Interactions of ADF/cofilin, Arp2/3 complex, capping protein and profilin in remodeling of branched actin filament networks

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Background: Cellular movements are powered by the assembly and disassembly of actin filaments. Actin dynamics are controlled by Arp2/3 complex, the Wiskott–Aldrich syndrome protein (WASp) and the related Scar protein, capping protein, profilin, and the actin-depolymerizing factor (ADF, also known as cofilin). Recently, using an assay that both reveals the kinetics of overall reactions and allows visualization of actin filaments, we showed how these proteins co-operate in the assembly of branched actin filament networks. Here, we investigated how they work together to disassemble the networks.

Results: Actin filament branches formed by polymerization of ATP–actin in the presence of activated Arp2/3 complex were found to be metastable, dissociating from the mother filament with a half time of 500 seconds. The ADF/cofilin protein actophorin reduced the half time for both dissociation of γ-phosphate from ADP–P_i–actin filaments and debranching to 30 seconds. Branches were stabilized by phalloidin, which inhibits phosphate dissociation from ADP–P_i–filaments, and by BeF_3, which forms a stable complex with ADP and actin. Arp2/3 complex capped pointed ends of ATP–actin filaments with higher affinity (K_d ~40 nM) than those of ADP–actin filaments (K_d ~1 μM), explaining why phosphate dissociation from ADP–P_i–filaments liberates branches. Capping protein prevented annealing of short filaments after debranching and, with profilin, allowed filaments to depolymerize at the pointed ends.

Conclusions: The low affinity of Arp2/3 complex for the pointed ends of ADP–actin makes actin filament branches transient. By accelerating phosphate dissociation, ADF/cofilin promotes debranching. Barbed-end capping proteins and profilin allow dissociated branches to depolymerize from their free pointed ends.

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Background

The assembly of actin filament networks at the leading edge of motile cells pushes forward the plasma membrane [1,2]. Arp2/3 complex nucleates actin filament branches and links the pointed end of the new daughter filament to the side of the mother filament, a mechanism that has been called dendritic nucleation [3,4]. Highly purified Arp2/3 complex nucleates poorly [3,5] but can be activated by ActA, the surface protein of the bacterial pathogen *Listeria monocytogenes* [6], or by the carboxyl termini of members of the Wiskott–Aldrich syndrome protein (WASp)/Scar family [7,8]. WASp/Scar proteins also bind a number of proteins thought to provide signals for actin polymerization [5].

A new assay using light microscopy to visualize branched actin filaments generated by the activated Arp2/3 complex reveals that these branches are metastable [4]. Here, we established that phosphate release from aged actin filaments favors dissociation of Arp2/3 complex from the pointed ends of filaments by showing that proteins of the actin depolymerizing factor (ADF, also known as cofilin) family promote both dissociation of γ-phosphate from ADP–P_i-bound actin (ADP–Pi–actin) filaments and debranching. We also established that Arp2/3 complex has a lower affinity for the pointed ends of ADP-bound actin (ADP–actin) filaments than those of ATP-bound actin (ATP–actin) filaments. The products of debranching were long unbranched filaments, as short dissociated branches annealed rapidly unless they were capped at one end. We found that, if a barbed-end capping protein was present to block annealing and profilin was present to block addition of dissociated actin subunits at free pointed ends, ADF/cofilin could depolymerize the free branches. We also found that ADF/cofilin binds Arp2/3 complex, another possible mechanism to promote disassembly.

Results

Using single turnover experiments [9,10], we followed the time course of the dissociation of subunits from the ends of actin filaments. Filaments were assembled from actin monomers with a nucleotide (εATP) that has a higher fluorescence when bound to actin monomers or filaments than when free. When labeled subunits dissociate from

filaments into buffer containing excess unlabeled ATP, these monomers exchange εADP for unlabeled ATP, reducing the fluorescence regardless of whether or not the monomers repolymerize. All single turnover experiments reported here began with steady-state samples of approximately 7.4 μM actin with capped barbed ends and free pointed ends at steady state. The rate of change of the fluorescence depends on the concentrations of free barbed and pointed ends and the concentration of unpolymerized actin available to add to these ends. In the simplest case for Mg–ATP monomers and filaments with no free barbed ends,

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

For Mg–ATP–actin in polymerization buffer, $k_+ = 1.3\ \mu M^{-1}\ sec^{-1}$ and $k_- = 0.8\ sec^{-1}$ [11], so at steady state the concentration of free Mg–ATP–actin monomer is 0.6 μM, the concentration required to give equal association and dissociation rates. A positive rate indicates elongation, a negative rate shortening.

