Cell, Vol. 112, 453–465, February 21, 2003, Copyright ©2003 by Cell Press

Cellular Motility Driven by Assembly and Disassembly of Actin Filaments

Review

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Motile cells extend a leading edge by assembling a branched network of actin filaments that produces physical force as the polymers grow beneath the plasma membrane. A core set of proteins including actin, Arp2/3 complex, profilin, capping protein, and ADF/cofilin can reconstitute the process in vitro, and mathematical models of the constituent reactions predict the rate of motion. Signaling pathways converging on WASp/Scar proteins regulate the activity of Arp2/3 complex, which mediates the initiation of new filaments as branches on preexisting filaments. After a brief spurt of growth, capping protein terminates the elongation of the filaments. After filaments have aged by hydrolysis of their bound ATP and dissociation of the γ phosphate, ADF/cofilin proteins promote debranching and depolymerization. Profilin catalyzes the exchange of ADP for ATP, refilling the pool of ATP-actin monomers bound to profilin, ready for elongation.

Directional motility is a fundamental cellular process essential for embryonic development, wound healing, immune responses, and development of tissues. For example, wiring the human brain requires the laying down of about 1 million miles of neurites, all proceeding through the crawling motility of growth cones. The motility mechanism is ancient, with key molecular components functionally conserved between protozoa and vertebrates, thus dating its origin to more than 1 billion years ago. Almost universally, crawling motility involves a cycle of four steps: protrusion of the leading edge (Figure 1), adhesion to the substratum, retraction of the rear, and de-adhesion. Remarkably, directional motility is not only manifest by intact cells but seems to be an autonomous property of the leading lamellum, the part of the cellular cortex, also called a lamellipodium, that expands forward as the cell moves. Lamellar fragments of keratocytes, which lack nuclei, centrosomes, microtubules, and most organelles, nevertheless retain the property of directional motility (Euteneuer and Schliwa, 1984); (Verkhovsky et al., 1999). Thus, the capacity of the motility machine to self-organize represents a "molecular autopilot," the rules for which we are challenged to decipher. Recent excitement in the motility field is focused on a convergence of structural, biochemical, genetic,and theoretical studies which have, for the first time, provided a coherent, semi-quantitative model for the molecular mechanism of protrusion of lamellae and how cells might respond to external signals.

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Actin filaments are, by mass, the dominant structural component of the lamellipodium and, indeed, actin is the most abundant protein in many eukaryotic cells. The filaments are double helical polymers of globular subunits all arranged head-to-tail to give the filament a molecular polarity (Figure 2). Based on the arrowhead pattern created by decoration with myosin, one end is called the barbed end and the other the pointed end. This polarity is key to the mechanism of actin assembly in cells. The barbed end is favored for growth and actin filaments in cells are strongly oriented with respect to the cell surface, barbed ends outward (Small et al., 1978). Accordingly, when permeabilized cells are provided with fluorescent actin subunits, they add to barbed ends at the leading edge of the lamellum (Symons and Mitchison, 1991; Chan et al., 2000).

Marking experiments, by photoactivating caged-fluorescent actin (Theriot and Mitchison, 1991) showed that in fast moving cells, like fish epidermal keratocytes, actin filaments remain stationary while the cell advances, thus demonstrating that protrusion of the leading edge occurs concomitantly with polymerization of actin. Alternatively, if a cell is stationary, like disc-shaped sea urchin coelomocytes, actin filaments assemble at the margin of the cell and move away from the edge (Wang, 1985; Edds, 1993; Henson et al., 1999), reflecting the same relationship to the cell surface as in locomotion. More commonly, as in fibroblasts, actin polymerization is transformed partially into protrusion and partially into retrograde flow (Theriot and Mitchison, 1992; Lin and Forscher, 1995). Analysis of single actin molecules tagged with green fluorescent protein revealed that fibroblasts incorporate actin into filaments not only at the margin of the cell, but also throughout the leading lamella (Watanabe and Mitchison, 2002). Rapid fading of fluorescent-actin marks (Theriot and Mitchison, 1991), fluorescent speckle microscopy (Salmon et al., 2002), and single molecule fluorescence microscopy (Watanabe and Mitchison, 2002) show that actin filaments at the leading edge turn over on a time scale of tens of seconds, recycling subunits for subsequent polymerization.

Amazingly, intracellular "rocketing" motility first discovered for *Listeria* (Tilney et al., 1992) shows a similar coupling of movement and actin polymerization (Theriot et al., 1992). *Listeria* recruits the motility machinery from the cytoplasm of infected cells to aid in its attempts to infect neighboring cells without subjecting itself to immune surveillance. It is now appreciated that not only bacteria, but also viruses (Frischknecht et al., 1999), endosomes (Merrifield et al., 1999), and endogenous vesicles (Rozelle et al., 2000) employ actin-polymerization for intracellular motility.

