

Focusing on Unpolymerized Actin

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A fascinating feature of cell locomotion is that net actin polymerization and depolymerization occur simultaneously at different locations in the cytoplasm, while the total concentration of polymerized actin remains roughly constant. Net polymerization occurs primarily at the cell front and net depolymerization occurs, depending on cell type, throughout or at the rear of the lamella (Wang, 1985; Symons and Mitchison, 1991; Theriot and Mitchison, 1992; Zigmond, 1993). The actin flux is particularly remarkable since the rates of elongation and of depolymerization can be very high. Specifically, the rate of filament elongation is at least as high as the rate of cell protrusion and the half life of monomers in a filament can be less than 1 min in a keratocyte and 10 s in a neutrophil (Theriot and Mitchison, 1991; Cassimeris et al., 1990). In contrast, in a test tube, a concentration of monomeric actin high enough to cause rapid elongation would inhibit depolymerization.

How then does a cell maintain high rates of net polymerization and net depolymerization simultaneously at different sites in its cytoplasm? The answer is probably complex involving: (a) distinct properties of actin molecules containing different intermediates of ATP hydrolysis (e.g., ATP, ADP-P_i, ADP); (b) the effects of proteins that bind to monomeric or filamentous actin; (c) the spatial distribution of monomeric actin, actin binding proteins, and their modulators in the cell. Several recent studies provide new insights into these issues.

In this issue of *The Journal of Cell Biology*, Cao et al. report the use of a fluorescent derivative of vitamin D-binding protein as a specific probe for unpolymerized actin in cultured vertebrate cells, enabling direct comparisons of the distribution of unpolymerized actin to that of microinjected fluorescent actin (total actin) and to actin filaments stained with phalloidin (Cao et al., 1993). The remarkable observation is that some of the unpolymerized actin is localized in discrete foci as shown by labeling with vitamin D-binding protein and lack of labeling with phalloidin. Similar punctate foci are also observed in the leading edge of living epithelial cells injected with fluorescently labeled actin. These appear to move rearward as locomotion proceeds. After fixation, these foci stain with vitamin D-binding protein. Actin in discrete foci within the leading lamella of cultured cells have also been seen with antibodies specific for the β isotype of actin (Hoock et al., 1991). While foci of highly localized unpolymerized actin have been observed in the gametes of marine invertebrates (Tilney et al., 1973; Tilney, 1976; Spudich et al., 1988; Bonder et al., 1989), they had not been ob-

served in vertebrate cells. Before considering the functional significance of these foci, a bit of background is required.

Fundamentals of Actin Polymerization

The total actin pool in cells is composed of filamentous and unpolymerized actin. Only a small fraction of the unpolymerized actin in cells is truly "free actin," i.e., not complexed with other proteins. At steady state, the amount of polymerized actin will depend on the total actin present (At) minus the free monomeric actin (A*), minus the actin bound to each of the different monomer-binding proteins. The concentration of free monomeric actin in steady state with filaments is called the critical concentration. The critical concentration is related to the affinity of the filament for monomer. It varies depending on ionic conditions, the adenine nucleotide bound to actin, and can be modified by some actin-binding proteins (see below).

Each actin monomer contains a bound adenine nucleotide. The nucleotide on monomeric actin can exchange with those in the medium. Thus, it has been assumed that most of the unpolymerized actin in the cytoplasm is bound to ATP, since the concentration of free ATP present is about ten times that of ADP and under physiological conditions monomeric actin has a slightly higher affinity for ATP than for ADP (Wanger and Wegner, 1983). After polymerization, the ATP bound to actin is rapidly hydrolyzed to ADP and P_i. Because the nucleotide on the filament does not exchange with that in the medium, actin monomers released during depolymerization probably contain bound ADP. The ADP-actin monomers have a lower affinity for filament ends than ATP-actin: a much higher concentration of ADP- than ATP-actin is required for polymerization at either end of the actin filament (Pollard, 1986; Korn et al., 1987; Carlier, 1991). Thus, monomers are thought to exchange bound ADP for ATP before repolymerizing.

