Regulation of Actin Filament Assembly by Arp2/3 Complex and Formins

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cortactin, profilin, Scar, WASp, WAVE

Abstract

This review summarizes what is known about the biochemical and biophysical mechanisms that initiate the assembly of actin filaments in cells. Assembly and disassembly of these filaments contribute to many types of cellular movements. Numerous proteins regulate actin assembly, but Arp2/3 complex and formins are the focus of this review because more is known about them than other proteins that stimulate the formation of new filaments. Arp2/3 complex is active at the leading edge of motile cells, where it produces branches on the sides of existing filaments. Growth of these filaments produces force to protrude the membrane. Crystal structures, reconstructions from electron micrographs, and biophysical experiments have started to map out the steps through which proteins called nucleation-promoting factors stimulate the formation of branches. Formins nucleate and support the elongation of unbranched actin filaments for cytokinesis and various types of actin filament bundles. Formins associate processively with the fast-growing ends of filaments and protect them from capping.

Contents

INTRODUCTION

Actin filaments not only provide mechanical support for cells but also participate in a wide variety of biological movements, including amoeboid cell locomotion and cytokinesis. Actin polymerization is a classic example of self-assembly, but to keep self-assembly from running amuck, cells tightly regulate all aspects of actin assembly by using a fascinating repertoire of proteins. Regulation is essential, because cytoplasm contains a high concentration of actin subunits that are capable of polymerization. This is demonstrated in cell extracts by simply adding some filaments with free barbed ends, which elongate rapidly until they are capped (81, 135). Multiple mechanisms control the pool of assembly-ready actin, including sequestration by thymosin-β4, binding to profilin (which inhibits nucleation but allows barbed end growth), and capping proteins (which block most barbed ends).

Cells use several mechanisms to generate actin filaments locally in response to signals. I focus on the biochemical and biophysical mechanisms of proteins that initiate new actin filaments, especially actin-related protein (Arp)2/3 complex and formins (**Figure 1**). Arp2/3 complex produces branched filaments to push forward the leading edge of motile cells and for endocytosis (93). Arp2/3 complex anchors the new filament to the preexisting actin network. The free end of the new filament elongates until a capping protein

Actin: protein building block of microfilaments

Profilin: protein that binds actin and poly-L-proline sequences

Actin-related protein (Arp)2/3 complex: protein assembly that forms actin filament branches

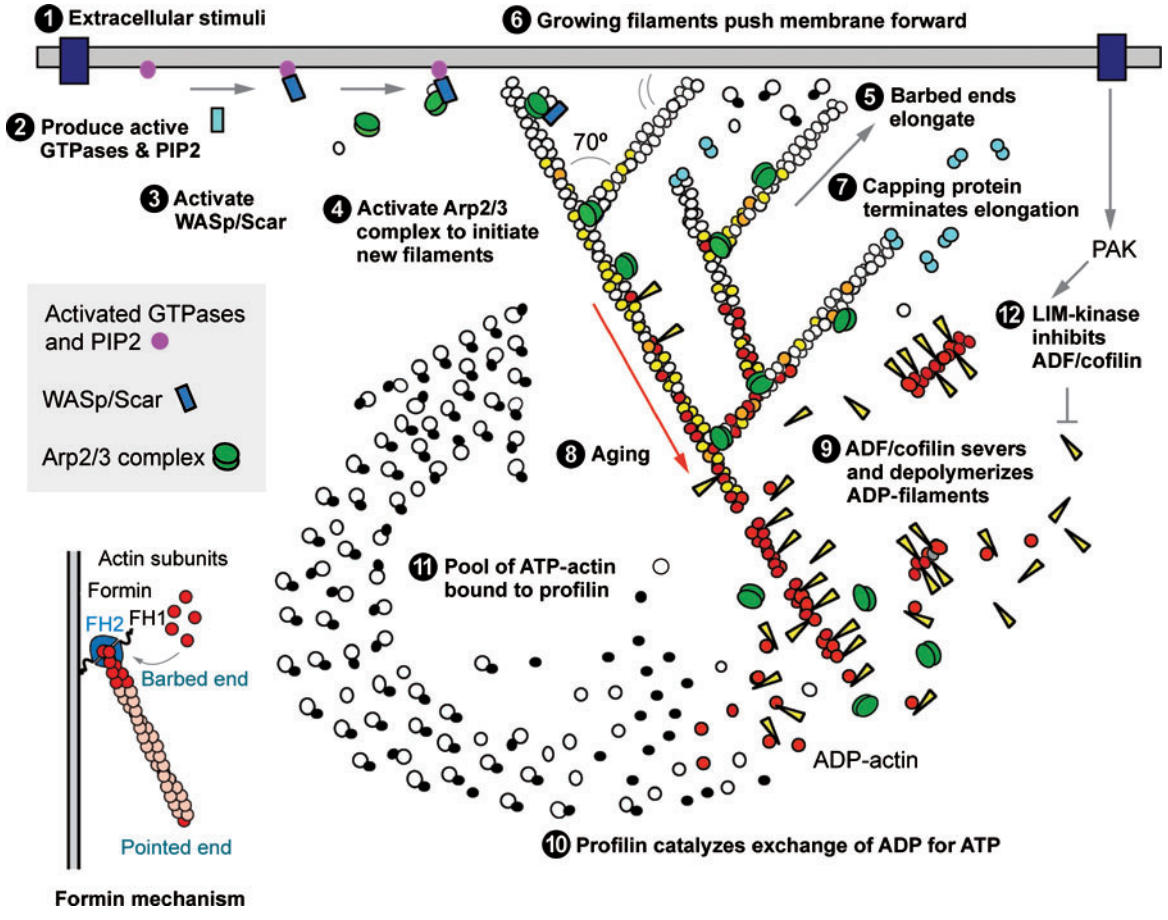

Figure 1

Overviews of the functions of Arp2/3 complex and formins. Dendritic nucleation hypothesis for the assembly of actin filaments at the leading edge of motile cells. Nucleation-promoting factors such as WASp and Scar/WAVE bring together Arp2/3 complex with an actin monomer on the side of a filament to nucleate a branch. The free barbed end of the branch grows until it is capped. Modified, with permission, from References 91 and 93. Bottom left-hand corner: Formins nucleate unbranched filaments and remain attached to their barbed ends as they elongate. Processive actin polymerization in association with a formin FH2 domain. Modified, with permission, from Reference 58.

terminates growth. Formins produce unbranched filaments for actin bundles found in filopodia and the cytokinetic contractile ring (123). The formin remains associated with the growing end of the filament, providing an anchor and protection against capping.

The field has reached a point where sophisticated quantitative assays are required to investigate mechanisms. Routine applications of simple assays are not up to this task. Typically an investigator adds their proteins to unpolymerized actin monomers (with a trace of the actin molecules labeled on cysteine 374 with pyrene) and records the change in fluorescence over time. The fluorescence of pyrenyl-actin is ~20 times higher when polymerized, so the signal-to-noise ratio is outstanding, but the only parameter directly available from this assay is the concentration of polymerized actin. If one also knows either the concentration of filament ends or the rate at which they

Formin: a member of a family of proteins with a formin homology-2 (FH2) domain that interacts with the barbed end of the actin filament

Leading edge: front end of motile cells, pushed forward by actin polymerization in the underlying cytoplasm

Actin-depolymerizing factor (ADF)/cofilin: a protein that binds actin monomers and severs actin filaments

grow, the bulk polymerization rate will give the other parameter. Direct observation of single filaments by fluorescence microscopy (2, 30) reveal elongation rates, but this approach has not yet reached general use. This microscopic assay is not without its limitations and potential artifacts (61, 69). Some proteins such as cofilin quench the fluorescence pyrenyl-actin fluorescence when they bind filaments, leading researchers to misinterpret cofilin binding to actin filaments as depolymerization.

Many investigators devise clever assays to detect interactions between proteins but report only qualitative observations such as binding or no binding. Usually the same assay could be used to measure affinities or reaction rates. For example, if a receptor is immobilized on a bead, simply using a range of concentrations of bead-bound receptors and measuring the concentration of bound ligand in the bead pellet or free ligand in the supernatant will often give the equilibrium constant (111). Similarly, if beads with bound ligand are diluted into buffer, the ligand will dissociate. The rate of dissociation can be measured by pelleting samples of the diluted beads at intervals. These two experiments, with materials already in hand in most labs, will also give the association rate constant from the ratio of dissociation rate constant to the dissociation equilibrium constant.

