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MOLECULAR MECHANISMS CONTROLLING ACTIN FILAMENT DYNAMICS IN NONMUSCLE CELLS

Thomas D. Pollard,[1] Laurent Blanchoin,[1] and R. Dyche Mullins[2]

[1]Structural Biology Laboratory, Salk Institute for Biological Studies, La Jolla, California 92037; e-mail: pollard@salk.edu; [2]Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California 94143; e-mail: dyche@itsa.ucsf.edu

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■ **Abstract** We review how motile cells regulate actin filament assembly at their leading edge. Activation of cell surface receptors generates signals (including activated Rho family GTPases) that converge on integrating proteins of the WASp family (WASp, N-WASP, and Scar/WAVE). WASP family proteins stimulate Arp2/3 complex to nucleate actin filaments, which grow at a fixed 70° angle from the side of preexisting actin filaments. These filaments push the membrane forward as they grow at their barbed ends. Arp2/3 complex is incorporated into the network, and new filaments are capped rapidly, so that activated Arp2/3 complex must be supplied continuously to keep the network growing. Hydrolysis of ATP bound to polymerized actin followed by phosphate dissociation marks older filaments for depolymerization by ADF/cofilins. Profilin catalyzes exchange of ADP for ATP, recycling actin back to a pool of unpolymerized monomers bound to profilin and thymosin-β4 that is poised for rapid elongation of new barbed ends.

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INTRODUCTION

One important function of the actin cytoskeleton in eukaryotic cells is to drive locomotion by the extension of pseudopods. Unicellular organisms use pseudopods for directed motility to find and engulf food. In multicellular organisms, many different processes depend on cell locomotion, including morphogenetic movements during embryonic development, movement of neurites during development and remodeling of the nervous system, chemotactic movements of immune cells, and fibroblast migration during wound healing. Depending on their morphology and the cellular context, pseudopods, are called (rather loosely) lamellipods, leading lamellae, growth cones, or ruffles.

Pseudopod extension requires assembly of a specialized network of actin filaments at the forward or leading edge of the cell. Actin filaments are polarized. Based on the arrowhead pattern created when myosin binds actin filaments, the rapidly growing end of a filament is called the barbed end. The slowly growing end is called the pointed end. Elongation of the barbed end of actin filaments drives membrane protrusion (133). To generate directed motility in response to external cues like chemoattractants, cellular signaling pathways must control actin polymerization and depolymerization. Within seconds such stimuli can initiate directed movements from any site on the surface of a motile cell. When a stimulus ceases or changes direction, pseudopod extension stops just as quickly (41).

At first glance, the filament network underlying the leading edge of motile cells appears chaotic (Figure 1). Careful examination, however, reveals a remarkable degree of order and a number of important structural features that appear to be conserved across eukaryotic phyla. The best studied examples are the leading edges of the fish and amphibian keratocytes (126, 130, 128). These cells are ideal for both light and electron microscopy. They undergo rapid, constitutive motility and assemble a large, extremely thin lamellipod at the leading edge. The actin filament network in these lamellae is organized into two roughly orthogonal arrays, with the rapidly growing barbed ends pointing toward the membrane. The filament network is especially dense near the inner surface of the membrane, and the actin filaments are connected in a highly cross-linked, branching arbor with short filaments apparently growing from the sides of other filaments (130). These general features are conserved in vertebrate tissue culture cells (6, 130) and protozoans (28). The concentration of polymerized actin is about 1000 μM (1a).

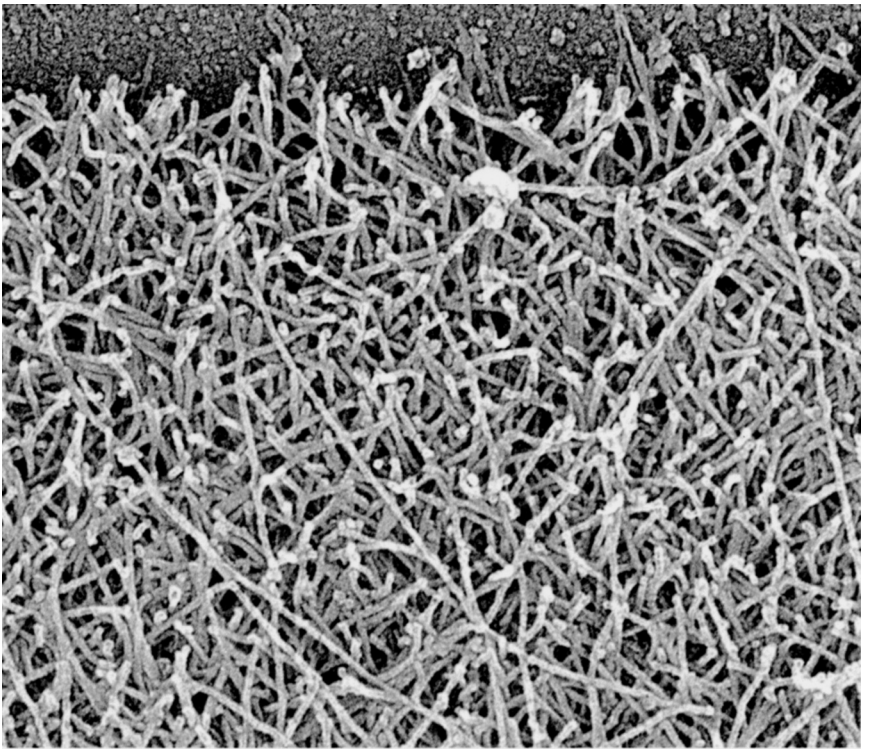

Figure 1 Electron micrograph of the leading edge of a migrating keratocyte prepared by detergent extraction and rotary shadowing. (Modified from 130.)

In a test tube, polymerizing actin with a cross-linking protein like α-actinin produces either an isotropic gel of filaments or parallel bundles (137), so how do cells use these proteins to assemble an ordered and polarized structure? The key to ordered network assembly is a cellular component that initiates polymerization and cross-linking in a highly localized manner. In the past 2 years, the Arp2/3 complex (65, 67, 142, 145) has emerged as the long sought cellular nucleator of actin filaments. This review focuses on a hypothesis that explains how Arp2/3 complex participates in the assembly and disassembly of the leading edge (Figure 2).

Arp2/3 complex is a stable complex of seven subunits—two actin-related proteins Arp2 and Arp3 with five novel proteins—p40 (ARPC1), p35 (ARPC2), p19 (ARPC3), p18 (ARPC4), and p14 (ARPC5) (Figure 3). The Arp2/3 complex was discovered in protozoans by affinity chromatography on the actin-binding protein profilin (67, 86). It is abundant (58), essential (124), and conserved (141) across eukaryotic phyla. In vitro, the complex also attaches the slowly growing pointed end of an actin filament to the side of another filament, producing a 70° branch exactly like those observed in cells (83). Immunoelectron microscopy has recently

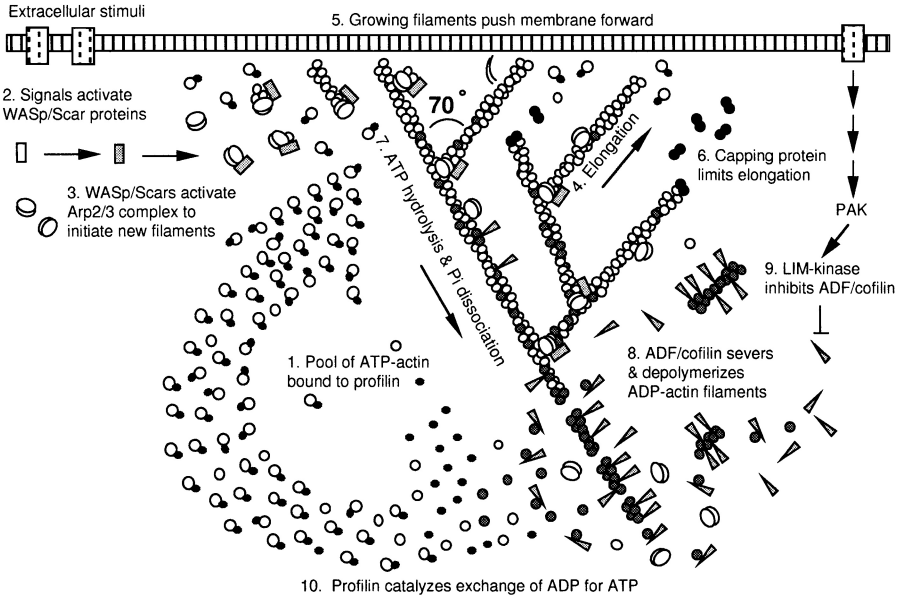

Figure 2 Dendritic nucleation model. The 10 numbered steps correspond to the sections of this review. The location of step 3, actin filament nucleation by activated Arp2/3 complex, is not settled. In vitro, free Arp2/3 complex can nucleate polymerization, but secondary activation by filaments strongly favors nucleation on the sides of pre-existing filaments, coupling nucleation and branching. (Modified from 83.)

