending step 1, cannot operate without the presence of Na^+ and K^+. Hence, an ATPase involved in Na^+/K^+ transport must depend on the concentrations of these two ions. In addition, since cardiac glycosides inhibit the transport, any ATPase activity reflecting this transport must be inhibited. ATPases from many systems have been found to fulfill these requirements, as shown in Table 5 (Bonting and Caravaggio, 1963), and, today, it is generally assumed that they correspond to the Na^+/K^+ pump.

The dependence of the membrane ATPase on Na^+ and K^+ is represented in Fig. 8a and b (Post et al., 1960). The ATPase activity of erythrocyte ghosts is expressed as P_i released per hour in the ordinate. The total osmotic pressure of the solution was maintained constant by varying the proportion of the two cations, as shown in the abscissa. Figure 8a represents the experiment over a wide range of Na^+ concentrations (0-100 mM) with K^+ varied accordingly from 20 to 140 mM. The experiment essentially provides an approximate constant (apparent K_m, 24 mM) for Na^+, as indicated by the half-maximal concentration of Na^+. The variation in the K^+ concentration in part *a* does not interfere with the results, because saturation levels of K^+ are used. Figure 8b shows the variation of activity with K^+ concentration, which is varied over a much narrower range (0-25 mM). Accordingly, Na^+ is at high concentration (120-145 mM) again at saturation levels. This experiment provides an apparent K_m, of 3 mM for K^+.

The dependence of the Na^+ transport on Na^+ concentration is shown in Fig. 8c. The constant for this process (K_m = 20 mM) is approximately the same as that for ATPase. Thus, the ATPase depends on the Na^+ and K^+ concentrations as required by the model of Fig. 7. The similar K_m confirm that the transport

and ATPase activity are coupled. The apparent Michaelis-Menten kinetics of Fig. 8 are probably coincidental, since the ATPase generally shows sigmoidal kinetics (Robinson, 1970); nevertheless, the details of the kinetics do not alter the interpretation.

Table 5 Comparison of Cation Fluxes and ATPase Activities[a]

Tissue	Temperature (°C)	Cation flux (average) (10^{-14}mol $cm^{-2}s^{-1}$)	Na^+,K^+-ATPase activity[a] (10^{-14}mol $cm^{-2}s^{-1}$)	Ratio Cation/ATP
Human erythrocytes	37	3.87	1.38±0.36 (4)	2.80
Frog muscle	17	985	530±94 (4)	1.86
Squid giatn axon	19	1, 200	400±79 (5)	3.00
Frog skin	20	19, 700	6,640±1,100 (4)	2.97
Toad bladder	27	43, 700	17,600±1,640 (15)	2.48
Electric eel noninnervated membrane Sachs organ	23	86, 100	38,800±4,160 (3)	2.22 ——— 2.56±0.19

Reproduced from S.L. Bonting and L.L. Caravaggio, *Arch. Biochem. Biophys.* 101: 37-46, 1963 with permission.

[a] Means ±standard errors and number of determinations in parentheses.

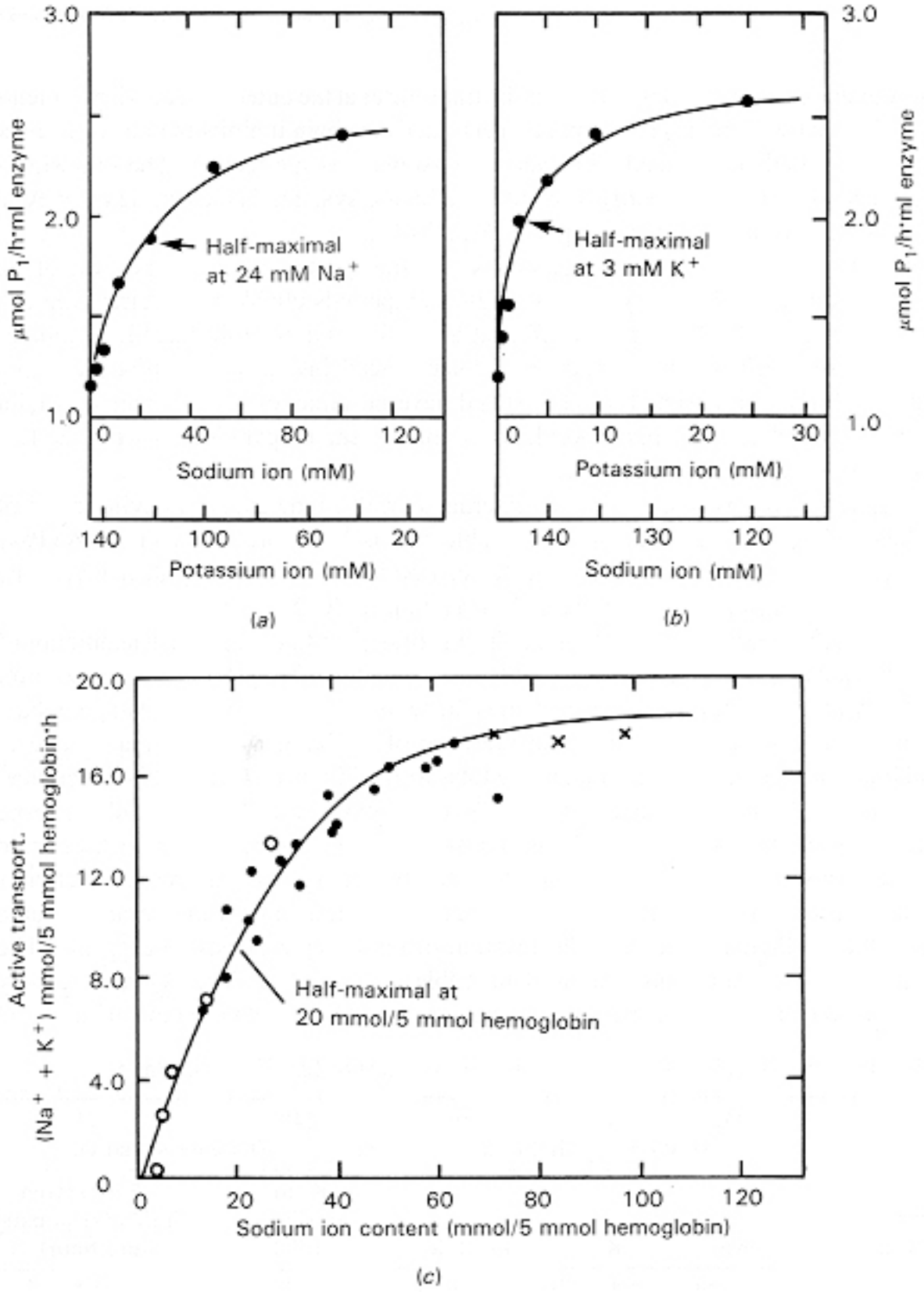


Fig. 8 (a and b) Effect of varying Na^+ and K^+ concentrations on the ATPase activity of a preparation of human erythrocyte membranes. Isotonicity was maintained by maintaining Na^+ and K^+ constant. (c) Influence of cell Na^+ content on rate of active transport in intact cells. The millimoles of hemoglobin correspond to millimolar concentrations. The sum of the active transport rates of sodium plus potassium in samples taken at 2-h intervals is plotted against the mean Ma content. External K^+ was always more than

30 mM and was not rate limiting. The different symbols indicate three different experiments. Passive transport corrections were less than 7% of the corresponding active rates. All the results plotted for the last experiment were arbitrarily multiplied by 1.5 to obtain a smooth continuation of the curve obtained in the first experiments with fresher cells. This was done because cells stored in the cold for a long period have regularly had a lower pumping capacity than fresher cells. Reproduced by permission from R. L. Post, et al., *Journal of Biological Chemistry*, 235:1789-1802. Copyright ©1960 American Society of Biological Chemists, Inc.

One of the significant features of the model of Fig. 7 is the asymmetry of the transport. In this model, the Na^+ combines with the energized carrier at the inner surface of the membrane, whereas the K^+ combines with a different form of the transporter at the outer surface. Experiments already discussed do show some asymmetry. As mentioned, ouabain inhibits the reactions only when placed on the outside of the cells; injected into axons, it is ineffective. The opposite is true of ATP: it must be present internally to be utilized by the system. The asymmetry of ATP hydrolysis is consistent with the model (step 1 of Fig. 7).

We have seen that it is possible to vary the internal, as well as the external environment of resealed red blood cell ghosts (see Fig. 3 and Table 6). Therefore, whether the ATPase system is asymmetric in relation to the Na^+ and K^+ can be tested in a forthright manner. The internal and external environments can be varied in relation to the Na^+ and K^+.

The results of such experiments are shown in Table 6 (Whittam, 1962). The amount of ATP hydrolyzed is measured by following the increase in P_i. Since the system is rather impure, the ATPase activity that is irrelevant to transport must be ignored. This is done by using ouabain in parallel experiments; the P_i liberated in the presence of ouabain and, presumably due to nonspecific ATP hydrolysis, is subtracted from the total formed in its absence. This difference, shown in column 5 of Table 6, represents the ATPase sensitive to ouabain and, therefore, presumed to be associated with transport. This view is reasonable, since the effect of ouabain has approximately the same constants in relation to either the transport or the ATPase activity. In addition, we have seen that ouabain blocks the ATPase of the neuronal membranes (Table 4) as well as the ATP-energized Na^+ translocation of erythrocyte ghosts (Fig. 4). The variation in external K^+ is shown in column 4 of Table 6. The ATPase activity (column 5) depends heavily on the external K^+ concentration. On the other hand, the external Na^+ level does not seem to matter, since KCl or choline chloride medium (items 2, 3, and 5) is as effective as Na^+ media (items 1 and 4). As shown in part B, the ATPase activity depends on the internal Na^+ (shown in column 1). Compare for example, 4a and 5c in column 5.

Table 6 Dependence of Na^+ and K^+ on Ouabain-Sensitive (0.1 mM) ATPase

	(1)	(2)	(3)	(4)	(5)

	Cations inside (mM)			Oubain sensitive	
Main solute (140-150 mM)	**Na^+**	**K^+**	**$Na^+ + K^+$**	**K^+in medium (mM)**	**ΔP_i liberated (mM/liter ghosts per hour)**
A 1. NaCl	a. 149 b.	18	167	0 10	0.4 1.5
2 . Choline chloride	c. 115 d.	20	135	0 10	0.5 1.4
3. KCl	e. -------	-------	------	150	1.9
B 4. NaCl	a. 100 b. 83 c. 41	75 14 79	175 97 120	10 10 10	1.9 1.7 1.2
5. Choline chloride	a. 80 b. 84 c. 14	16 82 81	96 166 95	10 10 10	1.6 1.6 1.0

Whittam (1962) Reproduced by permission from *Biochemistry Journal* 84: 110-118, copyright ©1962 The Biochemical Society.

In summary, all available evidence indicates that the Na^+/K^+ ATPase is present in most, if not all, plasma membranes and they support the model of Fig. 7.

The molecular organization of the transport ATPases and their topological arrangement will be discussed in the next chapter.

V. SECONDARY TRANSPORTS

Several transport systems of the plasma membrane are powered by the hydrolysis of ATP. These are considered primary transports. In vertebrates, the primary transport is generally the Na^+-K^+ discussed in this chapter. However, the transports of many other solutes against their electrochemical gradient are coupled to the favorable flux of other solutes in the direction of their gradients (see Chapter 12 and above). The transport systems, coupled in this manner, are frequently referred to as *secondary transports*. Secondary transport systems play a preeminent role in both prokaryotes and eukaryotes. The primary transports store the energy needed by the secondary transports in the form of a favorable gradient and indirectly pay the cost of the secondary transports. There are many secondary transports of significance.

In the plasma membrane of most cells, the efflux of Ca^+ is coupled to the influx of Na^+. Along with the Ca^+-ATPase system, this transporter helps in keeping the Ca^{2+} concentration in the cytoplasm vanishingly low, so that Ca^{2+} can be used as a sensitive intracellular signal (see Chapter 7).

In the renal proximal tubules, the transport of anions, such as dicarboxylates and sulfate, is also powered by the Na^+ gradient (e.g., see Pajor et al., 1998).

There are multiple pathways for the transport of amino acids in non-polar cells, such as Ehrlich cells or mouse fibroblasts (see Guidotti and Gazzola, 1993). Similar pathways were found in the intestine (e.g., Stevens, 1993), kidney (Schwegler et al., 1993) and in other tissues. These include Na^+-dependent and -independent pathways. The transmembrane Na^+-gradient has been shown to drive the Na^+-dependent pathways. The glutamate-transporter (see Worrall and Williams, 1994) is of particular importance since glutamate is the primary excitatory neurotransmitter in the mammalian brain.

In mammals, the transport of glucose in the intestinal and kidney epithelial cells is also powered by the Na^+-electrochemical gradient. The Na^+, K^+-ATPase of the basolateral membrane of epithelial cells provides the appropriate electrochemical gradient by pumping Na^+ out of the cells and K^+ into the cells. In enterocytes, the transport of peptides is linked to H^+. In this case, the pH gradient is provided by the Na^+/H^+-exchanger in the brush-border membrane that pumps H^+ out. The latter is also eventually powered by the Na^+, K^+-ATPase. The Na^+-glucose transporters (SGLTs) and the peptide transporters (PEPTs) (see Steel and Hediger, 1998) are discussed in the rest of this section.

Na^+-Glucose Transporters (SGLTs)

Glucose transport mediated by SGLT1 has a stoichiometry of 2 Na^+: 1 glucose and is electrogenic (see Wright et al., 1994). The system was studied with electrophysiological techniques with voltage clamping using two electrodes (see Chapter 22).

The importance of these transport systems is shown by human transport defects. A defect in SGLT1 produces familial glucose-galactose malabsorption (GGM) with subsequent severe diarrhea when on a

diet containing glucose and galactose. In addition, an increase in $Na^+$and glucose reabsorption by SGLT1 and SGLT2 is probably responsible for the pathogenesis of kidney disease in diabetes mellitus. SGLT1 in the brush-border intestinal membrane is responsible for the osmotic reabsorption of water and may actually be involved in the transport directly (Meinild et al., 1998). The study of mutants either present in the human population or produced in vitro in cultured cells, has been able to pin-point domains involved in substrate binding and cotransporter binding (e.g., see Panayotova-Heiermann et al., 1997).

SGLTs, as well as the peptide transporters, have been sequenced. Both SGLTs (Hediger et al., 1987) and H^+-peptide transporters (Fei et al., 1994) have twelve domains that are most likely to be transmembrane.

H^+-Peptide-Transporters

The hydrolysis of proteins in the gastro-intestinal tract produces a rich mixture of peptides and amino acids. These are transported in the intestinal and renal-brush border membranes. The transporters involved can transport almost any di- and tri-peptide, regardless of amino acid composition or charge but cannot transport individual amino acids or larger peptides (see Meredith and Boyd, 1995). They can also transport therapeutically active compounds (e.g., amino penicillin, cyclacillin, etc.) and facilitate their absorption.

Studies with vesicles from the membranes of intestinal and renal tissues have led to the identification of an electrogenic H^+-oligopeptide transporter. PepT1 has been shown to be coupled to H^+ movement (e.g., Fei et al., 1994). A similar protein, PepT2 (Liu et al., 1995) is present in a number of tissues including kidney (but not intestine).

The transport follows 1:1 stoichiometry for neutral or cationic dipeptides. The stoichiometry was found to be 2:1 (charge to substrate) for anionic dipeptides (Steel et al., 1997). For cationic or anionic dipeptides the transport is electrogenic (e.g., Fei et al., 1994).

SUGGESTED READING

Inesi, G.(1994) Teaching active transport at the turn of the twenty first century: recent discoveries and conceptual changes, *Biophys. J.* 66:554-560 http://www.biosci.umn.edu/biophys/OLTB/BJ/Inesi.pdf Adobe Acrobat from www.adobe.com is required for reading pdf files. (Medline)

Pedersen, P. L. and Carafoli, E. (1987) Ion motive ATPases. Part I. Ubiquity, properties and significance to cell function, *Trends Biochem. Sci.* 12:146-150.

Pedersen, P. L. and Carafoli, E. (1987) Ion motive ATPases. Part II. Energy, coupling and work output, *Trends Biochem. Sci.* 12:186-189.

Stein, W. D. (1986) *Transport and Diffusion Across Cell Membranes*, Chapter 6, Academic Press, New York.

Stekhoven, F. S. and Booting, S. L. (1981) Transport adenosine triphosphatase: properties and functions. *Physiol. Rev.* 61:1-76.

REFERENCES

Search the textbook

Back to Chapter 20

REFERENCES

Atkinson, A., Gatenby, A. D. and Lowe, A. G. (1971) Transport ATPase-subunit structure analyzed, *Nature New Biol.* 233:145-146.(Medline)

Bonting, S. L. and Caravaggio, L. L. (1963) Studies on sodium potassium activated adenosine triphosphatase. V. Correlation of enzyme activity with cation flux in six tissues, *Arch. Biochem. Biophys.* 101:37-46.

Bonting, S. L., Simon, K. A. and Hawkins, N. M. (1961) Studies on sodium-potassium-activated adenosine triphosphatase. I. Quantitative distribution in several tissues of the cat, *Arch. Biochem. Biophys.* 95:416-423.

Caldwell, P. C., Hodgkin, A. L., Keynes, R. D. and Shaw, T. I. (1960) The effects of injecting "energy-rich" phosphate compounds on the active transport of ions in the giant axons of Loligo, *J. Physiol. (London)* 152:561-590.

Christian, J. H. B. and Waltho, I. A. (1962) Solute concentrations within cells of halophilic and nonhalophilic bacteria, *Biochim. Biophys. Acta* 65:506-508.

Craig, W. S. and Kyte, J. (1980) Stoichiometry and molecular weight of the minimum asymmetric unit of canine renal sodium and potassium ion-activated adenosine triphosphatase, *J. Biol. Chem.* 255:6262-6269.(Medline)

Cummins, J. and Hyden, H. (1962) Adenosine triphosphate levels and adenosine triphosphatases in neurons, glia and neuronal membranes of the vestibular nucleus, *Biochim. Biophys. Acta* 60:271-283.

Fei, Y.J, Kanai, Y., Nussberger, S., Ganapathy, V., Leibach, F.H., Romero, M.F., Singh, S.K., Boron, W.F. and Hediger, M.A. (1994) Expression cloning of a mammalian proton-coupled oligopeptide transporter, *Nature* 368:563-566.(Medline)

Freytag, J.W. (1983) The (Na^+, K^+) ATPase exhibits enzymic activity in the absence of the glycoprotein subunit, *FEBS Lett.* 159:280-284.(Medline)

Glynn, I. M. (1957) The ionic permeability of the red cell membrane, *Prog. Biophys. Mol. Biol.* 8:241-307.

Guidotti, G.G. and Gazzola, G.C. (1993) Amino acid transporters: systematic approach and principles of

control, in *Mammalian Amino Acid Transport: Mechanisms and Control* (Kilberg, M.S. and Häussinger, D. ed.), Plenum Press, New York and London, pp.3-29.

Hediger, M.A., Coady, M.J., Ikeda, T.S. and Wright, E.M. (1987) Expression cloning and cDNA sequencing of the Na+/glucose co-transporter, *Nature* 330:379-381.(Medline)

Hoffman, J.F. (1962) Cation transport and structure of the red-cell plasma membrane, *Circulation* 26:1201-1213.

Hokin, L. E. (1981) Reconstitution of "carriers" in artificial membranes, *J. Membr. Biol.* 60:77-93.(Medline)

Jorgensen, P. L., Hansen, O., Glynn, I. M. and Cavieres, J. O. (1973) Antibodies to pig kidney (Na^+-K^+)-ATPase inhibit the sodium pump in human red cells provided they have access to the inner surface of the cell membrane, *Biochim. Biophys. Acta* 291:795-800.(Medline)

Kyte, J. (1971) Purification of the sodium- and potassium-dependent adenosine triphosphatase from canine renal medulla, *J. Biol. Chem.* 246:4157-4165.(Medline)

Kyte, J. (1974) Properties of the two polypeptides of sodium- and potassium-dependent adenosine triphosphatase, *J. Biol. Chem.* 247:7642-7649.

Lai, Y. F. and Thomson, J. E. (1971) The preparation and properties of an isolated plant membrane fraction enriched in (Na^+-K^+)-stimulated ATPase, *Biochim. Biophys. Acta* 233:84-90.(Medline)

Liu, W., Liang, R., Ramamoorthy, S., Fei, Y.J., Ganapathy, M.E., Hediger, M.A., Ganapathy, V. and Leibach, F.H. (1995) Molecular cloning of PEPT 2, a new member of the H+/peptide cotransporter family, from human kidney, *Biochim Biophys Acta* 1235:461-466.(Medline)

Lubin, M. (1964) Cell potassium and the regulation of protein synthesis. In, *The Cellular Functions of Membrane Transport* (Hoffman, J. F., ed.), pp. 193-209. Prentice-Hall, Englewood Cliffs, N. J.

Lubin, M. and Ennis, H. L. (1964) On the role of intracellular potassium in protein synthesis, *Biochim. Biophys. Acta* 80:614-631.

McDonough, A. A., Geering, K. and Farley, R. A. (1990) The sodium pump needs its beta subunit, *FASEB J.* 4:1598-1605.(Medline)

McLennan, D. H. (1969) Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum, *J. Biol. Chem.* 245:4508-4515.

Meinild, A., Klaerke, D.A., Loo, D.D., Wright, E.M. and Zeuthen, T. (1998) The human Na^+-glucose cotransporter is a molecular water pump, *J. Physiol. (London)* 508:15-21.(Medline)

Meredith, D. and Boyd, C.A. (1995) Oligopeptide transport by epithelial cells, *J. Membr. Biol.* 145:1-12.(Medline)

Mullins, L. J. and Brinley, F. J., Jr. (1967) Some factors influencing sodium extrusion by internally dialyzed squid axons, *J. Gen. Physiol.* 50:2333-2355.(Medline)

Nakamoto, R.K. and Slayman, C.W. (1989) Molecular properties of the fungal plasma-membrane [H^+]-ATPase. *J. Bioenerg. Biomembr.* 21:621-632. (MedLine)

Ohta, H., Matsumoto, J., Nagano, K., Fujita, M. and Nakao, M. (1971) The inhibitions of Na^+, K^+-activated adenosine triphosphatase by a large molecule derivative of p-chloromercuribenzoic acid at the outer surface of the human red cell, *Biochem. Biophys. Res. Commun.* 42:1127-1133.(Medline)

Pajor, A.M, Sun, N., Bai, L., Markovich, D. and Sule, P. (1998) The substrate recognition domain in the Na+/dicarboxylate and Na+/sulfate cotransporters is located in the carboxy-terminal portion of the protein, *Biochim. Biophys. Acta* 1370):98-106.(Medline)

Panayotova-Heiermann, M., Eskandari, S., Turk, E., Zampighi, G.A. and Wright, E.M. (1997) Five transmembrane helices form the sugar pathway through the Na+/glucose cotransporter, *J. Biol. Chem.* 272:20324-20327.(Medline)

Post, R. L., Merritt, C. R., Kinsolving, C. R. and Albright, C. D. (1960) Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte, *J. Biol. Chem.* 235:1796-1802.

Robinson, J.D. (1970) Interactions between monovalent cations and the (Na^+K^+)-dependent adenosine triphosphatase, *Arch. Biochem. Biophys.* 139:17-27.(Medline)

Sachs, G., Chang, H. H., Rabon, E., Schackman, R., Lewin, M. and Saccomani, G. (1976) A nonelectrogenic H^+ pump in plasma membranes of hog stomachs, *J. Biol. Chem.* 251:7690-7698.(Medline)

Scarborough, G. A. (1980) Proton translocation catalyzed by the electrogenic ATPase in the plasma-membrane of neurospora, *Biochemistry* 19:2925-2931.(Medline)

Schwegler, J.S., Sibernagl, S. and Tamarappoo, B.K. (1993) Amino acid transport in the kidney, in *Mammalian Amino Acid Transport: Mechanisms and Control* (Kilberg, M.S. and Häussinger, D., ed.),

Plenum Press, New York and London, pp. 233-260.

Steel, A. and Hediger, M.A, (1998) The molecular physiology of sodium- and proton-coupled solute transporters, *News Physiol. Sci.* 13:123-130.

Steel, A., Nussberger, S., Romero, M.F., Boron ,W.F., Boyd, C.A and Hediger, M.A (1997) Stoichiometry and pH dependence of the rabbit proton-dependent oligopeptide transporter PepT1, *J. Physiol. (London)* 498:563-569.(Medline)

Steinbach, H. B. (1963) Comparative biochemistry of the alkali metals. In, *Comparative Biochemistry* (Florkin, M, and Mason, H. S., eds.), Vol. 4, Part B, pp. 677-720. Academic Press, New York.

Stevens, B.R. (1993) Amino acid transport in the intestine, in *Mammalian Amino Acid Transport: Mechanisms and Control* (Kilberg, M.S. and Häussinger, D., ed.) Plenum Press, New York and London, pp. 149-163. York.

Tosteson, D. C. and Hoffman, J. F. (1960) Regulation of cell volume by active cation transport in high and low potassium sheep red cells, *J. Gen. Physiol.* 44:169-194.

Whittam, R. (1962) The asymmetrical stimulation of a membrane adenosine triphosphatase in relation to active cation transport, *Biochem. J.* 84:110-118.

Worrall, D.M. and Williams, D.C. (1994) Sodium ion-dependent transporters for neurotransmitters: a review of recent developments, *Biochem. J.* 297:425-436.(Medline)

Wright, E.M., Loo, D.D., Panayotova-Heiermann, M., Lostao, M.P., Hirayama, B.H., Mackenzie, B., Boorer, K. and Zampighi, G. (1994) 'Active' sugar transport in eukaryotes, *J. Exp. Biol.* 196:197-212.(Medline)

21. Transport of Ions:

Mechanisms and Models

Examining simple models and possible alternatives sometimes can provide insights into biological processes. This approach has proved very useful in sorting out the data on ion transport and their possible interpretation, and it provides the perspective of this chapter.

Section I examines data obtained in a study of Na^+, K^+-ATPase and, for discussion, uses the model represented in Fig. 1 based on the experiments presented in Chapter 20. This model undoubtedly will require extensive modification and elaboration, but it is a useful summary. Very similar data are available from studies of the Ca^{2+}-ATPase and a similar model could also be drawn for the transport of Ca^{2+}. Section II examines some of the characteristics of the phosphorylation of ADP by inorganic phosphate, catalyzed by transport ATPases in the absence of ionic gradients. These phenomena may reveal some new features of the ATPases and perhaps have some bearing on our understanding of the synthesis of ATP by the ATP synthase of mitochondria, chloroplasts and bacteria. Section III concentrates on possible molecular mechanisms of ion transport and discusses the information gained from knowledge of the amino acid sequences and the reconstruction of the structure of the Ca^{2+}-ATPase.

I. COUPLING BETWEEN ATP HYDROLYSIS AND TRANSPORT

The evidence reviewed in Chapter 20 unmistakably demonstrates that the active efflux of Na^+ and the influx of K^+ are coupled to the hydrolysis of ATP. As suggested in step 1 of Fig. 1, the coupling between the translocation of the ions and the hydrolysis of ATP may result from the required phosphorylation of the transporter molecule. The incubation of membrane preparations with ATP labeled with [^{32}P] in its terminal position, labels the membranes. The phosphate, and not the whole ATP molecule, is incorporated since [^{14}C]ATP does not label the membranes. Table 1 (Post et al., 1965) summarizes the incorporation of [^{32}P] into kidney plasma membranes as a function of the cation present in the medium. When Na^+ is present, the incorporation is highest, 97 pmoles/mg protein, compared to the incorporation in its absence (between 14 and 29 pmoles). Even in the presence of Na^+, the incorporation may not seem

very large. This is because the Na^+,K^+-ATPase is a minor component of the cell membrane (see below). Much higher values can be obtained for membrane fragments containing the Ca^{2+}-ATPase, which represents a very large proportion of the total protein of the sarcoplasmic reticulum. Actually, in both cases the amount of [^{32}P] incorporated corresponds to one per ATPase molecule. In step 1 of the model of Fig. 1, the phosphorylation of X produces Y~P. Different letters, X and Y, are used to denote the two forms because they have very different properties. Y is able to bind Na^+ (step 2) and transfer it to the external membrane interface (step 3), from which it is released (step 4). X-P is generated from Y~P (step 5) and it binds K^+ (step 6), transfers it to the internal membrane interface (step 7), and releases it to the cell's interior (step 8) with hydrolysis of X-P.

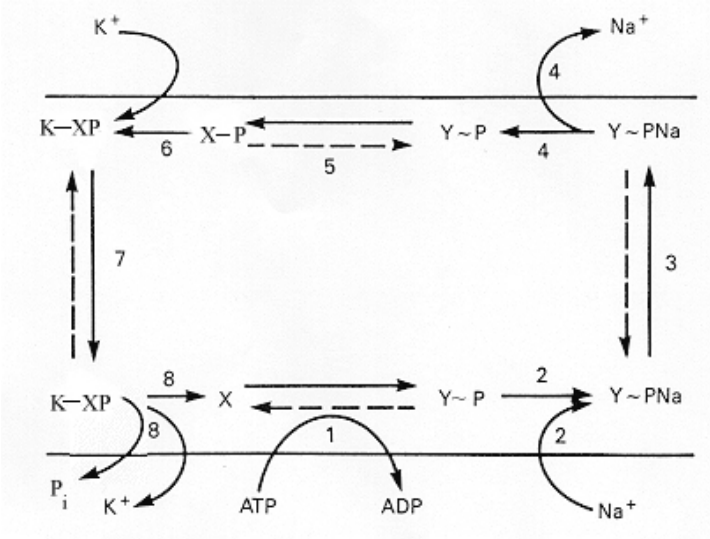


Fig. 1 Early model of the functioning of the Na^+,K^+-ATPase. The step corresponds to the following: step 1, the phosphorylation of the ATPase indicated as Y; step 2, the binding of Na^+ to Y~P; step 3, the movement of the binding group from the cytoplasmic side of the membrane to the outside; step 4, the release of Na^+; step 5, the hydrolysis of Y~P to form a different form of Y, X; step 6, the binding of K^+; step 7, its displacement to the cytoplasmic side of the membrane; and step 8, its release into the cytoplasm.

Table 1 Effect of Monovalent Cations on Labeling of Kidney Membranes After Incubation with Mg^{2+} and [^{32}P]ATP

Addition	Labelling (pmol ^{32}P/mg protein)
None	26
Li^+	20

Na^+	97
K^+	16
NH_{4+}	14
Rb^+	18
Cs^+	14
$Tris^+$	19

This scheme suggests that the formation of Y~P requires the presence of Na^+, as shown by the results in Table 1. Other univalent cations cannot substitute. Fig. 2 shows the dependence of the phosphorylation expressed as % of the maximum (ordinate) on the concentration of Na^+ (abscissa). The phosphorylation is related to transport, as shown by the inhibition of a large portion of the Na^+-dependent phosphorylation by ouabain (Post et al., 1965) which blocks the transport of Na^+ and K^+. In Chapter 20 we saw that ouabain is an inhibitor of the Na^+,K^+-ATPase. The scheme also predicts that K^+ would favor the hydrolysis of Y~P, as shown by the experiment represented in Fig. 3 (Post et al., 1965). In this experiment the membranes were first labeled with radioactive [^{32}P]ATP. Then after the addition of unlabelled ATP, they were incubated in the presence of K^+. Although the [^{32}P] is released even in the absence of K^+, the release is sharply accelerated when K^+ is present. The rates of phosphorylation and dephosphorylation are comparable to those of the ATPase activity (e.g., Kyte, 1974) which, in turn, correspond very closely to the moles of ions being transported, as shown in Table 6 of Chapter 20.

The nature of the phosphorylated ATPase has also been examined in relation to its sensitivity to ADP. The increased hydrolysis favored by K^+ also appears in the results of the experiment of Fig. 4 (curve 1), carried out with the same protocol as in Fig. 3, but with the addition of K^+ or ATP after a 5 min incubation (Post et al., 1965). In this experiment, K^+ decreases the radioactivity as expected (curve 1), whereas the addition of ADP (curve 2) has no effect, suggesting that the phosphorylated ATPase is no longer in a high-energy form. In its high energy form, the phosphorylation of the enzyme would be reversible.

The results are different when the ATPase has first been treated with *N*-ethylmaleimide (NEM), which

reacts with sulfhydryl groups. Treatment of the ATPase with NEM, blocks the ATPase activity but not the phosphorylation. As shown in Fig. 5 (Post et al., 1965), the NEM-treated ATPase is not sensitive to K^+ but is sensitive to ADP. These results suggest that the ATPase may be present in two distinct forms: a form with a high and another with a low phosphate group transfer potential, the latter corresponding to a K^+-sensitive form. NEM blocks the conversion of the high energy form to the low-energy form. This scheme is consistent with the following reactions:

$$Na_i^+ + E_1 + ATP \leftrightarrow E_1{\sim}P.Na^+ \quad (1)$$

$$E_1{\sim}P.Na^+ \leftrightarrow E_2\text{-}P + Na_o^+ \quad (2)$$

$$E_2\text{-}P + K_o^+ \leftrightarrow E_2\text{-}P.K^+ \quad (3)$$

$$E_2\text{-}P.K^+ \leftrightarrow E_2 + K^+_i + P_i \quad (4)$$

where E represents the transporter molecule. The subscripts are used to distinguish the various molecular configurations of the enzyme; E_1 and E_2 correspond to the Y and X of Fig. 1, respectively.

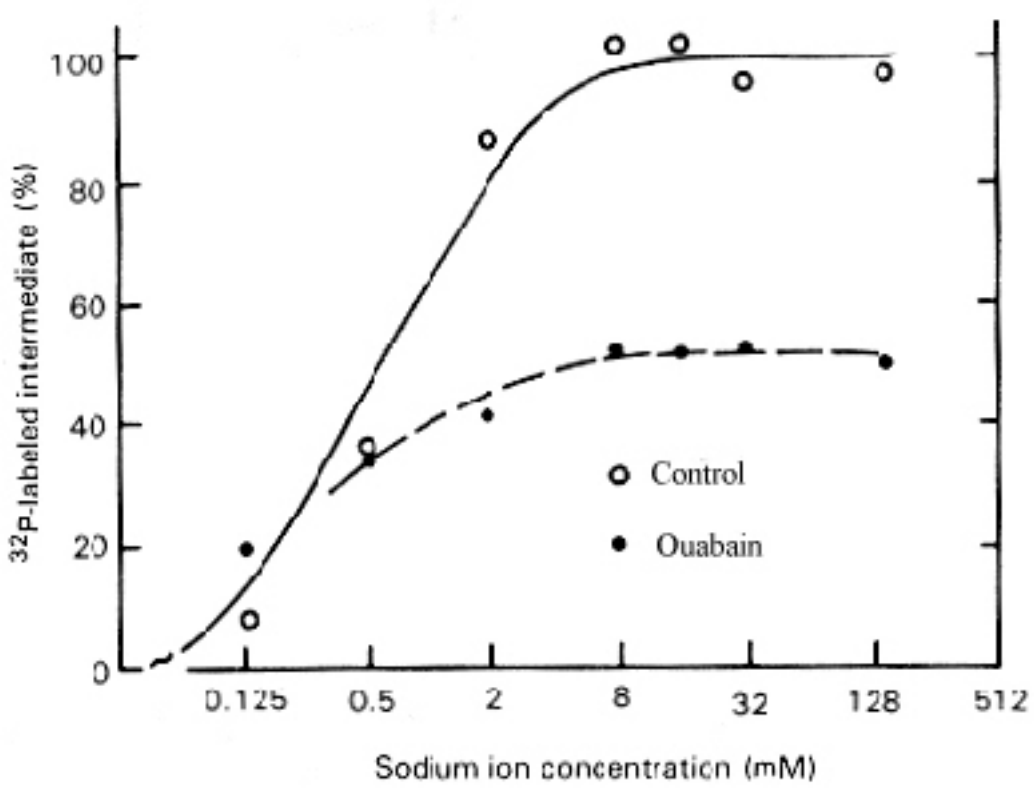


Fig. 2 Effect of ouabain on the sensitivity of the [^{32}P]-labeled intermediate to the concentration of sodium ion. The concentration of ouabain was 2.5 x 10^{-4} M, and that of Mg-ATP was 0.1 mM. Incubation was for 12 s at 23C. The results are the average of two experiments. Reproduced with permission from R. L. Post, et al., *Journal of Biological Chemistry*, 240:1437-1445. Copyright ©1965 The American Society for Biochemistry and Molecular Biology.

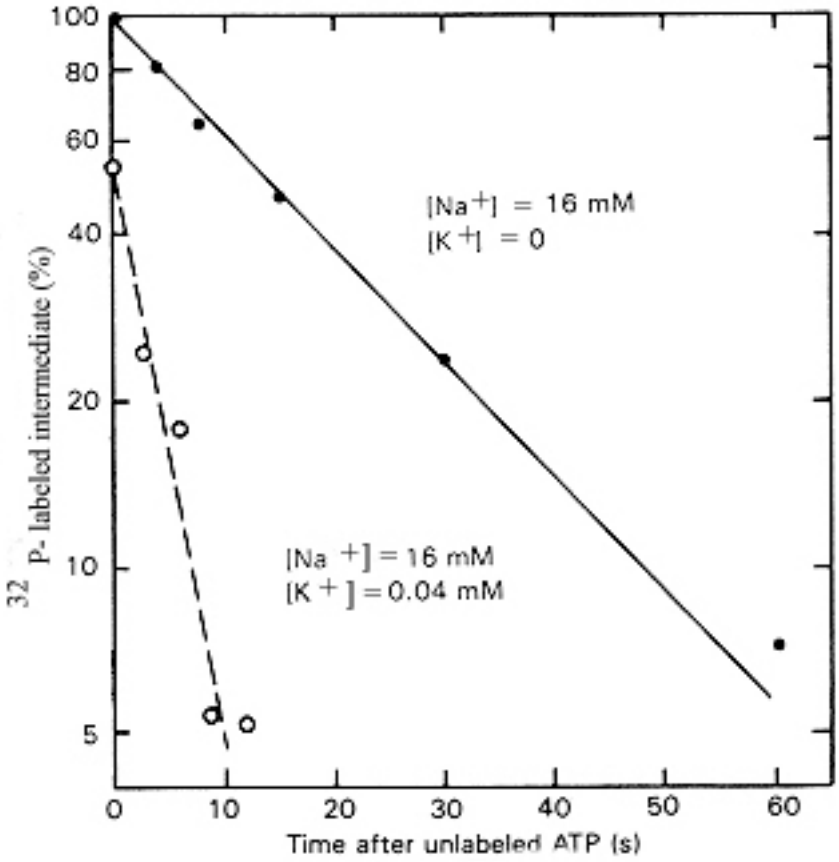


Fig. 3 Influence of K^+ on the rate of breakdown of the [^{32}P]-labeled intermediate. Kidney membranes were stirred with 0.04 mM Mg-ATP labeled with [^{32}P] for 2 min at 8.5C in the presence of 16 mM Na^+ in a volume of 1.0 ml. (●) K^+ absent; (○) K^+ present at 0.04 mM. Then 0.1 ml of 20 mM unlabeled (Tris) ATP was added to reduce the specific activity of the labeled ATP to 2% of its initial value. After the time intervals on the horizontal axis the reaction was stopped with acid. The solid line indicates exponential disappearance with a time constant of 21 s. The dashed line is similar, with a time constant of 4 s. Reproduced with permission from R. L. Post, et al., *Journal of Biological Chemistry*, 240:1437-1445.

The estimates of size of the molecule, together with estimates of the turnover number of the transport ATPase [i.e., moles of product x (moles of enzyme x minutes)$^{-1}$], permit a number of interesting approximations. The turnover number was calculated to be about 12,000, based on the phosphate hydrolyzed. 1 mmol of P_i per hour is hydrolyzed from the ATP by 1 liter of cells. If we assume that there are 1.1 x 10^{13} cells per liter, there must be 1.3 x 10^{-22} moles of enzyme per cell. Multiplied by Avogadro's number (the number of molecules in one mole) this value corresponds to about 80 transporter molecules per cell. Assuming that the volume of each transporter molecule is 3.2 x 10^{-19} cm^3, the total volume of transporter per cell is 80 X (3.2 x 10^{-19}) = 2.6 x 10^{-17} cm^3. The red blood cell surface area is about 1.55 X $l0^{-6}$ cm^2 and its thickness is approximately 5 nm, therefore, the volume of the membrane is about 0.78 x 10^{-12} cm^3. Thus, the transport ATPase occupies about 0.0003% of the membrane volume, an extremely tiny portion of the cell membrane. In the red cell ghost, the Na^+,K^+-ATPase has been found by a cytochemical electron microscopic method (Charnock et al., 1972) to be distributed evenly over the membrane surface. However, other membranes are quite different. The Ca^{2+}-ATPase is the major protein

present in the sarcoplasmic reticulum. In addition, the distribution may not be even, the Na^+, K^+-ATPase of polar cells such as epithelial cells, is present only on one surface, the apical surface.

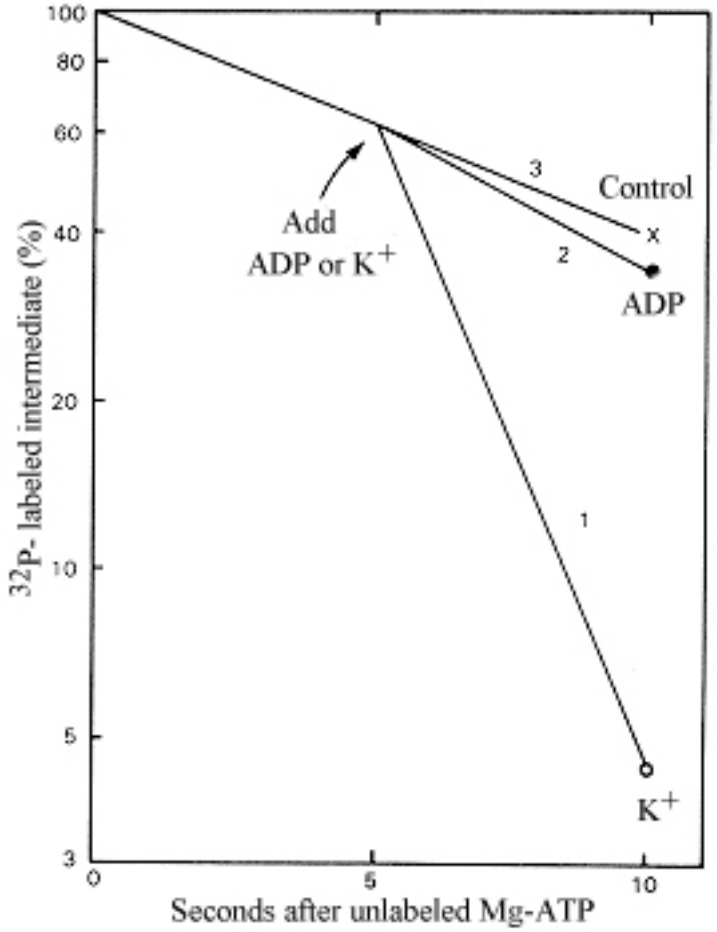


Fig. 4 Sensitivity of the phosphorylated intermediate of the native enzyme to ADP and K^+. The ATPase was labeled with [^{32}P]ATP. At zero time the radioactivity of the ATP was chased using a 100-fold excess of unlabeled ATP. From Post et al. (1969). Reproduced from *The Journal of General Physiology*, by copyright © permission of the Rockefeller University Press.

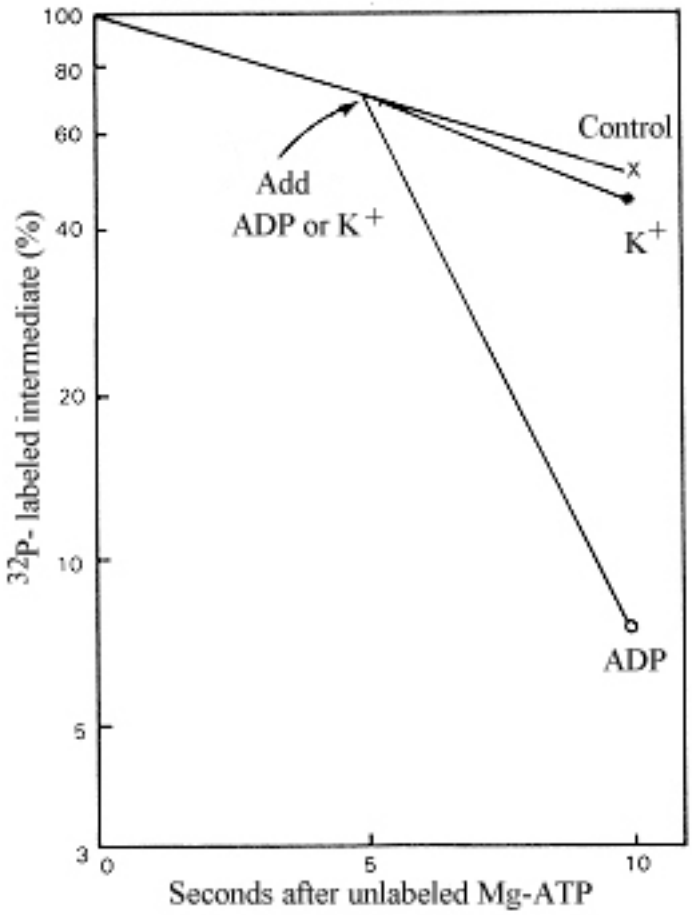


Fig. 5 Sensitivity of the phosphorylated intermediate of the Na^+,K^+-ATPase to ADP and K^+ after treatment with *N*-ethylmaleimide. From Post et al. Reproduced from *The Journal of General Physiology*, by copyright permission ©1969 of the Rockefeller University Press.

II. SYNTHESIS OF ATP BY TRANSPORT ATPases

As we saw in Chapter 10, when ion pumps are run in reverse, ATP can be synthesized from ADP and P_i. These findings have certain implications related to the model of Fig. 1. ATP can be synthesized only if the phosphorylated form of Y(Y~P) is a high-energy form (high phosphate group transfer potential); i.e., the G for its hydrolysis is sufficiently low to support the synthesis of ATP from ADP. However, as described above, the evidence indicates that the usual phosphorylated form of the ATPase is hydrolyzed with the addition of K^+, but not ADP. As already noted, a possible explanation is that there are two phosphorylated forms of the transporter molecule: a high-energy form involved in the transport of Na^+ and a low-energy form that interacts with K^+ (X-P). Furthermore, since X-P is a low-energy form, it should be possible to phosphorylate the molecule with P_i in the absence of Na^+, and this was found to be the case (Post et al., 1965; Schoot et al., 1977; Sen et al., 1969). In the formulation of Fig. 1, Y~P would then be the precursor of X-P. The Na^+,K^+-ATPase is present in two forms, E_1 and E_2, which differ in conformation, as shown by a variety of techniques means such as exposure of regions of the molecule at the membrane surface to tryptic digestion (see below). The Y~P and X-P would then correspond, respectively, to the phosphorylated forms of E_1 and E_2 (Jorgensen and Petersen, 1979). Digestion of the

enzyme phosphorylated with either ATP or P_i, produces identical electrophoretic patterns (Bontig et al., 1979; Siegel et al., 1969).

However, it is not necessary to have an ion gradient to synthesize ATP in the case of either the Na^+,K^+-ATPase (Post et al., 1974) or the Ca^{2+}-ATPase (Knowles and Racker, 1975). The in vitro synthesis is carried out in two steps. First, the ATPase is phosphorylated; we saw that this can be done in the case of the Na^+,K^+-ATPase by incubation with P_i. Then ATP is synthesized when ADP is added in the presence of a high concentration of Na^+. Obviously, this proceeds only for a single turnover.

The sequence of events perhaps can be understood best by examining the reactions in some detail. If K^+ is ignored, the reactions would be as shown in Eqs. (5) to (7):

$$E_2 + P_i \leftrightarrow E_2\text{-}P \quad (5)$$

$$E_2\text{-}P + Na^+ \leftrightarrow E_1P.Na^+ \quad (6)$$

$$E_1P.Na^+ + ADP \leftrightarrow E_1 + ATP + Na^+ \quad (7)$$

These reactions represent the reverse of the normal sequence of active transport. The passage from Eq. (5) to Eq. (7) would be highly improbable unless the Na^+ concentration was raised sufficiently, which is predictable from the law of mass action. However, as discussed more fully in Section III, the phosphorylation of the ATPase by ATP, presumably reaction (7) run from right to left, decreases the binding constant of the cation -- and the effect is reversible. When the binding of one component (e.g., the phosphorylation) to a protein capable of undergoing conformational change affects the binding of another (e.g., the cation), the inverse will be true. The nature of the enzyme-phosphate bond will thereby be affected by the binding of the cation (Weber, 1972, Weber, 1974). Presumably, the binding of Na^+ would then convert the low-energy bond into a high-energy bond.

The possibility of obtaining ATP from the reverse of ion transport can be explained by the considerations discussed in this section. The high Na^+ present on the outside of the cell will permit the formation of the high-energy phosphate [reaction of Eq. (6)]. The phosphorylation of ADP removes the phosphate and the enzyme can be used again for another cycle of phosphorylation.

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

III. MODELS OF ION TRANSPORT AND STRUCTURE

The simplest model for transport of an ion would include the following steps: (1) binding of the ion by specific binding groups on the transporter molecule at the loading site; (2) movement of the complex from one interface to the other; and, (3) release of the ion at the discharge site of the membrane. This model ignores a membrane potential to avoid complications unnecessary for our present discussion. The Ca^{2+} pump of the sarcoplasmic reticulum imports 2 Ca^{2+} and exports 1 H^+per ATP hydrolyzed; the Na^+/K^+ pump exports from the cell 3 Na^+ and simultaneously imports 2 K^+ per ATP hydrolyzed.

The model would carry out the net transport of the ion. Active transport, i.e., transport against an electrochemical gradient, could take place in this same model when two other conditions are met: (1) the affinity of the binding group changes from high at the loading interface to low at the discharge interface and (2) the free energy of the sequence of reactions decreases. In a transport ATPase the energy is provided by the coupled hydrolysis of ATP.

Models capable of carrying active transport can be constructed without postulating a change in binding constants. However, all transport systems known have been shown to have this feature (see Table 2). For simplicity, in the present discussion we assume that the transport of all ions occurs by the same basic process. This approach is not unreasonable because, as we saw in Chapter 20, there is considerable evidence that the transport functions are analogous for the Na^+,K^+-ATPase, Ca^{2+}-ATPase, the H^+,K^+-ATPase and the H^+-ATPase of plants and *Neurospora*. Furthermore, the properties of these molecules are very similar.

A mechanism of active transport, involving the phosphorylation of the transporter and changes in binding constants, is supported by a variety of observations. The experiments discussed here (Ikemoto, 1976) were carried out with a stop-flow apparatus (Fig. 6), which delivers reactants and enzyme (from syringes shown at A) into the same chamber (B) with very rapid mixing in relation to the time course of the reaction. Then the flow is stopped, also very rapidly. The light absorption of the contents of the chamber can be recorded (E). An oscillosope (D) is required to record very rapid reactions. These experiments used a purified preparation of Ca^{2+}-ATPase from the sarcoplasmic reticulum and the Ca^{2+} indicator Arsenazo III, which changes color when it binds Ca^{2+}. The record of Fig. 7 represents the light absorption with time. The two sets of panels differ in the time scale: set I shows fast changes (intervals correspond to 50 ms) and set II shows slower changes (intervals represent 5 s). The downward deflections reflect increases in the concentration of Ca^{2+}. The concentration of ATP added is shown at the left in the records. In the control (IA and IA), no ATP was added and no Ca^{2+} was released. The Ca^{2+}

released increases with the concentration of ATP added (compare B and D) until the system appears saturated (compare D and E), as would be expected because the amount of ATPase is finite. As shown by the longer time scale in set II, the release is temporary; eventually the Ca^{2+} is bound again, presumably when all the ATP is hydrolyzed. The results show that the ATPase binds Ca^{2+} and that activation by ATP reversibly decreases the binding. Fig. 8 shows the level of phosphorylation of the enzyme (determined after rapid filtration, curve 1) compared to the Ca^{2+} release (curve 2) calculated from Fig. 7. The two panels represent identical results plotted on two different time scales. The changes in phosphorylation of the ATPase precede the release of the Ca^{2+}, suggesting that phosphorylation is responsible for the change in binding constants. These results indicate the Ca^{2+} is bound more tightly (larger binding constant) before activation. Similar data are available for other transport systems, such as Na^+,K^+-ATPase (Masui and Homareda, 1982; Yamaguchi and Tonomura, 1980). The binding constants on the two sides of the membrane for different transport systems are shown in Table 2 (Tanford, 1983).

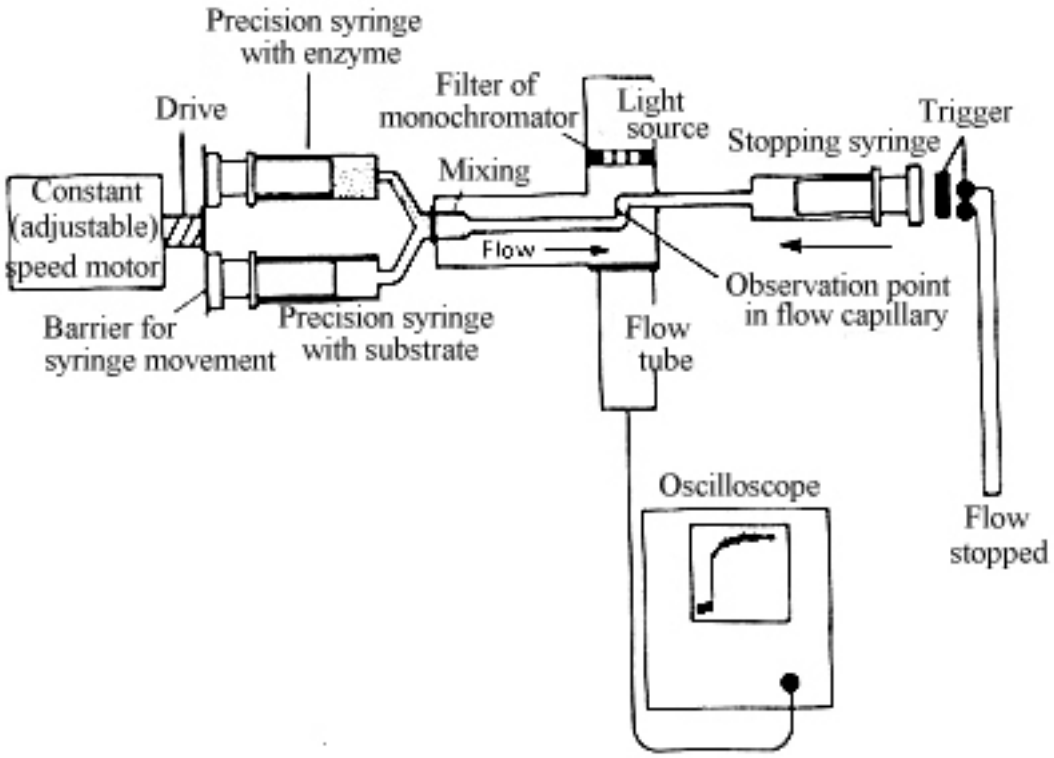


Fig. 6 Stop-flow apparatus.

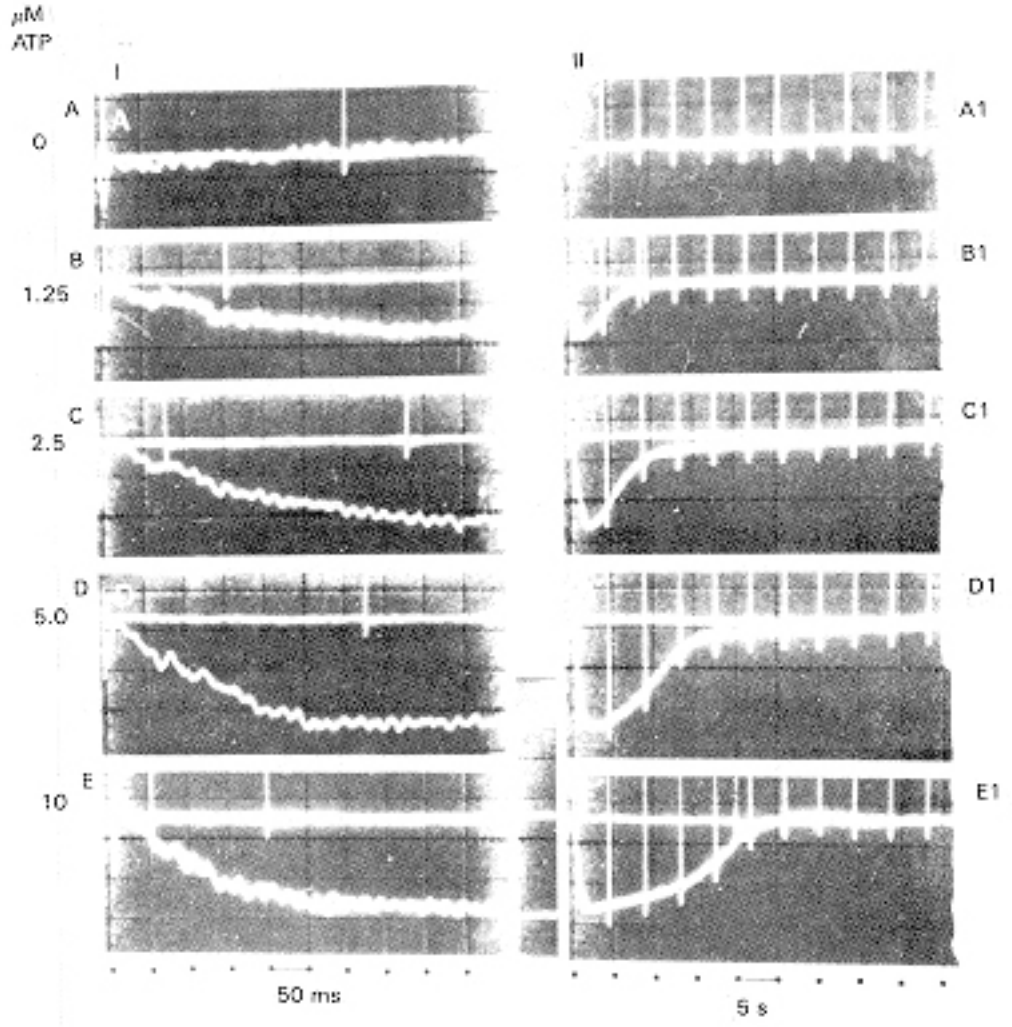


Fig. 7 ATPase-coupled changes in Ca^{2+} binding to purified Ca^{2+}-ATPase of the sarcoplasmic reticulum. From Ikemoto (1976), with permission.

The transport process seems to involve precise stoichiometry. As mentioned, 2 Ca^{2+} are transported per ATP hydrolyzed. Furthermore, 2 Ca^{2+} are bound per phosphorylated transporter molecule (Inesi et al., 1980). In the case of active transport, the affinity of the binding groups of the transporter for the ligand, decreases when the transporter molecule is phosphorylated and this lower affinity should represent the state of the transporter on the side with the higher concentration at steady state. A model of active transport involving ion binding sites and shuttling of ions across the plasma membrane is consistent with the data.

However, these considerations do not resolve how the binding sites can move from one interface to the other without a major movement of the transporter. Integral proteins have distinct domains corresponding to the two different membrane surfaces. It follows that the transporter molecule does not flip or rotate. Furthermore, the Na^+,K^+-ATPase continues to function even when anchored at one interface with an antibody (Kyte, 1974). These difficulties could be resolved by proposing that the binding sites do not traverse the whole membrane thickness, but rather move over much shorter distances. This would be possible if the binding sites were inside a channel traversing the membrane.

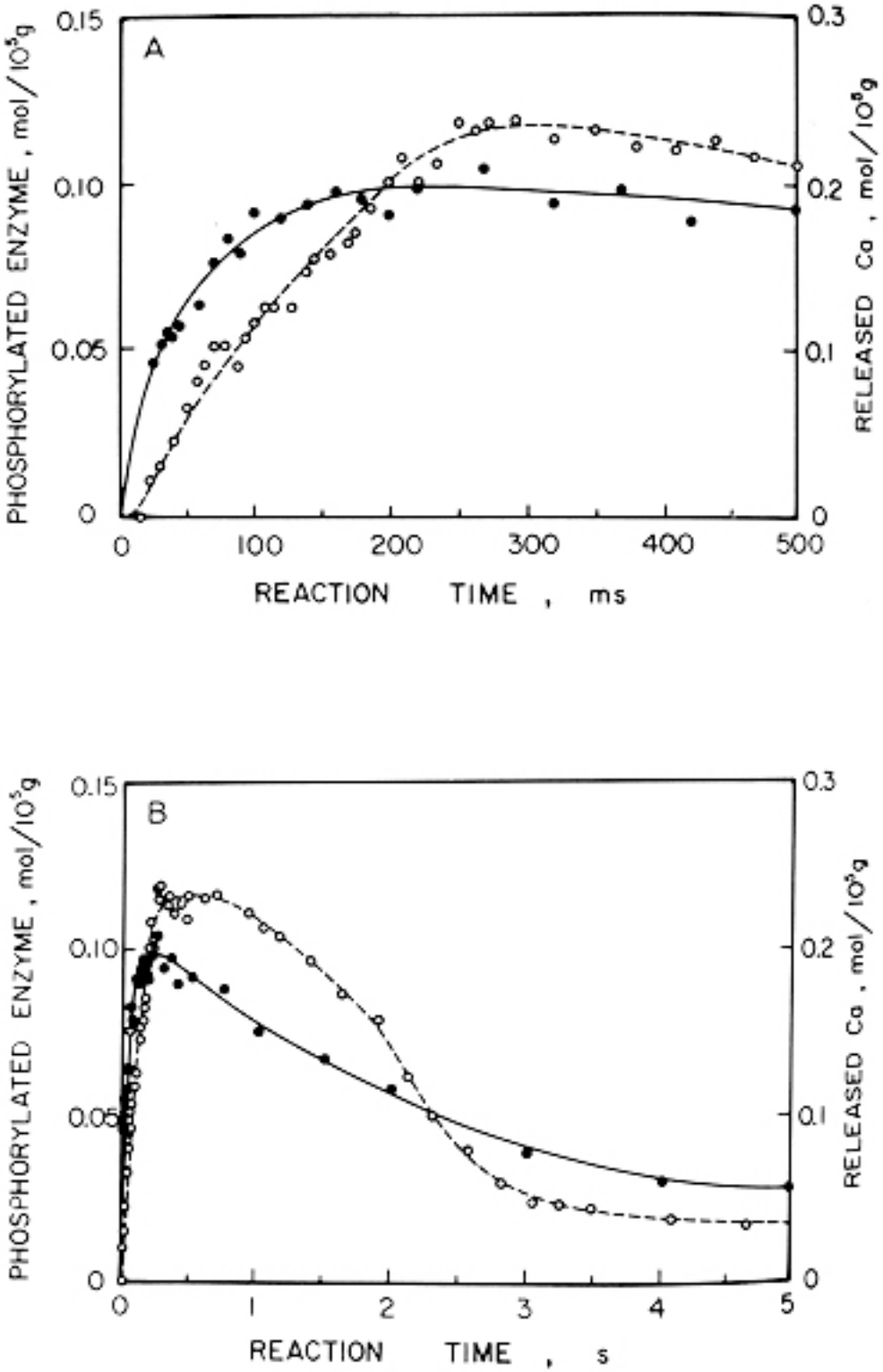


Fig. 8 Relationship between Ca^{2+} release and rebinding and the formation and decay of the phosphorylated intermediate. (○) Ca^{2+} release; (●) P in enzyme. Reproduced with permission from N. Ikemoto, *Journal of Biological Chemistry*, 251:7275-7277. Copyright &copy1976 The American Society of Biological Chemistry and Molecular Biology.

Table 2 Binding Constants for Transported Ions

		Binding constant, $K_{eq}(M^{-1})$	
Protein	**Ion**	**Uptake side**	**Discharge side**
SR Ca^{2+} pump	Ca^{2+}	10^7-10^8	300
Na^+ pump	Na^+	4 X 10^3	<20
Chloroplast F_oF_1	H^+	>10^8	<10^6
Na^+/ Ca^{2+} exchange	Ca^{2+}	2 x 10^5-10^6	400

Tanford (1983), reproduced with permission from the *Annual Review of Biochemistry* Vol. 52, copyright &copy1983 by Annual Reviews Inc.

There are, in fact, many indications that the transporters can act as channels, at least when reconstituted in artificial membrane systems. Addition of purified Ca^{2+}-ATPase to a bilayer (in this case a bilayer made of oxidized cholesterol) changes its conductivity (Shamoo and MacLennan, 1975). Under some conditions, the Na^+, K^+-ATPase incorporated into planar bilayers of phospholipid shows electrical conductance transitions typical of channels (Last et al., 1983). The anion transporter (Giebel and Passow, 1960) also behaves like a channel. In this case, the selectivity of the transport system appears to depend on the size of the molecule, suggesting a channel 0.8 to 0.9 nm in diameter. The channel behavior is related to the transport process of the native systems and not some irrelevant coincidence. This is shown by the sensitivity of the channel behavior to inhibitors of transport. $HgCl_2$ inhibits both the Ca^{2+}-ATPase activity and the Ca^{2+} conductivity in parallel. Ouabain and vanadate, both inhibitors of the Na^+/K^+ transport, inhibit the Na^+, K^+-ATPase channel behavior.

Evidence of conformational rearrangements comes from many kinds of experiments. The sensitivity of the transporter molecule to proteolytic digestion differs at different stages of transport (e.g., see Chapter 4). Gresalfi and Wallace (1984) have examined the circular dichroism (CD) spectra of purified and membrane-attached Na^+,K^+-ATPase in its E_1 and E_2 forms, obtained by introducing either Na^+ or K^+. The spectra for the peptide backbone (190-240 nm) were consistent with extensive conformational differences between E_1 and E_2. The changes appear to be reversible when the ion composition is altered. Phosphorylation of the ATPase is accompanied by fluorescence changes of its tryptophan residues (Nakamura et al., 1994), indicating a conformational change.

The involvement in transport of rearrangements within the ATPase is also shown by x-ray diffraction studies of packed membranes from the sarcoplasmic reticulum containing Ca^{2+}-ATPase (Blasie et al., 1985). A significant portion of the ATPase juts into the cytoplasmic phase, as also shown by three-dimensional reconstruction of negatively stained crystals in sarcoplasmic reticulum membranes (see below). Activation of the ATPase produces a conformational change with a displacement of the structure into the bilayer. Both the binding of Ca^{2+} (DeLong and Blasie, 1993; Cheong et al., 1996) and the phosphorylation of the enzyme (Blasie et al., 1985, Pascolini et al., 1988) were found to change the conformation of the enzyme as seen using X-ray diffraction. The experiments correlating specific steps in the transport used flash photolysis of *caged ATP*, a compound which releases ATP in response to a flash of light, assuring rapid and synchronous activation.

Important details have been provided by other studies, some of them more recent. Ten transmembrane helices (M1 to M10, shown by the numbers in Fig. 9) have been proposed based on the amino acid sequence (MacLennan et al., 1985) and confirmed by high resolution EM (8-Å, Zhang et al., 1998). The functional portions of the cytosolic domains of the head region of the molecule are distinct. The P domain is involved in the phosphorylation, the N domain contains the nucleotide binding site and the A or actuator domain (also called the transducer domain) is thought to have a special role in the transduction (see Toyoshima et al., 2000; Toyoshima and Nomura, 2002). These are represented in the diagram of Fig. 9 and discussed below. Fig. 9 was drawn from models derived from X-ray diffraction data (Toyoshima et al., 2000).

The more recent crystallographic study (Toyoshima and Nomura, 2002) with a resolution of 3.1 Å has provided data on the Ca^{2+}-ATPase in its E_2-state (with no Ca^{2+}, but protonated with 2 H^+).The conformation of E_2 was found to differ from that of E_1 (with 2 bound Ca^{2+}) as follows. In E_1 the three domains (P, N and A domains) are widely separated. In E_2 they form a compact structure as shown in Fig. 9. In addition, six out of the ten transmembrane segments also undergo conformational changes when assuming the E_2 conformation. These changes might be required for the release of the Ca^{2+} into the SR lumen by opening a channel for the passage of Ca^{2+} and the permitting the counter-transport of 2 H^+ to cytoplasm in exchange for 1 Ca^{2+} per 1 ATP hydrolyzed .(see fig. 2 of Green and MacLennan, 2002) as indicated in the model of Fig. 16 below.

The structure deduced for the Ca^{2+}-ATPase allowed the construction of an atomic homology model of the H^+-ATPase of *Neurospora* by comparing it to an 8 Å map of the *Neurospora* proton pump derived from electron microscopy (Kühlbrandt et al., 2002). The model, shows the probable path of the proton through the membrane and indicates that the nucleotide-binding domain rotates by approximately 70º to deliver ATP to the phosphorylation site of the ATPase. This model differs somewhat from that proposed for the Ca^{2+}-ATPase.

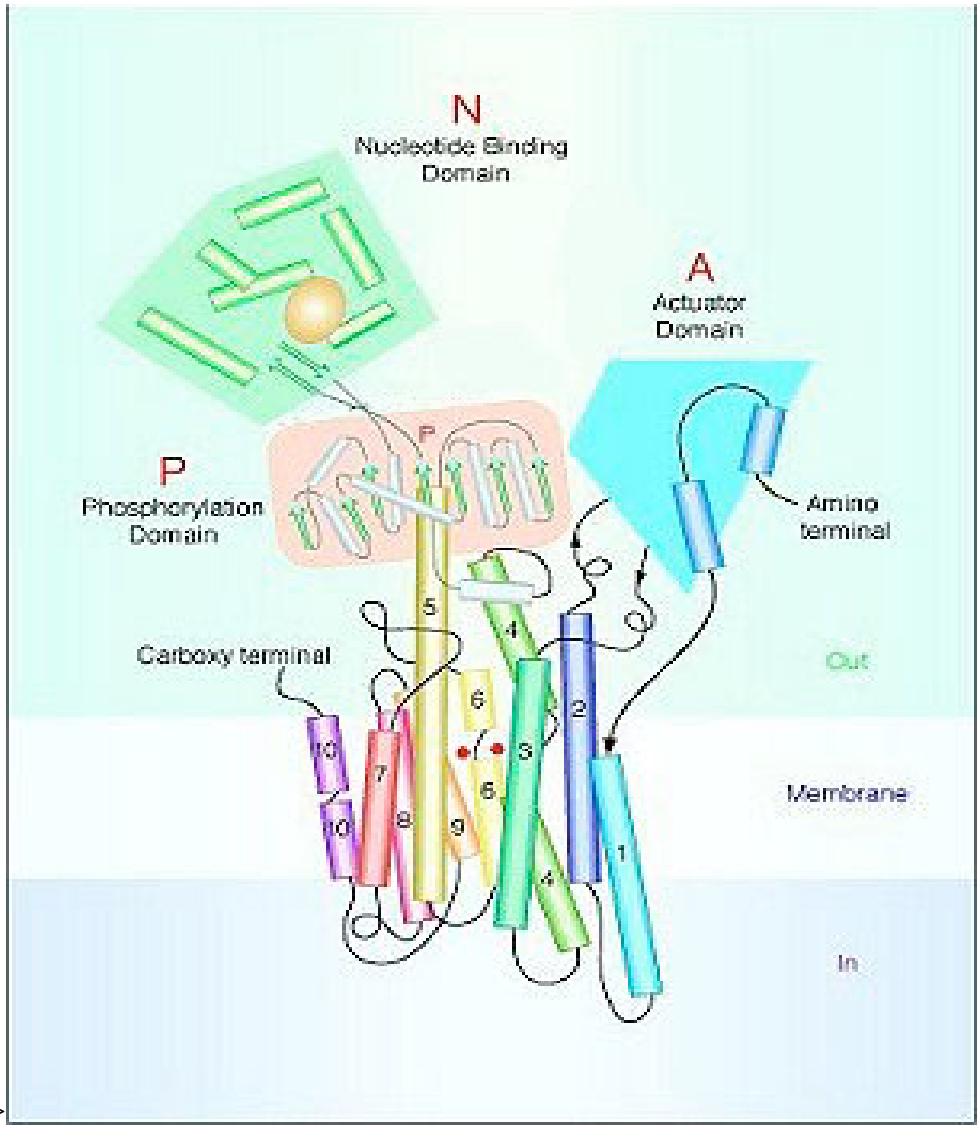
align="center"> 

Fig. 9 Schematic representations of the Ca^{2+}-ATPase in the Ca^{2+}-binding configuration, based on the reconstruction of Toyoshima et al. (2000). The red P in the P-domain represents the phosphorylated site. The yellow oval represent the site of nucleotide binding in the N-domain. The ten helices traversing the membrane are represented by cylinders and the two red dots represent the bound Ca^{2+}.

In summary, it appears that the transport of ions proceeds by binding the ions to specific sites. These sites are probably present in a channel of the transporter that traverses the membrane. The translocation is associated with some movement of the binding sites, so that the sites are exposed first to one, and then to the other side of the membrane and major rearrangments of the large cytoplasmic domains of the transporter.

How can this information be put together in a single model? The presence of a conventional channel would only allow passive flow in the direction of the gradient and could not carry out transport against an electrochemical gradient. For this reason, the models generally considered propose alternating access (see Fig. 10), in which a small conformational change (in this case a rotation) exposes the binding sites first to the water phase on one side of the membrane, and then to the water phase on the other side (Tanford, 1983). The channel would remain closed at all times, but would alternate using two different

"gates", comparable to the gates in a lock connecting two bodies of water of different heights. The movement of the binding groups could increase the distance between them, as represented in the diagram and, therefore, could also account for the change in the affinity for the transported ion. During the working cycle of the alternating access pump, when both "gates" are closed, the ion is unavailable for exchange. The ion in the transporter molecule is said to be *occluded*. Occlusion suggests the presence of an intermediate position of the binding sites, apart from their location at either the uptake or the discharge site (see Glynn and Karlish, 1990). An alternating access model of the Ca^{2+}-ATPase is shown in Fig.16.

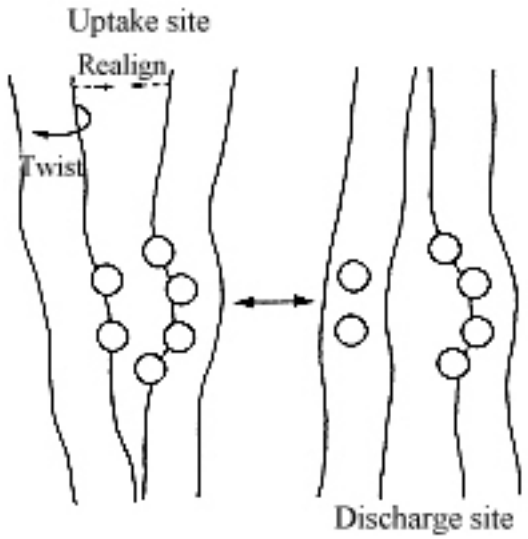


Fig. 10 Representation of the alternate-access model of transport. The structures represent polypeptide chains traversing the phospholipid bilayer of the plasma membrane. The circles indicate the binding sites of the transported ion. The closeness of the binding groups on the left accounts for the high-affinity binding, the separation on the right for the decrease in affinity. The slight rotation of the polypeptides accounts for the access of the binding sites from either the uptake site (left) or the discharge site (right).

Mutational studies have identified amino acids critical for transport (see MacLennan et al., 1997). Site-specific mutagenesis substitutes amino acids at defined locations in the molecule and delineates the functional role of amino acids or amino acid clusters in the transport. In some of these experiments, mutant DNA was incorporated into COS cells, a transformed simian cell line, using a vector and then assayed for function (see MacLennan, 1990). These studies have identified amino acids critical for transport (see MacLennan et al., 1997). Negatively charged residues in M4, M5, M6 and M8 are thought to constitute high affinity Ca^{2+}-binding site (Clarke et al., 1989). The two Ca^{2+}-binding sites are formed by the juxtaposition of acidic and oxygen containing amino acids next to each other in the middle of the four transmembrane helices (Clarke et al., 1989; see Andersen, 1995 and MacLennan et al., 1997) as represented in Fig. 15. Small changes in the position of the helices forming this cluster would disrupt these binding sites. The study of (Toyoshima et al., 2000) suggests the pathway lined by oxygen atoms, allowing for the in-and-out passage of Ca^{2+} and shows the disruption of structure of the M4 and M6 helices to provide a Ca^{2+}-binding cavity. In addition, they identified mutation-sensitive carbonyl groups in the M4 helix.

The properties common to at least some of the transport systems are summarized in Table 3. Some of these examples correspond to active transport, others do not. For all transport systems, the transporter binds the transported substrate. Furthermore, the transporters have been shown to undergo a conformational change. Channel behavior has been shown, at least under some conditions, for some of the transporters.

Table 3 Summary of the Properties of Some Transport Systems[a]

Ion or Solute	Active Transport	Binding demonstrated	Channel Properties	Conformational change of transporter
Anion Exchanger	No	Yes[b]	Yes[c]	Yes[d]
Na^+, K^+ (ATPase)	Yes[e]	Yes (Table 2)	Yes[f]	Yes[g]
Ca^{2+} (ATPase)	Yes	Yes (Table 2)	Yes[h]	Yes[i]
H^+ (ATP synthase)	Yes	Yes (Table 2)	Yes[j]	Yes (see Chapter 17)
Na^+-glucose (cotransporter)	Yes	--	--	Yes[k]
Na^+-amino acid (cotransporter)	Yes	--	--	Yes[l]

[a] Yes indicates that the phenomenon has been observed for the solute or the transporter [b] Falke et al. (1984b); [c] Giebel and

Passow (1960); [d] Falke et al (1984b);[e] Yamaguchi and Tonomura (1980);[f] Last et al. (1983); [g]Jorgensen (1975); Karlish and Yates (1978); Koepsell (1972) [h] Shamoo and MacLennan (1975) [i] Imamura et al. (1984); [j] Tanford (1983); [k] Peerce and Wright (1984);[l] Wright and Peerce, 1984.

Many years of collected evidence support the alternate access model represented in Fig. 10. This model, adapted to reflect the various experimental findings for the Ca^{2+}-ATPase, is shown in Fig. 11. In this figure, 2 Ca^{2+} are shown to be bound sequentially. One of these is not readily accessible from either side of the ATPase-channel (occlusion). ATP phosphorylates the ATPase so that the two Ca^{2+} are released sequentially. In this figure the stripes indicate the Ca^{2+} which is bound to the ATPase first (reaction 1-2) and the dotted circles represent the second Ca^{2+} bound in reaction 3. As shown, the first Ca^{2+} is not readily available from the outside or from inside the vesicle. It will equilibrate slowly with Ca^{2+} in the medium. However, phosphorylation of the enzyme (reaction 4) allows the sequential discharge of Ca^{2+} to the inside of the vesicles: the first Ca^{2+} to be bound is released into the vesicles first (reaction 5); the second Ca^{2+} to be bound is released second (reaction 7) and corresponds to the Ca^{2+} which is readily exchangeable with $^{40}[Ca^{2+}]$ before phosphorylation.

An alternate access model for the Ca^{2+}-ATPase highlighting the structural aspects is shown in Fig. 16 (Inesi, 1987).

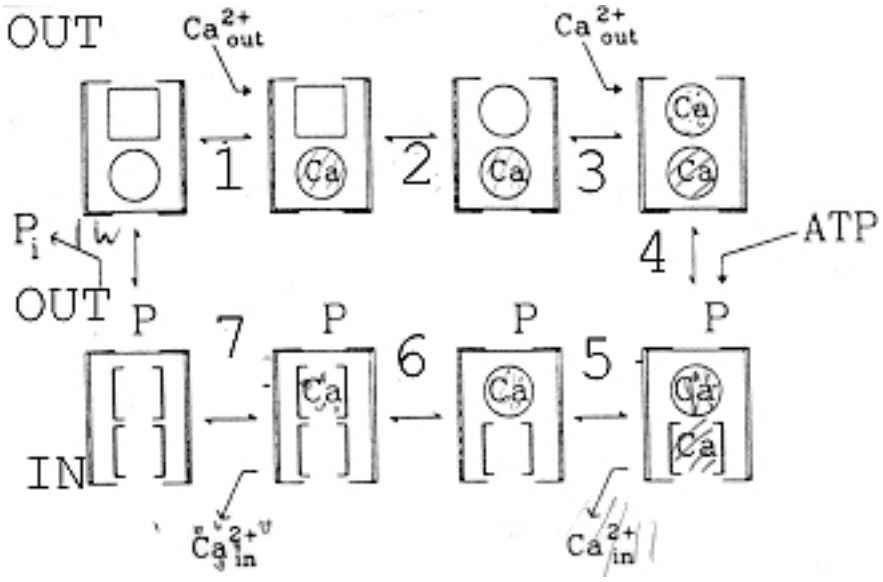


Fig. 11 Diagram representing the sequential mechanism of calcium binding and translocation upon ATP hydrolysis by SR ATPase. From Inesi, 1987. Reproduced by permission. Copyright &copy1987 The American Society for Biochemistry and Molecular Biology.

Inesi (1987) explored details of the Ca^{2+}-ATPase mediated transport of the SR with a pulse chase technique. In one experiment, SR vesicles containing Ca^{2+}-ATPase were first equilibrated with the radioactive isotope $[^{45}Ca]^{2+}$. This incubation was then followed by a chase with nonradioactive $[^{40}Ca]^{2+}$. The time course of the release of the labelled Ca^{2+} at low temperature is shown in Fig. 12 (Inesi, 1987).

In this figure, bound radioactive Ca^{2+} in the ordinate is shown as a function of the time after addition of the nonradioactive Ca^{2+}. The total initial binding corresponds to 2 Ca^{2+} per enzyme molecule. A rapid initial release, over in about 0.2 s, is followed by a much slower one, which cannot be seen with the time scale used. The vastly different rates of release are in agreement with the sequential model of Fig. 11. The faster release corresponds to the Ca^{2+} which is bound second and readily accessible from the outside medium.

La^{3+} displaces all bound Ca^{2+}, so that in the presence of ATP, any Ca^{2+} not displaced by La^{3+} represents Ca^{2+} which has been occluded or transported into the vesicle. The relationship between translocation and binding was examined in an experiment whose results are represented in Fig. 13. The experimental design is shown diagrammatically on the left side of the figure. Curve A represents results obtained without a chase. $[^{45}Ca]^{2+}$ was first bound to the ATPase of the vesicles and ATP added subsequently. La^{3+} was added at the various times indicated in the abscissa. The Ca^{2+} translocated into the vesicle is first very rapid, corresponding to the translocation of Ca^{2+} initially bound to the ATPase. This is followed by a slower transport that represents the Ca^{2+} subsequently transported into the vesicle. When ATP is added simultaneously to a chase with $[^{40}Ca]^{2+}$, the amount transported (curve B) corresponds exactly to that bound (2 Ca^{2+}/enzyme); no additional translocation of the radioactive Ca^{2+} can take place because of the chase. As indicated by Fig. 12, a chase of 0.2 s with $[^{40}Ca]^{2+}$ removes the molecule of Ca^{2+} that was bound second by the ATPase. The transport of the remaining Ca^{2+} ion (the first to be taken up) can, therefore, be followed by introducing ATP after a 0.2 s chase with the non-radioactive Ca^{2+} (curve C). After the 0.2 seconds chase, only half of the radioactive Ca^{2+} was transported into the vesicle (curve C). This indicates that the Ca^{2+} which occupies the position closer to the outside, is transported first into the vesicle, as predicted from a sequential model of Fig. 11.

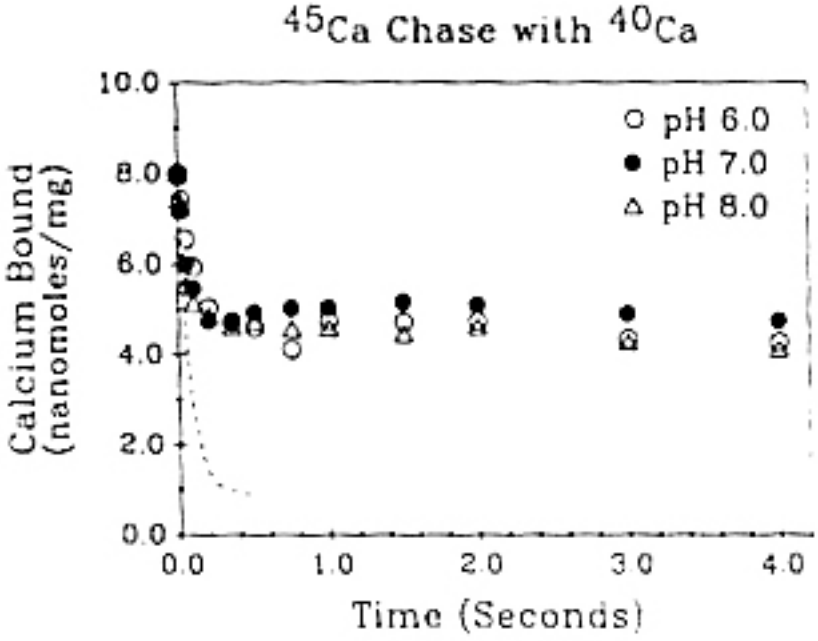


Fig. 12 Isotopic exchanges of bound Ca^{2+}. From Inesi, 1987. Reproduced by permission. Copyright &copy1987 The American Society for Biochemistry and Molecular Biology.

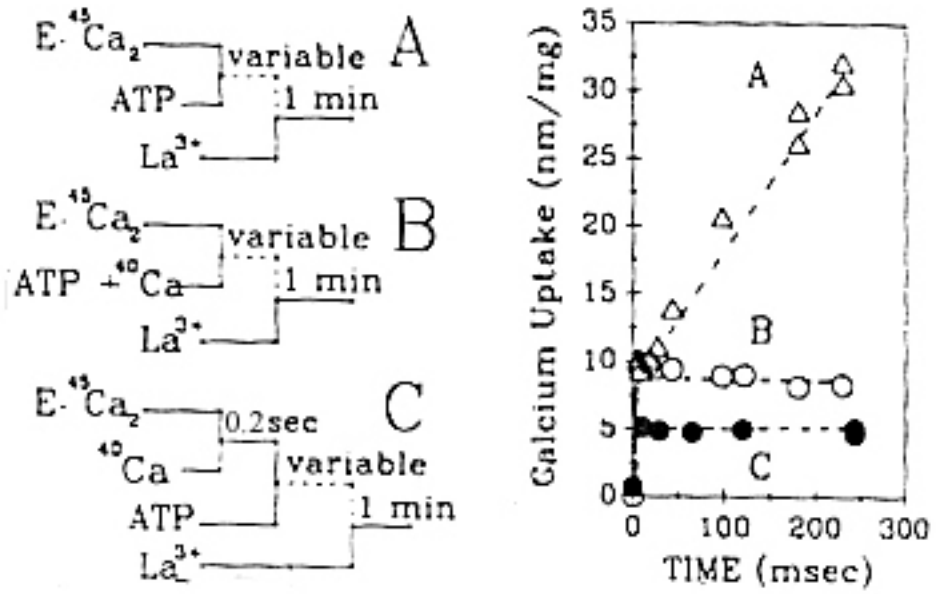


Fig. 13 Quench-flow measurements of ATP-dependent calcium uptake. From Inesi, 1987. Reproduced by permission. Copyright &copy1987 The American Society for Biochemistry and Molecular Biology.

The Ca^{2+} uptake of the initial burst (Fig. 13A) may include Ca^{2+} that is not exchangeable and is trapped in the ATPase, i.e. occluded. A different experimental design can differentiate between bound Ca^{2+} and occluded Ca^{2+}. When ADP is added in the presence of a Ca^{2+}-chelator [ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA)], the phosphorylation of the transporter is reversed. ADP is phosphorylated and the occluded Ca^{2+} is released into the medium. Only one single cycle of the enzyme is possible because there is no P_i present. In contrast, the Ca^{2+} transported into the vesicles would be retained (and would not be released by La^{3+}). The results of this experiment are shown in Fig. 14, which shows the radioactive Ca^{2+} uptake in the ordinate. The time shown in the abscissa represents the time of addition of ADP + EGTA which is then followed by the addition of La^{2+}. In curve A, the preparation is preincubated in $[^{45}Ca]^{2+}$. Then $[^{40}Ca]^{2+}$ and ATP are added simultaneously. In this case, the Ca^{2+} uptake after the ADP+EGTA addition represents the transported Ca^{2+} (amount taken up + amount occluded). At the earlier times of addition of ADP + EGTA, 4 to 5 nanomoles of Ca^{2+} are taken up per mg, compared to 9 to 10 without the ADP + EGTA treatment (Fig. 14A). Therefore, approximately half of the original Ca^{2+} taken up is in the occluded form. When ATP is added after the 0.2 s of $[^{40}Ca]^{2+}$ chase (which removes the more external Ca^{2+}) (Fig. 14B), half of the Ca^{2+} uptake has already become ADP + EGTA insensitive. This shows that the insensitive Ca^{2+} (released into the vesicles) is the one that was bound first (see Fig. 11). These results elaborate and support the alternating-site model and indicate a sequential release of the Ca^{2+}.

Much the same information is available for the Na^+, K^+-ATPase from entirely different experiments. As we have seen, 3 Na^+ and 2 K^+ bind to separate sites of the protein. First they become occluded (i.e., trapped inside the transporter) and then are released to the other side (see Post et al., 1972; Beaugé and Glynn, 1979). In the absence of K^+, Na^+ is still translocated (Garrahan and Glynn, 1967) and the translocation is electrogenic (Fendler et al., 1985; Nakao and Gadsby, 1986). The electrical signal during the ion pumping corresponds to the movement of the ions across the channel that traverses the membrane (e.g., Hilgemann, 1994) and is associated with charge movements. The rate of these electrogenic reactions is dependent on the membrane potential, so that enzymes conformations can be shifted. High speed voltage jumps can be used to initiate this redistribution. Three phases are apparent (Holmgren et al., 2000), reflecting the de-occlusion of the three ions. The results indicate that three are released one at a time, in order.

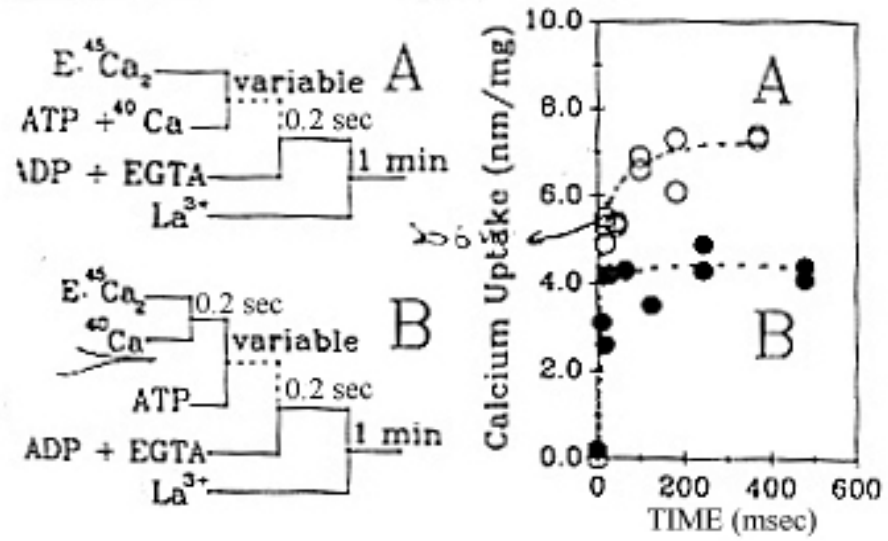
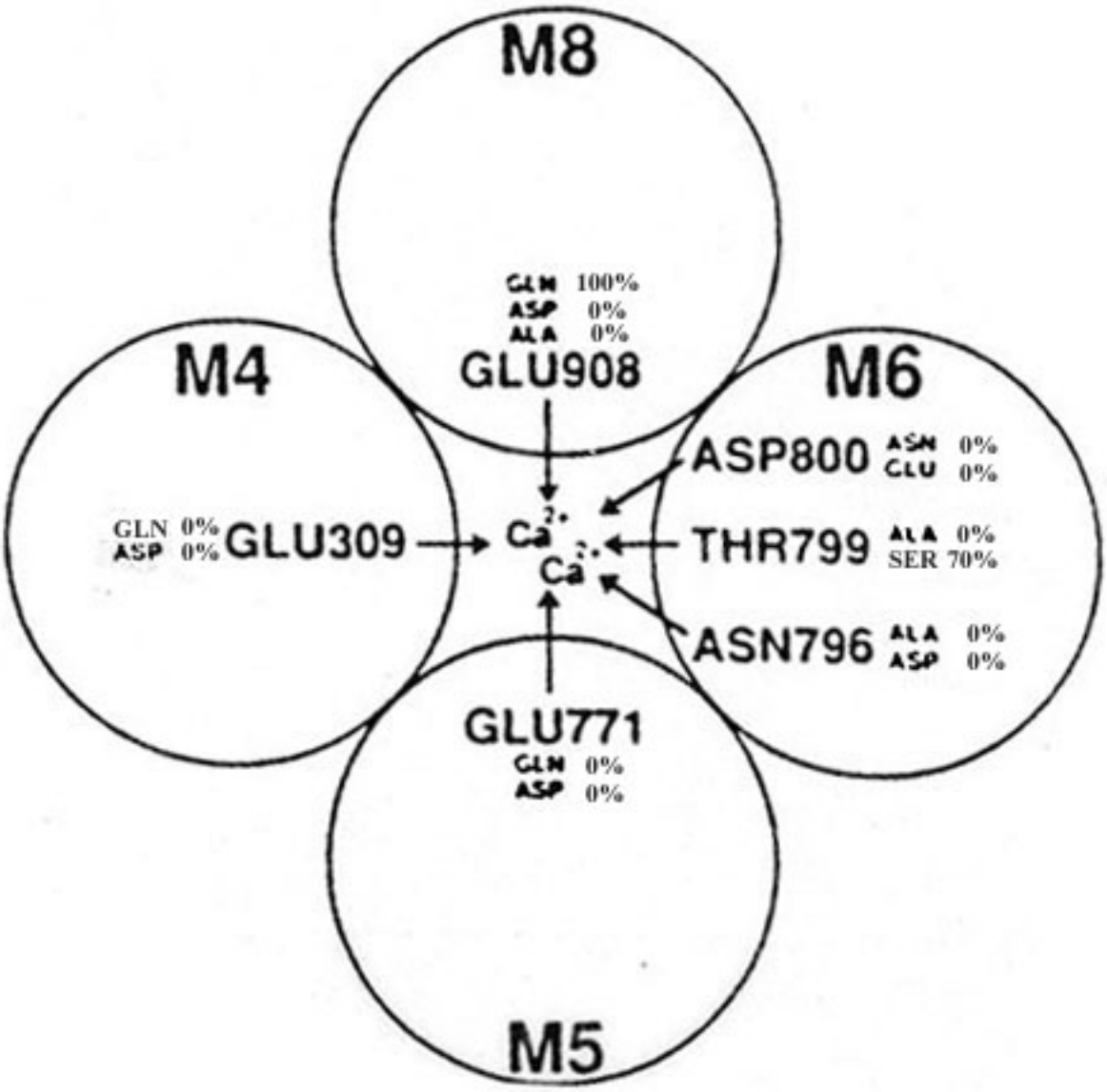


Fig. 14 ADP reversal of ATP-induced calcium translocation. From Inesi, 1987, reproduced by permission.

Analyses of the phosphorylating reactions were also carried out. Either ATP or P_i can phosphorylate the enzyme. The ATP phosphorylation depends on high affinity Ca^{2+} binding. In contrast, the phosphorylation by P_i is blocked by Ca^{2+}.

The Ca^{2+} occlusion was studied on detergent solubilized SR vesicles in the presence of CrATP. CrATP allows occlusion without the hydrolysis of ATP and it also stabilizes the Ca^{2+}-enzyme complex. A HPLC-molecular sieve procedure was used (see Chapter 1) to separate the proteins from free Ca^{2+}. Mutations at the sites thought to bind Ca^{2+}, prevented occlusion (Vilsen and Andersen, 1992, Andersen and Vilsen, 1994).



Summary of amino acid substitution introduced into the predicted Ca^{2+}-binding domain. Glu309, Glu771, Asn796, Thr799, Asp800, and Glu908 are thought to be in the transmembrane segments M4, M5, M6 and M8 respectively. From Clarke et al., 1990b. Reproduced by permission.

As already discussed, there is considerable evidence that the ATPase pumps require a channel-like structural arrangement. Modeling of the four helices thought to be involved in Ca^{2+} binding and which are amphiphilic, show that polar and charged residues are predominantly in one face of each helix with the hydrophobic residues in the opposite face. The hydrophilic components could therefore form hydrophilic clusters in the internal surfaces, thereby forming a channel. The hydrophobic residues, on the other hand, could interact with the bilayers providing the transmembrane arrangement.

Present information (e.g., Inesi et al., 1992; Toyoshima et al. 2000) indicates that the Ca^{2+}-binding domain and the catalytic domain are separated by 50 Å. This spatial arrangement would require that any interaction would be indirect, via a conformational change. We have seen that conformational changes have been demonstrated (see Fig. 9). A possible mechanism for the transport of Ca^{2+} involving the

transmembrane helices is indicated in Fig. 16 (MacLennan, 1990). The shift from E_1 to the E_2 form accompanying phosphorylation, would shift the negatively charged binding groups from the outer to the inner interface. Furthermore, the conformational shift would disrupt the arrangement of the high affinity binding groups to produce low affinity binding sites.

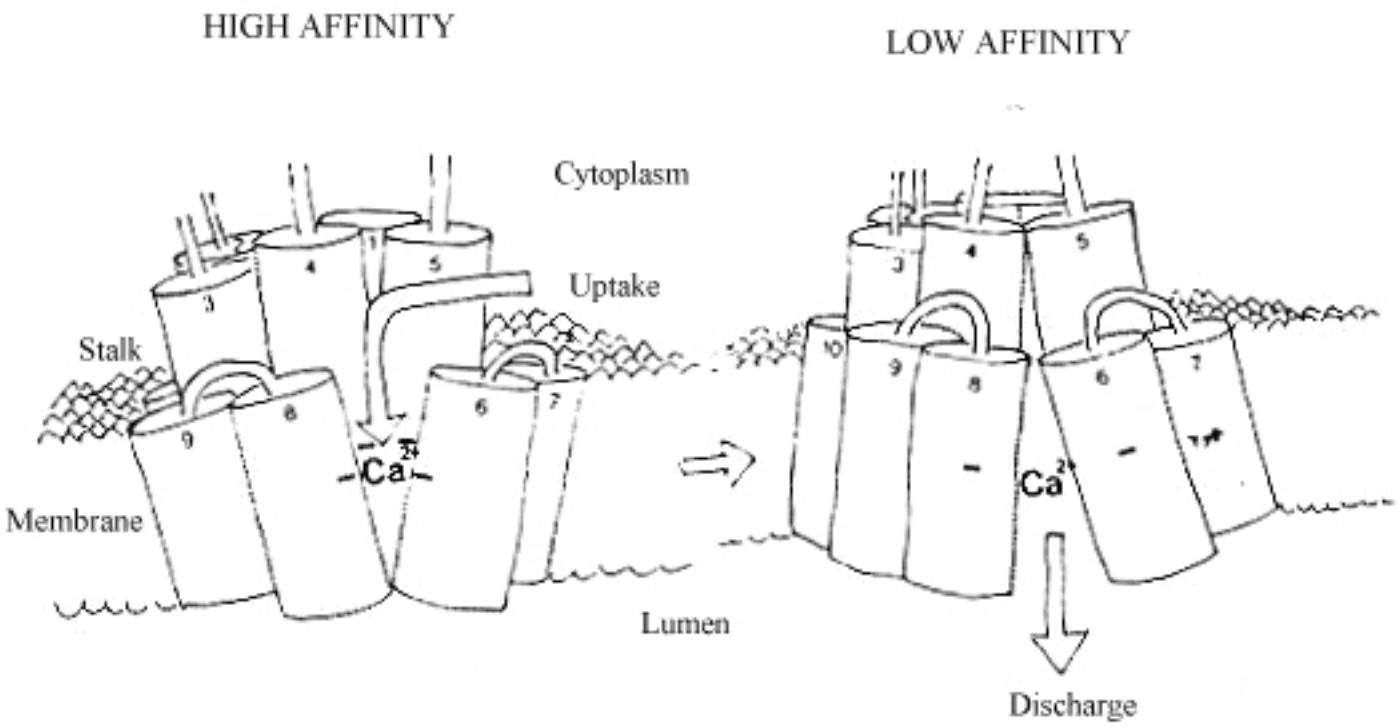


Fig. 16 Model illustrating the possible mechanism of Ca^{2+} transport by the Ca^{2+}-ATPase. In the E_1 configuration, high affinity Ca^{2+}-binding sites are accessible to the cytoplasmic Ca^{2+}. ATP hydrolysis induces the E_2 configuration, in which the access of the binding groups from the cytoplasmic side is blocked and their configuration of the binding groups is disrupted. The disruption results in a low affinity binding. From MacLennan, 1990, reproduced by permission.

SUGGESTED READING

Inesi, G., Zhang, Z., Sagara, Y. and Kirtley, M.E. (1994) Intracellular signaling through long-range linked functions in Ca^{2+} ATPase, *Biophys. Chem.* 50:129-138. (Medline)

MacLennan, D.H., Rice, W.J. and Green, N.M. (1997) The mechanism of Ca2+ transport by sarco(endo)plasmic reticulum Ca^{2+}-ATPases, *J. Biol. Chem.* 272:28815-28818. (MedLine)

Stein, W.D. and Lieb, W.R. (1986) *Transport and Diffusion Across Cell Membranes*, Chapter 6, pp. 475-

612. Academic Press, New York.

Tanford, C. (1984) The sarcoplasmic reticulum calcium pump. Localization of free energy transfer to discrete steps of the reaction cycle, *FEBS Lett.* 166:1-7. (Medline)

General Reviews

Inesi, G.(1994) Teaching active transport at the turn of the twenty first century: recent discoveries and conceptual changes, *Biophys. J.* 66:554-560. http://www.biosci.umn.edu/biophys/OLTB/BJ/Inesi.pdf Adobe Acrobat from www.adobe.com is required for reading pdf files.

Lauger, P. (1984) Channels and multiple conformational states: interrelations with carriers and pumps, *Curr. Top. Membr. Transport* 21:309-326.

REFERENCES

Search the textbook

Back to Chapter 21

REFERENCES

Andersen, J.P. and Vilsen, B. (1994) Amino acids Asn^{796} and Thr^{799} of Ca^{2+}-ATPase of sarcoplasmic reticulum bind Ca^{2+} at different sites, *J. Biol. Chem.* 269:15931-15936. (MedLine)

Beaugé, L.A. and Glynn, I.M. (1979) Occlusion of K ions in the unphosphorylated sodium pump, *Nature* 280:510-512. (Medline)

Blasie, J. K., Herbette, L. G., Pascolini, D., Skita, V., Pierce, D. H. and Scarpa, A. (1985) Time resolved x-ray diffraction of sarcoplasmic reticulum membrane during active transport, *Biophys. J.* 48:9-l8. (Medline)

Bontig, S. I., Schuurmans Stekhoven, F. M. A. H., Swarts, H. G.P. and dePont, J. J. H. H. M. (1979) The low-energy phosphorylated intermediate of Na^+,K^+-ATPase. In *Na,K-ATPase, Structure and Kinetics* (Skou, J. C., and Nϕrby, J. G., eds.), pp. 317-330. Academic Press, New York.

Charnock, J. S., Trebilcock, H. A. and Casley-Smith; J. R. (1972) Demonstration of transport adenosine triphosphatase in the plasma membranes of erythrocyte ghosts by quantitative electron microscopy, *J. Histochem. Cytochem.* 20:1069-1080. (Medline)

Cheong, G.W., Young, H.S., Ogawa, H., Toyoshima, C. and Stokes, D.L. (1996) Lamellar stacking in three-dimensional crystals of Ca(2+)-ATPase from sarcoplasmic reticulum, *Biophys. J.* 70:1689-1699. (MedLine)

Clarke, D.M., Maruyama, K., Loo, T.W., Leberer, E., Inesi, G. and MacLennan, D.H. (1989) Functional consequences of glutamate, aspartate, glutamine and aspargine mutations in the stalk section of the Ca^{2+}-ATPase of sarcoplasmic reticulum, *J. Biol. Chem.* 264:11246-11251. (Medline)

Clarke, D.M., Loo, T.W. and MacLennnan, D. (1990a) The epitope for monoclonal antibody A20 (amino acids 870-890) is located in the luminal surface of the Ca^{2+}-ATPase of sarcoplasmic reticulum, *J. Biol. Chem.* 265:17405-17408. (Medline)

Clarke, D.M., Loo, T.W. and MacLennnan, D. (1990b) Functional consequences of alterations to amino acids located in the nucleotide binding domain of the $Ca^{2+\text{-ATPase,}}$ of sarcoplasmic reticulum, *J. Biol. Chem.* 265:6262-6267. (Medline)

DeLong, L.J. and Blasie, J.K. (1993) Effect of Ca^{2+} binding on the profile structure of the sarcoplasmic reticulum membrane using time-resolved x-ray diffraction, *Biophys. J.* 64:1750-1759. (Medline)

Fendler, K., Grell, E., Haubs, M. and Bamberg, E. (1985) Pump currents generated by the purified Na^+K^+-ATPase from kidney on black lipid membranes, *EMBO J.* 4:3079-3085. (Medline)

Garrahan, P.J. and Glynn, I.M. (1967) The behaviour of the sodium pump in red cells in the absence of external potassium, *J. Physiol.* (London) 192:159-174. (MedLine)

Giebel, O. and Passow, H. (1960) Die permeabilität der eythrocytemembran für organische anionen, Pfluegers Arch. 271:378-388.

Glynn, I. M. and Karlish, S. J. D. (1990) Occluded cations in active transport, *Annu. Rev. Biochem.* 59:171-205. (Medline)

Green, N.M. and MacLennan, D.H. (2002) Calcium callisthenics, *Nature* 418:598-599.

Gresalfi, T.J. and Wallace, B.A. (1984) Secondary structural composition of the Na/K-ATPase E1 and E2 conformers, *J. Biol. Chem.* 259:2622-2628. (MedLine)

Hilgemann, D.W. (1994) Channel-like function of the Na,K pump probed at microsecond resolution in giant membrane patches, *Science* 263:1429-1432. (Medline)

Holmgren, M., Wagg, J., Bezanilla, F., Rakowski, R.E., De Weer, P. and Gadsby, D.C. (2000) Three distinct and sequential steps in the release of sodium ions by the Na^+/K^+-ATPase, *Nature* 403:898-901. (Medline)

Ikemoto, N. (1976) Behavior of Ca^{2+} transport sites linked with the phosphorylation reaction of ATPase purified from the sarcoplasmic reticulum, *J. Biol. Chem.* 251:7275-7277. (Medline)

Inesi, G. (1987) Sequential mechanism of calcium binding and translocation in sarcoplasmic reticulum adenosine triphosphatase, *J. Biol. Chem.* 262:16338-16342. (Medline)

Inesi, G., Kurzmack, M., Coan, C. and Lewis, E. (1980) Cooperative calcium binding and ATPase activation in sarcoplasmic reticulum vesicles, *J. Biol. Chem.* 255:3025-3031. (Medline)

Inesi, G., Lewis, D., Nikic, D. and Kirtely, M.E. (1992) Long range intramolecular linked functions in calcium transport ATPase, in *Advances in Enzymology*. Meister, A., ed.. Wiley and Sons, New York, pp. 185-215. (Medline)

Inesi, G., Zhang, Z., Sagara, Y. and Kirtley (1994) Intracellular signaling through long-range linked functions in Ca^{2+} ATPase, *Biophys. Chem.* 50:129-138. (Medline)

Jorgensen P. L., and Petersen, J. (1979) Protein conformations of the phosphorylated intermediates of purified Na^+,K^+-ATPase studied with tryptic digestion and intrinsic fluorescence as tools. In *Na^+,K^+ATPase Structure and Kinetics* (Skou, J. C., and Nϕrby, J. G., eds.), pp. 143-155. Academic Press, New York.

Knowles, A. F. and Racker, R. (1975) Formation of adenosine triphosphate from P_i and adenosine triphosphate by purified Ca^{2+}-adenosine triphosphatase, *J. Biol. Chem.* 250:1949-1951. (Medline)

Kühlbrandt, W., Zeelen, J. and Dietrich, J. (2002) Structure, mechanism, and regulation of the *Neurospora* plasma membrane H^+-ATPase, *Science* 297:1692-1696. (MedLine)

Kyte, J. (1971) Purification of the sodium- and potassium-dependent adenosine triphosphatase from canine renal medulla, *J. Biol. Chem.* 246:4157-4165. (Medline)

Kyte, J. (1974) The reactions of sodium and potassium ion activated adenosine triphosphatase with specific antibodies, *J. Biol. Chem.* 249:3652-3660. (Medline)

Kyte, J. (1981) Molecular considerations relevant to the mechanism of active transport, *Nature* 292:201-204. (Medline)

Last, T. A., Gantzer, M. L. and Tyler, C. D. (1983) Ion-gated channel induced in planar bilayers by incorporation of (Na^+,K^+)-ATPase, *J. Biol. Chem.* 258:2399-2404. (Medline)

MacLennan, D. H. (1990) Molecular tools to elucidate problems in excitation-contraction coupling, *Biophys. J.* 58:1355-1365. (Medline)

MacLennan, D.H., Brandl, C.J., Korczak, B. and Green, N.M. (1985) Amino-acid sequence of a Ca^{2+} + Mg^{2+}-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence, *Nature* 316:696-700. (MedLine)

MacLennan, D.H., Rice, W.J. and Green, N.M. (1997) The mechanism of Ca^{2+} transport by sarco(endo)plasmic reticulum Ca2+-ATPases, *J. Biol. Chem.* 272:28815-28818. (MedLine)

Masui, H. and Homareda, H. J. (1982) Interaction of sodium and potassium ions with Na^+,K^+-ATPase. I. Ouabain-sensitive alternative binding of three Na^+ or two K^+ to the enzyme, *J. Biochem.* 92:193-217.

Nakamura, S., Suzuki, H. and Kanazawa, T. (1994) The ATP- induced change in tryptophan fluorescence reflects a conformational change upon formation of ADP-sensitive phosphoenzyme in the sarcoplasmic reticulum Ca^{2+}-ATPase, *J. Biol. Chem.* 269:16015-16019. (Medline)

Nakao, M. and Gadsby, D.C. (1986) Voltage dependence of Na translocation by the Na/K pump, *Nature* 323:628-630. (Medline)

Pascolini, D., Herbett, L.G., Skita, V., Asturias, F., Scarpa, A. and Blasie, J.K. (1988) Changes in the sarcoplasmic reticulum membrane profile induced by enzyme phosphorylation to the E_1P at 16 resolution via time-resolved X-ray diffraction, *J. Biophys.* 54:679-688. (Medline)

Peerce, B.E. and Wright, E.M. (1984) Sodium-induced conformational changes in the glucose transporter of intestinal brush borders, *J. Biol. Chem.* 259:14105-14112. (Medline)

Post, R.L., Hegyvary, C. and Kume, S. (1972) Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase, *J. Biol. Chem.* 247:6530-6540. (Medline)

Post, R. L., Sen, A. K. and Rosenthal, A. S. (1965) A phosphorylated intermediate in adenosine triphosphate-dependent sodium and potassium transport across kidney membranes, *J. Biol. Chem.* 240:1437-1445.

Post, R. L., Kume, S., Tobin, T., Orgutt, R., and Shu, A. K. (1969) Flexibility of an active center in sodium plus potassium adenosine triphosphatase, *J. Gen. Phys.* 54:306s-326s.

Post, R. L., Taniguchi, K. and Toda, G. (1974) Synthesis of adenosine triphosphate by Na^+,K^+-ATPase, *Ann. N.Y. Acad. Sci.* 242:80-91. (Medline)

Schoot, B. M., Schoots, A. F. M., dePont, J. J. H. H. M., Schuurmans Stekhoven, F. M. A. H., and Bonting, S. L. (1977) Studies on (Na^+-K^+) activated ATPase. XVI. Effects of *N*-ethylmaleimide on overall and partial reactions, *Biochim. Biophys. Acta* 483:181-192. (Medline)

Sen, A., Tobin, T. and Post, R. L. (1969) A cycle for ouabain inhibition of sodium- and potassium-dependent adenosine triphosphatase, *J. Biol. Chem.* 244:6596-6604. (Medline)

Shamoo, A. and MacLennan, D. H. (1975) Separate effects of mercurial compounds on the ionophoric and hydrolytic functions of the (Ca^{2+} Mg^{2+})-ATPase of sarcoplasmic reticulum, *J. Membr. Biol.* 25:65-74. (Medline)

Siegel, G. J., Koval, G. J. and Albers, R. W. (1969) Sodium- potassium-activated adenosine

triphosphatase, *J. Biol. Chem.* 244:3264-3269. (Medline)

Tanford, C. (1983) Mechanism of free energy: coupling in active transport, *Annu. Rev. Biochem.* 52:379-409. (Medline)

Tokoshima, M., Sasabe, H. and Stokes, D.L. (1993) Three-dimensional cryo-electron microscopy of the calcium ion pump in the sarcoplasmic reticulum membrane, *Nature* 362:469-471.

Toyoshima, C., Nakasako, M., Nomura,H. and Ogawa, H. (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution, *Nature* 405:647-655. (MedLine)

Toyoshima C. AND Nomura, H. (2002) Structural changes in the calcium pump accompanying the dissociation of calcium, *Nature* 418:605-611. (MedLine)

Vilsen, B. and Andersen, J.P. (1992) CrATP-induced Ca^{2+} occlusion in mutants of the Ca^{2+}-ATPase of the sarcoplasmic reticulum, *J. Biol. Chem.* 267:25739-25743. (Medline)

Weber, G. (1972) Ligand binding and internal equilibria in proteins, *Biochemistry* 11:864-878. (Medline)

Weber, G. (1974) Addition of chemical and osmotic energies by ligand protein interactions, *Ann. N.Y. Acad. Sci.* 227:486-496. (Medline)

Wright, E.M. and Peerce, B.E. (1984) Identification and conformational changes of the intestinal proline carrier, *J. Biol. Chem.* 259:14993-14996. (Medline)

Yamaguchi, M. and Tonomura, Y. (1980) Binding of monovalen cations to Na^+,K^+-dependent ATPase purified from porcine kidney, *J. Biochem.* 88:1365-1375. (Medline)

Yu, X., Carroll, S., Rigaud, J.L., and Inesi, G. (1993) H^+ countertransport and electrogenicity of the sarcoplasmic reticulum Ca2+ pump in reconstituted proteoliposomes, *Biophys. J.* 64:1232-1242. (MedLine)

Zhang, P., Toyoshima, C., Yonekura, K., Green, N.M., and Stokes, D.L. (1998) Structure of the calcium pump from sarcoplasmic reticulum at 8-Å resolution, *Nature* 392:835-839. (MedLine)

22. Excitation and Conduction

Signals between cells and within cells are necessary for most physiological functions. These signals are composed of chemical and electrical events of some complexity.

In one way or another, signaling frequently makes use of the electrochemical gradient across the cell membrane. This is the case not only for excitable cells, the primary topic of this chapter, but also for

other cells. For example, the entry of Ca^{2+} from either the medium or internal stores, driven by the Ca^{2+} electrochemical gradient, has a role in the release of secretory products and in the release of neurotransmitters, which transmit signals between nerve cells. Electrical signals are also coupled to the release of Ca^{2+} needed for the contraction of muscle or less organized contractile systems (seeChapter 23). Resting membrane potentials are present in most cells, and even in some plant cells dynamic electrical changes take place on stimulation (discussed later in this chapter). The precise physiological role of these electrical events in plants is still not clear.

Nerve cells (and muscle cells) are specialized for conducting signals through the transmission of an electrical event (the *impulse* or *action potential*). A variety of stimuli are effective in initiating impulses. Perhaps the most direct way to elicit a nerve impulse in the laboratory is by means of an electrical current of an intensity above a critical level (the *threshold*). Once initiated, the nerve impulse can be propagated without loss of intensity over the entire length of the nerve cell, which can be 1 m long or even longer.

Although generally similar events underlie the conduction of impulses, even in nerves, the speed of conduction varies widely. Some mammalian nerve fibers can conduct with speeds as high as l00 m/s (over 200 miles per hour!), while others are much slower, with a conduction rate as low as 0.1 m/s.

The response to stimulation of nerves can be relatively direct and readily observable. One stimulating event in a motor nerve may result in one contraction of a striated muscle. However, the response may also be complex and subtle. This is particularly true where nerve cells interact, as in ganglia or in the central nervous system, or where the effector cell responds in a complex manner (e.g., smooth muscle does not respond with a single contraction).

I. NEURONS: UNITS OF CONDUCTION

Many kinds of cells can propagate an electrical event or an electrical impulse. However, nerve cells (*neurons*) function primarily to transmit impulses.

Some of the kinds of neurons encountered in the cerebellum are shown in Fig. 1 (Mugnaini and Floris, 1994). In general, different shapes are related to different functions of these neurons, such as, where they receive inputs and send their output signals. Many neurons have a characteristic stellate shape. The cell has many small branches, the *dendrites*, and a long process, the *axon*. Generally, the cell bodies are present in ganglia or in the central nervous system. The axon, a nerve fiber, is the portion that conducts the nerve impulse over long distances.

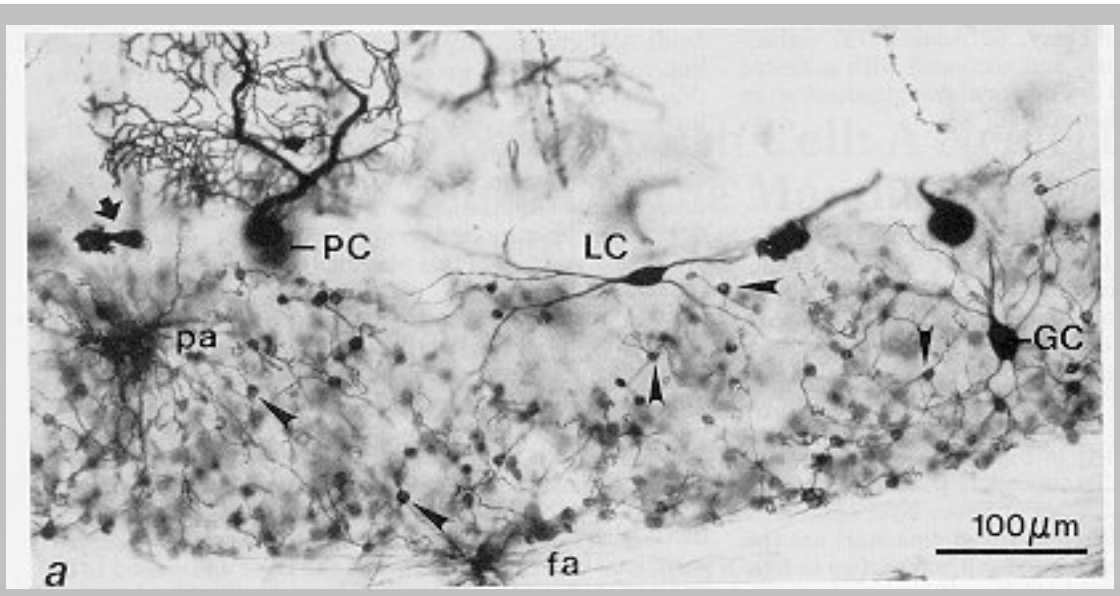


Fig. 1 Cells present in a section of mammalian cerebellar cortex shown to illustrate the variety of sizes and shapes of neurons and associated cells. PC = Purkinje cell: this is an inhibitory output neuron. Arrows= granular cells: the only excitatory cells present, GC = Golgi cell: these are inhibitory cells that can shut the granular cell excitatory output, LC = Lugaro cells: their function is not clear at this time; pa and fa are astrocytes (from Mugnaini and Floris, 1994). Reproduced by permission.

As shown in Fig. 1, neurons assume many shapes and forms. The size and morphology of the cell body (*soma*) of neurons vary widely. In the mammalian retina alone, a minimum of twenty two distinct cell types have been found (MacNeil and Masland, 1998). *Globulus* cells of invertebrates can be smaller than 3 μm in diameter. However, neurons can also be huge: for example, in gastropods they can be larger than 800 μm and therefore visible with the naked eye. Even within the same organism the variation in size and shape is surprising. Generally, the smaller cells have very little cytoplasm and larger cells have a good deal of it. The size of the cell is only roughly correlated with the size of the axon that originates from it.

Fig. 2 shows a leech segmental ganglion stained by immunofluorescence using an antibody to the neurotetrapeptide FMRFamide (Phe-Met-Arg-Phe-NH_2) (Kuhlman et al., 1985). The neurons, which occupy specific positions, differ not only in size or morphology but also in their capacity to interact with the antibody. In Fig. 2B, the neuron that was indicated in Fig. 2A by the arrow has been microinjected with the water-soluble fluorescent dye Lucifer Yellow. Diffusion of the dye inside the cell permits tracing the various extensions corresponding to the same cell.

At specialized junctions, the *synapses*, a nerve impulse in one cell can be communicated to another cell, such as another neuron. Synapses are usually on the surface of the dendrites or on the cell body but occasionally may be on the axon of the cell receiving the impulse. There are also synaptic connections between nerve fibers and effector organs, such as muscle and glands. The mechanism of synaptic transmission may be quite different depending on the synapse, as discussed later in the chapter. In some neuronal synapses, the transmission is electrical through specialized junctions (the *gap junctions*), resembling the transmission in the nerve fibers themselves (*electrical synapses*). In others, the transmission is carried out by the release of a neurotransmitter, which then interacts with postsynaptic

cells and may have an excitatory or an inhibitory effect (the *chemical synapses*).