An additional consequence of the ATP hydrolysis is that the "barbed-end" of a filament has a higher affinity for ATP-actin than the "pointed end" (Wegner, 1976; Bonder et al., 1983). With pure actin, in a physiological salt solution containing ATP, the barbed- and pointed-end critical concentrations are ~ 0.1 and $0.5 \mu\text{M}$, respectively (Pollard, 1986). It is this difference in affinity that causes actin filaments at steady state to treadmill, i.e., add monomers at the barbed ends and lose them at the pointed ends. The treadmill observed with pure actin in vitro appears too slow to account for the rate of actin flux seen in vivo (Wang,

1985). Moreover, a concentration of free monomeric actin high enough to promote barbed-end elongation at the leading edge at the rate observed in vivo would prevent concurrent pointed-end depolymerization. Rather, it would cause elongation at both ends. The spatially regulated actin flux occurring in cells must be due to the presence of actin-binding proteins.

A large fraction of the unpolymerized actin in cells is bound to various actin monomer-binding proteins which prevent its polymerization. This large monomer reserve can provide a source of actin for the rapid assembly occurring during cell movements. The monomer-binding proteins also rebind monomer released by depolymerization. The amount of unpolymerized actin bound to any given monomer-binding protein depends on the amount of that protein present, its affinity for monomeric actin, and the concentration of free monomeric actin present: $[PA] = ([Pt][A^*])/([A^*] + K_d)$ where $[PA]$ is the concentration of the complex of monomer-binding protein with monomeric actin, $[Pt]$ is the concentration of the total amount of a monomer-binding protein present, K_d is its equilibrium constant for monomeric actin, and (A^*) is the concentration of free monomeric actin. At steady state, the concentration of free monomeric actin will be determined by the critical concentration of the F-actin present.

Proteins that bind to actin filaments and change their critical concentration may affect the extent of actin polymerization in cells. For example, proteins that cap the barbed ends, raising the critical concentration to that of the pointed ends, could cause net depolymerization (reviewed by Weeds and Maciver, 1993). Proteins that bind to the sides of filaments, but not to monomeric actin, could lower the critical concentration and cause net polymerization (Benfenati et al., 1992; Broschat et al., 1989; Weight et al., 1990).

The effect of changes in the critical concentration on the amount of polymerized actin can be greatly amplified by monomer-binding proteins that create a reservoir of actin. Were there no pool of actin bound to monomer-binding proteins, the actin polymerization induced by uncapping all the barbed ends and changing the critical concentration from 0.5 to 0.1 μM would be small, e.g., an initial concentration of 60 μM filamentous actin would increase to only 60.4 μM . In contrast, the amount of filamentous actin could be increased dramatically if, in response to a decrease in critical concentration, the monomer-binding proteins would release a large amount of actin.

The amount of actin released depends on the affinity of the binding protein for monomeric actin (greatest for a monomer-binding protein that binds monomeric actin with a K_d in the range of the critical concentration change) and the size of the monomer reservoir (determined by the total concentration of binding protein). For example, if the K_d of the monomer-binding protein for monomeric actin equals the critical concentration, then $[PA]/[Pt] = 0.5$, i.e., 50% of the monomer-binding protein is complexed with actin. Lowering the critical concentration fivefold (such as might occur upon uncapping of barbed ends) would cause $[PA]/[Pt]$ to decrease to 0.165, i.e., more than 2/3 of the bound actin would be released. If $[Pt] = 250 \mu\text{M}$, then the filamentous actin could increase from 60 to 143 μM . If the K_d were lower or higher, the change would be less. This need to "buffer" free monomeric actin near the critical concentration probably ex-

plains why the K_d s of most intracellular monomeric actin-binding proteins are in the range of 0.2 to 1 μM . In contrast, extracellular monomer-binding proteins have K_d s in nanomolar range and thus appear designed to sequester, not buffer, monomer (Lee and Galbraith, 1992).

Local sites of net polymerization and net depolymerization may be achieved by spatially separating actin filaments with high monomer affinity from filaments with low monomer affinity. Thus, for example, in the presence of homogeneous-free monomeric actin at a concentration between the critical concentration of the two filament ends, net polymerization would occur at sites where filaments have free barbed ends (and blocked or free pointed ends). Net depolymerization would occur at sites where there were no free barbed ends. The pool of unpolymerized actin bound to monomer-binding proteins would also amplify this spatial effect. Careful consideration of the variety of monomer-binding proteins present in cells is needed for an understanding of these processes.

