Virtual University

Advance Cell Biology

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Cell Junctions

Specialized cell junctions occur at points of cell-cell and cell-matrix contact in all tissues, and they are particularly plentiful in epithelia. Cell junctions are best visualized using either conventional or freeze-fracture electron microscopy, which reveals that the interacting plasma membranes (and often the underlying cytoplasm and the intervening intercellular space as well) are highly specialized in these regions.

Cell junctions can be classified into three functional groups:

1. Occluding junctions seal cells together in an epithelium in a way that prevents even small molecules from leaking from one side of the sheet to the other.

2. Anchoring junctions mechanically attach cells (and their cytoskeletons) to their neighbors or to the extracellular matrix.

3. Communicating junctions mediate the passage of chemical or electrical signals from one interacting cell to its partner.

Occluding Junctions Form a Selective Permeability Barrier Across Epithelial Cell Sheets

All epithelia have at least one important function in common: they serve as selective permeability barriers, separating fluids on either side that have a different chemical composition. This function requires that the adjacent cells be sealed together by occluding junctions. Tight junctions have this barrier role in vertebrates, as we illustrate by considering the epithelium of the mammalian small intestine, or gut.

The epithelial cells lining the small intestine form a barrier that keeps the gut contents in the gut cavity, the lumen. At the same time, however, the cells must transport selected nutrients across the epithelium from the lumen into the extracellular fluid that permeates the connective tissue on the other side. From there, these nutrients diffuse into small blood vessels to provide nourishment to the organism. This transcellular transport depends on two sets of membrane-bound membrane transport proteins. One set is confined to the apical surface of the epithelial cell (the surface facing the lumen) and actively transports selected molecules into the cell from the gut. The other set is confined to the basolateral (basal and lateral) surfaces of the cell, and it allows the same molecules to leave the cell by facilitated diffusion into the extracellular fluid on the other side of the epithelium. To maintain this directional transport, the apical set of transport proteins must not be allowed to migrate to the basolateral surface of the cell, and the basolateral set must not be allowed to migrate to the apical surface. Furthermore, the spaces between epithelial cells must be tightly sealed, so that

the transported molecules cannot diffuse back into the gut lumen through these spaces.

The tight junctions between epithelial cells are thought to have both of these roles. First, they function as barriers to the diffusion of some membrane proteins (and lipids) between apical and basolateral domains of the plasma membrane. Mixing of such proteins and lipids occurs if tight junctions are disrupted, for example, by removing the extracellular Ca^{2+} that is required for tight junction integrity. Second, tight junctions seal neighboring cells together so that, if a low-molecular-weight tracer is added to one side of an epithelium, it will generally not pass beyond the tight junction. This seal is not absolute, however. Although all tight junctions are impermeable to macromolecules, their permeability to small molecules varies greatly in different epithelia. Tight junctions in the epithelium lining the small intestine, for example, are 10,000 times more permeable to inorganic ions, such as Na^{+}, than the tight junctions in the epithelium lining the urinary bladder. These differences reflect differences in tight junction proteins that form the junctions.

Epithelial cells can transiently alter their tight junctions to permit an increased flow of solutes and water through breaches in the junctional barriers. Such paracellular transport is especially important in the absorption of amino acids and monosaccharides from the lumen of the intestine, where their concentration can increase enough after a meal to drive passive transport in the desired direction.

When tight junctions are visualized by freeze-fracture electron microscopy, they seem to be composed of a branching network of sealing strands that completely encircles the apical end of each cell in the epithelial sheet. In conventional electron micrographs, the outer leaflets of the two interacting plasma membranes are seen to be tightly apposed where sealing strands are present. The ability of tight junctions to restrict the passage of ions through the spaces between cells is found to increase logarithmically with increasing numbers of strands in the network, suggesting that each strand acts as an independent barrier to ion flow.

Each tight junction sealing strand is composed of a long row of transmembrane adhesion proteins embedded in each of the two interacting plasma membranes. The extracellular domains of these proteins join directly to one another to occlude the intercellular space. The major transmembrane proteins in a tight junction are the claudins, which are essential for tight junction formation and function and differ in different tight junctions. A specific claudin found in kidney epithelial cells, for example, is required for Mg^{2+} to be resorbed from the urine into the blood. A mutation in the gene encoding this claudin results in excessive loss of Mg^{2+} in the urine. A second major transmembrane protein in tight junctions is occludin, the function of which is uncertain. Claudins and occludins associate with intracellular peripheral membrane proteins called ZO proteins (a

tight junction is also known as a zonula occludens), which anchor the strands to the actin cytoskeleton.

In addition to claudins, occludins, and ZO proteins, several other proteins can be found associated with tight junctions. These include some that regulate epithelial cell polarity and others that help guide the delivery of components to the appropriate domain of the plasma membrane. Thus, the tight junction may serve as a regulatory center to help in coordinating multiple cell processes.

In invertebrates, septate junctions are the main occluding junction. More regular in structure than a tight junction, they likewise form a continuous band around each epithelial cell. But their morphology is distinct because the interacting plasma membranes are joined by proteins that are arranged in parallel rows with a regular periodicity. A protein called Discs-large, which is required for the formation of septate junctions in Drosophila, is structurally related to the ZO proteins found in vertebrate tight junctions. Mutant flies that are deficient in this protein not only lack septate junctions but also develop epithelial tumors. This observation suggests that the normal regulation of cell proliferation in epithelial tissues may depend, in part, on intracellular signals that emanate from occluding junctions.

Anchoring Junctions Connect the Cytoskeleton of a Cell Either to the Cytoskeleton of Its Neighbors or to the Extracellular Matrix

The lipid bilayer is flimsy and cannot by itself transmit large forces from cell to cell or from cell to extracellular matrix. Anchoring junctions solve the problem by forming a strong membrane-spanning structure that is tethered inside the cell to the tension-bearing filaments of the cytoskeleton.

Anchoring junctions are widely distributed in animal tissues and are most abundant in tissues that are subjected to severe mechanical stress, such as heart, muscle, and epidermis. They are composed of two main classes of proteins. Intracellular anchor proteins form a distinct plaque on the cytoplasmic face of the plasma membrane and connect the junctional complex to either actin filaments or intermediate filaments. Transmembrane adhesion proteins have a cytoplasmic tail that binds to one or more intracellular anchor proteins and an extracellular domain that interacts with either the extracellular matrix or the extracellular domains of specific transmembrane adhesion proteins on another cell. In addition to anchor proteins and adhesion proteins, many anchoring junctions contain intracellular signaling proteins that enable the junctions to signal to the cell interior.

Anchoring junctions occur in two functionally different forms:

1. Adherens junctions and desmosomes hold cells together and are formed by transmembrane adhesion proteins that belong to the cadherin family.

2. Focal adhesions and hemidesmosomes bind cells to the extracellular matrix and are formed by transmembrane adhesion proteins of the integrin family.

On the intracellular side of the membrane, adherens junctions and focal adhesions serve as connection sites for actin filaments, while desmosomes and hemidesmosomes serve as connection sites for intermediate filaments.

Adherens Junctions Connect Bundles of Actin Filaments from Cell to Cell

Adherens junctions occur in various forms. In many nonepithelial tissues, they take the form of small punctate or streaklike attachments that indirectly connect the cortical actin filaments beneath the plasma membranes of two interacting cells. But the prototypical examples of adherens junctions occur in epithelia, where they often form a continuous adhesion belt (or zonula adherens) just below the tight junctions, encircling each of the interacting cells in the sheet. The adhesion belts are directly apposed in adjacent epithelial cells, with the interacting plasma membranes held together by the cadherins that serve here as transmembrane adhesion proteins.

Within each cell, a contractile bundle of actin filaments lies adjacent to the adhesion belt, oriented parallel to the plasma membrane. The actin is attached to this membrane through a set of intracellular anchor proteins, including catenins, vinculin, and α-actinin, which we consider later. The actin bundles are thus linked, via the cadherins and anchor proteins, into an extensive transcellular network. This network can contract with the help of myosin motor proteins, and it is thought to help in mediating a fundamental process in animal morphogenesis—the folding of epithelial cell sheets into tubes and other related structures.

The assembly of tight junctions between epithelial cells seems to require the prior formation of adherens junctions. Anti-cadherin antibodies that block the formation of adherens junctions, for example, also block the formation of tight junctions.

Desmosomes Connect Intermediate Filaments from Cell to Cell

Desmosomes are buttonlike points of intercellular contact that rivet cells together. Inside the cell, they serve as anchoring sites for ropelike intermediate filaments, which form a structural framework of great tensile strength. Through desmosomes, the intermediate filaments of adjacent cells are linked into a net that extends throughout the many cells of a tissue. The particular type of intermediate filaments attached to the desmosomes depends on the cell type:

they are keratin filaments in most epithelial cells, for example, and desmin filaments in heart muscle cells.

The junction has a dense cytoplasmic plaque composed of a complex of intracellular anchor proteins (plakoglobin and desmoplakin) that are responsible for connecting the cytoskeleton to the transmembrane adhesion proteins. These adhesion proteins (desmoglein and desmocollin), like those at an adherens junction, belong to the cadherin family. They interact through their extracellular domains to hold the adjacent plasma membranes together.

The importance of desmosome junctions is demonstrated by some forms of the potentially fatal skin disease pemphigus. Affected individuals make antibodies against one of their own desmosomal cadherin proteins. These antibodies bind to and disrupt the desmosomes that hold their skin epithelial cells (keratinocytes) together. This results in a severe blistering of the skin, with leakage of body fluids into the loosened epithelium.

Anchoring Junctions Formed by Integrins Bind Cells to the Extracellular Matrix: Focal Adhesions and Hemidesmosomes

Some anchoring junctions bind cells to the extracellular matrix rather than to other cells. The transmembrane adhesion proteins in these cell-matrix junctions are integrins—a large family of proteins distinct from the cadherins. Focal adhesions enable cells to get a hold on the extracellular matrix through integrins that link intracellularly to actin filaments. In this way, muscle cells, for example, attach to their tendons at the myotendinous junction. Likewise, when cultured fibroblasts migrate on an artificial substratum coated with extracellular matrix molecules, they also grip the substratum at focal adhesions, where bundles of actin filaments terminate. At all such adhesions, the extracellular domains of transmembrane integrin proteins bind to a protein component of the extracellular matrix, while their intracellular domains bind indirectly to bundles of actin filaments via the intracellular anchor proteins talin, α-actinin, filamin, and vinculin.

Hemidesmosomes, or half-desmosomes, resemble desmosomes morphologically and in connecting to intermediate filaments, and, like desmosomes, they act as rivets to distribute tensile or shearing forces through an epithelium. Instead of joining adjacent epithelial cells, however, hemidesmosomes connect the basal surface of an epithelial cell to the underlying basal lamina. The extracellular domains of the integrins that mediate the adhesion bind to a laminin protein (discussed later) in the basal lamina, while an intracellular domain binds via an anchor protein (plectin) to keratin intermediate filaments. Whereas the keratin filaments associated with desmosomes make lateral attachments to the desmosomal plaques, many keratin filaments associated with hemidesmosomes have their ends buried in the plaque.

Although the terminology for the various anchoring junctions can be confusing, the molecular principles (for vertebrates, at least) are relatively. Integrins in the plasma membrane anchor a cell to extracellular matrix molecules; cadherin family members in the plasma membrane anchor it to the plasma membrane of an adjacent cell. In both cases, there is an intracellular coupling to cytoskeletal filaments, either actin filaments or intermediate filaments, depending on the types of intracellular anchor proteins involved.

Gap Junctions Allow Small Molecules to Pass Directly from Cell to Cell

With the exception of a few terminally differentiated cells such as skeletal muscle cells and blood cells, most cells in animal tissues are in communication with their neighbors via gap junctions. Each gap junction appears in conventional electron micrographs as a patch where the membranes of two adjacent cells are separated by a uniform narrow gap of about 2–4 nm. The gap is spanned by channel-forming proteins (connexins). The channels they form (connexons) allow inorganic ions and other small water-soluble molecules to pass directly from the cytoplasm of one cell to the cytoplasm of the other, thereby coupling the cells both electrically and metabolically. Dye-injection experiments suggest a maximal functional pore size for the connecting channels of about 1.5 nm, implying that coupled cells share their small molecules (such as inorganic ions, sugars, amino acids, nucleotides, vitamins, and the intracellular mediators cyclic AMP and inositol trisphosphate) but not their macromolecules (proteins, nucleic acids, and polysaccharides). This cell coupling has important functional implications, many of which are only beginning to be understood.

Evidence that gap junctions mediate electrical and chemical coupling has come from many experiments. When, for example, connexin mRNA is injected into either frog oocytes or gap-junction-deficient cultured cells, channels with the properties expected of gap-junction channels can be demonstrated electrophysiologically where pairs of injected cells make contact.

The mRNA injection approach has been useful for identifying new gap-junction proteins. Genetic studies in the fruit fly Drosophila identified the gene shaking B, which, when mutated, resulted in flies that failed to jump in response to a visual stimulus. Although these flies had defective gap junctions, the sequence of the Shaking B protein did not resemble a connexin, and the function of the protein was unclear. An injection of the shaking B mRNA into frog oocytes, however, led to the formation of functional gap-junction channels, just like those formed by connexins. Shaking B thus became the first member of a new family of invertebrate gap-junction proteins called innexins. There are more than 15 innexin genes in Drosophila and 25 in the nematode C. elegans.

A Gap-Junction Connexon Is Made Up of Six Transmembrane Connexin Subunits

Connexins are four-pass transmembrane proteins, six of which assemble to form a channel, a connexon. When the connexons in the plasma membranes of two cells in contact are aligned, they form a continuous aqueous channel that connects the two cell interiors. The connexons hold the interacting plasma membranes at a fixed distance apart—hence the gap.

Gap junctions in different tissues can have different properties. The permeability of their individual channels can vary, reflecting differences in the connexins that form the junctions. In humans, for instance, there are 14 distinct connexins, each encoded by a separate gene and each having a distinctive, but sometimes overlapping, tissue distribution. Most cell types express more than one type of connexin, and two different connexin proteins can assemble into a heteromeric connexon, the properties of which differ from those of a homomeric connexon constructed from a single type of connexin. Moreover, adjacent cells expressing different connexins can form intercellular channels in which the two aligned half-channels are different. Each gap junction can contain a cluster of a few to many thousands of connexons.

Gap Junctions Have Diverse Functions

In tissues containing electrically excitable cells, coupling via gap junctions serves an obvious purpose. Some nerve cells, for example, are electrically coupled, allowing action potentials to spread rapidly from cell to cell, without the delay that occurs at chemical synapses. This is advantageous when speed and reliability are crucial, as in certain escape responses in fish and insects. Similarly, in vertebrates, electrical coupling through gap junctions synchronizes the contractions of both heart muscle cells and the smooth muscle cells responsible for the peristaltic movements of the intestine.

Gap junctions also occur in many tissues that do not contain electrically excitable cells. In principle, the sharing of small metabolites and ions provides a mechanism for coordinating the activities of individual cells in such tissues and for smoothing out random fluctuations in small molecule concentrations in different cells. In the liver, for example, the release of noradrenaline from sympathetic nerve endings in response to a fall in blood glucose levels stimulates hepatocytes to increase glycogen breakdown and release glucose into the blood. Not all the hepatocytes are innervated by sympathetic nerves, however. By means of the gap junctions that connect hepatocytes, the signal is transmitted from the innervated hepatocytes to the noninnervated ones. Thus, mice with a mutation in the major connexin gene expressed in the liver fail to mobilize glucose normally when blood glucose levels fall.

The normal development of ovarian follicles also depends on gap-junction-mediated communication—in this case, between the oocyte and the surrounding

granulosa cells. A mutation in the gene that encodes the connexin that normally couples these two cell types causes infertility.

Cell coupling via gap junctions also seems to be important in embryogenesis. In early vertebrate embryos, beginning with the late eight-cell stage in mouse embryos, most cells are electrically coupled to one another. As specific groups of cells in the embryo develop their distinct identities and begin to differentiate, they commonly uncouple from surrounding tissue. As the neural plate folds up and pinches off to form the neural tube, for instance, its cells uncouple from the overlying ectoderm. Meanwhile, the cells within each group remain coupled with one another and therefore tend to behave as a cooperative assembly, all following a similar developmental pathway in a coordinated fashion.

The Permeability of Gap Junctions Can Be Regulated

Like conventional ion channels, individual gap-junction channels do not remain continuously open; instead, they flip between open and closed states. Moreover, the permeability of gap junctions is rapidly (within seconds) and reversibly reduced by experimental manipulations that decrease the cytosolic pH or increase the cytosolic concentration of free Ca2+ to very high levels. Thus, gap-junction channels are dynamic structures that can undergo a reversible conformational change that closes the channel in response to changes in the cell.

The purpose of the pH regulation of gap-junction permeability is unknown. In one case, however, the purpose of Ca^{2+} control seems clear. When a cell is damaged, its plasma membrane can become leaky. Ions present at high concentration in the extracellular fluid, such as Ca^{2+} and Na+, then move into the cell, and valuable metabolites leak out. If the cell were to remain coupled to its healthy neighbors, these too would suffer a dangerous disturbance of their internal chemistry. But the large influx of Ca^{2+} into the damaged cell causes its gap-junction channels to close immediately, effectively isolating the cell and preventing the damage from spreading to other cells.

Gap-junction communication can also be regulated by extracellular signals. The neurotransmitter dopamine, for example, reduces gap-junction communication between a class of neurons in the retina in response to an increase in light intensity. This reduction in gap-junction permeability helps the retina switch from using rod photoreceptors, which are good detectors of low light, to cone photoreceptors, which detect color and fine detail in bright light.

In the most apical portion of the cell, the relative positions of the junctions are the same in nearly all vertebrate epithelia. The tight junction occupies the most apical position, followed by the adherens junction (adhesion belt) and then by a special parallel row of desmosomes; together these form a structure called a junctional complex. Gap junctions and additional desmosomes are less regularly organized.

In Plants, Plasmodesmata Perform Many of the Same Functions as Gap Junctions

The tissues of a plant are organized on different principles from those of an animal. This is because plant cells are imprisoned within rigid cell walls composed of an extracellular matrix rich in cellulose and other polysacharides, as we discuss later. The cell walls of adjacent cells are firmly cemented to those of their neighbors, which eliminates the need for anchoring junctions to hold the cells in place. But a need for direct cell-cell communication remains. Thus, plant cells have only one class of intercellular junctions, plasmodesmata (singular, plasmodesma). Like gap junctions, they directly connect the cytoplasms of adjacent cells.

In plants, however, the cell wall between a typical pair of adjacent cells is at least 0.1 μm thick, and so a structure very different from a gap junction is required to mediate communication across it. Plasmodesmata solve the problem. With a few specialized exceptions, every living cell in a higher plant is connected to its living neighbors by these structures, which form fine cytoplasmic channels through the intervening cell walls. The plasma membrane of one cell is continuous with that of its neighbor at each plasmodesma, and the cytoplasm of the two cells is connected by a roughly cylindrical channel with a diameter of 20–40 nm. Thus, the cells of a plant can be viewed as forming a syncytium, in which many cell nuclei share a common cytoplasm.

Running through the center of the channel in most plasmodesmata is a narrower cylindrical structure, the desmotubule, which is continuous with elements of the smooth endoplasmic reticulum in each of the connected cells. Between the outside of the desmotubule and the inner face of the cylindrical channel formed by plasma membrane is an annulus of cytosol through which small molecules can pass from cell to cell. As each new cell wall is assembled during the cytokinesis phase of cell division, plasmadesmata are created within it. They form around elements of smooth ER that become trapped across the developing cell plate. They can also be inserted de novo through pre-existing cell walls, where they are commonly found in dense clusters called pit fields. When no longer required, plasmadesmata can be readily removed.

In spite of the radical difference in structure between plasmodesmata and gap junctions, they seem to function in remarkably similar ways. Evidence obtained by injecting tracer molecules of different sizes suggests that plasmo-desmata allow the passage of molecules with a molecular weight of less than about 800, which is similar to the molecular-weight cutoff for gap junctions. As with gap junctions, transport through plasmodesmata is regulated. Dye-injection experiments, for example, show that there can be barriers to the movement of even low-molecular-weight molecules between certain cells, or groups of cells, that are connected by apparently normal plasmodesmata; the mechanisms that restrict communication in these cases are not understood.

During plant development, groups of cells within the shoot and root meristems signal to one another in the process of defining their future fates. Some gene regulatory proteins involved in this process of cell fate determination pass from cell to cell through plasmodesmata. They bind to components of the plasmodesmata and override the size exclusion mechanism that would otherwise prevent their passage. In some cases, the mRNA that encodes the protein can also pass through. Some plant viruses also exploit this route: infectious viral RNA, or even intact virus particles, can pass from cell to cell in this way. These viruses produce proteins that bind to components of the plasmodesmata to increase dramatically the effective pore size of the channel. As the functional components of plasmodesmata are unknown, it is unclear how endogenous or viral macromolecules regulate the transport properties of the channel to pass through it.

Cell-Cell Adhesion

To form an anchoring junction, cells must first adhere. A bulky cytoskeletal apparatus must then be assembled around the molecules that directly mediate the adhesion. The result is a well-defined structure—a desmosome, a hemidesmosome, a focal adhesion, or an adherens junction—that is easily identified in the electron microscope. Indeed, electron microscopy provided the basis for the original classification of cell junctions. In the early stages of cell junction development, however, before the cytoskeletal apparatus has assembled, cells often adhere to one another without clearly displaying these characteristic structures; in the electron microscope, one may simply see two plasma membranes separated by a small gap of a definite width. Functional tests show, nevertheless, that the two cells are stuck to each other, and biochemical analysis can reveal the molecules responsible for the adhesion.

The study of cell-cell junctions and the study of cell-cell adhesion were once quite distinct endeavors, originating from two different experimental approaches—junctions through electron microscopic description, and adhesion through functional tests and biochemistry. Only in recent years have these two approaches begun to converge in a unified view of the molecular basis of cell junctions and cell adhesion. In the previous section, we concentrated on the structures of mature cell junctions. In this section, we turn to functional and biochemical studies of the cell-cell adhesion mechanisms that operate when cells migrate over other cells and when they assemble into tissues—mechanisms that precede the construction of mature cell-cell anchoring junctions. We begin with a critical question for embryonic development: what mechanisms ensure that a cell attaches to appropriate neighbors at the right time?

Animal Cells Can Assemble into Tissues Either in Place or After They Migrate

Many simple tissues, including most epithelial tissues, derive from precursor cells whose progeny are prevented from wandering away by being attached to the extracellular matrix, to other cells, or to both. But the accumulating cells do not simply remain passively stuck together; instead, the tissue architecture is generated and actively maintained by selective adhesions that the cells make and progressively adjust.

Selective adhesion is even more essential for the development of tissues that have more complex origins involving cell migration. In these tissues, one population of cells invades another and assembles with it, and perhaps with other migrant cells, to form an orderly structure. In vertebrate embryos, for example, cells from the neural crest break away from the epithelial neural tube, of which they are initially a part, and migrate along specific paths to many other regions. There they assemble with other cells and with one another to differentiate into a variety of tissues, including those of the peripheral nervous system.