The response of this steady state to the addition of excess unlabeled ATP depends on many factors. When unlabeled ATP was added to a steady state sample of filaments, the fluorescence changed only slowly (Figure 1a, open triangles), as exchange of unlabeled for labeled subunits at pointed ends is slow. On the other hand, when ATP was added with an excess of profilin (Figure 1a, filled circles), the filaments depolymerized at a rate equal to k_- (pointed ends), as profilin binds tightly to ATP–actin monomers [12] and the complex does not elongate pointed ends [13]. Subunits dissociated but did not reassociate. The relevant dissociation rate constant was that for Mg–ADP–actin, 0.3 sec^{-1}. Addition of a mixture of ATP, profilin and Arp2/3 complex (to cap pointed ends) slowed but did not stop profilin-induced dissociation of Mg–ADP–actin subunits from gelsolin-capped filaments (Figure 1a, open circles), so Arp2/3 complex does not cap pointed ends tightly under these conditions.

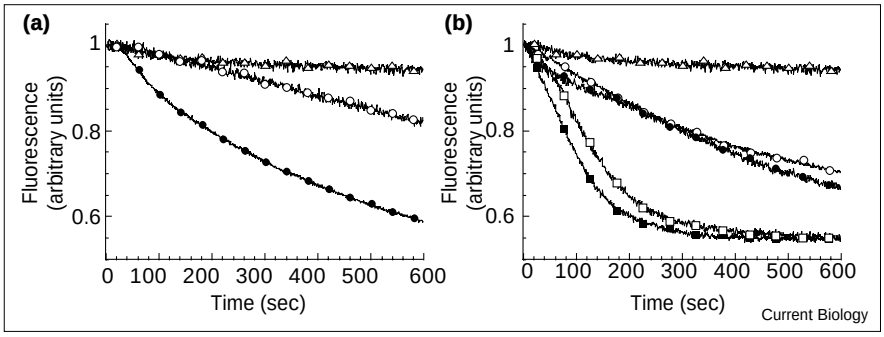
The ADF/cofilin protein actophorin also accelerated disassembly of gelsolin-capped filaments containing fluorescent actin subunits (Figure 1b, filled circles). Severing (increasing the concentration of ends; [14]) and/or faster subunit dissociation [15] may increase this rate. The fluorescence decreased faster when profilin was included with ATP and actophorin (Figure 1b, filled squares), as profilin catalyzes exchange of εADP for unlabeled ATP on monomers and blocks dissociated monomers from elongating pointed ends [9,16]. Remarkably, 100 nM Arp2/3 complex had no effect on the time course of fluorescent subunit dissociation by actophorin (Figure 1b), either in the absence (open circles) or presence (open squares) of profilin.

Thus, in the presence of profilin and/or actophorin, Arp2/3 complex caps depolymerizing ADP–pointed ends poorly, in spite of its ability to cap both polymerizing and depolymerizing pointed ends [3]. We considered two possibilities: first, the nucleotide on subunits near the pointed end influences binding of Arp2/3 complex to the pointed end of actin filaments; second, either actophorin or profilin may directly inhibit binding of Arp2/3 complex to pointed ends.

Effect of nucleotide on capping of pointed and barbed filament ends

We re-investigated capping of pointed ends by Arp2/3 complex using filaments capped on their barbed ends with gelsolin to isolate events at the pointed end. The amoeba Arp2/3 complex inhibits elongation of gelsolin-capped actin filaments by amoeba or muscle Mg–ATP–actin monomers in a concentration-dependent manner. The pointed end elongation rate of either amoeba and muscle Mg–ATP–actin as a function of Arp2/3 concentration gave an apparent K_d of 41 ± 7 nM for amoeba complex and a