Thus, the simplest view is that protrusive motility pro-

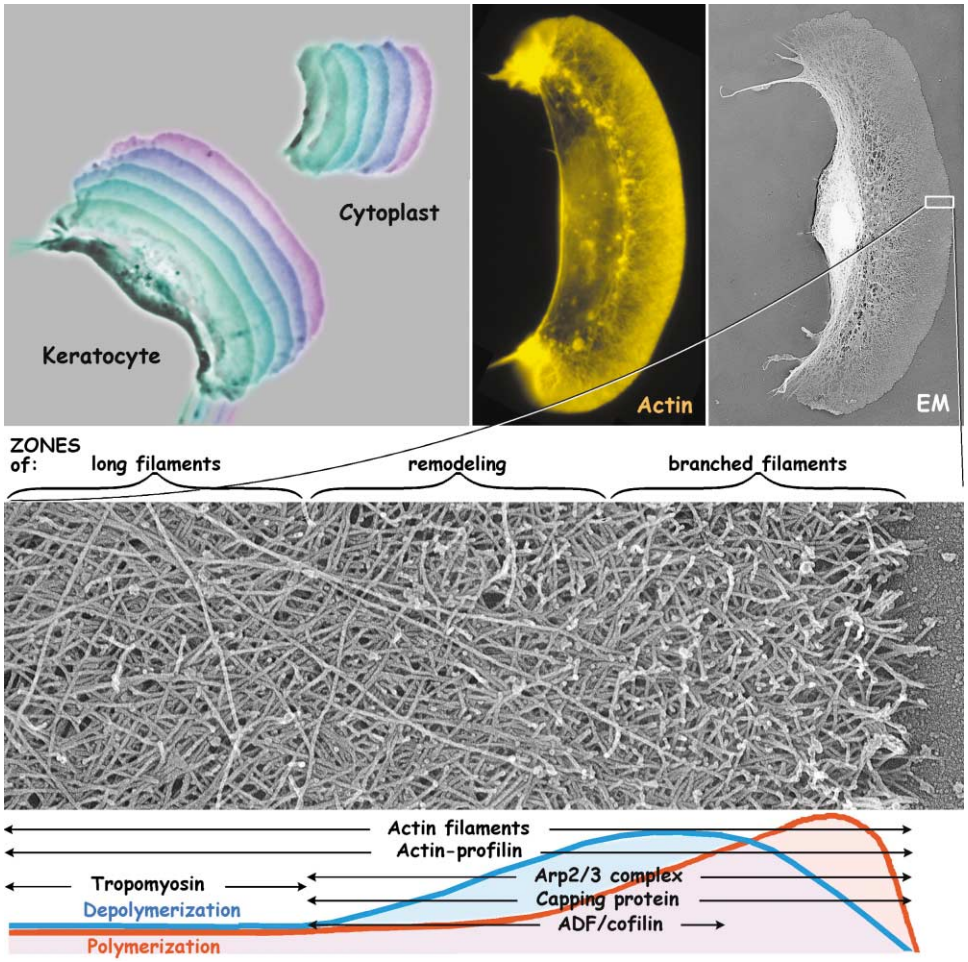

Figure 1. Light and Electron Micrographs of Motile Keratocytes

(Top left) Overlays of two series of phase contrast micrographs taken at intervals of 15 s showing the motility of a keratocyte and a keratocyte cytoplast. (Top middle) Fluorescence micrograph of a keratocyte stained with rhodamine phalloidin to label the actin filaments. (Top right) Transmission electron micrograph of a keratocyte prepared by Triton extraction in phalloidin, fixation, critical point drying, and metal shadowing. Detail of region shown below with the three zones of actin filament organization labeled (micrographs provided by Tanya Svitkina, Northwestern University School of Medicine). The schematic diagram indicates the locations of key proteins. The curves (actin subunits per unit time) indicate actin filament assembly (red) as reflected by imaging of single GFP-actin molecules (Watanabe and Mitchison, 2002) and disassembly (blue) as adapted from quantitative analysis (Mogilner and Edelstein-Keshet, 2002). Areas under curves were made equal to denote steady state.

ceeds by a treadmilling-type reaction—addition of subunits at the barbed end and loss of subunits at the pointed end. The insufficiency of such a simple view can be better understood after considering some basic actin biochemistry.

Basic Actin Biochemistry

Spontaneous assembly of pure actin monomers is unfavorable owing to the instability of actin dimers and trimers, but once started, filaments grow rapidly (Figure 2). Subunit addition at the barbed end is diffusion limited, meaning that the rate of growth is determined by the rate that subunits collide with the end. About 2% of such collisions are oriented correctly for binding (Drenckhahn and Pollard, 1986). The rate of elongation is directly proportional to the concentration of monomers in the solution with a rate constant for the barbed end of 11 μM^{-1} s^{-1} (Pollard, 1986). Thus, 10 μM actin monomers elongate barbed ends at 110 subunits (0.3 μm) per second. The ratio of the rate constants for dissociation and association ($k-/k+$) is the dissociation equilibrium constant for subunit binding at the end of a polymer, also known as the critical concentration. All actin above the critical concentration polymerizes, leaving the critical concentration of subunits exchanging with the end of the polymer. For the physiologically relevant species, Mg-ATP actin, the critical concentration is lower at the barbed end (0.1 μM) than at the pointed end (0.7 μM). As a consequence, the steady-state concentration of monomers in ATP is slightly above the critical concentration at the barbed end and below that at the pointed end.

Mg-ATP bound in a deep cleft in actin stabilizes the molecule but is not required for polymerization per se (De La Cruz et al., 2000a). Instead, hydrolysis of ATP by polymerized actin and dissociation of the γ-phosphate

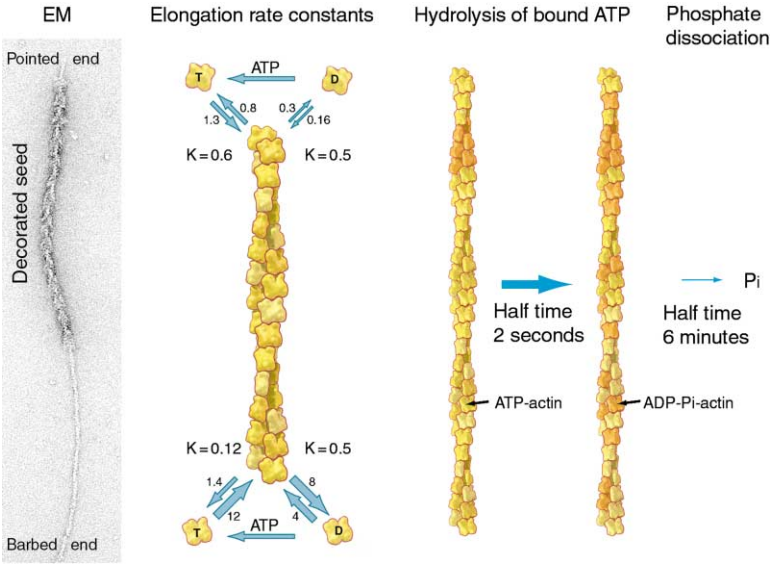

Figure 2. Actin Filament Elongation, ATP Hydrolysis, and Phosphate Dissociation

The EM shows an actin filament seed decorated with myosin heads and elongated with ATP-actin. The association rate constants have units of $\mu M^{-1}\ s^{-1}$. Dissociation rate constants have units of s^{-1}. The ratio of the dissociation rate constant to the association rate constant gives K, the dissociation equilibrium constant with units of μM. Note that the equilibrium constants for ATP-actin differ at the two ends, giving rise to slow steady state treadmilling. Hydrolysis of ATP bound to each subunit is fast, but dissociation of the γ phosphate is very slow. Modified from original artwork by Graham Johnson in "Cell Biology" by T.D. Pollard and W.C. Earnshaw, W.B. Saunders, 2002.

appear to be an internal timer that indicates the age of a filament and triggers processes that disassemble actin filaments in cells. ATP hydrolysis is irreversible (Carlier et al., 1988) and fast with a half time of about 2 s (Blanchoin and Pollard, 2002). Phosphate dissociation is much slower with a half time of 350 s (Carlier and Pantaloni, 1986), so ADP-P_i-actin is a relatively long-lived intermediate in freshly assembled filaments. Every known property of ADP-P_i actin is identical to ATP-actin. ADP-actin subunits dissociate faster from the barbed end than ATP-actin subunits, but both ATP- and ADP-actin dissociate slowly at the pointed end (Pollard, 1986). A consequence of these kinetic constants is that in the steady state, ATP-actin associates at the barbed end and ADP-actin dissociates from the pointed end, leading to very slow treadmilling of subunits from the barbed end to the pointed end, which has now been visualized directly by fluorescence microscopy (Fujiwara et al., 2002b). ATP hydrolysis in the filament is essential to maintain treadmilling.

