Actin Binding Proteins: 
Regulation of Cytoskeletal Microfilaments

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of these are likely to help us understand and distinguish between the structural models of actin microfilaments. In particular, 1) the structure of actin was resolved from crystals in the absence of cocrystallized actin binding proteins (ABPs), 2) the prokaryotic ancestral gene of actin was crystallized and its function as a bacterial cytoskeleton was revealed, and 3) the structure of the Arp2/3 complex was described for the first time. In this review we selected several ABPs (ADF/cofilin, profilin, gelsolin, thymosin β4, DNase I, CapZ, tropomodulin, and Arp2/3) that regulate actin-driven assembly, i.e., movement that is independent of motor proteins. They were chosen because 1) they represent a family of related proteins, 2) they are widely distributed in nature, 3) an atomic structure (or at least a plausible model) is available for each of them, and 4) each is expressed in significant quantities in cells. These ABPs perform the following cellular functions: 1) they maintain the population of unassembled but assembly-ready actin monomers (profilin), 2) they regulate the state of polymerization of filaments (ADF/cofilin, profilin), 3) they bind to and block the growing ends of actin filaments (gelsolin), 4) they nucleate actin assembly (gelsolin, Arp2/3, cofilin), 5) they sever actin filaments (gelsolin, ADF/cofilin), 6) they bind to the sides of actin filaments (gelsolin, Arp2/3), and 7) they cross-link actin filaments (Arp2/3). Some of these ABPs are essential, whereas others may form regulatory ternary complexes. Some play crucial roles in human disorders, and for all of them, there are good reasons why investigations into their structures and functions should continue.

I. INTRODUCTION

A. The Cytoskeleton

The internal cytoskeleton of eukaryotic cells is composed of actin microfilaments, microtubules, and intermediate filaments. The cytoskeleton is dynamic and strong, ever ready to adapt to demands on the cell. An important property of actin is its ability to produce movement in the absence of motor proteins. At the cell membrane microfilament assembly protrudes the membrane forward producing the ruffling membranes in actively moving cells. Microfilaments can also play a passive structural role by providing the internal stiffening rods in microvilli, maintaining cell shape, and anchoring cytoskeletal proteins. The major focus of this review is to examine how actin binding proteins (ABPs) control these processes. Finally, we raise some interesting and challenging questions for future research.

B. What Do We Exclude From This Review?

Actin microfilaments provide the “rails” along which myosin “motors” perform work in a variety of cellular functions. A major review of myosin motors (263) has been published, and they are excluded from this review. Microfilaments cooperate with microtubules via microtubule-associated proteins (MAPs) during the transport of vesicles and organelles, and this interesting aspect of microfilament function was recently reviewed (34). Actin filaments also interact with intermediate filaments, a function that may play an important role in enabling extracellular stimuli to be transmitted to key targets like ribosomes and chromosomes deep within the cell. This field is an emerging one. It will be covered by Quinlan et al. (235) and is beyond the scope of this review.

II. STRUCTURE AND DYNAMICS
OF MONOMERIC ACTIN

Actin is only found in eukaryotes. It comprises a highly conserved family of proteins that fall into three broad classes: α-, β-, and γ-isoforms. It is mainly located in the cytoplasm, but it is also present in the nucleus where it may or may not have motor-associated functions. The highest concentrations (~20% of the total protein) of actin are in striated muscles; however, significant quantities of actin are present in nonmuscle cells where it plays a variety of roles including myosin-independent changes of cells shape, motor-based organelle transport, regulation of ion transport, and receptor-mediated responses of the cell to external signals.

A. The First Actin Crystals

Few people in the field of actin remember that Christine Oriol-Audit was the first to report the crystallization of actin in the absence of an actin-binding protein (212). These crystals were achieved in the presence of polyethylene glycol but were too small to be useful for X-ray diffraction at that time. If today’s high-powered X-ray beam facilities were available then, they may well have yielded the atomic structure of actin in the absence of an ABP. Perhaps it was a cruel twist of fate that she sadly died early in 2001 without knowing that the first structure of uncomplexed actin was soon to be published (216). The principal obstacle to crystallizing actin was its propensity to spontaneously self-assemble under solvent conditions conventionally used to grow protein crystals.

B. Actin Microcrystals and Tubes

In their pioneering book on the biophysics of protein polymerization, Fumio Oosawa and Sho Asakura (210)
predicted that actin, like tubulin, would form helically tubular crystalline assemblies. We subsequently showed that these structures could be formed in the presence of the trivalent lanthanide ions, particularly Gd\(^{3+}\) (3, 87). These lanthanide-induced actin tubes (composed of a single layer of monomers) and microcrystals (bilayered sheets) provided the first views of the shape of the actin monomer (3, 73). The computed average image of the monomer was “pear” shaped, having a “large” and a “small” domain. Electron diffraction studies revealed structure out to at least 14.5-Å resolution, but this was simply not good enough to provide reliable molecular details (74). In solution, actin filaments can form supramolecular assemblies called paracrystals (99) as well as forming liquid crystalline arrays (67).

C. Actin Monomer Structure

Although the cocrystallization of actin with bovine pancreatic DNase I was first reported in the late 1970s (176), 13 years passed before the first atomic resolution (2.4 Å) structure of actin was reported (137). The actin monomers used in these crystals were lightly digested with trypsin to remove the COOH-terminal three residues before crystallization. Because the crystal structure of DNase I had already been determined at 2.5-Å resolution (272), it was a relatively simple task to subtract its structure from the actin-DNase I complex to obtain a clear view of the actin monomer. The resulting structure (illustrated in ribbon form in Fig. 1A) produced a number of surprises. For example, the large and small domains described at low resolution contained nearly equal numbers of residues and were nearly equal in size. Both the NH\(_2\) and COOH termini were located in the same subdomain. DNase I makes crystal contacts across the “top” of the nucleotide cleft, thus explaining how it inhibits nucleotide exchange. However, since these were cocrystals, did this structure represent a monomer conformation when it was not complexed to DNase I?

Monomeric (G-)actin has dimensions of \(\sim 67 \times 40 \times 37\) Å and a molecular mass of nearly 43,000 Da. The overall features of the structure include four quasi-subdomains (here they are numbered 1–4 but an alternative nomenclature of IA, IB, IIA, and IIB has an historical basis, Ref. 266), each having a repeating motif comprising a multi-stranded \(\beta\)-sheet, a \(\beta\)-meander, and a right-handed \(\beta\alpha\beta\)-unit. About 40% of the structure is \(\alpha\)-helical. In Figure 1A, the monomer is oriented so that the top has about the same orientation it would have in the filament with its “pointed” end directed at the top of the page and the “barbed” end toward the bottom.

A tightly bound nucleotide lies in a deep cleft in the center of G-actin. This site is usually occupied by ATP or ADP-P\(_i\), rather than ADP, which binds with \(\sim 10\)-fold lower affinity to Mg-G-actin and \(\sim 200\)-fold weaker binding with Ca-G-actin (147). ATP binds as a complex with either Mg\(^{2+}\) [dissociation constant \((K_d)\) 1.2 nM] or Ca\(^{2+}\) \((K_d)\) 0.12 nM] (79, 270). Mg\(^{2+}\), the dominant cation in vivo, determines how tightly or weakly the nucleotide binds. Six or seven relatively low-affinity divalent cation-binding sites have been identified that appear to be important for actin paracrystal (94, 271), and actin microcrystal formation (73), but their physiological importance is not well understood. A notable feature of the structure is a two-stranded “hinge” at residues 140 and 338 joining the large and small domains. Tirion et al. (283) describe a “propeller” motion between the two domains that produces an opening and closing of the nucleotide cleft. This putative slewing mo-
tion is permitted because of this hinge and is viewed as a rigid body movement between the subdomains.

There are several other atomic structures of actin. In 1993, McLaughlin’s group at the Medical Research Council (MRC) in Cambridge (186) reported the structure of actin complexed with gelsolin segment 1. This segment binds to the bottom of the monomer, making contacts with subdomains 1 and 3 (see discussion below). Unlike the DNase I-actin structure, the COOH-terminal three residues were not cleaved before crystallization. However, the DNase I binding loop was not well resolved, presumably because it was not sufficiently stabilized in the absence of DNase I. In the same year Schutt et al. (259) reported the structure of spleen actin (a β-isofrom) complexed to profilin at a resolution of 2.55 Å. This group had been refining their structure for many years and demonstrated that, although there was broad similarity to the structure of McLaughlin et al. (186), there were significant differences that they attributed to the sequence differences between the actin isoforms.

The Schutt/Lindberg consortium that captured actin in a different conformation subsequently reported a fourth actin-profilin structure. They again used bovine β-actin cocryrstallized with bovine pancreatic profilin (60). They named the two structures the open and closed states and used them to argue that the transition between the two states represented a structural change that could convert their “ribbon” polymer into conventional F-actin.

In 2001, the inevitable happened. Dominguez et al. at the Boston Biomedical Research Institute succeeded in producing small, high-quality crystals of actin that were complexed to ABPs (Fig. 1B). This finding was reported at the Boston meeting of the Biophysical Society colleagues (216) and was subsequently published in Science (217). They achieved the result that had eluded so many others by modifying the COOH-terminal cysteinyl (Cys-374) with a rhodamine label that suppressed the tendency of actin to polymerize.

Dominguez and colleagues (217) were also the first to crystallize actin with ADP present in the nucleotide-binding cleft. This was important because we know that the critical concentration of ADP-G-actin is about an order of magnitude higher than for ATP or ADP-Pi-G-actin (241). Kabsch et al. (137) had also produced crystals of ADP-G-actin and showed that the ADP structure was not remarkably different from the ATP form. However, the Kabsch crystals were formed in the presence of ATP, and the nucleotide was cleaved to ADP subsequent to crystallization.

The structure of Dominguez et al. significantly differs from the structure of Kabsch et al., particularly in subdomain 2 where DNase I binds. However, it could be argued that this Boston/Heidelberg difference is because ADP was present before the crystals were formed, i.e., before the crystal contact points were established.

A significant structural difference actin crystallized alone and complexed with an ABP protein is relatively large coil-to-α-helix conversion and a 10° rotation at the top of subdomain 2. Although it is not clear if these differences represent native cellular G-actin, Dominguez et al. are currently completing the analysis of an uncomplexed ATP-G-actin structure that should satisfy their critics.

The substantial conformational change reported by Otterbein et al. (217) is well supported by other laboratories including our own. Dedova et al. (81) recently demonstrated, using fluorescence spectroscopy, that significant (1–4 Å) changes occur in subdomains 1 and 2 when coflin and DNase I bind either separately or as a ternary complex. Several other authors have suggested that actin can undergo allosteric conformational changes, and this is discussed in more detail in section IV. The major challenge for structural biologists now lies in relating the structural changes to functional changes.

D. MreB, an Ancestral Actin

Actin is an essential and ubiquitous cytoskeletal component of all eukaryotic cells and is absent from prokaryotic cells. However, although it has long been suspected that the evolutionary origins of actin lie in their prokaryotic ancestors, until 2001 no one had reported a credible candidate. The first suggestion that MreB may be an ancestral actin gene was published 10 years ago (30), and recently (136), it was localized in Bacillus to form distinct filaments located close to the cell surface. The atomic structure of these filaments was more recently reported by Fusinta van den Ent and colleagues (289) at the MRC, Cambridge, who provided convincing evidence that MreB is indeed an ancestral actin gene. This actin look-alike is present in all nonspherical bacteria.

Amino acid sequence homology to actin is limited to 15% (289), and although its overall size and shape strongly resemble actin, there are differences. The sequence corresponding to the actin DNase I-binding loop in subdomain 2 is larger, and the COOH terminus, located in subdomain 1, is 20 residues longer. A 2.1-Å resolution crystal structure reveals that, like actin, MreB has two major domains separated by a nucleotide-binding cleft. Also, like actin, each domain has two subdomains with essentially the same topology. The structure of MreB is shown in Figure 2.

MreB can self-assemble into 51-Å-wide filaments that are about half the diameter of actin microfilaments, but unlike F-actin, they are linear (nonhelical) polymers resembling the linear polymers seen in actin crystalline sheets first reported by ourselves (87) and later by others (3). The axial repeat for MreB microfilaments is 51.1 Å, somewhat less than the axial repeat of F-actin (55 Å) (Fig.
3). These polymers form spirals beneath the bacterial cell wall, and although their precise function remains elusive, it is likely that MreB filaments behave like microfilament analogs and control the shape of bacteria. However, we know nothing of how assembly of this structure is regulated, or whether it binds to motor proteins. Figure 3 illustrates the striking similarities between the crystal structure of protofilaments of actin (right) and MreB (left). Nearly identical molecular orientations and contacts are seen between monomers for the two proteins. Conversion of a pair of actin protofilaments into F-actin is achieved by simply twisting a pair of actin protofilaments to conform to the actin filament symmetry (see below).

E. Models of F-actin

The field has made substantial progress in understanding the structure of F-actin. Nearly 40 years ago Jean Hanson and Jack Lowy were the first to describe the helical nature of actin filaments (116). In this model, F-actin can be viewed either as a single-start, left-handed tightly wound helix of monomers (the so-called genetic helix) or as a two-start, right-handed long-pitch helix. The conventional view of F-actin is the two-start helix because it is probable that the monomer-monomer affinity is stronger along the two long-pitch strands than between those strands (128).

No atomic structure has yet been determined for F-actin, although highly plausible models have been proposed based on the model of Holmes et al. (128). Figure 4 illustrates five monomers in a filament. Viewed along the
genetic helix, monomers rotate by $-166^\circ$ and have an axial translation of 27.5 Å. There are 13 monomers in 6 turns with a pitch of 59 Å yielding a filament diameter of $\sim 90–100$ Å (128). Contacts between the monomers along this helix occur across the diameter of the filament. The two-start long-pitch helix is right-handed with a somewhat variable half pitch of 360–390 Å comprising 12–14 monomers per half turn. In this model each monomer is still surrounded by four others. The center-to-center distance between monomers along this helix is $\sim 55$ Å. These filaments have a distinct structural polarity that was first noticed when the filaments were “decorated” with myosin fragments, but it can also be seen in good images of F-actin.

Thus, despite the passage of nearly 40 years and the best efforts of laboratories in Germany, the United Kingdom, the United States, Japan, and elsewhere, the atomic structure of F-actin remains elusive. A major part of the problem is the inherent disorder in linear aggregations of filaments. Even if they could be aligned with the same polarity, it is difficult to pack F-actin complexed to phalloidin (a 7-residue peptide that dramatically stabilizes actin filaments) so there are precise contacts between filaments. Devices like the innovative trumpet-shaped...
quartz tubes used by David Popp et al. (233) achieved greatly improved filament alignment that yielded the first low-angle X-ray diffraction data. More recently, Yuichiro Maeda (who also worked in Heidelberg and has since returned to the RIKEN SPring8 Institute synchrotron facility in Japan) and colleagues achieved even better alignment of F-actin without the help of phalloidin using very high (13.5 Tesla) magnetic fields. They already have structural data out to 5-Å resolution (207), and we can expect them to report further progress in the near future.