Figure 1



Effects of profilin, actophorin and Arp2/3 complex on the time course of dissociation of actin subunits from filaments capped on their barbed ends by gelsolin. The starting samples were filaments polymerized to steady state in 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM $CaCl_2$, 0.5 mM DTT and 10 mM imidazole pH 7.0 from a mixture of 8 μM amoeba Mg–εATP–actin and 4 nM gelsolin-capped actin seeds. Reactions were initiated at time zero by adding 500 μM unlabeled ATP and various other factors. (a) Actin filaments alone (open triangles), or together with 20 μM profilin (filled circles), or 20 μM profilin and 100 nM Arp2/3 complex (open circles). (b) Actin filaments alone (open triangles), or together with 4 μM actophorin (filled circles), or 4 μM actophorin and 100 nM Arp2/3 complex (open circles), or 4 μM actophorin and 20 μM profilin (filled squares), or 4 μM actophorin, 20 μM profilin and 100 nM Arp2/3 complex (open squares).

capping efficiency at saturation of 95% (Figure 2a; filled triangles and squares), which is in agreement with previous observations [3].

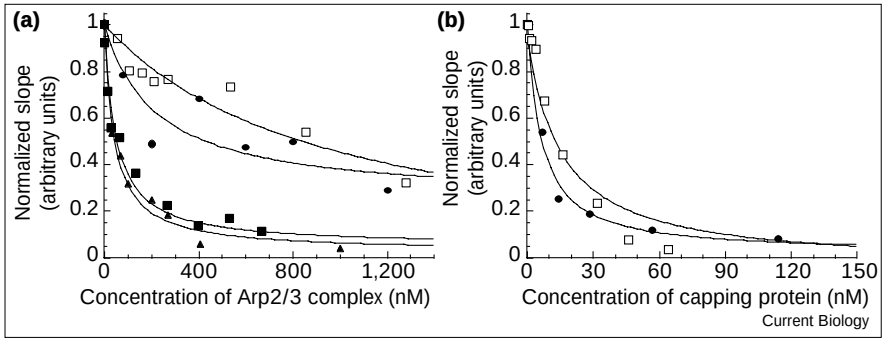
A fluorescence microscopy assay [4] confirmed efficient capping of pointed ends. We started with short gelsolin-capped filaments labeled red with rhodamine–phalloidin and added muscle actin monomers with Alexa green–phalloidin to label new filaments green. The bicolor filaments in the control without Arp2/3 complex (Figure 3a) showed that actin elongated only the free pointed ends. The average length of the elongated green filaments was 0.7 μm. A saturating concentration of amoeba Arp2/3 complex (above the dissociation equilibrium constant for pointed ends) prevented detectable growth from 90% of gelsolin-capped filaments. Microscopy revealed a side reaction that accounted for the residual actin polymerization of gelsolin-capped filaments saturated with Arp2/3 complex to cap their pointed ends. A few filaments formed spontaneously, separate from the added capped seeds (perhaps stimulated by the high concentration of Arp2/3 complex), grew long and aggregated into bundles [17]. These green filaments were so long that they must have grown from barbed ends. As they had no red segments, they did not form by annealing of seeds. As pointed ends grow so slowly, this side reaction gave an appreciable background of polymerization (Figure 2a) even though most of the seeds were capped on both ends by gelsolin and Arp2/3 complex.

Bovine Arp2/3 complex also inhibited pointed-end elongation by muscle ATP–actin monomers with a K_d of 220 ± 100 nM and an efficiency of 60% (Figure 2a, filled circles). The microscopy assay confirmed this inhibition. The number of filaments growing (15%) and the length grown (< 0.4 μm) in the presence of bovine Arp2/3 complex were very similar to the results with the amoeba Arp2/3 complex. As with the amoeba Arp2/3 complex, a few long filaments formed independently of the seeds in the presence of the bovine Arp2/3 complex. This side reaction may explain the residual 40% polymerization rate observed with seeds capped with gelsolin and bovine Arp2/3 complex. A similar experiment with bovine Arp2/3 complex by Ressad *et al.* [10] generated new barbed ends rather than capping pointed ends. In our hands and others [3,7–18,19], neither amoeba nor bovine Arp2/3 complex generated many new barbed ends without an activator like the WASp-WA domain. The Ressad Arp2/3 complex, which was prepared by affinity chromatography on a WASp column [10], may have been contaminated by a glutathione-S-transferase (GST)–WA fusion protein.