The key point related to cellular motility is that pure actin filaments, at steady state in vitro under physiological ion conditions but in the absence of regulatory proteins, treadmill very slowly whereas cells can advance quickly. In the steady-state, growth at the barbed end is limited by dissociation at the pointed end, which is $\sim 0.2\ s^{-1}$, which corresponds to 0.04 μm/min, in contrast to keratocytes or "rocketing" microbes, which can move at 10 μm/min, more than two orders of magnitude faster. Therefore, regulatory proteins are required to explain the physiological behavior.

Cells are endowed with a rich variety of actin binding proteins, falling into more than 60 classes (Pollard, 1999). Actin and a limited subset of actin binding proteins can reconstitute bacterial motility in a purified system (Loisel et al., 1999). These proteins are actin, ADF/cofilin (Bamburg et al., 1999), capping protein (Cooper and Schafer, 2000), Arp2/3 complex (Pollard and Beltzner, 2002), an activator of Arp2/3 complex (Weaver et al., 2003), and profilin (Schluter et al., 1997). Without doubt, many other proteins participate in the process, but for purposes of simplicity, we will concentrate on these core proteins (Figure 3).

A Quantitative Hypothesis to Explain the Properties of the Leading Edge

The behavior of the actin filament network at the leading edge poses several key questions. How do actin filaments grow fast? How do cells initiate and terminate the growth of new filaments? How do actin filaments push forward the membrane at the leading edge? How are proteins in the actin filament network recycled? How do environmental and internal signals control these reactions? Answers to these questions are provided in this review by the dendritic nucleation/array treadmilling hypothesis (Figure 3). Cells contain a pool of unpolymerized actin monomers bound to profilin and sequestering proteins such as thymosin-β4. New filaments arise when signaling pathways activate nucleation-promoting factors such members of the WASp/Scar family of proteins. (The first family member discovered, WASp, is the product of the gene mutated in a human bleeding disorder and immunodeficiency, Wiskott-Aldrich Syndrome [Rengan et al., 2000]). Active nucleation-promoting factors then stimulate Arp2/3 complex to initiate a new filament as a branch on the side of an existing filament. Fed by actin-profilin from the subunit pool, new branches grow rapidly and push the membrane forward. Each filament grows only transiently, since capping proteins terminate growth. Actin subunits in this branched network hydrolyze their bound ATP quickly but dissociate the γ-phosphate slowly. Dissociation of γ-phosphate initiates disassembly reactions by inducing debranching and binding of ADF/cofilin, which, in turn, promotes severing and dissociation of ADP-subunits from filament ends. Profilin is the nucleotide exchange factor for actin, catalyzing exchange of ADP for ATP and returning subunits to the ATP-actin-profilin pool, ready for another cycle of assembly. In addition to their role in recycling actin subunits during steady state movement, ADF/cofilins may

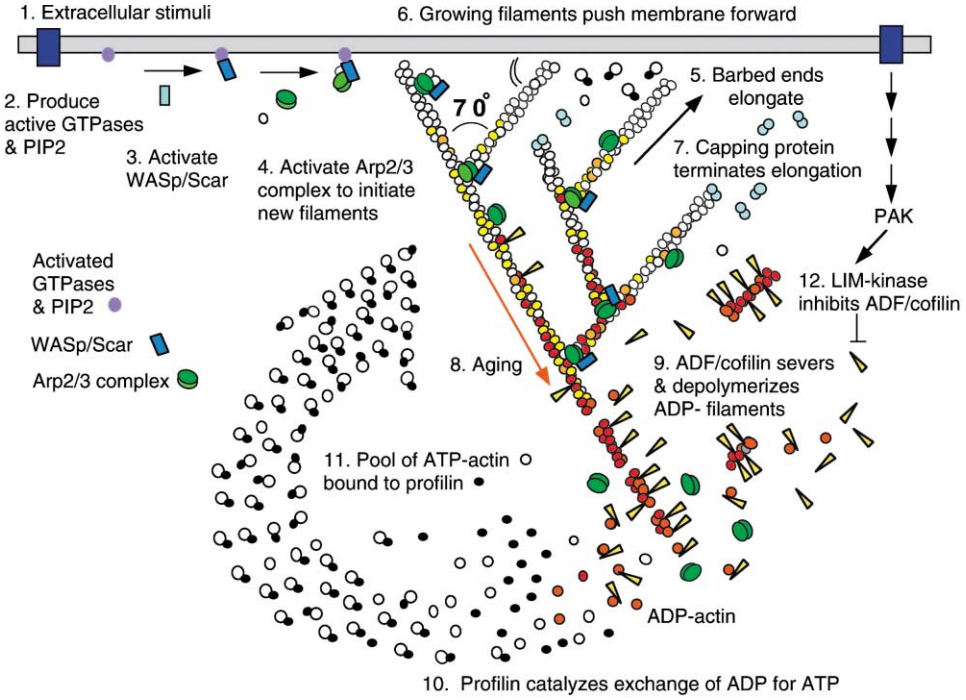

Figure 3. Dendritic Nucleation/Array Treadmilling Model for Protrusion of the Leading Edge

(1) Extracellular signals activate receptors. (2) The associated signal transduction pathways produce active Rho-family GTPases and PIP2 that (3) activate WASp/Scar proteins. (4) WASp/Scar proteins bring together Arp2/3 complex and an actin monomer on the side of a preexisting filament to form a branch. (5) Rapid growth at the barbed end of the new branch (6) pushes the membrane forward. (7) Capping protein terminates growth within a second or two. (8) Filaments age by hydrolysis of ATP bound to each actin subunit (white subunits turn yellow) followed by dissociation of the γ phosphate (subunits turn red). (9) ADF/cofilin promotes phosphate dissociation, severs ADP-actin filaments and promotes dissociation of ADP-actin from filament ends. (10) Profilin catalyzes the exchange of ADP for ATP (turning the subunits white), returning subunits to (11) the pool of ATP-actin bound to profilin, ready to elongate barbed ends as they become available. (12) Rho-family GTPases also activate PAK and LIM kinase, which phosphorylates ADF/cofilin. This tends to slow down the turnover of the filaments. (Redrawn from a figure in Pollard et al., 2000). Reprinted with permission from the Annual Review of Biophysics and Biomolecular Structure, Volume 29, copyright 2000 by Annual Reviews, www.annualreviews.org.

also help to initiate protrusions by severing filaments to expose barbed ends for elongation (Zebda et al., 2000).

In this model, an individual actin filament does not treadmill in the sense of simultaneous growth at one end and shortening at the other. A filament is born as a branch point and grows at its barbed end while its pointed end is capped at the branch; the filament becomes capped at its barbed end, debranches and shortens after being severed. Thus, the actin filament array as a whole treadmills by reproducing itself at the cell front while dismantling itself some distance from the leading edge.