Cell motility and cell adhesion combine to bring about these kinds of morphogenetic events. The process requires some mechanism for directing the cells to their final destination. This may involve chemotaxis or chemorepulsion, the secretion of a soluble chemical that attracts or repels migrating cells, respectively, or pathway guidance, the laying down of adhesive or repellent molecules in the extracellular matrix or on cell surfaces to guide the migrating cells along the right paths. Then, once a migrating cell has reached its destination, it must recognize and join other cells of the appropriate type to assemble into a tissue. How this latter process occurs can be studied if cells of different embryonic tissues are artificially mingled, after which they often spontaneously sort out to restore a more normal arrangement, as we discuss next.

Dissociated Vertebrate Cells Can Reassemble into Organized Tissues Through Selective Cell-Cell Adhesion

Unlike adult vertebrate tissues, which are difficult to dissociate, embryonic vertebrate tissues are easily dissociated. This is usually done by treating the tissue with low concentrations of a proteolytic enzyme such as trypsin, sometimes combined with the removal of extracellular Ca^{2+} and Mg^{2+} with a divalent-cation chelator (such as EDTA). These reagents disrupt the protein-protein interactions (many of which are divalent-cation-dependent) that hold cells together. Remarkably, the dissociated cells often reassemble in vitro into structures that resemble the original tissue. Such findings reveal that tissue structure is not just a product of history; it is actively maintained and stabilized by the system of affinities that cells have for one another and for the extracellular matrix.

A striking example of this phenomenon is seen when dissociated cells from two embryonic vertebrate organs, such as the liver and the retina, are mixed together and artificially formed into a pellet: the mixed aggregates gradually sort out according to their organ of origin. More generally, disaggregated cells are found to adhere more readily to aggregates of their own organ than to aggregates of other organs. Evidently there are cell-cell recognition systems that make cells of the same differentiated tissue preferentially adhere to one another; these adhesive preferences are presumably important in stabilizing tissue architecture.

Cells adhere to each other and to the extracellular matrix through cell-surface proteins called cell adhesion molecules (CAMs)—a category that includes the transmembrane adhesion proteins we have already discussed. CAMs can be cell-cell adhesion molecules or cell-matrix adhesion molecules. Some CAMs are Ca^{2+}-dependent, whereas others are Ca2+-independent. The Ca^{2+}-dependent CAMs seem to be primarily responsible for the tissue-specific cell-cell adhesion seen in early vertebrate embryos, explaining why these cells can be disaggregated with Ca^{2+}-chelating agents.

CAMs were initially identified by making antibodies against cell-surface molecules and then testing the antibodies for their ability to inhibit cell-cell adhesion in a test tube. Those rare antibodies that inhibit the adhesion were then used to characterize and isolate the adhesion molecule recognized by the antibodies.

Cadherins Mediate Ca2+-dependent Cell-Cell Adhesion

The cadherins are the major CAMs responsible for Ca^{2+}-dependent cell-cell adhesion in vertebrate tissues. The first three cadherins that were discovered were named according to the main tissues in which they were found: E-cadherin is present on many types of epithelial cells; N-cadherin on nerve, muscle, and lens cells; and P-cadherin on cells in the placenta and epidermis. All are also found in various other tissues; N-cadherin, for example, is expressed in fibroblasts, and E-cadherin is expressed in parts of the brain. These and other classical cadherins are related in sequence throughout their extracellular and intracellular domains. There are also a large number of nonclassical cadherins, with more than 50 expressed in the brain alone. The nonclassical cadherins include proteins with known adhesive function, such as the desmosomal cadherins discussed earlier and the diverse protocadherins found in the brain. They also include proteins that appear to have nonadhesive functions, such as T-cadherin, which lacks a transmembrane domain and is attached to the plasma membrane of nerve and muscle cells by a glycosylphosphatidylinositol (GPI) anchor, and the Fat protein, which was first identified as the product of a tumor-suppressor gene in Drosophila. Together, the classical and nonclassical cadherin proteins constitute the cadherin superfamily.

Cadherins are expressed in both invertebrates and vertebrates. Virtually all vertebrate cells seem to express one or more cadherins, according to the cell type. They are the main adhesion molecules holding cells together in early embryonic tissues. In culture, the removal of extracellular Ca^{2+} or treatment with anti-cadherin antibodies disrupts embryonic tissues, and, if cadherin-mediated adhesion is left intact, antibodies against other adhesion molecules have little effect. Mutations that inactivate the function of E-cadherin cause mouse embryos to fall apart and die early in development.

Most cadherins are single-pass transmembrane glycoproteins about 700–750 amino acids long. Structural studies suggest that they associate in the plasma membrane to form dimers or larger oligomers. The large extracellular part of the polypeptide chain is usually folded into five or six cadherin repeats, which are structurally related to immunoglobulin (Ig) domains. The crystal structures of E- and N-cadherin have helped to explain the importance of Ca^{2+} binding for cadherin function. The Ca^{2+} ions are positioned between each pair of cadherin repeats, locking the repeats together to form a stiff, rod like structure: the more Ca^{2+} ions that are bound, the more rigid the structure is. If Ca^{2+} is removed, the

extracellular part of the protein becomes floppy and is rapidly degraded by proteolytic enzymes.

Cadherins Have Crucial Roles in Development

E-cadherin is the best-characterized cadherin. It is usually concentrated in adherens junctions in mature epithelial cells, where it helps connect the cortical actin cytoskeletons of the cells it holds together. E-cadherin is also the first cadherin expressed during mammalian development. It helps cause compaction, an important morphological change that occurs at the eight-cell stage of mouse embryo development. During compaction, the loosely attached cells, called blastomeres, become tightly packed together and joined by intercellular junctions. Antibodies against E-cadherin block blastomere compaction, whereas antibodies that react with various other cell-surface molecules on these cells do not.

It seems likely that cadherins are also crucial in later stages of vertebrate development, since their appearance and disappearance correlate with major morphogenetic events in which tissues segregate from one another. As the neural tube forms and pinches off from the overlying ectoderm, for example, neural tube cells lose E-cadherin and acquire other cadherins, including N-cadherin, while the cells in the overlying ectoderm continue to express E-cadherin. Then, when the neural crest cells migrate away from the neural tube, these cadherins become scarcely detectable, and another cadherin (cadherin-7) appears that helps hold the migrating cells together as loosely associated cell groups. Finally, when the cells aggregate to form a ganglion, they re-express N-cadherin.

If N-cadherin is overexpressed in the emerging neural crest cells, the cells fail to escape from the neural tube. Thus, not only do cell groups that originate from one cell layer exhibit distinct patterns of cadherin expression when separating from one another, but these switches in cadherin expression seem to be intimately involved in the separation process.

Cadherins Mediate Cell-Cell Adhesion by a Homophilic Mechanism

How do cell-cell adhesion molecules such as the cadherins bind cells together? Three possibilities are (1) in homophilic binding, molecules on one cell bind to other molecules of the same kind on adjacent cells; (2) in heterophilic binding, the molecules on one cell bind to molecules of a different kind on adjacent cells; (3) in linker-dependent binding, cell-surface receptors on adjacent cells are linked to one another by secreted multivalent linker molecules. Although all three mechanisms have been found to operate in animals, cadherins usually link cells by the homophilic mechanism. In a line of cultured fibroblasts called L cells, for example, the cells neither express cadherins nor adhere to one another. When L cells are transfected with DNA encoding E-cadherin, the transfected cells become

adherent to one another by a Ca^{2+}-dependent mechanism, and the adhesion is inhibited by anti-E-cadherin antibodies. Since cadherin proteins can bind directly to one another and the transfected cells do not bind to untransfected L cells, one can conclude that E-cadherin binds cells together through the interaction of two E-cadherin molecules on different cells.

If L cells expressing different cadherins are mixed together, they sort out and aggregate separately, indicating that different cadherins preferentially bind to their own type, mimicking what happens when cells derived from tissues that express different cadherins are mixed together. A similar segregation of cells occurs if L cells expressing different amounts of the same cadherin are mixed together. It therefore seems likely that both qualitative and quantitative differences in the expression of cadherins have a role in organizing tissues.

In the nervous system especially, there are many different cadherins, each with a distinct but overlapping pattern of expression. As they are concentrated at synapses, they are thought to have a role in synapse formation and stabilization. Some of the nonclassical cadherins, such as the protocadherins, are strong candidates for helping to determine the specificity of synaptic connections. Like antibodies, they differ in their N-terminal (variable) regions but are identical in their C-terminal (constant) regions. The extracellular variable region and intracellular constant region are encoded by separate exons, with the variable-region exons arranged in tandem arrays upstream of the constant-region exons. The diversity of protocadherins is generated by a combination of differential promoter usage and alternative RNA splicing, rather than by site-specific recombination as occurs in antibody diversification.

Cadherins Are Linked to the Actin Cytoskeleton by Catenins

Most cadherins, including all classical and some nonclassical ones, function as transmembrane adhesion proteins that indirectly link the actin cytoskeletons of the cells they join together. This arrangement occurs in adherens junctions. The highly conserved cytoplasmic tail of these cadherins interacts indirectly with actin filaments by means of a group of intracellular anchor proteins called catenins. This interaction is essential for efficient cell-cell adhesion, as classical cadherins that lack their cytoplasmic domain cannot hold cells strongly together.

As discussed earlier, the nonclassical cadherins that form desmosomes interact with intermediate filaments, rather than with actin filaments. Their cytoplasmic domain binds to a different set of intracellular anchor proteins, which in turn bind to intermediate filaments.

Some cells can regulate the adhesive activity of their cadherins. This regulation may be important for the cellular rearrangements that occur within epithelia when these cell sheets change their shape and organization during animal development. The molecular basis of this regulation is uncertain but may involve

the phosphorylation of anchor proteins attached to the cytoplasmic tail of the cadherins.

Some cadherins can help transmit signals to the cell interior. Vascular endothelial cadherin (VE-cadherin), for example, not only mediates adhesion between endothelial cells but also is required for endothelial cell survival. Although endothelial cells that do not express VE-cadherin still adhere to one another via N-cadherin, they do not survive. Their survival depends on an extracellular signal protein called vascular endothelial growth factor (VEGF), which binds to a receptor tyrosine kinase that uses VE-cadherin as a co-receptor.

Selectins Mediate Transient Cell-Cell Adhesions in the Bloodstream

White blood cells lead a nomadic life, moving to and fro between the bloodstream and the tissues, and this necessitates special adhesive properties. These properties depend on selectins. Selectins are cell-surface carbohydrate-binding proteins (lectins) that mediate a variety of transient, Ca^{2+}-dependent, cell-cell adhesion interactions in the bloodstream. There are at least three types: L-selectin on white blood cells, P-selectin on blood platelets and on endothelial cells that have been locally activated by an inflammatory response, and E-selectin on activated endothelial cells. Each selectin is a transmembrane protein with a highly conserved lectin domain that binds to a specific oligosaccharide on another cell.

Selectins have an important role in binding white blood cells to endothelial cells lining blood vessels, thereby enabling the blood cells to migrate out of the bloodstream into a tissue. In a lymphoid organ, the endothelial cells express oligosaccharides that are recognized by L-selectin on lymphocytes, causing the lymphocytes to loiter and become trapped. Conversely, at sites of inflammation, the endothelial cells switch on expression of selectins, which recognize the oligosaccharides on white blood cells and platelets, flagging the cells down to help deal with the local emergency. Selectins do not act alone, however; they collaborate with integrins, which strengthen the binding of the blood cells to the endothelium. The cell-cell adhesions mediated by both selectins and integrins are heterophilic: selectins bind to specific oligosaccharides on glycoproteins and glycolipids, while integrins bind to specific proteins.

Selectins and integrins act in sequence to let white blood cells leave the bloodstream and enter tissues. The selectins mediate a weak adhesion because the binding of the lectin domain of the selectin to its carbohydrate ligand is of low affinity. This allows the white blood cell to adhere weakly and reversibly to the endothelium, rolling along the surface of the blood vessel propelled by the flow of blood. The rolling continues until the blood cell activates its integrins (discussed later), now causing the cell to bind strongly to the endothelial cell surface and to crawl out of the blood vessel between adjacent endothelial cells.

Members of the Immunoglobulin Superfamily of Proteins Mediate Ca2+-independent Cell-Cell Adhesion

Cadherins, selectins, and integrins all depend on extracellular Ca^{2+} (or Mg^{2+} for some integrins) to function in cell adhesion. The molecules responsible for Ca^{2+}-independent cell-cell adhesion belong mainly to the large and ancient immunoglobulin (Ig) superfamily of proteins. These proteins contain one or more Ig-like domains that are characteristic of antibody molecules. One of the best-studied examples is the neural cell adhesion molecule (N-CAM), which is expressed by a variety of cell types, including most nerve cells. N-CAM is the most prevalent of the Ca^{2+}-independent cell-cell adhesion molecules in vertebrates, and, like cadherins, it is thought to bind cells together by a homophilic mechanism (between N-CAM molecules on adjacent cells). Some Ig-like cell-cell adhesion proteins, however, use a heterophilic mechanism. Intercellular adhesion molecules (ICAMs) on endothelial cells, for example, bind to integrins on blood cells when blood cells migrate out of the bloodstream, as just discussed.

There are at least 20 forms of N-CAM, all generated by alternative splicing of an RNA transcript produced from a single gene. In all forms, the large extracellular part of the polypeptide chain is folded into five Ig-like domains. Some forms of N-CAM carry an unusually large quantity of sialic acid (with chains containing hundreds of repeating sialic acid units). By virtue of their negative charge, these long polysialic acid chains hinder cell adhesion, and there is increasing evidence that N-CAM heavily loaded with sialic acid serves to prevent adhesion, rather than cause it.

Although cadherins and Ig family members are frequently expressed on the same cells, the adhesions mediated by cadherins are much stronger, and they are largely responsible for holding cells together, segregating cell collectives into discrete tissues, and maintaining tissue integrity. N-CAM and other members of the Ig family seem to contribute more to the fine-tuning of these adhesive interactions during development and regeneration. In the developing rodent pancreas, for example, the formation of the islets of Langerhans requires cell aggregation, followed by cell sorting. Whereas inhibition of cadherin function prevents cell aggregation and islet formation, loss of N-CAM only impairs the cell sorting process, so that disorganized islets form.

Similarly, whereas mutant mice that lack N-cadherin die early in development, mutant mice that lack N-CAM develop relatively normally, although they do have some defects in neural development. Mutations in other genes that encode Ig-like cell adhesion proteins, however, can cause more severe neural defects. L1 gene mutations in humans, for example, cause mental retardation and other neurological defects resulting from abnormalities in the migration of nerve cells and their axons.

The importance of Ig-like cell adhesion proteins in connecting the neurons of the developing nervous system has been demonstrated dramatically in Drosophila. An N-CAM-like protein called fasciclin III (FAS3) is expressed transiently on some motor neurons, as well as on the muscle cells they normally innervate. If FAS3 is genetically removed from these neurons, they fail to recognize their muscle targets and do not make synapses with them. Conversely, if motor neurons that normally do not express FAS3 are made to express this protein, they now synapse with FAS3-expressing muscle cells to which they normally do not connect. It seems that FAS3 mediates these synaptic connections by a homophilic "matchmaking" mechanism.

Like the cadherins, some Ig-like proteins do more than just bind cells together. They can also transmit signals to the cell interior. Some forms of N-CAM in nerve cells, for example, associate with Src family cytoplasmic tyrosine kinases, which relay signals onward by phosphorylating intracellular proteins on tyrosines. Other Ig family members are transmembrane tyrosine phosphatases that help guide growing axons to their target cells, presumably by dephosphorylating specific intracellular proteins.

Multiple Types of Cell-Surface Molecules Act in Parallel to Mediate Selective Cell-Cell Adhesion

A single type of cell utilizes multiple molecular mechanisms in adhering to other cells. Some of these mechanisms involve organized cell junctions, while others do not. Each cell in a multicellular animal contains an assortment of cell-surface receptors that enables the cell to respond specifically to a complementary set of soluble extracellular signal molecules, such as hormones and growth factors. Likewise, each cell in a tissue has a particular combination (and concentration) of cell-surface adhesion molecules that enables it to bind in its own characteristic way to other cells and to the extracellular matrix. And just as receptors for soluble extracellular signal molecules generate intracellular signals that alter the cell's behavior, so too can cell adhesion molecules, although the signaling mechanisms they use are generally not as well understood.

Unlike receptors for soluble signal molecules, which bind their specific ligand with high affinity, the receptors that bind to molecules on cell surfaces or in the extracellular matrix usually do so with relatively low affinity. These low-affinity receptors rely on the enormous increase in binding strength gained through the simultaneous binding of multiple receptors to multiple ligands on an opposing cell or in the adjacent matrix. One could call this the "Velcro principle."

We have seen, however, that the interaction of the extracellular binding domains of these cell-surface molecules is not enough to ensure cell adhesion. At least in the case of cadherins and, as we shall see, integrins, the adhesion molecules must also attach (via anchor proteins) to the cytoskeleton inside the cell. The cytoskeleton is thought to assist and stabilize the lateral clustering of the

adhesion molecules to facilitate multipoint binding. The cytoskeleton is also required to enable the adhering cell to exert traction on the adjacent cell or matrix (and vice versa). Thus, the mixture of specific types of cell-cell adhesion molecules present on any two cells, as well as their concentration, cytoskeletal linkages, and distribution on the cell surface, determine the total affinity with which the two cells bind to each other.

Nonjunctional Contacts May Initiate Cell-Cell Adhesions That Junctional Contacts Then Orient and Stabilize

We have seen that adhesive contacts between cells play a crucial part in organizing the formation of tissues and organs in developing embryos or in adult tissues undergoing repair after injury. Most often, these contacts do not involve the formation of organized intercellular junctions that show up as specialized structures in the electron microscope. The interacting plasma membranes are simply seen to come close together and run parallel, separated by a space of 10–20 nm. This type of "nonjunctional" contact may be optimal for cell locomotion—close enough to provide traction and to allow transmembrane adhesion proteins to interact, but not so tight, or so solidly anchored to the cytoskeleton, as to immobilize the cell.

A reasonable hypothesis is that nonjunctional cell-cell adhesion proteins initiate cell-cell adhesions, which are then oriented and stabilized by the assembly of full-blown intercellular junctions. Many of the transmembrane proteins involved can diffuse in the plane of the plasma membrane and, in this or other ways, can be recruited to sites of cell-cell (and cell-matrix) contact, enabling nonjunctional adhesions to enlarge and mature into junctional adhesions. This has been demonstrated for some integrins and cadherins, which help initiate cell adhesion and then later become integral parts of cell junctions. The migrating tip of an axon, for example, has an even distribution of cadherins on its surface, which helps it adhere to other cells along the migration pathway. It also has an intracellular pool of cadherins in vesicles just under the plasma membrane. When the axon reaches its target cell, it is thought to release the intracellular cadherin molecules onto the cell surface, where they help form a stable contact, which matures into a chemical synapse.

As discussed earlier, antibodies against adherens junction proteins block the formation of tight junctions, as well as adherens junctions, suggesting that the assembly of one type of junction can be a prerequisite for the formation of another. An increasing number of monoclonal antibodies and peptide fragments have been produced that can block a single type of cell adhesion molecule. Moreover, an increasing number of genes encoding these cell-surface proteins have been identified, creating new opportunities for manipulating the adhesive machinery of cells in culture and in experimental animals. It is now possible, therefore, to inactivate the various cell-cell adhesion proteins in combinations—a

requirement for deciphering the rules of cell-cell recognition and binding used to build complex tissues.

Summary

Cells dissociated from various tissues of vertebrate embryos preferentially reassociate with cells from the same tissue when they are mixed together. This tissue-specific recognition process in vertebrates is mediated mainly by a family of Ca^{2+}-dependent cell-cell adhesion proteins called cadherins, which hold cells together by a homophilic interaction between these transmembrane proteins on adjacent cells. For this interaction to be effective, the cytoplasmic part of the cadherins must be linked to the cytoskeleton by cytoplasmic anchor proteins called catenins.

Two other families of transmembrane adhesion proteins have major roles in cell-cell adhesion. Selectins function in transient Ca2+-dependent cell-cell adhesions in the bloodstream by binding to specific oligosaccharides on the surface of another cell. Members of the immunoglobulin superfamily, including N-CAM, mediate Ca^{2+}-independent cell-cell adhesion processes that are especially important during neural development.

Even a single cell type uses multiple molecular mechanisms in adhering to other cells (and to the extracellular matrix). Thus, the specificity of cell-cell (and cell-matrix) adhesion seen in embryonic development must result from the integration of several different adhesion systems, of which some are associated with specialized cell junctions, while others are not.

Integrins

The linkage of the extracellular matrix to the cell requires transmembrane cell adhesion proteins that act as matrix receptors and tie the matrix to the cell's cytoskeleton. Although we have seen that some transmembrane proteoglycans function as co-receptors for matrix components, the principal receptors on animal cells for binding most extracellular matrix proteins—including collagens, fibronectin, and laminins—are the integrins. These constitute a large family of homologous transmembrane, cell-matrix adhesion receptors. In blood cells, as we have seen, integrins also serve as cell-cell adhesion molecules, helping the cells bind to other cells, as well as to the extracellular matrix.

Integrins, like other cell adhesion molecules, differ from cell-surface receptors for hormones and for other extracellular soluble signal molecules in that they usually bind their ligand with lower affinity and are usually present at about tenfold to a hundredfold higher concentration on the cell surface. If the binding were too tight, cells would presumably become irreversibly glued to the matrix and would be unable to move—a problem that does not arise if attachment depends on large numbers of weak adhesions. This is an example of the "Velcro principle" mentioned earlier. Like other transmembrane cell adhesion proteins, however, integrins do more than just attach a cell to its surroundings. They also activate intracellular signaling pathways that communicate to the cell the character of the extracellular matrix that is bound.

Integrins Are Transmembrane Heterodimers

Integrins are crucially important because they are the main receptor proteins that cells use to both bind to and respond to the extracellular matrix. An integrin molecule is composed of two noncovalently associated transmembrane glycoprotein subunits called α and β. Because the same integrin molecule in different cell types can have different ligand-binding specificities, it seems that additional cell-type-specific factors can interact with integrins to modulate their binding activity.

The binding of integrins to their ligands depends on extracellular divalent cations (Ca^{2+} or Mg^{2+}, depending on the integrin), reflecting the presence of divalent-cation-binding domains in the extracellular part of the α and β subunits. The type of divalent cation can influence both the affinity and the specificity of the binding of an integrin to its ligands.

Many matrix proteins in vertebrates are recognized by multiple integrins. At least 8 integrins bind fibronectin, for example, and at least 5 bind laminin. A variety of human integrin heterodimers are formed from 9 types of β subunits and 24 types of α subunits. This diversity is further increased by alternative splicing of some integrin RNAs.

β1 subunits form dimers with at least 12 distinct α subunits. They are found on almost all vertebrate cells: α5β1, for example, is a fibronectin receptor and α6β1 a laminin receptor on many types of cells. Mutant mice that cannot make any β1 integrins die at implantation, whereas mice that are only unable to make the α7 subunit (the partner for β1 in muscle) survive but develop muscular dystrophy (as do mice that cannot make the laminin ligand for the α7β1 integrin).

The β2 subunits form dimers with at least four types of α subunit. They are expressed exclusively on the surface of white blood cells, where they have an essential role in enabling these cells to fight infection. The β2 integrins mainly mediate cell-cell rather than cell-matrix interactions, binding to specific ligands on another cell, such as an endothelial cell. The ligands, sometimes referred to as counterreceptors, are members of the Ig superfamily of cell-cell adhesion molecules discussed earlier. The β2 integrins enable white blood cells, for example, to attach firmly to endothelial cells at sites of infection and migrate out of the bloodstream into the infected site. Humans with the genetic disease called leucocyte adhesion deficiency are unable to synthesize β2 subunits. As a consequence, their white blood cells lack the entire family of β2 receptors, and they suffer repeated bacterial infections.