In contrast to the situation in ATP, the amoeba Arp2/3 complex weakly inhibited elongation of gelsolin-capped filaments by amoeba Mg–ADP–actin monomers (Figure 2a, open squares). The concentration dependence of inhibition of pointed-end elongation gave an apparent K_d of ~1 μM for amoeba Arp2/3 complex blocking pointed ends. Therefore, the affinity of the amoeba Arp2/3 complex for amoeba ADP–actin filament ends was more than 20-fold lower than for amoeba ATP–actin filament ends. The microscopy assay revealed that, in ADP, either 1.1 μM of amoeba (Figure 3c) or bovine (data not shown) Arp2/3 complex blocked growth at the pointed end of only 70% of the gelsolin-capped actin seeds.

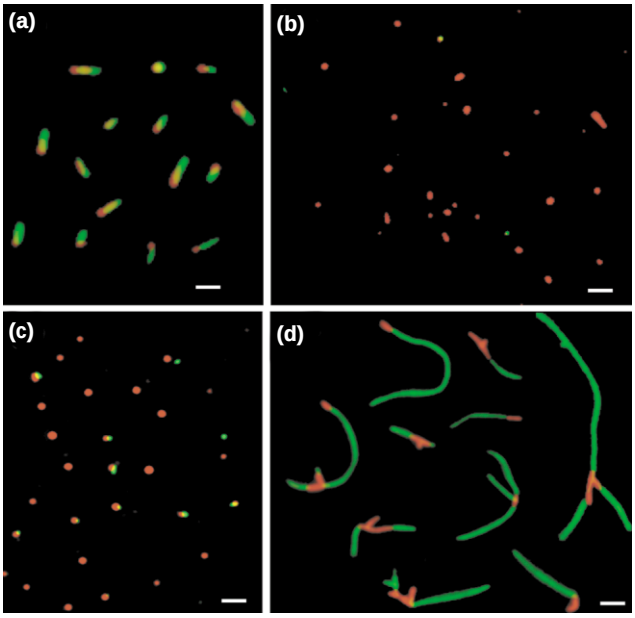
These results provide the following insights about events at the pointed end. In ADP, Arp2/3 complex will dissociate from and not rebind actin filament pointed ends during depolymerization. Even in ATP, Arp2/3 complex will not rebind providing that ATP–actin monomers are prevented from binding pointed ends by profilin. These properties explain both the lack of effect of Arp2/3 complex on depolymerization in the presence of

Figure 2



Effect of nucleotide on capping of actin filament ends by Arp2/3 complex and capping protein. **(a)** Dependence of pointed-end capping on the concentration of Arp2/3 complex. Freshly prepared seeds consisting of short actin filaments capped on their barbed ends with gelsolin with 5 nM free pointed ends were incubated with a range of concentrations of bovine or amoeba Arp2/3 complex for 1 min in 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM $CaCl_2$, 0.5 mM DTT, 10 mM imidazole pH 7.0, with either 0.2 mM ATP or ADP. The time course of elongation was recorded after adding 1.4 μM Mg–ATP–pyrenyl–actin or 1.4 μM Mg–ADP–pyrenyl–actin to this mixture. The initial rate of increase of pyrene fluorescence was plotted as a function of the concentration of bovine Arp2/3 complex in ATP (filled circles), or amoeba Arp2/3 complex in ATP (filled squares, amoeba actin; triangles, muscle actin) or ADP (open squares, amoeba actin). **(b)** Capping of barbed ends by *Acanthamoeba* capping proteins in ATP (filled circles) and ADP (open squares). The conditions were as in (a) except that the seeds were spectrin–actin seeds at 1 nM.