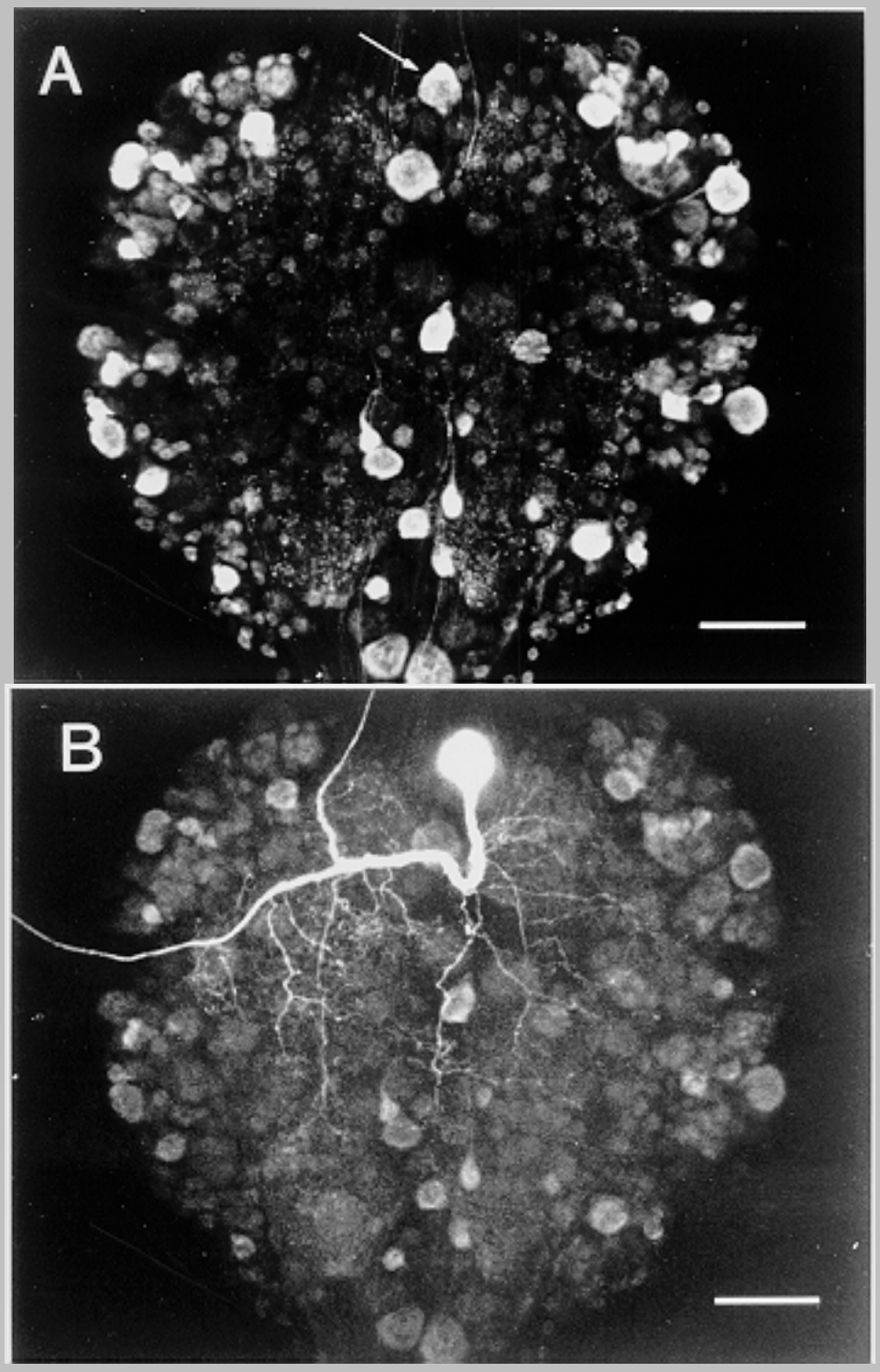


Fig. 2 Leech segmental ganglion stained with an antiserum directed against the neuropeptide FMRFamide. Primary antiserum is visualized by indirect immunofluorescence. (A) Demonstration of several cell bodies with an antigen sensitive to the antibody. (B) The same preparation but after intracellular injection of the water-soluble dye Lucifer Yellow. The arrow in (A) indicates the neuron (the penile evertor motor neuron) that is microinjected with Lucifer Yellow. The bar represents 0.1 mm. Courtesy of Ronald C. Calabrese.

In invertebrates, the cell bodies are on the outside layer of the ganglia (the *rind*), whereas axons, synapses

and dendrites are in the *core* of the ganglia. In the vertebrate central nervous system, the cell bodies are in the *gray matter* and the axons predominantly in the *white matter*. In the vertebrate central nervous system, in portions that are considered more primitive (e.g., in the cerebellum), the gray matter is frequently on the inside of the tissues; in higher brain regions (e.g., in the cerebral cortex), the gray matter is on the outside.

Many invertebrates and some primitive vertebrates have giant axons. In some organisms, each giant axon is formed from a single cell. In others, it is formed by the fusion of axons from many separate cells. Giant axons such as those of the squid, can be as much as 1 mm in diameter. Their size and hardiness, when excised, have made them a favorite experimental preparation.

In ganglia and in tissues of the central nervous system, specialized cells (*neuroglia*) form sheaths around one or more cells and also act as packing between the cells. They probably have an important maintenance role. In vertebrates, an insulating layer of structured lipoprotein, called *myelin*, surrounds the larger axons. The myelin layer is interrupted every millimeter or so by deep constrictions or breaks, called the *nodes of Ranvier*, where impulse conduction takes place. In some axons, the covering is simpler and may consist of a single layer of glial cells.

In most vertebrate nerves, the axons are held together in bundles that are enclosed in sheaths. A single bundle of nerves may contain fibers from neurons with very distinct functions. For example, some of the axons may be from *motor* neurons, which control striated muscle contraction, and others may be from *sensory* neurons, which transmit information from a receptor. Similar arrangements can also occur in invertebrates, but these animals usually have far fewer nerve fibers.

In most cells, there is an electrical potential difference (the *resting potential*) between the internal cytoplasm and the external environment. Generally, the inside is negative relative to the outside; the cell membrane is said to be *polarized*. In excitable cells, this potential is poised to permit the release of a nerve impulse at a speed unmatched by most other cellular events. This nerve impulse, once initiated, is propagated along the axon without decrement.

For purposes of discussion, we shall regard a nerve impulse as the discharge of the stored (resting) electrical potential (a *depolarization*). As we shall see, it is actually much more complex than this and is due to a transient reversal of polarization (the inside becomes positive).

Resting potentials and changes therein can be examined fruitfully in terms of ionic gradients, ionic channels and ionic movements across the membrane, although some of the mechanisms underlying these events still remain obscure.

II. IONIC ORIGINS OF THE RESTING POTENTIAL

When two different concentrations of a solution of the same salt come in contact with each other, there is a net flow of both ions from the area of high concentration to that of low concentration. If the mobilities

of the two component ions differ significantly, the cation and the anion tend to separate. The electrical potential produced by the separation opposes the diffusional forces and the actual amount of separation is limited and, in effect, the potential accelerates the diffusion of the slower ion. The potential generated by the diffusional events is the *diffusion* or *junction potential*. The charge of the more dilute phase corresponds to that of the more mobile ion, which moves ahead of the oppositely charged ion.

For the passage of 1 equivalent of charge from one phase (phase 1) to the other (phase 2), the change in free energy is

$$\Delta G = t_+ RT \ln a^+_2/a^+_2 + t_- RT \ln a^-_1/a^-_1 \quad (1)$$

where *a* refers to the activity of the ion in question and generally can be approximated by the concentration. The subscripts 1 or 2 refer to the phase, *t* is the transference number defined in terms of the mobility of the ions (*u*),

$$t_+ = u_+/(u_+ + u_-) \quad (1a)$$

$$t_- = u_-/(u_+ + u_-) \quad (1b)$$

and the subscript signs (+ and -) refer to the charge of the ion.

As discussed in Chapter 12, the electrical potential corresponds to $\Delta G/zF$. The diffusion potential, $\Delta\Psi_d$, will therefore take the form

$$\Delta\Psi_d = t_+ RT/zF \ln a^+_2/a^+_1 + t_-RT/zF \ln a^-_2/a^-_1 \quad (2)$$

$a^+_2 \sim a^-_2$ and $a^+_1 \sim a^-_1$ because in each phase, the concentration of the cation must be virtually the same as that of the anion. Therefore, for monovalent ions, the relationship can be written in a simpler form:

$$\Delta\Psi_d = (t_+ + t_-) RT/F \ln a_2/a_1 \quad (3)$$

or

$$\Delta\Psi_d = (1-2t_-) RT/F \ln a_2/a_1 \quad (4)$$

When the mobility of one of the ions is much greater than that of the other, only the faster ion needs to be considered. Supposing that *u* approaches 0, then *t* approaches 0 [Eq. (1b)] and 1 - 2*t* approaches 1, so that $\Delta\Psi_d$ is now independent of the mobilities. This point becomes rather significant in relation to biological potentials. Where the mobility of one ion surpasses that of the other, Eq. (4) becomes (5), the familiar

Nernst equation, which we have encountered before.

$$\Delta \Psi_d = RT/zF \ln a_2/a_1 \quad (5)$$

$$= -58 \text{ mV } \log_{10} a_2/a_1 \text{ for } T=20^oC$$

The resting potentials of most cells are governed by the principles that have been outlined. Generally, the permeability of the membrane to K^+ is far greater than the permeability to the other ions. In frog sartorius muscle, the permeability to K^+ is 100 times greater than to Na^+, and in the freshwater alga, *Nitella*, it is 52 times greater. Therefore, Eq. (5) can be used and *a* in the equation corresponds approximately to the concentration of K^+.

In some freshwater cells, such as the algae *Nitella* and *Chara*, the internal concentrations of both Na^+ and K^+ are much higher than those in the surroundings. However, in most complex multicellular organisms the total ionic concentrations of intra- and extracellular fluids are nearly equal. Nevertheless, there are steep gradients of ions, notably K^+ (high on the inside) and Na^+ (high on the outside).

The efflux of K^+ and the influx of Na^+ is compensated by the transport activities of the cell membrane. In the steady state, the internal ionic composition is maintained by the balance between the movement of ions in the direction of the electrochemical gradient and the active transport of the ions against the electrochemical gradient, so that the internal [K^+] remains high and the internal [Na^+] low (see Chapter 20).

Clearly, the cell membrane plays a fundamental role in the maintenance of the resting potential. However, as already discussed, a potential difference between two phases does not require the presence of a membrane. When one of the ionic components is restrained (e.g., is part of the cell structure), the mobile ions follow a Donnan distribution and the potential may still be considerable (see Collins and Edwards, 1971) even in the absence of a membrane.

The ionic compositions of the internal and external media of some cells have been studied extensively. The values for frog sartorius muscle are represented in Table 1 (Conway, 1957). It is possible to predict the magnitude of the potential between the external and internal phases, the resting potential, by means of Eq. (5), remembering the higher mobility of K^+. At l8°C, this value is calculated to be 102 mV, with the inside negative. The potential can be measured directly by inserting an electrode with a tip of microscopic dimensions (a microelectrode) into the muscle fibers. With an external concentration of about 2.5 mM K^+, the measured value is 80 to 92 mV, which is not very different from the calculated value.

It is possible to study the resting potential over a wide range of external K^+ concentrations. This has been done by placing the muscle fiber in various concentrations of KCl or a K^+ salt of a nonpermeable anion (e.g., acetate). The results of such experiments yield the relationship represented in Fig. 3 (Conway,

1957). The straight line (line A) corresponds to a slope of 57 mV for a 10-fold change in K^+ concentration. The value predicted from Eq. (5) is 58 mV. Thus, the equation has very good predictive value.

Similar results are obtained with nerve and other cells as well. The resting potential of the fresh water alga, *Chara*, measured directly, is - 181 mV (Gaffey and Mullins, 1958). The value calculated from the K^+ concentration and Eq. (5) is -184 mV, in good agreement with the actual measurement.

Clearly, the results indicate that in general the differential distribution of K^+ between the intracellular and extracellular phases is responsible for the resting potential.

Table 1 Intrafiber Composition[a]

Constituent	Muscle	Plasma	Concentration in fiber water
K	83.8	2.15	124
Na	23.9	103.8	3.6
Ca	4.0	2.0	4.9
Mg	9.6	1.2	14.0
Cl	10.7	74.3	1.5
HCO_2	11.6	25.4	12.4
Phosphate	5.3	3.1	7.3
Sulfate	0.3	1.9	0.4
Phosphor-creatine	23.7		35.2
Carnosine	11.0		14.7
Amino acids	6.8	6.9	8.8
Creatine	5.3	2.1	7.4
Lactate	3.1	3.3	3.9
ATP	2.7		4.0
Hexose monophosphate	1.7		2.5
Glucose	0.5	3.9	
Protein	1.5	0.6	2.1
Urea	1.6	2.0	2.0
Water (g/kg)	800	954	
Interspace water (g/kg)	127		

Source: Conway (1957). Reproduced by permission.
[a] Frog muscle and plasma (moles/kg), except where noted

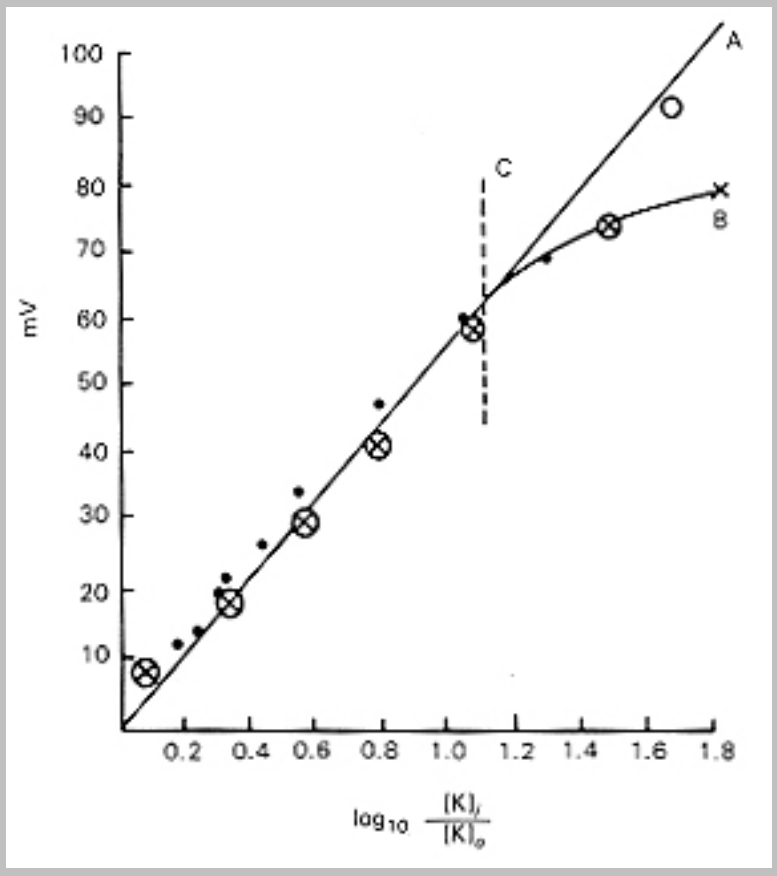


Fig. 3 Mean E_m values of frog sartorii immersed in Ringer-Barkan fluid with addition of external KCl. (●) Averages of results after overnight immersion at 0-3, then brought to room temperature. (⊗) Immediate results from isotonic mixtures with acetate ion replacing chloride and bicarbonate. (○) Average of observations taken immediately with Ringer Conway fluid containing 2.5 mM K^+. The vertical line C at 1.11 on the abscissa. Reproduced with permission from E.J. Conway, Physiological Review, 37:84-132, Copyright &copy1957 The American Physiological Society.

III. DYNAMICS OF THE MEMBRANE POTENTIAL

The resting potentials of nerve and muscle fibers depend on the permeability properties of the plasma membranes to K^+. The permeability of cells can be studied by following the entry or exit of the ions with time, usually using radioactive isotopes. In addition, the electrical resistance of the membrane is related to the permeability of the membrane to ions. This resistance can be determined by measuring the voltage produced by a current pulse between a microelectrode inserted into the cytoplasm and a reference electrode in the medium. An analogous, but more complex method, allows calculation of the resistance of the membrane by the passage of alternating currents through cell suspensions. The resistance of either the external medium or the cytoplasm is much lower than that of the cell membrane. The resistance

measurements, therefore, indicate the presence of a specialized structure of high electrical resistance and, hence, low permeability to ions at the surface of the cells.

Many experiments support the view that the cell membrane has an important role in excitation. Most of the axoplasm can be squeezed out from one end of a cut giant axon and be replaced with an artificial medium, such as a KF solution. Yet the excitability and the potentials of the axon remain undisturbed as long as the membrane remains undamaged.

A diagrammatic representation of the potential difference across the membrane (+ on the outside and - inside), the resting potential, is shown in Fig. 4a. A nerve impulse, or *action potential*, involves in part a depolarization, represented in Fig.4b. Nerve conduction corresponds to this depolarizing event and the propagation of depolarization.

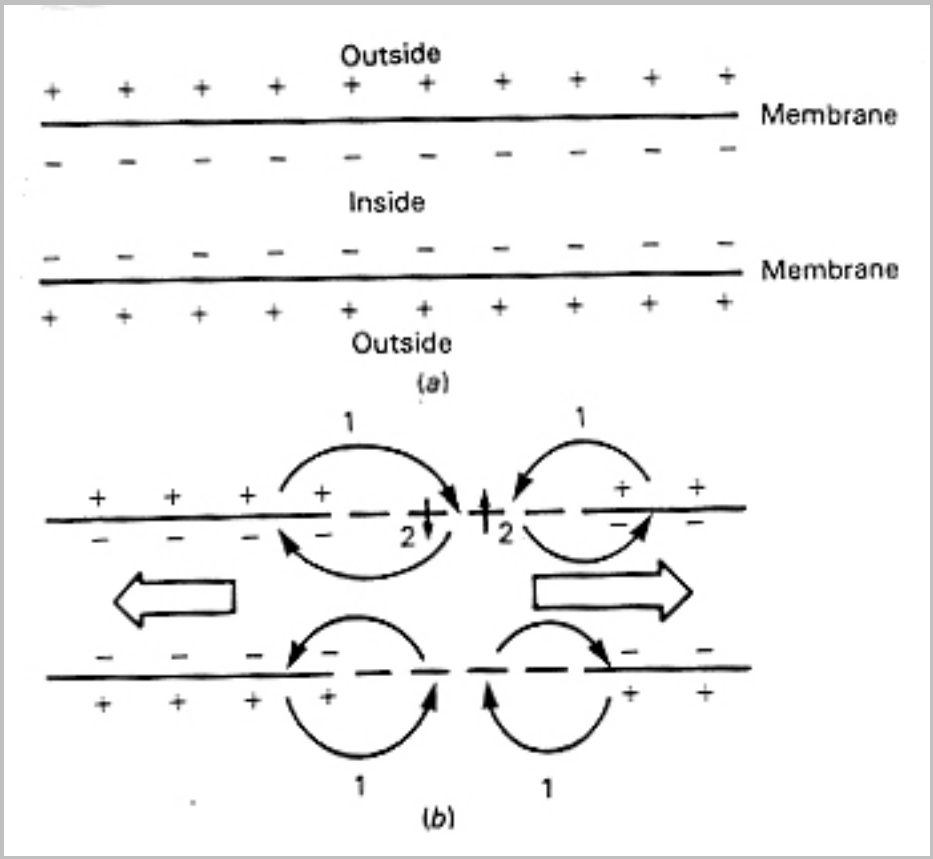
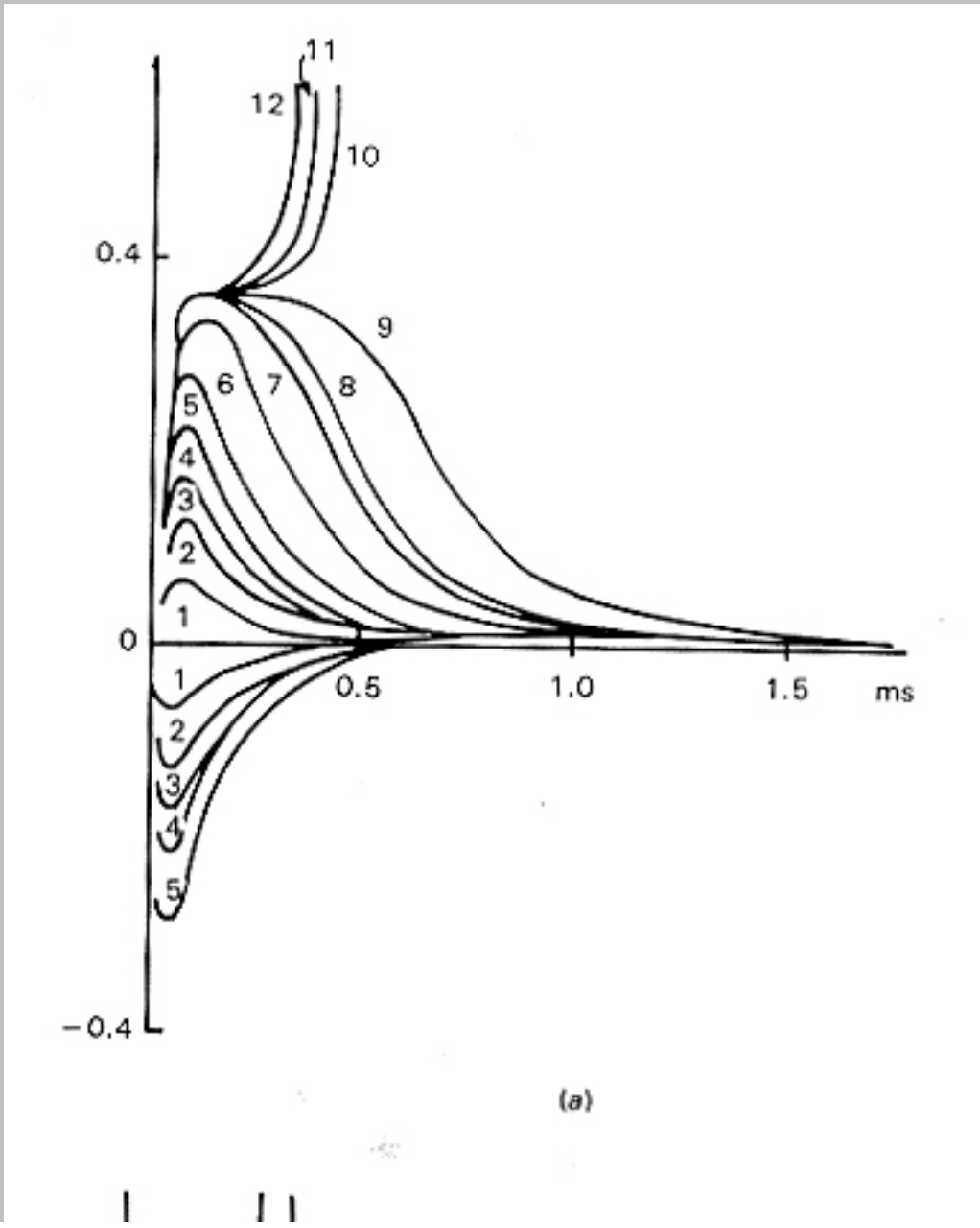


Fig. 4 Polarized (a) and conducting (b) fibers. The large arrows represent the direction of the propagation after artificial depolarization. The small arrows indicate the passage of ions in the longitudinal direction (1) and across the cell membrane (2).

The action potential can be initiated by an electric current at the site of the negatively charged electrode (*cathode*) placed at the outer surface of the nerve fiber. These events can be observed most readily with appropriate electrical amplification and recording equipment, such as amplifiers and an oscilloscope.

A stimulus must be above a minimal value (the threshold) to elicit an action potential. It is possible to study in detail what happens to the membrane potential with stimuli that vary in intensity above and

below the threshold (Fig. 5a) (Hodgkin, 1939). The results for squid axon represent the potentials recorded at the cell surface at the site of an external cathode (negatively charged electrode, curves above the axis) and at the anode (positively charged electrode, curves below the axis). The ordinate is in units relative to the action potential taken as unity. At very low intensity, the pattern is the same at the two stimulating electrodes. Naturally, the potentials are opposite in sign. There will be an enhancement of the potential difference between the inside and the outside at the anode. The system is said to be *hyperpolarized*. Under the cathode, a partial depolarization will take place. However, at higher intensities (curves 6, 7, 8, etc.) the curves representing the potential at the cathode change in shape. There is a depolarization beyond the direct electrode effect that is greater in both magnitude and duration. The responses of the nerve to these depolarizations can be shown by subtracting the direct effect of the stimulus (which mirrors the anodal response) from the total depolarization. This difference is shown in Fig. 5b. At sufficiently high depolarization, the potential is unstable and can give rise to an action potential (e.g., curves 10-12). The action potential is self-sustained, since it is independent of the input of the stimulating current, and it is also self-propogated. It turns out to be the same in magnitude at all points along the fiber.



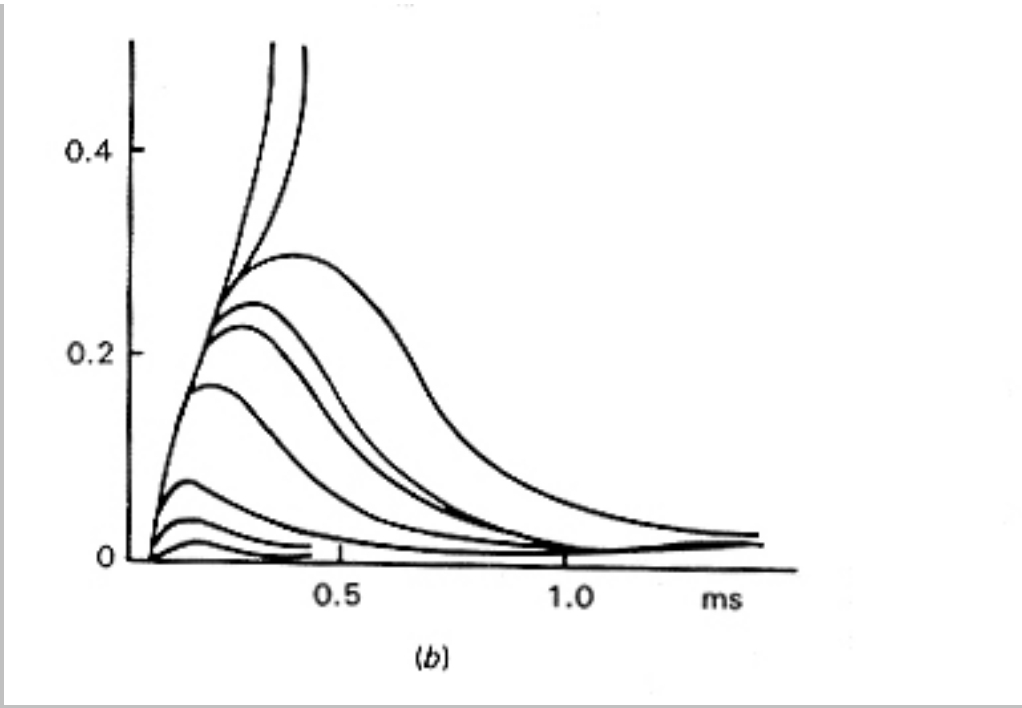


Fig. 5 (a) Electrical changes at stimulating electrode produced by shocks with relative strengths, successively from above, 1.00 (upper 6 curves), 0.96, 0.85, 0.71, 0.57, 0.43, 0.21, -0.21, -0.43, -0.57, -0.71, and -1.00. The ordinate scale gives the potential as a fraction of the propagated spike, which was about 40 mV in amplitude. The 0.96 curve is thicker than the others because the local response had begun to fluctuate very slightly at this strength. The width of the line indicates the extent of the fluctuation. (b) Responses produced by shocks with strengths, successively from above, 1.00 (upper 5 curves), 0.96, 0.85, 0.71, and 0.57; obtained from curves in (a) by subtracting anodic changes from corresponding cathodic curves. Two of the anodic curves necessary for this analysis were recorded but are not shown in (a). Ordinate, as in (a). Reproduced from A. L. Hodgkin, *Proceedings of Royal Society Series B.*, 148:1-37, with permission. Copyright &copy1958 The Royal Society, London.

A propagated wave of depolarization (or action potential) can be recorded from a nerve or muscle following each stimulation above threshold, provided that the interval between the stimuli is greater than the *refractory period*. This is the period during and immediately after an action potential when new action potentials cannot be elicited because the channels that allow Na^+ to enter are inactivated (see Section IIIC). An action potential recorded from an electrode inside the squid axon is shown in Fig. 6. The zero level on the scale represents the point at which there is no potential difference between the inside and the outside of the fiber; i.e., there is no potential difference across the membrane. The initial level (-50 mV) is the resting potential, where the inside is negative in relation to the outside. The upward swing that follows is the action potential, or spike.

In addition to involving a depolarization, the action potential reverses the polarity of the fiber (Fig. 6). Then the resting potential is reestablished rapidly after a period of hyperpolarization (Hodgkin, 1951) and the nerve can be stimulated again. Therefore, a process must exist that repolarizes the fibers very rapidly. A discussion of the ionic basis for the depolarization phenomenon and the repolarization follows. Analogous events take place in muscle.

The depolarization underlying the nerve impulse (the action potential or spike) causes a flow of current from the depolarized areas to the adjacent polarized areas. This current flow depolarizes the polarized region, setting up an action potential there. In this way, an action potential is propagated, or conducted, along an axon. The heavy arrows of Fig. 4b represent the direction of propagation of the action potential and the broken line represents the change in membrane resistance. Normally, the action potential is conducted in a single direction since it originates from the cell body. Because the portion behind the depolarized area is in the refractory state, the impulse cannot travel backward. When elicited artificially in the middle of the fiber, the action potential progresses in either direction.

A. Ionic Basis of Depolarization

Depolarization might be explained partially by proposing that the permeability of the membrane to all ions increases during the action potential so that the flux of ions causes the electrochemical gradient to collapse. Measurements of resistance do support this view. The resistance during the peak of the action potential is at best a small fraction of the resting resistance. However, an increase in the permeability to all ions would cause the membrane to move toward 0 mV and would not account for the overshoot, which is typically to +50 mV. Moreover, experiments in which different external ions substitute for the usual medium indicate that Na^+ has to be present to allow an action potential. Is the influx of Na^+ sufficient to account for the current of the action potential? The number of moles of ion required for a given change in potential is calculated with Eq.(6).

$$\text{Moles} = (C\,\Delta\Psi_m)/F \quad (6)$$

where *C* is the membrane capacitance in farads, or amount of charge (coulombs) across the membrane per volt, $\Delta\Psi_m$, is the maximum change in membrane potential during the rising phase of the impulse, and *F* is Faraday's constant (96,500 coulombs per mole of monovalent ion). The action potential of the axon corresponds to about 100 mV and the membrane capacitance is about 1.5 µF/cm^2. Therefore, the influx of Na^+ cannot be less than 1.6 x 10^{-12} mol/cm^2. This can only be a minimum value, as it ignores the possibilities of accompanying leakage of K^+ or entrance of Na^+ during the falling phase of the action potential.

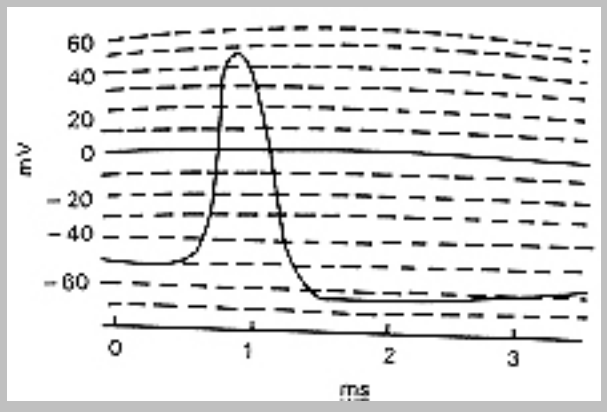


Fig. 6 Resting and action potential in giant squid axon at 18.5ºC. Reproduced with permission from A.L.

Hodgkin, *Biological Review of Cambridge Philosophical Society*, 26:339-409.

The quantity of Na^+ taken up per stimulus is so small that it cannot be detected; however, it can be calculated when the total uptake resulting from repeated stimuli is divided by the number of impulses. In the giant axon of the squid, *Loligo*, 3.5×10^{-12} mol/cm^2 of Na^+ is taken up per nerve impulse. Therefore, the entry of Na^+ suffices to account for the action potential. Table 2 summarizes similar results obtained for other systems.

These results imply that the membrane permeability to Na^+ during the rising phase of the action potential is greater than the permeability to other ions, and the relationship between action potential and [Na^+] should be quantitatively predictable from Eq. (5) using the appropriate [Na^+]. This is shown more clearly by Eq. (7), where [Na^+_{ext}] is the external Na^+ level, which is changed experimentally, and [Na^+_{st}] is the normal value in the extracellular medium.

$$\Delta\Psi_{ext} - \Delta\Psi_{st} = (58 \text{ mV}) \log_{10} (Na^+_{st})/(Na^+_{ext}) \quad (7)$$

Eq. (7) assumes that the internal Na^+ level is not changed by this manipulation of the external medium.

Results for different tissues are shown in Fig. 7 (Hodgkin, 1951). The measured membrane potentials (ordinate) at each Na^+ concentration (abscissa) are represented by the circles. Curve 1 of each graph represents the resting potential, which is hardly affected by the Na^+ concentration. Curve 2 represents the results calculated from Eq. (7). The predictions are close, although significant deviations do occur for the squid giant axon. These deviations may result from the approximations assumed in deriving Eqs. (5) and (7). All the results support the idea that Na^+ is responsible for carrying the depolarizing current during the rising phase of the action potential.

Action potentials also take place in freshwater algae, such as *Nitella* and *Chara*. The functional significance of these potentials is not clear, although they seem to be related to the movements of the cytoplasm. There is no external cation to carry the current. In this case the current is carried by the efflux of Cl^-. However, the measured efflux is well in excess of the calculated value. This excess may result from unrelated efflux of K^+ and Cl^- during the slow action potential of *Chara*.

For nerve and muscle, the reversal of polarity and the Na^+ permeation can be summarized as in Fig. 8. The small arrow shown perpendicular to the surface indicates the Na^+ influx, the large arrows, the direction of the action potential.

As mentioned, the depolarization of the action potential is followed almost immediately by repolarization of the axon (Fig. 6). In fact, the whole cycle of depolarization and repolarization generally takes place in 1 ms or so. Repolarization would be accomplished most rapidly by removal of the excess positive charge that has entered the nerve or muscle cell. This could be done most simply by a rapid efflux of K^+ in the

direction of the electrochemical gradient. The efflux of K^+ shown in Table 2 (column 2) is of the same order of magnitude as the influx of Na^+, as required by the fact that the two should represent equal but opposite phenomena. The changes in permeability for specific ions are likely to be the result of opening and closing of protein-lined channels, as discussed in Section IIIC. The diagram of Fig. 9 summarizes the ionic exchanges accompanying the action potential and the repolarization by the K^+ efflux.

Table 2 Na^+ and K^+ Exchanges During Nerve Excitation

Material	**(1)**	**(2)**	**Reference**
	Na^+ influx/impulse (10^{-12}mol/cm^2)	**K^+ efflux/impulse (10^{-12} mol/cm^2)**	
Carcinus maenas	-----	1.7	*a*
Carcinus maenas	-----	2.5	*b*
Sepia officinalis	-----	3.4	*c*
Sepia officinalis	3.7	4.3	*d*
Sepia officinalis	3.8	3.6	*e*
Loligo pealli	3.5	3.0	*f*
Loligo pealli	4.5	-------	g
Loligo pealli	4.4	-------	

[a]Hodkin and Huxley (1947) [b]Keynes (1951a) [c]Weidmann (1951) [d]Keynes (1951b) [e]Keynes and Lewis (1951) [f]Rothenberg (1950) [g]Grundfest and Nachmansohn (1950) Reproduced by permission.

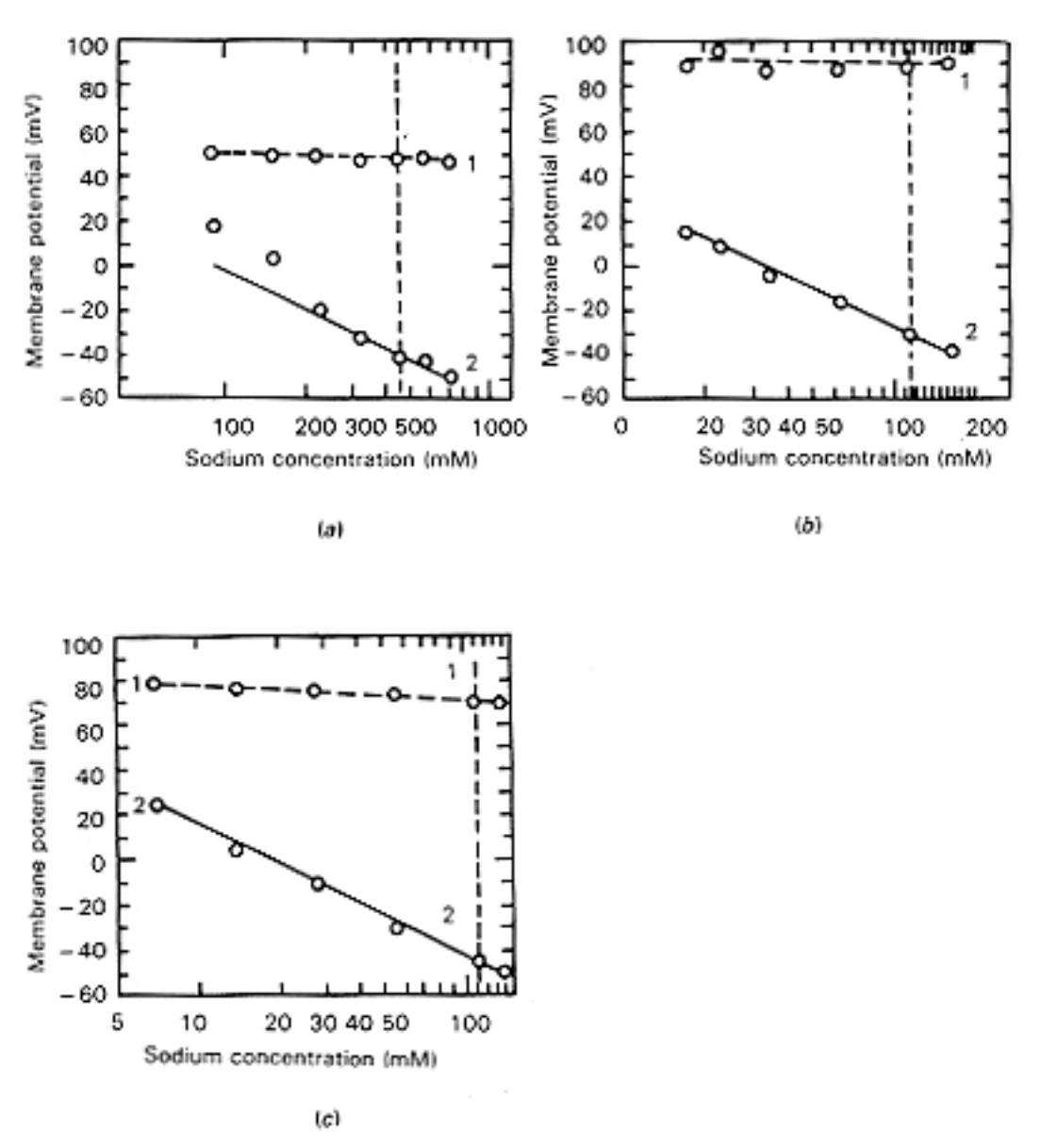


Fig. 7 Relation between sodium concentration in external solution and potential difference across resting and active membrane. (a) Squid giant axon (from work of Hodgkin and Katz); (b) frog sartorius muscle (from work of Nastuk and Hodgkin); (c) frog myelinated nerve (from work of Huxley and Stampfli). Abscissa: sodium concentration on logarithmic scale (dashed line shows concentration in Ringer's fluid or seawater). Ordinate: potential difference across membrane (outside potential minus inside potential) at rest (1) and at crest of action potential (2). The solid line is drawn with a slope of 58 mV for a tenfold change in sodium concentration. The points in these curves were obtained by adding the original author's values for the resting potential or for the reversed potential difference across the active membrane. Reproduced with permission from A. L. Hodgkin, *Biological Review of Cambridge Philosophical Society*, 26:339-409.

Both Na^+ and K^+ can shift across the nerve membrane with action potentials. How can the gradients for these ions be maintained? In part, the answer rests on the fact that each impulse does not change the concentration of the ions significantly. We have seen that many stimuli are needed to detect any change. The K^+ loss associated with one stimulus corresponds to about 4×10^{-12} mol/cm^2 (Table 2). Since the axon is about 500 µm in diameter, the loss per liter of axoplasm is approximately 1×10^{-5} mol, less than 1 part in 10,000. Although the loss is small, eventually work must be carried out to restore the internal K^+

and Na^+ levels. In Chapter 20, we discussed a transport system responsible for pumping Na^+ out and pumping K^+ in. It is this system that maintains the internal Na^+ and K^+ concentrations.

Although the Na^+ currents generally underlie the action potential in most animal tissues, there are exceptions. Crustacean muscle depends on Ca^{2+} for conduction. This is most readily demonstrable in muscles in which the K^+ channels have been blocked (e.g., with TEA). The involvement of Ca^{2+} fluxes was demonstrated with [^{45}Ca] using giant muscle fiber from a barnacle in which the internal Ca^{2+} was reduced. During induced action potentials the Ca^{2+} was found to correspond to 2-6 pmoles/µF, where 0.5 pmoles/µF are needed to depolarize the membrane by 100mV (Hagiwara and Naka, 1964).

B. Membrane Mechanisms: Voltage Clamping

The behavior of the membrane of an axon we examined so far can be outlined simply :

1 The unequal distribution of K^+ is responsible for the resting potential.

2 A small depolarization beyond a critical value leads to a change in permeability to Na^+, which, in turn, leads to Na^+ flow into the fiber. This influx is responsible for the reversal of the resting potential. The initial depolarization could be the result of an electrical stimulus or the depolarization of an adjoining area.

3 The immediate recovery of the resting potential is the consequence of an increase in the permeability to K^+, causing movement of K^+ in the direction of the electrochemical gradient.

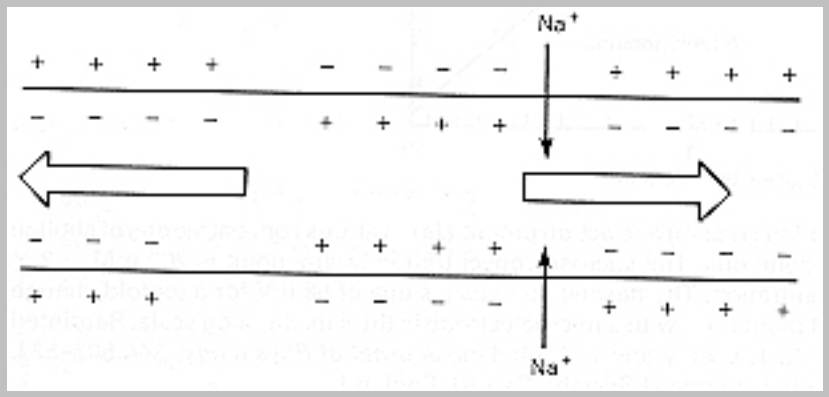


Fig. 8 Diagram representing the membrane potential during the action potential (center) and at rest. The large arrows represent the direction of the action potential (the nerve is stimulated in the middle). The smaller arrows represent the direction of the ion movement.

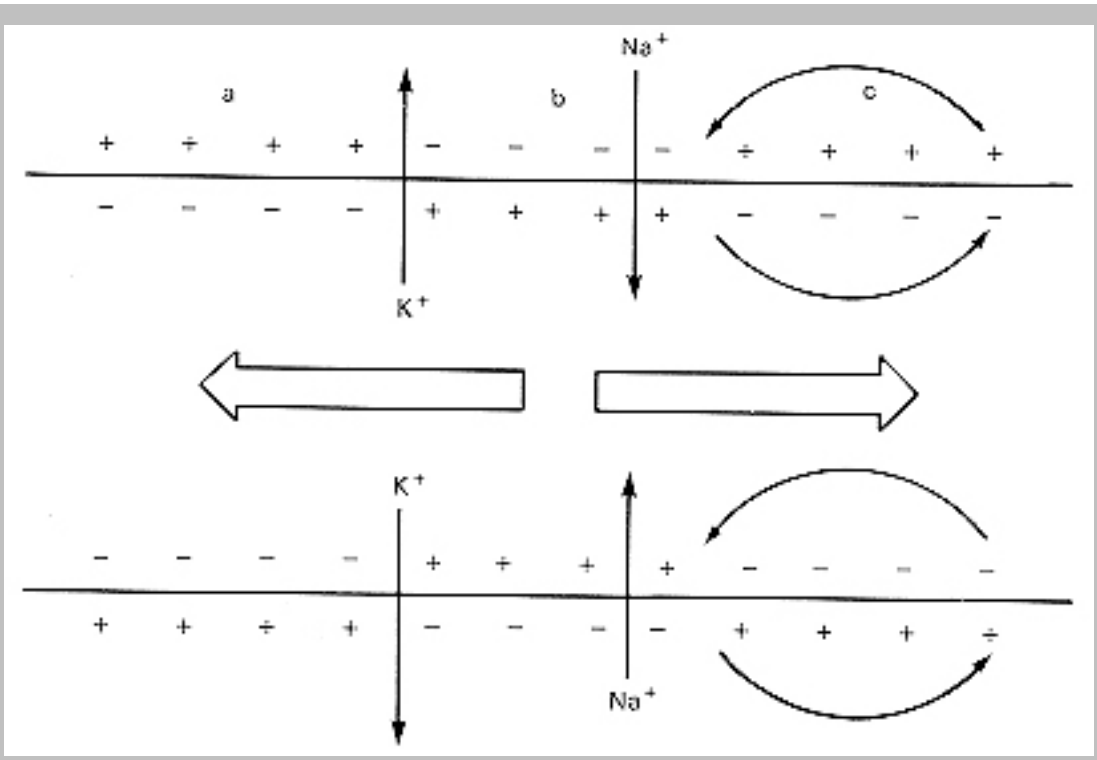


Fig. 9 Diagram representing the ionic basis of propagation of the action potential followed by recovery. The large arrows represent the direction of the action potential (the nerve is stimulated in the middle). The smaller arrows represent the direction of the ion movement.

If this model is correct, it seems possible to manipulate the electrical potentials of the axons and to test these premises independently. The potential difference between the inside and the outside can be set at any level by means of an external electrode, a microelectrode implanted in an axon, and appropriate electronics. In the *voltage clamp* technique, the membrane potential is kept constant at any desired level by passing a current equal and opposite to that generated by the flow of ions across the membrane.

With this method, it has been possible to test whether the potentials trigger the changes in flux necessary for the polarization-depolarization cycles. Increases in the potential difference (hyperpolarization) cause an inflow of current. This is expected from the passive resistive properties of the membrane. A small decrease in the potential causes an outflow of current, as expected. A decrease in the potential difference beyond a critical value has a very different effect (Fig. 10) (Hodgkin, 1958). Initially there is a large inward flow of current (dashed line, Fig. 10a); however, it is followed quickly by an outward current (full line), which in the absence of a clamp, would repolarize the nerve.

The inward current should correspond to the Na^+ influx that would take place during depolarization (in the absence of a clamp). Experimental tests of this point show that this current is, in fact, critically dependent on the presence of Na^+; replacement of the Na^+ in the external fluid by choline (which does not penetrate), blocks this event completely (Fig. 10b). The outward current that follows is likely to be carried by K^+, which normally would repolarize the nerve. A test of whether K^+ is involved can be carried out by measuring the current flow and the K^+ efflux simultaneously. The results of this experiment are shown in Fig. 11 (Hodgkin and Huxley, 1953). The efflux of K^+ and the outflow of

current, correspond quantitatively.

These potentials can be illustrated in a rather convenient form as shown in Fig. 12a (Narahashi et al., 1964). The inflowing current (the Na^+ current) and the outflowing current (the K^+ current) can be plotted as a function of the voltage at which the membrane potential is clamped. The current can be corrected for leakage, by assuming that the leakage current is a linear function of the potential (dashed line) and subtracting it from the total current.

A meaningful application of this system is shown in Fig. 12b, where the Na^+ passage is blocked by the toxic drug tetrodotoxin. Tetrodotoxin is a toxin extracted from certain organs of the puffer fish (the poison of the *fugu* fish of James Bond and more recently Homer Simpson fame). The Na^+ current is blocked completely, whereas the K^+ current remains unaffected.

In contrast to tetrodotoxin, tetraethylammonium interferes with the passage of K^+ and, hence, with the recovery of the resting potential after stimulation (not shown).

The results we have examinedm support the idea that the phenomenon of excitation can be explained entirely by underlying ionic currents. However, the ionic currents must be a reflection of events occurring in the structure of the membrane, and these events are beginning to be studied in detail.

C. Molecular Mechanisms and Channels

Resting potential, action potential and the recovery of the resting potential all depend on changes in the permeability of the membrane to Na^+ and K^+. What accounts for these changes? The lipid portion of the membrane is not likely to allow the passage of ions at the high rates that have been measured. The activation energy for such passage is of the order of 250 kJ/mol (Parsegian, 1969). In contrast, passage through channels would lower the activation energy for K^+, to about 20 kJ/ mol (Frankenhaeuser and Moore, 1963). Furthermore, it is difficult to visualize the lipid components being regulated to vary the passage of ions in response to a membrane potential. For these reasons, the presence of protein-lined channels has been considered for some time.

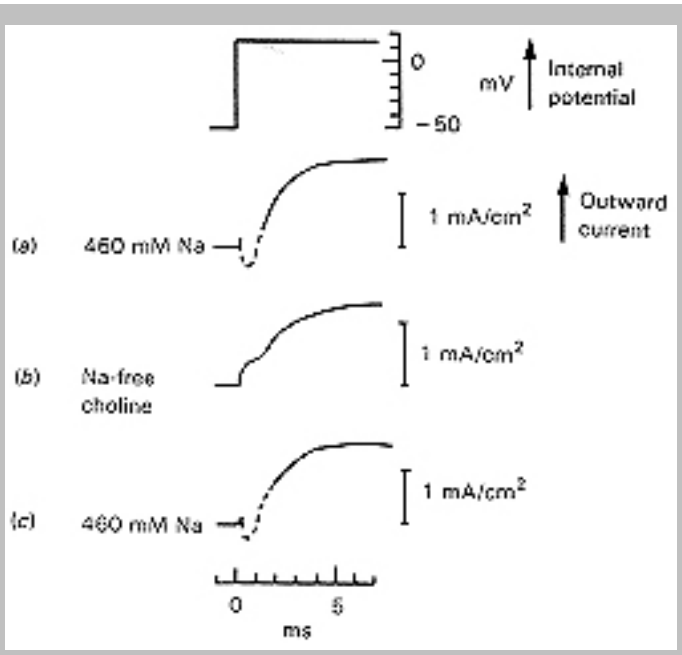


Fig. 10 Membrane currents associated with depolarization of 65 mV in presence and absence of external sodium ions. The change in membrane potential is shown at the top; the lower three records give the membrane current density; 11º C, outward current and internal potential shown upward. Reproduced from A. L. Hodgkin, *Proceedings of Royal Society Series B*, 148:1-37, with permission. Copyright © The Royal Society, London.

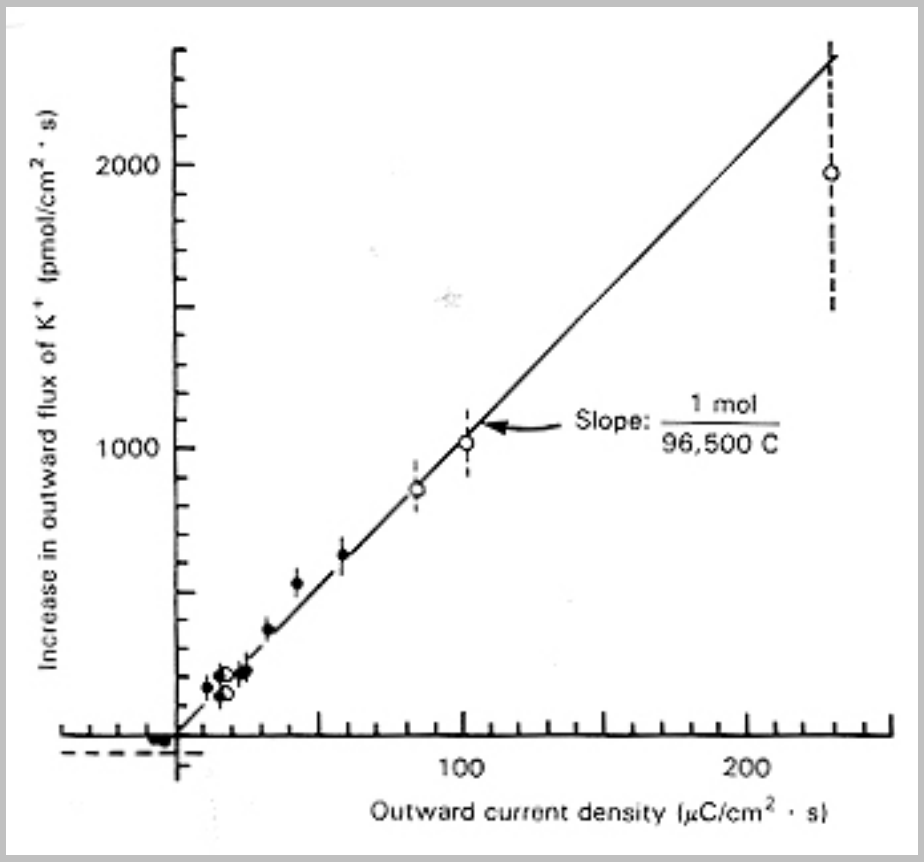


Fig. 11 Relation between membrane current density and potassium efflux when a Sepia axon is depolarized. The axon was depolarized by an applied current for periods of 60 to 600 s. Vertical lines show ±2 x SE; the horizontal line is drawn at a level corresponding to complete suppression of the average

resting efflux. Reproduced from A. L. Hodgkin and A. F. Huxley, *Journal of Physiology*, 121:403-414, with permission. Copyright &copy1953 The Physiological Society, Oxford, England.

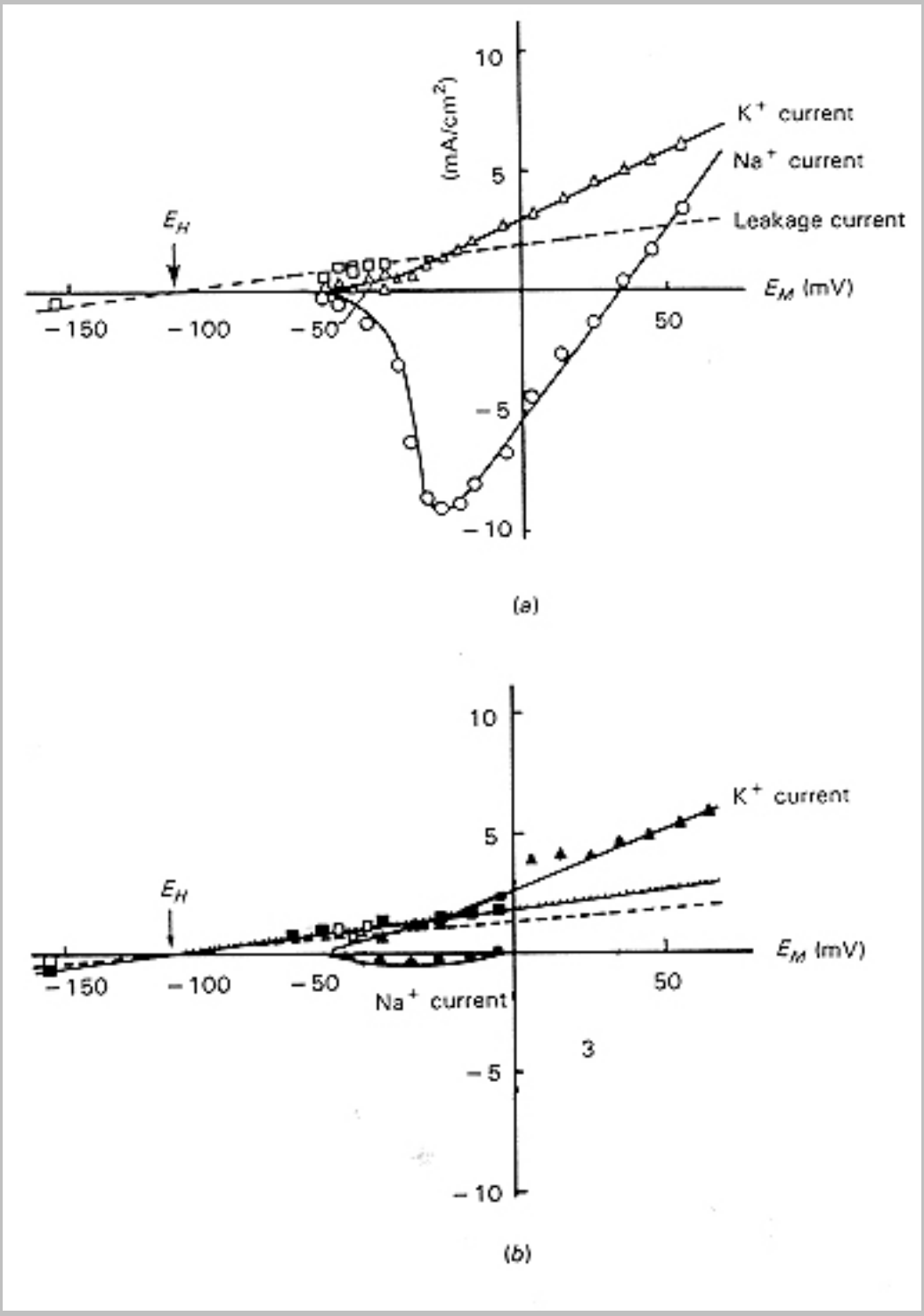


Fig. 12 (a) Current-voltage relations before treatment with tetrodotoxin. Circles refer to peak Na^+ current corrected for leakage current, triangles refer to steady-state K^+ current corrected for leakage current, and squares refer to leakage current; *I*, designated component of the membrane current (inward direction negative); E_M, membrane potential; E_H holding potential. (b) Current-voltage relations during treatment with tetrodotoxin 3 x 10^{-5} g/ml. Reproduced from the *Journal of General Physiology*, 47:965-974, &copy1964, by copyright permission of the Rockefeller University Press.

Membrane channels are integral proteins that span the lipid bilayer. As we discussed throughout this book, channels have far-reaching roles in cell function. Ion channels have an important role in hormone secretion, visual transduction, transepithelial ion transport and the activation of contractile mechanisms. Ion channels are frequently present as oligomers and they operate to regulate the passage of ions. The channels which concern us at this time are involved in the propagation of the electrical impulses in excitable cells and for postsynaptic responses to neurotransmitters, which are discussed later. In the latter case, the receptors that are part of the ion channels bind the neurotransmitter. Upon binding, the channels favor an open configuration.

Ion channels display specificity for certain ions, saturation kinetics in relation to ion concentration, competitive inhibition by analogs and conformational changes (to go from the open to the closed configuration) - all characteristic of enzyme activity such as that of transport proteins. However, they also exhibit behavior that differs from that of enzymes. The rate of ion transport is orders of magnitude higher than enzyme turnover and the temperature dependence of the ion passage is very low (Latorre and Miller, 1983). Furthermore, saturation kinetics is exhibited only at extremely high concentrations. As we shall see, proteins that have the appropriate characteristics have been isolated and functional channels reconstituted into bilayers.

Channels have been studied in their native state by *patch-clamping*. Patch refers to the fact that, by this technique, a "patch" of membrane is studied independently of the rest of the membrane. Clamping refers to maintaing the voltage electronically at a fixed value, that is, voltage clamped. With patch-clamping, a small heat-polished pipette is sealed against the cell membrane. The pipette is filled with an electrolyte solution. In effect, a small patch of membrane has been isolated and can be studied electrically independently of the rest of the membrane. This tight seal between pipette and membrane has been referred to as a *giga* seal (*giga* meaning a billion, referring to the fact that the pipette and the sealed patch, together, have a resistance in the range of 10^9 to 10^{11} ohms). When the voltage is clamped, a recording of the current permits the study of a single channel in the membrane (Hamill et al., 1981). Each deflection from the base line represents the opening of a channel. The relationship between voltage and current can be studied over a wide range of clamped voltages. In the neurotransmitter-regulated channels of the synapse, discussed later in this chapter, the effects of agonists (activators) and antagonists (blockers) can be studied at the level of the simplest unit. The relationship between current passing through a single channel and clamped voltage is shown in Fig. 13 (Hamill et al., 1981). The larger the voltage, the larger the currents seen as a deflection from the base line. The relationship between current deflections and voltage is linear, reflecting a constant conductance of individual channels. Note that at any given voltage, the levels of current in all openings are constant and, therefore, characteristic of those channels. *Conductance* is the reciprocal of resistance and is expressed in *siemens*, S, (1/ohms)(formerly called mhos). S = $ohms^{-1}$. Typical ion channels range in conductance between 5 to 100 pS.

The patches can also be isolated mechanically on the micropipette, either an inside-out or right-side out (i.e., outside-out) configuration. Alternatively, when the pipette is still attached to the cell, the patch can be ruptured, providing a direct connection between the cytoplasm and the fluid inside the pipette.

Maintaining voltage at a selected constant values by voltage-clamping, provides a means of examining the behavior of channels at these voltages. From the characteristics of the action potential and the curves depicted in Fig.12, we would expect the Na^+ channels to open at depolarizing voltages. Similarly, we would expect the K^+ channels to open during repolarization. This is found to be the case. The time spent in an open conformation is longer.

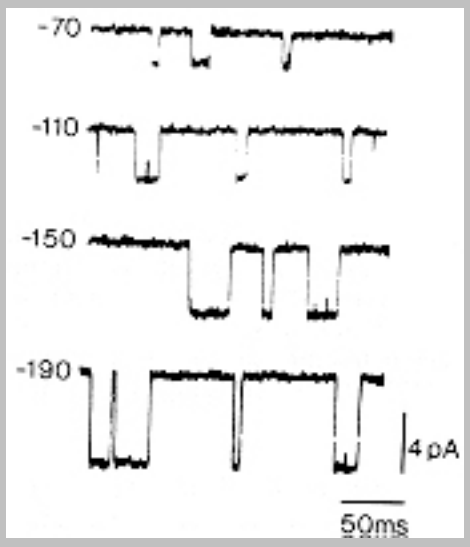


Fig. 13 Single-channel current recordings when the patch is clamped at the indicated voltage. Reproduced from O. P. Hamill, et al., *European Journal of Physiology*, 391:85-100, with permission. Copyright &copy1981 Springer-Verlag, Heidelberg.

The ability to open or close in response to voltage changes across the membrane is known as *voltage gating*. Voltage gating is presumably the result of conformational changes in the proteins constituting the channels. Many different kinds of channels that differ in ionic specificity have been recognized. Most of these are voltage gated. At any one time, regardless of voltage, some channels will be open, others closed. The behavior of a sector of the membrane will be a summation of the activity of individual channels.

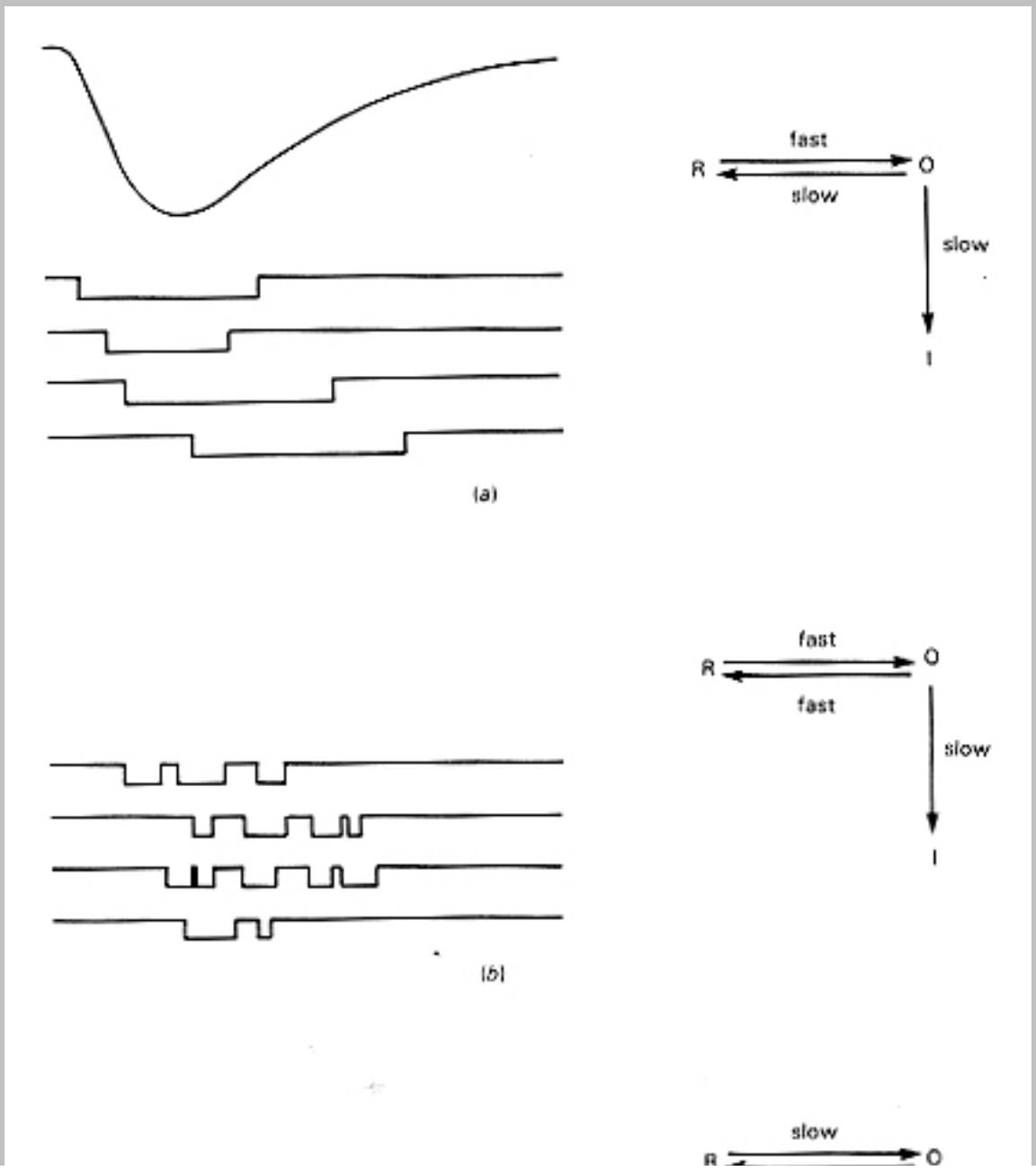
The whole cell current (macroscopic) after a voltage pulse, is represented at the top of Fig. 14 (Aldrich, 1986) for a bovine chromaffin cell of the adrenal medulla in tissue culture. These cells secrete epinephrine and norepinephrine and are excitable. The diagrams below (a to c) represent hypothetical voltage clamp records for individual channels, where each of the three lines represents the current recorded for a single channel.

The individual channels that are specific for Na^+ open in response to a depolarization. Three different states have been reported: resting states (R), which are present at hyperpolarizing voltages (no ions go through); open states (O), which allow the passage of Na^+ and which have opened as the result of an initial depolarization; and inactivated states (I), which are closed and cannot be opened with further depolarization. The latter correspond to the state during the refractory period.

The Na^+ current represented at the top of Fig. 14 can be generated by the behavior of the channels shown

under the curve and summarized by the schemes shown at the right in the figure. Figure 14a (top) corresponds to a long-maintained opening; more channels open in the rising current phase and close in the falling phase. This effect could result from a fast transition to the open state (0) followed by a slow transition to both the resting (R) and inactivated (I) states. Figure l4b shows how short-lived open states could generate the same pattern. In this case, the rate of opening and returning to the resting state must be high and the rate of conversion to the inactive state low; otherwise repeated opening and closing would not be possible. The open state would nevertheless predominate when the current passage is maximal. Similarly, the pattern could be explained by Fig. l4c, in which the opening is short-lived as the result of slow transitions from the open to the resting state and a rapid inactivation. The last case appears to be correct (Aldrich et al., 1983).

Much is now known about the proteins constituting the Na^+-channel (see Catterall, 2000). Most recently a detailed 3D-reconstruction using cryoelectron microscopy and tomographic analysis (Sato et al., 2001) has been carried out. For a more complete discussion see addendum



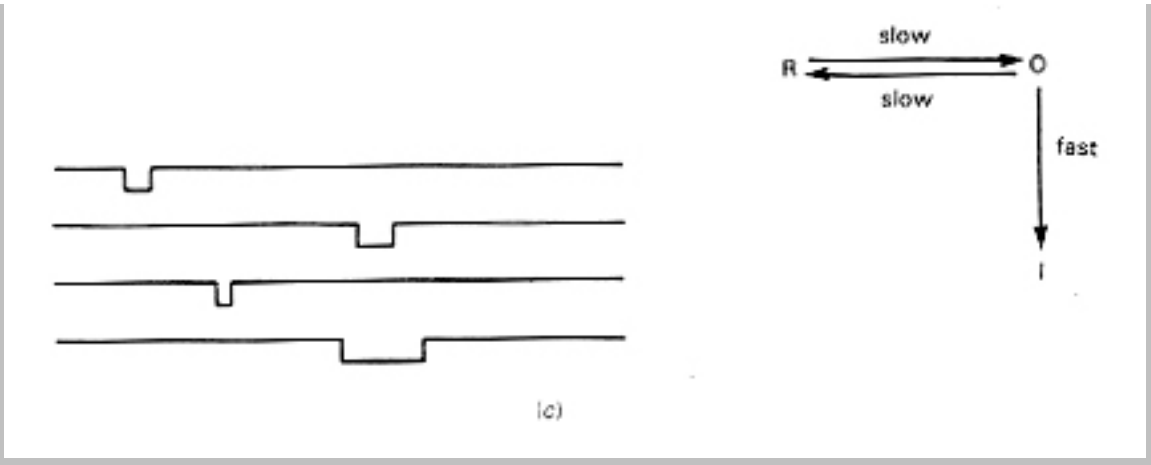


Fig. 14 Three possibilities for sodium channel gating that predict identical macroscopic sodium currents but different single-channel behavior. Reproduced from R. W. Aldrich, *Trends in Neurosciences*, 9:82-85, with permission. Copyright &copy1986 Elsevier Science Publishers, England.

The appropriate behavior for the K^+ channels has also been shown using patch clamping of internally perfused giant squid axons. Since the repolarization of the axon after the action potential depends on an increase in K^+ permeability, we would expect the frequency of opening the K^+-specific channels to increase with depolarization. The results of Fig. 15 (Conti and Neher, 1980) show exactly this behavior. In Fig. 15a, the voltages at which the patches are clamped are listed at the left of each record. Depolarization increases from the bottom of the figure to the top. The frequency of opening is minimal when the axon is polarized (see lower two records). It increases with depolarization, and then the channels close again. The increased conductance at constant voltage, is an indication of the number of channels that are open. Figure 15b compares the variances observed experimentally (the points) to those calculated from theoretical considerations. The agreement between the points and the line shows that the observed current changes correspond to random open-close transitions of the same channels.

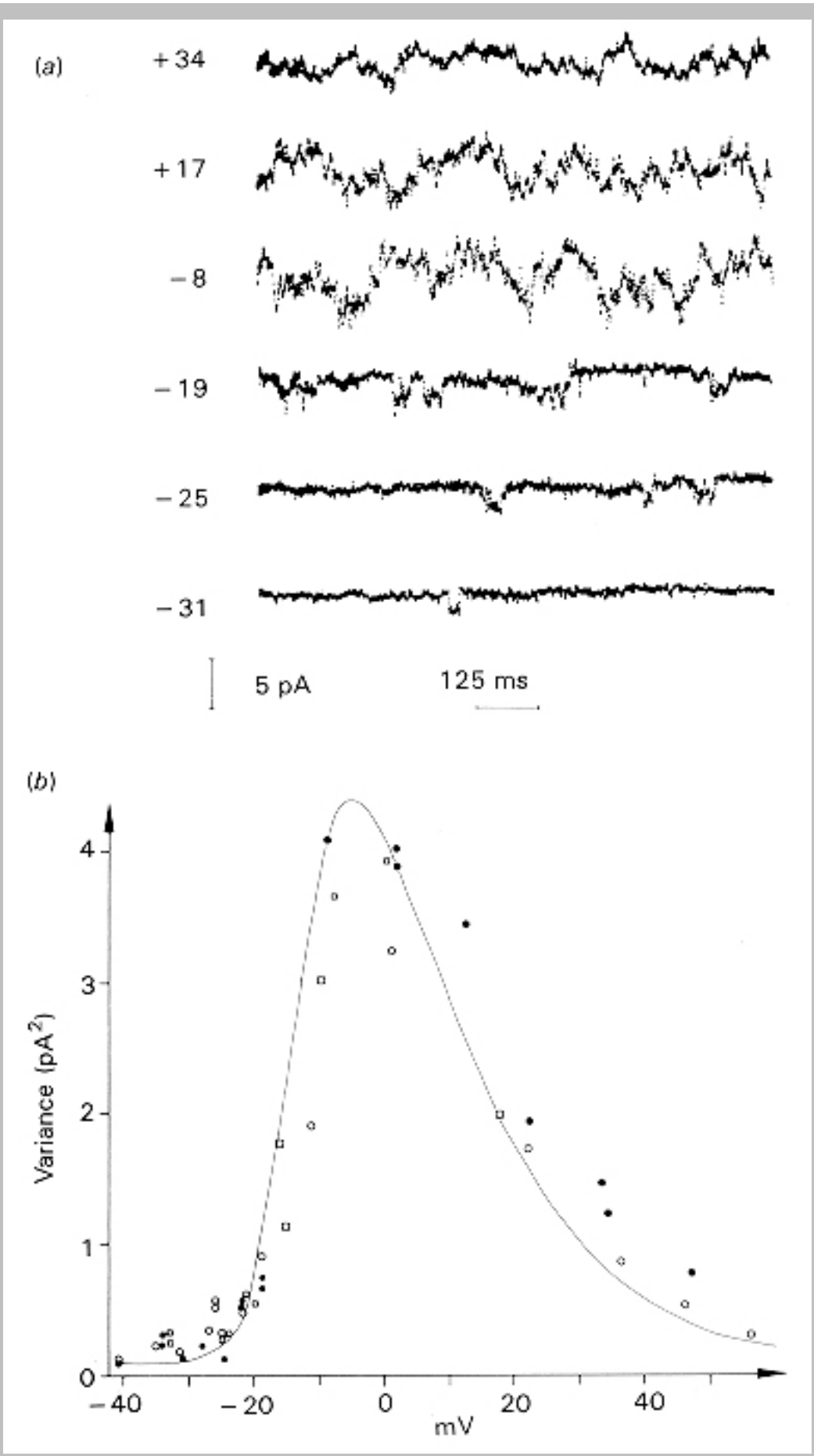


Fig. 15 (a) Recordings of patch current at different membrane potentials. From Conti and Neher (1980).

(b) Variance as a function of voltage. The points are experimental points and the line has been calculated from the observed probability of opening on the assumption that there are seven channels in the patch. Reproduced by permission from *Nature* 285:140-143, copyright &copy1980 Macmillan Magazines Ltd.

K^+-channels are very diverse (e.g., see Wei et al., 1990). Four different voltage-gated K^+-channels have been found in *Drosophila* , fourteen in rats and more are likely to be present. The *Drosophila* Shaker channel is an integral membrane protein of 70,200 daltons containing seven potential membrane-spanning sequences (Tempel et al., 1987) and probably assembling into a channel containg four subunits (see MacKinnon, 1991; Doyle et al., 1998). Several of these channels function during the action potential-recovery cycle: they open following depolarization (e.g., brought about by the action potential) and then close again (known as inactivation). In Shaker-type K^+-channels, the inactivation gate, responsible for the corresponds to the channel's cytoplasmic amino terminal. The data suggest that inactivation is part of a sequential process (Zhou et al., 2001). First the gate binds to the cytoplasmic channel surface and then penetrates the pore, blocking its opening.

Until recently, K^+ channel subunits were found to have one pore forming P domain with the characteristic TXGYG sequence (Yellen et al., 1991; Heginbotham et al., 1994). The P domain was identified after site-directed mutagenesis (see Chapter 1) identified in the *Drosophila* Shaker channel an amino acid residue that specifically affects the affinity for intracellular tetraethylammonium (TEA) (Yellen et al., 1991; Heginbotham et al., 1994).We have seen that TEA blocks K^+-channels. The single P-domain channels studied include the voltage-gated K^+ (Kv) (e.g., Abbott et al., 2001), the inward rectifying (Kir) (e.g., Krapivinsky et al., 1995) and KCNQ channels (e.g., Schroeder et al., 2000).

The *KCNK* channels comprise a family of potassium-selective leak channels possessing two pores. KCNK2 is involved in maintaining the resting potential (see Goldstein et al., 2001). The channel becomes voltage dependent during the rise of the action potential thereby promoting recovery of the resting potential. The phosphorylation of the channel by protein kinase A reversibly switches it into a voltage dependent channel (Bockenhauer et al., 2001).

The *large-conductance* K^+ (BK_{Ca}) channels (see Vergara et al., 1998) are activated by membrane depolarization or intracellular Ca^{2+}. They are involved in the regulation of neuronal excitability, secretion, and vascular tone. In most tissues, their stimulation results in a non-inactivating hyperpolarizing K^+ current that reduces excitability. These channels have four identical α subunits coded by the *Slo* gene. Although only one gene codes the pore forming α subunit of the channel, the properties can be quite different mostly because of alternative splicing (e.g., Tseng-Crank et al., 1994; see Shipston et al., 2001). The splice variants depend on the particular cell-type and cause significant changes in excitability and cell function (e.g., Tian et al., 2001). In addition, in some tissues the activity can also be modified by binding to the β subunits (see Toro et al., 1998; Gribkoff et al., 2001) and phosphorylation (see Levitan, 1999) via the cAMP-dependent protein kinase (PKA). The phosphorylation activates the channels in some tissues (e.g., smooth muscle and some neurons) and deactivates them in others (e.g.,

endocrine cells of the anterior pituitary). The *stress axis regulated exon* (STREX) of the α subunit plays a particularly significant role. STREX codes a 59 residue segment responsible for Ca^{2+} and voltage sensitivity. STREX is present in excitable cells. In other tissues, the splice variants are determined by physiological conditions (presence or absence of hormones, depolarization of the cell, etc.) (e.g., see Xie and McCogg, 1998; Xie and Black, 2001).

IV. ELECTROGENIC PUMPS

The electrical potential across the plasma membrane of many cells can be considered a K^+ diffusion potential. However, the transport of ions can generate potential. They are said to be electrogenic. One turnover of the Na^+ pump is responsible for the uptake of 3 Na^+ for each 2 K^+ extruded (Chapter 20). Therefore it should be electrogenic. Although in these cells the primary determinant of the resting potential is the relatively high permeability to K^+, hyperpolarizations have been observed in a number of cases (e.g., snail ganglion cells and striated muscle) during periods of rapid Na^+ extrusion. These potentials have been considered to be the result of the transport mechanism.

In some fungal and plant cells, electrogenic pumps play an important role in maintaining the resting potential. In *Neurospora* hyphae, part of the resting potential across the membrane responds to K^+ concentration and part responds rapidly and reversibly to interference with metabolism by inhibitors of respiration, such as azide or carbon monoxide (Slayman, 1965). In this case (Slayman and Tatum, 1965), an electrogenic pump is the major component. The pump, a 100 kDa protein, is a H^+-ATPase of the P-type (see Nakamoto and Slayman, 1989; Scarborough, 1996). The structure of the ATPase has been studied using electron crystallography (Auer et al., 1998).

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

V. TRANSMISSION OF EXCITATION BETWEEN CELLS

Excitable cells can communicate with each other, generally through specialized regions of contact, the *synapses*. In vertebrates, the synaptic connections form a network of great complexity. It has been estimated that there are 4 X 10^{11} synapses per gram of guinea pig cerebral cortex.

Many of the complexities of neural behavior are thought to be a reflection of the organization of neuronal networks and the properties of the synapses. The convergence of different terminals can produce complex effects because many excitatory and inhibitory influences are summed and integrated. Furthermore, the various connections and their properties are dynamic and continuously modified by experience. These dynamics have implications in sensation, memory, and learning.

A very intriguing interaction between neurons and glial cells which may have implications in the modulation and transmission of signals is discussed in Chapter 7.

A. The Synapse

Typically a vertebrate central nervous system synapse connects a presynaptic axon terminal to a postsynaptic dendrite. The synaptic cleft of about 200-300 Å separates the two. The synaptic gap is filled with electron-dense material, containing *cell adhesion molecules* (CAMs) (see Chapter 6) discussed below. Presynaptic terminals are filled with vesicles containing neurotransmitters either glutamate at most excitatory synapses and GABA or glycine at inhibitory ones. The junction resembles the *adherens junction* of epithelial cells (see Chapter 11).

One of the most studied synapses is that between nerve and striated muscle, the *neuromuscular junction*, represented diagrammatically in Fig. 16 (Whittaker, 1968). At this junction the nerve terminal expands into a baglike arrangement: nerve and muscle are separated by a space. Transmission occurs by the release of a transmitter, *acetylcholine* (Ach), at the presynaptic terminals. Ach induces a depolarization of the specialized portion of the muscle membrane, the *end-plate*. Presumably, the transmitter attaches to receptor sites on the end-plate. These are chemically gated channels, which open upon binding Ach. The application of Ach to the junction with a micropipette, generates postjunctional potential changes. The postjunctional potentials induced by the action potential of the nerve fiber can be blocked by certain drugs that compete with Ach for binding to the receptors. Curare, the poison used on arrow tips by South American Indians, acts in this manner. At rest, there is an occasional release of small packets or quanta of Ach from the undisturbed cells. This release produces the small postjunctional potentials (*miniature*

end-plate potentials) (Fatt and Katz, 1952; Miledi, 1966). The size of these potentials can be calculated to correspond to the release of 1,000 to 10,000 molecules of acetylcholine. It has been suggested that they correspond to the release of the contents of acetylcholine-containing vesicles that are visible with the electron microscope (for a discussion, see Auerback, 1972). These vesicles, the *synaptic vesicles*, have been isolated and shown to contain acetylcholine.

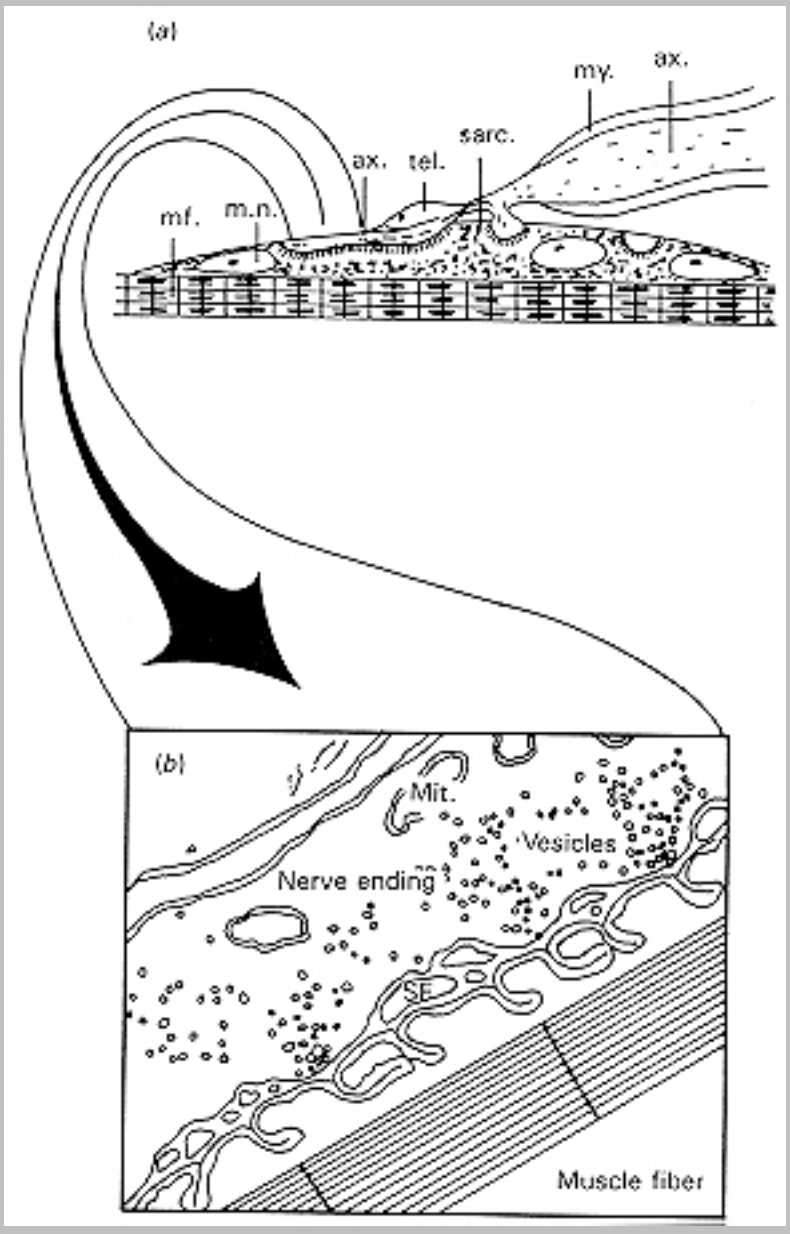


Fig. 16 (a) Diagram of a motor end-plate showing axoplasm (ax) and myelin (my) of motor nerve, and saclike terminals (arrows) lying in gutter-like depressions of the mitochondrion-rich muscle sarcoplasm (sarc). The terminals are protected by teloglia (tel). Muscle nuclei (m.n.) and myofibrils (mf) are also diagrammed. (b) Tracing of an electron micrograph of a portion of the nerve terminal similar to that between the arrows in (a). Note the highly folded postsynaptic membrane extending into the muscle sarcoplasm, the fingerlike projections of teloglia, and the numerous vesicles and mitochondria (Mit.) in the terminal cytoplasm. Reproduced with permission from V. P. Whittaker, *Proceedings of the National Academy of Sciences*, 60:1082, 1084, 1968.

Graded end-plate potentials can be produced by applying electrical currents locally to a nerve-muscle preparation, in which the nerve and the muscle are rendered unresponsive with tetrodotoxin (Katz and Miledi, 1967a; Katz and Miledi, 1967b). Conversely, the end-plate potential can be blocked by hyperpolarization of the nerve terminal by means of a current pulse delivered locally by a microelectrode. The neurotransmitter release requires external Ca^{2+}. Therefore, the results suggest a scenario in which the action potential arriving at the presynaptic terminal opens voltage gated Ca^{2+}-channels. The influx of Ca^{2+} triggers the release of neurotransmitter.

Neuronal synapses also have specialized regions. The part of the cell through which incoming events influence the synapse is called the *presynaptic terminal*. The presynaptic terminal stimulates the *postsynaptic cell*. The synaptic membranes may be tightly apposed, with only a small space between them forming a gap junction (Pappas and Bennett, 1966). Gap junctions have been discussed in some detail in Chapter 4. Presynaptic and postsynaptic cells are connected by channels. In gap junctions transmission occurs by purely electrical events. Other junctions resemble the neuromuscular junction. The distance between the membranes is greater and the transmission is thought to require a neurotransmitter. Fig. 17 (Pappas and Bennett, 1966) shows an electron micrograph of a section of the spinal cord of the toadfish, in which the two kinds of synaptic endings are present side by side. The two types of synapses are represented diagrammatically in Fig. 18 (Whittaker, 1968).

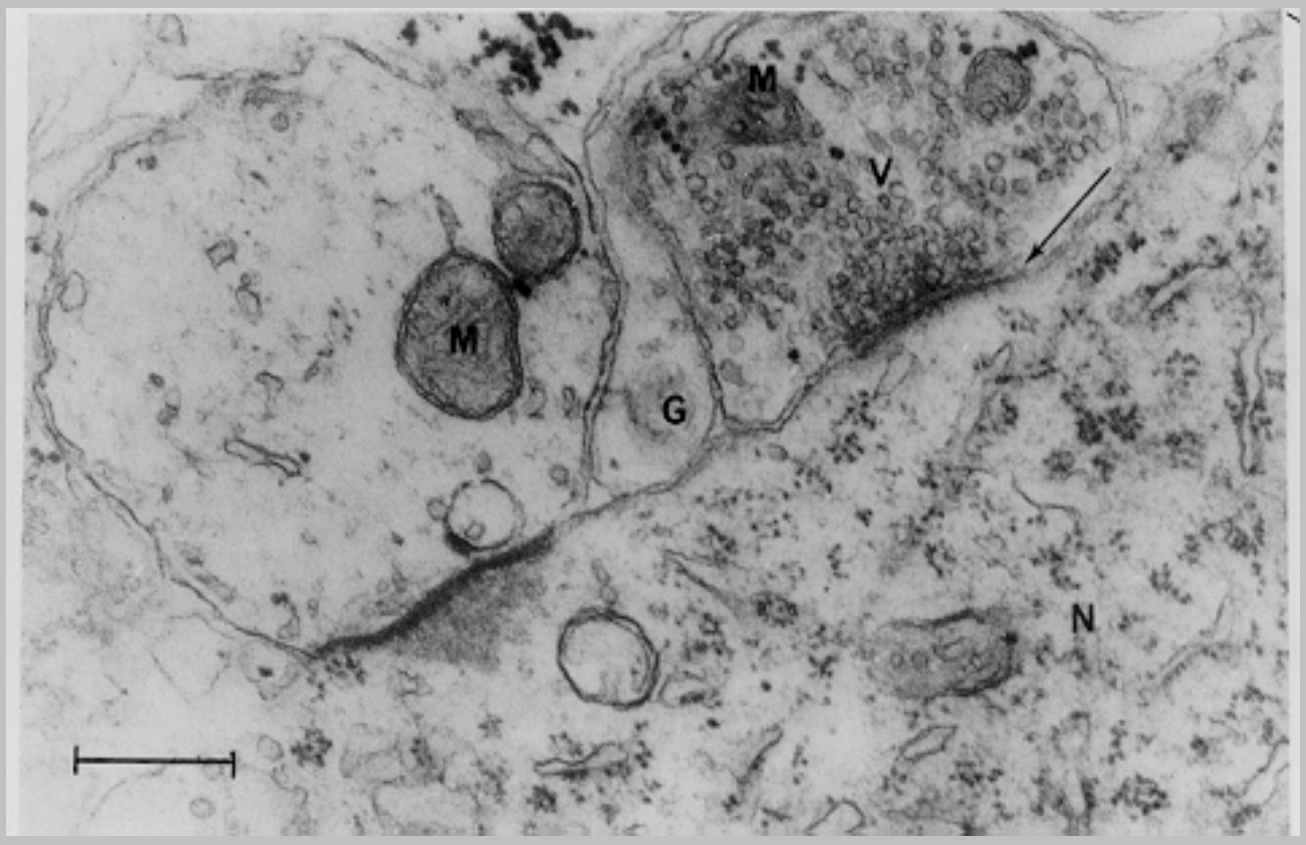


Fig. 17 Electron micrograph of a section from the spinal cord of the toadfish. Profiles of two synaptic endings can be seen separated by a glial cell process (G). The two synaptic endings form contact on a neuronal cell body (N). In the chemically transmitting synapse, vesicles (V) are clustered close to the presynaptic membrane. The pre- and postsynaptic membranes are distinctly separated by a 20-nm space

(arrow) at the chemically transmitting synapse. At the electrical synapse (at the left) the apposing membranes are very close and no space is discernible. M, Mitochondria. Bar corresponds to 714 nm. Reproduced from Pappas and Bennett (1966), with permission.

By inserting a stimulation electrode into one kind of cell (i.e., presynaptic or postsynaptic) and a recording electrode into the other, one can follow the various electrical events. The results confirm the presence of two distinct kinds of synapses. In gap synaptic junctions, both depolarizations and hyperpolarizations can be transmitted from one cell to the other in either direction (Bennett et al., 1967). Consequently, presynaptic cells can be stimulated from a postsynaptic action potential. The gap junction synapses also lack neurotransmitter vesicles, in agreement with the notion that in these cases the model of transmission is electrical.

The presynaptic terminals of the chemical synapses are full of vesicles that correspond to the synaptic vesicles isolated from brain and containing neurotransmitter, supporting the idea that the transmission is mediated by a neurotransmitter. In contrast to the gap junction, postsynaptic potentials cannot stimulate presynaptic cells. However, the postsynaptic potentials can be evoked electrically even when the presynaptic action potentials are blocked by tetrodotoxin or tetraethylammonium (Katz and Miledi, 1967a). As discussed above, tetrodotoxin blocks the Na^+ channels, whereas tetraethylammonium blocks the passage of K^+ needed for repolarization (Katz and Miledi, 1967c). Therefore, presynaptic electrical events are not directly involved in stimulating the postsynaptic cell. Small spontaneous postsynaptic depolarizations have been observed and are thought to reflect a continuous subthreshold release of packets of neurotransmitter. Certain pharmacological agents can also block these synapses by preventing neurotransmitter binding. Much of this evidence suggests a similarity to neuromuscular transmission.

Although acetylcholine is a synaptic neurotransmitter, many other chemicals have been implicated. At some junctions, the transmitters may be noradrenaline, dopamine, 5-hydroxy-tryptamine or serotonin (the *monoamines*). Some of the drugs that produce hallucinations (e.g., LSD and mescaline) and some tranquilizers (e.g., thalidomide) are thought to produce their effects by their structural similarity to neurotransmitters. There is some evidence that different synapses on the same cell may involve different transmitters (Gerschenfeld et al., 1967).

Glutamate is the major excitatory neurotransmitter in mammalian brain, although it can also be inhibitory acting via an inhibitory post-synaptic potential (Fiorillo and Williams, 1998). There are several kinds of postsynaptic receptors that respond to glutamate. Two of these are ligand-gated ion channels, the NMDA and AMPA receptors. The initials are based on pharmacological ligands: *N*-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxasole propionate, respectively. (see Bettler and Mulle, 1995). In contrast, the *metabotropic* receptors belong to the seven transmembrane receptor family. Their action is much slower because they are mediated by GTP-binding proteins and they depend on second messengers.

The NMDA receptors become activated only when the glutamate signal arrives after the postsynaptic neuron has been activated by another signal. Therefore, NMDA receptors are well suited to respond to

closely timed signals, supposedly a feature important for learning (see Section D, below). The AMPA receptors are ligand-gated cation channels responsible for the fast component of excitatory postsynaptic currents in the central nervous system (see Hollmann and Heinemann, 1994). They have been implicated in long term potentiation and depression (see Section D, below). The NMDA receptors have a double function (Hayashi et al., 1999). In addition to acting as ion channels, they act as signal transducers (see Chapter 7) by interacting with the Lyn tyrosine kinase, a protein of the Src-family non-receptor proteins. After activation, Lyn activates the pathway involving the mitogen-activated protein kinase (MAPK) and it increases the expression of brain derived-neurotrophic factor (BDNF) mRNA. Therefore, AMPA receptors can act as signals that may contribute to the expression of BDNF with consequent effects on synaptic plasticity (see Section D, below). The NMDA-glutamate receptors are activated by membrane depolarization and the simultaneous binding of ligand. They promote the post-synaptic influx of Ca^{2+} (MacDermott et al., 1986). A role of these receptors in forming synapses during development has been shown using specific antagonists, such as D,L(-)-2-amino-5-phosphonovaleric acid (APV).

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter which is bound postsynaptically to either $GABA_A$, $GABA_B$ or $GABA_C$ receptors (see Macdonald and Olsen, 1994; Lukasiewicz, 1996). The $GABA_C$ receptors are almost exclusively in the retina of adult vertebrates on bipolar cell axon terminals. The $GABA_A$ receptor forms a ligand-gated Cl^-channel (Barnard et al., 1987). Its inhibitory effect results from the hyperpolarization that follows opening of the channel (from the Cl^--diffusion potential, see Section II). $GABA_B$ receptors act more slowly through a GTP-binding protein, which activates or inhibits adenyl cyclase and activates adjacent K^+-channels and closes voltage gated Ca^{2+} channels. The effects are slower, but longer lasting. There are also $GABA_B$ presynaptic receptors usually in axons that release GABA. Their stimulation decreases the release of neurotransmitters, possibly through effects on Ca^{2+} or K^+ channels (Thompson et al., 1993).

The GABA receptors are probably held in place by interactions with the microtubules. The *microtubule associated protein 1B* (MAP-1B) interacts specifically with the $GABA_C$ receptor subunit ρ1 subunit, but not with $GABA_A$ receptors (Hanley at al., 1999). A cytoplasmic protein, *$GABA_A$-receptor-associated protein* (GABARAP), binds to the γ2 subunit of the receptor. GABARAP has sequence similarity with subunits of MAP proteins and has a putative tubulin-binding motif (Wang et al., 1999).

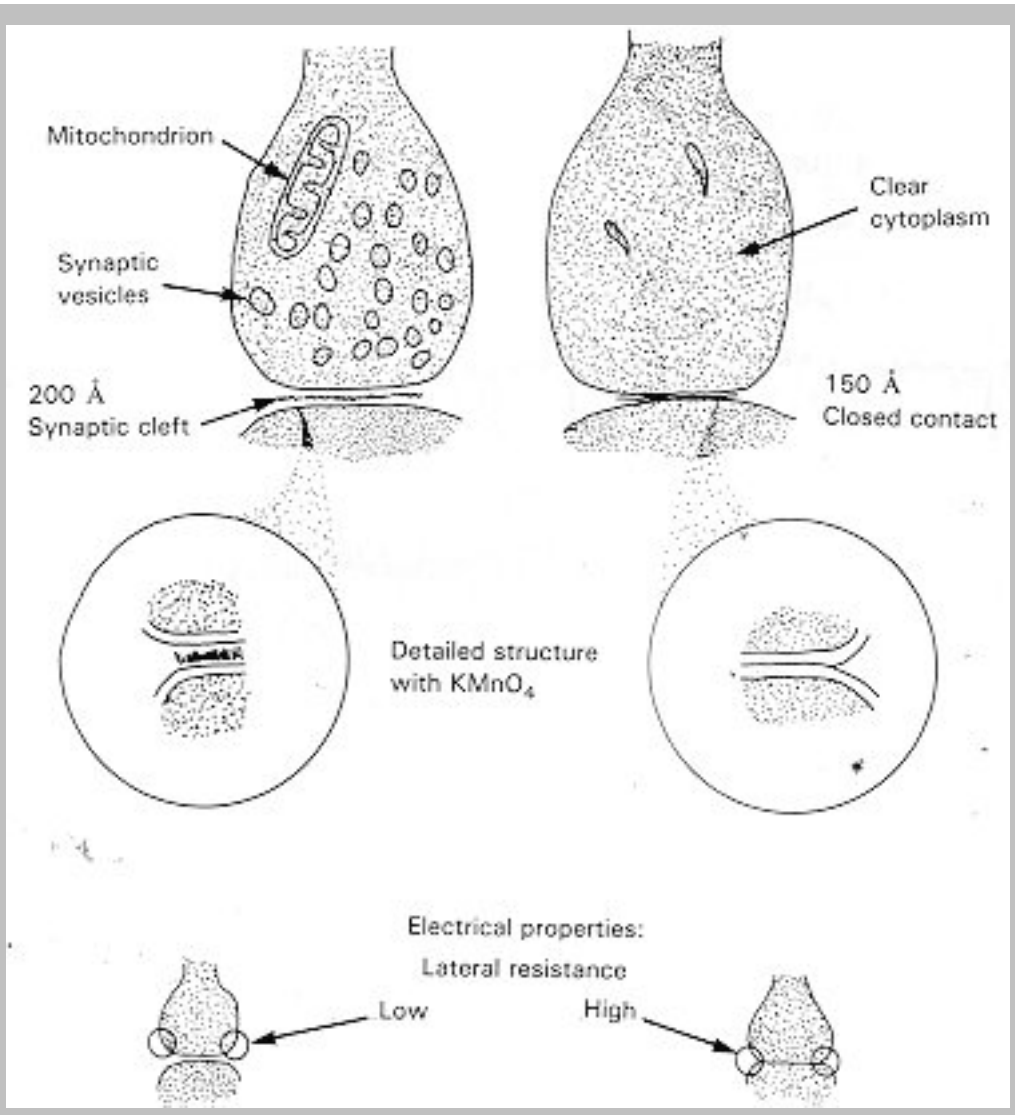


Fig. 18 Characteristics of a chemical synapse (left) and an electrical synapse (right). Note in the latter synapse the absence of both synaptic vesicles and cleft and the invasion of the postsynaptic elements by action currents, which in the chemical synapse are short-circuited by the low-resistance cleft. Reproduced with permission from V. P. Whittaker, *Proceedings of the National Academy of Sciences*, 60:1082, 1084, 1968.

Excitatory neurotransmitters depolarize the postsynaptic membrane. As might be expected from our previous examination of depolarization, this process involves the opening of channels. In this case, however, the opening depends on binding of the neurotransmitters; i.e., the channels are chemically gated. Postsynaptic depolarization has been studied with patch clamping techniques primarily in cells in culture or freshly dissociated neurons, because the native structures are enveloped in protective sheets.

One of the most versatile techniques involves the reconstitution of extracted channel proteins in bilayers or in the lipid at the tip of a patch pipette. This approach has been used extensively with many proteins, including Na^+ and K^+ channels and acetylcholine-activated channels.

The receptors have been isolated most commonly from the electric organ of the ray *Torpedo*. The acetylcholine-activated channels increase the permeability of the membrane to a variety of ions when

they bind acetylcholine as well as other agonists. The four distinct glycoproteins are present as a pentamer (Montal et al., 1986) with the composition $\alpha_2\ \beta\gamma\ \delta$. The activation of single channels reconstituted into a bilayer is shown in Fig. 19 (Montal et al., 1984).

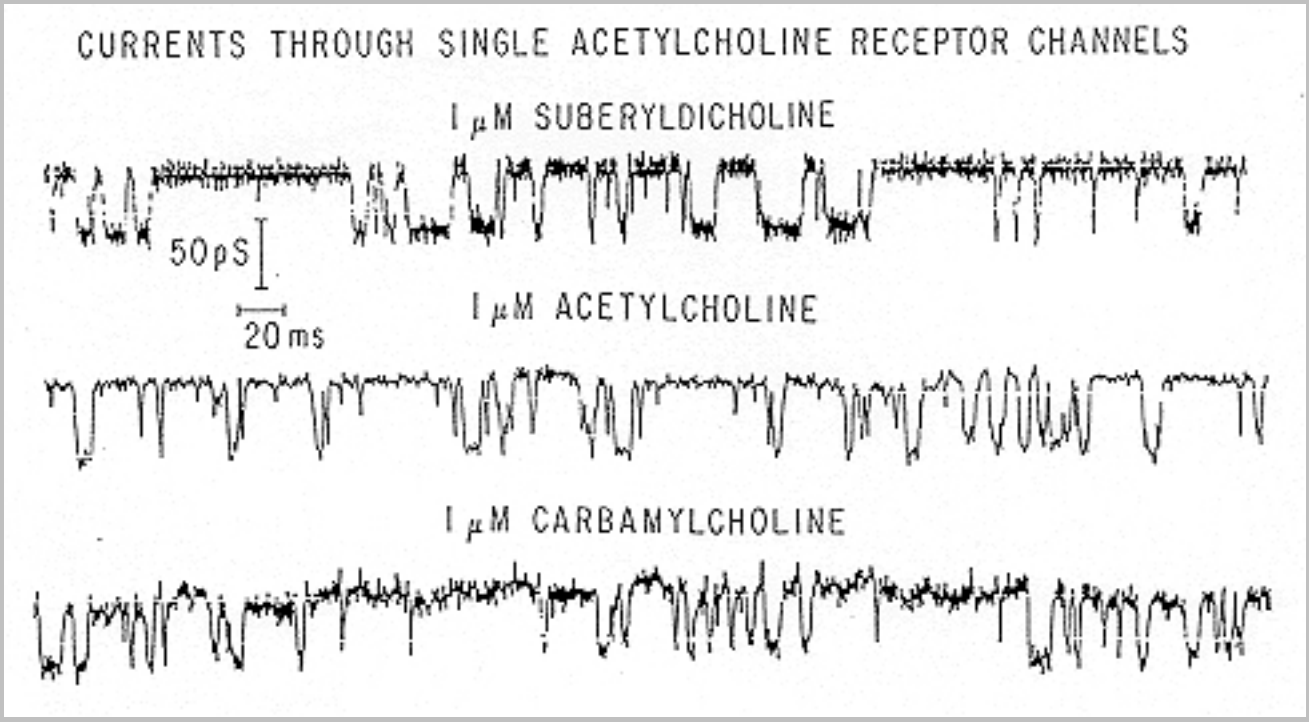


Fig. 19 Single acetylcholine receptor channel currents activated by different cholinergic agonists in planar lipid bilayers. Reproduced from *Biophysical Journal*, 45:165-174, &copy1984, by copyright permission of Rockefeller University Press.

Postsynaptic membrane receptors are anchored to structures connecting them to the cytoskeleton. In the nervous system, the assembly of these structures is thought to have a role in neuronal plasticity (see Section D, below). A common theme is the attachment of the receptor to an adaptor protein which attaches directly or indirectly to cytoskeletal elements.

Acetylcholine receptors (AchR) and sodium channels (NaCh) clustering at the neuromuscular junction (see Colledge and Forhner, 1998) depend on *agrin*. Agrin is a large heparan sulfate proteoglycan, a protein synthesized and secreted by motor neurons, that becomes incorporated in the synaptic basal lamina. AchRs are attached to the adaptor protein *rapsyn* of 43 kDa. In contrast, the NaChs are associated with *syntrophins* which bind to *dystrophin* complexes. Dystrophin, in turn, is connected to cortical actin and a transmembrane complex that interacts with the extracellular matrix.

Agrin also mediates the formation of immunological synapses (see Trautmann and Vivier, 2001). As in the case of the neuromuscular junction, the immunologivcal synapse involves signaling between cells via surface receptors and these receptors are clustered. Agrin is found at T-cell receptor sites during activation of primary immune cells. Furthermore, activated purified agrin triggers lipid raft clustering (Khan et al., 2001) and the clustering of antigen-specific T cell receptors

Gepheryn plays a role similar to that of rapsyn, but in certain inhibitory synapses where glycine is the neurotransmitter. Gephyrin binds to glycine receptors (GlyRs) (see Meyer et al., 1995) and mediates the binding to tubulin (Kirsch and Betz, 1995). *Gene targeting* mutations in mice showed that gephyrin is required for synaptic clustering of GlyRs and in non-neuronal tissue molybdoenzyme activity (Feng et al., 1998). Gene targeting was carried out by deleting the upstream sequences responsible for initiating transcription and translation.

Glutamate receptors also bind to adaptor proteins (see O'Brien et al. 1998). The latter, in turn, bind to the cytoskeleton by attaching to α-actinin-2 (an actin binding protein) (e.g., Wyszynski et al., 1997).

Go to Part 3

REFERENCES

Search the textbook

Back to Part 2

B. Neurotransmitters: Discharge and Recovery

This section will focus on the mechanisms of discharge and recovery of neurotransmitters. The discharge is by exocytosis as in other secretory events (see Chapter 10). However, synaptic transmission, whether at an electrical or a chemical synapse, must be very quick: in the millisecond range. In a chemical synapse, the speed of discharge is needed for rapid communication. The quick recovery is also essential to permit continued excitability. Other secretory events are similar to that of neurotransmitter. However, in these cases, speed is not as important. Therefore, presynaptic terminal of chemical synapses must differ in some way. What are these differences? In synaptic transmission, the vesicles in close apposition to the presynaptic membrane are thought to be in a rapidly releasable pool (see Fig. 17). Vesicles that are a short distance away are thought to be held in reserve and be able to replace quickly the vesicles that have been discharged.

Recent studies using total-internal-reflection microscopy (see Chapter 1) allowed following the process of exocytotic discharge of single vesicles in the goldfish retinal bipolar neurons (Zenisek et al., 2000), where the synaptic vesicles were labeled with a fluorescent lipid. The results are in agreement with the scenario of two different pools of vesicles. In these experiments the synaptic terminal attaches tightly to a coverslip. The brightness of a fluorescently labeled vesicle increases as it approaches this site. At the fusion, the fluorescence is incorporated into the plasma membrane. The fusion was found to occur at specific spots in the membrane probably corresponding to Ca^{2+}-channel clusters. Vesicles close to the membrane fused quickly. In contrast, vesicles held 20 nm away from the cell membrane were allowed to advance to the exocytotic sites and could fuse with the plasma membrane 0.25 s later

The recovery from the neurotransmitter discharge, although much slower than the exocytotic events, is nevertheless relatively quick occurring in about 10 s (Borges et al., 1995; Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996). Ca^{2+} and the synaptic vesicles' attachment-detachment from cytoskeletal elements are thought to play an important role in all these events.

Involvement of Ca^{2+}

As in other kinds of exocytosis, Ca^{2+} plays a role at the synapse. Presynaptic depolarization discharges neurotransmitter only when Ca^{2+} is present. Chemicals that block Ca^{2+} channels (e.g., Cd^{2+} or Mn^{2+}) also block chemical synapses. The role of presynaptic Ca^{2+} can also be demonstrated more directly.

Electrophysiological measurements of Ca^{2+}-current have shown that Ca^{2+} influx is followed by

neurotransmitter release within 200 µsec (Llinas et al., 1981). One of the pieces of evidence is shown in Fig. 21, discussed later. In this figure, the middle trace corresponds to the Ca^{2+} current which precedes the postsynaptic response, well within the time needed for synaptic transmission. Other data involving chelators agree with this notion. The intracellular presynaptic microinjection of the slowly binding chelator EGTA is ineffective in blocking neurotransmitter release (Adler et al., 1991). However, the fast-binding chelator BAPTA (1,2-bis (2-amino phenoxy) ethane-N,N,N,N-tetraacetic acid), which has similar binding constants, blocks synaptic transmission.

The rapid response of the presynaptic processes suggests that the machinery for the release must be already in place. These experiments also suggest the Ca^{2+} concentration that triggers release must be 100 µM or more (Adler et al., 1991). The resting concentration of Ca^{2+} is below the µM range. Many effects of Ca^{2+} are thought to be triggered by concentrations in the range of 1-5 µM. However, Ca^{2+} concentrations as high as 100 µM are entirely possible in microdomains of the presynaptic processes. The photoemission of the protein aequorin (see Chapter 1) when injected into nerve terminals indicates Ca^{2+} concentrations in this range (Llinas et al., 1992). A high local concentration could be explained by the proximity of the system delivering the Ca^{2+} to the vesicles. Freeze-fracture studies indicate that vesicle fusion takes place close to or at the location of intramembranous particles (Heuser, et al., 1974) that are thought to correspond to the Ca^{2+} channels. Other observations also indicate that the association between Ca^{2+} flux and neurotransmitter release is an intimate one. Measurements of Ca^{2+}-current and appearance of neurotransmitter using patch-clamping techniques in the study of chick ciliary ganglia suggest that the opening of a single Ca^{2+}-channel is sufficient for the release of a quantum of acetylcholine (Stanley, 1993).

Role of the cytoskeleton and the synapsins

Synaptic vesicles are attached to the cytoskeleton. Quick-freeze, deep-etch EM (Hirokawa et al., 1989, Gotow et al., 1991) found it to consist mainly of microtubules and actin filaments. The actin filaments form a network frequently associated with the presynaptic plasma membrane and extending to the cortical areas devoid of microtubules (Landis et al., 1988; Hirokawa et al., 1989). The attachment of the vesicles to the actin is mediated at least in part by proteins of the synapsin family (Valtorta et al., 1992, Greengard et al., 1993), derived from differential splicing of the transcript of two synapsin genes (one for synapsin I and the other for synapsin II). Synapsins are present in virtually all nerve terminals, where they are exclusively associated with the cytoplasmic surface of the synaptic vesicle membrane. Synapsins could be recognized using immunogold EM techniques, or from their distinctive morphology with a head region 14 nm in diameter and a filamentous portion 33 nm long.

Single synapsin molecules were found to cross-link actin filaments to other actin filaments and microtubules throughout the head region. The tail region was found to bind to the vesicles. The connecting portion was found to be about 30 nm in length. Longer and thinner strands (about 100 nm in length) were also found associated with vesicles and the plasma membrane.

Does the association of the vesicles to the cytoskeleton play a role in transmitter release? Many biological processes are regulated by the phosphorylation and dephosphorylation of proteins (e.g., Chapters 13). Phosphorylation-dephosphorylation cycles of the synapsins are thought to function in determining the availability of the vesicles loaded with neurotransmitter. The phosphorylation of synapsin was found to increase under all conditions that promote Ca^{2+}-dependent release of neurotransmitter, as summarized in Table 3 (Nestler and Greengard, 1984).

The properties of synapsin Ia and Ib are summarized in Table 4 (Nestler and Greengard, 1984). Part A of the table summarizes the physicochemical properties, part B, the protein kinase specificity and part C the site of their occurrence. The phosphorylation is catalyzed by either cAMP or Ca^{2+} -dependent protein kinases. As indicated in Table 4, synapsin I is phosphorylated by Ca^{2+}-calmodulin dependent protein kinase II (CaM kinase II). This latter phosphorylation is very rapid during physiological activity of neurons. Phosphorylation causes a conformational change in the synapsin I molecule (Benfenati et al., 1990). A variety of experiments suggests that unphosphorylated synapsin constrains the vesicles so that they fail to provide neurotransmitter; this constraint is removed by phosphorylation. Therefore, phosphorylation would be the trigger for neurotransmitter release. Micromanipulation experiments support this view.

Microinjection of unphosphorylated synapsin I into neurons inhibits neurotransmitter release (Llinas et al., 1985). In contrast, injection of the phosphorylated form has no effect. In addition, the introduction of CaM kinase II either has no effect or increases neurotransmitter release. We have seen that Ca^{2+} is involved in the release of the vesicles. Therefore, it would seem possible that Ca^{2+} triggers the phosphorylation of the synapsin, most likely by acting via CaM kinase II. Several experiments support this view.

Table 3 Physiological and pharmacological regulation of synapsin From Nestler and Greengard, 1984. Reproduced by permission.

---

1. In synaptosomes and slices of nervous tissues, depolarizing agents and cyclic AMP increases the phosphorylation

2. In specific anatomical regions of central and peripheral nervous system, the relevant neurotransmitter (serotonin, dopamine, norepinephrine, adenosine) increase state of phosphorylation

3. In isolated peripheral nervous tissue and in posterior pituitary

4. In whole animals, convulsants increase and depressants decrease state of phosphorylation in cerebrum

5. In whole animals, neurotransmitters and hormones increase total amount in specific brain regions

The relationship between internal Ca^{2+}, synapsin and neurotrasmitter release was shown in experiments of Llinas et al., (1991) on the squid giant synapse. The amount of presynaptic neurotransmitter release was followed by measuring postsynaptic potential, which is proportional to the concentration of neurotransmitter. The presynaptic potential and the Ca^{2+} current were also recorded. Fig. 20 (Llinas et al., 1991) shows presynaptic potentials (in this case action potentials) and the postsynaptic potentials, before and after injection of dephosphorylated synapsin I in the presynaptic cell. The numbered curves represent the postsynaptic potentials at the time in minutes indicated by the number. As shown, the presynaptic potential is not affected. In contrast, the postsynaptic potential is almost completely blocked 24 minutes after microinjection. Fig. 21 (Llinas et al., 1991) represents the postsynaptic potential (upper curves) and the presynaptic Ca^{2+} currents (middle curve) when the presynaptic voltage (lower record) is clamped at 25 mV. The numbers on the left of the figure represent the time after microinjection of dephosphorylated synapsin I. The postsynaptic potentials are inhibited by the microinjection. As indicated, the Ca^{2+} current is not affected, showing that the defect is between the internal Ca^{2+} signal (which is normal) and the release of the neurotransmitter.

Whereas unphosphorylated synapsin blocks exocytosis of the neurotransmitter, the Ca^{2+}-dependent phosphorylation facilitates release, as shown by the effect of the injection of CaM kinase II. Similar results were obtained in experiments using isolated *synaptosomes* (vesicles corresponding to neuron terminals) from the rat brain. Synapsin I was introduced into the vesicles by transient permeabilization using freeze-thawing (Nichols et al., 1992). Unphosphorylated synapsin I decreased the K^+-induced release of the neurotransmitter glutamate. Increased external K^+ depolarizes the vesicle membranes. In contrast, the introduction of CAM kinase II was without effect as expected for a system where the synapsin is already phosphorylated. Other experiments showed that dephosphorylated synapsin I, when injected presynaptically, decreases the spontaneous miniature postsynaptic potentials (mESPs) in goldfish Mauthner axons (Hackett et al. 1990). These potentials correspond to spontaneous quantal release of neurotransmitter as mentioned in Section V, Part A.

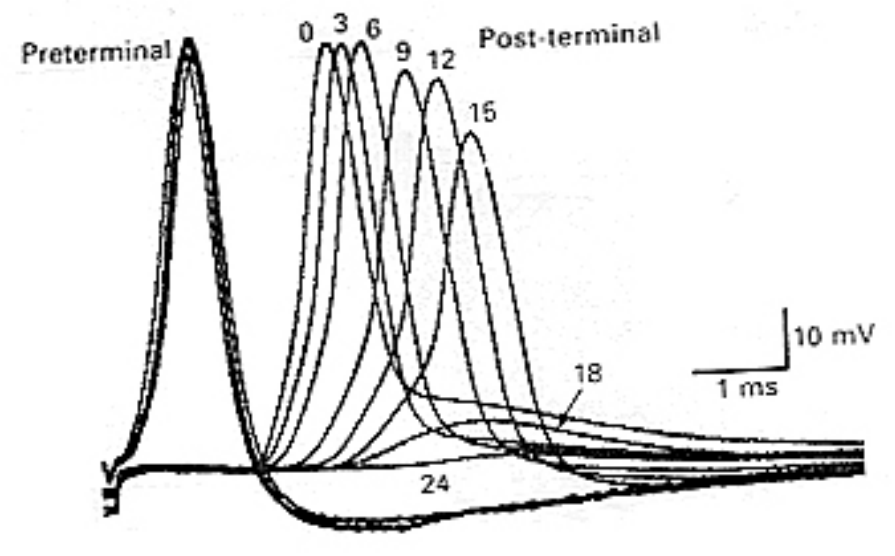


Fig. 20. Potentials from the squid giant axon synapse before and after injection of synapsin I. No significant changes in presynaptic voltages was observed. However, synaptic transmission was competely blocked after 24 min. Reproduced from Llinás et al., 1991, by permission.

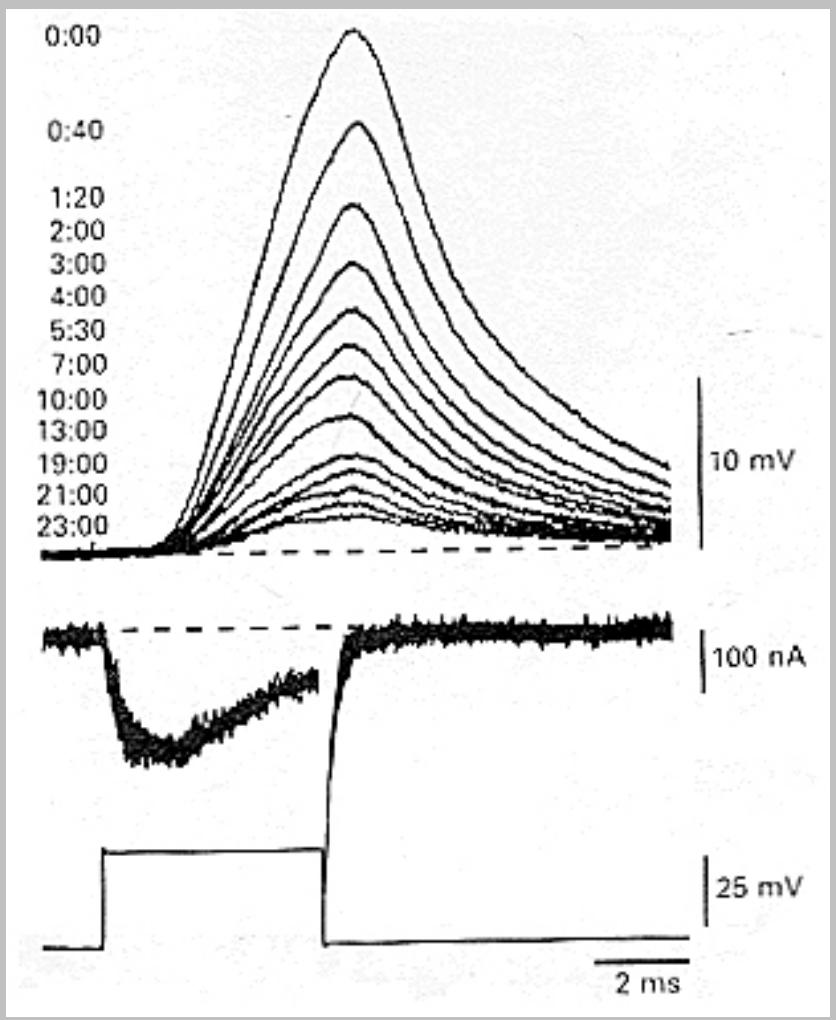


Fig. 21 Effect of presynaptic injection of synapsin I. Top: postsynaptic voltage. Middle: presynaptic Ca^{2+}-current. Bottom: Constant presynaptic voltage pulses were delivered at regular intervals before and after

injection. The numbers at left give the time intervals at which the presynaptic responses were recorded. From Llinas et al., 1991, reproduced by permission.

How can synapsin control the fate of synaptic vesicles? The model represented in Fig. 22 (Greengard et al., 1993) summarizes the mechanism that is implicated by current data. Before stimulation, synapsin cross-links the reserve pool of vesicles to other vesicles or actin (1). Upon stimulation, the influx of Ca^{2+} activates CaM kinase II, which catalyzes the phosphorylation of synapsin I (2). The dissociation vesicles and actin allow the vesicles to become part of the readily releasable pool (3). After exocytotic discharge, the vesicles are recycled (4,5). When a phosphatase dephosphorylates synapsin, the dephosphorylated synapsin again crosslinks actin and the vesicles (6), to reinitiate the cycle.