In the meantime, we must content ourselves with the available models of F-actin based on data from low-resolution electron microscope images. The original model (128) was created by fitting the atomic structure of the actin monomer (137) to models based on the limited-resolution X-ray diffraction data from aligned filaments. This model is illustrated in Figure 4. Subsequently, Michael Lorenz et al. (167) refined the Holmes (128) model using data from actin mutations. In the same year Tirion et al. (283) described the propeller motion of the two major domains about the hinge and then refined the F-actin model to take into account domain motion. Thus it emerged that the actin monomer was not the rigid, static (double strand of pearls) structure so commonly depicted. Monomers in this model are positioned slightly tangential to the filament axis, with subdomain 1 (the major myosin S-1 binding site) being located at the highest radius.

F-actin displays an arrowhead-like appearance when decorated with myosin subfragment 1 (S-1) (131, 188). For this reason the opposite ends of the filament have been named the “barbed” and “pointed” ends. These ends correspond to exposed subdomains 1 and 3 and subdomains 2 and 4, respectively.

Quite a different model was proposed by Schutt, Lindberg, and colleagues (259) based on crystallographic principles and known examples from other structures in biology. In profilin-actin crystals, a “ribbon” structure can be seen in which monomer subdomains 1 and 2 lie close to the ribbon axis. Alternating monomers displayed front and back views. They proposed that monomers were reoriented to produce F-actin. In essence, this group turned the model of Holmes et al. (128) inside out by rotating the actin monomer about 180° in the plane of the filament axis. The effect of this was to locate subdomains 1 and 2 closer to the filament axis, leaving subdomains 3 and 4 at higher radii. These authors further suggested that the subdomains were able to undergo significant movements relative to each other and that this could feasibly be built into a mechanism of contraction (258).

Low-resolution (25–30 Å) electron microscope studies have revealed that the actin filament can exist in multiple conformations depending on the type of bound cation and nucleotide, the isoform of actin (213, 214), and the presence of other proteins bound to actin (182, 218). Thus it is now more common to view F-actin as a dynamic, responsive structure than a passive structural element.

F. Assembly of Actin Filaments

The most authoritative dissertation on the biophysics of F-actin is Oosawa and Asakura’s slim volume, *Thermodynamics of the Polymerization of Protein* (210). Despite its age, this book continues to provide a comprehensive theoretical background for understanding the assembly of actin into filaments and higher order assemblies. The conditions under which actin monomers self-assemble in vitro are well documented (211), although it is fair to say they are less well understood for in vivo assembly.

Polymerization is essentially a condensation reaction. The main features of this process are 1) a slow initial association to a dimer that is more likely to rapidly dissociate to monomers than to assemble; 2) the formation of a stable trimer that represents the nucleus of polymerization, a state where actin assembly is more likely than disassembly; and 3) the elongation phase during which actin monomers are rapidly assembled. Solvent conditions that promote polymerization include high ionic strength (KCl concentrations >50 mM), neutral or slightly acidic pH, high Mg2+ (rather than high Ca2+), and elevated temperature (9, 111, 294), in other words, the conditions found in cells.

In addition to these three processes, actin filaments are in a continuous state of assembly/disassembly. As a consequence, in the steady state, a small but finite concentration of actin monomers will be present in any filament population (140, 211). This concentration of free monomers in equilibrium with a population of actin filaments is referred to as the critical concentration and is dependent on solvent conditions, particularly on the presence of certain ABPs and/or actin ligands such as phalloidin (80) or latrunculin A (193).

G. Elongation and Annealing of F-actin

Elongation involves association and dissociation of monomers from the filament. These processes can occur at either end of the filament, but association predominantly occurs at the barbed end and dissociation at the pointed end. The nucleotide binding site of monomeric actin is almost exclusively associated with ATP in vivo. Conversely, the majority of F-actin subunits contain bound ADP. While the G-actin bound ATP is readily exchangeable with solvent nucleotides, the ADP of F-actin is essentially nonexchangeable (121). Hydrolysis of bound ATP was originally thought to be tightly coupled to the polymerization process (210, 300); however, subsequent investigations (44, 46, 77, 221) revealed that a time lag
exists between the incorporation of ATP-G-actin onto the filament end and hydrolysis of the bound nucleotide. The kinetics of ATP hydrolysis and release of its product, P_i (43), suggests that the bound ATP of newly added actin subunits is hydrolyzed through two distinct sequential steps as illustrated in the following reaction scheme

\[
\text{ATP-G-actin} \leftrightarrow \text{ATP-F-actin} \rightarrow \text{Fast} \quad \text{ADP-P_i-F-actin} \leftrightarrow \text{ADP-F-actin} + P_i \quad \text{Slow}
\]

The rate of ATP hydrolysis was calculated to be 10 times higher than the rate of P_i release, suggesting that ADP-P_i-F-actin is the major intermediate in the nucleotide hydrolysis process. Conversion of ATP to ADP-P_i and then to ADP + P_i is not random but primarily occurs at the growing (barbed) ends of filaments (47).

Depolymerization of actin is not simply the opposite of polymerization, principally because actin cannot regenerate ATP from ADP and P_i (45). Instead, dissociated ADP-actin subunits rapidly exchange their bound ADP for ATP in solution (200), a process that is accelerated by profilin (see below). Polymerization of actin is not dependent on nucleotide hydrolysis. It is possible under special circumstances to form F-actin from G-actin containing either no bound nucleotide (141, 227) or a nonhydrolyzable analog of ATP (63). However, nucleotide hydrolysis is required for the normal function of F-actin.

The structural polarity of the actin filament and the irreversible nature of ATP hydrolysis during actin assembly have implications for the rate and direction of filament growth at opposite ends of F-actin. The critical concentration for the pointed end is 12- to 15-fold higher than for the barbed end under physiological conditions (301). This difference may result in the unidirectional growth of the actin filament due to a continual flux of actin subunits from the pointed to the barbed end of the filament. This reaction is called “treadmilling” (300).

Under in vivo conditions one might expect that all or most of the actin exists in an assembled (filamentous) form. Slow addition at the barbed end and even slower dissociation at the pointed end of the filaments produces a rate of treadmilling (300) of monomers (~2 \( \mu \text{m}/\text{h} \)) that is ~200-fold slower than observed in vivo. Monomers move progressively along the filament from the barbed end (the end associated with Z-disks of the sarcomere, or the cell membrane where ruffling occurs in motile cells) toward the free pointed end of the filament (located in the middle of the sarcomere or oriented away from the cell membrane). ABPs present in vivo regulate different aspects of the assembly/disassembly process. These include filament stabilizers (e.g., tropomyosin), capping proteins (e.g., CapZ, tropomodulin), ABPs that promote branching (e.g., Arp2/3), and ABPs that sequester G-actin and thus maintain a pool of monomers in solution (e.g., thymosin \( \beta_4 \), profilin).

Finally, filament lengths are affected by fragmentation and annealing. Fragmentation can arise from thermal motion in vitro, but in vivo much of this will be constrained by the other contents of the cytoplasm. Annealing occurs when an existing filament binds to the appropriate end of a second filament. This has been observed directly using fluorescent-phalloidin-labeled single filaments (Ishiwata, personal communication) and has been quantified (2.2 \( \mu \text{M}^{-1} \cdot \text{s}^{-1} \)) under similar conditions (145). Arp2/3 (discussed below) is believed to create branch points in actin microfilaments by “capturing” existing filaments.

III. ACTIN BINDING PROTEINS

Actin binds a substantial number of proteins collectively called ABPs. Some years ago, Pollard and Cooper (231) identified a large number of ABPs, and recently we counted 162 distinct and separate proteins without including their many synonyms or isoforms. No doubt more will be identified. Many of the known ABPs bind to the same loci on the surface of actin and therefore can be expected to compete. A few bind with positive cooperativity and tend to form ternary complexes (see below) but rather more bind with negative cooperativity. In myofibrils, at least eight sarcomeric proteins bind to the thin filaments. At least 12 ABPs are membrane-associated proteins, and another nine are membrane receptors or ion transporters. Thirteen ABPs cross-link actin filaments, whereas others enable filaments to interact with other elements of the cytoskeleton. Microfilaments probably do not interact directly with microtubules and/or intermediate filaments but do so via linker proteins.

Actin also binds ~30 other ligands including drugs and toxins. Thus the sheer number of ligands that have a significant affinity for actin strongly suggests there is probably a large number of binding sites that cover much of the exposed surface of the molecule. A comprehensive list is available from Actin (266), the Guidebook to the Cytoskeletal and Motor Proteins (151), and most recently from the two-volume Molecular Interactions of Actin (88). Attempts to classify these ABPs leave many “orphans” that do not fit into families, so any attempt to group them is bound to be somewhat arbitrary. Classifications according to the function of ABPs or their consensus sequences can also be problematic.

Several types of ABPs facilitate disassembly and assembly. Below is a review of the most common ABPs. Classification of these ABPs can be reduced to seven...
groups. 1) Monomer-binding proteins sequester G-actin and prevent its polymerization (e.g., thymosin β4, DNase I). (Note: C. E. Schutt and Uno Lindberg are completing an extensive review of Actin Monomer Binding Proteins to be published in the Protein Profile series of Oxford University Press.) 2) Filament-depolymerizing proteins induce the conversion of F- to G-actin (e.g., CapZ and coflin). 3) Filament end-binding proteins cap the ends of the actin filament preventing the exchange of monomers at the pointed end (e.g., tropomodulin) and at the barbed end (e.g., CapZ). 4) Filament severing proteins shorten the average length of filaments by binding to the side of F-actin and cutting it into two pieces (e.g., gelsolin). (Note: H. Hinssen is completing an extensive review on the Gelsolin Family to be published in the Protein Profile series of Oxford University Press.) 5) Cross-linking proteins contain at least two binding sites for F-actin, thus facilitating the formation of filament bundles, branching filaments, and three-dimensional networks (e.g., Arp2/3). 6) Stabilizing proteins bind to the sides of actin filaments and prevent depolymerization (e.g., tropomyosin). 7) Motor proteins that use F-actin as a track upon which to move (e.g., the myosin family of motors). Here we consider only groups 1–4.

ABPs are not limited to one class, for example, gelsolin is capable of severing and capping the barbed end of actin filaments, and the Arp2/3 complex can nucleate filament formation, elongate filaments, and establish branch points in actin networks (69). In this review we have selected eight ABPs that are biologically relevant and have atomic structures or good working models.

The rapid assembly of actin filaments is the principal driving force behind many forms of cell locomotion. Cells can migrate at rates up to ~0.5 μm/s (264). This means that filaments must have a net rate of elongation that is slightly less than 200 monomers/s. Principally this occurs at their barbed ends where MgATP-actin assembles about five times faster than MgADP-actin (149). The exchange of ATP for ADP in filaments is so slow (of the order of days, Ref. 201) it can be ignored. At steady state, filament length is constant, but there is a slow turnover (~2 μm/h) (264) of monomers due to a treadmilling process that involves the hydrolysis of ATP to ADP-P_7-actin and then a slower dissociation of P_1 leaving most of the monomers in the filament with bound MgADP. Thus, if pure actin filaments treadmilling very slowly and if actin assembly is the driver of cell motility, then the intrinsic rate of treadmilling must increase in vivo. This is the task of the ABPs.

A. ADF/Cofilin Family

Unlike so many of the ABPs, the actin depolymerizing factor (ADF)/cofilin family of proteins is expressed in virtually all eukaryotic cells. They are relatively small (15–19 kDa) proteins that exist in multiple isoforms. Their main functions include the rapid recycling of actin monomers associated with membrane ruffling and with cytokinesis. Different genes encode for ADF and coflin, but although it is common to regard them as synonymous, they are distinctly different.

1. Members of the family

ADF/cofilin was first discovered and purified in 1980 from embryonic chick brain extracts by Bamburg et al. (20). Since then, the family has grown to include a number of related proteins, including invertebrate depacin (named because it depolymerizes actin) (173); porcine ADF or destrin (destroys F-actin) (191); coflin (cosediments with filamentous actin) (1), Acanthamoeba acto-cin (236); Dictostelium coactosin (82), Drosophila twinstar or D-61 (91); unc-60A and unc-60B from Caenorhabditis (185); Xenopus Ac (or XAC1 and XAC2) (2); and finally Toxoplasma ADF (4). All of these share considerable (30–40%) amino acid sequence identity. Furthermore, two other major protein families are related to ADF through the presence of an ADF homology domain. One protein has a duplication of this domain and is consequently called twinfilin; the other contains a single ADF homology domain linked to another motif and encodes the drebin family of proteins (16).

Despite this somewhat confusing array of homologs, vertebrates have genes for only two forms, ADF and coflin. The names alone suggest that one depolymerizes F-actin (ADF) while coflin cosediments with F-actin, but actually both can elevate the levels of monomeric actin and both can bind to F-actin. Only one isoform of ADF is known in mammals (and birds), whereas two are known for coflin. ADF and coflin are clearly different but related proteins.