Understanding mechanisms is genuinely difficult. Differences in interpretations or theories are sometimes depicted as controversies, but the real issue is often the quality of the experimental design, the strength of the data, and the assumptions used to interpret the data. On the other hand, some false or questionable conclusions have become beliefs through repetition in print rather than confirmation in the laboratory. The field is blessed with a growing number of mathematical models that allow one to make quantitative comparisons of theory with experiment (76), but this strategy is still rarely used to test hypotheses regarding mechanisms.

ACTIN ASSEMBLY THERMODYNAMICS AND KINETICS

Nucleation of filaments by pure actin monomers is unfavorable owing to the extreme instability of small actin oligomers (108), so cells initiate new actin filaments with proteins including formins, Arp2/3 complex, and Spire. On the other hand, elongation of actin filaments is favorable, particularly at the fast-growing barbed end, where actin subunits associate with a diffusion-limited rate constant (90). ATP bound to each subunit is hydrolyzed to ADP with a half time of 2 s (12), and γ-phosphate dissociates slowly with a half time of 6 min (11, 74). ADP-actin dissociates from barbed ends more rapidly than does ATP-actin (90), so ATP hydrolysis and γ-phosphate dissociation may prepare filaments for disassembly in some circumstances. ADP bound to actin filaments has never been observed to exchange with ATP in the medium, so nucleotide exchange occurs only on actin monomers. Actin-depolymerizing factor (ADF)/cofilins and thymosin-β4 inhibit nucleotide exchange; profilin promotes nucleotide exchange.

Wegner (126) predicted that ATP hydrolysis in actin filaments would give the two ends different critical concentrations (the actin monomer concentration with no net association or dissociation) and showed that the subunits in filaments turn over in a way that is consistent with treadmilling or subunit flux. The two ends have different critical concentrations in buffer with millimolar Mg^{2+} and ATP, 0.1 μM at the barbed end and 0.7 μM at the pointed end, so subunits are expected to treadmill slowly (0.1 subunits per s) onto the barbed end and off the pointed end at steady state (90). This treadmilling rate is so slow that it is unlikely to contribute to actin filament turnover in cells, but understanding the mechanism has fascinated the field for years.

A full set of rate constants for ADP-actin, ADP-P_i-actin, and ATP-actin (31) provide new insights into how the two ends behave

differently at steady state in buffer containing ATP (**Figure 2**). The simplest interpretation of the effects of phosphate on depolymerization is that phosphate binds and dissociates on ADP-actin subunits at both ends of filaments much more rapidly than in the middle of the filament. In addition the affinity of pointed ends for phosphate appears to be lower than the rest of the filament. The mechanism of this surprising thermodynamic difference is not understood, but it must arise from cooperative interactions among the subunits near the end of the filament. Many features of this mechanism await investigation, such as the rates of phosphate binding and dissociation on ADP-actin monomers and along the filament.

The new parameters can be used with mathematical models (8, 121) to calculate the distribution of nucleotide states along the length of filaments and the flux of subunits through filaments at steady state. Depending on the assumptions, the ends of filaments may also fluctuate in length at steady state (121). The small values of the rate constants for dissociation of ADP-actin and ADP-P_i-actin limit the steady-state behavior to slow treadmilling and small fluctuations in length compared with the large fluctuations during dynamic instability of microtubules (23). One exception in the actin family is the bacterial actin homolog ParM (32). At steady-state ParM filaments undergo dramatic fluctuations in length. ParM is expressed from a plasmid along with an adapter protein that links ParM filaments to a DNA element similar to a centromere. Assembly and shortening of the ParM filaments help to segregate copies of the plasmid into the two daughter cells when they divide—a stripped down, actin-based mitotic apparatus for plasmids.

ADF/cofilins have been proposed to increase the rate of treadmilling (16). However, new work shows that these proteins inhibit subunit addition and dissociation at barbed ends and increase dissociation at pointed ends only up to the ADP-actin rate (3).

MECHANISM OF ACTION OF Arp2/3 COMPLEX

Background

Arp2/3 complex initiates actin filament branches on the sides of existing mother actin filaments. Arp2/3 complex anchors the pointed end of the daughter filament to the mother filament as the free barbed end of the daughter grows away from the complex. In motile cells Arp2/3 complex generates a network of branched actin filaments that grows like twigs on a bush. The tips of the branches push the cell membrane forward to protrude a pseudopod. Previous reviews provide the biological context for the functions of Arp2/3 complex (93) and a historic context for new work on mechanisms (91).

Arp2/3 complex consists of seven subunits: two actin-related proteins, Arp2 and Arp3, stabilized in an inactive state by five other subunits (**Figure 3**). The accepted nomenclature for these subunits is ARPC1 (for the 40-kDa subunit), ARPC2 (35-kDa subunit), ARPC3 (21-kDa subunit), ARPC4 (20-kDa subunit), and ARPC5 (16-kDa subunit). Metazoans, fungi, amoebae, and plants express all of these subunits; none are present in the genomes of algae, microsporidia, or apicomplexa (80), all of which have at least one gene for a formin, another actin filament-nucleating protein (see below).

Regulatory proteins called nucleation-promoting factors (NPFs), actin filaments, and an actin monomer cooperate to stimulate the intrinsically inactive Arp2/3 complex to nucleate a branch (reviewed in Reference 127). Wiskott-Aldrich syndrome protein (WASp), Scar/WAVE, and other NPFs usually include three short functional segments: a V motif (verprolin homology; also called WH2 for WASp homology 2), a C motif (connecting; erroneously called cofilin homology in early work), and an A motif (acidic) (**Figure 4**). Free VCA domains are largely unfolded (71) but assume secondary structure when bound to actin (20) or Arp2/3 complex

Nucleation-promoting factors (NPFs): proteins that bring together actin monomers and actin filaments with Arp2/3 complex to make a branch

Wiskott-Aldrich syndrome protein (WASp): an NPF and product of the gene mutated in an X-linked human immunodeficiency and bleeding disorder