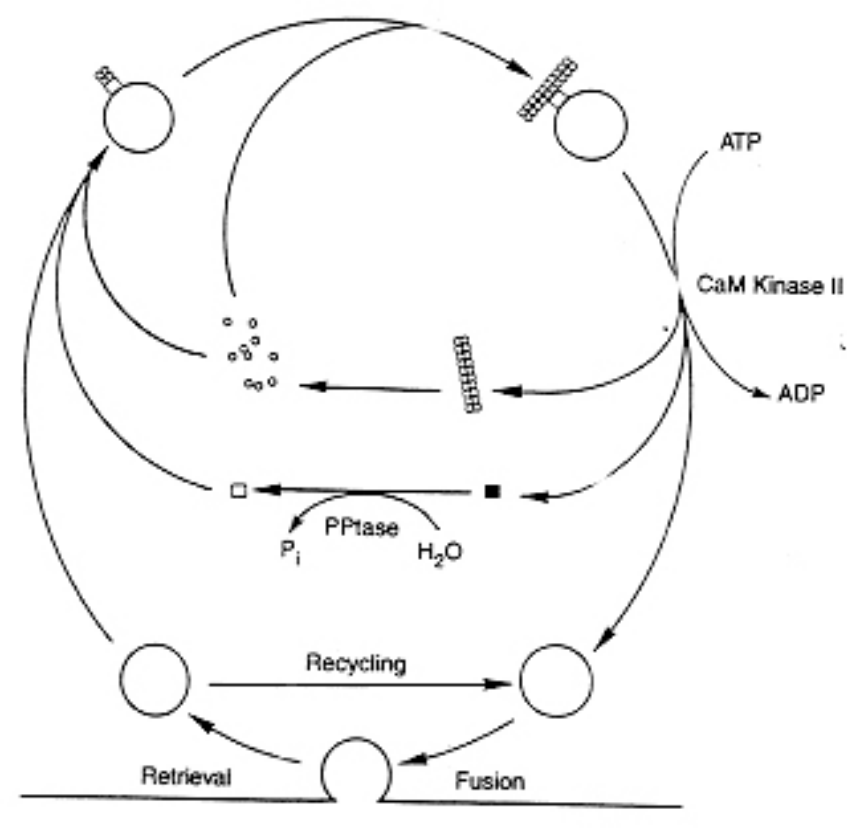


Fig. 22. Model illustrating how the state of phosphorylation of synapsin I regulates the availability of synaptic vesicles to exocytosis. 1. Resting conditions: synapsin I () cross links actin to vesicles . 2.Activation of CaM kinase II phosphorylates synapsin I () .3. The complex is disrupted. 4. The vesicle fuses with plasma membrane. 5. Retrieval of vesicle. 6. Phosphatase dephosphorylates synapsin I, which can now cross-link the vesicle to the actin. Reproduced with permission from Greengard, P., Valtorta, F., Czernik, A.J. and Benfenati, F. (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function, *Science* 259: 780-785. Copyright &copy1993 American Association for the Advancement of Science.

Table 4 Physicochemical properties, protein kinase specificity, and distribution of Synapsin I. From Netler and Greengard, 1984. Reproduced by permission.

A. Physico-chemical Properties

	Synapsin Ia	Synapsin Ib
Molar proportion	1	2
Molecular weight	86,000	80,000
Isoelectric point	10.3	10.2
Stokes radius	59	59
Sedimentation coefficient	2.9 S	2.9 S
Frictional ratio	2.2	2.2
Acid soluble	yes	yes
Amino acids	rich in proline and glycine	rich in proline and glycine
Other structural features	a collagenase-insensitive domain and a proline-rich collagenase sensitive domain	a collagenase-insensitive domain and a proline-rich collagenase sensitive domain

---

B. Protein kinase specificity

Synapsin I undergoes multisite phosphorylation
1. One serine residue (site 1) in the collagenase-insensitive domain of Synapsin I is phosphorylated by cAMP-dependent protein kinase and by Ca^{2+}/calmodulin-dependent protein kinase I.

2. Two serine residues (sites 2 and 3) in the collagenase-sensitive domain of Synapsin I are phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II.

3. Not an effective substrate for cyclic cGMP-dependent protein kinase or Ca^{2+}/phosphatidylserine-dependent protein kinase.

C. Distribution

1. Present in nervous system (both central and peripheral)
2. Within nervous system, present only in neurons
3. Within neurons, concentrated in presynaptic terminals
4. Within terminals, associated with synaptic vesicles
5. Present in virtually all synapses

6. Not present in adrenal chromaffin cells
7. Appears simultaneously with synapse formation during development

---

This model is supported by several findings. Synapsin binds to synaptic vesicles with high affinity. The binding is saturable, indicating the presence of specific binding sites. The binding affinity is decreased when synapsin I is phosphorylated by CaM kinase II (Huttner et al., 1983, Schiebler et al., 1986). In resting cells, 96-97% of the synapsin I is bound to vesicles (Schiebler et al., 1986, Benfenati et al., 1991). Synapsin I interacts in vitro with cytoskeletal proteins, including actin (Baines and Bennett, 1985, 1986; Goldenring et al., 1986; Bähler and Greengard, 1987; Petrucci and Morrow, 1987). The binding of synapsin II to actin produces actin bundles in vitro; this bundling is decreased when the synapsin is phosphorylated by CaM kinase II (Bähler and Greengard, 1987, Petrucci and Morrow, 1987). Therefore, synapsin is probably responsible for the trapping of the vesicles in the cytoskeletal network, and synapsin can release the vesicles when phosphorylated in response to an increase in Ca^{2+}. A physiological cycle of this kind is supported by computer simulations (Benfenati et al., 1991).

The carboxyl terminal of the synapsin bound to a vesicle protein has been implicated in the phosphorylation-dephosphorylation binding cycle (Benfenati et al., 1989a, 1989b). One of the proteins binding to synapsin has been shown to be the subunit of CaM kinase II (Benfenati et al., 1992), which would be consistent with the rapid response required for synaptic transmission.

Myosin IIA and IIB and myosin V are thought to be involved in the passage of vesicles from the reserve zone to the fusion competent vesicles. Myosin V has been implicated in the transport of post-Golgi secretory vesicles (e.g., Govindan et al., 1995), and the tail region of brain myosin V binds to the synaptic vesicle proteins synaptobrevin II and synaptophysin (Prekeris and Terrian, 1997). Myosin II has been shown to be present in presynaptic terminals. The heavy chains of myosin IIA and IIB bind to acid phospholipids, are capable of assembly and may play a role in neurotransmitter release (Mochida et al., 1994).

Membrane fusion

Neurotransmitter release requires targeting of the vesicles to the presynaptic plasma membrane followed by fusion of the membranes (see Jahn and Südhof, 1999). Membrane fusion and secretion are general processes of cells and many of the components have been found to be very similar, from yeast to mammalian neurons. Many of the details have been elucidated for the synaptic vesicle fusion with the presynaptic plasma membrane.

A cell-free system has been developed suited for the systematic study of exocytosis in a neuroendocrine cell line (Avery et al., 2000). In this assay, cells are loaded with acridine orange. Sonication produces flat vesicles with attached vesicles. The release of the dye and the disappearance of labeled vesicles allow

following exocytosis This kind of assay could accelerate progress in this area by finding what factors could substitute for cytosol. The formation of vesicles was demonstrated with EM and atomic force microscopy. In vitro systems (for a review see Rothman and Orci, 1992) have provided enough information to allow the formulation of models that suggest what might be taking place in the various cells and organisms. These propose the interaction of several proteins. The *N*-ethylmaleimide sensitive factor (NSF) and α, β and γ-SNAP (soluble NSF attachment protein) form a 20 S complex required for all fusion events (see Chapter 11). The fusion would require specific receptors both in the vesicle (v-SNARE) and the target membrane (t-SNARE). Each receptor would be specific for the vesicle and appropriate target.

The proteins isolated by affinity chromatography (Söllner et al., 1993a) using NSF and α-SNAP, were identified as syntaxin a and b, SNAP-25 and synaptobrevin. Syntaxins are associated with the plasma membrane identifying them as t-SNARE. Synaptobrevin is associated with vesicles identifying it as a v-SNARE. SNAP-25 is thought to bind to syntaxin. Syntaxin is also intimately connected to Ca^{2+} channels (Yoshida et al., 1992, Sheng et al., 1994), suggesting that the release of the neurotransmitter from a docked vesicle could occur almost instantaneously.

The sequence of events could be as diagrammed in Fig. 23 (Söllner et al., 1993b). The specific association of the components of the vesicle or plasma membrane, only requires the presence of t- and v-SNARES (syntaxin, SNAP25 and vSNARE). In the absence of SNAPs and NSF, the three SNARE proteins form a complex that can bind synaptotagmin. α-SNAP can displace synaptotagmin from its binding site (reaction 1). In this model, synaptotagmin acts as a clamp, preventing the fusion of membranes. When synaptotagmin is displaced by α-SNAP, the complex can bind NSF needed for the fusion reaction (reaction 2). ATP hydrolysis displaces the complex and initiates fusion (reaction 3). We saw that Ca^{2+} is needed for exocytosis. It might have a role in binding to synaptotagmin so that α-SNAP will be able to bind to the active site releasing the clamp. Contradicting this model, however, the addition of Ca^{2+} to the in vitro system was not found to release synaptotagmin from the SNARE complex. However, this might indicate that an additional component is still to be found. Synaptotagmins constitute a family of proteins with at least twelve isoforms with a unique amino-terminal domain and a conserved carboxy-terminal domain (see Schiavo et al., 1998; Craxton and Goedert, 1999).

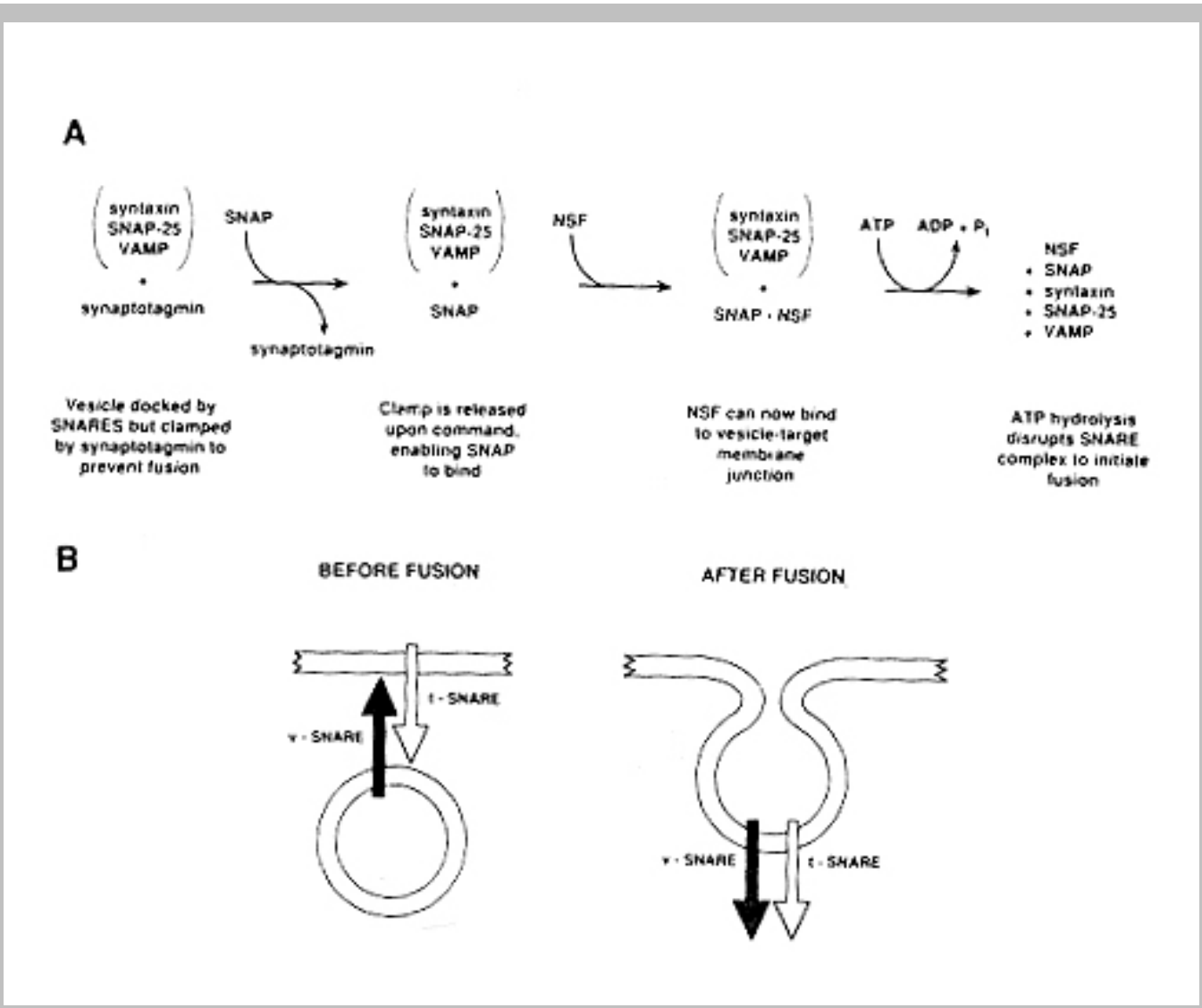


Fig. 23 Model of vesicle fusion to the plasma membrane during exocytosis. From Söllner et al., 1993b, reproduced by permission.

The release of neurotransmitter from the presynaptic terminals may also be modulated by second messengers. Stanley and Miroznik (1996), using patch-clamp techniques on chick ciliary ganglia, were able to show that the opening of the Ca^{2+}-channels depended on the activation of a GTP-binding protein. The response required intact syntaxin.

Recycling and recovery

The release of neurotransmitter by exocytosis has to be matched by the recovery of vesicles to be reloaded with neurotransmitter (see Cremona and De Camilli, 1997). The most widely accepted model proposes retrieval by clathrin mediated endocytosis (e.g., see Heuser and Reese, 1973). For a general discussion of endocytosis, see Chapter 9. However, other models have been formulated and the various likely mechanisms are not mutually exclusive. An alternative mechanism that also can coexist with clathrin-mediated endocytosis, is the so-called *"kiss and run"* model (see Fesce et al., 1994). In this model, vesicles never lose their identity and the rapid closure of the temporary fusion pore reconstitutes the

vesicle. There are arguments that support this view. There are intermediate structures before the exocytotic pore is open entirely and these are capable of releasing secretory products (Chow et al., 1993; Alvarez de Toledo, 1993) (see also Chapter 10). The exocytotic pore can close again after transient opening (Fernandez et al., 1984) and is capable of flickering between open and closed states (Breckenridge and Almers, 1987). An additional mechanism has been proposed involving internalization via large vacuoles (Artalejo et al., 1995).

A role of clathrin-coated vesicles in neuronal endocytosis is supported by genetic studies in invertebrates. The component of the clathrin coats of neuron terminals include clathrin, the clathrin adaptor AP-2 (a heterotrimer composed of α, β, μ and σ subunits) and the protein AP180. In *Drosophila*, mutation of the α-adaptin gene blocks synaptic vesicle formation in nerve terminals (Gonzalez-Gaitan and Jackle, 1997). Mutations of a gene with homology to the μ subunit of AP-2, contributes to lethality in *Drosophila* (Petrovich et al., 1993).

Several other proteins have been found to be involved in synaptic endocytosis. Endophilin I is a brain tissue protein. It contains an src-homology domain (SH3). This domain binds to the proline rich region of dynamin and two other proteins found at the synapse: *synaptojanin* and *amphiphysin* (Micheva et al., 1997; Ringstad et al., 1997). In fact, clathrin, dynamin and endophilin I are required to form vesicles in a system reconstituted from perforated rat neuroendocrine PC12 cells in a mixture containing cytosol and ATP (Schmidt and Hutter 1998). A deletion mutant lacking the SH3 domain of endophilin I cannot interact with dynamin and is incapable of mediating the formation of vesicles (Schmidt et al., 1999). Endophilin I is a lysophosphatic acid acyl transferase which catalyzes the formation of phosphatidic acid (two acyl chains) from lysophosphatic acid (one acyl chain) and arachidonoylCoA. Endophilin I is present in the cytoplasmic leaflet of the plasma membrane. This is an interesting finding because it implicates the complex in controlling the lipid components of the membrane. Furthermore, endophilin I may play a role in the formation of vesicles by altering the membrane curvature to facilitate invagination during endocytosis (see Schmidt et al., 1999). Presumably this would be accomplished by increasing the surface area of the cytoplasmic leaflet (to become the external leaflet of the vesicle).

The recycling of neurotransmitter at the presynaptic terminal also differs from the recycling of conventional secretion. Rather than linking endocytosis to retrograde intracellular transport, the presynaptic vesicles recovered by endocytosis remain at the terminal, where they are rapidly reloaded with neurotransmitter. The synaptic vesicles that are synthesized in the cell body are transported to the terminals by the microtubular system and assembled from two different types of precursor vesicles (Okada et al., 1995). The synthesis of neurotransmitter does not take place at the cell body (see Siegel et al., 1994), where other secretory products are produced. Rather, neurotransmitter synthesis involves several biochemical reactions that may occur in different compartments close to or at the terminal. Synaptic organelles, such as mitochondria (e.g., in the production of glutamate), or even the glial cells, may be involved.

The reloading of the vesicles with the neurotransmitter requires active transport mediated by V-ATPase.

This ATPase resembles the mitochondrial F_1-ATPase. In contrast to F_1, however, the V-ATPase is not an ATP-synthase. It links neurotransmitter translocation to proton transport (McMahon and Nicholls, 1991). There are four transporters, one each for acetylcholine and glutamate, one for biogenic amines, and another for amino butyric acid (GABA) and glycine (Nelson, 1992, Fordac, 1992).

C. Nitric Oxide

NO mediates many signals in the peripheral or central nervous system, either as an intracellular second messenger or as a neurotransmitter. NO differs from other neurotransmitters because it does not have an elaborate machinery for its release and can diffuse rapidly to its target. In addition, it has a short half life and can only affect nearby targets. NO binds to the iron of heme groups and can produce conformational changes in proteins, such as guanylyl cyclase. This enzyme contains a heme, responsible for its activation by NO (see Wolin et al., 1982).

NO synthase (NOS) synthesizes NO from L-arginine. NOSs are induced by various cytokines and bacterial products and are extensively present in the central nervous system.

That NO is a neuronal messenger was shown in experiments in which N-methyl-D-aspartate (NMDA) added to granule cells increased cGMP. NMDA is a ligand for glutamate channel receptors. NO was implicated because the increase was blocked by NOS inhibitors and hemoglobin (Garthwaite, 1988).

NO is produced postsynaptically, in response to Ca^{2+} influx following the activation of the NMDA channel receptors (NMDARs) or release from internal stores. Ca^{2+} binds calmodulin that, in turn, activates NOS. The newly formed NO then can act as a retrograde signal on the presynaptic process.

As a retrograde transmitter, NO increases cGMP synthesis and potentiates synaptic transmission in the brain (Garthwaite et al., 1988; O'Dell et al., 1991; Schuman and Madison, 1994). NO activates *cyclic-nucleotide-gated channels* (CGN) and triggers the release of glutamate (Savchenko et al., 1997).

NMDA receptor channels are an unusual kind of glutamate receptors that usually require depolarization to activate Ca^{2+} fluxes. NO-releasing compounds have been shown to reduce NMDA-induced currents. NO is thought to act as an oxidizing agent on closely spaced cysteine residues of NMDA. In this way, NO acts as a feedback inhibitor. This feedback inhibition may have a role in *long-term potentiation* (LTP) (see Schuman and Madison, 1994) discussed in the next section.

VI. Plasticity of Synapses

Transmission of impulses through the neuron by action potentials is largely an all-or-none effect that, within limits, does not change significantly. Events at the synapse are capable of being modified. The efficacy of a synapse does depend on previous experience. This phenomenon goes by several names such as *plasticity*, and involves neurotransmitter release and responses to the neurotransmitter. This is the topic

of this section. The properties of synapses have suggested the involvement of pre- and postsynaptic events in memory and learning. The actual de novo formation of synapses may also come into play and is discussed at the end of this section. The magnitude and significance of this plasticity can only be appreciated if we consider that cortical projections in adult animals are continuously being changed by experience (see Buonomano and Merzenich, 1998). This cortical map reorganization results from synaptic plasticity. For example, sensory deprivation was found to alter short-term synaptic excitatory pathways within the supragranular cortex (Finnerty et al., 1999).

Potentiation and depression

Learning is the modification of behavior by experience. Memory is the retention of sensory experiences or learning. Since the early discoveries of Ramón y Cajal, the cellular organization of the nervous system has suggested an involvement of events at the neuronal level. The discovery that the *strength* of synaptic potential (i.e., extent of response following a signal) is plastic and varies as a function of activity and use (Feng, 1941) has suggested possible mechanisms. The increase in strength has been termed *potentiation*. The opposite phenomenon has been termed *depression*. Brief periods of high frequency presynaptic activity (elicited by high frequency electrical stimulation called *tetanus*) can enhance the synaptic potential for minutes or even hours. Similarly, the synaptic potential can be depressed by low frequency presynaptic activity (Lloyd, 1949; for a review see Bear and Malenka, 1994).

Certain aspects of this problem have been studied in the large marine snail, *Aplysia*. Genetic approaches have used *Drosophila*. The hippocampal region of mammalian brain has also been found useful. This region is responsible for the initial storage of memory (Squire, 1992). In the mammalian system, many observations support the *consolidation theory* of memory, according to which newly acquired sensory information is transmitted from the cortex to the hippocampus. In the hippocampus learning is established first and then transmitted later, repetitively, to the cortex. In the cortex, memories are stabilized (i.e. consolidated) and available even if the hippocampus is removed (see McGaugh, 1966; Squire 1992). Presumably, the hippocampus is necessary for *declarative* (recognition) memory: the ability to recollect everyday events and factual knowledge. These memories are distinct from nondeclarative memory in that *nondeclarative* (implicit) memory such as skills and habits, simple conditioning, and the phenomenon of priming abilities which do not require the hippocampus. The hippocampus is needed temporarily to coordinate events taking place in the neocortex that together represent a whole memory.

Long term potentiation (LTP) has been considered a good model for learning (see Bliss and Collingridge, 1993). A single brief tetanus increases synaptic strength but then in a few hours it decays back to the original level. Longer duration tetanus produces an LTP that lasts for 8 hours in hippocampal slices and longer in intact animals (*late LTP*, LLTP, defined as the potentiation that lasts more than 3 hours). The phenomenon opposite to LTP, produced by low frequency stimulation, has been termed *long-term depression* (LTD) (see Linden and Connor, 1995).

Basic aspects of potentiation

As we have seen for many biological processes, insights into their mechanism have been provided by the study of simple organisms. This is also the case in the study of synaptic plasticity. Many seminal studies of plasticity have been carried out on the synapse between siphon sensory neurons and gill and siphon motor neurons of *Aplysia* where the withdrawal reflex, the so-called sensitization is induced by stimulation of the tail. The model (see Bailey et al., 1996) that has emerged from the various studies indicates that the neurotransmitter, serotonin initiates the short-term potentiation by binding to its receptors at the surface of the presynaptic cell. The activation of adenylyl cyclase that follows, produces cAMP which activates *cAMP activated protein kinase* (PKA). PKA first phosphorylates K^+ channels which increases Ca^{2+} influx and increases the magnitude of the action potential. In addition, the exocytosis of neurotransmitter is also increased. These changes do not require new synthesis of macromolecules. In contrast prolonged stimulation or release of neurotransmitter lead to the transfer of PKA to the nucleus and the activation of transcription factors. Similar observations have been made in *Drosophila*

As just outlined, short term changes do not depend on macromolecular synthesis (Schwartz et al., 1971) either in culture or in the intact animal. In contrast, inhibitors of transcription and translation block long term changes both in animals (Castellucci et al., 1989) and in culture (Montarolo et al., 1986). In *Aplysia* as well as other invertebrates and vertebrates there is a critical time period for the required protein and RNA synthesis (see Davis and Squire, 1984, Montarolo et al., 1986). One of the consequences of LTP has been found to be synaptic growth (Bailey and Chen, 1988a and b).

The steps necessary to produce LTP have been delineated by activating the cascade of events without stimulation. Since stimulation of the presynaptic cell is know to result in the release of neurotransmitter, it would seem that the initial steps in either short or long potentiations must involve neurotransmitter. In fact, in the case of *Aplysia*, exposure to serotonin appears to be responsible for LTP in the absence of stimulation either in intact animals (Glanzman et al, 1989; Montarolo et al., 1986) or in cultures (Rayport and Schacher, 1986). In addition, synaptic growth can be induced by microinjection of cAMP (a second messenger for serotonin) in presynaptic cells (Nazif et al., 1991). The level of cAMP increases upon exposure to serotonin and this in turn increases the active form of protein kinase A (PKA). With repeated stimulation, PKA enters the nucleus and phosphorylates *cAMP-responsive element binding protein*(CREB)-related transcription factors (Bacskai et al., 1993) which act by binding to the *cAMP response elements* (CRE) (see Chapter 7). Present evidence indicates that CREB1 activates the transcription factors involved in LTP whereas CRB2 (the latter not induced by serotonin) is inhibitory.

The synthesis of proteins near synaptic sites (e.g., Aakalu et al., 2001) plays a major role in plasticity (see Jiang and Schuman, 2002). Apparently, the periphery is independent of the cell bodies for this function. As might be expected, the components of the translational machinery have been found in dendrites (e.g., Gardiol ete al., 1999). In agreement with the notion that this machinery plays a role in plasticity, some of the mRNAs present at dendrites are involved in synaptic function (see Jiang and Schuman., 2002). For example, *NR1*-mRNA which codes for a subunit of NMDAR has been found in dendrites. This receptor is needed of LTP and LTD (e.g., Tsien et al., 1996). Similarly, mRNA which codes the α subunit of the

calcium dependent protein kinase II (CaMKII) needed for LTP and mRNA for the *brain-derived neurotrophic factor* (BDNF) and its receptor, TrkB, have been found in post-synaptic locations. Activation of TrkB is needed for LTP. Studies on RNA distribution in the cytoplasm are discussed in Chapter 2 and those of the events accompanying RNA transport at specific cellular sites in Chapter 10.

The mammalian system

As already mentioned hippocampus and the cortex have separate roles. This conclusion is supported by experiments in which one copy of the two genes of coding for α-calcium-calmodulin-dependent protein kinase type II (CAMKII) (see below) is inactivated in mice. The mutation does not interfere with learning, although memory is impaired. The mutation also does not interfere with LTP in the hippocampus or the cortex. However, the LTP decays very rapidly in the cortex (Frankland et al., 2001). These experiments suggest that learning takes place in the hippocampus in pre-existing synapses. However, for the consolidation phase, the neurons of the cortex have to make new connections dependent on hippocampal replay.

Many of the features just discussed for *Aplysia* are to be found in the mammalian systems. Inhibitors of transcription and protein synthesis (e.g., Frey et al., 1996; Nguyen et al., 1994) block long lasting LTP so that it decays as fast as the LTP produced by a brief tetanus. Therefore, synthesis of proteins is thought to play a role in this consolidation (Nguyen et al., 1994). The occurrence in two steps, a short term potentiation and a longer lasting LTP, suggest that a change occurs at the synapse first independent of protein synthesis, then followed by a second effect dependent on protein synthesis. This second effect is necessarily slow, because proteins are synthesized in the cell body and are transported the length of the axon to the synapse only slowly.

Two different kinds of LTP have been recognized (Nicoll and Malenka, 1995). One involves the activation of postsynaptic *N*-methyl-D-aspartate receptors (NMDARs), the other, the mossy fiber LTP, has a main presynaptic component and does not involve NMDARs. The mossy fiber LTP is initiated by a rise in presynaptic Ca^{2+} and requires the activation of protein kinase A and the GTPase Rab3A as shown in knockout mice mutants (Castillo et al., 1997) and in addition, the RIMα, an active zone protein that binds to Rab3A and which is a substrate for protein kinase A (Castillo et al., 2002; Schoch et al., 2002). The active zone corresponds to the part of the cytoplasm from which synaptic vesicles are discharged. Mossy fiber LTP has been recognized in hippocampus, mossy fiber, cerebellar and corticothalamic synapses.

Frey and Morris (1997) examined in hippocampal neurons whether newly synthesized proteins could also consolidate the signal in another synapse of the same neuron. The experiment was carried out as follows. The first synapse was stimulated with repeated tetani. The second synapse was examined to see whether LLTP could be induced by a single tetanus. The wave of protein synthesis induced by the repeated tetani of the first synapse permits consolidation of the LTP of the second synapse. However, it had to be stimulated within 90 minutes of the initial repeated tetani. Therefore, the new proteins are available to all

synapses of the same neuron. The results suggest that only a transient change in gene expression is necessary to provide a prolonged response. Apparently the induction of LTP creates a synaptic specific receptor (called "tag" by Frey and Morris) that accepts the gene product. In the presence of the receptor, the material supplied by the gene leads to consolidation of the synaptic enhancement.

The complex events that lead to LTD or LTP postsynaptically, are initiated by the activation of NMDARs (activated by tetanus) (see Section A, above). It would therefore seem logical to examine changes in behavior of NMDARs or receptor associated proteins accompanying LTP or LTD. The NMDARs are formed by the assembly of NR1 subunits with one or more NR2 subunits (see Hollman and Heinemann, 1994; Nakanishi, 1994) which assemble into a channel responsible for conducting Ca^{2+} into the cell. The molecular size of native NMDA receptors is approximately 605-850 kDa, consistent with the assembly of four to five subunits (e.g., Blahos and Wenthold, 1996). There are several possible NR2 subunits (A to D) whose deployment is developmentally and regionally regulated. The subunits determine the properties of the channel. For example, the NR2B subunits (implicated in LTP and learning, see below) permit a higher amount of Ca^{2+} to enter cells. The NR2 subunits have long carboxy-terminals in the cytoplasm that contain phosphorylative and protein interaction sites (Smart, 1997). The NMDARs initiate the events of synaptic development and plasticity in the central nervous system by allowing the entry of Ca^{2+} (see Ghosh and Greenberg, 1995). The Ca^{2+}-binding protein, calmodulin (see Gnegy, 2000) and calcineurin have been implicated in these mechanisms (see below).

An involvement of NMDARs in LTP and learning is supported by experiments in rats where a drug (D,L-2-amino-5-phosphonopentanoic acid, AP5), acting as an NMDAR antagonist, blocks the induction of LTP and interferes with the rats' ease of finding their way around a maze (Morris et al., 1986). Furthermore, deletion (see Chapter 1) of the NMDAR1 gene in the CA1 pyramidal cells of the hippocampus, produced a mouse strain (Tsien et al., 1996) that had lost the NMDAR-mediated synaptic currents and LTPs in the CA1 synapses. The mice also exhibited impaired spatial memory but unimpaired nonspatial learning. These experiments indicate that the NMDA receptors of the CA1 synapses are needed for the acquisition of spatial memories. More recent experiments lend strong support to the involvement of the NMDA receptors. Transgenic mice that overexpress NR2B show an increase in NMDAR mediated current (Tang et al., 1999). Furthermore, they have and enhanced level of LTP and learning (such as fear conditioning to a shock paired to a sound).

Although NMDARs have an important role in LTD or LTP, the deployment of AMPAR receptors (AMPARs) (see above) is responsible for the post-synaptic changes in excitability. The AMPARs are hetero-oligomers of three subunits in different combinations (GLUR1-GLUR4). In the adult hippocampus, the combinations of AMPAR subunits are GluR1/GluR2 or GluR2/GluR3. GluR4 is expressed mostly in early development. The number of receptors at the cell surface result from a steady state between insertion and removal, the latter via chlathrin dependent endocytosis (see Chapter 9). The receptors are removed or added to synapses depending on whether these are weakened or strengthened (Carroll et al., 1999; Zamanillo et al., 1999; Shi, et al., 1999). A role of these receptors in LTP or LTD is supported by the decrease in the AMPAR during LTD (Carroll et al., 1999) as shown by immunological

microscopic techniques (see Chapter 1). In other experiments, an increase of post-synaptic AMPAR at the synapse accompanying LTP was actually demonstrated (Shi et al., 1999). Neurons of cultured brain slices were transfected with a gene coding for one of the units of the AMPARs, fused to green fluorescent protein (see Chapter 1). Two photons laser scanning microscopy (see Chapter 1) revealed the AMPARs clustering at dendritic spines. After LTP was induced, more AMPARs were inserted at the tips of the spines, supposedly the site of the synapses. In addition, it has been shown (Zamanillo et al., 1999) that the absence of functional AMPARs precludes LTP in at least one kind of neurons. In this study, mice strains were produced lacking one of the four subunits of AMPAR. The other three subunits can form functional receptor. However, LTP cannot be elicited from CA1 neurons generally used in LTP experiments. Since other neurons are able to undergo LTP, it can be argued that the result are the consequence of a reduced supply of AMPARs in the mutant mice. Later work allowed more clear distinctions in hippocampal neurons (Shi et al, 2001). AMPAR GluR1/GluR2 receptors were found to be added to synapses as a function of activity, where GluR1 and PDZ domain proteins interact. These receptors are likely to have a role in plasticity. GluR2/GluR3 receptors at synapses were found to follow a different pattern. They were inserted at post-synaptic membranes that already have AMPARs and replaced existing synaptic receptors continuously, an interaction which required GluR2, NSF and PDZ domain proteins. These receptors were not regulated by activity.

How are the events that occur at the synapse orchestrated? Apparently the calcium/calmodulin-dependent protein kinase II (CaMKII) mediate some of the effects of the glutamate receptors. The sequence of events is initiated by the activation of the NMDAR channels. Ca^{2+} enters the cells via the NMDARs, activating CAMKII . In turn, CAMKII induces the insertion of AMPAR at synapses (Hayashi et al., 2000b) and increases their conductance (Derkach et al., 1999). CaMKII and the NMDAR are bound together (Gardoni et al., 1998).The CAMKII binding to NMDAR permits it to be transferred to synaptic sites where it would be most effective and in remaining in an active configuration (Bayer et al., 2001). CaMKII remains active after the Ca^{2+} concentration returns to a lower level.

The phosphatase *calcineurin* has been implicated in the molecular basis of LTD and LTP (e.g., Lu et al., 1996; Mansuy et al., 1998; Mulkey et al., 1994). This pathway resembles one that activates a number of genes in immunological responses (e.g., Clipstone and Crabtree, 1992; Hoey et al., 1995) (discussed in Chapter 7). In mammalian hippocampal neurons, calcium entry into L-type voltage gated calcium channels is triggered by depolarization of the postsynaptic potential (see Chapter 7) and the increased Ca^{2+} has been shown to activate calcineurin. The calcineurin supposedly activates the transcription factor ARc4/NF-AT3, which is then translocated from the cytoplasm to the nucleus (blocked by the immunosuppressive drugs cyclosporin A and FK506) and initiates NF-AT-dependent transcription (see Beals et al., 1997; Graef et al., 1999). Exit from the nucleus occurs when the Ser/Thr kinase *glycogen synthase kinase-3*(GSK-3) phosphorylates ARc4/NF-AT3 (Graef et al., 1999). These results indicate that gene expression mediated by NF-AT is involved in some manner in LTD and LTP.

The cytoplasmic postsynaptic protein, PSD-95, contains domains that participate in protein-protein

interactions including binding to the carboxy-terminal of the NR2 subunits of the NMDAR (Kornau et al., 1995; Niethammer et al., 1996). For this and other reasons, PSD-95 was thought to have a role in targeting and clustering of the NMDA receptors (e.g., see Kim et al., 1996) and, therefore, potentiation or depression. However, experiments using PSD-95 nul mice (see Chapter 1) challenge this view and suggest a much more subtle role of PSD-95. In PSD-95 nul mice (Migaud et al., 1998) the frequency function of NMDA-dependent LTP and LTD is shifted producing an increase in LTP at different frequencies of synaptic stimulation. The frequency shift is accompanied by severely impaired spatial learning. These findings indicate that PSD-95 does not have a role in the targeting and clustering of NMDA receptors, since these are still functional. However, it plays an important, still to be elucidated role in plasticity. In contrast, the NMDA receptor action has been shown to be intimately involved with the Eph receptors and ephrin system discussed below with other cell adhesion molecules.

As in *Aplysia cAMP-responsive element-binding protein* (CREB) play a role, the involvement of a cAMP-response element (CRE) (see Chapter 7) is suspected in LTP, because CRE-mediated gene expression occurs in hippocampal neurons during repeated tetani (Impey et al., 1996). This was demonstrated in mice transgenic for a CRE-regulated reporter construct (CRE-lacZ, whose expression can be recognized by the activity of β-galactosidase). CRE-mediated gene expression was increased after late-LTP, but not after decremental LTP. The effect required activation of PKA. Inhibitors of PKA blocked late-LTP and associated gene expression (but not decremental LTP). The signaling required for Late-LTP (but not D-LTP) was found sufficient to stimulate CRE-mediated transcription. Furthermore, cAMP is generated at tetanized synapses (Chetkovich and Sweatt, 1993). Mice with a targeted (see Chapter 1) disruptive mutation in the CREB are deficient in long term memory, with normal short term memory and a corresponding deficiency in the induction of the late LTP with repeated tetani in hippocampal slices (Bourtchuladze et al., 1994). In addition, permeable analogs of cAMP produce a non-specific synaptic strengthening that resembles late LTP in hippocampal slices (Frey et al., 1993).

Cell adhesion molecules

Cell adhesion molecules (CAMs) are responsible for cell-cell and cell-ECM interactions (see Chapter 6). At the synapse they produce structural changes induced by long-term nerve activity (see Fields and Itoh, 1996). As we saw, intracellular-signaling systems are generated by nerve activity. These are thought to have effects on CAM expression. In *Aplysia*, for example, the presence of CAM at the surface of a motor neuron is affected by the neuropeptide FMFamide (Peter et al., 1994) and 5-Hydroxytryptamine (Mayford et al., 1992).

CAMs have been shown to play a role in both short term and lasting plasticity (see Benson et al. 2000). Several kinds of CAMs are found at synapses, including integrins, immunoglobulin superfamily members, cadherins and the brain specific neurexins and neuroligins (see Benson et al., 2000). An involvement of CAMs in post-tetanic potentiation of short duration (E-LTP) has been shown on hippocampal slices by applying antibodies against CAMs (e.g., Tang et al., 1998; Yamagata et al., 1999)

or the use of synthetic peptides antagonistic to dimerization of cadherins (e.g., Tang et al., 1999) or other means (e.g., Muller et al., 1996). Similarly, N-cadherin has been shown to have a role in L-LTP. In addition to all these findings, learning and memory are impaired by interfering with cell-adhesion function (e.g., see Grotewiel et al., 1998). Neural cell adhesion molecules (NCAMs) knock-out mice show impaired spatial learning (Cremer et al., 1994)

CAMs of the immunoglobulin superfamily play a role in development of the nervous system but also in synaptic plasticity in the adult. The *receptor-like protein tyrosine phosphatases* (RRTPs) have similar properties and their role in the development of neurons has been demonstrated with *Drosophila* mutants. Three axonal RPTPs were shown to be required for routing and making appropriate neuronal connections (Krueger et al., 1996; Desai et al., 1996). RPTPs may also have a role in plasticity (see Peles et al., 1998).

The role of the NMDA receptors in synaptic development and plasticity have been found to involve the *Eph family of receptor* tyrosine kinases/*ephrin* system. The Eph family of receptor tyrosine kinases at the surface of a cell bind to ephrin ligands attached to membranes of other cells. The two are important in regulating cell-cell interactions. The signaling between the receptor bearing cell and the ephrin bearing cell is bidirectional (Holland et al., 1996) so that information is exchanged between Eph-expressing and the ephrin-expressing cells. It was recognized early that ephrin and its receptors are regulators of axon pathfinding and neuronal cell migration during embryogenesis (e.g., see Flanagan and Vanderhaeghen, 1998). More recently the two have been found to have a role in angiogenesis, tissues such as specialized epithelia (e.g., Frisén et al., 1999) and as outlined below in neuronal plasticity. The crystal structure of the Eph receptor-ephrin complex has been elucidated (Himanen et al., 2001).

The *ephrins* constitute a family of membrane attached ligands. Ephrins and Eph receptor tyrosine kinases are responsible for activity independent processes during development and are involved in synaptic plasticity.(see Klein 2001). Present thought suggests that in the post-synaptic neuron, NMDA activated by glutamate recruit EphB2 receptors which are in turn activated by ephrinB of the pre-synaptic terminals. The two form a bridge between the presynaptic and the postsynaptic neurons (see Klein, 2001). The action of the receptors is independent of their protein kinases activity since a truncated form of EphB2 lacking kinase activity rescued the EphB2 phenotype in null mutants (Grunwald et al, 2001). A function of the ephrins and Eph receptor system in plasticity is indicated by experiments in which mice lacking EphB2 receptor had reduced LTP at hippocampal CA1 and dentate gyrus synapses. In addition, stimuli known to induce changes in synaptic structure increase EphB2 activity (Henderson et al., 2001). The EphB receptor tyrosine kinases are found in clusters at NMDA receptors. In neurons, ephrinB2 activates EphB and facilitates the NMDA receptor-dependent influx of Ca^{2+}. This effect results in the phosphorylation of tyrosines in NMDA receptors by activating the Src family of tyrosine kinases and increases the activation of NMDAR dependent genes (Takasu et al., 2002). The role of this system in actin polymerization accompanying the formation of growth cones and the extension of other processes important in increasing synaptic connections (see next section) is discussed in Chapter 23).

The formation of synapses during development involve signals exchanged between presynaptic and

postsynaptic cells, in both directions. We saw that the ephrin-Eph system operates bidredctionally. In addition, one of these signals is retrograde, from post-synaptic to pre-synaptic cells. *Wnt proteins*, synthesized by the post-synaptic cells have a role in the development of the pre-synaptic processes (Hall et al., 2000). In the cerebellar cortex granule cell of mammals (GC) neurons receive synapses from mossy fibers from other sections of the brain. Cultured GC neurons secrete factors that have profound effects on the structure of mossy fibers (Hall et al., 2000). Conversely, mice with mutations in Wnt-7a exhibit changes in the development of the mossy fibers. The effect is not as pronounced as might be expected, probably because other Wnts are expressed in the cerebellum and may compensate for the defect. A Wnt-7 antagonist blocks the changes and these are mimicked by the presence of Wnt-7a. Cultured GC neurons from mice, release factors affecting mossy fiber axons and growth cones before innervation. Wnts are therefore likely to have a significant role in synapse formation. Synapse formation in relation to plasticity is discussed in the next section. Wnt proteins are a family of sixteen or more cysteine-rich molecules of the extracellular matrix. They have a role in determining cell polarity and embryonic patterning (e.g., see Eastman and Grossdl, 1999) and in synapse formation during development and possibly in plasticity. These proteins bind to integral membrane proteins with seven transmembrane segments, the *Frizaleds proteins*. One of these proteins (GSK3β) is a serine/threonine kinase with multiple targets. GSK3β activity is blocked by binding to Wnt. When active, this kinase phosphorylates microtubular associated proteins and β-catenin. When the kinase is held in check by Wnt, β-catenin is allowed to accumulate. The binding of β-catenin to a family of HMG proteins (LEF-1/TFCs) activates the Wnt target genes. *High-mobility group* (HMG) proteins are nonhistone nuclear proteins that play an important role in the regulation of chromatin structure and function. Catenins are proteins which link cytoskeletal proteins, such as actin, to integral proteins, such as cadherins, which interact with proteins external to the cell (e.g., see Chapter 11).

Increase in synaptic connections

The story of synaptic alterations in memory and learning appears to have another layer of complexity in a variety of organisms. In the marine mollusc *Aplysia*, long term sensitization (for more than three weeks) of the gill withdrawal reflex results in an increase in presynaptic terminals of identified neurons, as seen in serial sections (Bailey and Chen, 1988). New dendritic filapodial extensions or *spines* have been observed in chicks after learning an avoidance task (Patel et al., 1988), in rats after spatial learning (Moser et al., 1994) and after the induction of LTP in anesthetized rats (Andersen et al., 1996). Dendritic spines are the only known postsynaptic connections at least in the excitatory hippocampal pathway (Andersen et al., 1966).

The synapse, or more precisely neurotransmitter receptors are also likely to be the target of hormones (see Wehling, 1997) with possible effects on behavior and learning. Inhibitory and excitatory effects of progesterone on receptors of neurotransmitters have been reported (Valera et al., 1992).

Some neurons receive thousands of synaptic inputs through their dendrites. It is well recognized that Na^+ and Ca^{2+} channels (e.g., Johnston et al., 1996; Yute and Tank, 1996) and more recently K^+ (Hoffman et

al., 1997) are present at dendrites. Accordingly, they are capable of modifying postsynaptic responses and they are likely to play an important role in the propagation and integration of information.

How do connections between neurons come about? This question is of great significance not only in memory and learning, but also in the development of the nervous system and in its regeneration. The electrical activity and its temporal and spatial characteristics, shape the synaptic connections in early development (see Katz and Shatz, 1996). Similarly, memory is likely to be connected to similar morphological correlates of activity (see Bailey and Kandel, 1993).

Living dissociated neurons in culture exhibit motility at the growing tips (growth cones) and filopodia (see Chapter 23, Section IV C) that can initiate both cell-to-cell contact and the formation of synapses (e.g., Ziv and Smith, 1996, Fischer et al., 1998). This motility may be regulated by neurotransmitters and second messengers (Kater and Rehder, 1995).

The use of fluorescent dyes and confocal microscopy (see Chapter 1) on tissue slices confirm the results in cell culture and show extension and retraction of filopodia in whole embryos and brain tissues regulated by electrical activity or the presence of neurotransmitters (e.g., Dailey and Smith, 1996). In a recent study (Maletic-Savatic et al., 1999), neurons in developing rat hippocampal slices were transfected with a virus containing the green fluorescent protein (GFP) gene (Chapter 1, Section II B). The fluorescence was followed using a two-photon laser scanning microscope (see Chapter 1). The results confirm the presence of filopodial protrusions in dendrites and demonstrated that firing action potentials increases filopodia formation (or spines) and probably formation of synapses (as seen with the light microscope). This activity was blocked by an antagonist for NMDA receptors (APV), implicating the latter (see Section A, above). A similar study was carried out on the developing pyramidal neurons of rats where neuronal filapodia in the postsynaptic neurons form connections which depend on sensory experience. The filopodia appeared, disappeared or changed shape rapidly (minutes) (Lendvai et al., 2000). Similar results were obtained by Engert and Bonhoffer (1999) using thin slices cultured for several days. The neurons were traced after the microinjection of a fluorescent dye. An electron microscope study was carried out with mouse hippocampal slices in organotypic cultures after high-frequency stimulation (Toni et al., 1999). The study found transient remodelling of the postsynaptic membrane, followed by an increase in the proportion of axon terminals contacting two or more dendritic spines. Pharmacological blockade of LTP prevented these morphological changes.

Calcium-calmodulin-dependent protein kinase II (CaMKII) plays a role in the maintenance of the synaptic architecture (see also below). This is not surprising since CAMKII is intimately involved in synaptic transmission (see above) and CAMKII is present at excitatory synapses (e.g., Lisman et al., 1997; Braun and Schulman, 1996). CaMKII overexpression (e.g., by transfection) stabilizes dendritic structure in maturing neurons. In contrast, CaMKII inhibition increases their dendritic growth (Wu and Cline, 1998). CAMKII is required for LTP (Kennedy, 1988; Lisman, 1994). The incorporation of GluR1-containing AMPARs (see above) into synapses, seems to involved in the plasticity produced by CAMKII and LTP

(Hayashi et al., 2000). However, the target of CAMKII activity involved in LTP is still to be identified.

As indicated in the next section, growth factors (neurotrophins) are essential for the proper functioning of neuronal networks (see Section VI). Indeed, neurotrophins can regulate the morphology of nerve processes (e.g., Ming et al., 1997) and the efficiency of the transmission across synapses (e.g., Sala et al., 1998; Gottschalk et al., 1998). Electrical activity can regulate the synthesis (e.g., Funakoshi et al., 1995) and release (e.g., Wang and Poo, 1997) of neurotrophins. Current evidence indicates that *brain-derived neurotrophic factor* (BDNF), is transmitted from the pre-to the postsynaptic cells (e.g., Fawcett et al., 2000). Some of the evidence is direct. In these experiments, cDNAs of green fluorescent protein (GFP)-tagged BDNF and red fluorescence protein were microinjected into the nucleus of single neurons (Kohara et al., 2001) . BDNF was found to move in the anterograde and to some extent in the retrograde direction. A transfer of the GFP-BDNF to postsynaptic neurons was found to be activity dependent and probably represents a secretion. In contrast the red-fluorescence protein was not transferred postsynaptically indicating that the translocation of the GFP-BDNF is specific.These observations have obvious implications for neuronal plasticity (see McAllister et al., 1999). Other observations strengthen this conclusion. LTP is reduced in the hippocampus when *brain-derived neurophic factor* (BDNF) is depleted (e.g., Kang et al., 1997) or in BNDF-knockout mice (e.g., Korte et al., 1995) and the deficit is rescued by exogenous BDNF (Patterson et al., 1996). These observation suggest that neurotrophins mediate at least part of the mechanism of synaptic plasticity (e.g., see Berninger and Poo, 1996; Bonhoeffer, 1996). The neurotrophic activity and the facilitation of BDNF potentiation by presynaptic activity was found to be inhibited by cyclic AMP (cAMP) inhibitors (see Chapter 7), suggesting a possible pathway for the action of BDNF (Boulanger and Poo, 1999). cAMP can trigger many processes including transcription of mRNA for receptors and neurotrophins (see Condorelli et al., 1994) and has been implicated in LTP and LTD in other experiments (see discussion above). An involvement of NMDA receptors in the production of BDNF has been discussed above (see above).

Surprisingly, BDNF and neurotrophin-4/5 delivered to cell bodies of various types of neurons have been found to elicit action potentials as rapidly as the neurotransmitter glutamate (Kafitz et al., 1999). The depolarization is the consequence of the opening of sodium channels and the conductance is blocked by a protein kinase blocker of tyrosine kinase Trk receptors. Trk kinase receptors are the signaling receptors for neurotrophins (see Barbacid, 1995; see Chapter 7). These observations suggest a possible positive reinforcement mechanism between neuronal activity, increased neurotrophin secretion, followed by increased neuronal activity.

A role of glial cells in the formation of synapses, at least in cell culture, suggests an involvement of these cells in the plasticity of neurons. Glial astrocytes are present at synapses throughout the central nervous system. They serve as support and in the removal of ions and neurotransmitters. In the presence of glial cells, central nervous system neurons were found to produce functional synapses seven times as frequently as in their absence (Ullian et al., 2001). These findings indicate a possible role of glia in the development of the nervous system as well as in learning and memory.

A plasticity possibly related to all the events described in this section is the apoptotic elimination of some neurons during fetal or early neonatal development (see Chapter 2). At least in the fetal or early neonatal development of rats, pharmacological blockade of NMDA glutamate receptors for only a few hours triggered apoptosis of neurons suggesting that glutamate stimulation of NMDA receptors controls neuronal survival (Ikonomidou et al., 1999).

It is now clear that new neurons can be formed in adult mammals at least in regions involved in smell and memory (e.g., see Kuhn et al., 1996). The possibility that the production of new neurons may be involved in plasticity has to be seriously considered. There is also some evidence that new neurons are formed in the hippocampus for animals to be able to associate stimuli that are separated in time (*trace conditioning*) (Shors et al., 2001). In this study, neuronal divisions were estimated through the incorporation of bromodeoxyuridine into DNA (which requires cell division). Bromodeoxyuridine incorporation was detected using fluorescent microscopy and immunological techniques (see Chapter 1). Treatment with an inhibitor of neurogenesis was found to block trace conditioning but not other forms of learning.

VII. NEUROTROPHINS

We briefly discussed the role of neurotrophin in LTP (see above. In addition, there are interactions between neurons or neurons and their target cells that areessential for their maintenance of function. Neurons degenerate when their target cellsare damaged. Similarly, post-synaptic neurons degenerate when their presynaptic neuronsare damaged. What mechanisms could possible explain these effects? Neurotrophins play thecentral role in these interactions. Neurotrophins are growth factors that promote survivaland development of many kinds of neurons. They act on presynaptic neurons by retrogradesignaling by the post-synaptic cell (that can be a post-synaptic neuron). This is shown,for example, in <a href=" 1part3.html#homo">null mutations unable to synthesize the neurotrophin, or when the target cell is destroyed. Both cases result in losses of presynaptic cells (e.g., Jones et al., 1994). However, adding neurotrophins blocks this effect.

A less understood effect is anterograde degeneration. The destruction of a presynaptic cell or cutting its axon results in a degeneration in the postsynaptic neuron. That a similar mechanism is operative is indicated by the prevention of anterograde degeneration by neurotrophins (see Cowan and Clarke, 1970). However, until recently, there was little known about the details of this process.

Altar et al., 1997 showed with an immunological method (see Chapter 1) that the neurotrophin, *brain-derived neurotrophic factor* (BDNF), is present in neuronal terminals and, furthermore, it is present in parts of the brain that lack BDNF-mRNA but are innervated by neurons that produce both BDNF and BDNF-mRNA. In addition, interference with axonal transport prevented the presence of the BDNF in the post-synaptic cells (in the striatum) and accumulated the neurotrophin in the cell bodies of the presynaptic neurons (cortical neurons).

Obviously, the targeting of neurotrophins plays a very significant role in the maintenance of the nervous

system.

Neurons and glia cells are closely associated in the nervous system of vertebrates. Glial cells surround neurons and have a role not only in providing insulation and a controlled environment, but also in neuronal signaling and plasticity (see e.g., Barres, 1991; Pfreiger and Barres, 1997). There are many indications that there is an interaction between glial cells and the synaptic region of neurons (e.g., Keyser and Pellmar, 1994). In co-culture, glia doubled the number of synapses as seen with the EM. At the same time, glia cells were responsible for increasing spontaneous postsynaptic electrical activity. Some of these effects could be reproduced in purified retinal ganglion cells in culture exposed to medium previously conditioned by glial cells (Pfreiger and Barres, 1997). These observations may indicate the secretion of neurotrophins by the glial cells. The secretion of *neurexins* by glia may fulfill some of this role. Neurexins are neuronal cell surface proteins encoded in *Drosophila* by the gene, *axo*. First secreted by glial cells, they are subsequently transferred to axons. Temperature sensitive null mutations (Yuan and Ganetzy, 1999) caused a blockade in axonal conduction.

Much is still to be learned about these fascinating topics.

SUGGESTED READING

General

Kandel, E.R., and Schwartz, J.H. (1985) *Principles of Neural Science*, 2d ed., Chapters 2-8, pp. 14-90. Elsevier, New York.

Kuffler, S.W., Nicholls, J.G., and Martin. A.R. (1984). *From Neuron to Brain: A Cellular Approach to the Function of the Nervous system*. Chapters 4-7 and 9, pp. 99-186 and 207-240, Sinauer Associates, Sunderland, Mass.

McGeer, P.L., Eccles, J.C., and McGeer, E.G. (1987) *Molecular Biology of the Mammalian Brain*, 2d ed. Chapters 1-5, pp. 1-174, Plenum, New York.

Shepherd, G.M. (1988) *Neurobiology*, 2d ed., Chapters 1-8, pp. 1-176. Oxford Univ. Press, New York.

Channels

Aidley, D.J. and Stanfield, P.R. (1996) *Ion Channels. Molecules in Action*, Cambridge University Press, 307 pp. Chapters 2-6.

Catterall. W. A. (1988) Structure and function of voltage-sensitive ion channels. *Science* 242:50-61. (Medline)

Hille, B. (1989) Voltage-gated sodium channels since 1952, in *Ion Transport* (Keeling, D. and Benham, C., eds.). pp. 57-71, Academic Press. New York.

Hille. B. (1992) *Ionic Channels of Excitable Membranes*, 2d ed., Chapters 1-6, Sinauer Associates Inc., Sunderland, MA.

Montal, M. (1990) Molecular anatomy and molecular design of channel proteins, *FASEB J.* 4:2623-2635. (Medline)

Synaptic Vesicles, Exocytosis, Endocytosis

Brodin, L., Low, P. and Shupliakov, O. (2000) Sequential steps in clathrin-mediated synaptic vesicle endocytosis, *Curr. Opin. Neurobiol.* 10:312-320. (MedLine)

Greengard, P., Valtorta, F., Czernik, A.J. and Benfenati, F. (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function, *Science* 259: 780-785. (Medline)

Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, Geramanos, S., Tempst, P. and Rothman, J.E. (1993a), SNAP receptors implicated in vesicle targeting and fusion, *Nature* 362: 318-324. (Medline)

Neurons, Synapses and Plasticity

Bailey, C.H., Bartsch, D. and Kandel, E.R. (1996) Toward a molecular definition of long-term memory storage, *Proc. Natl. Acad. Sci. USA* 93:13445-13552. (MedLine)

Cohen-Cory, S. (2002) The developing synapse: construction and modulation of synaptic structures and circuits, *Science* 298:770-776. (MedLine)

Gnegy, M.E. (2000) Ca^{2+}/calmodulin signaling in NMDA-induced synaptic plasticity, *Crit. Rev. Neurobiol.* 14:91-129. (MedLine)

Jiang, C. and Schuman, E. (2002) Regulation and function of local protein synthesis in neuronal dendrites, *Trends Biochem. Sci.* 27:506-513. (MedLine)

Klein, R. (2001) Excitatory Eph receptors and adhesive ephrin ligands, *Curr. Opin. Cell Biol.* 13:196-203. (MedLine)

Levitan, I.B. and Kaczamarek, L.K. (1997) *Neuron. Molecular Biology*, Oxford University Press, 543 pp. Chapters III and IV.

Sheng, M. and Kim, M.J. (2002) Postsynaptic signaling and plasticity mechanisms, *Science* 298:776-780. (MedLine)

REFERENCES

Search the textbook

Back to Chapter 22

REFERENCES

Aakalu, G., Smith, W.B., Nguyen, N., Jiang, C. and Schuman, E.M. (2001) Dynamic visualization of local protein synthesis in hippocampal neurons, *Neuron* 30:489-502. (MedLine)

Abbott, G.W., Butler, M.H., Bendahhou, S., Dalakas, M.C., Ptacek, L.J. and Goldstein, S.A. (2001) MiRP2 forms potassium channels in skeletal muscle with Kv3.4 and is associated with periodic paralysis, *Cell* 104:217-231. (MedLine)

Adler, E.M., Augustine, G.J., Duffy, S.N. and Charlton, M.P. (1991) Alien intracellular calcium chelators attenuate neurotransmitter release at the squid giant synapse, J. Neurosci. 11: 1496-1507. (MedLine)

Aldrich, R.W. (1986) Voltage-dependent gating of sodium channels: towards an integrated approach, *Trends Neurosci.* 9:82-86.

Aldrich, R. W., Corey, D. P., and Stevens, C. F. (1983) A reinterpretation of mammalian sodium channel gating based on single channel recording, *Nature* 306:438-441. (MedLine)

Altar, C.A., Cai, N., Bilven, T., Juhasz, M., Conner, J.M., Acheson, A.L., Lindsay, R.M. and Wiegand, S.J. (1997) Anterograde transport of brain-derived neurotrophic factor and its role in the brain, *Nature* 389:856-860. (MedLine)

Alvarez de Toledo, G., Fernandez-Chacon, R., and Fernandez, J.M. (1993) Release of secretory products during transient vesicle fusion, *Nature* 363:554-558.

Andersen, P., Blackstad, T.W. and Lomo, T. (1966) Location and identification of excitatory synapses on hippocampal pyramidal cells, *Exp. Brain Res.* 1:236-248. (MedLine)

Andersen, P., Moser, E., Moser, M.B. and Trommald, M. (1996) Cellular correlates to spatial learning in the rat hippocampus, *J. Physiol., Paris* 90:349. (MedLine)

Artalejo, C.R., Henley, J.R., McNiven, M.A. and Palfrey, H.C. (1995) Rapid endocytosis coupled to exocytosis in adrenal chromaffin cells involves Ca^{2+}, GTP, and dynamin but not clathrin, *Proc. Natl. Acad. Sci. USA* 92:8328-8332. (MedLine)

Auer, M., Scarborough, G.A. and Kühlbrandt, W. (1998) Three-dimensional map of the plasma membrane H^+-ATPase in the open conformation, *Nature* 392:840-843. (MedLine)

Auerback, A. (1972) Transmitter release at chemical synapses. In *Structure and Function of Synapses* (Pappas, G. D., and Purpura D.P., eds.), pp. 137-159. Raven, New York.

Avery, J., Ellis, D.J., Lang, T., Holroyd, P., Riedel, D., Henderson, R.M., Edwardson, J.M. and Jahn, R. (2000) A cell-free system for regulated exocytosis in PC12 cells, *J. Cell Biol.* 148:317-324. (MedLine)

Bacskai, B.J., Hochner, B., Mahaut-Smith, M., Adams, S.R., Kaang, B.K., Kandel, E.R. amd Tsien, R.Y. (1993) Spatially resolved dynamics of cAMP and protein kinase A subunits in *Aplysia* sensory neurons, *Science* 260:222-226. (MedLine)

Bailey, C.H. and Kandel, E.R. (1993) Structural changes accompanying memory storage, *Annu. Rev. Physiol.* 55:397-426. (MedLine)

Bailey, C.H. and Chen, M. (1988) Long-term memory in *Aplysia* modulates the total number of varicosities of single identified neurons, *Proc. Natl. Acad. Sci. USA* 85:2373-2377. (MedLine)

Bailey, C.H., Bartsch, D. and Kandel, E.R. (1996) Toward a molecular definition of long-term memory storage, *Proc. Natl. Acad. Sci. USA* 93:13445-13452. (MedLine)

Baines, A.J. and Bennett, V. (1985) Synapsin I is a spectrin-binding protein immunologically related to erythrocyte protein 4.1, *Nature* 315: 410-413. (MedLine)

Baines, A.J. and Bennett, V. (1986) Synapsin I is a microtubule-bundling protein, *Nature* 319: 145-147. (MedLine)

Bahler, M. and Greengard, P. (1987) Synapsin I bundles F-actin in a phosphorylation dependent manner, *Nature* 326:704-707. (MedLine)

Barbacid, M. (1995) Neurotrophic factors and their receptors, *Curr. Opin. Cell. Biol.* 7:148-155. (MedLine)

Barnard, E.A., Darlison, M.G. and Seeburg, P. (1987) Molecular bioogy of the $GABA_A$ receptor/channel superfamily, *Trends in Neuroscie.* 10:502-508.

Barres, B.A. (1991) New roles for glia, *J. Neurosci.* 11:3685-3694. (MedLine)

Bayer, K.U., De Koninck, P., Leonard, A.S., Hell, J.W. and Schulman, H. (2001) Interaction with the

NMDA receptor locks CaMKII in an active conformation, *Nature* 411:801-805. (MedLine)

Beals, C.R., Clipstone, N.A., Ho, S.N. and Crabtree, G.R. (1997) Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction, *Genes Dev.* 11:824-834. (MedLine)

Bear, M.F. and Malenka, R.C. (1994) Synaptic plasticity: LTP and LTD, *Curr. Opin. Neurobiol.* 4:389-399. (MedLine)

Benfenati, F., Bähler, M., Jahn, R. and Greengard, P. (1989a) Interactions of synapsin I with small synaptic vesicles: distinct sites in synapsin I bind to vesicle phospholipids and vesicle proteins, *J. Cell Biol.* 108: 1863-1972. (MedLine)

Benfenati, F., Greengard, P.Brunner, J. and Bähler, M. (1989b) Electrostatic and hydrophobic interactions of synapsin I and synapsin I fragments with phospholipid bilayers, *J. Cell Biol.* 108: 1851-1862. (MedLine)

Benfenati, F., Valtorta, F. and Greengard, P. (1991) Computer modeling of synapsin I binding to synaptic vesicles and F-actin: implications for the regulation of neurotransmitter release, *Proc. Natl. Acad. Sci. USA* 88: 575-579. (MedLine)

Benfenati, F., Valtorta, F., Rubenstein, J.L., Gorelick, F.S., Greengard, P. and Czernick, A.J. (1992) Synaptic-vesicle associated Ca^{2+}-calmodulin-dependent protein kinase II is a binding protein for synapsin I, *Nature* 359: 417-420. (MedLine)

Bennett, M. V. L., Nakajima, Y., and Pappas, G. D. (1967) Physiology and ultrastructure of electrotonic junctions. I. Supramedullary neurons, *J. Neurophysiol.* 30:161-179. (MedLine)

Benson, D.L., Schnapp, L.M., Shapiro, L. and Huntley, G.W. (2000) Making memories stick: cell-adhesion molecules in synaptic plasticity, *Trends Cell Biol.* 10:473-482. (MedLine)

Berninger, B.and Poo, M.-m. (1996) Fast actions of neurotrophic factors, *Curr. Opin. Neurobiol.* 6:324-330. (MedLine)

Bettler, B. and Mule, C. (1995) Review; neurotrasmitter receptors II, AMPA and kainate receptors, *Neuropharm.* 34:123-139. (MedLine)

Blahos, J. and Wenthold, R.J. (1996) Relationship between N-methyl-D-aspartate receptor NR1 splice variants and NR2 subunits, *J. Biol. Chem.* 271:15669-15674. (MedLine)

Bliss, T.V.P. and Collingridge, G.L. (1993) A synaptic model of memory-long term potentiation in the hippocampus, *Nature* 361:31-39. (MedLine)

Bockenhauer, D., Zilberberg, N. and Goldstein, S.A.N. (2001) KCNK2: reversible convesion of a hippocampal potassium leak into a voltage-dependent channel, *Nature Neuroscie.* 4:486-491. (MedLine).

Bonhoeffer, T. (1996) Neurotrophins and activity-dependent development of the neocortex, *Curr. Opin. Neurobiol.* 6:119-126. (MedLine)

Borges, S., Gleason, E., Turelli, M. and Wilson, M. (1995) The kinetics of quantal transmitter release from retinal amacrine cells, *Proc. Natl. Acad. Sci. USA* 92:6896-6900. (MedLine)

Boulanger, L. and Poo M.-m. (1999) Gating of BDNF-induced synaptic potentiation by cAMP, *Science* 284:1982-1984. (MedLine)

Bourtchuladze R., Frenguelli, B., Blendy, J., Cioffi, D., Schulz, G. and Silver, A.J. (1994) Deficient long-term memory in mice with a targeted mutation of cAMP-responsive element-binding protein, *Cell* 79:59-68. (MedLine)

Braun, A.P. and Schulman, H. (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function, *Annu. Rev. Physiol.* 57:417-445. (MedLine)

Breckenridge, L.J. and Almers, W. (1987) Currents through the fusion pore that forms during exocytosis of a secretory vesicle, *Nature* 328:814-817. (MedLine)

Buonomano, D.V. and Merzenich, M.M. (1998) Cortical plasticity: from synapses to maps, *Annu. Rev. Neurosci.* 21:149-186. (MedLine)

Carroll, R.C., Lissin, D.V., von Zastrow, M., Nicoll. R.A. and Malenka, R.C. (1999) Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures, *Nature Neurosci.* 2:454-460. (MedLine)

Castellucci, V.F., Blumenfeld, H., Goelet, P. and Kandel, E.R. (1989) Inhibitor of protein synthesis blocks long-term behavioral sensitization in the isolated gill-withdrawal reflex of *Aplysia*, *J. Neurobiol.* 20:1-9. (MedLine)

Castillo, P.E., Janz, R., Sudhof, T.C., Tzounopoulos, T., Malenka. R.C. and Nicoll, R.A. (1997) Rab3A is essential for mossy fibre long-term potentiation in the hippocampus, *Nature* 388:590-593. (MedLine)

Castillo, P.E., Schoch, S., Schmitz, F., Südhof, T.C. and Malenka, R.C. (2002) RIM1α is required for

presynaptic long-term potentiation, *Nature* 415:327-330. (MedLine)

Catterall, W.A. (2000) From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels, *Neuron* 26:13-25. (MedLine)

Chetkovich, D.M. and Sweatt, J.D. (1993) NMDA receptor activation increases cyclic AMP in area CA1 of the hippocampus via calcium/calmodulin stimulation of adenyl cyclase, *J. Neurochem.* 61:1933-1942. (MedLine)

Chow, R.H., von Ruden, L. and Neher, E. (1992) Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells, *Nature* 356:60-63. (MedLine)

Clipstone, N.A. and Crabtree, G.R. (1992) Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation, *Nature* 357:695-697. (MedLine)

Cole, K. S., and Curtis, H. J. (1938) Electrical impedence of *Nitella* during activity, *J. Gen. Physiol.* 22:37-64.

Cole, K. S., and Curtis, H. J. (1939) Electrical impedence of squid giant axon during activity, *J. Gen. Physiol.* 22:649-670.

Colledge, M. and Froehner, S.C. Signals mediating ion channel clustering at the neuromuscular junction, *Curr. Opin. Neurobiol.* 8:357-363. (MedLine)

Collins, E. W., Jr., and Edwards, C. (1971) Role of Donnan equilibrium in the resting potentials in glycerol extracted muscle, *Am. J. Physiol*. 221:1130-1133. (MedLine)

Condorelli, D.F., Dell'Albani, P., Mudo, G., Timmusk, T. and Belluardo, N. (1994) Expression of neurotrophins and their receptors in primary astroglial cultures: induction by cyclic AMP-elevating agents, *J. Neurochem.* 63:509-516. (MedLine)

Conti, F., and Neher, E. (1980) Single channel recording of K currents in squid axons, *Nature* 285:140-143. (MedLine)

Conway, E. J. (1957) Nature and significance of concentration relations of K^+ and Na^+ ions in skeletal muscle, *Physiol. Rev.* 37:84-132.

Cowan, W.M. and Clarke, P.G.H. (1970) *Anterograde and Retrograde Degeneration in the Central and Peripheral Nervous System*, 217-251, Springer, New York.

Craxton, M. and Goedert M. (1999) Alternative splicing of synaptotagmins involving transmembrane

exon skipping, *FEBS Lett.* 460:417-422. (MedLine)

Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P., Scheff, S., Barthels, D., Rajewsky, K. and Wille, W. (1994) Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning, *Nature* 367:455-459. (MedLine)

Cremona,O. and De Camilli, P. (1997) Synaptic vesicle endocytosis, *Curr. Opin. Neurobiol.* 7: 323-330. (MedLine)

Dailey, M.E. and Smith, S.J. (1996) The dynamics of dendritic structure in developing hippocampal slices, *J. Neurosci.* 16:2983-2994. (MedLine)

Davis, H.P. and Squire, L.R. (1984) Protein synthesis and memory: a review, *Psychol. Bull.* 96:518-559. (MedLine)

Derkach, V., Barria, A. and Soderling, T.R. (1999) Ca^{2+}/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors, *Proc. Natl. Acad. Sci. USA* 96:3269-3274. (MedLine)

Desai, C.J., Gindhart, J.G. Jr., Goldstein, L.S. and Zinn, K. (1996) Receptor tyrosine phosphatases are requires for motor axon guidance in Drosophila embryos, *Cell* 84:599-609. (MedLine)

Doyle, D.A., Morais Cabral, J., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T. and MacKinnon, R. (1998) The structure of the potassium channel: molecular basis of K^+ conduction and selectivity, *Science* 280:69-77. (MedLine)

Eastman, Q. and Grosschedl, R. (1999) Regulation of LEF-1/TCF transcription factors by Wnt and other signals, *Curr. Opin. Cell Biol.* 11:233-240. (MedLine)

Fatt, P., and Katz, B. (1952) Spontaneous subthreshold activity of motor nerve endings, *J. Physiol (London)* 117:109-128.

Fawcett, J.P., Alonso-Vanegas, M.A., Morris, S.J., Miller, F.D., Sadikot, A.F. and Murphy, R.A. (2000) Evidence that brain-derived neurotrophic factor from presynaptic nerve terminals regulates the phenotype of calbindin-containing neurons in the lateral septum, *J. Neurosci.* 20:274-282. (MedLine)

Feng, T.P. (1941) Studies on the neuromuscular junction.XXVI. The changes of end-plate potential during and after prolonged stimulation, *Chin. J. Physiol.* 16:341-372.

Feng, G., Tintrup, H., Kirsch, J., Nichol, M.C., Kuhse, J., Betz, H. and Sanes, J.R. (1998) Dual requirement for gephyrin in glycine receptor clustering and molybdoenzyme activity, *Science* 282:1321-1324. (MedLine)

Fernandez, J.M., Neher, E. and Gomperts, B.D. (1984) Capacitance measurements reveal stepwise fusion events in degranulating mast cells, *Nature* 312:453-455. (MedLine)

Fesce, R., Grohovaz, F., Valtorta, F. and Meldolesi, J. (1994) Neurotransmitter release: fusion or kiss and run?, *Trends Cell Biol.* 4:1-4.

Fields, R.D. and Ito, K. (1996) Neural adhesion molecules in activity-dependent development and synaptic plasticity, *Trends Neurosci.* 19:473-480. (MedLine)

Finnerty, G.T., Roberts, L.S. and Connors, B.W. (1999) Sensory experience modifies the short-term dynamics of neocortical synapses, *Nature* 400:367-371. (MedLine)

Fiorillo, C.D. and Williams, J.T. (1998) Glutamate mediates an inhibitory postsynaptic potential in dopamine neurons, *Nature* 394:78-84. (MedLine)

Fischer, M., Kaech, S., Knutti, D. and Matus, A. (1998) Rapid actin-based plasticity in dendritic spines, *Neuron* 20:847-854. (MedLine)

Flanagan, J.G. and Vanderhaeghen, P. (1998) The ephrins and Eph receptors in neural development, *Annu. Rev. Neurosci.* 21:309-345. (MedLine)

Fordac, M. (1992) Structure, function and regulation of the coated vesicle V-ATPase, *J. Exp. Biol.* 172: 155-169.

Frankenhaeuser, B., and Moore, L. E. (1963) The effect of temperature on the sodium and potassium permeability changes in myelinated nerve fibers of *Xenopus laevis*, *J. Physiol. (London)* 169:431-437.

Frankland, P.W., O'Brien, C., Ohno, M., Kirkwood, A. and Silva, A.J. (2001) α-CaMKII-dependent plasticity in the cortex is required for permanent memory, *Nature* 411:309-313. (MedLine)

Frey, U. and Morris, R.G.M (1997) Synaptic tagging and long term-potentiation, *Nature* 385:533-536. (MedLine)

Frey, U., Huang, Y.-Y., and Kandel, E.R. (1993) Effects of cAMP stimulate a late stage of LTP hippocampal CA1 neurons, *Science* 260:1661-1664. (MedLine)

Frey, U., Frey, S., Schollmeier, F. and Krug, M. (1996) Influence of actinomycin D, a RNA synthesis inhibitor, on long-term potentiation in rat hippocampal neurons *in vivo* and *in vitro*, *J. Physiol.* 490:703-711. (MedLine)

Frisén, J., Holmberg, J. and Barbacid, M. (1999) Ephrins and their Eph receptors: multitalented directors of embryonic development, *EMBO J.* 18:5159-5165. (MedLine)

Funakoshi, H., Belluardo, N., Arenas, E., Yamamoto, Y., Casabona, A., Persson, H. and Ibáñez, C.F. (1995) Muscle-derived neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons, *Science* 268:1495-1499. (MedLine)

Gaffey, C. T., and Mullins, L. J. (1958) Ion fluxes during the action potential in *Chara*, *J. Physiol.* (London) 144:505-524.

Gardiol, A., Racca, C. and Triller A. (1999) Dendritic and postsynaptic protein synthetic machinery, *J. Neurosci.* 19:168-179. (MedLine)

Gardoni, F., Caputi, A., Cimino, M., Pastorino, L., Cattabeni, F. and Di Luca, M. (1998) Calcium/calmodulin-dependent protein kinase II is associated with NR2A/B subunits of NMDA receptor in postsynaptic densities, *J. Neurochem* 71:1733-1741. (MedLine)

Garthwaite, J., Charles, S.J. and Chess-Williams, R. (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain, *Nature* 336:385-388. (MedLine)

Gerschenfeld, H. M., Ascher, P., and Tauc, L. (1967) Two different excitatory transmitters acting on a single molluscan neurone, *Nature* 213:358-359. (MedLine)

Ghosh, A. and Greenberg, M.E. (1995) Calcium signaling in neurons: molecular mechanisms and cellular consequences, *Science* 268:239-247. (MedLine)

Glanzman, D.L., Mackey, S.L., Hawkins, R.D., Dyke, A.M., Lloyd, P.E. and Kandel, E.R. (1989) Depletion of serotonin in the nervous system of *Aplysia* reduces the behavioral enhancement of gill withdrawal as well as the heterosynaptic facilitation produced by tail shock, *J. Neurosci.* 9:4200-4213. (MedLine)

Gnegy, M.E. (2000) Ca^{2+}/calmodulin signaling in NMDA-induced synaptic plasticity, *Crit. Rev. Neurobiol.* 14:91-129. (MedLine)

Goldenring, J.R.. Lasher, R.S., Vallano, M.L., Ueda, T., Naito, S., Sternberger, N.H., Stenberger, L.A. and DeLorenzo, R.J. (1986) Association of synapsin I with neuronal cytoskeleton. Identification of

cytoskeletal preparations *in vitro* and immunocytochemical localization in brain of synapsin I, *J. Biol. Chem.* 261: 8495-8504. (MedLine)

Goldin, A.L., Barchi, R.L., Caldwell, J.H., Hofmann, F., Howe, J.R., Hunter, J.C., Kallen, R.G., Mandel, G., Meisler, M.H., Netter, Y.B., Noda, M., Tamkun, M.M., Waxman, S.G., Wood, J.N. and Catterall, W.A. (2000) Nomenclature of voltage-gated sodium channels, *Neuron* 28:365-368.

Goldstein, S.A, Bockenhauer, D., O'Kelly, I. and Zilberberg, N. (2001) Potassium leak channels and the KCNK family of two-P-domain subunits, *Nature Rev. Neurosci.* 2:175-184. (MedLine)

Gonzalez-Gaitan, M. and Jackle, H. (1997) Role of Drosophila α-adaptin in presynaptic vesicle recycling, *Cell* 88:767-776. (MedLine)

Gotow, T., Miyaguchi, K. and Hashimoto, P.H. (1991) Cytoplasmic architecture of the axon terminal: filamentous strands specifically associated with synaptic vesicles, *Neuroscience* 40: 587-598. (MedLine)

Gottschalk, W., Pozzo-Miller, L.D., Figurov, A. and Lu, B. (1998) Presynaptic modulation of synaptic transmission and plasticity by brain-derived neurotrophic factor in the developing hippocampus, *J. Neurosci.* 18:6830-6839. (MedLine)

Govindan, B., Bowser, R. and Novick, P. (1995) The role of Myo2, a yeast class V myosin, in vesicular transport, *J. Cell Biol.* 128:1055-1068. (MedLine)

Graef, I.A., Mermelstein, P.G., Stankunas, K., Neilson, J.R., Deisseroth, K., Tsien, R.W. and Crabtree GR (1999) L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons, *Nature* 401:703-708. (MedLine)

Greengard, P., Valtorta, F., Czernik, A.J. and Benfenati, F. (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function, *Science* 259: 780-785. (MedLine)

Gribkoff, V.K., Starrett, J.E. Jr. and Dworetzky, S.I. (2001) Maxi-K potassium channels: form, function, and modulation of a class of endogenous regulators of intracellular calcium, *Neuroscientist* 7:166-177. (MedLine)

Grotewiel, M.S., Beck, C.D., Wu, K.H., Zhu, X.R., Davis, R.L. (1998) Integrin-mediated short-term memory in *Drosophila*, *Nature* 391:455-460. (MedLine)

Grundfest, H, and Nachmansohn, D. (1950) Increased sodium entry into squid giant axons during activities at high frequencies and during reversible inactivation of cholinesterase, *Fed. Proc. Fed Amer, Soc. Exp. Biol*, 9:53.

Grunwald, I.C., Korte, M., Wolfer, D., Wilkinson, G.A., Unsicker, K., Lipp, H.P., Bonhoeffer, T. and Klein, R. (2001) Kinase-independent requirement of EphB2 receptors in hippocampal synaptic plasticity, *Neuron* 32:1027-1040. (MedLine)

Hackett, J.T., Cochran, S.L., Greenfield, J. Jr, Brosius, D.C. and Ueda, T. (1990) Synaptin I injected presynaptically into goldfish Mauthner axons reduces quantal synaptic transmission, *J. Neurophysiol.* 63: 701-706. (MedLine)

Hagiwara, S., and Naka, K. I. (1964) The initiation of the spike potential in barnacle muscle fibers under low intracellular Ca^{2+}, *J. Gen. Physiol.* 48:141-161.

Hall, A.C., Lucas, F.R. and Salinas PC. (2000) Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling, *Cell* 100:525-535. (MedLine)

Hamill, A. M., Marty, A., Neher, H., Sakman, B., and Sigworth, F. J. (1981) Improved patch-clamp techniques for high resolution current recording from cells and cell free membrane patches, *Eur. J.Physiol.* 391:85-100. (MedLine)

Hanley, J.G., Koulen, P., Bedford, F., Gordon-Weeks, P.R. and Moss, S.J. (1999) The protein MAP-1B links $GABA_C$ receptors to the cytoskeleton at retinal synapses, *Nature* 397:66-69. (MedLine)

Hayashi, T., Umemori, H., Mishina, M. and Yamamoto, T. (1999) The AMPA receptor interacts with and signals through the protein tyrosine kinase Lyn, *Nature* 397:72-76. (MedLine)

Hayashi, Y., Shi, S.H., Esteban, .J.A., Piccini, A., Poncer, J.C. and Malinow, R. (2000a) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction, *Science* 287:2262-2267. (MedLine)

Hayashi, Y., Shi, S.H., Esteban, J.A., Piccini, A., Poncer, J.C. and Malinow, R. (2000b) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction, *Science* 287:2262-2267. (MedLine),/a>

Heginbotham, L., Lu, Z., Abramson, T. and MacKinnon, R. (1994) Mutations in the K^+ channel signature sequence, *Biophys. J.* 66:1061-1067. (MedLine)

Henderson, J.T., Georgiou, J., Jia, Z., Robertson, J., Elowe, S., Roder J.C. and Pawson, T. (2001) The receptor tyrosine kinase EphB2 regulates NMDA-dependent synaptic function, *Neuron* 32:1041-1056. (MedLine)

Heuser, J.E. and Reese, T.S. (1973) Evidence for recycling of synaptic vesicle membrane during

transmitter release at the frog neuromuscular junction, *J. Cell Biol.* 57:315-344. (MedLine)

Heuser, J.E. and Reese, T.S. (1987) in *Handbook of Physiology: The Nervous System*, ed. by Kendel, E.K. 1 (part I) : 99-136. Amer. Physiol. Soc.

Heuser, J.E., Reese, T.S. and Landis, D.M.D. (1974) Functional changes in frog neuromuscular-junctions studied with freeze fracture, *J. Neurocytol.* 3:109-131. (MedLine)

Himanen, J.P., Rajashankar, K.R., Lackmann, M., Cowan, C.A., Henkemeyer, M. and Nikolov, D.B. (2001) Crystal structure of an Eph receptor-ephrin complex, *Nature* 414:933-938. (MedLine)

Hirokawa, N., Sobue, K., Kanda, K., Harada, A., and Yorifuji, H. (1989) The cytoskeletal architecture of the presynaptic terminal and molecular structure of synapsin I, *J. Cell. Biol.* 108: 111-126. (MedLine)

Hodgkin, A. L. (1939) The subthreshold potentials in a crustacean nerve fibre, *Proc. R. Soc. London. Ser. B* 126:87-121.

Hodgkin, A. L. (1951) The ionic basis of electrical activity in nerve and muscle, *Biol. Rev. Cambridge Philos. Soc*. 26:339-409.

Hodgkin, A. L. (1958) Ionic movements and electrical activity in giant nerve fibres, *Proc. R. Soc. London Ser. B* 148:1-37.

Hodgkin, A. L., and Huxley, A. F. (1947) Potassium leakage from an active nerve fibre, *J. Physiol. (London)* 106:341-367.

Hodgkin, A. L., and Huxley, A. F. (1953) Movement of radioactive potassium and membrane current in a giant axon, *J. Physiol. (London)* 121:403-414.

Hodgkin, A. L., and Katz, B. (1949) The effect of sodium ions on the electrical activity of the giant axon of the squid, *J. Physiol. (London)* 108:33-77.

Hoey, T., Sun, Y.L., Williamson, K. and Xu, X. (1995) Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins, *Immunity* 2:461-472. (MedLine)

Hoffman, D.A., Magee, J.C., Colbert, C.M. and Johnston, D. (1997) K^+ channel regulation of signal propagation in dendrites of hippocampal pyramidal neurons, *Nature* 387:869-875. (MedLine)

Holland, S.J., Gale, N.W., Mbamalu, G., Yancopoulos, G.D., Henkemeyer, M. and Pawson T. (1996) Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands, *Nature* 383:722-725. (MedLine)

Hollman, M. and Heinemann, S. (1994) Cloned glutamate receptors, *Annu. Rev. Neurosci.* 17:31-108. (MedLine)

Huttner, W.B., Schiebler, W., Greengard, P., De Camilli, P. (1983) Synapsin I (protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation, *J. Cell Biol.* 96: 1374-1388. (MedLine)

Huxley, A. F., and Stämfli, R. (1951) Effects of potassium and sodium on resting and action potentials of single myelinated fibres, *J. Physiol. (London)* 112:496-508.

Ikonomidou, C., Bosch, F., Miksa, M., Bittigau, P., Vockler, J., Dikranian, K., Tenkova, T.I., Stefovska, V., Turski, L., Olney, J.W. (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain, *Science* 283:70-74. (MedLine)

Impey, S., Mark, M., Villacres, E.C. Poser, S., Charkin, C. and Storm, D.R. (1996) Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus, *Neuron* 16:973-982. (MedLine)

Jahn, R. and Südhof, T.C. (1999) Membrane fusion and exocytosis, *Annu. Rev. Biochem.* 68:863-911. (MedLine)

Jiang, C. and Schuman, E. (2002) Regulation and function of local protein synthesis in neuronal dendrites, *Trends Biochem. Sci.* 27:506-513. (MedLine)

Johnston, D., Magee, J.C., Colbert, C.M. and Christie, B.R. (1996) Active properties of neuronal dendrites, *Annu. Rev. Neurosci.* 19:165-186. (MedLine)

Jones, K.R., Fari&ntildeas, I., Backus, C. and Reichardt, L.F. (1994) Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor development, *Cell* 76:989-999. (MedLine)

Kafitz, K.W., Rose, C.R., Thoenen, H. and Konnerth, A. (1999) Neurotrophin-evoked rapid excitation through TrkB receptors, *Nature* 401:918-921. (MedLine)

Kang, H., Welcher, A.A., Shelton, D. and Schuman, E.M. (1997) Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation, *Neuron* 19:653-664. (MedLine)

Kater, S.B. and Rehder, V. (1995) The sensory-motor role of growth cone filopodia, *Curr. Opin. Neurobiol.* 5:68-74. (MedLine)

Katz, B. (1969) *The Release of Neurotransmitter Substances,* Sherrington Lectures, Vol. 10. Thomas, Springfield, Ill.

Katz, B., and Miledi, R. A. (1967a) A study of synaptic transmission in the absence of nerve impulses, *J. Physiol. (London)* l92:407-436. (MedLine)

Katz, B., and Miledi, R. (1967b) Tetrodotoxin and neuromuscular transmission, *Proc. R. Soc. London Ser. B* 167:8-22. (MedLine)

Katz, B., and Miledi, R. (1967c) The release of acetylcholine from nerve endings by graded electric pulses, *Proc. R. Soc. London Ser. B* 167:23-28. (MedLine)

Katz, L.C. and Shatz, C.J. (1996) Synaptic activity and the construction of cortical circuits, *Science* 274:1133-1138. (MedLine)

Kennedy, M.B. (1988) Cellular neurobiology. Synaptic memory molecules, *Nature* 335:770-772. (MedLine)

Keyser, D.O. and Pellamer, T.C. (1993) Synaptic transmission in the hippocampus: critical role for glial cells, *Glia* 10:237-243. (MedLine)

Keynes, R. D. (1951a) Leakage of radioactive potassium from stimulated nerve, *J. Physiol. (London)* 113:99-114.

Keynes, R. D. (1951b) The ionic movements during nervous activity, *J. Physiol. (London)* 114:119-150.

Keynes, R. D., and Lewis, P. R. (1951) The sodium and potassium content of cephalopod nerve fibers, *J. Physiol. (London)* 114:151-182.

Khan, A.A., Bose, C., Yam, L.S., Soloski, M.J. and Rupp, F. (2001) Physiological regulation of the immunological synapse by agrin, *Science* 292:1681-1686. (MedLine)

Kim, E., Cho, K.O., Rothschild, A. and Sheng, M. (1996) Heteromultimerization and NMDA receptor-clustering activity of Chapsyn-110, a member of the PSD-95 family of proteins, *Neuron* 17:103-113. (MedLine)

Kirsch, J. and Betz, H. (1995) The postsynaptic localization of the glycine receptor-associated protein gephyrin is regulated by the cytoskeleton *J. Neurosci.* 15:4148-4156. (MedLine)

Klein, R. (2001) Excitatory Eph receptors and adhesive ephrin ligands, *Curr. Opin. Cell Biol.* 13:196-

203. (MedLine)

Kohara, K., Kitamura, A., Morishima, M. and Tsumoto, T. (2001) Activity-dependent transfer of brain-derived neurotrophic factor to postsynaptic neurons, *Science* 291:2419-2423. (MedLine)

Kornau, H.C., Schenker, L.T., Kennedy, M.B. and Seeburg, P.H. (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95, *Science* 269:1737-1740. (MedLine)

Korte, M., Carroll, P., Wolf, E., Brem, G., Thoenen, H. and Bonhoeffer, T. (1995) Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor, *Proc. Natl. Acad. Sci. USA* 92:8856-8860. (MedLine)

Krapivinsky, G., Gordon, E.A., Wickman, K., Velimirovic, B., Krapivinsky, L. and Clapham, D.E. (1995) The G-protein-gated atrial K^+ channel IKACh is a heteromultimer of two inwardly rectifying K(+)-channel proteins, *Nature* 374:135-141. (MedLine)

Krueger, N.X., Van Vector, D., Wan, H.I., Gelbaret, W.M., Goodman, C.S. and Saito, H. (1996) The transmembrane tyrosine phosphatase DLAR controls motor axon guidance in Drosophila, *Cell* 84:611-622. (MedLine)

Kuhlman, J. R., Li, C., and Calabrese, R. L. (1985) FMRF-amide-like substances in the leech: immunocytochemical localization, *J. Neurosci.* 5:2301-2309. (MedLine)

Kuhn, H.G., Dickinson-Anson, H. and Gage, F.H. (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation, *J. Neurosci.* 16:2027-2033. (MedLine)

Lander, E.S. et al. (2001) Initial sequencing and analysis of the human genome, *Nature* 409, 860 -921. (MedLine)

Landis, D.M., Hall, A.K., Weinstein, L.A. and Reese, T.S. (1988) The organization of cytoplasm at the presynaptic active zone of a central nervous system synapse, *Neuron* 1:201-209. (MedLine)

Latorre, R., and Miller, C. (1983) Conduction and selectivity in potassium channels. *J. Membr. Biol.* 71:11-30. (MedLine)

Lendvai, B., Stern, E.A., Chen, B. and Svoboda, K. (2000) Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex in vivo, *Nature* 404:876-881. (MedLine)

Levitan, I.B. (1999) Modulation of ion channels by protein phosphorylation. How the brain works, *Adv.*

Second Messenger Phosphoprotein Res. 33:3-22. (MedLine)

Linden, D.J. and Connor, J.A. (1995) Long-term synaptic depression, *Annu. Rev. Neuroscie.* 18:319-357. (MedLine)

Lisman, J. (1994) The CaM kinase II hypothesis for the storage of synaptic memory, *Trends Neurosci.* 17:406-412. (MedLine)

Lisman, J., Malenka, R.C., Nicoll, R.A. and Malinow, R. (1997) Learning mechanisms: the case for CaM-KII, *Science* 276:2001-2002. (MedLine)

Llinas, R., Steinberg, I.Z. and Walton. K. (1981) Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse, *Biophys. J.* 33: 323-351. (MedLine)

Llinas, R., McGuiness, T. L., Leonard, C. S., Sugimori, M., and Greengard, P. (1985) Intraterminal injection of synapsin-I or calcium calmodulin dependent protein kinase-II alters neurotransmitter release at the squid giant synapse, *Proc. Natl. Acad. Sci. U.S.A.* 82:3035-3039. (MedLine)

Llinas, R., Gruner, J.A., Sugimori, M. McGuiness, T.L.and Greengard, P. (1991) Regulation by synapsin I and Ca^{2+}-calmodulin-dependent protein kinase II of transmitter release in squid giant synapse, *J. Physiol.* 436: 257-282. (MedLine)

Llinas R., Sugimori, M., and Silver, R.B. (1992) Microdomains of high calcium concentration in a presynaptic terminal, *Science* 256:677-679. (MedLine)

Lloyd, D.P.C. (1949) Post-tetanic potentiation of response in monosynaptic reflex pathways of the spinal cord, *J. Gen. Physiol.* 33:147-170.

Lu, Y.F., Tomizawa, K., Moriwaki, A., Hayashi, Y., Tokuda, M., Itano, T., Hatase, O. and Matsui, H. (1996) Calcineurin inhibitors, FK506 and cyclosporin A, suppress the NMDA receptor-mediated potentials and LTP, but not depotentiation in the rat hippocampus, *Brain Res.* 729:142-146. (MedLine)

Lukasiewicz, P.D. (1996) $GABA_C$ receptors in the vertebrate retina, *Mol. Neurobiol.*. 12:181-194. (MedLine)

MacDermott, A.B., Mayer, M.L., Westbrook, G.L., Smith, S.J., and Barker, J.L. (1986) NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones, *Nature* 321:519-522. (MedLine)

Macdonald, R.L. and Olsen, R.W. (1994) $GABA_A$ receptor channels, *Annu. Rev. Neurosci* 17:569-602.

(MedLine)

MacKinnon, R. (1991) Determination of the subunit stoichiometry of a voltage-activated potassium channel, *Nature* 350:232-235. (MedLine)

MacNeil, M.A. and Masland, R.H. (1998) Extreme diversity among amacrine cells: implications for function, *Neuron* 20:971-982. (MedLine)

Maletic-Savatic, M., Malinow, R. and Svoboda, K. (1999) Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity, *Science* 283:1923-1927. (MedLine)

Mansuy, I.M., Mayford, M., Jacob, B., Kandel, E.R. and Bach, M.E. (1998) Restricted and regulated overexpression reveals calcineurin as a key component in the transition from short-term to long-term memory, *Cell* 92:39-49. (MedLine)

Mayford, M., Barzilai, A., Keller, F., Schacher, S. and Kandel, E.R. (1992) Modulation of an NCAM-related adhesion molecule with long-term synaptic plasticity in *Aplysia*, *Science* 256:638-644. (MedLine)

McAllister, A.K., Katz, L.C., Lo, D.C. (1999) Neurotrophins and synaptic plasticity, *Annu. Rev. Neurosci.* 22:295-318. (MedLine)

McGaugh, J.L. (1966) Time-dependent processes in memory storage, *Science* 153:1351-1358. (MedLine)

McMahon, H.T. and Nicholls, D.G. The bioenergetics of neurotransmitter release, (1991) *Biochim. Biophys. Acta* 1059: 243-264. (MedLine)

Meyer, G., Kirsch, J., Betz, H. and Langosch, D. (1995) Identification of a gephyrin binding motif on the glycine receptor β subunit, *Neuron* 15:563-572. (MedLine)

Micheva, K.D., Kay, B.K. and McPherson, P.S. (1997) Synaptojanin forms two separate complexes in the nerve terminal. Interactions with endophilin and amphiphysin, *J. Biol. Chem.* 272:27239-27245. (MedLine)

Migaud, M,, Charlesworth, P., Dempster, M., Webster, L.C., Watabe, A.M., Makhinson, M., He, Y., Ramsay, M.F., Morris, R.G., Morrison, J.H., O'Dell, T.J. and Grant, S.G. (1998) Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein, *Nature* 396:433-439. (MedLine)

Miledi, R. (1966) Miniature synaptic potentials in squid nerve cells, *Nature* 212:1240-1242.

Ming, G-I, Lohof, A.M. and Zheng, J.Q. (1997) Acute morphogenic and chemotropic effects of neurotrophins on cultured embryonic *Xenopus* spinal neurons, *J. Neurosci.* 17:7860-7871. (MedLine)

Mochida, S., Kobayashi, H., Matsuda, Y., Yuda, Y., Muramoto, K. and Nonomura, Y. (1994) Myosin II is involved in transmitter release at synapses formed between rat sympathetic neurons in culture, *Neuron* 13:1131-1142. (MedLine)

Montal, M., Labarca, P., Fredkin, D. R., and Isla, B. A. (1984) Channel properties of the purified acetylcholine receptor from *Torpedo californica*. Reconstituted in planar lipid bilayer membranes, *Biophys. J.* 45:165-174. (MedLine)

Montal, M., Aholt, R., and Labarca, P. (1986) The reconstituted acetylcholine receptor. In *Ion Channel Reconstitution* (Miller, C., ed.). Plenum, New York.

Montarolo, P.G., Goelet, P., Castellucci, V.F., Morgan, J., Kandel, E.R. and Schacher, S. (1986) A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*, *Science* 234:1249-1254. (MedLine)

Morris, R.G., Anderson, E., Lynch, G.S. and Baudry, M. (1986) Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5, *Nature* 319:774-776. (MedLine)

Moser, M.B., Trommald, M. and Andersen, P. (1994) An increase in dendritic spine density on hippocampal CA1 pyramidal cells following spatial learning in adult rats suggests the formation of new synapses, *Proc. Natl. Acad. Sci. USA* 91:12673-12675. (MedLine)

Mugnaini, E. and Floris, A. (1994) The unipolar brush cell: A neglected neuron of mammalian cerebellar cortex, *J. Comp. Neurobiol.* 339:174-180. (MedLine)

Mulkey, R.M., Endo, S., Shenolikar, S. and Malenka, R.C. (1994) Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression, *Nature* 369:486-488. (MedLine)

Muller, D., Wang, C., Skibo, G., Toni, N., Cremer, H., Calaora, V., Rougon, G. and Kiss, J.Z. (1996) PSA-NCAM is required for activity-induced synaptic plasticity, *Neuron* 17:413-422. (MedLine)

Nakamoto, R.K. and Slayman, C.W. (1989) Molecular properties of the fungal plasma-membrane [H+]-ATPase, *J. Bioenerg. Biomembr.* 21:621-632. (MedLine)

Nakanishi, S, (1994) Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity, *Neuron* 13:1031-1037. (MedLine)

Narahashi, T., Moore, J. W., and Scott, W. R. (1964) Tetrodotoxin blockage of sodium conductance increase in lobster giant axons, *J. Gen. Physiol.* 47:965-974.

Nastuk, W. L., and Hodgkin, A. L. (1950) The electrical activity of single muscle fibres, *J. Cell Comp. Physiol.* 35:39-73.

Nazif, F.A., Byrne, J.H. and Cleary, L.J. (1991) cAMP induces long-term morphological changes in sensory neurons of *Aplysia Brain Res.*539:324-327. (MedLine)

Nestler, E.J. and Greengard, P. (1984) *Protein Phosphorylation in the Nervous System*, Wiley and Sons, New York, see pp.128-157.

Nelson, N. (1992) The vacuolar H^+-ATPase - one of the most fundamental ion pumps in nature, *J. Exp. Biol.* 172: 19-27. (MedLine)

Nguyen, P.V., Abel, T. and Kandel, E.R. (1994) Requirement of a critical period of transcription for induction of a late phase of LTP, *Science* 265:1104-1107. (MedLine)

Nichols, R.A., Chilcote, T.J., Czernik, A.J. and Greengard, P. (1992) Synapsin I regulates glutamate release from rat brain synaptosomes, *J. Neurochem.* 58: 783-785. (MedLine)

Nicoll, R.A. and Malenka, R.C. (1995) Contrasting properties of two forms of long-term potentiation in the hippocampus, *Nature* 377:115-118. (MedLine)

Niethammer, M., Kim, E, and Sheng, M. (1996) Interaction between the C terminus of NMDA receptor subunits and multiple members of the PSD-95 family of membrane-associated guanylate kinases, *J. Neurosci.* 16:2157-2163. (MedLine)

Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, H., Kanaoka, Y., Minamino, N. (1984) Primary structure of Electrophorus electricus sodium channel deduced from cDNA sequence, *Nature* 312(5990):121-127. (MedLine)

O'Brien, R.J., Lau, L.F. and Huganir, R.L. (1998) Molecular mechanisms of glutamate receptor clustering at excitatory synapses, *Curr. Opin. Neurobiol.* 8:364-369. (MedLine)

O'Dell, T.J., Hawkins, R.D., Kandel, E.R. and Arancio, O. (1991) Tests of the role of two diffusible substances in long term potentiation: evidence for nitric oxide as a possible early retrograde messenger, *Proc. Natl. Acad.Sci.* 88:11285-11289. (MedLine)

Okada, Y., Yamazaki, H., Sekine-Aizawa, Y. and Hirokawa, N. (1995) The neuron-specific kinesin

superfamily protein KIF1A is a unique monomeric motor for anterograde transport of synaptic vesicles precursors, *Cell* 81:769-780. (MedLine)

Pappas, G. D., and Bennett, M. V. L. (1966) Specialized junctions involved in electrical transmission between neurons, *Ann. N.Y. Acad. Sci.* 137:495-508. (MedLine)

Parsegian, A. (1969) Energy of an ion crossing a low dielectric membrane: solutions to four relevant static problems, *Nature* 221:844-846. (MedLine)

Patel, S.N., Rose, S.P. and Stewart, M.G. (1988) Training induced dendritic spine density changes are specifically related to memory formation processes in the chick, *Gallus domesticus Brain Res.* 463:168-173. (MedLine)

Patterson, S.L., Abel, T., Deuel, T.A., Martin, K.C., Rose, J.C. and Kandel, E.R. (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice, *Neuron* 16:1137-1145. (MedLine)

Peles, E., Schlessinger, J. and Grumet, M. (1998) Multi-ligand interactions with receptor-like protein tyrosine phosphatase β: implications for intercellular signaling, *Trends Biochem. Sci.* 23:121-124. (MedLine)

Peter, N., Aronoff, B., Wu, F. and Schacher, S. (1994) Decrease in growth cone-neurite fasciculation by sensory or motor cells in vitro accompanies down-regulation of Aplysia cell adhesion molecules by neurotransmitters, *J. Neurosci.* 14:1413-1421. (MedLine)

Petrovich, T.Z., Merakovsky, J. and Kelly, L.E. (1993) A genetic analysis of the *stoned* locus and its interaction with *dunce*, *shibire* and suppressor of *stoned* variants of *Drosophila melanogaster*, *Genetics* 133: 955-965. (MedLine)

Petrucci, T.C. and Morrow, J.S. (1987) Synapsin I, an actin bundling protein under phosphorylation control, *J. Cell Biol.* 105:1355-1363. (MedLine)

Pfreiger, F.W. and Barres, B.A. (1997) Synaptic efficacy enhanced by glial cells *in vitro*, *Science* 277:1684-1687. (MedLine)

Prekeris, R. and Terrian, D.M. (1997) Brain myosin V is a synaptic vesicle-associated motor protein: evidence of a Ca^{2+}-dependent interaction with the synaptobrevin-synaptophysin complex, *J. Cell Biol.* 137:1589-1601. (MedLine)

Rayport, S.G. and Schacher, S. (1986) Synaptic plasticity in vitro: cell culture of identified *Aplysia*

neurons mediating short-term habituation and sensitization, *J. Neurosci.* 6:759-763. (MedLine)

Ringstad, N., Nemoto, Y. and De Camilli, P.(1997) The SH3p4/Sh3p8/SH3p13 protein family: binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain, *Proc. Natl. Acad. Sci. USA.* 94:8569-8574. (MedLine)

Rosenmund, C. and Stevens, C.F. (1996) Definition of the readily releasable pool of vesicles at hippocampal synapses, *Neuron* 16:1197-1207. (MedLine)

Rothenberg, M. A. (1950) Studies on permeability in relation to nerve function. II. Ionic movements across axonal membranes, *Biochim. Biophys. Acta* 4:96-114.

Rothman, J.E. and Orci, L. (1992) Molecular dissection of the secretory pathway, *Nature* 355: 409-415. (MedLine)

Sala, R., Viegi. A., Rossi, F.M., Pizzorusso, T., Bonanno, G., Raiteri, M. and Maffei, L, (1998) Nerve growth factor and brain-derived neurotrophic factor increase neurotransmitter release in the rat visual cortex, *Eur. J. Neurosci.* 10:2185-2191. (MedLine)

Sato, C., Ueno, Y., Asai, K., Takahashi, K., Sato, M., Engel, A. and Fujiyoshi, Y. (2001) The voltage-sensitive channel is a bell-shaped molecule with several cavities, *Nature* 409:1047-1051.

Savchenko, A., Barnes, S. and Kramer, R.H. (1997) Cyclic-nucleotide-gated channels mediate synaptic feedback by nitric oxide, *Nature* 390:694-698. (MedLine)

Scarborough, G.A. (1996) in *Handbook of Biological Physics* vol. 2, Konings, W.N., Kaback, H.R. and Lolkema, J.S. eds., Elsevier Science, Amsterdam, pp. 75-92.

Schiavo, G., Osborne, S.L. and Sgouros, J.G. (1998) Synaptotagmins: more isoforms than functions?, *Biochem. Biophys. Res. Commun.* 248:1-8. (MedLine)

Schiebler, W., Jahn, R., Doucet, J.-P., Rothlein, J. and Greengard, P. (1986) Characterization of synaptin I binding to small vesicles, *J. Biol. Chem.* 261: 8383-8390. (MedLine)

Schoch, S., Castillo, P.E., Jo, .T, Mukherjee, K., Geppert, M., Wang, Y., Schmitz, F., Malenka, R.C. and Sudhof, T.C. (2002) RIM1α forms a protein scaffold for regulating neurotransmitter release at the active zone, *Nature* 415:321-326. (MedLine)

Schroeder, B.C., Waldegger, S., Fehr, S., Bleich, M., Warth, R., Greger, R. and Jentsch, T.J. (2000) A constitutively open potassium channel formed by KCNQ1 and KCNE3, *Nature* 403:196-199. (MedLine)

Shipston, M.J. (2001) Alternative splicing of potassium channels: a dynamic switch of cellular excitability, *Trends Cell Biol.* 11:353-358. (MedLine)

Schmidt, A. and Huttner, W.B. (1998) Biogenesis of synaptic-like microvesicles in perforated PC12 cells, *Methods* 16:160-169. (MedLine)

Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A.V., Witke, W., Huttner, W.B. and Soling, H.D. (1999) Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid, *Nature* 401:133-141. (MedLine)

Schuman, E.M. and Madision, D.V., (1994) Nitric oxide and synaptic function, *Annu. Rev. Neurosci.* 17:153-183. (MedLine)

Schwartz, J.H., Castellucci, V.F. and Kandel, E.R. (1971) Functioning of identified neurons and synapses in abdominal ganglion of *Aplysia* in absence of protein synthesis, *J. Neurophysiol.* 34:939-953. (MedLine)

Shi, S.H., Hayashi, Y., Petralia, R.S., Zaman, S.H., Wenthold, R.J., Svoboda, K. and Malinow, R. (1999) Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation, *Science* 284:1811-1816. (MedLine)

Shi, S., Hayashi, Y., Esteban, J.A. and Malinow, R.(2001) Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons, *Cell* 105:331-343. (MedLine)

Shors, T.J., Miesegaes, G., Beylin, A., Zhao, M., Rydel, T. and Gould E. (2001) Neurogenesis in the adult is involved in the formation of trace memories, *Nature* 410:372-376. (MedLine)

Siegel, G.J., Agranoff, B.W., Albers R.W. and Molinoff, P.B., eds (1994) *Basic Neurochemistry. Molecular, Cellular and Medical Aspects.* Raven Press, New York. For acetylcholine see Taylor, P. and Brown, J.H., Acetylcholine, pp. 231-260; for glutamate, Dingledine, R. and McBain, C.J., Excitatory amino acid transmitters, pp. 367-387; for GABA and glycine, DeLorey, M. and Olsen, R.W., GABA and glycine, pp.389-387.

Slayman, C. L. (1965) Electical properties of *Neurospora crassa* respiration and intracellular potential, *J. Gen. Pysiol.* 49:93-116. (MedLine)

Slayman, C. L., and Tatum E. l. (1965) Potassium Transport in *Neurospora*. II. Measurements of steady-state potassium fluxes, *Biochim. Biophys. Acta* 102:149-160. (MedLine)

Smart, T.G. (1997) Regulation of excitatory and inhibitory neurotransmitter-gated ion channels by protein

phosphorylation, *Curr. Opin. Neurobiol.* 7:358-367. (MedLine)

Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, Geramanos, S., Tempst, P. and Rothman, J.E. (1993a), SNAP receptors implicated in vesicle targeting and fusion, *Nature* 362:318-324. (MedLine)

Söllner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H. and Rothman, J.E. (1993b) A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation and fusion, *Cell* 75: 409-418. (MedLine)

Sheng, Z.-H., Rettig, J., Takahashi, M. and Catteral, W.A. (1994) Identification of syntaxin-binding sites on N-type calcium channels, *Neuron* 13:1303-1313. (MedLine)

Squire, L.R. (1992) Memory and the hippocampus: a synthesis from findins with rats, monkeys, and humans, *Psych. Rev.* 99:195-231. (MedLine)

Stanley, E.F. (1993) Single calcium channels and acetylcholine release at presynaptic nerve terminal, *Neuron* 11:1007-1011. (MedLine)

Stanley, E.F. and Miroznik, R.R. (1996) Cleavage of syntaxin prevents G-protein regulation of presynaptic calcium channels, *Nature* 385:340-342. (MedLine)

Stevens, C.F. and Tsujimoto, T. (1995) Estimates for the pool size of releasable quanta at a single central synapse and for the time required to refill the pool, *Proc. Natl. Acad. Sci.* USA 92:846-849. (MedLine)

Sweatt, D. and kandell, E.R. (1989) Persistent and transcriptionally-dependent increase in protein phosphorylation in long term facilitation in *Aplysia* sensory neurons, *Nature* 339:51-54. (MedLine)

Takasu, M.A., Dalva, M.B., Zigmond, R.E. and Greenberg, M.E. (2002) Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors, *Science* 295:491-495. (MedLine)

Tang, L., Hung, C.P. and Schuman, E.M. (1998) A role for the cadherin family of cell adhesion molecules in hippocampal long-term potentiation, *Neuron* 20:1165-1175. (MedLine)

Tang, Y.P., Shimizu, E., Dube, G.R., Rampon, C., Kerchner, G.A., Zhuo, M., Liu, G. and Tsien, J.Z. (1999) Genetic enhancement of learning and memory in mice, *Nature* 401:63-69. (MedLine)

Tempel, B.L., Papazian, D.M., Schwarz, T.L., Jan, Y.N. and Jan L.Y. (1987) Sequence of a probable potassium channel component encoded at Shaker locus of Drosophila, 237:770-775. 9MedLine)

Thompson, S.M, Capogna, M. and Scanziani, Y. (1993) Presynaptic inhibition in the hippocampus, *Trends in Neuroscie.* 16:222-226. (MedLine)

Tian, L., Duncan, R.R., Hammond, M.S., Coghill, L.S., Wen, H., Rusinova, R., Clark, A.G., Levitan, I.B. and Shipston, M.J. (2001) Alternative splicing switches potassium channel sensitivity to protein phosphorylation, *J. Biol. Chem.* 276:7717-7720. (MedLine)

Toni, N., Buchs, P.A., Nikonenko, I., Bron, C.R. and Muller, D. (1999) LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite, *Nature* 402:421-425. (MedLine)

Toro, L., Wallner, M., Meera, P. and Tanaka, Y. (1998) Maxi-K(Ca), a Unique Member of the Voltage-Gated K Channel Superfamily, *News Physiol. Sci.* 13:112-117. (MedLine)

Trautmann, A. and Vivier, E. (2001) Immunology. Agrin--a bridge between the nervous and immune systems, *Science* 292:1667-1668. (MedLine)

Tseng-Crank, J., Foster, C.D., Krause, J.D., Mertz, R., Godinot, N., DiChiara, T.J. and Reinhart, P.H. (1994) Cloning, expression, and distribution of functionally distinct Ca^{2+}-activated K^{+} channel isoforms from human brain, *Neuron* 13:1315-1330. (MedLine)

Tsien, J.Z., Huerta, P.T. and Tonegawa, S. (1996) The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory, *Cell* 87:1327-1338. (MedLine)

Ullian, E.M., Sapperstein, S.K., Christopherson, K.S. and Barres, B.A. (2001) Control of synapse number by glia, *Science* 291:657-661. (MedLine)

Valera, S., Ballivet, M. and Bertrand, D. (1992) Progesterone modulates a neuronal nicotinic acetylcholine receptor, *Proc. Natl. Acad. Sci. USA* 89:9949-9953. (MedLine)

Valtorta, F., Benfenati, F. and Greengard, P. (1992) Structure and function of synapsins, *J. Biol. Chem.* 267: 7195-7198. (MedLine)

Vergara, C., Latorre, R., Marrion, N.V. and Adelman, J.P (1998) Calcium-activated potassium channels, *Curr. Opin. Neurobiol.* 8:321-329. (MedLine)

Wang X.-h. and Poo, M.-m. (1997) Potentiation of developing synapses by postsynaptic release of neurotrophin-4, *Neuron* 19:825-835. (MedLine)

Wang, H., Bedford, F.K., Brandon, N.J., Moss S.J. and Olsen, R.W. (1999) $GABA_A$-receptor-associated protein links $GABA_A$ receptors and the cytoskeleton, *Nature* 397:69-72. (MedLine)

Wehling, M. (1997) Specific, nongenomic actions of steroid hormones, *Annu. Rev. Physiol.* 59:365-393. (MedLine)

Wei, A., Covarrubias, M., Butler, A., Baker, K., Pak, M. and Salkoff, L. (1990) K^+ current diversity is produced by an extended gene family conserved in Drosophila and mouse, *Science* 248:599-603. (MedLine)

Weldman, S. (1951) Electrical characteristics of *Sepia* axons, *J. Physiol. (London)* 114:372-381.

Whittaker, V. P. (1968) Synaptic trasmission, *Proc. Natl. Acad. Sci. U.S.A.* 60:1081-1091. (MedLine)

Whittaker, V. P. and Sheridan, M. N. (1965) The morphology and acetylcholine content of isolated cerebral cortical synaptic vesicles, *J. Neurochem.* 12:363-372.

Wolin, M.S., Wood, K.S. and Ignarro, L.J. (1982) Guanylate cyclase from bovine lung. A kinetic analysis of the regulation of unpurified soluble enzyme by protoporphyrin IX heme, and nytrosyl heme, *J. Biol. Chem.* 257:11312-11320.

Wu, G.Y. and Cline, H.T. (1998) Stabilization of dendritic arbor structure in vivo by CaMKII, *Science* 279:222-226. (MedLine)

Wyszynski, M., Lin, J., Rao, A., Nigh, E., Beggs, A.H., Craig, A.M. and Sheng, M. (1997) Competitive binding of α-actinin and calmodulin to the NMDA receptor, *Nature* 385:439-442. (MedLine)

Xie, J. and McCobb, D.P. (1998) Control of alternative splicing of potassium channels by stress hormones, *Science* 280:443-446. (MedLine)

Xie, J. and Black, D.L. (2001) A CaMK IV responsive RNA element mediates depolarization-induced alternative splicing of ion channels, *Nature* 410:936-939. (MedLine)

Yamagata, K., Andreasson, K.I., Sugiura, H., Maru, E., Dominique, M., Irie, Y., Miki, N., Hayashi, Y., Yoshioka, M., Kaneko, K., Kato, H. and Worley, P.F. (1999) Arcadlin is a neural activity-regulated cadherin involved in long term potentiation, *J. Biol. Chem.* 274:19473-19479. (MedLine)

Yang, N., George, A.L. and Horn, R. (1996) Molecular basis of charge movement in voltage-gated sodium channels, *Neuron* 16:113-122. (MedLine)

Yellen, G., Jurman, M.E., Abramson, T. and MacKinnon, R. (1991) Mutations affecting internal TEA blockade identify the probable pore-forming region of a K+ channel, *Science* 251:939-942. (MedLine)

Yoshida, A., Oho, C., Omori, A., Kuwara, R., Ito, T. and Takahashi, M. (1992) HPC-1 is associated with synaptotagmin and -conotoxin receptor, *J.Biol.Chem.* 267:24925-24928. (MedLine)

Yuan, L.-L. and Ganetzy, B. (1999) A glial-neuronal signaling pathway revealed by mutations in neurexin-related proteins, *Science* 283:1343-1345. (MedLine)

Yuste, R. and Tank, D.W. (1996) Dendritic integration in mammalian neurons, a century after Cajal, *Neuron* 16:701-716. (MedLine)

Zamanillo, D., Sprengel, R., Hvalby, O., Jensen, V., Burnashev, N., Rozov, A., Kaiser, K.M., Koster, H.J., Borchardt, T., Worley, P., Lubke, J., Frotscher, M., Kelly, P.H., Sommer, B., Andersen, P., Seeburg, P.H. and Sakmann, B. (1999) Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning, *Science* 284:1805-1811. (MedLine)

Zenisek, D., Steyer, J.A. and Almers, W. (2000) Transport, capture and exocytosis of single synaptic vesicles at active zones, *Nature* 406:849-854. (MedLine)

Zhou, M., Morais-Cabral, J.H., Mann, S. and MacKinnon, R. (2001) Potassium channel receptor site for the inactivation gate and quaternary amine inhibitors, *Nature* 411:657-661. (MedLine)

Ziv, N.E. and Smith, S.J. (1996) Evidence for a role of dendritic filopodia in synaptogenesis and spine formation, *Neuron* 17:91-102. (MedLine)

23. Mechanochemical Coupling in Various Systems

Whether we are concerned with single cells, multicellular organisms, or populations, biological motion is of fundamental importance. Movement plays obvious roles in feeding, avoidance, digestion, respiration, circulation, and reproduction. Contractile proteins are likely to be involved in the shape of a cell and its changes. The continuous flow of the cytoplasm of plant cells is analogous to circulation in a multicellular organism. The movement and the rearrangement of cells are fundamental to morphogenesis.

Motility may be the result of the action of special structures, such as cilia, flagella, or muscle fibers. It also takes place in the cytoplasm of cells, where the contractile machinery, not readily apparent, may involve the assembly and dissociation of contractile units.

Regardless of details, the displacement of matter will require the performance of work. In the living organism, metabolic and photosynthetic events generally make energy available in a chemical form, such as ATP or some other compound of high phosphate group transfer potential. In producing movement, the hydrolysis of high-energy compounds is coupled to the mechanical events.

I. HIGH-ENERGY PHOSPHATE AND MOVEMENT

The hydrolysis of high-energy phosphate, generally ATP, is involved in motility. ATP has frequently been implicated in experiments in which most of the soluble components of cells or contractile structures were extracted either with cold glycerol solutions (in a procedure known as *glycerination*) or in more recent procedures with detergents. The extraction leaves the contractile apparatus intact. The addition of ATP, normally extracted along with the other soluble components, induces contraction.(e.g., Szent-György, 1949; Summers and Gibbons, 1971).

In the case of muscle, either the force generated or the amount of work performed can be readily measured. The muscle can be attached by a lever to an appropriate transducer and the tension generated is recorded by measuring the current generated by the transducer. Where work has to be measured, the muscle can be allowed to shorten and lift a weight or shorten against a force exerted by the apparatus. In addition to a role of ATP in contraction of extracted muscle, the direct involvement of ATP hydrolysis in the contraction of intact striated muscle has been shown. In muscle, phosphocreatine is usually present at higher concentrations than ATP and acts as a high-energy phosphate reserve. In the reaction of Eq. (1), catalyzed by creatine phosphotransferase, the enzyme transfers the phosphate from creatine phosphate (Cr~P) to ADP.

$$Cr\sim P + ADP \rightleftharpoons ATP + Cr \qquad (1)$$

In Eq. (1) ~P represents a high phosphate group transfer potential. An involvement of ~P in contraction is shown by the decrease in the level of phosphocreatine when the synthesis of new ATP is blocked by adding an uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (DNP). In the experiment of Fig. 1 (Cain et al., 1962), the frog's rectus abdominis muscle is stimulated electrically and contracts against a constant load. The amount of phosphocreatine hydrolyzed is directly proportional to the amount of work performed (displacement x mass).

To test for involvement of ATP requires blocking creatine phosphotransferase, which can be inhibited by 1-fluoro-2,4-dinitrobenzene (FDNB). Table 1 (Cain et al., 1962) shows the constancy of Cr~P in a system in which metabolism is blocked by DNP in the presence of FDNB. In these experiments, matched pairs of muscles from the same animal were used. All were treated with DNP and all except the last with FDNB. One muscle served as control and was allowed to rest, whereas the other carried out work as tabulated in column 3. Column 4 shows the difference in Cr~P concentration between control and experimental muscles. The difference is small unless FDNB is left out (last experiment). Although the hydrolysis of Cr~P is blocked by FDNB, the contractions

produce inorganic phosphate (column 5, boxed value), suggesting that hydrolysis of some other high-energy phosphate, presumably ATP, is taking place. Table 2 (Cain et al., 1962) shows that the amount of ATP is indeed decreased during contraction to produce ADP and AMP. The reactions involved can be expressed as

$$ATP \rightleftharpoons ADP + P_i + work \qquad (2)$$

$$ATP \rightleftharpoons ADP + AMP \qquad (3)$$

Not all the energy is expended as work; a significant portion is lost in the form of heat.

The reaction depicted by Eq.(3) is catalyzed by adenylate kinase. Aside from a role in regenerating ATP, it also has an important role in the regulation of muscle metabolism (see Chapter 14).