Figure 3



Visualization by fluorescence microscopy of the products generated by elongation of red-labeled (a–c) gelsolin-capped actin filament seeds or (d) branched filaments nucleated by Arp2/3 complex. The reaction contained 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 0.1 mM $CaCl_2$, 0.2 mM ATP or ADP, 3 mM NaN_3 and 10 mM imidazole pH 7. Phalloidin conjugated to Alexa green (Alexa green–phalloidin) was present during the elongation to stabilize the new filaments. Samples were prepared by dilution and application to a cover slip. (a) Gelsolin-capped seeds (5 nM) stabilized with rhodamine–phalloidin were incubated with 1 μM Mg–ATP–actin for 15 min in the presence of Alexa green–phalloidin and with an additional 1 μM Mg–ATP–actin for 15 min. (b) Gelsolin-capped red seeds were pre-incubated for 1 min with 1080 nM amoeba Arp2/3 complex, then with a mixture of 1 μM Mg–ATP–actin monomers and 1 μM Alexa green–phalloidin 15 min, followed by an additional 1 μM Mg–ATP–actin for 15 min. (c) Gelsolin-capped red seeds were pre-incubated with 1080 nM amoeba Arp2/3 complex, then with 1 μM Mg–ADP–actin monomers and Alexa green–phalloidin for 15 min, followed by an additional 1 μM Mg–ADP–actin for 15 min. (d) Branched actin filaments nucleated by Arp2/3 complex, labeled with rhodamine–phalloidin (4.8 nM of barbed ends) and capped with 100 nM capping protein, were incubated for 20 min with 4 μM Mg–ATP–actin monomers in the presence of Alexa green–phalloidin. The scale bars represent 0.6 μm.

actophorin (Figure 1b, open circles) and the partial inhibition of pointed-end depolymerization by Arp2/3 complex in the presence of profilin (Figure 1a, open circles). They also highlight the importance of ATP hydrolysis and the rate-limiting release of phosphate from ADP–P_i–actin subunits for polymer stability in the presence of regulatory proteins. The situation at the barbed end was dramatically different. First, although capping protein bound better in ATP than ADP (Figure 2b), this 2-fold difference was small compared with the 40-fold difference in affinity of Arp2/3 complex for ATP– and ADP–pointed ends. This emphasizes the sensitivity of Arp2/3 complex to the nucleotide on subunits at pointed ends. Second, barbed ends were more likely to have ATP than pointed ends in the presence of profilin because of exchange of ATP–subunits and exchange of ATP from the medium with the terminal subunits at the barbed end [20].

Another series of microscopy experiments showed that the bovine Arp2/3 complex blocked all pointed ends of the daughter filaments at the branch point but that barbed-end capping by capping protein was intermittent (Figure 3d). Experiments started with branched filaments labeled red with rhodamine–phalloidin. In the presence of 100 nM capping protein, muscle ATP–actin monomers elongated pointed ends rapidly and barbed ends slowly (labeled green with Alexa green–phalloidin). Pointed ends growing faster than barbed ends is opposite to what happens with uncapped filaments. We never observed new filament growth at the pointed end of filaments at branch points. Thus, pointed ends residing at branches are capped more effectively by Arp2/3 complex than unbranched pointed ends. This observation also shows that branches rarely, if ever, form by Arp2/3 complex joining the pointed end of a preformed filament (even a newly polymerized ATP–pointed end) to the side of another filament.

The fact that some barbed ends had short green extensions (Figure 3d) means that capping protein dissociates intermittently from barbed ends to allow for elongation or annealing. As growth on these barbed ends was short, recapping must be efficient or else barbed-end growth would have greatly exceeded pointed-end growth. From the concentration of capping protein and the association rate constant [21], we estimated that the half time for capping protein to rebind was about 2 seconds, which is in agreement with the limited growth of barbed ends. Slow growth in the presence of saturating capping protein suggests that capping protein may dissociate from barbed ends faster than proposed previously ($t_{1/2}$ = 30 minutes, [21]).

Dissociation of branches during aging and depolymerization of dendritic networks

As Arp2/3 complex bound ADP–pointed ends much more weakly than ATP–pointed ends, we examined how three factors that affect phosphate dissociation — phalloidin, BeF_3 and actophorin — affect the stability of actin filament branches formed by Arp2/3 complex on the side of actin filaments (Figure 4). Phalloidin inhibits phosphate release [22] whereas actophorin promotes phosphate release [23]. BeF_3 substitutes for γ-phosphate, forming a much more stable complex with ADP and actin than phosphate [24]. We assembled branched networks from concentrations of pure actin and activated Arp2/3 complex that yielded branching density and lengths suitable for light microscopy. To follow the time course of any rearrangements in these samples, we terminated the reactions at intervals by adding rhodamine–phalloidin and

diluting samples to a concentration appropriate for fluorescence microscopy.