Scar/WAVE: nucleation-promoting factors

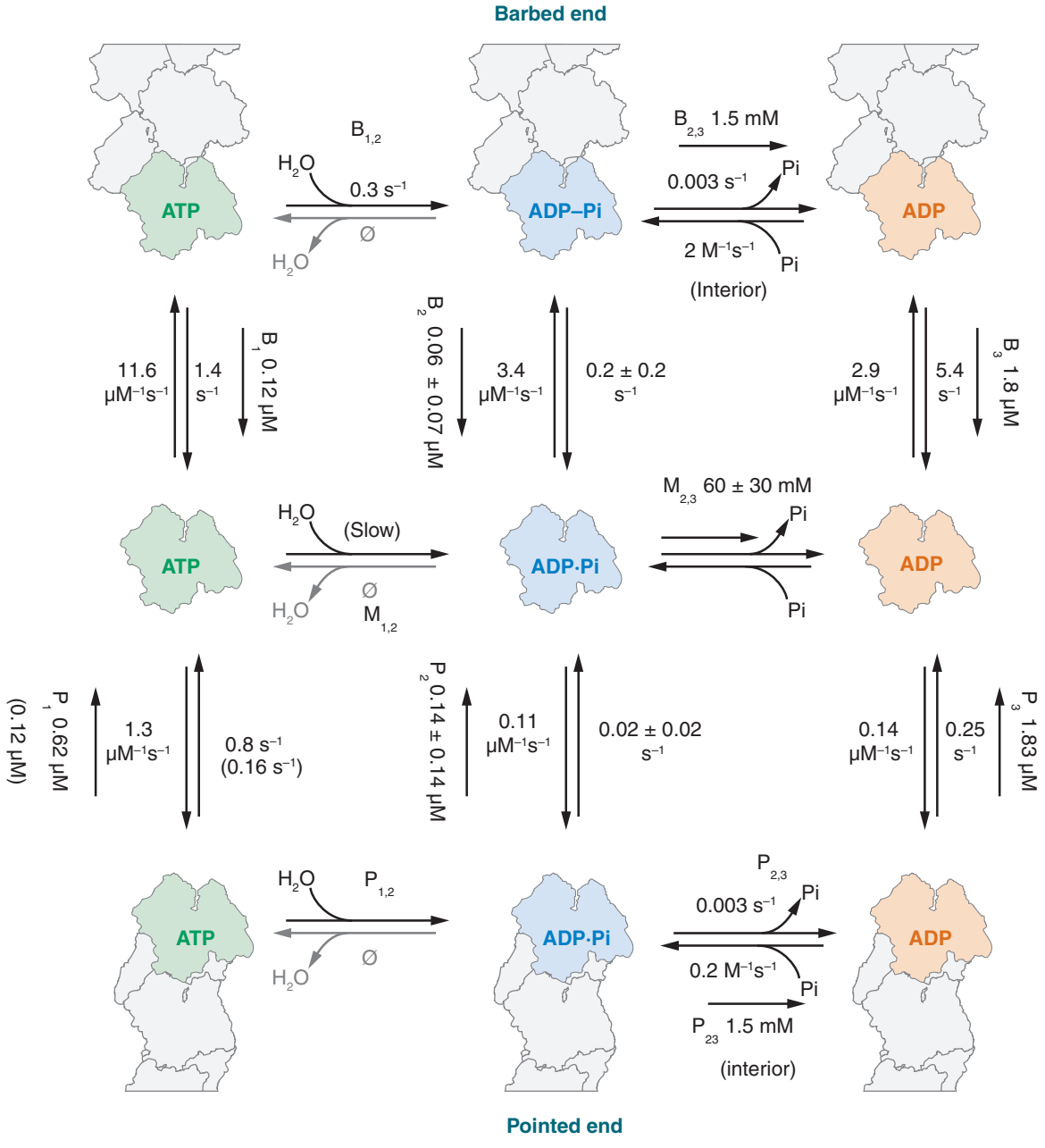

Figure 2

Actin filament elongation reactions. Rate and equilibrium constants for association and dissociation of ATP-actin, ADP-P_i-actin, and ADP-actin subunits at both ends of actin filaments. Reproduced, with permission, from figures in Reference 31.

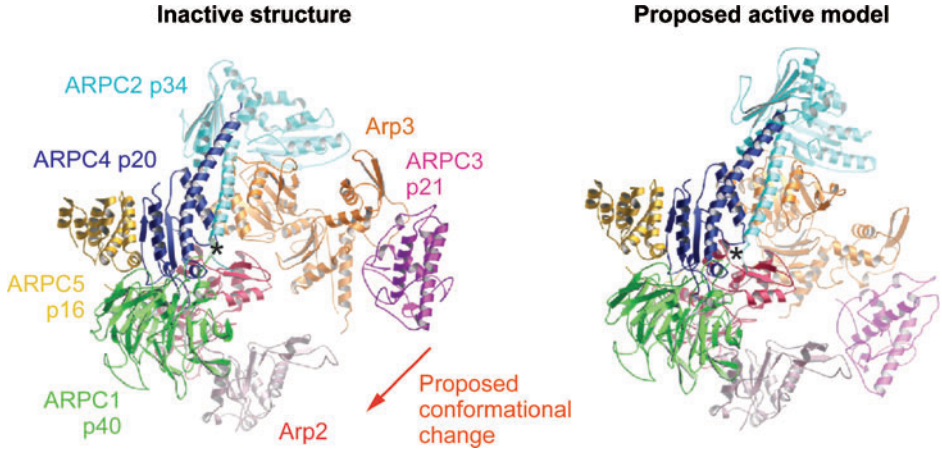

Figure 3

Ribbon diagrams of bovine Arp2/3 complex. Left: Crystal structure of inactive Arp2/3 complex. Right: Hypothesis for the structure of active Arp2/3 complex with the Arps arranged like two subunits along the short-pitch helix of an actin filament. Reproduced, with permission, from Reference 99.

(86). By virtue of the affinity of VC for actin and of CA for Arp2/3 complex, VCA domains bring together Arp2/3 complex and the first subunit in the daughter filament.

A prime but unmet goal has been to establish the pathway of branch formation. Reaching this goal requires structures of the reactants and the product (the branch junction) as well as the rate and equilibrium constants for the steps on the assembly pathway. We know the structures of the reactants: actin monomer (50), actin filament (46), inactive Arp2/3 complex (99), and examples of activators (20, 54). Electron microscopy has provided the first views of the conformation of Arp2/3 complex in branch junctions. Crystallography and biochemical experiments have characterized interactions of NPFs with Arp2/3 complex and actin. Fragments of the branch assembly pathway have been characterized kinetically, but many key parameters are still unknown.

Structure of the Branch Junction

Two-dimensional reconstructions of electron micrographs of branches with molecular tags on subunits of Arp2/3 complex (27) confirmed the hypothesis that Arp2 and Arp3 are the first two subunits in the daughter filament. The Arps are separated in the crystal structures of inactive Arp2/3 complex (82, 99), so a substantial conformational change is required to bring them together like two subunits in an actin filament (**Figure 3*b***). However, in the absence of a high-resolution structure of either active Arp2/3 complex or the branch junction, the pathway from reactants to a branch was merely a matter for speculation.

A new three-dimensional reconstruction of actin filament branches (105) provides the first glimpse of the rearrangements required to make a branch (**Figure 5**). A tilt series of electron micrographs of negatively stained branch junctions was used to make a tomographic reconstruction with a resolution of ~2.5 nm. Crystal structures of the subunits of Arp2/3 complex and actin filaments were used to build a model within the envelope of density provided by the reconstruction. I assume for this discussion that the model of the branch junction is the structure of active Arp2/3 complex. Confirmation of some details of this model requires a high-resolution crystal structure of the active conformation of the complex.

The branch model confirms that Arp3 and Arp2 are the first two subunits at the pointed end of the daughter filament. The surfaces of Arp3 and Arp2 that bind the first and second subunits in the daughter filament are conserved and similar to actin (7), so these interactions are favorable once the Arps are brought together in the active complex.