The β3 integrins are found on a variety of cells, including blood platelets. They bind several matrix proteins, including fibrinogen. Platelets interact with fibrinogen during blood clotting, and humans with Glanzmann's disease, who are genetically deficient in β3 integrins, bleed excessively.

Integrins Must Interact with the Cytoskeleton to Bind Cells to the Extracellular Matrix

Integrins function as transmembrane linkers (or "integrators"), mediating the interactions between the cytoskeleton and the extracellular matrix that are required for cells to grip the matrix. Most integrins are connected to bundles of actin filaments. The α6β4 integrin found in hemidesmosomes is an exception: it is connected to intermediate filaments. After the binding of a typical integrin to its ligand in the matrix, the cytoplasmic tail of the β subunit binds to several intracellular anchor proteins, including talin, α-actinin, and filamin. These anchor proteins can bind directly to actin or to other anchor proteins such as vinculin, thereby linking the integrin to actin filaments in the cell cortex. Given the right conditions, this linkage leads to a clustering of the integrins and the formation of focal adhesions between the cell and the extracellular matrix, as discussed earlier.

If the cytoplasmic domain of the β subunit is deleted using recombinant DNA techniques, the shortened integrins still bind to their ligands, but they no longer mediate robust adhesion, and they fail to cluster at focal adhesions. It seems that integrins must interact with the cytoskeleton to bind cells strongly to the matrix, just as cadherins must interact with the cytoskeleton to hold cells together

efficiently. The cytoskeletal attachment may help cluster the integrins, providing a stronger aggregate bond; it may also lock the integrin in a conformation that allows the integrin to bind its ligand more tightly.

Just as cadherins can promote cell-cell adhesion without forming mature adherens junctions, integrins can mediate cell-matrix adhesion without forming mature focal adhesions. In both cases, however, the transmembrane adhesion proteins may still bind to the cytoskeleton. For integrins, this kind of adhesion occurs when cells are spreading or migrating, and it results in the formation of focal complexes. For such focal complexes to mature into the focal adhesions that are typical of many well-spread cells, the activation of the small GTPase Rho is required. The activation of Rho leads to the recruitment of more actin filaments and integrins to the contact site.

Cells Can Regulate the Activity of Their Integrins

We discuss below how integrin clustering activates intracellular signaling pathways. But signaling also operates in the opposite direction: signals generated inside the cell can either enhance or inhibit the ability of integrins to bind to their ligand outside the cell. This regulation is poorly understood, but it may involve the phosphorylation of the cytoplasmic tails of the integrins, the association of the tails with activating cytoplasmic proteins, or both.

The ability of a cell to control integrin-ligand interactions from within is termed inside-out signaling. It is particularly important in platelets and white blood cells, where integrins usually have to be activated before they can mediate adhesion. In most other cells, integrins are usually maintained in an adhesion-competent state. Regulated adhesion allows white blood cells to circulate unimpeded until they are activated by an appropriate stimulus. Because the integrins do not need to be synthesized de novo, the signaled adhesion response can be rapid. Platelets, for example, are activated either by contact with a damaged blood vessel or by various soluble signal molecules. In either case, the stimulus triggers intracellular signaling pathways that rapidly activate a β3 integrin in the platelet membrane. This induces a conformational change in the extracellular domain of the integrin that enables the protein to bind the blood-clotting protein fibrinogen with high affinity. The fibrinogen links platelets together to form a platelet plug, which helps stop bleeding.

Similarly, the weak binding of a T lymphocyte to its specific antigen on the surface of an antigen-presenting cell triggers intracellular signaling pathways in the T cell that activate its β2 integrins. The activated integrins then enable the T cell to adhere strongly to the antigen-presenting cell so that it remains in contact long enough to become stimulated fully. The integrins may then return to an inactive state, allowing the T cell to disengage.

There are occasions, especially during development, when cells other than blood cells also regulate the activity of their integrins. If a constitutively active integrin (made by deleting the cytoplasmic tail of the α subunit) is expressed in a developing Drosophila embryo, for example, it disrupts normal muscle development. The muscle precursor cells expressing the activated integrin cannot disengage from the extracellular matrix and therefore cannot migrate normally.

Integrins Activate Intracellular Signaling Pathways

We have already discussed how integrins function as transmembrane linkers that connect extracellular matrix molecules to actin filaments in the cell cortex and thereby regulate the shape, orientation, and movement of cells. But the clustering of integrins at the sites of contact with the matrix (or with another cell) can also activate intracellular signaling pathways. Signaling is initiated by the assembly of signaling complexes at the cytoplasmic face of the plasma membrane, much as in signaling by conventional signaling receptors.

Whereas activated integrins, like activated conventional signaling receptors, can induce global cell responses, often including changes in gene expression, activated integrins are especially adept at stimulating localized changes in the cytoplasm close to the cell-matrix contact. This may be a fundamental feature of signaling by transmembrane cell adhesion proteins in general. In the developing nervous system, for example, the growing tip of an axon is guided mainly by its responses to local adhesive (and repellent) cues in the environment that are recognized by transmembrane cell adhesion proteins. The primary effects of the adhesion proteins are thought to result from the activation of intracellular signaling pathways that act locally in the axon tip, rather than through cell-cell adhesion itself or signals conveyed to the cell body.

Many of the signaling functions of integrins depend on a cytoplasmic protein tyrosine kinase called focal adhesion kinase (FAK). Focal adhesions are often the most prominent sites of tyrosine phosphorylation in cells in culture, and FAK is one of the major tyrosine-phosphorylated proteins found in focal adhesions (although it can also associate with conventional signaling receptors). When integrins cluster at sites of cell-matrix contact, FAK is recruited to focal adhesions by intracellular anchor proteins such as talin, which binds to the integrin β subunit, or paxillin, which binds to one type of integrin α subunit. The clustered FAK molecules cross-phosphorylate each other on a specific tyrosine, creating a phosphotyrosine docking site for members of the Src family of cytoplasmic tyrosine kinases. These kinases then phosphorylate FAK on additional tyrosines, creating docking sites for a variety of intracellular signaling proteins; they also phosphorylate other proteins at focal adhesions. In this way, the signal is relayed into the cell.

One way to analyze the function of FAK is to examine focal adhesions in cells from mutant mice that lack the protein. FAK-deficient fibroblasts still adhere to fibronectin and form focal adhesions. Suprisingly, they form too many focal adhesions rather than too few; as a result, cell spreading and migration are slowed. This unexpected finding suggests that FAK normally helps disassemble focal adhesions and that this loss of adhesions is required for normal cell migration. By interacting with both conventional signaling receptors and focal adhesions, FAK can couple migratory signals to changes in cell adhesion. Many cancer cells have elevated levels of FAK, which may help explain why they are often more motile than their normal counterparts.

Integrins and conventional signaling receptors can work together in several ways. The signaling pathways activated by conventional signaling receptors can increase the expression of integrins or extracellular matrix molecules, while those activated by integrins can increase the expression of conventional signaling receptors or the ligands that bind to them. The intracellular signaling pathways themselves can also interact and reinforce one another. While some conventional signaling receptors and integrins activate the Ras/MAP kinase pathway independently, for example, they often act together to sustain the activation of this pathway long enough to induce cell proliferation. Integrins and conventional signaling receptors cooperate to stimulate many types of cell response. Many cells in culture, for example, will not grow or proliferate in response to extracellular growth factors unless the cells are attached via integrins to extracellular matrix molecules. For some cell types, including epithelial, endothelial, and muscle cells, even cell survival depends on signaling through integrins. When these cells lose contact with the extracellular matrix, they undergo programmed cell death, or apoptosis. This dependence on attachment to the extracellular matrix for survival and proliferation may help ensure that the cells survive and proliferate only when they are in their appropriate location, which may protect animals against the spread of cancer cells. Attachment-dependent cell survival is exploited for special purposes in embryonic development, as shown in. The signaling pathways that integrins activate to promote cell survival are similar to those activated by conventional signaling receptors.

Summary

Integrins are the principal receptors used by animal cells to bind to the extracellular matrix. They are heterodimers and function as transmembrane linkers between the extracellular matrix and the actin cytoskeleton. A cell can regulate the adhesive activity of its integrins from within. Integrins also function as signal transducers, activating various intracellular signaling pathways when activated by matrix binding. Integrins and conventional signaling receptors often cooperate to promote cell growth, cell survival, and cell proliferation.

The Extracellular Matrix of Animals

Tissues are not made up solely of cells. A substantial part of their volume is extracellular space, which is largely filled by an intricate network of macromolecules constituting the extracellular matrix. This matrix is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organized meshwork in close association with the surface of the cell that produced them.

Whereas we have discussed cell junctions chiefly in the context of epithelial tissues, our account of the extracellular matrix concentrates on connective tissues. The extracellular matrix in connective tissue is frequently more plentiful than the cells it surrounds, and it determines the tissue's physical properties. Connective tissues form the framework of the vertebrate body, but the amounts found in different organs vary greatly—from cartilage and bone, in which they are the major component, to brain and spinal cord, in which they are only minor constituents.

Variations in the relative amounts of the different types of matrix macromolecules and the way in which they are organized in the extracellular matrix give rise to an amazing diversity of forms, each adapted to the functional requirements of the particular tissue. The matrix can become calcified to form the rock-hard structures of bone or teeth, or it can form the transparent matrix of the cornea, or it can adopt the ropelike organization that gives tendons their enormous tensile strength. At the interface between an epithelium and connective tissue, the matrix forms a basal lamina, which is important in controlling cell behavior.

The vertebrate extracellular matrix was once thought to serve mainly as a relatively inert scaffold to stabilize the physical structure of tissues. But now it is clear that the matrix has a far more active and complex role in regulating the behavior of the cells that contact it, influencing their survival, development, migration, proliferation, shape, and function. The extracellular matrix has a correspondingly complex molecular composition. Although our understanding of its organization is still incomplete, there has been rapid progress in characterizing many of its major components.

We focus on the extracellular matrix of vertebrates, but the origins of the extracellular matrix are very ancient and virtually all multicellular organisms, make it; examples include the cuticles of worms and insects, the shells of mollusks, and, as we discuss later, the cell walls of plants.

The Extracellular Matrix Is Made and Oriented by the Cells Within It

The macromolecules that constitute the extracellular matrix are mainly produced locally by cells in the matrix. As we discuss later, these cells also help to organize the matrix: the orientation of the cytoskeleton inside the cell can control

the orientation of the matrix produced outside. In most connective tissues, the matrix macromolecules are secreted largely by cells called fibroblasts. In certain specialized types of connective tissues, such as cartilage and bone, however, they are secreted by cells of the fibroblast family that have more specific names: chondroblasts, for example, form cartilage, and osteoblasts form bone.

Two main classes of extracellular macromolecules make up the matrix: (1) polysaccharide chains of the class called glycosaminoglycans (GAGs), which are usually found covalently linked to protein in the form of proteoglycans, and (2) fibrous proteins, including collagen, elastin, fibronectin, and laminin, which have both structural and adhesive functions. We shall see that the members of both classes come in a great variety of shapes and sizes.

The proteoglycan molecules in connective tissue form a highly hydrated, gel-like "ground substance" in which the fibrous proteins are embedded. The polysaccharide gel resists compressive forces on the matrix while permitting the rapid diffusion of nutrients, metabolites, and hormones between the blood and the tissue cells. The collagen fibers both strengthen and help organize the matrix, and rubberlike elastin fibers give it resilience. Finally, many matrix proteins help cells attach in the appropriate locations.

Glycosaminoglycan (GAG) Chains Occupy Large Amounts of Space and Form Hydrated Gels

Glycosaminoglycans (GAGs) are unbranched polysaccharide chains composed of repeating disaccharide units. They are called GAGs because one of the two sugars in the repeating disaccharide is always an amino sugar (N-acetylglucosamine or N-acetylgalactosamine), which in most cases is sulfated. The second sugar is usually a uronic acid (glucuronic or iduronic). Because there are sulfate or carboxyl groups on most of their sugars, GAGs are highly negatively charged. Indeed, they are the most anionic molecules produced by animal cells. Four main groups of GAGs are distinguished according to their sugars, the type of linkage between the sugars, and the number and location of sulfate groups: (1) hyaluronan, (2) chondroitin sulfate and dermatan sulfate, (3) heparan sulfate, and (4) keratan sulfate.

Polysaccharide chains are too stiff to fold up into the compact globular structures that polypeptide chains typically form. Moreover, they are strongly hydrophilic. Thus, GAGs tend to adopt highly extended conformations that occupy a huge volume relative to their mass, and they form gels even at very low concentrations. Their high density of negative charges attracts a cloud of cations, most notably Na^+, that are osmotically active, causing large amounts of water to be sucked into the matrix. This creates a swelling pressure, or turgor, that enables the matrix to withstand compressive forces (in contrast to collagen fibrils, which resist stretching forces). The cartilage matrix that lines the knee

joint, for example, can support pressures of hundreds of atmospheres in this way.

The GAGs in connective tissue usually constitute less than 10% of the weight of the fibrous proteins. But, because they form porous hydrated gels, the GAG chains fill most of the extracellular space, providing mechanical support to the tissue. In one rare human genetic disease, there is a severe deficiency in the synthesis of the dermatan sulfate disaccharide. The affected individuals have a short stature, prematurely aged appearance, and generalized defects in their skin, joints, muscles, and bones.

It should be emphasized, however, that, in invertebrates and plants, other types of polysaccharides often dominate the extracellular matrix. Thus, in higher plants, as we discuss later, cellulose (polyglucose) chains are packed tightly together in ribbonlike crystalline arrays to form the microfibrillar component of the cell wall. In insects, crustaceans, and other arthropods, chitin (poly-N-acetylglucosamine) similarly forms the main component of the exoskeleton. Together, cellulose and chitin are the most abundant biopolymers on Earth.

Hyaluronan Is Thought to Facilitate Cell Migration During Tissue Morphogenesis and Repair

Hyaluronan (also called hyaluronic acid or hyaluronate) is the simplest of the GAGs. It consists of a regular repeating sequence of up to 25,000 nonsulfated disaccharide units, is found in variable amounts in all tissues and fluids in adult animals, and is especially abundant in early embryos. Hyaluronan is not typical of the majority of GAGs. In contrast with all of the others, it contains no sulfated sugars, all its disaccharide units are identical, its chain length is enormous (thousands of sugar monomers), and it is not generally linked covalently to any core protein. Moreover, whereas other GAGs are synthesized inside the cell and released by exocytosis, hyaluronan is spun out directly from the cell surface by an enzyme complex embedded in the plasma membrane.

Hyaluronan is thought to have a role in resisting compressive forces in tissues and joints. It is also important as a space filler during embryonic development, where it can be used to force a change in the shape of a structure, as a small quantity expands with water to occupy a large volume. Hyaluronan synthesized from the basal side of an epithelium, for example, often serves to create a cell-free space into which cells subsequently migrate; this occurs in the formation of the heart, the cornea, and several other organs. When cell migration ends, the excess hyaluronan is generally degraded by the enzyme hyaluronidase. Hyaluronan is also produced in large quantities during wound healing, and it is an important constituent of joint fluid, where it serves as a lubricant.

Many of the functions of hyaluronan depend on specific interactions with other molecules, including both proteins and proteoglycans—molecules consisting of

GAG chains covalently linked to a protein. Some of these molecules that bind to hyaluronan are constituents of the extracellular matrix, while others are integral components of the surface of cells.

Proteoglycans Are Composed of GAG Chains Covalently Linked to a Core Protein

Except for hyaluronan, all GAGs are found covalently attached to protein in the form of proteoglycans, which are made by most animal cells. The polypeptide chain, or core protein, of a proteoglycan is made on membrane-bound ribosomes and threaded into the lumen of the endoplasmic reticulum. The polysaccharide chains are mainly assembled on this core protein in the Golgi apparatus. First, a special link tetrasaccharide is attached to a serine side chain on the core protein to serve as a primer for polysaccharide growth; then, one sugar at a time is added by specific glycosyl transferases. While still in the Golgi apparatus, many of the polymerized sugars are covalently modified by a sequential and coordinated series of reactions. Epimerizations alter the configuration of the substituents around individual carbon atoms in the sugar molecule; sulfations increase the negative charge.

Proteoglycans are usually easily distinguished from other glycoproteins by the nature, quantity, and arrangement of their sugar side chains. By definition, at least one of the sugar side chains of a proteoglycan must be a GAG. Whereas glycoproteins contain 1–60% carbohydrate by weight in the form of numerous relatively short, branched oligosaccharide chains, proteoglycans can contain as much as 95% carbohydrate by weight, mostly in the form of long, unbranched GAG chains, each typically about 80 sugars long. Proteoglycans can be huge. The proteoglycan aggrecan, for example, which is a major component of cartilage, has a mass of about 3 × 106 daltons with over 100 GAG chains. Other proteoglycans are much smaller and have only 1–10 GAG chains; an example is decorin, which is secreted by fibroblasts and has a single GAG chain.

In principle, proteoglycans have the potential for almost limitless heterogeneity. Even a single type of core protein can vary greatly in the number and types of attached GAG chains. Moreover, the underlying repeating pattern of disaccharides in each GAG can be modified by a complex pattern of sulfate groups. The heterogeneity of these GAGs makes it difficult to identify and classify proteoglycans in terms of their sugars. The sequences of many core proteins have been determined with the aid of recombinant DNA techniques, and they, too, are extremely diverse. Although a few small families have been recognized, no common structural feature clearly distinguishes proteoglycan core proteins from other proteins, and many have one or more domains that are homologous to domains found in other proteins of the extracellular matrix or plasma membrane. Thus, it is probably best to regard proteoglycans as a diverse group of highly glycosylated glycoproteins whose functions are mediated by both their core proteins and their GAG chains.

Proteoglycans Can Regulate the Activities of Secreted Proteins

Given the great abundance and structural diversity of proteoglycan molecules, it would be surprising if their function were limited to providing hydrated space around and between cells. Their GAG chains, for example, can form gels of varying pore size and charge density; one possible function, therefore, is to serve as selective sieves to regulate the traffic of molecules and cells according to their size, charge, or both. Evidence suggests that a heparan sulfate proteoglycan called perlecan has this role in the basal lamina of the kidney glomerulus, which filters molecules passing into the urine from the bloodstream (discussed below).

Proteoglycans are thought to have a major role in chemical signaling between cells. They bind various secreted signal molecules, such as certain protein growth factors, and can enhance or inhibit their signaling activity. For example, the heparan sulfate chains of proteoglycans bind to fibroblast growth factors (FGFs), which stimulate a variety of cell types to proliferate; this interaction oligomerizes the growth factor molecules, enabling them to cross-link and activate their cell-surface receptors, which are transmembrane tyrosine kinases. Whereas in most cases the signal molecules bind to the GAG chains of the proteoglycan, this is not always so. Some members of the transforming growth factor β (TGF-β) family bind to the core proteins of several matrix proteoglycans, including decorin; binding to decorin inhibits the activity of the growth factors.

Proteoglycans also bind, and regulate the activities of, other types of secreted proteins, including proteolytic enzymes (proteases) and protease inhibitors. Binding to a proteoglycan could control the activity of a secreted protein in any of the following ways: (1) it could immobilize the protein close to the site where it is produced, thereby restricting its range of action; (2) it could sterically block the activity of the protein; (3) it could provide a reservoir of the protein for delayed release; (4) it could protect the protein from proteolytic degradation, thereby prolonging its action; (5) it could alter or concentrate the protein for more effective presentation to cell-surface receptors.

Proteoglycans are thought to act in all these ways to help regulate the activities of secreted proteins. An example of the last function occurs in inflammatory responses, in which heparan sulfate proteoglycans immobilize secreted chemotactic attractants called chemokines on the endothelial surface of a blood vessel at an inflammatory site. In this way, the chemokines remain there for a prolonged period, stimulating white blood cells to leave the bloodstream and migrate into the inflamed tissue.

GAG Chains May Be Highly Organized in the Extracellular Matrix

GAGs and proteoglycans can associate to form huge polymeric complexes in the extracellular matrix. Molecules of aggrecan, for example, the major proteoglycan

in cartilage, assemble with hyaluronan in the extracellular space to form aggregates that are as big as a bacterium.

Moreover, besides associating with one another, GAGs and proteoglycans associate with fibrous matrix proteins such as collagen and with protein meshworks such as the basal lamina, creating extremely complex structures. Decorin, which binds to collagen fibrils, is essential for collagen fiber formation; mice that cannot make decorin have fragile skin that has reduced tensile strength. The arrangement of proteoglycan molecules in living tissues is generally hard to determine. As the molecules are highly water-soluble, they may be washed out of the extracellular matrix when tissue sections are exposed to aqueous solutions during fixation. In addition, changes in pH, ionic, or osmotic conditions can drastically alter their conformation. Thus, specialized methods must be used to visualize them in tissues.

Cell-Surface Proteoglycans Act as Co-receptors

Not all proteoglycans are secreted components of the extracellular matrix. Some are integral components of plasma membranes and have their core protein either inserted across the lipid bilayer or attached to the lipid bilayer by a glycosylphosphatidylinositol (GPI) anchor. Some of these plasma membrane proteoglycans act as co-receptors that collaborate with conventional cell-surface receptor proteins, in both binding cells to the extracellular matrix and initiating the response of cells to some extracellular signal proteins. In addition, some conventional receptors have one or more GAG chains and are therefore proteoglycans themselves.

Among the best-characterized plasma membrane proteoglycans are the syndecans, which have a membrane-spanning core protein. The extracellular domains of these transmembrane proteoglycans carry up to three chondroitin sulfate and heparan sulfate GAG chains, while their intracellular domains are thought to interact with the actin cytoskeleton in the cell cortex.

Syndecans are located on the surface of many types of cells, including fibroblasts and epithelial cells, where they serve as receptors for matrix proteins. In fibroblasts, syndecans can be found in focal adhesions, where they modulate integrin function by interacting with fibronectin on the cell surface and with cytoskeletal and signaling proteins inside the cell. Syndecans also bind FGFs and present them to FGF receptor proteins on the same cell. Similarly, another plasma membrane proteoglycan, called betaglycan, binds TGF-β and may present it to TGF-β receptors.

The importance of proteoglycans as co-receptors is illustrated by the severe developmental defects that can occur when specific proteoglycans are inactivated by mutation. In Drosophila, for example, signaling by the secreted signal protein Wingless depends on the protein's binding to a specific heparan

sulfate proteoglycan co-receptor called Dally on the target cell. In mutant flies deficient in Dally, Wingless signaling fails, and the severe developmental defects that result are similar to those that result from mutations in the wingless gene itself. In some tissues, inactivation of Dally also inhibits signaling by a secreted protein of the TGF-β family called Decapentaplegic (DPP).

Collagens Are the Major Proteins of the Extracellular Matrix

The collagens are a family of fibrous proteins found in all multicellular animals. They are secreted by connective tissue cells, as well as by a variety of other cell types. As a major component of skin and bone, they are the most abundant proteins in mammals, constituting 25% of the total protein mass in these animals.