Cofilin is diffusely distributed in the cytoplasm of quiescent cells. However, in active cells, it translocates to cortical regions where the actin cytoskeleton is highly dynamic and drives the ruffling of membranes of motile cells (18, 309), the cleavage furrow of dividing cells (197), the advancing of neuronal growth cones (18, 198), and myobrilllogenesis (206). ADF and coflin are not necessarily expressed at the same level in all tissues. In adults, ADF is relatively highly expressed in nerves, intestine, kidney, and testes (18), whereas coflin levels are higher in hematopoietic tissues, bone osteoclasts, and fibroblasts (309). In baby hamster kidney cells, ADF constitutes 0.4% of the soluble protein, while coflin accounts for 1.3% (148). The total ADF and coflin amounts to 20 μM in cells where the total actin concentration is more than three times higher (67 μM) (148) (see Table 1).
<table>
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<tr>
<th>Actin Binding Protein</th>
<th>Actin</th>
<th>ADF/Cofilin</th>
<th>Profilin</th>
<th>Gelsolin</th>
<th>Thymosin β4</th>
<th>DNase I</th>
<th>CapZ</th>
<th>Arp2/3 (to Filaments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relevant figures</td>
<td>Figs. 1, 4, 6 (row 1)</td>
<td>Figs. 5B, 6 (row 1), 7B, 8, 10</td>
<td>Figs. 6 (row 2), 9, 10</td>
<td>Figs. 6 (row 3), 7C, 11, 12A, 12B, 13</td>
<td>Figs. 6 (row 2), 14</td>
<td>Figs. 6 (row 1), 15</td>
<td>Figs. 6 (row 4), 16</td>
<td>Fig. 17</td>
</tr>
<tr>
<td>Amino acids; molecular mass</td>
<td>375 amino acids; 43 kDa (288)</td>
<td>165–168 amino acids; 18-19 kDa (261)</td>
<td>125–153 amino acids; 138 amino acids 14.9 kDa (183)</td>
<td>720–730 amino acids in 6 subunits, 80 kDa (86)</td>
<td>42 amino acids, 4,963 Da (248)</td>
<td>260 amino acids, 29 kDa (222)</td>
<td>a = 286, β = 277 amino acids; 59 kDa (38)</td>
<td>384 (Arp2) + 391 (Arp3) 7 subunit protein 220 kDa (172)</td>
</tr>
<tr>
<td>Concentration in cells</td>
<td>65–70 μM (19); 300 μM (290) 20% of total muscle protein (266)</td>
<td>20 μM (0.44% of total protein) (18)</td>
<td>20–100 μM (230)</td>
<td>–5 μM</td>
<td>Up to 500 μM (249)</td>
<td>(Not known)</td>
<td>0.6 μM (1,500 actin monomers) (305)</td>
<td>2 μM Arp2, 5 μM Arp3 (41, 143)</td>
</tr>
<tr>
<td>Binding to G-actin</td>
<td>Weak</td>
<td>pH 8, ATP-GA K₈ s μM; ADP-GA K₈ s 0.1 μM (107, 240)</td>
<td>Mg-ATP K₈ 0.5 μM; Mg-ADP K₈ 5 μM (261); Mg-ATP K₈ 0.1 (6)</td>
<td>Segment 1 to barbed end K₄ 1 μM; segments bind to 2 GAs 0.07 μM (257)</td>
<td>Mg-ATP-GA K₄ 1.7 μM; Mg-ADP-GA = 80 μM (42)</td>
<td>0.05 nM (177)</td>
<td>Only the β-subunit can bind GA (53)</td>
<td>Binds weakly to actin dimers (242)</td>
</tr>
<tr>
<td>Binding to actin filaments</td>
<td>(ATP-GA) K₄ 0.1 μM at the barbed end; K₄ 0.6 μM at the pointed end (225)</td>
<td>pH =7, ADP-FA (03 μM); ATP-FA (10 μM) (42, 202)</td>
<td>Delivers ATP-GA to the barbed end of FA</td>
<td>5–60 nM at barbed end (146); 50 nM (202)</td>
<td>Weak (41)</td>
<td>K₄ 0.1 mM (177)</td>
<td>1:1, K₄ 0.5–1.5 nM (53)</td>
<td>K₄ 10 μM (242, 262)</td>
</tr>
<tr>
<td>Pointed end effect</td>
<td>Critical concentration is 0.6 μM (228); ATP on rate 0.5 μM/s; off rate 1 μM/s; ADP on rate 0.1 μM/s; off rate 0.2 μM/s</td>
<td>Accelerates off rate at pointed end, 9 s⁻¹ (240)</td>
<td>Very weak</td>
<td>Yes</td>
<td>? Yes</td>
<td>Yes (dissassembles FA)</td>
<td>Kᵣ 10 nM (242, 262)</td>
<td></td>
</tr>
<tr>
<td>--------------------</td>
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<tr>
<td>Barbed end effect</td>
<td>Critical concentration is 0.1 μM (228); ATP on rate 5 μM/s; off rate 1 μM/s; ADP on rate 1 μM/s; off rate 6 μM/s</td>
<td>Inserts monomers 0.15 μM (262). No change in off rate</td>
<td>With coflin, disassembly is fast: 125 s⁻¹ (85)</td>
<td>No</td>
<td>? Yes</td>
<td>No</td>
<td>Blocks exchange (Kᵣ 1 nM) (255)</td>
<td></td>
</tr>
<tr>
<td>Caps FA Nucleates assembly</td>
<td>N/A</td>
<td>Nucleus is an actin trimer</td>
<td>No</td>
<td>Yes</td>
<td>Inhibits nucleation</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Cross-links filaments Severs FA Side binding to Factin Important functional ligands</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Important functional ligands</td>
<td>Mg-ATP (Kᵣ 1.2 nM) (79)</td>
<td>PIP₂ inhibits depolymerization (283); inhibits nucleotide exchange (27)</td>
<td>Promotes nucleotide exchange (203); PIP₂ inhibits severing (275)</td>
<td>PIP₂ inhibits nucleotide exchange (275)</td>
<td>Inhibits nucleotide exchange (107)</td>
<td>PIP₂ inhibits nucleotide exchange (127); inhibits binding to actin (130)</td>
<td>PIP²/PIP₃ inhibit binding of CapZ to actin filaments (123)</td>
<td>Arp2 binds profilin (143); activated by WASP, Scar 1 (103)</td>
</tr>
</tbody>
</table>

ABP, actin binding protein; Kᵣ, dissociation constant; ADF, actin depolymerizing factor.
Recently, Vartiainen et al. (291) clarified the ADF/cofilin nomenclatures for vertebrates. Using mice, they proposed that cofilin 1 is expressed in most tissues of embryonic and adult cells, cofilin 2 is expressed in muscle cells, and ADF is limited to epithelial and endothelial cells. More importantly, they defined the functional similarities and differences between them.

2. Structure of ADF/cofilin

Porcine destrin was the first structure in this family of proteins to be solved by NMR spectroscopy (118). Subsequently, atomic structures of three more members of the ADF/cofilin family have been determined. A ribbon representation of yeast cofilin is shown in Figure 5A.

**FIG. 5.** A: the atomic structure of yeast cofilin solved at 1.8 Å showing the NH$_2$ and COOH termini. Putative sites for binding F-actin and G-actin (purple helix) are located in the COOH-terminal region of the molecule. The solid spheres identify residues that are essential for actin binding. [Modified from Fedorov et al. (95).] B: the crystal structure of yeast cofilin (red) was fitted using molecular dynamics to the actin monomer structure (grey) using energy minimization. This approximate orientation is preserved in views of subsequent actin-ABP complexes. (Model kindly provided by Willy Wriggers.)
solved at 1.8 Å (95). Crystal structures have also been
determined for both Acanthamoeba actophorin (161) and
a plant ADF from Arabidopsis thaliana (32). No atomic
structure has been published for any vertebrate cofilin or
for any muscle ADF/cofilin, but the atomic coordinates
for a high-resolution NMR structure for chick cofilin from
embryonic skeletal muscle were recently deposited in the
BioMagRes Bank (http://bmrb.wisc.edu) under BMRB ac-
cession number 5177 (11).

Indirect methods have been used to examine the sites
on cofilin that bind to actin. Despite the best efforts of
several groups, these two proteins have not yet been
cocrystallized, and consequently, unlike DNase I, gelsolin
segment 1, and profilin, the precise contact sites between
cofilin and actin are not known. Structural homologies
between cofilin, gelsolin, and profilin strongly suggest
they bind to the same region of G-actin (i.e., to subdo-
mains 1 and 3). However, the functional products of sys-
tematic mutagenesis of yeast cofilin indicate that the
cofilin-actin interaction is distinctively different.

Competitive binding of a synthetic dodecapeptide
(residues 104–115 of vertebrate cofilin) has implicated
this region in the interaction of cofilin with monomeric
but not with filamentous actin (308). This peptide is
largely conserved in other members of the ADF/cofilin
family including destrin and depactin, suggesting that it
may be a consensus sequence essential for actin binding
and depolymerizing activities.

Mutagenesis of surface residues for both yeast (158)
and chick (154) cofilin have implicated the NH2-terminal
region in G-actin binding. Zero-length cross-linking of
G-actin to vertebrate cofilin by 1-ethyl-3-(3-dimethylamino-
propyl)carbodiimide (308) revealed that either Lys-112
and/or Lys-114 of cofilin is in direct contact with actin.
Mutations of these cross-linked residues (192), or the
stained electron micrographs are considered essential. Despite the absence of cocystals, the
cofilin structure has been “docked” onto the Kabsch et al.
(137) actin structure (Fig. 5B) using energy minimization
techniques (304). This figure provides a clear view of the
structural relationship between these two proteins and is
presented so it can be compared with the binding sites for
profilin and DNase I (see below).

Bamburg (16) recently stressed the importance of
switching from an ADF/cofilin system to a tropomyosin-
based regulation of F-actin function. Tropomyosin binds
along the grooves of the long pitch helix of F-actin. In
myofibrils, where tropomyosin is constitutively ex-
pressed, the binding of tropomyosin and cofilin to actin
filaments is mutually exclusive and competitive (311).
Thus the depolymerizing activity of cofilin is inhibited by
the presence of muscle and nonmuscle tropomyosins
(17). Tropomyosin is located deeper into the cortex from
the site of active actin assembly and its presence probably
inhibits cofilin binding (28). Cofilin and phallolidin also
compete for binding to F-actin (120), even though their
binding sites are separated by 15–20 Å. McGough et al.
(182) proposed that an allosteric change in the twist of
F-actin induced by cofilin may inhibit phallolidin from
binding between monomers close to the filament axis.

The blue residues in Figure 6 represent the probable
minimal molecular contacts for ADF/cofilin on actin. The
principal motif shown in Figure 5, A and B (a 4-stranded
β-sheet surrounded by 4 α-helices), is also found in an
unrelated sequence of gelsolin segment 1. It is therefore
not surprising that cofilin competes with gelsolin segment
1 and profilin (85) for binding to G-actin. Both profilin and
gelsolin segment 1 bind to the barbed end of the actin
monomer (subdomains 1 and 3), even though only Glu-
167 and Tyr-169 (see Table 1) are common binding sites.
Because cofilin competes with both proteins, it is possible
that it too interacts with residues in the 162–176 loop of
actin subdomain 3 (see Fig. 1A).

The structural sites to which cofilin binds along the
outside of the filament are not known in atomic detail, so
the blue residues in Figure 6 are based on chemical
cross-linking and other data. At low resolution (25 Å),
ADF/cofilin bridges subdomains 1 and 3 of an upper actin
monomer to subdomains 1 and 2 of a lower actin in a filament. The major axis of cofilin makes a 30° angle with the plane
total by increasing (by 30-fold) the off rate (9 s−1, Ref. 240)
in the presence of stoichiometric cofilin (Fig. 7a). We are
grateful to Roger Craig for providing these micrographs.

3. Cellular functions

A) Treadmilling. The ADF/cofilin family is almost sin-
gle-handedly credited for the high rate of treadmilling of
monomers in actin filaments in vivo. This is achieved in
part by increasing (by 30-fold) the rate of treadmilling of
monomers at the pointed ends of filaments without changing the off
rate at the barbed ends (42). However, in the presence of
profilin (see sect. mB), the rate of pointed-end disassem-
bly is even faster (125 s−1, Ref. 85). The resulting elevated
concentration of coflin-G-actin in the cytoplasm can be rapidly recycled at the barbed end of filaments, provided that ATP can replace the actin-bound nucleotide. This concept is illustrated in Figure 8. In vivo, the concentration of ADF/cofilin is low relative to other ABPs such as profilin and thymosin β4 (see discussion below). ADF/cofilin “decoration” of actin filaments is probably restricted to their “aged” ends where ATP has been converted to ADP, i.e., some distance from the membrane surface where the filaments are actively growing (see below).

B) DEPOLYMERIZATION. At steady state, increasing the rate of dissociation at the pointed end would not depolymerize actin filaments if the released monomers were able to reassemble at the barbed end. However, if a barbed-end capping protein (e.g., CapZ) blocks reassembly, then coflin will depolymerize actin filaments.

C) NUCLEATION OF POLYMERIZATION. ADF/cofilin can nucleate the assembly of actin, and this function is likely to be especially important in the presence of barbed-end blockers like CapZ. The ability to nucleate assembly is probably pH dependent and may vary between different isoforms of ADF/cofilin.

D) ADP-ACTIN. Binding of ADF/cofilin to actin is regulated, at least in part, by the state of the nucleotide bound to actin. It binds to ADP-actin with about two orders of magnitude greater affinity than to ATP-actin (Table 1) or ADP-Pi-actin, and this is true for both the G- and F-actin (42) at pH 7.8. Thus coflin not only promotes the disassembly of ADP-actin monomers from filaments, but it also binds to released ADP-actin monomers and inhibits the exchange of their bound nucleotide (202). The off rate at the pointed ends of filaments is probably the rate-limiting step in the recycling of disassociated actin subunits (281). The extent of depolymerization by ADF/cofilin depends on several factors; for example, different isoforms may have differing pH sensitivities (240, 183), and other ABPs can influence the binding of coflin to actin because they share binding sites.

E) SEVERING. Marie-France Carlier et al. (42) argue that the kinetic effects of ADF/cofilin are specific for the ends of filaments and therefore it cannot sever. However, the rate of filament fragmentation would not have to be very great to increase the disassembly rate from ~125 s⁻¹ in vitro to the rate required to match the speed of motility (~200 s⁻¹). Evidence based on light microscopy (119) suggests that filaments are rapidly depolymerized by ADF at slightly alkaline pH. Also, there is a difference in severing activities of recombinant and native coflin where the native form is more active than the recombinant coflin. Recombinant coflins can vary in their severing activity and can give the impression that they have only weak severing activity (132). Thus it remains unclear whether ADF/cofilin can sever actin filaments like true severing proteins or whether the highly cooperative bind-

FIG. 6. The molecular contact sites between actin (shown here as Van der Waals representations) and ABP sites. "Front" (A) and "back" (B) views of the monomer are shown where the binding sites are colored: row 1: yellow, actin monomer-monomer interaction sites; light green, DNase I; and blue, ADF/cofilin; row 2: dark green, thymosin β4; and red, profilin; row 3: pink, gelsolin segment 1; and black, vitamin D-binding protein; row 4: red, CapZ sites. There is significant overlap between the actin binding sites for ADF/cofilin, profilin, gelsolin segment 1, and CapZ and are therefore shown on separate figures. These are the minimal contacts and are based on chemical cross-linking and mutagenesis data.
ing of coflin at substoichiometric ratios makes the filaments “brittle” at the points where the decorated and undecorated regions meet (19). Recently, Ed Egelman and colleagues (104) reported filament severing in the presence of two coflins (yeast) per actin subunit. These were seen by electron microscopy as two distinct sites in image reconstructions. This raises the question of whether one or two coflins bind to actin monomers, a feature which may depend on the isoform of coflin. Thus the question is whether coflin accelerates treadmilling by severing or depolymerizing F-actin. Current opinion seems to favor the severing ability of coflin.

4. Cellular concentrations

The cellular concentrations of ADF/cofilin are substantially lower than the concentration of actin, so it is unlikely there is sufficient ADF/cofilin present in cells (20 μM, Ref. 148) to bind to all polymerized and unpolymerized actins (65 μM; see Table 1), even taking into account its low affinity for ATP- or ADP-P_i-actins. Furthermore, the binding of ADF/cofilin to filaments is cooperative (182). If there is not enough ADF/cofilin to act as a monomer-sequestering protein, this role probably belongs to profilin and thymosin β4 (see below).

Relatively few ABPs bind to the pointed compared with the barbed end of a filament. Cofilin can compete with 1) spectrin that stabilizes short-actin oligomers (see below) (254); 2) tropomodulin that caps the pointed end of tropomyosin-coated actin filaments in muscle and non-muscle cells (97); 3) DNase I, which binds very tightly to the pointed end of actin monomers but only weakly to actin filaments (see sect. mE); and 4) new pointed-end ABPs that are likely to be identified from proteins that currently are either orphans or have not yet been identified in gene arrays and two-dimensional gel electrophoresis.