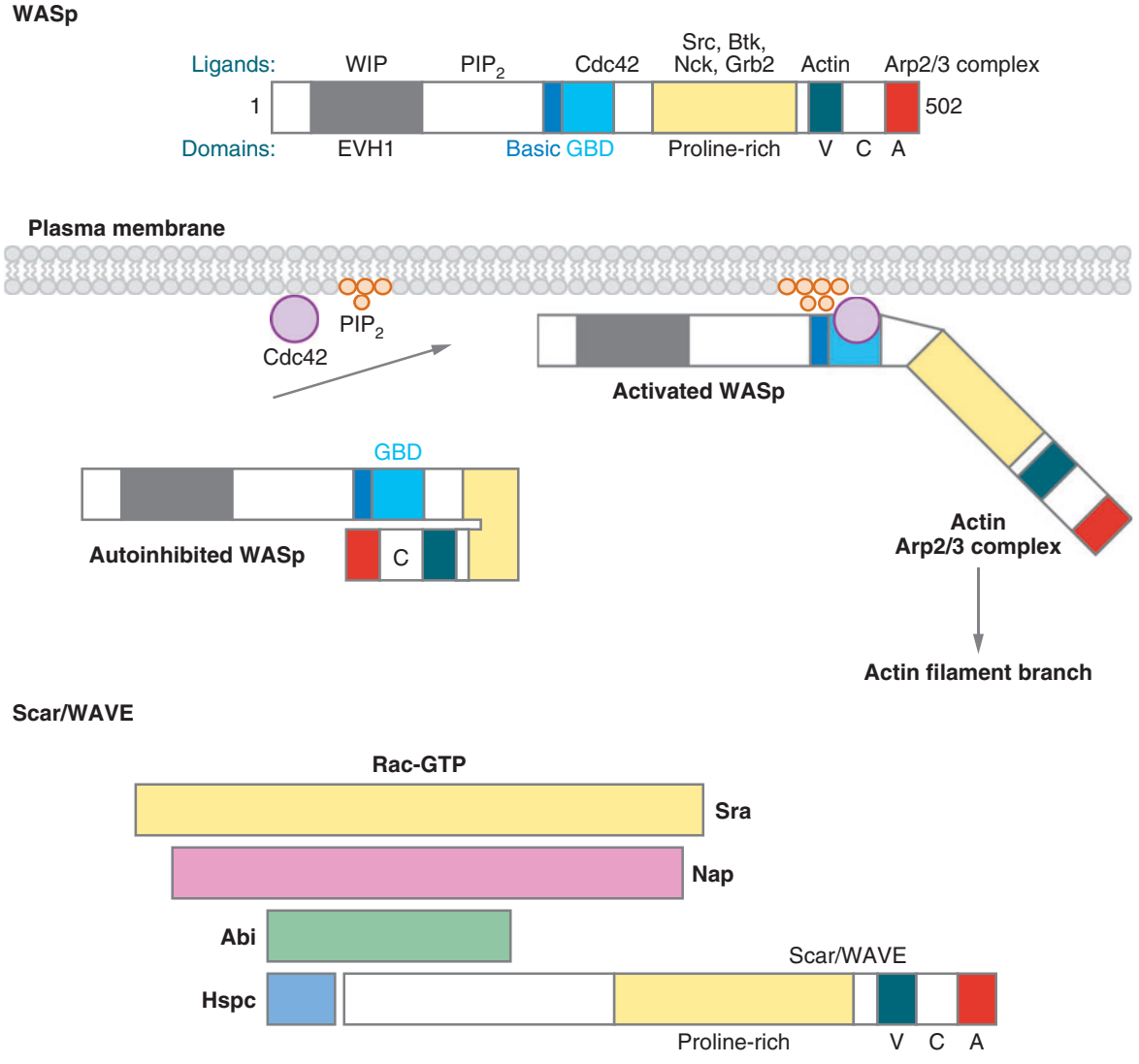

Figure 4

Domain organization and regulatory mechanisms of nucleation-promoting factors. Top: WASp, bottom: Scar/WAVE.

Bringing the Arps together to initiate the daughter filaments requires a substantial conformational change. The branch model moves Arp2 ~2 nm from its position in the crystal structure to overlap by 50% with Arp3 similar to two successive subunits along the short-pitch helix of an actin filament. Subdomains 3 and 4 of Arp3 and ARPC3 rotate modestly from their positions in the crystal structure, whereas the rest of the complex fits into the reconstruction in the conformation of the inactive crystal structure. This movement of Arp2 requires dissociation of Arp2 from ARPC1 and ARPC4, but this is conceivable if the N terminus of ARPC5 acts like a flexible tether between Arp2 and the

rest of the complex. Alternatively, bending motions may reposition Arp2 next to Arp3 without dissociating Arp2 from ARPC1 and ARPC4.

Remarkably, all seven subunits of Arp2/3 complex interact with the mother filament in the branch model. The interface between the complex and the mother filament buries 9000 Å^2 of surface, consistent with the rigidity of the junction (10). The extensive contacts of ARPC2/ARPC4 with the mother filament were anticipated by the demonstration that this dimer binds actin filaments (38). The reconstruction shows that the conformations of two of five mother filament subunits that interact with Arp2/3 complex are unconventional. These changes open up surfaces for Arp2/3 complex to contact the mother filament.

Interactions of NPFs with Actin Monomers

V and C motifs of NPFs interact simultaneously with an actin monomer. This actin monomer is believed to become the first subunit in the daughter actin filament. V motifs consist of approximately 20 residues, which form a three-turn, amphipathic α-helix followed by an extended chain. Crystal structures show that the α-helix binds the barbed end of actin monomers between subdomains 1 and 3 (20), where it competes with profilin and inhibits nucleotide exchange on actin. The extended chain C-terminal to the helix binds along the surface of actin between subdomains 2 and 4. Many actin-binding proteins have V motifs. WASp, Scar/WAVE, and WASp-interacting protein (WIP) have one V motif and N-WASP has two. The protein Spire has a tandem repeat of four V motifs that interact with multiple actin monomers to nucleate filaments in vitro and in vivo (97). C motifs bind both actin and Arp2/3 complex (71). These interactions are independent but mutually exclusive (53), so the C motif is not sandwiched between Arp2/3 complex and actin during branch formation.

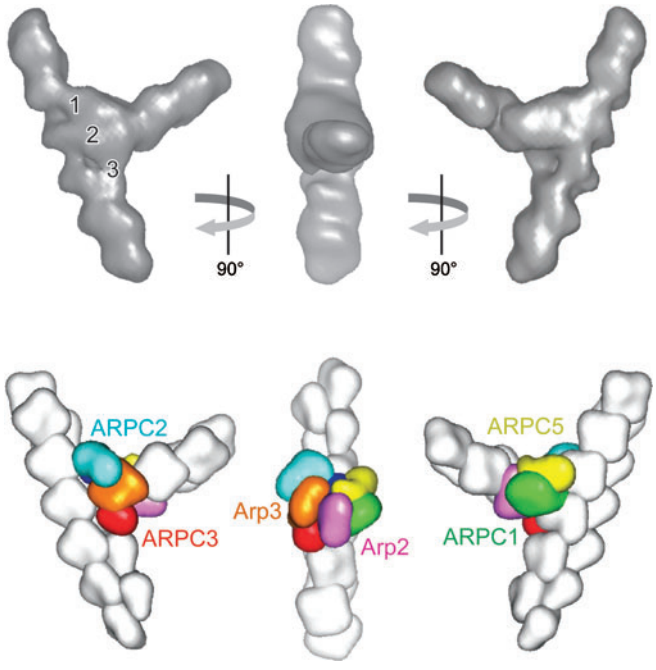

Figure 5

Reconstruction from electron tomograms of an actin filament branch junction formed by amoeba Arp2/3 complex. Reproduced, with permission, from Reference 105.

Interactions of NPFs with Arp2/3 Complex

Cross-linking and NMR experiments established that the CA segment of NPFs spans multiple subunits of Arp2/3 complex. Cross-linking and NMR of spin-labeled VCA show that the C terminus of the A motif binds Arp3 near to ARPC3 (60, 124, 133). This interaction may involve the insertion of the penultimate tryptophan of the A motif into a conserved hydrophobic hole on Arp3 near the bound nucleotide (7). Because the C motif can be cross-linked to ARPC1 (53), it must also bind near the interface of Arp2 and ARPC1. Residues in the C motif that bind Arp2/3 complex were identified by NMR and were interpreted to form an amphipathic α-helix on the basis of the pattern of hydrophobic residues and the effects of mutagenesis (86).

Three-dimensional reconstructions of Arp2/3 complex from electron micrographs show at low resolution that CA segments

WIP: N-WASP-interacting protein

N-WASP: isoform of WASp expressed in the nervous system and other tissues

Cortactin: a nucleation-promoting factor

of WASp and Scar bind Arp2/3 complex differently than does cortactin (129). CA bridges the pointed ends of both Arps with a connection to ARPC1. Cortactin bridges the pointed end of Arp3 to ARPC2, ARPC4, and ARPC5, consistent with cross-linking and competition experiments (124). These interactions are appropriate to stabilize an active conformation of the Arps, with their barbed ends ready to accept the first subunit in the daughter filament.

How Do NPFs Activate Arp2/3 Complex?

An equilibrium exists among the various conformations of Arp2/3 complex. The nucleation activity of bovine and amoeba Arp2/3 complex differs by orders of magnitude in the absence and presence of saturating NPFs (68), suggesting that less than one in a hundred complexes is active at equilibrium, but the equilibrium constant has never been measured. Several factors appear to contribute to the structural basis of this strong bias toward the inactive state. Extensive interactions with ARPC2 and ARPC4 position Arp2 and Arp3 apart from each other. The residues at the polymer interface between Arp2 and Arp3 are divergent from actin and between species, so the Arps may have a low affinity for each other in the absence of NPFs (7). Furthermore, Arp3 appears to interfere sterically with subdomain 2 of Arp2, preventing closure of the cleft around a bound nucleotide in the inactive complex (83). Budding yeast Arp2/3 complex may be an exception, because it has substantial nucleation activity without NPFs (37, 128).

NPFs, mother filaments, and actin monomers cooperate to influence this equilibrium, but individually none of them can activate the complex. Spectroscopy and electron microscopy have documented conformational changes, but the structural, thermodynamic, and kinetic details of the activation process are still incomplete.