The primary feature of a typical collagen molecule is its long, stiff, triple-stranded helical structure, in which three collagen polypeptide chains, called α chains, are wound around one another in a ropelike superhelix. Collagens are extremely rich in proline and glycine, both of which are important in the formation of the triple-stranded helix. Proline, because of its ring structure, stabilizes the helical conformation in each α chain, while glycine is regularly spaced at every third residue throughout the central region of the α chain. Being the smallest amino acid (having only a hydrogen atom as a side chain), glycine allows the three helical α chains to pack tightly together to form the final collagen superhelix.

So far, about 25 distinct collagen α chains have been identified, each encoded by a separate gene. Different combinations of these genes are expressed in different tissues. Although in principle more than 10,000 types of triple-stranded collagen molecules could be assembled from various combinations of the 25 or so α chains, only about 20 types of collagen molecules have been found. The main types of collagen found in connective tissues are types I, II, III, V, and XI, type I being the principal collagen of skin and bone and by far the most common. These are the fibrillar collagens, or fibril-forming collagens, with the ropelike structure. After being secreted into the extracellular space, these collagen molecules assemble into higher-order polymers called collagen fibrils, which are thin structures (10–300 nm in diameter) many hundreds of micrometers long in mature tissues and clearly visible in electron micrographs. Collagen fibrils often aggregate into larger, cablelike bundles, several micrometers in diameter, which can be seen in the light microscope as collagen fibers.

Collagen types IX and XII are called fibril-associated collagens because they decorate the surface of collagen fibrils. They are thought to link these fibrils to one another and to other components in the extracellular matrix. Types IV and VII are network-forming collagens. Type IV molecules assemble into a feltlike sheet or meshwork that constitutes a major part of mature basal laminae, while type VII molecules form dimers that assemble into specialized structures called anchoring fibrils. Anchoring fibrils help attach the basal lamina of multilayered

epithelia to the underlying connective tissue and therefore are especially abundant in the skin.

There are also a number of "collagen-like" proteins, including type XVII, which has a transmembrane domain and is found in hemidesmosomes, and type XVIII, which is located in the basal laminae of blood vessels. Cleavage of the C-terminal domain of type XVIII collagen yields a peptide called endostatin, which inhibits new blood vessel formation and is therefore being investigated as an anticancer drug.

Many proteins that contain a repeated pattern of amino acids have evolved by duplications of DNA sequences. The fibrillar collagens apparently arose in this way. Thus, the genes that encode the α chains of most of these collagens are very large (up to 44 kilobases in length) and contain about 50 exons. Most of the exons are 54, or multiples of 54, nucleotides long, suggesting that these collagens arose by multiple duplications of a primordial gene containing 54 nucleotides and encoding exactly 6 Gly-X-Y repeats.

Collagens Are Secreted with a Nonhelical Extension at Each End

Individual collagen polypeptide chains are synthesized on membrane-bound ribosomes and injected into the lumen of the endoplasmic reticulum (ER) as larger precursors, called pro-α chains. These precursors not only have the short amino-terminal signal peptide required to direct the nascent polypeptide to the ER, they also have additional amino acids, called propeptides, at both their N- and C-terminal ends. In the lumen of the ER, selected prolines and lysines are hydroxylated to form hydroxyproline and hydroxylysine, respectively, and some of the hydroxylysines are glycosylated. Each pro-α chain then combines with two others to form a hydrogen-bonded, triple-stranded, helical molecule known as procollagen.

Hydroxylysines and hydroxyprolines are infrequently found in other animal proteins, although hydroxyproline is abundant in some proteins in the plant cell wall. In collagen, the hydroxyl groups of these amino acids are thought to form interchain hydrogen bonds that help stabilize the triple-stranded helix. Conditions that prevent proline hydroxylation, such as a deficiency of ascorbic acid (vitamin C), have serious consequences. In scurvy, the disease caused by a dietary deficiency of vitamin C that was common in sailors until the nineteenth century, the defective pro-α chains that are synthesized fail to form a stable triple helix and are immediately degraded within the cell. Consequently, with the gradual loss of the preexisting normal collagen in the matrix, blood vessels become extremely fragile and teeth become loose in their sockets, implying that in these particular tissues the degradation and replacement of collagen occur relatively rapidly. In many other adult tissues, however, the turnover of collagen (and other extracellular matrix macromolecules) is thought to be very slow. In bone, to take an extreme example, collagen molecules persist for about 10 years before they

are degraded and replaced. By contrast, most cell proteins have half-lives of hours or days.

After Secretion, Fibrillar Procollagen Molecules Are Cleaved to Collagen Molecules, Which Assemble into Fibrils

After secretion, the propeptides of the fibrillar procollagen molecules are removed by specific proteolytic enzymes outside the cell. This converts the procollagen molecules to collagen molecules, which assemble in the extracellular space to form much larger collagen fibrils. The propeptides have at least two functions. First, they guide the intracellular formation of the triple-stranded collagen molecules. Second, because they are removed only after secretion, they prevent the intracellular formation of large collagen fibrils, which could be catastrophic for the cell.

The process of fibril formation is driven, in part, by the tendency of the collagen molecules, which are more than a thousandfold less soluble than procollagen molecules, to self-assemble. The fibrils begin to form close to the cell surface, often in deep infoldings of the plasma membrane formed by the fusion of secretory vesicles with the cell surface. The underlying cortical cytoskeleton can therefore influence the sites, rates, and orientation of fibril assembly.

When viewed in an electron microscope, collagen fibrils have characteristic cross-striations every 67 nm, reflecting the regularly staggered packing of the individual collagen molecules in the fibril. After the fibrils have formed in the extracellular space, they are greatly strengthened by the formation of covalent cross-links between lysine residues of the constituent collagen molecules. The types of covalent bonds involved are found only in collagen and elastin. If cross-linking is inhibited, the tensile strength of the fibrils is drastically reduced; collagenous tissues become fragile, and structures such as skin, tendons, and blood vessels tend to tear. The extent and type of cross-linking vary from tissue to tissue. Collagen is especially highly cross-linked in the Achilles tendon, for example, where tensile strength is crucial.

Given the large number of enzymatic steps involved, it is not surprising that there are many human genetic diseases that affect fibril formation. Mutations affecting type I collagen cause osteogenesis imperfecta, characterized by weak bones that fracture easily. Mutations affecting type II collagen cause chondrodysplasias, characterized by abnormal cartilage, which leads to bone and joint deformities. Mutations affecting type III collagen cause Ehlers-Danlos syndrome, characterized by fragile skin and blood vessels and hypermobile joints.

Fibril-associated Collagens Help Organize the Fibrils

In contrast to GAGs, which resist compressive forces, collagen fibrils form structures that resist tensile forces. The fibrils have various diameters and are

organized in different ways in different tissues. In mammalian skin, for example, they are woven in a wickerwork pattern so that they resist tensile stress in multiple directions. In tendons, they are organized in parallel bundles aligned along the major axis of tension. In mature bone and in the cornea, they are arranged in orderly plywoodlike layers, with the fibrils in each layer lying parallel to one another but nearly at right angles to the fibrils in the layers on either side. The same arrangement occurs in tadpole skin.

The connective tissue cells themselves must determine the size and arrangement of the collagen fibrils. The cells can express one or more genes for the different types of fibrillar procollagen molecules. But even fibrils composed of the same mixture of fibrillar collagen molecules have different arrangements in different tissues. How is this achieved? Part of the answer is that cells can regulate the disposition of the collagen molecules after secretion by guiding collagen fibril formation in close association with the plasma membrane. In addition, as the spatial organization of collagen fibrils at least partly reflects their interactions with other molecules in the matrix, cells can influence this organization by secreting, along with their fibrillar collagens, different kinds and amounts of other matrix macromolecules.

Fibril-associated collagens, such as types IX and XII collagens, are thought to be especially important in this regard. They differ from fibrillar collagens in several ways.

1. Their triple-stranded helical structure is interrupted by one or two short nonhelical domains, which makes the molecules more flexible than fibrillar collagen molecules.

2. They are not cleaved after secretion and therefore retain their propeptides.

3. They do not aggregate with one another to form fibrils in the extracellular space. Instead, they bind in a periodic manner to the surface of fibrils formed by the fibrillar collagens. Type IX molecules bind to type-II-collagen-containing fibrils in cartilage, the cornea, and the vitreous of the eye, whereas type XII molecules bind to type-I-collagen-containing fibrils in tendons and various other tissues.

Fibril-associated collagens are thought to mediate the interactions of collagen fibrils with one another and with other matrix macromolecules. In this way, they have a role in determining the organization of the fibrils in the matrix.

Cells Help Organize the Collagen Fibrils They Secrete by Exerting Tension on the Matrix

Cells interact with the extracellular matrix mechanically as well as chemically, with dramatic effects on the architecture of the tissue. Thus, for example,

fibroblasts work on the collagen they have secreted, crawling over it and tugging on it—helping to compact it into sheets and draw it out into cables. When fibroblasts are mixed with a meshwork of randomly oriented collagen fibrils that form a gel in a culture dish, the fibroblasts tug on the meshwork, drawing in collagen from their surroundings and thereby causing the gel to contract to a small fraction of its initial volume. By similar activities, a cluster of fibroblasts surrounds itself with a capsule of densely packed and circumferentially oriented collagen fibers.

If two small pieces of embryonic tissue containing fibroblasts are placed far apart on a collagen gel, the intervening collagen becomes organized into a compact band of aligned fibers that connect the two explants. The fibroblasts subsequently migrate out from the explants along the aligned collagen fibers. Thus, the fibroblasts influence the alignment of the collagen fibers, and the collagen fibers in turn affect the distribution of the fibroblasts. Fibroblasts presumably have a similar role in generating long-range order in the extracellular matrix inside the body—in helping to create tendons and ligaments, for example, and the tough, dense layers of connective tissue that ensheathe and bind together most organs.

Elastin Gives Tissues Their Elasticity

Many vertebrate tissues, such as skin, blood vessels, and lungs, need to be both strong and elastic in order to function. A network of elastic fibers in the extracellular matrix of these tissues gives them the required resilience so that they can recoil after transient stretch. Elastic fibers are at least five times more extensible than a rubber band of the same cross-sectional area. Long, inelastic collagen fibrils are interwoven with the elastic fibers to limit the extent of stretching and prevent the tissue from tearing.

The main component of elastic fibers is elastin, a highly hydrophobic protein (about 750 amino acids long), which, like collagen, is unusually rich in proline and glycine but, unlike collagen, is not glycosylated and contains some hydroxyproline but no hydroxylysine. Soluble tropoelastin (the biosynthetic precursor of elastin) is secreted into the extracellular space and assembled into elastic fibers close to the plasma membrane, generally in cell-surface infoldings. After secretion, the tropoelastin molecules become highly cross-linked to one another, generating an extensive network of elastin fibers and sheets. The cross-links are formed between lysines by a mechanism similar to the one discussed earlier that operates in cross-linking collagen molecules.

The elastin protein is composed largely of two types of short segments that alternate along the polypeptide chain: hydrophobic segments, which are responsible for the elastic properties of the molecule; and alanine- and lysine-rich α-helical segments, which form cross-links between adjacent molecules. Each

segment is encoded by a separate exon. There is still controversy, however, concerning the conformation of elastin molecules in elastic fibers and how the structure of these fibers accounts for their rubberlike properties. In one view, the elastin polypeptide chain, like the polymer chains in ordinary rubber, adopts a loose "random coil" conformation, and it is the random coil structure of the component molecules cross-linked into the elastic fiber network that allows the network to stretch and recoil like a rubber band.

Elastin is the dominant extracellular matrix protein in arteries, comprising 50% of the dry weight of the largest artery—the aorta. Mutations in the elastin gene causing a deficiency of the protein in mice or humans result in narrowing of the aorta or other arteries as a result of excessive proliferation of smooth muscle cells in the arterial wall. Apparently, the normal elasticity of an artery is required to restrain the proliferation of these cells.

Elastic fibers are not composed solely of elastin. The elastin core is covered with a sheath of microfibrils, each of which has a diameter of about 10 nm. Microfibrils are composed of a number of distinct glycoproteins, including the large glycoprotein fibrillin, which binds to elastin and is essential for the integrity of elastic fibers. Mutations in the fibrillin gene result in Marfan's syndrome, a relatively common human genetic disease affecting connective tissues that are rich in elastic fibers; in the most severely affected individuals, the aorta is prone to rupture. Microfibrils are thought to be important in the assembly of elastic fibers. They appear before elastin in developing tissues and seem to form a scaffold on which the secreted elastin molecules are deposited. As the elastin is deposited, the microfibrils become displaced to the periphery of the growing fiber.

Fibronectin Is an Extracellular Protein That Helps Cells Attach to the Matrix

The extracellular matrix contains a number of noncollagen proteins that typically have multiple domains, each with specific binding sites for other matrix macromolecules and for receptors on the surface of cells. These proteins therefore contribute to both organizing the matrix and helping cells attach to it. The first of them to be well characterized was fibronectin, a large glycoprotein found in all vertebrates. Fibronectin is a dimer composed of two very large subunits joined by disulfide bonds at one end. Each subunit is folded into a series of functionally distinct domains separated by regions of flexible polypeptide chain. The domains in turn consist of smaller modules, each of which is serially repeated and usually encoded by a separate exon, suggesting that the fibronectin gene, like the collagen genes, evolved by multiple exon duplications. All forms of fibronectin are encoded by a single large gene that contains about 50 exons of similar size. Transcription produces a single large RNA molecule that can be alternatively spliced to produce the various isoforms of fibronectin. The main type of module, called the type III fibronectin repeat, binds to integrins. It is about 90 amino acids long and occurs at least 15 times in each subunit. The type

III fibronectin repeat is among the most common of all protein domains in vertebrates.

One way to analyze a complex multifunctional protein molecule like fibronectin is to chop it into pieces and determine the function of its individual domains. When fibronectin is treated with a low concentration of a proteolytic enzyme, the polypeptide chain is cut in the connecting regions between the domains, leaving the domains themselves intact. One can then show that one of its domains binds to collagen, another to heparin, another to specific receptors on the surface of various types of cells, and so on. Synthetic peptides corresponding to different segments of the cell-binding domain have been used to identify a specific tripeptide sequence (Arg-Gly-Asp, or RGD), which is found in one of the type III repeats, as a central feature of the binding site. Even very short peptides containing this RGD sequence can compete with fibronectin for the binding site on cells, thereby inhibiting the attachment of the cells to a fibronectin matrix. If these peptides are coupled to a solid surface, they cause cells to adhere to it.

The RGD sequence is not confined to fibronectin. It is found in a number of extracellular proteins, including, for example, the blood-clotting factor fibrinogen. Fibrinogen peptides containing this RGD sequence have been useful in the development of anti-clotting drugs that mimic these peptides. Snakes use a similar strategy to cause their victims to bleed: they secrete RGD-containing anti-clotting proteins called disintegrins into their venom.

RGD sequences are recognized by several members of the integrin family of cell-surface matrix receptors. Each integrin, however, specifically recognizes its own small set of matrix molecules, indicating that tight binding requires more than just the RGD sequence.

Fibronectin Exists in Both Soluble and Fibrillar Forms

There are multiple isoforms of fibronectin. One, called plasma fibronectin, is soluble and circulates in the blood and other body fluids, where it is thought to enhance blood clotting, wound healing, and phagocytosis. All of the other forms assemble on the surface of cells and are deposited in the extracellular matrix as highly insoluble fibronectin fibrils. In these cell-surface and matrix forms, fibronectin dimers are cross-linked to one another by additional disulfide bonds.

Unlike fibrillar collagen molecules, which can be made to self-assemble into fibrils in a test tube, fibronectin molecules assemble into fibrils only on the surface of certain cells. This is because additional proteins are needed for fibril formation, especially fibronectin-binding integrins. In the case of fibroblasts, fibronectin fibrils are associated with integrins at sites called fibrillar adhesions. These are distinct from focal adhesions, in that they are more elongated and contain different intracellular anchor proteins. The fibronectin fibrils on the cell surface are highly stretched and under tension. The tension is exerted by the cell

and is essential for fibril formation, as we discuss below. Some secreted proteins function to prevent fibronectin assembly in inappropriate places. Uteroglobin, for example, binds to fibronectin and prevents it from forming fibrils in the kidney. Mice that have a mutation in the uteroglobin gene accumulate insoluble fibronectin fibrils in their kidneys.

The importance of fibronectin in animal development is dramatically demonstrated by gene inactivation experiments. Mutant mice that are unable to make fibronectin die early in embryogenesis because their endothelial cells fail to form proper blood vessels. This defect is thought to result from abnormalities in the interactions of these cells with the surrounding extracellular matrix, which normally contains fibronectin.

Intracellular Actin Filaments Regulate the Assembly of Extracellular Fibronectin Fibrils

The fibronectin fibrils that form on or near the surface of fibroblasts are usually aligned with adjacent intracellular actin stress fibers. In fact, intracellular actin filaments promote the assembly of secreted fibronectin molecules into fibrils and influence fibril orientation. If cells are treated with the drug cytochalasin, which disrupts actin filaments, the fibronectin fibrils dissociate from the cell surface (just as they do during mitosis when a cell rounds up).

The interactions between extracellular fibronectin fibrils and intracellular actin filaments across the fibroblast plasma membrane are mediated mainly by integrin transmembrane adhesion proteins. The contractile actin and myosin cytoskeleton thereby pulls on the fibronectin matrix to generate tension. As a result, the fibronectin fibrils are stretched, exposing a cryptic (hidden) binding site in the fibronectin molecules that allows them to bind directly to one another. In addition, the stretching exposes more binding sites for integrins. In this way, the actin cytoskeleton promotes fibronectin polymerization and matrix assembly.

Extracellular signals can regulate the assembly process by altering the actin cytoskeleton and thereby the tension on the fibrils. Many other extracellular matrix proteins have multiple repeats similar to the type III fibronectin repeat, and it is possible that tension exerted on these proteins also uncovers cryptic binding sites and thereby influences their polymerization.

Glycoproteins in the Matrix Help Guide Cell Migration

Fibronectin is important not only for cell adhesion to the matrix but also for guiding cell migrations in vertebrate embryos. Large amounts of fibronectin, for example, are found along the pathway followed by migrating prospective

mesodermal cells during amphibian gastrulation. Although all cells of the early embryo can attach to fibronectin, only these migrating cells can spread and migrate on fibronectin. The migration is inhibited by an injection into the developing amphibian embryo of various ligands that disrupt the ability of the cells to bind to fibronectin.

Many matrix proteins are believed to have a role in guiding cell movements during development. The tenascins and thrombospondins, for example, are composed of several types of short amino acid sequences that are repeated many times and form functionally distinct domains. They can either promote or inhibit cell adhesion, depending on the cell type. Indeed, anti-adhesive interactions are as important as adhesive ones in guiding cell migration,.

Basal Laminae Are Composed Mainly of Type IV Collagen, Laminin, Nidogen, and a Heparan Sulfate Proteoglycan

As mentioned earlier, basal laminae are flexible, thin (40–120 nm thick) mats of specialized extracellular matrix that underlie all epithelial cell sheets and tubes. They also surround individual muscle cells, fat cells, and Schwann cells (which wrap around peripheral nerve cell axons to form myelin). The basal lamina thus separates these cells and epithelia from the underlying or surrounding connective tissue. In other locations, such as the kidney glomerulus, a basal lamina lies between two cell sheets and functions as a highly selective filter. Basal laminae have more than simple structural and filtering roles, however. They are able to determine cell polarity, influence cell metabolism, organize the proteins in adjacent plasma membranes, promote cell survival, proliferation, or differentiation, and serve as specific highways for cell migration.

The basal lamina is synthesized largely by the cells that rest on it. In some multilayered epithelia, such as the stratified squamous epithelium that forms the epidermis of the skin, the basal lamina is tethered to the underlying connective tissue by specialized anchoring fibrils made of type VII collagen molecules. The term basement membrane is often used to describe the composite of the basal lamina and this layer of collagen fibrils. In one type of skin disease, these connections are either absent or destroyed, and the epidermis and its basal lamina become detached from the underlying connective tissue, causing blistering.

Although its precise composition varies from tissue to tissue and even from region to region in the same lamina, most mature basal laminae contain type IV collagen, the large heparan sulfate proteoglycan perlecan, and the glycoproteins laminin and nidogen (also called entactin).

Type IV collagens exist in several isoforms. They all have a more flexible structure than the fibrillar collagens; their triple-stranded helix is interrupted in 26 regions, allowing multiple bends. They are not cleaved after secretion, but

interact via their uncleaved terminal domains to assemble extracellularly into a flexible, sheetlike, multilayered network.

Early in development, basal laminae contain little or no type IV collagen and consist mainly of laminin molecules. Laminin-1 (classical laminin) is a large, flexible protein composed of three very long polypeptide chains (α, β, and γ) arranged in the shape of an asymmetric cross and held together by disulfide bonds. Several isoforms of each type of chain can associate in different combinations to form a large family of laminins. The laminin γ-1 chain is a component of most laminin heterotrimers, and mice lacking it die during embryogenesis because they are unable to make a basal lamina. Like many other proteins in the extracellular matrix, the laminin in basement membranes consists of several functional domains: one binds to perlecan, one to nidogen, and two or more to laminin receptor proteins on the surface of cells.

Like type IV collagen, laminins can self-assemble in vitro into a feltlike sheet, largely through interactions between the ends of the laminin arms. As nidogen and perlecan can bind to both laminin and type IV collagen, it is thought that they connect the type IV collagen and laminin networks. In tissues, laminins and type IV collagen preferentially polymerize while bound to receptors on the surface of the cells producing the proteins. Many of the cell-surface receptors for type IV collagen and laminin are members of the integrin family. Another important type of laminin receptor is the transmembrane protein dystroglycan, which, together with integrins, may organize the assembly of the basal lamina.

Basal Laminae Perform Diverse Functions

As we have mentioned, in the kidney glomerulus, an unusually thick basal lamina acts as a molecular filter, preventing the passage of macromolecules from the blood into the urine as urine is formed. The heparan sulfate proteoglycan in the basal lamina seems to be important for this function: when its GAG chains are removed by specific enzymes, the filtering properties of the lamina are destroyed. Type IV collagen also has a role, as a human hereditary kidney disorder (Alport syndrome) results from mutations in type IV collagen α-chain genes.

The basal lamina can also act as a selective barrier to the movement of cells. The lamina beneath an epithelium, for example, usually prevents fibroblasts in the underlying connective tissue from making contact with the epithelial cells. It does not, however, stop macrophages, lymphocytes, or nerve processes from passing through it. The basal lamina is also important in tissue regeneration after injury. When tissues such as muscles, nerves, and epithelia are damaged, the basal lamina survives and provides a scaffold along which regenerating cells can migrate. In this way, the original tissue architecture is readily reconstructed. In some cases, as in the skin or cornea, the basal lamina becomes chemically altered after injury—for example, by the addition of fibronectin, which promotes the cell migration required for wound healing.

A particularly striking example of the instructive role of the basal lamina in regeneration comes from studies on the neuromuscular junction, the site where the nerve terminals of a motor neuron form a chemical synapse with a skeletal muscle cell. The basal lamina that surrounds the muscle cell separates the nerve and muscle cell plasma membranes at the synapse, and the synaptic region of the lamina has a distinctive chemical character, with special isoforms of type IV collagen and laminin and a heparan sulfate proteoglycan called agrin.

This basal lamina at the synapse has a central role in reconstructing the synapse after nerve or muscle injury. If a frog muscle and its motor nerve are destroyed, the basal lamina around each muscle cell remains intact and the sites of the old neuromuscular junctions are still recognizable. If the motor nerve, but not the muscle, is allowed to regenerate, the nerve axons seek out the original synaptic sites on the empty basal lamina and differentiate there to form normal-looking nerve terminals. Thus, the junctional basal lamina by itself can guide the regeneration of motor nerve terminals.