A number of isoforms of coflin have been described, and it is possible that not all members of the family perform the same functions under the same conditions (e.g., divalent cations and pH) (128). Some coflins bind tightly to actin monomers while others do not (Nosworthy, unpublished observations). Some cosediment with F-actin while others have only a low affinity (120). Some promote actin assembly while others rapidly depolymerize it (128). Human coflin seems to be a better nucleator of assembly whereas ADF is a better polymerizing agent,
and this is reflected in their respective $K_d$ values (42, 240) (Table 1).

If different isoforms of coflin have different effects on microfilament assembly, are they differentially expressed within a cell? Mouse myocyte cells lines express significant amounts of ADF. Elevated expression of actin causes a downregulation of ADF and decreases spreading of these cells without changing the levels of coflin (256). Regulating ADF/cofilin expression is likely to be slow (on a time scale of tens of minutes/hours) and, apart from being energetically wasteful, it is unlikely to be an effective way of enabling microfilaments to respond quickly to environmental stimuli. However, because most ABPs bind with moderate to low affinity (in the micromolar range), elevated levels of ADF/cofilin could, over time, subtly shift the balance of ABPs and, by mass action, alter the state of assembly of cytoskeletal microfilaments.

5. Phosphorylation of ADF/cofilin

The ability of coflin to bind G-actin is also regulated by phosphorylation of Ser-3, which is conserved in most members of the ADF/cofilin family. When ADF/cofilin is phosphorylated, there is a sharp fall in its affinity for actin (190). ADF exchange in G-actin is strongly inhibited by coflin, but once coflin is phosphorylated, the released ADF-actin monomer can exchange with cytoplasmic ATP, and it is now ready for reincorporation at the barbed end of a growing filament. Typically, this occurs at the interface of the microfilaments and the membrane at the leading edge of the moving cell. Thus cellular microfilament turnover can potentially be regulated by cycles of phosphorylation and dephosphorylation. As we will see below, profilin plays an important role here.

We know that in yeast, phosphorylation is not a regulatory factor whereas it is in most vertebrates. An important question is, How much of total cellular ADF/cofilin is phosphorylated? For most cells, we simply do not have this information, but in amoeba~30% of actophorin is phosphorylated (29). On balance, it seems likely that, since not all of ADF/cofilin is constitutively active, there may be mechanisms other than phosphorylation that control actin assembly-disassembly in vertebrates.

Phosphorylation of vertebrate coflin is achieved by LIM-kinase proteins 1 and 2 (LIMK-1 and LIMK-2). LIMK-1 is predominantly neuronal, whereas LIMK-2 is more widespread (26, 187). Although the signaling pathway for activation of the LIM-kinases is not yet fully understood, it is known that LIMK-1 is under the control of the small GTPase Rac (307), whereas LIMK-2 is regulated by the GTPase cdc42 and rho (21) (see discussion below). LIMKs are themselves regulated by phosphorylation of a Thr residue (90). In Acanthamoeba, activation of LIM-kinase by Pak1 (91) results in phosphorylation of Ser-1 (the Acanthamoeba version of Ser-3) of actophorin (29).

When expressed in cultured cells, LIMK-1 induces actin reorganization and reverses coflin-induced depolymerization (307). Expression of inactive LIMK-1 results in the accumulation of F-actin filaments (7). Phosphorylation of coflin appears to increase with pH-induced activation of coflin, consistent with a compensating homeostatic mechanism (27).

Although NH$_2$-terminal Ser residues (Ser-1, -3, -4, and/or -6 in different species) are recognized as the site for phosphorylation, it is not clear whether the resulting inhibition of actin binding involves a steric or conformational change. Recently, Blanchin et al. (29) examined this question by crystallizing Acanthamoeba actophorin (ADF/cofilin) in the phosphorylated state. Their structure was essentially identical to unphosphorylated ADF/cofilin, and they concluded that inhibition of activity by LIM-kinase was not due to a conformational change at the ADF/cofilin-actin interface. Their reported steric blocking of actin binding was consistent with molecular dynamics predictions (Fig. 5B) and with site-directed mutant studies. Frustratingly, Ser-1 residue in these crystals could not be resolved.

Until recently, the mechanism by which coflin is dephosphorylated was not well understood. Slingshot, a protein phosphatase with F-actin binding ability, was shown by Niwa et al. (204) to dephosphorylate coflin in cultured cells and in cell-free assays. In Drosophila, loss of this enzyme results in a dramatic increase in cellular levels of both F-actin and phosphorylated coflin.

6. Inhibition by phosphatidylinositol 4,5-bisphosphate

The activity of ADF/cofilin can be regulated by the membrane lipids phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (309). These bind to and inhibit the actin-binding domain of ADF/cofilin at residues 104–115 and the NH$_2$-terminal region (154) and suggest that transmembrane signaling by PIP$_2$ can regulate the function of ADF/cofilin. Little is known of the molecular nature of this binding site.

7. pH effect

The ability of ADF/cofilin to assemble or disassemble F-actin is pH dependent in vitro (311). Acidic conditions (less than pH 6.8) enhance the ability of ADF/cofilin to stabilize F-actin while at more alkaline pH (>7.3), coflin can rapidly depolymerize F-actin. Furthermore, the critical concentration of the coflin-actin complex is known to be pH dependent, being low (~2 μM) at pH 6.5 and significantly higher (7 μM) at pH 8.2 (240). Conversely, it has been suggested that the severing activity of coflin is pH independent (89). The physiological significance of this pH dependency is that actin filaments may be stabilized in the vicinity of membrane Na$^+$-H$^+$ antiports (16). A more recent report (27) focused on the role of pH in vivo.
Using pH-sensitive fluorescent probes introduced into mouse fibroblasts (Swiss 3T3 cells), they (27) showed that by lowering intracellular pH, ADF colocalized with actin filaments and that when the pH was increased, ADF partitioned more with monomeric actin. In contrast, coflin is much less responsive to altered pH, and there are no reports of structural changes induced in either ADF or coflin, despite the fact that its structure has been determined by both NMR spectroscopy (11) and crystallography (158).

8. Nuclear translocation sequence

All vertebrate ADF/cofilin (destrin) sequences contain a putative nuclear localization sequence (NLS) that enables it to migrate from its normal location in the cytoplasm to the nucleus under conditions of cellular stress (203). Heat shock and other forms of stress probably affect a few residues immediately preceding the NLS, which expose it for subsequent binding to a nuclear transport factor and thus passage through the nuclear pores (32). Transport is an active process, and actin accompanies the coflin into the nucleus, although the significance of this is not clear.

B. Profilin Family

The profilin family of ABPs is found in eukaryotic cells from Acanthamoeba through to human and in many but not all species there are two or more profilin genes (230). Profilins are small proteins with an approximate molecular mass of 19 kDa (6). They are among the most highly expressed (20–100 μM, Ref. 37) of the cytoplasmic proteins and are distributed throughout the cytoplasm. Like coflin and DNase I, profilin can be denatured in 8 M urea and then renatured by dialysis (138), a property used during its purification.

1. Atomic structure

X-ray diffraction and NMR atomic resolution structures are available for no less than six isoforms of profilin. All are very similar. Profilin has also been cocrystallized with actin in two different conformations, the so-called “closed” and “open” states (60). It was on the basis of these two structures that Schutt et al. (259) developed their model of F-actin (discussed above). Within the profilins, the NH2-terminal sequence is conserved whereas the COOH-terminal region of certain isoforms resembles gelsolin (see below). The atomic structure of the profilin–γ-actin complex is illustrated in Figure 9. Profilin binds to subdomains 1 and 3 at loci that substantially overlap the binding sites of gelsolin segment-1 (259). Its main crystal contacts with actin are as follows: subdomain 1, 113, 354, 355, 361, 364, 369, 371, 373, 375; subdomain 3, 166, 167, 169, 171–173, 284, 286–288, 290 (see Fig. 6).

2. Cellular functions

Profilin has several cellular functions. It is essentially a high-affinity (Kₐ = 10⁷ M⁻¹) monomer-binding protein (225). It is best known for its ability to promote the exchange of nucleotide in actin monomers released from filaments (109) and, because the high (millimolar) concentrations of Mg-ATP in the cytoplasm, it catalyzes the exchange of ADP for ATP. It achieves this by binding to subdomains 1 and 3 near the hinge between the two major domains (see Figs. 6 and 9) and modulates the opening of the nucleotide cleft. Profilin enhances filament turnover in the presence of coflin (85) because the two proteins act at opposite ends of a filament, with profilin adding ATP-actin to the barbed end and coflin dissociating ADP-actin from the pointed end (see Fig. 10). Profilin also inhibits the hydrolysis of ATP bound to actin, thus maintaining actin monomers in a state where they retain a high affinity for the growing barbed end of filaments (6).

While profilin acts as an effective buffer for high monomer concentrations in cells, it can promote polymerization by transporting monomers to the fast-growing barbed ends of filaments. Profilin binds to a site on actin (see row 2 in Fig. 6) that is inaccessibly in the Holmes model of the filament (128) (see Fig. 4). Thus, after deliv-
ering a monomer to the growing end of the filament, it must either move to a new site that does not block assembly or completely dissociate. This can occur even when actin is complexed to thymosin (see below) because of the high exchange rates for both thymosin and profilin (219). It is not clear why some cells employ thymosin and ADP when actin is complexed to thymosin (see below) be-

earings of actin monomers and oligomers are then strongly sequestered by vitamin D-binding protein (DBP) and ultimately removed from the circulation in the liver (124). Plasma gelsolin is slightly larger (83 kDa) than cytosolic gelsolin and is derived by alternative splicing of a single gene resulting in a short peptide appended to its NH₂-terminus (155). Otherwise, the sequences of plasma and intracellular gelsolins are identical.

1. Structure of gelsolin and gelsolin/F-actin

Gelsolin is an 80-kDa protein consisting of two tandem homologous halves (segments 1–3 and 4–6), each containing threefold repeats (Fig. 11). Its structure in the absence of Ca²⁺ has been determined at 2.5-Å resolution (35). The isolated NH₂-terminal half can bind to (cap) two actin monomers and can sever F-actin without the need for free Ca²⁺. In contrast, the COOH-terminal half binds a single actin and is Ca²⁺ dependent. The molecule is compact and globular having dimensions of ~85 × 36 × 55 Å. Segment 1 cocrystallized with monomeric actin is shown in Figure 12A. It has two bound Ca²⁺ (186). Gelsolin segments 4–6 also bind to monomeric actin, and their relationship to the actin monomer is illustrated in Figure 12B.

McGough et al. (181) had earlier suggested a mechanism in which segments 2 and 3 bind to the actin filament while segment 1 wedges itself between two adjacent actin monomers along the longitudinal axis. This step requires a rearrangement of the interface between segments 1 and 2 to lengthen the linker between these segments. Segments 4–6 reach across the filament to bind a monomer in the other strand. This step would also require extension of the convoluted linker between segments 3 and 4.

The following year, on the basis of crystallographic data, Robinson et al. (243) concluded that Ca²⁺ binding induces a conformational rearrangement in which segment 6 is flipped over and translated by ~40 Å relative to segments 4 and 5. The structural reorganization tears apart the continuous β-sheet core of segments 4 and 6. This exposes the actin-binding site on segment 4, enabling severing and capping of actin filaments to proceed (Fig. 13). Severing of F-actin occurs only when sufficient actin-actin bonds are broken (36). The severing of F-actin by gelsolin is illustrated in Figure 7C where only short segments of actin filaments are observed in the presence of recombinant gelsolin (electron micrograph courtesy of...
Roger Craig, University of Massachusetts Medical Center, Worcester, MA). Here the molar ratio of gelsolin (segments 1–3) to actin is 0.05:1.

The crystal structure of the F-actin binding domain of severin, the gelsolin homolog from Dictyostelium discoideum, has recently been described at high (1.75 Å) resolution (243). The structure reveals an α-helix-β-sheet sandwich that contains two cation-binding sites and F-actin binding residues in the equivalent region of gelsolin segment 2 (234). This segment also mediates the binding of gelsolin to tropomyosin (172).

2. Functional properties in vitro

A) ACTIN SEVERING. Gelsolin certainly lives up to its name. It rapidly dissolves actin gels in vitro by severing, capping, and nucleating growth. Like coflin, it can dissociate phalloidin from F-actin (262). These functions are achieved by attacking filaments from their barbed ends. Gelsolin is an important ABP because of its high affinity for actin filaments ($K_d$ 50 nM) (229).

Segments 1–3 and 4–6 of intact gelsolin both play an active role in the severing of F-actin. Binding of gelsolin

**FIG. 11.** A ribbon diagram of the atomic structure of inactive gelsolin showing segment 1 (red) (containing the NH$_2$ terminus), segments 2 and 3 (green, yellow), and segments 4–6 (magenta, green, gold). (Figure kindly provided by Bob Robinson.)

**FIG. 12.** A: crystal ribbon structure of gelsolin segment 1 (red) and actin. B: gelsolin segments 4, 5, and 6 and actin. These figures are shown in the same orientation as Figs. 5B and 9A. Colors are those used in Fig. 11. (Figures kindly provided by Bob Robinson.)
trigger for events that involve the cytoskeleton, and gelsolin is also the only known Ca\(^{2+}\)-dependent severing protein with a \(K_d\) for Ca\(^{2+}\) in the micromolar range. The six motifs contain two sites that bind to G-actin and one that binds to filaments. The Ca\(^{2+}\)-dependent monomer-binding site in the COOH-terminal half plays a critical role both in the cooperative binding to actin by gelsolin and in its nucleating activity. Actin binding was localized to segment 4 by expressing segments 4, 4–5, 5, and 5–6 in Escherichia coli (232). The nonbinding segments play an important role in the Ca\(^{2+}\) regulation of actin binding and other activities of gelsolin (232). The segmental arrangements are shown in Figure 10.

An analysis of the gelsolin construct lacking the COOH-terminal helix indicated the importance of this part of molecule for Ca\(^{2+}\) regulation and supported the so-called “tail helix latch” mechanism of activation by Ca\(^{2+}\) (170). This latch is primarily responsible for transmitting Ca\(^{2+}\) binding from the COOH-terminal half to the NH\(_2\)-terminal half of gelsolin. Occupancy of this site primes gelsolin for severing, but it is unable to sever because the tail latch is still engaged. Unlatching the tail helix by micromolar Ca\(^{2+}\) releases the final constraint to initiate the severing cascade (163).