Goley et al. (35) used fluorescence resonance energy transfer (FRET) to examine the effect of NPFs on the conformation of human Arp2/3 complex. They tagged the C termini of ARPC1 and ARPC3 with cyan fluorescent protein and yellow fluorescent protein (both pairs). The tagged complex isolated from SF9 cells was partially functional in polymerization assays and supported *Listeria* motility in cell extracts. Binding of nucleotides increased the energy transfer between the fluorescence probes. Providing that ADP or ATP was bound to the Arps, VCA further increased the energy transfer. Binding of nucleotides and VCA are coupled thermodynamically (21, 35, 63), such that nucleotide binding favors VCA binding and vice versa. Binding actin filaments or a CA construct with a mutation that allows binding but compromises activation (86) did not change the energy transfer. Therefore the FRET signal comes from a conformational change. Mutations showed that nucleotide binding to both Arps is important for branch nucleation, but only nucleotide binding to Arp3 is required for the change in energy transfer. Additional information is required to understand the nature of these conformation changes. If ATP binding simply moved the fluorescent probes closer together, the conformational changes would have to be larger than the differences observed between crystals of Arp2/3 complex without nucleotide and with bound ATP or ADP (82).

Two studies used two- and three-dimensional reconstructions of single particles of Arp2/3 complex from electron micrographs to examine the influence of NPFs and mutations on the conformation of the complex. Rodal et al. (101) examined budding yeast and bovine Arp2/3 complex; Xu et al. (129) compared budding yeast and amoeba Arp2/3 complex. Both sets of investigators prepared the specimens by negative staining. Rodal et al. found equal parts of three populations of particles in preparations of wild-type yeast Arp2/3 complex: an open

state into which the inactive crystal structure fit well; an intermediate state; and a more compact closed state. The bovine complex was divided between the intermediate and closed states. The intermediate and closed states have a mass corresponding to subdomains 1 and 2 of Arp2 that is missing from the open state. These subdomains of Arp2 are mobile in crystals (99). Binding of coronin (an inhibitor of Arp2/3 complex activation) or inactivating mutations in ARPC2 favor the open state. Binding of full-length yeast WASp or activating mutations in ARPC2 favor the closed state, which Rodal et al. interpreted to be active. Xu et al. found that Scar-VCA converted a uniform population of inactive Arp2/3 complex shaped like the crystal structure into a uniform population of particles, with the nucleotide-binding cleft of Arp2 closed and the two Arps moved closer together like subunits in an actin filament. These changes are consistent with the effects of NPFs on the positions of fluorescent tags on ARPC1 and ARPC3 (35).

Pathway of Branch Formation

The assembly pathway must be complicated, given the extensive conformational changes required for Arp2/3 complex and an actin filament to make a branch junction. Branch formation requires the cooperation of VCA, Arp2/3 complex, the first actin monomer, and the mother filament. Mother filaments alone do not activate Arp2/3 complex (35), but without mother filaments nucleation by Arp2/3 complex, VCA, and actin monomers is slow (10).

Biochemical reconstitution experiments indicate that the rate-limiting steps in branch formation occur after NPFs have equilibrated with Arp2/3 complex and actin monomers. The reactions of VCA domains with actin monomers and Arp2/3 complex are favorable, rapid equilibria (71). Thus the pathway is expected to begin with an equilibrium mixture of three species: VCA bound to actin, VCA bound to Arp2/3 complex, and a ternary complex of Arp2/3 complex linked to an actin monomer by VCA. In vitro the absolute rate of branch nucleation is directly proportional to the concentration of bovine Arp2/3 complex in the presence of excess VCA, actin monomers, and actin filaments (69). Assuming that Arp2/3 complex was nearly saturated with VCA and actin in this experiment, the second-order association rate constant of the ternary complex with actin filaments was 0.002 $\mu M^{-1}\ s^{-1}$, the same as the rate constant for fission yeast Arp2/3 complex saturated with VCA to bind actin filaments (6). As appropriate for the extensive interface between Arp2/3 complex and the mother filament, dissociation is also slow ($10^{-3}\ s^{-1}$), resulting in a dissociation equilibrium constant of 0.5 μM (6).

The thermodynamic or kinetic barriers to branch nucleation are unknown, but several ideas deserve consideration. One possibility is that the conformations of Arp2/3 complex and actin filaments required for interaction are poorly populated even under favorable conditions. For example, the dominant conformation of Arp2/3 complex with a bound NPF in solution may differ substantially from the conformation in a branch junction. Furthermore, few segments of actin filaments are expected be in the distorted conformation found in branch junctions (105). Alternatively, even after formation of a collision complex between Arp2/3 complex, NPFs, and the mother filament, slow first-order rearrangements may be required to make a branch. This explanation is consistent with several lines of evidence. Because interactions of C motif with actin and Arp2/3 complex are mutually exclusive, and because both interactions are required for branching, the C motif must interact sequentially with these two ligands (53). Kelly et al. (53) suggest that the C motif first helps to activate Arp2/3 complex and then delivers the first actin subunit to the daughter filament. Knowledge of the rates of these reactions with cellular concentrations of the

Actin patch: cluster of actin filaments associated with endocytosis in fungi

reactants (including profilin) will be required to define the pathway.

Because NPFs attached to the surface of bacteria (67) activate Arp2/3 complex to nucleate branched filaments and then stay behind as polymerization pushes the bacterium forward, the immobilized NPFs must dissociate from Arp2/3 complex during branch formation or soon thereafter. Transient tethering of Arp2/3 complex to the bacterium by the NPF may contribute to the drag observed between these particles and the actin network (34, 119). Similarly, in yeast actin patches, Arp2/3 complex and actin move away from NPFs on the plasma membrane within seconds (51, 52, 111). The same is expected to be true at the leading edge of motile cells. VCA dissociates from both Arp2/3 complex and actin monomers on a subsecond timescale (71), but the timing of dissociation relative to branch formation and ATP hydrolysis by Arp2/3 complex is not yet established.

Role of Nucleotides Bound to Arps

Both Arps have nucleotide-binding sites similar to those of actin. When ATP or ADP binds to nucleotide-free Arp3, subdomains rotate about two different axes (82). These motions close the nucleotide cleft by 6 to 8 Å, resulting in slight constriction of the complex. These subtle changes increase the affinity of Arp2/3 complex for VCA by an order of magnitude (21, 35, 63). ADP and ATP bind to the surface of subdomains 3 and 4 in the nucleotide-binding cleft of Arp2, but their presence does not immobilize subdomains 1 and 2 in the inactive complex (82) or change energy transfer between ARPC1 and ARPC3 (35).

Role of ATP Hydrolysis by Arp2/3 Complex in Branch Formation

ATP hydrolysis by Arp2/3 complex and actin influence the formation and stability of branches. Both Arps have a histidine comparable to a histidine implicated in hydrolysis of ATP by actin (122). Like monomeric actin, neither Arp hydrolyzes ATP in the inactive complex. The mobility of subdomains 1 and 2 of Arp2 may preclude ATP hydrolysis until a branch is formed. The low ATPase activity of Arp3 may result from the displacement of the catalytic histidine from its position in actin by a hydrogen bond to a neighboring histidine (82).

Three groups have investigated the role of ATP hydrolysis by Arp2/3 complex in branch formation (21, 22, 35, 63, 64, 72, 73). The investigators agree that Arp2/3 complex with bound ATP binds NPFs with higher affinity than does Arp2/3 complex with bound ADP. They agree that ATP binding to Arp2/3 complex is required for nucleation of daughter filaments and that Arp2 hydrolyzes ATP much faster than does Arp3. They disagree on the rate of ATP hydrolysis by Arp2 and the relation of ATP hydrolysis to nucleation and dissociation of branches from mother filaments. Readers should appreciate that these experiments are challenging, because ATP is freely exchangeable between the buffer, actin, and Arp2/3 complex and because ATP hydrolysis by actin exceeds by far ATP hydrolysis by Arp2/3 complex in most experiments. Consequently, these experiments are done with radiolabeled ATP covalently cross-linked to the Arps and with unlabeled ATP in the buffer and on actin.