(128) localized Arp2/3 to filament branches at the leading edge. In 1999 several laboratories (70, 115, 144, 146) discovered that WASp/Scar proteins regulate the nucleation activity of Arp2/3 complex, providing for the first time a plausible link between many cell surface receptors and de novo actin assembly.

Based on these new discoveries, we postulate a concrete, quantitative mechanism for the assembly and disassembly of the leading edge (Figure 2), which we have called the dendritic-nucleation model (83). The model proposes that, in the absence of free barbed ends, cytoskeletal components are held in a metastable state, poised for assembly (step 1). Activation of WASp family proteins (step 2) activates Arp2/3 complex to create new barbed ends at a constant rate (step 3). These filaments grow rapidly (step 4) and push the membrane forward (step 5). After a short time, growth of barbed ends is terminated by capping (step 6). Consequently, the system requires continuous activation of new Arp2/3 complex, because it is consumed by incorporation into a network that grows for a limited time. If the rate of nucleation drops to zero, capping stops polymerization automatically (84). Constitutive ATP hydrolysis within actin filaments and dissociation of phosphate (step 7) trigger severing and depolymerization of older filaments by ADF/cofilins (step 9) at a rate that is controlled by some of the same signals that stimulate assembly. Nucleotide exchange catalyzed by profilin (step 10) recycles ADP-actin

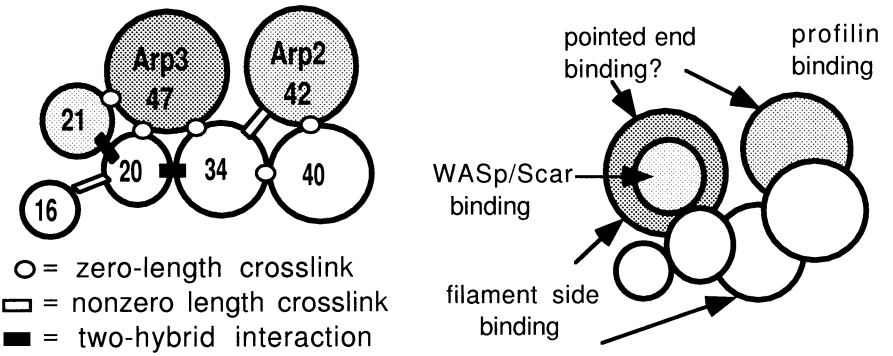

Figure 3 Subunit topology and binding partners of Arp2/3 complex. (Modified from 51.)

subunits back to the ATP-actin monomer pool. In a continuously moving cell, assembly and disassembly are balanced.

This is a minimal model with just 5 of the >60 families of actin-binding proteins (101). We focus on these proteins (profilin and thymosin-β4, Arp2/3 complex, capping protein, and ADF/cofilin), because they are highly conserved, well characterized, and sufficient to induce self-sustaining assembly of dynamic actin filament networks that drive motility of pathogenic, intracellular bacteria (63). Most eukaryotic cells use the same abundant set of cytoskeletal proteins to construct pseudopods (Table 1). Yeasts have similar proteins in dynamic actin patches. Because the building blocks, the overall structure, and (probably) the mechanism of assembly are so highly conserved, we regard the leading edge of a motile cell as a discrete cytoskeletal structure, one whose function and design principles were specified early in eukaryotic evolution.

TABLE 1 Cellular concentrations (μM) of key proteins in the actin system of diverse cells

Protein	Acanthamoeba	Dictyostellium	Neutrophil unactivated	Xenopus cgg extract	Platelet unactivated	S. cerevisiae
Polymerized actin	100	90	100	4	330	2
Unpolymerized actin	100	160	300	12	220	0.01
Profilin	100			5		present
Thymosin-β4	? absent	? absent		20	550	absent
ADF/cofilin	20	<100		3	30	present
Arp2/3 complex	2–4	present	10		9	present
Capping protein	1	1	1–2		5	1
Gelsolin	? absent				5	
α-actinin	4	3				
Filamin	? absent				6	
ABP120	? absent	6				

References: *Acanthamoeba* (43, 134, 30, 58, 106); *Dictyostelium* (John Condeelis, Albert Einstein College of Medicine); neutrophil (32, Cano 91, 38, 50); platelet (John Hartwig, Harvard Medical School, 87, 9, 46); *S. cerevisiae* (John Cooper, Washington University).

This review evaluates each step in the proposed mechanism, emphasizing what is known and yet to be discovered about the molecular interactions. A number of recent reviews cover the organization of the leading-edge (129) structure of the key proteins in the system (102), the structure of the Arp2/3 complex (85), and activation of Arp2/3 complex by WASp/Scar (51, 69, 140). Earlier reviews of pseudopod extension (28, 29, 79) provide access to the older literature before the discovery of Arp2/3 complex and WASp/Scar proteins.

HOW DO CELLS MAINTAIN A POOL OF UNPOLYMERIZED ACTIN SUBUNITS?

Studies dating back 25 years (18, 43) documented that nonmuscle cells maintain a high concentration of unpolymerized actin, estimated to be $\leq$100 μM in some cells (Table 1). Most of this monomer pool has bound ATP (117) and is presumed to have bound Mg^{2+}, given the high physiological concentration of Mg^{2+} (millimolar) and the low concentration of Ca^{2+} (0.1 μM). This large pool of Mg-ATP-actin monomers is remarkable because, under physiological conditions, pure ATP-actin from muscle or nonmuscle cells has a critical concentration for polymerization (K_d) of 0.1 μM at the barbed end and 0.6 μM at the pointed end (Table 2). At 100 μM, pure actin in physiological concentrations of salt polymerizes in a few seconds, leaving only 0.1 μM monomer.

Elongation of an actin filament is a bimolecular reaction between monomers and filament ends. It can be regulated by controlling the ability of either monomers or filament ends to participate in the reaction. Cells evolved both mechanisms—proteins that bind monomeric actin and modify its polymerization properties and capping proteins that prevent monomers from adding to filament ends. Alone, neither mechanism is sufficient to account for the extremely large actin monomer pool in the cytoplasm. The combination creates a metastable state with a huge pool of unpolymerized actin, poised for explosive growth when a cell produces free barbed ends.