Four Families of Actin Monomer-binding Proteins Have Distinct Functions

The intracellular proteins that bind to actin monomers can be grouped into four families, based on their amino acid sequences: β -thymosins, profilins, actin depolymerizing factors (actin depolymerizing factor (ADF),¹ actophorin, cofilin, depactin, and destrin), and actobindin (so far purified only from *Acanthamoeba*). A picture is emerging in which each family not only binds actin monomers, but also plays distinct roles in the regulation of actin dynamics in cells. The β -thymosins are primarily responsible for monomer buffering. Profilins can help to sustain high rates of assembly. The ADF family of proteins have severing activity that may speed filament disassembly. Actobindin is particularly active as an inhibitor of spontaneous nucleation of actin assembly.

The thymosins contribute the bulk of actin-buffering activity in the cytoplasm in many cells (Safer 1992; Yu et al., 1993; Hannappel and Wartenberg, 1993). Thus, the combination of its abundance (as high as 560 μM in platelets, twice the concentration of unpolymerized actin) and its affinity for actin monomer (K_d of 0.4–0.7 μM) suggests that thymosin β_4 is bound to most of the unpolymerized actin in platelets (assuming that the critical concentration in the cell is close to 0.5 μM) (Nachmias, 1993). Thymosin β_4 binds and releases monomeric actin rapidly, allowing it to effectively "buffer" the actin monomer concentration even during episodes of rapid assembly of new actin filaments (Goldschmidt-Clermont et al., 1992; Cassimeris et al., 1992). The effects of thymosin β_4 appear to be due solely to its ability to bind actin monomers; it does not possess the special abilities to enhance filament elongation, to sever filaments, or to inhibit nucleation (Weber et al., 1992). Consistent with its ability to maintain a pool of monomeric actin, injection of cultured cells with thymosin β_4 induces a decrease in the quantity of filamentous actin (Sanders et al., 1992).

Thymosin β_4 inhibits exchange of the bound nucleotide on unpolymerized actin (Goldschmidt-Clermont et al., 1992). Thus, it was proposed that in cells exhibiting rapid actin flux, thymosin β_4 , by inhibiting nucleotide exchange, might promote accumulation of actin monomers with bound ADP.

1. Abbreviation used in this paper: ADF, actin depolymerization factor.

This model has now been seriously questioned by the finding that thymosin $\beta 4$ has a much higher affinity (50-fold) for ATP- than for ADP-actin monomers (Carlier et al., 1993). This means that thymosin $\beta 4$ will selectively bind the actin subunits with bound ATP and will not increase the fraction of ADP-actin present. Interestingly, its relative affinity for ATP- and ADP-actin roughly parallels the relative affinities of actin filaments for these monomeric forms. This may allow thymosin $\beta 4$ to serve as a physiological buffer for both ATP- and ADP-actin.

Profilins are unique in having the ability not only to bind monomers, but also to contribute to filament elongation at the barbed but not the pointed end. In vitro, profilin-actin complex (reconstituted from purified proteins) can bind to the barbed end of a filament and, following rapid dissociation of the profilin, result in net elongation of the filament (Tilney et al., 1983; Pollard and Cooper, 1984; Pring et al., 1992). Thus, if present in sufficiently high concentrations, profilin-actin complexes have the potential to drive the rate of filament elongation at the barbed end to the high rates observed in vivo. These rates could not be achieved by the relatively low concentration of free monomeric actin thought to be present in cells. Profilins may also enhance elongation rates by maintaining the pool of ATP actin both by inhibiting hydrolysis of ATP by monomeric actin (Tobacman and Korn, 1982) and by increasing the rate of exchange of the bound nucleotide on actin monomers thus speeding exchange of ADP for ATP (Mockrin and Korn, 1980; Nishida, 1985; Goldschmidt-Clermont et al., 1991). However, if present at high enough concentrations, free profilin might inhibit elongation both by capping the barbed end and by lowering the concentration of free monomeric actin (Pollard and Cooper, 1984). The ability of profilin in vitro either to inhibit or enhance barbed end elongation agrees with the observations that a decrease or increase of actin filament assembly in cells is induced by microinjection of profilin or profilin-actin complex, respectively (Cao et al., 1992).