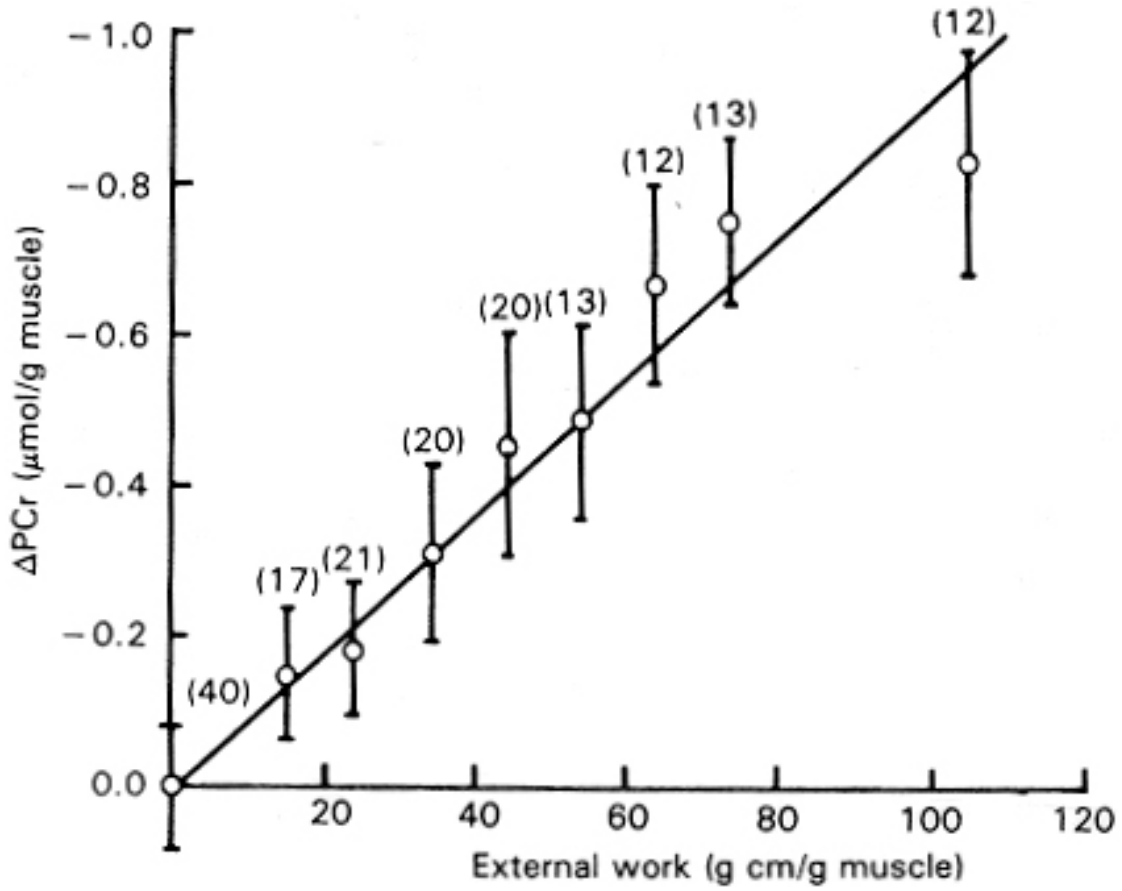


Fig. 1 Relationship of phosphocreatine breakdown to the amount of external work. Frog rectus abdominis muscle contract once or twice against a constant load to different degrees and for different times at 0oC. From(Cain et al. (1962)). Reproduced by permission from *Nature* 196:214-217, copyright ©1962 Macmillan Magazines Ltd.

Table 1 Production of Inorganic Phosphate (P_i) without Change in Phosphocreatine (PCr) after Three Small Contractions at 0oC in Frog Rectus Abdominis Muscles Pretreated with Dinitrophenol (DNP) Plus Fluorodinitrobenzene (FDNB)[a]

(1) Type of experiment	(2) Pairs of muscles	(3) External work (g cm/g muscle)	(4) PCr (μmol/g muscle)	(5) P_i (μmol/g muscle)
Control minus contractions	27	81±5	-0.10±0.08	---------
Control minus contractions	12	79±8	-0.17±0.08	+1.23±0.48
Control minus control	4	0	+0.17±0.17	--------
Control minus contractions (DNP but not FDNB)	4	76±8	-1.02±0.28	---------

From Cain et al (1962)). Reproduced by permission from *Nature* 196: 214-217, copyright ©1962, MacMillan Magazines Ltd.

ATP hydrolysis also provides the energy for ciliary and flagellar motion. Fig. 2 (Brokaw, 1967) represents an experiment in which the beating of flagella of glycerinated sea urchin sperm is observed. The frequency of beat (Fig. 2a) is shown as a function of ATP concentration. The higher the ATP concentration, the greater the frequency. At a sufficiently low concentration, the flagella do not beat at all. Breakdown of the added ATP is clearly involved, since the beat frequency is linearly related to the ATP hydrolyzed (Fig. 2b). The contractile machinery functions as an ATPase.

The molecular assemblies responsible for the energy transduction are referred to as *motors*. All eukaryotic motors discovered to date are powered by ATP hydrolysis.

The experiments discussed in this section demonstrate that the energy expenditure for these two

kinds of movement is provided by the hydrolysis of ATP. Unfortunately, this conclusion says little about the mechanism of the movement. Some of the best understood events in cell movement are those involved in the contraction of striated muscle, where the structured elements are fixed and regular, and for this reason they are discussed first.

Table 2 Breakdown of Adenosine Triphosphate (ATP) to Form Adenosine Diphosphate (ADP) and Adenosine Monophosphate (AMP) during Contraction of Frog Rectus Abdominis Muscles after Treatment with Fluorodinitrobenzene (FDNB)[a]

	ATP (μmol/g muscle)	ADP (μmol/g muscle)	AMP (μmol/g muscle)
Single contraction			
Control	1.25	0.64	0.10
After one contraction	0.81	0.90	0.24
Change ± SE for 9 pairs	-0.44±0.046	+0.26±0.023	+0.14±0.027
Double contraction			
Control	1.24	0.61	0.07
After two contractions	0.59	0.88	0.41
Change ±SE for 3 pairs	-0.65±0.061	+0.27±0.051	+0.34±0.037

From (Cain et al., 1962). Reproduced by permission from *Nature* 196: 214-217, copyright ©1962, MacMillan Magazines Ltd.

[a]External work 100 gcm per g of muscle per contraction

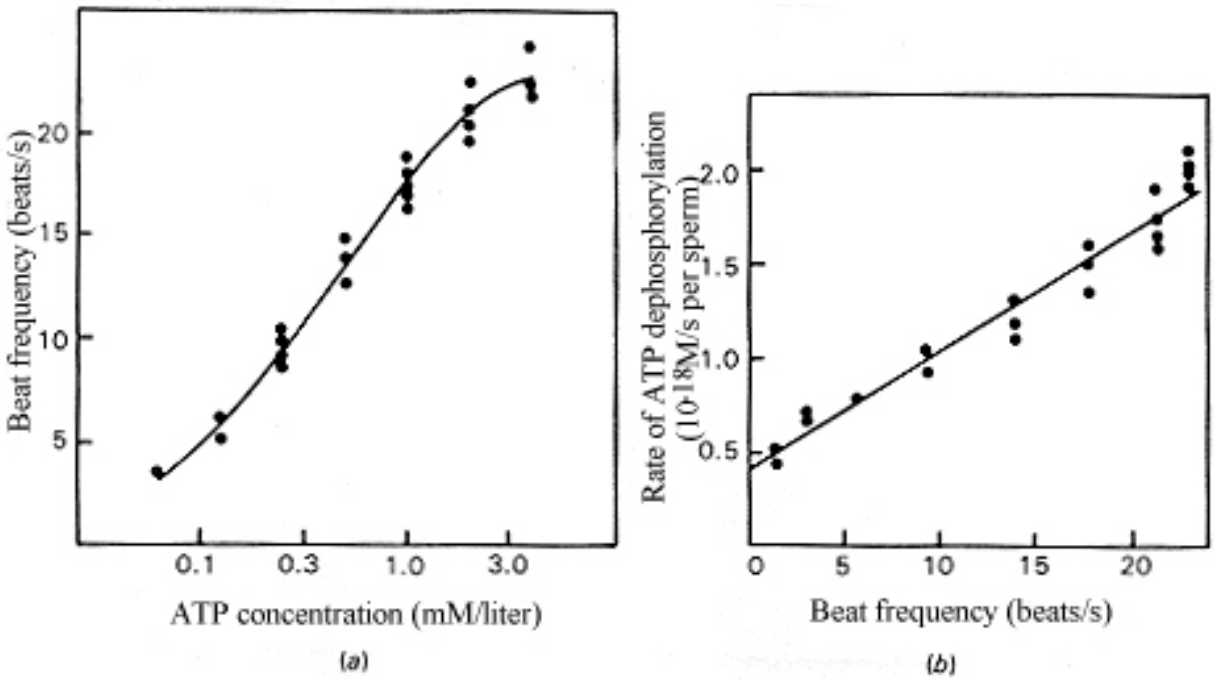


Fig. 2 (a) Effect of ATP concentration on beat frequency of glycerinated sea urchin spermatozoa. Each point represents the average of measurements of 20 spermatozoa. (b) Hydrolysis of ATP by sperm suspensions at various beat frequencies obtained by varying the ATP concentration. Each point represents a single measurement of the rate of ATP dephosphorylation. The line was obtained by the method of least squares. From (Brokaw 1967), *Science* 156:76-78, copyright ©1967 by the AAAS.

II. CONTRACTION IN STRIATED MUSCLE

The structural regularity of striated muscle approaches that of a paracrystalline state: contraction events and structural states can be directly correlated. Striated muscle shortens on electrical, mechanical, or chemical stimulation; when loaded, it can perform work.

Striated muscle is made up of longitudinal elements. A diagrammatic view of vertebrate striated muscle at different levels of organization is shown in Fig. 3 (Huxley, 1960). The smallest functional element is the myofibril. The repeating unit of the myofibril is the sarcomere, which extends from Z line (or disk) to Z line. The structure of the myofibril shown in the figure is based on observations with both the light microscope (Fig. 4) (Hanson and Huxley, 1955) and the electron microscope (Fig. 7, below). As shown, the striations are the result of the presence of dark or anisotropic bands (*A bands*) and light or isotropic bands (*I bands*). Anisotropy and isotropy refer to behavior in relation to polarized light. The isotropic bands transmit incident polarized light at the same velocity regardless of the light's direction. The anisotropic bands transmit light at different velocities depending on its direction; the bands are birefringent.

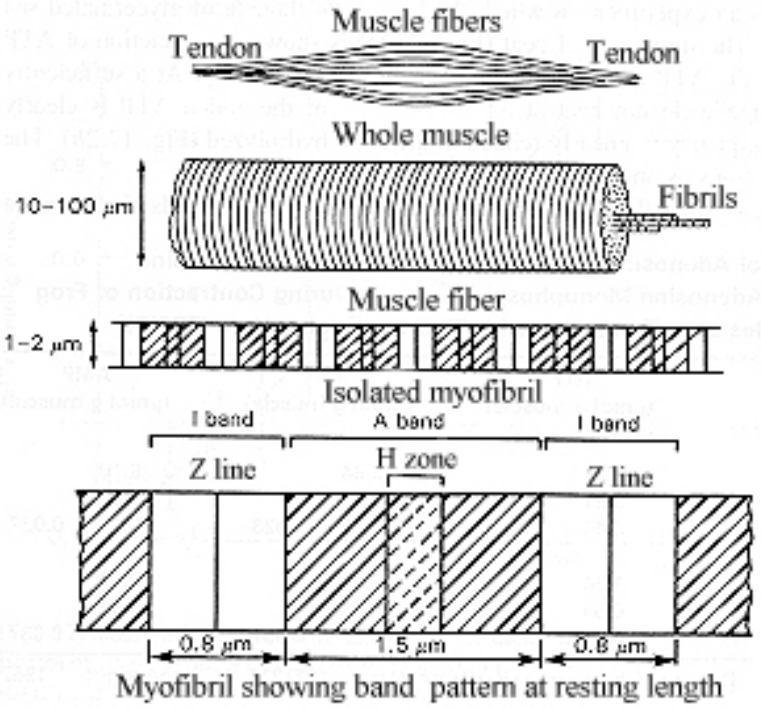


Fig. 3 Diagram representing the structure of striated muscle at different levels of organization; dimensions shown are those for rabbit psoas muscle. Reproduced from H. E. Huxley, *The Cell*, Vol. 4(1):365-481, with permission. Copyright ©1960 Academic Press.

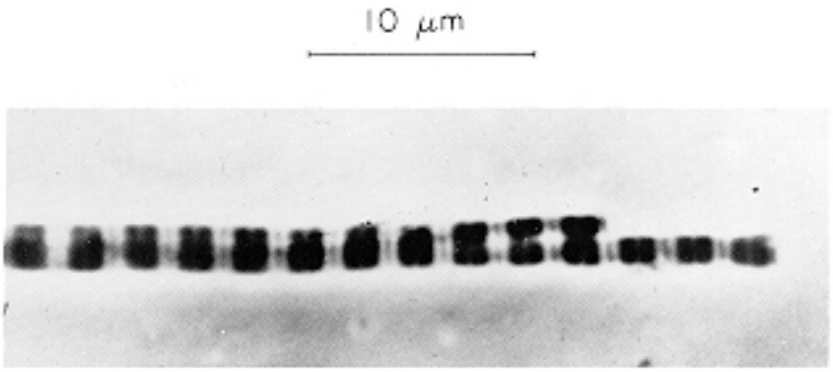
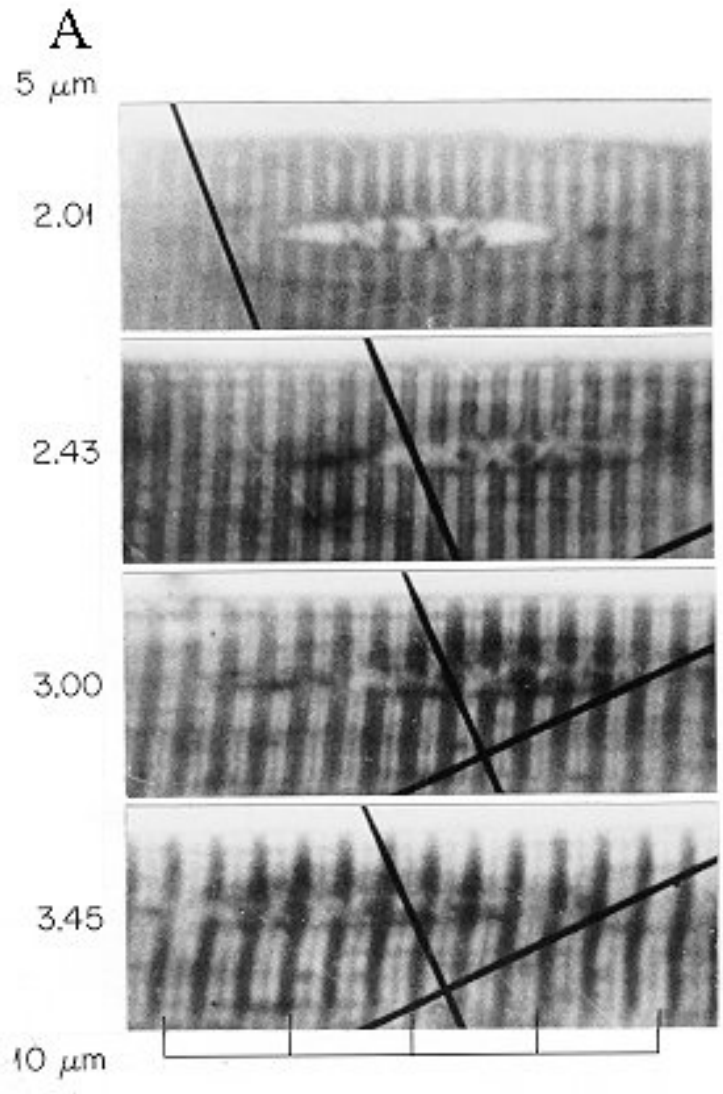


Fig. 4 Isolated myofibril from rabbit psoas muscle (glycerinated) in phase-contrast illumination with positive contrast. Reproduced from Hanson and Huxley (1955), with permission.

The changes occurring in isolated muscle fibers during contraction and stretching may well approximate the events occurring in the living muscle. Observations with the light microscope provide some insight, posing the problem in a more meaningful manner. The results obtained with the interference microscope are shown in Fig. 5 (Huxley and Niedergerke, 1954). In this figure, the sarcomere lengths under the different conditions are shown at the left. The sarcomere length is about 2 to 3 μm at rest. The *A* band remains largely unchanged by stretching (Fig. 5A) or contraction (Fig. 5B) of the muscle fiber. However, the *I* band is wider when the fiber is stretched, and it is narrow when the myofibril contracts. A model capable of explaining the basic organization of the muscle fibril should be able to explain these observations.

First, some knowledge of the components of the fibers and how they are put together is necessary. Extraction of the fibers by different procedures can provide a good deal of information. The myofibrils are composed largely of proteins. The solubility properties of the major protein components are fairly well known, making it possible to extract selectively one protein at a time. Myosin makes up about 55%, actin about 20%, tropomyosin about 5%, and troponin about 3 to 4% of the fiber.



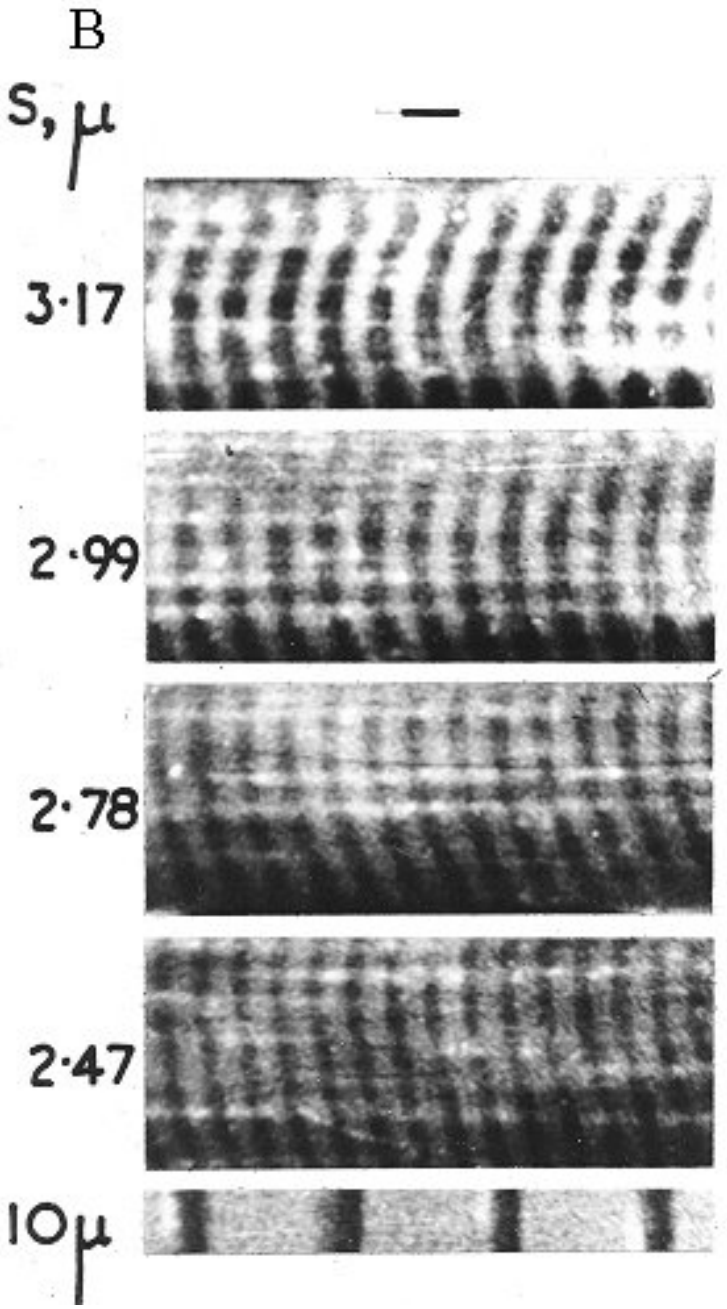


Fig. 5 Sarcomere lengths indicated beside the photographs. Almost all the change of length is in the *I* bands (light). A. Passive stretch of a muscle fiber, positive contrast (*A* bands, dark). B. Muscle fibers during isotonic tetanus (fiber is maintained at constant load and stimulated at high frequency) From Huxley and Niedergerke (1954). Reproduced by permission from *Nature* 173:971-973, copyright ©1954 MacMillan Magazines Ltd.

We have seen that myofibrils extracted with glycerol can still contract when ATP is added to the medium. The glycerinated system seems to be a suitable system to analyze because most irrelevant components have probably been removed. Myosin can be extracted differentially with 0.6 M KCl, 0.01 M pyrophosphate and 10 mM $MgCl_2$. The material treated in this fashion loses its *A* bands (Fig. 6) (Hanson and Huxley, 1955). Treatment with Kl, on the other hand, removes the actin and the *I* band simultaneously. These results indicate that the *A* band corresponds predominantly to myosin and the *I* band to actin. It is likely that other components are also present; the fact that the

organization of the remaining components is not disrupted when the bands are extracted speaks for the persistence of some other component.

Electron micrographs of longitudinal sections of muscle show that the sarcomeres are made up of thick and thin fibers that are interdigitated (Fig. 11a) (Huxley, 1960). The thick filaments are predominantly in the *A* bands and the thin filaments in the *I* bands. On the basis of the differential disappearance of the bands with the extraction procedures, we can assign myosin to the thick fibers and actin to the thin fibers. Such sarcomeres can be represented diagrammatically as shown in Fig. 7b, right hand.

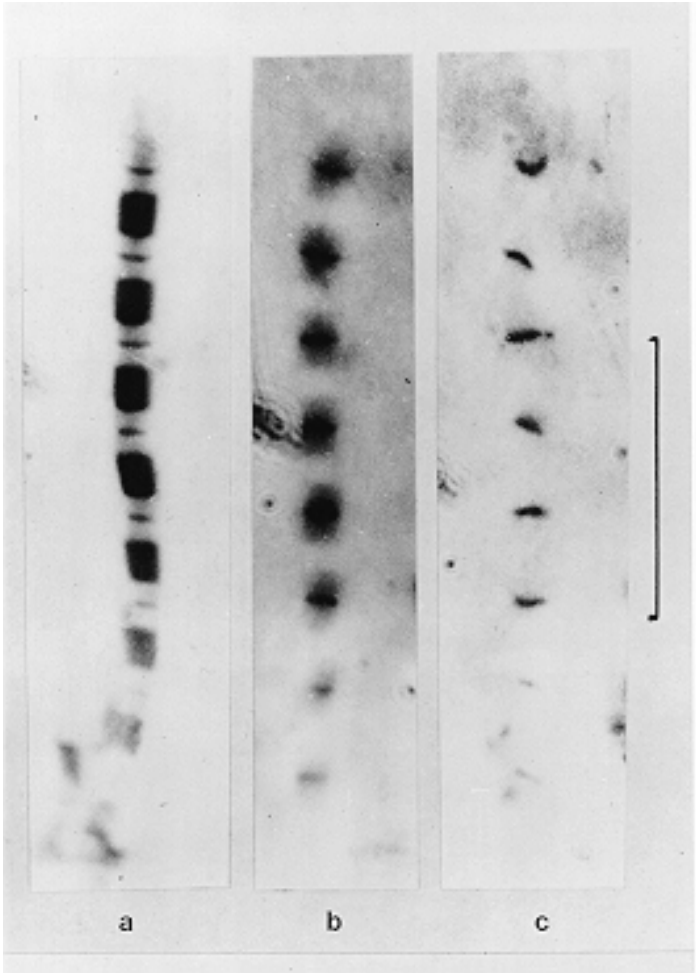


Fig. 6 Isolated myofibrils from glycerinated rabbit psoas muscle in phase contrast illumination: (a) intact, (b) after actin extraction, (c) after myosin extraction. A large amount of the secondary material disappears when actin extraction takes place, leaving behind the Z zones connected together by some residual backbone substance. Bar corresponds to 10 μm. Reproduced from (Hanson and Huxley, 1955), with permission.

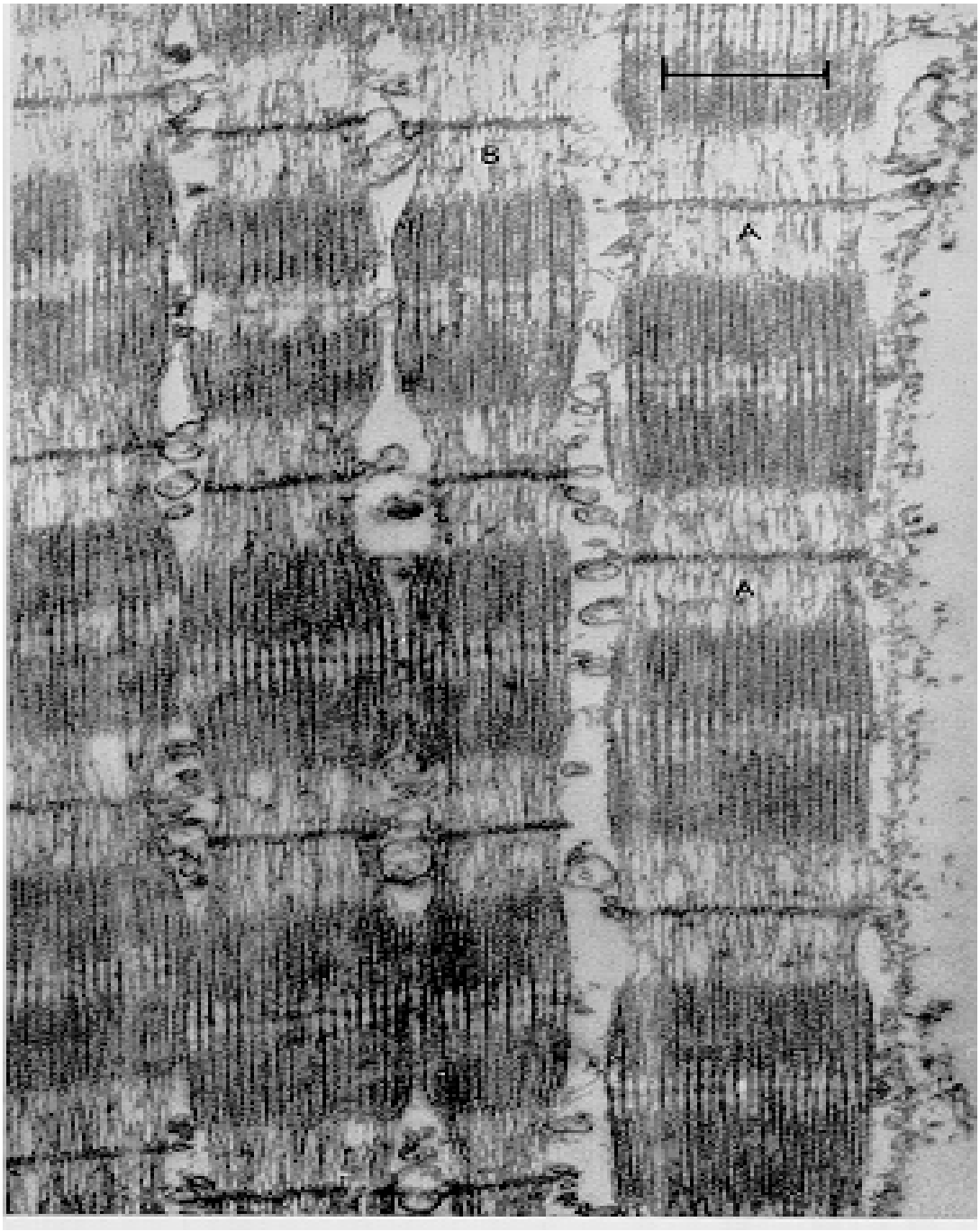


Fig. 7 Electron micrograph of a section through part of a muscle fiber; the hexagonal arrays of filaments in different myofibrils and in different sarcomeres have their axes oriented in a variety of directions. Bar corresponds to 350 nm. Reproduced from (Huxley, 1960), with permission.

The interdigitating structure of the sarcomere fibers suggests that contraction could take place if the thin filaments slide over the thicker filaments, shortening the sarcomere. The *I* band would then be shortened with no change in width of the *A* band, as was observed. This proposal is known as the sliding-filament model of muscle contraction. Such effects should be visible with the electron microscope in sections perpendicular to the long axis of the fiber. Figs. 8 (Huxley (1960) and 9 (Carlsen et al., 1961) show that electron microscopic observations of thick sections are consistent with this view. Figure 8a illustrates a stretched muscle and Fig. 8b a resting muscle. Figure 9 shows a preparation that was fixed at rest (Fig. 9a) or in the contracted state where the myofibril is held at

constant length (Fig. 9b). Figure 9c shows a contracted myofibril. The sliding is thought to correspond to a rearrangement of the bonds between thick and thin filaments at the bridges visible with the electron microscope (Fig. 10) (Huxley, 1960)).

Many studies have suggested additional complexities not yet fully understood. Nevertheless, the events that now need to be described in terms of molecular rearrangements and forces, are sufficiently known to permit the construction of realistic molecular models.

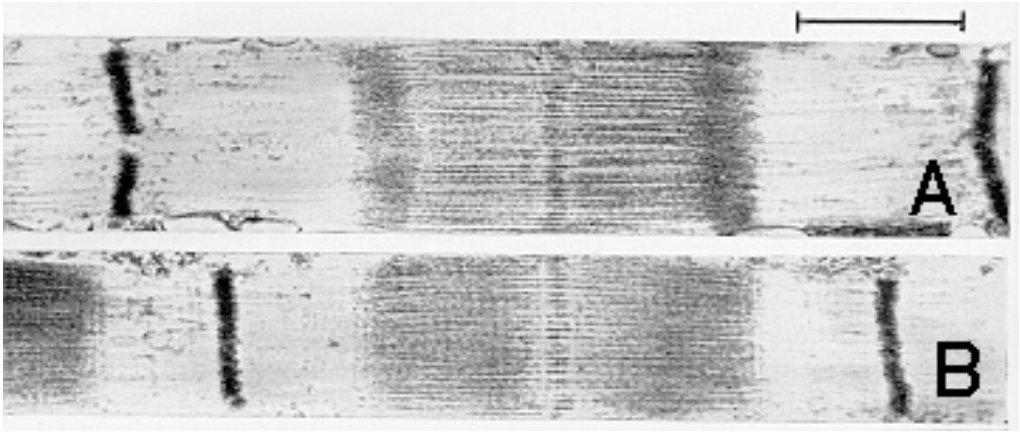


Fig. 8 Changes in band pattern at different muscle lengths as seen in the electron microscope (thick sections) oriented for sectioning so as not to foreshorten band lengths. Bar corresponds to 670 nm. (a) Stretched muscle showing long *I* bands and *H* zone. (b) Rest length muscle, showing decrease in length of *I* bands and *H* zone and constancy of length of *A* band. From (Huxley, 1960), reproduced with permission.

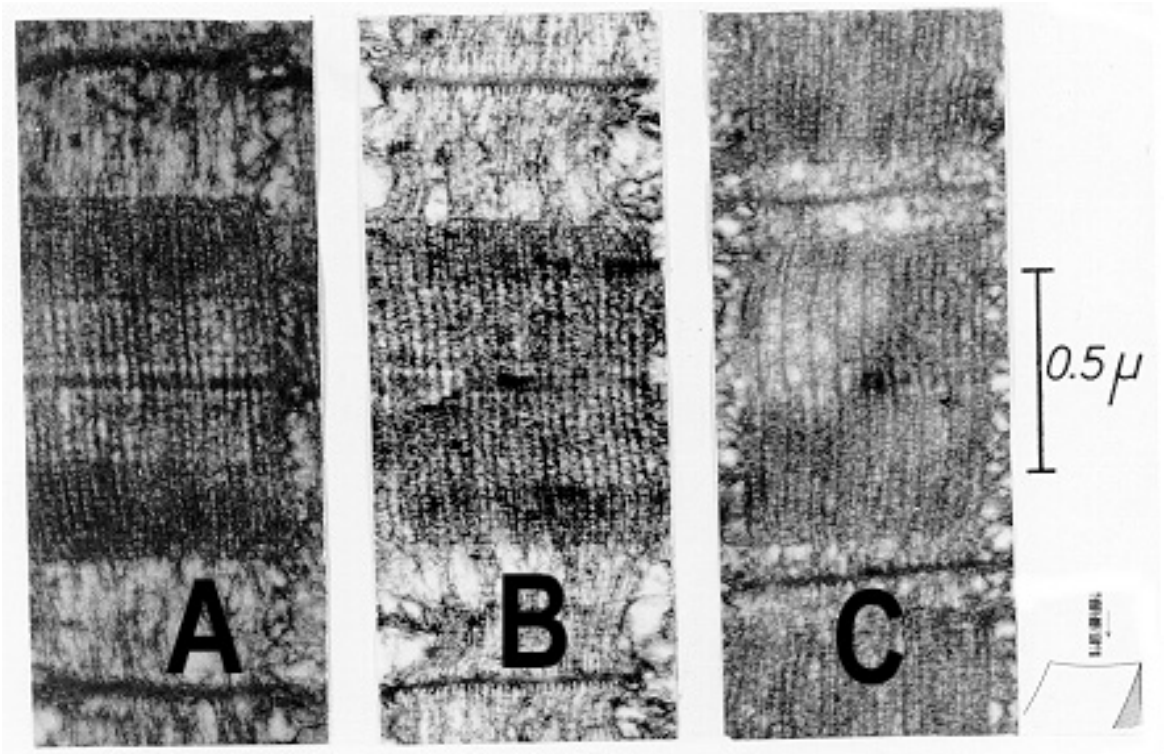


Fig. 9 Electron micrographs of (a) sarcomere at rest, (b) sarcomere contracting but held at constant

rest length (isometric contraction), and (c) sarcomere shortening 20%. The insert on the right shows the orientation of the myofibril in relation to the sectioning glass knife. From Carlsen et al. (1961), reproduced with permission.

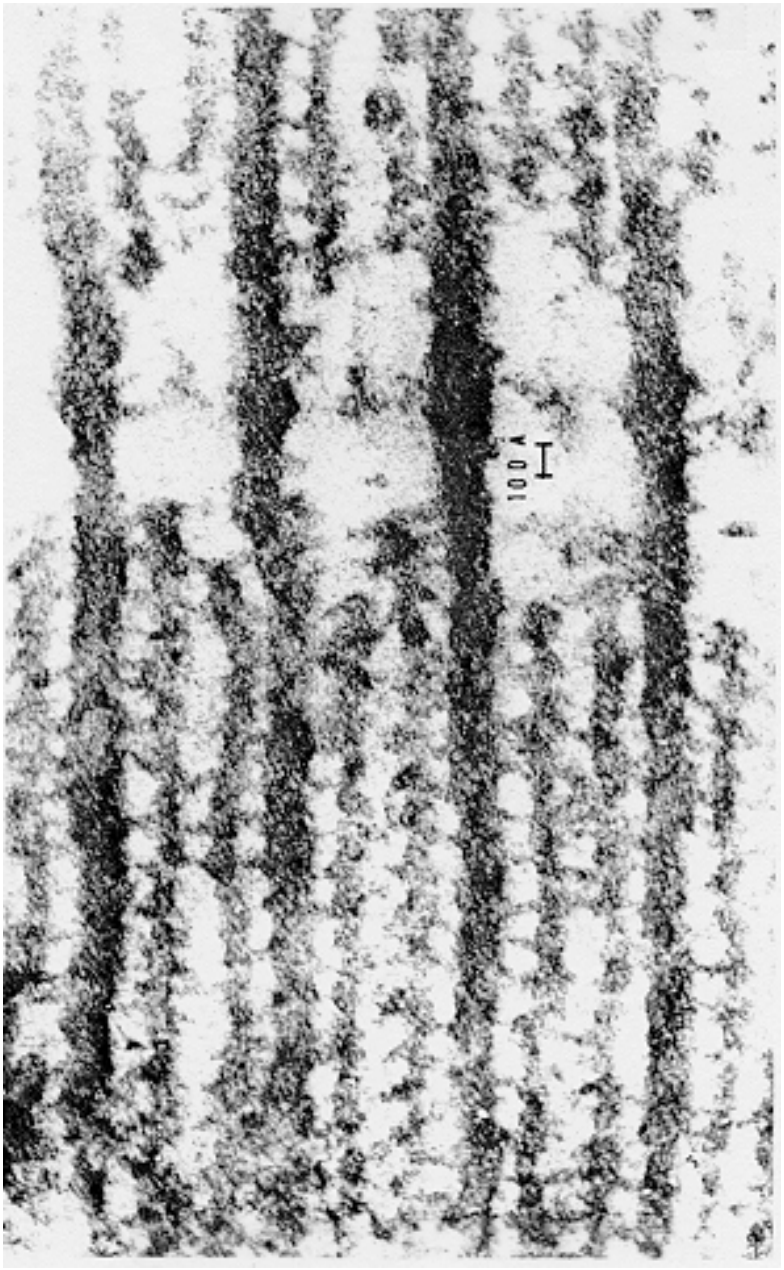


Fig. 10 Highly magnified electron micrograph through *A* band of myofibril from rabbit psoas muscle, showing the system of cross connections between the large and small filaments. x418,000. From Huxley (1960), reproduced with permission.

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

III. CILIA AND FLAGELLA

Motile cell processes are known as cilia and flagella. Generally, they are called cilia when they are short and numerous, and flagella when they are long in relation to cell size and few in number. Although the motion of flagella is frequently undulant and that of cilia pendular, this is not always the case.

Cilia may form a composite of many shafts. The shafts may have their own individual membranes (as in the compound cilia of some ctenophores and protozoans). Rarely (as in the ctenophore *Beroe*), they are enclosed by the same membrane.

Cilia and flagella are fundamental to the motility and feeding activity of many unicellular organisms. In more complex organisms they play basic roles in respiration, circulation, digestion, and reproduction.

The processes are usually attached to specialized structures -- the basal bodies -- which can vary considerably in structure. Throughout completely unrelated phyla, the internal arrangement of the component parts of cilia and flagella is constant, and the basic mechanism responsible for motility is the same.

So far, all cilia and flagella studied have nine pairs of longitudinal tubules, the doublets, which are arranged around two central tubules. The central tubules are not always present and may not be essential. The component tubules of the doublets differ and are known as A and B to distinguish them (see Fig. 11). Together, the tubular components of a cilium or a flagellum are known as the *axoneme*. Coarse fibers are also present in vertebrate sperm, but their function is likely to be only indirectly related to motion. The ubiquity of the peripheral tubules suggests that they are involved in movement.

Fig. 11 (Johnson, 1985) summarizes the structure of an axoneme of a cilium. It represents a reconstruction based on electron micrographs of intact and disrupted cilia. The structures labeled D correspond to the arms that have been found to be *dynein* complexes, which are attached to the A tubule of a doublet and are in a position to interact with the B tubule of an adjacent doublet.

As we saw previously, flagellar motion is powered by the hydrolysis of ATP. ATPase activity has also been demonstrated with the electron microscope by precipitating the phosphate produced by the hydrolysis of ATP with a heavy metal. ATPase activity shown by these studies appears to be localized in the peripheral tubules (Nelson, 1958).

When a protein fraction with ATPase activity is extracted from cilia of *Tetrahymena*, the "arms" of the

tubules, i.e., the dynein complex (Fig. 12) (Gibbons, 1963), are also removed along with the ATPase activity. When the Mg^{2+} and soluble factors are replaced after the extraction the typical ciliary structure with arms is reconstituted.

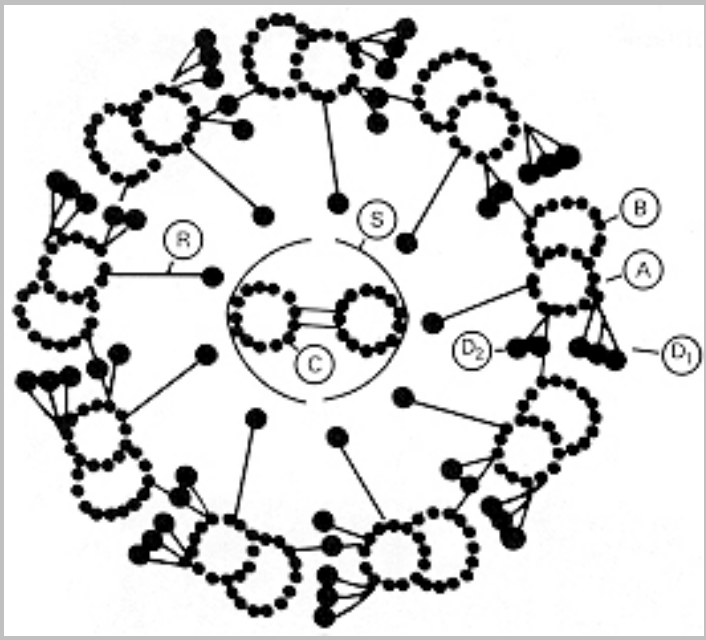
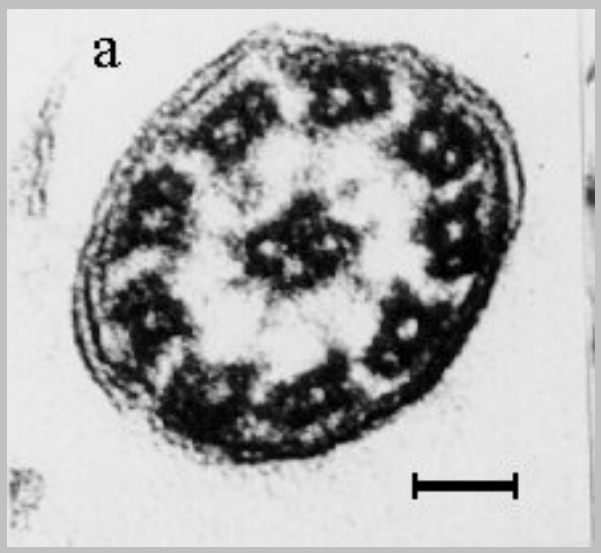


Fig. 11 Cross-sectional view of a ciliary axoneme represented schematically. D_1: outer dynein arm; D_2: inner dynein arm; A: A tubule; B: B tubule; C: central tubule; R, radial spoke; S: central sheath. From Johnson, 1985. Reproduced, with permission, from the *Annual Review of Biophysical Chemistry*, Volume 14, 1985 by Annual Reviews Inc.



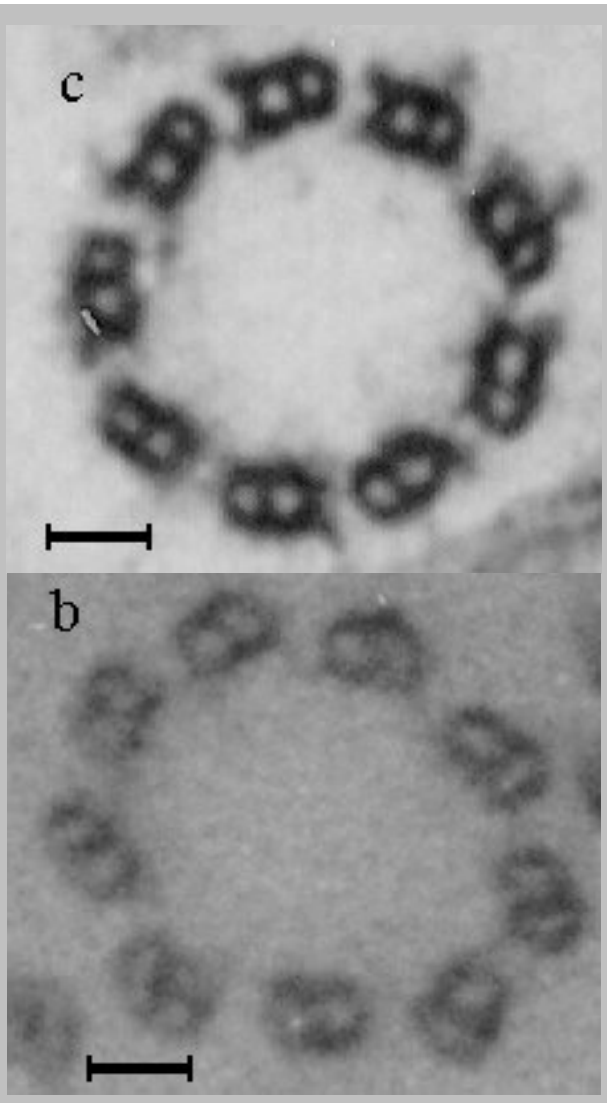


Fig. 12

(a) Freshly isolated cilia,(bar corresponds to 59 nm).
(b) Structures remaining after extraction (bar corresponds to 128 nm).
(c) reconstitution of the fraction (bar corresponds to 62.5 nm).

Selected views from Gibbons, 1963, reproduced by permission.

One would think that during motion the cilium must bend. Observations with the electron microscope (Horridge, 1965; Satir, 1968) suggest that the tubules in cilia do not buckle or become deformed; they may be sliding in relation to each other. The sliding out of a tubule preferentially on one side of the cilium could produce a bending in that direction, as shown in Fig. 13 (Satir, 1968). Detailed analyses of the motion of flagella and cilia tend to agree with a mechanism based on sliding filaments (Bryan and Wilson, 1971; Warner and Mitchell, 1981).

A sliding-filament mechanism is supported more directly by a variety of experiments carried out with isolated components containing the axonemes prepared from the flagella. Fig. 14 shows results obtained with sea urchin (*Tripneustes gratilla*) sperms. The sea urchin sperm axonemes treated with trypsin remain

largely intact, although they have become detached from other structures. Addition of ATP causes the axonemes to dissociate. The process can be followed by dark-field light microscopy (in which the light reflected by the specimen is observed). The axonemes elongate by a process in which the tubules appear to be extruded. These observations are shown in Fig. l4a and b (Summers and Gibbons, 1971). In Fig. l4a the successive photographs represent progressive changes. The markers show a stationary position in the field. Apparently, part of the axoneme is stuck to the slide and does not move from its position next to the lower stationary marker. However, some of the filaments move in relation to the upper marker. This result indicates that the tubules are sliding in relation to each other.

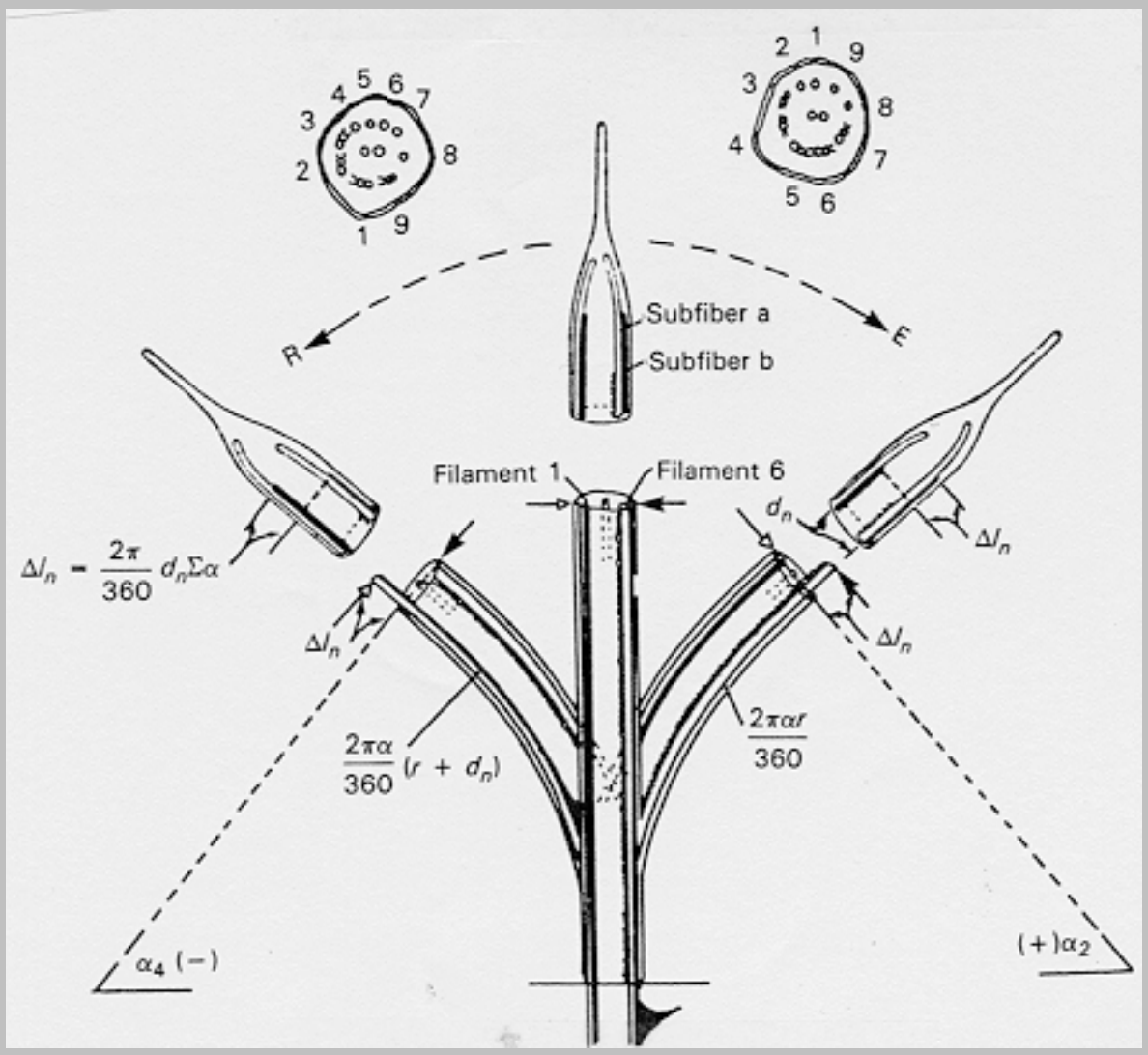


Fig. 13 Sliding-filament model of ciliary motility. Behavior of two doublet peripheral filaments (1 and 6) at the tip and base is illustrated when a cilium is bent to either side of a straight position (center). In the neutral position subfibers *b* of the filaments end together at one level at the tip (i.e., are equally long) while subfibers *a* continue onward as naked singlet microtubules to different termination points (i.e., not equally long). The arrows with open and solid arrowheads mark equal shaft lengths from the basal plate (base line) on projections of the two filaments in the plane of the diagram (plane of beat). When the cilium bends, a circular arc arises at the base. A cross section through the tip at a level where some of the filaments are doublet and some are singlet is shown for both E and R cilia at the top of the diagram. The eye indicates that the view in both cases is from the abfrontal (effective) side of the cell. Axis tilt is neglected.(From Satir, 1968. (&copy1968). Reproduced from *The Journal of Cell Biology*, by copyright permission of the

Rockefeller University Press.

The electron micrograph of a similar preparation from *Tetrahymena* is shown in Fig. 15 (Warner and Mitchell, 1981). The tubules, originally aligned, slid in relation to each other in the direction of the arrow after the addition of MgATP. This micrograph also implicates the dynein cross-bridges, which form the connection between the doublets.

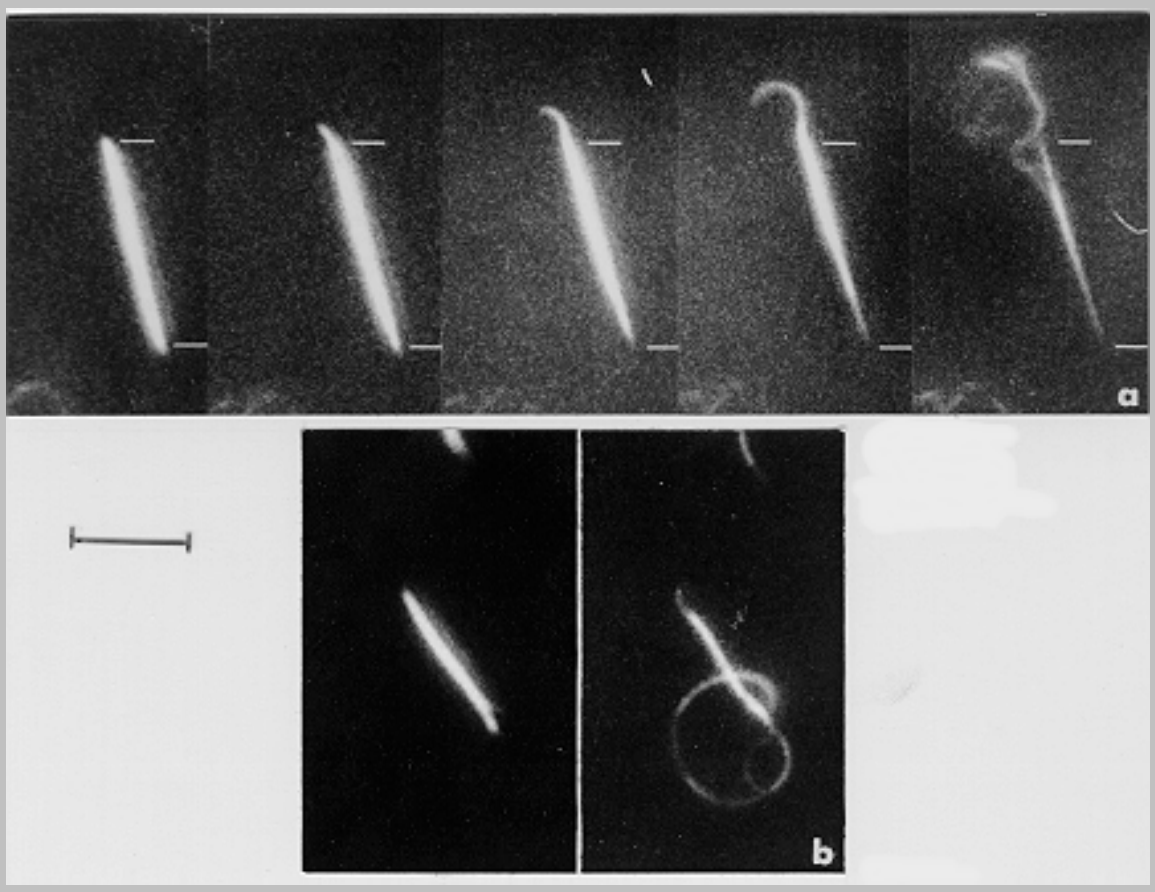


Fig. 14 Dark-field light micrographs of trypsin-treated axonemes after the addition of ATP. Bar corresponds to 8.7 μm. (a) Each successive micrograph was taken after 12 to 30 s intervals. (b) Initial and final frames in an experiment similar to that of (a). In this preparation, a group of tubules slide down the attached tubules and loop around, forming a circle of tubules. The final figure is more than three times longer than the original fragment. From Summers and Gibbons, 1971, reproduced with permission.

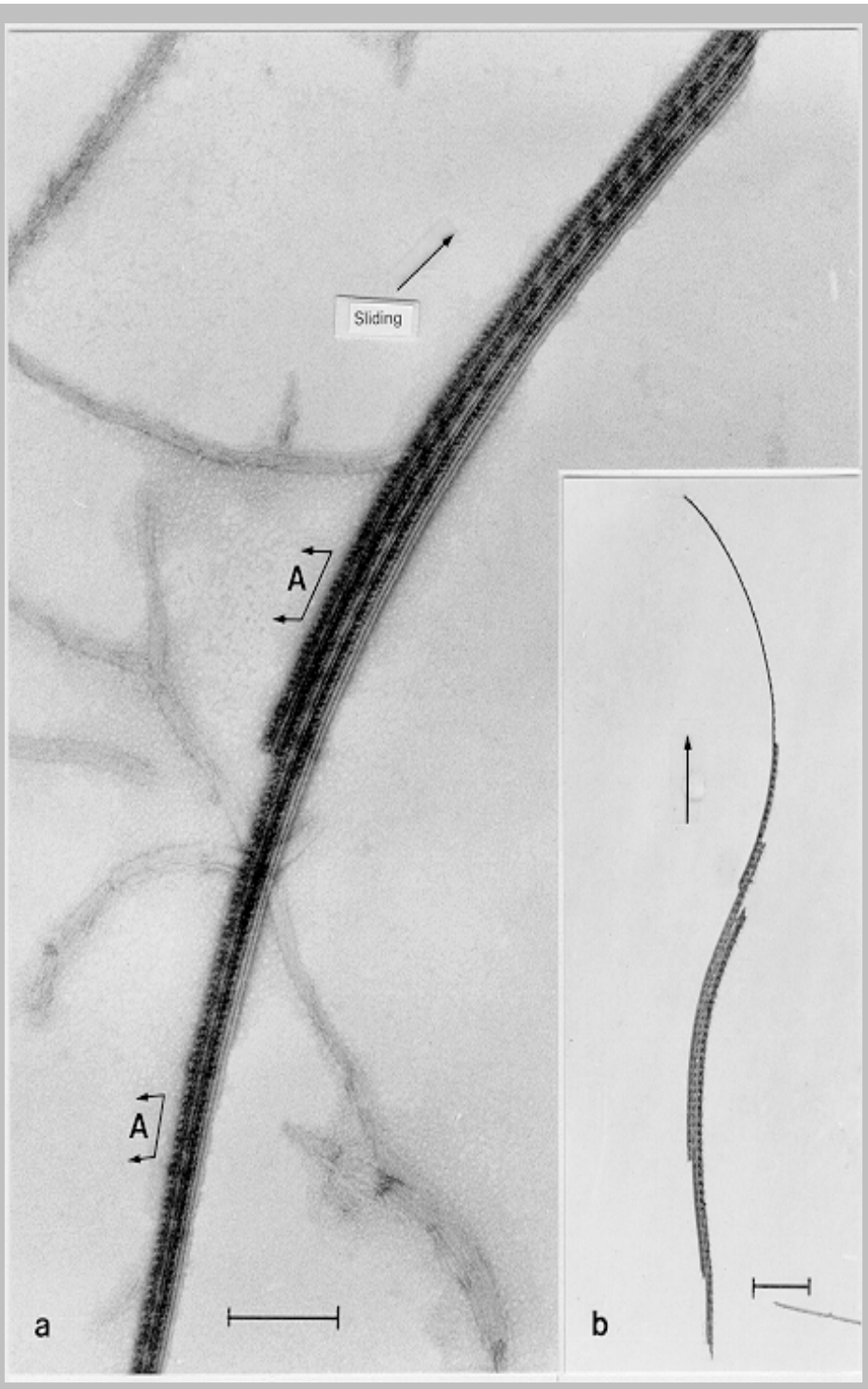


Fig. 15 Isolated *Tetrahymena* cilia reactivated with 0.1 mM MgATP to cause sliding disintegration. Typical sliding figures are recognized as partially overlapping doublets cross-bridged by the dynein arms. Free dynein arms are polarized and tilt uniformly toward the base of the cilium (bracketed arrows), away from the direction of active sliding. (a) Bar corresponds to 390 nm;(b) bar corresponds to 8.3µm. From Warner and Mitchell, 1981 (&copy1981). Reproduced from the *Journal of Cell Biology*, by copyright permission of the Rockefeller University Press.

IV. MOVEMENT IN OTHER SYSTEMS

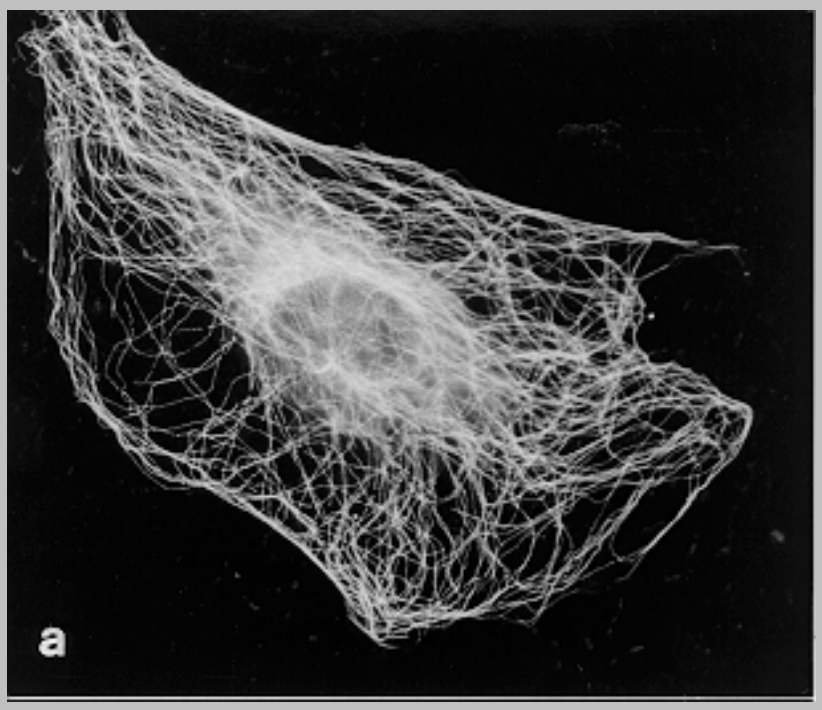
In many cases of cell movement the relationship between structure and function is less obvious than for

the cases already discussed. Frequently, newer approaches and techniques were needed to provide the necessary information.

Movement displaces mass vectorially. A direction can be imposed if the displacement occurs along a linear structure. Not surprisingly, linear elements frequently containing actin or tubulin have been found to be associated with cell movement. In many cases, these molecules have been shown to be present in linear structures by *immunofluorescence*. In immunofluorescence, antibodies conjugated to fluorescent dyes are used to identify or trace proteins. Antibodies to the appropriate purified protein (e.g., tubulin or actin) may be labeled. The structures to which they bind appear fluorescent when viewed with the appropriate microscope. In indirect immunofluorescence, the antibody is not labeled and a second fluorescent antibody capable of binding to the first (a different species can be used to produce this second antibody), is used to locate the antigen.

In mouse 3T3 fibroblasts (Fig. 16a) microtubular bundles seem to radiate from the nucleus. On the other hand, actin bundles seem to be distributed longitudinally (Fig. 16b). The pattern differs significantly with the cell type, and the intricacies of detail and arrangement of the individual fibers can be examined only with the electron microscope. The emergence of the use of high-voltage electron microscopy of thick sections, tilting of sections, and computer-aided image processing, provides a wealth of detail (see Chapter 1).

The arrangement of fibers in the cytoplasm has been seen as a three-dimensional network, the so-called *cytoskeleton*. In many respects this term is a misnomer because the system not only is not rigid but it changes from moment to moment. A more detailed discussion of movement in various cells is presented below.



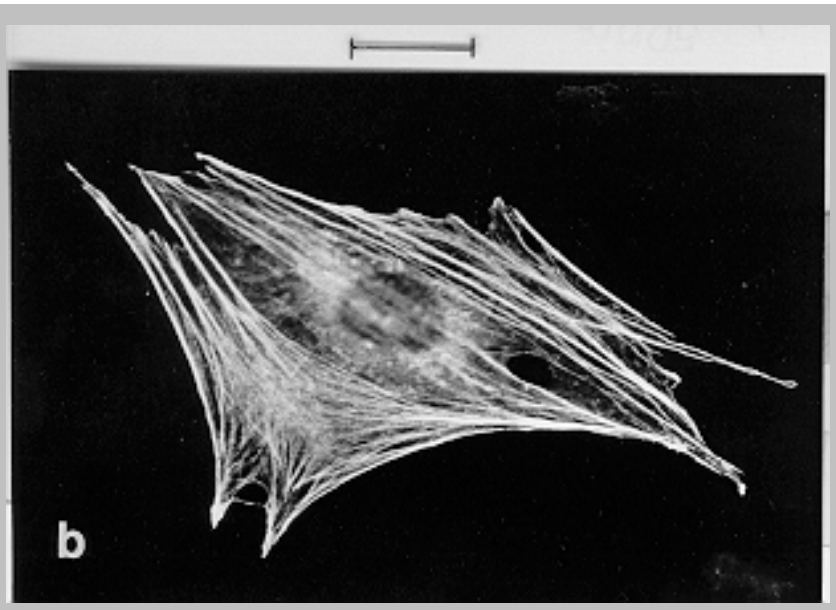


Fig. 16 (a) Distribution of microtubules in mouse 3T3 cells as visualized by immunofluorescence. Microtubules appear as an intricate network of fine fibers radiating from the region of the nucleus. (b) Distribution of microfilaments in mouse 3T3 cells as visualized by immunofluorescence. Microfilaments form bundles that lie parallel to the long axis of the cell. Bar corresponds to 23 μm. From Osborn and Weber (1977), reproduced with permission.

A. Plant Cells

The cytoplasm of some plant cells flows between an ectoplasmic static layer and the central vacuole in a rotational manner (cyclosis), as shown in Fig. 17 (Hayashi, 1964). A pair of indifferent zones in which there are no chloroplasts separates the two opposing streams.

A velocity profile of particles present in the cytoplasm of the internodal cells of *Nitella* is very revealing (Fig. 18) (Kamiya and Kuroda, 1956). The bulk of the endoplasm flows at a constant rate regardless of the location. The ectoplasm does not flow at all. These results differ from those found for slime mold (see below), and they suggest that the motion is the result of some interaction in a region between endoplasm and ectoplasm. In experiments in which the chloroplasts have been detached or removed, it is possible to observe longitudinally arranged fibers (Kamiya and Kuroda, 1957). These fibers disappear when the motion is arrested by the passage of an electrical current, and they return after motion is reestablished. Therefore, the fibers are likely to be involved in movement.

B. Slime Molds

Acellular slime molds are easy to manipulate and give a good rate of cytoplasmic flow. The cytoplasm flows in channels (endoplasm). The direction of flow can reverse rhythmically. Streaming cytoplasm appears more fluid and less gel-like than the surrounding cytoplasm (ectoplasm). The two cytoplasmic states are dynamic, and transitions from one form to the other may occur rapidly.

The system behaves as if it were in a state of tension, since cutting a channel produces a spurt of material.

Movement can be interpreted as an increase in tension at some site accompanied by a weakening of gel-like structures where new channels are formed. The movement of the endoplasm appears to be completely passive. This has been demonstrated in experiments using an apparatus in which two separate chambers are connected by a small tube and the pressure in the two chambers can be readily manipulated. The normal cytoplasmic flow shows the velocity gradient represented in Fig. 19 (Kamiya and Kuroda, 1958). The pattern of flow is identical whether the flow is endogenous (Fig. 19a) or imposed by an external pressure (Fig. 19b). The results can therefore be interpreted in terms of models in which contraction occurs at a site other than the endoplasm and the passive flow is a result of the increase in pressure.

Since the hydrolysis of ATP is likely to be involved in motion and fibrillar structures must be involved for directional movement, it may be particularly important to examine the distribution of both ATPase activity and fibers. The ATPase activity visualized by the precipitation of inorganic phosphate formed from ATP hydrolysis appears in the ectoplasm and, more specifically, where the fibers are located.

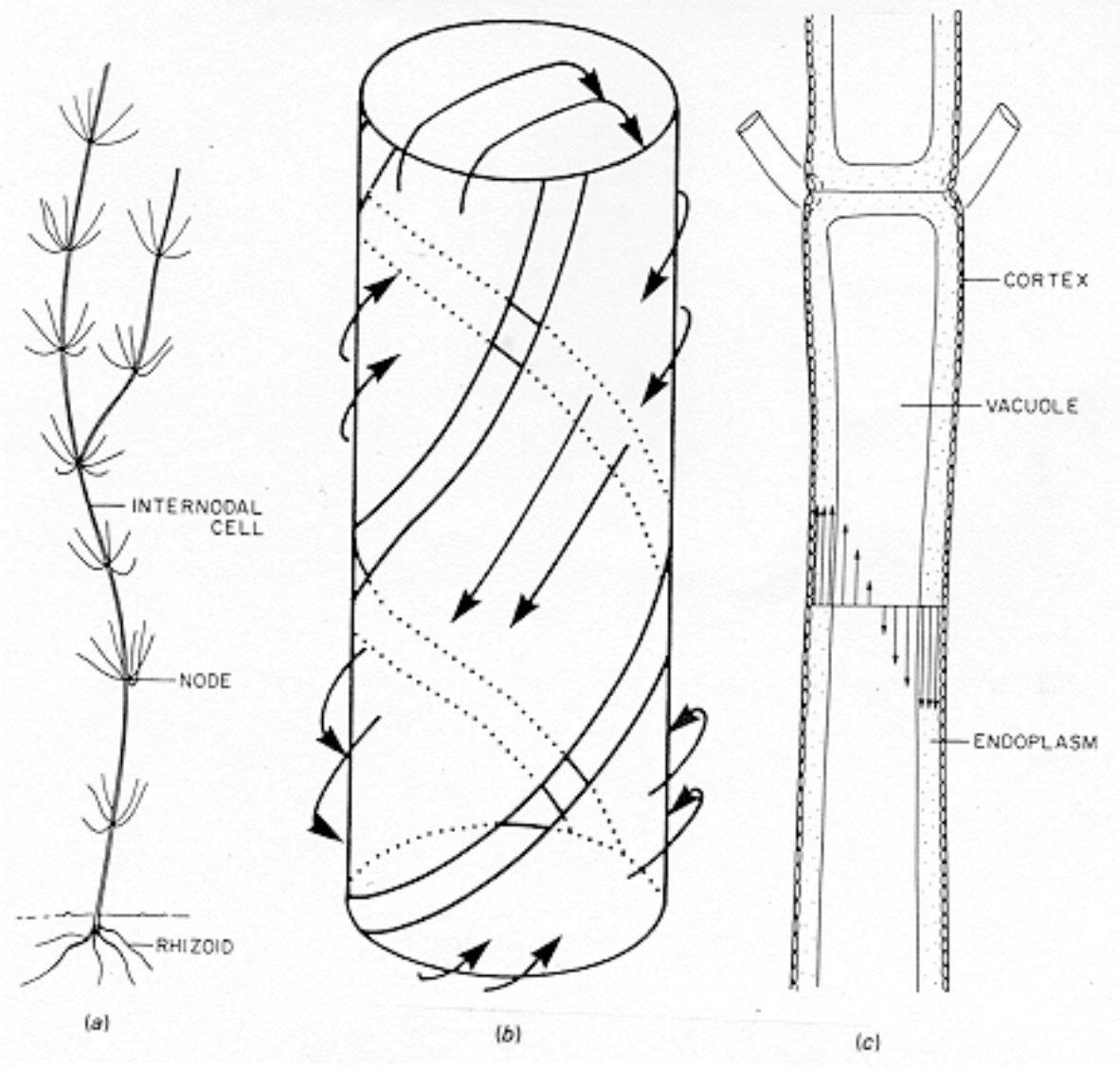


Fig. 17 (a) The whole plant of *Chara braunnii*. The internodal cells are about 0.5 to 2 cm long. (b) Diagram illustrating the flow in the intact internodal cell. (c) Longitudinal section of (b). In part from T. Hayashi, *Primitive Motile Systems in Cell Biology*, with permission. Copyright &copy1964 Academic

Press.

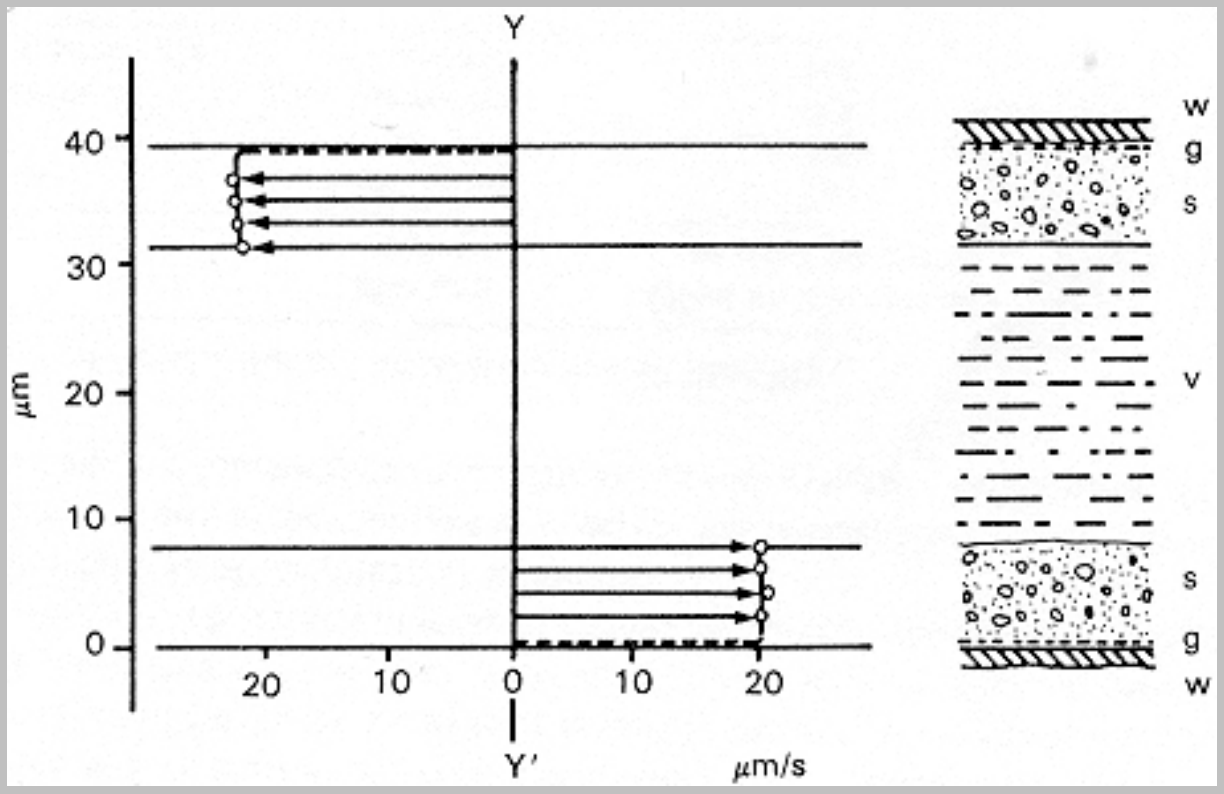


Fig. 18 Velocity distribution of the rotational streaming in a rhizoid cell of *Nitella flexilis*. Part of the rhizoid cell as seen under the microscope is shown on the right: w, cell wall; g, plasmagel layer (ectoplasm); s, flowing plasmasol (endoplasm); v, vacuole. From N. Kamiya and K. Kuroda, *Botanical Magazine*, 69:544-554, with permission. Copyright &copy1956 Botanical Society of Japan, Tokyo.

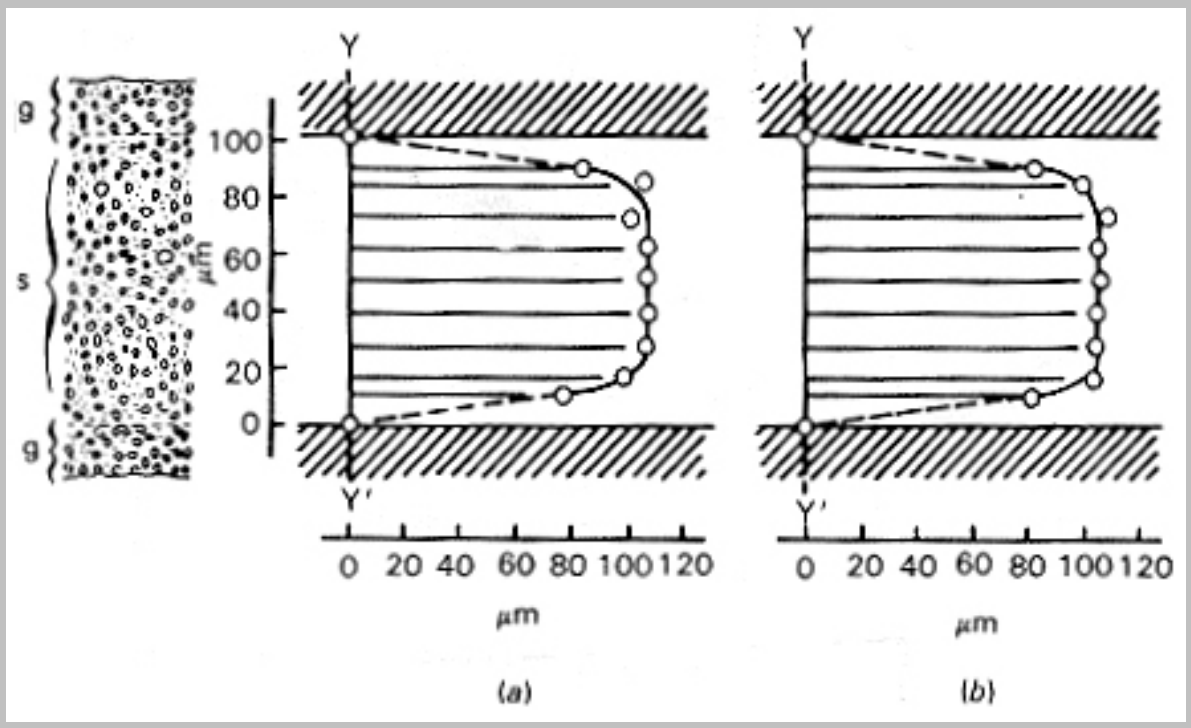


Fig. 19 Velocity distributions of the protoplasmic streaming in a strand of the plasmodium. Circles show

positions of the granules that were in cross sections *YY'* 3 s ago. (a) Spontaneous streaming under natural condition. (b) Artificially induced streaming when pressure difference of 15 mm of water was established between the two ends of the strand 5 mm long. Microscopic view of part of the strand is shown on the left; g, plasmagel forming the wall of the capillary tube; s, plasmasol involved in streaming. From N. Kamiya and K. Kuroda, *Protoplasma*, 49:1-4, with permission. Copyright &copy1958 Springer-Verlag, Heidelberg.

Slime molds have remarkable properties and various experimental manipulations can be carried out that are difficult or impossible with other organisms. The cytoplasmic threads can be hung up and shown to flow in the direction of gravitational pull to form a droplet. In this case, little work is performed to maintain tension (Wohlfarth-Bottermann, 1964). On the other hand, the flow can oppose the pull of gravity so the system must be performing work. Fibers appear only when work is performed. The endoplasmic fibers are oriented in the direction of flow, whereas the ectoplasmic fibers associated with ATP hydrolysis are perpendicular to the direction of flow. Results consistent with this are obtained in the study of birefringence of the threads. When fibrous material is oriented all in one direction, the threads act as a polarizer and polarize light. This birefringence can be detected with the appropriate polarizer inserted in the microscopic system.

Tension could be developed by contraction of the fibers that are anchored to the ectoplasm and laid across the endoplasmic tube, producing the pressure described above and the flow in the channels of the more fluid endoplasm. The mechanism of the contraction could be similar to the sliding-filament mechanism. Perhaps filaments temporarily oriented in a particular direction slide in relation to each other.

C. Crawling

Many kinds of cells, including ameba, fibroblasts, macrophages, epithelial cells and embryonic neurons, move by a process which has been referred to as *crawling* (see Mitchison and Cramer, 1996; Heidemann and Buxbaum, 1998). These cells move in response to surface stimulation whether it be from soluble factors or from extracellular matrix components. Consequently in metazoan organisms, crawling plays a fundamental role in embryonic development, inflammatory immune response, wound repair as well as tumor formation and metastasis (see Lauffenburger and Horwitz, 1996).

Crawling involves basically four stages: (a) extension of the leading margin, (b) attachment of the new extension to the substratum through a special contact attachment, the *focal contact* sites, (c) presumably a contraction, which draws the cell forward using the point of attachment as an anchorage, and (d) detachment of the cell from the focal contact site at the tail end of the cell. The last two stages may be missing in cells or structures that remain in place. The extensions of the cells may be as *filopodia*, protrusions that appear as thin cylinders. Filipodia have been studied mostly in neuronal growth cones but are also present in other cells. Growth cones of neurons are the ends of axons that are growing during development and extending away from the cell body. However, generally, the extensions at the leading margins of cells are not filipodia, but thin lamellae that extend in the direction of the movement. Lamellae (the thin sheet-like portions are the *lamellipodia*) may have a smooth or serrated anterior edge. Temporary pulling back of the lamellopodia frequently makes them rise up over the dorsal surface of the cell. This

produces an apparent flickering referred to as *ruffling*. Typical lamellipodia are shown in Fig. 20 (Heath and Holifield, 1991). The upper view is a light micrograph using phase contrast (see Chapter 1). The bottom view corresponds to that obtained with a scanning EM (see Chapter 1). In Fig. 21 (Heath and Holifield, 1991) the image obtained with DIC (part A) is compared with the fluorescence of rhodamine phalloidin which stains actin. The spikes referred to as *microspikes* (or *ribs*) show considerable fluorescence indicating a large presence of actin. They are analogous to filopodia. Ameboid cells have larger protrusions, the pseudopods. We are beginning to gain insights into all these processes. However, only a few will be dicussed here.

The cell adhesion protein CD44 and its main ligand hyaluronic acid (HA) (both ECM components, external to the cell) (see Chapter 6) have a role in cytoskeletal rearrangement. The local application of HA to CD44 in mouse mammary epithelial cells in culture promotes the formation of lamellopodia in the direction of the stimulus. The process is inhibited by pretreatment with monoclonal anti-CD44 antibodies or dominant negative recombinant mutant Rac1. These results can be interpreted to mean that process initiates a cascade involving the small GTPase, Rac1 and resulting in actin rearrangement and reorientation of cells (Oliferenko et al., 2000).

The lamellipodia reversibly attach to the substratum. The focal contacts are frequently associated with actin bundles, the *stress fibers*. In fibroblasts, the extracellular proteins *fibronectin* and *vitronectin* bind cell surface receptors and are responsible for the attachments. Like fibronectin, vitronectin is a multifunctional glycoprotein present in blood and in the extracellular matrix (see Euteneuer et al., 1999). Contractions which propel the cell body forward are thought to occur at the junction of the lamella to the cell body and at the rear of the cell. Most of the features of crawling are exhibited by isolated lamellae which are capable of directed movement (Malawista and De Boisfleury Chevance, 1982; Euteneuer and Schliwa, 1984), indicating that they have all the components needed for locomotion. Similarly, when an ameba is split inside a capillary, flow occurs in the front. In addition, the heat released is greater at the front end of the ameba. In contrast, suction at the rear end of an amoeba does not interfere with the flow in the pseudopods (Allen et al., 1971). However, the forward movement of the cell body is a separate event from the formation of lamellipodia, as shown by movement in the absence of forward protrusion (Anderson et al., 1996).

As a cell migrates, the focal complexes at the front become larger and more organized to form the focal adhesions that serve as points of traction. Release of the adhesions at the rear, allow the cell to be displaced in the forward direction. Focal adhesions are highly motile in stationary fibroblasts but stationary in motile cells (Smilenov et al. 1999).

Current thought proposes that adhesive complexes form at sites at which small clusters of ligand bound integrins have assembled. These sites assemble structural and signaling molecules with a distinct chronology. Presumably, these complexes proceed to form larger complexes (e.g., see Yamada and Miyamoto, 1995) . Another alternative scenario proposes that integrins associate to preassembled cytoskeletal elements (Izzard, 1988).

The dynamics of the assembly and disassembly of adhesions have been followed using proteins fused to the green fluorescent protein (GFP) (see Chapter 1). α 5 integrin-, α-actinin-, and paxillin-GFP were used (Laukaitis et al., 2001) (see below). After the binding of integrins at the cell surface to its ligand, the formation of the focal adhesion complex include activation of focal adhesion kinase (FAK) and protein-protein interactions between focal adhesion components. Paxillin binds to FAK (see Hanks and Polte, 1997) and is phosphorylated upon integrin activation. In the study of Laukaitis et al. (2001), the paxillin was shown to assemble at new protrusion sites from older adhesions at the leading edge. Although, integrin could not be demonstrated, possibly because present at very low concentrations at these sites, it was required since anti-integrin antibodies blocked the assembly of paxillin. The binding of paxollin was followed by the appearance α-actinin. The adhesions were translocated to the cell center and the paxillin turnover was inhibited. Then, α 5 integrin associated with the complex . α 5-GFP was also found in endocytic vesicles forming at the leading edge of protrusions. As cells migrated, α5 integrin vesicles moved from a perinuclear region to the base of the lamellipodium. During the detachment at the rear, α 5 integrin-GFP was found in fibrous structures attached to the ECM behind the cells. Instead, α-actinin-GFP and paxillin-GFP moved up the lateral edge of retracting cells as organized structures and then were disassembled.

The degree of tension on the areas of contact with the substratum has been found to determine the strength of the adhesions and the organization of actin. As a consequence, the nature of the substratum as well as the contractility of the cell, play a role in the assembly of components. The role of cellular contractility is discussed below. When the cells attach to an adhesive substrates, they generate a good deal of tension and have highly organized actin filaments. The role the tension determined by the substratum has been illustrated in experiments in which fibronectin coated beads in contact with the cells were held motionless by a laser tweezer (see Chapter 1) (Felsenfeld et al., 1999). The cells responded with a local, proportional increase in cytoskeletal attachment, shown by the need of increased force to displace the beads. Attachment of fluorescent beads to collagen coated sheets serving as substratum also gave an accounting of the forces involved (Pelham and Wang, 1999). With this experimental design, the forces from the cell's cytoskeleton caused the substratum to move. In cells in the process of crawling, the forces near the leading edge are strong but transient. Those in the rear are weaker and last longer. These observations suggest a contraction behind the leading edge as a major element in cellular movement.

Movement of focal adhesions has been observed by fusing the cytoplasmic and transmembrane domain of integrin receptors to the green fluorescent protein (GFP) (see Chapter 1). In fibroblasts, the integrins first accumulate at focal adhesions (Smilenov et al., 1999). In moving cells, the integrin molecules remain in place while the cytoplasm moves over them, in agreement with the notion that they act as traction points. In contrast, in non-motile cells the adhesions move toward the center of the cell. The rate of movement varies with the tension and are produced by the actin-myosin system, since they can be inhibited by an inhibitor of myosin contraction (2,3-butanedione monoxoime). These observations are puzzling and suggest the presence of mechanisms regulating traction separately from contraction. When not coupled to cell movement, these factors produce movement of the integrin toward the center of the cells.

The Rho family-GTPases (see Hall 1998; also Chapter 11 and below), Cdc42 and Rac, in particular, regulate the formation of protrusions, formation of new adhesions and stabilization of existing adhesions. As discussed, the GTPases function in actin polymerization, responsible for lamellopodia formation.

Fish keratocytes have been used repeatedly in the study of crawling. Keratocytes are unpigmented epithelial cells. Each cell contains a single broad, thin lamella constituting the whole front of the cytoplasm and forming a half-moon lamellopodium. The smaller rear part, or cell body, contains the nucleus. The movement is fast (10 μm/min) and continuous, possibly the consequence of perfectly coordinated protrusion, traction and retraction phases (Mitchison and Cramer, 1996). Most of the force is exerted near the lamellar sides (Lee et al., 1994) (seen as two-dimensional displacements of small beads embedded in the plane of an elastic substratum). The cytoplasm, the organelles of the cell body and beads applied to the rear dorsal surface of the cell body rotate as the cells advance (Anderson et al., 1996).

Another special and important form of crawling is that of *growth cones*. Growth cones are the structures at the tip of developing or regenerating axons. The embryonic development of the nervous system depends on the formation of precise connections. The connections require the directed movement of growth cones. The growth cones are highly motile and are directed through their environment by both attractive or repulsive clues (see Goodman, 1996; Tessier-Lavigne and Goodman, 1996) including contact and chemical signals acting simultaneously and in a coordinated manner. The cytoskeleton of the growth cone is involved in the response to short-range signals and extracellular matrix molecules (Tanaka and Sabry, 1995). In contrast to fish kerocytes, during movement, growth cones change shape and have a stop-and-go behavior. The events occurring in neurons have been examined in several systems including cultured bag cell neurons of the marine gastropod *Aplysia*.

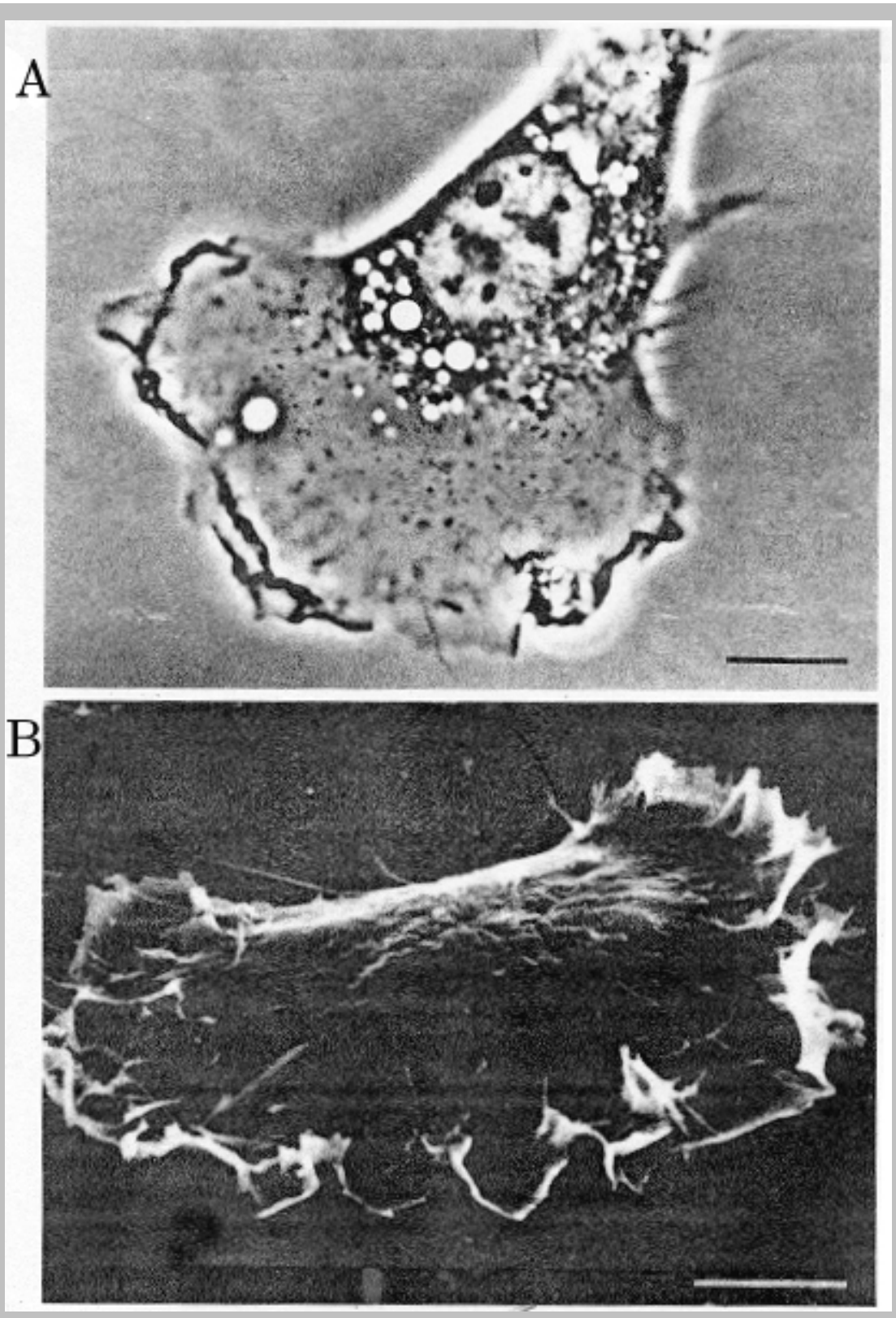


Fig. 20. Views of a fibroblast. The marker indicates 10 µm. From Heath and Holifield, 1991, reproduced by permission.

A. Phase contrast micrograph of a mouse fibroblast showing the fan shaped leading lamella and ruffling of the lamellipodia.

B. Scanning electron micrograph of a human fibroblast showing ruffling lamellipodia.

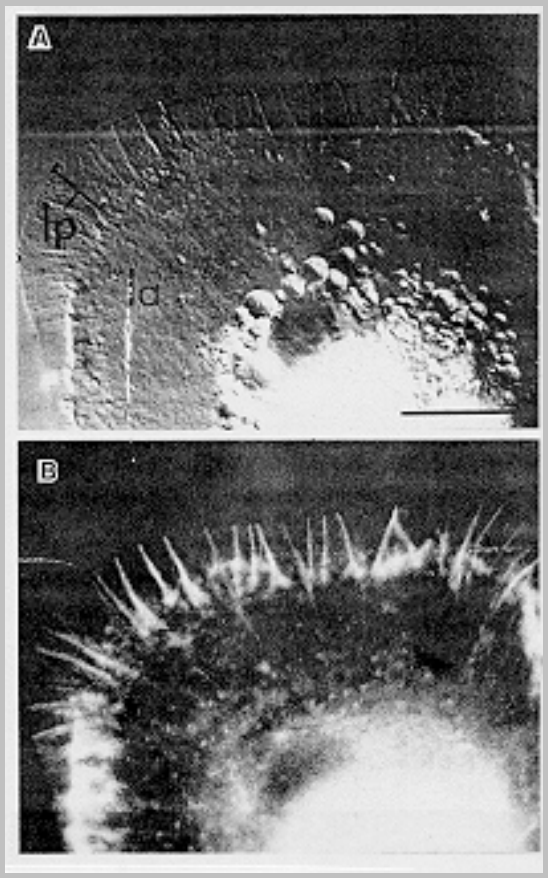


Fig. 21. Comparison of the view of a human fibroblast (A) DIC of leading lamellae (la) and lamellipodia region (lp) and (B) a similar view after fixation and staining with rhodamine phalloidin. Bar corresponds to 20 µm. From Heath and Holifield, 1991, reproduced by permission.

Actin is involved in crawling, as indicated by its presence in the microspikes. However, the molecular events responsible for the movement are far from clear. Although their role is thought to be indirect, microtubules also appear to be involved in the formation and maintenance of focal adhesion (Waterman-Storer et al., 1999; Kaverina et al., 1998, 1999) possibly by having a role in the transport of needed components.

Actin assembly

Actin appears in a thin layer close to the plasma membrane. This cortex is most prominent in cells capable of migrating. The cortex excludes organelles. In the case of human macrophages (Hartwig and Shevlin, 1986), the cortex is 0.2 to 0.5 µm in thickness. Electron microscopy using freeze etching reveals a dense three dimensional network of actin filaments, which can be identified with either antibodies or S1 myosin fragments (Heuser and Kischner, 1980; Lewis and Bridgman 1992). The actin is generally associated with actin-binding proteins such as *filamin* (also called actin-binding protein or ABP), *cofilin*, *fragmin* and *gelsolin* (see Luna et al., 1990). The actin filaments are highly branched in the leading lamellae, an arrangement favored by ABP.

Actin filaments are polar. The two ends of the filaments are called *barbed* and *pointed* ends. This refers to their appearance when they bind the S1 myosin fragment. The barbed end is the fast growing end (also called the plus-end). Free barbed ends of actin localize at the growing margin of activated cells (Hartwig, 1992; Symons and Mitchinson, 1991). Removal of *capping* proteins (see Chapter 24) at the barbed ends is thought to activate assembly. This may occur in response to lipid second messengers (polyphosphoinositides or diacylglycerol) or other agents. Most frequently, fiber elongation takes place by the addition of ATP-G-actin-profilin (called profilactin) complexes at the barbed ends of F-actin. After incorporation the ATP is hydrolyzed. The ADP-actin subunits can then bind to cofilin which favors disassembly (see Bamburg et al., 1999). The assembly may be the driving force for movement.

Besides polymerizing to form filaments, actin can also form a network where cross-linking proteins bind to actin. α-*actinin* is such a linker. It is composed of a dimer which associates in antiparallel manner. The amino terminal of both can bind actin and thereby cross-link two actin molecules to each other. This process can convert an actin solution from a viscous fluid to a gel.

In an initial step initiating actin assembly at the plasma membrane, cytoskeletal proteins can bind directly to phospholipids (e.g., talin and ponticulin) (e.g., see Isenberg and Niggli, 1998). The binding or proteins to lipid components of membranes is also discussed in Chapter 4. In addition, cytoskeletal proteins can also bind to membranes by the mediation of transmembrane proteins such as *ezrin, radixin and moesin* (ERM) (see Mangeat et al., 1999; Critchley, 2000). These proteins act as linkers between the plasma membrane and the cytoskeleton acting similarly to integrins that mediate the interaction between ECM and the cytoskeleton (see Chapter 6).

The ERM family (part of the 4.1 superfamily) (see Mangeat et al., 1999) has a domain of approximately 300 amino acids the *4.1 ERM* (FERM) domain. ERM proteins are found at cell-cell adhesion sites, microvilli, and cleavage furrows, where actin filaments are densely associated with plasma membranes. They can bind to the plasma membrane either directly or indirectly through the FERM domain. In addition, they anchor actin filaments. The function of the various proteins was elucidated using antisense nucleotide inactivation. Ezrin, radixin and moesin were found to be needed for both cell-cell, cell-substrate adhesion and the disappearance of microvilli. Ezrin or radixin were needed for the initial step of formation of cell-cell and cell-substrate adhesion but had no effect on the microvilli structures. Moesin was needed only for formation of microvilli. (Takeuchi et al., 1994). In primary neurons in cell culture, radixin and moesin were found to play a significant role in the formation of axon growth cones and neurite formation (Paglini et al., 1998). Inactivation of ezrin was carried out by chromophore-assisted laser inactivation in Fos-transformed Rat-1 cells and normal Rat-1 cells. In chromophore-assisted laser proteins are inactivated by irradiation with a laser of appropriate wavelength when labeled with microinjected, non-blocking antibodies conjugated to a dye. The Fos oncogene induced expression of ezrin. Ezrin elimination at tips of membrane extensions produced membrane retraction. Elimination of the ezrin cytosolic pool caused a massive retraction even though the leading edge of the cells was not irradiated (Lamb et al., 1997).

Some proteins such as profilin or the *myristoylated alanine-rich C kinase substrate* (MARCKS) were found to have a role in delivering actin to the plasma membrane bu functioning as actin shuttles (see Isenberg and Niggli, 1998). MARCKS uses a myristoyl electrostatic switch for a reversible interaction with membranes (Wang et al., 2001). The myristoyl tail insert into the inner lipid leaflet and is secured by basic amino acid residues. When phosphorylated at serine and threonine residues the lipid interactions are switched off (see McLaughlin and Aderem, 1995).

The assembly of actin filaments results from concerted interactions between several components notably the Arp2/Arp3 complex, the Rho family of small GTPases, the *Wiscott-Aldrich syndrome proteins* (WASPs) and membrane phospholipids (e.g., see see Mullins, 2000). WASPs have similarity in amino acid sequence to the protein mutated in the case of the Wiscott-Aldrich syndrome, an immunodeficiency disorder.

Arp2/3 is a complex which is concentrated in lamellopodia and pseudopodia where it is recruited in response to growth factors or chemotaxis (e.g., Machesky et al., 1997; Bailly et al., 1999). In permeabilized cells the complex recruits actin to lamellipodia and pseudopodia (Bailly et al., 1999; Weiner et al., 1999). In vitro, the complex nucleates actin filaments elongating at their barbed ends and can nucleate actin at the cell surface of *Listeria monocytogenes*, bacteria that exhibits actin polymerization resembling the formation of lamellopodia (Dramsi and Cossart, 1998). The Arp2/3 complex can cap actin at the pointed end and laterally on an actin filament so that it can produce branches (Mullins et al., 1998a,b). The Arp2/3 complex has been shown to be present at filament junctions in the actin network of lamellopodia (Svitkina et al., 1997; Bailly et al., 1999; Svitkina and Borisy, 1999).

Arp2/3 contains five polypeptides (p40, p35, p19, p18, and p14) and in addition two molecules, Arp2 and Arp 3, that are very similar to actin have been identified (Machesky et al., 1994). This complex was originally isolated from *Acanthamoeba castellanii*. The seven polypeptides are present in eukaryotes (see Mullins and Pollard, 1999a; Machesky and Gould, 1999; Welch, 1999) with a 1:1:1:1:1:1:1 stoichiometry (Mullins et al., 1997). As seen with the EM, the complex has a bilobed horseshoe shape with outer dimensions of 13 x 10 nm. (Mullins et al., 1997). The action of Arp2/3 is closely associated with GTPases.

The Rho family of small GTPases have distinct roles in initiating changes in the organization of the actin cytoskeleton). Rho, Rac and Cdc42 trigger the formation of stress fibers, lamellopodia and filopodia (see Hall, 1998; Schmitz et al., 2000). Rho induces the reorganization of pre-existing filaments. In contrast, Rac and Cdc42 induce the polymerization of new actin filaments by stimulating actin nucleation or either the uncapping or severing of filaments (Machesky and Insall, 1999). Cdc42 is thought to initiate the formation of filopodia in the front of the cell, whereas Rac is though to control the formation of lamellopodia. The formation of focal adhesion is coupled to both Cdc42 and Rac. Rho and Rac are regulated together and this regulation depends on the location inside the cell (see Rottner et al., 1999). Rac is needed for a new contact with the substrate at the front of the cell to form lamellopodia. Rho appears to have a role in the maturation of existing contacts. The formation of contacts require the

presence of actin and myosin.

There are other indications that various components are active in different aspects of actin-polymerization. The *WASP-family verprolin homologous proteins* (WAVEs) are similar to other WASP proteins discussed above. However, they do not have a CRIB domain which is used by WASPs to bind to Cdc42/Rac1. Nevertheless, WAVEs move to membrane ruffles when these are induced by Rac1 (Miki et al., 1998) and ectopically expressed WAVE induces the formation of actin filament clusters. WAVE1 is present in a complex which is inactive (Eden et al., 2002). It has been proposed that it has to be liberated from the complex before it becomes active. A WAVE mutant unable to induce actin reorganization blocks ruffle formation induced by Rac (Miki et al., 1998). However, it has no effects on Cdc42-induced actin-microspike formation. This argues for two separate modes for actin nucleation, one involved in ruffle formation and the other in microspike formation.

How does Rac function to produce lamellopodia? The effect of Rac on actin structures include uncapping of F-actin, the polymerization of new actin filaments, the control of depolymerization and the severing of filaments already present. Two protein kinases, *p21-activated kinase* (Pak1) and *LIM-domain-kinase* (LIM-kinase) are regulated by Rac. The uncapping of actin is thought to take place via the Rac-stimulated generation of phosphatidylinositol-4,5-bisphosphate (see Ren and Schwartz, 1998). This phospholipid catalyzes the removal of capping proteins from actin filaments. Rac is also responsible for the formation of new filaments perhaps by recruiting the actin polymerizing Arp2/3 complex possibly by recruiting it into the lamellopodia using the protein WAVE or SCAR (Miki et al., 1998). In addition, Rac indirectly inhibits the actin-depolymerizing protein *cofilin*. (Cofilin is an ubiquitous actin-binding protein that increases the depolymerization at the pointed end of actin and cuts pre-existing filaments) (Bamburg et al., 1999). Cellular activities involving rapid actin turnover (see Theriot, 1997; Lappalainen et al., 1997) and other processes such as endocytosis (Lappalainen and Drubin 1997) and cytokinesis (Abe et al., 1996) require cofilin. Actin polymerization is regulated by cofilin phosphorylation-dephosphorylation cycles. Cofalin binding to actin is inhibited by its phosphorylation and the inhibition is overcome by dephosphorylation (see Moon and Drubin, 1995).

Besides actin dynamic organization, the GTPases are involved in many other functions (e.g., see Chapter 7). For example, Rac has also a role in control of morphology, transcriptional activation and the initiation of apoptosis (e.g., Kjoller and Hall, 1999). What determines the specific function of Rac? The specificity may be due to the regulation of its cellular localization. The GTP exchange factors which are required for the function of the small GTPases have a variety of localization domains which can regulate the signaling of Rac (Zhou et al., 1998). The evidence of studies using *fluorescent resonance energy transfer* (FRET) (see Chapter 1) supports the notion that specificity is determined by the location inside the cell. FRET can be used by introducing a fluorescently labelled biosensor into the cell along with a protein construct of Rac and *green fluorescent protein* (GFP). The biosensor was labelled with an acceptor dye (Alexa 546) suitable for resonance energy transfer to GFP (Kraynov et al., 2000). The biosensor used was a fragment of the p21-activated kinase 1, a GTP-Rac target protein which binds to GFP-Rac only when Rac is activated . The binding produces a FRET signal that gives the amount and location of the activated

protein. The method has been referred to as *fluorescence activation indicator for Rho proteins* (FLAIR). FLAIR revealed a spatial control of growth factor-induced Rac activation, in membrane ruffles and in a gradient of activation at the leading edge of motile cells (Kraynov et al. 2000). Similar experiments were carried out with different probes and growth factors (Mochizuki, 2001)

In actin polymerization, both Rac and Cdc42 have been found to act through WASPs (see Mullins, 2000). The requirement for Cdc42 in Arp2/3-induced actin assembly (Ma et al., 1999) has been shown in *Xenopus* egg extracts. Similarly in *Acanthamoeba* extracts, immunoprecipitation of the complex blocks the polymerization induced by Cdc42 in the presence of GTP analog, GTPγS (Mullins and Pollard, 1999b).

The WASPs have a role as adaptors needed for coupling the GTPases to the Arp2/3 complex. A related protein has been referred to as WAVE (e.g., see Ramesh et al., 1999). One of the domains of WASP and related proteins is a *pleckstrin homology (PH) domain* which binds to proline rich ligands (e.g., Prehoda et al., 1999) and membranes (Miki et al., 1998). The action is enhanced by the presence of phosphatidylinositol (4,5)-bisphosphate (PIP_2) (Rohatgi et al., 1999) implicating membrane components in the actin polymerization by this mechanism. Similar associations of actin filaments to membrane components are discussed below.

The details of the events leading to the nucleation of actin are not entirely clear. The results of Rohatgi et al.(2000) carried out with neuronal WASP (N-WASP) indicate that N-WASP is a weak activator of Arp2/3 complex. Cdc42 and PIP_2 enhance this activity. The carboxy terminal of N-WASP binds Arp2/3 during this process. The binding of the amino terminal to the effector carboxy terminal within the same N-WASP molecule blocks the Arp2/3 binding site, thereby inhibiting actin nucleation. Cdc42 and PIP_2 reduce the affinity between the terminals of the N-WASP molecule. In contrast, purified WASP from bovine thymus does not produce nucleation even in the presence of Arp2/3, Cdc42 and GTP. However, the presence of PIP_2 (or phosphatidyl serine) micelles permits the activation of by WASP, resulting in actin polymerization next to the micelles. The presence of Cdc42 and GTP increased this effect (Higgs and Pollard, 2000). The difference between the two sets of experiment may well reflect quantitative rather than qualitative differences (e.g., a weak effect may be missed).

(PIP_2) is formed at specific locations in the cell because the enzyme mediating to its formation is targeted to specific sites on the cell. Phosphatidylinositol phosphate kinase type 1 γ (PIK1 γ), a phosphatidylinositol-4-phosphate 5-kinase, is found in synapses. A splice variant (see Chapter 3) of this enzyme binds to the FERM domain of talin which activates the kinase. Talin has been shown to be concentrated in focal adhesion plaques in non-neuronal cells. The product of the kinase, PIP_2, has been shown to be essential for plaque formation (Di Paolo et al., 2002; Ling et al., 2002).

Integrins are also involved in the formation of actin-containing structures. Integrins are integral proteins bridging the ECM and proteins associated with the cytoskeleton (see Chapter 6). The extracellular domain

of these proteins binds to ECM components, whereas their cytoplasmic domain binds to a complex of proteins which includes vinculin, talin, paxillin, tensin and many other molecules (Critchley, 2000). Talin is a dimeric protein in which each subunit corresponds to 270 kDa which can bind to integral membrane proteins, actin and focal adhesion kinases (FAK). It is a long flexible molecule (60 nm). In some cell types the function of talin is carried out by filamin or α-actinin. Vinculin, a 130 kDa protein may bind to talin and actin to stabilize their interaction. In addition, it can interact with phospholipid bilayers. Paxillin, a protein with a molecular weight of 61 kDa (calculated from cDNA), is a multifunctional docking protein involved in cell adhesion. Proteins of the filamin family cross link actins and are thought to mediate effects of integrin (see Critchley, 2000). A complete presentation relating to the linkers to actin will be found in the book edited by Kreis and Vale (1999).

In cultured cells the molecular complexes form small dot like contacts (focal complexes) at the edges of lamellipodia (Nobes and Hall, 1995) and actin-myosin containing bundles, focal adhesions (stress fibers) (e.g., Zamir et al., 2000). The formation of punctate focal complexes requires the activity of the GTPase Rac (e.g., Rottner et al., 1999). Rho is needed for the formation of focal contacts and stress fibers. Rho acts through *Rho associated kinase* (ROCK) and mDia1 which are needed for mediating the interaction between matrix adhesion and the actin cytoskeleton (Watanable et al., 1999).

Cellular contractility is also required for the assembly of these components (e.g., Helfman et al., 1999). Since contraction of cells attached to the substrate produce tension at the adhesion sites (e.g., Dembo and Wang, 1999), it would seem possible that the tension itself affects the interaction to produce focal contacts. This premise was put directly to a test. Mechanical force applied centripetally to vinculin-containing dot-like adhesions at the cell edge using a micropipette, cause further assembly and elongation at the site eventually forming focal contacts (Riverline et al., 2001). The assembly of components was demonstrated by following the movement of GFP-tagged vinculin or paxillin (see Chapter 1). These studies were supplemented by observations using interference reflection microscopy.

In disrupted cells the plasma membrane and the actin-network can be shown to be attached. Interactions of the cell membranes with the cytoskeleton is in part explained by the tethering of cytoskeletal components to *phosphoinositides* (PIs). The PIs are thought to be present at specific sites in the plasma membrane (see Chapter 4). Immunocytochemistry (see Chapter 1) localized PIP_2 to a central region of cells, around nuclei (possibly Golgi) and in addition at microfilament bundles, and focal contacts, where α-actinin and vinculin are present (Fukami et al., 1994). α-actinin and vinculin are usually associated with actin and they are present in PIP_2-bound form. An involvement of *phosphoinositide phosphates* (PIPs) with elements of the cytoskeleton has been shown more directly. In platelets, Rac (see below) activation results in increases in actin polymerization which requires PIP_2. This effect may result from Rac stimulation of PIP-kinase (Hartwig et al., 1996). The increased actin polymerization is thought to result from PIP inhibition of capping proteins where different PIPs trigger distinct protrusions (Hartwig et al., 1996). Many actin binding proteins bind PIPs [e.g., α-actinin, (Fukami et al., 1996), gelsolin (Janmey and Stossel, 1987), vinculin (Gilmore and Burridge, 1996) and profilin (Lassing and Lindberg, 1985)] so that

they bridge PIP and actin . Microinjection of antibodies against PIP_2 inhibits assembly of stress fibers and focal adhesions. PIP_2 is produced by the activation of PI-5 kinase by Rho (Gilmore and Burridge, 1996).

The movement or retraction of the neuronal growth cones during development are responsible for molding of neuronal pathways. These dynamics require the interaction of a variety of extracellular factors and their surface receptors in the growth cones. The factors act through actin assembly and disassembly. The membrane-bound ephrin (Eph) ligands and the Eph receptors are involved in the regulation of actin dynamics. In the growth cones, changes in the activity of certain Rho GTPases have been linked to Eph receptor activation. Presumably, the process is triggered by some external stimulus. The Eph receptors and Eph mediate cell contact-dependent retraction and the interference with cell intermingling (see Xu et al., 2000). Eph-A ligands are glycosylphosphatidyl inositol-anchored, whereas Eph-B ligands are transmembrane proteins. The receptors are tyrosine kinases and constitute a family of 14 members. Eph are required for proper formation of specific axon projections (see Frisén et al, 1999). They induce the collapse of neuronal growth cones and favor cell adhesion. The collapse is mediated by activation of the small GTPase Rho and its downstream effector Rho kinase (Wahl et al., 2000). A guanine nucleotide exchange factors (GEFs) for Rho GTPases, *Eph-interacting protein* (ephexin), was found to interact with the Eph receptor (Shamah et al., 2001). The activity of ephexin is regulated by EphA stimulation of EphA receptors activating RhoA. Ephexin is responsible for a highly localized effect on growth cone motility.

The role of the ephrin system in neuronal plasticity is discussed in Chapter 22.

Actin and movement

How actin is involved in the intimate mechanisms of movement is still far from being settled. However, a good deal is known and will be discussed in this section.

Vectorial actin turnover in the lamellae with a net addition at the front end and a net loss at the rear end is thought to explain vectorial lamellar progressions as a form of treadmilling. The various components of these processes are complex (see Chapter 24 and Carlier and Pantaloni, 1997).

Polymerization of actin is a major player in protrusion and provides enough force to overcome resistance from cell membrane structures (e.g., see Mogliner and Oster, 1996). Certain myosin I isoforms are abundant in protrusive structures (e.g., see Baines et al., 1992; Wagner et al., 1992) and may play a role, possibly by moving actin forward.

There is a continuous backward flow of lamellopodia components toward the cell body. This movement is accompanied by a backward flow of actin filaments (e.g., see Wang, 1985; Forscher and Smith, 1988). Wang (1985) labeled actin with the fluorescent dye iodoacetamidotetramethyl rhodamine. After incorporation of the labeled actin into the lamellipodium, FRAP (see Chapter 4) revealed the pattern of fluorescence dynamics. When only part of the lamellipodium was photobleached, the bleached spot moved toward the cell's center and through an area unbleached by the laser beam. The movement of

molecules attached to both actin and the substratum may exert force and displace the cell forward. Whether this mechanism is significant in exerting force is not clear (however, see experiments of Suter et al., 1998). Large particles attached to the cell surface by lectin or antibody crosslinking (e.g., Kucik et al., 1991) consistently show a movement to the rear. The particles are thought to have associated with integral proteins and cortical actin (see below).

In the study of Symons and Mitchison (1991) actin labelled with a fluorescent dye was also injected into cells. Initial incorporation was in the lamellopodia, with a decreasing intensity from tip to base. The results suggest a growth of the polymerized actin in the front and a displacement and disassembly toward the rear.

During retrograde transport in the growth cone of neurons, cortical actin originates from the margin of growth cone, moves across the thin lamella and then presumably disassembles at the thicker microtubule rich central cytoplasm. Beads that attach to the cell surface move at the speed of actin (Forscher and Smith, 1988; Lin and Forscher, 1995). Pharmacological evidence suggests an involvement of myosin in this movement (Lin et al., 1996). When growth cones contact one another the actin flow slows at a rate inversely proportional to the growth cone advance (Lin and Forscher, 1995) suggesting that the same mechanism is operative for both cortical flow and growth cone advance. The growth cone advance depends on engagement and disengagement of cell surface receptors with the actin network (Lin et al., 1996; Mitchison and Cramer, 1996)

(Suter et al. (1998) have examined the flow of cortical actin at the surface of growth cone of *Aplysia* bag neurons using beads coated with *Aplysia cell adhesion molecules* (ApCAM) and antibodies to ApCAM. They were able to restrain the movement of the beads using glass needles. These approaches revealed two different states of actin. ApCAM beads attached to the growth cone follow retrograde transport. Initially, the glass needles could restrain the beads but the retrograde transport of actin would continue (shown by the movement of smaller indicator, unrestrained ApCAM beads). However, after a latency period, the needles in contact with the beads were bent backwards indicating the backward exertion of force. In addition, both kinds of beads, including the "indicator" small beads were restrained. This is evidence that the beads and the actin had bonded strongly and that a good deal of force can be generated by the system. During these events protrusion and traction of the growth cone were taking place. The actin assembly at the margin was unaffected by retrograde transport. The slowing down of the retrograde transport produced an increase in forward progression. While the beads were restrained, the microtubule-rich central cytoplasm moved forward. Such a movement is an essential feature of cone crawling (Goldberg and Burmeister, 1986). Conversely, the release of the beads was immediately followed by retreat of the central cytoplasm and revival of the retrograde transport. This suggests that the forward movement and the actin retrograde transport result from the same mechanism [also supported by other observations that retrograde transport is inhibited at a rate inversely proportional to the movement of the central cytoplasm (Lin and Forscher, 1995)]. The function of *retrograde* flow is far from clear (e.g. see Mitchison and Crane, 1996).

The geometry responsible for the action of actin is difficult to visualize because most actin filaments form a criss-cross network (Lewis and Bridgman, 1992), although they are predominantly oriented with the barbed end toward the leading edge.

As described above, the movements in fish keratocytes are characterized by exertion of force at the lamellar sides and in addition a rotation of the cytoplasm as the cell moves forward. In these cells, the actin filaments are almost uniform in polarity, with barbed ends forward. The observations suggest that both forward and retrograde movement in the lamellipodia are driven by contraction of an actin-myosin network in the lamellipodial/cell body transition zone. The actin is present in a gradient with a high density at the lamellipodia, less density at the cell body and the highest density in the region between cell body and lamellopodia (Small et al., 1995; (Svitkina et al., 1997). Myosin II is also distributed in a gradient, with the highest concentration at the transition region. The following mechanism was proposed (Svitkina et al., 1997). Actin is assembled at the lamellar margin and disassembled near the nucleus forming a meshwork. The actomyosin contracting at the transition zone pulls the cell body forward and causes the molecules at the transition zone to form bundles. This results in forward movement but also a lateral exertion of the force. The concentration of cytoskeletal molecules near the bottom of the cell produce a drag force. Drag force together with the forward momentum cause the cell to rotate (see Svitkina et al., 1997; Heidemann and Buxbaum, 1998).

The phosphorylation of cofilin is catalyzed by LIM-kinase 1 (Frangiskakis et al., 1966; Arber et al., 1998; Yang et al., 1998). Deletion of the gene coding for LIMK-1 causes serious cognitive problems in humans associated with *Williams syndrome*. LIM-kinase mutants block Rac-induced lamellopodia formation (but not Cdc42 filopodia). Pak1 phosphorylates LIM-kinase after being activated by Rac and Cdc42 (Manser et al., 1994; Edwards et al., 1999). This increases the LIM-kinase phosphorylation of cofilin and thereby blocks the depolymerization effect of cofilin (Edwards et al., 1999).

Role of myosin

Myosin is involved in the cell body forward movement. Myosin II-less *Dictyostelium* mutants (so called *knockout* mutants) have a dramatic drop in cell movement (Wessels et al., 1988). In a medium of increased resistance, the cells are immobilized (Doolittle et al., 1995; Jay et al., 1995).

When attached, myosin has been shown repeatedly to produce a sliding of actin filaments in relation to each other. These aspects of myosin-actin interactions will be discussed in more detail in Chapter 24. In artificial gels this can produce streaming (Kane, 1983). The motor molecules myosin I and II, present in cytoplasmic extracts, can generate a form of streaming in actin gels.

In *Dictyostelium*, myosin I is located mostly in the front end of the cell, associated with microspikes and ruffles (e.g., Morita et al., 1996). We already noted that the microspikes are actin bundles. In contrast, myosin II is mainly at the rear of the cell (e.g., Chu and Fukui, 1996). Similarly, in keratocyes, immunofluorescence (Anderson et al., 1996; Svitkina et al., 1997) and immunogold EM (Svitkina et al.,

1997) localizes myosin II at the transition zone between lamellipodia and cell body suggeting that a contractile event may take place at this location during forward cell body movement. Therefore, myosin I may be associated with the movement of actin toward the leading edge. Whereas myosin II is likely to have a role in contraction at the interface between cell body and lamella.

Besides their role in the assembly, Cdc42 and Rac also regulate nonmuscle myosin though phosphorylation of its light chains. Rac and Cdc42 in GTP-bound state stimulate the *p21-activated kinase*, PAK, a serine/threonine kinase which controls the phosphorylative state of the myosin light chain (see Bresnick, 1999; Bagrodia and Cerione, 1999). This phosphorylation allows myosin to interact with actin. This kinase and other related proteins constitute a family of PAK-proteins. PAK has been found to be involved in nuclear events that result in gene expression and cytoskeletal dynamics. Phosphorylation of the myosin light chains by myosin light chain kinase favors dimerization and the interaction with actin to produce contraction.

Role of Ca^{2+}

Ca^{2+} has been shown to be associated in some way with crawling, although its actual role is difficult to pin down. Free cytoplasmic Ca^{2+} increases with the activation of movement, with a gradient of lower concentration at the moving end and higher concentration at the rear (e.g., Brundage et al., 1991; Hahn et al, 1992). However, in permeabilized cells (cells which have been rendered leaky by rupturing the plasma membrane), actin assembly does not seem to implicate Ca^{2+} (Downey et al., 1990).

Go to Part 3

REFERENCES

Search the textbook

Back to Part 2

D. Tubules and Cytoplasmic Transport

The microtubules

Tubules resembling those present in cilia and flagella, the *microtubules* (MTs), have been identified in the cytoplasm of many cells. A number of observations have suggested a direct or indirect involvement of these microtubules in movement within the cytoplasm.

MTs generally are straight cylinders, 25 to 16 nm in diameter. The tubules may be hollow, although central cores have been described for some. Cytoplasmic MTs, like their counterparts in the axonemes of cilia and flagella, are polar assemblies of α- and β-*tubulin*, proteins of 55 kDa. The two subunits alternate in the formation of *protofilaments* (i.e., the individual threads). There are generally 13 protofilaments per MT. Prominent in axons, MTs have also been found in most if not all cells (Porter, 1966), in cytoplasmic spiky projections, and adjacent to the walls of some plant cells. MTs are present in the mitotic spindle, which has been estimated to contain as many as 5,000 to 10,000 MTs in *Haemanthus catherinae*.

The organization of MTs in cells is determined by the centrosomes the main *microtubular organizing center* (MTOC). (see discussion below) The centrosome serves as a nucleation center for microtubules and thereby organizes them into arrays characteristic of non-dividing cells. In addition, it is capable of duplication with each cell division. During mitosis the centrosomes determine the position of the poles of the mitotic spindle. The centrosomes are made up of two centrioles at right angle to each other (see Urbani and Stearns, 1999).

Colchicine prevents chromosomes from separating, apparently by binding to the spindle protein. This observation led to the idea that colchicine might be used as a marker of microtubule subunits (the *tubulins*) in various systems. By using [^{3}H]-labeled colchicine to identify the molecules through fractionation procedures, proteins isolated from several sources were shown to have very similar properties (Adelman et al., 1968). These molecules are the building blocks of microtubules and the isolated subunits are able to reassemble into microtubules.

The tubulins constitute a family of closely related proteins, each *isotubulin* encoded by a different gene. In addition, the tubulins are modified postranslationally (see Ludveña, 1998). As many as 17 isotubulins may be present in nerve tissue (George et al., 1981), and their presence or absence is strongly influenced by the developmental state (Dahl and Weibel, 1979;Denoulet et al., 1982;Gozes and Littauer, 1978). Interestingly, a single neuron may have as many as nine isotubulins (Gozes and Sweadner, 1981) and

there are indications that their location within the cell is specific. A much smaller number of isotubulins has been detected in liver cells, where their presence does not seem as dependent on developmental state (Donges and Roth, 1972).

Some of the tubulin isotypes have different physiological roles and in vitro display different properties . The covalent modifications may influence the stability or interactions of tubulin with other proteins. Despite these differences, the tubulins from different organisms and tissues are similar in molecular weight and amino acid composition and have many properties in common. They bind to colchicine and the antitumor drug vinblastine, and they react similarly to common antitubulin antibodies (e.g., Dales, 1972; Donges and Roth, 1972; Fulton et al., 1971).

The MTs of cilia and flagella are involved in cell movement. It would be surprising, if the MTs in the cytoplasm of other cells were not responsible in some way for cell movement. In support of this view, colchicine and vinblastine were found to block cell movement and simultaneously interfere with microtubular function. The effect of colchicine seems to be relatively specific. Colchicine binds almost exclusively to microtubules. In contrast, vinblastine also binds a number of other cell components. However, taken together these findings are strong evidence for the involvement of microtubules in movement. The effects of these two drugs are summarized in Table 3. Although sensitivity to these drugs suggests involvement of MTs, there is more direct evidence for this premise, as discussed in the rest of this section.