Monomer-Binding Proteins

The main actin monomer-binding proteins in vertebrate cells are thymosin-β4 and profilin. Thymosin-β4, a small peptide of 43 residues (118), competes with

TABLE 2 Actin filament elongation rate constants in 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.0 (from 101)

	ATP-actin Barbed end	ATP-actin Pointed end	ATP-actin Barbed end	ATP-actin Pointed end
k_+ ($\mu M^{-1}\,s^{-1}$)	11.6	1.3	3.8	0.16
k_- (s^{-1})	1.4	0.8	7.2	0.27
K_d (μM)	0.12	0.62	1.9	1.7

profilin, a small globular protein of 125–139 residues, for binding to overlapping sites on actin (119, 123). Only profilin has been found in protozoa, slime molds, and fungi. (Budding yeasts have no thymosin gene.) Thymosin-β4 is a true sequestering protein that binds about 50-fold more strongly to ATP-actin (K_d, 1.2–1.6 μM) than to ADP-actin (22, 57a, 94, 136). The concentration of thymosin-β4 varies from 600 μM in platelets to 20 μM in *Xenopus* eggs (Table 1) and is thought to account for a large fraction of the unpolymerized actin in these cells. Actin bound to thymosin does not polymerize (22, 42, 57a), but profilin competes with thymosin-β4 for binding ATP-actin (22, 57a, 136) and can shuttle actin away from thymosin onto the barbed end of actin filaments (94, 103). Exchange is rapid, owing to the high dissociation rate constants (5 s^{-1} for profilin and 2.5 s^{-1} for thymosin-β4). ATP-actin partitions between profilin and thymosin-β4 based on the affinities and concentrations of the binding proteins (57a, 97, 136).

Profilin is the main protein that binds ATP-actin monomers in protozoa, slime molds, and fungi, and is present at concentrations of $\leq$100 μM (Table 1). Profilins bind cytoplasmic ATP-actin monomers ($K_d = 0.1$ μM) better than cytoplasmic ADP-actin monomers ($K_d = 0.5$ μM) and muscle ATP-actin monomers ($K_d = 0.5$ μM) (96, 136). The affinity of profilin for actin filaments is low, because the binding site on the barbed end of actin is buried in the filament structure (123).

Profilin-Mg-ATP-actin complexes elongate the barbed end of actin filaments nearly as quickly as free actin, but do not form nuclei or elongate the pointed end of actin filaments (94, 103, 109). High concentrations of profilin slow barbed-end elongation of Mg-ATP-actin $\sim$20% (44, 57), an effect that was originally attributed to rate limiting dissociation of profilin from the barbed end of actin filaments (103). On thermodynamic grounds, it has been argued that profilin should enhance the rate of ATP hydrolysis on the terminal subunit and that this should promote dissociation of profilin (44), but we have not been able to detect an effect of profilin on ATP hydrolysis (15). Thus the mechanism of the small effect of profilin on barbed-end elongation by Mg-ATP-actin is not yet understood. Similarly, we cannot explain how excess profilin completely inhibits elongation by Ca-ATP-actin (44, 57).

Because both free actin monomers and profilin-actin monomer complexes contribute equally to elongation of barbed ends (57a), the concentration of free actin required for growth is less in the presence of profilin than the critical concentration of free actin alone. Profilin is thus said to lower the critical concentration at the barbed end (94). In fact, the critical concentration is the same in the presence of profilin (57a), with both actin and actin-profilin contributing to the critical concentration.

Capping

Profilin (and thymosin-β4 when present) maintains a pool of Mg-ATP-actin that is ready to elongate any available actin filament barbed ends. If the barbed ends of cellular actin filaments were free, elongation would rapidly deplete this pool of

actin monomers in a few seconds. Thus, the concentration of free barbed ends must be low. We do not know what fraction of cellular actin filaments has free barbed ends, but the micromolar concentration of capping protein (also called CapZ in muscle) and its affinity for barbed ends ($K_d = 0.1$ nM) are sufficient to cap most of the barbed ends (122). This is true in cellular extracts (32, 84) in which high concentrations of unpolymerized actin monomers are stable in the presence of actin filaments. The addition of uncapped filaments to such extracts results in the explosive polymerization of the actin pool. The rate and extent of growth of these new filaments are limited by capping, which follows a pseudo-first-order reaction (84).

Thus, both monomer binding by profilin and capping of barbed ends are required to maintain a pool of actin monomers. In cells with thymosin-β4, profilin serves as a carrier between the thymosin-ATP-actin pool and the barbed ends of actin filaments (94). Because neither actin-profilin nor actin-thymosin-β4 elongates pointed ends, pointed-end capping is not required to maintain a pool of unpolymerized subunits. Nevertheless, the micromolar concentration of Arp2/3 complex and its nanomolar affinity for pointed ends suggest that most pointed ends are also capped (83).

HOW ARE SIGNALS DIRECTED TO ARP2/3 COMPLEX?

External stimuli drive the assembly of the cortical actin filament network, acting through receptors and multiple signal transduction pathways, several of which converge on WASp/Scar proteins and Arp2/3 complex (Figure 4). The molecular pathways from receptors to WASp/Scar are not well established, but enough is known to postulate that WASp/Scar proteins integrate diverse signals, including those carried by Rho family GTPases, Rac, and Cdc42 (69). Downstream, the ability of active WASp/Scar to stimulate actin filament nucleation by Arp2/3 complex is firmly established (see below), so it is possible that Arp2/3 complex is the

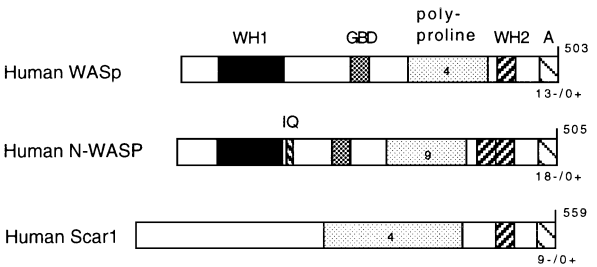

Figure 4 Domains of WASp/Scar proteins. Abbreviations: WH1, WASp homology domain 1, also called EVH1 domain; WH2, WASp homoloogy domain 2; GBD, GTPase-binding domain; A, acidic domain; IQ, IQ domain. The numbers within the polyproline domains indicate the number of polyproline sequences. The numbers below the A domains indicate the number of acidic (−) and basic (+) residues. (Modified from 51.)

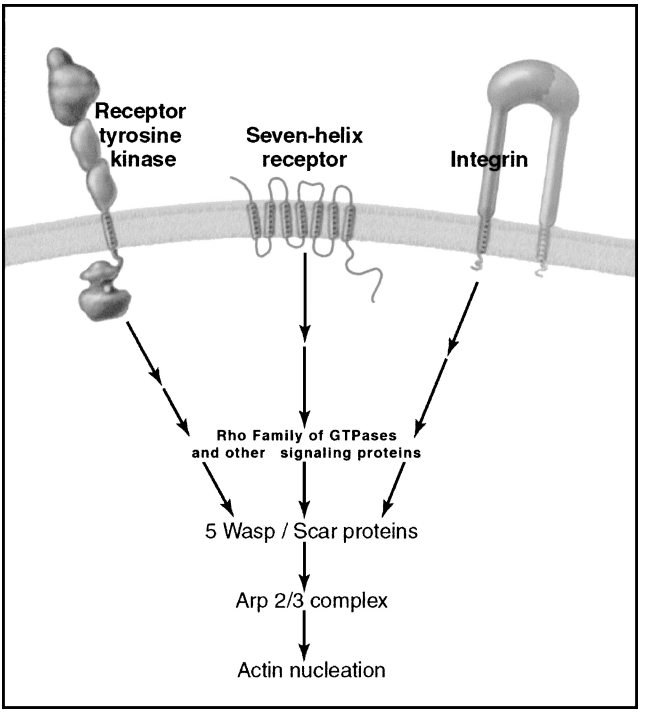

Figure 5 Signaling pathways through WASp/Scar to Arp2/3 complex.

final common effector for all of the signals that impinge on WASp/Scar. Intrinsic signals are also likely to exist for constitutive pseudopod formation but have not been identified.

WASp, the founding member of the WASp/Scar family (Figure 4), is the protein defective in Wiskott-Aldrich syndrome, a human genetic disease with deficiencies in the actin cytoskeleton of platelets and leukocytes (92). WASp is expressed in platelets and white blood cells. N-WASP is expressed in brain and many other tissues. Scar was discovered in *Dictyostelium discoideum* as a suppressor of a mutation in a seven-helix cyclic-AMP (cAMP)-receptor (11). The vertebrate homolog was discovered independently and named WAVE (78). In no case do we know the cellular concentration of a WASp/Scar protein, but anecdotal evidence suggests that the quantities are limiting compared with the abundant Arp2/3 complex. If so, activation of WASp/Scars is likely to be the limiting factor driving actin assembly.