How Do Actin Filaments Grow Fast?

Since the rate of actin filament elongation is proportional to the concentration of subunits, this question is equivalent to asking how cells maintain a pool of polymerization-ready actin at a high concentration. The maximum rates of cellular protrusion or rocketing motility require a concentration of polymerization-competent subunits at least one hundred times the critical concentration for pure actin filaments. In addition to maintaining a pool of subunits sufficient to sustain fast protrusion in the steady state, cells also keep a pool of subunits in reserve in the event that explosive or non-steady-state protrusion is required. Proteins that bind actin monomers and other proteins that cap filament ends cooperate to maintain the pool of unpolymerized actin.

The first mechanism is association of ATP-actin monomers with a small protein called profilin. Profilin binds the barbed end of an actin monomer, allowing actin-profilin to elongate barbed ends of filaments at rates similar to free actin monomers but blocking binding to filament point ends. Profilin also inhibits spontaneous nucleation of actin filaments. All eukaryote cells appear to express profilin. In amoeba, the concentration of profilin and its affinity for actin are sufficient to account for the entire pool of unpolymerized actin monomers (Kaiser et al., 1999; Vinson et al., 1998). Profilin also binds proline-rich sequences in proteins such as formins, VASP, and N-WASP. Ability to bind both actin and poly-L-proline are essential for viability, at least in fungi (Lu and Pollard, 2001) and for actin assembly in extracts (Yang et al., 2000).

The second mechanism is association of ATP-actin monomers with thymosin-β4, a small protein of 43 residues. The pool of unpolymerized actin in human platelets and leukocytes exceeds the concentration of profilin, but sufficient thymosin-β4 is present to make up the difference (Safer and Nachmias, 1994; De La Cruz et al., 2000b). However, sequestering actin monomers

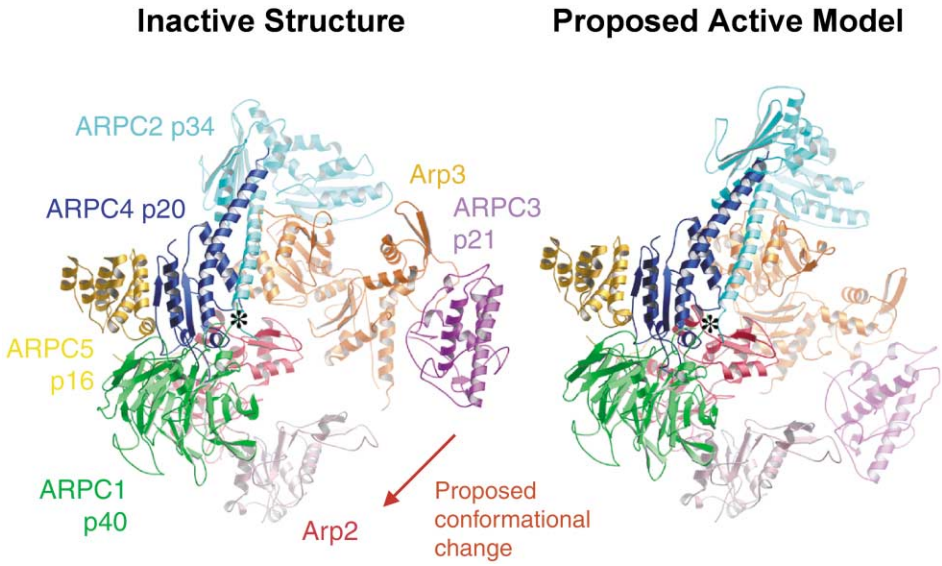

Figure 4. Ribbon Model of the Crystal Structure of Bovine Arp2/3 Complex

The inactive structure from the crystal has the actin-related proteins, Arp2 and Arp3, separated too far to initiate a new filament. The model on the right shows a hypothetical conformation with the Arps positioned like two subunits in an actin filament. Such a conformation is proposed to be stabilized by binding of nucleation promoting factors and to the side of an actin filament. The new actin filament is proposed to grow toward the southwest form the barbed ends of the Arps (modified from Robinson et al., 2001). Reprinted with permission from Robinson et al., 2001. Copyright 2001 American Association for the Advancement of Science.

with thymosin-β4 creates a problem, namely thymosin-β4 blocks all actin assembly reactions including nucleation and growth at both ends. The solution is competition between profilin and thymosin-β4 for binding actin. Both proteins exchange on and off actin on a sub-second time scale, but profilin binds actin monomers tighter than thymosin-β4. This allows profilin to maintain a pool of actin ready to elongate barbed ends, while thymosin-β4 holds the rest of the monomers in reserve. Some lower eukaryotes, including fungi, lack genes for thymosin-β4 and apparently survive without this second actin buffering system.

Rapid growth at barbed ends would deplete the unpolymerized actin pool, resulting in progressively slower elongation. At steady state, two mechanisms, which are discussed in more detail in subsequent sections, compensate for this depletion. One mechanism is capping of many barbed ends, which effectively reduces the rate of drawdown on the pool. The second is mediated by ADF/cofilin proteins, which accelerate actin depolymerization and thus replenish the monomer pool. Together with profilin (and thymosin-β4 in higher eukaryotes), ADF/cofilin and capping of barbed ends allow cells to maintain a high concentration of unpolymerized actin far from equilibrium. This pool sustains high rates of filament elongation at the steady state and is available to add explosively to barbed ends when they become available. Thus, to a large extent, regulation of polymerization reduces to regulation of the availability of free barbed ends.

Initiation and Termination of the Growth of New Filaments

In principle, cells might produce new barbed ends by any or all of three hypothetical mechanisms: severing existing filaments, uncapping existing filaments, or de novo nucleation (Condeelis, 1993; Zigmond, 1996). De novo creation of new barbed ends is now considered to be the dominant mechanism in the leading edge (Zigmond, 1996), but contributions from severing and uncapping should not be neglected. In particular, ADF/cofilin severing proteins appear to contribute to polymerization in vertebrate cells (Zebda et al., 2000), and uncapping of gelsolin-capped filaments occurs during the polymerization of actin that follows activation of platelets (Falet et al., 2002). Although supported by indirect evidence, analysis of de novo nucleation lagged until recently, owing to the lack of a cellular factor to promote formation of barbed ends. Discovery that the Arp2/3 complex has this activity refocused the field and generated an explosion of research. More recently, formins have emerged as a second cellular factor capable of initiating new actin filaments (Pruyne et al., 2002; Sagot et al., 2002).