3. Comparison with ADP/cofilin

ADP/cofilin competes with gelsolin segments 2 and 3 for binding to actin filaments. This suggests they share a common structural topology for binding to actin (290), and this was subsequently confirmed by molecular dynamics simulation (304). Both proteins alter the conformation of the actin filament, dissociate phalloidin, sever, and cap F-actin. They both bind to ADP-G-actin with higher affinity than to ATP-G-actin, and the binding of both to actin is inhibited by PIP\(_2\) (275). Recent expression studies suggest both proteins play an important role in regulating actin dynamics in vivo (208). However, despite the more powerful severing action of gelsolin, ADP/cofilin is currently favored as the major regulatory mechanism of actin cytoskeleton reorganization (16).

4. Knock-out and overexpression

Transgenic gelsolin-null mice have normal embryonic development and longevity but suffer subtle changes suggesting that it is needed for rapid changes in cell motility in hemostasis (reduced platelet function), inflammation (delayed neutrophil migration), and wound healing (fibroblasts move more slowly) (303). In these null mice, gelsolin is an essential effector of rac-mediated actin dynamics, acting downstream of rac recruitment to the membrane (10). Gelsolin also appears to play a role in the initiation of filopodial retraction. An absence of gelsolin delays its retraction and produces “studded” filopodia along growing neurites (169).
Overexpression of gelsolin in cultured mouse fibroblasts enhances their migration rate by \( \sim 125\% \) via gelsolin-promoted actin assembly and disassembly (72). Control of the rate of cell migration is closely linked to the rates of actin filament severing. In fibroblasts that overexpress gelsolin, there is a direct correlation between movement velocity and actin turnover rates. This demonstrates that gelsolin is an important control mechanism for actin cycling in cells (184). Overexpression of gelsolin in cells also inhibits phospholipase C-\( \gamma \) activity by competing with it for available PIP\(_2\). A rise in free Ca\(^{2+}\) increases the affinity of gelsolin for PIP\(_2\) and as the level and availability of PIP\(_2\) changes during signaling, crosstalk between PIP\(_2\)-regulated proteins (e.g., gelsolin and phospholipase C-\( \gamma \)) provides a mechanism for positive or negative regulation of the signal transduction cascade (274).

D. Thymosins

Thymosin \( \beta 4 \) (T\( \beta 4 \)) is a small (molecular mass \( \sim 5 \) kDa) protein that belongs to a family of highly conserved small proteins originally isolated from the thymus gland but now known to be more widely distributed (93). It contains 43 residues, \( \sim 50\% \) of which are charged. The amino acid composition of T\( \beta 4 \) is remarkably deficient in hydrophobic residues (168), which suggests that T\( \beta 4 \) probably has very little defined structure in solution.

1. Structure

Until very recently, the structure of T\( \beta 4 \) was based on a model constructed by Dan Safer et al. (251) who docked it to the crystal structure of monomeric actin solved in the presence of DNase I (Fig. 14). These authors regard this protein as having an essentially unfolded conformation in solution and, although there may be an increase in its \( \alpha \)-helical content, it remains unfolded once it binds to actin (251). Based on a range of data (250), it has been suggested that T\( \beta 4 \) drapes itself around nearly half the perimeter of the actin. The molecular contact points between T\( \beta 4 \) and the actin monomer investigated using zero-length cross-linkers show that Lys-3 of T\( \beta 4 \) is located close to Glu-167 (in subdomain 3 near the hinge), and that Lys-18 of T\( \beta 4 \) cross-links to one of the four NH\(_2\)-terminal acidic residues of actin (Asp-1 through Glu-4). These two contacts flank the \( \alpha \)-helical region of T\( \beta 4 \) at the barbed end of the monomer. The COOH-terminal region of T\( \beta 4 \) (Lys-38) was cross-linked to Gln-41 at the top of actin subdomain 2, a known DNase I binding site.

The NMR structure of free T\( \beta 4 \) in solution (75) exhibits a range of conformational preferences. In water it appears to lack a folded conformation, but a preferential \( \alpha \)-helical conformation can be observed in solution at different temperatures and pH. Residues 5–16 putatively bind to the NH\(_2\)-terminal region of actin and have characteristic temperature-dependent conformations (75). These data indicate that many parts of T\( \beta 4 \) are not in tight contact with actin. Similarly, circular dichroism spectra indicate that free T\( \beta 4 \) is predominantly unstructured but increases its \( \alpha \)-helical content when it binds to actin. At least one research group has made progress with a crystal structure of T\( \beta 4 \). De La Cruz et al. (78) recently concluded that T\( \beta 4 \) changes the conformation and structural dynamics (the so-called “breathing”) of actin monomers. The conformational change may reflect the unique ability of T\( \beta 4 \) to sequester actin monomers and inhibit nucleotide exchange (compared with profilin).

2. Cellular distribution and functions

\( \beta \)-Thymosins are widely distributed in nature. Translational levels can vary from tissue to tissue (262). In chick brain, concentrations are an order of magnitude higher than profilin and coflin. It is abundant in neural tissue as well as in circulating cells such as platelets, leukocytes, and macrophages. T\( \beta 4 \) binds stoichiometrically to monomeric actin and inhibits polymerization. In the developing chick brain, the ratio of ADF to coflin to T\( \beta 4 \) increases between embryonic days 13 and 17. Because no other thymosin isoforms were detected in brain extracts, it is probable that it is the major actin-sequestering protein in this tissue (84). T\( \beta 4 \) mRNA is mainly expressed in neurons of the hippocampus, neocortex, and the amygdala, as well as in oligodendrocytes and probably has a function in the production and remodeling of neu-
ronal processes (48). Tβ4 is upregulated in the pyramidal neurons of the hippocampus where its function may be related to restoration of neurite circuits after focal ischemic damage (292).

Tβ4 is present in some cells at concentrations as high as 0.5 mM (249). High expression levels have been observed in young embryonic muscles but not in adult skeletal muscles. Consequently, it seems that the G-actin pool in young embryonic skeletal muscle is mainly regulated by Tβ4 and profilin (206). However, Tβ4 may become less important as muscle develops (199). In contrast, there is evidence that it plays a role in angiogenesis in coronary smooth muscle, acting as a chemoattractant by stimulating the migration of endothelial cells in vivo (174).

In quiescent NIH 3T3 cells, Tβ4 was undetectable, but after restoring serum to the culture medium where cells were induced to proliferate, there was a pronounced increase in Tβ4 mRNA (316). Overexpression in these cells increases F-actin levels while maintaining a constant level of total actin (273). In contrast, others reported that a twofold increase in Tβ4 in these same cells doubled the total actin expression but maintained the ratio of G- to F-actin (110). Unexpectedly, other cytoskeletal proteins such as myosin IIA, α-actinin, and tropomyosin also increased nearly twofold (273). The Tβ4 cell lines spread more fully and adhered to the dish more strongly than vector controls, suggesting a coordinated regulation of the cytoskeleton by Tβ4. In addition, transcriptional levels of several related cytoskeletal proteins also increased. These observations suggest there is a coordinated cytoskeletal expression of this protein (110).

Like several other ABPs, thymosins bind to and coordinately control the availability of actin monomers for incorporation into filaments. Tβ4 is a contender with profilin for the title of the universal actin-monomer binding protein. It has a putative actin-binding motif (LKHAET) (287) found in other ABPs including actobindin and myosin. Nachmias (196), and more recently Chen et al. (54), reviewed the discovery and primary structure of Tβ4.

Acting alone, Tβ4 inhibits polymerization, and like ADF/cofilin it also inhibits the exchange of the actin-bound nucleotide (107). This contrasts with the action of profilin which, acting alone, facilitates the exchange of ADP for ATP and promotes actin assembly (109). Thus, while it acts as a passive buffer of actin monomers, preventing their polymerization, Tβ4 can cooperate with profilin to regulate actin assembly (273). However, Tβ4 is not a simple G-actin-sequestering protein because, at least at high concentrations, it also interacts with F-actin (40).

The actin-binding sites of Tβ4 resemble that of another small ABP, actobindin, which also inhibits polymerization. Their binding loci on monomeric actin have been localized using peptide mimetics (286). Both have an NH₂-terminal hexapeptide motif forming the major electrostatic contact site with actin. A nonconserved NH₂-terminal segment preceding the motif exerts an inhibitory activity on actin polymerization probably by steric hindrance. Substitution of a His or Lys for a Glu at the third position in the motif converts the peptide from an inhibitor of polymerization into a stimulator. Thus subtle sequence changes in ABPs can have remarkable effects on their functions.

3. Molecular interaction with actin

Binding of Tβ4 to actin is coupled to the dissociation of bound water molecules, which is greater for CaATP-actin than MgATP-actin monomers. The COOH terminus of Tβ4 makes contact with actin at His-40 near the mouth of the nucleotide cleft and binding slows the ³H exchange rate, suggesting there are changes in conformation of actin monomers when it binds. This may reflect the ability of Tβ4 to both sequester actin monomers and inhibit nucleotide exchange (78).

Gelsolin and Tβ4 compete for binding to actin, indicating that Tβ4 binds to a site close or identical to the gelsolin segment 1-binding site (13). Figure 6 shows that the binding loci for gelsolin and Tβ4 do not precisely coincide but that there must be sufficient overlap to explain the competition. If Tβ4 is to be a major cellular actin-sequestering factor, it should bind, among other things, with a slightly higher affinity (0.7 μM) than gelsolin (−1 μM) (262), thus subtly shifting the assembly/disassembly equilibrium of actin toward the monomeric state. The interactions of these two proteins with actin are complex and require the participation of several complementary peptide sequences. A common motif (I,V)EKFD in these two proteins exerts a major inhibitory effect on salt-induced actin polymerization (96).

To make matters more complex, Tβ4 and profilin (and presumably gelsolin) compete with WASP (an activator of Arp2/3) for binding to actin monomers and presumably have overlapping binding sites (see sect. mG).

DNase I and Tβ4 bind to sites on actin that partially overlap. The apparent Kd of the actin-Tβ4 complex generated from a preformed actin-DNase I complex is 160 μM. A fivefold excess of DNase I over the preformed actin-Tβ4 complex is necessary to observe a comparable Kd (129). Thus DNase I can displace Tβ4 from the monomer (and probably vice versa).

Chemical cross-linking experiments also indicate that profilin competes with Tβ4 for actin binding. In the presence of Mg²⁺, profilin promotes assembly of Tβ4-actin but in the presence of Ca²⁺, profilin was unable to initiate this polymerization (12). Phalloidin, rabbit skeletal muscle myosin S1, and chicken intestinal myosin I all polymerize actin from the Tβ4-actin complex (15). Thus, despite the very high affinity of DNase I for actin, its binding can be inhibited by a lower affinity Tβ4. The
explanation for this may be an allosteric conformational change in the actin monomer as reported by us recently (81) for DNase I and cofillin.

Competitive binding data suggest that Tβ4 also makes contact with the myosin binding site as well as with the bottom of subdomain 3 where it overlaps the binding sites of several ABPs including DBP (Fig. 6, row 3) at the barbed end of the monomer. Therefore, the distance between the pointed and barbed ends requires the COOH-terminal half of Tβ4 to be in a predominantly extended conformation (247). Point mutations revealed that the middle section of Tβ4 (residues 17–22) also interacts with actin (267).

Although Tβ4 is widely recognized as a monomer binding protein, at least three reports have demonstrated its ability to bind to F-actin (14, 40, 273). Addition of 200 μM Tβ4 during actin assembly decreases the critical concentration of free actin. This is consistent with the in vivo observation that overexpression of Tβ4 increases stress fibers and decreases the amount of monomeric actin (273). A chemically cross-linked binary complex of Tβ4 actin is incorporated into F-actin only when both phalloidin and gelsolin are present (14). Tβ4 increases the long-pitch helical repeat (from 35 to 40.5 nm determined from 3-dimensional helical reconstructions) of F-actin. Like its pitch helical repeat (from 35 to 40.5 nm determined from 3-dimensional helical reconstructions) of F-actin. Like its

4. Other thymosins

Thymosin isoforms are distributed throughout the vertebrate phyla (247). While Tβ4 is the predominant expressed isoform in mammalian cells, many species produce at least two Tβ isoforms, in some cases in the same cell (49). Differences in the distributions of the mRNAs encoding Tβ10 and Tβ4 suggest there are different roles for these peptides during mouse early embryogenesis (49). The levels of Tβ10 mRNA strongly increase during early postimplantation mouse embryogenesis, suggesting that Tβ10 plays an important role in early development. Changes in Tβ10 and Tβ4 mRNA expression are also important in the control of actin dynamics in developed neurons and glia since Tβ10 mRNA is present in different regions of the rat forebrain, including hippocampus, neocortex, and several brain nuclei (48). Tβ10 is also downregulated in failing human hearts with dilated cardiomyopathy (Tsubakihara, unpublished data).

The underlying mechanism for these cellular changes remains to be elucidated. However, plasmid-driven overexpression of the Tβ10 gene is known to increase the susceptibility of apoptosis in transfected fibroblasts (115). These proapoptotic properties of thymosins may be mediated by G-actin sequestering and involve interaction with DNase I (114). NIH 3T3 cells that overexpress (x3) Tβ10 have been shown to have 23–33% more polymerized actin than control cells, and their microfilaments appear thicker. There is no change in total actin, profilin, or cofillin/ADF content. These cells are more motile, spread faster, and have higher chemotactic and wound healing activity (273). Immunofluorescent labeling of peritoneal macrophages showed that both Tβ4 and Tβ10 were uniformly distributed within the cytoplasm. Overexpression in fibroblasts induces extensive loss of stress fibers, indicating that Tβ4 and Tβ10 are functionally similar and that both are effective regulators of actin filament dynamics in living cells (313).

Both Tβ4 and Tβ10 are elevated in a human prostate cancer cell line and correlate positively with the grade of tumor. Transfection with antisense Tβ15 constructs into rat prostatic carcinoma cells demonstrates that the Tβ4 peptide positively regulates cell motility. Increased motility is a critical component of the metastatic pathway (22). Tβ15 is also upregulated in nonmetastatic breast cancer and even in ductal carcinoma (compared with benign breast tissue). Consequently, it might represent a potential early marker for breast malignancy (106).

Tβ9 and Tβmet9 (the minor variants of Tβ4) inhibit polymerization of ATP-actin with identical Kd values (0.7–0.8 μM). Like Tβ4, these thymosins bind to ADP-G-actin with a 100-fold lower affinity than to ATP-G-actin (134).

E. DNase I

DNase I is a secretory glycoprotein with an endonuclease activity that cleaves double-stranded DNA to yield 5'-phosphorylated polynucleotides. It has a requirement for both Ca2+ and Mg2+. Bovine pancreatic DNase I is the best characterized of a group of DNases. It has a molecular mass of 31 kDa and an optimum pH of 7.8 (152). It was first purified from pancreas and parotid gland that contained four DNases (A, B, C, and D), differing according to their carbohydrate side chain and polypeptide components. DNase I is glycosylated at Asn-18 (189). These factors, together with the presence of sialic acid, result in a charge heterogeneity that can be detected by isoelectric focusing (162).