WASp/Scar proteins share similar C-terminal WH2 and A domains and a proline-rich central region, but differ in the N-terminal third (Figure 5). The A domain consists of ∼30 conserved residues, including a large fraction of acidic residues, and it interacts with the ARPC3 subunit of the Arp2/3 complex (68). The WH2 domain is a 30-residue motif first identified in verprolin (135). This domain

binds actin monomers, blocking their addition to pointed ends but not barbed ends of filaments, similar to profilin (37, 51). Unlike profilin, the WH2 domain does not catalyze nucleotide exchange (50). Both the WH2 and A domains are required for efficient activation of the Arp2/3 complex and together they are sufficient. All WASp family proteins contain a conserved, proline-rich domain with four to nine clusters of polyproline. This domain binds SH3-containing proteins (127) and may bind multiple molecules of profilin. On the N-terminal side of the proline-rich domain, WASp and N-WASP have a GBD domain, a consensus sequence for binding the small G-protein Cdc42. Scar appears to be an effector for Rac but not Cdc42, although it lacks a recognizable GBD. The WH1 domain (also called the EVH1 domain) near the N-terminus is folded like a PH domain but binds polyproline-containing ligands. Crystal structures are available of an EVH1 domain (39, 108) and a GBD bound to Cdc42 (1). N-WASP also has a calmodulin-binding IQ motif. This domain structure gives WASp/Scar proteins the potential to integrate a wide variety of signals, but little is known about the conformation of the full-length proteins, not even an estimate of the native molecular weight. So, much is still to be learned about signal transduction at this level.

A strong case can be made for WASp and N-WASP as intermediaries between Rho family GTPases and Arp2/3 complex. In vertebrate cells, Rac is required for cell motility, and Cdc42 is required for chemotaxis (3, 45). WASp and N-WASP bind Cdc42 and are required for Cdc42-stimulated polymerization (115, 146). Cdc42-induced actin polymerization in cell extracts (84, 148, 149) depends on Arp2/3 complex (65, 84). A simple model, supported by cellular studies with dominant-active and dominant-negative Cdc42 (78), is that WASp/N-WASP is inactive until activated by GTP-Cdc42. Scar may be downstream of Rac (78), but because it lacks a binding site for Rho family GTPases, it may respond to different unidentified activators, perhaps downstream from the seven helix receptors.

HOW DO CELLS CREATE ACTIN FILAMENTS WITH FREE BARBED ENDS?

Profilin and thymosin-β4 strongly inhibit spontaneous nucleation of unpolymerized actin, so the limiting factor in actin polymerization in vivo is the creation of free barbed ends. Cells could either initiate new filaments (the focus of this section) or uncap or sever existing filaments, allowing them to elongate.

Uncapping

The two most abundant barbed-end–capping proteins, gelsolin and capping protein, both cap filament ends with nanomolar affinity, and both can be removed by interacting with polyphosphoinositides [e.g. PI(4, 5)P_2 (56, 122)]. In platelets, uncapping of gelsolin-capped filaments appears to generate free barbed ends and

contribute to actin polymerization (47). In other cells selective removal of capping protein from filaments may help filaments to grow persistently toward the membrane and to organize the three-dimensional structure of the actin network at the leading edge (see below). At least in filopodia growth of existing filaments persists at low rates for long times (74a).

Severing

Severing existing filaments into multiple smaller ones can generate new ends. Platelet activation is the best example, in which severing is a major mechanism of inducing actin polymerization (10). Platelet activation increases cytoplasmic Ca^{2+}, which activates gelsolin to sever actin filaments and tightly cap their barbed ends. Membrane PIP_2 can remove the gelsolin cap and contribute to the burst of polymerization (47). However, gelsolin is not absolutely required, because platelets from mice lacking gelsolin still polymerize and cap new actin filaments when activated. In fibroblasts the reactions mediated by gelsolin appear to be downstream of Rac (5). ADF/cofilins also sever actin filaments (discussed below), but most evidence suggests that this is associated with depolymerization rather than assembly (23, 61, 116). Nevertheless, cofilin-mediated severing has been proposed to generate short actin oligomers that are subsequently stabilized and cross-linked by Arp2/3 complex (35). This interesting possibility requires further study.

De Novo Filament Formation

In a many cell types, actin polymerization appears to be initiated de novo by assembly of nuclei from actin monomers and nucleation-promoting factors. Although this has long been considered, no barbed-end nucleating factor was known before Arp2/3 complex was shown to have this activity. The following section summarizes what has been learned about nucleation mediated by Arp2/3 complex, but we caution at the outset that an open mind is necessary about the possibility of other factors with such activity.

Spontaneous nucleation of actin alone is unfavorable because of the instability of actin dimers and trimers, which are obligate intermediates on the path to longer filaments. The details of these reactions are still murky, because no assays are available for dimers or trimers. Their properties have been inferred from complete polymerization time courses (reviewed in 104), using kinetic simulation to estimate equilibrium constants; $K_d = \sim$100,000 μM for dimers and $\sim$10–100 μM for trimers. Filament elongation is a rapid, diffusion-limited reaction (see below). If monomer association reactions are also diffusion limited, the lifetimes of dimers and trimers are exceedingly brief, in the submillisecond range. Nevertheless, under physiological salt conditions, pure actin does form nuclei spontaneously, if slowly, in a very concentration-dependent reaction. Nucleation reactions deserve new work with new approaches.

The term nucleation is sometimes used loosely and inappropriately in the primary literature. Nucleation is the initiation of a new actin filament by assembly

from monomers. Accessory proteins can promote or inhibit these reactions. Addition of subunits to the ends of existing actin filaments, which are simply added to a reaction or are uncapped or severed to expose new ends, is elongation, not nucleation.

Highly purified Arp2/3 complex nucleates filaments with free barbed ends and capped pointed ends, but the mechanism is extremely inefficient, because it depends on the capture of spontaneously formed actin dimers (83). Cells use at least two synergistic regulatory mechanisms to turn on the intrinsically inactive Arp2/3 complex. WASp/Scar proteins are primary activators, and actin filaments are powerful secondary activators, an effect that promotes branching. Certain bacteria have evolved their own activators of Arp2/3 complex, such as *Listeria* ActA (143).

WASP-family proteins activate Arp2/3 complex to generate new actin filaments. Constructs consisting of the WH2 and A domains (WA) of all WASp/Scars activate actin filament nucleation by Arp2/3 complex, independently of the rest of the protein (70, 115, 144, 146). Under optimal conditions, each WA-activated Arp2/3 complex initiates a new filament (50). WA constructs from WASp and Scar interact with Arp2/3 subunits in affinity chromatography and yeast two-hybrid assays, and overexpression of WA peptides in vertebrate cells delocalizes Arp2/3 and inhibits actin reorganization (68).

The mechanism of nucleation by WA-activated Arp2/3 complex is being investigated. Activation may simply be allosteric—a conformational change induced in the complex by WA (85). WH2 domains also bind actin monomers (68) with submicromolar affinity, so they may actively recruit actin monomers to Arp2/3 complex to form a nucleus (37, 50). After nucleation, Arp2/3 complex remains attached to the pointed end of the filament, so it is incorporated into the growing actin filament network (83).

Actin filaments are powerful secondary activators of nucleation by Arp2/3 complex and WA. Inclusion of filaments with Arp2/3 complex and WA can eliminate the lag at the outset of an in vitro polymerization experiment (70). The synergism between WA and filaments biases the initiation of new filaments to the sides of existing actin filaments, where Arp2/3 complex anchors end-to-side branches at a fixed angle of 70° (12a, 83). Examination of the products by light microscopy revealed that branching occurs during rather than after nucleation (12a). This coupling between nucleation and branching explains the morphology of the leading edge. The situation must differ in filopodia, which depend on Cdc42 for initiation (78), but which consist of a parallel bundle of filaments rather than a branching network.