Dendritic networks of filaments assembled from muscle Mg–ATP–actin and activated bovine Arp2/3 complex in ATP buffer de-branched spontaneously with a half time of about 500 seconds (Figure 4a–c,g; open circles). The fraction of branched filaments at each time point varied between experiments because of experimental factors that we cannot yet fully control, but the mean values gave a reliable estimate of the time course, which is similar to the time course of phosphate release under these conditions [23,25]. Remarkably, short branched filaments were converted to long unbranched filaments in this experiment. The most likely mechanism to account for the change of length is rapid end-to-end annealing of the short dissociated branches, as the concentration of polymerized actin is constant during this time [19]. The alternative mechanism of subunit redistribution between filaments is unlikely because of the slow rate of ADP–subunit dissociation (8 sec^{-1} at barbed ends and < 1 sec^{-1} at pointed ends). As annealing requires a free barbed end and a free pointed end, pointed ends must dissociate from Arp2/3 complex during debranching.

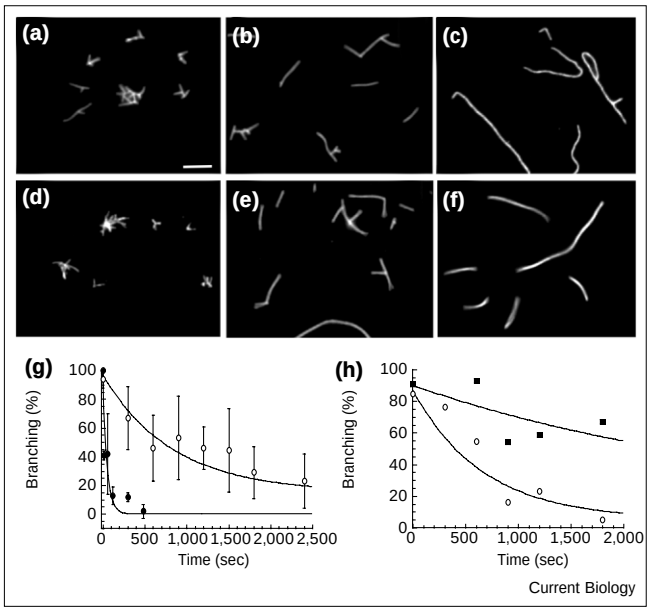
Two factors that stabilized the ADP–P_i state of actin polymers, BeF_3 (Figure 4h) and phalloidin [4], slowed the time course of de-branching. BeF_3 extended the half time for de-branching to over 2000 seconds. Phalloidin stabilized branches for hours [4]. These control experiments show that two agents that reduce the rate of phosphate release from actin filament also slow dissociation of branches.

Actophorin accelerated de-branching more than 10-fold (Figure 4g, filled circles) compared with controls. Again, densely branched networks of short filaments were rapidly converted to long unbranched filaments (Figure 4d–f), suggesting that in these experiments annealing of short filaments is faster than severing or depolymerization. Actophorin promotes dissociation of γ-phosphate from ADP–P_i–actin filaments [23]. Under the conditions of these de-branching experiments, the MESG assay for phosphate release [23,25,26] confirmed that actophorin promotes phosphate dissociation from filament networks formed by activated Arp2/3 complex, similar to the effect of actophorin on unbranched filaments. The half time for phosphate release with 40 μM actophorin was about 30 seconds.

Binding of actophorin to Arp2/3 complex

We studied the interaction of actophorin with the amoeba Arp2/3 complex by chemical cross-linking and analytical ultracentrifugation. Using conditions known to cross-link profilin to Arp2/3 complex [17], the zero-length cross-linker EDC together with NHS (see Materials and methods) cross-linked ^{35}S-labeled actophorin to Arp2/3 complex (Figure 5). The major cross-linked band containing