Similar experiments show that the basal lamina also controls the localization of the acetylcholine receptors that cluster in the muscle cell plasma membrane at a neuromuscular junction. If the muscle and nerve are both destroyed, but now the muscle is allowed to regenerate while the nerve is prevented from doing so, the acetylcholine receptors synthesized by the regenerated muscle localize predominantly in the region of the old junctions, even though the nerve is absent. Thus, the junctional basal lamina apparently coordinates the local spatial organization of the components in each of the two cells that form a neuromuscular junction. Some of the matrix proteins have been identified. Motor neuron axons, for example, deposit agrin in the junctional basal lamina, where it triggers the assembly of acetylcholine receptors and other proteins in the junctional plasma membrane of the muscle cell. Conversely, muscle cells deposit a particular isoform of laminin in the junctional basal lamina. Both agrin and this isoform of laminin are essential for the formation of normal neuromuscular junctions.

The Extracellular Matrix Can Influence Cell Shape, Cell Survival, and Cell Proliferation

The extracellular matrix can influence the organization of a cell's cytoskeleton. This can be vividly demonstrated by using transformed (cancerlike) fibroblasts in culture. Transformed cells often make less fibronectin than normal cultured cells and behave differently. They adhere poorly to the culture substratum, for example, and fail to flatten out or develop the organized intracellular bundles of actin filaments known as stress fibers. The decrease in fibronectin production and adhesion may contribute to the tendency of cancer cells to break away from the primary tumor and spread to other parts of the body.

In some cases, fibronectin deficiency seems also to be at least partly responsible for this abnormal morphology of cancer cells: if the cells are grown on a matrix of organized fibronectin fibrils, they flatten out and assemble intracellular stress fibers that are aligned with the extracellular fibronectin fibrils. This interaction between the extracellular matrix and the cytoskeleton is reciprocal in that intracellular actin filaments can promote the assembly and influence the orientation of fibronectin fibrils, as described earlier. Since the cytoskeleton can exert forces that orient the matrix macromolecules the cell secretes and the matrix macromolecules can in turn organize the cytoskeleton of the cells they contact, the extracellular matrix can in principle propagate order from cell to cell, creating large-scale oriented structures, as described earlier. The integrins serve as the main adaptors in this ordering process, mediating the interactions between cells and the matrix around them.

Most cells need to attach to the extracellular matrix to grow and proliferate—and, in many cases, even to survive. This dependence of cell growth, proliferation, and survival on attachment to a substratum is known as anchorage dependence, and it is mediated mainly by integrins and the intracellular signals they generate. The physical spreading of a cell on the matrix also has a strong influence on intracellular events. Cells that are forced to spread over a large surface area survive better and proliferate faster than cells that are not so spread out, even if in both cases the cells have the same area making contact with the matrix directly. This stimulatory effect of cell spreading presumably helps tissues to regenerate after injury. If cells are lost from an epithelium, for example, the spreading of the remaining cells into the vacated space will stimulate them to proliferate until they fill the gap. It is still uncertain, however, how a cell senses its extent of spreading so as to adjust its behavior accordingly.

The Controlled Degradation of Matrix Components Helps Cells Migrate

The regulated turnover of extracellular matrix macromolecules is crucial to a variety of important biological processes. Rapid degradation occurs, for example, when the uterus involutes after childbirth, or when the tadpole tail is resorbed during metamorphosis. A more localized degradation of matrix components is required when cells migrate through a basal lamina. This occurs when white blood cells migrate across the basal lamina of a blood vessel into tissues in response to infection or injury, and when cancer cells migrate from their site of origin to distant organs via the bloodstream or lymphatic vessels—the process known as metastasis. Even in the seemingly static extracellular matrix of adult animals, there is a slow, continuous turnover, with matrix macromolecules being degraded and resynthesized.

In each of these cases, matrix components are degraded by extracellular proteolytic enzymes (proteases) that are secreted locally by cells. Thus, antibodies that recognize the products of proteolytic cleavage stain matrix only around cells. Many of these proteases belong to one of two general classes. Most

are matrix metalloproteases, which depend on bound Ca^{2+} or Zn^{2+} for activity; the others are serine proteases, which have a highly reactive serine in their active site. Together, metalloproteases and serine proteases cooperate to degrade matrix proteins such as collagen, laminin, and fibronectin. Some metalloproteases, such as the collagenases, are highly specific, cleaving particular proteins at a small number of sites. In this way, the structural integrity of the matrix is largely retained, but cell migration can be greatly facilitated by the small amount of proteolysis. Other metalloproteases may be less specific, but, because they are anchored to the plasma membrane, they can act just where they are needed.

The importance of proteolysis in cell migration can be shown by using protease inhibitors, which often block migration. Moreover, cells that migrate readily on type I collagen in culture can no longer do so if the collagen is made resistant to proteolysis by mutating the collagenase-sensitive cleavage sites. The proteolysis of matrix proteins can contribute to cell migration in several ways: (1) it can simply clear a path through the matrix; (2) it can expose cryptic sites on the cleaved proteins that promote cell binding, cell migration, or both; (3) it can promote cell detachment so that a cell can move onward, or (4) it can release extracellular signal proteins that stimulate cell migration.

Three basic mechanisms operate to ensure that the proteases that degrade the matrix components are tightly controlled.

Local activation: Many proteases are secreted as inactive precursors that can be activated locally when needed. An example is plasminogen, an inactive protease precursor that is abundant in the blood. It is cleaved locally by other proteases called plasminogen activators to yield the active serine protease plasmin, which helps break up blood clots. Tissue-type plasminogen activator (tPA) is often given to patients who have just had a heart attack or thrombotic stroke; it helps dissolve the arterial clot that caused the attack, thereby restoring bloodflow to the tissue.

Confinement by cell-surface receptors: Many cells have receptors on their surface that bind proteases, thereby confining the enzyme to the sites where it is needed. A second type of plasminogen activator called urokinase-type plasminogen activator (uPA) is an example. It is found bound to receptors on the growing tips of axons and at the leading edge of some migrating cells, where it may serve to clear a pathway for their migration. Receptor-bound uPA may also help some cancer cells metastasize.

Secretion of inhibitors: The action of proteases is confined to specific areas by various secreted protease inhibitors, including the tissue inhibitors of metalloproteases (TIMPs) and the serine protease inhibitors known as serpins. These inhibitors are protease-specific and bind tightly to the activated enzyme, blocking its activity. An attractive idea is that the inhibitors are secreted by cells

at the margins of areas of active protein degradation in order to protect uninvolved matrix; they may also protect cell-surface proteins required for cell adhesion and migration. The overexpression of TIMPs inhibits the migration of some cell types, indicating the importance of metalloproteases for the migration.

Summary

Cells in connective tissues are embedded in an intricate extracellular matrix that not only binds the cells together but also influences their survival, development, shape, polarity, and behavior. The matrix contains various protein fibers interwoven in a hydrated gel composed of a network of glycosaminoglycan (GAG) chains.

GAGs are a heterogeneous group of negatively charged polysaccharide chains that (except for hyaluronan) are covalently linked to protein to form proteoglycan molecules. They occupy a large volume and form hydrated gels in the extracellular space. Proteoglycans are also found on the surface of cells, where they function as co-receptors to help cells respond to secreted signal proteins.

Fiber-forming proteins strengthen the matrix and give it form. They also provide surfaces for cells to adhere to. Elastin molecules form an extensive cross-linked network of fibers and sheets that can stretch and recoil, imparting elasticity to the matrix. The fibrillar collagens (types I, II, III, V, and XI) are ropelike, triple-stranded helical molecules that aggregate into long fibrils in the extracellular space. The fibrils in turn can assemble into a variety of highly ordered arrays. Fibril-associated collagen molecules, such as types IX and XII, decorate the surface of collagen fibrils and influence the interactions of the fibrils with one another and with other matrix components.

In contrast, type IV collagen molecules assemble into a sheetlike meshwork that is a crucial component of all mature basal laminae. All basal laminae are based on a mesh of laminin molecules. The collagen and laminin networks in mature basal laminae are bridged by the protein nidogen and the large heparan sulfate proteoglycan perlecan. Fibronectin and laminin are examples of large, multidomain matrix glycoproteins. By means of their multiple binding domains, such proteins help organize the matrix and help cells adhere to it.

Matrix proteins such as collagens, laminins, and fibronectin are assembled into fibrils or networks on the surface of the cells that produce them by a process that depends on the underlying actin cortex. The organization of the matrix can reciprocally influence the organization of the cell's cytoskeleton and can mechanically influence cell spreading. The matrix also influences cell behavior by binding to cell-surface receptors that activate intracellular signaling pathways.

Matrix components are degraded by extracellular proteolytic enzymes. Most of these are matrix metalloproteases, which depend on bound Ca^{2+} or Zn^{2+} for

activity, while others are serine proteases, which have a reactive serine in their active site. Various mechanisms operate to ensure that the degradation of matrix components is tightly controlled. Cells can, for example, cause a localized degradation of matrix components to clear a path through the matrix.

The Cytoskeleton

Cells have to organize themselves in space and interact mechanically with their environment. They have to be correctly shaped, physically robust, and properly structured internally. Many of them also have to be able to change their shape and move from place to place. All of them have to be able to rearrange their internal components as they grow, divide, and adapt to changing circumstances. All these spatial and mechanical functions are developed to a very high degree in eucaryotic cells, where they depend on a remarkable system of filaments called the cytoskeleton.

The cytoskeleton pulls the chromosomes apart at mitosis and then splits the dividing cell into two. It drives and guides the intracellular traffic of organelles, ferrying materials from one part of the cell to another. It supports the fragile plasma membrane and provides the mechanical linkages that let the cell bear stresses and strains without being ripped apart as the environment shifts and changes. It enables some cells, such as sperm, to swim, and others, such as fibroblasts and white blood cells, to crawl across surfaces. It provides the machinery in the muscle cell for contraction and in the neuron to extend an axon and dendrites. It guides the growth of the plant cell wall and controls the amazing diversity of eucaryotic cell shapes.

The varied functions of the cytoskeleton center on the behavior of three families of protein molecules, which assemble to form three main types of filaments. Each type of filament has distinct mechanical properties and dynamics, but certain fundamental principles are common to them all. These principles provide the basis for a general understanding of how the cytoskeleton works.

In this chapter, we begin by describing the three main types of filaments, the basic principles underlying their assembly and disassembly, and their individual peculiarities. We then describe how other proteins interact with the three main filament systems, enabling the cell to establish and maintain internal order, to shape and remodel its surface, to move organelles in a directed manner from one place to another, and—when appropriate—to move itself to new locations.

The Self-Assembly and Dynamic Structure of Cytoskeletal Filaments

Three types of cytoskeletal filaments are common to many eucaryotic cells and are fundamental to the spatial organization of these cells. Intermediate filaments provide mechanical strength and resistance to shear stress. Microtubules determine the positions of membrane-enclosed organelles and direct intracellular transport. Actin filaments determine the shape of the cell's surface and are necessary for whole-cell locomotion (Panel 16-1). But these cytoskeletal filaments would be ineffective on their own. Their usefulness to the cell depends on a large number of accessory proteins that link the filaments to other cell components, as well as to each other. This set of accessory proteins is essential for the controlled assembly of the cytoskeletal filaments in particular locations, and it includes the motor proteins that either move organelles along the filaments or move the filaments themselves.

Cytoskeletal systems are dynamic and adaptable, organized more like ant trails than interstate highways. A single trail of ants may persist for many hours, extending from the ant nest to a delectable picnic site, but the individual ants within the trail are anything but static. If the ant scouts find a new and better source of food, or if the picnickers clean up and leave, the dynamic structure rearranges itself with astonishing rapidity, to deal with the new situation. In a similar way, large-scale cytoskeletal structures can change or persist, according to need, lasting for lengths of time ranging from less than a minute up to the lifetime of the cell. The individual macromolecular components that make up these structures are in a constant state of flux. Thus, like the alteration of an ant trail, a structural rearrangement in a cell requires little extra energy when conditions change. In this section, we discuss the remarkable mechanisms that cause cytoskeletal filaments to be dynamic, and thereby able to respond rapidly to any eventuality.

Regulation of the dynamic behavior and assembly of the cytoskeletal filaments allows eucaryotic cells to build an enormous range of structures from the three basic filament systems. Microtubules, which are frequently found in a star-like cytoplasmic array emanating from the center of an interphase cell, can quickly rearrange themselves to form a bipolar mitotic spindle during cell division. They can also form motile whips called cilia and flagella on the surface of the cell, or tightly aligned bundles that serve as tracks for the transport of materials down long neuronal axons. Actin filaments form many types of cell-surface projections. Some of these are dynamic structures, such as the lamellipodia and filopodia that cells use to explore territory and pull themselves around. Others are stable structures such as the regular bundles of stereocilia on the surface of hair cells in the inner ear, which tilt as rigid rods in response to sound. Inside cells, actin filaments can also form either transient or stable structures: the contractile ring, for example, assembles transiently to divide cells in two during cytokinesis; more stable arrays allow cells to brace themselves against an underlying substratum

and enable muscle to contract. Intermediate filaments line the inner face of the nuclear envelope, forming a protective cage for the cell's DNA; in the cytosol, they are twisted into strong cables that can hold epithelial cell sheets together, help neuronal cells to extend long and robust axons, or allow us to form tough appendages such as hair and fingernails.

Each Type of Cytoskeletal Filament Is Constructed from Smaller Protein Subunits

Cytoskeletal structures frequently reach all the way from one end of the cell to the other, spanning tens or even hundreds of micrometers. Yet the individual protein molecules of the cytoskeleton are generally only a few nanometers in size. The cell is able to build the large structures by the repetitive assembly of large numbers of the small subunits, like building a skyscraper out of bricks. Because these subunits are small, they can diffuse rapidly within cytoplasm, whereas the assembled filaments cannot. In this way, cells can undergo rapid structural reorganizations, disassembling filaments at one site and reassembling them at another site far away.

Intermediate filaments are made up of smaller subunits that are themselves elongated and fibrous, whereas actin filaments and microtubules are made of subunits that are compact and globular—actin subunits for actin filaments, tubulin subunits for microtubules. All three types of cytoskeletal filaments form as helical assemblies of subunits, which self-associate, using a combination of end-to-end and side-to-side protein contacts. Differences in the structures of the subunits and the strengths of the attractive forces between them produce critical differences in the stability and mechanical properties of each type of filament.

Many biological polymers—including DNA, RNA, and proteins—are held together by covalent linkages between their subunits. In contrast, the three types of cytoskeletal "polymers" are held together by weak noncovalent interactions, which means that their assembly and disassembly can occur rapidly, without covalent bonds being formed or broken.

Within the cell, hundreds of different cytoskeleton-associated accessory proteins regulate the spatial distribution and the dynamic behavior of the filaments. These accessory proteins bind to the filaments or their subunits to determine the sites of assembly of new filaments, to regulate the partitioning of polymer proteins between filament and subunit forms, to change the kinetics of filament assembly and disassembly, to harness energy to generate force, and to link filaments to one another or to other cellular structures such as organelles and the plasma membrane. In these processes, the accessory proteins bring cytoskeletal structure under the control of extracellular and intracellular signals, including those that trigger the dramatic transformations of the cytoskeleton that occur during the cell cycle. Acting together, the accessory proteins enable a eucaryotic cell to maintain a highly organized but flexible internal structure and, in many cases, to move.

Filaments Formed from Multiple Protofilaments Have Advantageous Properties

In general, the linking of protein subunits together to form a filament can be thought of as a simple association reaction. A free subunit binds to the end of a filament that contains n subunits to generate a filament of length n + 1. The addition of each subunit to the end of the polymer creates a new end to which yet another subunit can bind. However, the robust cytoskeletal filaments in living cells are not built by simply stringing subunits together in this way in a single straight file. A thousand tubulin monomers, for example, lined up end to end, would be enough to span the diameter of a small eucaryotic cell, but a filament formed in this way would not have enough strength to avoid breakage by ambient thermal energy, unless each subunit were bound very tightly to its neighbor. Such tight binding would limit the rate at which the filaments could disassemble, making the cytoskeleton a static and less useful structure.

Cytoskeletal polymers combine strength with adaptability because they are built out of multiple protofilaments—long linear strings of subunits joined end to end—that associate with one another laterally. Typically, the protofilaments twist around one another in a helical lattice. The addition or loss of a subunit at the end of one protofilament makes or breaks one set of longitudinal bonds and either one or two sets of lateral bonds. In contrast, breakage of the composite filament in the middle requires breaking sets of longitudinal bonds in several protofilaments all at the same time. The large energy difference between these two processes allows most cytoskeletal filaments to resist thermal breakage, while leaving the filament ends as dynamic structures in which addition and loss of subunits occur rapidly.

As with other specific protein-protein interactions, the subunits in a cytoskeletal filament are held together by a large number of hydrophobic interactions and weak noncovalent bonds. The locations and types of subunit-subunit contacts are different for the different cytoskeletal filaments. Intermediate filaments, for example, assemble by forming strong lateral contacts between α-helical coiled coils, which extend over most of the length of each elongated fibrous subunit. Because the individual subunits are staggered in the filament, intermediate filaments tolerate stretching and bending, forming strong rope-like structures. Microtubules, by contrast, are built from globular subunits held together primarily by longitudinal bonds, and the lateral bonds holding the 13 protofilaments together are comparatively weak. For this reason, microtubules break much more easily than do intermediate filaments.

Nucleation Is the Rate-limiting Step in the Formation of a Cytoskeletal Polymer

There is an important additional consequence of the multiple-protofilament organization of cytoskeletal polymers. Short oligomers composed of a few subunits can assemble spontaneously, but they are unstable and disassemble

readily because each monomer is bonded only to a few other monomers. For a new large filament to form, subunits must assemble into an initial aggregate, or nucleus, that is stabilized by many subunit-subunit contacts and can then elongate rapidly by addition of more subunits. The initial process of nucleus assembly is called filament nucleation, and it can take quite a long time, depending on how many subunits must come together to form the nucleus. The instability of smaller aggregates creates a kinetic barrier to nucleation, which is easily observed in a solution of pure actin or tubulin—the subunits of actin filaments and microtubules, respectively. When polymerization is initiated in a test tube containing a solution of pure individual subunits (by raising the temperature or raising the salt concentration), there is an initial lag phase, during which no filaments are observed. During this lag phase, however, nuclei are assembling slowly, so that the lag phase is followed by a phase of rapid filament elongation, during which subunits add quickly onto the ends of the nucleated filaments. Finally, the system approaches a steady state at which the rate of addition of new subunits to the filament ends is exactly balanced by the rate of subunit dissociation from the ends. The concentration of free subunits left in solution at this point is called the critical concentration, Cc. As explained in Panel 16-2, the value of the critical concentration is equal to the rate constant for subunit loss divided by the rate constant for subunit addition—that is, Cc = koff/kon.

The lag phase in filament growth is eliminated if preexisting nuclei (such as filament fragments that have been chemically cross-linked) are added to the solution at the beginning of the polymerization reaction. The cell takes great advantage of this nucleation requirement: it uses special proteins to catalyze filament nucleation at specific sites, thereby determining the location where new cytoskeletal filaments are assembled. Indeed, this type of regulation of filament nucleation is a primary way that cells control their shape and movement.

The Tubulin and Actin Subunits Assemble Head-to-Tail, Creating Filaments that Are Polar

Microtubules are formed from protein subunits of tubulin. The tubulin subunit is itself a heterodimer formed from two closely related globular proteins called α-tubulin and β-tubulin, tightly bound together by noncovalent bonds. These two tubulin proteins are found only in this complex. Each α or β monomer has a binding site for one molecule of GTP. The GTP that is bound to the α-tubulin monomer is physically trapped at the dimer interface and is never hydrolyzed or exchanged; it can therefore be considered to be an integral part of the tubulin heterodimer structure. The nucleotide on the β-tubulin, in contrast, may be in either the GTP or the GDP form, and it is exchangeable. As we see, the hydrolysis of GTP at this site to produce GDP has an important effect on microtubule dynamics.

A microtubule is a stiff, hollow cylindrical structure built from 13 parallel protofilaments, each composed of alternating α-tubulin and β-tubulin molecules. When the tubulin heterodimers assemble to form the hollow cylindrical microtubule, they generate two new types of protein-protein contacts. Along the longitudinal axis of the microtubule, the "top" of one β-tubulin molecule forms an interface with the "bottom" of the α-tubulin molecule in the adjacent dimer subunit. This interface is very similar to the interface holding the α and β monomers together in the dimer subunit, and the binding energy is strong. Perpendicular to these interactions, lateral contacts are formed between neighboring protofilaments. In this dimension, the main lateral contacts are between monomers of the same type (α-α and β-β). Together, the longitudinal and lateral contacts are repeated in the regular helical lattice of the microtubule. Because most of the subunits in a microtubule are held in place by multiple contacts within the lattice, the addition and loss of subunits occurs almost exclusively at the microtubule ends.

Each protofilament in a microtubule is assembled from subunits that all point in the same direction, and the protofilaments themselves are aligned in parallel. Therefore, the microtubule itself has a distinct structural polarity, with α-tubulins exposed at one end and β-tubulins exposed at the other end.

The actin subunit is a single globular polypeptide chain and is thus a monomer rather than a dimer. Like tubulin, each actin subunit has a binding site for a nucleotide, but for actin the nucleotide is ATP (or ADP) rather than GTP (or GDP). As for tubulin, the actin subunits assemble head-to-tail to generate filaments with a distinct structural polarity. The actin filament can be considered to consist of two parallel protofilaments that twist around each other in a right-handed helix. Actin filaments are relatively flexible compared with the hollow cylindrical microtubules. But in a living cell, actin filaments are cross-linked and bundled together by a variety of accessory proteins (see below), making these large-scale actin structures much stronger than an individual actin filament.

The Two Ends of a Microtubule and of an Actin Filament Are Distinct and Grow at Different Rates

The structural polarity of actin filaments and microtubules is created by the regular, parallel orientation of all of their subunits. This orientation makes the two ends of each polymer different in ways that have a profound effect on filament growth rates. Addition of a subunit to either end of a filament of n subunits results in a filament of n + 1 subunits. In the absence of ATP or GTP hydrolysis, the free energy difference, and therefore the equilibrium constant (and the critical concentration), must be the same for addition of subunits at either end of the polymer. In this case, the ratio of the forward and backward rate constants, kon/koff, must be identical at the two ends, even though the absolute values of these rate constants may be very different at each end.

In a structurally polar filament, the kinetic rate constants for association and dissociation—kon and koff, respectively—are often much greater at one end than at the other. Thus, if an excess of purified subunits is allowed to assemble onto marked fragments of preformed filaments, one end of each fragment elongates much faster than the other. If filaments are rapidly diluted so that the free subunit concentration drops below the critical concentration, the fast-growing end also depolymerizes fastest. The more dynamic of the two ends of a filament, where both growth and shrinkage are fast, is called the plus end, and the other end is called the minus end.

On microtubules, α subunits are exposed at the minus end, and β subunits are exposed at the plus end. On actin filaments, the ATP-binding cleft on the monomer points toward the minus end. (For historical reasons, the plus ends of actin filaments are usually referred to as "barbed" ends, and minus ends as "pointed" ends, because of the arrowhead appearance of myosin heads when bound along the filament.)