Different preparations of profilin vary in their affinity for actin suggesting the existence of regulatory mechanisms not yet fully understood (Carlsson et al., 1977; Markey et al., 1978; Southwick and Young, 1990; Katakami et al., 1992). The K_d for actin of isolated profilin, purified by different laboratories, ranges between 0.5 and 10 μM (Goldschmidt-Clermont et al., 1991; Pring et al., 1992; Pollard and Cooper, 1984). In contrast, complexes of profilin-actin isolated intact from cells are of high affinity, K_d is $\sim 2 \times 10^{-8}$ M (Carlsson et al., 1977; Markey et al., 1978; Lassing and Lindberg, 1985; Katakami et al., 1992). In this high-affinity form, profilin cannot contribute to barbed end elongation; rather its role is probably to sequester monomer. Phosphoinositides can dissociate profilin-actin complexes and thus decrease the amount of profilin available for actin binding (Lassing and Lindberg, 1985, 1988; Goldschmidt-Clermont et al., 1990).

Members of the actin depolymerizing factor family of monomer-binding proteins enhance the rate of actin depolymerization beyond that predicted from their ability to bind actin monomers (K_d of actophorin for monomeric actin is $\sim 0.2 \mu\text{M}$; Maciver et al., 1991). The enhanced rate of depolymerization appears to be mediated by their ability to cut filaments and thus to increase the number of filament ends undergoing depolymerization (Mabuchi, 1983; Nishida et

al., 1984a,b; Nishida et al., 1985; Cooper et al., 1986; Maciver et al., 1991). The presence of active filament-severing proteins at molar ratios to actin as high as 0.1 to 0.64 (Mabuchi, 1983; Cooper et al., 1986; Bamberg and Bray, 1987) may contribute to the rapid turnover of filaments in cells. Cofilin, like thymosin $\beta 4$, inhibits exchange of nucleotide on monomeric actin (Nishida, 1985). ADF and cofilin appear to be regulated by phosphorylation (Koffer et al., 1988; Morgan et al., 1993; Ohta et al., 1989). The phosphorylated form of ADF lacks the ability to bind monomeric actin, and to affect the rate and extent of actin assembly. The fraction of ADF in the phosphorylated form varies from 0.15 to 0.6 in different cells and tissues (Morgan et al., 1993). In addition, phosphoinositides inhibit actin binding by at least some members of this family (Yonezawa et al., 1990). Null mutations of cofilin are lethal in yeast in support of the general physiological significance of members of the ADF family (Moon et al., 1993).

The cutting activity of some members of the ADF family appears to be directed away from rapidly growing filaments and toward older or slowly growing filaments. Shortly after polymerization actin monomers in the filament contain ADP-P_i rather than ADP since following the very rapid hydrolysis of ATP on actin subunits assembled onto filaments, inorganic phosphate is released quite slowly ($t_{1/2} = 2$ min) (Korn et al., 1987; Carlier, 1991). The rapid rate of subunit flux into and out of actin filaments in cells can exceed the measured rate of phosphate release in vitro, and filaments containing ADP-P_i actin subunits would be expected to accumulate in some regions. The ability of actophorin to cut an actin filament is inhibited by addition of phosphate (which is thought to restore the ADP-P_i intermediate), suggesting that actophorin is not able to sever filaments containing subunits with bound ADP-P_i (Maciver et al., 1991). Severing and disassembly of actin filaments by destrin and ADF (but not by depactin) is also inhibited or slowed by tropomyosin (Bernstein and Bamberg, 1982; Mabuchi, 1982; Nishida et al., 1985) providing a mechanism for long-term protection from severing and disassembly.

A fourth family of monomer-binding proteins, represented by actobindin (Lambooy and Korn, 1986) is present at 15–25 μM in *Acanthamoeba*. Actobindin which has an affinity for monomeric actin of 3–5 μM is unusually effective at inhibiting the nucleation step of polymerization (Bubb et al., 1991; Lambooy and Korn, 1988). This suppression of spontaneous nucleation may help limit polymerization to sites where nuclei in the form of free barbed end are available. No regulation of actobindin is known.