Le Clainche et al. (64) reported that bovine Arp2 hydrolyzes bound ATP on a minute timescale well after branches form, while others found that amoeba and yeast Arp2 hydrolyze ATP rapidly in less than 1 min with no lag in time relative to branch formation (21, 22, 72). Dayel & Mullins (22) showed that binding Arp2/3 complex with an NPF to a mother actin filament does not trigger ATP hydrolysis by Arp2 until the daughter filament starts to grow. Addition of the first actin subunit in the daughter filament stimulates Arp2 to hydrolyze ATP. This is similar to subunit interactions within actin filaments stimulating ATP hydrolysis. No one has measured the actual rate constant for ATP hydrolysis by Arp2, but if the reaction is similar to actin (half time

of 2 s), only a short lag between the binding of the first subunit of the daughter filament and ATP hydrolysis by Arp2 is expected. Martin et al. (72) agree that Arp2 hydrolyzes cross-linked ATP during the course of branch formation. They find that Arp2 with the H161A mutation is defective in ATP hydrolysis but nucleates branches, so, as in actin, hydrolysis is associated with but not required for polymerization.

Role of ATP Hydrolysis by Actin in Branch Formation and Stability

Fluorescence microscopy showed more branches on younger parts of mother filaments assembled from ATP-actin (2, 47). This might have resulted from the nucleotide in mother filaments influencing the branching reaction. However, Arp2/3 complex, VCA, and actin monomers form branches at the same rate on ADP-actin mother filaments, ADP-P_i mother filaments, and aging ATP mother filaments (69).

The most common way to document branch dissociation is to assemble branched filaments and then stabilize (and stain) the filaments at intervals with rhodamine-phalloidin for observation by fluorescent microscopy (10). In this assay the density of branches declines exponentially over time (13, 125), with the same half time (6 min) as that for dissociation of phosphate from ADP-P_i-actin filaments (74). Inorganic phosphate, or BeF_3, slows the dissociation of branches. It was not known if phosphate dissociation from Arp2/3 complex, mother filaments, or daughter filaments was associated with debranching. Le Clainche et al. (64) observed that ATP hydrolysis by Arp2 and branch dissociation took place over several hundred seconds, so they suggested that they are causally related, but others found that Arp2 hydrolyzes ATP on a timescale of seconds during branch formation (21, 22, 72). Martin et al. (72) reported that branches formed by budding yeast Arp2/3 complex dissociate slowly (half time of 27 min) and fivefold slower with an Arp2 mutant that is defective in ATP hydrolysis. None of these studies compared the number of branches formed in solution to the number of branches observed in the microscope. This approach is also complicated by the fact that phalloidin not only stabilizes actin filaments, but also interacts with both Arp2/3 complex and VCA NPFs and strongly promotes nucleation (70).

A comparison of the polymerization time course in bulk samples with real time fluorescence microscopy of single filaments showed that many more branches form in solution than observed by microscopy (69). The difference was attributed to rapid dissociation of branches that never grow long enough to be distinguished from the mother filament. One population of branches dissociates rapidly from segments of mother filaments containing mixtures of ADP-P_i and ADP subunits. Other filaments dissociate in parallel with phosphate dissociation from ADP-P_i filaments. Consequently, more branches are observed on ATP and ADP-P_i mother filaments than on ADP mother filaments. More work is required to characterize these dissociation events.

Technical Matters

Mechanistic interpretation of some published work is limited by three factors. First, some investigators report qualitative raw data rather than calculate quantitative parameters. They use the fluorescence of pyrene-labeled actin to measure the concentration of polymer in bulk samples and report raw polymerization data in arbitrary units. This data should be used to calculate the concentration of barbed ends over time from the rate of polymerization (moles of polymer formed per s), the actin monomer concentration (from the difference between the total actin monomer at the start of the reaction and the concentration of polymer), and the barbed end elongation rate constant (10 $\mu M^{-1}\ s^{-1}$). Publications should also include the rate of end formation relative to the concentrations of Arp2/3 complex, actin filaments (an essential activator), and NPF.

Second, the rates of spontaneous polymerization of some actin preparations are high, most likely due to the presence of oligomers. Low concentrations of truly monomeric Mg-ATP-actin nucleate at such a low rate that the background polymerization is nearly zero. Third, many papers neglect the fact that mother filaments are required for Arp2/3 complex to nucleate new filaments. Instead of varying the concentration of polymerized actin in assays, the influence of the filaments is usually neglected.

Biochemical assays of Arp2/3 complex with muscle actin or without profilin may be misleading. Wen et al. (128) found that budding yeast Arp2/3 complex nucleates polymerization of yeast actin without NPFs but that nucleation of muscle actin requires NPFs. Bovine Arp2/3 complex requires NPFs to nucleate both muscle and yeast actin. With rare exceptions (21, 22), experiments with yeast, platelet, or brain Arp2/3 complex have been done with muscle actin. The participation of profilin is also generally ignored. Profilin and VCA compete for binding actin monomers (26, 43), so profilin inhibits branching nucleation by Arp2/3 complex in vitro (68) and presumably is an important factor in cells.

REGULATION OF NUCLEATION-PROMOTING FACTORS

Work on NPFs has advanced rapidly owing to the discovery and characterization of proteins that regulate their activity. Readers can consult recent reviews for additional details (4, 52, 115, 127).

Regulation of WASp and N-WASP

Intramolecular interactions of a GTPase-binding domain (GBD) with the C motif of VCA autoinhibit WASp and N-WASP (**Figure 4**). Rho-family GTPases (Cdc42 and Rac) cooperate with phosphatidylinositol 4,5-bis-phosphate (PIP_2) to overcome autoinhibition by binding the GBD and displacing VCA, which can then interact with actin and Arp2/3 complex. Leung & Rosen (65) put this mechanism on a firmer thermodynamic basis. Cdc42-GTP is a better activator of WASP than is Cdc42-GDP, because the difference in its affinity for active and inactive WASp is greater than that of Cdc42-GDP. Even at saturation Cdc42-GDP only partially activates WASp. In animals SH3 domains of Nck and Grb2 are an alternative to Cdc42 to activate N-WASP (17, 102), but in budding yeast SH3 domain proteins Bbc1p and Sla1p inhibit Las17p, which is otherwise constitutively active (100).

Verprolin and WIP

A family of proline-rich proteins called verprolins in yeast and WASp-interacting proteins (WIPs) in animals bind WASp. In yeast genetics, live-cell imaging and biochemistry established that verprolin coordinates the activities of WASp with type I myosins to activate actin assembly by Arp2/3 complex in actin patches during clathrin-dependent endocytosis (111, 117). Animal WASp and N-WASP also interact with proteins that have established roles in endocytosis and podosome formation (52).

Mammals have two proteins related to verprolin, widely expressed WIP and brain-specific CR16. These proteins have N-terminal WH2 domains, a central domain rich in proline, and a C-terminal domain that binds N-WASP. WIP inhibits the nucleation-promoting activity of N-WASP. Cdc42, PIP_2, and SH3 proteins overcome this inhibition and allow N-WASP to stimulate actin assembly by Arp2/3 complex (44, 77). On the other hand, CR16 binds actin monomers and filaments but has no effect on actin assembly stimulated by N-WASP (with Cdc42-GTP and PIP_2) and Arp2/3 complex (45). Indirect evidence that WIP is required for WASP function in vivo comes from the finding that human WASp can replace Las17p (WASp) in budding yeast only if WIP is coexpressed (98).

PCH-Family Proteins

The founding member of this family of proteins is fission yeast Cdc15p, a protein that participates in both endocytosis at actin patches during interphase and cytokinesis (18). The budding yeast PCH protein Bzz1p forms a complex with WASP (Las17p), type I myosins (Myo3p, Myo5p), and verprolin (Vrp1p), all of which are concentrated in actin patches (112). Bzz1p has an N-terminal FCH domain, two coiled-coils, and two C-terminal SH3 domains, which bind polyproline tracks of Las17p. Bzz1p is not required for viability, but deletion of both Bzz1p and Myo3p is lethal. The mammalian family of PCH proteins includes Toca-1 (transducer of Cdc42-dependent actin assembly), FBP17 (formin-interacting protein), CIP4 (Cdc42-interacting protein), and syndapin-1 (an endocytosis protein). Toca-1 has an N-terminal FCH domain, two regions of coiled-coils, an HR1 domain that binds Cdc42, and a C-terminal SH3 domain that binds N-WASP (44). Toca-1 and Cdc42-GTP activate the N-WASP-WIP complex to stimulate actin polymerization by Arp2/3 complex as part of a complicated regulatory network that is still being characterized.