Filament elongation proceeds spontaneously when the free energy change (ΔG) for addition of the soluble subunit is less than zero. This is the case when the concentration of subunits in solution exceeds the critical concentration. Likewise, filament depolymerization proceeds spontaneously when this free energy change is greater than zero. A cell can couple an energetically unfavorable process to these spontaneous processes; thus, the free energy released during spontaneous filament polymerization or depolymerization can be used to do mechanical work—in particular, to push or pull an attached load. For example, elongating microtubules can help push out membranes, and shrinking microtubules can help pull mitotic chromosomes away from their sisters during anaphase. Similarly, elongating actin filaments help protrude the leading edge of motile cells.

Filament Treadmilling and Dynamic Instability Are Consequences of Nucleotide Hydrolysis by Tubulin and Actin

Thus far, our discussion of filament dynamics has ignored a critical fact that applies to both actin filaments and microtubules. In addition to their ability to form noncovalent polymers, the actin and tubulin subunits are both enzymes that can catalyze the hydrolysis of a nucleoside triphosphate, ATP or GTP, respectively. For the free subunits, this hydrolysis proceeds very slowly; however, it is accelerated when the subunits are incorporated into filaments. Shortly after incorporation of an actin or tubulin subunit into a filament, nucleotide hydrolysis occurs; the free phosphate group is released from each subunit, but the nucleoside diphosphate remains trapped in the filament structure. Thus, two different types of filament structures can exist, one with the "T form" of the nucleotide bound (ATP for actin, GTP for tubulin), and one with the "D form" bound (ADP for actin, GDP for tubulin).

When the nucleotide is hydrolyzed, much of the free energy released by cleavage of the high-energy phosphate-phosphate bond is stored in the polymer lattice, making the free energy change upon dissociation of the D-form polymer higher than the free energy change upon dissociation of the T-form polymer. Consequently, the equilibrium constant for dissociation KD = koff/kon for the D-form polymer, which is numerically equal to its critical concentration [Cc(D)], is larger than the corresponding equilibrium constant for the T-form polymer. Thus, Cc(D) is greater than Cc(T). For certain concentrations of free subunits, D-form polymers will therefore shrink while T-form polymers grow.

In living cells, most of the free subunits are in the T form, as the free concentration of both ATP and GTP is much higher than that of ADP and GDP. The longer the time that subunits have been in the polymer lattice, the more likely they are to have hydrolyzed their bound nucleotide. Whether the subunit at the very end of a filament is in the T or the D form will depend on the relative rates of hydrolysis and subunit addition. If the rate of subunit addition is high, that is if the filament is growing rapidly, then it is likely that a new subunit will add on to the polymer before the nucleotide in the previously added subunit has been hydrolyzed, so that the tip of the polymer remains in the T form, forming an ATP cap or GTP cap. However, if the rate of subunit addition is low, hydrolysis may occur before the next subunit is added, and the tip of the filament will then be in the D form.

The rate of subunit addition at the end of a filament is the product of the free subunit concentration and the rate constant kon. The kon is much faster for the plus end of a filament than for the minus end because of a structural difference between the two ends. At an intermediate concentration of free subunits, it is therefore possible for the rate of subunit addition to be faster than nucleotide hydrolysis at the plus end, but slower than nucleotide hydrolysis at the minus end. In this case, the plus end of the filament remains in the T conformation, while the minus end adopts the D conformation. As just explained, the D form has a higher critical concentration than the T form; in other words, the D form leans more readily toward disassembly, while the T form leans more readily toward assembly. If the concentration of free subunits in solution is in an intermediate range—higher than the critical concentration of the T form (that is, the plus end), but lower than the critical concentration of the D form (that is, the minus end)—the filament adds subunits at the plus end, and simultaneously loses subunits from the minus end. This leads to the remarkable property of filament treadmilling.

During treadmilling, subunits are recruited at the plus end of the polymer in the T form and shed from the minus end in the D form. The ATP or GTP hydrolysis that occurs along the way gives rise to the difference in the free energy of the association/dissociation reactions at the plus and minus ends of the actin filament or microtubule and thereby makes treadmilling possible. At a particular intermediate subunit concentration, the filament growth at the plus end is exactly

balanced by the filament shrinkage at the minus end. Now, the subunits cycle rapidly between the free and filamentous states, while the total length of the filament remains unchanged. This "steady-state treadmilling" requires a constant consumption of energy in the form of nucleoside triphosphate hydrolysis. While the extent of treadmilling inside the cell is uncertain, the treadmilling of single filaments has been observed in vitro for actin, and a phenomenon that looks like treadmilling can be observed in live cells for individual microtubules.

The kinetic differences between the behavior of the T form and the D form have another important consequence for the behaviors of filaments. If the rate of subunit addition at one end is similar in magnitude to the rate of hydrolysis, there is a finite probability that this end will start out in a T form, but that hydrolysis will eventually "catch up" with the addition and transform the end to a D form. This transformation is sudden and random, with a certain probability per unit time. Thus, in a population of microtubules, at any instant some of the ends are in the T form, and some are in the D form, with the ratio depending on the hydrolysis rate and the free subunit concentration.

Suppose that the concentration of free subunits is intermediate between the critical concentration for a T-form end and the critical concentration for a D-form end (that is, in the same range of concentrations where treadmilling is observed). Now, any end that happens to be in the T form will grow, whereas any end that happens to be in the D form will shrink. On a single filament, an end might grow for a certain length of time in a T form, but then suddenly change to the D form and begin to shrink rapidly, even while the free subunit concentration is held constant. At some later time, it might then regain a T-form and begin to grow again. This rapid interconversion between a growing and shrinking state, at a uniform free subunit concentration, is called dynamic instability. The change to rapid shrinkage is called a catastrophe, while the change to growth is called a rescue. In cells, dynamic instability is thought to predominate in microtubules, whereas treadmilling may predominate in actin filaments.

For microtubules, the structural difference between a T-form end and a D-form end is dramatic. Tubulin subunits with GTP bound to the β-monomer produce straight protofilaments that make strong and regular lateral contacts with one another. But the hydrolysis of GTP to GDP is associated with a subtle conformational change in the protein, which makes the protofilaments curved. On a rapidly growing microtubule, the GTP cap constrains the curvature of the protofilaments, and the ends appear straight. But when the terminal subunits have hydrolyzed their nucleotides, this constraint is released, and the curved protofilaments spring apart. This cooperative release of the energy of hydrolysis stored in the microtubule lattice results in the curled protofilaments peeling off rapidly, and rings and curved oligomers of GDP-containing tubulin are seen near the ends of depolymerizing microtubules.

Treadmilling and Dynamic Instability Require Energy but Are Useful

Both dynamic instability and treadmilling allow a cell to maintain the same overall filament content, while individual subunits constantly recycle between the filaments and the cytosol. How dynamic are the microtubules and actin filaments inside a living cell? Typically, a microtubule, with major structural differences between its growing and shrinking ends, switches between growth and shrinkage a few times per minute. The ends of individual microtubules can therefore be seen in real time to exhibit such dynamic instability. For actin filaments, by contrast, the difference between the two types of ends is not so extreme, and the dynamic instability of an individual filament cannot be observed directly with the light microscope. With appropriate techniques based on fluorescence microscopy, however, one can show that actin filament turnover is typically rapid, with individual filaments persisting for only a few minutes.

At first glance, this dynamic behavior of filaments seems like a waste of energy. To maintain a constant concentration of actin filaments and microtubules, most of which are undergoing a process of either treadmilling or dynamic instability, the cell must hydrolyze large amounts of nucleoside triphosphate. As we explained with our ant-trail analogy at the beginning of the chapter, the advantage to the cell seems to lie in the spatial and temporal flexibility that is inherent in a structural system with constant turnover. Individual subunits are small and can diffuse very rapidly; an actin or tubulin subunit can diffuse across the diameter of a typical eucaryotic cell in a second or two. As noted above, the rate-limiting step in the formation of a new filament is nucleation, so these rapidly diffusing subunits tend to assemble either on the ends of preexisting filaments or at special sites where the nucleation step is catalyzed by special proteins. The new filaments in either case are highly dynamic, and unless specifically stabilized, they have only a fleeting existence. By controlling where filaments are nucleated and selectively stabilized, a cell can control the location of its filament systems, and hence its structure. It seems that the cell is continually testing a wide variety of internal structures and only preserving those that are useful. When external conditions change, or when internal signals arise (as during the transitions in the cell cycle), the cell is poised to change its structure rapidly.

In certain specialized structures, particularly in various specialized cells in a multicellular organism, parts of the cytoskeleton become less dynamic. In a terminally differentiated cell such as a neuron, for example, it is desirable to maintain a consistent structure over time, and many of the actin filaments and microtubules are stabilized by association with other proteins. However, when new connections are made in the brain, as when the information you are reading here is transferred into long-term memory, even a cell as stable as a neuron can grow new processes to make new synapses. To do this, a neuron requires the inherently dynamic and exploratory activities of its cytoskeletal filaments.

Other Polymeric Proteins Also Use Nucleotide Hydrolysis to Couple a Conformational Change to Cell Movements

It is remarkable that actin and tubulin have both evolved nucleoside triphosphate hydrolysis for the same basic reason—to enable them to depolymerize readily after they have polymerized. Actin and tubulin are completely unrelated in amino acid sequence: actin is distantly related in structure to the glycolytic enzyme hexokinase, whereas tubulin is distantly related to a large family of GTPases that includes the heterotrimeric G proteins and monomeric GTPases such as Ras.

Several other types of protein polymers bind and hydrolyze nucleoside triphosphates. For example, dynamin assembles in helical coils around the base of membrane invaginations, helping to pinch off the invaginations to form vesicles. Hydrolysis of GTP by dynamin generates a cooperative conformational change in the dynamin coil and provides the energy for the constriction. Bacteria and archaea have a tubulin homolog called FtsZ that is essential for cell division. A band of FtsZ protein forms at the site of septation, where the new cell wall is to form. Constriction and disassembly of the FtsZ band then help to pinch the two daughter cells apart. In vitro, FtsZ forms protofilaments and rings reminiscent of tubulin, and it also binds and hydrolyzes GTP. The increase in ring curvature and disassembly of the FtsZ filaments is similar to the disassembly of curved GDP-tubulin protofilaments described earlier, and it may occur by a similar mechanism.

These examples point to a second general way in which protein polymers can use the energy of nucleotide hydrolysis to affect cell structure and dynamics. In a linear protein polymer, a modest hydrolysis-linked conformational change by a few tenths of a nanometer in each subunit can be amplified by the thousands of subunits acting in parallel, so as to drive movements over tens or hundreds of nanometers. For dynamin and FtsZ, apparently, the effect of the GTP-dependent conformational changes is to bend lipid bilayers.

Tubulin and Actin Have Been Highly Conserved During Eucaryotic Evolution

Tubulin is found in all eucaryotic cells, and FtsZ has been found in all bacteria and archaea examined. Rod-shaped and spiral-shaped bacteria also contain an actin homolog, MreB, that forms filaments and regulates bacterial length. Thus, the principle of organizing cell structure by a self-association of nucleotide-binding proteins seems to be used in all cells.

Tubulin molecules themselves come in multiple isoforms. In mammals, there are at least six forms of α-tubulin and a similar number of forms of β-tubulin, each encoded by a different gene. The different forms of tubulin are very similar, and they generally copolymerize into mixed microtubules in the test tube, although they can have distinct locations in the cell and perform subtly different functions. As a particularly striking example, the microtubules in six specialized touch-sensitive neurons in the nematode Caenorhabditis elegans contain a specific

form of β-tubulin, and mutations in the gene for this protein result in the loss of touch-sensitivity with no apparent defect in other cell functions.

Yeast and human tubulins are 75% identical in amino acid sequence. Most of the variation among different isoforms of tubulin is found in the amino acids near the C-terminus, which form a ridge on the surface of the microtubule. Thus, variations among isoforms are expected to affect primarily the association of accessory proteins with the surface of the microtubule, rather than microtubule polymerization per se.

Like tubulin, actin is found in all eucaryotic cells. Most organisms have multiple genes encoding actin; humans have six. Actin is extraordinarily well conserved among eucaryotes. The amino acid sequences of actins from different species are usually about 90% identical. But, again like tubulin, small variations in actin amino acid sequence can create significant functional differences. In vertebrates, there are three subtly different isoforms of actin, termed α, β, and γ, that differ slightly in their amino acid sequences. The α-actin is expressed only in muscle cells, while β and γ are found together in almost all nonmuscle cells. Yeast actin and Drosophila muscle actin are 89% identical, yet the expression of yeast actin in Drosophila results in a fly that looks normal but is unable to fly.

What is the explanation for the unusually strict conservation of actin and tubulin in eucaryotic evolution? Most other cytoskeletal proteins, including intermediate filament proteins and the large families of accessory proteins that bind to actin or tubulin, are not particularly well conserved at the level of amino acid sequence. The likely explanation is that the structure of the entire surface of an actin filament or microtubule is constrained because so many other proteins must be able to interact with these two ubiquitous and abundant cell components. A mutation in actin that could result in a desirable change in its interaction with one other protein might cause undesirable changes in its interactions with a number of other proteins that bind at or near the same site. Genetic and biochemical studies in the yeast Saccharomyces cerevisiae have demonstrated that actin interacts directly with dozens of other proteins, and indirectly with even more. Over time, evolving organisms have found it more profitable to leave actin and tubulin alone, and alter their binding partners instead.

Intermediate Filament Structure Depends on The Lateral Bundling and Twisting of Coiled Coils

All eucaryotic cells contain actin and tubulin. But the third major type of cytoskeletal filament, the intermediate filament, is found in only some metazoans, including vertebrates, nematodes, and molluscs. Even in these organisms, intermediate filaments are not required in every cell type. The specialized glial cells (called oligodendrocytes) that make myelin in the vertebrate central nervous system, for example, do not contain intermediate filaments. Cytoplasmic intermediate filaments are closely related to their ancestors, the much more

widely used nuclear lamins. The nuclear lamins are filamentous proteins that form a meshwork lining the inner membrane of the eucaryotic nuclear envelope, where they provide anchorage sites for chromosomes and nuclear pores. Several times during metazoan evolution, lamin genes have apparently duplicated, and the duplicates have evolved to produce rope-like, cytoplasmic intermediate filaments. These filaments have mechanical properties that are especially useful to soft-bodied animals such as nematodes and vertebrates that do not have an exoskeleton. Intermediate filaments are particularly prominent in the cytoplasm of cells that are subject to mechanical stress, and their major function seems to be to impart physical strength to cells and tissues.

The individual polypeptides of intermediate filaments are elongated molecules with an extended central α-helical domain that forms a parallel coiled coil with another monomer. A pair of parallel dimers then associates in an antiparallel fashion to form a staggered tetramer. This tetramer represents the soluble subunit that is analogous to the αβ-tubulin dimer, or the actin monomer.

Since the tetrameric subunit is made up of two dimers pointing in opposite directions, its two ends are the same. The assembled intermediate filament therefore lacks the overall structural polarity that is critical for actin filaments and microtubules. The tetramers pack together laterally to form the filament, which includes eight parallel protofilaments made up of tetramers. Each individual intermediate filament therefore has a cross section of 32 individual α-helical coils. This large number of polypeptides all lined up together, with the strong lateral hydrophobic interactions typical of coiled-coil proteins, gives intermediate filaments a rope-like character. They can be easily bent but are extremely difficult to break.

Less is understood about the mechanism of assembly and disassembly of intermediate filaments than of actin filaments and microtubules, but they are clearly highly dynamic structures in most cell types. Under normal conditions, protein phosphorylation probably regulates their disassembly, in much the same way that phosphorylation regulates the disassembly of nuclear lamins in mitosis. As evidence for a rapid turnover, labeled subunits microinjected into tissue culture cells rapidly add themselves onto the existing intermediate filaments within a few minutes, while an injection of peptides derived from a conserved helical region of the subunit induces the rapid disassembly of the intermediate filament network. Interestingly, the latter injection can induce the disassembly of the microtubule and actin filament networks in some cells that contain all three networks revealing that there is a fundamental mechanical integration among the different cytoskeletal systems in these cells.

Intermediate Filaments Impart Mechanical Stability to Animal Cells

Intermediate filaments come in a wide variety of types, with substantially more sequence variation in the subunit isoforms than occurs in the isoforms of actin or

tubulin. A central α-helical domain has 40 or so heptad repeat motifs that form an extended coiled-coil. This domain is similar in the different isoforms, but the N- and C-terminal globular domains can vary a great deal.

Different families of intermediate filaments are expressed in different cell types. The most diverse intermediate filament family is that of the keratins: there are about 20 found in different types of human epithelial cells, and about 10 more that are specific to hair and nails. A single epithelial cell may produce multiple types of keratins, and these copolymerize into a single network (. Every keratin filament is made up of an equal mixture of type I (acidic) and type II (neutral/basic) keratin chains; these form heterodimers, two of which then join to form the fundamental tetrameric subunit. Cross-linked keratin networks held together by disulfide bonds may survive even the death of their cells, forming tough coverings for animals, as in the outer layer of skin and in hair, nails, claws, and scales. The diversity in keratins is clinically useful in the diagnosis of epithelial cancers (carcinomas), as the particular set of keratins expressed gives an indication of the epithelial tissue in which the cancer originated and thus can help to guide the choice of treatment.

Mutations in keratin genes cause several human genetic diseases. For example, when defective keratins are expressed in the basal cell layer of the epidermis, they produce a disorder called epidermolysis bullosa simplex, in which the skin blisters in response to even very slight mechanical stress, which ruptures the basal cells. Other types of blistering diseases, including disorders of the mouth, esophageal lining, and the cornea of the eye, are caused by mutations in the different keratins whose expression is specific to those tissues. All of these maladies are typified by cell rupture as a consequence of mechanical trauma and a disorganization or clumping of the keratin filament cytoskeleton. Many of the specific mutations that cause these diseases alter the ends of the central rod domain, underlining the importance of this particular part of the protein for correct filament assembly.

A second family of intermediate filaments, called neurofilaments, is found in high concentrations along the axons of vertebrate neurons. Three types of neurofilament proteins (NF-L, NF-M, NF-H) coassemble in vivo, forming heteropolymers that contain NF-L plus one of the others. The NF-H and NF-M proteins have lengthy C-terminal tail domains that bind to neighboring filaments, generating aligned arrays with a uniform interfilament spacing. During axonal growth, new neurofilament subunits are incorporated all along the axon in a dynamic process that involves the addition of subunits along the filament length, as well as the addition of subunits at the filament ends. After an axon has grown and connected with its target cell, the diameter of the axon may increase as much as fivefold. The level of neurofilament gene expression seems to directly control axonal diameter, which in turn controls how fast electrical signals travel down the axon.

The neurodegenerative disease amyotrophic lateral sclerosis (ALS, or Lou Gehrig's Disease) is associated with an accumulation and abnormal assembly of neurofilaments in motor neuron cell bodies and in the axon, which may interfere with normal axonal transport. The degeneration of the axons leads to muscle weakness and atrophy, which is usually fatal. The overexpression of human NF-L or NF-H in mice results in mice that have an ALS-like disease.

The vimentin-like filaments are a third family of intermediate filaments. Desmin, a member of this family, is expressed in skeletal, cardiac, and smooth muscle. Mice lacking desmin show normal initial muscle development, but adults have a variety of muscle cell abnormalities, including misaligned muscle fibers.

Thus, intermediate filaments in general seem to serve as the ligaments of the cell, whereas microtubules and actin filaments seem to serve, respectively, as the bones and muscles of the cell. Just as we require our ligaments, bones, and muscles to work together, so all three cytoskeletal filament systems must normally function collectively to give a cell its strength, its shape, and its ability to move.

Filament Polymerization Can Be Altered by Drugs

Because the survival of eucaryotic cells depends on a balanced assembly and disassembly of the highly conserved cytoskeletal filaments formed from actin and tubulin, the two types of filaments are frequent targets for natural toxins. These toxins are produced in self-defense by plants, fungi, or sponges that do not wish to be eaten but cannot run away from predators, and they generally perturb the filament polymerization reaction. The toxin binds tightly to either the filament form or the free subunit form of a polymer, driving the assembly reaction in the direction that favors the form to which the toxin binds. For example, the drug latrunculin, extracted from the sea sponge Latrunculia magnifica, binds to and stabilizes actin monomers; it thereby causes a net depolymerization of actin filaments. In contrast, phalloidin, from the fungus Amanita phalloides (death cap), binds to and stabilizes actin filaments, causing a net increase in actin polymerization. (One remedy for Amanita mushroom poisoning is to eat a large quantity of raw meat: the high concentration of actin filaments in the muscle tissue binds the phalloidin and thereby reduces phalloidin's toxicity.) Either change in actin filaments is very toxic for cells. Similarly, colchicine, from the meadow saffron (or autumn crocus), binds to and stabilizes free tubulin, causing microtubule depolymerization. In contrast, taxol, extracted from the bark of a rare species of yew tree, binds to and stabilizes microtubules, causing a net increase in tubulin polymerization.

Drugs like these have a rapid and profound effect on the organization of the cytoskeleton in living cells. They provided early evidence that the cytoskeleton is a dynamic structure, maintained by a rapid and continual exchange of subunits

between the soluble and filamentous forms and that this subunit flux is necessary for normal cytoskeletal function.

Both microtubule-depolymerizing drugs such as vinblastine and microtubule-polymerizing drugs such as taxol preferentially kill dividing cells, since both microtubule assembly and disassembly are crucial for correct function of the mitotic spindle. These drugs efficiently kill certain types of tumor cells in a human patient, although not without toxicity to rapidly dividing normal cells, including those in the bone marrow, intestine, and hair follicles. Taxol in particular has been widely used to treat some specific cancers that are resistant to other chemotherapeutic agents.

The common laboratory reagent acrylamide, used as the precursor in making polyacrylamide gels for size separation of proteins and nucleic acids, is also a cytoskeletal toxin. By an unknown mechanism, it causes the disassembly or rearrangement of intermediate filament networks. Acryl-amide can be absorbed through the skin and acts as a potent neurotoxin by dismantling the neurofilament bundles in peripheral nerve axons. This is why it causes an unpleasant tingling sensation when spilled on bare skin and why it should always be handled cautiously.

Summary

The cytoplasm of eucaryotic cells is spatially organized by a network of protein filaments known as the cytoskeleton. This network contains three principal types of filaments: microtubules, actin filaments, and intermediate filaments. All three types of filaments form as helical assemblies of subunits that self-associate using a combination of end-to-end and side-to-side protein contacts. Differences in the structure of the subunits and the manner of their self-assembly give the filaments different mechanical properties. Intermediate filaments are rope-like and easy to bend but hard to break. Microtubules are strong, rigid hollow tubes. Actin filaments are the thinnest of the three and are hard to stretch but easy to break.

In living cells, all three types of cytoskeletal filaments undergo constant remodeling through the assembly and disassembly of their subunits. Microtubules and actin filaments add and lose subunits only at their ends, with one end (the plus end) growing faster than the other. Tubulin and actin (the subunits of microtubules and actin filaments, respectively) bind and hydrolyze nucleoside triphosphates (tubulin binds GTP and actin binds ATP). Nucleotide hydrolysis underlies the characteristic dynamic behavior of these two filaments. Actin filaments in cells seem to predominantly undergo treadmilling, where a filament assembles at one end while simultaneously disassembling at the other end. Microtubules in cells predominantly display dynamic instability, where a microtubule end undergoes alternating bouts of growth and shrinkage.