Arp2/3 complex (Machesky et al., 1994) is a stable assembly of two actin-related proteins, Arp2 and Arp3, with five novel subunits (Figure 4). Found in all parts of eukaryote phylogeny, Arp2/3 complex is present at remarkably high concentrations, almost 10 μM in the cytoplasm of human leukocytes (Higgs et al., 1999), for example. A crystal structure of inactive Arp2/3 complex (Robinson et al., 2001) revealed that the p34 ARPC2 and p20 ARPC4 subunits form the core of the complex, anchoring the two Arps, the p40 ARPC1 β-propeller subunit, and two novel α-helical subunits, p21 ARPC 3 and p16 ARPC5. This form of the complex is inactive, because the two Arps are too far apart to form the first two subunits of a new actin filament.

Biochemical and microscopic experiments established that the Arp2/3 complex caps pointed ends and

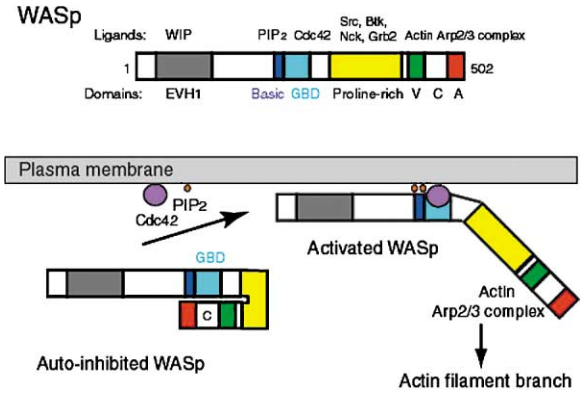

Figure 5. Domain Organization and Regulation of WASp

Linear model with identified domains and examples of interacting ligands. Proposed activation mechanism. WASp and N-WASP are intrinsically autoinhibited by the interaction of the GTPase binding domain (GBD) with the C region. Interaction of the basic region, GBD and proline-rich domain with Cdc42, PIP2, and SH3 domain proteins (such as Nck and Grb2) free the C-terminal VCA domain to interact with an actin monomer and Arp2/3 complex to initiate an actin filament branch.

initiates growth in the barbed direction as 70° branches (Mullins et al., 1998) identical to the actin filament branches at the leading edge of the fish keratocyte (Svitkina et al., 1997) (Figure 1). The correspondence of branching structures in situ and in vitro extended beyond their morphological appearance. Polarity experiments with myosin S1 decoration indicated that barbed ends in lamellipodia were numerous near the leading edge (Small et al., 1978), but pointed ends were involved in end to side junctions with the sides of other filaments, resulting in y-shaped branches (Svitkina et al., 1997). Immunogold labeling with antibodies to components of the Arp2/3 complex showed that the complex was located at the branch of the y junctions (Svitkina and Borisy, 1999), as predicted if the complex functioned as a nucleator of polymerization and remained bound to the pointed end. The complex links the branch to the side of the mother filament in two-dimensional reconstructions from electron micrographs (Volkmann et al., 2001). Structural analysis of comet tails (Cameron et al., 2001) revealed a similar branched actin filament system with pointed ends and Arp2/3 complex at the branch points. Thus, the supramolecular organization of both lamellipodia and comet tail motility systems is fully consistent with the in vitro results and the concept that the Arp2/3 complex functions as a nucleator of actin filaments upon preexisting actin filaments, i.e., dendritic nucleation.

As purification of Arp2/3 complex improved, its nucleation activity declined, and it is now accepted that the complex itself is intrinsically inactive, dependent upon extrinsic activators. The first established activator of Arp2/3 complex was ActA (Welch et al., 1998), a surface protein of *Listeria* and the only gene product required for the bacterium to usurp a cell's actin system to form a comet tail (Kocks et al., 1992; Smith et al., 1995). Yeast two-hybrid screens and biochemical binding assays revealed that the C termini of WASp/Scar proteins (Figure 5) bind Arp2/3 complex (Machesky and Insall, 1998), and reconstitution experiments established that these C-terminal VCA domains of WASp/Scar proteins from protozoa, fungi, and animals all activate actin filament nucleation by Arp2/3 complex (Machesky et al., 1999; Rohatgi et al., 1999; Yarar et al., 1999; Winter et al., 1999; Egile et al., 1999). Since actin filaments are coactivators of Arp2/3 complex (Machesky et al., 1999; Pantaloni et al., 2000), the reaction proceeds autocatalytically with newly formed actin filaments promoting the initiation of subsequent generations of filaments. The relative contributions of nucleation-promoting factors and preexisting filaments to activation of Arp2/3 complex have not yet been sorted out.

Rapidly emerging biochemical and genetic evidence established the existence of additional nucleation-promoting factors for Arp2/3 complex (Weaver et al., 2003). Vertebrate cortactin also binds (Weed et al., 2000) and activates (Uruno et al., 2001; Weaver et al., 2001) Arp2/3 complex, as well as stabilizing branches. A fungal protein called Abp1p has some some similar features (Goode et al., 2001). Fungal myosin-I genes acquired a C-terminal A domain soon after the divergence of fungi from animals. Myosin-I from both budding yeast (Lechler et al., 2000; Evangelista et al., 2000) and fission yeast (Lee et al., 2000) bind Arp2/3 complex, and the tail of fission yeast myosin-I activates Arp2/3 complex. Genetic interactions and biochemical analysis show that these myosin-Is have overlapping functions with the yeast WASp homologs and verprolin in regulating actin assembly. The budding yeast homolog of Eps15, Pan1p, also activates Arp2/3 complex (Duncan et al., 2001).

Mechanistic studies show that WASp/Scar proteins cooperate with Arp2/3 complex and actin filaments to stimulate formation of new barbed ends (Blanchoin et al., 2000a; Higgs et al., 1999; Pantaloni et al., 2000; Marchand et al., 2001; Zalevsky et al., 2001b; Amann and Pollard, 2001), linking nucleation and branching. The C-terminal VCA domains of WASp/Scar proteins are constitutively active for activating Arp2/3 complex. This nucleation-promoting activity depends on a verprolin homology domain (V; also called a WASp homology, WH-2 domain) that binds an actin monomer (Miki and Takenawa, 1998) and a terminal acidic (A) domain that binds Arp2/3 complex (Machesky and Insall, 1998). A connecting sequence (C), originally named cofilin-homology sequence in spite of little sequence homology and no structural homology, contributes to binding both actin and Arp2/3 complex (Marchand et al., 2001). VCA domains are not folded compactly until they bind an actin monomer and/or Arp2/3 complex (Marchand et al., 2001). VCA binds actin monomers and Arp2/3 complex with submicromolar affinity in rapidly reversible equilibria (Marchand et al., 2001). Actin filaments enhance VCA binding to Arp2/3 complex, so from thermodynamic considerations, VCA binding to Arp2/3 complex is expected to enhance the affinity of the complex for the side of an actin filament. One hypothesis to explain these results is that VCA and actin interactions both favor a compact conformation of Arp2/3 complex with Arp3 and Arp2 juxtaposed like two subunits in an actin filament (Figure 4), ready to initiate a new filament (Robinson et al., 2001). This is supported by the finding that VCA can be chemically crosslinked to ARPC1, Arp2, and Arp3 (Zalevsky et al., 2001a; Weaver et al., 2002).