The role of microtubules in the transport of materials has been explored extensively in the *axopodia* of heliozoans and foraminifera. Axopodia are slender cytoplasmic processes radiating from the main cytoplasmic mass. They are a few micrometers thick, but they can extend as far as half a millimeter (Fig. 22a) (Travis and Allen, 1981). The material within the axopodia exhibits cytoplasmic streaming. The internal structure of the axopodia, the axoneme, contains longitudinally oriented microtubules (Fig. 22b and c) (Travis and Bowser, 1988). The electron micrographs also show cross-bridges between microtubules and vesicles. The interaction between microtubules and vesicles is discussed in Chapters 11 and 24. In the axoneme, cytoplasmic particles may stream independently and even in opposite directions (MacDonald and Kitching, 1967). A direct involvement of microtubules is indicated most clearly by experiments in which keratocytes cultured from the corneas of frogs were studied with light microscopy and video enhancement techniques (Allen et al., 1981). Keratocytes are cells of mesenchymal origin. Motion of particles was observed in linear elements visible in the thin parts of the cells. Although individual microtubules and even microtubular bundles may be below the level of resolution (see Chapter 1), they can be perceived with the light microscope as structures in the range of 100 to 200 nm forming linear arrays. The involvement of linear structures in the movement of particles can be shown by recording the image over a period of time (Hayden et al., 1983). Fig. 23 (a-d) shows the movement of the particle marked by the arrow in successive photographs of video images. The linear arrays correspond to microtubules for the following reasons. An array of microtubules and the moving particles were observed in living cells. The cell was then lysed and fixed under continuous observation to ensure that no structural rearrangement took place (Fig. 23 e-g). Indirect immunofluorescence using anti-tubulin antibodies (Fig.

23h) identified the arrays as MTs.

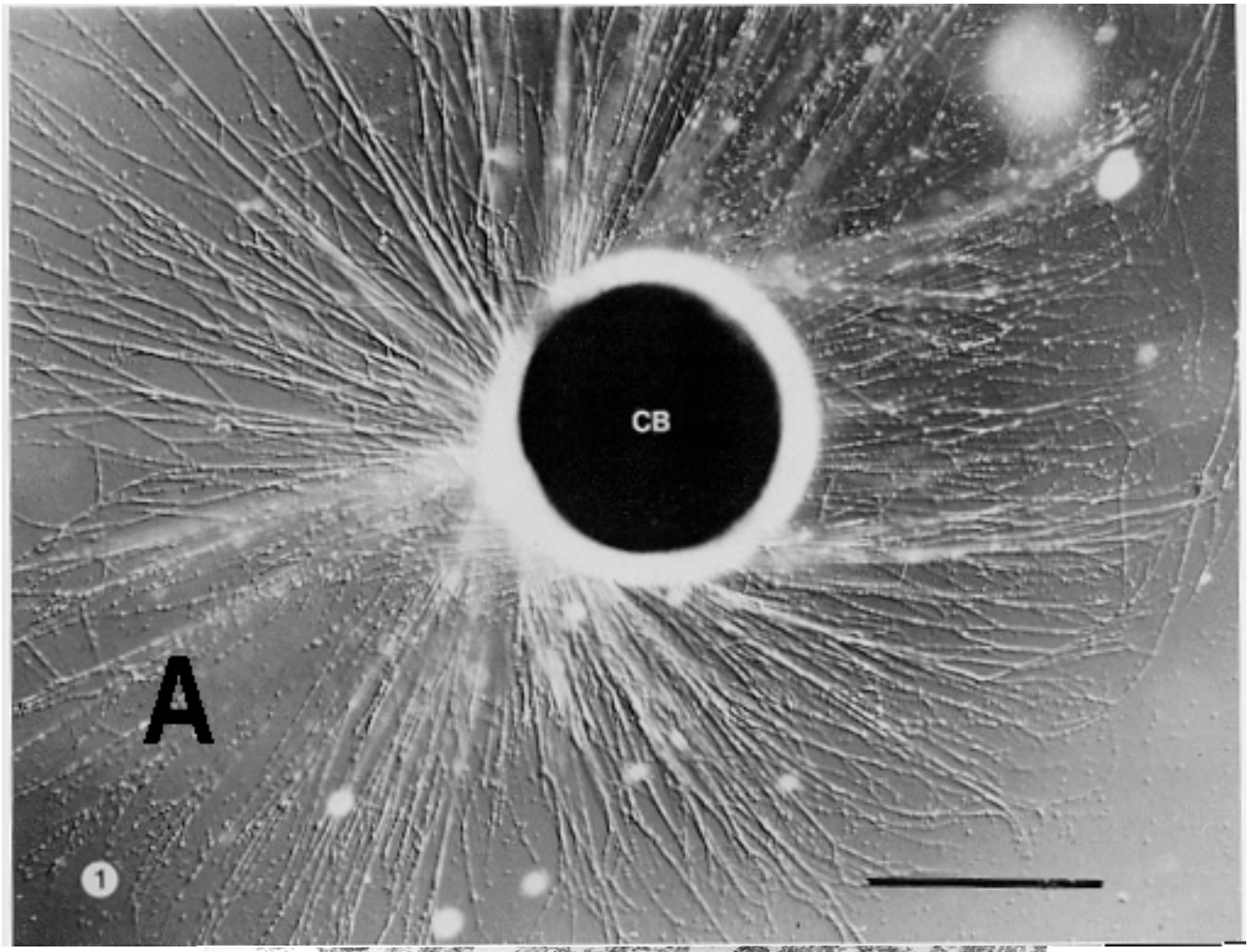
The study of MT dynamics has been aided in recent years by *fluorescent speckle microscopy* (FSM). This approach is based on the fact that incorporation of fluorescent subunits into a fibers is discontinuous when the proportion of fluorescent molecules is low. This results in the formation of a discontinuous pattern of fluorescence (Waterman-Storer and Salmon, 1998; 1999; Keating and Borisy, 2000) or speckles. FSM allows following a very large area and the study of single microtubules. For MTs assembled in vitro, speckles containing one fluorophore can be detected (Waterman-Storer and Salmon, 1998) although a larger number is usually required in vivo. This method has been particularly useful in the study of movement and treadmilling of MTs.

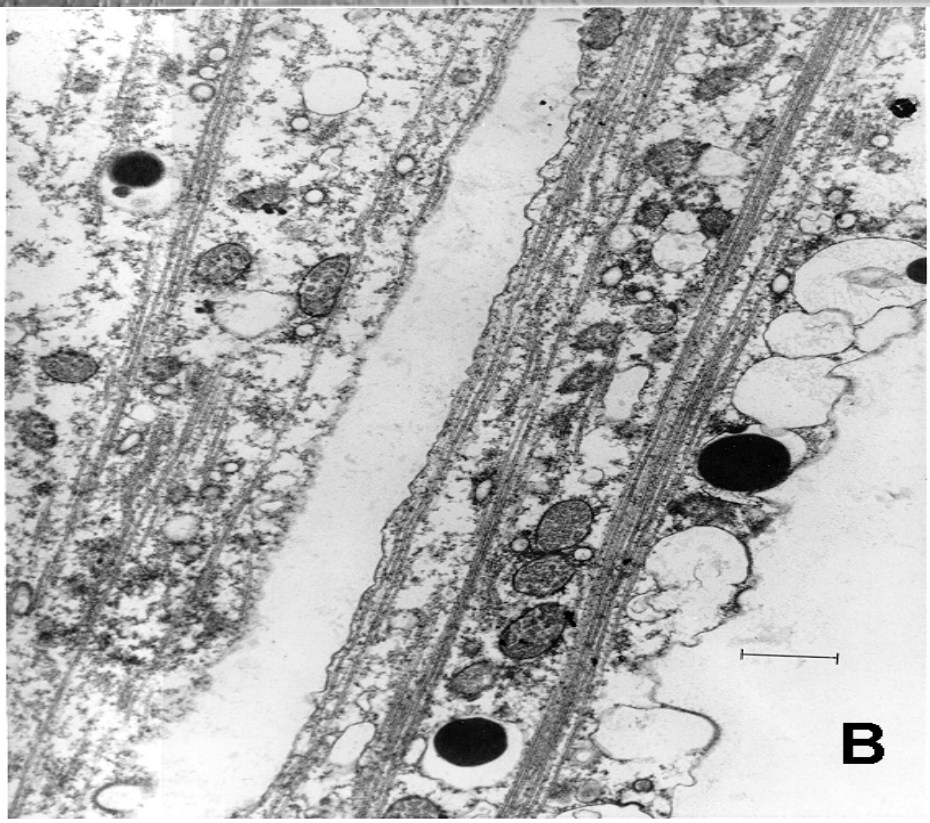
FSM studies confirmed that microtubules move through the cytoplasm either by motor based translocation or by treadmilling, where one end of the microtubule grows while the other shortens. Treadmilling was shown in the lamellae of migrating epithelial cells (Waterman-Storer and Salmon, 1997) and in enucleated cells (Rodionov et al., 1999).

Table 3 Effect of Colchicine or Vinblastine in Cell Motility

Tissue	Blocking agent	Effect on microtubules or oriented fibers	References
Chick nerve endings	Vinblastine	Yes	a
Crayfish neurons	Vinblastine	Yes	b
Hypothalamic neurons	Colchicine	No	c
Cultured hamster kidney cells	Colchicine	Yes	d

[a]Feit et al. (1971)
[b]Fernandes et al. (1971)
[c]Flament-Durant (1972)
[d]Goldman (1971)


CB
A
1


B

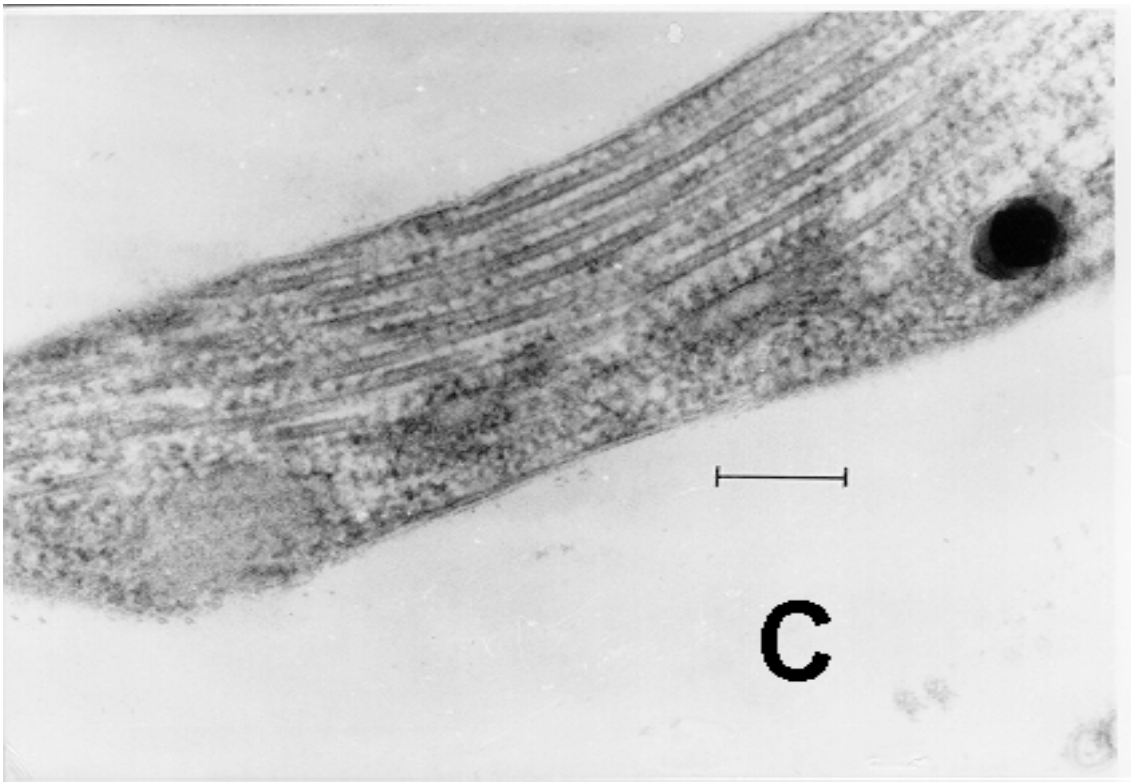
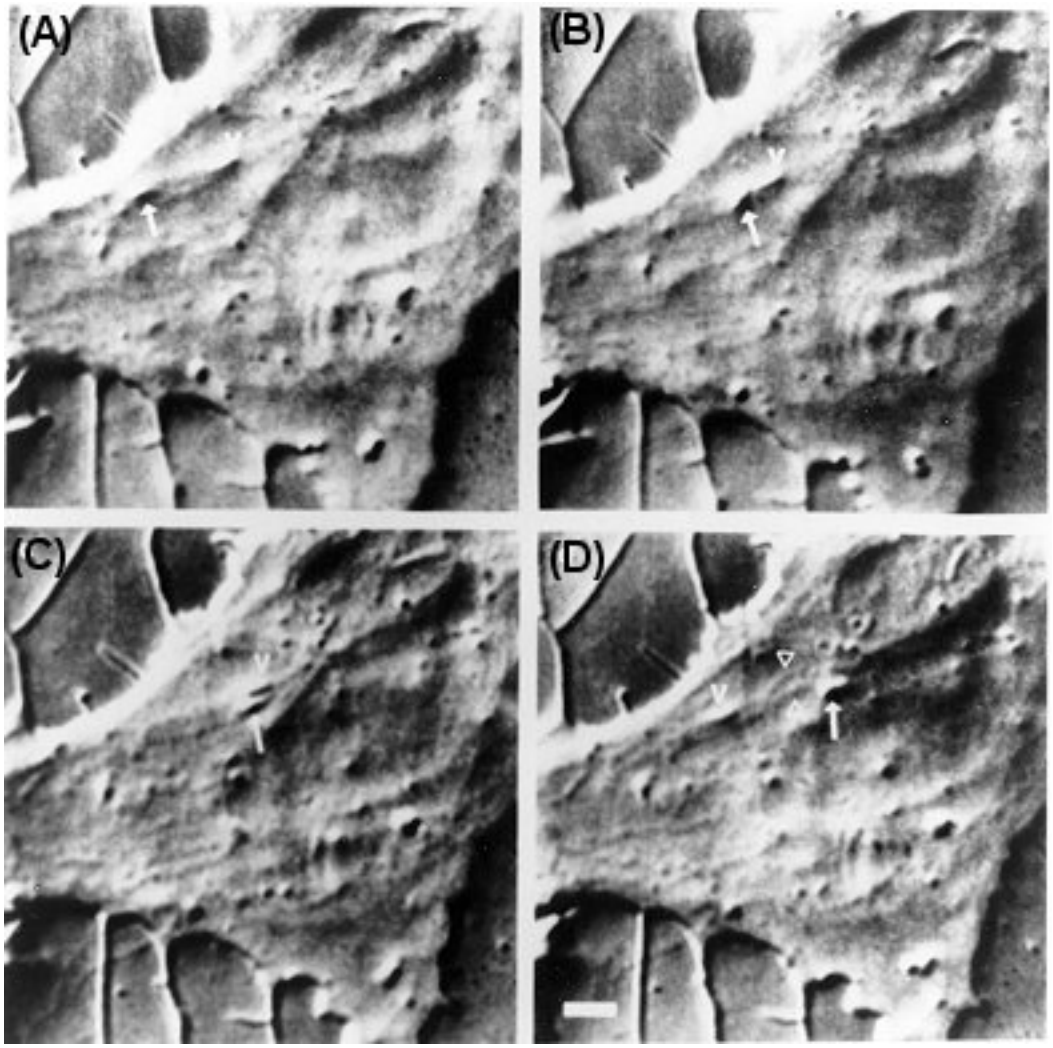


Fig. 22 (a) *Allogromia laticollaris*, a typical foraminiferan. This single-celled organism consists of a spherical cell body that contains the nucleus and an elaborate network of interconnected pseudopods. The pseudopodial networks may become quite expansive, with the main trunks attaining lengths of nearly 1 cm. Food particles, such as bacteria and diatoms, bind to the pseudopodial membrane and are transported along the outside of the plasma membrane until they accumulate near the cell body. The pseudopodial movements are driven by movements of the cytoskeletal microtubules. Bidirectional intracellular transport of organelles occurs throughout the network. This transport, as well as the cell surface transport of food particles, occurs only along the cytoplasmic microtubules. x350; bar = 100 µm. From Travis, J. L., and Allen, R. D. (&copy1981). Reproduced from *J. Cell Biol.* by copyright permission of the Rockefeller University Press.

(b) Conventional thin-section transmission electron micrograph through a foraminiferan pseudopod, showing the close association between the microtubules and the transported organelles. Mitochondria, coated vesicles, and other membranous organelles move along the microtubules. In this figure, several organelles appear to be linked to the microtubules by cross-bridge structures. Bar = 1µm. Unpublished micrograph from J. L. Travis. (c) A two-step lateral translation of a thin-section electron micrograph of a foraminiferan pseudopod. This technique enhances the periodic cross-bridge structures that link adjacent microtubules. Note that similar side arms serve to attach the microtubules to the plasma membrane. Bar = 0.2 µm.(From Travis and Bowser, 1988), with permission.

Like actin filaments, MTs are polar: one end of the microtubule differs from the other end functionally and in molecular terms, since the front and the back ends have different properties. In vitro, tubulin polymerizes to form microtubules. The rate of growth differs, however. The end that grows faster is called the *plus* end; the slower growing end is the *minus* end. The direction of movement of different motors, kinesin and cytoplasmic dynein(Chapter 24), in relation to the two ends also differs. The polarity of microtubules can be demonstrated experimentally by two different approaches. Dynein attaches to

microtubules with its arms directed toward the *microtubule-organizing center* (MTOC), the nucleation center which initiates MT-polymerization. As discussed in more detail in Chapter 24, dynein is one of the "motors" associated with movement. Tubules are also capable of attaching to more tubulin subunits, thereby forming incomplete tubules. These have been found to form arms with the appearance of right- or left-handed hooks. In cross section, right-handed hooks indicate that the positive end faces up.



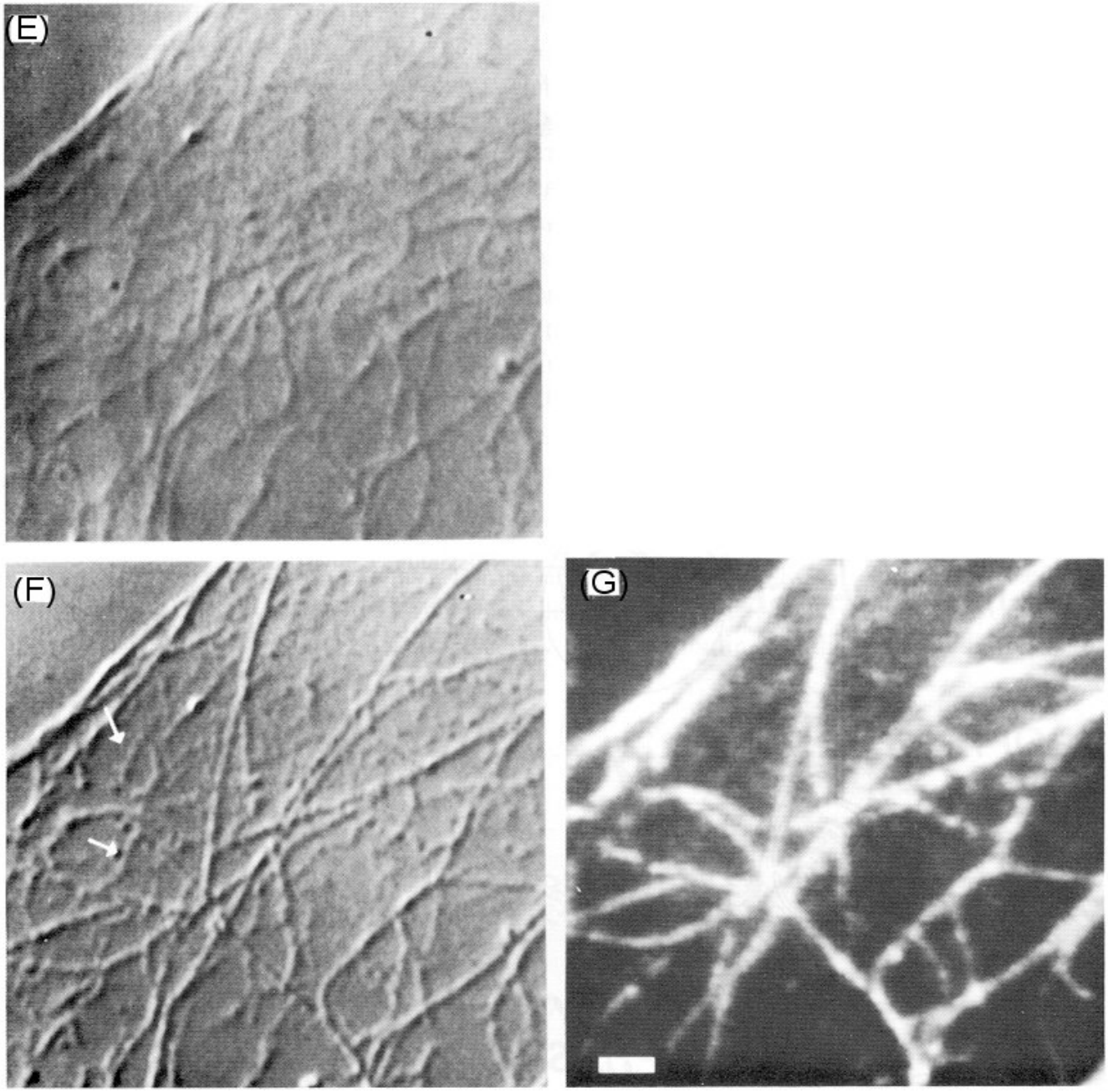


Fig. 23 Demonstration of movement involving linear elements (a-d) and demonstration that the linear elements correspond to microtubules in keratocytes. Video-enhanced images using differential interference contrast (AVEC-DIC) and immunofluorescence. Bar corresponds to 2 µm. (a) One particle in motion (arrow) approached a stationary particle (arrowhead). (b) The particles collided and motion stopped. (c) The particle in motion moved to the side of the stationary particle. (d) Both particles moved in opposite directions along different linear elements (LE). Bar corresponds to 2µm. (e)-(f) demonstrate that the LEs are microtubules. (e) An intact cell where movement was demonstrated is lysed in stabilizing solution. (f) Image of cell after rabbit antitubulin antibody is added, and after the addition of goat antibody (to the rabbit antitubulin antibody) which is labelled with a fluorescent dye. (g) Immunofluorescence demonstration that the linear elements are microtubules (Hayden et al., 1983, reproduced with permission).

MTs can shrink or grow at both ends (e.g., Walker et al., 1988). The growth is referred to as *rescue* and

the shrinkage as *catastrophe*. Even at steady state, there is a rapid exchange of subunits between microtubules and the soluble tubulin, in vitro and in intact cells (Saxton et al., 1984). The continuous addition of subunits at one end and removal from the other has been called *treadmilling*.

Studies of the kinetics of polymerization in cells or *Xenopus* extracts have indicated that catastrophe and shrinkage rates are much higher than those obtained with purified components, suggesting the presence of factors controlling these events (e.g., Cassimeris et al., 1988; Belmont et al., 1990), the *microtuble-associated proteins* (MAPs). Purification of *Xenopus* extracts led to the isolation of XMAP215 (also called XMAP) a 215 KDa protein that stimulates growth eighfold at the plus end (Vasquez et al., 1994) and decreases the rate of catastrophe (Tournebize et al., 2000) . In addition, two catastrophe factors opposing the effect of XMAP215 have been found: XKCM1 (e.g., Tournebize et al., 2000) and Op18/stathmin (e.g., see Cassimeris, 2002). XKCM1 is a protein of the kinesin family which doe not have motor activity. Op18/stathmin is a small heat stable protein. Inhibition of XKCM1 suggests that it is the major factor in catastrophe (slowing 4-7x), with Op18/stathmin as a minor factor (slowing 2x). XMAP215 is an elongated molecule, about 60 nm in length, long enough to span seven to eight tubulin dimers along a protofilament. (Cassimeris et al., 2001). The XMAP215 protein was found to be a member of a family of proteins (Dis/XMAP215) which have similar functions. They are target of regulation by phosphorylation-dephosphorylation (e.g., Vasquez et al., 1999). Similarly, Op18/strathmin when phosphorylated is less active (see Cassimeris, 2002) Regulation of the polymerization has very important roles in the organization of the cytoskeleton and cell polarity and at least some of this regulation results from the physiologically controlled phosphorylation of MAPs by *microtubule-affinity regulating kinases* (MARKS). MARKs are serine/threonine kinases that phosphorylate the tubulin binding domain of MAPs (e.g. see Drewes, 1997).

Inside cells, the assembly is highly regulated spatially and temporally. For example, the microtubules that will form the mitotic apparatus (see next section) must assemble in a particular location (that is, at the two poles) and at the appropriate time (i.e., at the beginning of mitosis) to create the proper structure. Conversely, its disassembly must be organized. The same regulation should take place at other sites of microtubular assembly.

MTs are constituted by α and β subunits. The structure of the two subunits are almost identical. Both have a core of two β-sheets surrounded by α-helices. The structure has been elucidated using electron crystallography (e.g., Nogales et al., 1998).

The α and β subunits bind guanine nucleotides. The nucleotides are exchangeable when bound to the β but not the α subunit.

The polymerization of MTs occurs between tubulin dimers (α and β) and is generally initiated at *microtubule-organizing centers* (MTOCs) with the minus end of the microtubule attached to the MTOC. The MTOCs are frequently diverse structures. In animal cells the basal bodies of cilia and flagella and the centrioles are MTOCs. Assembly of MTs can also occur spontaneously in vitro. In the test tube, the

assembly produces MTs with 14 *protofilaments* (i.e. the filaments formed by the assembly of α and β tubulin dimers so that 14 tubulin dimers appear in cross section). In contrast, in the presence of a MTOC, the assembled MT has 13 protofilaments.

The assembly of MTs proceeds by the addition of GTP-bound tubulin molecules to the growing ends. The GTP is bound to the β-tubulin of the αβ heterodimer. Upon polymerization the GTP molecule is hydrolyzed to GDP (see Erickson and O'Brien, 1992 and Mitchison, 1993). A stable GTP molecule is also bound to the α component but it has no role in the polymerization.

γ-Tubulin first discovered in *Aspergillus nidulans* has been shown to be present in virtually all eukaryotes and to be highly concentrated in MTOCs (Oakley et al., 1990; Stearns et al., 1991; Zheng et al., 1991). These findings led to the proposal that the γ-tubulins held in the MTOC could be the template for polymerization. γ-Tubulin is present in a high molecular weight complex (Stearns and Kirschner, 1994). This complex has been isolated and shown to accelerate polymerization dramatically (Zheng et al., 1995). EM studies with 3D-immuno tomographic techniques show the presence of a helical open ring structure which approximately corresponds in width to the MTs and which are decorated with anti-γ-tubulin antibodies (Moritz et al., 1995). The γ-tubulin-ring complex (γ-TuRC) has a minimum of seven polypeptides two of which are α and β-tubulin. The authors estimate 10 to 13 γ-tubulin per complex and 1 to 2 α and β-tubulin. Zheng et al., proposed an arrangement as shown in Fig 24. In this model the non-tubulin component forms a helical scaffold on which 13 γ-tubulin molecules are arranged. These 13 molecules determine the number and polarity of the protofilaments. The endogenous α-β dimer is postulated to form a stabilizing side-by-side interactions with the first exogenous dimer joining the complex.

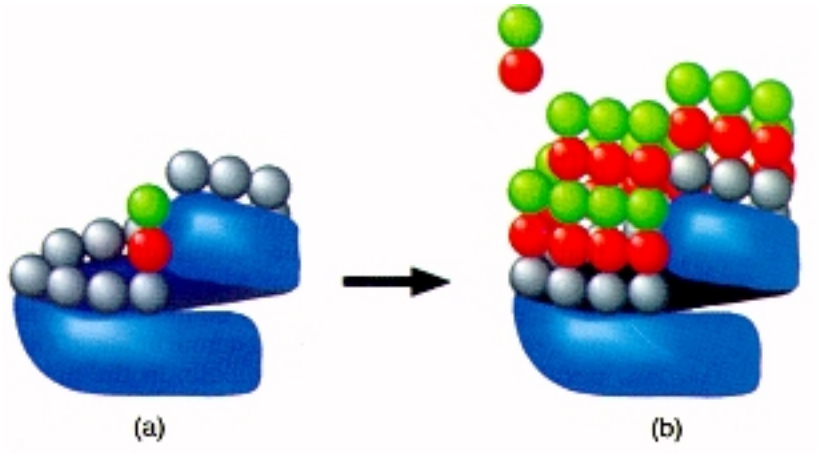


Fig. 24 Model for the nucleation by the γ-tubulin ring complex (γ-TuRC). (a) Proposed structure of the purified γ-TuRC. Blue: helical scaffold; grey: γ-tubulin; green and red: α-β dimers. (b) Nucleation by the helical arrangement of γ-tubulin molecules on the surface of the ring. Reproduced from Raff, J.W., (1996) *Trends in Cell Biology* Centrosomes and microtubules: wedded with a ring, 6:248-251. Copyright &copy1996 with permission from Elsevier Science.

Data-base searches in the human genome for sequences homologous to other tubulins (Chang and Stearns, 2000) found a DNA coding for tubulin (δ-tubulin) and one called ε-tubulin. The human δ-tubulin is 40% identical to the *Chlamydomonas* δ-tubulin and has a predicted molecular mass of 51 kDa. Immunofluorescence experiment using cells in culture localized the two within the centrosome but distinct from each other and γ-tubulin. δ-tubulin was most visible between centrioles within the centrosome. ε-Tubulin colocalized with γ-tubulin. However, ε-tubulin has a cell-cycle specific localization. The single centrosome of G1 cells possess ε-tubulin. The new centrosome acquires ε-tubulin later in what could be regarded a maturation process.

Transport in axons and dendrites

As we saw in Chapter 22, neurons are functionally and anatomically unique cells. The axons extend from the cell body for the length of the nerve. Because most synthetic and assembly processes take place in the cell body (the exception being neurotransmitter synthesis and packaging, see Chapter 22), the distant regions can be supplied only by a special transport system. Conversely, to be removed from the periphery, continuous transport in the direction of the cell body is needed. Movement from the cell body out into the axon is referred to as *anterograde*. Movement in the opposite direction is called *retrograde*.

When neuronal cell bodies are labelled by radioactive amino acids (e.g., by injection into a ganglion), labelled proteins are detected along the axon as a function of time and distance. The proteins can be separated by SDS-PAGE electrophoresis (see Chapter 1). This approach has shown that different proteins travel at different rates (see Grafstein and Forman, 1980).

Rapid anterograde transport [approximately 200 to 400 mm per day (2-5 μm s^{-1})] is responsible for the movement of vesicle, endoplasmic reticulum, synaptic vesicles, and plasma membrane components. Slow movement [0.3 to 8 mm per day (0.003-0.09 μm s^{-1})], on the other hand, concerns the movement of cytoskeletal elements and cytoplasmic enzymes of intermediate metabolism. Retrograde transport resembles fast movement and seems to correspond to movement of vesicles in the endocytotic pathway (see Chapters 9 and 11).

Fast axonal transport involves microtubules. Virtually all microtubules are oriented with the plus end toward the axon terminal and the minus end toward the cell body (e.g., Heidemann et al., 1981). In axons the plus end corresponds to the direction of the anterograde transport. Transport of materials along the microtubules in one direction is different from transport in the other direction -- structurally and kinetically. Gliding in either direction along single axonal microtubules has been observed (Allen et al., 1985), and two major mechanochemical transducing enzymes (or so-called motors), *dynein* and *kinesin*, are responsible for transport, dynein toward the plus and kinesin toward the minus end. For a recent review see Hirokawa, 1998. The involvement of microtubules and these motors in movement will be discussed in more detail in Chapter 24.

Recent studies indicate that the mechanism of the slow movement can be accounted for by conventional motors acting on microtubular tracks. The slowness of the process is a consequence of the discontinuity of the movement. The movement of neurofilaments (NFs) in neurons in culture has been followed by labelling the filaments with green fluorescent protein (see Chapter 1) (Wang et al., 2000; Roy et al., 2000). NF transport in both directions exhibited a broad range of rates with averaged to approximately 0.6-0.7 $\mu m\ s^{-1}$. The movement of individual NFs was intermittent with prolonged pauses, explaining the slow rate of movement (on the average only 20% of the NFs are in movement at any one time). These experiments also show that the movement is that of assembled filaments (a source of controversy at one time). In vitro the NFs (Shah et al., 2000) or vimentin filaments in intact fibroblasts (Prahlad et al., 1998) move rapidly (0.1-1 $\mu m\ s^{-1}$ for NFs and 0.55 $\mu m\ s^{-1}$ for vimentin) on microtubular tracks. The vimentin filaments colocalize with conventional kinesin as shown by immunofluorescence. In the case of the NFs, the movement was mediated in part by the dynein/dynactin motor complex (minus end directed motion) and several kinesin-like proteins (plus end directed motion) . These motors were found associated with the NFs after their isolation and were implicated using immunological methods.

Clearly microtubules and microtubular motors are involved in organelle and vesicular transport in axons. However, this may not be the whole story. Movement on fibers other than microtubules has been shown in extruded cytoplasm (Kutznetsov et al., 1992). However, the fibers were formed after delays of as much as thirty minutes, suggesting that they were not present in the intact axon.

In dendrites, the microtubules have a mixed pattern of polarity (Baas et al., 1988) apparently established by motor proteins that transport the microtubules from the cell body to the appropriate location (see Sharp et al., 1995). A motor protein, with the properties needed to intercalate minus end-leading microtubules to arrays of plus-oriented microtubules, has been identified and localized to dendrites of developing neurons by immunofluorescence (Sharp et al., 1997).

Generally there is no movement of the MTs themselves in axons (e.g., Hollenbeck and Bamburg, 1999, or with FSM: Chang et al., 1999) except in growth cones and in developing interstitial branches (Dent et al., 1999).

E. Movements in the Mitotic Spindle

The complexity, precision, and drama (it has all but a surprise ending) of cell division have fascinated many investigators from the very first days of the study of cells. Microtubules are heavily involved in many of the processes. This section can only cover some of the salient points. The first part will discuss the general pattern of mitosis, followed by some of the details of chromosome movement.

Mitosis follows a specific choreography that differs somewhat in detail from one kind of cell to another (for details see e.g., Hyams and Brinkley, 1989). The cells enter mitosis, that is, the M phase from the G_2 phase (see Chapter 8). At *prophase* (Fig. 25), the chromatin that is present in a diffuse form at interphase and has duplicated during the preceding S-phase (the phase before G_2) to form sister chromatids,

condenses to form chromosomes, which remain attached at the *centromere* (see below). The centromere is the part of the chromosome that serves as an organizer in the formation of the *kinetochores*. The microtubules, disassembled from the cytoskeleton, begin assembling to form an *aster* at the *microtubule organizing centers* (MTOC) corresponding to the *centrioles* in animal cells. The centrioles have originated from the duplication of the original centriole pair (the *centrosome*) and eventually arrange themselves at opposite ends of the cells at the *poles*. At *prometaphase*, the nuclear envelope breaks up into many vesicles and the microtubules of the nascent spindle extend into the nuclear region. Some of the microtubules attach to kinetochores, the kinetochore microtubules (K-MT in Fig 25). The kinetochores are specialized structure formed from the centromeres, a specific portion of each chromosome.

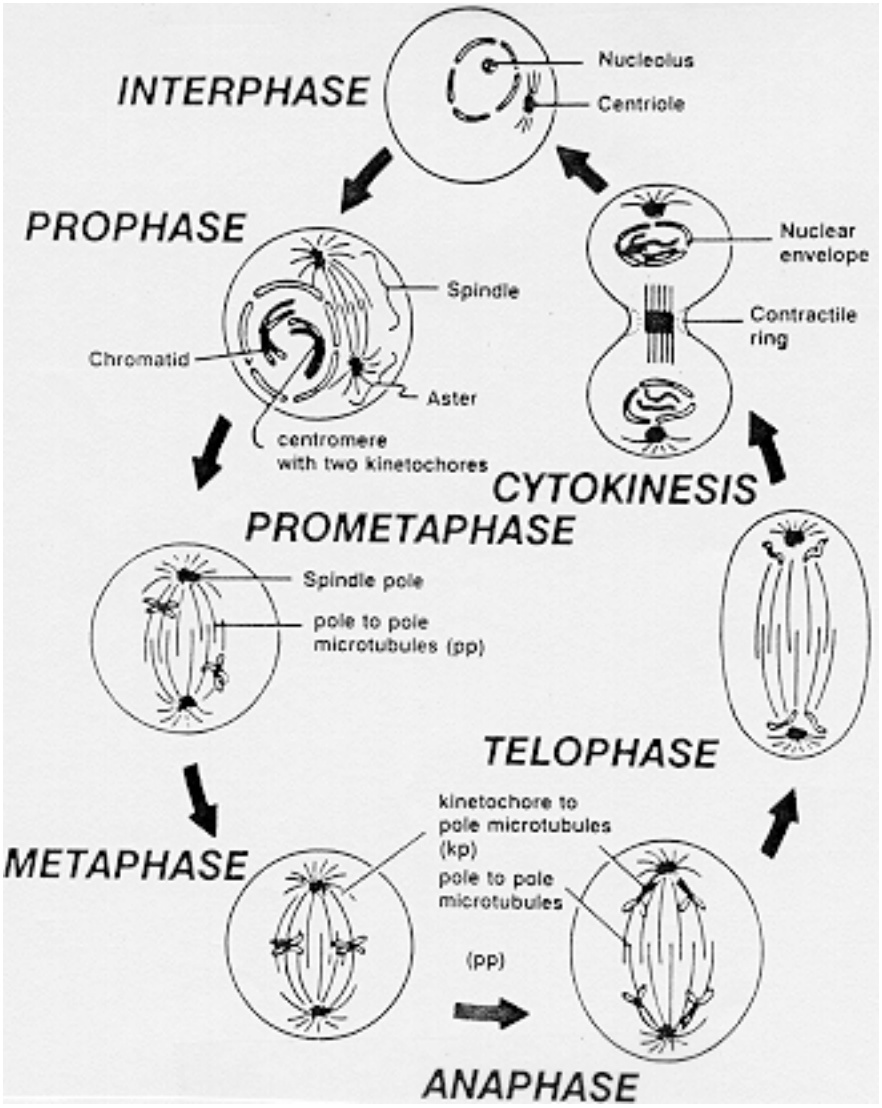


Fig. 25 Diagrammatic representation of the processes occurring during mitosis.

The polar microtubules (P-MTs in Fig. 25 and 26) overlap with microtubules originating from the opposite pole in the spindle equator. Other microtubules remain in the original aster and may play a role during the movement of the centrioles in cell elongation during anaphase (see below). At *metaphase*, the chromosomes become aligned in the *metaphase plate*. The splitting of the centromeres initiates

anaphase, in which the kinetochore microtubules (KMTs) shorten and the sister chromatids move in opposite directions. At anaphase, the spindle elongates when the poles of the spindle are pulled apart and the P-MTs elongate by polymerization. During *telophase*, the separated daughter chromatids arrive at the poles and the K-MTs dissociate, whereas the P-MTs continue elongating. The nuclear envelope then reforms and the chromatids expand from the condensed configuration. At *cytokinesis*, the two newly formed cells separate in a process which includes the contraction of the *contractile ring*, based on an actomyosin system (Schroeder, 1973; Mabuchi and Okuno, 1977).

During some of these stages, the plus end of the MTs are connected to the kinetochores and their minus end to the poles (K-MTs) (see e.g., Euteneur and McIntosh, 1981). The two kinetochores of each sister chromatid are attached to K-MT that are connected to opposite poles. Tubulin is thought to be continuously incorporated at the kinetochore during metaphase. During anaphase, when the K-MT shorten and the kinetochores have moved toward the poles, tubulin is lost from the kinetochore region. These exchanges have been followed by injecting fluorescently labelled tubulin into dividing cells (see below). The fluorescent tubulin gets incorporated in the MTs.

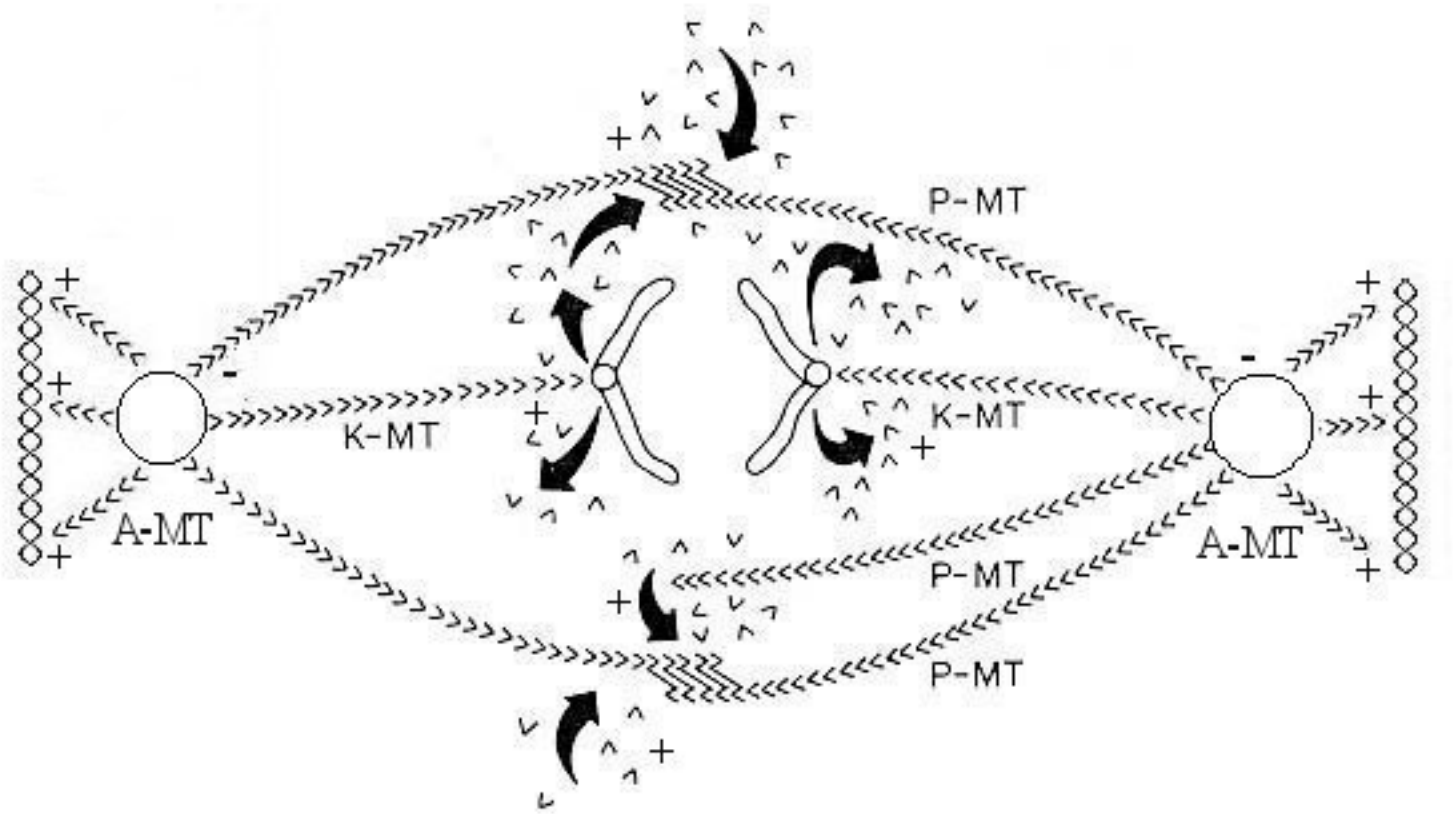


Fig. 26 Diagrammatic representation of events during anaphase. Only one chromosome pair is shown. The

circles represent centrosomes, K-MTs correspond to the kinetochore microtubules, P-MTs correspond to the polar microtubuIes and A-MT to the astral microtubules. The arrowheads represent the tubulin subunits that form the microtubules. The heavy arrows indicate the direction of the polymerization of the P-MTs or the K-MTs. The lines between the oppositely oriented P-MTs represent cross-links. The + and - signs indicate the polarity of the microtubules. The meshworks on the distal sides of the centrosomes represent the cytoplasmic cortex.

The details of mitosis are far more complex than those presented here. This section can only outline the major aspects. There are a number of questions that may be asked. How do the microtubules attach to the kinetochores? Using same-cell correlative video-DIC light microscopy, immunofluorescence, and electron microscopy, Rieder and Alexander (1990) studied the attachment of chromosomes that were initially positioned many micrometers from the polar region in early prometaphase. Attachment occurred when a single microtubule became associated with one of the kinetochores. The kinetochore then moved poleward along the surface of the microtubule, suggesting a mechanism of movement similar to the other microtubule based motor-driven movements, as discussed for the axon. Before attachment, the kinetochore lacks microtubules (shown by EM and immunofluorescence) so that it cannot serve as a nucleation site. Another important question is the mechanism of alignment of the chromosomes in the metaphase plate. This is thought to be the result of a balance between the pull by the K-MTs attached kinetochores connected to opposite poles. The force appears to be proportional to the length of the attached microtubule, tending to keep the chromosome on the equatorial region. What initiates the anaphase movements? Ample evidence suggests that anaphase is triggered by a sudden increase in the Ca^{2+} concentration released by vesicles in the spindle (see Hepler, 1980; Hepler and Callahan, 1987).

The events of anaphase have two components. At anaphase A, the K-MTs shorten and the sister chromatids are pulled to opposite poles (Gorbsky et al., 1988). In contrast, at anaphase B, the poles move apart and the P-MTs lengthen (Masuda and Gande, 1987, Pickett-Heaps, 1986). The two are clearly distinct processes because they can take place independently. For example, chloral hydrate blocks anaphase B but has no effect on chromosome movement.

Movements of the kinetochores in anaphase could be generated by several possible mechanisms. Apparently, the actomyosin system is not involved since antimyosin or antiactin applied to isolated spindles, lysed cells, or microinjected into cells, does not interfere with anaphase movements (Sakai et al., 1976), whereas they block cytokinesis. Of several possible alternatives, two of these are considered the most likely. One involves protein motors and is discussed more fully in the next chapter (Chapter 24). The other corresponds to the disassembly of the microtubules.

At least eight distinct motors have been found to be involved in mitosis (see Chapter 24). Two motors with opposite polarity have been demonstrated on kinetochores of isolated chromosomes (e.g., Hyman and Mitchison, 1991). Experiments using various concentrations of ATPγS, an ATP analog, have implicated phosphorylation in regulating the motors. ATPγS can phosphorylate but cannot be hydrolyzed, that is they cannot provide energy for movement. Different concentrations of ATPγS were able to vary the direction of the movement. In addition, the presence of two motors with opposite polarity suggests a

possible role of the kinetochore in a variety of chromosome movements indicated in Fig. 26. A plus-end motor (i.e., acting away from the poles) could be involved at or before metaphase. The minus-end motor (i.e., acting toward the poles) would be involved in the anaphase A chromosome movement. In support of this position, antibody to one of the proteolytic fragments of dynein was found to block the process (Sakai et al., 1976). Since no K-MTs remain on the equatorial side of the chromosomes as the kinetochores are moved toward the poles, the K-MTs must depolymerize at the kinetochore. Such a depolymerization has been demonstrated (Gorbsky et al., 1988) using fluorescent tubulin (derivatized with the dye X-rhodamine) injected into cells. The MTs are homogeneously labelled. A photobleached spot on a K-MT remains stationary while the microtubule shortens. This could only happen if the K-MTs were depolymerizing at the kinetochore end. Note that this observation implies a dissociation at the plus-end of the microtubules.

The evidence strongly supports the involvement of a motor in the movement of the kinetochores. However, the depolymerization could provide the needed energy by itself as demonstrated with model systems (Coue et al., 1991). In this study, lysed and extracted *Tetrahymena* cells were used. Microtubules were polymerized from ordered arrays of basal bodies so that the minus end is fixed. They could be depolymerized simply by perfusing with a tubulin-free medium. Attached chromosomes introduced in the system were found to move in the direction of the basal bodies upon depolymerization. The movement appears not to require ATP (e.g. it occurred in the presence of orthovanadate which blocks ATP hydrolysis or apyrase, an enzyme which hydrolyses ATP), and under appropriate conditions it exhibited constant velocity.

Anaphase B spindle elongation involves the P-MTs. In this case, isolated spindles from diatoms were shown to incorporate fluorescently labelled tubulin at the spindle midzone (Masuda and Cande, 1987). However, the actual elongation requires ATP hydrolysis and might involve a motor in a mechanism resembling the sliding of axonemes in disrupted cilia or flagella. An overlap between P-MTs originating from the two different poles has been shown with electron microscopy (McDonald et al., 1979). Cross-bridges between P-MTs have been shown in isolated mitotic spindles using a colloidal gold-labelled monoclonal antibody to flagellar dynein (Hirokawa et al., 1985). In contrast to cilia or flagella (see Fig. 26), the sliding tubules are antiparallel. However, dynein cross-links are possible with either parallel or antiparallel configuration (Warner and Mitchell, 1981), suggesting that MTs could still slide past each other despite this orientation.

F. Cytokinesis

In cell division, the formation of two daughter cells requires an equal distribution of the newly formed chromosomes and the various cellular components. The process by which two daughter cells become separated from the mother cell is referred to a *cytokenesis*. The formation of a *cleavage furrow* is one of the early manifestations of cytokinesis.

The division of the chromosomes and cytokinesis are well coordinated. In animal cells, the possibility that the mitotic apparatus plays a physical role in cytokinesis has been experimentally eliminated.

However, the position of the asters determines the eventual position of the cleavage furrow (Rappaport, 1971).

The actual details of how the cleavage furrow components are assembled or how cytokinesis takes place is still not well understood. Actomyosin is involved and actomyosin is thought to be present throughout the cell cortex. A separation of the two cells could take place as a consequence of a contraction at the location of the furrow or as a consequence of a relaxation at the poles (see Rappaport, 1971). Experiments involving micromanipulation implicate a contraction at the furrow (e.g., Rappaport, 1967; Ohtsubo and Hiramoto, 1985).

An experiment of Burton and Taylor (1997) also shows an equatorial location for development of force. The traction of cells adhering to a substratum of silicon rubber can be detected by the wrinkles produced by the force generated by the cells. The magnitude and direction of the force can be estimated from these deformations. In sheets sensitized by exposure to ultraviolet light, the length of the wrinkles is proportional to the force exerted. With this method it was possible to demonstrate that traction is exerted at the cell equator, where the cleavage furrow forms and the traction increases with time. It drops sharply after the two cells have separated. Similarly the force also increases slightly in the rest of the cell and at the poles until the cells divide. The actual contractile mechanism is still under discussion.

V. CONCLUDING REMARKS

The examples presented make it clear that motility occurs in a variety of ways that may be based on fundamentally different molecular processes. On the other hand, as we shall see, there are a number of similarities in the behavior of the macromolecules extracted from these motile systems. For this reason, a number of investigators have used as their working hypotheses the idea that the different motile systems are different facets of similar molecular events.

SUGGESTED READING

General

Bray, D. (1992) *Cell Movements*, Garland Publishing Inc., New York and London, pp. 406.

The centrosome

Urbani, L. and Stearns, T. (1999) The centrosome, *Curr. Biol.* 9:R315-317. (MedLine)

Plants

Kamiya, N. (1981) Physical and chemical basis of cytoplasmic streaming, *Annu. Rev. Plant Physiol.* 32:205-236.

Kamiya, N. (1986) Cytoplasmic streaming in giant algal cells: a historical survey of experimental approaches. *Bot. Mag. (Tokyo)* 99:44l-467.

Actin polymerization and crawling

Machesky, L.M. and Gould, K.L. (1999) The Arp2/3 complex: a multifunctional actin organizer, *Curr. Opin. Cell Biol.* 11:117-121. (MedLine)

Mullins, R.D. (2000) How WASP-family proteins and the Arp2/3 complex convert intracellular signals into cytoskeletal structures, *Curr. Opin. Cell Biol.*12:91-96. (MedLine)

Small, J.V., Stradal, T., Vignal, E. and Rottner, K. (2002) The lamellipodium: where motility begins, *Trends Cell Biol.* 12:112-120. (MedLine)

Stossel, T.P. (1993) On the crawling of animal cells, *Science* 260:1086-1094. (MedLine)

Movement in Axons

Vallee, R.B., and Bloom, G.S, (1991) Mechanisms of fast and slow axonal transport, *Annu. Rev. Neuroscie.* 14:59-92. (MedLine)

Microtubules during Interphase and in Mitosis

Joshi, H.C. (1998) Microtubule dynamics in living cells, *Curr. Opin. Cell Biol.* 10:35-44. (Medline)

Lane, J. and Allan, V. (1998) Microtubule-based membrane movement, *Biochim. Biophys. Acta* 1376:27-55. (MedLine)

McIntosh, J. R., and Pfarr, C. M. (1991) Mitotic motors, *J. Cell Biol.* 115:577-585. (MedLine)

Sawin, K. E., and Scholey, J. M. (1991) Motor proteins in cell division, *Trends in Cell Biol.* 1:123-129.

Shroer, T. A., and Sheetz, M. P. (1991) Functions of microtubule based motors, *Annu. Rev. Physiol.* 53:629-652.

WEB RESOURCES

Barth, A. and de Hostos, E.L., Filaments on the Move: Cells Expressing GFP-Actin or Tubulin. http://www-bioc.rice.edu/~hostos/gfptubMDCK.html

Brown, A. et al. (2000) movies of neurofilaments on the move
http://www.biosci.ohiou.edu/faculty/brown/pages/movies.html

Carminati, J. Microtubules orient the mitotic spindle in yeast through dynein-dependent interactions with the cell cortex. http://www-leland.stanford.edu/~stearns/carminati.html

Cell Migration Consortium. http//www.cellmigration.org.html See Cell Migration Science section.

Kaech, S., Ludin, B. and Matus, A. (1996) Cytoskeletal plasticity in cells expressing neuronal microtubule-associated proteins *Neuron* 17:1189-1199.
http://www.fmi.ch/groups/AndrewMatus/Video.html

Olmsted, J.B. The hows and whys of cellular morphogenesis.
http://www.rochester.edu/College/BIO/olmstedlab/olmstedhp.html

Waterman-Storer, C., Microtubule dynamics in migrating cells.
http://www.unc.edu/depts/biology/salmon.html

REFERENCES

Search the textbook

Back to Chapter 23

REFERENCES

Abe, H., Obinata, T., Minamide, L.S. and Bamburg, J.R. (1996) *Xenopus laevis* actin-depolymerizing factor/cofilin: a phosphorylation-regulated protein essential for development, *J. Cell Biol.* 132:871-885.(MedLine)

Adelman, M.R., Borisy, G.C., Shelanski, N.L., Weisenberg, R. C., and Taylor, E.W. (1968) Cytoplasmic filaments and tubules, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 27:1186-1193.

Allen, R.D., Francis, D. and Zeh, R. (1971) Direct test of the positive pressure gradient theory of pseudopod extension and retraction in amoebae, *Science* 174:1237-1240.(MedLine)

Allen, R. D., Allen, N. S., and Travis, J. L. (1981) Video-enhanced contrast, differential interference contrast (AVEC-DIC) microscopy: a new method capable of analyzing microtubule-related motility in the reticulopodial network of *Allogromia laticollaris*, *Cell Motil.* 1:291-302.(MedLine)

Allen, R. D., Weiss, D. G., Hayden, J. H., Brown, D.T., Fujiwake, H., and Simpson, M. (1985) Gliding movement of and bidirectional transport along single native microtubules from squid axoplasm: evidence for an active role of microtubules in cytoplasmic transport, *J. Cell Biol.* 100:1736-1752.(MedLine)

Anderson, K.I., Wang, Y.-L. and Small, J.V. (1996) Coordination of protrusion and translocation of the keratocyte involves rolling of the cell body, *J. Cell Biol.* 134:1209-1218.(MedLine)

Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O. and Caroni, P. (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase, *Nature* 393:805-809..(MedLine)

Baas, P.W., Deitch, J.S., Black, M.M., and Banker, G.A. (1988) Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite, *Proc. Natl. Acad. Sci. USA* 85:8335-8339..(MedLine)

Bagrodia, S. and Cerione, R.A. (1999) Pak to the future, *Trends Cell Biol.* 9:350-355.(MedLine)

Bailly, M., Macaluso, F., Cammer, M., Chan, A., Segall, J.E. and Condeelis, J.S. (1999) Relationship between Arp2/3 complex and the barbed ends of actin filaments at the leading edge of carcinoma cells after epidermal growth factor stimulation, *J. Cell Biol.* 145:331-345.(MedLine)

Baines, I.C., Brzeska, H. and Korn, E.D. (1992) Differential localization of Acanthamoeba myosin I isoforms, *J. Cell Biol.* 119:1193-1203. (MedLine)

Bamburg, J.R., McGough, A. and Ono, S. (1999) Putting a new twist on actin: ADF/cofilins modulate actin dynamics, *Trends Cell Biol.* 9:364-370.(MedLine)

Belmont, L.D., Hyman, A.A., Sawin, K.E. and Mitchison, T.J. (1990) Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts, *Cell* 62:579-589. (MedLine)

Bré, M.H., and Karsenti, E. (1990) Effect of brain microtubule-associated protein on microtubule dynamics and nucleating activity of centrosomes, *Cell Motil. Cytosk.* 15:88-98.(MedLine)

Bresnick, A.R. (1999) Molecular mechanisms of nonmuscle myosin-II regulation, *Curr. Opin. Cell Biol.* 11:26-33.(MedLine)

Brokaw, C.J. (1967) Adenosine triphosphate usage by flagella, *Science* 156:76-78.(MedLine)

Brundage, R.A., Fogarty, K. E., Tuft, R.A., and Fay, F.S. (1991) Calcium gradients underlying polarization and chemotaxis in eosinophils, *Science* 254:703-706.(MedLine)

Bryan, J., and Wilson, L. (1971) Are cytoplasmic microtubules heteropolymers? *Proc. Natl. Acad. Sci. U.S.A.* 68:1762-1766.(MedLine)

Burton, K., and Taylor, D.L. (1997) Traction forces of cytokinesis measured with optically modified elastic substrate, *Nature* 385:450-454.(MedLine)

Cain, D.F., Infante, A.A., and Davies, R.E. (1962) Chemistry of muscle contraction: adenosine triphosphate and phosphorylcreatine as energy supplies for single contractions of working muscle, *Nature* 196:214-217.

Carlier, M.-F. and Pantaloni, D. (1997) Control of actin dynamics in cell motility, *J. Mol. Biol.* 269:459-467.(MedLine)

Carlsen, F., Knappeis, G.G., and Buchtal, F. (1961) Ultrastructure of the resting and contracted striated muscle fiber at different degrees of stretch, *J. Biophys. Biochem. Cytol.* 11:95-117.

Cassimeris, L. (2002) The oncoprotein 18/stathmin family of microtubule destabilizers, *Curr. Opin. Cell Biol.* 14:18-24. (MedLine)

Cassimeris, L., Pryer, N.K. and Salmon, E.D. (1988) Real-time observations of microtubule dynamic

instability in living cells, *J. Cell Biol.*107:2223-2231. (MedLine).

Cassimeris L, Gard D, Tran PT, Erickson HP. (2001) XMAP215 is a long thin molecule that does not increase microtubule stiffness, *J. Cell Sci.* 114:3025-3033. (MedLine)

Chang, P. and Stearns, T. (2000) δ-Tubulin and ε-tubulin: two new human centrosomal tubulins reveal new aspects of centrosome structure and function, *Nature Cell Biol.* 2:30-35. (MedLine)

Chang, S., Svitkina, T.M., Borisy, G.G., Popov, S.V. (1999) Speckle microscopic evaluation of microtubule transport in growing nerve processes, *Nature Cell Biol.* 1:399-403.(MedLine)

Chu, Q. and Fukui, Y. (1996) In vivo dynamics of myosin II in *Dictyostelium* by fluorescent analogue cytochemistry, *Cell Motil. Cytosk.* 35:254-268. (MedLine)

Coue, M., Lombillo, V.A., and McIntosh, J. R. (1991) Microtubule depolymerization promotes particle and chromosome movement in vitro, *J. Cell Biol.* 112:1165-1175.(MedLine)

Dahl, J. L., and Weibel, V.J. (1979) Changes in tubulin heterogeneity during postnatal development of rat brain, *Biochem. Biophys. Res. Commun.* 86:822-828.(MedLine)

Dales, S. (1972) Concerning the universality of a microtubule antigen in animal cells, *J. Cell Biol.* 52:748-754.(MedLine)

de Heuvel, E., Bell, A.W., Ramjaun, A.R., Wong, K., Sossin, W.S. and McPherson, P.S. (1997) Identification of the major synaptojanin-binding proteins in brain, *J. Biol. Chem.* 272:8710-8716.(MedLine)

Denoulet, P., Jentet, C.,and Gros, F. (1982) Tubulin microheterogenicity during mouse liver development, *Biochem. Biophys. Res. Commun.* 105:806-813.(MedLine)

Dent, E.W., Callaway, J.L., Szebenyi, G., Baas, P.W. and Kalil, K. (1999) Reorganization and movement of microtubules in axonal growth cones and developing interstitial branches, *J. Neurosci.* 19:8894-8908.(MedLine)

Di Paolo, G., Pellegrini, L., Letinic, K., Cestra, G., Zoncu, R., Voronov, S., Chang, S., Guo, J., Wenk, M.R. and De Camilli, P. (2002) Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 γ by the FERM domain of talin, *Nature* 420:85-89. (MedLine)

Donges, S., and Roth, E. A. (1972) Serological similarities of microtubule protein, *Naturwissenschaften* 59:372.

Doolittle, K.W., Reddy, I. and McNally, J.G. (1995) 3D analysis of cell movement during normal and myosin-II-null cell morphogenesis in *Dictyostelium*, *Dev. Biol.* 167:118-129.(MedLine)

Downey,G.P., Chan, C.K., Trudel, S. and Grinstein, S. (1990) Actin assembly in electropermeabilized neutrophils: role of intracellular calcium, *J. Cell Biol.* 110:1975-1982.

Dramsi, S. and Cossart, P. (1998) Intracellular pathogens and the actin cytoskeleton, *Annu. Rev. Cell Dev. Biol.* 14:137-166.(MedLine)

Drewes, G., Ebneth, A, Preuss, U., Mandelkow, E.M. and Mandelkow, E. (1997) MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption, *Cell* 89:297-308.(MedLine)

Eden, S., Rohatgi, R., Podtelejnikov, A.V., Mann, M. and Kirschner, M.W. (2002) Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck, *Nature* 418:790-793. (MedLine)

Edwards, D.C., Sanders, L.C., Bokoch, G.M. and Gill, G.N. (1999) Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeleton dynamics, *Nature Cell Biol.* 1:253-259.(MedLine)

Erickson, H.P. and O'Brien, E.T. (1992) Microtubule dynamic instability and GTP hydrolysis *Annu. Rev. Biophys. Biomol. Str.* 21:145-166.(MedLine)

Euteneuer, S.E. and Schliwa, M. (1984) Persistent, directional motility of cells and cytoplasmic fragments in the absence of microtubules, *Nature* 310:58-61.(MedLine)

Felsenfeld, D.P., Schwartzberg, P.L., Venegas, A., Tse, R. and Sheetz, M.P. (1999)Selective regulation of integrin--cytoskeleton interactions by the tyrosine kinase Src, *Nature Cell Biol* 1:200-206.(MedLine)

Forscher, P. and Smith, S.J.(1988) Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone, *J. Cell Biol.* 107:1505-1516.(MedLine)

Frangiskakis, J.M., Ewart, A.K., Morris, C.A., Mervis, C.B., Bertrand, J., Robinson, B.F., Klein, B.P., Ensing, G.J., Everett, L.A., Green, E.D., Proschel, C., Gutowski, N.J., Noble, M., Atkinson, D.L., Odelberg, S.J. and Keating, M.T. (1996) LIM-kinase 1 hemizygosity implicated in impaired visuospatial constructive cognition, *Cell* 86:59-69.(MedLine)

Fukami, K., Endo, T., Imamura, M. and Takenawa, T. (1994) α-actinin and vinculin are PIP2-binding proteins involved in signaling by tyrosine kinase, *J. Biol. Chem.* 269:1518-1522.(MedLine)

Fukami, K., Sawada, N., Endo, T. and Takenawa, T. (1996) Identification of a phosphatidylinositol 4,5-bisphosphate-binding site in chicken skeletal muscle α-actinin, *J. Biol. Chem.* 271:2646-2650.(MedLine)

Fulton, C., Kane, R.E., and Stephens, R.E. (1971) Serological similarities of flagellar and mitotic microtubules, *J.Cell Biol.* 50:762-773.(MedLine)

George, H.J., Misra, L., Field, D.J., and Lee, C. (1981) Polymorphism of brain tubulin, *Biochemistry* 20:2402-2409.(MedLine)

Gibbons, J.R. (1963) Studies on the protein components of cilia. from *Tetrahymena pyriformis*, *Proc. Natl. Acad. Sci. USA.* 50:1002-1010.

Gilmore, A.P. and Burridge, K. (1996) Regulation of vinculin binding to talin and actin by phosphatidyl-inositol-4-5-bisphosphate, *Nature* 381:531-535. (MedLine)

Goldberg, D.J. and Burmeister, D.W. (1986) Stages in axon formation: observations of growth of Aplysia axons in culture using video-enhanced contrast-differential interference contrast microscopy, *J. Cell Biol.* 103:1921-1931.(MedLine)

Goodman, C.S. (1996) Mechanisms and molecules that control growth cone guidance, *Annu. Rev. Neurosci.* 19:341-377.(MedLine)

Gorbsky, G.J., Sammak, P.J., and Borisy, G.G. (1988) Microtuble dynamics and chromosome motion visualized in living anaphase cells, *J. Cell Biol.* 106:1185-1192.(MedLine)

Gozes, I., and Littauer, U.Z. (1978) Tubulin microheterogeneity increase with rat brain maturation, *Nature* 276:411-413.(MedLine)

Gozes, I., and Sweadner, K.J. (1981) Multiple tubulin forms are expressed by a single neurone, *Nature* 294:477-480.(MedLine)

Grafstein, B., and Forman, D.S. (1980) Intracellular transport in neurons, *Physiol. Rev.* 60:1167-1283.(MedLine)

Hahn, K,, DeBiasio, R.L., and Taylor, D.L. (1992) Patterns of elevated calcium and calmodulin activation in living cells, *Nature* 359: 736-738.(MedLine)

Hall, A. (1998) Rho GTPases and the actin cytoskeleton, *Science* 279:509-514.(MedLine)

Hanson, J., and Huxley, H.E. (1955) The structural basis of contraction in striated muscle, *Symp. Soc.*

Exp. Biol. 9:228-264.

Hartwig, J.H. and Shevlin, P. (1986) The architecture of actin filaments and the ultrastuctural location of actin-binding proteins in the periphery of lung macrophage, *J. Cell Biol.* 103:1007-1020.(MedLine)

Hartwig, J.H., Kung, S., Kovacsovics, T., Janmey, P.A., Cantley, L.C., Stossel, T.P. and Toker, A. (1996) D3 phosphoinositides and outside-in integrin signaling by glycoprotein IIb-IIIa mediate platelet actin assembly and filopodial extension induced by phorbol 12-myristate 13-acetate, *J. Biol. Chem.* 271:32986-32993.(MedLine)

Hayashi, T. (1964) Role of the cortical gel layer in cytoplasmic streaming, In *Primitive Motile systems in Cell Biology* (Allen, R. D., and Kamiya, N., eds.), pp. 19-29. Academic Press, New York.

Hayden, J.H., Allen, R.D., and Goldman, R.D. (1983) Cytoplasmic transport in keratocytes; direct visualization of particle translocation along microtubules, *Cell Motil.* 3:1-19.(MedLine)

Heath, J.P. and Dunn, G.A. (1978) Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interence-reflexion and high voltage electron-microscope study, *J. Cell Biol.* 29:197-212.(MedLine)

Heath, J.P. and Holifield, B.F. (1991) Cell locomotion: new research tests old ideas on membrane and cytoskeletal flow, *Cell Motil. Cytoskel.* 18:245-257.(MedLine)

Heidemann, S.R., Landers, J.M., and Hamburg, M.A. (1981) Polarity orientation of axonal microtubules, *J. Cell Biol.*91:661-665.(MedLine)

Heidemann, S.R. and Buxbaum, R.E. (1998) Cell crawling: first the motor, now the transmission, *J. Cell Biol.* 141:1-4. (Medline)

Hepler, P.K. (1980) Membranes in the mitotic apparatus of barley cells, *J. Cell Biol.* 86:490-499.(MedLine)

Hepler, P.K. and Callahan, D.A. (1987) Free calcium increases during anaphase in stamen hair cells of *Tradescantia, J. Cell Biol.* 105:2137-2143.(MedLine)

Heuser, J.E. and Kischner, M.S. (1980) Filament organization revealed in platinum replicas of freeze-dried cytoskeletons, *J. Cell Biol.* 86:212-234.(MedLine)

Higgs, H.N. and Pollard, T.D. (2000) Activation by Cdc42 and PIP(2) of Wiskott-Aldrich syndrome protein (WASp) stimulates actin nucleation by Arp2/3 complex, *J. Cell Biol.* 150:1311-1320. (MedLine)

Hirokawa, N., Takemura, R., and Hisanaga, S.-I. (1985) Cytoskeletal architecture of isolated mitotic spindle with special reference to microtubule-associated proteins and cytoplasmic dynein, *J. Cell Biol.* 101:1858-1870.(MedLine)

Hirokawa, N. (1998) Kinesin and dynein superfamily proteins and the mechanism of organelle transport, *Science* 279:519-526. (MedLine)

Hollenbeck, P.J. and Bamburg, J.R. (1999) Axonal microtubules stay put, *Nature Cell Biol.* 1:E171-E173.(MedLine)

Horridge, G. A. (1965) Macrocilia with numerous shafts from the lips of the ctenophore *Beroe, Proc. R. Soc. London Ser. B* 162:351-364.

Huxley, A. F., and Niedergerke, R. (1954) Structural changes in muscle during contraction, *Nature* 173:971-973.

Huxley, H. E. (1960) Muscle cells. In *The Cell* (Brachet, J., and Mirsky, A. E., eds.), Vol. 4, Part 1, pp. 365-481. Academic Press, New York.

Hyams, J. S., and Brinkley, B. R. (1989) *Mitosis*, pp. 1-350. Academic Press, Orlando, Fl.

Hyman, A. A., and Mitchison, T. J. (1991) Two different microtubule-based motor activities with opposite polarities in kinetochores, *Nature* 351:206-211.(Medline)

Janmey, P.A. and Stossel, T.P. (1987) Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate, *Nature* 325:362-364.(Medline)

Jay, P.Y., Pham. P.A., Wong, S.A. and Elsone, E.L. (1995) A mechanical function of myosin II in cell motility, *J. Cell Scie.* 108:387-393.(MedLine)

Johnson, K. A. (1985) Pathway of the microtubule-dynein ATPase and the structure of dynein: a comparison with actomyosin, *Annu. Rev. Biophys. Biophys. Chem.* 14:161-188.(MedLine)

Kaech, S., Ludin, B.and Matus, A. (1996) Cytoskeletal plasticity in cells expressing neuronal microtubule-associated proteins, *Neuron* 17:1189-1199.(MedLine)

Kamiya, N., and Kuroda, K. (1956) Velocity distribution of the protoplasmic streaming in *Nitella* cells. I. Velocity distribution of protoplasm in a rhizoid cell, *Bot. Mag.* 69:544-555. <p>Kamiya, N., and Kuroda, K. (1957) Cell operation in *Nitella*. II. Behavior of isolated endoplasm, *Proc. Jpn. Acad.* 33:201-205.

Kamiya, N., and Kuroda, K. (1958) Studies on the velocity distribution of the protoplasmic streaming in the myxomycete *Plasmodium, Protoplasma* 49:1-4.

Kane, R.E. (1983) Interconversion of structural and contractile actin gels by insertion of myosin during assembly, *J. Cell Biol.* 97:1745-1752.(MedLine)

Kaverina, I., Rottner, K. and Small, J.V. (1998) Targeting, capture, and stabilization of microtubules at early focal adhesions, *J. Cell Biol.* 142:181-190.(MedLine)

Kaverina, I., Krylyshkina, O. and Small, J.V. (1999) Microtubule targeting of substrate contacts promotes their relaxation and dissociation, *J. Cell Biol.* 146:1033-1044.(MedLine)

Keating, T.J. and Borisy, G.G. (2000) Speckle microscopy: when less is more, *Curr. Biol.* 10:R22-R24.(MedLine)

Kucik,, D.F., Kuo, S.C., Elson, E.L. and Sheetz, M.P. (1991) Preferential attachment of membrane glycoproteins to the cytoskeleton at the leading edge of the lamella, *J. Cell Biol.* 114:1029-1036.(MedLine)

Kutznetsov, S. A., Langford, G.M. and Weiss, D.G. (1992) Actin-dependent organelle movement in the squid axoplasm, *Nature* 356:722-725.(MedLine)

Lassing, I. and Lindberg, U. (1985) Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin, *Nature* 314:472-474.(MedLine)

Lappalainen, P. and Drubin, D.G. (1997) Cofilin promotes rapid actin filament turnover in vivo, *Nature* 388:78-82.(MedLine)

Lappalainen, P., Fedorov, E.V., Fedorov, A.A., Almo, S.C. and Drubin, D.G. (1997) Essential functions and actin-binding surfaces of yeast cofilin revealed by systematic mutagenesis, *EMBO J.* 16:5520-5530.(MedLine)

Lauffenburger, D.A. and Horwitz, A.F. (1996) Cell migration: a physically integrated molecular process, *Cell* 84:359-369.(MedLine)

Lee, J., Leonard, M., Oliver, T., Ishihara, A. and Jacobson, K. (1994) Traction forces generated by locomoting keratocytes, *J. Cell Biol.* 127:1957-1964.(MedLine)

Lewis, A.K. and Bridgman, P.C. (1992) Nerve growth cone lamellipodia contain two populations of actin filaments that differ in organization and polarity, *J. Cell Biol.* 119: 1219-1243.(MedLine)

Lin, C.H. and Forscher, P. (1995) Growth cone advance is inversely proportional to retrograde F-actin flow, *Neuron* 14:763-771.(MedLine)

Lin, C.H., Espreafico, E.M., Mooseker, M.S. and Forscher, P. (1996) Myosin drives retrograde F-actin flow in neuronal growth cones, *Neuron*16:769-782.(MedLine)

Ling, K., Doughman, R.L., Firestone, A.J., Bunce, M.W. and Anderson, R.A. (2002) Type I γ phosphatidylinositol phosphate kinase targets and regulates focal adhesions, *Nature* 420:89-93. (MedLine)

Ludveña, R.F. (1998) The multiple forms of tubulin: different gene products and covalent modifications, *Int. Rev. Cytol.* 178:207-275.

Luna, A.J., Wuestehube, L.J., Ingalls, H.M. and Chia, C.P. (1990) The *Dictyostelium discoideum* plasma membrane: a model system for the study of actin-membrane interactions, *Adv. Cell Biol.* 3:1-33.

Ma, L., Rohatgi, R. and Kirschner, M.W. (1998) The Arp2/3 complex mediates actin polymerization induced by the small GTP-binding protein Cdc42, *Proc. Natl. Acad. Sci. USA* 95:15362-15367.(MedLine)

Mabuchi, I. and Okuno, M. (1977) The effect of myosin antibody on the division of starfish blastomeres, *J.Cell Biol.* 74:251-263.(MedLine)

MacDonald, A. C. and Kitching, J. A. (1967) Axopodial filament of heliozoa. *Nature* 215:99-100.

Machesky, L.M. and Gould, K.L. (1999) The Arp2/3 complex: a multifunctional actin organizer, *Curr. Opin. Cell Biol.* 11:117-121.(MedLine)

Machesky, L.M. and Insall, R.H. (1999) Signaling to actin dynamics, *J. Cell Biol.* 146):267-272. (MedLine)

Machesky, L.M., Atkinson, S.J., Ampe, C., Vandekerckhove, J. and Pollard, T.D. (1994) Purification of a cortical complex containing two unconventional actins from *Acanthamoeba* by affinity chromatography on profilin-agarose, *J. Cell Biol.* 127:107-115.(MedLine)

Machesky, L.M., Reeves, E., Wientjes, F., Mattheyse, F.J., Grogan, A., Totty, N.F., Burlingame, A.L., Hsuan, J.J. and Segal, A.W. (1997) Mammalian actin-related protein 2/3 complex localizes to regions of lamellipodial protrusion and is composed of evolutionarily conserved proteins, *Biochem. J.* 328:105-112.(MedLine)

Malawista, S.E. and De Boisfleury Chevance, A. (1982) The cytokinetoplast: purified, stable and

functional motile machinery for human blood polymorphonuclear leukocytes, *J. Cell Biol.* 95:960-973.(MedLine)

Manser, E., Leung, T., Salihuddin, H., Zhao, Z.S. and Lim, L. (1994) A brain serine/threonine protein kinase activated by Cdc42 and Rac1, *Nature* 367:40-46.(MedLine)

Masuda, H. and Cande, W.Z. (1987) The role of tubulin polymerization during spindle elongation, *Cell* 49:193-202.(MedLine)

McDonald, K.L., Edwards, M.K. and McIntosh, J.R. (1979 Cross-sectional structure of the central mitotic spindle of Diatoma vulgare. Evidence for specific interactions between antiparallel microtubules, *J. Cell Biol.* 83:443-461. (MedLine)

Micheva, K.D., Ramjaun, A.R., Kay, B.K. and McPherson, P.S. (1997) SH3 domain-dependent interactions of endophilin with amphiphysin, *FEBS Lett.* 414:308-312.(MedLine)

Miki, H., Suetsugu, S. and Takenawa, T. (1998) WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac, *EMBO J.* 17:6932-6941.(MedLine)

Mitchison, T.J. (1993) Localization of an exchangeable GTP binding site at the plus end of microtubules *Science* 261:1044-1047.(MedLine)

Mitchison, T.J. and Cramer, L.P. (1996) Actin-based cell motility and cell locomotion, *Cell* 84:371-379.(MedLine)

Mogliner, A. and Oster, G. (1996) Cell motility driven by actin polymerization, *Biophys J.* 71:3030-3045. (MedLine)

Moon, A.and Drubin, D.G. (1995) The ADF/cofilin proteins: stimulus-responsive modulators of actin dynamics, *Mol. Biol. Cell* 6:1423-1431.(MedLine)

Morita, Y.S., Jung, G., Hammer, J.A. 3rd and Fukui, Y. (1996) Localization of *Dictyostelium* myoB and myoD to filopodia and cell-cell contact sites using isoform-specific antibodies, *Eur. J Cell Biol.* 71:371-379. (MedLine)

Moritz, M., Braunfeld, M.B., Sedat, J.W., Alberts, B. and Agard, D.A. (1995) Microtubule nucleation by γ-tubulin-containing rings in the centrosome, *Nature* 378: 638-640.(MedLine)

Mullins, R.D. (2000) How WASP-family proteins and the Arp2/3 complex convert intracellular signals into cytoskeletal structures, Curr. Opin. Cell Biol. 12:91-96. (MedLine)

Mullins, R.D. and Pollard, T.D. (1999a) Structure and function of the Arp2/3 complex, *Curr. Opin. Struct. Biol.* 9:244-249.(MedLine)

Mullins, R.D. and Pollard, T.D. (1999b) Rho-family GTPases require the Arp2/3 complex to stimulate actin polymerization in *Acanthamoeba* extracts, *Curr. Biol.* 9:405-415.(MedLine)

Mullins, R.D., Stafford, W.F. and Pollard, T.D. (1997) Structure, subunit topology, and actin-binding activity of the Arp2/3 complex from *Acanthamoeba*, *J. Cell Biol.* 136:331-343.(MedLine)

Mullins, R.D., Heuser, J.A. and Pollard, T.D. (1998a) The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments, *Proc. Natl. Acad. Sci. USA* 95:6181-6186.(MedLine)

Mullins, R.D., Kelleher, J.F., Xu, J. and Pollard, T.D. (1998b) Arp2/3 complex from Acanthamoeba binds profilin and cross-links actin filaments, *Mol. Biol. Cell.* 9:841-852.(MedLine)

Nelson, L. (1958) Cytochemical studies with the electron microscope. I. Adenosine triphosphatase in rat spermatozoa. *Biochim. Biophys. Acta* 27:634-641.

Nobes, C.D. and Hall, A. (1999) Rho GTPases control polarity, protrusion, and adhesion during cell movement, *J. Cell Biol.* 144:1235-1244.(MedLine)

Nogales, E., Wolf, S.G. and Downing, K.H., (1998) Structure of the αβ tubulin dimer by electron crystallography, *Nature* 391:199-203.(MedLine)

Oakley, B.R., Oakley, C.E., Yoon, Y. and Jung, M.K. (1990) γ-Tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*, *Cell* 61: 1289-1301.(MedLine)

Ohtsubo, M. and Hiramoto, Y. (1985) Regional differences in mechanical properties of the cell surface in dividing echinoderm eggs, *Develop. Growth and Differ*, 27:371-383.

Oliferenko, S., Kaverina, I., Small, J.V. and Huber, L.A. (2000) Hyaluronic acid (HA) binding to CD44 activates rac1 and induces lamellipodia outgrowth, *J. Cell Biol.* 148:1159-1164. (MedLine)

Osborn, M., and Weber, K. (1977) The detergent-resistant cytoskeleton of tissue culture cells includes the nucleus and the microfilament bundle. *Exp. Cell Res.* 106:339-349.(MedLine)

Pelham, R.J. Jr. and Wang, Y. (1999) High resolution detection of mechanical forces exerted by locomoting fibroblasts on the substrate, *Mol. Biol. Cell* 10:935-945.(MedLine)

Pickett-Heaps, J. D. (1986) Mitotic mechanisms: an alternative view, *Trends in Biochem. Sci.* 11:504-507.

Porter, K. R. (1966) Cytoplasmic microtubules and their function. Principles of biomolecular organization, *CIBA Found. symp.* 1965, pp. 308-345.

Prahlad, V., Yoon, M., Moir, R.D., Vale, R.D. and Goldman, R.D. (1998) Rapid movements of vimentin on microtubule tracks: kinesin-dependent assembly of intermediate filament networks, *J. Cell Biol.* 143:159-170. (MedLine)

Prehoda, K.E., Lee, D.J. and Lim, W.A. (1999) Structure of the enabled/VASP homology 1 domain-peptide complex: a key component in the spatial control of actin assembly, *Cell* 97:471-480.(MedLine)

Raff, J.W. (1996) Centrosomes and microtubules: wedded with a ring, *Trends in Cell Biol.* 6: 248-251.

Ramesh, N., Anton, I.M., Martinez-Quiles, N. and Geha, R.S. (1999) Waltzing with WASP, *Trends Cell Biol.* 9:15-19.(MedLine)

Rappaport, R. (1967) Cell division: direct measurement of maximum tension exerted by furrow of echinoderms, *Science* 156:1241-1243.(MedLine)

Rappaport, R. (1971) Cytokinesis in animal cells, *Inter. Rev. Cytol.* 31:169-213.(MedLine)

Ren, X.D. and Schwartz, M.A. (1998) Regulation of inositol lipid kinases by Rho and Rac *Curr. Opin. Genet. Dev.* 8:63-67.(MedLine)

Rieder, C. L., and Alexander, S. P. (1990) Kinetochores are transported poleward along a single astral microtubule during chromosome attachment to the spindle in newt lung cells, *J. Cell Biol.* 110:81-95.(MedLine)

Ringstad, N., Nemoto, Y. and De Camilli, P. (1997) The SH3p4/Sh3p8/SH3p13 protein family: binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain, *Proc. Natl. Acad. Sci. USA* 94:8569-8574.(MedLine)

Rodionov, V., Nadezhdina, E. and Borisy, G. (1999) Centrosomal control of microtubule dynamics, *Proc. Natl. Acad. Sci. USA*, 96:115-120.(Medline)

Rohatgi, R., Ho, H.Y. and Kirschner, M.W. (2000) Mechanism of N-WASP activation by CDC42 and phosphatidylinositol 4, 5-bisphosphate, *J. Cell Biol.* 150:1299-1310. (MedLine)

Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T. and Kirschner, M.W. (1999) The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly, *Cell* 97:221-231.(MedLine)

Rottner, K., Hall, A. and Small, J.V. (1999) Interplay between Rac and Rho in the control of substrate contact dynamics, *Curr. Biol.* 9:640-648.(MedLine)

Roy, S., Coffee, P., Smith, G., Liem, R.K., Brady, S.T. and Black, M.M. (2000) Neurofilaments are transported rapidly but intermittently in axons: implications for slow axonal transport, *J. Neurosci.* 20:6849-6861. (MedLine)

Sakai, H., Mabuchi, I., Shimoda, S., Kuriyama, R., Ogawa, K., and Mohri, H. (1976) Induction of chromosome motion in the glycerol-isolated mitotic apparatus: nucleotide specificity and effects of antidynein and myosin sera in motion, *Dev.Growth Diff.* 18:211-219.

Satir, P. (1968) Studies on cilia. III. Further studies on the cilium tip and a "sliding filament" model of ciliary motility, *J. Cell Biol.* 39:77-94.(MedLine)

Saxton. W. M., Stemple, D. L., Leslie, R. J., Salmon, E. D., Zavortink, M., and McIntosh, J. R. (1984) Tubulin, dynamics in cultured mammalian cells. *J. Cell Biol.* 99:2175-2186.(MedLine)

Scales, S.J. and Scheller, R.H. (1999) Lipid membranes shape up, *Nature* 401:123-124.(MedLine)

Schmidt, A. and Huttner, W.B. (1998) Biogenesis of synaptic-like microvesicles in perforated PC12 cells, *Methods: A Companion to Methods in Enzymology* 16:160-169.(MedLine)9

Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A.V., Witke, W., Huttner, W.B.and Soling, H.D. (1999) Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid, *Nature* 401:133-141.(MedLine)

Schroeder, T. E. (1963) Actin in dividing cells: contractile ring filaments bind heavy meromyosin, *Proc. Natl. Acad. Sci. USA.* 70:1688-1692.

Schvartz, I., Seger, D. and Shaltiel, S. (1999) Vitronectin, *Int. J. Biochem. Cell Biol.* 31:539-544(MedLine)

Shah, J.V., Flanagan, L.A., Janmey, P.A. and Leterrier, J.F. (2000) Bidirectional translocation of neurofilaments along microtubules mediated in part by Dynein/Dynactin, *Mol. Biol. Cell* 11:3495-3508. (MedLine)

Sharp, D.J., Yu, W. and Baas, B.W. (1995) Transport of dendritic microtubules establishes the

nonuniform polarity orientation, *J. Cell Biol.* 130:93-104.(MedLine)

Sharp, D.J., Yu, W., Ferhat, L., Kuriyama, R., Rueger, D.C., and Baas, P.W. (1997) Identification of a microtubule-associated motor protein essential for dendritic differentiation, *J. Cell Biol.* 138:833-843.(MedLine)

Small, J.V., Herzog, M., Anderson, K.A (1995) Actin filament organization in the fish keratocyte lamellipodium, *J. Cell Biol.* 129:1275-1286.(MedLine)

Smilenov, L.B., Mikhailov, A., Pelham, R.J., Marcantonio, E.E. and Gundersen, G.G. (1999) Focal adhesion motility revealed in stationary fibroblasts, *Science* 286:1172-1174.(MedLine)

Stearns, T. and Kirschner, M. (1994) In vitro reconstitution of centrosome assembly and function: the central role of γ-tubulin, *Cell* 76: 623-637.(MedLine)

Stearns, T., Evans, L. and Kirschner, M. (1991) γ-Tubulin is a highly conserved component of the centrosome, *Cell* 65: 825-836.(MedLine)

Suen, P.W., Ilic, D., Caveggion, E., Berton, G., Damsky, C.H. and Lowell, C.A. (1999) Impaired integrin-mediated signal transduction, altered cytoskeletal structure and reduced motility in Hck/Fgr deficient macrophages, *J. Cell Sci.* 112:4067-4078.(MedLine)

Summers, K. E., and Gibbons, I. R. (1971) Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagellae of sea urchin sperm, *Proc. Natl. Acad. Sci. USA* 68:3092-3096.(MedLine)

Suter, D.M., Errante, L.D., Belotserkovsky, V. and Forscher, P. (1998) The Ig superfamily cell adhesion molecule, apCAM, mediates growth cone steering by substrate-cytoskeletal coupling, *J. Cell Biol.* 141:227-240.(MedLine)

Svitkina, T.M. and Borisy, G.G. (1999) Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia, *J. Cell Biol.* 145:1009-10026.(MedLine)

Svitkina, T.M., Verkhovsky, A.B., McQuade, K.M. and Borisy, G.G. (1997) Analysis of actin-myosin II system in fish epidermal keratocytes: mechanism of cell body translocation, *J. Cell Biol.* 139:397-415.(MedLine)

Symons, M.H. and Mitchinon, T.J. (1991) Control of actin polymerization in live and permeabilized fibroblasts, *J. Cell Biol.* 114:503-513.(MedLine)

Szent-György, A. (1949) Free energy relations and contraction of actomyosin, *Biol. Bull.* 96:140-161.

Tanaka, E. and Sabry, J. (1995) Making the connection: cytoskeletal rearrangements during growth cone guidance, *Cell* 83:171-176.(MedLine)

Tessier-Lavigne, M. and Goodman, C.S. (1996) The molecular biology of axon guidance, *Science* 274:1123-1133.(MedLine)

Theriot, J.A. (1997) Accelerating on a treadmill: ADF/cofilin promotes rapid actin filament turnover in the dynamic cytoskeleton, *J. Cell. Biol.* 136:1165-1168.(MedLine)

Tournebize, R., Popov, A., Kinoshita, K., Ashford, A.J., Rybina, S., Pozniakovsky, A., Mayer, T.U., Walczak, C.E., Karsenti, E. and Hyman, A.A. (2000)Control of microtubule dynamics by the antagonistic activities of XMAP215 and XKCM1 in Xenopus egg extracts, *Nature Cell Biol.* 2:13-19. (MedLine)

Travis, J.L., and Allen, R.D. (1981) Studies on the motility of foraminifera. I. Ultrastructure of the reticulopodial network of *Allogromia laticollaris* (Arnold), *J. Cell Biol.* 90:211-221.(MedLine)

Travis, J.L., and Bowser, S.S. (1988) Optical approaches to the study of foraminifera, *Cell Motil. Cytoskel.* 10:126-236.(MedLine)

Urbani, L. and Stearns, T. (1999) The centrosome, *Curr. Biol.* 9:R315-317.(MedLine)

Vasquez, R.J., Gard, D.L. and Cassimeris, L. (1994) XMAP from Xenopus eggs promotes rapid plus end assembly of microtubules and rapid microtubule polymer turnover, *J. Cell Biol.* 127:985-993. (MedLine)

Vasquez, R.J., Gard, D.L. and Cassimeris, L. (1999) Phosphorylation by CDK1 regulates XMAP215 function *in vitro*, *Cell Motil. Cytoskeleton* 43:310-321. (MedLine)

Wagner, M.C., Barylko, B. and Albanesi, J.P. (1992) Tissue distribution and subcellular localization of mammalian myosin I, *J. Cell Biol.* 119:163-170.(MedLine)

Walker, R.A., O'Brien, E.T., Pryer, N.K., Soboeiro, M.F., Voter, W.A., Erickson, H.P. and Salmon, E.D. (1988) Dynamic instability of individual microtubules analyzed by video light microscopy: rate constants and transition frequencies, *J. Cell Biol.* 107:1437-1448. (MedLine)

Wang, Y.L. (1985) Exchange of actin subunits at the leading edge of living fibroblasts: possible role in treadmilling, *J. Cell Biol.* 101:597-602.(MedLine)

Wang, L., Ho, C.L., Sun, D., Liem, R.K. and Brown A. (2000) Rapid movement of axonal neurofilaments

interrupted by prolonged pauses, *Nature Cell Biol.* 2:137-141. (MedLine)

Warner, F. D., and Mitchell, D. R. (1981) Polarity of dynein-microtubule interactions *in vitro*: cross linking between parallel and antiparallel microtubules, *J. Cell Biol.* 89:35-44.(MedLine)

Waterman-Storer, C.M. and Salmon, E.D. (1997) Actomyosin-based retrograde flow of microtubules in the lamella of migrating epithelial cells influences microtubule dynamic instability and turnover and is associated with microtubule breakage and treadmilling, *J. Cell Biol.* 139:417-434.(MedLine)

Waterman-Storer, C.M. and Salmon, E.D. (1998) How microtubules get fluorescent speckles, *Biophys. J.* 75:2059-2069.(MedLine)

Waterman-Storer, C.M. and Salmon, E.D. (1999) Fluorescent speckle microscopy of microtubules: how low can you go? *FASEB J.* 13 Suppl 2:S225-230.(MedLine)

Waterman-Storer, C.M., Desai, A., Bulinski, J.C. and Salmon, E.D. (1998) Fluorescent speckle microscopy, a method to visualize the dynamics of protein assemblies in living cells, *Curr. Biol.* 8:1227-1230.(MedLine)

Waterman-Storer, C.M., Worthylake, R,A,, Liu, B.P., Burridge, K. and Salmon, E.D. (1999) Microtubule growth activates Rac1 to promote lamellipodial protrusion in fibroblasts, *Nature Cell Biol.* 1:45-50.(MedLine)

Weber, K. (1976) Biochemical anatomy of microfilaments in cells in tissue culture using immunofluorescence microscopy. In *Contractile systems in Non-Muscle Tissues* (Perry, S.V., ed.), pp. 51-66. Elsevier/North-Holland, New York.

Weber, K., Pollack, R., and Bibring, T. (1975) Antibody against tubulins: the specific visualization of cytoplasmic microtubules in tissue culture cells, *Proc. Natl. Acad. Sci. USA* 72:459-463.(MedLine)

Weiner, O.D., Servant, G., Welch, M.D., Mitchison, T.J., Sedat, J.W. and Bourne, H.R. (1999) Spatial control of actin polymerization during neutrophil chemotaxis, *Nature Cell Biol.* 1:75-81.(MedLine)

Welch, M.D. (1999) The world according to arp: regulation of actin nucleation by the Arp2/3 complex, *Trends Cell Biol.* 9:423-427.(MedLine)

Wessels, D., Soll, D.R., Knecht, D., Loomis, W.F., De Lozannne, A. and Spudich, J. (1988) Cell motility and chemotaxis in *Dictyostelium* amebae lacking myosin heavy chain, *Dev. Biol.* 128:164-177.(MedLine)

Wohlfarth-Bottermann, K.E. (1964) Differentiations of the ground cytoplasm and their significance for the generation of the motive force of ameboid movement. In *Primitive Motile Systems in Cell Biology*

(Allen, R., and Kamiya, N., eds.), pp. 79-109. Academic Press, New York.

Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E. and Mizuno, K. (1998) Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization, *Nature* 393:809-812.(MedLine)

Zheng, Y., Yung, M.K. and Oakley, B.R. (1991) γ-Tubulin is present in Drosophila melanogaster and Homo sapiens and is associated with the centrosome, *Cell* 65: 817-823.(MedLine)

Zheng, Y.X., Wong, M.L., Alberts, B., and Mitchison, T. (1995) Nucleation of microtubule assembly by a γ-tubulin-containing ring complex, *Nature* 378: 578-583.(MedLine)

24. Mechanochemical Coupling:

Molecular Basis

Web Resources
References

Back to List of Chapters

At times the extraction and purification of the molecular components of a system leads to a deeper understanding of its intimate mechanisms. Ideally, it should be possible to reassemble the various component molecules and reestablish function. Only then can the significance of the molecular elements of the system be certain and perhaps lead us to decipher the mechanism of the action at the molecular level. The experimental approach to contraction need not be different from the studies carried out with enzymes and multienzyme complexes. Conformational rearrangements of the molecules accompany many enzymatic reactions. Similarly, movement, perhaps in the form of a conformational change, is likely to play a role in the transport across cell membranes. Contraction could be considered a conformational change involving macromolecular assemblies and magnified by a variety of means. The task of elucidating the nature of events underlying contraction may be more complex than those involved in other conformational changes: in most cases intermediate compounds are not readily evident, and more ephemeral structural states may perhaps be involved.

The viewpoint is emerging that many processes are similar to the movement mediated by conventional molecular motors (see Vale, 1996). A strong argument can be made that the elongation factor G involved in the movement of ribosomes along the mRNA molecule is similar to that of the motors discussed in this chapter (see Cross, 1996, Rodnina et al., 1996).

Skeletal muscle as well as cilia and flagella may be considered the prototypes of cell movement because they were the first to be studied and a good deal is known about them. For this reason, actin and myosin (section I and II) and tubulin and dynein of these systems are addressed first (section III). Analogous molecules have been found to be associated with other forms of cellular movement and are discussed in section IV.

I. MOLECULAR BASIS OF CONTRACTION IN STRIATED MUSCLE

The study of the molecular mechanism of contraction requires some indication of activity. Since the system transduces chemical energy into mechanical energy (contraction), it could be possible to examine preparations for their capacity to contract. However, in some respects it may be more practical to examine activities that must be associated with mechanical activity, e.g., the Ca^{2+} dependence of ATPase that is characteristic of the myofibril system or the capacity of components to complex with other macromolecules. Several other useful assays will be described.

A. Myosin and Actin

The myofibrils contain protein associated with contraction: 55% of this is myosin, 20% actin, 5% tropomyosin, and 3 to 4% troponin. We will have to focus our attention on these components. Two of

these, actin and myosin, can be recombined to form *actomyosin*. The complex can be made into a fiber by releasing it into distilled water or into a dilute solution. The fiber shortens in the presence of ATP. Because the actomyosin molecules are randomly distributed, the shortening occurs in all directions and the thread cannot displace a weight. But when actomyosin is appropriately oriented, by spreading it on a surface, for example, it will contract only longitudinally on the addition of ATP and is thereby capable of lifting weights. This experiment is shown in Fig. 1 (Hayashi, 1952). The contractions are slow -- it takes several minutes to reach the maximal level-in contrast to the very fast contraction of intact muscle. This slowness may be the result of the slow diffusion of ATP into the relatively thick fibers. It is also possible that not all the appropriate components of the system are present. During the contraction, ATP is hydrolyzed, as estimated from parallel experiments carried out under the same conditions.

Events thought to correspond only indirectly to the primary events of contraction have also been found useful in studying actomyosin. For example, it has been found that the viscosity of the complex decreases on addition of ATP and then returns to normal, indicating dissociation and reformation of a complex. The viscosity depends on the shape of the macromolecules in solution. The longer the molecule, the higher the viscosity. The dissociation of the rodlike actomyosin produces a decrease in viscosity, because the axial ratios (i.e., the length to the width) of actin and myosin are much lower than the axial ratio of actomyosin. As we shall see, the dissociation of actomyosin and its subsequent reassociation from actin and myosin are likely to be part of the contractile mechanism. Actomyosin gels decrease in volume on addition of ATP (the superprecipitation reaction), a change that has also been used as an index of contractility.

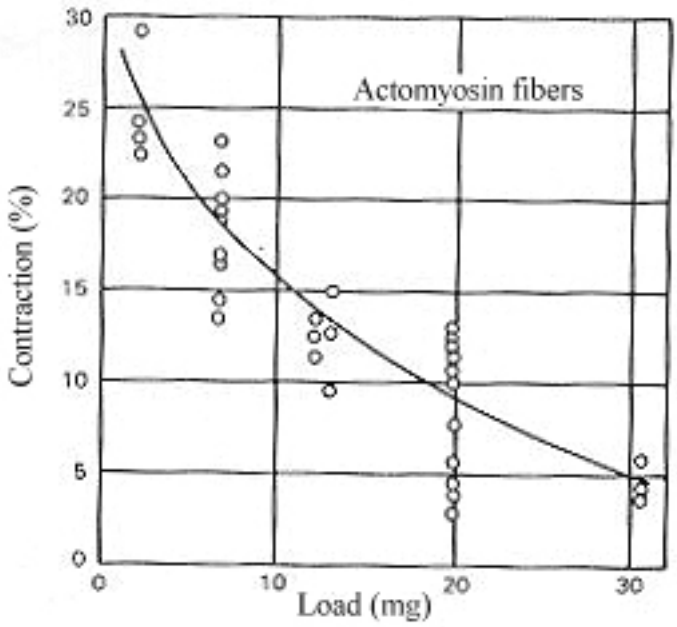


Fig. 1 Percent contraction with load of actomyosin fibers. Each point represents a 15-minute contraction. From (Hayashi, 1952). Reproduced from *The Journal of General Physiology*, ©1952, 36: 139-151, by copyright permission of the Rockefeller University Press.

As seen with the electron microscope after negative staining, skeletal muscle myosin is a long molecule, about 160 nm in length and 20-40 nm in diameter. A single myosin unit has a molecular mass of about

500 kDa and contains two heavy chains of about 230 kDa and four light chains of 16 to 20 kDa. Electron microscopic views of the monomers are shown in Fig. 2 (H.E. Huxley, 1963). When either the pH or the ionic strength is reduced, myosin monomers associate (i.e., polymerize) into thick filaments remarkably similar to the thick filaments of the myofibrils. As we saw in Chapter 23, there is evidence that the thick filaments of striated muscle are made up of myosin. In the fibers that polymerize from myosin monomers, the tail ends of the molecules cling together and the head pieces jut out. The molecules align in the filament in such a way that some of the head pieces face in one direction while others face in the other direction, forming a symmetrical composite. Figure 3a (Huxley, 1963) shows such polymerized filaments. Figure 3b is a diagrammatic representation of how these filaments are probably formed.

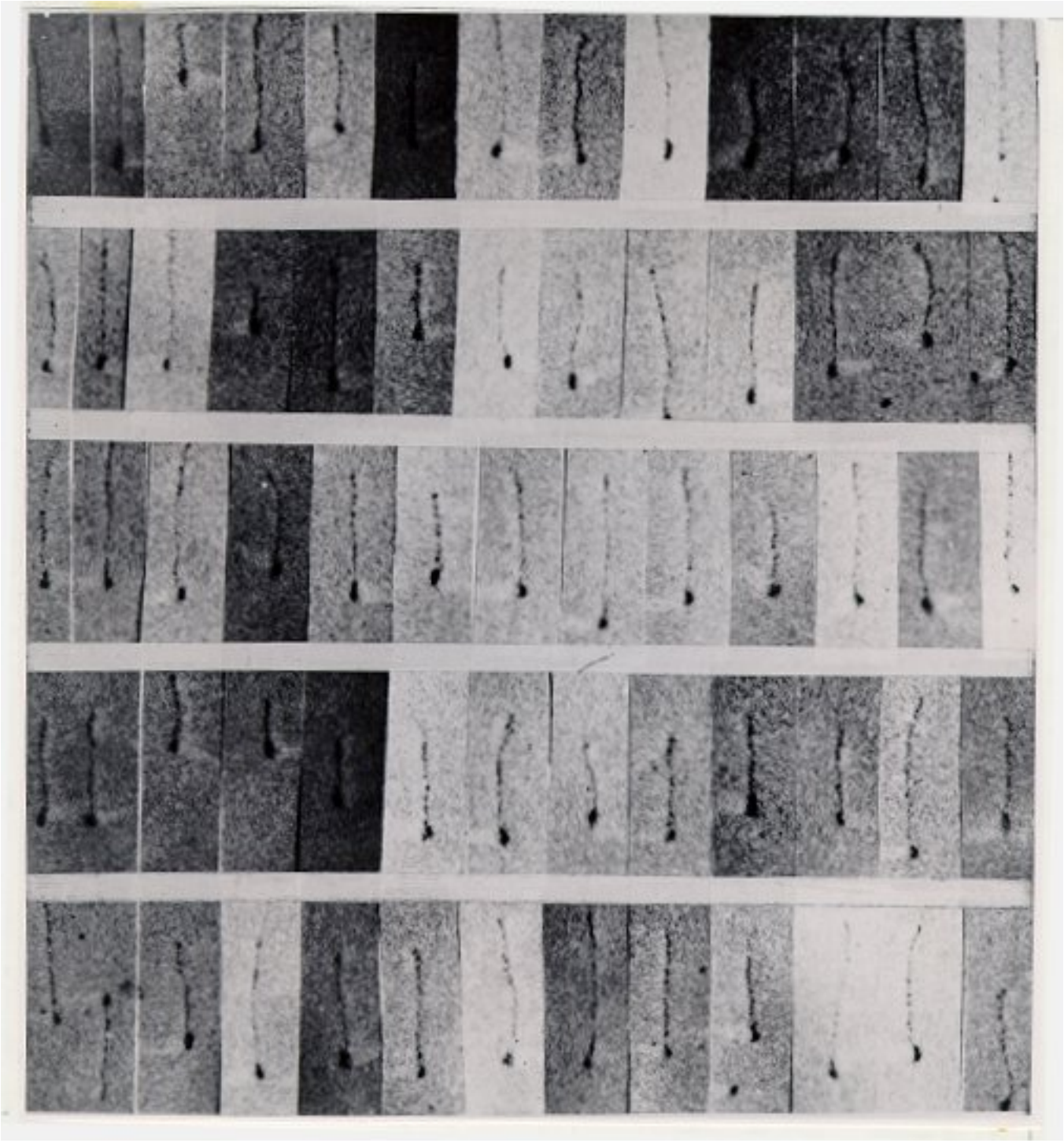
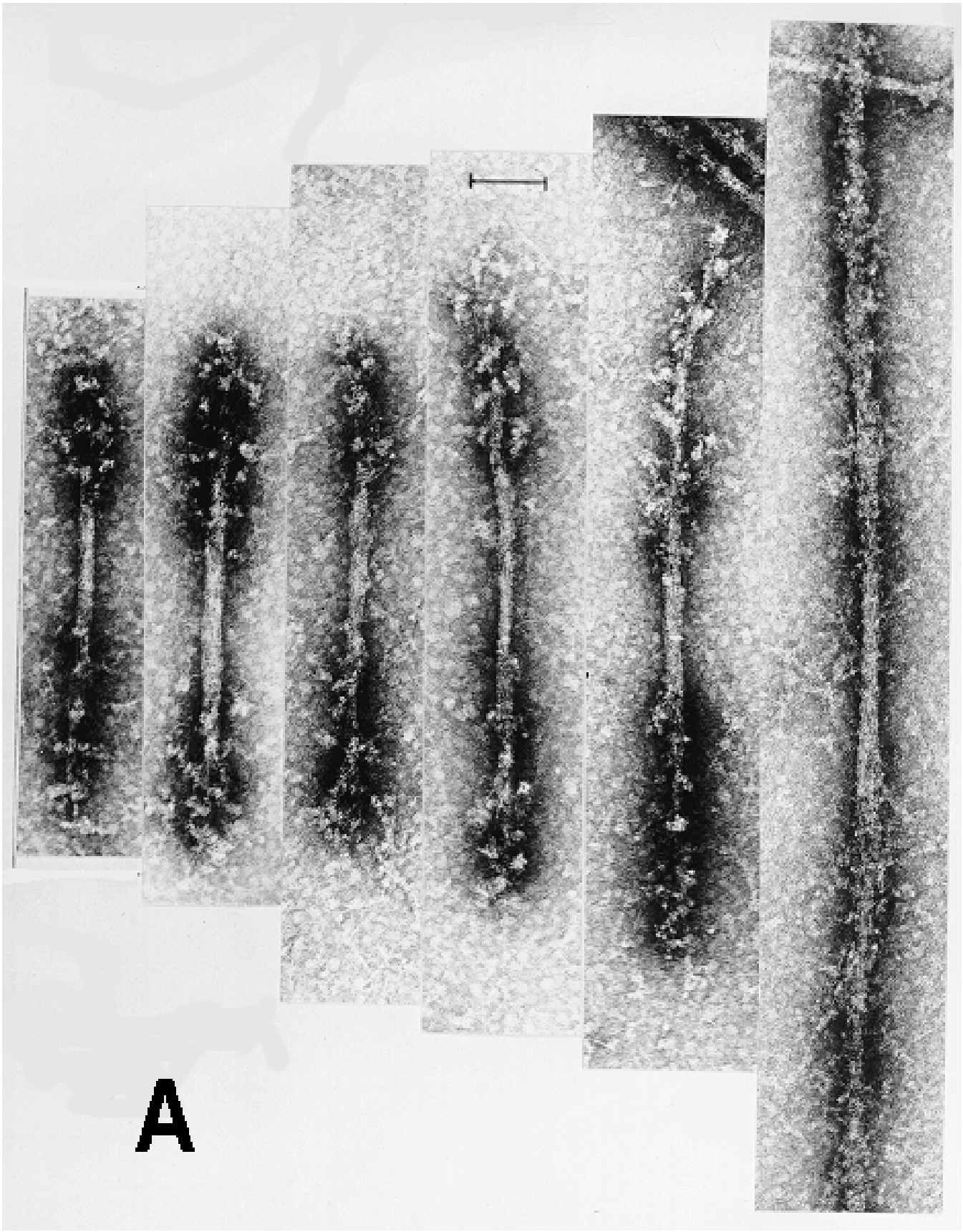


Fig. 2 Myosin monomers seen with the electron microscope. Reproduced with permission from (Huxley, H. E., 1969) The mechanism of muscular contraction. *Science* 164:1356-1366. Copyright ©1963 by the American Association for the Advancement of Science.



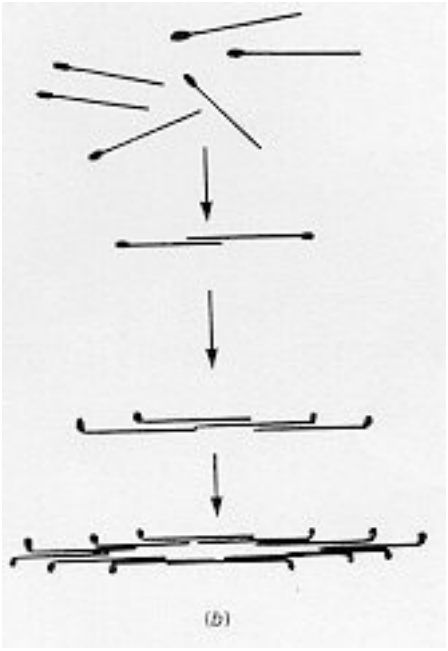


Fig. 3 (a) Myosin aggregates formed in vitro as seen with the electron microscope after negative staining. (Bar corresponds to 77 nm.) (b) Scheme showing mechanism of formation of fibrous aggregates from myosin monomers. Reproduced with permission from (Huxley, H. E., 1969) The mechanism of muscular contraction. *Science* 164:1356-1366. Copyright ©1963 by the American Association for the Advancement of Science.

The head portion of the individual myosin molecules at the amino-terminal appears to correspond to bridges between thick and thin filaments seen with the electron microscope in sections of sarcomeres. The head portions can be isolated after partial proteolytic digestion of the myosin as heavy meromyosin (HMM). Their ability to then combine with actin supports the view that they correspond to bridges observed with the electron microscope. The HMM fragment can be further cleaved into two smaller *subfragments*, *S1* and *S2*.

The myosin head pieces seen with the electron microscope after shadowing appear to be formed by two subunits (Slayter and Lowry, 1967). A model incorporating our present knowledge is shown in Fig. 4 (Stebbins and Hyams, 1979).

The addition of HMM (or S1) to actin produces an appearance of many neatly arranged arrowheads. This arrowhead configuration could occur only if the HMM fragments were specifically oriented by the thin filaments. It is therefore considered to be a specific test for the presence of actin filaments and indicates that the actin itself has polarity. The ends of the actin have been named after the appearance of the arrowheads, i.e., pointed (P) and barbed (B). The actin with oriented arrowheads, as seen in the negatively stained preparation shown in Fig. 5, is referred to as *decorated*. The two views shown in the figure represent electron micrographs at slightly different tilts, and they permit visualization of the arrangement in three dimensions using a simple standard or homemade stereoscope.

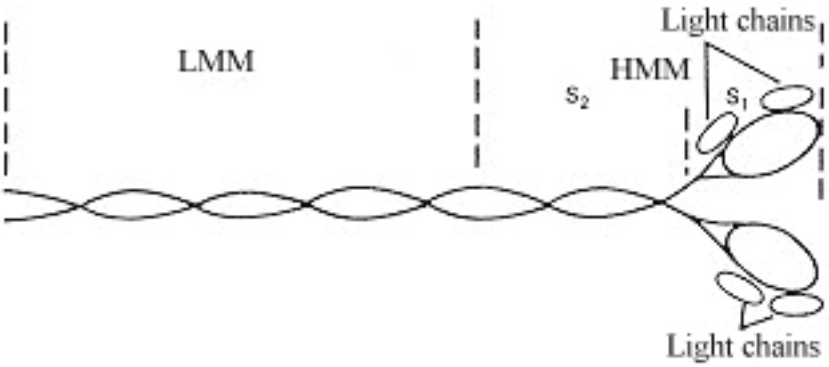


Fig. 4 Structure of myosin. Light meromyosin (LMM) and heavy meromyosin (HMM) and the subfragments of HMM, S1 and S2, are shown. The broken lines indicate the cleavage sites of the proteolytic digestion. From H. Stebbins and J. S. Hyams *Cell Motility*, with permission. Copyright ©1979 Longman Group Ltd., Edinburgh.

Actin is a smaller molecule than myosin; it has a molecular mass of 42 kDa. In its globular, monomeric conformation (G-actin), it is about 5.5 nm in diameter. In this conformation, 1 mole of actin contains 1 mole of ATP and 1 mole of divalent cation, Ca^{2+} or Mg^{2+}. G-actin polymerizes in a process in which ATP is hydrolyzed to form a long thread of F-actin, a double helix 7-8 nm in diameter (Fig. 6) (Hanson and Lowy, 1963). Both the ADP derived from the ATP and the divalent cation remain attached to the actin. The divalent cation is thought to be involved in regulation of the polymerization rate.

Since actomyosin threads formed from the combination of actin and myosin contract, the two components and the complex must have properties that play a fundamental role in contraction. Myosin, for example, hydrolyzes ATP. We have seen that ATP is probably the energy source for contraction. In fact, there is a good correlation between the speed of contraction of various striated muscles and their ATPase activity (Maddox and Perry, 1966).

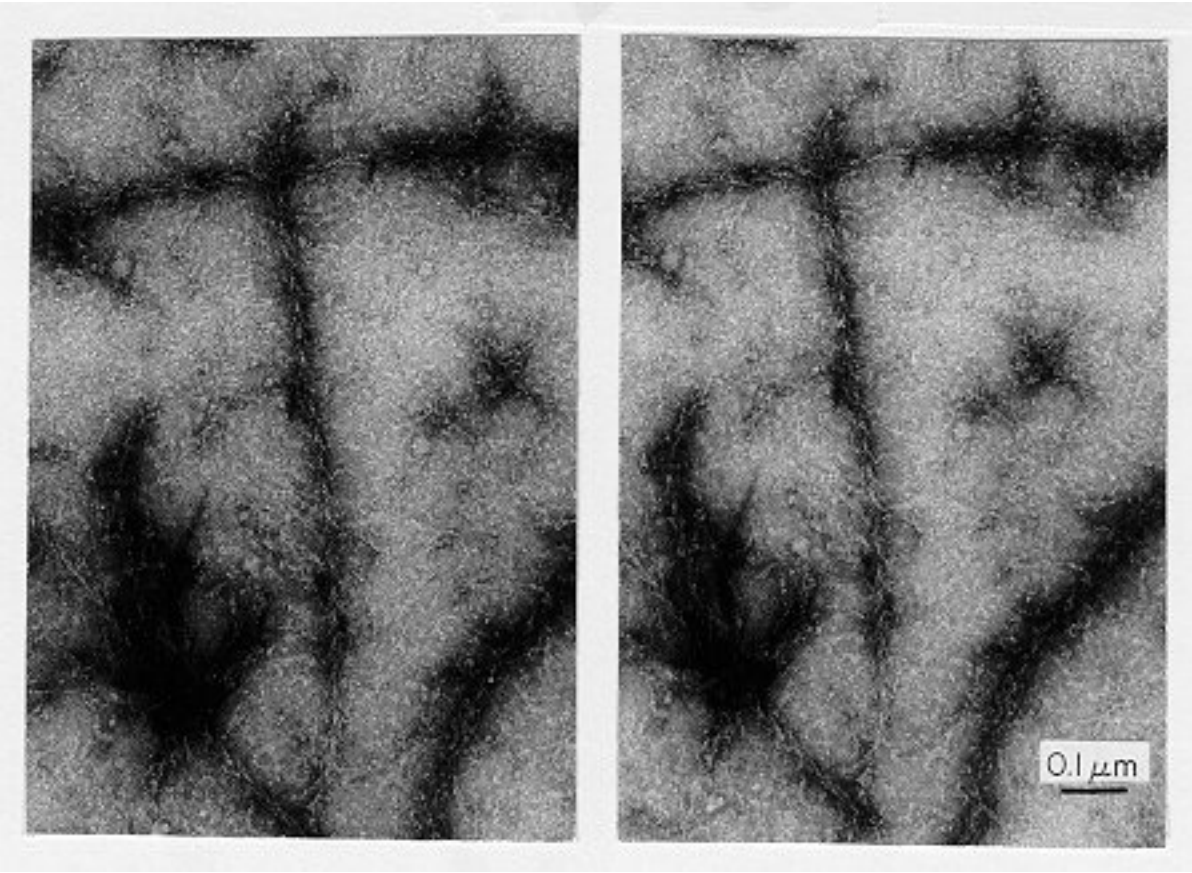


Fig. 5 Rabbit actin complexed with heavy meromyosin (HMM), negatively stained with 1% aqueous uranyl acetate. Stereophotographs at +6 and -6 tilt. For three dimensional depth, view with a standard stereoscope or a simple homemade prism stereoscope [see E. G. Gray and R. A. Willis, *J. Cell Sci.* 3:309-326 (1968)]. Toward upper part of filament note clear demonstration of arrowhead orientation in relation to the filament. Electron microscopy by Barry S. Eckert and S. M. McGee-Russell, Department of Biological Sciences, S.U.N.Y. at Albany. Reproduced by permission.

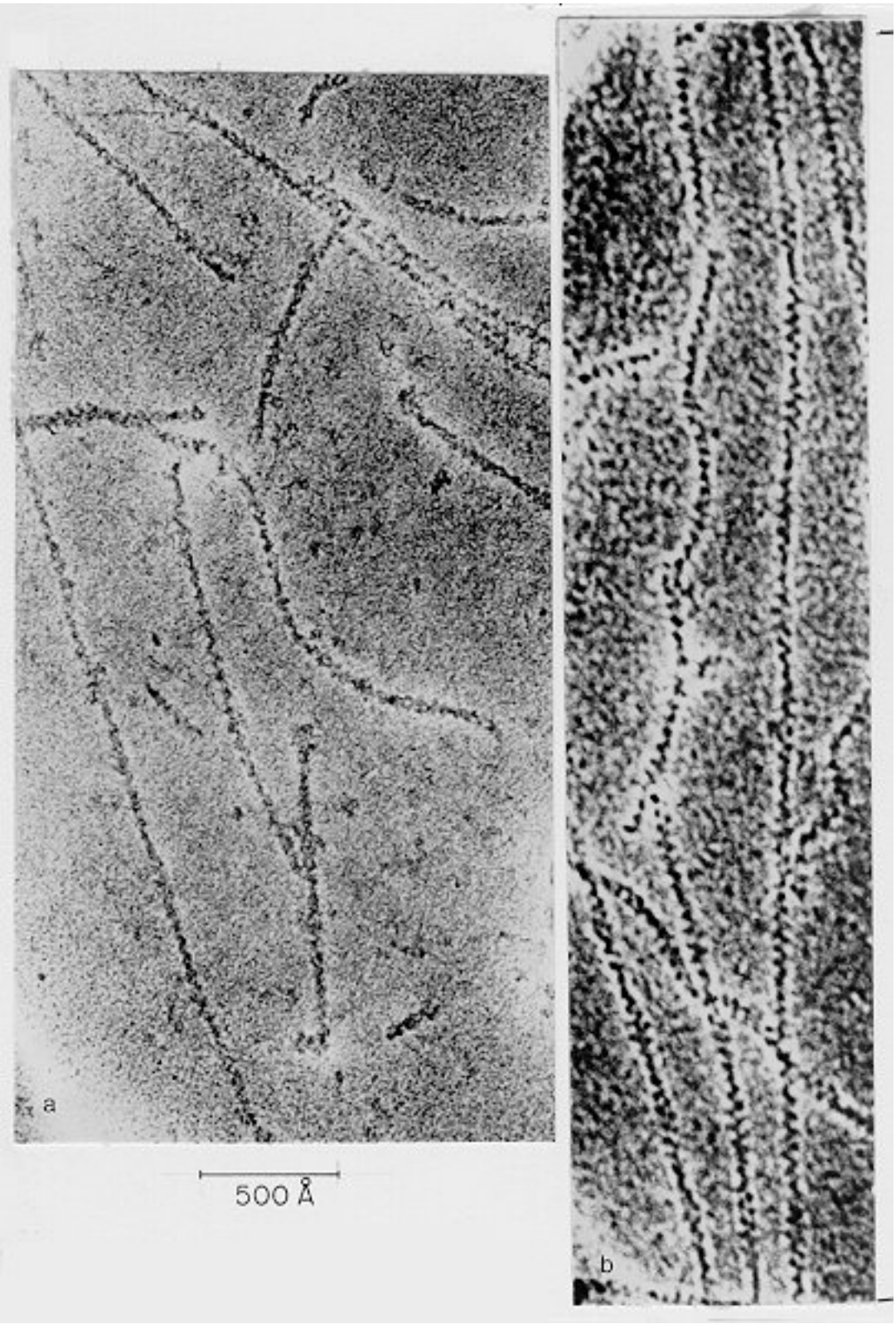


Fig. 6 (a) Electron micrograph of actin filaments extracted with water-glycerol in the presence of Mg-ATP and EDTA. Negative staining with uranyl acetate. x36,125. (b) Electron micrograph similar to that in (a). However, the preparation corresponds to F-actin. The electron micrograph permits counting the number of globular units per turn of the two-stranded helix. x44,625. From Hanson and Lowy (1963), reproduced with permission.