Whereas tubulin and actin have been strongly conserved in eucaryotic evolution, the family of intermediate filaments is very diverse. There are a variety of tissue-specific forms, including keratin filaments in epithelial cells, neurofilaments in nerve cells, and desmin filaments in muscle cells. In all these cells, the primary job of intermediate filaments is to provide mechanical strength.

How Cells Regulate Their Cytoskeletal Filaments

Microtubules, actin filaments, and intermediate filaments are much more dynamic in cells than they are in the test tube. The cell regulates the length and stability of its cytoskeletal filaments, as well as their number and the geometry. It does so largely by regulating their attachments to one another and to other components of the cell, so that the filaments can form a wide variety of higher-order structures. Some filament properties are regulated by direct covalent modification of the filament subunits, but most of the regulation is performed by accessory proteins that bind to either the filaments or their free subunits.

This section focuses on how these accessory proteins modify the dynamics and structure of cytoskeletal filaments. We begin with a discussion of how microtubules and actin filaments are nucleated in cells, as this plays a major part in determining the overall organization of the cell's interior.

Microtubules Are Nucleated by a Protein Complex Containing γ-tubulin

While α- and β-tubulins are the regular building blocks of microtubules, another type of tubulin, called γ-tubulin, has a more specialized role. Present in much smaller amounts than α- and β-tubulin, this protein is involved in the nucleation of microtubule growth in organisms ranging from yeasts to humans. Microtubules are generally nucleated from a specific intracellular location known as a microtubule-organizing center (MTOC). Antibodies against γ-tubulin stain the MTOC in virtually all species and cell types thus far examined.

Microtubules are nucleated at their minus end, with the plus end growing outward from each MTOC to create various types of microtubule arrays. A γ-tubulin ring complex (γ-TuRC) has been isolated from both insect and vertebrate cells and is an impressively efficient nucleator of microtubule growth in a test tube. Two proteins, conserved from yeasts to humans, bind directly to the γ-tubulin, along with several other proteins that help create the ring that can be seen at the minus ends of the microtubules nucleated by γ-TuRC. This ring of γ-tubulin molecules is therefore thought to serve as a template that nucleates a microtubule with 13 protofilaments.

Microtubules Emanate from the Centrosome in Animal Cells

In most animal cells, there is a single, well-defined MTOC called the centrosome, located near the nucleus. From this focal point, the cytoplasmic microtubules emanate in a star-like, "astral" conformation. Microtubules are nucleated at the centrosome at their minus ends, so the plus ends point outward and grow toward the cell periphery. A centrosome is composed of a fibrous centrosome matrix that contains more than fifty copies of γ-TuRC. Most of the proteins that form this matrix, remain to be discovered, and it is not yet known how they recruit and activate the γ-TuRC.

Embedded in the centrosome is a pair of cylindrical structures arranged at right angles to each other in an L-shaped configuration. These are the centrioles, which become the basal bodies of cilia and flagella in motile cells (described later). The centrioles organize the centrosome matrix (also called the pericentriolar material), ensuring its duplication during each cell cycle as the centrioles themselves duplicate. The centrosome duplicates and splits into two equal parts during interphase, each half containing a duplicated centriole pair. These two daughter centrosomes move to opposite sides of the nucleus when mitosis begins, and they form the two poles of the mitotic spindle. A centriole consists of a short cylinder of modified microtubules, plus a large number of accessory proteins. The molecular basis for its duplication is not known.

In fungi and diatoms, microtubules are nucleated at an MTOC that is embedded in the nuclear envelope as a small plaque called the spindle pole body. Higher-plant cells seem to nucleate microtubules at sites distributed all around the nuclear envelope. Neither fungi nor most plant cells contain centrioles. Despite these differences, all these cells contain γ-tubulin and seem to use it to nucleate their microtubules.

In animal cells, the astral configuration of microtubules is very robust, with dynamic plus ends pointing outward toward the cell periphery and stable minus ends collected near the nucleus. The system of microtubules radiating from the centrosome acts as a device to survey the outlying regions of the cell and position the centrosome at its center, and it does this even in artificial enclosures. Even in an isolated cell fragment lacking the centrosome, dynamic microtubules interacting with membraneous organelles and motor proteins arrange themselves into a star-shaped array with the microtubule minus ends clustered at the center. This ability of the microtubule cytoskeleton to find the center of the cell establishes a general coordinate system, which is then used to position many organelles within the cell.

Actin Filaments Are Often Nucleated at the Plasma Membrane

In contrast to microtubule nucleation, which occurs primarily deep within the cytoplasm near the nucleus, actin filament nucleation most frequently occurs at the plasma membrane. Consequently, the highest density of actin filaments in most cells is at the cell periphery. These actin filaments in the layer underlying the plasma membrane, called the cell cortex, determine the shape and movement of the cell surface. For example, depending on their attachments to one another and to the plasma membrane, actin structures can form many strikingly different types of cell surface projections. These include spiky bundles such as microvilli or filopodia, flat protrusive veils called lamellipodia that help move cells over solid substrates, and the phagocytic cups in macrophages.

The nucleation of actin filaments at the plasma membrane is frequently regulated by external signals, allowing the cell to change its shape and stiffness rapidly in response to changes in its external environment. This nucleation is catalyzed by a complex of proteins that includes two actin-related proteins, or ARPs, each of which is about 45% identical to actin. Analogous to the function of the γ-TuRC, the ARP complex (also known as the Arp 2/3 complex) nucleates actin filament growth from the minus end, allowing rapid elongation at the plus end. However, the complex can also attach to the side of another actin filament while remaining bound to the minus end of the filament that it has nucleated, thereby building individual filaments into a treelike web. The ARP complex is localized in regions of rapid actin filament growth such as lamellipodia, and its nucleating activity is regulated by intracellular signaling molecules and components at the cytosolic face of the plasma membrane.

Both γ-tubulin and ARPs are evolutionarily ancient, and they are conserved among a wide variety of eucaryotic species. Their genes seem to have arisen by early duplication of the gene for the microtubule or actin filament subunit, respectively, followed by divergence and specialization of the gene copies so that they encode proteins with a special nucleating function. That a similar strategy has evolved for two separate cytoskeletal systems underlines the central importance of regulated filament nucleation as a general organizing principle in cells.

Filament Elongation Is Modified by Proteins That Bind to the Free Subunits

Once cytoskeletal filaments have been nucleated, they generally elongate by the addition of soluble subunits. In most nonmuscle vertebrate cells, approximately 50% of the actin is in filaments and 50% is soluble. The soluble monomer concentration is typically 50–200 μM (2–8 mg/ml), which is surprisingly high, given the low critical concentration observed for pure actin in a test tube (less than 1 μM). Why does the soluble actin not polymerize into filaments? The reason is that the abundant subunit pool contains special proteins that bind to the actin monomers, thereby making polymerization much less favorable (the action is similar to that of latrunculin). A small protein called thymosin is the most abundant of these proteins. Actin monomers bound to thymosin are in a locked

state, where they cannot associate with either the plus or minus ends of actin filament and cannot hydrolyze or exchange their bound nucleotide.

How do cells recruit actin monomers from this sequestered pool and use them for polymerization? One might imagine that the thymosin itself could be regulated by signal transduction pathways, but this has not been found to be the case. Instead, recruitment depends on another monomer-binding protein, profilin. Profilin binds to the face of the actin monomer opposite the ATP-binding cleft, blocking the side of the monomer that would normally associate with the filament minus end. However, the profilin-actin complex can readily add onto a free plus end. As soon as this addition occurs, a conformational change is induced in the actin that reduces its affinity for profilin, so the profilin falls off, leaving the actin filament one subunit longer. Profilin competes with thymosin in binding to individual actin monomers, and a local activation of profilin molecules moves actin subunits from the sequestered thymosin-bound pool onto filament plus ends.

Several types of intracellular mechanisms regulate the activity of profilin, including profilin phosphorylation and profilin binding to inositol phospholipids. These mechanisms can define the sites where profilin acts. Profilin's ability to move sequestered actin subunits onto the growing ends of filaments is critical for filament assembly at the plasma membrane, for example. Profilin is localized at the cytosolic face of the plasma membrane because it binds to acidic membrane phospholipids there. At this location, extracellular signals can produce explosive local actin polymerization and the extension of actin-rich motile structures such as filopodia and lamellipodia (see below). Besides binding to actin and phospholipids, profilin also binds to various other intracellular proteins that have domains rich in proline; these proteins may also help to localize profilin to sites where rapid actin assembly may be required.

Like actin monomers, unpolymerized tubulin subunits are sequestered in the cell to maintain the subunit pool at a level substantially higher than the critical concentration. One molecule of the small protein stathmin binds to two tubulin heterodimers and prevents their addition onto the ends of microtubules. Stathmin thus decreases the effective concentration of tubulin subunits that are available for polymerization (the action is analogous to that of colchicine). High levels of active stathmin in a cell decrease the elongation rate of microtubules, since the elongation rate is just the product of the concentration of available tubulin subunits and the rate constant kon. The slower elongation rate also has a second remarkable effect. Since the transition from the growing state to the shrinking state for a microtubule undergoing dynamic instability depends on the race between GTP hydrolysis and filament elongation, slowing the elongation rate by sequestering tubulin subunits can increase the frequency of microtubule shrinkage. Thus, a protein that inhibits tubulin polymerization can have the secondary effect of dramatically increasing the dynamic turnover of microtubules in living cells.

Proteins That Bind Along the Sides of Filaments Can Either Stabilize or Destabilize Them

Once a cytoskeletal filament is formed by nucleation and elongated from the subunit pool, its stability and mechanical properties are often altered by a set of proteins that bind along the sides of the polymer. Different filament-associated proteins use their binding energy to either lower or raise the free energy of the polymer state, and they thereby either stabilize or destabilize the polymer, respectively.

Proteins that bind along the sides of microtubules are collectively called microtubule-associated proteins, or MAPs. Like the drug taxol, MAPs can stabilize microtubules against disassembly. A subset of MAPs can also mediate the interaction of microtubules with other cellular components. This subset is prominent in neurons, where stabilized microtubule bundles form the core of the axons and dendrites that extend from the cell body. These MAPs have at least one domain that binds to the microtubule surface and another that projects outward. The length of the projecting domain can determine how closely MAP-coated microtubules pack together, as demonstrated in cells engineered to overproduce different MAPs. Cells overexpressing MAP2, which has a long projecting domain, form bundles of stable microtubules that are kept widely spaced, while cells overexpressing tau, a MAP with a much shorter projecting domain, form bundles of more closely packed microtubules.

The microtubule-binding domain of several MAPs, including tau and MAP2, includes multiple copies of a tubulin-binding motif. When such MAPs are added to a solution of pure unpolymerized tubulin, they greatly accelerate nucleation, presumably because they stabilize the small tubulin oligomers that form early in polymerization. MAPs are the targets of several protein kinases, and the resulting phosphorylation of a MAP can have a primary role in controlling both its activity and localization inside cells.

Whereas MAP2 and tau are confined to selected cell types in vertebrates, there are other MAPs that seem to have a central role in microtubule dynamics in nearly all eucaryotic cells. In particular, a ubiquitous protein called XMAP215 has close homologs in organisms that range from yeast to humans (XMAP stands for Xenopus microtubule-associated protein, and the number refers to its molecular weight). This protein binds along the sides of microtubules, but, as discussed later, it also has a special ability to stabilize free microtubule ends and inhibit their switch from a growing to a shrinking state. The phosphorylation of XMAP215 during mitosis inhibits this activity, making a substantial contribution to the tenfold increase in the dynamic instability of microtubules observed during mitosis.

Actin filaments are likewise strongly affected by the binding of accessory proteins along their sides. Selected actin filaments in most cells are stabilized by the binding of tropomyosin, an elongated protein that binds simultaneously to seven adjacent actin subunits in one protofilament. The binding of tropomyosin along an actin filament can prevent the filament from interacting with other proteins; for this reason, the regulation of tropomyosin binding is an important step in muscle contraction.

Another important actin-filament binding protein present in all eucaryotic cells is cofilin, which destabilizes actin filaments. Also called actin depolymerizing factor, cofilin is unusual in that it binds to actin in both the filament and free subunit forms. Cofilin binds along the length of the actin filament, forcing the filament to twist a little more tightly. This mechanical stress weakens the contacts between actin subunits in the filament, making the filament brittle and more easily severed. In addition, it makes it easier for an ADP-actin subunit to dissociate from the minus end of the filament. Because the rate of actin filament treadmilling is normally limited by the slow dissociation rate at the minus end, cofilin binding causes a large increase in the rate of actin filament treadmilling. As a result, most of the actin filaments inside cells are much shorter-lived than are filaments formed from pure actin in a test tube.

Cofilin binds preferentially to ADP-containing actin filaments rather than to ATP-containing filaments. Since ATP hydrolysis is usually slower than filament assembly, the newest actin filaments in the cell still contain mostly ATP and are resistant to depolymerization by cofilin. Cofilin therefore efficiently dismantles the older filaments in the cell, ensuring that all actin filaments turn over rapidly.

Intermediate Filaments Are Cross-linked and Bundled Into Strong Arrays

Each individual intermediate filament forms as a long bundle of tetrameric subunits. Many intermediate filaments further bundle themselves by self-association; for example, the neurofilament proteins NF-M and NF-H contain a C-terminal domain that extends outward from the surface of the assembled intermediate filament and binds to a neighboring filament. Thus groups of neurofilaments form robust parallel arrays that are held together by multiple lateral contacts, giving strength and stability to the long cell processes of neurons.

Other types of intermediate filament bundles are held together by accessory proteins, such as filaggrin, which bundles keratin filaments in differentiating cells of the epidermis to give the outermost layers of the skin their special toughness. Plectin, which makes bundles of vimentin, is a particularly interesting cross-linking protein. Besides bundling intermediate filaments, it also links the intermediate filaments to microtubules, actin filament bundles, and filaments of the motor protein myosin II (discussed below), as well as helping to attach intermediate filament bundles to adhesive structures at the plasma membrane.

Mutations in the gene for plectin cause a devastating human disease that combines epidermolysis bullosa (caused by disruption of skin keratin filaments), muscular dystrophy (caused by disruption of desmin filaments), and neurodegeneration (caused by disruption of neurofilaments). Mice lacking a functional plectin gene die within a few days of birth, with blistered skin and abnormal skeletal and heart muscles. Thus, although plectin may not be necessary for the initial formation and assembly of intermediate filaments, its cross-linking action is required to provide cells with the strength they need to withstand the mechanical stresses inherent to vertebrate life.

Cross-linking Proteins with Distinct Properties Organize Different Assemblies of Actin Filaments

Actin filaments in animal cells are organized into two types of arrays: bundles and weblike (gel-like) networks. Actin filament cross-linking proteins are accordingly divided into two classes, bundling proteins and gel-forming proteins. Bundling proteins cross-link actin filaments into a parallel array, while gel-forming proteins hold two actin filaments together at a large angle to each other, thereby creating a looser meshwork. Both types of cross-linking protein generally have two similar actin-filament-binding sites, which can either be part of a single polypeptide chain or contributed by each of two polypeptide chains held together in a dimer. The spacing and arrangement of these two filament-binding domains determines the type of actin structure that a given cross-linking protein forms.

Fimbrin and α-actinin are widely distributed actin-bundling proteins. Fimbrin is a small crosslinker, with two actin-binding domains close together in a single polypeptide chain. It is enriched in the parallel actin filament bundles in filopodia at the leading edge of cells, and it is presumably responsible for the tight association of these actin filaments. α-actinin contains two actin-binding domains that are further apart; it is concentrated in stress fibers, where it is responsible for the relatively loose cross-linking of actin filaments in these contractile bundles. It also helps form the structure that holds stress fiber ends in focal contacts at the plasma membrane (see below).

Each type of bundling protein determines which other molecules can interact with an actin filament. Myosin II (discussed later) is the protein in stress fibers and other contractile arrays that is responsible for their ability to contract. The very close packing of actin filaments caused by fimbrin apparently excludes myosin, and thus filopodia are not contractile; on the other hand, the looser packing caused by α-actinin allows myosin molecules to enter, making stress fibers contractile. Because of the very different spacing between the actin filaments, bundling by fimbrin automatically discourages bundling by α-actinin, and vice-versa, so that the two types of bundling protein are themselves mutually exclusive.

Villin is another bundling protein that, like fimbrin, has two actin-filament-binding sites very close together in a single polypeptide chain. Villin (together with fimbrin) helps cross-link the 20 to 30 tightly bundled actin filaments found in microvilli, the finger-like extensions of the plasma membrane on the surface of many epithelial cells. A single absorptive epithelial cell in the human small intestine, for example, has several thousand microvilli on its apical surface. Each is about 0.08 μm wide and 1 μm long, making the cell's absorptive surface area about 20 times greater than it would be without microvilli. When villin is introduced into cultured fibroblasts, which do not normally contain villin and have only a few small microvilli, the existing microvilli become greatly elongated and stabilized, and new ones are induced. The actin filament core of the microvillus is attached to the plasma membrane along its sides by lateral sidearms made of myosin I (discussed later), which has a binding site for filamentous actin on one end and a domain that binds lipids on the other end. These two types of cross-linkers, one binding actin filaments to each other and the other binding these filaments to the membrane, seem to be sufficient to form microvilli on cells.

The various bundling proteins that we have discussed so far have straight, stiff connections between their two actin-filament-binding domains, and they tend to align filaments in parallel bundles. In contrast, those actin cross-linking proteins that have either a flexible or a stiff, bent connection between their two binding domains form actin filament webs or gels, rather than actin bundles.

A well-studied web-forming protein is spectrin, which was first identified in red blood cells. Spectrin is a long, flexible protein made out of four elongated polypeptide chains (two α subunits and two β subunits), arranged so that the two actin-filament-binding sites are about 200 nm apart (compared with 14 nm for fimbrin and about 30 nm for α-actinin). In the red blood cell, spectrin is concentrated just beneath the plasma membrane, where it forms a two-dimensional web held together by short actin filaments; spectrin links this web to the plasma membrane because it has separate binding sites for peripheral membrane proteins, which are themselves positioned near the lipid bilayer by integral membrane proteins. The resulting network creates a stiff cell cortex that provides mechanical support for the overlying plasma membrane, allowing the red blood cell to spring back to its original shape after squeezing through a capillary. Close relatives of spectrin are found in the cortex of most other vertebrate cell types, where they also help to shape and stiffen the surface membrane.

Any cross-linking protein that has its two actin-binding domains joined by a long bent linkage can form three-dimensional actin gels. Filamin promotes the formation of a loose and highly viscous gel by clamping together two actin filaments roughly at right angles. The actin gels formed by filamin are required for cells to extend the thin sheet-like membrane projections called lamellipodia that help them to crawl across solid surfaces. Filamin is lacking in some types of

cancer cells, especially some malignant melanomas (pigment-cell cancers). These cells cannot crawl properly, and instead they protrude disorganized membrane blebs. Losing filamin is bad news for the melanoma cells but good news for the melanoma patient; because of the cells' inability to crawl, melanoma cells that have lost filamin expression are less invasive than similar melanoma cells that still express filamin, and, as a result, the cancer is much less likely to metastasize.

Severing Proteins Regulate the Length and Kinetic Behavior of Actin Filaments and Microtubules

In some situations, a cell may break an existing long filament into many smaller filaments. This generates a large number of new filament ends: one long filament with just one plus end and one minus end might be broken into dozens of short filaments, each with its own minus end and plus end. Under some intracellular conditions, these newly formed ends nucleate filament elongation, and in this case severing accelerates the assembly of new filament structures. Under other conditions, severing promotes the depolymerization of old filaments, speeding up the depolymerization rate by tenfold or more. In addition, severing filaments changes the physical and mechanical properties of the cytoplasm: stiff, large bundles and gels become more fluid when the filaments are severed.

To sever a microtubule, thirteen longitudinal bonds must be broken, one for each protofilament. The protein katanin, named after the Japanese word for "sword," accomplishes this demanding task. Katanin is made up of two subunits, a smaller subunit that hydrolyzes ATP and performs the actual severing, and a larger one that directs katanin to the centrosome. Katanin releases microtubules from their attachment to a microtubule organizing center, and it is thought to have a particularly crucial role in the rapid microtubule depolymerization observed at the poles of a mitotic spindle during mitosis. It is also found in proliferating cells in interphase and in postmitotic cells such as neurons, where it may also be involved in microtubule release and depolymerization.

In contrast to microtubule severing by katanin, which requires ATP, the severing of actin filaments does not require an extra energy input. Most actin-severing proteins are members of the gelsolin superfamily, whose severing activity is activated by high levels of cytosolic Ca^{2+}. Gelsolin has subdomains that bind to two different sites on the actin subunit, one exposed on the surface of the filament and one that is normally hidden in the longitudinal bond to the next subunit in the protofilament. According to one model for gelsolin severing, gelsolin binds on the side of an actin filament and waits until a thermal fluctuation happens to create a small gap between neighboring subunits in the protofilament; gelsolin then insinuates its subdomain into the gap, breaking the filament. Once gelsolin has severed an actin filament, it remains bound to the plus end and acts as an effective capping protein. However, like several other

actin filament capping proteins, it can be removed from the filament end by a local rise in PIP2 concentration.

The process of platelet activation shows how a cell can regulate its actin accessory proteins that mediate severing, capping, and cross-linking to generate rapid and dramatic morphological changes. Platelets are tiny cells without a nucleus that circulate in the blood and help to form clots at sites of injury. The resting platelet is discoid in shape, and it contains short actin filaments capped by CapZ, surrounded by a large pool of actin monomer bound to profilin. When the platelet is activated by physical contact with the edge of a damaged blood vessel or by a chemical clotting signal such as thrombin, a rapid, intracellular, signal transduction cascade results in a massive influx of Ca^{2+} into the platelet cytosol. The Ca^{2+} activates gelsolin, which cleaves the capped filaments into tiny fragments, each now capped by gelsolin. With slower kinetics, the same signaling pathway causes a rise in PIP2 levels, which inactivates both gelsolin and CapZ, removing them from the filament plus ends. The large numbers of free plus ends generated by severing and uncapping are then rapidly elongated by the monomeric actin pool, forming many long filaments. Some of these long actin filaments are cross-linked into a gel by filamin, while others are bundled by α-actinin and fimbrin. This causes the activated platelet to extend lamellipodia and filopodia and to spread itself across the clot, attaching to the clot by transmembrane adhesion proteins called integrins. Once the PIP2 signal subsides, the CapZ returns to the ends of the filaments, rendering them stable against depolymerization and locking the platelet into its spread form. Finally, myosin II uses ATP hydrolysis to slide the long actin filaments relative to one another, causing a contraction of the platelet that pulls the edges of the wound together.

Molecular Motors

Perhaps the most fascinating proteins that associate with the cytoskeleton are the molecular motors called motor proteins. These remarkable proteins bind to a polarized cytoskeletal filament and use the energy derived from repeated cycles of ATP hydrolysis to move steadily along it. Dozens of different motor proteins coexist in every eucaryotic cell. They differ in the type of filament they bind to (either actin or microtubules), the direction in which they move along the filament, and the "cargo" they carry. Many motor proteins carry membrane-enclosed organelles—such as mitochondria, Golgi stacks, or secretory vesicles—to their appropriate locations in the cell. Other motor proteins cause cytoskeletal filaments to slide against each other, generating the force that drives such phenomena as muscle contraction, ciliary beating, and cell division.