Accessory Proteins that Regulate Scar/WAVE Proteins

In contrast to the autoinhibited WASp family of NPFs, full-length Scar/WAVE proteins are constitutively active (68). Regulation by the Rho-family GTPase, Rac, requires a complex of proteins (WAVE complex) originally isolated from brain along with WAVE1 (25). The subunits are Abi (Abl-interacting protein), Nap1 (Nck-associated protein), PIR121/Sra1 (binds Rac), and HSPC300 (a 9-kDa protein). The same proteins purify with mammalian WAVE2 (33, 49) and WAVE3 (114) as well as with Scar proteins from plants (24) and *Dictyostelium* (9). Nearest-neighbor analysis shows that Abi and HSPC300 bind WAVE, Abi binds Nap1, and Nap1 binds Sra1 (33, 49). Depletion of one WAVE complex subunit by RNAi results in the loss of the entire complex (49, 62).

Eden et al. (25) found that the WAVE complex inhibits the ability of Scar/WAVE proteins to activate Arp2/3 complex in an actin polymerization assay and that Rac-GTP overcomes this inhibition by dissociating the WAVE complex from WAVE1. On the other hand, Innocenti et al. (49) reported that Rac-GTP binds the WAVE complex without dissociating it from WAVE2. The nucleation-promoting activity of WAVE2 in their actin polymerization assays with Arp2/3 complex was the same in the presence and absence of WAVE complex or Rac-GTP. A third study (116) showed that another protein, IRSp53, enhanced the ability of Rac-GTP to stimulate the nucleation-promoting activity of the WAVE complex. A complicating factor in all these studies is that the WAVE preparations had little activity, producing under-optimal conditions, only 0.3 to 2 barbed ends for every 100 Arp2/3 complexes. Active NPFs produce nearly 1 end per Arp2/3 complex under the conditions used (43).

In HeLa cells most Abi is in the WAVE complex, but a small fraction is bound to N-WASP. This interaction of the SH3 domain of Abi activates N-WASP to stimulate actin polymerization by Arp2/3 complex in vitro (48) and during the development of *Drosophila* sensory organs (14).

Cortactin

Cortactin and WASp function synergistically, because they can bind Arp2/3 complex simultaneously. WASp is a stronger NPF, but cortactin also stabilizes branches (124) by virtue of binding sites on both Arp2/3 complex (129) and subdomain 1 of polymerized actin (87). A notable feature of actin filaments decorated with cortactin is a gap between long pitch strands of the filament that possibly distort filament in preparation for binding of Arp2/3 complex. RNAi has been used to deplete cortactin from several specialized mammalian

cells. Loss of cortactin in human fibrosarcoma cells reduces the persistence of their lamellipodia (15). Osteoclasts lose rings of actin filaments and podosomes, with the consequence that they fail to resorb bone (118). HeLa and 3T3 cells depleted of cortactin move normally and support the movement of *Listeria* in the cytoplasm, but uptake of *Listeria* by phagocytosis is compromised (5).

FUNCTIONS OF Arp2/3 COMPLEX AT THE CELLULAR LEVEL

Work on actin assembly directed by Arp2/3 complex in cells is advancing rapidly in parallel with the biochemical and biophysical studies reviewed here. Actin patches associated with endocytosis in yeast are one of the best-characterized examples. The combination of genetics and time-lapse fluorescence microscopy of fluorescent fusion proteins has mapped a detailed pathway associated with endocytosis (52). Proteins associated with clathrin-coated pits concentrate NPFs (including WASp and type I myosin) in an activator patch inside the membrane. Arp2/3 complex interacts with the NPFs for a few seconds, moves away from the NPFs on the membrane as actin polymerizes, and then disassembles over a few more seconds.

Signaling pathways regulate the local concentrations of active NPFs. Cells contain high concentrations of inhibited NPFs (micromolar range), actin monomers (tens of micromolar range), and Arp2/3 complex (micromolar range). Stimuli such as chemoattractants create local signals to activate NPFs and produce actin filaments. The combination of mRNA and protein depletion by RNAi and localization of fluorescent fusion proteins in live cells has been invaluable in helping researchers understand how these signaling pathways function in systems lacking genetics. To cite just two of many examples in the literature, WAVE2 and WAVE complex localize together at the leading edge in response to Rac activation (113). Cells depleted of the WAVE complex do not produce ruffles in response to growth factors or active Rac. On the other hand, depletion of N-WASP by RNAi has no effect on EGF-induced ruffles but compromises the formation of actin comet tails by intracellular endosomes (48).

MECHANISMS OF FORMINS

Background

Since 2002, formins have emerged as key regulators of actin polymerization and their mechanisms are already understood in some detail (see reviews in References 36, 42, and 55). Cells depend on formins for the assembly of a subset of their actin filaments, including actin filaments for the contractile ring that separates daughter cells during cytokinesis and for the unbranched bundles of actin filaments in filopodia (reviewed in Reference 123). The processive association of a formin with a growing barbed end precludes capping and allows persistent growth of these bundles of filaments (59, 79, 134).

In some cases the cellular functions of formin isoforms are so specific that they cannot substitute for each other. For example, the three formin isoforms of fission yeast nucleate actin filaments exclusively for three different structures: Cdc12p for the contractile ring (19), For3p for actin cables (29), and Fus1p for mating (89). None of these formins can substitute for each other, whereas Bni1p and Bnr1p of budding yeasts are interchangeable for the assembly of actin filament cables emanating from the bud (28, 106). Yeast actin cables assemble at spectacular rates, more than 100 subunits per s (131). The degree of specialization of the 15 vertebrate formins is less well understood (42).

Formin Structure and Influence on Actin Polymerization

Formins are homodimers of polypeptides with several characteristic domains (**Figure 6**). FH2 domains form donut-shaped

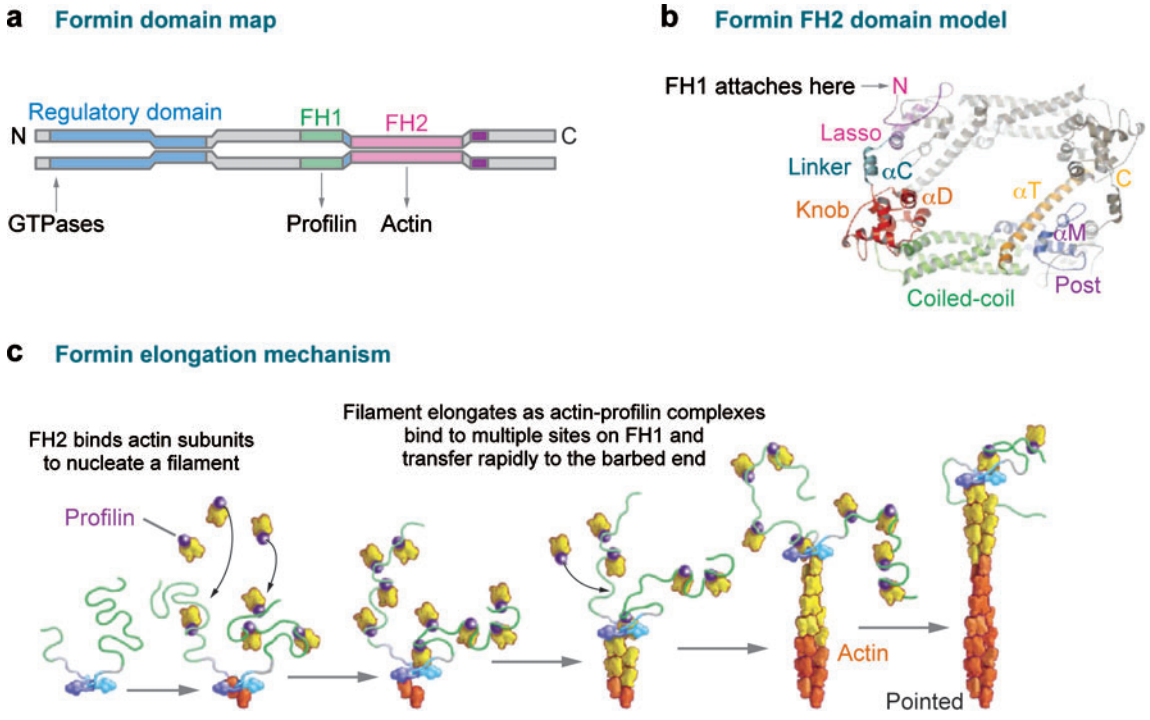

Figure 6

Formin structure and mechanism. (*a*) Domains of a generic formin. (*b*) Ribbon diagram of the structure of the FH2 domain from Bni1p. Coordinates are from Reference 130. (*c*) Elongation mechanism with actin-profilin binding to multiple sites on the FH1 domains and transferring rapidly to the barbed end growing in association with the FH2 domain. Reproduced, with permission, from Reference 94.

head-to-tail dimers (130) that nucleate actin polymerization and interact with the barbed ends of actin filaments (96, 107). A crystal structure shows that linker polypeptides connecting the two subunits can extend far enough for the FH2 homodimer of budding yeast formin Bni1p to wrap around two actin subunits, so FH2 dimers probably form a sleeve around the barbed end of the filament (85). The conformations of the FH2 domain on the end of a filament have yet to be established by electron microscopy or another approach.