1. Atomic structure

The structure of DNase I was solved crystallographically (272) at 2-Å resolution and was found to have two Ca2+ binding sites. DNase I has a high affinity for actin (Kd = 5 × 106 M−1) (177). The crystal structure of the actin-DNase I complex (with actin in the ATP form) has been determined at 2.8-Å resolution (137). Most ABPs interact with actin subdomain I, whereas DNase I binds to
levels in cells (71), although its function in cytoskeletal dynamics is yet to be elucidated. However, the tight binding and specificity of DNase I for actin and its wide distribution suggests it has a biological role. It binds very tightly to G-actin ($K_d$ in the nM range) (266) and essentially removes all free actin monomers from solution. It binds much more weakly to F-actin (100 $\mu$M) where it is a capping protein that increases the rate of dissociation from the pointed ends of filaments (298).

We recently examined the possible cellular codistribution of DNase I and coflin in cultured mammalian cells. Using fluorescent-labeled antibodies to coflin and DNase I, we found the cytoplasmic distributions of these two proteins overlap but are not identical (56). Like Tβ4, DNase I may act as a stabilizer of actin monomers by effectively removing them from the available pool of monomers available for assembly. In section IV we raise the possibility that it may also be a natural modulator of the action of coflin on the cytoskeleton.

**F. Capping Proteins**

Monomers in actin filaments “age” as they progress from the assembling (barbed) end to the disassembling (pointed) end of a filament. This aging process is driven by the chemical energy of Mg-ATP. Free monomers predominantly contain bound ATP that is hydrolyzed to ADP-P$_i$, only after incorporation at the barbed end, with the subsequent release of P$_i$. At the barbed end, the on rate for ATP monomers (5 $\mu$M/s) exceeds the off rate (1 $\mu$M/s) while for ADP monomers the corresponding on rate (1 $\mu$M/s) is slower than the off rate (6 $\mu$M/s). Thus, while monomers at the barbed end nearly all contain ATP, monomers at the pointed end mostly contain ADP. At the pointed end, the on rate for ATP monomers (0.5 $\mu$M/s) is slower than its off rate (1 $\mu$M/s) while the off rate for ADP (0.2 $\mu$M/s) is faster than its on rate (0.1 $\mu$M/s) (149). Because the rates of monomer exchange are 20 times faster at the barbed than at the pointed end, there is a high probability that an ATP monomer that adds to this end will be hydrolyzed before another exchange occurs. Clearly, capping proteins at these ends will alter monomer kinetics depending on their binding affinity and rate of exchange.

Many, perhaps most, cells control the length and number distribution of thin filaments under conditions where total actin concentration remains approximately constant. This assembly and disassembly of actin requires a dynamic equilibrium between actin monomers and the two, nonequivalent ends of actin filaments. Proteins that cap or prevent this exchange may variously nucleate, promote, stabilize, or inhibit assembly. They are therefore in a strong position to regulate actin polymerization. This simple concept is illustrated in Figure 16. Here we focus
assembly at the barbed ends of filaments, 4) elimination of annealing at the barbed ends of filaments (265), and 5) correct assembly of filaments at the Z-disk (254). CapZ does not affect the rate of fragmentation of actin filaments and does not bind to their pointed ends (53). Figure 16 shows CapZ at the barbed end of a filament.

CapZ is a heterodimer comprising an α-subunit (there are at least 2 isoforms in eukaryotic cells) and a β-subunit (2 or 3 isoforms), and both subunits are required for effective barbed-end capping of filaments (53). The α₁- and α₂-isoforms result from alternative gene expression (117). Capping proteins containing the α₁-isoform display fourfold higher affinity for actin than capping protein containing the α₂-isoform, although the physiological significance of this remains unclear. The β₁- and β₂-isoforms result from alternative splicing of a single mRNA transcript (255), and although both isoforms demonstrate similar affinity for actin, they are differentially expressed and distributed. The β₁-isoform is predominantly expressed in muscle cells and is localized to the Z-line in striated muscle, whereas the β₂ is located under the sarcolemma (51). Consequently, capping protein containing the β₁-isoform is often referred to as CapZ and is analogous to β-actinin (179). The β₂-isoform is predominantly expressed in nonmuscle cells where it is localized to the cell periphery (255).

B) STRUCTURE OF CAPZ. CapZ was recently crystallized by Yuichiro Maeda and colleagues, and a preliminary report was presented at the SPring'8 actin conference held in Japan in November 2001. Details of this structure are not yet available but are likely to be published later in 2003. Despite the fact there are no cocrystals of CapZ-actin, considerable detail is available on the molecular contacts between these proteins. The contact sites on actin are illustrated in Figure 6, row 4. Deletion of the COOH terminus of the β-subunit of CapZ eliminates its capping activity as does the binding of a monoclonal antibody directed against the COOH-terminal 12 residues (130). Later, this same group showed that 31–40%, respectively, of the α- and β-subunits are not required for strong binding to actin, suggesting that these regions may be important for other functions (53).

As its name suggests, capping protein binds tightly to the barbed end of actin filaments. In vitro, this capping prevents the exchange of actin subunits from the barbed end. Furthermore, capping protein facilitates actin polymerization by binding to and stabilizing monomers or oligomers of actin forming nuclei for elongation of filaments (51).

C) REGULATION OF CAPZ ACTIVITY. In vivo, the capping of actin filaments is regulated by second messengers, PIP and PIP₂. This is also true for other ABPs including coflin, destin and DNase I (309), profilin (108), and gelsolin (285) (see Table 1). Upon signal transduction, PIP and PIP₂ promote removal of capping protein from actin fila-

[FIG. 16. A cartoon representation of the interaction of a barbed-end (CapZ, purple) and a pointed-end capping protein, tropomodulin (brown) (Tmod). One CapZ binds to two monomers at the barbed end of an actin (yellow) filament. The NH₂ terminus of Tmod binds tightly to NH₂ terminus of tropomyosin (green) at the pointed end of filaments and also to one (or two) monomers. The on rate and off rates at the filament ends are indicated by lengths of the arrows. Two views of the Tmod molecule [originally published in Krieger et al. (153)], modeled from X-ray scattering data, are shown at the top of this figure.]

on the most abundant barbed-end capping protein CapZ and the predominant pointed-end capping protein tropomodulin.

1. CapZ, a barbed-end capping protein

Several different proteins can bind to the barbed end of filaments. Some of them like gelsolin (and its close relatives adseverin and fragmin) (254) and profilin have already been discussed above in a different context. Villin, CapG, tensin, and fragmin have been reviewed elsewhere (54).

CapZ is present in all eukaryotic cells (65). In skeletal muscle it is localized at the Z-disk where it is probably held onto the Z-lamellae by a unique protein (see review in Ref. 165). It is usually prepared from chicken or rabbit skeletal muscle but, unless G-actin is twice purified by gel filtration, traces of CapZ remain in the preparation and affect actin assembly characteristics (52). The difficulty in preparing actin free of CapZ can be appreciated when one realizes that one heterodimer of CapZ can cap a filament containing a thousand or more actin monomers (265).

A) FUNCTIONS OF CAPZ. The reported biological roles of CapZ are as follows: 1) nucleation of actin assembly, 2) capture of preexisting filaments, 3) regulation of actin assembly at the barbed ends of filaments, 4) elimination of annealing at the barbed ends of filaments (265), and 5) correct assembly of filaments at the Z-disk (254). CapZ does not affect the rate of fragmentation of actin filaments and does not bind to their pointed ends (53). Figure 16 shows CapZ at the barbed end of a filament.
ments, resulting in localized pockets containing a dramatic increase in the number of free barbed ends. This allows actin polymerization to proceed in localized regions of the cell. Capping protein is involved in a variety of biological roles including the activation of platelets and maintaining the proper assembly of actin filaments at the Z-line of sarcomere (254).

2. Tropomodulin, a pointed-end capping protein

Unlike barbed-end capping proteins, pointed-end capping proteins are something of a rarity. The name tropomodulin (Tmod) is apt because it binds strongly to actin in the presence of tropomyosin. With the exception of Arp2/3 (see sect. wG), no other protein binds to the pointed end of F-actin, and no other protein inhibits filament elongation at this end. Although Tmod binds to F-actin (it does not bind monomeric actin), its affinity is weak ($K_a \approx 0.1 \mu M$) compared with its affinity for tropomyosin-decorated actin filaments that is about three orders of magnitude stronger ($K_a$ is submicromolar) (297). For this reason, some authors originally referred to it as a tropomyosin binding protein (295).

Human Tmod has been cloned and is expressed in a wide range of tissues with high levels in skeletal and cardiac muscle (276). It contains 359 amino acids and has a molecular mass of 40,476 Da determined by mass spectrometry. Overexpression of Tmod in cardiomyocytes produces short, disorganized, and destabilized thin filaments (277). A Tmod overexpressing transgenic mouse is now available (278). A related, somewhat large (64 kDa) protein, leiomodin, is highly expressed in smooth as well as cardiac and skeletal muscle (62).

Yuichiro Maeda’s group (at the RIKEN SPring8 Institute, Japan) has reported crystals of the actin-binding COOH-terminal half of Tmod that diffract to 1.45 Å (153). This structure is now in press in the *Biophysical Journal*. The overall shape of a slightly truncated Tmod has been determined from low-angle X-ray scattering by Fujisawa et al. (101), also in Maeda’s group, Tmod is 115 Å long and consists of two domains, one 65 Å long, the other slightly smaller, where the long axes of the two are tilted by $\approx 40^\circ$ relative to each other and is illustrated in Figure 16. The authors proposed a model for Tmod in which the rod-shaped NH$_2$-terminal half binds to the NH$_2$ terminus of tropomyosin, and the COOH-terminal region protrudes from the pointed end of the actin filament and is slightly bent toward the actin subunit at the end, capping the pointed end (101). There appears to be 1–2 Tmod molecules/actin filament (98).

Both CapZ and Tmod can regulate filament length and assembly in vitro and in vivo. By studying the incorporation of rhodamine-labeled actin monomers into native thin filaments of cardiomyocytes, Littlefield et al. (164) concluded that both proteins can rapidly exchange, i.e., at their respective ends. Blocking barbed ends with cytochalasin D had no effect on filament length, whereas overexpression of Tmod reduced actin filament length, possibly because Tmod promotes the conversion of ATP or ADP-P$_i$ monomers to ADP with a consequent raising of the critical concentration from 0.1 to 1.0 µM (241).

G. The Arp2/3 Complex

Arp2 and Arp3 belong to a recently discovered family of proteins that are related, at least in a limited way, to the sequence and structure of actin. Their cellular concentrations are 2 and 5 µM, respectively (171). These two actin-like proteins are assembled into the Arp2/3 complex, which is known to occur in a wide range of organisms from yeast to mammals. The seven-subunit protein complex comprises Arp2 and Arp3 and five smaller (Arc) proteins that do not appear to be related to any proteins currently in the databases. Both Arp2 and Arp3 sequences have nucleotide-binding and divalent cation-binding regions, but otherwise they are divergent from each other and from actin (143). Arp2/3 is associated with dynamic cortical regions of mammalian cells that are rich in cytoskeletal actin (69). It colocalizes with the actin “comet tails” of *Listeria* and *Shigella* where actin polymerization drives filament assembly and propels these intracellular bacterial pathogens through the cytoplasm of the host cell at speeds of 10–90 µm/min (302).

1. Structure

The structure of the Arp2/3 complex at 2.0 Å resolution was only recently described (244). It is remarkable that a complex this big crystallized at all. Proteins were extracted from bovine thymus gland, and the isolated complex was in an inactive state when it was crystallized. Conversion to an active form is a matter of speculation but will probably involve a rearrangement of several subunits to allow the Arp2 and Arp3 to be repositioned so that they can act as nucleation site for the assembly of new filaments. This rearrangement will also be needed if the Arp2/3 complex is to “capture” preexisting filaments and incorporate them with the correct orientation toward the advancing cell membrane. The structure is now deposited in the Protein Data Bank (PDB accession number 1k8k).

Briefly, the structure has the following features. The overall shape of the complex is a flat ellipsoid with dimensions of 150 × 140 × 70–100 Å. The structures of Arp2 and Arp3 strongly resemble the actin monomer. Arp2 has a strong resemblance to subdomains 1 and 2 of actin except that the interdomain cleft is more open (by $\approx 18^\circ$) and contains no bound ATP. It also has a profilin binding domain (143). Arp3 contains large inserts into subdomains 2, 3, and 4 and has 42 residues more than actin. The
cleft is less open (~12°), a feature that lowers its affinity for ATP. Subdomains 1 and 2 were difficult to resolve in the crystals, and the authors replaced them with a polyalanine backbone. These and other features give the Arps distinctive surface features. In mammalian cells, the five associated proteins are p16, p20, p21, p34, and p40 (proteins are also called ARPC-5, -4, -3, -2, and -1, respectively) of which the last is a seven-blade propeller structure with a large insert that may be responsible, along with p20 and p21, for anchoring the complex to the side of the actin filament. p20 and p34 are located in the center of the structure and may move as a block relative to Arp2 (244).

2. Cellular functions

The principal function of Arp2/3 is to create branch points by nucleating the assembly of filaments near ruffling membranes, a process that needs micromolar concentrations of ATP (195). Arp2/3 is also considered to be a cross-linking protein, and some authors believe it can cap the pointed (depolymerizing) ends of actin filaments, although this is still controversial.

Nucleation of filament assembly by Arp2/3, first observed by Welch et al. (302), is unlike any other ABP. Extracellular signals such as growth factors are transmitted through G protein-coupled receptors (e.g., Cdc42) to Wiskott-Aldrich syndrome protein (WASP), the neuronal homolog of WASP (N-WASP), or suppressor of cAMP receptor 1 (Scar1). PIP₂ may be also involved in the activation of WASP (242). Activated WASP has sites that bind both an actin monomer and the Arp2/3 complex. Arp2/3 then changes its conformation so that it can nucleate the assembly of new actin filaments and assemble a "daughter" filament at an angle of ~70° to the "mother" filament (Fig. 17). As a consequence, Arp2/3 is localized at branch points in the cortical filament network, binding the pointed end of a new (uncapped) daughter filament to the mother filament thus leaving the barbed (growing) end available for filament elongation. It is not clear at this stage whether WASP dissociates from activated Arp2/3 at the branch point.