Bringing together an actin monomer with Arp2 and Arp3 on the side of an actin filament is postulated to form a stable trimer that acts as the nucleus to generate a branch, which grows in the barbed direction. A point mutation in a Wiskott-Aldrich syndrome patient revealed an additional activation step after the assembly of Arp2/3 complex, VCA, and actin, which may be rate limiting for nucleation (Marchand et al., 2001). The process underlying this activation step may be hydrolysis of ATP bound to one or both Arps (Le Clainche et al., 2001; Dayel et al., 2001). The mechanism by which *Listeria* ActA activates Arp2/3 complex appears to be similar to WASP/Scar VCA in spite of limited sequence homology (Skoble et al., 2000; Zalevsky et al., 2001a). Under optimal activation conditions, each Arp2/3 complex nucleates the formation of a single branch. Pantaloni et al. (2000) proposed that branches form preferentially on the barbed ends of actin filaments, but real time microscopy shows that most branches form on the sides of preexisting filaments (Amann and Pollard, 2001; Ichetovkin et al., 2002; Fujiwara et al., 2002a) with newly polymerized filaments favored over older filaments.

Filaments grow until they are capped. Heterodimeric capping protein (called CapZ in muscle) is an ubiquitous barbed end capping protein (Cooper and Schafer, 2000). Its concentration in the cytoplasm (about 1 μM) and the rate constant for binding barbed ends accounts for capping with a half time of about 1 s (Schafer et al., 1996). Growth for about 1 s at 0.3 μm per second accounts approximately for the lengths of branches observed at the leading edge (Svitkina et al., 1997). Gelsolin is another barbed end capping protein in higher eukaryotes (Sun et al., 1999).

Capping may appear counterproductive, since it antagonizes elongation of barbed ends, the driving force for motility. However, capping makes two important contributions to actin-driven motility. First, capping protein limits the lengths of the growing branches. Although this requires constant initiation of new filaments, short filaments are stiffer than long filaments and therefore more effective at pushing on the membrane. Second, capping controls where actin filaments "push." Since only those barbed ends in contact with the lamellipodial surface are effective in generating propulsive force, global capping of barbed ends avoid nonproductive consumption of actin subunits elsewhere in the cell (Cooper and Schafer, 2000) and funnels subunits from diverse points of disassembly to a limited number of growing barbed ends (Carlier and Pantaloni, 1997).

Local factors may inhibit capping and favor persistent growth of barbed ends pushing productively against the inner surface of the plasma membrane. Phosphatidylinositol 4, 5-bisphosphate displaces gelsolin (Janmey and Stossel, 1987) and capping protein (Schafer et al., 1996) from barbed ends. The GTPase Cdc42 may also help barbed ends to escape capping (Huang et al., 1999). VASP is a candidate inhibitor of capping, since it localizes to the extreme leading edge and antagonizes interaction of capping protein with barbed ends (Bear et al., 2002).

How Does Actin Push the Membrane Forward?

The leading lamellum of motile cells is a thin (0.1–0.2 μm), sheet-like protrusion filled with actin filaments at high density (100/μm of leading edge) (Abraham et al., 1999). Pure actin filaments in vitro are long and flexible. Long, flexible filaments cannot sustain a pushing force without buckling. Cells overcome this problem by creating a dense array of short-branched filaments (Svitkina and Borisy, 1999; Svitkina et al., 1997). Branches are often separated by less than 100 nm, presumably as a result of tightly coupled nucleation and crosslinking of actin filaments at the leading edge. Such coupling would allow nascent filaments to push against the membrane immediately after formation and provide the structural basis for polymerization-driven protrusion. Because of the dendritic nucleation pattern and the 70° angle between branches, filaments are not oriented perpendicular to the leading edge. Rather, they are distributed over a range of angles, the most favored being $\pm$35° with respect to the normal to the leading edge (Maly and Borisy, 2001).

Given the numerous filament barbed ends at the leading edge, how does polymerization actually generate a pushing force? All models require the polymerizing actin to be cross linked in some way or to be anchored to the substratum; otherwise, the force of polymerization would drive the filaments rearward instead of the surface forward. A major crosslinking component is likely to be the Arp2/3 complex itself but also supplemented by other crosslinkers, since cells lacking the crosslinking protein filamin have unstable lamellae (Cunningham et al., 1992).

A problem for polymerization models is to understand how a subunit can elongate a filament abutting a surface. A solution to this problem is the "elastic Brownian ratchet" model (Mogilner and Oster, 1996), which envisages the actin filament as a spring-like wire, which is constantly bending because of thermal energy. In fact, direct observation of "vibrating" branched filaments in vitro (Blanchoin et al., 2000a) suggests a Young's modulus equivalent to that of rigid plastic (Gittes et al., 1993). When bent away from the surface, a subunit can "squeeze" in, lengthening the filament. The restoring force of the filament straightening against the surface delivers the propulsive force. From the measured stiffness of actin filaments, Mogilner and Oster (1996) calculated that the length of the "pushing" actin filament (that is, the "free" length beyond the last crosslinking point) must be quite short, in the range 30–150 nm. Beyond this length, thermal energy is taken up in internal bending modes of the filament, and pushing is ineffective. These considerations are important because they imply a requirement for the cell to balance the relative rates of branching, elongation, and capping. Theoretical calculations (Mogilner and Edelstein-Keshet, 2002) suggest that the cell "tunes" these parameters to obtain rapid motility and that it uses negative feedback from capping to regulate the number of barbed ends close to optimal levels. VASP may play such a regulatory role since it antagonizes capping and its depletion leads to shorter filaments, while its targeting to the plasma membrane leads to longer filaments that push inefficiently (Bear et al., 2002).

Remodeling of the Branched Actin Filament Network

The network of short, branched filaments at the leading edge is generally narrow, less than 1 μm deep under the

plasma membrane (Svitkina and Borisy, 1999). Further from the membrane, all of the filaments are long and unbranched (Small et al., 1978). Given the rate of movement (0.2 μm per second) and the width of the zone of branched filaments, these branched filaments must be remodeled into long, unbranched filaments in about 5 s. The first step in this remodeling process must be dissociation of branches from the sides of their mother filaments.