Cytoskeletal motor proteins that move unidirectionally along an oriented polymer track are reminiscent of some other proteins and protein complexes discussed elsewhere in this book, such as DNA and RNA polymerases, helicases, and ribosomes. All of these have the ability to use chemical energy to propel themselves along a linear track, with the direction of sliding dependent on the structural polarity of the track. All of them generate motion by coupling nucleoside triphosphate hydrolysis to a large-scale conformational change in a protein.

The cytoskeletal motor proteins associate with their filament tracks through a "head" region, or motor domain, that binds and hydrolyzes ATP. Coordinated with their cycle of nucleotide hydrolysis and conformational change, the proteins cycle between states in which they are bound strongly to their filament tracks and states in which they are unbound. Through a mechanochemical cycle of filament binding, conformational change, filament release, conformational relaxation, and filament rebinding, the motor protein and its associated cargo move one step at a time along the filament (typically a distance of a few nanometers). The identity of the track and the direction of movement along it are determined by the motor domain (head), while the identity of the cargo (and therefore the biological function of the individual motor protein) is determined by the tail of the motor protein.

In this section, we begin by describing the three groups of cytoskeletal motor proteins. We then describe how they work to transport membrane-enclosed organelles or to change the shape of structures built from cytoskeletal filaments. We end by describing their action in muscle contraction and in powering the whiplike motion of structures formed from microtubules.

Actin-based Motor Proteins Are Members of the Myosin Superfamily

The first motor protein identified was skeletal muscle myosin, which is responsible for generating the force for muscle contraction. This myosin, called myosin II (see below) is an elongated protein that is formed from two heavy chains and two copies of each of two light chains. Each of the heavy chains has a globular head domain at its N-terminus that contains the force-generating machinery, followed by a very long amino acid sequence that forms an extended coiled-coil that mediates heavy chain dimerization. The two light chains bind close to the N-terminal head domain, while the long coiled-coil tail bundles itself with the tails of other myosin molecules. These tail-tail interactions result in the formation of large bipolar "thick filaments" that have several hundred myosin heads, oriented in opposite directions at the two ends of the thick filament.

Each myosin head binds and hydrolyses ATP, using the energy of ATP hydrolysis to walk toward the plus end of an actin filament. The opposing orientation of the heads in the thick filament makes the filament efficient at sliding pairs of oppositely oriented actin filaments past each other. In skeletal muscle, in which carefully arranged actin filaments are aligned in "thin filament" arrays surrounding the myosin thick filaments, the ATP-driven sliding of actin filaments results in muscle contraction. Cardiac and smooth muscle contain myosins that are similarly arranged, although they are encoded by different genes.

When a muscle myosin is digested by chymotrypsin and papain, the head domain is released as an intact fragment (called S1). The S1 fragment alone can generate filament sliding in vitro, proving that the motor activity is contained completely within the head.

It was initially thought that myosin was present only in muscle, but in the 1970's, researchers found that a similar two-headed myosin protein was also present in nonmuscle cells, including protozoan cells. At about the same time, other researchers found a myosin in the freshwater amoeba Acanthamoeba castellanii that was unconventional in having a motor domain similar to the head of muscle myosin but a completely different tail. This molecule seemed to function as a monomer and was named myosin I (for one-headed); the conventional myosin was renamed myosin II (for two-headed).

Subsequently, many other myosin types were discovered. The heavy chains generally start with a recognizable myosin motor domain at the N-terminus, and then diverge widely with a variety of C-terminal tail domains. The new types of myosins include a number of one-headed and two-headed varieties that are approximately equally related to myosin I and myosin II, and the nomenclature now reflects their approximate order of discovery (myosin III through at least myosin XVIII). The myosin tails (and the tails of motor proteins generally) have apparently diversified during evolution to permit the proteins to dimerize with other subunits and to interact with different cargoes.

Some myosins (such as VIII and XI) have been found only in plants, and some have been found only in vertebrates (IX). Most, however, are found in all eucaryotes, suggesting that myosins arose early in eucaryotic evolution. The yeast Saccharomyces cerevisiae contains five myosins: two myosin Is, one myosin II, and two myosin Vs. One can speculate that these three types of myosins are necessary for a eucaryotic cell to survive and that other myosins perform more specialized functions in multicellular organisms. The nematode C. elegans, for example, has at least 15 myosin genes, representing at least seven structural classes; the human genome includes about 40 myosin genes.

All of the myosins except one move toward the plus end of an actin filament, although they do so at different speeds. The exception is myosin VI, which moves toward the minus end.

The exact functions for most of the myosins remain to be determined. Myosin II is always associated with contractile activity in muscle and nonmuscle cells. It is also generally required for cytokinesis, the pinching apart of a dividing cell into two daughters (discussed in Chapter 18), as well as for the forward translocation of the body of a cell during cell migration. The myosin I proteins contain a second actin-binding site or a membrane-binding site in their tails, and they are generally involved in intracellular organization and the protrusion of actin-rich structures at the cell surface. Myosin V is involved in vesicle and organelle transport. Myosin VII is found in the inner ear in vertebrates, and certain mutations in the gene coding for myosin VII cause deafness in mice and humans.

There Are Two Types of Microtubule Motor Proteins: Kinesins and Dyneins

Kinesin is a motor protein that moves along microtubules. It was first identified in the giant axon of the squid, where it carries membrane-enclosed organelles away from the neuronal cell body toward the axon terminal by walking toward the plus end of microtubules. Kinesin is similar structurally to myosin II in having two heavy chains and two light chains per active motor, two globular head motor domains, and an elongated coiled-coil responsible for heavy chain dimerization. Like myosin, kinesin is a member of a large protein superfamily, for which the motor domain is the only common element. The yeast Saccharomyces cerevisiae has six distinct kinesins. The nematode C. elegans has 16 kinesins, and humans have about 40.

There are at least ten families of kinesin-related proteins, or KRPs, in the kinesin superfamily. Most of them have the motor domain at the N-terminus of the heavy chain and walk toward the plus end of the microtubule. A particularly interesting family has the motor domain at the C-terminus and walks in the opposite direction, toward the minus end of the microtubule. Some KRP heavy chains lack a coiled-coil sequence and seem to function as monomers, analogous to myosin I. Some others are homodimers, and yet others are heterodimers. At least one

KRP (BimC) can self-associate through the tail domain, forming a bipolar motor that slides oppositely oriented microtubules past one another, much as a myosin II thick filament does for actin filaments. Most kinesins carry a binding site in the tail for either a membrane-enclosed organelle or another microtubule. Many of the kinesin superfamily members have specific roles in mitotic and meiotic spindle formation and chromosome separation during cell division.

The dyneins are a family of minus-end-directed microtubule motors, but they are unrelated to the kinesin superfamily. They are composed of two or three heavy chains (that include the motor domain) and a large and variable number of associated light chains. The dynein family has two major branches (Figure 16-56). The most ancient branch contains the cytoplasmic dyneins, which are typically heavy-chain homodimers, with two large motor domains as heads. Cytoplasmic dyneins are probably found in all eucaryotic cells, and they are important for vesicle trafficking, as well as for localization of the Golgi apparatus near the center of the cell. Axonemal dyneins, the other large branch, include heterodimers and heterotrimers, with two or three motor-domain heads, respectively. They are highly specialized for the rapid and efficient sliding movements of microtubules that drive the beating of cilia and flagella (discussed later). A third, minor, branch shares greater sequence similarity with cytoplasmic than with axonemal dyneins but seems to be involved in the beating of cilia.

Dyneins are the largest of the known molecular motors, and they are also among the fastest: axonemal dyneins can move microtubules in a test tube at the remarkable rate of 14 μm/sec. In comparison, the fastest kinesins can move their microtubules at about 2–3 μm/sec.

Motor Proteins Generate Force by Coupling ATP Hydrolysis to Conformational Changes

Although the cytoskeletal motor proteins and GTP-binding proteins both use structural changes in their nucleoside-triphosphate-binding sites to produce cyclic interactions with a partner protein, the motor proteins have a further requirement: each cycle of binding and release must propel them forward in a single direction along a filament to a new binding site on the filament. For such unidirectional motion, a motor protein must use the energy derived from ATP binding and hydrolysis to force a large movement in part of the protein molecule. For myosin, each step of the movement along actin is generated by the swinging of an 8.5-nm-long α helix, or lever arm, which is structurally stabilized by the binding of light chains. At the base of this lever arm next to the head, there is a piston-like helix that connects movements at the ATP-binding cleft in the head to small rotations of the so-called converter domain. A small change at this point can swing the helix like a long lever, causing the far end of the helix to move by about 5.0 nm. These changes in the conformation of the myosin are coupled to changes in its binding affinity for actin, allowing the myosin head to release its grip on the actin filament at one point and snatch hold of it again at another. The

full mechanochemical cycle of nucleotide binding, nucleotide hydrolysis, and phosphate release (which causes the "power stroke") produces a single step of movement (Figure 16-58). In the myosin VI subfamily of myosins, which move backward (toward the minus end of the actin filament), the converter domain probably lies in a different orientation, so that the same piston-like movement of the small helix causes the lever arm to rotate in the opposite direction.

In kinesin, instead of the rocking of a lever arm, the small movements of switch loops at the nucleotide-binding site regulate the docking and undocking of the motor head domain to a long linker region that connects this motor head at one end to the coiled-coil dimerization domain at the other end. When the front (leading) kinesin head is bound to a microtubule before the power stroke, its linker region is relatively unstructured. On the binding of ATP to this bound head, its linker region docks along the side of the head, which throws the second head forward to a position where it will be able to bind a new attachment site on the protofilament, 8 nm closer to the microtubule plus end than the binding site for the first head. The nucleotide hydrolysis cycles in the two heads are closely coordinated, so that this cycle of linker docking and undocking can allow the two-headed motor to move in a hand-over-hand (or head-over-head) stepwise manner.

The coiled-coil domain seems both to coordinate the mechanochemical cycles of the two heads (motor domains) of the kinesin dimer and to determine its directionality of movement. Recall that whereas most members of the kinesin superfamily, with their motor domains at the N-terminus, move toward the plus end of the microtubule, a few superfamily members have their motor domains at the C-terminus and move toward the minus end. Since the motor domains of these two types of kinesins are essentially identical, how can they move in opposite directions? The answer seems to lie in the way in which the heads are connected. In high-resolution images of forward-walking and backward-walking members of the kinesin superfamily bound to microtubules, the heads that are attached to the microtubule are essentially indistinguishable, but the second, unattached heads are oriented very differently. This difference in tilt apparently biases the next binding site for the second head, and thereby determines the directionality of motor movement.

Although both myosin and kinesin undergo analogous mechanochemical cycles, the exact nature of the coupling between the mechanical and chemical cycles is different in the two cases (see Figure 16-60). For example, myosin without any nucleotide is tightly bound to its actin track, in a so-called "rigor" state, and it is released from this track by the association of ATP. In contrast, kinesin forms a rigor-like tight association with a microtubule when ATP is bound to the kinesin, and it is hydrolysis of ATP that promotes release of the motor from its track.

Thus, cytoskeletal motor proteins work in a manner highly analogous to GTP-binding proteins, except that in motor proteins the small protein conformational changes (a few tenths of a nanometer) associated with nucleotide hydrolysis are

amplified by special protein domains—the lever arm in the case of myosin and the linker in the case of kinesin—to generate large-scale (several nanometers) conformational changes that move the motor proteins stepwise along their filament tracks. The analogy between the GTPases and the cytoskeletal motor proteins has recently been extended by the observation that one of the GTP-binding proteins—the bacterial elongation factor G—translates the chemical energy of GTP hydrolysis into directional movement of the mRNA molecule on the ribosome.

Motor Proteins Mediate the Intracellular Transport of Membrane-enclosed Organelles

A major function of cytoskeletal motors in interphase cells is the transport and positioning of membrane-enclosed organelles. Kinesin was originally identified as the protein responsible for fast axonal transport, the rapid movement of mitochondria, secretory vesicle precursors, and various synapse components down the microtubule highways of the axon to the distant nerve terminals. Although organelles in most cells need not cover such long distances, their polarized transport is equally necessary. A typical microtubule array in an interphase cell is oriented with the minus ends near the center of the cell at the centrosome, and the plus ends extending to the cell periphery. Thus, centripetal movements of organelles toward the cell center require the action of minus-end-directed motor proteins such as cytoplasmic dynein, whereas centrifugal movements toward the periphery require plus-end-directed motors such as kinesins.

The role of microtubules and microtubule motors in the behavior of intracellular membranes is best exemplified by the part they play in organizing the endoplasmic reticulum (ER) and the Golgi apparatus. The network of ER membrane tubules aligns with microtubules and extends almost to the edge of the cell, whereas the Golgi apparatus is located near the centrosome. When cells are treated with a drug that depolymerizes microtubules, such as colchicine or nocodazole, the ER collapses to the center of the cell, while the Golgi apparatus fragments and disperses throughout the cytoplasm. In vitro, kinesins can tether ER-derived membranes to preformed microtubule tracks, and walk toward the microtubule plus ends, dragging the ER membranes out into tubular protrusions and forming a membranous web very much like the ER in cells. Likewise, the outward movement of ER tubules toward the cell periphery is associated with microtubule growth in living cells. Conversely, dyneins are required for positioning the Golgi apparatus near the cell center, moving Golgi vesicles along microtubule tracks toward minus ends at the centrosome.

The different tails and their associated light chains on specific motor proteins allow the motors to attach to their appropriate organelle cargo. For example, there is evidence for membrane-associated motor receptors, sorted to specific membrane-enclosed compartments, that interact directly or indirectly with the

tails of the appropriate kinesin family members. One of these receptors seems to be the amyloid precursor protein, APP, which binds directly to a light chain on the tail of kinesin-I and is proposed to be a transmembrane motor protein receptor molecule in nerve-cell axons. It is the abnormal processing of this protein that gives rise to Alzheimer's disease.

For dynein, attachment to membranes is known to be mediated by a large macromolecular assembly. Cytoplasmic dynein is itself a huge protein complex, and it requires association with a second large protein complex called dynactin to translocate organelles effectively. The dynactin complex includes a short actinlike filament that is made of the actin-related protein Arp1 (distinct from Arp2 and Arp3, the components of the ARP complex involved in the nucleation of conventional actin filaments). Membranes of the Golgi apparatus are coated with the proteins ankyrin and spectrin, which have been proposed to associate with the Arp1 filament in the dynactin complex to form a planar cytoskeletal array reminiscent of the erythrocyte membrane cytoskeleton. The spectrin array probably gives structural stability to the Golgi membrane, and—via the Arp1 filament—it may mediate the regulatable attachment of dynein to the organelle.

Motor proteins also have a significant role in organelle transport along actin filaments. The first myosin shown to mediate organelle motility was myosin V, a two-headed myosin with a large step size. In mice, mutations in the myosin V gene result in a "dilute" phenotype, in which fur color looks faded. In mice (and humans), membrane-enclosed pigment granules, called melanosomes, are synthesized in cells called melanocytes beneath the skin surface. These melanosomes move out to the ends of dendritic processes in the melanocytes, from where they are delivered to the overlying keratinocytes that form the skin and fur. Myosin V is associated with the surface of melanosomes, and it is able to mediate their actin-based movement in a test tube (Figure 16-64). In dilute mutant mice, the melanosomes are not delivered to the keratinocytes efficiently, and pigmentation is defective. Other myosins, including myosin I, are associated with endosomes and a variety of other organelles.

Muscle Contraction Depends on the Sliding of Myosin II and Actin Filaments

Muscle contraction is the most familiar and the best understood form of movement in animals. In vertebrates, running, walking, swimming, and flying all depend on the rapid contraction of skeletal muscle on its scaffolding of bone, while involuntary movements such as heart pumping and gut peristalsis depend on the contraction of cardiac muscle and smooth muscle, respectively. All these forms of muscle contraction depend on the ATP-driven sliding of highly organized arrays of actin filaments against arrays of myosin II filaments.

Muscle was a relatively late evolutionary development, and muscle cells are highly specialized for rapid and efficient contraction. The long thin muscle fibers of skeletal muscle are actually huge single cells that form during development by

the fusion of many separate cells. The many nuclei of the contributing cells are retained in this large cell and lie just beneath the plasma membrane, but the bulk of the cytoplasm inside is made up of myofibrils, which is the name given to the basic contractile elements of the muscle cell. A myofibril is a cylindrical structure 1–2 μm in diameter that is often as long as the muscle cell itself. It consists of a long repeated chain of tiny contractile units—called sarcomeres, each about 2.2 μm long, which give the vertebrate myofibril its striated appearance.

Each sarcomere is formed from a miniature, precisely ordered array of parallel and partly overlapping thin and thick filaments. The thin filaments are composed of actin and associated proteins, and they are attached at their plus ends to a Z disc at each end of the sarcomere. The capped minus ends of the actin filaments extend in toward the middle of the sarcomere, where they overlap with thick filaments, the bipolar assemblies formed from specific muscle isoforms of myosin II. When this region of overlap is examined in cross section by electron microscopy, the myosin filaments are seen to be arranged in a regular hexagonal lattice, with the actin filaments evenly spaced between them. Cardiac muscle and smooth muscle also contain sarcomeres, although the organization is not as regular as that in skeletal muscle.

Sarcomere shortening is caused by the myosin filaments sliding past the actin thin filaments, with no change in the length of either type of filament. Bipolar thick filaments walk toward the plus ends of two sets of thin filaments of opposite orientations, driven by dozens of independent myosin heads that are positioned to interact with each thin filament. There is no coordination among the movements of the myosin heads, so it is critical that they operate with a low processivity, remaining tightly bound to the actin filament for only a small fraction of each ATPase cycle so that they do not hold one another back. Each myosin thick filament has about 300 heads (294 in frog muscle), and each head cycles about five times per second in the course of a rapid contraction—sliding the myosin and actin filaments past one another at rates of up to 15 μm/sec and enabling the sarcomere to shorten by 10% of its length in less than 1/50th of a second. The rapid synchronized shortening of the thousands of sarcomeres lying end-to-end in each myofibril gives skeletal muscle the ability to contract rapidly enough for running and flying, and even for playing the piano.

Accessory proteins govern the remarkable uniformity in filament organization, length, and spacing in the sarcomere. As mentioned previously, the actin filament plus ends are anchored in the Z disc, which is built from CapZ and α-actinin; the Z disc caps the filaments (preventing depolymerization), while holding them together in a regularly spaced bundle. The precise length of each filament is determined by a template protein of enormous size, called nebulin, which consists almost entirely of a repeating 35-amino-acid actin-binding motif. Nebulin stretches from the Z disc to the minus end of each thin filament and acts as a "molecular ruler" to dictate the length of the filament. The minus ends of the thin filaments are capped and stabilized by tropomodulin. Thus, the actin filaments in

sarcomeres are remarkably stable, unlike the dynamic actin filaments characteristic of most other cell types.

The thick filaments are positioned midway between the Z discs by opposing pairs of an even longer template protein, called titin. Titin acts as a molecular spring, with a long series of immunoglobulin-like domains that can unfold one by one as stress is applied to the protein. A springlike unfolding and refolding of these domains keeps the thick filaments poised in the middle of the sarcomere and allows the muscle fiber to recover after being overstretched. In C. elegans, whose sarcomeres are longer than those in vertebrates, titin is also longer, suggesting that it too serves as a moleculer ruler, determining in this case the overall length of each sarcomere.

Cilia and Flagella Are Motile Structures Built from Microtubules and Dyneins

Just as myofibrils are highly specialized and efficient motility machines built from actin and myosin filaments, cilia and flagella are highly specialized and efficient motility structures built from microtubules and dynein. Both cilia and flagella are hair-like cellular appendages that have a bundle of microtubules at their core. Flagella are found on sperm and many protozoa. By their undulating motion, they enable the cells to which they are attached to swim through liquid media. Cilia tend to be shorter than flagella and are organized in a similar fashion, but they beat with a whip-like motion that resembles the breast stroke in swimming. The cycles of adjacent cilia are almost but not quite in synchrony, creating the wave-like patterns that can be seen in fields of beating cilia under the microscope. Ciliary beating can either propel single cells through a fluid (as in the swimming of the protozoan Paramecium) or can move fluid over the surface of a group of cells in a tissue. In the human body, huge numbers of cilia (109/cm2 or more) line our respiratory tract, sweeping layers of mucus, trapped particles of dust, and bacteria up to the mouth where they are swallowed and ultimately eliminated. Likewise, cilia along the oviduct help to sweep eggs toward the uterus.

The movement of a cilium or a flagellum is produced by the bending of its core, which is called the axoneme. The axoneme is composed of microtubules and their associated proteins, arranged in a distinctive and regular pattern. Nine special doublet microtubules (comprising one complete and one partial microtubule fused together so that they share a common tubule wall) are arranged in a ring around a pair of single microtubules. This characteristic arrangement is found in almost all forms of eucaryotic flagella and cilia from protozoans to humans. The microtubules extend continuously for the length of the axoneme, which can be 10–200 μm. At regular positions along the length of the microtubules, accessory proteins cross-link the mircotubules together. Molecules of ciliary dynein form bridges between the neighboring doublet microtubules around the circumference of the axoneme. When the motor domain of this dynein is activated, the dynein molecules attached to one microtubule doublet attempt to walk along the adjacent microtubule doublet, tending to force

the adjacent doublets to slide relative to one another, much as actin thin filaments slide during muscle contraction. However, the presence of other links between the microtubule doublets prevents this sliding, and the dynein force is instead converted into a bending motion.

Bacteria also swim using cell surface structures called flagella, but these do not contain microtubules or dynein and do not wave or beat. Instead, bacterial flagella are long, rigid helical filaments, made up of repeating subunits of the protein flagellin. The flagella rotate like propellers, driven by a special rotary motor embedded in the bacterial cell wall. The use of the same name to denote these two very different types of swimming apparatus is an unfortunate historical accident.

Structures called basal bodies firmly root eucaryotic cilia and flagella at the cell surface. The basal bodies have the same form as the centrioles that are found embedded at the center of animal centrosomes, with nine groups of fused triplet microtubules arranged in a cartwheel. Indeed, in some organisms, basal bodies and centrioles are functionally interconvertible: during each mitosis in the unicellular alga Chlamydomonas, for example, the flagella are resorbed, and the basal bodies move into the cell interior and become part of the spindle poles. New centrioles and basal bodies arise by a curious replication process, in which a smaller daughter is formed perpendicular to the original structure by a still mysterious mechanism.

In humans, hereditary defects in ciliary dynein cause Kartagener's syndrome. The syndrome is characterized by male sterility due to immotile sperm, a high susceptibility to lung infections owing to the paralyzed cilia in the respiratory tract that fail to clear debris and bacteria, and defects in determination of the left-right axis of the body during early embryonic.

Summary

Motor proteins use the energy of ATP hydrolysis to move along microtubules or actin filaments. They mediate the sliding of filaments relative to one another and the transport of membrane-enclosed organelles along filament tracks. All known motor proteins that move on actin filaments are members of the myosin superfamily. The motor proteins that move on microtubules are members of either the kinesin superfamily or the dynein family. The myosin and kinesin superfamilies are diverse, with about 40 genes encoding each type of protein in humans. The only structural element shared among all members of each superfamily is the motor "head" domain. These heads can be attached to a wide variety of "tails," which attach to different types of cargo and enable the various family members to perform different functions in the cell. Although myosin and kinesin walk along different tracks and use different mechanisms to produce force and movement by ATP hydrolysis, they share a common structural core, suggesting that they are derived from a common ancestor.