Full-length WASp/Scar proteins also activate Arp2/3 complex in vitro, but the details differ in the initial reports. Full-length Scar was as active as its WA domain (70), so the recombinant protein is constitutively active. Similarly, no GTPase was required for WASp to activate Arp2/3 complex (146). On the other hand, full-length N-WASP required both Cdc42 and lipid vesicles containing $PI(4,5)P_2$ to stimulate maximal nucleation activity (115). Oddly, both GTP-Cdc42 and GDP-Cdc42 were active in these experiments, whereas Egile et al (37) found that GTP-Cdc42 was required for N-WASP to activate Arp2/3 complex.

Functional Implications

Nucleation in cells appears to require a continuous supply of activated Arp2/3 complex, because activated complex is physically consumed by incorporation into the filament network and because capping protein stops polymerization after a few seconds (see below). A supply of barbed ends will be maintained in the cytoplasm only if they are created at the same rate as they are capped. For this reason, any cellular mechanism that produces sustained actin polymerization must induce a constant rate of nucleation rather than produce a fixed number of nuclei all at once. Production of active Arp2/3 complex at a constant rate appears to be the function of WASP-family proteins and the *Listeria* ActA. This dependence on the rate of nucleation may explain why filament initiation stops abruptly when stimuli, such as chemotactic signals, turn off.

Two lines of evidence suggest that activators interact only transiently with Arp2/3 complex. *Listeria* ActA stimulates nucleation by Arp2/3 complex but remains attached to the bacterial surface, whereas Arp2/3 complex is incorporated into the actin comet tail behind the bacterium (143). Similarly, addition of WASp-coated beads to cytosolic extracts induces actin polymerization that is dependent on Arp2/3 complex, which is incorporated into the actin network leaving WASp behind on the beads (146). Thus each molecule of ActA or WASp/Scar may activate multiple Arp2/3 complexes. If true, the concentration of activated WASP family proteins will induce a constant rate of nucleation, but more work is required on the rates of the various reactions. The site of activation is unknown but has been suggested to be either on the inner surface of the plasma membrane (37, 115, 143) or in the cortical-filament network (70).

Polarity and Coincidence Detection

Although far from complete, current evidence suggests that multiple signals converge at the same place to maximally activate nucleation. Therefore, the combination of N-WASP and Arp2/3 complex may act as a coincidence detector that responds maximally to the combination of active GTPase, phospholipids, actin filaments, and other signals. In addition to making filament formation responsive to a signaling pathway, this mechanism prevents nucleation away from the membrane surface and causes filaments to feed back positively on their formation. The result is tightly localized filament formation that, once initiated, accelerates rapidly. This is probably critical for the cell's ability to polarize rapidly in response to external signals and to maintain polarity during movement.

HOW DO NEW FILAMENTS ELONGATE?

Elongation of purified actin is the best characterized part of this system (Table 2), because robust assays are available to measure the elongation rates at both ends of filaments. The ratio of the dissociation rate constant to the association rate constant for each reaction gives the dissociation equilibrium constant, also known as

the critical concentration. The critical concentrations for ADP-actin are the same at both ends. (The apparent difference in Table 2 represents experimental error.) For Mg-ATP-actin, the critical concentration is considerably lower at the barbed end than at the pointed end, whether measured kinetically during rapid elongation or at steady state. This difference in critical concentrations at the two ends in ATP must be from ATP hydrolysis and/or phosphate dissociation, but the mechanism is not understood. It is not caused by different rates of ATP hydrolysis at the two ends (15).

Elongation of ATP-actin at the barbed end is diffusion limited by accepted physical-chemical criteria, namely that the reciprocal of the rate constant is proportional to the viscosity of the solution and extrapolates to an infinite rate constant at zero viscosity (33). Elongation at the pointed end is slower and not diffusion limited. Molecular dynamics simulations (125) revealed why the rate constants differ at the two ends; without taking electrostatics into consideration, elongation is favored at the pointed end, but electrostatic effects enhance elongation rates at the barbed end and inhibit elongation at the pointed end, as observed. Through electrostatic effects, proteins such as ADF/cofilins bound to actin monomers may accelerate elongation modestly [20%–50% (125)], but suggestions that other proteins enhance elongation by 10- or 20-fold (23) may not be physically possible.

Two opposing factors influence diffusion-limited reactions in cytoplasm. The high concentration of macromolecules slows diffusion of proteins the size of actin by a factor of about 3 (64), but this is compensated for by an excluded volume effect that increases reaction rates, including actin filament elongation (33). At the concentrations of unpolymerized actin found in cells (10–100 μM), elongation rates of barbed ends in dilute buffers are exceptionally fast, $\sim$100–1000 subunits/s, that is, $\sim$0.3–3 μm/s. In cytoplasm the rate may be lower, but even if twofold lower, the rate is adequate to account for the rate of pseudopod formation in rapidly moving cells, and it is much faster than required for slow cells like epithelial cells in tissue culture. Some authors argue that diffusion of actin to sites of elongation is rate limiting, but this is unlikely to be true in cells with a large pool of unpolymerized, freely diffusible actin.

In the foregoing, it was assumed that all of the unpolymerized actin is bound to profilin (Figure 6). This is true in amoebae, but part of the unpolymerized actin pool is bound to thymosin in vertebrate cells. This lowers the available monomer concentration and the rate of elongation. If the concentration of unpolymerized actin really varies as much as reported (Table 1), the rate of elongation will vary considerably.

HOW DO GROWING FILAMENTS PUSH THE MEMBRANE FORWARD?

The network of actin filaments at the leading edge of a motile cell is uniquely adapted to convert the free energy of monomer binding into mechanical energy. The idea that polymerization itself generates force to deform the plasma membrane

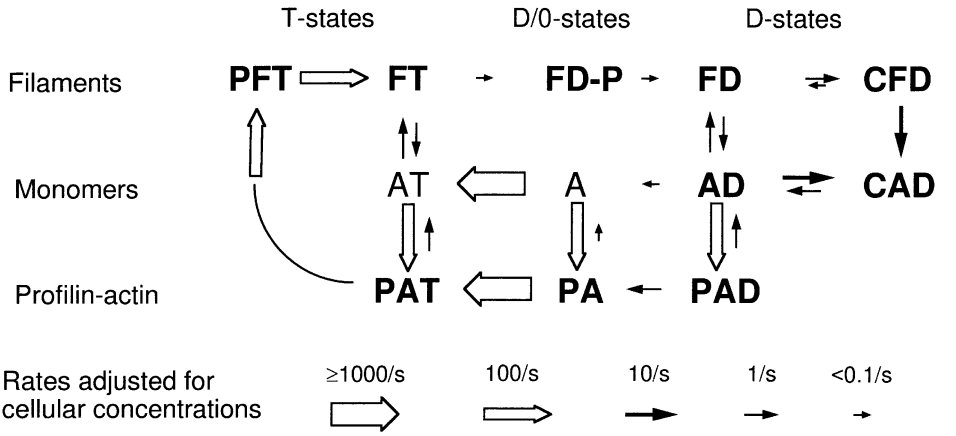

Figure 6 Actin monomer economy. A, actin monomer; F, actin filament; P, profilin; C, ADF/cofilin; T, ATP; D, ADP; D-P, ADP-P_i. The *arrows* indicate the approximate reaction rates based on rate constants and cytoplasmic concentrations of proteins.

was proposed by Tilney et al (133). It is consistent with most observations of living cells but has never been demonstrated experimentally to be the mechanism of membrane protrusion in vivo. The best experimental evidence for this mechanism comes from two sources: (*a*) studies in which monomeric actin encapsulated in giant liposomes is induced to polymerize (80) and (*b*) reconstitution of bacterial motility from purified components. In giant liposomes the polymerization of pure actin is sufficient to deform the membrane and, in the presence of actin cross-linking proteins, the deformation is quite severe (81). *Listeria* motility requires, in addition to actin, only three proteins (Arp2/3 complex, ADF/cofilin, and capping protein), none of which is a force-generating motor protein (63). Assembly and turnover of the actin comet tail appear to be sufficient to propel the bacterium forward.