Phosphate release from the subunits in the branch appears to be a prerequisite for debranching (Blanchoin et al., 2000b). After newly polymerized actin subunits hydrolyze their bound ATP, the γ phosphate dissociates with a half time of 6 min (Carlier, 1987). The half time for new branches to dissociate from their mother filament is also 6 min. ADF/cofilins accelerate both phosphate dissociation and debranching, while phalloidin or BeF_3 slow both reactions. Phosphate dissociation from branches weakens the attachment of the pointed end of the filament to Arp2/3 complex and promotes debranching. Once free of the Arp2/3 complex cap on their pointed end, debranched actin filaments can anneal with filaments with free barbed ends (Blanchoin et al., 2000b), perhaps contributing to the formation of the long, unbranched filaments found beyond the highly branched network at the leading edge (Figure 1). In cells, capping of barbed ends might inhibit annealing, but tropomyosin, which localizes to the deeper part of the lamella (Des-Marais et al., 2003) allows annealing even in the presence of gelsolin, a high-affinity barbed end capping protein (Nyakern-Meazza et al., 2002). A similar effect of tropomyosin on filaments capped with capping protein, might explain how the branched network of capped filaments can be remodeled rapidly by annealing. On the other hand, the protein cortactin binds both actin filaments and Arp2/3 complex and stabilizes branches (Weaver et al., 2001, 2002). Much remains to be learned about the mechanism of this rapid, dramatic remodeling of leading edge filaments in cells.

Recycling of Proteins in the Actin Filament Network

Since the cell is a bounded compartment, rapid polymerization of actin filaments cannot continue for long without being balanced by rapid depolymerization. Locomoting keratocytes and the rocketing motility of microbes are dramatic examples of rapid actin turnover. The keratocyte moves its body length (10 μm) in about 1 min, which requires complete turnover of the entire actin network within that time. In dramatic contrast to the rapid turnover of cellular actin filaments, pure actin filaments are intrinsically stable, undergoing only slow subunit exchange reactions at their ends and slow subunit flux (0.04 μm/min) through the polymer at steady state. Regulatory proteins must account for this difference. Most attention has focused on ADF/cofilins, an ubiquitous family of small proteins that bind ADP-actin filaments and promote their disassembly (Bamburg et al., 1999). ADF/cofilin also stimulates the turnover of Listeria comet tails (Rosenblatt et al., 1997; Carlier et al., 1997). ADF/cofilins concentrate just behind the leading edge of rapidly moving cells, where ADP-actin filaments are expected to occur (Svitkina and Borisy, 1999). ADF/cofilins bind only weakly to ATP or ADP-P_i actin filaments and do not depolymerize them, so ATP hydrolysis and phosphate dissociation are postulated to regulate actin filament disassembly (Maciver et al., 1991).

Since ATP hydrolysis is fast, irreversible, and not known to be affected by any actin binding protein, phosphate release is the most attractive candidate for a timer for depolymerization. However, spontaneous phosphate release is slower than actin filament turnover in vivo. Although ADF/cofilins have a low affinity for ADP-P_i actin filaments, they promote phosphate dissociation (Blanchoin and Pollard, 1999). Physiological concentrations of ADF/cofilin accelerate phosphate dissociation, so that it keeps pace with elongation and ATP hydrolysis.

The driving force for depolymerization is the higher affinity of ADF/cofilin for ADP-actin monomers than ADP-actin filaments, but the pathway of disassembly has been difficult to pin down. Binding of ADF/cofilins to ADP-actin filaments changes the twist of the actin helix (McGough et al., 1997) and promotes severing of the filaments into short segments (Maciver et al., 1991; Maciver et al., 1998; Blanchoin and Pollard, 1999; Ichetovkin et al., 2000). Severing is important, since the bulk rate of disassembly is proportional to the concentration of free filament ends. The Aip1 family of proteins appear to promote severing by ADF/cofilin since they block barbed ends and inhibit annealing, the reverse of the severing reaction (Okada et al., 2002). ADF/cofilins also promote disassembly of ADP-actin monomers bound to ADF/cofilin (Carlier et al., 1997). Attention has focused on dissociation of ADF/cofilin-ADP-actin from pointed ends, but dissociation from barbed ends should also be considered since ADP-actin dissociates much faster from barbed ends than pointed ends (Pollard, 1986). On the other hand, capping proteins may preclude barbed end dissociation in cells. One can imagine that these disassembly events involve a certain element of chance, with some short branches surviving as segments of long filaments, while others being lost, owing to disassembly (Theriot and Mitchison, 1991; Watanabe and Mitchison, 2002). Much important work remains to be done on the disassembly mechanisms both in vitro and in live cells. One unresolved puzzle is how ADF/cofilins can depolymerize filaments with bound tropomyosin, which blocks ADF/cofilin binding and inhibits depolymerization (Nishida et al., 1984; Maciver et al., 1991).

Tight binding of ADF/cofilin might trap ADP-actin monomers, but profilin reenters the cycle at this point as a nucleotide exchange factor for actin (Mockrin and Korn, 1980; Vinson et al., 1998). Profilin competes with ADF/cofilin for binding ADP-actin and promotes dissociation of ADP. Owing to the higher concentration of ATP in living cells and its higher affinity for actin than ADP, nucleotide-free actin preferentially binds ATP. Profilin binds ATP-actin more tightly than ADF/cofilin, thus returning the ATP-actin monomer to its polymerization ready state (Rosenblatt et al., 1995).

Disassembly of actin filaments by ADF/cofilin, including debranching, severing, and depolymerization, all follow inevitably after phosphate dissociation from the filaments. Phosphate dissociation by ADF/cofilin sets the tempo, so that the concentration of active ADF/cofilin is a crucial variable. LIM-kinase (Yang et al., 1998; Arber et al., 1998) controls the activity of most ADF/cofilins

by phosphorylation of a serine near the N terminus. This phosphoserine blocks interactions of ADF/cofilins with ADP-actin filaments and monomers without changing the atomic structure (Blanchoin et al., 2000c). A specific phosphatase called *slingshot* activates ADF/cofilin by removing the inhibitory phosphate (Niwa et al., 2002). Rho-family GTPases activate LIM-kinase indirectly via PAK (Edwards et al., 1999). The consequences of activating and inactivating ADF/cofilin are likely to be complicated. On one hand, Rho-family GTPases promote actin polymerization in two ways—activating nucleation by Arp2/3 complex and inhibiting depolymerization by ADF/cofilins. On the other hand, activation of severing by ADF/cofilin (for example by dephosphorylation) may jump-start the extension of a leading edge following stimulation (for example with EGF) by providing barbed ends for elongation (Zebda et al., 2000; Ichetovkin et al., 2002). Growth of these new ends might push forward directly or provide fresh filaments that are favored for branching by Arp2/3 complex.