The Distribution of Monomer-binding Proteins and Unpolymerized Actin May Contribute to the Spatial Arrangement of Actin Filament Flux

The interactions of the four classes of monomer-binding proteins with actin are now sufficiently well described to explain many of their effects on the assembly of actin in vitro, and to discern functionally distinct activities associated with each of the classes. Yet, this knowledge is not sufficient to explain spatial regulation of actin dynamics that characterizes cell movements. The distribution of actin depolymerizing factor appears to be relatively homogeneous (Bamberg and Bray, 1987), although the distribution of the active, un-

Table 1. Four Families of Actin Monomer-binding Proteins

	Profilin	ADF	Thymosin β 4	Actobindin
Subunit mol wt*	15,220	18,520	5,074	9,682
K_d actin monomer [‡]	0.02 [§] –1 μ M	0.2 μ M	0.4–0.7 μ M	3.3–5.0 μ M
Abundance [‡]	40 μ M platelet	<5 μ M muscle [†] 373 μ M aorta [†]	20 μ M brain 580 μ M platelet	15–25 μ M in <i>Acanthamoeba</i> Leading edge
Distribution [‡]	Elevated at sites of rapid filament turnover	Homogeneous	Homogeneous?	Leading edge
Special function [‡]	Enhance assembly Sequester monomer [§]	Sever filaments/ promote disassembly	Buffer monomer	Suppress nucleation

* GenBank Accession Numbers: J02912 (ADF) and M17733 (Thymosin); and PIR Accession Numbers: A03010 (Profilin) and A36614 (Actobindin).

[‡] See text for references.

[§] High-affinity form of profilin.

^{||} Low-affinity form of profilin.

[†] Calculated from values of 0.01 to 0.71% cell protein (Bamburg and Bray, 1987).

phosphorylated form is not known. The activity of the actin depolymerizing factors may be spatially defined by the distribution of filaments susceptible to cleavage by these factors: newly polymerized filaments at the leading edge which contain bound P_i may resist cleavage. Profilin can have a nonhomogeneous distribution, being concentrated at sites of sequestration in preparation for polymerization (Tilney et al., 1973; Tilney, 1976). Thus, unpolymerized actin in *Thyone* sperm is stored in a complex with profilin prior to the explosive assembly seen with the acrosome reaction. Profilin has been localized also on membranes (Hartwig et al., 1989), and in regions of rapidly turning over actin (Buß et al., 1992). It localizes at the site of actin polymerization in *Listeria* within cells, and has been implicated in the bacterium's motility (S. Theriot, personal communication). Subcellular localization of actobindin reveals striking concentration in pseudopods in *Acanthamoeba* (Dr. M. Bubb, personal communication). The consequences of the distribution of the monomer sequestering proteins depends of course on whether they contain bound actin. The properties of these actin binding proteins are summarized in Table 1.

What Role Is Played by Foci of Unpolymerized Actin?

Returning to the discovery of Cao et al. (1993) of foci of unassembled actin, what significance do these foci have? Localized monomeric actin can enable a cell, upon stimulation, to exhibit rapid polymerization, e.g., in *Thyone* sperm (Tilney, 1976) and in sea urchin eggs where unpolymerized actin is concentrated in the cortex (Spudich et al., 1988; Bonder et al., 1989). Thus, the punctate foci of unpolymerized actin may represent sites that locally promote polymerization. If the foci contain actin in a form able to polymerize (perhaps complexed to profilin), they might represent sites of high rates of polymerization.

However, the rearward movement of the foci observed in the study would not be predicted if the function is to promote polymerization of actin at the leading edge (although they might contribute to cortical actin polymerization). Thus, the foci may aid depolymerization by sequestering unpolymerized actin from the soluble pool. However, it is not obvious what the cell would gain by creating "sinks" of sequestered actin. It is possible, as the authors note, that the foci are not involved in the polymerization/depolymerization dynamics but play a role in the folding of actin during initial synthesis which for β actin is directed by its 3'UTR to the lamellipod (Kislauskis et al., 1993). Yet, microinjected actin becomes

incorporated into the structures, so the accumulated actin in the foci is not solely newly synthesized actin.

To understand the role of these intriguing foci we will need to answer a number of questions: What holds the monomeric actin in the foci? What fraction of the unpolymerized actin is present in punctate structures? Which monomer-binding proteins are present in the foci? Knowledge of monomeric actin—its distribution and binding to various cellular components—is needed to elucidate the spatial regulation and dynamics of actin during cell movements.

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