Zalevsky et al. (314) recently examined how N-WASP and Scar1 differentially activate the Arp2/3 complex to produce actin networks with different three-dimensional architectures. They found that nucleation was slowest for Scar1, 16-fold higher for WASP, and 70-fold higher still for the neuronal analog N-WASP. Their data fit a mathematical model in which one activated Arp2/3 complex, one actin monomer, and an actin filament combine into a preactivation complex. This then undergoes a first-order activation step to become a nucleus. They went on to

![Diagram of the molecular interactions Arp2/3. Components are as follows: activated Arp2/3 (red), inactive Arp2/3 (pink), N-WASP (green), Cdc42 (blue), phosphatidylinositol 4,5-bisphosphate (PIP₂) (yellow), and actin (orange). Inactive Arp2/3 is activated by N-WASP under the control of Cdc42 and PIP₂, and filament formation is nucleated. Actin filaments assemble by addition of monomers at the barbed end. The N-WASP-Arp2/3 complex can bind to the side of the "mother" filament to create a branch point that also polymerizes. This branching assembly creates an "out-pushing" of the cell membrane. The branching of "daughter" filaments occurs at an angle of 70° to the mother filament. The solid black arrows indicate the directions of the polymerization forces.](http://physrev.physiology.org/)

**Fig. 17.** A cartoon of the molecular interactions Arp2/3. Components are as follows: activated Arp2/3 (red), inactive Arp2/3 (pink), N-WASP (green), Cdc42 (blue), phosphatidylinositol 4,5-bisphosphate (PIP₂) (yellow), and actin (orange). Inactive Arp2/3 is activated by N-WASP under the control of Cdc42 and PIP₂, and filament formation is nucleated. Actin filaments assemble by addition of monomers at the barbed end. The N-WASP-Arp2/3 complex can bind to the side of the "mother" filament to create a branch point that also polymerizes. This branching assembly creates an "out-pushing" of the cell membrane. The branching of "daughter" filaments occurs at an angle of 70° to the mother filament. The solid black arrows indicate the directions of the polymerization forces.
show that WASP and Scar1 bind actin and Arp2/3 with nearly identical affinities but stimulate rates of actin nucleation that vary by almost 100-fold. Differences in nucleation rates were attributed to differences in the number of COOH-terminal acidic amino acids that are responsible for different rates of actin assembly. The authors therefore concluded that Arp2/3 is not regulated by a simple on-off switch.

In the vicinity of the ruffling membrane, new monomers are added to the barbed-end of the growing filament creating a polymerization-based “out-pushing” of the cell membrane (Fig. 17). The Y-shaped branch points produce a cross-linked meshwork of filaments that act as a platform against which the actin polymerization can push. The entire Arp2/3 complex along with WASP have been observed in electron microscopic images of branching filaments (293). Mullins et al. (195) refer to this process of branching filaments as “dendritic nucleation.” Assembly of monomers at the barbed ends of these filaments generates the force needed to push the ruffling membrane forward. Branching may occur at the barbed ends or along the side of the mother filament (or both). Ishiwata and co-workers (103) recently addressed this question by the directly visualizing actin assembly using total internal reflection fluorescence microscopy. In the presence of Arp2/3 and N-WASP, branching occurred predominantly along the sides of growing filaments.

Nucleation and branching are carried out close to the leading edge of motile cells. Arp2/3 is not generally associated with filaments deeper into the cortex of the cell where the actin filaments are known to bind tropomyosin. A recent report (28) demonstrated that tropomyosin located deeper into the cytoplasmic cortex inhibits branching and nucleation by WASP-activated Arp2/3, thus restricting its distribution to the cytoplasmic perimeter.

In Listeria, nucleation of new filaments occurs in response to the expression of ActA that mimics the function of WASP in recruiting Arp2/3. ActA contains 612 amino acids and is anchored into the bacterial membrane. In a fascinating series of experiments, Cameron et al. (39) showed that small microspheres coated with purified ActA could undergo movement generated by actin polymerization. Thus ActA alone can nucleate actin assembly. The minimum set of proteins and conditions needed for actin-based motility was recently defined for the propulsion of Listeria by Marie-France Carlier and colleagues (166). ATP, Arp2/3, actin filaments with bound ADP, ADF/cofilin, and a capping protein (e.g., CapZ) were all that was required. ADF/cofilin accelerates the treadmilling of monomers through the filaments (see above) and drives the Listeria through the actin-rich cytoplasm of its host cell. A review from this group (220) highlighted the complexity of the mechanism of actin assembly in cell biology. Clearly other intracellular factors such as profilin and α-actinin regulate the rates of actin assembly in these “comet tails” (the review by Pantaloni et al. is available at www.sciencemag.org/cgi/content/full/292/5521/1502/DC1).

The role of WASP/Scar proteins is critical for cytoplasmic organization in a range of organs during development in Drosophila (315) and presumably Arp2/3 and the WASP family of proteins play essential roles in the development of mammalian cells.

Several reviews have recently been published on the role of Arp2/3 in actin polymerization and the factors that control the functions of Arp2/3. These include the role of WASP family of proteins (50, 125, 242), and a review of the structure of Arp2/3 itself (31).

Thus Arp2/3 is a complex protein with an even more complicated method of activation. Single-headed myosin I motor proteins interact with Arp2/3 via their Scar-homology 3 (SH3) domain (160), but what are motor protein doing in a process that is usually regarded as an actin-only process? Certainly there is no evidence to suggest that Arp2/3 is involved in muscle contraction, although it clearly plays a role in actin-based motility.

Several other questions remain. What is the nature of the conformational changes needed to activate Arp2/3? Can Arp2/3 promote filament growth at the pointed ends of filaments? What is the role of profilin? What is the role of ATP hydrolysis, and is this related to changes in the putative nucleotide cleft in Arp2 or Arp3? Does activation of filament nucleation produce filament growth at the barbed or pointed end? Why do branch points occur where they do? How do Cdc42 and PIP2 cooperate to release the inhibitory effect of WASP on Arp2/3?

IV. ROLE OF TERNARY COMPLEXES IN REGULATING ACTIN CYTOSKELETON ASSEMBLY

The foregoing discussion has probably created the impression that regulation of assembly and disassembly of the actin cytoskeleton is complex. In fact, the complexity may be comparable to the system that controls gene expression in eukaryotic genes where a promoter element contains the promoter-proximal upstream control elements as well as enhancers and silencer elements. A further level of regulation is then provided by distal regulatory sequences. DNA-bound proteins that can alter the state of condensation of the chromatin DNA also influence these DNA sequences. The control of actin cytoskeleton assembly/disassembly may be even more complex.

Just as we do not have a precise understanding of the control of gene expression, we are also only beginning to understand the control of location and mechanism of assembly of the actin cytoskeleton. Not only are there potentially multiple ABPs, but they are also capable of simultaneous expression within a single cell. The presence of actin ligands such as ATP or ADP, Ca2+ or Mg2+ ...
can also be important, as well as pH. Under these conditions, it would be surprising if control of the state of assembly was left to single ABPs as suggested in the preceding sections. The next level of complexity to consider is whether ternary complexes play a role and if so how is this achieved. Of course, quaternary and higher orders of complexity may also exist. There can be no argument that complexes of more than one ABP control polymerization. The ternary complex may be destabilized by other ABPs such as profilin or gelsolin, which alter the bound nucleotide presumably via an allosteric conformational change. Such a regulatory mechanism would occur on a slow time scale equivalent to the rate of appearance and disappearance of DNase I and would depend on the balance of free ABPs, pH, and divalent cations present in the cell.

A. Cofilin, Actin, and DNase I

It is widely accepted that regulation of the rate of assembly/disassembly (treadmilling) of the actin cytoskeleton is achieved by a single ABP, probably ADF/cofilin (21). However, the widespread use of DNase I affinity chromatography to capture complexes of actin with other ABPs (cofilin, destrin, actobindin, adseverin, fimbrin, and radixin) suggests that ternary complexes exist, at least in vitro (133). We recently reported the formation of ternary complexes of actin, cofilin, and DNase I (57) using nondenaturing polyacrylamide gel electrophoresis. The affinity of DNase I for G-actin is high (nM $K_d$), significantly higher than for actin-cofilin ($\mu$M) (206). However, the affinity of cofilin for the binary DNase I-actin complex is higher than for actin alone, and the reverse is also true, namely, the affinity of DNase I is higher for the actin-cofilin complex than for actin alone. Thus when DNase I is present in a mixture of actin monomers and the cofilin-actin binary complex, it binds to the binary complex forming a ternary (cofilin-actin-DNase I) complex before it binds to the remaining free actin (205). This ternary complex was recently suggested based on colocalization in monkey kidney epithelial cells (56).

In a report only just completed, we (81) have documented the magnitude of actin conformational changes as a consequence of specifically binding cofilin and DNase I and in the ternary cofilin-actin-DNase I complex. We used fluorescence and fluorescence resonance energy transfer spectrosopy to demonstrate significant conformational changes in the small domain (subdomains 1 and 2) of monomeric actin. DNase I binding to actin increases by $\sim 1$ Å the distance between probes attached to Gln-41 and Cys-374. When cofilin alone binds to actin, this distance decreases by $\sim 3$ Å, but when both bind (ternary complex) the distance increases by $\sim 4$ Å. This effect is completely reversible and is independent of the order of addition. Thus the effects are not simply additive. We speculated that DNase I may act as a regulator of actin-cofilin interaction and that the ternary complex may therefore be an important element in the regulation of microfilament assembly (142), effectively removing cofilin from the cellular pool and thereby preventing its normal ability to polymerize. The ternary complex may be destabilized by other ABPs such as profilin or gelsolin, which alter the bound nucleotide presumably via an allosteric conformational change. Such a regulatory mechanism would occur on a slow time scale equivalent to the rate of appearance and disappearance of DNase I and would depend on the balance of free ABPs, pH, and divalent cations present in the cell.

B. Ternary Complexes of Actin With Two or More ABPs

The actin-DNase I binary complex is unusual but not unique in its ability to form a ternary complex with cofilin. Profilin can be chemically cross-linked to actin-DNase I; however, in the absence of cross-linking, a negative cooperativity was observed (12), i.e., profilin dissociates DNase I from actin, and vice versa. DNase I and gelsolin also bind to actin in a negatively cooperative way. Chemical cross-linking of Cys residues between Tβ4 and actin-DNase I also revealed the formation of a ternary complex (239). These authors found that the $K_d$ of Tβ4 for actin-DNase I was the same as for actin alone (see Table 1), and they concluded that the binding loci for these two ABPs do not overlap. Others (13) reported that Tβ4 competes with gelsolin segment 1, but both are dissociated by the binding of DNase I to actin. A similar negative cooperativity between spatially separated actin-binding sites was observed between DNase I and gelsolin segment 1 (12). Thus, although the binding site for DNase I is physically separated from the sites for gelsolin segment 1, Tβ4, and profilin, the negatively cooperative nature of their interactions suggests that an allosteric conformational change may be transmitted along the “length” of the small domain of actin.

Capping of the pointed ends of actin filaments by tropomodulin (Tmod, discussed above) appears to involve a ternary complex. Tmod binds only weakly to F-actin, but the affinity of Tmod for actin increases nearly three orders of magnitude in the presence of tropomyosin. Rapid exchange of Tmod at this end permits assembly and disassembly of these filaments. The same is probably true for CapZ and other proteins that cap the barbed end of filaments.

In a two-hybrid system, actin interacting protein 1 (Aip1; a 65-kDa protein) was shown to form a ternary complex with actin and ADF/cofilin (245). In yeast, Aip1 interacts with actin via subdomains 3 and 4 (5), and some of these residues do not overlap those for ADF/cofilin and...
other ABPs. AIP1 alone has little or no filament severing activity, but it exhibits a cooperative filament severing activity in the presence of Xenopus ADF/cofilin (245). This ability of Aip1 to recognize ADF/cofilin bound to filaments may be important in the modulation of ADF/cofilin activity.

Drugs like cytochalasin D act directly on the actin cytoskeleton by altering the rate of assembly/disassembly at both the pointed and barbed ends of the filaments. A recent structural analysis of latrunculin-A (193) revealed that it binds only to actin monomers and prevents polymerization by inducing specific structural changes. The binding site was not previously known to be an ABP binding locus. Crystals of actin-gelsolin segment 1 were soaked in latrunculin-A, and crystallographic refinement of the resulting ternary complex revealed that it tightly links subdomains 2 and 4 of actin. Latrunculin-A produces local rather than global structural changes in actin, allosterically altering the surfaces involved in the assembly of F-actin. This effect is achieved by restricting the rotations of subdomains 2 and 4. Thus ternary complexes involving actin appear to be important for the action of some drugs.

V. THE CYTOSKELETON AND PATHOLOGY

Many of the proteins discussed above are affected by mutations resulting in the development of significant pathology. Given the central role played by actin and the ABPs, this outcome is not surprising (see Ref. 21a).

A. Actin

Actin has a remarkably conserved amino acid sequence, probably more than any other protein. This alone suggests that mutations have been eliminated rather than tolerated by the genome. Because actins are essential for motility, force generation, and other important cellular functions, it is remarkable that so few diseases are associated with primary defects in microfilaments and their regulators. No doubt, part of the reason is that there are usually multiple actin genes, i.e., the problem is circumvented by redundancy.

Perhaps the same evolutionary pressures that maintained the highly conserved sequences of actins also protected them from being a major player in the disease process. Because of its pivotal role, defects or alterations in actin probably have dramatic effects on survival when no alternate actin gene is available (e.g., yeast has a single actin gene which is lethal when disrupted).

A major obstacle to studying the altered properties of mutant actins is the considerable difficulty cell biologists have had in expressing actin using standard protein expression systems. Bacteria have no actin of their own and are therefore a logical choice as an expression system. Only one report (100) suggested that bacterial expression could yield native actin, and the findings of this report have not been repeated. Yeast can be used to express mutant actins (246), although the yield of the inserted actin gene product is impractically low.

There are mouse fibroblast cell lines that express almost pure nonmuscle β-actin (252), but these are special cases. Most cells express more than one actin isoform and, although several methods have been described for separating them, for example, hydroxyapatite column chromatography (260), they are technically challenging. Some actin mutations (for example, removal of the NH2-terminal acidic residues) are not essential for the survival of yeast but are needed to activate myosin ATPase activity (64).

More than 150 papers have reported ~300 actin mutations (266). Only a handful deal with human actin mutations, and most of these are deletion mutants near the COOH terminus. Three at residues 336, 346, and 356 resulted in the production of nonfunctional actin. The remaining deletions at residues 366, 373, and 374 had significantly reduced functionality. In a recent review, Marston and Hodgkinson (178) observed that 13 actin mutations were known to cause hypertrophic cardiomyopathy (E99K, A331P, P164A, A295S), dilated cardiomyopathy (R312H, E361G), severe nemaline myopathy (R183C, R256H, N280K, V370F, L94P), and actin myopathy (G15R, V163L). Clearly, caution is needed in drawing sweeping conclusions about the functional effects of these structural changes. However, when these mutant actins can be expressed in substantial quantities, we can begin to understand the functional consequences of mutant actins in organs where more than one actin gene is coexpressed (25).

B. Gelsolin

If the actin cytoskeleton plays a significant role in disease, and in particular tumors (215), then we would also expect its associated ABPs to be involved in the disorder or disease. The functions of gelsolin have been described above, but it is also a substrate for the apoptotic enzyme caspase-3 (150) that cleaves the actin-binding domain of gelsolin from its calcium-binding domain. Physical separation of these two domains coincides with plasma membrane blebbing, one of the hallmarks of apoptosis. Overexpression of the Ca2+-independent NH2-terminal half of gelsolin induces apoptosis, whereas gelsolin null neutrophils have a delayed onset of apoptosis (150). Cancer cells characteristically lack apoptotic mechanisms and express less gelsolin (157), whereas expression of mutated (His-32I) gelsolin is associated with the loss of tumorigenicity of transformed cells (102).