The microscopic details of polymerization-driven motility are not immediately obvious. Peskin et al (98) proposed that, when the end of an actin filament contacts a load, elongation of the filament could act as a ratchet to rectify the thermal motions of the load. That is, if thermal motions cause the load to fluctuate away from the end of the filament, elongation of the filament into the gap will prevent the load from returning to its original position. This might apply to membranes, but not to bacteria, which are too large to undergo appropriate Brownian motions. Mogilner & Oster (82) combined this idea of a thermal ratchet with the elastic properties of an actin filament and proposed an "elastic Brownian ratchet" model. In this model, filaments behave like elastic springs with properties determined by the bending modulus of the filament and the angle it makes with the load. According to Mogilner & Oster, the thermal fluctuations of actin filaments are the most important. Thermal motion can displace a filament from the membrane, allowing room for additional monomers to add to the end of the filament. The

elastic restoring force of the filament tries to return the longer filament to its original position and results in deformation of the membrane. One important prediction of this model is that there is an optimal angle to transmit force from an actin filament to a load. In the force regime of the leading edge (~45 pN), this angle is ~45° as observed in cells (130; Figure 1). The branches formed by Arp2/3 complex are quite stiff (12a), in keeping with this model. The actin filament concentration is also consistent with protrusion being driven by polymerization (1a).

WHAT LIMITS THE GROWTH OF FILAMENTS?

Our dominant theme is that control of actin polymerization takes place at the barbed end of the filament; to make new filaments, cells create new barbed ends; and to limit filament growth, cells cap barbed ends rapidly (Figure 2). The supporting evidence is that most cells maintain a large pool of polymerizable actin that would disappear if ends were allowed to grow indefinitely; filaments injected into living cells do not elongate detectably (120), and actin seeds added to cell extracts grow for only a short time and do not deplete the pool of monomeric actin (53, 84, 149).

The factor thought to cap barbed ends in most cells is a ubiquitous heterodimeric protein called capping protein, discovered in amoebae (55). Capping protein is abundant in most cell types, with cytoplasmic concentrations in the micromolar range (Table 1). The muscle isoform of capping protein (CapZ) caps the barbed ends of the actin filaments at the Z-disk (26). Injection of function-blocking anti-CapZ antibodies into developing muscle cells profoundly disrupts sarcomere formation (121). Genetic deletion of capping protein in nonmuscle cells alters the balance between polymerized and unpolymerized actin (4, 54) and immunodepletion of capping protein from nonmuscle cell extracts removes almost all of the barbed-end capping activity (32).

Capping protein binds the barbed end of actin filaments very tightly [K_d = 0.1 nM (122)] and prevents both association and dissociation of monomers (55). Capping protein binds barbed ends with an association rate constant of 3 μM s^{-1}. At cellular concentrations of capping protein, a free barbed end will have a half-life of about a quarter of a second before it is capped, but, because of the high cellular concentration of monomeric actin, the filament will have elongated by >200 monomers during this time. The high affinity of capping protein for barbed ends is a consequence of its extremely slow dissociation rate [5×10^{-4} s^{-1} (122)]. The half-time for uncapping is >1000 s, much longer than the lifetime of dynamic actin filaments in cells (27, 132), so, unless it is actively uncapped or severed, a capped filament probably never elongates again.

If a new filament is cross-linked into the network with its barbed end pointing away from the membrane, it will probably remain capped until it is disassembled by ADF/cofilin. If, however, a capped barbed end collides with a membrane, interaction with poly phosphoinositides like PIP_2 and PIP_3 may remove capping

protein and allow one or more additional rounds of growth and capping. In this way, localized uncapping may bias the growth of filaments and produce more long filaments with barbed ends oriented toward the leading edge. This might explain why the barbed ends of all long filaments in the keratocyte leading edge point forward. New experiments are required to test this idea. Another area for research is to learn how activated Cdc42 protects growing actin filaments from capping in leukocyte extracts (53). Elsewhere in this review, we consider further consequences of uncapping.

HOW ARE FILAMENTS MARKED FOR DEPOLYMERIZATION?

Hydrolysis of ATP bound to actin subunits, subsequent to their incorporation into filaments, and the dissociation of the gamma-phosphate are postulated to mark filaments for depolymerization by ADF/cofilin proteins (74; Figure 2). New actin filaments at the leading edge of a cell are built with ATP actin subunits, because ATP-actin is the predominate form of unpolymerized actin (117). Phosphate dissociation is an effective timer for destruction, because the depolymerizing proteins, ADF/cofilins (next section), bind polymerized ADP-actin subunits more strongly than ATP-actin or ADP-P_i actin subunits (Table 3). This explains why BeF_3 (14, 23) or millimolar concentrations of phosphate (14, 23, 72, 74) protect ADP-actin filaments from ADF/cofilins in binding, low-shear-viscosity, and depolymerization experiments.

ATP hydrolysis by polymerized actin is irreversible (24), but phosphate dissociates slowly. By several criteria including critical concentration, ADP-P_i actin is very similar to ATP-actin (113). ADP-actin is distinctly different. The dissociation equilibrium constant for P_i from polymerized ADP-P_i subunits is in the low millimolar range (19), so some ADP-actin subunits may retain the gamma-phosphate in vivo. The nucleotide bound to polymerized actin subunits is not exchangeable along the length of the filament (105), but ATP does exchange rapidly with ADP on the terminal subunits at the barbed ends of filaments, possibly contributing to the stability of barbed ends (131). By inhibiting this exchange ADF/cofilin might destabilize barbed ends in cells. Nothing is known about nucleotide exchange on pointed ends.

TABLE 3 Dissociation equilibrium constants (μM) for ADF/cofilins binding actin monomers and filaments

ADF/cofilin	Mg-ATP-monomer	Mg-ADP-monomer	Mg-ADP-P-filament	Mg-ADP-filament
Actophorin, amoeba actin[a]	5.9	0.15		5.6
Actophorin, muscle actin[b]	4.5	0.14	≈20	0.49
Plant ADF, muscle actin[c]	8	0.1		0.3
Human ADF, muscle actin[d]		0.09		

References: a. 14, b. 15, c. 23, d. 112.

TABLE 4 ATP hydrolysis rate constants (s^{-1}) by polymorized actin calculated assuming random hydrolysis

Report	Mg-ATP-actin	Ca-ATP-actin	Li-ATP-actin
Pardee & Spudich, 1982 (95)		~0.0005 ($t_{1/2}$ = 30 min.)	
Pollard & Weeds, 1984 (107)	0.07*	0.08	
Carlier et al, 1986 (25)	0.08	0.02	
Pieper & Wegner, 1996 (99)	0.02	0.01	
Blanchoin & Pollard, 2000 (13)	0.10	0.06	0.10

*This was Ca-ATP-actin polymerized in 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA. We now know that much of the actin incorporated into polymer was Ca-ATP-actin.

ATP hydrolysis and gamma-phosphate dissociation are both first-order reactions. The simplest model for these reactions is that each subunit in a polymer acts independently (52), so that all have an equal probability of hydrolyzing ATP and dissociating phosphate. The time courses of hydrolysis and phosphate dissociation are consistent with but do not prove this model. Alternatively, it is reasonable to think that the local environment in the polymer might influence either or both reactions. The most extreme version of such thinking is a vectorial hydrolysis model in which ATP hydrolysis occurs only at the interface between the central core of ADP-P_i-actin subunits and ATP-actin subunits at either end of a growing filament (25). If this mechanism is correct, the hydrolysis rate at the interface of ADP- and ATP-subunits would have to be very high to account for the observed hydrolysis rates, because so few subunits would be eligible for hydrolysis. Kinetics experiments cannot prove or disprove this hypothesis, but an experiment of Pieper & Wegner (99) argues against vectorial hydrolysis. They found that copolymerization of ATP-actin with a range of ADP-actin concentrations did not affect the rate of ATP hydrolysis by the polymerized actin.