FH1 domains have one or more short polyproline sequences that interact with profilin (19). Because profilin can bind simultaneously to polyproline and to an actin monomer, FH1 domains can tether multiple profilin-actin complexes near the end of a growing filament. The other formin domains appear to be more diverse. Crystal structures show that the N-terminal domains of mDia1 form a dimer (84, 104) that interacts intramolecularly with a segment of the polypeptide distal to the FH2 dimer (1, 66, 109). This intramolecular interaction autoinhibits the actin-nucleating activity of the paired FH2 domains. Rho-family GTPases activate formins by binding the N terminus, displacing the C terminus, and overcoming this autoinhibition.

Formins appear to have a wide range of behaviors, so it was possible that formin genes diverged sufficiently during evolution from a common ancestor to encode fundamentally different proteins. One documented difference is that some formins cross-link actin filaments (75, 78, 40). However, direct comparisons of constructs consisting of FH1 and FH2

domains (56) showed that diverse formins have a common mechanism but with substantial quantitative differences in some of the reaction parameters. The fission yeast cytokinesis formin, Cdc12p (19), lies at one extreme. Recombinant protein constructs consisting of the FH2 domain alone or both the FH1 and FH2 domains nucleate actin polymerization, but filaments grow only at their pointed ends (57). Including profilin inhibits nucleation to some extent but allows Cdc12p(FH1FH2) to nucleate filaments that grow at both ends, similar to free actin filaments. This gating of capping requires that a profilin can bind both polyproline and actin monomers. At the other extreme, mouse formin mDia1(FH1FH2) slows barbed end growth only 10% and profilin can increase the rate of elongation up to five times that of actin alone (56, 103). Elongation is processive with and without profilin. In between these extremes, FH1FH2 constructs of mouse mDia2 and budding yeast Bni1p slow down elongation by actin alone and also speed up elongation with profilin. The following mechanistic matters remain under active investigation.

Nucleation Mechanism

The effect of FH2 dimers on spontaneous assembly of actin can be simulated by a mechanism in which the formin stabilizes actin dimers during the nucleation process (95). This mechanism is supported by the ability of FH2 dimers to form a stable complex with two actins and by the association of a formin dimer with two actins in the crystal structure (85). The nucleation mechanism should be revisited now that the elongation mechanism is better understood.

Processivity

The pioneering papers on formin-mediated actin polymerization suggested that formin FH2 domains remain bound to the growing barbed end of the filament (96, 107, 134). This was called processive capping. The persistent association of FH2 domains with growing barbed ends was confirmed by direct observations of GFP-tagged formins on growing actin filaments (41) and of actin filaments growing on the surface of microscope slides coated with FH1FH2 domains (58). Bleaching of the fluorescent actin revealed that new (bright) subunits added at the barbed end of the filament associated with the formin on the surface. Formins remained associated with a single barbed end during the addition of thousands of subunits over 1000 s or more. One consequence of this processivity is that the presence of a formin FH2 dimer on the barbed end precludes binding of capping protein (39, 59, 79, 134). We still do not know how actin subunits insert themselves between a formin and the barbed end of a filament.

Zigmond et al. (134) and Xu et al. (130) proposed that formins walk in a stair-step fashion along actin filaments. However, when a filament grows between a formin FH1FH2 dimer and an inactivated myosin, both of which are immobilized on a glass slide, it buckles but never supercoils (58). Hence the growing actin helix must rotate relative to the formin similar to a shaft in a bearing, or the formin must rotate relative to the substrate. A theory exists to explain how an FH2 dimer might rotate on the end of a filament (110), but we still do not know how an FH2 dimer remains bound to an elongating barbed end without tracking with the actin helix.

Gating of Elongation

A formin FH2 dimer on the barbed end of an actin filament slows down the addition of actin subunits, but the degree of inhibition varies widely from only 10% for mDia1 to nearly 100% for Cdc12p (56). One interpretation is that the FH2 domain has at least two states on the end of a filament: an open state that allows an actin subunit to bind and a capped state that does not. The equilibrium between these states varies from ~0 for Cdc12p to ~0.9

for mDia1. A formin may have to visit the capped state transiently after the addition of each new subunit in order to maintain its position on the end of the filament. Otomo et al. (85) proposed plausible models of the open and capped states, but additional work is required to understand the structures, kinetics, and thermodynamics of FH2 domains on the end of the filament.

Acceleration of Elongation by Profilin

Profilin increases the elongation rate of filaments associated with formin FH1FH2 dimers (57, 103). The maximum rate of elongation occurs when the actin monomer pool is saturated with profilin (56). Elongation is slower at higher concentrations of profilin owing to free profilin competing with profilin-actin for binding to FH1 sites. The extent of acceleration by profilin depends on the number of profilin-binding sites in the FH1 domain (88). Romero et al. (103) discovered that actin filament elongation with a formin and profilin can exceed the rate of a free barbed end, a remarkable finding considering that elongation is a diffusion-limited process. For mDia1 the elongation rate at the optimal profilin concentration is four to five times higher than the elongation rate at the same concentration of actin (56). One model for this mechanism is that multiple profilin-binding sites on flexible FH1 domains allow profilin to concentrate actin near the FH2 domain and to transfer actin rapidly to the barbed end. A mathematical model based on this hypothesis is consistent with the available data (120). Understanding how profilin accelerates growth will depend on further insights into processive elongation and on a deeper understanding of FH2 interactions with the end of the filament and of profilin itself (132).

Requirement for ATP Hydrolysis

Romero et al. (103) presented evidence that processive elongation with mDia1p is coupled to ATP hydrolysis by the actin, but Kovar et al. (56) found that ADP-actin supports processive elongation by mDia1 and Cdc12p. Additional work is required to understand how ATP hydrolysis contributes to elongation.

Technical Matters

Because formins not only stimulate the formation of new filaments but also affect the rate of elongation of barbed ends, interpretation of bulk polymerization assays is difficult or impossible. Without knowledge of the number of growing ends, it is impossible to calculate elongation rates from the time course of polymerization in bulk samples. The only reliable way to know the elongation rate is by direct observation of single filaments by fluorescence microscopy. The nucleation rate (the change in filament ends over time) can be calculated from the time course of polymerization in a bulk sample if the elongation rate as a function of actin monomer concentration (and profilin concentration) is known.

SUMMARY POINTS

1. Proteins called NPFs bring together Arp2/3 complex, an actin monomer, and an actin filament to initiate an actin filament branch that grows by the addition of actin subunits to the newly formed barbed end. Growing actin filaments power cellular movements by pushing against the inside of the plasma membrane at the leading edge of the cell. The branching process builds the new filaments into the pre-existing network of actin filaments, providing a scaffold to support the forces produced by their polymerization.

2. Signaling pathways regulate actin filament assembly temporally and spatially in cells by activating NPFs. NPFs are held in an inactive state by autoinhibition or accessory proteins and activated locally in cells by Rho-family GTPases, SH3 domain proteins, and polyphosphoinositides.
3. A family of proteins called formins initiate and regulate the growth of unbranched filaments by a mechanism different than the Arp2/3 complex. A donut-shaped dimer of formin FH2 domains remains attached to the growing barbed end of the filament for hundreds of seconds as actin subunits add at rates up to at least 100 subunits per second. Profilin enhances the rate of elongation well beyond the diffusion limit by concentrating actin on multiple sites located on flexible FH1 domains and transferring the actin rapidly to the end of the filament.

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