Normally, plasma gelsolin functions as a part of the
actin-scavenging system to assemble and disassemble actin filaments. The D187N and D187Y gelsolin mutations generate a 71-residue fragment that forms amyloid fibrils in humans. These mutations were also associated with familial amyloidosis of the Finnish type (FAF) (139). The symptoms of FAF patients include the accumulation in their tissues of amyloid derived from plasma gelsolin (285). Neither the recombinant wild-type nor the D187N FAF-associated gelsolin fragments self-associate into amyloid at pH 7.5, but incubation of either fragment at low pH values (6.0–4.0) produces well-defined fibrils (237).

C. Tropomodulin

Changes in gene expression of this thin filament capping protein produce a surprisingly extensive range of phenotypes. Increases in Tmod expression levels are associated with the dilation (rather than the hypertrophic) component in human dilated cardiomyopathy (300). In a transgenic mouse model, inhibition of Tmod expression prevents dilation (279) but not hypertrophy of the myocardium. Tmod is a promising development in our understanding of this baffling disorder (278).

Graves’ disease is a complex autoimmune disease characterized by multiple susceptible loci involving cytotoxic T cells, HLA antigens, and thyroid-stimulating hormone receptors (299). Human Tmod has ~40% identity at the NH₂ and COOH termini of the 64-kDa autoantigen protein identified in Graves’ disease. The insertion of several homologous repeats in the midsection of the Graves’ protein together with an extension of the COOH terminus of Tmod accounts for the differences in length between the Graves’ protein (572 residues) and tropomodulin (359 residues) (299). It is therefore likely the two genes evolved from a common ancestral gene (276). The gene for Tmod was assigned to human chromosome 9 in a region also known to contain several other genes and disease loci, for example, an unrelated cytoskeletal gene for gelsolin.

Finally, two genes encoding for Tmod that map next to another on human chromosome 15 make them candidates for amyotrophic lateral sclerosis and a dyslexia gene as well as a candidate gene for limb girdle muscular dystrophy (70). At this stage it is not clear how these syndromes are related to Tmod expression disorders, but the clear link suggests that Tmod is a key gene.

D. Connections Between the Extracellular Matrix and the Nucleus

Maniotis et al. (175) showed that a mechanical tug on cell surface receptors can immediately change the organization of proteins in the cytoplasm and the nucleus. They showed that by specifically pulling on cell surface integrin receptors, the nucleus was elongated in the direction of the pull. Furthermore, they showed that this effect on the nuclear membrane was mediated by the actin cytoskeleton. The actin cytoskeleton also provides a physical connection between ion channels such as CIC3 chloride ion channels in the cell membrane and the interior of the cell (33).

Defects, deficiencies, or deletions of any component of the linkage between the extracellular matrix (laminin-2, α-dystroglycan), trans-sarcomemal proteins (α-, β-, and γ-sarcoglycans, β-dystroglycan), the cytoskeleton (dystrophin, dystrobrevin, syntrophin, actin), and the nucleus (emerin, lamin A/C) would dramatically alter this putative mechanical signaling (175) and thus affect the mechanical stability of cardiomyocytes.

Altered expression of cytoskeletal, linkage, and extracellular proteins has been reported in hypertrophied cardiomyocytes (280, 282) and also in failing myocytes (61, 83, 122). Changes in expression levels of fibronectin, laminin, fibrillin, fibrulin SC1, and decorin, all components of the extracellular matrix, are upregulated in rat experimental myocardial infarctions (269). A number of monogenic cardiomyopathies have recently been detailed in a selected number of families, which include a range of different genes involved in the surface-nucleus pathway (55). Thus cytoskeletal proteins may be involved in the progressive defects leading to heart failure. Hypertrophic cardiomyopathy appears to be a final common outcome for gene defects involving an astonishing variety of cytoskeletal and sarcomeric proteins.

E. Heart Failure

Familial hypertrophic cardiomyopathy (FHC) is an autosomal dominant disorder resulting in increased myocardial mass and myofibrillar disarray. It is the most common cause of sudden death in the young. Linkage studies and candidate-gene approaches have revealed disease-causing mutations in more than eight different genes encoding sarcomeric proteins. More than 100 mutations have already been identified, and they involve practically every protein component in the sarcomere. Mutations are observed in β-myosin heavy chain (105), troponin I (144, 92), troponin T (238), α-tropomyosin (282), myosin-binding protein C (296), regulatory and essential myosin light chains (226), cardiac α-actin (209), titin (253), and desmin (122, 253). However, these mutations are present in only half the patients (296), and consequently, there must be other gene mutations that cause FHC, some of which will probably involve the cytoskeleton. The common phenotype resulting from a wide range of cytoskeletal/sarcomeric proteins may well prove to be a reflection of the functional linkage of these proteins to both the extracellular matrix and the nucleus in cardiomyocytes.
Studies of human dilated cardiomyopathy (DCM) (306) and of mouse models (279) of DCM also point to a critical role of cytoskeletal elements at the Z-disks in chamber dilation. Mice lacking the muscle LIM-domain protein (MLP), a Z-disk protein that interacts directly with \( \alpha \)-actinin, displayed many of the features of human DCM (8). Mutations (usually more than one per protein) in other proteins associated with \( \alpha \)-actinin, cardiac \( \alpha \)-actin, desmin, and titin were subsequently found in familial forms of DCM (76, 209, 253). Also, mutations of other Z-disk components in the mouse result in DCM (55, 59). Mechanical stress on the extracellular matrix leads to defects in Z-disk formation, sarcomeric stabilization, and altered muscle-cell signaling, thereby driving the onset of chamber dilation and heart failure (58). In a dog model of heart failure, cytoskeletal actin was upregulated while desmin (a protein in the intercalated disks) was down-regulated (122).

F. Gene Arrays

Yang et al. (306) used gene array technology to examine altered genes in failing human hearts. They observed an increased expression of gelsolin and a significant upregulation of \( \alpha \)-B-crystallin, a desmin-associated protein that protects or chaperones actin and desmin filaments from stress-induced damage. \( \alpha \)-B-crystallin is a member of 27-kDa heat shock protein family. These data (using Affymetrix gene arrays) were confirmed by us (using NEN arrays) (135). They support the view that the actin cytoskeleton is damaged in DCM. Samples of failing left ventricles have been studied by two-dimensional gel electrophoresis of human DCM patients (68) and of an animal model of DCM (122). In another form (familial hypertrophic) of human heart failure, the expression of \( \alpha \)-actin, plasma gelsolin, thymosin \( \beta 4 \), and several other cytoskeletal proteins (23) changed more than twofold. Finally, gene arrays have been used to examine an experimental myocardial infarction using failing rat hearts (269). Cytoskeletal actin and several ABPs (\( \alpha \)-actin, thymosin \( \beta 4 \) and \( \beta 19 \), Arp2/3 and a number of other ABPs not reviewed here) were upregulated. The data clearly implicate the involvement of cytoskeletal and myofibrillar genes in human heart failure. Thus there is growing evidence for altered expression of cytoskeletal proteins in heart failure. It is likely that similar results will be reported for the failure of other organs.

G. Single Nucleotide Polymorphisms, Diseases, and the Cytoskeleton

A relatively new method of determining the connections between human diseases and gene mutations involves analysis of single nucleotide polymorphisms (SNPs) from human populations. The idea here is that SNPs (unlike most other analytical methods) are potentially capable of discovering the cause of disease.

A number of companies have developed technologies for analyzing SNPs, but the most challenging aspect of the technology is its ability to accurately detect very small differences between DNA restriction fragments in which only one base pair has been replaced (e.g., T instead of A or G instead of a C base pair, or vice versa). Sequenom (www.sequenom.com) has developed a method that very accurately “weighs” the DNA fragments using mass spectrometry which achieves precisions of >99.9%. It is debatable whether this accuracy is really needed, although their approach was both unusual and effective. They have identified at least three cytoskeletal proteins that are statistically associated with specific diseases in specific human populations. These diseases have a late onset and are not associated with mortality in infants, juveniles, or young adults.

H. Cell Signaling and Actin Microfilaments

In mammals, there is a well-defined connection between extracellular signals (like insulin), their membrane receptors, intracellular signals (Ras and Rac), and the regulators of actin microfilament assembly (Arp2/3, gelsolin, ADF/cofilin). Because the binding of ligands to some surface receptors is known to activate genes, the cytoskeletal links may extend from the membrane all the way to the nuclei and perhaps even to the chromosomes. The control of these connections in yeast, Drosophila, and mammalian cells was recently summarized by Hall and colleagues (24). The relationships between components of the signaling pathways are complex and are beyond the scope of this review.

VI. UNRESOLVED ISSUES

A. Atomic Structure of F-actin

It is common to assume that because we know all or most of the structural atomic details of the actin monomer, the available models of F-actin are equally valid. How good is this assumption?

It is true that the known atomic structures of monomeric actins are very similar. However, significant differences are beginning to emerge. Nearly all of the available atomic structures were achieved using actin co-crystallized with an ABP. Recently, a new structure of actin was reported (217) that is not complexed to an ABP. These authors observed “a dramatic conformational change in subdomain 2” compared with previous structures. It will therefore be of considerable interest to see the effect the
new structure from Dominguez et al. (217) has on a model of F-actin with its bound ADP. Bound nucleotide can have dramatic effects on the structure of actin and on the affinities of ABPs for actin. Three-dimensional reconstructions of electron micrographs of actin filament are based on low-resolution structural data. Thus the available models make assumptions about the arrangement and orientation of the monomers in the filament. To date, most filament models (128, 167, 283) have been constructed using the structures from actin cocrystals with ABPs, and there is an assumption that this is unaltered in the filament. Arguably, filaments are the most biologically relevant form of actin in cells, so there is a real need to resolve their structure at high resolution. Several groups are currently working on this most difficult, perhaps intractable, structural problem.

B. Functional Differences Between Actin Isoforms

The great majority of the several thousand references on this topic have used rabbit skeletal muscle actin. Only very small sequence differences exist between different actin isoforms, and these probably have functional consequences. For example, the myocardium of small mammals like mice and rats expresses almost pure cardiac α-actin, whereas cardiomyocytes in larger animals (including humans) express about equal amounts of cardiac and skeletal α-actin (25, 113). Why do larger hearts express skeletal muscle actin? γ-Actin isoforms are located just beneath the sarcolemma, whereas only γ-actins are incorporated into sarcomeres (113). We do not know if isoforms can coassemble or whether such filaments have different effects on activities such as myosin ATPase. Very little is known about the functional differences between actin isoforms. Even less is known about whether certain ABPs bind to particular actin isoforms and whether differences in actin isoform structure determine which ABPs bind.

The situation is no clearer for ADF/cofilin. We know there are functional differences (nucleation, elongation, fragmenting, binding ratios to actin) between ADFs and cofilins, but comparisons based on existing literature are difficult because groups investigating these properties often use different (yeast, chick, human) isoforms. There may also be differences between isoforms within the cofilins and ADFs, and these will have subtle but important functional effects.

C. Ternary Complexes of Monomeric Actin With ABPs

We have reviewed the evidence for a ternary complex between actin monomers and two ABPs, cofilin and DNase I. Although there is good evidence for a ternary complex involving monomeric actin in vitro, is there a role for ternary complexes in vivo? The more details we learn about the ABPs, the more they seem to fit into families with common cellular functions. Not all DNases bind actin, so it is still not clear if they can play a universal role.

D. Undiscovered ABPs

Despite the proliferation of ABPs over recent years (65 new proteins have been discovered over the past 5 years, Ref. 151), there are still significant areas of the actin monomer surface that do not appear to be the site for ABPs (see Fig. 6). Some of this surface may come to be occupied by known but not yet fully characterized ABPs. It is unlikely that we have discovered all of the ABPs, and it seems a safe bet to suggest that as the human genome and other genomes become refined, at least some of the “orphan” genes will turn out to be new ABPs.

E. Cooperative Binding of ABPs Along Filaments

There is a spatial separation and organization of filament nucleation, elongation, branching, and other processes involving actin assembly and disassembly, but little or nothing is known about the controls of these processes. For example, we know that Arp2/3 produces a regular branching of actin microfilaments and that this regular branching is important for the protrusion of cytoplasmic blebs and fingers (195), but what controls the distribution of Arp2/3? In muscle thin filaments, binding of a myosin head at one point alters the probability of a second head binding up to several monomers along the filament. What conformational changes are involved here, and how are they propagated along the filaments? ADF/cofilin binds cooperatively to F-actin, but does this cooperativity depend on the actin isoform?

F. Cytoskeletal Proteins in the Nucleus

A number of cytoskeletal proteins are found in the nucleus including cofilin (56, 203) (which has a nuclear localization peptide) and actin (which lack this sequence). We do not know the functions of these proteins or how they are regulated.

G. Disease and the Cytoskeleton

The role of the cytoskeleton and ABPs in disease is an emerging story. There can be little doubt that several cytoskeletal proteins are positively associated with significant human disease. Sequenom of San Diego and Gemini Genomics PLC of Cambridge recently discovered three of
these proteins, but their identities have not yet been disclosed. However, it is possible that identification of specific disease-associated genes may not produce a complete understanding of the disease process for we know that a single phenotype like hypertrophic cardiomyopathy can be the product of mutations in almost one of the myofibrillar proteins.

H. Prokaryotic Cytoskeletal Elements

It seems likely that the ancestor of eukaryotic actin is the bacterial protein MreB. This protein can self-assemble and contributes to structures that resemble straightened actin rails. This discovery prompts questions like: Are there MreB-binding proteins, equivalent to ABPs, that control filament assembly? Do bacteria have motor proteins that employ the MreB “rails”?1

We are grateful to Roger Craig, Roberto Dominguez, Shin’ichi Ishiwata, Glenn King, Steve Marston, Takashi Obinata, Tetsura Fujisawa, Alla Kostyukova, Yuichiro Maeda, Bob Robinson, Dan Safer, Fusinita van den Ent, and Willy Wriggers for many of the figures used here and for valuable contributions on the manuscript and for discussions.

We are grateful for the support of the National Health and Medical Research Council of Australia, the Australian Research Council, the National Heart Foundation of Australia, and the Medical Foundation of the University of Sydney.

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REFERENCES


29. BLANCHON L, ROBINSON RC, CHOE S, AND POLLARD TD. Phosphoryla-


45. Collins JF, Pavloski-Dair D, Davis MG, Ball N, Dorn GW II, and Wals RA. The role of the cytoskeleton in left ventricular pressure overload hypertrophy and failure. J Mol Cell Cardiol 28: 1435–1443, 1996.


61. De la Cruz EM, Oost EM, Brundage RA, Riedy KS, Sweeney HL,


134. JEAN C, RIEGER K, BLANCHOIN L, CARLIER MF, AND PAN-


239. Rechert A, Hertz D, Eichner H, Voelter W, and Faulstich H. The