The range of ATP hydrolysis rates reported over the years is wide (Table 4). Some differences are caused by technical limitations of the early work, because the experiments were done before we knew how to make good Mg-ATP-actin. The most recent work with quench-flow methods shows that Mg-ATP-actin (and Li-ATP-actin) hydrolyzed ATP about twice as quickly as Ca-ATP-actin. Atomic structures explain the different rates, because Mg^{2+} and Li^{+} position the attacking water more favorably than Ca^{2+} (S Almo, personal communication).

Both groups reporting on the rate of gamma-phosphate dissociation from Mg-ADP-P_i-actin subunits agree that the rate constant is about $0.002\ s^{-1}$ (14, 20, 77), but this half time of >5 min is too slow to account for the fastest turnover of actin filaments in vivo [$t_{1/2}$ = 23 s (132)]. One resolution of this difference is that ADF/cofilin increases the rate of phosphate release by a factor of >10, although ADF/cofilins bind only weakly to ADP-P_i-actin subunits (14). This is reasonable thermodynamically, because, if phosphate reduces the affinity of ADF/cofilin for actin, ADF/cofilin must reduce the affinity of phosphate for actin.

Thus phosphate dissociation is the most plausible timer for actin polymer destruction by ADF/cofilin. If true, the lag between rapid actin polymerization in vivo and the enhanced rate of phosphate release by ADF/cofilin should influence the thickness of the network of ATP-actin filaments near the membrane. Careful observations of ADF/cofilin localization revealed differences between highly motile keratocytes and slower fibroblasts (128). Keratocytes exclude ADF/cofilin from a zone near the membrane, whereas in fibroblasts ADF/cofilin is present throughout the cortex, up to the front of the leading edge. This difference may reflect more rapid growth of the actin network or a longer lag between actin polymerization and phosphate release in keratocytes than fibroblasts.

What about long-lived actin filaments? The best example is striated muscle, in which ADP-actin filaments are stable for hours to days. These filaments are protected from depolymerization by tropomyosin, which inhibits ADF/cofilin binding and depolymerization (12, 91). Some stable actin filaments in nonmuscle cells, including stress fibers, are also protected by tropomyosin (62). The brush border of intestinal epithelial cells is another example, with two populations of stable actin filaments (49). Those in the terminal web are probably stabilized by tropomyosin. Those in the microvilli lack tropomyosin, so they must be stabilized by the other associated proteins.

HOW DO FILAMENTS DEPOLYMERIZE?

Filaments turn over rapidly during pseudopod extension (27, 132) and during the centripetal flow of the cortical actin filament network in some stationary cells (40, 138). How fast must subunits dissociate from individual filaments to account for these rates of polymer turnover? If all depolymerization were in one zone at a fixed distance from the front of the cell, the rates would have to be very fast, hundreds of subunits per second, because lamellipodia move up to 1 μm s^{-1} (370 subunits/μm) and centripetal flow can be $\leq$0.8 μm s^{-1}. However, depolymerization takes place across a broad zone (132), so no one knows how quickly individual filaments shorten.

The situation is more complicated than usually acknowledged. Making the reasonable assumption that subunit dissociation is restricted to the ends of filaments, the rate of depolymerization of a population of polymerized actin depends directly on the intrinsic rate of subunit dissociation (k_-) and the concentration of free ends minus the rate of subunit association:

$$\text{Depolymerization rate} = k_-(\text{ends}) - k_+(\text{ends})(\text{actin monomer}).$$

Dissociation rate constants for Mg-ADP-actin are 7 s^{-1} at the barbed end and a very slow 0.3 s^{-1} at the pointed end (Table 2). For a given mass of polymer, the concentration of ends depends inversely on their lengths, so the bulk depolymerization rate is inversely proportional to polymer length. This is why severing is so important to consider as part of the depolymerization mechanism. In a cell, some barbed ends and some pointed ends will be free and others capped. All of these

parameters appear to be subject to control in cells. In no live cell are all of these parameters known.

Proteins of the ADF/cofilin family are thought to promote recycling of actin (for review, see 21), because they enhance the dynamics of actin filaments in *Listeria* comet tails (23, 116) and promote depolymerization of pure actin filaments (7, 14, 23, 30, 34, 66, 72, 74, 90). A major point of disagreement is whether ADF/cofilin proteins enhance depolymerization by severing actin filaments and creating more ends, by increasing the rate of subunit dissociation from one or both ends, or by both severing and rapid subunit dissociation (for review, see 71).

As explained in the following paragraphs, most investigators favor a dual mechanism with ADF/cofilins severing filaments to increase the number of ends and also increasing the rate constant for subunit dissociation at one or both ends (Figure 6, large vertical arrow at the top right). Carlier et al (23) and Ressad et al (112) have argued that the effects of ADF/cofilin on actin filaments might be explained entirely by a 30-fold-higher rate of subunit dissociation at the pointed ends of filaments. Much remains to be learned about the depolymerization mechanism.

Binding of ADF/cofilins to ADP-actin filaments is still incompletely understood. One basic uncertainty is whether association is cooperative as observed for all ADF/cofilins tested (including amoeba-actophorin) binding to muscle actin filaments or not cooperative as observed for amoeba-actophorin binding to amoeba-actin filaments, the only homogeneous system tested to date. Resolving this question is important for understanding cells, because polymerized actin exceeds the concentration of ADF/cofilins, and cooperativity would focus this limited ADF/cofilin locally on filaments and might promote subsequent reactions. For the homogeneous, noncooperative amoeba system, the association rate constant is exceptionally slow, 0.03 $\mu M^{-1}\,s^{-1}$, but, for the cooperative system of actophorin and muscle actin, the rate constant increases with saturation from 0.008 $\mu M^{-1}\,s^{-1}$ to $>0.075\ \mu M^{-1}\,s^{-1}$ (14). The rates of cooperative binding of plant ADF to muscle actin are orders of magnitude faster (111). Both the very slow binding in the homogeneous system and the cooperative binding suggest that few subunits in bare filaments are in a conformation that favors binding.

Saturating concentrations of ADF/cofilins change the twist of the long pitch helix of muscle actin filaments by 5° per subunit (75), a structural change that is suggested to promote severing and subunit dissociation. Binding of ADF/cofilins to pyrene-labeled actin filaments quenches the fluorescence down to the low level of actin monomers (14, 23), a valuable signal to measure binding that does not yet have a structural interpretation. Both tropomyosin (12, 74) and phalloidin (14, 74) inhibit the binding of ADF/cofilins to actin filaments.

A variety of evidence suggests that ADF/cofilin proteins sever actin filaments: direct visualization by fluorescence microscopy (48, 74), electron microscopy (30, 72), viscometry (30, 73), kinetics of spontaneous polymerization (34, 74, 110), measurement of ends by elongation assays (14, 72), and fluorescence recovery after photobleaching (34). The extent of severing depends on the concentration of ADF/cofilin and time, but the number of breaks is much lower than the number of

ADF/cofilins bound. For example, mixing 4 μM actophorin with 2 μM polymerized actin severs each filament of ~2000 subunits into ~10 shorter filaments (14). In contrast, gelsolin severs actin filaments with high efficiency, with one break per binding event (139), so ADF/cofilins are said to sever weakly.

Other experiments show that ADF/cofilins promote the dissociation of subunits from the ends of actin filaments. The most extensive study (23) showed that filaments depolymerize about 30-fold more quickly from their pointed ends in the presence of plant ADF, assuming that ADF had no effect on the number of ends. The same study found a 20-fold increase in the rate of elongation at the barbed end of actin filaments, a result that can be explained only by an increase in the concentration of barbed ends (125). Thus the higher rate of dissociation at the pointed end may be explained in part by a higher concentration of ends produced by severing.

One unresolved issue is how filaments can depolymerize in the presence of high concentrations of unpolymerized actin and proteins that cap both ends with high affinity. Why do bare filament ends created by severing or uncapping become shorter rather than growing or being capped? How is depolymerization made processive enough that an entire filament can disappear on a time scale of tens of seconds?