Regulation of Leading Edge Assembly and Disassembly by Extrinsic Factors

Some cells are spontaneously motile and little is known about the intrinsic factors driving the extension of lamella. Much more is known about how environmental cues guide cells toward attractants and away from repellents. Examples include the attraction of white blood cells to bacterial peptides and chemokines and of the slime mold Dictyostelium to cAMP. Chemoattractants activate a variety of receptors including seven-helix receptors and receptor tyrosine kinases. A remarkable feature of these signaling systems is their ability to convert a shallow spatial gradient of attractant into a steep gradient of signals inside the cell (Bourne and Weiner, 2002).

Given the low nucleating activity of pure Arp2/3 complex, nucleation-promoting factors appear to drive the whole actin assembly-disassembly system. This feature focuses attention on how external stimuli are converted into signals that regulate nucleation-promoting factors (Figure 5). WASp and N-WASP are intrinsically inactive, owing to strong autoinhibition, which is overcome by signaling molecules including Rho-family GTPases, PIP_2, profilin, and the SH3 domain proteins Grb2 and Nck (reviewed by Higgs and Pollard, 2001). Extensive biochemical and structural data supports the hypothesis that N-terminal domains of WASp and N-WASP autoinhibit the constitutive nucleation-promoting activity of the C-terminal VCA domain (Kim et al., 2000; Miki and Takenawa, 1998). An NMR structure (Kim et al., 2000) clearly defines the interaction between GBD and the C motif in VCA. Strong autoinhibition is important given the high concentrations of some of these proteins, such as 10 μM WASp in human neutrophils (Higgs and Pollard, 2000). A basic sequence and a GTPase binding domain (GBD) bind intramolecularly to VCA (Higgs and Pollard, 2000; Kim et al., 2000; Rohatgi et al., 2000; Rohatgi et al., 2001; Prehoda et al., 2000). Membrane-associated PIP_2 and GTP-Cdc42 compete with VCA for binding to the basic/GBD region, freeing VCA to activate Arp2/3 complex. Micrographs of the reaction products show dense arrays of actin filaments surrounding lipid vesicles containing PIP_2 and prenylated GTP-Cdc42 (Higgs and Pollard, 2000). PIP_2 and GTP-Cdc42 cooperate to overcome autoinhibition of WASp and N-WASP. Requiring two signaling inputs of different natures is attractive, because it may make the WASp/N-WASP transduction mechanism sensitive to coincident signals (Prehoda et al., 2000). SH3 domains of the adaptor proteins Grb2 (Carlier et al., 2000) and Nck (Rohatgi et al., 2001) bind the poly-proline rich domain of N-WASP and cooperate with PIP_2 to overcome the autoinhibition of N-WASP. Similarly, profilin binding to the proline rich domain cooperates with Cdc42 to activate N-WASP (Yang et al., 2000).

Scar/WAVE was proposed to transmit signals from activated Rac to stimulate formation of lamellipodia, although no direct interaction between Rac and WAVE was found (Miki et al., 1998). Full-length Scar/WAVE proteins are intrinsically active with respect to stimulating Arp2/3 complex (Machesky et al., 1999) but can be inhibited by a complex of four other proteins (Eden et al., 2002). Membrane bound Rac-GTP overcomes this inhibition by dissociating the inhibitory proteins from Scar/WAVE. A substrate for insulin receptor, IRSp53, binds activated Rac to WAVE2 (Miki et al., 2000).

Given that membrane bound GTPases, a membrane lipid, and SH3 adaptor proteins that associate with activated receptor tyrosine kinase cooperate to activate WASp/N-WASP and Scar/WAVE and given that rapidly growing filaments are secondary activators of Arp2/3 complex, new filaments most likely arise at sites where existing filaments push against the inner surface of the plasma membrane. This would account for the localized expansion of the branched network against the plasma membrane. However, no direct evidence at the cellular level yet supports the details of this hypothesis.

After initiating a new branch near the membrane, the fate of WASp/N-WASP and Scar/WAVE is unclear. The nucleation-promoting factors are presumed to be bound at least transiently to their activators on the inner surface of the plasma membrane. However, activated WASp and N-WASP also bind Arp2/3 complex and thus might have some tendency to associate with the actin network. Since ActA can promote nucleation by Arp2/3 complex while remaining attached to *Listeria*, WASp/N and WASP are speculated to dissociate from Arp2/3 complex shortly after a branch forms. The rapid rate of VCA dissociation from both actin and Arp2/3 complex (Marchand et al., 2001) is consistent with this idea. Cortactin can bind Arp2/3 complex simultaneously with VCA and is proposed to stabilize the branch after dissociation of WASp/N-WASP (Weaver et al., 2002).

Rho-family GTPases also influence the disassembly of actin filaments. The Rho-family GTPase Rac stimulates p21-activated kinase (PAK) to activate LIM kinase (Edwards et al., 1999) to phosphorylate ADF/cofilin. This inactivates ADF/cofilin and should tend to prolong the lifetime of newly formed actin filaments. Thus, activating small GTPases stimulates both formation and stabilization of new filaments.

Key Features of the Protrusion Process

The system is poised far from equilibrium with a high concentration of unpolymerized actin maintained by a

combination of profilin and capping of filament barbed ends. This system is ready to respond explosively when new barbed ends are created. Extracellular stimuli such as chemoattractants trigger transduction pathways that generate spatially restricted accumulations of signaling molecules associated with the plasma membrane including Rho-family GTPases, PIP_2, and SH3 adaptor proteins. Individually and cooperatively, these molecules activate nucleation-promoting factors including WASp, N-WASP, and Scar/WAVE, making them available to activate in turn Arp2/3 complex. Active Arp2/3 complex binds the side of filaments and initiates a branch that grows rapidly in the barbed direction. Growing filaments push the plasma membrane forward until they are capped. The filaments then age automatically by hydrolysis of bound ATP and dissociation of the γ phosphate. ADF/cofilins accelerate phosphate release, the rate-limiting step. After phosphate release, branches dissociate from Arp2/3 complex and their mother filament and become targets for severing and depolymerization by ADF/cofilins.

Many intriguing mysteries still remain to be solved. We still do not know the identity of the intrinsic signals that cells use to stimulate actin assembly in the absence of external signals. No pathway from a chemoattractant or repellent has been fully mapped and characterized dynamically. Integration of signals from multiple signaling pathways at the level of WASp/Scar proteins is an attractive concept that needs fuller documentation, especially the physical arrangement of signaling molecules, actin filaments, and Arp2/3 complex on the inner surface of the plasma membrane. Remodeling of the branched network and recycling of all of the proteins is far from understood. The relation (if any) of dendritic nucleation to motility of particular cells, especially growth cones, remains to be proven. We also have yet to learn how bundles of actin filaments in filopodia and microvilli form and grow.

Acknowledgments

We thank John Cooper (Washington University Medical School) and Tatyana Svitkina (Northwestern University Medical School) for critical reading of the manuscript. Supported by NIH Grants GM26338 to TDP and GM62431 to GGB.

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