We saw in Chapter 23 that contraction and ciliary motion involves the sliding of filaments in relation to

each other. What are the minimum requirements of such a model at the molecular level? The events accompanying contraction, according to the sliding-filament model, must be cyclic. At least four events must take place: (1) binding between actin and myosin, (2) the pulling of the actin filaments from the two sides of the sarcomere toward each other, (3) coupling of the hydrolysis of ATP to the appropriate structural changes, and (4) breaking of the bond between actin and myosin. Since a sarcomere shortens more than the distance between side chains or cross-bridges, the cyclic breaking and remaking of bonds must occur over a distance corresponding to several cross-bridges. In frog muscle the shortening corresponds to 30% of the sarcomere length, or about 370 nm, whereas the myosin projections are approximately 40 nm apart. Let us examine some of the properties of the actomyosin system with these steps in mind.

As mentioned, it is possible to fragment the myosin molecule by proteolytic digestion. The individual fragments can be separated out and tested for various properties. The ATPase activity resides in only a portion of the myosin molecule, the heavy meromyosin associated with the thicker part of the molecule (Jones and Perry, 1966; Mueller and Perry, 1962). A basic role of the interaction between actin and myosin predicted by the sliding-filament model is supported by the observation that actin and myosin have to be complexed for shortening to occur. It is interesting to note that the complexing ability also resides in the myosin fragment that has the ATPase activity. The actin and the ATP binding sites reside in different parts of the molecule and are nearly 4 nm apart.

B. Troponin, Tropomyosin and Paramyosin

Myofibril contractility requires the presence of Ca^{2+}. Muscle contraction is initiated by the release of Ca^{2+} from the sarcoplasmic reticulum. In vertebrates and arthropods, the Ca^{2+} sensitivity of actomyosin apparently depends on the presence of two proteins, troponin (Tn) and tropomyosin (Tm). In some other organisms, such as mollusks, brachiopods, and some worms (Lehman et al., 1972), a light chain of myosin seems to be directly involved in this control mechanism (Kendrick-Jones, 1974). In annelids (e.g., the sandworm *Nereis*), both mechanisms are present (Lehman et al., 1972).

In vertebrates, the Ca^{2+}-activation of the contractile mechanism depends on binding of Ca^{2+} to Tn (see Zot and Potter, 1987). Tn and Tm are located periodically along the thin filament of the myofibril (Ebashi et al., 1972; Flicker et al., 1982). Tm dimers are present in a coil-coil configuration. A representation of the possible arrangement of these components in relation to actin is shown in Fig. 7 (Payne and Rudnick, 1989).

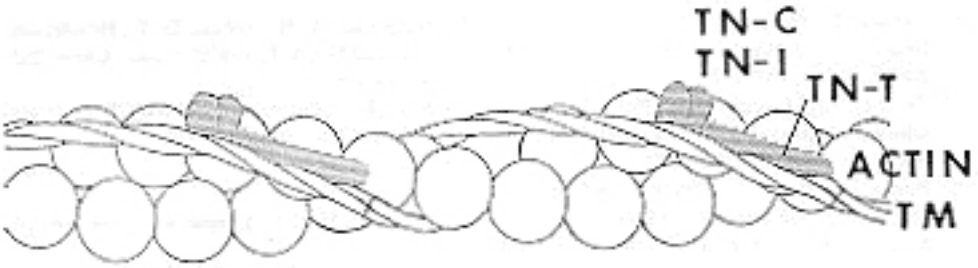


Fig. 7 Probable arrangement of Tn, Tm and actin in the thin filament of a myofibril. Reproduced from *Trends in Biochemical Science*, vol. 14, Payne, M.R. and Rudnick, S.E., Regulation of vertebrate striated muscle contraction, pp.357-360. Copyright ©1989 with permission from Elsevier Science.

Binding of Ca^{2+} to the regulatory site on the TnC subunit alters the interactions between Tn and Tm and actin. These structural changes increase the interaction between actin and myosin, increase the ATPase activity of actomyosin, and produce contraction (see Payne and Rudnick, 1989). Supposedly, these effects are reversed when the free Ca^{2+} concentration decreases. The details on how this is achieved are still not entirely clear.

Actin, Tn, and Tm in 7:1:1 molar ratios form the thin filament anchored to the Z-line (Yates and Greaser, 1983). When actin polymerizes, forming a double stranded helix, the Tm molecule polymerizes head-to-tail so that one Tm molecule spans seven actin monomers. One Tn molecule contains three different subunits (1 TnC:1 TnT and 1 TnI) present one third of the distance from the carboxy terminal end of each Tm molecule.

TnC is composed of 159 amino acids and contains four Ca^{2+} binding sites. Two bind Ca^{2+} with low affinity and are unable to bind Mg^{2+}. Two others bind both Ca^{2+} and Mg^{2+} with high affinity. The four binding sites are thought to correspond to four homologous regions that conform to a helix-loop-helix-Ca^{2+} domain referred to as an EF hand (see Collins, 1976). The low affinity sites are those thought to have a regulatory role.

Paramyosin is found in certain invertebrate muscles that are capable of maintaining tension over long periods of time with a minimum expenditure of energy (*catch muscle*). In some muscles paramyosin may represent as much as 30% of the total protein. Paramyosin forms threads 133 nm long and 2 nm in diameter. Intact muscle fibers, probably corresponding to paramyosin, are intermeshed with thin filaments that probably correspond to actin (Hanson and Lowy, 1961). This arrangement is thought to have some role in contraction and the catch phenomenon.

C. Cross-Bridges and Contraction

As we have seen, the reactions involved in contraction must be cyclic; the association and dissociation involves several cross-bridges. The isometric tension (i.e., tension at constant length) developed during

contraction at various sarcomere lengths (Gordon et al., 1966), correlates well with the degree of overlap of thick and thin filaments, which in turn allows the maximum number of cross-bridges. Figure 8a (Gordon et al., 1966) shows a diagrammatic representation of the overlap in filaments based on studies with the electron microscope. Figure 8b is a graphical summary of the results. The tension developed is expressed as a percentage of the maximal value (ordinate). The sarcomere length, from Z line to Z line, is represented on the abscissa. The numbered arrows correspond to the numbers in the diagram (Fig. 8a). Comparison of two diagrams shows that maximal tension is developed when the overlap of the cross-bridges is maximal. The tension falls when the myofibrils are stretched beyond this length and fewer bridges are in register with the corresponding point of attachment in the actin fibers. The tension also drops sharply when the myofibril is shorter than this optimal value; apparently in this case the thin filaments overlap (Fig. 8a).

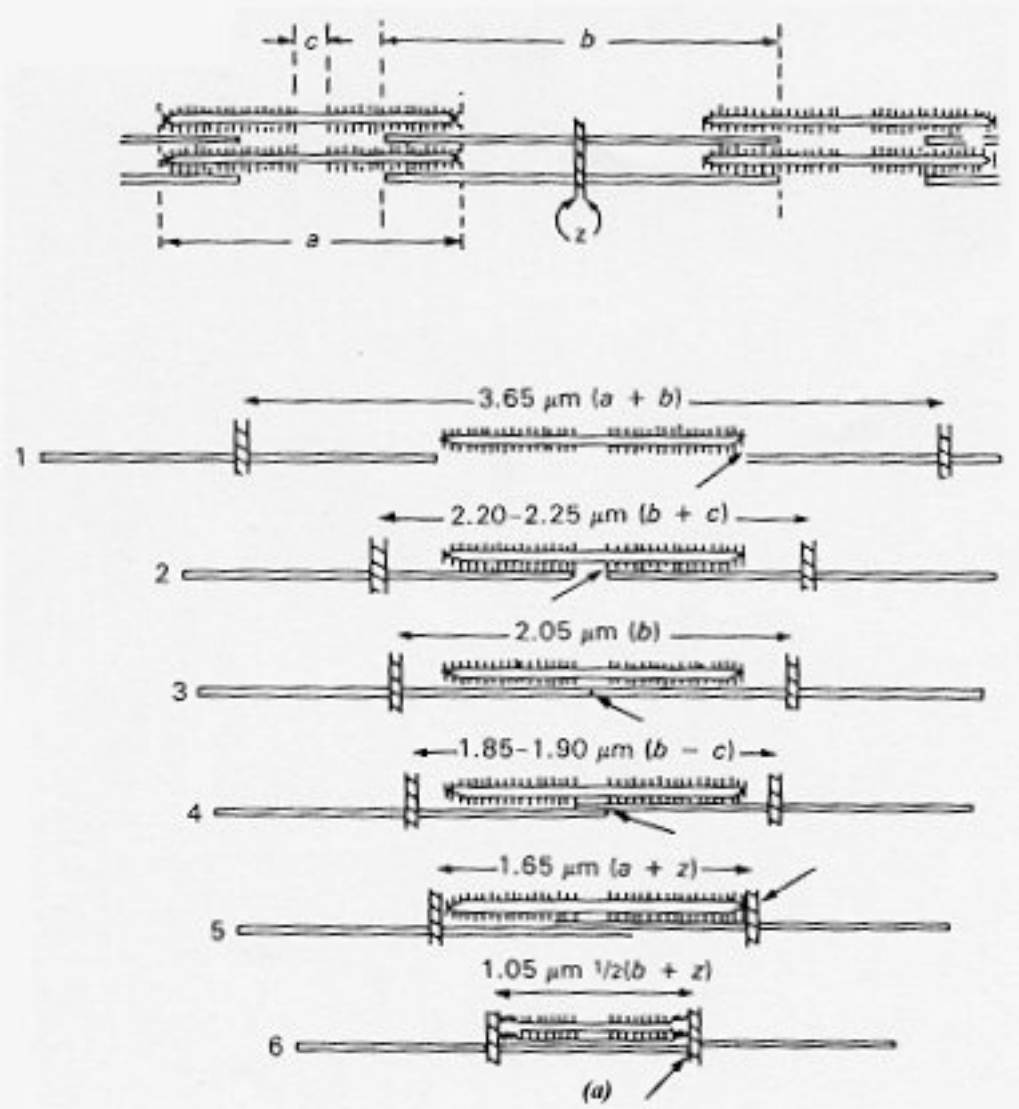


Fig 8 *(a)* Schematic diagram of the filaments. The cross-bridges are represented by the small lines on the thick filaments.

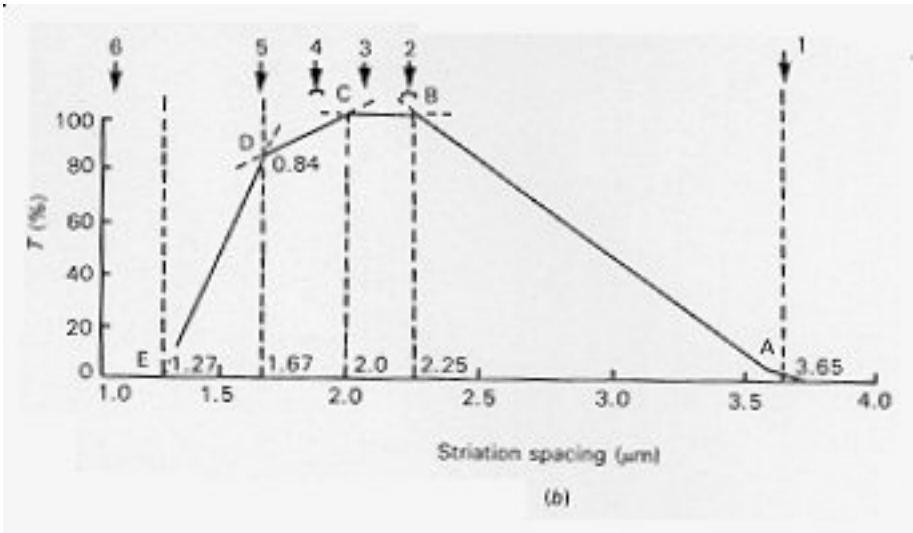


Fig. 8 *Continued (b)* Schematic summary of results. *T* = percent tension. The arrows along the top are placed opposite the striation spacings at which the critical stages of overlap of filaments occur, numbered as in *(a)*. From Gordon et al., *Journal of Physiology (London)* 184:170-182, with permission. Copyright © 1966 The Physiological Society, Oxford, England.

. The *swinging myosin cross bridge* hypothesis of muscle contraction (see Lymn and Taylor, 1971) summarized succinctly much of the evidence available until recently. The hypothesis proposed a contractile cycle starting with the binding of myosin to actin through a myosin cross-bridge, the so-called rigor position, (1) in Fig. 9 (Holmes, 1997). Then ATP dissociates the actin-myosin complex by binding to the ATPase site (2), and after the hydrolysis of the ATP, myosin forms a stable complex with ADP and P_i (3). Actin then rebinds at the myosin cross-bridge (4), and the bridge undergoes a conformational change or power stroke. ADP and P_i dissociate from the myosin and the myosin-actin complex is reformed at a new position (1). This model provided a useful framework to explain many observations of molecular events complementing the formulation of the sliding filament mechanism. In general, the details of this model were difficult to demonstrate because in a myofibril only a small fraction of the total myosin heads were involved at one time. However, a change in the conformation of the S1 fragment elicited by ATP was shown by several studies (e.g., Wakabayashi et al., 1992, using X-ray scattering).

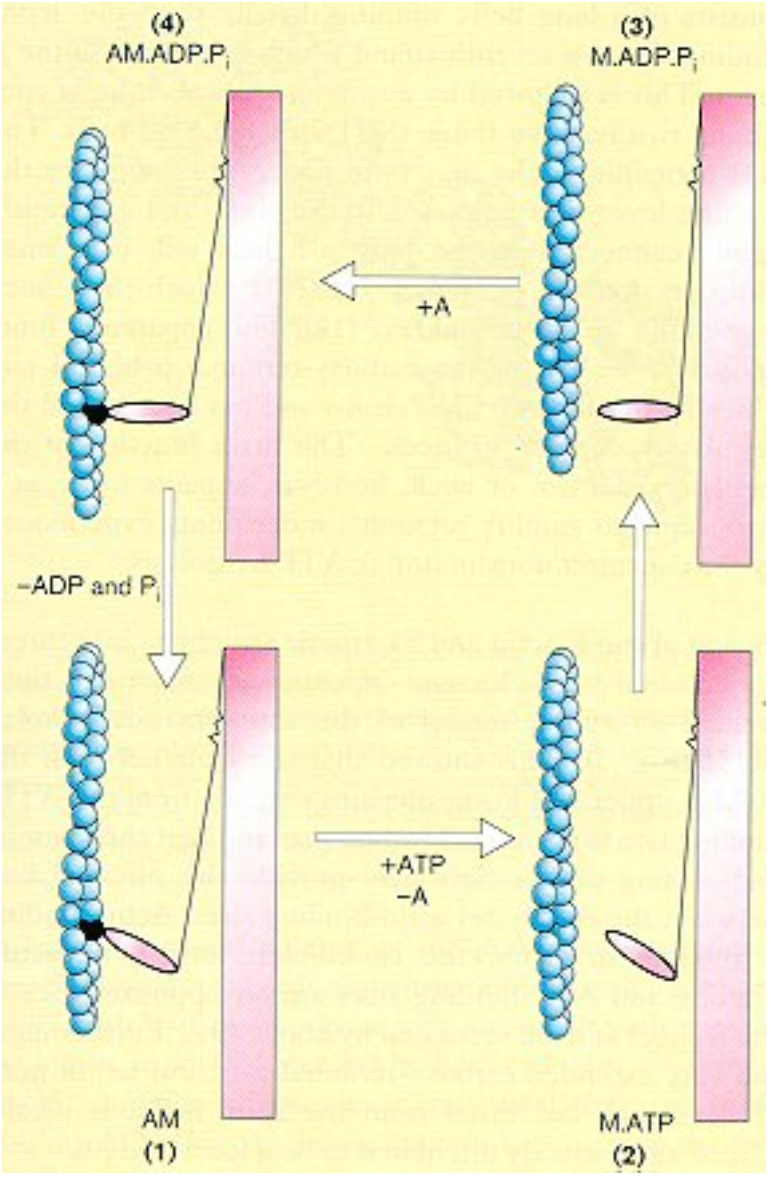


Fig. 9 Lymn-Taylor cycle of swinging cross-bridges. The spheres represent actin monomers. The vertical bar represents the myosin thick filament (M). The nucleotide-free cross-bridge (the myosin head) is shown attached to the actin in the 45º position (in relation to the actin filament), the rigor state (1). The binding of ATP releases the actin from the cross-bridge (2), still in the 45º position. The hydrolysis of ATP puts the cross bridge at the 90º conformation (3). The release of the products of hydrolysis that puts the myosin head in the 45º position (return to 1) is the power stroke (from Holmes, 1997). Reproduced by permission.

In vitro studies of movement of myosin and actin filaments, X-ray crystallography and cryoelectron microscopy have fueled recent advances. In vitro motility assays have shown that only the myosin head (subfragment 1 or S1) is required for in vitro movement on actin filaments (e.g., Kishino and Yanagida, 1988). X-ray crystallographic studies (see Fig. 10) revealed not only the structure of S1 but suggested how movement may take place (Rayment et al., 1993a and b; Dominguez,et al. 1998) (see Fig. 11).

S1 contains a globular motor domain (MD) and a long (8.5 nm)α-helical tail region at the carboxy terminal (Fig. 10). The MD contains the ATP and actin binding sites. The tail region binds the light regulatory chains [containing the *calmodulin-like essential* (ELC) and *regulatory light chains* (RLC)].

The tail can act as a lever arm capable of magnifying small conformational changes in the MD corresponding to different nucleotide induced states (Rayment et al., 1993b; Xie et al., 1994) (e.g. as shown in Fig. 11).

Although we discuss the various myosin together in this section the discussion should be viewed with caution. There are several kinetic, structural and mechanical differences between the various myosins (e.g. see Jontes et al., 1997; Gollub et al., 1996). The systems that have been studied include the *brush border myosin-I* (BBMI) (see section IVB), rat liver myosin I (the myr-1a gene product), smooth muscle myosin II and striated muscle. In BBMI and smooth muscle there is a conformational change upon binding ADP that does not take place in skeletal muscle myosin. The angular swing induced by binding ADP is greater for BBM-I than for smooth muscle S1 (31º vs 23º). Smooth muscle lacks the rotational component of the movement found in BBM-I.

In the in vitro studies, a laser trap system (see Chapter 1) has shown that movement does proceed in steps of 10-20 nm (11±1.2, mean±SD) and forces of up to 7 pN (3.4±1.2 pN, mean±SD) (Finer et al., 1994). 10 nm steps are consistent with the stroke size predicted for a swinging cross-bridge model (Huxley, 1969). The distance between cross-bridges (corresponding to S1) is 14 nm. However, other studies indicate a much shorter working stroke, one of about 4 nm (Molloy et al., 1995b; see also Block, 1995) with a force of about 1.7 pN under isometric conditions. The longer values could be the result of the inclusion of Brownian motion of the actin filament.

A new instrument was developed that can manipulate individual myosin S-1 fragments using a scanning probe. Single S-1 fragments could be seen using a fluorescent label. The data of experiments with striated muscle S-1 are consistent with single steps of 5.3 nm. Two to five steps were also found to take place in succession consistent with the completion of a single ATP cycle (Kitamura et al., 1999). In this study, the S-1 fragments were attached to a fluorescent probe and biotinylated at their regulatory chain. A scanning probe was attached to a glass needle mounted on a 3-D piezoelectric scanner. The probe was coated with streptavidin so that it could attach to a single biotinylated S-1 unit. The fluorescent probe at the tip of the scanning probe were visualized with total internal reflection microscopy (Tokunaga et al., 1997) (see Chapter 1). This was accomplished in a relatively simple manner by switching from epi-fluorescence microscopy to objective-type achieved by translation of a single mirror in the system. Clear images of single molecules were obtained with a fluorescence-to-background ratio of 12, using a conventional high aperture objective.

Different results were obtained using the single headed myosins, myr-1 from rat liver and BBMI from chicken brush-border epithelium (Veigel et al., 1999). In these experiments, an actin filament was suspended between two 1 μm plastic beads held by optical tweezers. A myosin molecule bound to nitrocellulose was brought in contact with the filament. Veigel et al. found an initial displacement of 6 nm followed by a 5.5 nm interaction 100-300 ms later. The interval between the two steps was independent of ATP concentration. However, the interval between the second step and the end of the interaction was shorter at higher ATP concentrations. In contrast, with S-1 from skeletal muscle only one

5.5 ms step was detected. The substeps may correspond to intermediate steps in the ATP-hydrolysis cycle such as the release of P_i followed by the release of ADP. This view is in agreement with the image reconstruction of BMM-I head attached to actin filaments (Jontes and Milligan, 1997).

The data assembled so far is consistent with the model depicted in Fig. 11 (Holmes, 1997). The proposed mechanism predicts that the length of the tail will determine the length of the power stroke. Mutants of *Dictyostelium* with different lever lengths were produced. In vitro motility tests of this premise found this prediction to be correct, including one case in which the lever was longer than in the wild type (Uyeda et al., 1996). Surprisingly, the α-helical lever can be replaced by the rigid structure of γ-actinin repeats (Anson et al., 1996). These findings suggest a role for the portion of the molecule near the base of the tail in producing the mechanical movement. The electron microscope data shows this to be the hinge region (Fisher et al., 1995a). In addition, many mutations in *Dictyostelium* myosin that interfere with movement, occur in this region (Patterson and Spudich, 1996). This portion of the molecule, intimately involved in the energy transduction, is referred to as the *converter region* (see Trayer and Smith, 1997).

Crystal structures of S1 with bound analogues of ADP and Pi have provided insight into how ATP hydrolysis could produce mechanical work (e.g., see Fisher et al., 1995b; Smith and Rayment, 1996). Combining F-actin and S1 atomic structures (Rayment et al., 1993b; Schroeder, et al., 1993) permits arriving at a three dimensional model represented in Fig. 10 (Holmes, 1997). Cryoelectron micrograph reconstructions of actin decorated with BB myosin (Whittaker et al., 1995) or smooth muscle (Jontes and Milligan, 1997), are consistent with a rotation of the lever arm away from the rigor position on binding ADP. The movement of the lever arm during the power stroke has been referred to as the *swinging lever arm model*, represented in Fig. 11 (Holmes, 1997). The precise dimensions of the power stroke could not be arrived by either cryoelectron microscopy or X-ray diffraction because only the the structures of the nucleotide free and transitional conformations had been studied. Dominguez et al. (1998) have reported the crystallographic structure of myosin from chicken gizzard smooth muscle (see Fig. 12). They examined the structure of the motor domain of myosin (MD) and a complex of the motor domain attached to its ELC (MDE) while bound to nucleotide analogs. They found that tail structure shows a 70^o rotation as compared to the nucleotide free S1 (Rayment et al. 1993a). This corresponds to a potential displacement of approximately 10 nm during the power stroke. The presence of the ELC changes the position of the converter region and hence that of the lever arm.

Most myosins move toward the plus end of the actin filament. Myosin VI moves in the opposite direction (Wells et al., 1999). Cryo-electron microscopy and image analysis of myosin VI found an ADP-mediated conformational change in the lever arm that is in the opposite direction of that occurring in other myosins. The class VI myosins have large insertion at the converter domain, confirming the importance of this sector in the motor function of the myosins. Models can be constructed that can explain simply how direction can be reversed in a myosin that essentially has a conventional structure (see Schliwa, 1999).

The movement of the lever arm was also followed by a technique devised for measuring motion in protein domains. This method consists in introducing two cysteine residues at a defined location in the protein. The cysteines are then crosslinked to bifunctional rhodamine (Corrie et al., 1998). The polarization of the rhodamine fluorescence can then be used to measure the orientation of the rhodamine dipole. When returned to its native environment the orientation of the protein domain can be calculated from observations on sets of these dipoles. Changes in orientation can be measured in the sub-millisecond range. Applied to the myosin-light chain domain located in the lever arm region, this approach shows that there is increase in tilt angle and a decrease in twist angle (Corrie et al., 1999). Generally, the findings are in agreement with the crystallographic models.

In current models, as indicated in Fig. 9 [(4) to (1)], the force generation is precisely coupled to the release of ADP and P_i from the myosin head. Experiments combining two very different approaches (measurement of nucleotide binding combined to the measurement of the displacement; Ishijima et al., 1998), have indicated that the two need not be coupled. Myosin must be able to store energy so that the working stroke can occur later. Fluorescence was detected from a single molecule of a fluorescent ATP analog bound to myosin (Funatsu et al., 1995). With this technique (total fluorescence reflection) a stationary fluorescent spot indicates binding of ATP (or ADP) to the attached myosin (see Chapter 1). Disappearance of the spot indicates dissociation from myosin of either ATP or the ADP produced by hydrolysis. The simultaneous displacement of an actin filament caused by a single myosin molecule attached to a slide was measured using the optical trap method (see Chapter 1) where beads held in optical traps were attached to the ends of a single actin filament which was held firmly. Binding of the actin filament to myosin is indicated by a reduction in the Brownian motion of the beads. The use of the two techniques simultaneously confirmed that release of actin from striated muscle myosin coincided with ATP binding ([Fig. 9, (1) to (2)]. Free myosin bound ATP (or ADP). The ATP was released when actin and myosin combined [Fig. 9, (4) to (1)] during the working stroke. However, approximately 50% of the time, there was a long delay (several 0.1 s intervals) between the disappearance of the fluorescence (release of the nucleotide) on the one hand, and the decrease in Brownian motion (indicating binding of the myosin to the actin) and the displacement on the other. Presumably, myosin hydrolyses ATP and releases ADP and P_i before attaching to actin but can still carry out the working stroke when it finally binds to actin.

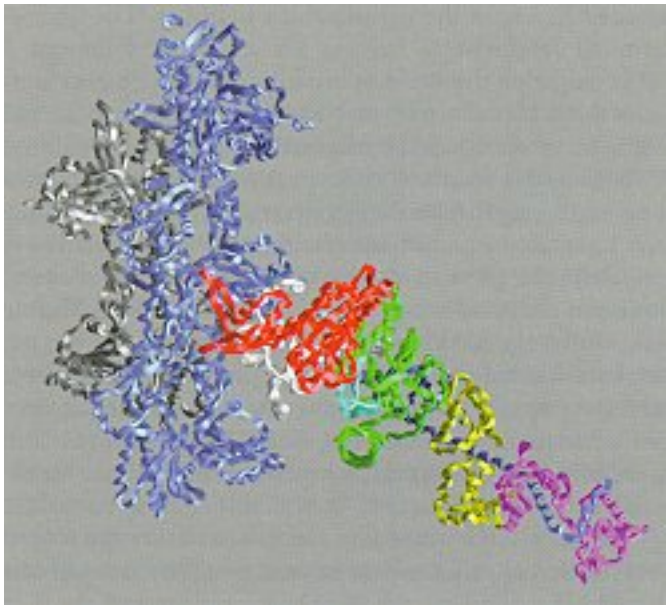


Fig. 10 The structure of the actin-myosin complex (Holmes, 1997). On the left, five actin monomers in an actin helix (blue and grey strands). On the right, a myosin S1. The 25 kDa domain is green, the 50 kDa upper domain is red and the lower domain is white. Part of the 20 kDa domain is light blue (including the SH2 domain). The SH1 helix, converter domain and carboxy-terminal is dark blue. The regulatory light chain is magenta and the essential light chain is yellow. Reproduced by permission of *Current Biology*. (Available in the BioMedNet library at: http://biomednet.com/cbiology/cub)

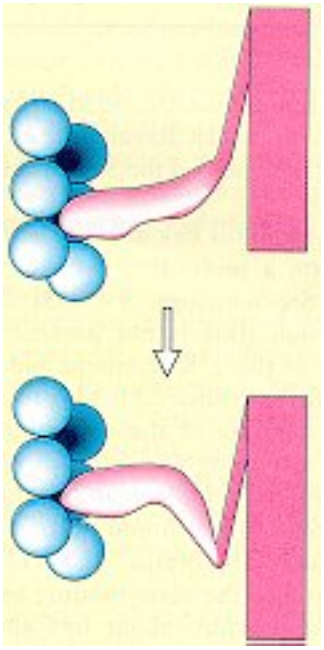


Fig. 11 Swinging lever arm model of movement. Only part of the tail of the myosin head acts as a swinging lever (Holmes, 1997). Reproduced by permission of *Current Biology*. (Available in the BioMedNet library at: http://biomednet.com/cbiology/cub)

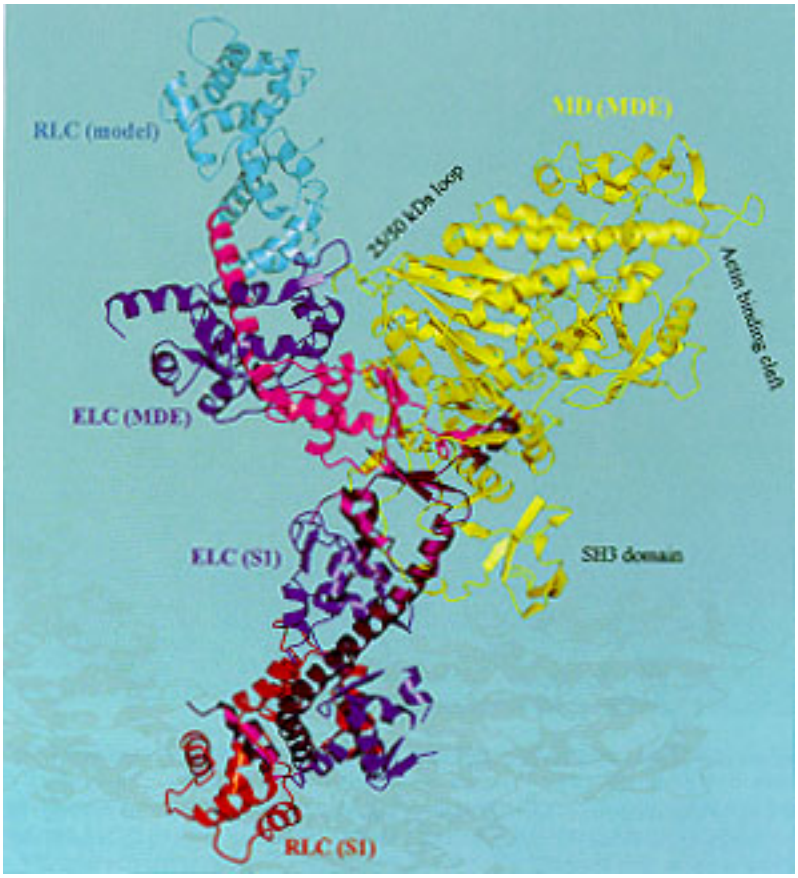


Fig. 12 Ribbon diagram representing the two different orientations of the lever arm. Beginning of the power stroke (the upper position of the lever)[observed in a motor domain-essential light chain complex (MDE) with either MgADP.AlF_4^- or MgADP-BeF_x bound at the active site] and the end of the power stroke (the lower position of the lever) observed in the nucleotide free S1). In the figure the motor domain up to the hinge point (yellow) is shown only for the MDE since they superimpose in this representation. After this the two structures diverge at an angle of about 70º. The carboxyterminal of the heavy chain of skeletal S1 is magenta and that of the MDE structure is pink. In both structures the motor domain essential chain (ELC) is in blue. The RLC of the MDE is from a model and is greenish-blue (cyan). The RLC of S1 is red. From Dominguez et al. (1998) *Cell* 94:559-571, Copyright ©1998 by Cell Press. Reproduced by permission.

As summarized in Fig. 11 and 12, most current information on muscle contraction has suggested models in which movement is provided by the lever arm of the light chain domain of myosin, while the actin-binding motor domain has a single orientation (e.g., see Holmes, 1997). However, the possibility of a change in conformation also in the motor domain is supported by crystallographic data (e.g., see Bershitsky et al., 1997; Tsaturyan et al., 1999) and electron tomography (see Chapter 1) of quickly frozen insect flight muscle (Taylor et al., 1999).

Details of the interaction of myosin components as the consequence of events following nucleotide binding are discussed in Section V.

D. Attachment of the Thin Filaments: α-Actinin and Vinculin

The contractile event, expressed in the shortening of the sarcomere, requires that the thin actin filament be attached to the Z disks. The latter are probably mainly made up of a rod-shaped protein of about 95 kDa, α-*actinin*. The location of the α-actinin in the Z disk has been demonstrated with immunofluorescence using anti-α-actinin antibodies (Lazarides and Burridge, 1975; Masaki et al., 1967). Since α-actinin cross-links in vitro with actin, it is presumed to constitute the attachment site for the thin filaments.

In smooth muscle another protein, *vinculin*, of 130 kDa (Geiger, 1979) seems to play a similar role in connecting the actin filaments to the dense plaques (Geiger et al., 1980).

E. Titin and Nebulin

Titin and *nebulin* are huge molecules of myofibrils. Titin, also known as *connectin*, is as large as 3,000 kDa in skeletal muscle (for a review see Murayama, 1997) and nebulin is approximately 800 kDa. Their size precluded their early discovery because these molecules cannot enter most polyacrylamide gels that are generally used to study proteins.

Titin

Members of the *titin* family, also known as *connectins*, are giant proteins in the molecular weight range of 1,000 kDa or more (e.g. see Trinick and Tskhovrebova, 1999). Titins are modular proteins constituted in large part by tandems of immunoglobin- and fibronectin-like domains. Some of the titins are intracellular and are thought to provide scaffolding (e.g., Gregorio et al., 1999) that directs the correct assembly of contractile proteins. Extracellular titins are involved in adhesion and recognition. Titin is also suspected to be present in chromosomes (Machado et al., 1998).

Vertebrate muscle titins (see Labeit and Komer, 1995a; Murayama, 1997) and non-muscle titins (Eilersten and Keller, 1992) are 3,000 kDa in size. The titin-like molecules of invertebrate smooth and striated muscle are smaller, about 800 kDa and go by various names such *twichins* (e.g., Benian et al., 1989), *projectins* (Daley et al., 1998) and *mini-titins* (Nave and Weber, 1990). The larger titins may also be present in some invertebrates (e.g., Vibert et al., 1996).

All titins occur in various isoforms produced by differential splicing. In vertebrates, most of the diverging sequences are toward the amino-terminal of the protein producing molecules with different extensible properties responsible for the elasticity of the muscle. Cardiac muscle has a stiff titin. Other muscles have isoforms that are less stiff and therefore more easily stretched.

In vertebrate striated muscle titin is about 1 μm long and about 4 nm wide. Each titin molecule extends for half a sarcomere with the amino terminal in the Z-disc and carboxy-terminal in the M-line (Labeit et al., 1992; Furst et al., 1988) (see Fig. 13). Most of its domains are immunoglobulin (Ig, I-set) and

fibronectin (Fn, type III) repeats, each with about 100 amino acid residues folded in β-sheets sandwich. In the A-band, titin interacts with the thick filaments and M-line proteins and the amino terminal interacts with actin and Z-disc proteins. The Ig and Fn domains of the titin adjacent to the A-band are present in periodic patterns (Labeit et al., 1992; Labeit and Kolmerer, 1995a). The long-range repeat has been called a *super-repeat* and is 43-44 nm long. This distance (the myosin helix repeat of the thick filament) suggests that titin is bound to the thick filament. The observation that myosin binds to titin (Soteriou et al., 1993) or to constructs produced from cDNA encoding titin (Labeit et al., 1992), supports this view. The interaction appears to involve mostly the light meromycin part of the molecule, the backbone of the thick filament. Titin can therefore be considered an integral part of the thick filament. The I-band portion has mostly Ig domains as well as sequences rich in prolines, glutamates, valines and lysines (the PEVK region). Close to the carboxy-terminal (and the M-line) there is a protein kinase domain.

The A-band portion of titin is thought to control the assembly of the thick filament (see Whiting et al., 1989). At the I-band, titin connects Z-disc and A-band and is thought to provide the muscle passive tension since it is elastic. The titin in the I-band maintains the distance between A-band and Z-disc. In the absence of titin, there would be imbalances between the opposing halves of the sarcomere (Horowits and Podolsky, 1987). In highly shortened muscles such as cardiac myocytes, it provides a sarcomere lengthening force (Helmes et al., 1996). When the elastic segment of titin in the I band was removed from the sarcomere by trypsin treatment, the restoring force of myocytes was found to be depressed.

In *Caenorhabditis elegans*, twichin which also has a protein kinase domain, phosphorylates myosin (see Heierhorst et al., 1996). However, this is not the case for vertebrate titin. Its protein kinase domain phosphorylates a 22 kDa protein, *telethonin* (Mayans et al., 1998). Telethonin is a Z-disc protein, about 1 μm away from the kinase domain (Mues et al., 1998). How titin could phosphorylate telethonin was intitally a mystery. However, the phosphorylation is likely to occur in differentiating myocytes before the myofibrils are formed where the amino terminal and telethonin appear together (Mues et al., 1998) as shown by immunofluorescence (see Chapter 1) with antibodies against the amino-terminal region of titin and telethonin, which detect both proteins at the Z-disc of human myotubes. Therefore the kinase domain is thought to function in sarcomere assembly. The kinase activity has to be activated by Ca^{2+}-calmodulin and phosphorylation of a tyrosine (Mayans et al., 1998).

Titin is a major component of the Z-disc (see Gregorio et al., 1999). The titin amino-terminal contains several copies of a residue, the Z-repeat of 45 amino acid residues. These Z-repeats bind to the carboxy terminal of α-actinin. A domain in the central region of α-actinin binds to a region of titin close to the Z-repeats (Young et al., 1998). The titin from adjacent sarcomeres overlap for the full width of the Z-disc as shown by immunoelectron microcopy (Gregorio et al., 1998; Young et al., 1998).

The configuration of titin is likely to vary when the muscle is stretched. When the sarcomere is relaxed, the I-band part of titin is likely to be coiled. When the sarcomere is stretched, the molecule is straightened out with a minimum of force (e.g., see Higuchi, 1996). After this however, extension

requires unfolding and, therefore, high forces (50 pN or higher) as shown using atomic force microscopy (Rief et al., 1997).

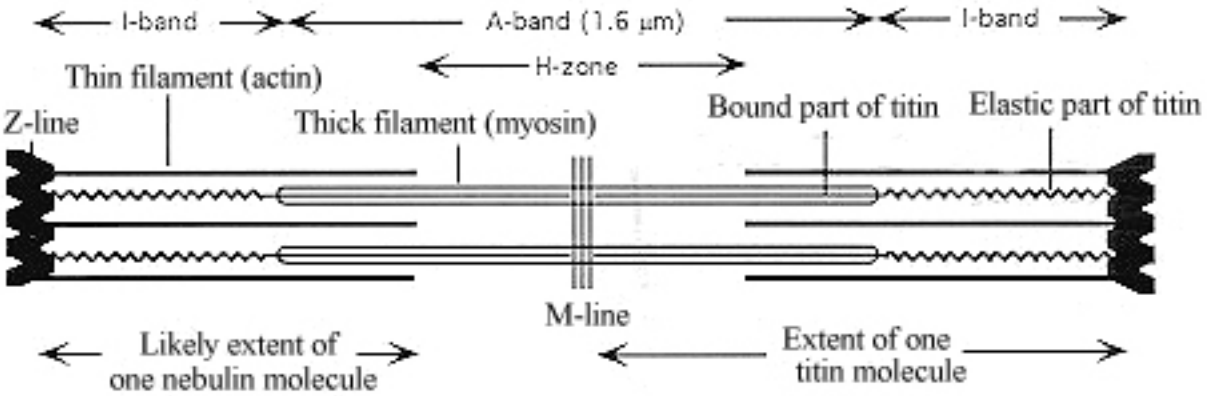


Fig. 13 The sarcomere showing the extent and location of titin and the probable location of nebulin. Reproduced from *Trends in Biochemical Science* , vol. 19, Trinick, J., Titin and nebulin: protein rulers of muscle? pp.405-409. Copyright ©1994 with permission from Elsevier Science.

Nebulin

Nebulin is a large actin binding protein of skeletal muscle, where a single polypeptide extends along the entire length of the thin filament with its carboxy terminal at the Z-line and the amino-terminal at the pointed filament end. Some of the probable features of nebulin in relation to the structure of the myofibril are also displayed in the model of Fig. 13 (Trinick, 1994). In different skeletal muscles the size of the nebulin differs and correlates with the length of the free portion of the thin filament. Consequently, it ranges from a molecular mass of 600 to 800 kDa (e.g., Wang and Wright, 1988; Kruger et al., 1991). A portion close to the carboxy terminal is located in the periphery of the Z-disc (Wright et al., 1993) as shown by immunoelectron microscopic localization (see Chapter 1) of epitopes of a number of site-specific monoclonal antibodies.

cDNA studies of nebulin revealed repeat modules (of 35 amino acid residues) and sets of modules (185 or more) (Labeit and Komerer, 1995b). These modules can bind actin and stabilize the filaments. The modules are present in approximately 20 tandem super-repeats, each containing 7 different modules. Near the amino and carboxy terminals these super-repeats are flanked by single-repeat regions containing 8 modules (Labeit and Kolmerer, 1995; Wang et al., 1996b; Zhang et al., 1996). Each repeat is predicted to have an α-helical configuration and contains an SDXXYK sequence (Labeit et al., 1991). The seven-fold periodicity in the super-repeat reflects that of the actin filament which contains a 385 Å unit composed of seven actin molecules and one complex of troponin/tropomyosin. Single repeats with the central SDXXYK motif are the smallest unit that can bind to actin (Chen et al., 1993; Pfuhl et al., 1994). The binding involves both charges as well as hydrophobic interactions. Nebulin has been proposed to form a stable extended configuration along the actin filament by a zipper-like actin binding mechanism,

(Chen et al., 1993; Pfuhl et al, 1996). The binding of nebulin to actin suggests a role as a "ruler" to specify the length of the actin filament (Kruger et al., 1991; Labeit et al., 1991) (i.e. a progressive polymerization by addition of individual G-actin molecules binding to nebulin modules). Chemical cross-linking (Shih et al., 1997) was used to define the molecular contacts between actin and ND8, a two-module nebulin fragment that promotes actin polymerization and inhibits depolymerization by binding to both G- and F-actin. A complex with a stoichiometry of 1:1 complex between ND8 and G-actin was demonstrated. Cross-linking of ND8 to F-actin occurred at the amino terminal of actin. The binding of nebulin to the amino terminal of actin is likely to have a role in actin polymerization.

A much smaller protein (100 kDa), *nebulette*, has been found whose carboxy-terminal portion resembles nebulin. Nebulin and nebulette are coded by separate gene. A subgroup of repeats at the carboxy terminal of both nebulin and nebulette molecules, depends on the tissue and stage of development so that a variety of isoforms of both molecules are produced (Millevoi et al., 1998). Nebulette is likely to correspond to a nebulin-like protein of cardiac muscle (which lacks nebulin).

Go to Part 2

REFERENCES

Search the textbook

Back to Part 1

II. MECHANISMS IN OTHER MUSCLES

We have seen that much of the information available for striated muscle points to contraction occurring as a consequence of the sliding of actin filaments in relation to myosin filaments. Some clues are presented as to how this might occur at the molecular level. As previously discussed, the striations are the consequence of the presence of fibers of myosin in the thick filaments of the denser anisotropic bands and the presence of actin in the thin filaments spanning the less dense isotropic bands. The thin filaments interdigitate with the thick filaments of the A bands. The sarcomeres are in register, so that the A or I bands of one sarcomere are adjacent to those of a sarcomere located next to it.

Striated muscle is found in vertebrates and a number of arthropods. Presumably, the same contractile mechanism operates in all these striated muscles, although certain details may differ. Other muscles are arranged differently from striated muscle.

Some, such as the body wall muscle of the earthworm, are obliquely striated (Fig. 14) (Heumann and Zebe, 1967); others, such as smooth muscles, have no apparent striations at all. Do these muscles contract in a different manner? The answer is not entirely clear. Smooth or obliquely striated muscles generally have some properties that distinguish them from striated muscle. For example, although striated muscle generally contracts to about 80% of its resting length, some smooth muscles can contract to 30% of their resting length (Hasselbach and Ledermair, 1958; Winton, 1926). Are these manifestations of fundamental differences or do they represent minor modifications of the same basic mechanism? Close examination of at least some of the cases indicates that usually it is not necessary to propose a different mechanism. Where shortening is extreme, the change in length could take place only if the filaments did not abut against a barrier, such as a Z disk. In this extreme, the thick filaments would have to slide relative to other thick filaments, as has been observed. An example of extreme contraction, that of the body wall muscle of the earthworm, is shown in Fig. 14. Different types of atypically striated or nonstriated muscle are listed in Table 1 (Rüegg, 1968). The table summarizes the type of muscle, the Z line structure, the proteins in the thin and thick filaments, and where these muscles are present.

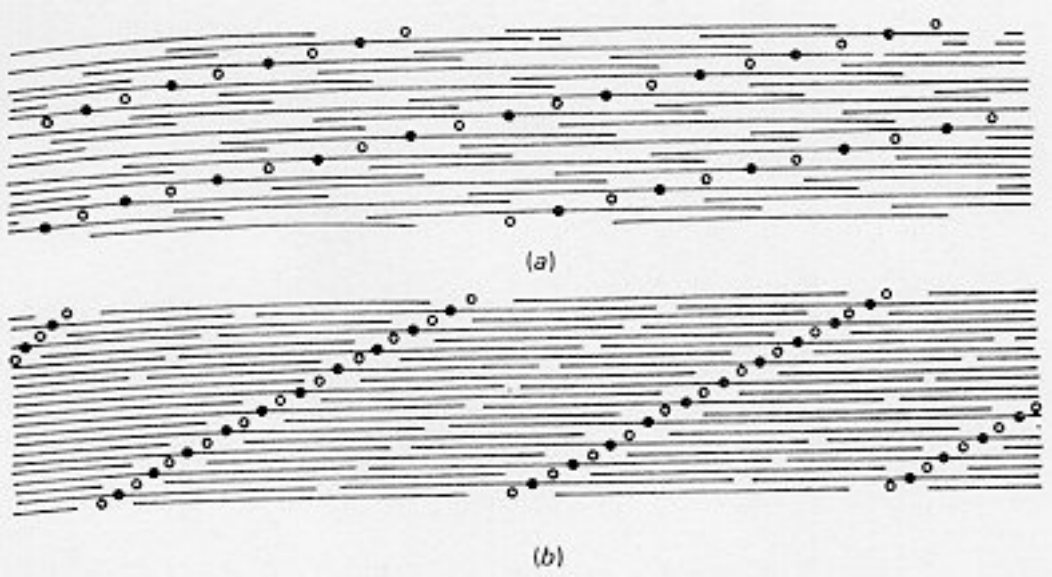


Fig. 14 Model of the sliding-filament mechanism in the obliquely striated body wall muscle of the earthworm. (a) Relaxed state; (b) contracted state. Note that the thick filaments slide not only relative to thin filaments but also relative to each other. From H. G. Heumann and E. Zebe, *Zeitschrift feur Zellforschung und Mikroskopische Anatomie*, 78:131-150, with permission. Copyright &copy1967 Springer-Verlag, Heidelberg.

All the systems that have been studied seem to have both actin and myosin and may well work by a similar mechanism, although differences must occur. Not surprisingly, the properties of the component molecules are different. This is particularly true of myosin, which differs depending on the system from which it has been isolated. Evidence as to whether sliding actually occurs or whether the thick and thin filaments actually correspond to actin and myosin is not always readily available. In addition, the thick filaments have been difficult to demonstrate in vertebrate smooth muscle by means of electron microscopic techniques. Smooth muscle contraction has been recently reviewed (Horowitz et al., 1996).

Table 1 The Diversity of Smooth Muscle

		Proteins of myofilaments		
Type of muscle	**Z-line structure**	**Thin filaments**	**Thick filaments**	**Examples**
Helical smooth or obliquely striated	Z-column or dense bodies	Actin	Myosin and tropomyosin A (paramyosin)	Earthworm body wall; oyster yellow adductor

Invertebrate smooth type I (paramyosin muscle)	Dense bodies	Actin	Myosin, very much paramyosin	Muscle anterior byssal retractor, oyster opaque adductor
Invertebrate smooth type II (classical smooth)	Dense bodies	Actin	Myosin	Pharynx retractor, snail
Vertebrate smooth muscle	Dense bodies	Actin	Thick fibers difficult to see, no paramyosin	Uterus, taenia coli, chicken gizzard

From J.C. Rüegg, *Symposia of the Society for Experimental Biology, XXII*: *Aspects of Cell Motiity*, with permission. Copyright &copy1968 Academic Press.

As already discussed, in striated muscle the arrangement of cross-bridges is bipolar. The cross-bridges have the same polarity in one half of the filament and the opposite polarity in the other half. They are arranged helically with an axial spacing of 14.5 nm between one level of cross-bridges and the other. This allows for contractions consistent with the structure of the sarcomeres.

Smooth muscle could be arranged in the same way as striated muscle. Another possible alternative is an arrangement in a non-helical side polar structure. Fig. 15 (Xu et al., 1996a) presents these two alternatives in diagrammatic form. EM studies of a variety of smooth muscles indicate that the myosin filaments of smooth muscle are side-polar (Xu et al., 1996). This arrangement allows for a much more extensive contraction because myosin can move along actin filaments in a much longer path.

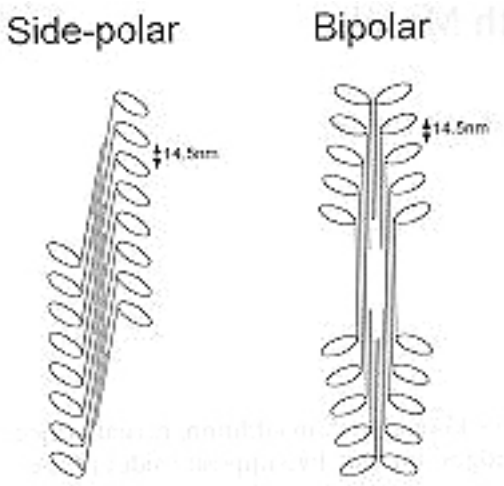


Fig. 15 Schematic comparison of non-helical, side-polar filaments with helical, bipolar filaments. From

III. MOVEMENT IN CILIA AND FLAGELLA

In Chapter 23 we saw that the bending of cilia or flagella may occur through a mechanism analogous to the sliding filament of striated muscles. Nevertheless, the details of the mechanism are likely to differ significantly. As discussed in that chapter, the MTs of cilia or flagella are made up of *tubulin* α and β, as are the MTs present in the cytoplasm. Dynein, responsible for the ATPase activity, corresponds to the cross-bridges in the tubular doublets (see Chapter 23). Tubulin seems to be analogous to actin, and dynein to myosin. However, tubulin differs from actin, and dynein does not correspond to myosin. The details of the interactions between the two proteins making up the microtubular system of cilia or flagella are still not well understood. *Dynein* is made up of several polypeptides (Johnson, 1985). In *Tetrahymena* cilia and in *Chlamydomonas* flagella the dynein is three-headed, whereas in sea urchin sperm flagella the dynein is double-headed. A comparison of myosin and dynein is shown in Fig. 16 (Johnson, 1985). The base of the dynein is anchored to one microtubule, the so-called A subfiber, by ionic interactions. The head, which contains the ATP binding site, is free to interact with the B subfiber of the adjacent doublet, as shown in Chapter 23. Although this arrangement is entirely analogous to the one in actomyosin, the sliding is between microtubules and the dynein heads are much larger.

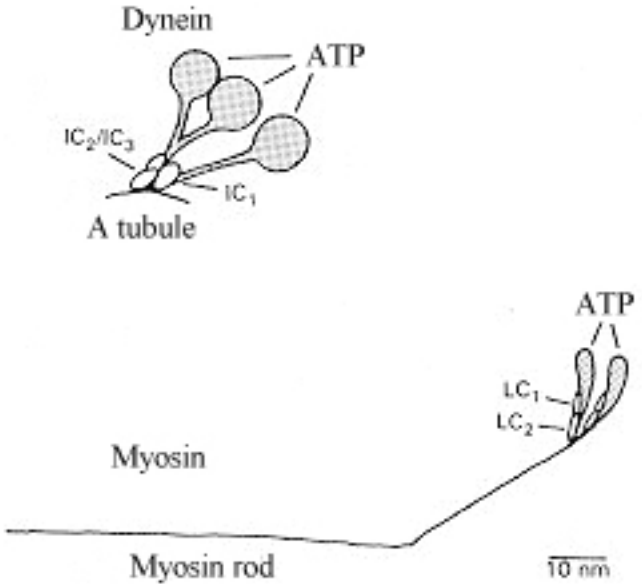


Fig. 16 Structures of dynein and myosin. The structure of *Tetrahymena* 22S dynein is schematically compared to that of myosin, which is drawn to the same scale. The dynein structure combines information obtained from the three dynein sources (see text). IC, Dynein intermediate chains; LC, myosin light chains. From Johnson (1985). Reproduced, with permission, from the *Annual Review of Biophysics and Biophysical Chemistry*, Vol. 14, copyright &copy1985 by Annual Reviews Inc.

Dynein forms a so-called rigor bond with the B subfiber and is released by the addition of ATP. The rate of this dissociation suggests that the microtubule-dynein complex may operate in essentially the same

way as the actomyosin complex, with the same cycle of dissociation, movement of bridge, ATP hydrolysis, formation of bond, and generation of force as outlined for actomyosin.

IV. MOVEMENT IN THE CYTOPLASM

In myofibrils, the structure of the contractile elements is obvious. Many other systems lack apparent contractile structures. Perhaps the proper molecular arrangement could be assembled when needed and then disassembled (e.g., mitotic apparatus) or could actually be labile and present only for a short while. In slime molds, the flow of the cytoplasm reverses direction continuously (Chapter 23). Fibers could produce contraction of the kind observed in slime molds if arranged across the flowing cytoplasmic channel at one location, and could then be disassembled while another contractile structure is formed elsewhere.

The idea that the underlying mechanisms of motility are similar is reinforced by the fact that microtubules or fibers have been found in several contractile systems and in some cases actomyosin and actin molecules have been isolated.

A. Microfilaments and Microfilament Bundles

As mentioned, filaments and tubules are present in the cytoplasm. Not only tubules but also filaments are prominent in neurons (*neurofilaments*). These are 8-10 nm in diameter (Davidson and Taylor, 1960) and appear to be formed of globular subunits. Filaments also appear in many other cell systems. Generally the filaments fall into two groups. Some of the fibers, the *microfilaments* range from 5-7 nm in diameter and correspond in size to actin filaments. Others, such as the neurofilaments, are in the range of 8-10 nm. These *intermediate filaments*, thought to have a structural role, are discussed below in Section IVD. There is extensive evidence that the neurofilaments form a network with microtubules. They form cross-bridges, possibly through the so-called *microtubule-associated proteins* (MAPs) (Leterrier et al., 1982).

This section examines the possible role of the actomyosin system of cells other than muscle and then the role of actin-binding proteins.

The actomyosin system

In *Nitella* and *Chara*, as we saw in Chapter 23, the pattern of flow of the cytoplasm suggests that the motile force is produced in the boundary between the stationary cortex and the outer edge of the cytoplasm. Fibers in this zone have been recognized with the light microscope (Ishikawa et al., 1969; Kamitsubo, 1966, 1972) and the electron microscope (Nagai and Rebhun, 1966). The fibrils are 5-6 nm thick, which suggests that they are actin filaments. Immunofluorescence techniques (Williamson and Toh, 1979) and HMM decoration (Korn and Hammer, 1988) confirm that actin is in fact involved. In *Nitella*, myosin has been isolated (Kato and Tonomura, 1977) suggesting that an actomyosin contractile system is present.

The mechanism of cytoplasmic flow implied by the boundary model described above for *Nitella* and *Chara*, together with the location of the filaments in the cortex, suggests that whereas actin is in the cortex, myosin is most likely to be present in the moving endoplasm. This model is supported by experiments in which cortex and endoplasm are separated out by centrifugation of these very large cells. Centrifugation at low speed collects the endoplasm in the centrifugal end of the cell, whereas the cortex remains in place. The two components are differentially treated and then reassembled by centrifugation in the reverse direction (Chen and Kamiya, 1975; Nagai and Kamiya, 1977). *N*-Ethylmaleimide (NEM) is known to interfere with the F-actin-activated ATPase activity of myosin. In the separation-reassembly experiments, treatment the cortex with NEM did not interfere with streaming after reconstitution. However, treatment of the endoplasm blocked the streaming. Similarly, cytochalasin B, which reacts with actin, blocked streaming when the cortex alone was treated. These observations seem to be in agreement with the model previously proposed for motility in this system, discussed in Chapter 23 Another experiment supports this interpretation. The ectoplasmic fibers were isolated by cutting open the cell and washing away the cytoplasm. The oriented fibers and the chloroplasts stayed behind. Fluorescent beads 0.7 μm in diameter were coated with HMM and then placed on these fibers, on which they proceeded to move unidirectionally along the array of filaments. This motion required the presence of ATP and did not take place when the HMM was inactivated (Sheetz and Spudich, 1983a). Therefore, all indicators support the models that were proposed.

Actins

The actins are ubiquitous in cells. Their polymerization is thought to play a significant role not only in cytoplasmic movement, but also in the structure and mechanical properties of the cytoplasm.

G-actins, the monomeric forms, correspond to a family of globular protein. Like the skeletal muscle counterpart, they are of approximately 42 kDa and are composed of 374 to 375 amino acids, depending on the variant. Various isoforms are present in mammalian tissues. The actins have been conserved to a remarkable extent. Animal and fungal actins are very similar. These, however, differ significantly from the plant actins. Most actins have been modified by post-transcriptional acylation of the amino-terminal and methylation of a histidine residue.

The structure of actin has been studied with electron microscopy and X-ray diffraction. For X-ray diffraction of G-actin, it was necessary to study the structure of the actin-DNase I complex (Kabsch et al., 1990). This complex, by blocking actin polymerization, allows the formation of crystals necessary for the X-ray diffraction. The reconstruction of the structure of actin is shown in Fig. 17 (Bremer and Aebi, 1992). The molecule has two domains, one slightly larger than the other. Actin contains centrally located bound ATP and a divalent cation.

G-actin polymerizes to form actin filaments (*F-actin*). The structure of these filaments, was studied with electronmicroscopy obtained with frozen-hydrated or negatively stained fibers and also studied by X-ray diffraction of the phalloidin stabilized filaments (Holmes et al, 1990). A model is shown in Fig. 18

([Bremer and Aebi, 1992](#)). Polymerization of G-actin first requires the formation of a nucleating seed, then monomers assemble at either end. One end generally grows faster (the *plus*-end) than the other (the *minus*-end). The two can be distinguished by decorating with HMM or the S1 fragment of myosin. As discussed in [Chapter 23](#), the barbed end (B-end) is the *plus*-end and the pointed end (P-end) is the *minus* end. The polymerization is much faster in the presence of ATP. Under appropriate conditions, while G-actin is added at one end, it is removed from the other, eventually reaching a steady state. Although the length and appearance of the filament does not change, the actin molecules are continuously exchanged with the medium, with a migration of each individual molecular from the plus to the minus end. This is known as *treadmilling*.

The polymerization of actin can be blocked using drugs such as the *cytochalasins* (alkaloids of fungal origin), which block the ends of the actin filaments, or the *latrunculins* (derived from the red sea sponge), which bind to G-actin monomers. *Phalloidin* (a compound present in the deadly mushroom *Amanita phalloides*) complexes to filaments and prevents them from depolymerizing. Combined with rhodamine it can be used as a fluorescent stain for actin filaments.

Apart from its binding to myosin, actin binds to many other proteins.

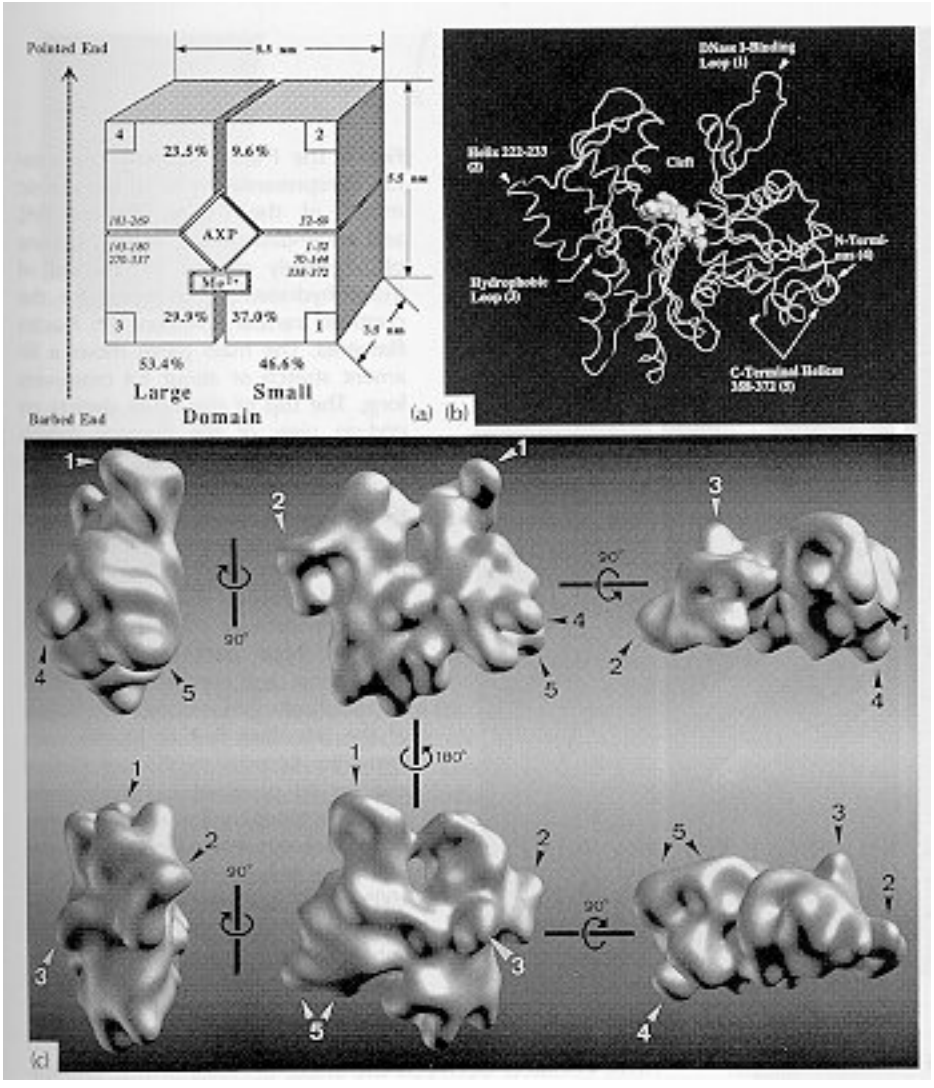


Fig. 17 The actin molecule. (a) A schematic view of G-actin. The arrow defines the orientation of the molecule relative to the myosin subfragments-1 decoration pattern of the F-actin filament. (b) Folding of the actin molecule represented by ribbon tracing of the α-carbon atoms. An ATP molecule with its associated Ca^{2+} atom (bottom) are shown in a Van-der-Waals-radius representation. The cleft separating the two domains, the DNase I-binding loop, the hydrophobic loop, α-helix 222-223, the amino terminus, and the two carboxyl-terminal α-helices 358-372 are marked. The orientation is the same as in (a). (c) Different views of the atomic structure of the actin molecule reduced to 1.0 nm resolution. From Bremer and Aebi, 1992. Reproduced by permission. (Available in the BioMedNet library at: http://biomednet.com/cbiology/cel)

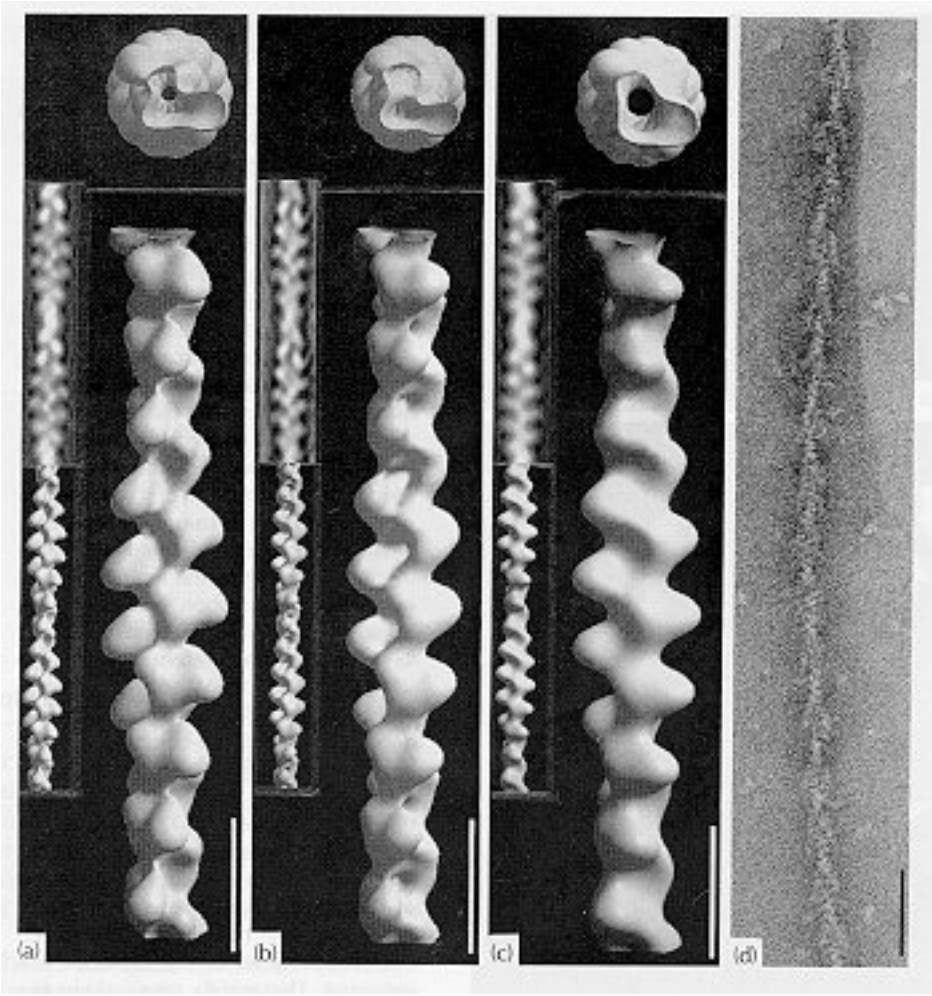


Fig. 18 The F-actin filament. Low resolution representation of (a) the atomic model of the F-actin filament and three-dimensional reconstruction of negatively stained (b) and of frozen-hydrated (c: corrected for the contrast transfer function) F-actin filaments. The main panel shows a filament stretch of about 1.4 crossovers long. The top of the figure depicts an end-on view of the filament stretch shown in the main panel. To the left is a comparison of the three-dimensional filament model (bottom half) with a computed projection perpendicular to the filament axis (top half). (d) Myosin subfragment-1-decorated filament stretch, negatively stained with uranyl formate. Scale bars are 10nm (for a-c; main panel and top inset) and 50nm (for d). Reproduced from Bremer and Aebi, 1992 by permission. (Available in the BioMedNet library at: http://biomednet.com/cbiology/cel)

Actin-binding proteins

The proteins involved in actin-polymerization in relation to the lamellopodia and pseudopodia such as the Arp2/3 complex and α-actinin are also discussed in Chapter 23. Several different groups of proteins bind actin (see Ayscough, 1998; Pollard and Cooper, 1986). The *capping proteins* generally bind to the barbed end of the filaments and thereby interfere with the binding of monomers at that end. They can then grow with their barbed ends attached to particles or surfaces coated with capping proteins such as *villin* or *severin*. The capping proteins in the presence of Ca^{2+} favor the polymerization of actin monomers by facilitating nucleation to form short filaments, possibly by severing longer segments.

Capping may stabilize filament length by stopping *treadmilling*, that takes place in vitro and in the intact cell. By facilitating the formation of short segments, they favor the sol state and not the gel state and hence presumably favor the flow of cytoplasm.

Some low molecular weight proteins, the *severing proteins*, bind to actin monomers and sever longer actin filaments to produce shorter ones.

Various *bundling proteins*, which are present as dimers, cross-link actin filaments to form bundles. Generally, three to five actin molecules are cross-linked by one bundling protein molecule. Bundling proteins play a role in intact cells; in the brush border of the intestinal microvilli, for example, *fimbrin* and *villin* cross-link actins to form bundles. In addition, cross-linking favors the formation of a gel. The role of these actin-binding proteins is summarized in Fig. 19 (Craig and Pollard, 1982).

Much of the recent information on the actin-based interactions has been derived by primary amino acid sequence data of the binding proteins, determined by using recombinant DNA techniques. The search for common sequences has allowed the recognition of actin-binding domains. Many of these actin-binding proteins mediate interactions by binding to yet other proteins. Many of the actin-binding proteins can bind directly to polyphosphoinosides (i.e., a phospholipid component of the membrane); others to integral membrane proteins, possibly implicating the actin system in the regulation of both structure and metabolism. For example, *profilin* (see Chapter 23) inhibits the hydrolysis of PIP by some phospholipases C and, therefore, has a potential regulatory role in the production of second messenger of the inositol system (Chapter 7).

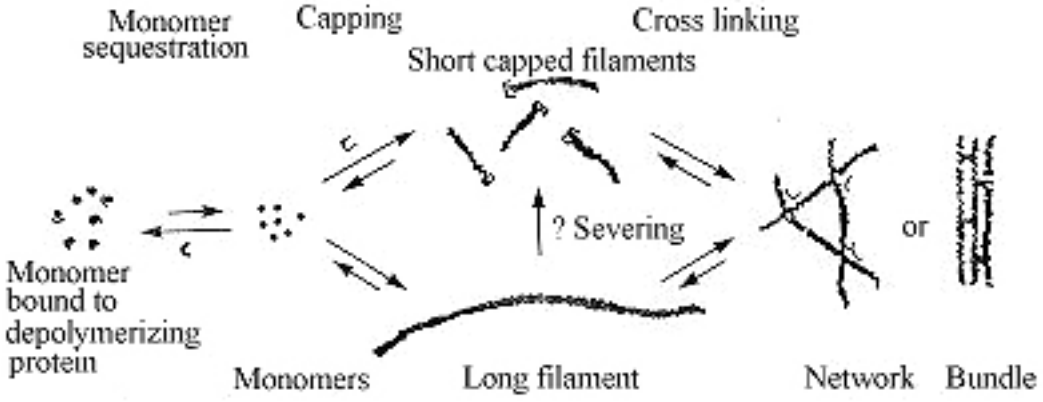


Fig. 19 Regulation of actin assembly by three classes of actin-binding proteins. Class I cross-links filaments into networks or bundles. Class II caps an end of the filament and may sever preformed filaments. Class III inhibits polymerization by binding to actin monomers. From S. W. Craig and T. D. Pollard, *Trends in Biochemical Sciences*, with permission. Copyright &copy1982 Elsevier Science Publishers, England.

The known actin-binding proteins share a 270 amino acid region that is presumed to be the actin-binding domain (see Hartwig and Kwiatkowski, 1991). Subfragments of these domains bind actin (e.g., a 17 kDa fragment of *Dictyostelium* ABP-120, Bresnick et al., 1990). These molecules generally are rod-shaped, and apart from at least one actin-binding domain, the rest of the rod contains tandems of structural repeats of variable lengths, presumably to distance the actin from specific binding sites to other proteins that are also present. For example, spectrin has binding sites in the rod region for calmodulin, ankyrin, and band 4.1. Many of these proteins also contain domains that have homology to sequences of the Ca^{2+}-binding proteins (e.g., Vandekerkhove, 1990), suggesting a regulatory role of Ca^{2+}. Such a region is at the carboxy-terminal of α-actinin (Noegel et al., 1987) and of α-spectrin (Wasenius et al., 1990), and at the amino-terminal region of fimbrin (de Arruda et al., 1990).

Except for fimbrin/plastin, the actin-binding proteins are thought to be in the form of dimers in antiparallel subunit chains, so that the actin-binding domains, generally present at the amino-terminal, are at both ends of the composite rods (Fig. 20, Hartwig and Kwiatkowski, 1991). The subunits are either side by side (e.g., spectrin, α-actinin, and ABP-120), or end-to-end (e.g., filamin). Spectrin is formed by α and β chains that self-associate in overlapped antiparallel alignment to form heterodimers 100 nm in length; these associate end to end to form tetramers (see Fig. 20).

The rate of treadmilling of F-actin in intact lamellipodia is much greater than that in vitro (e.g., see Wang, 1985). This is because the in situ system has accessory proteins that are missing in vitro. Actin binding proteins such as capping proteins and ADF/cofilin (see Theriot, 1997) regulate the rate of turnover. The details of the various interactions between accessory proteins, ATP, ADP, G-actin and F-actin have acquired sufficient complexity to require detailed kinetic analysis facilitated by the use of computers (e.g., Dufort and Lumsden, 1996, see Carlier and Pantaloni, 1997). Profilin binding to an actin monomers blocks the pointed but not the barbed end and facilitates the exchange of ATP for the bound ADP (Dufort and Lumsden, 1996). ATP-bound actin is the form that is added at the barbed end (Pantaloni and Carlier, 1993). Capping proteins also decrease the total number of growing barbed ends "funneling" polymerization to the remaining free barbed ends. In contrast the *cofilin/actin-depolymerizing factors* (ADFs) increase the depolymerization at the pointed end (Carlier et al., 1997) without affecting the barbed ends. In this process the increase in ATP-bound G-actin speeds up the association at the barbed ends. The balance of the various actions together produce a continuous movement of actin molecules through the actin thread.

The proteins of the *gelsolin* family sever and cap the barbed ends of actin filaments (see Ayscough, 1998). Gelsolins are required for rapid motile responses of cells. Ca^{2+} favors the binding of gelsolin to F-

actin. In contrast PIP_2 (phospatidylinositol 4,5 bisphosphate) dissociates gelsolin from actin.

Tropomyosin have been found in many kinds of cells and it is thought to have a role in stabilizing actin filaments. Several isoforms have been found suggesting that they may have different roles.

Coronin was first found in an actin-myosin complex (de Hostos, 1991). Unlike mutants of genes coding for ABP-120, α-actinin or severin that only have slightly altered phenotypes, mutants of the gene coding for *coronin* of the slime mold *Dictyostelium*, were found to be impaired in cell locomotion and cytokinesis (de Hostos et al., 1993; Fukui et al., 1999) phagocytosis (Maniak et al., 1995) and macropinocytosis (Hacker et al., 1997). Members of the coronin family were found in a variety of organisms including mammals (see de Hostos et al., 1999).

Coronin, a protein of 55 kDa, binds to actin in vitro (de Hostos et al., 1991). Immunofluorescence localizes the protein in actin-rich crown-like extension of the cytoplasm. Coronin has five WD (trp-Asp) repeats similar to the β subunit of the GTP-heterotrimeric binding protein (or G-protein) (see Chapter 7, Section II). These are flanked by other domains. WD proteins have been found only in eukaryotes where they function in cell division, cell-fate determination, gene transcription, transmembrane signalling, mRNA modification and vesicle fusion (Neer, 1994). Close to the amino-terminal, the molecule forms a β propeller structure similar to that of the β-subunit of G-proteins (see de Hostos, 1999). Generally the last 25 to 40 amino acids at the carboxy-terminal are likely to form a coiled-coil configuration. Between the WD-containing regions and the coiled-coil domain there is a unique region (Goode et al., 1999) of variable length (22 to 50 amino acids) which differs in the various coronins.

The coronin present in *Saccharomyces cerevisiae* (Crn1p) (Goode et al., 1999) is a component of the cortical actin cytoskeleton and binds to F-actin with high affinity. Crn1p promotes the rapid barbed-end assembly of actin filaments and cross-links filaments into bundles and more complex networks, but does not stabilize them. Filament cross-linking depends on the coiled-coil domain suggesting that dimerization is required. Assembly-promoting activity is independent of cross-linking. Crn1p also binds to microtubules in vitro in the unique region not found in other coronins and which is much longer than in other organisms. This region is homologous to the microtubule binding region of MAP1B. The binding of microtubules to Crn1p is enhanced by the presence of actin filaments.

The possibility that actin polymerization alone may have a role in some movements inside cells has been suggested. The pathogenic bacteria *Listeria monocytogenes* move in the absence of motor molecules (e.g., Loisel et al., 1999). The bacteria propel themselves in the cytoplasm of the host cells by nucleating actin filaments at surface of their outer membrane. The actin filaments rearrange into a tail that varies in length and trails behind the moving bacterium (the so-called "comet tail"; see Cossart, 1995). The actin filaments cross-link and the actin polymers are rapidly disassembled by depolymerizing factors. The actin-based propulsion is linked to actin polymerization dependent on ATP hydrolysis, but not requiring myosin. An activated Arp2/3 complex, actin depolymerizing factor (ADF, or cofilin) and capping protein

(see above) are also required for motility. These maintain a high level of G-actin required for the unidirectional growth of actin filaments at the surface of the bacterium. Similar mechanisms of motion have been shown for *Shigella*, as well as Rickettsia and vaccinia virus (Higley and Way, 1997). Is it possible that a similar mechanism operates in cells? Although the role of this "comet tail" mechanism in movements of vesicles in the cytoplasm is probably not common, recent evidence suggests that it does occur.

The possible involvement of the actin-assembly mechanism in the movement of some cytoplasmic organelles was examined in *Xenopus* eggs (Taunton et al., 2000). Dynamic actin "comet tails" were found on a portion of cytoplasmic vesicles which exhibited movement and contained protein kinase C (PKC). The process was enhanced by the activator of PKC, phorbol myristate acetate (PMA). The phenomenon was also found in a cell free system. In vitro, N-WASP (*N-Wiscott-Aldrich syndrome proteins*) was found to be recruited to every vesicle with a comet tail. WASPs are thought to have a role as adaptors needed for coupling the GTPases to the Arp2/3 complex (see Chapter 23) and N-WASP, a protein homologous to WASP, is thought to transmit signals that lead to rearrangements of cortical actin filaments (see Miki et al., 1996). These findings suggest, that in these experiments, N-WASP recruits Arp2/3 which, in turn, nucleates actin. The vesicles exhibiting this behavior were found to be probably endosomes and lysosomes, because they accumulate acridine orange (indicating an internal acid pH). In addition., endosomes and lysosomes isolated from mammalian cells also nucleated actin and moved in the *Xenopus* extracts. The formation of comets has been implicated in endocytosis involving caveolae (see Chapter 9)

Other experiments using mouse fibroblasts reveal more facets to the role of actin, implicating membrane components in actin nucleation and the formation of comets. Phosphatidylinositol 4,5-bisphosphate (PIP_2) (see Chapters 4 and 7) stimulates actin polymerization by activating the pathway of the WASP and the actin-related protein complex Arp2/3 (see Chapter 23 and Higgs and Pollard, 1999). The binding of phosphoinositides to actin has been discussed in Chapter 23. In addition, actin polymerization is initiated at cholesterol-sphingolipid-rich membrane sites, the 'rafts' (see Chapter 4), in a process requiring tyrosine phosphorylation. Overexpression of type I phosphatidylinositol phosphate 5-kinase (PIP5KI), which synthesizes PIP_2, initiates actin polymerization from vesicles to form motile actin comets (Rozelle et al., 2000). The vesicles attached to comets, were enriched in PIP5KI and tyrosine phosphoproteins. WASP-Arp2/3 were involved as shown with WASP-null mutants. Extraction of cholesterol reduced comet formation, suggesting an involvement of the rafts. The formation of comets may be an exaggeration of a normal process where actin assembles around transport vesicles or may represent an actual movement of vesicles under certain conditions.

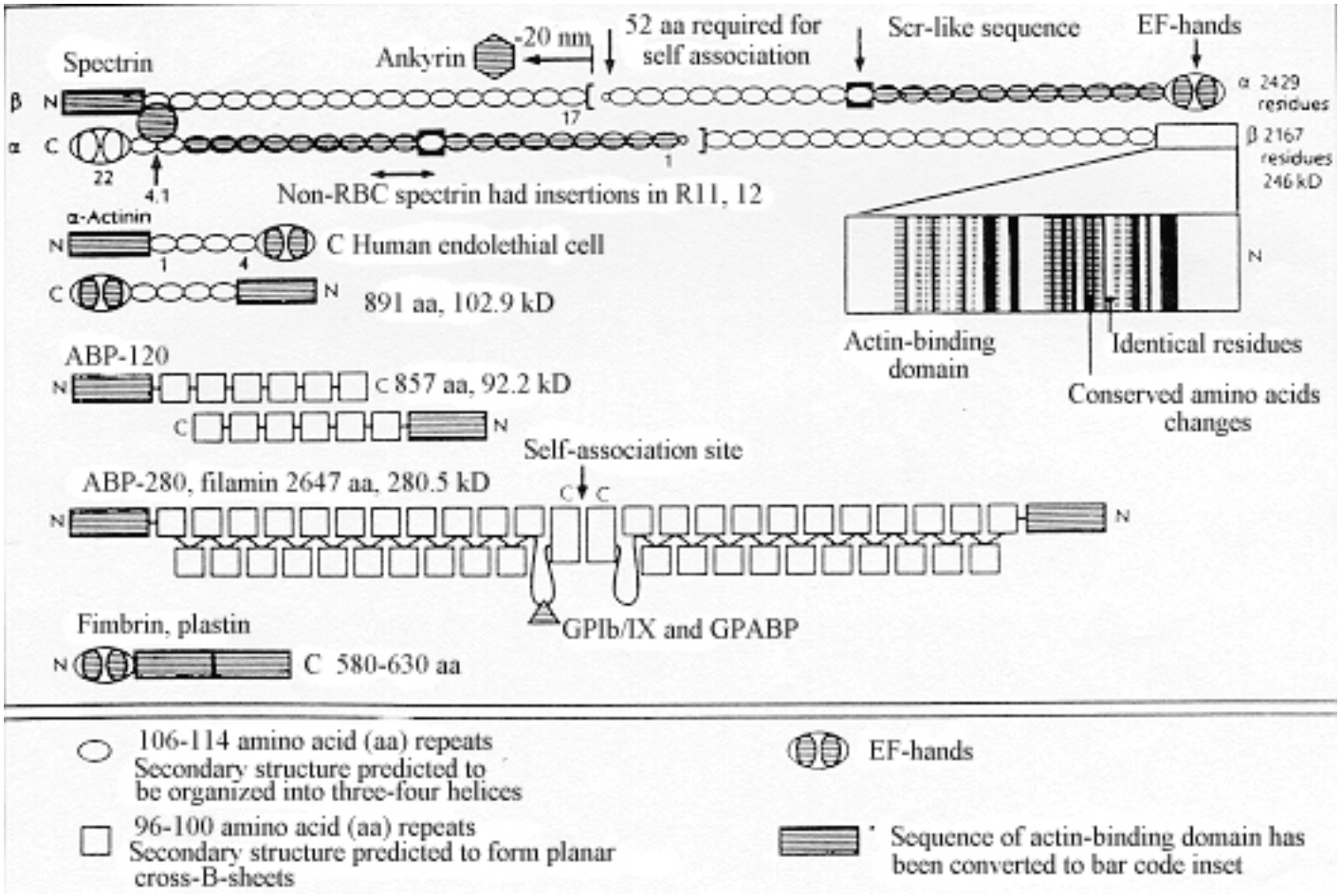


Fig. 20 Comparison of predicted structure of actin-binding proteins sharing a common actin-binding domain. Reproduced from J. H. Hartwig and D. J. Kwiatkowski, *Current Opinion in Cell Biology*, 3:87-97. Copyright &copy1991 Current Biology Ltd., by permission. (available in the BioMedNet library at: http://biomednet.com/cbiology/cel)

The assembly of cytoskeletal elements containing actin has been examined in detail in the red blood cells (see Bennett, 1990; Anderson and Marchesi, 1985) and in intestinal microvilli. In the red blood cell, a meshwork of spectrin tetramers interacting with F-actin is attached to the plasma membrane through two high affinity associations: a binding to ankyrin, which attaches to band 3 (anion exchanger) protein, and a binding to band 4.1 attached to glycophorin, an association favored by polyphosphoinositides.

In the brush border of microvilli, a myosin I (see next section) complex containing calmodulin subunits attaches to actin, while at the carboxy-terminal it binds to acidic phospholipids (Hayden et al., 1990). The complex also contains the bundling proteins villin and fibrin. These four proteins can assemble in vitro to form the microvillar core (Coluccio and Bretscher, 1989). A model of this association is shown in Fig. 21 (Bretscher, 1991).

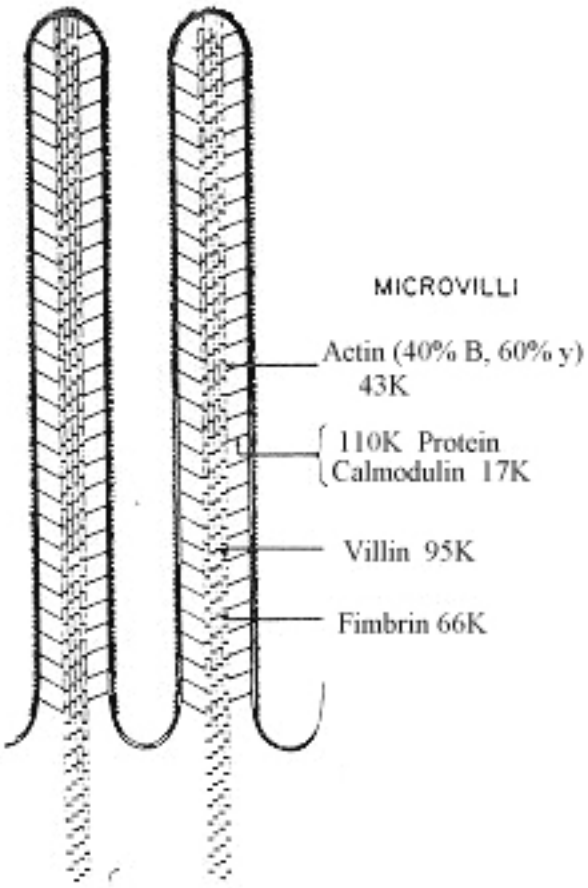


Fig. 21 Model of the molecular organization of the microvillus cytoskeleton. The microvilli are approximately 100 nm in diameter. The 100 kDa-calmodulin-17 kDa is a myosin I (BB myosin I). Reproduced from the *Annual Review of Cell Biology*, copyright &copy1991 by Annual Reviews Inc.

B. Myosins

As previously discussed, skeletal muscle myosin is double-headed and composed of two approximately 200 kDa heavy chains with an amino-terminal globular domain. A single unit of the latter (S_1), which corresponds to one head, is responsible for the ATPase activity and, furthermore, has all the machinery needed to generate force (Toyoshima et al., 1987; Kishino and Yanagida, 1988). However, myosin molecules represent a family of proteins of which striated muscle myosin, *myosin II*, is just one. Other myosins have been referred to as *unconventional myosins* (e.g. see Titus, 1997).

Generally the various myosins move toward the plus (barbed) end of F-actin (e.g., see Sellers and Goodson, 1995). The exception, however, is a myosin VI which moves in the opposite direction (Wells et al., 1999). Myosin VI is supposedly involved in the cytoplasmic transport of vesicles (Mermall et al., 1994) and is probably involved in endocytosis (see Chapter 9).

A diagrammatic summary of the myosins is shown in Fig. 22 (Cheney and Mooseker, 1992).

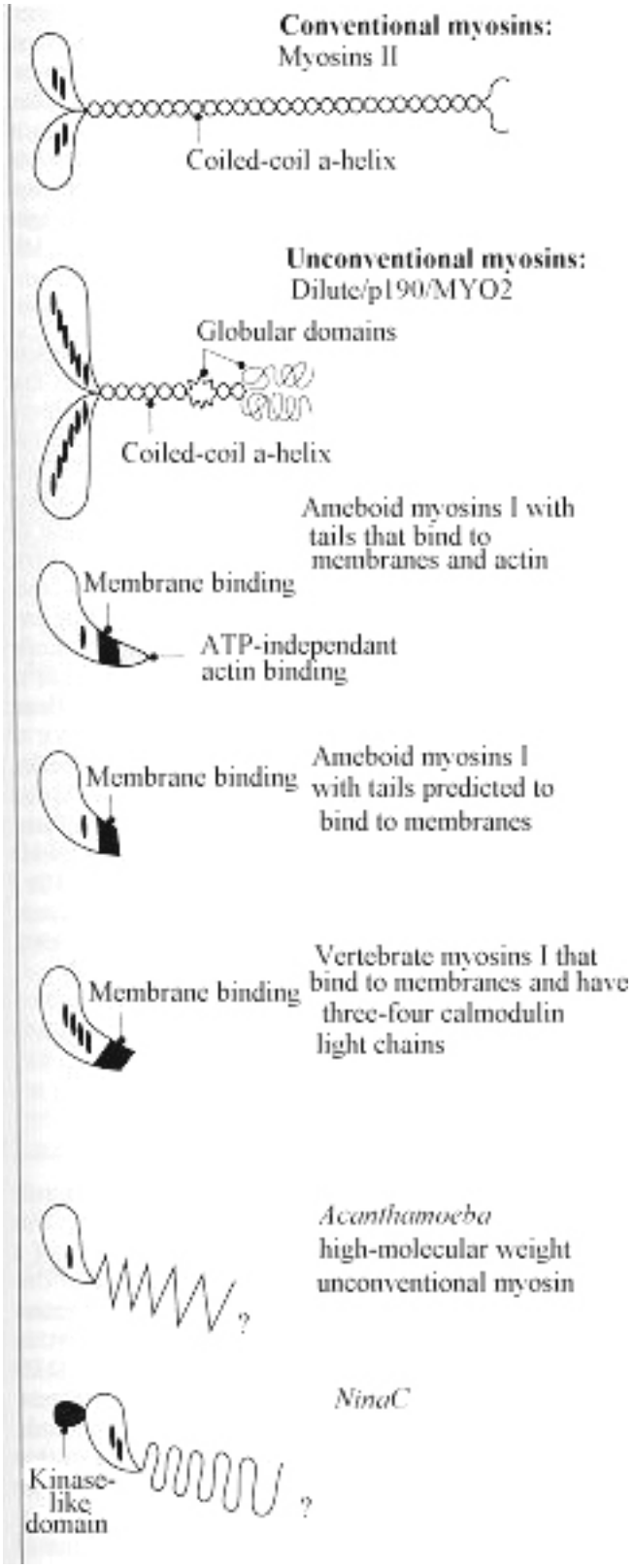


Fig. 22 The myosin motor superfamily. Schematic diagrams of the several classes of myosins are shown with the myosin head domains indicated by large open ovals and the various myosin light chains by small filled ovals. From Cheney and Mooseker, 1992, reproduced by permission.

Unconventional myosins became the focus of interest with the demonstration that *Dictyostelium* cells lacking myosin II retain many actin based functions such as phagocytosis, pseudopod extension and cell

movement (see Pollard et al., 1991). The possibility of a role in these functions for myosin I was suggested by the localization of this protein in phagocytotic cups and the leading margins of locomoting cells (Fukui et al, 1989, 1993a,b). Mooseker and Cheney (1995) have grouped the currently known myosins into 13 classes, with the understanding that this classification may have to be revised as our knowledge expands (see Mooseker and Cheney, 1995, Cope et al., 1996). There is evidence for 25 myosin genes in vertebrates. At least half of these are thought to be distinct from myosin II and considered "*unconventional*". A single vertebrate cell has been reported to express mRNA or protein for at least 11 distinct myosins, 9 of them unconventional (Bement et al., 1994a). Unconventional myosins are thought not to form filaments in contrast to myosin II.

The myosins have a head domain at the amino-terminal, a neck domain which is the site of light-chain binding, and a class specific tail domain. A current working hypothesis proposes that the tail domains dictate subcellular localization and function because these sectors are the most variable from myosin to myosin. However, there is evidence for a role of the tail region for only one myosin (class III, NINAC, Porter et al., 1992). The head domains have some specific features and have been shown to have a role in localization (Porter and Montell, 1993; Ruppert et al., 1995; Durrbach et al., 1996).

Some unconventional myosins have large insertions which would alter the motor activity considerably. The neck regions have repeat motifs referred as IQ motifs, which are thought to bind the light chains of myosin II and calmodulin. The neck domain might also act as a lever (e.g. Spudich, 1994). The tail domains of some unconventional myosins are thought to be able to interact with membranes. Some also have SH3, kinase, PH and GTPase-activating domains associated with signal transduction.

Phosphorylation of the myosin light chains has a regulatory function in striated muscle, increasing isometric tension (Persechini et al., 1985). Smooth muscle and nonmuscle myosin assembly depends on the phosphorylation of the light regulatory subunits for their ability to assemble in filaments; in addition, the phosphorylated form has a high ATPase activity (see Trybus, 1991). In at least some cases, phosphorylation has been shown to be in response to physiological stimuli (Devreotes et al., 1987). A regulation of myosin and actin filaments assembly by phosphorylation-dephosphorylation of the myosin light chains has been demonstrated by injection of a protein phosphatase into living human fibroblasts (Fernandez et al., 1990). The microinjection resulted in a disassembly of the actin network, as seen by immunofluorescence. After long incubations, the cells' actin returned to the original distribution. Neutralization of the phosphatase by microinjection of the corresponding antibody prevented the disruption. In contrast to this function in vertebrate cells, in lower eukaryotes (such as the cellular slime mold *Dictyostelium discoideum*), phosphorylation of the tail inhibits assembly and the assembly of myosin II of *Acanthamoeba castellanii* is not affected by phosphorylation (see Trybus, 1991).

Unconventional myosins and vesicle movement

We saw that the movement of vesicles inside the cell is often on microtubular-based motors (Chapter 10, 11 and 23, see also below, Section IVB). The role of actin-based motors are beginning to be studied (see

Langford, 1995; Hasson and Mooseker, 1995). Information has been obtained in a variety of systems. In squid axoplasm (Kuznetsov, et al. 1992, 1994; Bearer et al., 1993), where the role of microtubules is well established, a given organelle can move on both types of filaments (Kuznetov et al., 1992). At this time, it would seem likely that the microtubular system provides movement over long distances, whereas the actin-myosin system provides movement to local sites (Atkinson et al., 1992; Langford, 1995). This idea is supported by the organization of the two kinds of filaments in axons. Microtubules are long (25 μm or more) and are oriented longitudinally in parallel to each other. In contrast, actin filaments are short (less than 1 μm) and form a cross-linked filamentous network (Fath and Lasak, 1988). Furthermore, the kinetic properties of myosin I suggest that it is suited only as a short duty motor (Ostap and Pollard, 1996) and may function in locations where actin is clustered.

However, there is considerable evidence as well for the involvement of unconventional myosins in vesicular transport. An association between the microtubular and the actin-based system is suggested by the demonstration of interactions between actin and microtubules (e.g. Griffith and Pollard, 1982) and the finding of a protein, p150 ^{glued} which is a component of the dynactin complex. The dynactin complex is an activator of dynein-mediated vesicle movement. p150 ^{glued} is attached to an actin-like filament (Schafer et al., 1994).

In yeast, the malfunction of MYO2 (a myosin-V) can be overcome by the overexpression of kinesin (Lillie and Brown, 1992) suggesting some interaction between the microtubular and the actin-based systems that is still not understood. In addition, there is evidence that myosin V and microtubular systems interact in the transport of vesicles (e.g., see Rogers and Gelfand, 1998; Huang et al. 1999 and discussion below).

Unlike the microtubular-based movements that have been shown to be bidirectional, actin-based movements have been shown to be only unidirectional, toward the barbed end (e.g. Bearer et al., 1993) with the exception of myosin VI (see above).

The idea that unconventional myosins are involved in vesicular transport is supported by the inhibition of the movement of endogenous vesicles on actin filaments by antimyosin I and not antimyosin II in *Acanthamoeba* cell extracts (Adams and Pollard, 1986). When absorbed to lipid surfaces, myosin I can also move actin filaments along the surface (Zot et al., 1992). Furthermore, the localization of myosin I, as well as the phenotype of myosin I mutants of *Aspergillus*, *Dictyostelium* and *Saccharomyces cerevisiae*, have implicated this class of motors in the control of the activity of the cell cortex, rich in actin. Myosin I is present on the plasma membrane and the actin-rich cortex (McGoldrick et al., 1995; Goodson et al., 1996). Loss of myosin I function produces defects in pseudopod formation, actin rearrangement, endocytosis and secretion (McGoldrick et al, 1995; Novak et al., 1995; Geli and Riezman, 1996; Goodson et al., 1996; Jung et al., 1996; Wessels et al., 1996).

A question that remains unresolved is whether a single kind of myosin is involved in all vesicular or

organelle transport or each vesicle has a different motor. Multiple motors for different vesicles is possible, considering that the same cell hosts several distinct myosins. This idea is supported in a study of *Dictyostelium discoideum* (Titus et al., 1989). The genome of *Dictyostelium* was probed with DNA fragments containing parts of the conserved region of conventional myosin. These experiments identified several genes coding for other actin-based motors.

The role of myosin I is still unclear. Ameboid-type myosin-I is generally associated with motile cells. However, one of these is present in motile and non-motile cells as well (Bement et al., 1994b). The function of another characterized subclass that includes brush border myosin I (BBMI) is unknown. BBMI itself cross-links the microvillar core bundle to the plasma membrane.

A myosin I, similar or identical to BB-myosin I, has been isolated in rat kidney brush border (Coluccio, 1991) and a similar but slightly different protein has been isolated from brain and the adrenal cortex (Barylko et al., 1992).

An interaction of myosin I with the microtubular system in Golgi vesicle transport is suspected. BBMI is present in the cytoplasmic face of Golgi derived vesicles (Fath and Burgess, 1993, Fath et al., 1994). Dynein, a microtubular motor, is present in a portion of these vesicles. As discussed above microtubules are likely to be involved in the transport of the vesicles over long distances. Therefore this observation suggests that microtubules deliver the vesicles to actin arrays. Following this, fusion with the apical plasma membrane may then involve BB-myosin I moving on actin fibers.

Myosin IC is associated with the contraction of the contractile vacuole of *Acanthamoeba* (Doberstein et al., 1993). Microinjection of antibodies to myosin IC have been shown to interfere with vacuole contraction.

Myosin V has been found in yeast and vertebrate cells. In vitro, myosin V acts as a molecular motor (Cheney, et al., 1993a). In mammals myosin V is most abundant in secretory and nervous tissue. It is also found in microvilli.

The indications that myosin V is involved in vesicular traffic are many. In brain myosin V is associated with vesicles and can be activated to function on actin filaments (Evans et al., 1998) and ER vesicles have been found to be transported in neurons by myosin V (Tabb et al., 1998). *Saccharomyces cerevisiae* lacking one of the two genes for MYO2 (coding for myosin V), accumulate small vesicles in the mother cells and vacuolar inheritance is lost (Johnston et al., 1991, Govindan, 1995, Hill et al., 1996). Myo2p is needed for polarized secretion (e.g., Santos and Snyder, 1997; Catlett and Weisman, 1998; Schott et al., 1999). It attaches to secretory vesicles and transports them on actin filaments to the site of secretion (Schott et al., 1999) (see Chapter 11). Cultured melanocytes from the mice mutant *dilute*, alleles at the dilute unconventional myosin heavy chain locus (corresponding to the gene for myosin V), accumulate melanosomes around the nucleus (Provance et al., 1996). Myosin V localizes with melanosome markers

(Provance et al., 1996) and with tubovesicular organelles (Tabb et al., 1996) in extracts of squid axons. In addition, dilute-lethal mice and dilute-opisthonus rats have an abnormal distribution of endoplasmic reticulum in Pukinje cells of the brain (Dekker-Ohno et al., 1996; Takagishi et al., 1996). Melanosomes purified from *Xenopus* melanophores move on microtubules. They have also been shown to move along actin filaments as well (Rogers and Gelfand, 1998). Immunological techniques identify myosin V as the motor associated with the organelles. Isolated ER vesicles (Tabb et al., 1998) were found to be capable of moving on actin filaments adsorbed to coverslips. Immunogold EM techniques (see Chapter 1) using an antibody to squid myosin V showed that this myosin localized to the vesicles. In addition, dual labeling with a squid myosin V antibody and a kinesin heavy chain antibody showed that the two motors colocalized on the same vesicles. A more direct interaction between the myosin based systems and the microtubular system is indicated by the finding that myosin VA can interact directly with a kinesin (KhcU) to form a complex (Huang et al. 1999). This observation suggests that the two motors may move together and switch from microtubules to actin tracks without any complicated transition.

Drosophila 95F myosin of the myosin VI class has been shown to be associated with vesicle movement in cells of Drosophila embryos. The injection of antibody to this protein was found to block particle movement (Mermall et al., 1994).

Unconventional myosins and cell movement

Besides a role in vesicle and organelle transport, the association of myosin I with membranes and actin suggests that myosin I may be able to support pseudopod extension and membrane ruffling. The tail-region actin-binding site allows cross-linking with actin to form gels that condense when phosphorylated by special kinases (Fujisaki et al., 1985). This phenomenon suggests a mechanochemical function that could play a role in phagocytosis and the extension of pseudopods. For example, some myosins I from *Acanthamoeba* (IA, IB and IC) or *Dictyostelium* (IB and IC) have a tail region that binds the acidic phospholipids or membranes and a second actin-binding domain independent of ATP binding (see Pollard et al., 1991). The myosin, therefore, can not only be involved in movement but also interact with membranes and cross-link with the actin structural framework of the cytoplasm. Apart from the well recognized actin-binding domain of the motor, myosin of *Dictyostelium* has three other domains. An approximately 200 amino acid domain is rich in basic residues (*tail homology domain 1*, TH-1) and binds membranes. An adjacent region (*tail homology domain 2*, TH-2 or GPA domain) is rich in glycine, proline and alanine. A third domain (TH3) of approximately 50 amino acids, homologous to src protein, is present either at the tail or within TH-2 and constitutes a second actin-binding domain. src is a membrane associated protein kinase that phosphorylates tyrosine and is coded by an oncogene.

Immunofluorescence microscopy shows that *Acanthamoeba* myosin I is associated with membranes and is likely to play a role in vesicle transport, phagocytosis and cell movement. Phosphorylation of ameboid myosin I greatly increases the activity of this protein. The phosphorylation may correspond to autophosphorylation or a response to the presence of acidic phospholipids such as phosphatidylinositol (which also has a role as a second messenger). *Dictostylium* myosin IA and IE are similar to other myosins I but lack the ATP-independent actin binding site.

Insights to the relative roles of myosin I and II in vivo are provided by experiments in which the expression or the function of one of these myosins is blocked. In prectice, it has been easiest to block myosin II (e.g., in *Dictyostelium*, there is a single gene corresponding to this myosin, whereas there are five for myosin I). *Dictyostelium* cells lacking myosin II can be produced by genetic manipulation (e.g., DeLozanne and Spudich, 1987) or by using antisense RNA (which by hybridizing to the mRNA blocks its translation) (e.g., Knecht and Loomis, 1987). These myosin II-deficient cells are unable to carry out cytokinesis and receptor capping and to maintain cortical tension (as determined by deformability) (Pasternak et al., 1989). Capping of certain receptors is a sign of mobility of the receptor, shown by an accumulation due to cross-linking to multivalent ligands (e.g., concanavalin A). However, cell locomotion, formation of pseudopods, and phagocytosis remain unaffected. A similar strategy was used by injecting an excess of antimyosin II (Sinard and Pollard, 1989) which slows down but does not stop motility. Therefore, locomotion is believed to be driven by myosin I. Other properties, such as cytokinesis and receptor capping, are likely to depend on myosin II.

In yeast, myosin I has been found to associate with WASP-like adapter proteins and components of the Arp2/3 actin nucleation complex (Evangelista et al., 2000; Lechler et al., 2000). These findings suggests that these molecules bridge interactions between actin and myosin and play a role in actin polymerization. The Arp2/3 complex is also discussed in relation to formation of lamellopodia and pseudopodia in Chapter 23.

The binding of myosin I was shown by the two-hybrid system and immunoprecipitation (Evangelista et al., 2000), as well as affinity chromatography and mass spectroscopic analysis (Lechler et al., 2000). The carboxy-terminal tail of the yeast myosin-I (Myo3p and Myo5p) contains an Src homology domain 3 (SH3) followed by an acidic domain. The SH3 domain binds to the yeast homologues of human *Wiskott-Aldrich syndrome protein* (WASP) and the *Wiskott-Aldrich syndrome protein-interacting protein* (WIP). These are adapters linking actin assembly and signaling molecules. In contrast, the acidic domain binds to Arp2/3 complex subunits which are associated with Arp2/3-mediated actin nucleation. In addition, assays with permeabilized yeast cells showed that the WASP-like components and phosphorylation of the myosin [by p21-activated kinases (PAKs)] are required for actin polymerization which also requires the action of the small GPPase, Cdc42p (Lechler et al., 2000).

A role of myosin V in the formation of filapodia is demonstrated in experiments using *chromophore-assisted laser inactivation* (CALI) with myosin V-specific antibodies. These antibody-targeted laser inactivations implicate myosin V in the formation of filopodial extensions (Wang et al., 1996a).

Three distinct monoclonal antibodies to chicken brush border myosin were injected into chicken fibroblasts (Höner et al., 1988). The antibodies were to three distinct epitopes of the tail region of the molecule. The microinjection resulted in the loss of stress fibers, change in shape, and increased locomotory activity. Other changes were consistent with the interpretation that there was an increase in fluidity in the cells.

Sensory role of myosins

Movement appears to be only one of the functions of the myosins. Myosin-Iβ is associated with the sensory hair cells of the frog sacculus (Gillespie et al., 1993). This myosin is thought to have a role in an actin based motor regulating mechanochemically Ca^{2+} channels. These channels present within the hair bundle respond to mechanical stimulation (Assad and Corey, 1992; Gillespie et al, 1993).

Myosin VII is also thought to have a role in sensory transduction. It is found in inner and outer hair cells of the inner ear and in the pigmented epithelium of the retina (Hasson et al., 1995). Mutations can produce deafness and blindness (e.g. Gibson et al., 1995).

Class III myosin *ninaCs* of *Drosophila melanogaster* are involved in phototransduction. The *ninaC* gene codes for two proteins, p132 and p174, by alternative splicing. Each has a protein serine/threonine domain fused to a myosin head domain (Montell and Rubin, 1988). The tail domains of the two proteins differ and are thought to be responsible for the localization of p174 in the microvillar rhabdomeres, whereas p132 are in the sub-rhabdomeral cell bodies (Porter et al., 1992). In the case of p174 the head domain is thought to have a role in its localization. In p174 (Porter and Montell, 1993; Porter et al., 1993, 1995), the kinase domain is required for normal electrophysiology. The head domain has a role in phototransduction and rhabodomere maintenance. The neck region binds calmodulin at two sites. Both domains are required in vivo to terminate phototransduction.

Myosin and GTP-binding proteins

Class IX myosins have been found to have a domain homologous to GTPase activating proteins (see Lamarche and Hall, 1994; Hall, 1994). They are presently thought to be a link between rearrangements of the actin-based cytoskeleton which are modulated by the rho family of GTPases, such as membrane ruffling and stress fiber formation.

Obviously, the interactions between the myosins and actin are very complex and many questions still remain unanswered.

The structural versatility and the dynamics of all cells are in large part the result of dynamic interactions of the components of the cytoskeleton. As we have seen some interactions require actin and actin associated proteins, and these are addressed in this section. Others are based on the system of microtubules and associated proteins discussed in the next section.

Go to Part 3

REFERENCES

Search the textbook

Back to Part 2

C. Microtubular Motors

Like actin filaments, microtubules are ubiquitous in cells and are thought to play a role in both structure and movement. The general role of microtubules in movement was briefly described in Chapter 23. Aside from their role in chromosome movement, microtubules are likely to be associated with Golgi stacks (Allan and Kreis, 1987), endoplasmic reticulum (Franke, 1971), mitochondria (Lindén et al., 1989), and lysosomes (Swanson et al., 1987). Chapter 10 examined intracellular transport and discussed evidence for an involvement of microtubules. Chapter 23 examined the role of microtubules in axonal movement and in cell division, and the dependence of microtubular transport on motors was noted. This section will take a closer look at the currently known motors and their possible modes of action.

Current studies have suggested two major kinds of microtubule-associated motors, the *dynein* and the *kinesin* classes. Conventional kinesin drives the transport of organelles from the minus to the plus end (see Fig. 23). In neurons and undifferentiated cells, this corresponds to the movement from the cell nucleus to the periphery (axon in the case of neurons). Kinesin can also translocate microtubules linearly in a minus direction when attached to a surface. Dynein drives movement in the opposite direction, translocating particles toward the minus end and moving microtubules in the plus direction (Vale et al., 1986). The experimental design where the motor molecules are attached and the microtubules are free to move has been referred to as the *microtubular-gliding assay*. The role of these motors is summarized in Fig. 23 (Vallee and Bloom, 1991). The two motors are also distinguishable by their different sensitivities to inhibitors or ATP-analogs as well as direction and rate of movement along microtubules. Categorizing motors according to directionality of movements can be too simplistic. Some kinesins move particles toward the minus end (see below). However, it is not yet clear whether it is the microtubules in the lysed cell model that are moving relative to one another. This is an important consideration because dynein can effect bidirectional microtubule sliding in the ciliary axoneme.

In the protist *Reticulomyxa* (related to foraminifera) the bidirectional traffic of organelles (Schliwa et al, 1991, Orokos and Travis, 1997) and surface transport of latex beads in the pseudopodia (Orokos et al., 1997; Orokos and Travis, 1997) are powered by the motor protein dynein on MT-tracks (see Chapter 23). These findings introduce an apparent paradox. In these pseudopodia the microtubules are oriented uniformly with the plus ends toward the distal ends of the pseudopodia (Euteneuer et al., 1989) and dynein is involved only with the unidirectional movement of organelles toward the minus end. This paradox can be resolved as indicated above by the observation that microtubules slide along each other (Chen and Schliwa, 1990). The microtubules with their cargo attached would then slide in both directions (see Orokos and Travis, 1997) as they do in reactivated demembranated sperm axonemes (Brokaw, 1991).

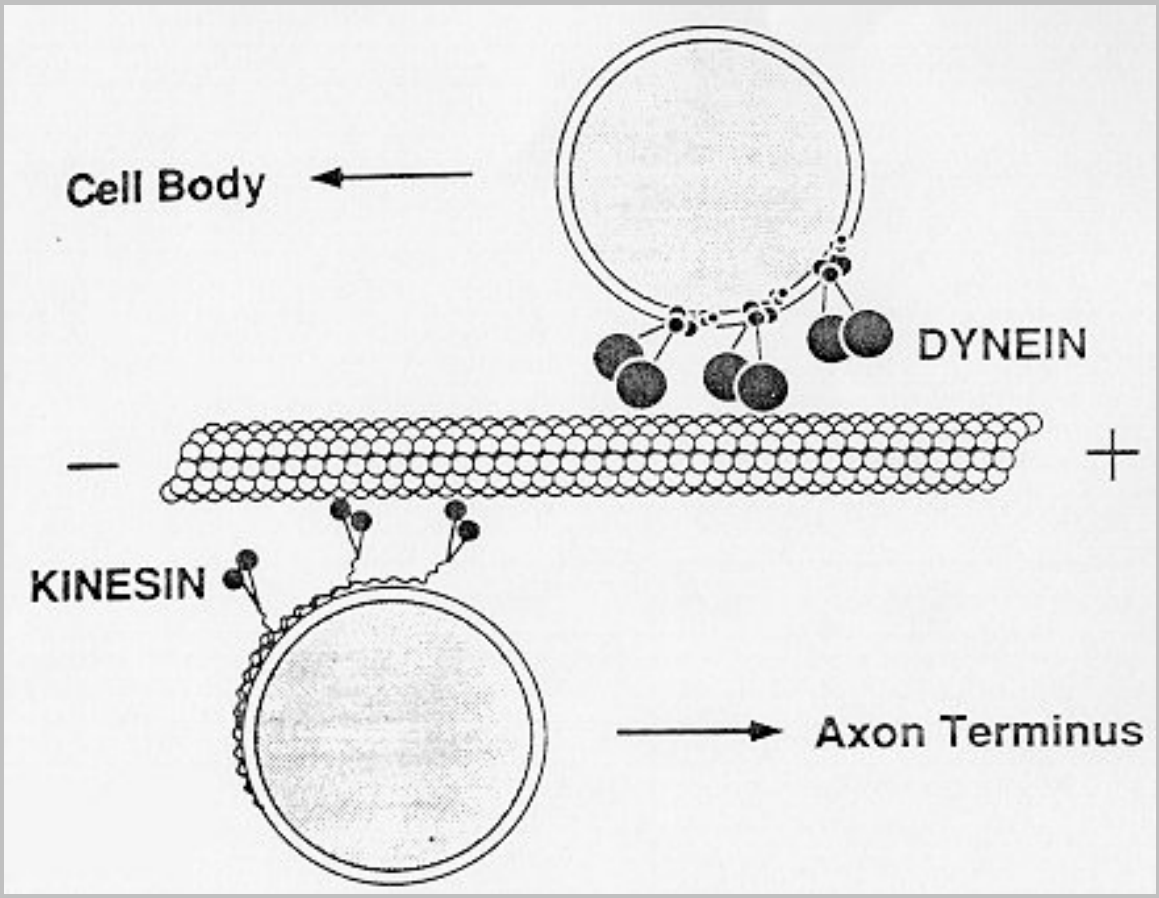


Fig. 23 Axonal transport. Organelles are depicted using either kinesin for anterograde transport or cytoplasmic dynein for retrograde transport. Vallee and Bloom (1991). Reproduced from the *Annual Review of NeuroSciences*, Volume 14, copyright &copy1991 by Annual Reviews Inc.

Apart from directional motion some cytoplasmic dynein and kinesin can produce torque, causing rotation of microtubules coincident to linear translocation. This is the case for the *nonclaret disfunctional protein*, Ncd (Walker et al., 1990) and ciliary dynein from *Tetrahymena* (Vale and Toyoshima 1988).

Cytoplasmic dynein

Dynein represents a family of motors (see King, 2000) related to the AAA (*ATPases Associated with a variety of cellular Activities*) family of of chaperone-like ATPases (e.g., Mocz and Gibbons, 2001). Cytoplasmic dynein is required for (a) the steady state localization of the Golgi cisternae and the endosomal vesicles toward the center of cells (Burkhardt et al., 1997; Harada et al., 1998), (b) the movement of cargo from ER to Golgi (Presley et al., 1997) and (c) microtubular organization at interphase (Quintyne et al., 1999) and mitosis (see Compton, 1998).

The structure and mechanism of movement of dynein has lagged behind that of other motors probably because of its complexity (see Vallee and Sheetz, 1996). Axonemal and cytoplasmic dynein are very similar. Cytoplasmic dynein was isolated from microtubules and found to bind microtubules in an ATP-dependent manner (Paschal et al., 1987). It was originally named microtubular associated protein 1C

(MAP1C), because its homology to the dynein of cilia and flagella was not immediately recognized. The protein had ATPase activity, markedly stimulated by binding to microtubules. When tested with the microtubule-gliding assay (Paschal and Valee, 1987), it was found to be a motor. When tested on isolated axonemes of *Chlamydomonas reinhardi*, a biflagellate single celled alga, the axonemes were found to move toward the plus end (so that the force exerted by the motor was toward the minus end). As indicated earlier, this polarity is opposite that of kinesin. Dynein has been found in a variety of cells and tissues.

MAP1C was identified as cytoplasmic dynein (DHC1) from its biochemical and physical properties. Perhaps most significantly, scanning transmission electron microscopy (STEM) (Vallee et al., 1988) revealed the same morphology and molecular mass as the two-headed forms of ciliary and flagellar dynein. Dyneins typically have two heads and in some cases three for a total molecular weight of 1.2 to 2 x 10^3 kDa. The native protein has two 410 kDa heavy chains, three 74 kDa subunits, and one subunit each of low molecular weight subunits ranging from 53 to 59 kDa (Vallee et al., 1988). The head domain of dynein (350 to 400 kDa) is much larger than that of kinesin (40 kDa) or myosin (95 kDa). Each head contains four putative ATP-binding domains (P-loop motifs) separated by approximately 300 amino acids (Holzbauer and Vallee, 1994). The heavy chains are responsible for ATPase activity and force production. A very large domain at the carboxy terminal is needed for ATP binding and the microtubule-binding domain is far removed from the ATP-binding domain (Gee et al., 1997). These properties suggest that dynein might function very differently from other motors. The structure of isolated dynein also argues for a distinct mechanism. Electron microscopy has shown that the heavy chain fold in such a way that it forms globular head with two elongated processes, the stalk and the stem (Goodenough et al., 1984, 1987; Goodenough and Heuser, 1989). Supposedly, the stalk binds the microtubules whereas the stem binds to the cargo as well as the intermediate and light chains. In the head region six AAA modules form a ring, 125 Å in diameter. Negatively stained dynein from *Dictyostelium* has been studied using transmission EM and image reconstruction techniques (Samsó et al., 1998; Burgess et al., 2003). Fig. 24A shows an EM view of purified cytoplasmic dynein and 24B a model showing attachment to the microtubule and a membrane bound vesicle (Gee et al., 1997) and possible power strokes.

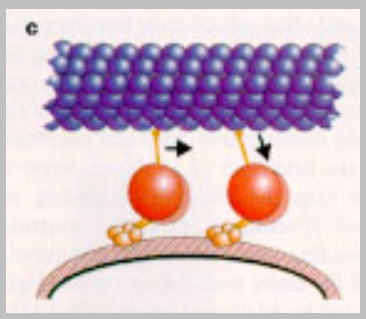


Fig. 24 Structure of cytoplasmic dynein. A. Cytoplasmic dynein seen with the EM after freeze-drying and

platinum replication. B. Models of cytoplasmic dynein showing attachment to microtubule through the stalk and to a vesicle. The arrows indicate possible direction of power stroke. Reproduced with permission from *Nature* , from Gee et al. 1997, Copyright &copy1997 MacMillan Magazines Ltd.

In addition to the structural similarity between axonemal and cytoplasmic dynein, microtubular gliding in vitro is supported by either kind of dynein. However, the rates of movement are different. For cytoplasmic dynein they are 1-2 μm/s (Paschal et al., 1987, Lye et al. 1987). The movement is much faster for outer arm dyneins (5-10 μm/s; Vale and Toyoshima, 1988) or inner arm dyneins (2-12 μm/s; Kagami and Kamiya, 1992). In contrast to kinesin, single headed dynein does not follow a protofilament but moves throughout a microtubular lattice (Wang et al., 1995) (see below).

The conformational changes that accompany cross-bridge cycles that must accompany movement have been studied in axonemes (Goodenough and Heuser, 1984; Burgess, 1995). In the study of Burgess (1995), after removal of the plasma membrane, cockerel sperm flagella were observed with freeze etching in three different nucleotide states: no added ATP (rigor); relaxed (1 mM ATP plus vanadate); and active (i.e. 1 mM ATP). Each state produced a statistically significant morphology of outer dynein arms. The relaxed and active morphologies differed only in the angulation of their heads. The dynein in the relaxed state were in a more tilted position. The rigor morphology showed a conformational change of 12 nm shift in the position of the dynein head in relation to the base, suggesting an ability to develop tension. Active flagella showed all three morphologies. In this case, no unattached stalks were observed suggesting a very prolonged duty cycle.

In a study of the dynein from the flagella of of the alga *Chlamydomonas reinhardtii* using the EM and an image averaging technique (Burgess et al., 2003), the stalk was found to be bent. The conformation in the presence of ADP and vanadate, supposedly being equivalent to ADP and phosphate right after the hydrolysis of ATP, was compared to that without ATP or ADP, supposedly the condition after the power stroke. The conformation changes so that in the latter state the angle between the stalk and stem shifts, a movement that would be reflected in a 15 nm movement at the tip of the stalk. In addition, the stalk becomes straighter. It is unclear how these changes are related to movement. The mechanism is thought to involve the AAA ring head structure changing its orientation in relation to the stem and of moving the stalk toward the plus end of the microtubules.

To produce force two possible mechanisms have been proposed (see Gee et al., 1997; Vallee and Gee, 1998). The stalk could act as a rigid lever arm amplifying the conformational changes in the rest of the dynein head. Alternatively the stalk could act as a passive tether where conformational changes in the head region could pull at the base of the stalk dragging the microtubule along.

Two dynein heavy chains that differ from the heavy chains of DHC1 have been identified. Both are present in mammalian cells that lack cilia or flagella. DHC3 is localized in a unidentified structures that may be transport intermediates. Microinjection of antibodies to DHC2 disrupts the Golgi apparatus, suggesting that this protein has a role in maintaining the organization of the Golgi (Vaisberg et al., 1996).

Dyneins bind to different partners where the heavy chain combines with various accessory subunits. These in turn bind to *dynactins* forming large complexes. These complexes distinguish different cargos and have a broad spectrum of functions. These include the endocytotic pathway, retrograde membrane transport and other functions during cell division (see Hirokawa et al., 1998). The dynein/dynactin complex is also involved in anchoring microtubules to centrosomes (Quintyne et al., 1999) (see Chapter 23).

Dynactin is part of the cytoplasmic dynein motor and is needed for dynein-based movement (e.g., see Holleran et al., 1998). Dynactin is believed to function as an adapter, mediating dynein attachment to cargo. It contains two domains. A side arm ($p150^{glued}$) interacts with dynein and binds to MTs. An actin like filament is thought to bind to cargo. Several subunits of dynactin have been identified and characterized (Eckley et al., 1999). Dynactin, acting as a cytoplasmic dynein activator, has been shown to bind microtubules and increases the average length of dynein movements while the velocity or ATPase kinetics remains the same (King and Schroer, 2000). Both the increase in microtubule binding and motility by dynactin are blocked by an antibody to dynactin's microtubule-binding domain.

The complexes are regulated by phosphorylation of the dynein light intermediate chain which reduces the level of membrane associated dynactin decreasing the rate of retrograde transport (Niclas et al., 1996). In addition, isoforms of $p150^{glued}$ bind to dynein without binding to microtubules, presumably down-regulating the motor by competing with the functioning dynein (Tokito et al., 1996).

A study using a chimera of the green fluorescent protein (GFP) and the $p150^{Glued}$ subunit of dynactin showed that dynactin interacts with the growing microtubule plus end (Vaughan et al., 2002). The binding is regulated by the phosphorylation of $p150^{Glued}$. Effectors of protein kinase A affected the binding suggesting a role for $p150^{Glued}$ phosphorylation in plus-end binding specificity. During the dynein-dependent transport of Golgi membranes the Golgi membranes temporarily interacted with GFP-p150(Glued)-labeled microtubules before transport toward the minus end was seen.