Binding most unpolymerized ATP-actin monomers to profilin (and thymosin-β4 when present) allows depolymerization of pointed ends, but strongly favors elongation at free barbed ends. This argues that depolymerization is most likely at pointed ends. However, the rate of ADP-actin dissociation from pointed ends is so slow (Table 2) that even a 30-fold increase in this rate (23) may not explain the high in vivo rates of turnover inferred from leukocyte or keratocyte movement (27, 132). ATP exchange on subunits at barbed ends of ADP-actin filaments makes depolymerization even less favorable (131) and may influence pointed-end depolymerization as well.

Thus, other cellular factors may be required to enhance actin filament turnover. One candidate is actin-interacting protein 1 (Aip1), a conserved 64-kDa protein that interacts with actin and cofilin. Aip1 localizes to dynamic regions of the cell cortex such as lamellipodia in *Dictyostelium discoideum* (60). This protein enhances the filament disassembly activity of ADF/cofilin (93, 114), but the published work has not revealed the mechanism. One hypothesis is that, at any given time, few subunits in actin filaments are in the conformation required to bind ADF/cofilin. By interacting with both actin filaments and ADF/cofilin, Aip1 may enhance the binding of ADF/cofilin to actin filaments.

Capping poses theoretical difficulties for depolymerization at both ends. All of the actin filaments in the dendritic arbor at the leading edge are associated at their pointed ends with the side of other filaments, presumably capped by Arp2/3 complex (128). Therefore, how does depolymerization get started? One answer is that ADF/cofilins sever filaments between the caps, as shown by experiments with filaments of pure actin capped on both ends with gelsolin and Arp2/3 complex. Such double capping does not inhibit depolymerization by ADF/cofilin (16, 111). A more difficult question is what prevents capping of free ends during

depolymerization? The micromolar cytoplasmic concentrations and nanomolar affinities of capping protein and Arp2/3 complex are theoretically sufficient to cap all ends (83, 122). The rate of capping protein binding to barbed ends is expected to leave them free only for brief intervals. Nothing is known about the rate of Arp2/3 complex binding to pointed ends, and in principle the large complex may bind so slowly that free pointed ends are left open for longer times. In addition, three potentially synergistic mechanisms might favor processive depolymerization of dendritic actin filament networks: (*a*) ADF/cofilin binding to Arp2/3 complex (16) may inhibit binding to pointed ends; (*b*) the conformational change induced in actin filaments by ADF/cofilin (75) may promote dissociation of cappers from one or both ends, particularly if it is cooperative; or (*c*) ATP hydrolysis and phosphate dissociation from terminal subunits may reduce the affinity of one or both cappers (12a). To account for the rapid turnover in vivo, it would be helpful if one or more of these mechanisms made depolymerization processive, because rapid recapping appears to have the potential to stop disassembly soon after an end is exposed.

HOW DO STABLE FILAMENTS SURVIVE IN CYTOPLASM?

In addition to the intrinsic ATP timer built into actin filaments, at least two mechanisms control the stability of actin filaments in cytoplasm: regulation of severing proteins, including both gelsolin and ADF/cofilins, and binding of stabilizing proteins like tropomyosin.

Phosphorylation of ADF/cofilins provides a mechanism for signaling pathways to regulate the stability of actin filaments. PAK (a kinase regulated by Rho family GTPases) activates LIM-kinase (36) to phosphorylate a serine near the N terminus of ADF/cofilins (2, 88). Phosphorylation has no effect on the atomic structure, but reduces the affinity of ADF/cofilins for actin monomers and filaments by about 2 orders of magnitude, presumably by steric and electrostatic effects (17). Substitution of an acidic residue for this serine has a much smaller effect (112). The extent of ADF/cofilin phosphorylation and rate of phosphate turnover depend on agonist stimulation (8, 76). This mechanism may allow signals flowing through Rho family GTPases to coordinate the initiation of new filaments (through WASp and Arp2/3 complex) with their rate of turnover (through PAK, LIM-kinase, and ADF/cofilins). It is not known whether this regulation of ADF/cofilins is global or local in cells.

Calcium regulates actin filament binding and severing by gelsolin (147). Free Ca^{2+} affects the rate but not the extent of actin filament severing by gelsolin (59). Significant binding of gelsolin to actin filaments is possible at physiological concentrations of free Ca^{2+} (0.1–1 μM) (59). In resting cells, the low concentration of free calcium may allow very slow severing and contribute to filament turnover. Transient increases in intracellular free calcium will promote both gelsolin binding and actin filament severing.

One unsolved mystery is how a population of filaments in lamellapodia survives to become very long. Behind the dense arbor of short filaments at the leading edge, the actin network is composed exclusively of long filaments that extend for several micrometers toward the nucleus (126, 128, 130). Tropomyosin or other stabilizing proteins seem likely, but they have not been shown to be present.

HOW ARE SUBUNITS RECYCLED TO THE ATP-ACTIN-PROFILIN POOL?

Acting together, profilin and ADF/cofilin enhance the turnover of actin filaments to a time scale nearly compatible with the one observed in vivo (13, 23, 31). Regardless of the mechanism, the species dissociating from filaments is likely to be Mg-ADP-actin, either free or bound to ADF/cofilin. Most unpolymerized actin has bound ATP (117), so nucleotide exchange is required (Figure 6). The rate-limiting step is dissociation of ADP ($k = 0.08\ s^{-1}$ for Mg-ADP-actin). However, in physiological salt, ADF/cofilins bind ADP-actin monomers with high affinity ($K_d = 0.15\ \mu M$) and slow ADP dissociation by >10-fold to $0.006\ s^{-1}$ (13, 23, 89). In the absence of other actin-binding proteins, a pool of ADF/cofilin-Mg-ADP-actin monomers would tend to accumulate. However, profilin competes with ADF/cofilin for binding ADP-actin and catalyzes the exchange of ADP for ATP (13). This process works, because both profilin and ADF/cofilins exchange rapidly with ADP-actin. When profilin binds, ADP dissociates rapidly, and the excess of ATP over ADP in cytoplasm favors rebinding of ATP. Mg-ATP-actin binds tightly to profilin and (when present) to thymosin-β4, restocking the pool of subunits ready to elongate uncapped barbed ends and releasing ADF/cofilins to recycle back to ADP-actin filaments for another round of severing and depolymerization.

CONCLUSIONS

Exciting new experimental results summarized in the dendritic-nucleation hypothesis have opened the way toward a molecular explanation for pseudopod extension. We have described what amounts to an enzymatic cycle that converts the energy of ATP hydrolysis into mechanical force through the polymerization and depolymerization of actin filaments. In the inactivated state, the system is poised for assembly, with a large supply of actin monomers ready for rapid elongation when new filaments are created by the activation of Arp2/3 complex. WASp/Scar proteins control the activity of Arp2/3 complex, which is consumed by incorporation into a branching network of filaments. Growth of each filament is transient, owing to rapid capping, so signaling pathways must supply active Arp2/3 complex at a constant rate to maintain polymerization and pseudopod extension. The system

tends automatically toward depolymerization, so that, when assembly stops, the balance is shifted to disassembly by ADF/cofilins. The direction of subunit flow through the system is created by irreversible hydrolysis of ATP bound to polymerized actin (and P_i release) and is driven by the higher concentration of ATP than ADP in the cytoplasm. Thus ATP hydrolysis is necessary to return polymerized actin back to the unpolymerized monomeric pool. The rates of the reactions explain why cells can change direction so quickly in response to chemotactic signals and why, in the absence of signals, pseudopods tend to collapse. The pace of these events can vary between cell types and in a single cell, depending on the rate of signal input and the concentrations and activities of a few key proteins.

Although attractive as an initial attempt at a mechanism, most of the ideas in the dendritic-nucleation hypothesis still require rigorous testing, especially in live cells. In particular, we lack essential information about signaling pathways, signal integration, WASp/Scar activation, nucleation mechanisms, and depolymerization mechanisms.

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