Kinesins

Movement of particles on single microtubules can be followed using DIC and enhanced contrast video technology (Chapter 23). Not surprisingly, giant axons were used in the early studies. The microtubules of extruded axoplasm from giant squid axons supported the fast movement of particles on microtubules in either direction and, the transport was found to require ATP (Brady et al., 1982, 1985). Kinesin was revealed when the ATP analog 5'adenylimidodiphosphate (AMP-PNP), that cannot be hydrolyzed, was used to block fast transport of vesicles in the axoplasm (Brady et al., 1985). The effect of the analog differed from that observed with either dynein or myosin. AMP-PNP weakens the association between motor and fibrous elements in the myosin-actin or dynein-microtubule systems. In contrast, it favors binding of kinesin (and therefore vesicles) to microtubules. This led to the realization that a new motor was involved and soon after to its isolation by extracting microtubules isolated in the presence of AMP-PNP. This protein, which was found later to be an ATPase, could be tested for motor activity readily, for example, by the microtubular gliding assay. In a different kind of assay, synthetic microspheres can be

coated with the protein and tested on oriented microtubules. Because of its involvement in movement this protein was called kinesin (e.g., Vale et al., 1985a). When tested on isolated microtubules assembled on a centrosome (see Chapter 23) (with the plus end out) using the microbead assay, the beads were found to move away from the centrosome (i.e., in the anterograde axonal direction, toward the plus end, as represented in Fig. 23) (Vale et al., 1985b). The involvement of kinesin is in vesicular transport is shown also by the block of the movement along microtubules provided by kinesin antibodies (Ingold et al., 1988; Brady et al., 1990).

A similar assay system that permits dissecting the various molecular components needed for transport has been developed. In essence, components of extracts to be tested are mixed with organelles and assembled microtubules (Pollock et al., 1999) and the movement is observed with video enhanced differential interference microscopy (see Chapter 1). This system has recognized two new kinesin motors involved in organelle movement in *Dictyostelium* (Pollock et al., 1999).

Green fluorescent protein (GFP) has been used to observe movement of motors such as kinesin-GFP constructs (see Chapter 1) moving along a microtubule (e.g., Romberg et al., 1998). This technique has allowed examining the role of components of the neck region in the directionality of the movement (see below).

The kinesin family of motors has over 140 proteins in organisms that include plants, protists, fungi and animals (see Hirokawa et al., 1998); www.blocks.fhcrc.org/~kinesin/). The 380 kDa protein is tetrameric, consisting of two copies of a 110-130 kDa (the *kinesin heavy chains*, KHCs) and two copies of a 60-65 kDa complex (the *kinesin light chains*, KLC) (Kuznetsov et al., 1988; Bloom et al., 1988). The heavy chains contain the motor domain. However, the kinesin light chains are essential for the functioning of the heavy chain motor domain (see below).

In conventional kinesin, the two KHCs subunits, (referred to as KIF5A and KIF5B or KIF5C) are parallel and form a coil-coil domain, so that the two amino terminals and the carboxy-terminal are adjacent (see Vale and Fletterick, 1997). In contrast, the two KLCs have an α-helical coiled-coil domain which associates with the carboxy-terminal of the KHC (e.g., Verhey et al., 1998; Diefenbach et al., 1998). A domain of about 590 amino acids at the amino terminal differs between different species and the sequence of the extreme carboxy-terminal differs between species and between splice variants (Cyr et al., 1991). In mammals, the polypeptide components of the KHC are encoded by three genes, kif5A, kif5B and kif5c (e.g., Xia et al., 1998) and the KLC genes by klc1, klc2 and klc3 (e.g., Rahman et al., 1999).

The structure of the motor domain of of conventional kinesin (Kull et al., 1996; Kozielski et al., 1997; Sack et al., 1997) and Ncd (Sablin et al., 1996; Sablin et al., 1998; Kozielski et al., 1999) has been determined by X-ray crystallography. Like other motors, conventional kinesin was found to be rod shaped with a pair of globular heads at one end (e.g., Hirokawa et al., 1989). cDNA corresponding to *Drosophila* kinesin has been isolated. Like myosin II or dynein from cilia and flagella, the globular portions of kinesin were found at the amino-terminal (however, note that the location of the globular motor domain differs in

different kinesins, see below). Kinesin binds to microtubules and hydrolyses ATP at a head site (see Goldstein, 1993). Like myosin, the kinesin molecule was found to have three distinct domains (e.g., Yang et al., 1989). The head domain alone was found to have the motor activity (Yang et al., 1990). A model of a kinesin molecule is shown in Fig. 25.

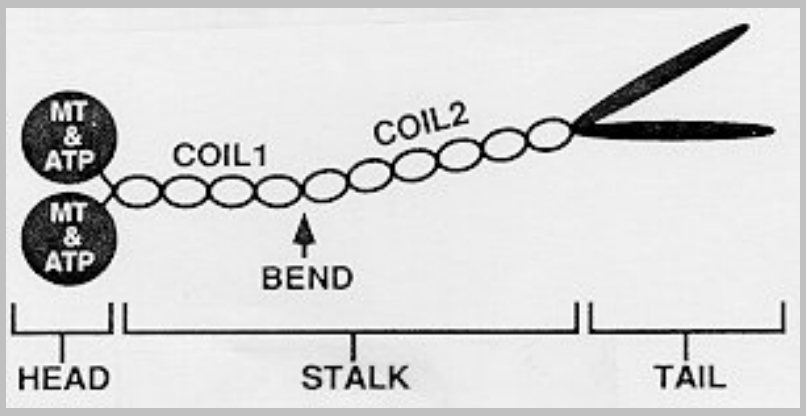


Fig. 25 Model of a kinesin molecule. The head domain binds MTs and ATP and generates the motor activity. Attached to the head in an α-helical coiled-coil stalk and a tail composed of heavy and light chains (solid elipses). From Goldstein, 1993. With permission from the *Annu. Rev. Gen.*, Vol. 27, copyright &copy1993, by Annual Reviews Inc.

Proteins of the kinesin family have been identified by determining the amino acid sequence through the techniques involving the isolation of cDNA and PCR amplification (Endow, 1991) (Chapter 1). Once the sequence was known, database searches for amino acid homologies were then carried out and many proteins belonging to this family were identified. The head portions are very similar for all kinesins. In contrast, the sequences of the tail portion have little in common. Hybridization of PCR-amplified cDNA produced from degenerate primers to the *Drosophila* polytene chromosomes (where the genome is amplified) suggests the existence of as many as 25 members of the kinesin family (Endow and Hatsumi, 1991). Phosphorylation is the only well documented post-transcriptional modification both in the heavy and light chains (Hollenbeck, 1993). Various members of the kinesin family are represented in Fig. 26.

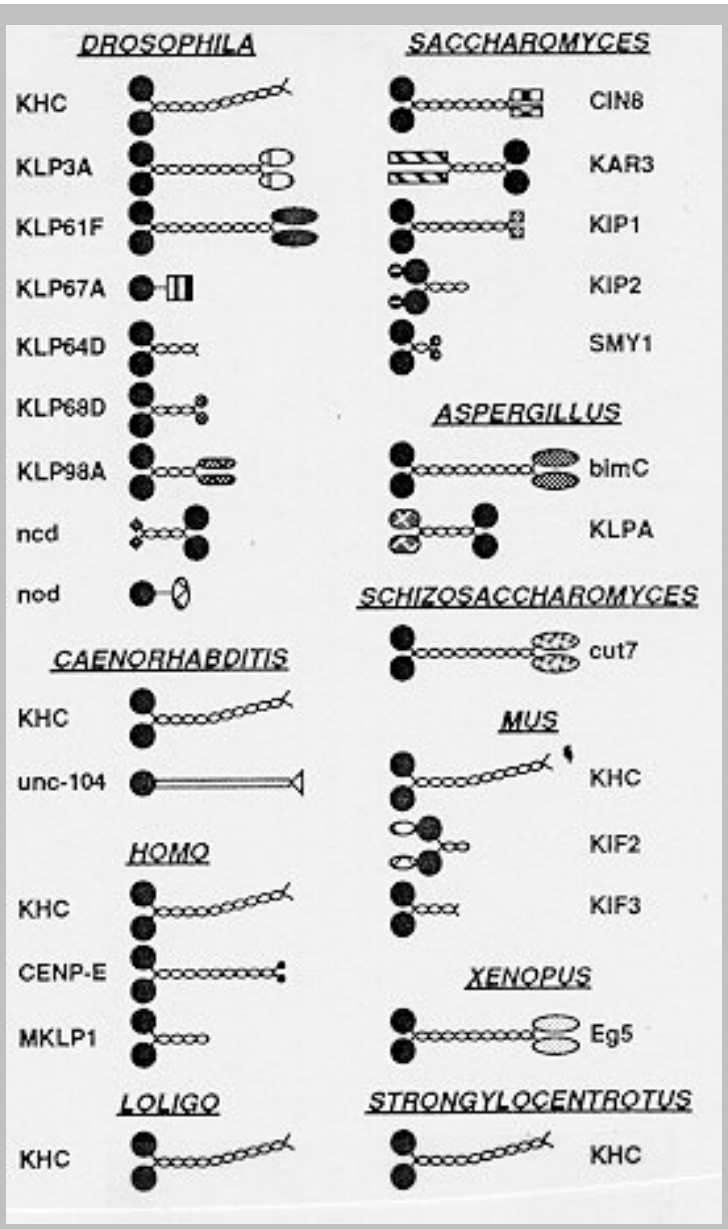


Fig. 26 Various members of the kinesin superfamily represented diagrammatically. The motor region (represented by the solid circle) is similar for all of the molecules. Proteins that are predicted to be α-helical coiled-coils in the tail domain are represented as dimers. The α-helical coiled-coil is represented as a chain of ovals. Other proteins are arbitrarily represented as monomers. From Goldstein, 1993. With permission from the *Annu. Rev. Gen.*, Vol. 27, copyright &copy1993, by Annual Reviews Inc.

Based on the position of their motor domain containing the ATP and the microtubules binding domains, the kinesin family proteins (KIF) are also referred to as amino-terminal type, middle-type and the carboxy-terminal-type. In all plus-directed kinesins the motor domain is attached to the amino-terminal (conventional kinesins) and all minus-directed kinesins have the motor attached to the carboxy-terminal (e.g., Ncd).

The kinesin KIF1A is an amino-terminal type of kinesin present in neurons. It moves microtubules at 1.5 μm/s . Mutants of a kinesin of the nematode *Caenorhabditis elegans*, similar to KIF1A retain vesicles in the cell bodies, and have few synapses suggesting the major role in the transport of synaptic vesicles

(Otsuka et al., 1991). KIF1B is a smaller amino-terminal type kinesin similar to KIF1A which occurs in many tissues and is involved in the transport of mitochondria (Nangaku et al., 1994). Alternative splicing of the Kif1b gene (which codes for KIF1B) produces eight kinesin isoforms (Gong et al., 1999). One of these isoforms, KIF1Bβ, has a different cargo binding domain (Zhao et al., 2001) and has been shown to bind to synaptic vesicle proteins and to be essential for proper development of neurons.

KIF3, also an amino-terminal kinesin, occurs as a heterodimer and associates with an associated protein (KAP3). KAP3 is approximately 11 nm in diameter and it is possibly involved in membrane binding of the KIF3 heterodimer (Yamazaki et al., 1996). Another member of the KIF3 family is associated with melanosomes in *Xenopus laevis* (Rogers et al., 1997).

The middle-type KIFs have a motor domain in the middle of the molecule. KIF2 forms homodimers that move at approximately 0.4 μm/s. KIF2 occurs in several cell types but primarily in neurons where its expression is controlled developmentally and appears to be present mostly in juvenile neurons (Noda et al., 1995) where it carries a receptor for the β *subunit of the insulin-like growth factor 1* receptor (Morfini et al., 1997).

Carboxy-terminal-type KIFs have varied functions including the transport of organelles (Saito et al., 1996; Harlon et al., 1997) as well as functioning in meiosis, mitosis and nuclear division. KIFC2 seems to function in transporting a multivesicular organelle functioning between early and late endosomes (Saito et al., 1996). At least some of these kinesins propel cargos toward the minus-end rather than the plus-end, notably Ncd, a carboxy-terminal type kinesin from *Drosophila* (see below).

The profusion of kinesin isoforms suggests that they might be targeted to different structures. In agreement with this notion, the carboxy-terminal of the light chain domains are similar to targeting domains (Cyr et al., 1991) (e.g., a mitochondrial import sequence). Furthermore, in axons, vesicles are transported at different rates. Vesicles propelled by KHC-A moved much faster than those using KHC-B (Elluru et al., 1995). In addition to this complex picture of kinesins, there are many genes related to that of kinesin which code for the kinesin-related proteins (KRPs) (see Goldstein, 1993). The attachment of kinesin to vesicles may require a receptor attached to the membranes (see Section VI). The polypeptide *kinectin* has been reported to bind to both kinesin and cytoplasmic dynein (Toyoshima et al., 1992) and might serve as a receptor attached to membranes.

What happens in the absence of cargo? In this case, the movement of the motors must be inhibited or the unneeded motion would be bioenergetically very expensive. It has been estimated to correspond to 100 ATP molecules/s per molecule of kinesin (Coy et al., 1999) (extrapolated for an individual to be comparable to the human basic metabolic rate!). In the case of kinesin the inhibition results from the kinesin tail binding to the motor domain (Coy et al., 1999; Friedman and Vale, 1999). The tail does not interfere with the attachment of the kinesin to the microtubule. The experiments of Coy et al.(1999) were carried out with KHC constructs derived from the DNA coding for heavy chain of the *Drosophila* kinesin.

The study of Friedman and Vale (1999) used constructs of the human KHC. A sequence in the globular tail domain which has been termed the IAK sequence is responsible for blocking the ATPase activity of the motor domain inactivating kinesin (see Hackney and Stock, 2000). Although the mechanism of inactivation of kinesin is clear, the mechanism or mechanisms responsible for activation of inactive kinesin is less well defined (see Verhey and Rapoport, 2001) and may be multiple. Activation may correspond simply to the binding of cargo to the tail of kinesin. Head-to-tail interactions have also been found to regulate several myosins (see Reilein et al., 2001).

A variety of arguments suggest that the KLC component of kinesin imparts specificity of binding to different cargoes (see Manning and Snyder, 2000). This is attested by the multiplicity of the KLC isoforms and in some cases the demonstration of specific binding to certain organelles or structural components. For example, five different KLC isoforms are present in a mammalian culture line, one of which binds only to mitochondria (Khodjakov et al., 1998). In addition, kinesin complexed to vimentin (see below) contains a specific KLC isoform (Liao and Gundersen, 1998). As discussed below, the binding of kinesin to cargo is mediated by scaffolding proteins.

Another possible role of the KLC is the regulation of the kinesin motor or the binding to microtubules. KLC has been proposed to inhibit the motor activity. The kinesin conformation change during movement brings the KHC tail domain in close contact with KLC (e.g., Hackney et al., 1992). Furthermore, removing KLC from the KHC increases the ATPase activity of the motor (Hackney et al., 1991). When KHC and KLC are cotransfected into COS cells (transformed kidney cells from the African green monkey), the binding to microtubules is inhibited (Verhey et al., 1998). These observations suggest that KLCs are involved in cargo recognition and the microtubule binding capacity. As discussed above, without a cargo, kinesin is probably in the folded conformation so that the KLC domain may bind to the motor domain blocking the ATPase.

Although the role of KLCs is uncertain, they are essential for function. In *Drosophila* disruption of a single *klc* gene is lethal (Gindhart et al., 1998) because of a failure axonal transport. In mice, knockout mutation of the *klc1* gene causes a defect in the activation and targeting of KIF5A and KIF5B (Rahman et al., 1999).

Motors and mitosis

Some of the details of mitosis were discussed in Chapter 23. The role of various motors will be discussed in this section. Since the motors act in concert with microtubules (MTs), these will have to be discussed as well. As we saw in Chapter 23, the organization of MTs is dynamic and plays a role in many functions of the mitotic apparatus. The spindle MTs are oriented so that their minus ends are toward the centrosomes and their plus ends toward the equator (see Chapter 23, Fig. 26). The astral MTs have a similar orientation, however, since they point in the opposite direction, their plus ends are toward the cytoplasmic cortex rather than the spindle. As we saw, some of the interpolar MTs overlap. Since they originate from different poles they are antiparallel. As discussed below, these MTs in conjunction with the appropriate motor(s) move the spindle poles away from each other. The MTs attached to the kinetochores (K-MT in the figure) and their motors move the condensed chromosomes toward the equator (prophase and prometaphase) or

the poles (anaphase). On the other hand, the astral MTs, attached to the cell's cortex and the spindle poles also function in pulling the poles apart and maintain the orientation of the spindle in relation to the cortex. In addition, a set of MTs connects the centrosomes to the arms of chromosomes and should also function in chromosomes movement.

At least eight distinct motors have been found to be involved in mitosis (see Sharp et al., 2000). It is generally agreed that movements related to the mitotic spindle involve the coordinated interaction of several motors that can operate antagonistically or in a complementary manner. These motors are thought to function in three different ways. When they form cross-bridges between MTs, they slide an MT in relation to adjacent MTs. Some may bridge MTs to other structure structures and also provide movement. MTs transport cargos along the surface of the spindle. In addition, they regulate the assembly and disassembly of the MTs.

Some members of the kinesin family form bipolar kinesin-like homotetramers with two motor domains at opposite ends of the kinesin molecule (Kashina et al., 1996; Gordon and Roof, 1996). The motors can attach to adjacent MTs and are likely to slide antiparallel spindle MTs in opposite directions. They are thought to have a role in the elongation of the spindle. For example, interference with these bipolar kinesins produce spindles with abnormally close poles (see Sharp et al., 1999). As seen with the EM, the *Saccharomyces cerevisiae* bipolar motor (Kip1p) has two globular domains 14 nm in diameter connected by a 73 nm long stalk (Gordon and Roof, 1999). Monopolar motor molecules can also produce a sliding. In this case, a domain at one end of the molecule attaches to an MT and the motor end is attached to an adjacent MT. Other motors (dynein/dynactin) act on MTs attached to the cell's cortex and are thought maintain the centrosomes in their appropriate position and play a role in spindle elongation.

The positioning of the chromosomes could be driven to the metaphase plate by plus-directed motors. In contrast, the minus-directed motors would drive the chromosomes toward the poles. The kinetochores are involved in these movements. The kinetochores can move bidirectionally on the surface of MTs (e.g., Hyman and Mitchison, 1991). In fact, a plus-end (CENP-E) (Yen et al., 1992) and a minus-cytoplasmic dynein (Steuer et al. 1990; Pfarr et al., 1990) have been shown to be located in the kinetochore. For example, immunological microscopic techniques (see Chapter 1) showed the presence of cytoplasmic dynein near or at kinetochores, centrosomes and spindle fibers during mitosis but found it distributed throughout the cytoplasm in interphase (Pfarr et al., 1990). CENP-E was found to be in the kinetochores (Yen et al., 1992) during the formation of the metaphase plate, in the spindle midzone at anaphase. Eventually, it is degraded at the end of mitosis. In agreement with a role of these motors in chromosome movement, inhibition of CENP-E produces defects in chromosome alignment (Wood et al., 1997) and mutations of genes coding for components of the dynein complex produce defective chromosome segregation (Bowman et al., 1999; Lee et al., 1999).

Several plus-end motors contain chromatin-binding motifs and are likely to bind to the chromosome arms rather than kinetochores. They are thought to be involved in the transport of the chromosomes in the formation of the metaphase plate (e.g., Molina et al., 1997).

There are indications that some motors regulate the assembly and disassembly of MTs. Chromosomes can move while attached to MTs that are shortening by depolymerization. In lysed *Tetrahymena* cells, antibodies to the dynein CENP-E were shown to block the movement of chromosome fragments toward the MTs minus-ends during MTs depolymerization (Lombillo et al., 1995). These observations suggest that CENP-E helps couple chromosomes to depolymerizing MTs. In a similar coupling activity, the MTs of the spindle may remain attached to kinetochores while their lengths change during both prometaphase and anaphase A.

D. Intermediate Filaments

Intermediate filaments (IFs) are cytoskeletal fibers generally 8-10 nm in diameter. They are intermediate in thickness between the thinner F-actin filaments (the microfilaments) and the thicker microtubules. In most cells the IFs form a basketlike array around the nucleus but also reach the periphery of the cell. They are present at specialized junctions, and they are prominent throughout the length of axons. However, they are not evenly distributed. *Neurofilaments* (IFs of neurons, or NFs) are six times more numerous at distal levels of the mouse optic axon (Nixon et al., 1994) or in the squid giant axon (Martin, 1996).

50 different IF genes are differentially expressed in various cells depending on tissue and developmental stage. In many cells IFs constitute only 1% of the total protein. However, in certain cells such as epidermal keratinocytes and neurons, IFs account for as much as 85% of the total protein of mature cells. Genetically characterized keratin disorders involve fourteen of the known keratins (see Fuchs and Cleveland, 1998).

The complexity and variety of IF proteins and tissue-specific expression suggest that they have an important role. They are most likely to play a role in specialized cell functions and in maintaining cell structure. However, a general housekeeping role seems unlikely because at least some cultured cells function well and even divide in the absence of IFs (although in these cases, lamins, the IFs of the nucleus are present) (see Steinert and Roop, 1988).

Strictly speaking, IFs are not part of the machinery responsible for movement. However, a discussion of IF is necessary in any consideration of movement. We have seen how difficult it is to separate a discussion of movement from that of structure (e.g., for the case of microvilli whose scaffolding is in part composed of actin and myosin). The neurofilaments are thought to function in the maintenance of the caliber of large myelinated axons, a factor with obvious implications in intra-axonal transport and in the structural and dynamic environment needed for other functions. It has been suggested that IFs are also implicated in signal transduction (Baribault et al., 1989; Skalli et al., 1992).

As is the case for MT and actin, IF filaments are also in a dynamic state. IF incorporate microinjected unpolymerized subunits. Fluorescence recovery studies (FRAP) (see Chapter 4) demonstrate that the polymerized IF and unpolymerized subunits are at a steady state (Vikstrom et al., 1992 and Okabe et al., 1992). Hyperphosphorylation of IF is frequently accompanied by disassembly (see Skalli et al., 1992).

As discussed below, the organization of the IF network is thought to be the result of interactions between

IF, IF-associated proteins (IFAPs), microtubules and actin filaments. IF networks have been studied in living cells transfected with DNA coded for a green fluorescent protein-vimentin construct (e.g., Yoon et al., 1998; Prahlad et al., 1998). Vimentin is one of the subunits of IF (see below). GFP-vimentin is incorporated into the IF network and accurately reports the movement of IFs. Interphase arrays of vimentin containing fibrils were found to constantly change in configuration even when the cells do not change in shape. Fluorescence recovery after photobleaching (FRAP) shows a rapid recovery of bleached spots indicating that the IFs are in continuous motion. Areas that are still bleached frequently move, indicating whole fibrils are moving. In view of the association between IF and microtubules and actin filaments the movements are thought to reflect to motion mediated by these components. The movement of small fragments containing vimentin were found to be inhibited by kinesin antibodies, indicating that this is the motor responsible for the movement of these short fibers (Prahlad et al., 1998).

The role of IFs has to be evaluated from contradictory data, probably because defects in IFs may be rather subtle and difficult to observe. Microinjection into cultured fibroblasts of peptides of the helix region of the IFs disrupts the IF, MT and microfilament network (Goldman et al., 1996). These so called *mimetic peptides* disassemble IF assemblies into small oligomeric complexes and monomers within 30 min at room temperature in vitro. These observations argue for a very important role of IFs in cell organization. In agreement with this interpretation disruption of proteins that cross-link IFs to other filamentous elements (including IFs) does have serious consequences (see below). However, earlier work in which IF organization was disrupted by anti-IF antibodies had no effect on other components (Quinlan et al., 1994). In addition, cell lines lacking IFs were found not to have a major disarray in actin filaments or MTs (Sarria et al., 1994; Colucci-Guyton et al., 1994).

Neurofilaments form bundles by combining with other neurofilaments by cross bridges in the axon as they do in vitro (Hirokawa, 1991) as also shown by the persistence of the spacing of 50-55 nm between neurofilaments corresponding to the side arm of the heavy chain of the filaments (NF-H) (Xu et al., 1996b). NFs are heteropolymers of NF-L, NF-M, and NF-H. Their carboxy-terminal tail form sidearms along the length of the fibers (Mulligan et al., 1991).

All indications are that the elements of the cytoskeleton form a cross-linked network. Generally, ultrastructural studies support this concept. They show that IFs are cross-linked to microtubules and F-actin (e.g., Hirokawa 1982; Yang et al., 1999). Furthermore, when microtubules depolymerize, the IF network collapses, a process which depends on actin (see Gard and Klymkowsky , 1998). In addition, disruption of microtubules and microfilaments produces a reorganization of keratin-type IFs (Gard and Klymkowsky , 1998).

The three-dimensional structuring of the cytoplasm requires the interaction of the various components of the cytoskeleton (see Chou et al., 1997). A variety of *intermediate-filament-associated proteins* (IFAP) cross-link the IFs to other IFs and to F-actin and MTs and cell membranes (see below).

IFs are unique among the cytoskeletal structure in that their protein subunits are fibrous (for a review on

IF, see Steinert and Roop, 1988). As previously discussed, F-actin and microtubules are formed from the polymerization of globular subunits. The IF proteins form long filaments of high-tensile strength. Five types of IF are recognized, spanning a size range between 40 to 130 kDa, and they have been classified from their amino acid sequences and assembly properties (see Parry, 1999, Herrmann and Aebi, 2000).

The central portions of all IF proteins (the so-called rod portions) of approximately 310 amino acids are highly homologous and in an α-helical configuration (see Fig. 27 for a diagrammatic representation; Steinert and Roop, 1988). Antiparallel association of the central region of two fibrous subunits produces a double-stranded coil (i.e., a dimer), a 3 nm *protofilament* (Fig. 28b). In the case of keratins, the two chains constituting the dimers are different (one of type I and the other of type II). Two of the dimers align in parallel to form a tetramer with two globular domains at each end, the 4.5 nm *protofibril* (Fig. 28c). Associations of these protofibrils produce the ~10 nm IF fiber. These further arranged in a staggered manner (Fig. 28d) produce a striated appearance when viewed with the EM.

Type I and II IFs (so-called *assembly group I*, see Herrmann and Aebi, 2000), the keratins (acid, basic, or neutral), are heteropolymers formed from an equal number of subunits from each subgroup. They are found predominantly in epithelial cells (besides hair and nails). There are at least 19 different types of keratins in human epithelia. The keratin network links the plasma membrane, the nucleus and other cytokeletal components. Keratin-null mutants in humans and mice show that it is an essential component neeed for the integrity of epithelial tissues (see Albers, 1996).

Type III (assembly group 2) proteins include *vimentin* (mesenchyme), *desmin* (striated and smooth muscle), and *glial fibrillar acidic proteins* (astrocytes and Schwann cells). These IF proteins assemble spontaneously in vitro to form homopolymers and heteropolymers. Desmin-null animals have severe muscle deficiencies such as misaligned muscle fibers (Li et al., 1996; Milner et al., 1996). Desmin is thought to be associated with the Z discs of muscles (see Sections I C and I D).

Type IV proteins are neurofilament proteins of neurons. In vertebrates the type IV IF proteins represent three different polypeptides. Type V are nuclear IF proteins, the *lamins*, that form a highly organized network, the *nuclear laminae*.

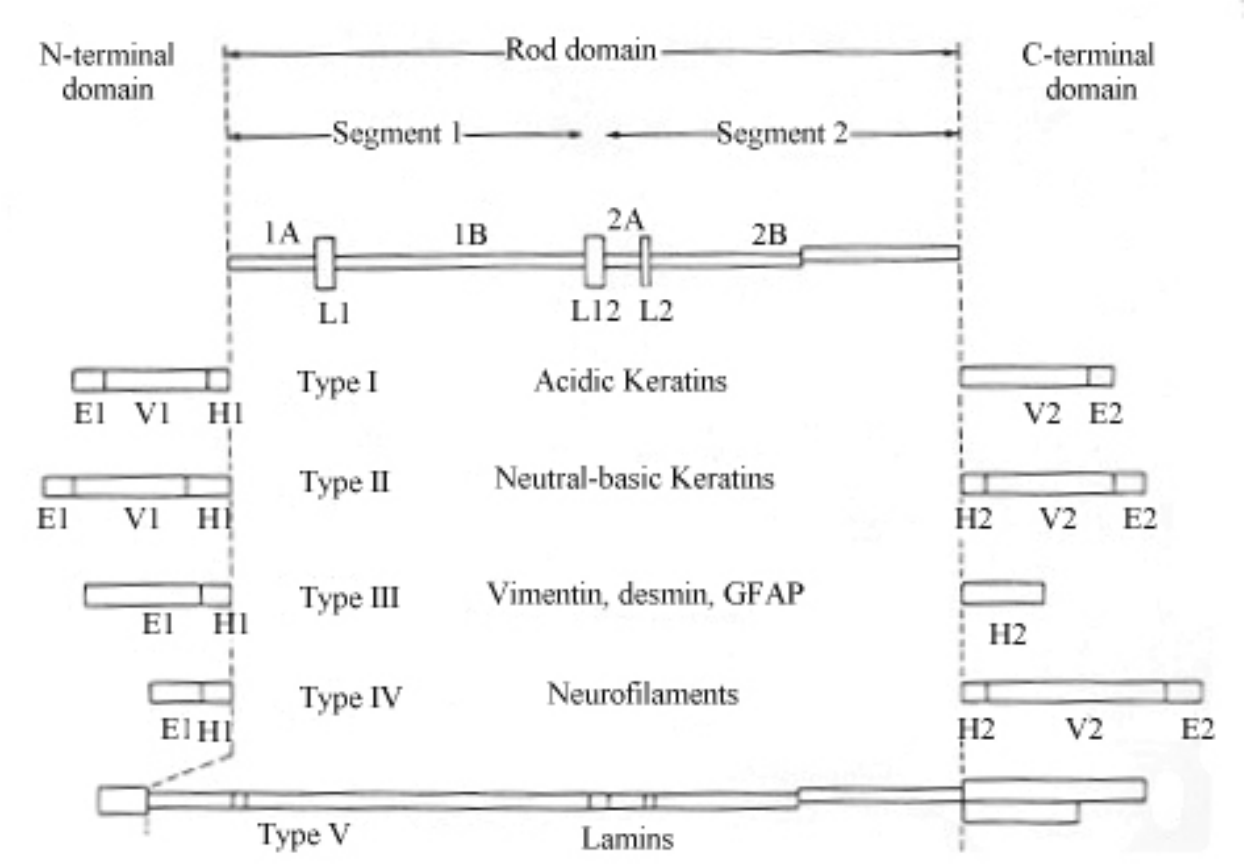


Fig. 27 Subdomains of IF chains. All central rod domains are flanked by end domains. For details see Steinert and Roop (1988). Reproduced from the *Annual Review of Biochemistry*, Volume 57, copyright &copy1988 by Annual Reviews Inc.

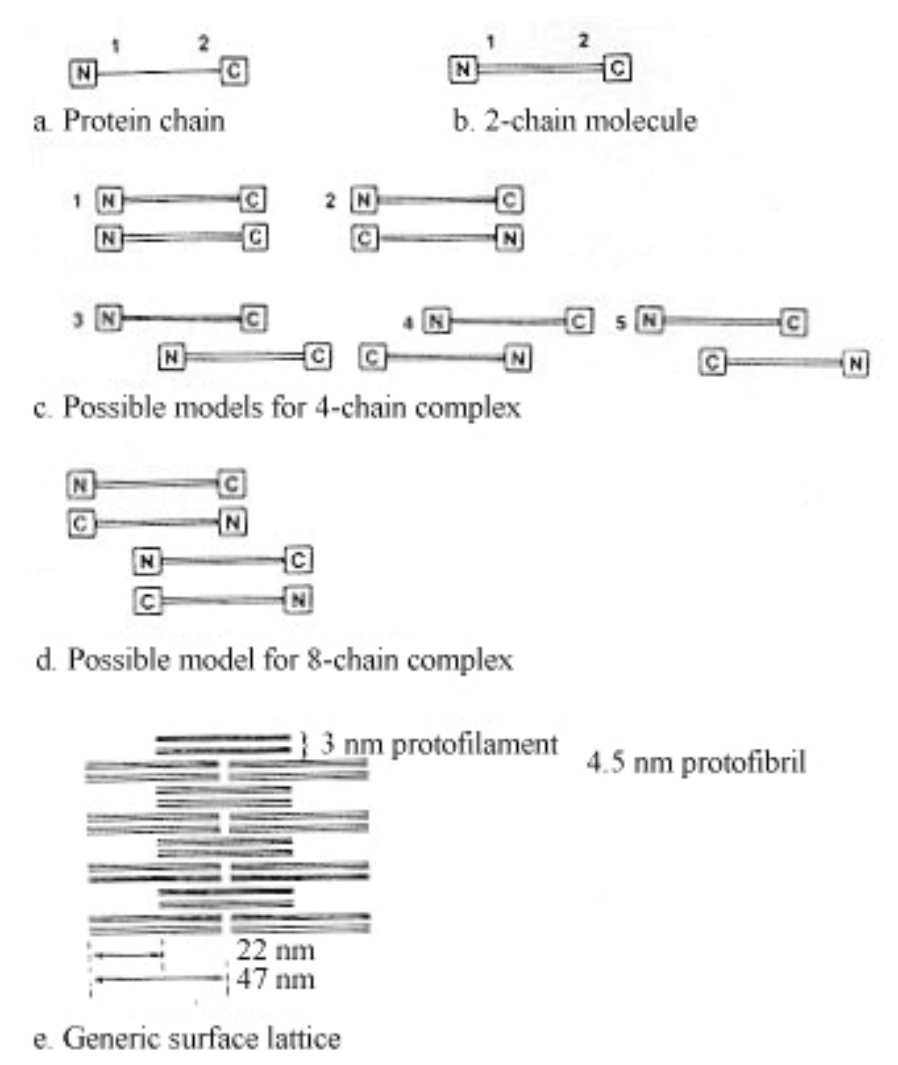


Fig. 28 IF chains and formation of oligomers. (a) IF chain, (b) two coiled-coil molecules in parallel and in register, (c) Proposed models for four chain complexes, (d) possible 8-chain complex and (e) lattice of an entire IF containing 32 chains 147 nm. The axial banding at 22 and 47 nm is that observed with the EM and X-ray diffraction. Steinert and Roop (1988). Reproduced from the *Annual Review of Biochemistry*, Volume 57, copyright &copy1988 by Annual Reviews Inc.

The view that is emerging with more studies suggests the IFs form a network that has a dynamic role in the arrangement of the cytoskeleton and attachment of the plasma membrane to the cytoskeleton (see Houseweart and Cleveland, 1998). IFs can contribute to rapid changes in the cell's cytoskeleton because they are present in readily available pools of subunits. The interactions involving IFs are thought to determine cell shape and resistance to mechanical stress.

Phosphorylation of specific domains in the IF proteins controls assembly. For example, vimentin (Inagaki et al., 1987) is disassembled by phosphorylation, whereas phosphorylation of desmin inhibits assembly (Geisler and Weber, 1988). In fact, a dynamic remodeling of the cytoskeleton during neuronal growth and the establishment of directionality is thought to depend on the phosphorylation of specific domains of neurofilament proteins (Nixon and Sihag, 1991).

E. The Plakin Family

The interdependence of the cytoskeletal components has been firmly established by the finding of cytoskeletal linkers, proteins of the *plakin* family (see Ruhrberg and Watt, 1997; Leung et al., 2002). Plakins crosslink fibrous elements of the cytoskeleton and junctional complexes (see (Ruhrberg and Watt, 1997; Leung et al., 2002). Plakins are expressed mostly in tissues subject to mechanical stress (e.g., epithelia, muscle, etc.). They contain a globular amino-and carboxy-domains separated by a central coiled-coil rod. All plakins possess one or more plakin domains (PRD) usually flanking the rod domain. Some members also have other domains such as the actin-binding, microtubule binding and spectrin-repeat domains.

Desmoplakins are to be found in junctional plaques (see Green and Bornslaeger, 1999) such as desmosomal plaques and they anchor IFs to the plasma membrane to form connections between cells. The desmoplakin gene produces two alternatively spliced forms DPI and DPII of 322 and 259 kDa respectively (Green et al., 1990; Virata et al., 1992).

The *plectins* are found in most tissues (except neurons). They are more than 500 kDa in size and they have been found associated with IFs (e.g., Clubb et al., 2000) and in stress fibers . They also connect IFs to microtubules (Svitkina et al., 1996). Homozygous mutants of the plectin gene are responsible for *epidermolysis bullosa simplex* which produces blistering of the epidermal basal layer and muscular dystrophy. *Bullous pemphigoid antigen 1* (BPAG-1) is present as two isoforms BP230 (BBPAG-1) and BP180 (BPAG-2). BP230 is cytoplasmic and associates with hemidesmosomal plaques, BP180 is a transmembrane glycoprotein. The *BPAG-1* gene gives rise to several distinct proteins with a distinct tissue distribution by alternative splicing. They are important for the maintenance of cell structure of neurons, muscle and epithelia. One of the plakin isoforms contains an actin-binding domain and some isoforms have been shown to link IFs and the actin cytoskeleton (Yang et al., 1996; Andra et al., 1998a). Immunoelectron microscopy has shown plectin cross-bridges between IFs and microtubules, IFs and actin filaments and IFs and myosin filaments (Svitkina et al., 1996). In BPGA1-null mice IFs fail to tether neurofilaments to the actin cytoskeleton (Yang et al., 1996) and the microtubules are disorganized. The mice exhibit *dystonia musculorum*, characterized by rapid degeneration of sensory neurons, and display a mild skin blistering, a reflection of disorganization of the IFs. In humans the homologous condition is *epidermolysis bullosa simplex* (EBS)-MD, a hereditary skin blistering disease with muscular dystrophy, caused by defects in the BPFGA-1 gene. A form of plakin (Yang et al. 1999) lacks the domain that binds to actin but is capable of binding to microtubules and appears to stabilize them. Plectin has been shown to bind to vimentin, keratins, Lamin B, MAPs, α-spectrin and neurofilament proteins (Foisner et al. 1988,1991) as well as actin (Yang et al., 1996). An involvement of plectin with the cell membrane and the actin network is shown by its ability to interact with G-actin in vitro in a phosphatidylinositol-4,5-biphosphate dependent manner and its association with actin stress fibers in living cells (Andra et al., 1998a).

Microtubule-actin cross linking factor (MACF) is a 600 kDa protein is thought to functionin providing

interations between actin and microtubules (e.g., Leung et al., 1999).

V. MECHANISMS OF MOVEMENT AND COMPARISON OF MOTORS

Myosin and kinesin have been studied much more intensely than cytoplasmic dynein. The mechanism of movement of myosin has been discussed in Section IA. For these reasons much of the discussion that follows is directed toward kinesin. However, the intimate mechanism linking the conformational changes to the nucleotide binding site are discussed in some detail for both myosin and kinesin.

A. The Movement

As in the case of myosin, the kinesin heads have been shown to change in conformation during the ATPase cycle involved in motion (Hirose et al, 1995). Kinesin molecules were found to move very slowly without a neck domain. However, they gained very rapid movement when joined to a very short (about 11 amino acids) flexible random chain (Inoue et al., 1997). This latter finding suggests that the mechanism of movement differs significantly from that of myosin, where a rigid lever arm is likely to play a role in magnifying the conformational changes.

Kinesin is a *processive* motor: it remains bound to the tubulin while undergoing multiple rounds of activity. This is probably because kinesin is double headed and moves *hand-over-hand* and at any one time one of the two heads is attached to the MTs. The hand-over-hand model proposes that the heads of double headed kinesin attach alternatively, with the head that is not bound moving to face the direction of the movement. However, simultaneous transient binding of both heads are required for processivity, otherwise the motor would detach entirely. For kinesin, processivity is shown by a variety of experiments. Single molecules typically travel over distance as long as 1 μm representing a 1% probability of dissociation per molecular step or about 100 enzymatic turnovers. It has been proposed that myosin V, which is two headed, is also a processive motor (e.g., Nascimento et al., 1996). Studies using an optical trapping system (see Chapter 1) have concluded that myosin V is indeed processive (see Mehta et al., 1999) and moves in large steps of about 36 nm.

Myosin II and dynein are are not processive. In the case of dynein, Wang et al.(1995) studied the movement on MTs of single or few cytoplasmic dynein molecules bound to each bead. In contrast to kinesin, dynein moved on more than one protofilament indicating that it frequently detaches from its original track. Similarly, the results obtained with subfragment 1 (a single head) and heavy meromyosin (double-headed) are approximately the same (Molloy et al., 1995b).

The hand-over-hand mechanism of kinesin movement is supported by a several experiments. In the absence of ATP one head is attached to the microtubule, the other is attached and bound to ADP. Addition of ATP releases the bound head and the ADP from the detached head (Hackney, 1994). Furthermore, the results of other studies of Ma and Taylor (1997) suggest that ATP binding to one head is required for the ADP release by the other head so that the two heads act cooperatively and alternate in their function.

Structural information is in harmony with the hand-over-hand model. Cryoelectron microsope images of kinesin attached to a MT show one head attached with the other detached and oriented at right angle to the filament (Arnal et al., 1996; Hirose et al., 1996).

If the hand-over-hand model were correct a single headed kinesin should not be processive, in contrast to the double headed variety. Furthermore, it is difficult to see how it could be directional (Inoue et al., 1997). The dimeric motor works at extremely low concentrations down to the single molecule level (Howard et al., 1989) and follows the track of protofilaments closely (Ray et al., 1993). In contrast, in the same assays, single headed monomeric constructs of *Drosophila* kinesin move slowly, do not follow straight tracks and require high concentration of the motor (Gelles et al., 1995). This simple picture is complicated by the observation that a supposedly single headed kinesin is processive, being able to move along a microtubule for as much as 1 μm (Okada and Hirokawa, 2000). This kinesin was a chimeric construct of conventional kinesin with the motor of KIF1A, a monomeric kinesin, supposedly unable to dimerize (Jiang et al., 1997). Apparently, the explanation for this behavior rests on the structure of the molecule. This protein possesses a lysine rich region in the motor domain referred to as a K-loop. The K-loop is thought to keep the motor in close contact with the negatively charged microtubule (Okada and Hirokawa, 2000).

Other experiments have also been directed toward this question. The properties of single headed kinesin containing the whole "rod" segment were compared to those of wild-type kinesin (Hancock and Howard, 1998). The microtubule-gliding assay was used, where the motor is attached to glass and the microtubules are free to move. The microtubules were labelled with a visible marker, in these experiments at the front end of the microtubule. The movement could then be recorded and analyzed. The procedure was carried out with various densities of the motor molecules. The Hill coefficient derived from a Hill plot provides information on cooperativity, i.e., how many kinesin molecules produce motion. The maximum value of *n* corresponds to the number of subunits or binding sites. Unity indicates independent action of each molecules. In the case of the double headed kinesin the Hill coefficient was unity indicating that only one was needed for motion. When the single headed kinesins were used, however, the Hill coefficient ranged from 4 to 6 indicating that motion required the participation of that many kinesin molecules. Furthermore, single heads remained attached for as long as 3 s. This indicates that the two heads act in concert in agreement with the hand-over-hand model.

The role of the neck region in kinesin motion has lead to a good deal of speculation. The neck region is needed to allow the two heads to interact during the cycle. In addition, the geometry of of MTs and kinesin in its most frequent conformation is such that both heads cannot be bound simultaneously at the required 8 nm distance between binding sites, whereas the two heads are 6 nm apart. Transient binding is needed for processivity (see Kozielski et al, 1997). Therefore, the neck region must undergo some conformational change to allow for the two heads to attach simultaneously. In contrast to conventional dimeric kinesin, Ncd (also dimeric) has low processivity (Case et al., 1997; Block, 1998). However, the two heads must move in relation to each other to allow for the conformation seen in the three dimensional reconstructions from cryoelectronmicroscopy (Hirose et al., 1996; Arnal et al., 1996; Hirose et al., 1998; Hirose et al., 1999). This conformation could correspond to a melting of the coiled-coil neck region to allow for the

separation of the two heads (Endow and Fletterick, 1998).

In most kinesins, the neck consists of two parts. At the amino-terminal approximately 10 amino acids form a β sheet (Case et al., 1997; Henningsen and Schliwa, 1997). The domain that follows forms heptad repeats of about 30 amino acids that form a coiled-coil structure (Huang et al., 1994; Morii et al., 1997; Tripet et al., 1997). The carboxy-terminal of kinesin has a coiled-coil stalk domain followed by a globular tail. In contrast, the Ncd neck is a coiled-coil continuous with the stalk (Sablin et al., 1998). The difference in structure of the two molecules leads to a different orientation of the heads in relation to the stalks. The structural differences suggest that two distinct mechanisms operate in the neck region.

Recent models of conventional kinesin suggest a cycle in which the neck serves to integrate the function of the heads (Hackney, 1994; Hirose et al., 1996, Tripet et al., 1997). At the beginning of the cycle the neck holds the two heads close together and so tightly that only one can bind to the microtubule. The binding of the nucleotide to the second head causes a segment of the coiled-coil to unwind. This unwinding alters the spacing between the two heads and allows the second head to reach a new binding site in the microtubule. Then the coiled-coil is reformed at the end of the cycle, a process that pulls the first head off the microtubule.

There is substantive evidence for a rearrangement of the two heads. Microtubules decorated with constructs of kinesin molecules (Hoenger et al., 1998) were examined with cryoelectron microscopy using image reconstruction techniques and X-ray crystallography. A monomeric construct with a short neck (insufficient for a coiled-coil formation) decorates microtubules with a stoichiometry of one kinesin/αβ tubulin dimer. A longer kinesin construct with a longer neck forms a helix coiled-coil is correspondingly dimeric. A we already saw, the stoichiometry was found to be unchanged corresponding to one kinesin head per tubulin heterodimer. In order for both head to bind simultaneoulsy they must be able to separate to a distance of 8 nm, beyond the 6 nm normal distance between the two.

The conformational change may not involve a rearrangement of the coiled-coil region. Romberg et al. (1998) tested the role of the neck region by deleting or altering it and then examining the effect on single molecule motility. Eliminating the coiled-coil region of the neck entirely, decreases processivity ten-fold but does not abolish it. Stabilizing the neck by replacing the neck region with a peptide sequence that forms a very stable coiled-coil, only reduced processivity 45% suggesting that unwinding of the coiled coil region does not play a significant role. Increasing the flexibility between the two heads by introducing a three residue glycine linker at the beginning of the neck reduced processivity by 60%. When the first heptad was duplicated processivity was enhanced ten-fold. These results suggest that unwinding of coiled-coil region is not involved in the motion but that the neck structure is still very important. Romberg et al. (1998) propose that the β sheet region is disrupted during the hydrolysis cycle. There is some structural support for this model. The β sheet of the neck have been found in a highly ordered (Kozielski et al., 1997) and in a disordered form (Kull et al., 1996) suggesting that it may be able to alternate between two different conformations.

An interesting approach (see Howard, 1997; note however that several aspects are still under discussion, Huxley, 1998 and Howard, 1998) that has considerable predictive value and may help understand the functioning of motors, is that of *duty ratio, r*, defined as fraction of the time a motor domain spends in contact with the corresponding filament (originally called duty cycle) (Eq.(1).

$$r = \frac{\tau_{on}}{\tau_{on} + \tau_{off}} \qquad (1)$$

A two headed molecule that stays attached most of the time will have an *r* that is high (e.g., 0.5), otherwise it will diffuse away. On the other hand, when many cross-bridges are involved as in the case of muscle myosin, the *r* can be quite small (approaching 0.01 or 0.1). A low value of *r* (0.1-0.2) was deduced by paramagnetic resonance spectroscopy which showed that only 20% of the heads were ordered as expected if bound to actin (Cooke et al., 1982) (the reciprocal of the number of cross bridges needed for continuous motion).

This simple relationship has several implications. If during a contractile event each head moves over a distance δ then $v = \delta/\tau_{on}$. where *v* is the velocity of the motor. However, we expect the total cycle time to be $\tau_{tot} = 1/V$, where *V* is the rate at which each head hydrolyzes ATP. Substituting these values in the original equation, assuming that 1 ATP is hydrolyzed per mechanical cycle, the ratio becomes:

$$r = \delta \frac{V}{v} \qquad (2)$$

This approach has promise because of its predictive value. For example, (see Howard, 1997) unloaded fast skeletal muscle glides twenty times faster than conventional kinesin per ATP hydrolyzed. In addition, the movement is for approximately 400 nm. This is because myosin has a low value of *r*, about 0.01 and an ATPase rate of 20 per head per second. From Eq. (2), $\delta = r.v/V$ or 0.01 x 8000 nm s^{-1}= 4 nm. 4nm is well within the size of the displacement of one myosin head. Basically this means that of one hundred myosin heads, each contributes only 4 nm of displacement. While the motor is detached, the other heads displace the filament for 400 nm. This will not be the case for kinesin where a distance of 8 nm and an ATPase rate of 50 s^{-1} permits a speed of only 800 nm s^{-1} because $r = 0.5$. The values of *r* are, in actual fact, limited because the displacement must conform to the presence of a binding site on the filament.

Another way of looking at *r* is in terms of distance (Eq. 3). If we consider *d* the path distance as the minimum distance between consecutive binding sites of the same motor, the *r* ought to equal the fraction of the distance to the next binding site. Where it is smaller than *d*, each individual crossbridge must spend a significant amount of time detached while other crossbridges move the filament. In this case, the duty ratio must be less than 1; it should be equal to the fraction of the distance to the next binding site. *n* can be

greater than 1 if a binding site is skipped.

$$r = \frac{\delta}{n.d} \qquad (3)$$

Does the definition formalized by Eq. (3) agree with the previous definition? In the case of kinesin there is one dimer binding site per kinesin molecule and the kinesin proceeds straight on the protofilament. Recent tracking studies have shown that the steps are probably 8 nm. Since $n = 2$ (one kinesin head has to skip one site occupied by the other head), r would be 0.5 consistent with previous considerations.

Myosin on actin filaments also proceed on a straight or near straight line. If the myosin head binds to correctly oriented actin subunits, the path distance should be 36 nm corresponding to the half pitch of the actin helix. When an actin filament is suspended between to beads held in optical traps, binding is observed in multiples of 40 nm (Molloy et al., 1995a).

B. Inside the Motors

Movement is linked to ATP hydrolysis. The intimate details of the mechanism of motion must involve the catalytic site of the motors. Therefore, it would seem necessary to examine the details of nucleotide binding and hydrolysis and their structural correlates in order to understand the mechanisms. Although a good deal has been found in recent years, the emerging picture is not always consistent (e.g., see Volkmann and Hanein, 2000)

Myosin, kinesin and G-protein (the heterotrimeric GTPase) (see Chapters 7 and 11) have several common structural features (see Smith and Rayment, 1996b; Kull et al., 1996, 1998) and many differences. The present discussion will center on myosin and kinesin.

Members of the kinesin family have catalytic core of about 330 amino acids. This core differs little from one kind of kinesin to another (see Vale and Fletterick, 1997) and is very similar to the myosin core (Kull et al., 1996; Kull et al., 1998). Despite the similarities between the kinesin and the myosin motor domains, there are significant differences in the mechanism of motion (for animation models for both myosin and kinesin see Vale and Milligan, 2000 www.sciencemag. org/feature/data/1049155.shl). As discussed above (Section IC), muscle myosin undergoes a large angular rotation of a long and relatively rigid element corresponding to an α-helix and associated light chains. Each ATP-hydrolyzed corresponds to a 10 nm displacement (note however that this may be controversial point; 6 nm is indicated by single molecule analysis, see above, however, in these experiments two steps are seen with a total of about 12 nm). ATP causes myosin to dissociate from actin and to undergo its recovery stroke. The release of P_i following nucleotide hydrolysis, produces a tight binding to actin and the forward swing of the lever arm. (Rayment et al., 1993a; Dominguez et al., 1998; Corrie et al., 1999; Suzuki et al., 1998; Houdusse et al., 1999). In contrast, in conventional kinesin, a conformational change occurs in the neck linker peptide of

approximately 15 amino acids. The neck linker is rigid only in the ATP/ADP.P_i-bound state. In the case of kinesin, ATP-binding is responsible for the power stroke in the form of a forward motion of the neck linker. ATP hydrolysis releases the binding of kinesin to the microtubule and detaches the head linker. The small conformational change of the head linker is amplified to produce an 8 nm movement by the swinging of the partner head to the next binding site.

Three segments of myosin have been traditionally referred to as 15, 50 and 20 kDa segments (in that order from the amino terminal) corresponding to the segments separated by tryptic digestion (see Rayment, 1996 and Fig. 10). The ATPase site is located in the cleft that splits the central 50 kDa segment into lower an upper domains. The bound nucleotide is surrounded by the P-loop and two loops in the 50 kDa segment (referred to as switch II and switch I loops). The switch I loop runs along the ATPase pocket and some ot its side chains are arranged toward the pocket to form non-covalent bonds with nucleotide and Mg^{2+}. One of the side chains of switch II points toward the ATPase pocket and is hydrogen bonded to the water surrounding the Mg. Other chains outside of the ATPase pocket connect the ATPase site and the long α-helix (the switch II helix).

In the myosin-MgADP/VO_4 complex (considered equivalent to myosin-ADP.P_i, the metastable state after hydrolysis; see Fig. 9), a residue of the switch I loop forms an ionic bond with with one of the switch II loop residues so that switch II is held close to the nucleotide. In contrast, when myosin is complexed to MgADP/BeFx (considered equivalent to ATP), these residues are separated and the conformation is open with the loops away from each other. In this process, switch II moves by almost 6 Å relative to switch I. The movement is propagated to the long adjacent helix and rigid loop (the *relay*), to the lever arm (Dominguez et al., 1998). A domain referred to as the *converter domain* which is connected to the lever arm has been shown to rotate about 70º, calculated to correspond to a 10 nm displacement of the lever arm.

Vale and Milligan (2000) view the mechanical factors to be in response to the γ-phosphate of ATP. A sensor of the γ-phosphate produces a change conformation when undergoing the transition from the ATP and ADP bound states. Other components link the catalytic site, the mechanical element and the filament binding sites. In myosin, the sensor would correspond to Switch I and Switch II which form hydrogen bonds with the γ-phosphate. In addition, they position a catalytic water and important side chains for cleavage of the β to γ-phosphate bond. Switch II swings in to interact with the γ-phosphate and swings out when the γ-phosphate is removed. This has been shown when the myosin structures were compared with and without ATP analogs (Fisher et al., 1995b; Smith and Rayment, 1996a). The switch mechanism in kinesin (see Kull et al., 1996) is almost identical and it also resembles that of GTPase proteins (see Vale and Fletterick, 1997; Kull et al., 1998).

The movements of the γ-phosphate sensor are transmitted via a long helix connected to the switch II loop at the amino terminal and to the filament binding sites at the carboxy-terminal. This helix is the *switch II helix* of kinesin and the *relay helix* of myosin. In myosin, the helix undergoes a conformational change similar to that of a piston. With ATP-ADP.P_i analogs, the motion of the switch II loop toward the γ-

phosphate tilts and translates the relay helix along its axis toward the upstroke position. In the absence of nucleotide, switch II swings away and the relay helix goes to the downstroke position (see Vale and Milligan, 2000). Similar conclusions can be reached for kinesin.

The movement of kinesin is produced by a conformational change of the 'neck linker" (Rice et al., 1999) which consists of a 15 amino acid region. In the absence of nucleotide or when it binds ADP, the neck linker is mobile when kinesin is attached to microtubules. When the attached kinesin binds an ATP analog, the neck linker becomes immobilized by attaching to the catalytic core with its carboxy-terminal pointing toward the plus-end of the microtubule. In this way, the binding of ATP drives the forward motion of the head linker and whatever is attached to the carboxy-terminal. The motion of one head is conveyed to the other via the coiled-coil dimerization domain, so that, the trailing head detaches and from its binding site and is moved forward to the next binding site.

A major advance has been the determination that 1 ATP is hydrolyzed for each step in the movement of kinesin on a microtubular track (Schnitzer and Block, 1997; Hua et al., 1997). In principle, without corrections for Brownian motion or statistical analysis, the ATP hydrolyzed per step can be computed directly. For a single dynein dimer, the velocity of the movement (nm/s) (measured experimentally) divided by the size of the steps (8 nm), will give us the number of steps per second (n s^{-1}). The rate of ATP hydrolysis per g of purified dynein (measured independently in the presence of tubulin) x the molecular weight of the dynein dimer will give us the rate of hydrolysis per molecule of dynein (ATP s^{-1}). Then the ATP/step = (ATP s^{-1})/ (n s^{-1}).

In one set of experiments, beads attached to a single molecule of kinesin were tracked with interferometry (Svoboda et al., 1993) (see Chapter 1 for the principles involved in interference microscopy). Statistical analysis of the interval between steps, together with measurement of motor speed as a function of ATP concentration allows the calculation that 1 ATP is hydrolyzed per 8 nm step. In another study carried out at the same time, a very low ATP concentration (150 nM) was used allowing for corrections of Brownian motion. The study also arrived at the same conclusion (Hua et al., 1997).

A study of motion of single kinesin molecules at various loads and ATP concentrations used an optical trap (see Chapter 1) which mantains the load constant, (Visscher et al., 1999). A kinesin molecule attached to a silica bead moves along an immobilized microtubule while an optical trap follows the movement. The steps were found to be tightly coupled to the hydrolysis of ATP with a single hydrolysis for each 8 nm step regardless of load. The force required to stall the kinesin depends on the ATP concentration. Increasing load not only reduces the velocity of the movement but also the K_m (in relation to ATP).

The in vitro properties of the motors need not reflect how they actually operate in vivo. A case in point is that of the bidirectional movement of lipid droplets in early *Drosophila* embryos. Each droplet is moved by multiple motor molecules (Welte et al. 1998). The minus motor is cytoplasmic dynein (shown by mutations that alter both the dynein molecules and the movement) and the positive motor is presumably, kinesin. Video-enhanced DIC microscopy and optical tweezers were used to track single droplets and measure the forces generated (Gross et al., 2000). The droplets travel shorter distances from what we

would expect from in vitro estimates of cytoplasmic dynein processivity. Usually, the interruption of movement results in an immediate switch in travel direction. The switching appears to occur similarly in either direction, suggesting the presence of a switch mechanism for both motors. The quick switch in direction suggests that both motors are present in the droplet and both are in contact with the switch.

C. Directionality of the Movement

In addition to a mechanism providing movement, the kinesin molecules must have the means to move on the microtubules in a specific direction. All plus-directed kinesins have their motor domain attached to the amino-terminal (e.g., conventional kinesins) and all minus-directed kinesins have the motor domain attached to the carboxy-end (e.g., Ncd). The Ncd neck is a coiled-coil, whereas this region in conventional kinesin corresponds to an interrupted β-strand (Sablin et al., 1998; Kozielski et al., 1999). At one time the direction of the microtubule-based movement was thought to be a property of the motor domain (e.g., Stewart, 1993) because replacing the other regions of the molecule did not alter the direction of the movement. However, the answer is more complex.

A clue to the mechanism responsible for the directionality of the movement is provided by the observation that Ncd and Nkin at any moment in time bind with only one of their two heads. As already mentioned, the head that does not bind faces the direction of the movement (Hirose et al., 1996; Arnal et al., 1996). This suggests a role of the neck region.

The involvement of the neck region was studied by producing hybrid proteins containing the Ncd motor domain fused to the kinesin heavy chain, α-helical coiled-coil stalk and containing the region just adjacent to the motor domain (Henningsen and Schliwa, 1997; Case et al., 1997). The Ncd stalk-neck region fused to a kinesin motor core reversed the directionality of kinesin. This reversed (minus) motor could go back to its original polarity (plus, however with slow motility) by the mutation of certain residues in the neck-motor junction (Endow and Waligora, 1998). These mutations are thought to inactivate the minus-end determinants and suggest the presence of plus-end determinants in the motor. In another study of the Ncd molecule, site directed mutations of 13 class-specific residues in the Ncd neck, next to the motor domain (Sablin et al., 1998), resulted in a reversal from a minus to a slow plus-end determinant indicating that the Ncd motor domain also contains directionality determinants. These findings identify residues in the Ncd neck that confer minus-directionality and show plus-end determinants in both the kinesin and Ncd motors.

Although residues needed for minus-end motility have been identified in the Ncd neck (Sablin et al., 1998; Endow and Waligora, 1998), this is not the case for kinesin. It might be possible that the neck region of kinesin only amplifies the plus-directed motility of the motor. This hypothesis would explain the slow plus-end movement of the Ncd-kinesin-heavy chain and Ncd neck mutants (Sablin et al., 1998; Endow and Waligora, 1998).

Interestingly, a mutations in a single amino acid in Ncd neck produce a motor that will move in either direction. In experiments using the gliding assay (see above), the microtubules were found to move either

in one direction or the other and at times reverse the direction (Endow and Higuchi, 2000). Half of the MTs will initially move in one direction and the other half in the other direction. In addition, single motor molecules attached to beads in contact with microtubules in a single beam optical trap indicated the same properties. These observations are not easy to explain. However, they emphasize the theme that the directionality depends on a neck-motor core interaction.

A similar situation occurs in the case of the myosins. The myosins generally move toward the plus end of actin (the barbed end). Recently, myosin VI has been found to move in the opposite direction (Wells et al., 1999). This property is associated with a large insertion in the converter domain (between head an tail) (see above). How the different structure can determine polarity of movement is not known, although simple mechanical models can be designed to account for the observations (e.g., see Schliwa, 1999).

VI. ASSOCIATION OF MOTORS WITH CARGO

The melanophore system of amphibians and fish (see Halmo and Thaler, 1994) has been one of the most useful in examining the role of the motor systems responsible for organelle movement. This system has revealed a role for microtubules as well as an intimate interaction between microtubules and actin. Melanophores can change in appearance by rearranging their pigment granules, the *melanosomes*. The distribution of melanosomes is hormonally regulated and is mediated by cytoplasmic cAMP. When the melanophores are dispersed, the cells appear dark. When aggregated, the cells appear light. In part the movement, either anterograde or retrograde, involves the microtubular motors dynein and kinesin (e.g., see Rogers, 1997) on uniformly polarized microtubules (Euteneur and McIntosh, 1981). Although the anterograde transport toward the periphery depends on microtubules. The dispersion in the cytoplasm is independent of microtubules. This distribution requires movement on actin filaments driven by myosin V (Radionov et al., 1998).

Motors can move not only a variety of vesicular elements but also protein and RNP complexes (see Karcher et al., 2002). The ability of the motors to carry such disparate cargoes is due to the divergence of their tail domains and their ability to bind cargo or proteins which can serve as mediators by binding to both cargo and motor. The interactions between motor proteins and their binding partners have been studied by standard biochemical methods which have identified such molecules as kinectin, dynactin and pericentrin. The yeast two-hybrid strategy (see Chapter 1) has revealed many other binding partners.

The motors have multiple domains that can bind to various receptors. For example, kinesin, can bind to several proteins through its *tricopeptide* (TPR) motifs of the light chains. TPRs are known to mediate protein-protein interactions (Gindhart and Goldstein, 1996).However, kinesin can also bind through its heavy chain (e.g. see Ong et al., 2000). Similarly, in myosin V from yeast, the tail contains subdomains so that random mutations of this region have different effect on the transport of various vesicles (e.g, Catlett et al., 2000).

When associated with vesicles, the motors were found to be located on the organelle surfaces (Langford et

al., 1987). In the native system, binding of the motor to the organelle is likely to require an integral membrane protein, as phospholipid vesicles or trypsinized organelles (i.e., with the surface proteins cleaved) do not move on microtubules (Gilbert et al., 1985; Vale et al. l985b). Specific binding to such motor receptors could determine the sorting out of vesicles accompanying intracellular transport (Chapter 10 and 11). A role of microtubules in this transport is supported by the observations that microtubules are associated with the endoplasmic reticulum (Franke, 1971), Golgi vesicles (Allan and Kreis, 1987), and lysosomes (Swanson et al., 1987).

The receptor for kinesin, kinectin, has been isolated only in the ER (Toyoshima et al., 1992). Kinesin also binds to *c-Jun N-terminal kinase (JNK)-interacting proteins* (JIPs) (Bowman et al., 2000; Verhey et al., 2001). JIPs are so named because they were first found to be scaffolding proteins for JNK. Mammalian *Sundaydriver* (SYD), a JIP, binds to the TPRs motifs of the kinesin light chain. Using the TPR motif and the two-hybrid procedure (see Chapter 1), two other JIP proteins were found to bind to kinesin (Verhey et al., 2001). In agreement with the notion that the TPRs have a role in cargo transport, monoclonal antibodies (see Chapter 1) against the third tandem repeat, blocked axonal transport and released kinesin from vesicles (Stenoien and Brady, 1997). The complex of kinesin and JIP was found to bind to *dual leucine zipper-bearing kinase* (DLK), part of the JNK pathway (see Chapter 7). In addition, it was attached to the ApoER2 (LR7/8B) complex, a receptor of the LDL receptor family highly expressed in neurons. These associations suggest a role of JIP and kinesin in signaling. Other proteins have been found to be possible adaptors for kinsine based transport (see Karcher et al., 2002).

An involvement of JIP-3 in vesicle transport is shown by the accumulation of organelles in axons in JIP-3 mutants, similar to what takes place in the kinesin heavy chain null mutants (Bowman et al., 2000). JIPs 1, 2 and 3 were also shown to bind to the TPR motif (Verhey et al., 2001) and kinesin-JIP combinations were found to coprecipitate using immunological techniques. Presumably, the motor proteins are linked to their membranous vesicles or organelles via these scaffolding proteins. Accordingly, in neurons, mutations in JIP-1 that abolish the interaction with kinesin light chains or the expression of dominant negative constructs of JIP-1, eliminate its localization at the terminals (Verhey et al., 2001). In addition to serving as links between vesicles and kinesin, the scaffolding proteins carry with them transmembrane signaling molecules (e.g., see Chapter 6). This finding indicates that signal transduction pathways may regulate motor activity or even distribute signaling molecules at definite locations in the cell (see Verhey and Rapoport, 2001).

Some experiments implicate the protein *fodrin* in providing a link between kinesin motors and vesicles. Fodrin is an actin/calmodulin-binding protein with similarities to spectrin (see Chapter 4, Fig. 7 and Fig. 8) and a protein of the intestinal epithelium brush border (see Cheney et al., 1983). Fodrin is composed of two polypeptide subunits of 235 and 240 kDa (Glenney and Weber, 1985). Fodrins are highly concentrated in the cortical cytoplasm of neurons and other tissues (e.g., skeletal muscle, uterus, intestinal epithelium) (Levine and Willard, 1981). The movement of fodrins has been demonstrated in neurons and in some other tissues. The redistribution of fodrin is accompanied by concomitant redistributions of actin, myosin, and

calmodulin, and the movements proceed at similar velocities (Cheney et al., 1983), suggesting that fodrin serves to link various organelles or proteins.

Other kinesins [so called *kinesin superfamily proteins* (KIFs)] play a role in vesicle transport and mitosis (see Goldstein, 2001). There is direct evidence of the involvement of fodrin in tethering the kinesin KIF3 to vesicles. KIF3, located in the nervous system, corresponds to a heterotrimer of KIF3A (e.g., Kondo et al., 1994) and either KIF3B (Yamazaki et al., 1995) or KIF3C (Muresan et al., 1998; Yang and Goldstein, 1998) and an associated protein, KAP3 (Yamazaki et al., 1995). Microinjection of antibodies against KIF3 in neurons was found to block fast axonal transport and to block neuronal processes extension (Takeda et al., 2000) implicating this kinesin as the primary microtubular based motor in this system. The yeast two-hybrid system (see Chapter 1) showed that KIF3 is attached to *fodrin* with KAP3 bridging the association (Takeda et al., 2000). Fodrin and KIF3 colocalized in the same vesicle as shown by immunoprecipitation and immunoelectron microcopy. The evidence supports a role of fodrin and KAP3 in tethering KIF3 to vesicles for transport in neurons.

In some cases, KIFs attach directly to the cargo proteins (e.g., an NMDA receptor subunit which is in vesicle membranes; Setou et al., 2000) or to the coat of vesicles, for example thorugh the β1 adaptin subunit of AP1 in the *trans*-Golgi. (Nakagawa et al., 2000). Some KIFs are linked to cargo through Rab proteins (see Echard et al., 1998).

KIF1A (Unc104), involved in vesicle transport, has been found to attach to membranes directly through its PH domain (see Chapter 4; Chapter 6 and linked discussion). This motor can transport artificial vesicles containing phosphatidylinositol(4,5)bisphosphate (PI(4,5)P_2) at high concentrations . Much lower concentrations are required when cholesterol and sphingomyelin are added to the vesicles suggesting a role of rafts in the transport (Klopfenstein et al., 2002).

Like kinesin, dynein often acts through other proteins. The dynein intermediate, light intermediate and light chains associate with the *synactin* complex, a dimer of 150-kDa required for most functions of dynein such as the transport of vesicles (Gill et al., 1991). Three isoforms of dynactin are coded by a single gene, probably equivalent to the *Drosophila* Glued. Synactin action is through spectrin, generally present in the membranes (Fath et al., 1997; Muresan et al., 2001). In addition, dynein binds directly to cargo proteins such as an isoform of visual rhodopsin and *pericentrin*, a centrosomal protein.

We have seen that myosin V is involved in the transport of vesicles (see Wilson et al., 2000) . The attachment of myosin V to membranes is mediated by Rab proteins (e.g., melanosome movement, e.g. Bahadoran et al., 2001). However, Myosin Va binds to synaptic vesicles through the protein VAMP/synaptobrevin (see Chapter 11), a member of the SNARE family of proteins (Prekeris and Teriian, 199). Another myosin V (myo4) transports ASH1 RNA (a yeast mRNA that localizes to the bud tip in *Saccharomyces cerevisiae*), possibly through an adaptor protein such as She3p (e.g., Bohl et al., 2000). Interestingly, conventional kinesin has been found to bind to myosin V (Huang et al., 1999), although an

actual role in vesicle transport has not been shown. Myosin VI colocalizes with clathrin and AP-2 structures and binds to clathrin coated vesicles (Buss et al., 2001)

The binding of motors to cargo is regulated, thereby controlling its transport. For example, the dephosphorylated form of dynein binds dynactin and phosphorylation abolishes the binding (Vaughan et al., 2001). Similarly, the kinesin Eg5, involved in spindle formation during mitosis, is phosphorylated when it binds to microtubules through the mediation of dynactin (Blangy et al., 1997). In addition, the phosphorylation of myosin V in the globular part of the tail by Ca^{2+}-calmodulin dependent protein kinase II dissociates the myosin from the transport of pigment organelles in *Xenopus* (Karcher et al., 2001).

Calculations of the energy required for movement on microtubules (Sheetz and Spudich, l983b) show that only a few of these motor molecules need to be attached to the organelles, and electron microscopic observations suggest only five cross-bridges between organelles and microtubules (Langford et. al., 1987).

VII. TRIGGERING OF CONTRACTION

The events that trigger contraction are reasonably well understood for vertebrate skeletal muscle. Several steps are interposed between the excitation of the nerve and contraction. The action potential of the nerve is transmitted to the muscle. The nerve terminals release acetylcholine. A specialized structure on the muscle fiber, the *end plate* responds to the arrival of acetylcholine by depolarizing, giving rise to the *end plate potential* (Chapter 22). The end-plate potential in turn triggers an action potential in the muscle, which results in contraction by processes discussed in this section. The sequence of events in other motile systems is less well understood. The coupling between stimulus events and motility in the various motor systems is the topic of this section.

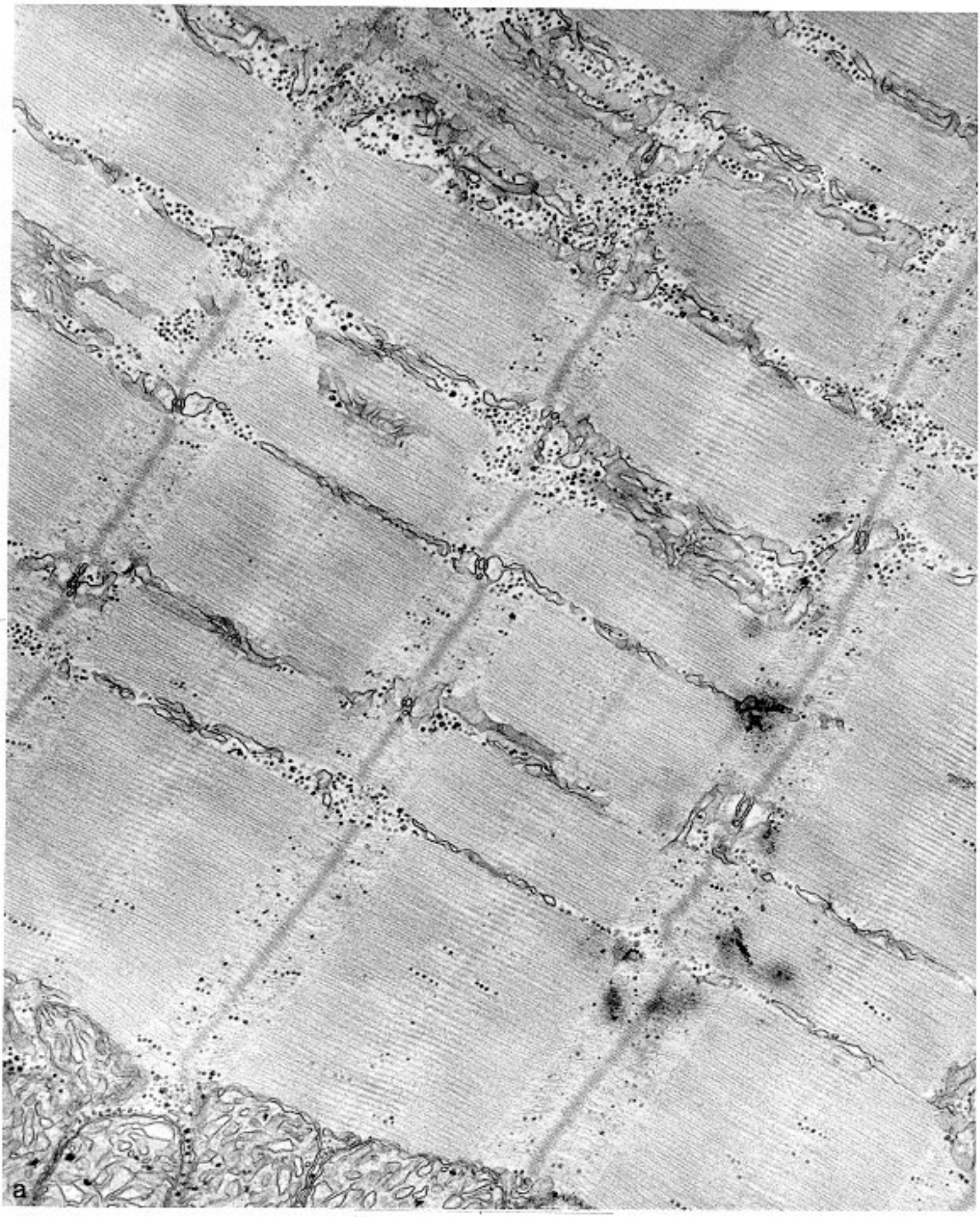
Calcium is necessary to couple excitation to contraction; it has been implicated in muscle contraction from the earliest experiments. Heart muscle, for example, exhibits an action potential but fails to contract when Ca^{2+} is absent from its external medium (Mines, 1913). The situation is somewhat more complex in the striated muscle of the frog, which fails to conduct or contract when stimulated in a medium lacking Ca^{2+} (Ishiko and Sato, 1957). Apparently, the two independent mechanisms, conduction and contraction, are affected by the lack of Ca^{2+}. When depolarization is induced by increasing the K^+ concentration in the medium, contraction still cannot be elicited without Ca^{2+} (Franck, 1960).

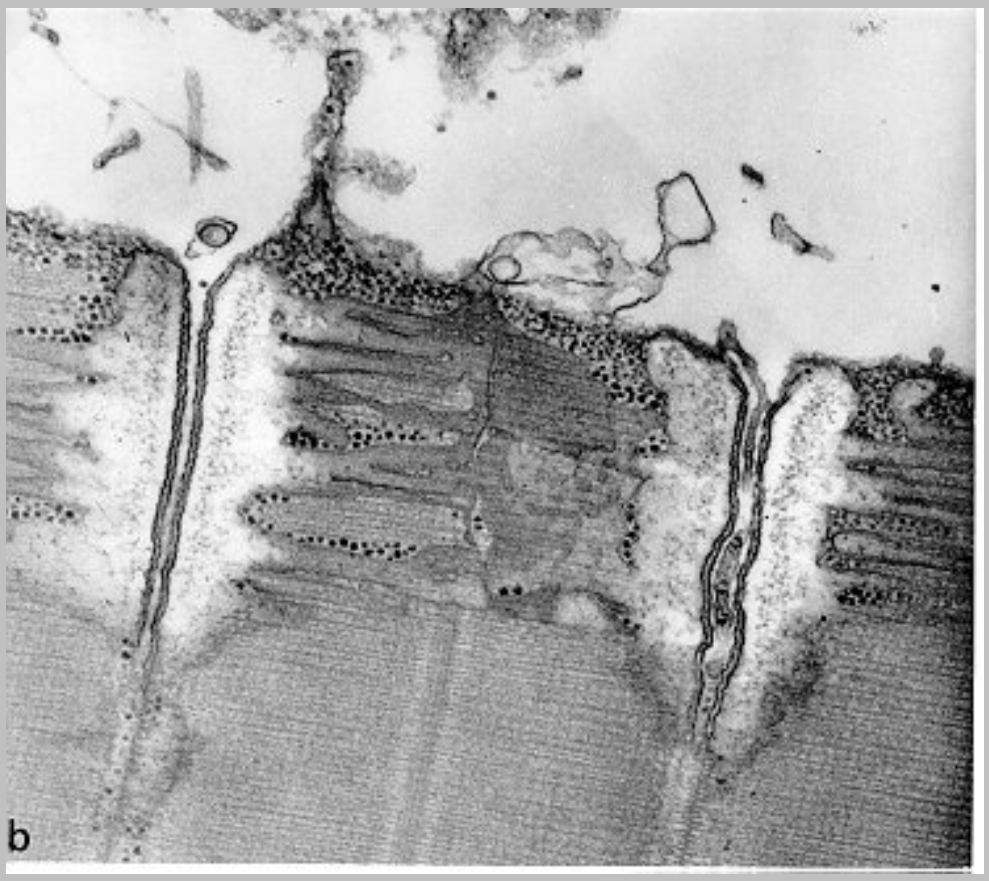
Although Ca^{2+} is necessary for contraction, muscle can contract in the absence of the action potential. Microinjection of Ca^{2+} into the fiber (Heilbrunn and Wiercinski, 1947; Portzehl et al., 1964) in concentrations as low as 0.3 to 1.5 μM (Portzehl et al., 1964) induces a contraction. In contrast, injections of Mg^{2+}, Na^+, K^+, ATP, AMP, arginine, and inorganic phosphate are ineffective (Caldwell and Walster, 1963; Heilbrunn and Wiercinski, 1947). The Ca^{2+} could then serve as a trigger for initiating contraction. This view is supported by the contraction elicited by delivering small amounts of Ca^{2+} with a micropipette on muscle fibers stripped of their surface membrane (skinned preparations) (Constantin et al., 1965). The

contraction is limited to a small area on which the Ca^{2+} has been delivered, and it is followed soon by relaxation. The requirement of Ca^{2+} for contraction, either in intact muscle or in stripped fibers, suggests that the release of Ca^{2+} into the fiber's interior induces contraction. Its subsequent removal by some special mechanism induces the contractile elements to relax.

The Ca^{2+} required for contraction does not originate from the medium external to the fiber. The influx of Ca^{2+} during activation is insufficient to provide enough for contraction (Franck, 1961; Winegrad, 1961). On the other hand, in vitro vesicles derived from the muscle's endoplasmic reticulum (the *sarcoplasmic reticulum*, the SR) can remove enough Ca^{2+} from the medium to induce isolated fibrils to relax after having been made to contract by the addition of ATP (Weber et al., 1963). Therefore, the SR could provide the system for removal and release of the Ca^{2+} necessary for the relaxation-contraction cycles of functional striated muscle.

The structural arrangement of the internal membranes is well suited for a role in triggering contraction. It is composed of a system of transverse tubules continuous with the surface membrane (Fig. 29a and b) and a longitudinal system, the lateral sacs, that reach each sarcomere (Fig. 30) (Peachey, 1965; Porter, 1961). The T tubules and the two associated cisternae at the sarcomere level constitute the *triad*. The continuity of the transverse system with the surface membrane has been demonstrated by electron microscopy (Fig. 29a) (Franzini-Armstrong, 1964). Similarly, the longitudinal and transverse systems are closely associated (Fahrenbach, 1965; Franzini-Armstrong 1964, 1973). The continuity of the transverse tubules (T tubules) with the surface membrane is supported by observation of the passage of large molecules, such as ferritin (Huxley, 1969; Page, 1964) or albumin (Hill, 1964), into the transverse system from the medium. Three-dimensional reconstructions are shown in Fig. 30 (Peachey, 1965).


a


b

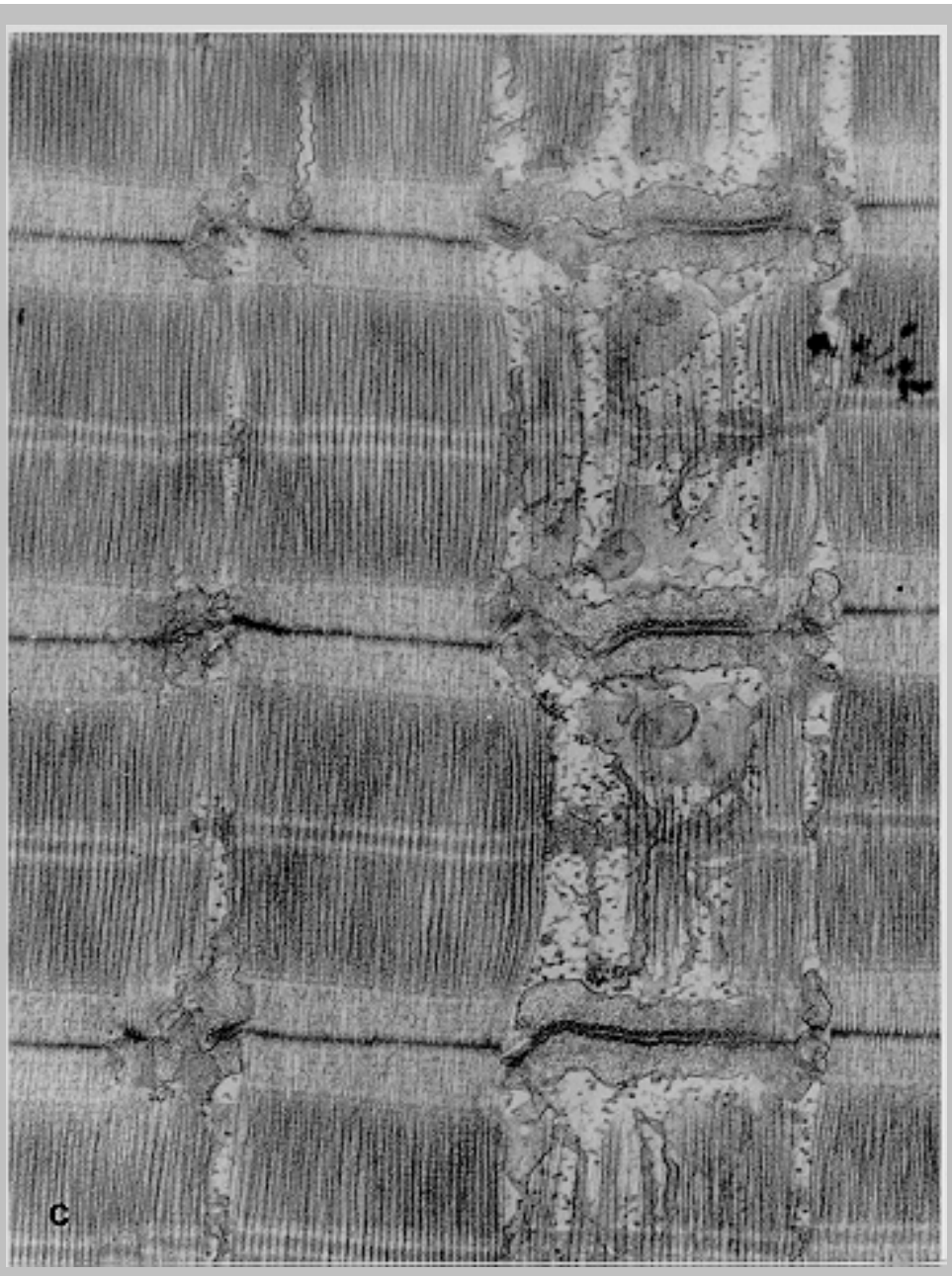


Fig. 29 (a) Tail myotome of guppy. The A and I bands and the Z line are labeled in the left corner at the bottom of the figure. At each Z line a *triad* [the central tubule (part of the T system) and the lateral sacs], is indicated by the triple arrows. The rest of the SR (single, large arrows) is probably continuous with the lateral sacs. The central tubule is convoluted and was originally present in and out of the section. It appears in this section as vesicles. Bar corresponds to 786 nm. From Franzini-Armstrong (1964), with permission. (b) Opening of the T-system to the outside showing continuity with the cell membrane (the plasmalemma or sarcolemma). White twitch fiber from the black mollie. Bar corresponds to 300 nm. From Franzini-Armstrong (1973), with permission. (c) Sarcoplasmic reticulum of frog sartorius muscle. From Peachey,&copy1965. Reproduced from *Journal of Cell Biology* by copyright permission of the Rockefeller University Press.

A reconstruction of the triad is shown in Fig. 31 (Franzini-Armstrong et al., 1987, Block et al., 1988). The T tubule (in the center of the image, toward the viewer) is shown with two terminal cisternae (TC). The lower one is shown occupied by *calsequestrin*, an acidic Ca2+-binding protein of skeletal, cardiac, and smooth muscle (MacLennan et al., 1983). Calsequestrin has a molecular mass of 42 kDa and can bind

approximately 50 Ca^{2+} per molecule. The surface of the TC shows projection of the Ca^{2+}-ATPase, visible also as intramembranous particles. The projections are lacking in the junctional portion of the triad. The large structures of four subunits in the junctional portion are the so-called *junctional feet* (JF) identified in other studies as ryanodine receptors. Calsequestrin binds Ca^{2+} with low affinity (K_d = 1 mM), so that the bound Ca^{2+} is readily avalable for release.

The formation of T-tubules is induced by amphiphysin 2 (Amph 2), a molecule previously shown to induce invaginations at endocytotic sites (see Chapter 9). When expressed in non-muscle cells, an isoform of Amph2 induced tubular structure resembling T-tubules and where it was found in high concentrations (Lee et al., 2002). The induction of tubular structures was found to depend on the presence of a phosphoinositide-binding module in the amphiphysin molecule (binding to 4,5 bisphosphate, PIP_2). Caveolin, thought to have a role in myogenesis is also recruited to the tubules (along with dynamin 2 and Amph 2).

That the SR is involved in triggering a contraction is supported by experiments in which small portions of the muscle fibers are given electrical subthreshold stimuli (Huxley and Taylor, 1958; Huxley, 1969) at the location of the T tubules and in experiments in which direct electrical stimulation is applied to fibers stripped of surface membranes (Constantin and Podolski, 1966; Csapo, 1959). In contrast to the effect of normal stimulation, the responses are local, not propagated, and graded depending on the strength of the stimulus.

The effectiveness of the stimulation varies dramatically with the position of the electrode. In frog muscle, stimulation at the Z line is effective (Huxley, 1959; Huxley and Taylor, 1958), and in the crab and lizard, stimulation between the A and I bands (Huxley, 1959) is effective. These susceptible spots correspond to the location of the openings of the transverse tubules in these animals. The portions of the sarcomeres that contract correspond in distribution to the longitudinal vesicles. These experiments support a role of the SR in the control of muscle contraction and also implicate the T tubules in conducting depolarization.

In the proposed mechanism, Ca^{2+} would be released from the SR to produce a contraction, whereas it would be transported into the vesicles during relaxation. Tests of this premise have been carried out with several approaches. The Ca^{2+} can be detected in a variety of ways. It can be traced by using a radioactive isotope such as $^{45}[Ca^{2+}]$. Alternatively, its concentration can be estimated using Ca^{2+} indicators. These can be dyes such as murexide, which complexes with Ca^{2+} and whose light absorption varies with the degree of binding. Ca^{2+} sensitive dyes were discussed previously in connection with a study of the interaction between Ca^{2+} and Ca^{2+}-transporting enzyme, the Ca^{2+}-ATPase. A convenient indicator is the protein aequorin, extracted from the luminous jellyfish, which fluoresces when complexed with Ca^{2+} so that the light emitted is proportional to the concentration of Ca^{2+} (see Chapter 1) .

The results obtained with these three approaches essentially agree. Muscle can be incubated in the presence of $^{45}[Ca^{2+}]$ and then fixed in either the contracted or in the relaxed state (Winegrad, 1965a,

1965b). The position of the radioactivity viewed with autoradiography then serves to locate the Ca^{2+}. At rest, the $^{45}[Ca^{2+}]$ was found in a position corresponding to the lateral sacs close to the I-band. During contraction it was found to shift to the A-band, where fiber overlap would be expected. The Ca^{2+} can also be followed using Ca^{2+} indicators. The experiments with murexide showed that the amount of free Ca^{2+} inside the muscle fiber increases with contraction (Jobis and O'Connor, 1966). In other experiments in which aequorin was injected into barnacle muscle fibers, the time course of the fluorescent emission preceded the contraction, and the decrease in fluorescence preceded the relaxation. The results are shown in Fig. 32 (Ashley and Ridgeway, 1970). Curve 1 indicates the changes in membrane potential that precede the release of Ca^{2+}, shown as light emission in trace 2. The isometric tension of the muscle, shown in trace 3 (the muscle is held at constant length), follows the release of Ca^{2+} and decreases after the decrease in internal Ca^{2+}. These experiments suggest that Ca^{2+} is indeed the trigger for muscle contraction.

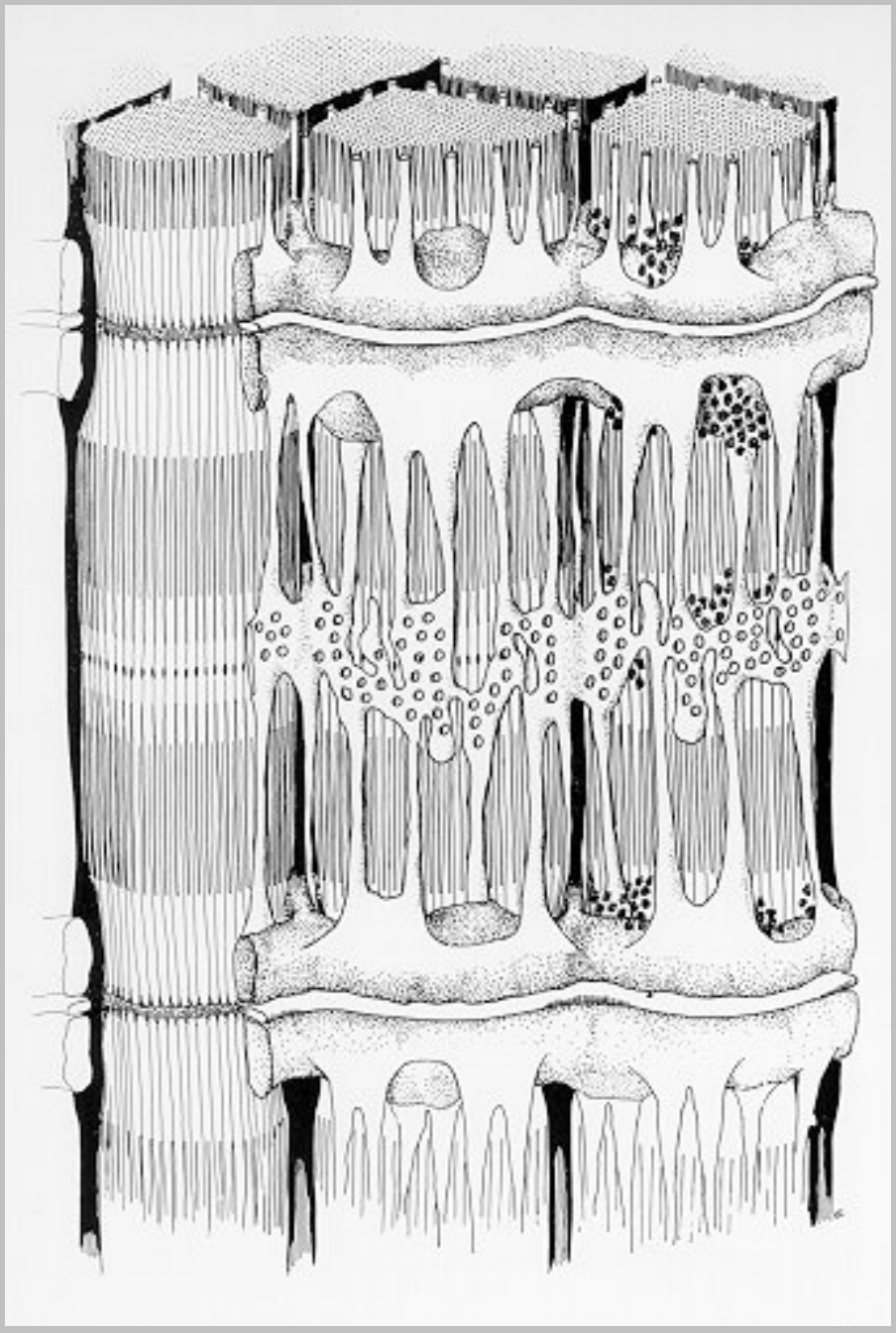


Fig. 30 Three-dimensional reconstruction of the sarcoplasmic reticulum of the frog. From Peachey (&copy1965). Reproduced from *The Journal of Cell Biology*, by copyright permission of the Rockefeller University Press.

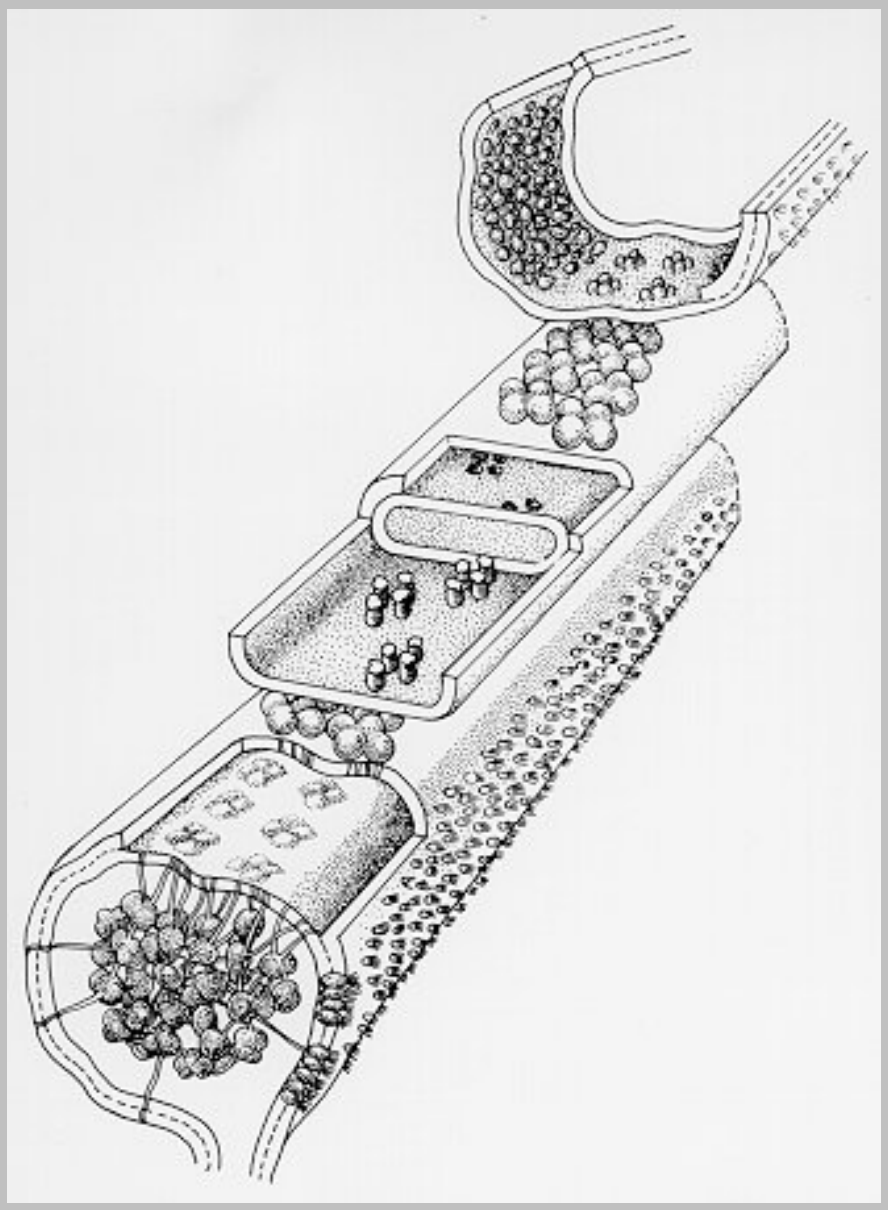


Fig. 31 Three-dimensional reconstruction of a triad. From Franzini-Armstrong et al., (1987) and Block et al., (&copy1988). Reproduced from *The Journal of Cell Biology*, by copyright permission of the Rockefeller University Press.

The portion of the SR that intimately controls the Ca^{2+} release-sequestration cycles must correspond to the *triad*. Physiologically, excitation-contraction must depend on three processes that correspond to the structures. There must be a mechanism of coupling the depolarization of the cell membrane and the release of Ca^{2+}. The release of Ca^{2+} needed for contraction of the myofibril is most likely to involve Ca^{2+} channels of the junctional SR. Removal of the Ca^{2+} by the terminal cisternae involves the operation of Ca^{2+}-ATPase, which we discussed in part in Chapters 20 and 24. In addition, calsequestrin helps retain the Ca^{2+} in the SR and serves as a Ca^{2+} store.

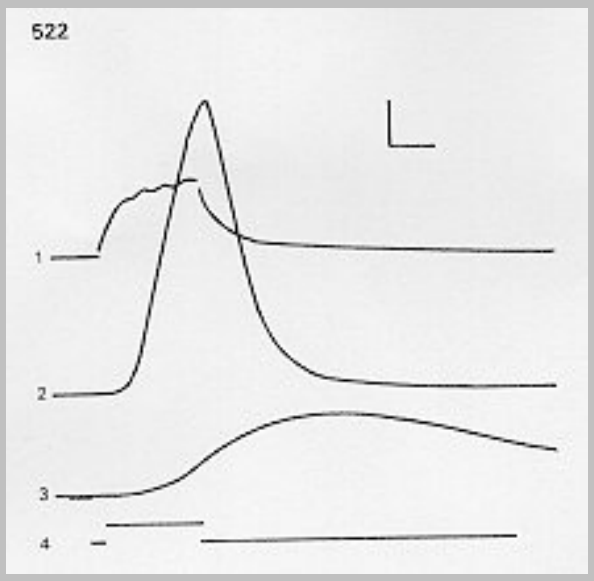


Fig. 32 Results of applying a single depolarizing pulse to a single barnacle muscle fiber. Curve 1, membrane potential; curve 2, light emission; curve 3, isometric tension; curve 4, stimulation marker. Calibration: ordinate, 20 mV (curve 1) or 5 g (curve 2); abscissa, 100 ms. From C. C. Ashley and E. B. Ridgeway, *Journal of Physiology*, 209:105-130, with permission. Copyright &copy1970 The Physiological Society, Oxford, England.

Electrical currents in the T tubules in every way analogous to action potentials are involved in excitation-contraction coupling. Voltage clamp experiments on isolated portions of muscle fibers have demonstrated an inward delayed Na^+ current that follows the current of the action potential as shown in Fig. 33, curve A (Mandrino, 1977). When the Na^+ in the medium is reduced (in this case to half the normal amount), the delayed Na^+ current is also reduced, as shown in Fig. 33, curve B. In agreement with this observation, tetrodotoxin, which blocks Na^+ channels, also delays the current. The delayed current disappears when the T tubules are removed by treatment with glycerol. The presence of Na^+ channels in the T tubules is also supported by the localization of monoclonal antibodies to the Na^+ channels in the T tubules (Haimovich et al., 1987).

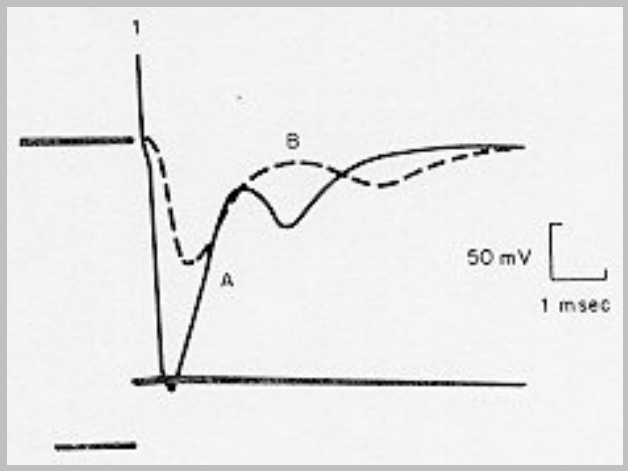


Fig. 33 Effects of decreasing the external sodium concentration on the inward currents. A, Record in normal

Ringer's solution; B, Record in a solution with 50% of the normal sodium concentration. The peak current is approximately 1 x 10^{-7} A. From M. Mandrino, *Journal of Physiology*, 269:605-625, with permission. Copyright &copy1977 The Physiological Society, Oxford, England.

The mechanism of coupling between the T tubules and the terminal cisternae has been studied in detail. The closeness of the association between the terminal cisternae and the T tubules suggests the possibility of a direct mechanical connection between the two. A molecular voltage sensor in the T tubules in contact with the Ca^{2+}-release channels could induce a conformational change in the latter, initiating the Ca^{2+} release. As we shall see, this is probably the main mechanism for the activation. The *voltage-dependent Ca^{2+} channel* (VDCC) (or *dihydropyridine receptor*, DHPR) composed of five subunits, is a voltage sensor (see Catterall, 1991) that undergoes a conformational change responsible for activating the Ca^{2+}-release channels.

The "foot structures" of the terminal cisternae of the SR (Franzini-Armstrong and Nunzi, 1983) are in contact with the transverse tubules. They function as Ca^{2+}-release channels (Hymel et al., 1988) that are blocked by the drug ryanodine (a neutral alkaloid derived from the plant *Ryana speciosa* and generally used as an insecticide) (see Fleischer and Inui, 1989). Binding of ryanodine provides a convenient way to recognize the channel protein during fractionation procedures. The channels have been referred to as *ryanodine receptors* (RyRs). In contrast, in other cells the inositol 1,4,5-tris phosphate receptors (IP_3Rs) act as Ca^{2+}-release channels of the SR and the endoplasmic reticulum (see Chapter 7, section IA or section IF).

The IP_3Rs are tetramers very similar to the RyRs, (see Chadwick et al., 1990). Most cells have a prevalence of one of the two kinds of SR-channels. Smooth muscle cells, cardiomyocytes and Purkinje cerebellar neurons have both at high levels but they have a different spacial distribution in each type of cell (Kijima et al., 1993; Walton et al., 1991). In cardiomyocytes, immunological methods showed the RyR to be at the transverse bands throughout the length of the cells, coincident with the triad junctions at the I-band. Immunogold particles localized the IP_3Rs at the intercalated discs (the structures containing gap junctions and connecting two adjacent cardiomyocytes) of rat ventricular and atrial cardiomyocytes (Kijima et al, 1993

Three different RyRs isoforms have been isolated and reconstituted in bilayers. RyR1 is the release channel of skeletal muscle, RyR2 is predominant in cardiac muscle and the brain, whereas RyR3 is present in other tissues including the brain. Smooth muscle has both RyR2 and RyR3 in minor amounts. The three RyRs have been cloned. They are arranged similarly in bilayers and have similar properties (see Marks, 1997).

The native Ca^{2+}-release channels of striated muscle are constituted by four RyR1 subunits of 565 kDa and four 12 kDa proteins (FKBP12) (Jayamaran et al., 1992). The combination of the two kinds of proteins allow the four subunits, each functioning as a channel, to open in a coordinate fashion to be fully open (Brillantes et al., 1994). Removing FKBP12 from RyR1 produces multiple subconductance levels,

indicating that each subunit is acting independently. FKB12 is also associated with the (IP3R) that has many similarities to RyR (Cameron et al., 1995).

In striated muscle, Ca^{2+} in the micromolar range and adenine nucleotides in the millimolar range activate RyRs channels. Calmodulin (CaM), present in the muscles, increases the open probability of RyRs at Ca^{2+} concentrations corresponding to relaxed muscle. At higher concentrations of Ca^{2+}, it has the opposite effect (see Schneider, 1994).

The cytoplasmic domain of RyR1 projects into the space between the transverse tubule and the SR. Activation of RyR during excitation-contraction coupling, requires the cytoplasmic domain of the α1 subunits of the VDCCs (Tanabe et al., 1990). The excitation-contraction coupling probably involves the physical interaction between these two kinds of channels because pieces of the α1 subunits of VDCCs (the cytoplasmic loop) can activate or inactivate the RyR1 channels (e.g., Lu et al., 1994, 1995; el-Hayek, 1995).

In the arrangement of VDCC and RyR1, four VDCC overlie only every other RyR1 channel (Franzini-Armstrong and Kish, 1995), so that the cytoplasmic loop of VDCC can only be in contact with half of the RyR1 channels. However, studies with RyR1 channels reconstituted in planar bilayers show that two channels are coupled by the presence of FKBP12 so that they open and close simultaneously (Marx et al., 1998).

The cardiac SR has three distinct continuous regions: the network SR, the interior and peripheral junctional SR (jSR) and the corbular SR (cSR) (e.g., Segretain et al., 1981). The network SR consists of vesicular tubules forming a network of connected compartments along the length of the sarcomere. The junctional and corbular SR represent specialized domains that contain electron dense material. The junctional SR is physically connected to either the T tubules or the sarcolemma via feet structures that correspond to the RyRs. The corbular SR is not connected in this way although it might have feet structures protruding into the cytoplasm (Sommer and Johnson, 1979). In agreement with this view the RyR, thought to correspond to the feet structures can be shown by immunofluorescence and immuno-gold techniques to be present in both (Jorgensen et al., 1993).

In the cardiac cells, the area of surface occupied by VDCC in the sarcolemma is equal to that occupied by feet structures in the SR (Protasi et al., 1996). However, they are not positioned in relation to the feet structures; the two sets of channels are close to each other but not intimately connected (Sham et al., 1995). As we shall see the VDCC in cardiac muscle allow entry of Ca^{2+} which in turn triggers the release from the SR.

Depolarization of the sarcolemma and T-tubes of cardiac muscle cells is not enough to produce the Ca^{2+} release from the sarcoplasmic reticulum. Depolarization triggers Ca^{2+}-release from cardiac SR only via activation of the VDCCs in the sarcolemma (e.g., Grantham et al., 1996). This influx of Ca^{2+}, directly or

indirectly induces the release of Ca^{2+} from the sarcoplasmic reticulum (SR) (e.g., Reuter, 1984).

In smooth muscle Ca^{2+} is also released from RyR receptors (see Marks, 1992; Nelson et al., 1995). The end result may be very indirect. For example, increases in local Ca^{2+} concentration can result in vasodilation of the vessels surrounded by the smooth muscle by activation of Ca^{2+}-dependent K^+ channels, an effect that leads to hyperpolarization.

In the mammalian intestine, smooth muscle contractions move from cell to cell in waves controlled by the local nervous system (Furness and Costa, 1987) and the *interstitial cells of Cajal* (Sanders, 1996). A key element in the control of the contractility resides in intracellular concentration of Ca^{2+} which is discharged in waves as shown using a fluorescent Ca^{2+} indicator (introduced as an ester which enters intact cell and is hydrolyzed by esterases, generating and trapping inside the intracellular calcium indicator) (Stevens et al., 1999).

In summary, in cardiac muscle and other tissues the activation of RyR are thought to be mediated not by a conformational change but by intracellular Ca^{2+} entering via the VDCCs. In certain cerebellar neurons, Ca^{2+}-channels activated by glutamate receptors are thought to play a similar role to the VDCCs (Chavis et al., 1996).

Fig. 34 (Wagenknecht et al., 1989) shows an image reconstruction of the isolated RyR1 protein from electron micrographs (see Chapter 1).

Table 2 Possible Role of Ca^{2+} in the Motility of Various Cells

Organisms or cell	Evidence	References
Vorticella	Ca^{2+} activation of contraction	a
Xenopus laevis	Cleavage of eggs requires Ca^{2+}	b
Spirostomum	Release of Ca^{2+} at contraction followed with aequorin	c
Fibroblasts	Ca^{2+} required for contraction, vesicular fraction inhibits contraction	d
Stentor	Contraction requires Ca^{2+}	e

Leukocytes	Demonstration of a contractile protein which requires Ca^{2+} for contraction	f
Amoeba	Ca^{2+} required for contraction of cytoplasm in ruptured cells	g
Physarum polycephalum	Ca^{2+}- requiring actinomyosin for contraction Ca^{2+}-requiring vacuoles Release of Ca^{2+} with electrical charge	h i j

[a] Amos, W.B., *Nature* 229: 127 (1971). [b]Baker, P.F. and Warner, A. E., *J. Cell Biol.*. 53: 579 (1972). [c]Ettienne, E.M., *J. Gen. Physiol.* 56: 168 (1970). [d]Kinoshita S. et al., *Biochim. Biophys. Acta* 79: 88 (1964). [e]Huang, B. and Pitelka, D.R., *J. Cell Biol.* 57: 704 (1973). [f]Shibata, N. et al., *Biochim. Biophys. Acta* 256: 565 (1972). [g]Taylor, D.L. et al., *J. Cell Biol.* 59: 378 (1973). [h]Kato, T. and Tonomura, Y. *J. Biochem.* 81: 207 (1977). [i]Rdigway, E.B. and Durham, A.C.H., *J. Cell Biol.*. 69: 223 (1976).

Many studies have suggested that Ca^{2+} activation also occurs in primitive motile systems. The freshwater protozoan *Spirostomum ambiguum*, for example, contracts when stimulated electrically. Release of Ca^{2+}, detected by the aequorin assay, coincides with the contraction. Removal of Ca^{2+} coincides with relaxation (Ettienne, 1970).

A role of Ca^{2+} is also indicated in the ciliary motion of *Paramecium* (Eckert, 1972) and in other systems as well, but the evidence is indirect. Some examples of the indirect evidence for Ca^{2+} involvement are shown in Table 2.

The initiation of chromosomal movement in the mitotic spindle also seems to be triggered by a release of Ca^{2+} from vesicles distributed in the mitotic spindle (Hepler, 1980; Hepler and Callahan, 1987).

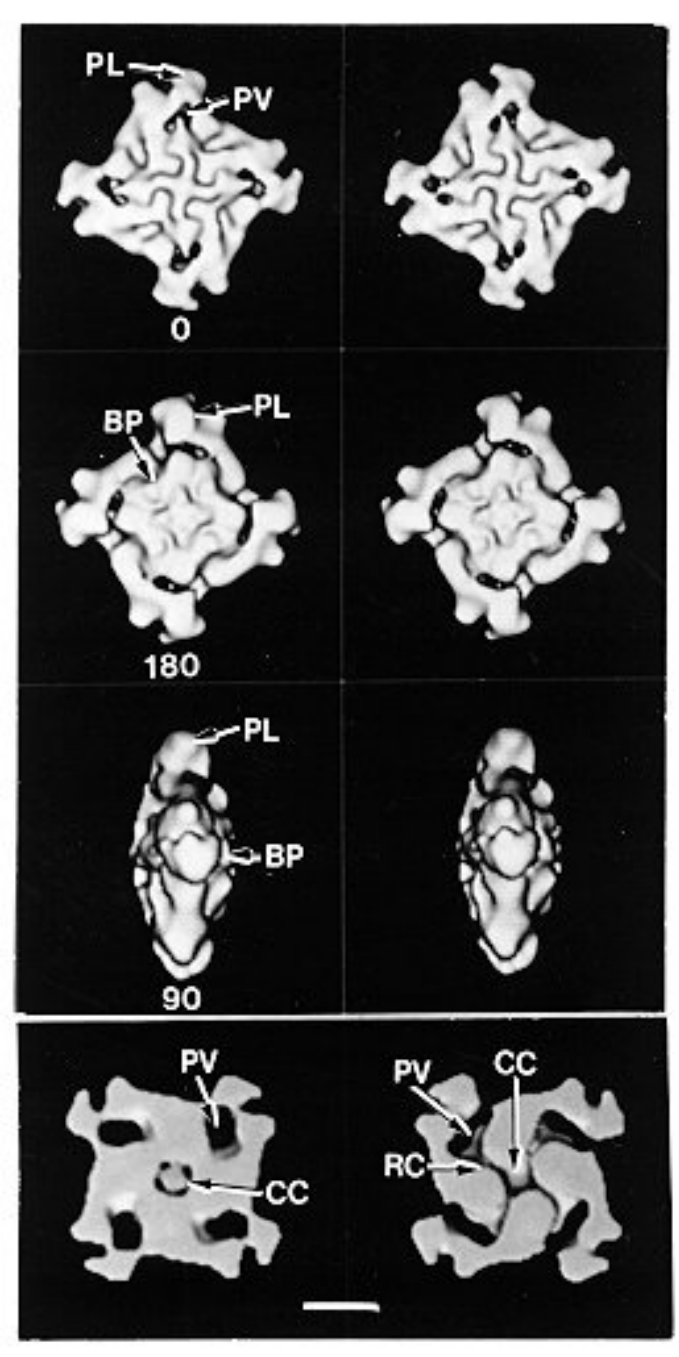


Fig. 34 Computer-generated surface representations of the three-dimensionally reconstructed junctional channel complex. (a-c) Stereo pairs of the reconstruction in various orientations related by rotation about a vertical axis. (d and e) The two complementary halves of the reconstruction after slicing it in half to reveal internal structural features. In (e) the view is from the interior of the channel toward the surface adsorbed to the grid (the "platform" side) and in (d) it is toward the T-tubule surface. Abbreviations: BP, base platform; PL, peripheral lobes; PV, peripheral vestibules; CC, central channel; RC, radial channels. Scale bar, 10 nm. Reproduced with permission from *Nature* , Wagenknecht, T., Grassucci, R., Frank, J., Saito, A., Inui, M. and Fleischer, S. (1989) Three-dimensional architecture of the calcium channel/foot structure of sarcoplasmic reticulum, *Nature* 338:167-170. Copyright &copy1989 MacMillan Magazines Ltd.

Relaxation follows the removal of Ca^{2+}. Ca^{2+} is removed by the Ca^{2+}-transport system of the SR, a process likely to be facilitated by the presence of parvalbumin in the sarcoplasm.

Parvalbumins constitute a family of soluble intracellular proteins of approximately 11 kDa, present in the cytoplasm of certain cells in high concentrations. In striated muscle of fast fish the parvalbumin concentration is in the range of 0.3-0.5 mM. One molecule of parvalbumin binds 2 Ca^{2+} with high affinity (10^8 M^{-1}) and therefore this protein can serve as a calcium buffer. Parvalbumin has a role in relaxation, at least in frog muscle, where Ca^{2+} replaces Mg^{2+} and acts in parallel with the sarcoplasmic Ca^{2+}-sequestering system (Rall, 1996).

SUGGESTED READING

General

Amos, L. A. and Amos, W. B. (1991) *Molecules of the Cytoskeleton*, The Garland, The Guilford Press, New York.

Adams, R. and Pollard, T. D. (1989) Prediction of common properties of particle translocation motors through comparison of myosin I, cytoplasmic dynein, and kinesin, In *Cell Movement*, Vol. 2. pp 3-10. Liss, New York.

Bárány,M. and Bárány, K. graduate course lectures, Biochemistry of muscle contraction. Updated: March 2000. http://www.uic.edu/classes/phyb/phyb516/

Block, S.M. (1995) One small step for myosin, *Nature* 378:132-133. (Medline)

Bray, D. (1992) *Cell Movements*, Garland Publishing Inc., New York and London, Chapters 6, 8, 11 and 16.

Vale, R.D. (1996) Switches, latches and amplifiers: common themes of G proteins and molecular motors, *J. Cell Biol.* 135:291-302. (Medline)

Vallee, R.B. and Sheetz, M.P. (1996) Targeting of motor proteins, *Science* 271:1539-1544. (Medline)

Actins and actin-binding proteins

Bretscher, A. (1991) Molecular aspects of microfilament structure and assembly. *Curr. Opin. Struct. Biol.* 1:281-287.

Hartwig. J. H. and Kwiatkowski, D. J. (1991) Actin-binding proteins. *Curr. Opin. Cell Biol.* 3:87-97. (Medline)

Myosins

Baker, J.P. and Titus, M.A. (1998) Myosins: matching function with motors, *Curr. Opin. Cell Biol.* 10:80-86. (Medline)

Irving, M. and Piazzesi, G. (1997) Motions of myosin heads that drive muscle contraction, *News in Physiol Scie.* 12:249-254.

Mermall V., Post P.L. and Mooseker M.S. (1998) Unconventional myosins in cell movement, membrane traffic, and signal transduction, *Science* 279:527-533. (Medline)

Microtubule-based motors

Endow, S.A. (1999) Determinants of molecular motor directionality, *Nature Cell Biol.* 1:E163-E167. (Medline)

Endow, S.A. and Fletterick, R.J. (1998) Reversing a 'backwards' motor, *BioEssays* 20:108-112.

Gee, M. and Vallee, R. (1998) The role of the dynein stalk in cytoplasm and flagellar motility, *Eur. Biophys. J.* 27:466-473. (Medline)

Hirokawa, N., Noda, Y. and Okada, Y. (1998) Kinesin and dynein superfamily proteins in organelle transport and cell division, *Curr. Opin. Cell Biol.* 10:60-73. (Medline)

Lane, J. and Allan, V. (1998) Microtubule-based membrane movement, *Biochim. Biophys. Acta* 1376:27-55. (Medline)

Excitation-contraction coupling

Caswell, A. H. and Brandt, N. R. (1989) Does muscle activation occur by direct mechanical coupling of tranverse tubules to the sarcoplasmic reticulum? *Trends Biochem. Sci.* 14:161-165. (Medline)

Franzini-Armstrong, C., Protasi, F. (1997) Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions, *Physiol. Rev.* 77:699-729. (Medline)

Martonosi, A. N. (1983) The regulation of cytoplasmic Ca^{2+} concentration in muscle and non-muscle cells. In *Muscle and Nonmuscle Motility*. Vol. 1, pp. 233-357 (Stracher, A., ed.). Academic Press, New York.

Rüegg, J. C. (1986) Vertebrate smooth muscle. In *Calcium in Muscle Activation*, Chapter 8, pp. 201-238. Springer-Verlag, Berlin.

Somlyo, A. P. (1984) Cellular site of calcium regulation. *Nature* 309:516-517. (Medline)

Somlyo, A. P. and Himpens, B. (1989) Cell calcium and its regulation in smooth muscle. *FASEB J.* 3:2266-2276. (Medline)

Molecular mechanisms of motion

Brokaw, C. J. and Johnson, K. A. (1989) Perspectives, dynein induced microtubule sliding and force generation. In *Cell Movement*, Vol. I, pp. 191-198. Liss, New York.

Huxley, H. E. (1990) Minireview: Sliding filaments and molecular motile system, *J. Biol. Chem.* 265:8347-8350. (Medline)

Huxley, A.F. (1998) Biological motors: Energy storage in myosin molecules, *Curr. Biol.* 8:R485-R488. (Medline)

Rayment I. (1996) The structural basis of the myosin ATPase activity, *J. Biol. Chem.* 271:15850-15853. (MedLine)

Rüegg, C., Veigel, C., Molloy, J.E., Schmitz, S., Sparrow, J.C. and Fink, R.H. (2002) Molecular motors: force and movement generated by single myosin II molecules, *News Physiol. Sci.* 17:213-218. (MedLine)

Satir, P. (1989) Mechanism of ciliary movement - what's new. *News Physiol. Sci.* 4:153-157.

Smith, C.A. and Rayment, I. (1996) Active site comparisons highlight structural similarities between myosin and other P-loop proteins, *Biophys. J.* 70:1590-1602. (MedLine)

Vale, R.D. and Milligan, R.A. (2000) The way things move: looking under the hood of molecular motor proteins, *Science* 288:88-95. (MedLine)

Online animated models of myosin and kinesin motors: www.sciencemag.org/feature/data/1049155.shl

Volkmann, N. and Hanein, D. (2000) Actomyosin: law and order in motility, *Curr. Opin. Cell Biol.* 12:26-34. (MedLine)

Intermediate filaments

Albers, K. and Fuchs, E. (1992) The molecular biology of intermediate filament proteins, *Int. Rev. Cytol.* 134:243-279. (Medline)

Fuchs, E. and Cleveland. D.W. (1998) A structural scaffolding of intermediate filaments in health and disease, *Science* 279:514-519. (MedLine)

Houseweart, M.K. and Cleveland, D.W. (1998) Intermediate filaments and their associated proteins: multiple dynamic personalities *Curr. Opin. Cell Biol.* 10:93-101. (Medline)

Nixon, R.A. (1998) The slow axonal transport of cytoskeletal proteins, *Curr. Opin. Cell Biol.* 10:87-92. (Medline)

WEB RESOURCES

Barth, A. and de Hostos, E.L Filaments on the move: cells expressing GFP-actin or tubulin. http://www-bioc.rice.edu/~hostos/gfptubMDCK.html

Doyle, T. Yeast actin cytoskeleton, GFP fusions to the yeast actin gene http://genome-www.stanford.edu/group/botlab/people/doyle.html

Duke University Medical Center: Movies created from data analysis of a fusion of the Ncd microtubule motor protein to the green fluorescent protein of jellyfish http://abascus.mc.duke.edu/moviepage.html

Greene, L. and Henikoff, S. Kinesin home page. www.blocks.fhcrc.org/~kinesin/.

Kaech, S., Ludin, B. and Matus, A. Cytoskeletal plasticity in cells expressing neuronal microtubule associated proteins. http://www.fmi.ch/groups/AndrewMatus/Video.html

Section of the Cell Biology & Cytoskeleton Group Division of Hematology, Brigham & Women's Hospital, Harvard Medical School, Research Overview : information and movies in relation to actin crosslinking, actin dynamics, actin severing and capping, cell motility and mechanics, cytoskeletal polymer physics and rheology, genetics of motility, interactions between cytoskeletal systems (e.g., neurofilaments-microtubules interactions and signaling to the actin cytoskeleton.

Vale, R.D. and Milligan, R.A. (2000) *Science* Online. Animated models of myosin and kinesin motors. http://www.sciencemag.org/feature/data/1049155.shl

Waddle et al. Movement of Cortical Actin Patches in Yeast
http://www.cooperlab.wustl.edu/Waddle_Ms:Waddle_Ms.html

REFERENCES

Search the textbook

Back to Chapter 24

REFERENCES

Adams, R. J. and Pollard, T. D. (1986) Propulsion of organelles isolated from Acanthamoeba along actin filaments by myosin I, *Nature* 322:754-756 (MedLine)

Albers, K.M. (1996) Keratin biochemistry, *Clin. Dermatol.* 14: 309-320. (MedLine)

Allan, V. J. and Kreis, T. E. (1987) A microtubule-binding protein associated with membranes of the Golgi apparatus, *J.Cell Biol.* 103:2229-2239. (MedLine)

Anderson, R. A. and Marchesi, V. T. (1985) Associations between glycophorin and protein 4.1 are modulated by polyphosphoinositides: a mechanism for membrane skeletal regulation, *Nature* 318:295-298. (MedLine)

Andra, K., Lassmann, H., Bittner, R., Shorny, S., Fassler, R., Propst, F. and Wiche G. (1997) Targeted inactivation of plectin reveals essential function in maintaining the integrity of skin, muscle, and heart cytoarchitecture, *Genes Dev.* 11:3143-3156. (MedLine)

Andra, K., Nikolic, B., Stocher, M., Drenckhahn, D. and Wiche, G. (1998a) Not just scaffolding: plectin regulates actin dynamics in cultured cells, *Genes Dev.* 12:3442-3451. (MedLine)

Andra, K., Lassmann, H., Bittner, R., Shorny, S., Fassler, R., Propst, F., Wiche, G. (1998b) Targeted inactivation of plectin reveals essential function in maintaining the integrity of skin, muscle, and heart cytoarchitecture, *Genes Dev.* 11:3143-3156. (MedLine)

Anson, M., Geeves, M.A., Kurzawa, S.E. and Manstein, D.J. (1996) Myosin motors with artificial lever arms, *EMBO J.* 15:6069-6074. (MedLine)

Arata, T. and Shimizu, H. (1981) Spin-label study of actin- myosin-nucleotide interactions in contracting glycerinated muscle fibers, *J. Mol. Biol.* 151:411-437. (MedLine)

Arnal, L., Metoz, E., DeBonis, S. and Wade, R.H. (1996) Three-dimensional structure of functional motor proteins on microtubules, *Curr. Biol.* 6:1265-1270. (MedLine)

Ashley, C. C. and Ridgeway, E. B. (1970) On the relationship between membrane potential, calcium

transient and tension in single barnacle muscle fibers, *J. Physiol. (London)* 209:105-130. (MedLine)

Assad, J.A. and Corey, D.P. (1992) An active motor model for adaptation by vertebrate hair cells, *J. Neurosci.* 12:3291-3309. (MedLine)

Atkinson S.J., Doberstein, S.K. and Pollard, T.D. (1992) Moving off the beaten tracks, *Curr. Biol.*2:326-328.

Ayscough KR. (1998) In vivo functions of actin-binding proteins, *Curr. Opin. Cell Biol.* 10:102-111. (MedLine)

Bahadoran, P., Aberdam, E., Mantoux, F., Busca, R., Bille, K., Yalman, N., de Saint-Basile, G., Casaroli-Marano, R., Ortonne, J.P. and Ballotti, R. (2001) Rab27a: A key to melanosome transport in human melanocytes, *J. Cell Biol.* 152:843-850. (MedLine)

Baribault, H.R., Blouin, R., Bourgon, L., and Marceau, N. (1989) Epidermal growth factor-induced selective phosphorylation of cultured rat hepatocyte 55 kD cytokeratin before filament reorganization and DNA synthesis, *J. Cell Biol.* 109:1665-1676. (MedLine)

Barylko, B., Wagner, M.C., Reizes, O. and Albanesi, J.P. (1992) Purification and characterization of a mammalian myosin I, *Proc. Natl. Acad. Sci. USA* 89:490-494. (MedLine)

Bearer, E.L., DeGriogis, J.A., Bodner, R.A., Kao, A., and Reese, T.S. (1993) Evidence for myosin motors on organelles in squid axoplasm, *Proc. Natl. Acad. Sci. USA* 90:11252-11256. (MedLine)

Bement, W.M., Hasson, T., Wirth, J.A., Cheney, R.E. and Mooseker, M.S. (1994a) Identification and overlapping expression of multiple unconventional myosins in vertebrate cell types, *Proc. Natl. Acad. Sci. USA* 91:6549-6553. (MedLine)

Bement, W.M., Wirth, J.A. and Mooseker, M.S. (1994b) Cloning and mRNA expression of human unconventional myosin-IC: a homolog of amoeboid myosin-I with a single IQ motif and an SH3 domain, *J. Mol. Biol.* 243:356-363. (MedLine)

Benian, G.M, Kiff, J.E., Neckelmann, N., Moerman, D.G. and Waterston, R.H. (1989) Sequence of an unusually large protein implicated in regulation of myosin activity in *C. elegans*, *Nature* 342:45-50. (MedLine)

Bennett, V. (1990) Spectrin: structural mediator between diverse plasma membrane proteins and the cytoplasm, *Curr. Opin. Cell Biol.* 2:51-56. (MedLine)

Berliner, E., Young, E.C., Anderson, K., Mahtani, H.K. and Gelles, J. (1995) Failure of a single-headed kinesin to track parallel to microtubule protofilaments, *Nature* 373:718-721. (MedLine)

Bernier, G., Brown, A., Dalpe, G., Mathieu, M., De Repentigny, Y. and Kothary, R. (1995) Dystonin transcripts are altered and their levels are reduced in the mouse neurological mutant dt24J, *Biochem. Cell Biol.* 73:605-609. (MedLine)

Bershitsky, S.Y., Tsaturyan, A.K., Bershitskaya, O.N., Mashanov, G.I., Brown, P., Burns, R. and Ferenczi, M.A. (1997) Muscle force is generated by myosin heads stereospecifically attached to actin, *Nature* 388:186-190. (MedLine)

Blangy, A., Arnaud, L. and Nigg, E.A. (1997) Phosphorylation by p34cdc2 protein kinase regulates binding of the kinesin-related motor HsEg5 to the dynactin subunit p150, *J. Biol. Chem.* 272(31):19418-19424. (MedLine)

Block, S.M. (1996) Fifty ways to love your lever: myosin motors, *Cell* 87:151-157. (MedLine)

Block, S.M. (1998) Kinesin: what gives? *Cell* 93:5-8. (MedLine)

Block, B. A., Imagawa. T., Campbell. K. P. and Franzini-Armstrong, C. (1988) Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle, *J. Cell Biol.* 107:2587-2600. (MedLine)

Block, S. M., Goldstein. L. S. B. and Schnapp, B. J. (1990) Dead movement by single kinesin molecules studied with optical tweezers, *Nature* 348:348-352. (MedLine)

Bloom, G. S., Wagner, M. C., Pfister, K. and Brady, S.T. (1988) Native structure and physical properties of bovine brain kinesin, and identification of the ATP-binding polypeptide, *Biochem.* 27:3409-3416. (MedLine)

Bohl, F., Kruse, C., Frank, A., Ferring, D. and Jansen, R.P. (2000) She2p, a novel RNA-binding protein tethers ASH1 mRNA to the Myo4p myosin motor via She3p, *EMBO J.* 19:5514-5524. (MedLine)

Bowman, A.B., Patel-King, R.S., Benashski, S.E., McCaffery, J.M., Goldstein, L.S. and King, S.M. (1999) *Drosophila* roadblock and *Chlamydomonas* LC7: a conserved family of dynein-associated proteins involved in axonal transport, flagellar motility, and mitosis, *J. Cell Biol.* 146:165-180. (MedLine)

Bowman, A.B., Kamal, A., Ritchings, B.W., Philp, A.V., McGrail, M., Gindhart, J.G. and Goldstein, L.S. (2000) Kinesin-dependent axonal transport is mediated by the sunday driver (SYD) protein, *Cell* 103:583-594. (MedLine)

Brady, S. T., Lasek, R. J. and Allen, R. D. (1982) Fast axonal transport in extruded axoplasm from squid giant axon, *Science* 218:1129-1131. (MedLine)

Brady, S. T., Lasek, R. J. and Allen, R. D. (1985) Video microscopy of fast axonal transport in extruded axoplasm: a new model for study of molecular mechanisms, *Cell Motil.* 5:81-101. (MedLine)

Brady, S.T., Pfister, K.K. and Bloom, G.S. (1990) A monoclonal antibody against kinesin inhibits both anterograde and retrograde fast axonal transport in squid axoplasm, *Proc. Natl. Acad. Sci. USA* 87:1061-1065. (MedLine)

Bremer, A. and Aebi, U. (1992) The structure of the F-actin filament and the actin molecule, *Curr. Opin. Cell Biol.* 4:20-26. (MedLine)

Bresnick, A., Warren, V. and Condeelis, J. (1990) Identification of a short sequence essential for actin binding by Dictyostelium ABP-120, *J. Biol. Chem.* 265:9236-9240. (MedLine)

Bretscher, A. (1991) Microfilament structure and functionin the cortical cytoskeleton, *Ann. Rev. Cell Biol.* 7:337-374. (MedLine)

Brillantes, A.B., Ondrias, K., Scott, A., Kobrinsky, E., Ondriasova, E., Moschella, M.C., Jayaraman, T., Landers, M., Ehrlich, B.E. and Marks, A.R. (1994) Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein, *Cell* 77:513-523. (MedLine)

Brokaw, C.J. (1991) Microtubule sliding in swimming sperm flagella: direct and indirect measurements on sea urchin and tunicate spermatozoa, *J. Cell Biol.* 114:1201-1215. (MedLine)

Burgess, S.A. (1995) Rigor and relaxed outer dynein arms in replicas of cryofixed motile flagella, *J. Mol. Biol.* 250:52-63. (MedLine)

Burgess, S.A., Walker, M.L., Sakakibara, H., Knight, P.J. and Oiwa, K. (2003) Dynein structure and power stroke, *Nature* 421:715-718.

Burkhardt, J.K., Echeverri, C.J., Nilsson, T. and Vallee, R.B. (1997) Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution, *J. Cell Biol.* 139:469-484. (MedLine)

Buss, F., Arden, S.D., Lindsay, M., Luzio, J.P. and Kendrick-Jones, J. (2001) Myosin VI isoform localized to clathrin-coated vesicles with a role in clathrin-mediated endocytosis, *EMBO J.* 20:3676-3784. (MedLine)

Caldwell, P. C. and Walster, G. (1963) Studies on the microinjection of various substances into crab muscle fibers, *J. Physiol. (London)* 169:353-373

Cameron, A.M., Steiner, J.P., Sabatini, D.M., Kaplin, A.I., Walensky, L.D. and Snyder, S.H. (1995) Immunophilin FK506 binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux, *Proc. Natl. Acad. Sci. USA* 92:1784-1788. (MedLine)

Carlier, M.F. and Pantaloni, D. (1997) Control of actin dynamics in cell motility, *J. Mol. Biol.* 269:459-467. (MedLine)

Carlier, M.F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G.X., Hong, Y., Chua, N.H. and Pantaloni, D. (1997) Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility, *J. Cell Biol.* 136:1307-1322. (MedLine)

Case, R.B., Pierce, D.W., Hom-Booher, N., Hart, C.L. and Vale, R.D. (1997) The directional preference of kinesin motors is specified by an element outside of the motor catalytic domain, *Cell* 90:959-966. (MedLine)

Catlett, N.L., Duex, J.E., Tang, F. and Weisman, L.S. (2000) Two distinct regions in a yeast myosin-V tail domain are required for the movement of different cargoes, *J. Cell Biol.* 150:513-526. (MedLine)

Catterall, W.A. (1991) Excitation-contraction coupling in vertebrate skeletal muscle: a tale of two calcium channels, *Cell* 64:871-874. (MedLine)

Catlett, N.L and Weisman, L.S. (1998) The terminal tail region of a yeast myosin-V mediates its attachment to vacuole membranes and sites of polarized growth, *Proc. Natl. Acad. Sci. USA* 95:14799-14804.

Chadwick, C.C., Saito, A. and Fleischer, S. (1990) Isolation and characterization of the inositol trisphosphate receptor from smooth muscle, *Proc. Natl. Acad. Sci. USA* 87:2132-2136. (MedLine)

Chavis, P., Fagni, L. and Lansman, J.B. (1996) Functional coupling between ryanodine receptors and L-type calcium channels in neurons, *Nature* 382:719-722. (MedLine)

Chen, J. C. W. and Kamiya, N. (1975) Localization of myosin in the internodal cell of Nitella as suggested by differential treatment with N-ethylmaleimide, *Cell Struct. Funct.* 1:1-9.

Chen, Y.-T. and Schliwa, M. (1990) Direct observation of microtubules dynamics in *Reticulomyxa*: unusually rapid length changes and microtubule sliding, *Cell Motil. Cytoskeleton* 17:214-226. (MedLine)

Chen, M.J., Shih, C.L. and Wang, K. (1993) Nebulin as an actin zipper. A two-module nebulin fragment

promotes actin nucleation and stabilizes actin filaments, *J. Biol. Chem.* 268:20327-20334. (MedLine)

Cheney, R.E. and Mooseker, M.S. (1992) Unconventional myuosins, *Current Opin. in Cell Biol.* 4:27-35. (MedLine)

Cheney, R.E., Oshea, M.K., Heuser, J.E., Coelho, M.V., Wolenski, J.S., Espreafico, E.M., Forscher, P., Larson, R.E. and Mooseker, M.S. (1993a) Brain myosin-V is a two headed unconventional myosin with motor activity, *Cell* 75:13-23. (MedLine)

Cheney, R., Hirokawa, N., Levine, J. and Willard, M. (1983b) Intracellular movement of fodrin, *Cell Motil.* 3:649-655. (MedLine)

Chou, Y.H., Skalli, O., Goldman, R.D. (1997) Intermediate filaments and cytoplasmic networking: new connections and more functions, *Curr. Opin. Cell Biol.* 9:49-53. (MedLine)

Clubb, B.H., Chou, Y.H., Herrmann, H., Svitkina, T.M., Borisy, G.G. and Goldman, R.D. (2000) The 300-kDa intermediate filament-associated protein (IFAP300) is a hamster plectin ortholog, *Biochem. Biophys. Res. Commun.* 273:183-187. (MedLine)

Collins, J.H. (1976) Structure and evolution of troponin C and related proteins, *Symp. Soc. Exp. Biol.* 30:303-334.

Colucci-Guyon, E., Portier, M.M., Dunia, I., Paulin, D., Pournin, S. and Babinet, C., (1994) Mice lacking vimentin develop and reproduce without an obvious phenotype, *Cell* 79:679-694. (MedLine)

Coluccio, L.M. (1991) Identification of the microbillar 110-kDa calmodulin complex (myosin-1) in kidney, *Eur. J. Cell Biol.*56:286-294. (MedLine)

Coluccio, L. M. and Bretscher, A. (1988) Mapping of the microvillar l00K-calmodulin complex: calmodulin-associated or -free fragments of the 110-kD polypeptide bind F-actin and retain ATPase activity, *J. Cell Biol.* 106:367-373. (MedLine)

Coluccio, L. M. and Bretscher, A. (1989) Reassociation of microvillar core proteins: making a microvillar core in vitro, *J. Cell Biol.* 108:495-502. (MedLine)

Compton, D.A. (1998) Focusing on spindle poles, *J. Cell Sci.* 111:1477-1481 (MedLine)

Constantin, L. L. and Podolosky, R. J. (1966) Evidence for the depolarization of the internal membrane system in activation of the frog semitendinosus muscle, *Nature* 210:483-486.

Constantin, L. L., Franzini-Armstrong, C. and Podolsky, R. J. (1965) Localization of calcium-accumulating structures in striated muscle fibers, *Science* 147:158-159.

Cooke, R., Crowder, M.S. and Thomas, D.D. (1982) Orientation of spin labels attached to cross-bridges in contracting muscle fibres, *Nature* 300:776-778. (MedLine)

Cope, M.J.T.V., Whisstock, J., Rayment, I. and Kendrick-Jones, J.K. (1996) Conservation within the myosin motor domain: implications for structure and function, *Structure* 4:969-987. (MedLine)

Corrie, J.E., Craik, J.S. and Munasinghe, V.R. (1998) A homobifunctional rhodamine for labeling proteins with defined orientations of a fluorophore, *Bioconjug. Chem.* 9:160-167. (MedLine)

Corrie, J.E., Brandmeier, B.D., Ferguson, R.E., Trentham, D.R., Kendrick-Jones, J., Hopkins, S.C., van der Heide, U.A., Goldman, Y.E., Sabido-David, C., Dale, R.E., Criddle, S. and Irving, M. (1999) Dynamic measurement of myosin light-chain-domain tilt and twist in muscle contraction, *Nature* 400:425-430. (MedLine)

Cossart, P. (1995) Actin-based bacterial motility, *Curr. Opin. Cell Biol.* 7:94-101. (MedLine)

Coy, D.L., Hancock, W.O., Wagenbach, M. and Howard, J. (1999) Kinesin's tail domain is an inhibitory regulator of the motor domain, *Nature Cell Biol.* 1:288-292. (MedLine)

Craig, S. W. and Pollard, T. D. (1982) Actin binding proteins, *Trends Biochem. Sci.* 7:88-91.

Cross, R.A. (1996) A protein-making motor protein, *Nature* 385:18-19. (MedLine)

Csapo, A. (1959) Studies on excitation-contraction coupling, *Ann. N.Y. Acad. Sci.* 81:453-467. (MedLine)

Cyr, J.L., Pfister, K.K., Bloom, G.S., Slaughter, C.A. and Brady, S.T. (1991) Molecular genetics of kinesin light chains: generation of isoforms by alternative splicing, *Proc. Natl. Acad. Sci. USA.* 88:10114-10118. (MedLine)

Daley, J., Southgate, R. and Ayme-Southgate. A. (1998) Structure of the *Drosophila* projectin protein: isoforms and implication for projectin filament assembly, *J. Mol. Biol.* 279:201-210. (MedLine)

Dalpe, G., Mathieu, M., Comtois, A., Zhu, E., Wasiak, S., De Repentigny, Y., Leclerc, N. and Kothary, R. (1999) Dystonin-deficient mice exhibit an intrinsic muscle weakness and an instability of skeletal muscle cytoarchitecture, *Dev. Biol.* 210:367-380. (MedLine)

Davidson, P. F. and Taylor, E. W. (1960) Physical-chemical studies of proteins of squid nerve axoplasm,

with special reference to the axon fibrous protein, *J. Gen. Physiol.* 43:801-803.

de Arruda, M. V., Watson, S., Lin, C.-S., Leavitt. J. and Matsudaira, P. (1990) Fimbrin is a homologue of cytoplasmic phosphoprotein plastin and has domains homologous with calmodulin and actin gelation proteins, *J.*Cell Biol. 111:1069-1079. (MedLine)

de Hostos, E.L. (1999) The coronin family of actin-associated proteins, *Trends in Cell Biol.* 9:345-349. (MedLine)

de Hostos, E.L., Bradtke, B., Lottspeich, F., Guggenheim, R. and Gerisch, G. (1991) Coronin, an actin binding protein of *Dictyostelium discoideum* localized to cell surface projections, has sequence similarities to G protein beta subunits *EMBO J.* 10:4097-4104. (MedLine)

de Hostos, E.L., Rehfuess, C., Bradtke, B., Waddell, D.R., Albrecht, R., Murphy, J. and Gerisch, G. (1993) *Dictyostelium* mutants lacking the cytoskeletal protein coronin are defective in cytokinesis and cell motility, *J. Cell Biol.* 120:163-173. (MedLine)

Dekker-Ohno, K., Hayasaka, S., Tagagishi, Y., Oda, S., Wakasugi, N., Mikoshiba, K., Inouye, M. and Yamamura, S (1996) Endoplasmic reticulum is missing in dendritic spines of Pukinje-cells in ataxic mutant rat, *Brain Res.* 714: 226-230. (MedLine)

DeLozanne, A. and Spudich, J.A. (1987) Disruption of *Dictyostelium* myosin heavy chain gene by homologous recombination, *Science* 236:1086-1091. (MedLine)

Devreotes, P., Fontana, D., Klein, P., Shening, J. and Theibert, A. (1987) Trans-membrane signaling in *Dictyostelium*, *Methods Cell Biol.* 28:489-496.

Diefenbach, R.J., Mackay, J.P., Armati, P.J. and Cunningham, A.L. (1998) The C-terminal region of the stalk domain of ubiquitous human kinesin heavy chain contains the binding site for kinesin light chain, *Biochemistry* 37:16663-16670. (MedLine)

Doberstein,S.K., Baines, I.C., Wiegand, G., Korn, E.D. and Pollard, T.D. (1993) Inhibition of contractile vacuole function *in vivo* by antibodies against myosin-I, *Nature* 365:841-843. (MedLine)

Dominguez, R., Freyzon, Y., Trybus, K.M. and Cohen, C. (1999) Crystal structure of a vertebrate smooth muscle myosin motor domain and its complex with the essential light chain: visualization of the pre-power stroke state, *Cell* 94:559-571. (MedLine)

Dufort, P.A. and Lumsden, C.J. (1996) How profilin/barbed-end synergy controls actin polymerization: a kinetic model of the ATP hydrolysis circuit, *Cell Motil. Cytoskel.* 35:309-330. (MedLine)

Durrbach, A., Collins, K., Matsudaira, P., Louvard, D. and Courdrier, E. (1996) Brush border myosin-I truncated in the motor domain impairs the distribution and the function of endocytotic compartments inan hepatoma cell line, *Proc. Natl. Acad. Sci. USA* 93:7053-7058. (MedLine)

Ebashi, S., Ohtsuki, J. and Mihashi, K. (1972) Regulatory proteins of muscle with special reference to troponin, *Cold Spring Harbor Symp. Quant. Biol.* 37:215-223.

Echard, A., Jollivet, F., Martinez, O., Lacapere, J.J., Rousselet, A., Janoueix-Lerosey, I. and Goud, B. (1998) Interaction of a Golgi-associated kinesin-like protein with Rab6, *Science* 279:580-585. (MedLine)

Eckert, R. (1972) Bioelectrical control of ciliary activity, *Science* 176:473-481. (MedLine)

Eckley, D.M., Gill, S.R., Melkonian, K.A., Bingham, J.B., Goodson, H.V., Heuser, J.E. and Schroer, T.A. (1999) Analysis of dynactin subcomplexes reveals a novel actin-related protein associated with the arp1 minifilament pointed end, *J. Cell Biol.* 147:307-320. (MedLine)

Eilersten, K.J. and Keller, T.C.S.3rd (1992) Identification and characterization of two huge protein components of the brush border cytoskeleton: evidence for a cellular isoform of titin. *J. Cell Biol.* 119:549-556. (MedLine)

el-Hayek, R., Antoniu, B., Wang, J., Hamilton, S.L and Ikemoto, N. (1995) Identification of calcium release-triggering and blocking regions of the II-III loop of the skeletal muscle dihydropyridine receptor, *J. Biol. Chem.* 270:22116-22118. (MedLine)

Elluru, R.G., Bloom, G.S. and Brady, S.T. (1995) Fast axonal transport of kinesin in the rat visual system: functionality of kinesin heavy chain isoforms, *Mol. Biol. Cell* 6:21-40. (MedLine)

Endow, S.A. (1991) The emerging kinesin family of microtubule motor proteins, *Trends Biochem. Sci.* 16:221-225. (MedLine)

Endow, S.A. (1999) Determinants of molecular motor directionality, *Nature Cell Biol.* 1:E163-167. (MedLine)

Endow, S.A. and Fletterick, R.J. (1998) Reversing a 'backwards' motor, *BioEssays* 20:108-112.

Endow, S.A. and Hatsumi, S. (1991) A multimember kinesin gene family in *Drosophila*, *Proc. Natl. Acad. Sci.USA.* 88:4424-4427. (MedLine)

Endow, S.A. and Higushi, H. (2000) A mutant of the motor protein kinesin moves in both diredtion on microtubules, *Nature* 406:913-916. (MedLine)

Endow, S.A. and Waligora, K.W. (1998) Determinants of kinesin motor polarity, *Science* 281:1200-1202. (MedLine)

Ettienne, E. M. (1970) Control of contractility in Spirostomum by dissociated calcium ions, *J. Gen. Physiol.* 56:168-179. (MedLine)

Euteneur, U. and McIntosh, J.R. (1981) Polarity of some motility-related microtubules, *Proc. Natl. Acad. Sci.* 78:372-376. (MedLine)

Euteneuer, U., Haimo, L.T. and Schliwa, M. (1989) Microtubule bundles of *Reticulomyxa* networks are of uniform polarity, *Eur. J. Cell Biol.* 49:373-376. (MedLine)

Evangelista, M., Klebl, B.M., Tong, A.H., Webb, B.A., Leeuw, T., Leberer, E., Whiteway, M., Thomas, D.Y. and Boone, C. (2000) A role for myosin-I in actin assembly through interactions with vrp1p, bee1p, and the Arp2/3 complex, *J. Cell Biol.* 148:353-362. (MedLine)

Evans, L.L., Lee, A.J., Bridgman, P.C. and Mooseker, M.S. (1998) Vesicle-associated brain myosin-V can be activated to catalyze actin-based transport, *J. Cell Sci.* 111:2055-2066. (MedLine)

Fabiato, A. and Fabiato, F. (1984) Calcium and cardiac excitation-contraction coupling, *Annu. Rev. Physiol.* 41:473-756. (MedLine)

Fahrenbach, W. H. (1965) Sarcoplasmic reticulum: ultrastructure of the triadic structure, *Science* 147:1308- 1309.

Fath, K.R. and Burgess, D.R. (1993) Golgi derived vesicles from developing epithelial cells bind actin filaments and possess myosin-I as a cytoplasmically oriented peripheral membrane protein, *J. Cell Biol.* 120:117-128. (MedLine)

Fath, K.R. and Lazak, R.J. (1988) Two classes of actin microfilaments associated with the inner cytoskeleton of axons, *J. Cell Biol.* 107:613-621. (MedLine)

Fath, K.R., Trimbur, G.M. and Burgess, D.R. (1994) Molecular motors are differentially distributed on Golgi membranes from polarized epithelial cells, *J. Cell Biol.* 126:661-675. (MedLine)

Fath, K.R., Trimbur, G.M. and Burgess, D.R. (1997) Molecular motors and a spectrin matrix associate with Golgi membranes in vitro, *J. Cell Biol.* 139:1169-1181. (MedLine)

Fernandez, A., Brautigan, D. L., Mumby, M. and Lamb, N. J. C. (1990) Protein phosphatase type-1, not type-2A, modulates actin microfilament integrity and myosin light chain phosphorylation in living

nonmuscle cells, *J. Cell Biol.* 111:103-112. (MedLine)

Finer, J.T., Simmons, R.M. and Spudich, J.A. (1994) Single myosin molecule mechanics: piconewton forces and nanometre steps, *Nature* 368:113-119. (MedLine)

Fisher, A.J., Smith, C.A., Thoden, J., Smith, R., Sutoh, K., Holden, H.M. and Rayment, I. (1995a) Structural studies of myosin:nucleotide complexes: a revised model for the molecular basis of muscle contraction, *Biophys. J.* 68:19s-28s. (MedLine)

Fisher, A.J., Smith, C.A., Thoden, J.B., Smith, R., Sutoh, K., Holden, H.M. and Rayment, I. (1995b) X-ray structures of the myosin motor domain of *Dictyostelium discoideum* complexed with MgADP.BeFx and MgADP.AlF^{4-}, *Biochemistry* 34:8960-8972. (MedLine)

Fleischer, S. and Inui, M. (1989) Biochemistry and biophysics of excitation-contraction coupling, *Annu. Rev. Biophys. Biophys. Chem.* 18:333-364 (MedLine)

Flicker, P.F., Phillips, G.N. and Cohen, C. (1982) Troponin and its interactions with tropomyosin: An electron microscope study, *J. Mol. Biol.* 162:495-501. (MedLine)

Foisner, R., Leichtfried, F.E., Herrmann, H., Small, J.V., Lawson, D., Wiche, G. (1988) Cytoskeleton-associated plectin: *in situ* localization, in vitro reconstitution, and binding to immobilized intermediate filament proteins, *J. Cell Biol.* 106:723-733 (MedLine)

Foisner, R., Traub, P. and Wiche, G. Protein kinase A- and protein kinase C-regulated interaction of plectin with lamin B and vimentin, *Proc. Natl. Acad. Sci. USA* 88:3812-3816. (MedLine)

Foisner, R., Malecz, N., Dressel, N., Stadler, C. and Wiche, G. (1996) M-phase-specific phosphorylation and structural rearrangement of the cytoplasmic cross-linking protein plectin involve p34cdc2 kinase, *Mol. Biol. Cell.* 7:273-288. (MedLine)

Franck, G. B. (1960) Effects of changes in extracellular calcium concentration and potassium induced contracture of frog's skeletal muscle, *J. Physiol. (London)* 151:518-538.

Franck, G.B. (1961) Role of extracellular calcium ub excitation-contraction coupling in skeletal muscle. In *Biophysics of Physiological and Pharmacological Action.* Publ. 69, pp. 293-307. American Association for the Advancement of Science. Washington. D.C.

Franke, W.W. (1971) Cytoplasmic microtubules linked to endoplasmic reticulum with cross bridges, *Exp. Cell Res.* 66:486-489. (MedLine)

Franzini-Armstrong, C. (1964) Fine structure of sarcoplasmic reticulum and transverse tubular systems in

muscle fibers, *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 23:887-895.

Franzini-Armstrong, C. (1973) Membranous systems in muscle fibers, In *Structure and Function of Muscle* (Bourn, G. H., ed.), pp. 531-619. Academic Press. New York.

Franzini-Armstrong, C. and Kish, J.W. (1995) Alternate disposition of tetrads in peripheral couplings of skeletal muscle, *J. Muscle Res. Cell Motil.* 16:319-324. (MedLine)

Franzini-Armstrong, C. and Nunzi, G. (1983) Junctional feet and particles in the triads of a fast-twitch muscle fibre, *J. Muscle Res. Cell Motil.* 4:233-252. (MedLine)

Franzini-Armstrong, C., Protasi, F. (1997) Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions, *Physiol. Rev.* 77:699-729. (MedLine)

Franzini-Armstrong, C., Kenney, L. J. and Vatriano-Marston, E. (1987) The structure of calsequestrin in triads of vertebrate skeletal muscle: a deep-etch study, *J. Cell Biol.* 105:49-56. (MedLine)

Friedman, D.S. and Vale, R.D. (1999) Single-molecule analysis of kinesin motility reveals regulation by the cargo-binding tail domain, *Nature Cell Biol.*1:293-297. (MedLine)

Fuchs, E. and Cleveland. D.W. (1998) A structural scaffolding of intermediate filaments in health and disease, *Science* 279:514-519. (MedLine)

Fujisaki, H., Albanesi, J. P. and Korn, E. D. (1985) Experimental evidence for contractile activities of Acanthoamoeba myosin IA and IB, *J. Biol. Chem.* 260:1183-1189. (MedLine)

Fukui, Y. (1993a) Toward a new concept of cell motility: cytoskeletal dynamics in ameboid movement and cell division, *Intern. Rev. Cyto.* 144:85-127. (MedLine)

Fukui, Y. (1993b) Composition, organization an *d* function of motor systems of free-living *Dictyostelium* amoeba, *Acta Protozool.* 32:201-210.

Fukui, Y., Lynch, T. J., Brzeska, H. and Korn, E. D. (1989) Myosin I is located at the leading edges of locomoting *Dictyostelium amoebae, Nature* 341:328-331. (MedLine)

Fukui, Y., Engler, S., Inoue, S. and de Hostos, E.L. (1999) Architectural dynamics and gene replacement of coronin suggest its role in cytokinesis, *Cell Motil. Cytoskeleton.* 42:204-217. (MedLine)

Funatsu, T., Harada, Y., Tokunaga, M., Saito, K. and Yanagida, T. (1995) Imaging of single fluorescent molecules and individual ATP turnovers by smyosin molecules in aqueous solution, *Nature* 374:555-559.

(MedLine)

Furness, J.B. and Costa, M. (1987) *The Enteric Nervous System*, Churchill-Livingston, Edinburgh.

Furst, D.O., Osborn, M., Nave, R. and Weber, K. (1988) The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z line extends close to the M line, *J. Cell Biol.* 106:1563-1572. (MedLine)

Gard, D.L. and Klymkowsky, M.W. (1998) in *Intermediate filaments* (Hermann, H. and Harris, J.R., eds), Plenum, New York, pp.35-69. (MedLine)

Gee, M.A., Heuser, J.E. and Vallee, R.B. (1997) An extended microtubule-binding structure within the dynein motor domain, *Nature* 390:636-639. (MedLine)

Geiger, B. (1979) A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells, *Cell* 18:193-205. (MedLine)

Geiger, Tokuyasu, K.T., Dutton, A.H. and Singer, S.J. (1980) Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell-membranes, *Proc. Natl. Acad. Sci. USA* 77:4127-4131. (MedLine)

Geisler, N. and Weber, K. (1988) Phosphorylation of desmin in vitro inhibits formation of intermediate filaments: identification of three kinase A sites in the aminoterminalhead domain, *EMBO. J.* 7:15-20. (MedLine)

Geli, M.I. and Riezman, H. (1996) Role of type I myosins in receptor-mediated endocytosis in yeast, *Science* 272:533-535. (MedLine)

Gelles, J., Berliner, E., Young, E.C., Mahtani, H.K., Perez-Ramirez, B.and Anderson K. (1995) Structural and functional features of one- and two-headed biotinated kinesin derivatives, *Biophys. J.* 68:276S-281S (MedLine)

Gibson, F., Walsh, J., Mburu, P., Varela, AS., Brown, K.A., Antonio M., Beisel, K.W., Steel, K.P. and Brown, S.D.M. (1995) A type VII myosin encoded by the mouse deafness gene shaker-1, *Nature* 374:62-64. (MedLine)

Gilbert, S. P., Slaboda, R. D. and Allen, R. D. (1985) Translocation of vesicles from squid axoplasm on flagellar microtubules, *Nature* 315:245-248. (MedLine)

Gill, S.R., Schroer, T.A., Szilak, I., Steuer, E.R., Sheetz, M.P. and Cleveland, D.W. (1991) Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic

dynein, *J. Cell Biol.* 115:1639-1650. (MedLine)

Gillespie, P.G., Wagner, M.C.and Hudspeth, A.J. (1993) Identification of a 120 kd hair-bundle myosin located near stereociliary tips, *Neuron* 11:581-594. (MedLine)

Gindhart, J.G. and Goldstein, L.S. (1996) Tetratrico peptide repeats are present in the kinesin light chain, *Trends Biochem Sci.* 21:52-53. (MedLine)

Gindhart, J.G. Jr., Desai, C.J., Beushausen, S., Zinn, K. and Goldstein, L.S. (1998) Kinesin light chains are essential for axonal transport in *DrosophilaJ. Cell Biol.* 141:443-454. (MedLine)

Glenney, J.R. Jr and Weber, K. (1985) Separation of fodrin subunits by affinity chromatography on calmodulin-Sepharose, *Anal. Biochem.*150:364-368. (MedLine)

Goldman, R.D., Khuon, S., Chou, Y.H., Opal, P. and Steinert, P.M. (1996) The function of intermediate filaments in cell shape and cytoskeletal integrity, *J. Cell Biol.* 134:971-983. (MedLine)

Goldstein, L.S.B. (1993) With apologies to Scheherazade: Tails of 1001 kinesin motors, *Ann. Rev. Gen.* 27:319-351. (MedLine)

Goldstein, L.S.B. (2001) Molecular motors: from one motor many tails to one motor many tales, *Trends Cell Biol.* 11:477-482. (MedLine)

Gollub, J., Cremo, C.R. and Cooke, R. (1996) ADP release produces a rotation of the neck region of smooth myosin but not skeletal myosin, *Nature Struct. Biol.* 3:796-802. (MedLine)

Gong, T.W., Winnicki, R.S., Kohrman, D.C. and Lomax, M.I. (1999) A novel mouse kinesin of the UNC-104/KIF1 subfamily encoded by the Kif1b gene, *Gene* 239:117-127. (MedLine)

Goode, B.L., Wong, J.J., Butty, A.C., Peter, M., McCormack, A.L., Yates, J.R., Drubin, D.G. and Barnes, G. (1999) Coronin promotes the rapid assembly and cross-linking of actin filaments and may link the actin and microtubule cytoskeletons in yeast, *J. Cell Biol.* 144:83-98. (MedLine)

Goodenough, U.W. and Heuser, J.E. (1984) Structural comparison of purified dynein proteins with in situ dynein arms, *J. Mol. Biol.* 180:1083-1118. (MedLine)

Goodenough, U.W. and Heuser, J.E. (1989) in *Cell Movement: the Dynein ATPases* vol. 1. (Eds. Warner, F.D., Satir, P. and Gibbons, I.R.) Alan Liss, New York, pp. 121-140.

Goodson, H.V., Anderson, B.L., Warrick, H.M., Pon, L.A. and Spudich, J.A. (1996) Synthetic lethality

screen identifies a novel yeast myosin I gene (MYO5): myosin I proteins are required for polarization of the actin cytoskeleton, *J. Cell Biol.* 133:1277-1291. (MedLine)

Gordon, A. M. Huxley, F. and Julian, F. J. (1966) The variation in isometric tension with sarcomere length in vertcbrate muscle fibers, *J. Physiol.* (London) 184:170-192. (MedLine)

Gordon, D.M. and Roof, D.M. (1999) The kinesin-related protein Kip1p of *Saccharomyces cerevisiae* is bipolar, *J. Biol. Chem.* 274:28779-28786. (MedLine)

Govindan, B., Bowser, R. and Novick, P. (1995) The role of Myo2, a yeast class V myosin, in vesicular transport, *J.Cell Biol.* 128:1055-1068. (MedLine)

Grantham, C.J. and Cannell, M.B. (1996) Ca^{2+} influx during the cardiac action potential in guinea pig ventricular myocytes, *Circ. Res.* 79:194-200. (MedLine)

Green, K.J., Parry, D.A., Steinert, P.M., Virata, M.L., Wagner, R.M., Angst, B.D. and Nilles, L.A. (1990) Structure of the human desmoplakins. Implications for function in the desmosomal plaque, *J. Biol. Chem.* 265:2603-2612. (MedLine).

Green, K.J. and Bornslaeger, E.A. (1999), Desmoplakin, in *Guidebook to the Extracellular Matrix, Anchor, and Adhesion Proteins* (Kreis, T. and Vale, R. eds.), pp. 103-105, Oxford University Press.

Gregorio, C.C., Trombitas, K., Centner, T., Kolmerer, B., Stier, G., Kunke, K., Suzuki, K., Obermayr, F., Herrmann, B., Granzier, H., Sorimachi, H. and Labeit, S. (1998) The NH_2 terminus of titin spans the Z-disc: its interaction with a novel 19-kD ligand (T-cap) is required for sarcomeric integrity, *J. Cell Biol.* 143:1013-1027. (MedLine)

Gregorio, C.C., Granzier, H., Sorimachi, H. and Labeit, S. (1999) Muscle assembly: a titanic achievement? *Curr. Opin. Cell Biol.* 11:18-25. (MedLine)

Griffith, L. M. and Pollard, T. D. (1982) The interaction of actin filaments with microtubules and microtubule-associated proteins, *J. Biol. Chem.* 257:9143-9151. (MedLine)

Gross, S.P., Welte, M.A., Block, S.M. and Wieschaus, E.F. (2000) Dynein-mediated cargo transport in vivo. A switch controls travel distance, *J. Cell Biol.* 148:945-956. (PubMed)

Gulick, A.M. and Rayment, I. (1997) Structural studies on myosin II: communication between distant protein domains, *BioEssays* 19:561-569. (MedLine)

Guo, L., Degenstein, L., Dowling, J., Yu, Q.C., Wollmann, R., Perman, B. and Fuchs, E. (1995) Gene

targeting of BPAG1: abnormalities in mechanical strength and cell migration in stratified epithelia and neurologic degeneration, *Cell* 81:233-243. (MedLine)

Hacker, U., Albrecht, R.and Maniak, M. (1997) Fluid-phase uptake by macropinocytosis in *Dictyostelium*, *J. Cell Sci.* 110:105-112. (MedLine)

Hackney, D.D. (1994) Evidence for alternating head catalysis by kinesin during microtubule-stimulated ATP hydrolysis, *Proc. Natl. Acad. Sci. USA.* 91:6865-6869. (MedLine)

Hackney, D.D. and Stock, M.F. (2000) Kinesin's IAK tail domain inhibits initial microtubule-stimulated ADP release, *Nature Cell Biol.* 2:257-260. (MedLine)

Hackney, D.D., Levitt, J.D. and Wagner, D.D. (1991) Characterization of α2 β2 and α2 forms of kinesin, *Biochem. Biophys. Res. Commun.* 174:810-815. (MedLine)

Hackney, D.D., Levitt, J.D. and Suhan, J. (1992) Kinesin undergoes a 9 S to 6 S conformational transition, *J. Biol. Chem.* 267:8696-8701. (MedLine)

Haimovich, B., Schotland. D. L., Fieles, W. E. and Barchi, R. L. (1987) Localization of sodium channel subtypes in adult rat skeletal muscle using channel-specific monoclonal antibodies, *J. Neurosci.* 7:2957-2966. (MedLine)

Hall, A. (1994) Small GTP binding proteins and the regulation of the actin cytoskeleton, *Ann. Rev. Cell Biol.*10:31-54. (MedLine)

Halmo, L.T. and Thaler, C.D. (1994) Regulation of organelle transport - lessons from color change in fish, *BioEssays* 16:727-733.

Hancock, W.O. and Howard, J. (1998) Processivity of the motor protein kinesin requires two heads, *J. Cell Biol.* 140:1395-1405. (MedLine)

Hanson, J. and Lowy, J. (1961) The structure of the muscle fibres in the translucent part of the adductor of the oyster Crassastrea angulata, *Proc. R. Soc. London Ser. B.* 154:173-196.

Hanson, J. and Lowy, J. (1963) The structure of Facto filaments isolated from muscle, *J. Mol. Biol.* 6:46-60.

Harada. A., Takei, Y., Kanai, Y., Tanaka, Y., Nonaka, S. and Hirokawa, N. (1998) Golgi vesiculation and lysosome dispersion in cells lacking cytoplasmic dynein, *J. Cell Biol.* 141:51-59. (MedLine)

Harlon, D.W., Yang, Z. and Goldstein, L.S.B (1997) Characterization of KIFC2, a neuronal kinesin superfamily membrane in mouse, *Neuron* 18:439-451. (MedLine)

Hartwig, J. H. and Kwiatkowski, D. J. (1991) Actin-binding proteins, *Curr. Opin. Cell Biol.* 3:87-97. (MedLine)

Hasselbach, W. and Ledermair, D. (1958) Der Kontraktionscyclus der Isolierten contractiles Strukturen der Uterusmuskulatur und Seine Besonderheiten, *Pfluegers Arch. Gesamte Physiol.* 267:532-542.

Hasson, T. and Mooseker, M.S. (1995) Molecular motors, membrane movements and physiology, *Curr. Opin. Cell Biol.* 7:587-594. (MedLine)

Hasson, T., Heintzelman, M.B., Santo-Sacchi, J., Corey, D.P. and Mooseker, M.S. (1995) Expression in cochlea and retina of myosin-VIIa, the gene defective in Usher Syndrome type 1B, *Proc. Natl. Acad. Sci. USA*92: 9815-9819. (MedLine)

Hayashi, T. (1952) Contractile properties of compressed monolayers of actomyosin, *J. Gen. Physiol.* 36:139-151.

Hayden, S. M., Wolenski, J. S. and Mooseker, M. S. (1990) Binding of brush border myosin to phospholipid vesicles, *J. Cell Biol.* 111:443-451. (MedLine)

Heilbrunn, L. V. and Wiercinski, F. J. (1947) The action of various cations on muscle protoplasm, *J. Cell Comp. Physiol.* 29:15-32.

Heierhorst, J., Kobe, B., Feil, S.C., Parker, M.W., Benian, G.M., Weiss, K.R. and Kemp, B.E. (1996) Ca^{2+}/S100 regulation of giant protein kinases, *Nature* 380:636-639. (MedLine)

Helmes, M, Trombitas, K. and Granzier, H. (1996) Titin develops restoring force in rat cardiac myocytes, *Circ. Res.* 79:619-626. (MedLine)

Henningsen, U. and Schliwa, M. (1997) Reversal of the direction of movement of a molecular motor, *Nature* 389:93-95. (MedLine)

Hepler, P. K. (1980) Membranes in the mitotic apparatus of barley cells, *J. Cell Biol.* 86:490-499. (MedLine)

Hepler, P. K. and Callahan, D. A. (1987) Free calcium increases during anaphase in stamen hair cells of *Tradescantia, J. Cell Biol.* 105:2137-2143. (MedLine)

Herrmann, H. and Aebi, U. (2000) Intermediate filaments and their associates: multi-talented structural elements specifying cytoarchitecture and cytodynamics, *Curr. Opin. Cell Biol.* 12:79-90. (MedLine)

Heumann, H. G. and Zebe, E. (1967) Über Feinbau und Funktionsweisse der Fasern aus dem Hautmuskelschlauch des Regenwurms *Lutnbricus terrestris, L. Z. Zelforsch. Mikrask. Anat.* 78:131-150.

Higgs, H.N. and Pollard, T.D. (1999) Regulation of actin polymerization by Arp2/3 complex and WASp/Scar proteins, *J. Biol. Chem.* 274:32531-32534. (MedLine)

Higley, S. and Way, M. (1997) Actin and cell pathogenesis, *Curr. Opin. Cell Biol.* 9:62-69. (MedLine)

Higuchi, H. (1996) Viscoelasticity and function of connectin/titin filaments in skinned muscle fibers, *Adv. Biophys.* 33:159-171. (MedLine)

Hill, D. K. (1964) The space accessible to albumin within the striated fiber of the toad, *J. Physiol. (London)* 175:275-294.

Hill, K.L., Catlett, N.L. and Weisman, L.S. (1996) Actin and myosin function in directed vacuole movement durig cell divisioin in *Saccharomyces cervesiae*, *J. Cell Biol.* 135:1535-1549. (MedLine)

Hirokawa, N. (1982) Cross-linker system between neurofilaments, microtubules, and membranous organelles in frog axons revealed by the quick-freeze, deep-etching method, *J. Cell Biol.* 94:129-142. (MedLine)

Hirokawa, N. (1991) Molecular architecture and dynamics of the neuronal cytoskeleton, in *The Neuronal Cytoskeleton* ed. by Burgoyne, R.D., Alan R. Liss, New York, pp. 5-74. (MedLine)

Hirokawa, N., Hisanaga, S. and Shiomura, Y. (1988) MAP2 is a component of crossbridges between microtubules and neurofilaments in the neuronal cytoskeleton: quick-freeze, deep-etch immunoelectron microscopy and reconstitution studies, *J. Neurosci.* 8:2769-2779. (MedLine)

Hirokawa, N., Pfister, K. K., Yorifuji, H., Wagner, M. C., Brady, S. T. and Bloom, G. S. (1989) Submolecular domains of bovine brain kinesin identified by electron microscopy and monoclonal antibody decoration, *Cell* 56:867-878. (MedLine)

Hirokawa, N., Noda, Y. and Okada, Y. (1998) Kinesin and dynein superfamily in organelle transport and cell division, *Curr. Opin. Cell Biol.* 10:60-73. (MedLine)

Hirose, K., Lockhart, A., Cross, R.A. and Amos, L.A. (1995) Nucleotide-dependent angular change in the kinesin motor domain bound to tubulin, *Nature* 276:277-279. (MedLine)

Hirose, K., Lockhart, A., Cross, R.A. and Amos, L.A, (1996) Three-dimensional cryoelectron microscopy of dimeric kinesin and *ncd* domains on microtubules, *Proc. Natl. Acad. Sci. USA* 93:9539-9544. (MedLine)

Hirose, K., Cross, R.A. and Amos, L.A. (1998) Nucleotide-dependent structural changes in dimeric NCD molecules complexed to microtubules, *J. Mol. Biol.* 278:389-400. (MedLine)

Hirose, K., Lowe, J., Alonso, M., Cross, R.A. and Amos, L.A. (1999) Congruent docking of dimeric kinesin and ncd into three-dimensional electron cryomicroscopy maps of microtubule-motor ADP complexes, *Mol. Biol. Cell.* 10:2063-2074. (MedLine)

Hoenger, A., Sack, S., Thormahlen, M., Marx, A., Muller, J., Gross, H. and Mandelkow, E. (1998) Image reconstructions of microtubules decorated with monomeric and dimeric kinesins: comparison with x-ray structure and implications for motility, *J. Cell Biol.* 141:419-430. (MedLine)

Hollenbeck, P. J. and Chapman, K. (1986) A novel microtubule associated protein from mammalian nerve shows ATP sensitive binding to microtubule, *J. Cell Biol.* 103:1539-1545. (MedLine)

Hollenbeck, P.J. (1993) Phosphorylation of neuronal kinesin heavy and light chain in vivo, *J. Neurochem.* 60:2265-2275. (MedLine)

Holleran, E.A., Karki, S. and Holzbaur, E.L. (1998) The role of the dynactin complex in intracellular motility, Int. Rev. Cytol. 182:69-109. (MedLine)

Holmes, K.C. (1997) The swinging lever-arm hypothesis of muscle contraction, *Current Biology* 7:R112-118. (MedLine)

Holmes, K.C., Popp, S., Gebhard, W. and Kabsch, W. (1990) Atomic model of the actin filament, *Nature* 347:44-49. (MedLine)

Holzbauer, E.L.F. and Vallee, R.B. (1994) Dyneins: molecular structure and cellular function, *Annu. Rev. Cell Biol.* 10:339-372. (MedLine)

Horowits, R. and Podolsky, R.J. (1987) The positional stability of thick filaments in activated skeletal muscle depends on sarcomere length: evidence for the role of titin filaments, *J. Cell Biol.* 105:2217-2223. (MedLine)

Horowitz, A., Menice, C.B., Laporte, R. and Morgan, K.C. (1996) Mechanism of smooth muscle contraction, *Physiol. Rev.* 76:967-1003. (MedLine)

Houdusse, A., Kalabokis, V.N., Himmel, D., Szent-Gyorgyi, A.G. and Cohen C. (1999) Atomic structure of scallop myosin subfragment S1 complexed with MgADP: a novel conformation of the myosin head, *Cell* 97:459-470. (MedLine)

Höner, B., Citi, S, Kendrick-Jones, J. and Jockusch, B. M. (1988) Modulation of cellular morphology and locomotory activity by antibodies against myosin, *J. Cell Biol.* 107:2181-2189. (MedLine)

Houseweart, M.K. and Cleveland, D.W. (1998) Intermediate filaments and their associated proteins: multiple dynamic personalities, *Curr. Opin. Cell Biol.* 10:93-101. (MedLine)

Howard, J., Hudspeth, A.J.and Vale, R.D. (1989) Movement of microtubules by single kinesin molecules, *Nature* 342:154-158. (MedLine)

Howard, J. (1997) Molecular motors: structural adaptations to cellular functions, *Nature* 389:561-567. (MedLine)

Howard, J. (1998) How molecular motors work, *Nature* 391:239-240.

Hua, W., Young, E.C., Fleming, M.L. and Gelles, J. (1997) Coupling of kinesin steps to ATP hydrolysis, *Nature* 388:390-393. (MedLine)

Huang, T.G., Suhan, J. and Hackney, D.D. (1994) Drosophila kinesin motor domain extending to amino acid position 392 is dimeric when expressed in *Escherichia coli, J. Biol. Chem.* 269:16502-16507. (MedLine)

Huang, J.D., Brady, S.T., Richards, B.W., Stenolen, D., Resau, J.H., Copeland, N.G. and Jenkins, N.A. (1999) Direct interaction of microtubule- and actin-based transport motors, *Nature* 397:267-270. (MedLine)

Huxley, A. (1998) How molecular motors work, *Nature* 391:239. (MedLine)

Huxley, A.F. and Simmons, R. (1971) Proposed mechanism of force generation in striated muscle, *Nature* 233:533-538. (MedLine)

Huxley, F. (1959) Local activation of muscle, *Ann. N.Y. Acad. Sci.* 81:446-452.

Huxley, F. and Taylor, R. E. (1958) Local activation of striated muscle fibres, *J. Physiol. (London)* 144:426-441.

Huxley, H. E. (1963) Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle, *J. Mol. Biol.* 7:281-308.

Huxley, H. E. (1969) The mechanism of muscular contraction, *Science* 164:1356-1366. (MedLine)

Hyman, A.A. and Mitchison, T.J. (1991) Two different microtubule-based motor activities with opposite polarities in kinetochores, *Nature* 351:206-211. (MedLine)

Hymel, L., Inui, M., Fleischer, S. and Schindler, H. (1988) Purified ryanodine receptor of skeletal muscle sarcoplasmic reticulum forms Ca^{2+}-activated oligomeric Ca2+ channels in planar bilayers, *Proc. Natl. Acad. Sci. USA.* 85:44l-445. (MedLine)

Inagaki, M., Nishi, Y., Nishizawa, K., Matsuyama, M. and Sato, C. (1987) Site-specific phosphorylation induces disassembly of vimentin filaments in vitro, *Nature* 328:649-652. (MedLine)

Ingold, A.L., Cohn, S.A. and Scholey, J.M. (1988) Inhibition of kinesin-driven microtubule motility by monoclonal antibodies to kinesin heavy chain, *J. Cell Biol.* 107:2657-2667. (MedLine)

Inoue, Y., Toyoshima, Y.Y., Iwane, A.H., Morimoto, A. and Higuchi, H. (1997) Movements of truncated kinesin fragments with a short or an artificial flexible neck, *Proc. Natl. Acad. Sci. USA* 94:7275-7280. (MedLine)

Ip, W., Hartzer, M. K., Pang, Y.-Y. S. and Robson, R. M. (1985) Assembly of vimentin in vitro and its implications concerning the structure of intermediate filaments, *J. Mol. Biol.* 183: 365-375. (MedLine)

Ishijima, A., Kojima, H., Funatsu, T., Tokunaga, M., Higuchi, H., Tanaka, H. and Yanagida, T. (1998) Simultaneous observation of individual ATPase and mechanical events by a single myosin molecule during interaction with actin, *Cell* 92:161-172. (MedLine)

Ishikawa, H., Bischoff, R. and Holtzer, H. (1969) Formation of arrowhead complexes with heavy meromyosin in a variety of cells, *J. Cell Biol.* 43:312-328. (MedLine)

Ishiko, N. and Sato, M. (1957) The effect of calcium ions on electrical properties of striated muscle fibres, *Jpn. J. Physiol* 7:51-63.

Jayaraman, T., Brillantes, A.M., Timerman, A.P., Fleischer, S., Erdjument-Bromage, H., Tempst, P. and Marks, A.R. (1992) FK506 binding protein associated with the calcium release channel (ryanodine receptor), *J. Biol. Chem.* 267:9474-9477. (MedLine)

Jiang, W., Stock, M.F., Li, X. and Hackney, D,D. (1997) Influence of the kinesin neck domain on dimerization and ATPase kinetics, *J. Biol. Chem.* 272:7626-7632. (MedLine)

Jobis, F. F. and O'Connor, M. J. (1966) Calcium release and reabsorption in the sartorius muscle of the toad, *Biochem. Biophys. Res. Commun.* 25:246-252.

Johnson, K. A. (1985) Pathway of the microtubules-dynein ATPase and the structure of dynein: a comparison with actomyosin, *Annu. Rev. Biophys. Biophys. Chem.* 14:161-188. (MedLine)

Johnston, G.C., Prendergast, J.A. and Singer, R.A. (1991) The *Saccharomyces cerevisiae MYO2* gene encodes an essential myosin for vectorial transport of vesicles, *J. Cell Biol.* 113:539-551. (MedLine)

Jones, J. M. and Perry, S. V. (1966) The biological activity of subfragments prepared from heavy meromyosin, *Biochem. J.* 100:120-130. (MedLine)

Jontes, J.D. and Milligan, R.A. (1997) Brush border myosin-I structure and ADP-dependent conformational changes revealed by cryoelectron microscopy and image analysis, *J. Cell Biol.* 139:683-693. (MedLine)

Jontes, J.D., Milligan, R.A., Pollard, T.D. and Ostap, E.M. (1997) Kinetic characterization of brush border myosin-I ATPase, *Proc. Natl. Acad. Sci. USA* 94:14332-14337. (MedLine)

Jorgensen, A.O., Shen, A.C., Arnold, W., McPherson, P.S. and Campbell, K.P. (1993) The Ca2+-release channel/ryanodine receptor is localized in junctional and corbular sarcoplasmic reticulum in cardiac muscle, *J. Cell Biol.* 120:969-980. (MedLine)

Jung, G., Wu, X., and Hammer III, J. A. (1996) Dictyostelium mutants lacking multiple classic myosin I isoforms reveal combinations of shared and distinct functions, *J. Cell Biol.* 133:305-323. (MedLine)

Kabsch, W., Mannherz, H. G., Suck, D, Pai, E.F. and Holmes, K.C. (1990) Atomic structure of the actin:DNase I complex, *Nature* 347:37-44. (MedLine)

Kagami, O. and Kamiya, R., (1992) Translocation and rotation of microtubules caused by multiple species of *Chlamydomonas* inner-arm dynein, *J. Cell Sci.* 103:653-664.

Kamitsubo, E. (1966) Motile protoplasmic fibrils in Characeae. II. Linear fibrillar structure and its bearing on protoplasmic streaming, *Proc. Jpn. Acad.* 42:640-643.

Kamitsubo, E. (1972) Motile protoplasmic fibrils in cells of Characeae, *Protoplasma* 74:53-70.

Karcher, R.L., Roland, J.T., Zappacosta, F., Huddleston. M.J., Annan, R.S., Carr, S.A. and Gelfand, V.I. (2001) Cell cycle regulation of myosin-V by calcium/calmodulin- dependent protein kinase II, *Science* 293:1317-1320. (MedLine)

Karcher, R.L., Deacon, S.W. and Gelfand, V.I. (2002) Motor-cargo interactions: the key to cargo specificity, *Trends in Cell Biol.* 12:21-27.

Kashina, A.S., Baskin, R.J., Cole, D.G., Wedaman, K.P., Saxton, W.M. and Scholey, J.M. (1996) A bipolar kinesin, *Nature* 379:270-272. (MedLine)

Kato, T. and Tonomura, Y. (1977) Identification of myosin in *Nitella flexilis, J. Biochem.* 82:777-782. (MedLine)

Kendrick-Jones J. (1974) Role of myosin light chains in calcium regulation, *Nature* 249:631-634. (MedLine)

Khodjakov, A., Lizunova, E.M., Minin, A.A., Koonce, M.P. and Gyoeva, F.K. (1998) A specific light chain of kinesin associates with mitochondria in cultured cells, *Mol. Biol. Cell* 9:333-343. (MedLine)

Kijima, Y., Saito, A., Jetyton, T.L., Magnuson, M. A. and Fleischer, S. (1993) Different intracellular localization of inositol 1,4,5-trisphosphate and ryanodine receptors in cardiomyocytes, *J. Biol. Chem.* 268:3499-3506. (MedLine)

King, S.M. (2000) The dynein microtubule motor, *Biochim. Biophys. Acta* 1496:60-75. (MedLine)

King, S.J. and Schroer, T.A. (2000) Dynactin increases the processivity of the cytoplasmic dynein motor, *Nature Cell Biol.* 2:20-24. (MedLine)

Kishino, A. and Yanagida, T. (1988) Force measurements by micromanipulation of a single actin filament by glass needles, *Nature*334:74-76 (MedLine)

Kitamura, K., Tokunaga, M.,. Iwane, A.H. and Yanagida, T. (1999) A single myosin molecule moves along an actin filament with regular steps of 5.3 nanometres, *Nature* 397:129-134. (MedLine)

Klopfenstein, D.R., Tomishige, M., Stuurman, N. and Vale, R.D. (2002) Role of phosphatitylinositol(4,5)bisphosphate organization in membrane transport by the Unc104 kinesin motor, *Cell* 109:347-358.

Knecht, O. A. and Loomis, W. F. (1987) Antisense RNA inactivation of myosin heavy-chain gene-expression in *Dictyostelium discoideum, Science* 236:1081-1086. (MedLine)

Kondo, S., Sato-Yoshitake, R., Noda, Y., Aizawa, H., Nakata, T., Matsuura, Y. and Hirokawa, N. (1994) KIF3A is a new microtubule-based anterograde motor in the nerve axon, *J. Cell Biol.* 125:1095-1107. (MedLine)

Korn, E.D. and Hammer, J.A. (1988) Myosins in nonmuscle cells, *Ann. Rev. Biophys.* 17:23-45. (MedLine)

Kozielski, F., Sack, S., Marx, A., Thormahlen, M., Schonbrunn, E., Biou, V., Thompson, A., Mandelkow, E.M. and Mandelkow, E. (1997) The crystal structure of dimeric kinesin and implications for microtubule-dependent motility, *Cell* 91:985-994. (MedLine)

Kozielski, F., De Bonis, S., Burmeister, W.P., Cohen-Addad, C. and Wade, R.H. (1999) The crystal structure of the minus-end-directed microtubule motor protein ncd reveals variable dimer conformations, *Structure* 7:1407-1416. (MedLine)

Kruger, M., Wright, J. and Wang K. (1991) Nebulin as a length regulator of thin filaments of vertebrate skeletal muscles: correlation of thin filament length, nebulin size, and epitope profile, *J. Cell Biol.* 115:97-107. (MedLine)

Kull, F.J., Sablin, E.P., Lau, R., Fletterick, R.J. and Vale, R.D. (1996) Crystal structure of the kinesin motor domain reveals a structural similarity to myosin, *Nature* 380:550-555. (MedLine)

Kull, F.J., Vale, R.D. and Fletterick, R.J. (1998) The case for a common ancestor: kinesin and myosin motor proteins and G proteins, *J. Muscle Res. Cell Motil.* 19:877-886. (MedLine)

Kuznetsov, S.A., Vaisberg, E.A., Shanina, N.A., Magretova, N.N., Chernyak, V.Y.and Gelfand, V.I. (1988) The quaternary structure of bovine brain kinesin, *EMBO J.* 7:353-356. (MedLine)

Kuznetsov, S.A., Langford, G.M. and Weiss, D.G. (1992) Actin-dependent organelle movement in squid axoplasm, *Nature* 356:722-725. (MedLine)

Kuznetsov, S.A., River, D.T., Severin, F.F., Weiss, D.G., and Lamarche, N. and Hall, A. (1994) GAPs for rho-related GTPases, *Trends Genet.* 10:436-440. (MedLine)

Labeit, S. and Kolmerer, B. (1995a) Titins: giant proteins in charge of muscle ultrastructure and elasticity. *Science* 270:293-296. (MedLine)

Labeit, S. and Kolmerer, B. (1995b) The complete primary structure of human nebulin and its correlation to muscle structure, J. Mol. Biol. 248:308-315. (MedLine)

Labeit, S., Barlow D.P., Gautel, M., Gibson, T., Holt, J., Hsieh, C.-L., Francke, U., Leonard, K., Wardale, J., Whiting, A. and Trinick, J.(1990) A regular pattern of two types of 100-residue motif in the sequence of titin, *Nature* 345:273-276. (MedLine)

Labeit, S., Gibson, T., Lakey, A., Leonard, K., Zeviani, M., Knight, P., Wardale, J. and Trinick, J. (1991) Evidence that nebulin is a protein-ruler in muscle thin filaments, *FEBS Lett.* 282:313-316. (MedLine)

Labeit, S., Gautel, M., Lakey, A. and Trinick, J. (1992) Towards a molecular understanding of titin, *EMBO J.* 11:1711-1716. (MedLine)

Lamarche, N. and Hall, A (1994) GAPs for rho-related GTPases, *Trends Genet.* 10:436-440. (MedLine)

Langford, G.M. (1994) Movement of axoplasmic organelles on actin filaments from skeletal muscle, *Cell Motil. Cytoskel.* 28:231-242. (MedLine)

Langford, G. M., Allen, R. D. and Weiss. D. G. (1987) Substructure of side arms on squid axoplasmic vesicles and microtubules visualized by negative contrast electronmicroscopy, *Cell Motil.* 7:20-30. (MedLine)

Lazarides, E. and Bunridge, K. (1975) α-Actinin: immunofluorescent localization of a muscle structural protein in non-muscle cells, *Cell* 6:289-298. (MedLine)

Lechler, T., Shevchenko, A. and Li, R. (2000) Direct involvement of yeast type I myosins in Cdc42-dependent actin polymerization, *J. Cell Biol.* 148:363-374. (MedLine)

Lee, S., Wisniewski, J.C., Dentler, W.L., Asai,D.J. (1999) Gene knockouts reveal separate functions for two cytoplasmic dyneins in *Tetrahymena thermophila* Mol. Biol. Cell 10:771-784. (MedLine)

Lee, E., Marcucci, M., Daniell, L., Pypaert, M., Weisz, O.A., Ochoa, G.C., Farsad, K., Wenk, M.R. and De Camilli, P. (2002) Amphiphysin 2 (Bin1) and T-tubule biogenesis in muscle, *Science* 297:1193-1196. (MedLine)

Lehman, W., Kendrick-Jones, J. and Szent-Gyorgyi, A. G. (1972) Myosin-linked regulatory systems: comparative studies, *Cold Spring Harbor Symp. Quant. Biol.* 37:319-330.

Leibler, S. and Huse, D.A. (1993) Porters versus rowers: a unified stochastic model of motor proteins, *J. Cell Biol.* 121: 1357-1368. (MedLine)

Leterrier, J. F., Liem, R. K. H. and Shelanski, M. L. (1982) Interactions between neurofilaments and microtubule-associated proteins: possible mechanism for intraorganellar bridging, *J. Cell Biol.* 95:982-986. (MedLine)

Leung, C.L., Sun, D., Zheng, M., Knowles, D.R. and Liem, R.K. (1999) Microtubule actin cross-linking factor (MACF): a hybrid of dystonin and dystrophin that can interact with the actin and microtubule

cytoskeletons, *J. Cell Biol.* 147:1275-1286. (MedLine)

Leung, C.L., Green, K.J., Liem, R.K.H. (2002) Plakins: a family of versatile cytolinker proteins, *Trends in Cell Biol.* 12:37-45.

Levine, J. and Willard, M. (1981) Fodrin: axonally transported polypeptides associated with the internal periphery of many cells, *J. Cell Biol.* 90:631-642. (MedLine)

Li, Z., Colucci-Guyon, E., Pincon-Raymond, M., Mericskay, M., Pournin, S., Paulin, D. and Babinet, C. (1996) Cardiovascular lesions and skeletal myopathy in mice lacking desmin, *Dev. Biol.* 175:362-366. (MedLine)

Liao, G. and Gundersen, G.G. (1998) Kinesin is a candidate for cross-bridging microtubules and intermediate filaments. Selective binding of kinesin to detyrosinated tubulin and vimentin, *J. Biol. Chem.* 273:9797-9803. (MedLine)

Lillie, S. and Brown, S. (1992) Suppression of a myosin defect by a kinesin related gene, *Nature* 356:358-361. (MedLine)

Lindén, M., Nelson, B. B., Loncar, D. and Leterrier, J.-F. (1989) Studies on the interaction between mitochondria and the cytoskeleton, *J. Bioeneg. Biomembr.* 21:507-518. (MedLine)

Loisel, T.P., Boujemaa, R., Pantaloni, D. and Carlier, M.F. (1999) Reconstitution of actin-based motility of *Listeria* and *Shigella* using pure proteins, *Nature* 401:613-616. (MedLine)

Lombillo, V.A., Nislow, C., Yen, T.J., Gelfand, V.I. and McIntosh, J.R. (1995) Antibodies to the kinesin motor domain and CENP-E inhibit microtubule depolymerization-dependent motion of chromosomes in vitro, *J. Cell Biol.* 128:107-115. (MedLine)

Lu, X., Xu, L. and Meissner, G. (1994) Activation of the skeletal muscle calcium release channel by a cytoplasmic loop of the dihydropyridine receptor, *J. Biol. Chem.* 269:6511-6516. (MedLine)

Lu, X., Xu, L., Meissner, G. (1995) Phosphorylation of dihydropyridine receptor II-III loop peptide regulates skeletal muscle calcium release channel function. Evidence for an essential role of the beta-OH group of Ser687, *J. Biol. Chem.* 270:18459-18464. (MedLine)

Lye, R.J., Porter, M.E., Scholey, J.M., McIntosh, J.R. (1987) Identification of a microtubule-based cytoplasmic motor in the nematode C.elegans, *Cell* 51:309-318. (MedLine)

Lymn, R.W. and Taylor, E.W. (1971) Mechanism of adenosine triphosphate hydrolysis by actomyosin,

Biochemistry 10:4617-4624. (MedLine)

Ma, Y.Z. and Taylor, E.W. (1997) Interacting head mechanism of microtubule-kinesin ATPase, *J. Biol. Chem.* 272:724-730. (MedLine)

Machado, C., Sunkel, C.E. and Andrew, D.J. (1998) Human autoantibodies reveal titin as a chromosomal protein, *J. Cell Biol.* 141: 321-333. (MedLine)

MacLennan, D. H., Campbell, K. and Reithmeier, R. A. F. (1983) Calsequestrin, In *Calcium and Cell Function.* Vol. 4, pp. 151-172. Academic Press, New York. (MedLine)

Maddox, C. R. and Perry, S. V. (1966) Differences in the myosins of the red and white muscles of the pigeon, *Biochem. J.* 99:8p-9p.

Mandrino, M. (1977) Voltage clamp experiments on frog single skeletal muscle fibres: evidence for a tubular sodium current, *J. Physiol. (London)* 269:605-625. (MedLine)

Maniak, M., Rauchenberger, R., Albrecht, R., Murphy, J. and Gerisch, G. (1995) Coronin involved in phagocytosis: dynamics of particle-induced relocalization visualized by a green fluorescent protein Tag, *Cell* 83:915-924. (MedLine)

Manning, B.D. and Snyder, M. (2000) Drivers and passengers wanted! the role of kinesin-associated proteins, *Trends Cell Biol.* 10:281-289. (MedLine)

Marks, A.R. (1992) Calcium channels expressed in vascular smooth muscle, *Circulation* 86 (6 Suppl): III61-III67.

Marks, A.R. (1997) Intracellular calcium-release channels: regulators of cell life and death, *Am. J. Physiol. (Heart Circ. Physiol.)* 41: H597-H605.

Martin R.(1996) The structure of the neurofilament cytoskeleton in the squid giant axon and synapse, *J. Neurocytol.* 25:547-554. (MedLine)

Marx, S.O., Ondrias, K. and Marks, A.R. (1998) Coupled gating between individual skeletal muscle Ca^{2+} release channels (Ryanodine receptors), *Science* 281:818-821. (MedLine)

Masaki, T., Endo, M., and Ebashi, 5. (1967) Localization of 6S component of α-actinin at Z-band, *Biochem. (Tokyo)* 62(5):630-632. (MedLine)

Mayans, O., van der Ven, P.F, Wilm, M., Mues, A., Young, P., Furst, D.O., Wilmanns, M. and Gautel, M.

(1998) Structural basis for activation of the titin kinase domain during myofibrillogenesis, *Nature* 395:863-869. (MedLine)

McGoldrick, C.A., Gruver, C., and May, G.S. (1995) MyoA of *Aspargillus nidulans* encodes an essential myosin I required for secretion and polarized growth, *J. Cell Biol.* 128:577-587. (MedLine)

Mehta, A.D., Finer, J.T. and Spudich, J.A. (1997) Detection of single-molecule interactions using thermal diffusion, *Proc. Natl. Acad. Sci. USA* 94:7927-7931. (MedLine)

Mehta, A.D., Rock, R.S., Rief, M., Spudich, J.A., Mooseker, M.S. and Cheney, R.E. (1999) Myosin-V is a processive actin-based motor, *Nature* 400:590-593. (MedLine)

Mermall, V., McNally, J.G. and Miller, K.G. (1994) Transport of cytoplasmic particles catalyzed by an unconventional myosin in living *Drosophila* embryos, *Nature* 369:560-562. (MedLine)

Miki, H., Miura, K. and Takenawa, T. (1996) N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases, *EMBO J.* 15:5326-5335. (MedLine)

Millevoi, S., Trombitas, K., Kolmerer, B., Kostin, S., Schaper, J., Pelin, K., Granzier, H. and Labeit, S. (1998) Characterization of nebulette and nebulin and emerging concepts of their roles for vertebrate Z-discs, *J. Mol. Biol.* 282:111-123. (MedLine)

Milner, D.J., Weitzer, G., Tran, D., Bradley, A. and Capetanaki, Y. (1996) Disruption of muscle architecture and myocardial degeneration in mice lacking desmin, *J. Cell Biol.* 134:1255-1270. (MedLine)

Mines, G. R. (1913) On the functional analysis of the action of electrolytes, *J. Physiol. (London)* 46:553-564.

Mocz, G. and Gibbons, I.R.. Model for the motor component of dynein heavy chain based on homology to the AAA family of oligomeric ATPases, *Structure* (Camb), 9:93-103. (MedLine)

Molina, I., Baars, S., Brill, J.A., Hales, K.G., Fuller, M.T. and Ripoll, P. (1997) A chromatin-associated kinesin-related protein required for normal mitotic chromosome segregation in *Drosophila J. Cell Biol.* 139:1361-1371. (MedLine)

Molloy, J.E., Burnes, J.E., Sparrow, J.C., Tregear, R.T., Kendrick-Jones, J. and White, D.C.S. (1995a) Single molecule mechanics of heavy meromyosin and S1 interacting with rabbit or *Drosophila* actins using optical tweezers, *Biophys. J.* 68:298s-305s. (MedLine)

Molloy, J.E., Burns, J.E., Kendrick-Jones, J., Tregear, R.T. and White, D.C.S. (1995b) Movement and

force produced by a single myosin head, *Nature* 378:209-212. (MedLine)

Montell, C. and Rubin, G.M. (1988) The Drosophila *ninaC* locus encodes two photreceptor cell specific proteins with domains homologous to protein kinases and the mysoin heavy chain head, *Cell* 52:757-772. (MedLine)

Mooseker, M.S. and Cheney, R.E. (1995) Unconventional myosins, *Ann. Rev. Cell. Biol.* 11:633-675. (MedLine)

Mooseker, M.S., Wolenski, J.S., Coleman, T.R. Hauden, S.M., Cheney, R.E., Espreafico, E.M., Heintzelman, M.B. and Peterson, M.D. (1991) Structural and functional dissection of a membrane-bound mechanoenzyme: brush border myosin I, *Curr. Topics Membr.* 38:31-55.

Morfini, G., Quiroga, S., Rosa, A., Kosik, K. and Caceres, A. (1997) Suppression of KIF2 in PC12 cells alters the distribution of a growth cone nonsynaptic membrane receptor and inhibits neurite extension, *J. Cell Biol.* 138:657-669. (MedLine)

Morii, H., Takenawa, T., Arisaka, F. and Shimizu, T. (1997) Identification of kinesin neck region as a stable alpha-helical coiled coil and its thermodynamic characterization, *Biochemistry* 36:1933-1942. (MedLine)

Mueller, H. and Perry, S. V. (1962) The degradation of heavy meromyosin by trypsin, *Biochem.* 85:431-439.

Mues, A., van der Ven, P.F., Young, P., Furst, D.O., Gautel, M. (1998) Two immunoglobulin-like domains of the Z-disc portion of titin interact in a conformation-dependent way with telethonin, *FEBS Lett.* 111-114. (MedLine)

Mulligan, L., Balin, B.J., Lee, V.M. and Ip, W. (1991) Antibody labeling of bovine neurofilaments: implications on the structure of neurofilament sidearms, *J. Struct. Biol.* 106:145-160. (MedLine)

Murayama, K. (1997) Connectin/titin, giant elastic protein of muscle, *FASEB J.* 11:341-345.

Muresan, V., Abramson, T., Lyass, A., Winter, D., Porro, E., Hong, F., Chamberlin, N.L. and Schnapp, B.J. (1998) KIF3C and KIF3A form a novel neuronal heteromeric kinesin that associates with membrane vesicles, *Mol. Biol. Cell* 9:637-652. (MedLine)

Muresan, V., Stankewich, M.C., Steffen, W., Morrow, J.S., Holzbaur, E.L. and Schnapp, B.J. (2001) Dynactin-dependent, dynein-driven vesicle transport in the absence of membrane proteins: a role for spectrin and acidic phospholipids, *Mol. Cell.* 7:173-183. (MedLine)

Murphey, D. B. and Tilney, L. G. (1974) The role of microtubules in the movement of pigment granules in teleost melanophores, *J. Cell Biol.* 61:757-779. (MedLine)

Nagai, R. and Kamiya, N. (1977) Differential treatment of Chara cells with cytochalasin B with special reference to its effect in cytoplasmic streaming, *Exp. Cell Res.* 108:231-237. (MedLine)

Nagai, R. and Rebhun, L. I. (1966) Cytoplasmic microfilaments in streaming Nitella cells, *J. Ultrastruct. Res.* 14:571-589. (MedLine)

Nakagawa, T., Setou, M., Seog, D., Ogasawara, K., Dohmae, N., Takio, K. and Hirokawa, N. (2000) A novel motor, KIF13A, transports mannose-6-phosphate receptor to plasma membrane through direct interaction with AP-1 complex, *Cell* 103:569-581. (MedLine)

Nangaku, M., Sato-Yoshitake, R.,Okada, Y., Noda, Y., Takemura, R., Yamazaki, H. and Hirokawa, N. (1994) KIF1B, a novel microtubule plus end directed monomeric motor protein for mitochondria transport, *Cell* 79:1209-1220. (MedLine)

Nascimento, A.A.C., Cheney, R.E., Tauhata, S.B.F, Larson, R.E. and Mooseker, M.S. (1996) Enzymatic characterization and functional domain mapping of brain myosin-V, *J. Biol. Chem.* 271:17561-17569. (MedLine)

Nave, R. and Weber, K.(1990) A myofibrillar protein of insect muscle related to vertebrate titin connects Z band and A band: purification and molecular characterization of invertebrate mini-titin, *J. Cell Scie.* 95:535-544. (MedLine)

Nave, R., Furst, D.O. and Weber, K. (1989) Visualization of the polarity of isolated titin molecules: a single globular head and a long thin rod as the M band anchoring domain? *J. Cell Biol.* 109:2177-2187. (MedLine)

Neer, E.J., Schmidt, C.J., Nambudripad, R. and Smith, T.F. (1994) The ancient regulatory-protein family of WD-repeat proteins, *Nature* 371:297-300. (MedLine)

Nelson, M.T., Cheng, H., Rubart, M., Santana, L.F., Bonev, A.D., Knot, H.J. and Lederer W.J. (1995) Relaxation of arterial smooth muscle by calcium sparks, *Science* 270:633-637. (MedLine)

Neuwald, A.F., Aravind, L., Spouge, J.L. and Koonin, E.V. (1999) AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes, *Genome Res.* 9:27-43. (MedLine)

Niclas, J., Allan, V.J. and Vale, R.D. (1996) Cell cycle regulation of dynein association with membranes modulates microtubule-based organelle transport, *J. Cell. Biol.* 133:585-593. (MedLine)

Nikolic, B., Mac Nulty, E., Mir, B. and Wiche G. (1996) Basic amino acid residue cluster within nuclear targeting sequence motif is essential for cytoplasmic plectin-vimentin network junctions, *J. Cell Biol.* 134:1455-1467 (MedLine)

Nixon, R.A., Paskevich, P.A., Sihag, R.K., Thayer, C.Y. (1994) Phosphorylation on carboxyl terminus domains of neurofilament proteins in retinal ganglion cell neurons in vivo: influences on regional neurofilament accumulation, interneurofilament spacing, and axon caliber, *J. Cell Biol.* 126: 1031-1046. (MedLine)

Nixon, R. A. and Sihag, R. K. (1991) Neurofilament phosphorylation: a new regulation and function, *Trends in Neurosci.* 14:501-506. (MedLine)

Noda, Y., Sato-Yoshitake, R., Kondo, S., Nangaku, M. and Hirokawa, N .(1995) KIF2 is a new microtubule-based anterograde motor that transports membranous organelles distinct from those carried by kinesin heavy chain or KIF3A/B, *J. Cell. Biol.* 129:157-167. (MedLine)

Noegel, A., Witke, W. and Schleicher, M. (1987) Calcium-sensitive non-muscle α-actinin contains EF-hand structures and highly conserved regions, *FEBS Lett.* 221:391-396. (MedLine)

Novak, K.D., Peterson, M.D., Reedy, M.C. and Titus, M.A. (1995) *Dictyostelium* myosin I double mutants exhibit conditional defects in pinocytosis, *J. Cell Biol.* 131:1205-1221. (MedLine)

Okabe, S., Muyasaka, H., and Hirokawa, N. (1993) Dynamics of neuronal intermediate filaments, *J. Cell Biol.* 121:375-386. (MedLine)

Okada, Y. and Hirokawa, N. (1999) A processive single-headed motor: kinesin superfamily protein KIF1A, *Science* 283:1152-1157. (MedLine)

Okada, Y. and Hirokawa, N. (2000) Mechanism of the single-headed processivity: Diffusional anchoring between the K-loop of kinesin and the C terminus of tubulin, *Proc. Natl. Acad. Sci. USA* 97:640-645. (MedLine)

Ong, L.L., Lim, A.P., Er, C.P., Kuznetsov, S.A. and Yu, H. (2000) Kinectin-kinesin binding domains and their effects on organelle motility, *J. Biol. Chem.* 275:32854-32860. (MedLine)

Orokos, D.D. and Travis, J.L. (1997) Cell surface and organelle transport share the same enzymatic properties in *Reticulomyxa*, *Cell Motil. Cytoskeleton* 38:270-277. (MedLine)

Orokos, D.D., Bowser, S.S. and Travis, J.L. (1997) Reactivation of cell surface transport in *Reticulomyxa*,

Cell Motil. Cytoskeleton 37:139-148. (MedLine)

Ostap, E.M. and Pollard, T.D. (1996) Biochemical kinetic characterization of the *Acanthamoeba* myosin-I ATPase, *J. Cell Biol.* 132:1053-1060. (MedLine)

Otsuka A.J., Jayaprakash, A., Annoveros-Garcia, J., Tang, L., Fisk, G., Hartshorne, T., Franco, R. and Born, T. (1991) The *C. elegans* unc-104 gene encodes putative kinesin heavy chain-like protein, *Neuron* 6:113-122. (MedLine)

Page, S. (1964) The organization of the sarcoplasmic reticulum in frog muscle, *J. Physiol. (London)* 175:10P-11P.

Pantaloni, D. and Carlier, M.F. (1993) How profilin promotes actin filament assembly in the presence of thymosin β4, *Cell* 75:1007-1014. (MedLine)

Parry, D.A.D. (1999) Structural features of IF proteins, in *Guidebook to the the Cytoskeleton and Motor Proteins* (ed. Kreis, T. and Vale, R.) Oxford University Press, Oxford and New York, pp. 285-291.

Paschal B.M. and Vallee, B.B. (1987) Retrograde transport by microtubule-associated portein MAP1C, *Nature* 330:181-183. (MedLine)

Paschal, B.M., Shetner, H.S. and Vallee, B.B. (1987) MAP-1C is a microtubule activated ATPase which translocates microtubules in vitro and has dynein-like preperties, *J. Cell Biol.* 105:1273-1282. (MedLine)

Pasternak, C., Spudich, J. A. and Elson, E. L. (1989) Capping of surface receptors and concomitant cortical tension are generated by conventional myosin, *Nature* 341:549-551. (MedLine)

Patterson, B. and Spudich, J.A. (1996) Cold-sensitive mutations of *Dictyostelium* myosin heavy chain highlight functional domains of the myosin motor, *Genetics* 143:801-810. (MedLine)

Payne, M.R. and Rudnick, S.E. (1989) Regulation of vertebrate striated muscle contraction, *Trends Biochem. Sci.* 14:357-360. (MedLine)

Peachey, L. D. (1965) The sarcoplasmic reticulum and transverse tubules of the frog's sartorius, *J. Cell Biol.* 25 (No. 3, Pt. 2):209-231. (MedLine)

Persechini, A., Stull, J. T. and Cooke, R. (1985) The effect of myosin phosphorylation on the contractile properties of skinned rabbit skeletal muscle fibers, *J. Biol. Chem.* 260:7951-7954. (MedLine)

Pfarr, C.M., Coue, M., Grissom, P.M., Hays, T.S., Porter, M.E. and McIntosh, J.R. (1990) Cytoplasmic

dynein is localized to kinetochores during mitosis, *Nature* 345:263-265. (MedLine)

Pfuhl, M., Winder, S.J. and Pastore, A. (1994) Nebulin, a helical actin binding protein, *EMBO J.* 13:1782-1789. (MedLine)

Pfuhl, M., Winder, S.J., Castiglione Morelli, M.A., Labeit, S. and Pastore, A. (1996) Correlation between conformational and binding properties of nebulin repeats, *J. Mol. Biol.* 257:367-384. (MedLine)

Pollard, T.D., Doberstein, S.K. and Zot, H.G. (1991) Myosin I, *Ann. Rev. Physiol.* 53:653-681. (MedLine)

Pollard, T. D. and Cooper, J. A. (1986) Actin and actin-binding proteins. A critical evaluation of mechanisms and function, *Annu. Rev. Biochem.* 55:987-1035. (MedLine)

Pollock, N., de Hostos, E.L., Turck, C.W. and Vale, R.D. (1999) Reconstitution of membrane transport powered by a novel dimeric kinesin motor of the Unc104/KIF1A family purified from *Dictyostelium*, *J. Cell. Biol.* 147:493-506. (MedLine)

Porter, K. R. (1961) The sarcoplasmic reticulum. Its recent history and present status, *J. Biophys. Biochem. Cytol.* l0(2):219-226.

Porter, J.A. and Montell, C. (1993) Distinct roles of *Drosophila* ninaC kinase and myosin domains revealed by systematic mutagenesis, *J. Cell Biol.* 122;601-612. (MedLine)

Porter, J.A., Hicks, J.C., Williams, P.C. and Montell, C. (1992) Differential localization and requirements for the two Drosophila *ninac* kinase/myosins in photoreceptor cells, *J. Cell Biol.* 116:683-695. (MedLine)

Porter, J.A., Yu, M., Doberstein, S.K., Pollard, T.D. and Montell, C. (1993) Dependence of calmodulin localization in the retina on ther ninaC unconventional myosin, *Science* 262:1038-1042. (MedLine)

Porter, J.A., Minke, B., and Montell, C. (1995) Calmodulin binding to *Drosophila* NinaC required for termination of phototransduction, *EMBO J.* 14:4450-4459. (MedLine)

Portzehl, H. E., Caldwell. P. C. and Rüegg, J. C. (1964) The dependence of contraction and relaxation of muscle fibres from the crab Maya squinado on the internal concentration of free calcium ions, *Biochim. Biophys. Acta* 79:581-591.

Prahlad, V., Yoon, M., Moir, R.D., Vale, R.D. and Goldman, R.D. (1998) Rapid movements of vimentin on microtubule tracks: kinesin-dependent assembly of intermediate filament networks, *J. Cell Biol.* 143:159-170 (MedLine)

Prekeris, R. and Terrian, D.M. (1997) Brain myosin V is a synaptic vesicle-associated motor protein: evidence for a Ca^{2+}-dependent interaction with the synaptobrevin-synaptophysin complex, *J. Cell Biol.* 137:1589-1601. 9MedLine)

Presley, J.F., Cole, N.B., Schroer, T.A., Hirschberg, K., Zaal, K.J. and Lippincott-Schwartz, J. (1997) ER-to-Golgi transport visualized in living cells, *Nature* 389:81-85. (MedLine)

Protasi, F., Sun, X.H., Franzini-Armstrong, C. (1996) Formation and maturation of the calcium release apparatus in developing and adult avian myocardium, *Dev. Biol.* 173:265-278. (MedLine)

Provance , D.W., Jr., Wei, M., Ipe, V. and Mercer, J.A. (1996) Cultured melanocytes from dilute mutant mice exhibit dendritic morphology and altered melanosome distribution, *Proc. Natl. Acad. Sci. USA* 93:14554-14558. (MedLine)

Pryer, N.K., Wadsworth, P. and Salmon, E.D. (1986) Polarized microtubule gliding and particle saltations produced by soluble factors from sea urchin eggs and embryos, *Cell Motil.* 6:537-548. (MedLine)

Quinlan, R., Hutchinson, C. and Lane, B. (1994) Intermediate filament proteins, *Protein Profile* 2:801-952.

Quintyne, N.J., Gill, S.R., Eckley, D.M., Crego, C.L., Compton, D.A. and Schroer, T.A. (1999) Dynactin is required for microtubule anchoring at centrosomes, *J. Cell Biol.* 147:321-334. (MedLine)

Radionov, V.I., Hope, A.J., Svitikina, T.M. and Borisy, G.G. (1998) Functional coordination of microtubule-based and actin-based motility in melanophores, *Current Biol.* 8:165-168. (MedLine)

Rahman, A., Kamal, A., Roberts, E.A. and Goldstein, L.S. (1999) Defective kinesin heavy chain behavior in mouse kinesin light chain mutants, *J. Cell Biol.* 146:1277-1288. (MedLine).

Rall, J.A. (1996) Role of parvalbumin in skeletal muscle relaxation, *News Physiol. Scie.* 11:249-255. (MedLine)

Ray, S., Meyhofer, E., Milligan, R.A. and Howard, J.(1993) Kinesin follows the microtubule's protofilament axis, *J. Cell Biol.* 121:1083-1093. (MedLine)

Rayment I. (1996) The structural basis of the myosin ATPase activity, *J. Biol. Chem.* 271:15850-15853. (MedLine)

Rayment, I., Rypniewski, W.R., Schmidt-Base, K., Smith, R., Tomchick, D.R., Benning, M.M., Winkelmann, D.A., Wesenberg, G. and Holden, H.M. (1993a) Three-dimensional structure of myosin

subfragment-1: a molecular motor, *Science* 261:50-58. (MedLine)

Rayment, I., Holden, H.M., Wittaker, M., Yohn, C.B., Lorenz, M., Holmes, K.C. and Milligan, R.A. (1993b) Structure of the actin-myosin complex and its implications for muscle contraction, *Science* 261:58-65. MedLine

Reilein, A.R., Rogers, S.L., Tuma, M.C. and Gelfand, V.I. (2001) Regulation of molecular motor proteins, *Int. Rev. Cytol.* 204:179-238. (MedLine)

Reuter, H. (1984) Ion channels in cardiac cell membranes, *Annu. Rev. Physiol.* 46:473-484. (MedLine)

Rice, S., Lin, A.W., Safer, D., Hart, C.L., Naber, N., Carragher, B.O., Cain, S.M., Pechatnikova, E., Wilson-Kubalek, E.M., Whittaker, M., Pate, E., Cooke, R., Taylor, E.W., Milligan, R.A. and Vale, R.D. (1999) A structural change in the kinesin motor protein that drives motility, *Nature* 402:778-784. (MedLine)

Rief, M., Gautel, M., Oesterhelt, F., Fernandez, J.M. and Gaub, H.E. (1997) Reversible unfolding of individual titin immunoglobulin domains by AFM, *Science* 276:1109-1112. (MedLine)

Rodnina, M.V., Savelbergh, A., Katunin, V.I. and Wintermeyer, W. (1996) Hydrolysis of GTP by elongation factor G drives tRNA movement on the ribosome, *Nature* 385:37-41. (MedLine)

Rogers, S.L. and Gelfand, V.I. (1998) Myosin cooperates with microtubule motors during organelle transport in melanophores, *Curr. Biol.* 8:161-164. (MedLine)

Rogers, S.L., Tint, I.S., Fanapour, P. and Gelfand, V.I. (1997) Regulated bidirectional motility of melanophores along microtubules in vitro, *Proc. Natl. Acad. Sci. USA* 94:3720-3725. (MedLine)

Romberg, L., Pierce, D.L. and Vale, R.D. (1998) Role of the kinesin neck region in processive microtubule-based motility, *J. Cell Biol.* 140:1407-1416. (MedLine)

Rozelle, A.L., Machesky, L.M., Yamamoto, M., Driessens, M.H., Insall, R.H., Roth, M.G., Luby-Phelps, K., Marriott, G., Hall, A. and Yin, H.L. (2000) Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3, *Curr. Biol.* 10:311-320. (MedLine)

Rüegg, J. C. (1968) Contractile mechanisms of smooth muscle, *Symp. Soc. Exp. Biol.* 22:45-66. (MedLine)

Ruhrberg, C. and Watt, F.M. (1997) The plakin family: versatile organizers of cytoskeletal architecture, *Curr. Opin. Genet. Dev.* 7:392-397. (MedLine)

Ruppert, C., Goddel, J., Müller, R.T., Kroschewski, R., Reinhard, J. and Bähler, M. (1995) Localization of the rat myosin I molecule myr1 and myr2 and *in vivo* targeting of their tail domains, *J. Cell Scie.* 108: in press (MedLine)

Sablin, E.P., Kull, F.J., Cooke, R., Vale, R.D. and Fletterick, R.J. (1996) Crystal structure of the motor domain of the kinesin-related motor ncd, *Nature* 380:555-559. (MedLine)

Sablin, E.P., Case, R.B., Dai, S.C., Hart, C.L., Ruby, A., Vale, R.D. and Fletterick, R.J. (1998) Direction determination in the minus-end-directed kinesin motor ncd, *Nature* 395:813-816. (MedLine)

Sack, S., Muller, J., Marx, A., Thormahlen, M., Mandelkow, E.M., Brady, S.T. and Mandelkow, E. (1997) X-ray structure of motor and neck domains from rat brain kinesin, *Biochemistry* 36:16155-16165. (MedLine)

Saito, N., Okada, Y., Noda, Y., Kinoshita, Y., Kondo, S. and Hirokawa, N. (1997) KIFC2 is a novel neuron-specific C-terminal type kinesin superfamily motor for dendritic transport of multivesicular body-like organelles, *Neuron* 18:425-438. (MedLine)

Samsó, M., Radermacher, M., Frank, J. and Koonce, M.P. (1998) Structural characterization of a dynein motor domain, *J. Mol. Biol.* 276:927-937. (MedLine)

Sanders, K.M. (1996) A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract, *Gastroenterology* 111:492-515. (MedLine)

Santos, B. and Snyder, M. (1997) Targeting of chitin synthase 3 to polarized growth sites in yeast requires Chs5p and Myo2p, *J. Cell Biol.* 136:95-110. (MedLine)

Sarria, A.J., Lieber, J.G., Nordeen, S.K. and Evans, R.M. (1994) The presence or absence of a vimentin-type intermediate filament network affects the shape of the nucleus in human SW-13 cells, *J. Cell Sci.* 107:1593-1607. (MedLine)

Schafer, D.A., Gill, S.R., Cooper, J.A., Heuser, J.E. and Schroer, T.A. (1994) Ultrastructural analysis of the dynactin complex: an actin-related protein is a component of a filament that resembles F-actin, *J. Cell Biol.* 126:403-412. (MedLine)

Schliwa, M. (1999) Myosin steps backwards, *Nature* 401:431-432. (MedLine)

Schliwa, M. Shimizu, T., Vale, R. D. and Euteneur, U. (1991) Nucleotide specificities of anterograde and retrograde organelle transport in Reticulomyxa are undistinguishable, *J. Cell Biol.* 112:1199-1203. (MedLine)

Schnapp, B. J., Crise, B., Sheetz, M. P., Reese, T. S. and Khan,S. (1990) Delayed start-up of kinesin-driven microtubule gliding following inhibition by adenosine 5'-[,-imido]triphosphate, *Proc. Natl. Acad. Sci. USA.* 87:10053-10057. (MedLine)

Schneider, M.F. (1994) Control of calcium release in functioning skeletal muscle fibers, *Annu. Rev. Physiol.* 56:463-484.

Schnitzer, M.J. and Block, S.M. (1997) Kinesin hydrolyses one ATP per 8-nm step, *Nature* 388:386-390. (MedLine)

Schott, D., Ho, J., Pruyne, D. and, Bretscher, A. (1999) The COOH-terminal domain of Myo2p, a yeast myosin V, has a direct role in secretory vesicle targeting, *J. Cell Biol.* 147:791-808. (MedLine)

Searle, A.G. (1952) A lethal allele of *dilute* in the house mouse, Heredity 6:395-401.

Segretain, D., Rambourg, A. and Clermont, Y. (1981) Three dimensional arrangement of mitochondria and endoplasmic reticulum in the heart muscle fiber of the rat, *Anat. Rec.* 200:139-151. (MedLine)

Sellers, J.R. and Goodson, H.V. (1995) Motor proteins 2: myosins, Protein Profiles 2:1323-1483. (MedLine)

Setou, M., Nakagawa, T., Seog, D.H. and Hirokawa, N. (2000) Kinesin superfamily motor protein KIF17 and mLin-10 in NMDA receptor-containing vesicle transport, *Science* 288:1796-1802. (MedLine)

Sham, J.S., Cleemann, L. and Morad, M, (1995) Functional coupling of Ca2+ channels and ryanodine receptors in cardiac myocytes, *Proc. Natl. Acad. Sci. USA* 92:121-125. (MedLine)

Sharp, D.J., Yu, K.R., Sisson, J.C., Sullivan, W. and Scholey, J.M. (1999) Antagonistic microtubule-sliding motors position mitotic centrosomes in Drosophila early embryos, *Nature Cell Biol.* 1:51-54. (MedLine)

Sharp, D.J., Rogers, G.C. and Scholey, J.M. (2000) Microtubule motors in mitosis, *Nature* 407:41-47. (MedLine)

Sheetz, M. P. and Spudich, J. A. (1983a) Movement of myosin- coated fluorescent structures on actin cables, *Cell Motil.* 3:485-489. (MedLine)

Sheetz, M. P. and Spudich, J. A. (1983b) Movement of myosin- coated fluorescent beads on actin cables in vitro, *Nature* 303:31-35. (MedLine)

Shih, C.L., Chen, M.J., Linse, K. and Wang, K. (1997) Molecular contacts between nebulin and actin: cross-linking of nebulin modules to the N-terminus of actin, *Biochemistry* 36:1814-1825. (MedLine)

Sinard, J. H. and Pollard, T. D. (1989) Microinjection into *Acanthamoeba castellanii* of monoclonal antibodies to myosin II slows but does not stop cell locomotion, *Cell Motil. Cytosk.* 12:42-52. (MedLine)

Skalli, O., Chou, Y.-H. and Goldman, R.D. (1992) Intermediate filaments: not so tough after all, *Trends in Cell Biol.* 2:308-312.

Slayter, H. S. and Lowry, S. (1967) Substructure of the myosin molecule as visualized by electron microscopy, *Proc. Natl. Acad. Sci. USA* 58:1611-1618. (MedLine)

Smith, C.A. and Rayment, I. (1996a) X-ray structure of the magnesium(II).ADP.vanadate complex of the *Dictyostelium discoideum* myosin motor domain to 1.9 Å resolution, *Biochemistry* 35:5404-5417. (MedLine)

Smith, C.A. and Rayment, I. (1996b) Active site comparisons highlight structural similarities between myosin and other P-loop proteins, *Biophys. J.* 70:1590-1602. (MedLine)

Sommer, J.R. and Johnson, E.A. (1979) Ultrastructure of cardiac muscle in *Handbook of Physiology Section 2: The Cardiovascular System*, Vol. 1, *The Heart*, ed. Berne, R.M. Sperekalis and Geiger, R.S., American Physiological Society, Bethesda, MD, pp. 113-187.

Soteriou, A., Gamage, M. and Trinick, J. (1993) A survey of interactions made by the giant protein titin, *J. Cell Sci.* 104:119-123. (MedLine)

Spudich, J.A. (1994) How molecular motors work, *Nature* 372:515- 518. (MedLine)

Squire, J.M. (1997) Architecture and function in the muscle sarcomere, *Curr. Opin. Struct. Biol.* 7:247-257. (MedLine)

Stebbins, H. and Hyams. J. S. (1979) In *Cell Motility* (Phillips, I. D.J., ed.), p. 192. Longman, London.

Steinert, P. M., and Roop, D. R. (1988) Molecular and cellular biology of intermediate filaments, *Annu. Rev. Biochem.* 57:593-626. (MedLine)

Stenoien, D.L. and Brady, S.T. (1997) Immunochemical analysis of kinesin light chain function, *Mol. Biol. Cell* 8:675-689. (MedLine)

Steuer, E.R., Wordeman, L., Schroer, T.A. and Sheetz, M.P. (1990) Localization of cytoplasmic dynein to

mitotic spindles and kinetochores, *Nature* 345:266-268. (MedLine)

Stevens, R.J., Publicover, N.G. and Smith, T.K. (1999) Induction and organization of Ca^{2+} waves by enteric neural reflexes, *Nature* 399:62-66.

Stewart, R.J., Thaler, J.P., and Goldstein, L.S.B. (1993) Direction of microtubular movement is an intrinsic property of the motor domain of kinesin heavy chain and *Drosophila ncd* protein, *Proc. Natl. Acad. Sci. USA* 90:5209-5213.

Suzuki, Y., Yasunaga, T., Ohkura, R., Wakabayashi, T. and Sutoh, K.(1998) Swing of the lever arm of a myosin motor at the isomerization and phosphate-release steps, *Nature* 396:380-383. (MedLine)

Svitkina, T.M., Verkhovsky, A.B. and Borisy, G.G. (1996) Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton, *J. Cell Biol.* 135:991-1007. (MedLine)

Svoboda, K., Schmidt, C.F., Schnapp, B.J. and Block, S.M. (1993) Direct observation of kinesin stepping by optical trapping interferometry, *Nature* 365:721-727. (MedLine)

Swanson, J., Bushnell, A. and Silverstein, S. C. (1987) Tubular lysosomes, morphology and distribution within macrophages depend on the integrity of cytoplasmic microtubules, *Proc. Natl. Acad. Sci. USA* 84:1921-1925. (MedLine)

Tabb, J.S., Harmon, K.O., DePina, A.S. and Langford, G.M. (1996) Localization of myosin on tubovesicular organelles in squid giant axon by immuno-EM, *Biol. Bull.* 191:274-275. (MedLine)

Tabb, J.S., Molyneaux, B.J., Cohen, D.L., Kuznetsov, S.A. and Langford, G.M. (1998) Transport of ER vesicles on actin filaments in neurons by myosin V, *J. Cell Sci.* 111:3221-3234. (MedLine)

Takagishi, Y., Oda, S., Hayasake, S., Dekker-ohno, K. Shikata, T. Inouye, M. and Yamamura, H. (1996) The *dilute-lethal* (d') gene attacks a Ca^{2+} store in the denditic spine of Purkinje cells in mice, *Neuroscie. Lett.* 215:169-172. (MedLine)

Takeda, S., Yamazaki, H., Seog, D.H., Kanai, Y., Terada, S. and Hirokawa, N. (2000) Kinesin superfamily protein 3 (KIF3) motor transports fodrin-associating vesicles important for neurite building, *J. Cell Biol.* 148:1255-1266. (MedLine)

Tanabe, T., Beam, K.G., Adams, B.A., Niidome, T. and Numa, S. (1990) Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling, *Nature* 346:567-569. (MedLine)

Taunton, J., Rowning, B.A., Coughlin, M.L., Wu, M., Moon, R.T., Mitchison, T.J. and Larabell, C.A.

(2000) Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP, *J. Cell Biol.* 148:519-530. (Medline)

Taylor, K.A., Schmitz, H., Reedy, M.C., Goldman, Y.E., Franzini-Armstrong, C., Sasaki, H., Tregear, R.T., Poole, K., Lucaveche, C., Edwards, R.J., Chen, L.F., Winkler, H. and Reedy, M.K. (1999) Tomographic 3D reconstruction of quick-frozen, Ca^{2+}-activated contracting insect flight muscle, *Cell* 99:421-431. (MedLine)

Theriot, J.A. (1997) Accelerating on a treadmill: ADF/cofilin promotes rapid actin filament turnover in the dynamic cytoskeleton, *J. Cell Biol.* 136:1165-1168. (MedLine)

Titus, M.A. (1997) Unconventional myosins: new frontiers in actin-based motors, *Trends in Cell Bio.* 7:119-123.

Titus, M.A., Warwick, H.M. and Spudich, J.A. (1989) Multiple actin-based motor genes in *Dictyostelium*, *Cell Regul.* 1:55-63. (MedLine)

Tokito, M.K., Howland, D.S., Lee. V.M. and Holzbaur, E.L (1996) Functionally distinct isoforms of dynactin are expressed in human neurons, *Mol. Biol. Cell* 7:1167-1180. (MedLine)

Tokunaga. M., Kitamura, K., Saito, K., Iwane, A.H. and Yanagida, T. (1997) Single molecule imaging of fluorophores and enzymatic reactions achieved by objective-type total internal reflection fluorescence microscopy, *Biochem. Biophys. Res. Commun.* 235:47-53. (MedLine)

Toyoshima, Y.Y., Kron, S.J., McNally, E.M., Niebling, K.R., Toyoshima, C. and Spudich. J.A. (1987) Myosin subfragment-1 is sufficient to move actin filament in vitro, *Nature* 328:536-539 (MedLine)

Toyoshima, I., Yu, H., Steuer, E.R. and Sheetz, M.P. (1992) Kinectin,a major kinesin-binding protein on ER, *J. Cell Biol.* 118:1121-1131. (MedLine)

Trayer, I.P. and Smith, J. (1997) Motoring down the highways of the cell, *Trends Cell Bio.* 7:259-263.

Trinick, J. (1994) Titin and nebulin: protein rulers of muscle? *Trends in Biochem. Sci.* 19:405-409. (MedLine)

Trinick, J. and Tskhovrebova, L. (1999) Titin:molecular control freak, *Trends in Cell Biol.* 9:377-380. (MedLine)

Tripet, B., Vale, R.D. and Hodges, R.S. (1997) Demonstration of coiled-coil interactions within the kinesin neck region using synthetic peptides: implications for motor activity, *J. Biol. Chem.* 272:8946-

8956. (MedLine)

Trybus, K. M. (1991) Assembly of cytoplasmic and smooth muscle myosins, *Curr. Opin. Cell Biol.* 3:105-111. (MedLine)

Tsaturyan, A.K., Bershitsky, S.Y., Burns, R. and Ferenczi, M.A. (1999) Structural changes in the actin-myosin cross-bridges associated with force generation induced by temperature jump in permeabilized frog muscle fibers, *Biophys. J.* 77:354-357 (MedLine)

Uyeda, T.Q.P., Abramson, P.D. and Spudich, J.A. (1996) The neck region of the myosin domain acts as a lever arm to generate movement, *Proc. Ntl. Acad. Sci. USA* 93:4459-4464. (MedLine)

Vaisberg, E.A., Grissom, P.M. and McIntosh, J.R. (1996) Mammalian cells express three distinct heavy chains that are localized in different parts of the cytoplasm, *J. Cell Biol.* 133: 831-842. (MedLine)

Online animated models of myosin and kinesin motors: www.sciencemag.org/feature/data/1049155.shl

Vale, R.D. (1996) Switches, latches and amplifiers: common themes of G proteins and molecular motors, *J. Cell Biol.* 135:291-302. (MedLine)

Vale, R.D. and Fletterick, R.J. (1997) The design plan of kinesin motors, *Annu. Rev. Cell Dev. Biol.* 13:745-777. (MedLine)

Vale, R.D. and Milligan, R.A. (2000) The way things move: looking under the hood of molecular motor proteins, *Science* 288:88-95. (MedLine)

Vale, R.D. and Toyoshima, Y.Y. (1988) Rotation and translocation of microtubules in vitro induced by dyneins from *Tetrahymena* cilia, *Cell* 52:459-469. (MedLine)

Vale, R. D., Reese, T. S. and Sheetz, M. P. (1985a) Identification of a novel force generating protein, kinesin, involved in microtubule-based motility, *Cell* 42:39-50. (MedLine)

Vale, R. D., Reese, T. S. and Sheetz, M. P. (1985b) Different axoplasmic proteins movement in opposite directions along microtubules in vitro, *Cell* 43:623-632. (MedLine)

Vale, R. D., Schnapp, B. J., Mitchision, T., Steuer, A., Reese, T. S. and Sheetz, M. P. (1986) Different axoplasmic proteins generate movement in different directions along microtubules in vitro, *Cell* 43:623-632. (MedLine)

Vallee, R. and Bloom, G. S. (1991) Mechanism of fast and slow axonal transport, *Annu. Rev. Neuroscie.*

14:59-92. (MedLine)

Vallee, R.B., and Gee, M.A. (1998) Make room for dynein, *Trends Cell Biol.* 8:490-494. (MedLine)

Vallee, R.B. and Sheetz, M.P. (1996) Targeting of motor proteins, *Science* 271:1539-1544. (MedLine)

Vallee, R. B., Wall, J. S., Paschal, B. M. and Sheptner. H. S. (1988) Microtubule associated protein 1C from brain is a two-headed cytosolic dynein, *Nature* 332:561-563. (MedLine)

Vandekerkhove, J. (1990) Actin-binding proteins, *Curr. Opin. Cell Biol.* 2:41-50.

Vaughan, P.S., Leszyk, J.D. and Vaughan, K.T. (2001) Cytoplasmic dynein intermediate chain phosphorylation regulates binding to dynactin, *J. Biol. Chem.* 276:26171-179. (MedLine)

Vaughan, P.S., Miura, P., Henderson, M., Byrne, B. and Vaughan, K.T. A role for regulated binding of p150^{Glued} to microtubule plus ends in organelle transport, *J. Cell Biol.* 158:305-319. (MedLine)

Veigel, C., Coluccio, L.M., Jontes, J.D., Sparrow, J.C., Milligan, R.A. and Molloy, J.E. (1999) The motor protein myosin-I produces its working stroke in two steps, *Nature* 398:530-533. (MedLine)

Verhey, K.J. and Rapoport, T.A. (2001) Kinesin carries the signal, *Trends Biochem. Sci.* 26:545-550. (MedLine)

Verhey, K.J., Lizotte, D.L., Abramson, T., Barenboim, L., Schnapp, B.J. and Rapoport, T.A. (1998) Light chain-dependent regulation of kinesin's interaction with microtubules, *J. Cell Biol.* 143:1053-1066. (MedLine)

Verhey, K.J., Lizotte, D.L., Abramson, T., Barenboim, L., Schnapp, B.J. and Rapoport, T.A. (1998) Light chain-dependent regulation of Kinesin's interaction with microtubules, *J. Cell Biol.* 143:1053-1066. (MedLine)

Verhey, K.J., Meyer, D., Deehan, R., Blenis, J., Schnapp, B.J., Rapoport, T.A. and Margolis, B. (2001) Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules, *J. Cell Biol.* 152:959-970. (MedLine)

Vibert, P., York, M.L., Castellani, L., Edelstein, S., Elliott, B. and Nyitray, L.(1996) Structure and distribution of mini-titins. *Adv. Biophys*. 33:199-209. (MedLine)

Vikstrom, K.L., Sim, S.S., Goldman, R.D. and Borisy, G.G. (1992) Steady state dynamics of intermediate filament networks, *J. Cell Biol.* 118:121-129. (MedLine)

Virata, M.L., Wagner, R.M., Parry, D.A. and Green, K.J. (1992) Molecular structure of the human desmoplakin I and II amino terminus, *Proc. Natl. Acad. Sci. USA* 89:544-548. (MedLine)

Visscher, K., Schnitzer, M.J. and Block, S.M. (1999) Single kinesin molecules studied with a molecular force clamp, *Nature* 400:184-189. (MedLine)

Volkmann, N. and Hanein, D. (2000) Actomyosin: law and order in motility, *Curr. Opin. Cell Biol.* 12:26-34. (MedLine)

Volpe, P., Di Virgilio, F., Pozzan, T. and Salviati, G. (1986) Role of 1,4,5-triphosphate in excitation-contraction coupling in skeletal muscle, *FEBS Lett.* 197:1-4.

Wagenknecht, T., Grassucci, R., Frank, J., Saito, A., Inui, M. and Fleischer, S. (1989) Three-dimensional architecture of the calcium channel/foot structure of sarcoplasmic reticulum, *Nature* 338:167-170. (MedLine)

Walker, R. A., Salmon. E. D. and Endow, S. A. (1990) The *Drosophila* claret segregation protein is a minus-end directed motor molecule, *Nature* 347:780-782. (MedLine)

Wakabayashi, K., Tokunaga, M., Kohno, I., Sugimoto, Y., Hamanaka, T.. Takezawa, Y., Wakabayashi, T. and Amemiya, Y. (1992) Small-angle synchrotron X-ray scattering reveals distinct shape changes of the myosin head during hydrolysis of ATP, *Science* 258:443-447. (MedLine)

Walton, P.D., Airey, J.A., Sutko, J.L., Beck, C.F., Mignery, G.A., Sudhof, T.C., Deerinck, T.J. and Ellisman, M.H. (1991) Ryanodine and inositol trisphosphate receptors coexist in avian cerebellar Purkinje neurons, *J. Cell Biol.* 113:1145-1157. (MedLine)

Wang, Y.L. (1985) Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling, *J. Cell Biol.* 101:597-602 (MedLine)

Wang, K. and Wright, J. (1988) Architecture of the sarcomere matrix of skeletal muscle: immunoelectron microcopic evidence that suggests a set of parallel inextensive nebulin filaments anchored at the Z line, *J. Cell Biol.* 107:2199-2212. (MedLine)

Wang, Z., Khan, S. and Sheetz, M.P. (1995) Single cytoplasmic dynein molecule movements: characterization and comparison with kinesin, *Biophys. J.* 69:2011-2023. (MedLine)

Wang, F.S., Wolenski, J.S., Cheney, R.E., Mooseker, M.S. and Jay, D.G. (1996a) Function of myosin-V in filopodia extension of neuronal growth cones, *Science* 273:660-663. (MedLine)

Wang, K., Knipfer, M., Huang, Q.Q., van Heerden, A., Hsu, L.C., Gutierrez, G., Quian, X.L. and Stedman H (1996b) Human skeletal muscle nebulin sequence encodes a blueprint for thin filament architecture. Sequence motifs and affinity profiles of tandem repeats and terminal SH3, *J. Biol. Chem.* 271:4304-4314. (MedLine)

Wasenius, V. M., Saraste, M., Salvén, P., Erämaa, M., Holm, L. and Lehto, V. P. (1989) Primary structure of brain -spectrin, *J. Cell Biol.* 108:79-93. (MedLine)

Weber, A., Hertz, R. and Reiss, I. (1963) On the mechanism of the relaxing effect of fragmented sarcoplasmic reticulum, *J. Gen. Physiol.* 46:679-702.

Wells, A.L., Lin A.W., Chen, L.-Q, Safer, D., Cain, S.M., Hasson, T., Garragher, B.O., Milligan, R.A. and Sweeny, H.L. (1999) Myosin VI is an actin-based motor that moves backwards, *Nature* 401:505-508. (MedLine)

Welte, M.A., Gross, S.P., Postner, M., Block, S.M. and Wieschaus, E.F. (1998) Developmental regulation of vesicle transport in *Drosophila* embryos: forces and kinetics, *Cell* 92:547-457. (MedLine)

Wessels, D., Titus, M.A. and Soll, D.R. (1996) A Dictyostelium myosin I plays crucial role in regulating the frequency of pseudopods formed on substratum, *Cell Motil. Cytoskeleton* 33:64-79. (MedLine)

Whiting, A., Wardale, J. and Trnick, J. (1989) Does titin regulate the length of muscle thick filaments? *J. Mol. Biol.* 205:263-268. (MedLine)

Whittaker, M., Wilson-Kubalek, E.M., Smith, J.E., Faust, L., Milligan, R.A. and Sweeney, H.L. (1995) A 35-Å movement of smooth muscle myosin on ADP release, *Nature* 378:748-751. (MedLine)

Williamson, R. E. and Toh, B. H. (1979) Motile models of plant cells and the immunofluorescent localization of actin in a motile Chara cell model, In *Cell Motility.* Molecules and Organization (Hatano, S., Ishikawa, H. and Sato, H., eds.), pp. 339-346. University Park Press. Baltimore.

Wilson, S.M., Yip, R., Swing, D.A., O'Sullivan, T.N., Zhang, Y., Novak, E.K., Swank, R.T., Russell, L.B., Copeland, N.G. and Jenkins, N.A. (2000) A mutation in Rab27a causes the vesicle transport defects observed in ashen mice, *Proc. Natl. Acad. Sci. USA* 97:7933-7938. (MedLine)

Winegrad, S. (1961) The possible role of calcium in excitation- contraction coupling of heart muscle, *Circulation* 24:523-529.

Winegrad, S. (1965a) Autoradiographic studies of intraccllular calcium in frog skeletal muscle, *J. Gen. Physiol.* 48:455-479.

Winegrad, S. (1965b) The location of muscle calcium with respect to the myofibrils, *J. Gen. Physiol.* 48:997-1002. (MedLine)

Winton, F. R. (1926) The influence of length on the responses of unstriated muscle to electrical and chemical stimulation and stretching, *J. Physiol. (London)* 61:368-382.

Wood, K.W., Sakowicz, R., Goldstein, L.S. and Cleveland, D.W. (1997) CENP-E is a plus end-directed kinetochore motor required for metaphase chromosome alignment, *Cell* 91:357-366. (MedLine)

Wright, J., Huang, Q.Q. and Wang, K. (1993) Nebulin is a full-length template of actin filaments in the skeletal muscle sarcomere: an immunoelectron microscopic study of its orientation and span with site-specific monoclonal antibodies, J. Muscle Res. Cell Motil. 14:476-483. (MedLine)

Xie, X., Harrison, D.H., Schlichting, I., Sweet, R.M., Kalabokis, V.N., Szent-Gyorgyi, A.G. and Cohen, C. (1994) Structure of the regulatory domain of scallop myosin at 2.8 Å resolution, *Nature* 368:306-312. (MedLine)

Xia, Ch., Rahman, A., Yang, Z. and Goldstein, L.S. (1998) Chromosomal localization reveals three kinesin heavy chain genes in mouse, *Genomics* 52:209-213. (MedLine)

Xu, J.-Q., Harder, B.A., Uman, P. and Craig, R. (1996a) Myosin filament structure in vertebrate smooth muscle, *J. Cell Biol.* 134:53-66. (MedLine)

Xu, Z., Marszalek, J.R., Lee, M.K., Wong, P.C., Folmer, J., Crawford, T.O., Hsieh, S.T., Griffin, J.W., and Cleveland, D.W. (1996b) Subunit composition of neurofilaments specifies axonal diameter, *J. Cell Biol.* 133:1061-1069. (MedLine)

Yamazaki, H., Nakata, T., Okada, Y. and Hirokawa, N. (1995) KIF3A/B: a heterodimeric kinesin superfamily protein that works as a microtubule plus end-directed motor for membrane organelle transport, *J. Cell Biol.* 130:1387-1399. (MedLine)

Yamazaki, H., Nakata, T., Okada, Y. and Hirokawa, N.(1996) Cloning and characterization of KAP3: a novel kinesin superfamily-associated protein of KIF3A/3B, *Proc. Natl. Acad. Sci. USA* 93: 8443-8448 (MedLine)

Yang, Z. and Goldstein, L.S. (1998) Characterization of the KIF3C neural kinesin-like motor from mouse, *Mol. Biol. Cell* 9:249-261. (MedLine)

Yang, J. T., Layman, R. A. and Goldstein, L. S. B. (1989) A three-domain structure of kinesin heavy chain revealed by DNA sequence and microtubule binding analysis, *Cell* 56:879-889.

Yang, J. T., Saxton, W. M., Stewart, R. J., Raff, E. C. and Goldstein, L. S. B. (1990) Evidence that the head of kinesin is sufficient for force generation and motility in vitro, *Science* 249:42-47. (MedLine)

Yang, Y., Dowling, J., Yu, Q.C., Kouklis, P., Cleveland, D.W. and Fuchs, E. (1996) An essential cytoskeletal linker protein connecting actin microfilaments to intermediate filaments, *Cell* 86:655-665. (MedLine)

Yang, Y., Bauer, C., Strasser, G., Wollman, R., Julien, J.P. and Fuchs, E. (1999) Integrators of the cytoskeleton that stabilize microtubules, *Cell* 98:229-238. (MedLine)

Yates, L.D. and Greaser, M.L. (1983) Troponin subunit stoichiometry and content of rabbit skeletal muscle and myofibrils, *J. Biol. Chem.* 258:5770-5774. (MedLine)

Yen, T.J., Li, G., Schaar, B.T., Szilak, I. and Cleveland, D.W. (1992) CENP-E is a putative kinetochore motor that accumulates just before mitosis, *Nature* 359:536-539. (MedLine)

Yoon, M., Moir, R.D., Prahlad, V. and Goldman, R.D. (1998) Motile properties of vimentin intermediate filament networks in living cells, *J. Cell Biol.* 143:147-157. (MedLine)

Young , P., Ferguson C., Banuelos, S. and Gautel, M. (1998), Molecular structure of the sarcomeric Z-disk: two types of titin interactions lead to an asymmetrical sorting of alpha-actinin, *EMBO J.* 17:1614-1624. (MedLine)

Young, E.C., Mahtani, H.K., and Gelles, J. (1998) One-headed kinesin derivatives move by a nonprocessive, low-duty ratio mechanism unlike that of two-headed kinesin, *Biochemistry* 37:3467-3479. (MedLine)

Zhang, J.Q., Luo, G., Herrera, A.H., Paterson, B. and Horowits, R. (1996) cDNA cloning of mouse nebulin. Evidence that the nebulin-coding sequence is highly conserved among vertebrates, *Eur. J. Biochem.* 239:835-841. (MedLine)

Zhao, C., Takita, J., Tanaka, Y., Setou, M., Nakagawa, T., Takeda, S., Yang, H.W., Terada, S., Nakata, T., Takei, Y., Saito, M., Tsuji, S., Hayashi, Y. and Hirokawa, N. (2001) Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bβ, *Cell* 105:587-597. (MedLine)

Zot, H.G., Doberstein, S.K. and Pollard, T.D. (1992) Myosin-I moves actin filaments on a phospholipid substrate: implications for membrane targeting, *J. Cell Biol.* 116:367-376. (MedLine)

Zot, A.S. and Potter, J.D. (1987) Structural aspects of troponin- tropomyosin regulation of skeletal muscle, *Ann. Rev. Biophys. Biophys. Chem.* 16:535-559. (MedLine)