Figure 4



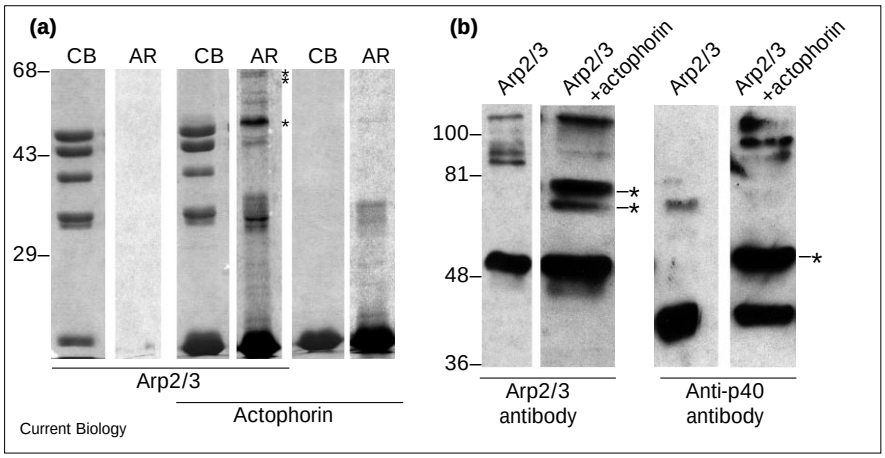
Time course of dissociation of actin filament branches. Actin (4 μM), Arp2/3 complex (23 nM) and WASp-WA (300 nM) were polymerized in 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, 3 mM NaN_3 and 10 mM imidazole pH 7 at 25°C for the times indicated below. Samples were prepared by stabilizing with rhodamine–phalloidin, dilution and application to a cover slip. **(a–f)** Fluorescence micrographs. (a–c) Actin, Arp2/3 complex and WASp-WA were incubated for a total of 20 min in polymerization buffer, and rhodamine–phalloidin added at (a) the onset of polymerization, or (b) 10 min or (c) 20 min after the onset of polymerization. (d–f) Actin, Arp2/3 complex, WASp-WA and 6 μM actophorin. Rhodamine–phalloidin was added at (d) the onset of polymerization, or (e) 30 sec or (f) 4 min after the onset of polymerization. **(g)** Time course of de-branching with actin, Arp2/3 complex and WASp-WA alone (open circles) or with 6 μM actophorin (filled circles). The fraction of branched actin filaments was measured by light microscopy. **(h)** Time course of de-branching with actin, Arp2/3 complex and WASp-WA alone (open circles) or in 150 μM BeF_3 (filled squares). The scale bar represents 1.5 μm.

^{35}S-labeled actophorin ran at ~58 kDa (Figure 5a) and reacted with antibodies to the p40 (ARPC1) subunit of Arp2/3 complex (Figure 5b), as expected from the size of the product. A fainter doublet of bands containing ^{35}S-labeled actophorin ran at ~68 kDa and reacted with antibodies to Arp2. Another band with ^{35}S-labeled actophorin at about 34 kDa could be actophorin cross-linked to the p14, p18 or p19 subunits of Arp2/3 complex. We used sedimentation equilibrium ultracentrifugation [17] to measure the affinity of rhodamine-labeled actophorin S88C mutant to Arp2/3 complex (data not shown). Least-squares fitting [17] gave a K_d of 12 μM for actophorin binding to Arp2/3 complex.

Discussion

Understanding how actin filaments disassemble and the subunits recycle in motile cells remains a conceptual and

Figure 5



Products of cross-linking *Acanthamoeba* actophorin to Arp2/3 complex. The reaction contained actophorin and Arp2/3 complex, 5 mM EDC, 5 mM NHS, 150 mM NaCl, 0.2 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM ATP, 1 mM DTT and 10 mM imidazole pH 7.5, and was incubated for 1 h at 25°C. (a) SDS–PAGE of cross-linked products visualized by Coomassie blue staining (CB) or by autoradiography (AR). The samples indicated by the horizontal bars were 3 μM Arp2/3 complex alone; 3 μM Arp2/3 complex + 23 μM ^{35}S-labeled actophorin; 23 μM ^{35}S-labeled actophorin alone. (b) Immunoblot of cross-linked products probed with antibodies against Arp2 and the p40 subunit of Arp2/3 complex. Molecular weights are in kDa. Asterisks indicate the main cross-linked products.

experimental challenge. As far as we know, all disassembly takes place at the ends of actin filaments where elongation and shortening are fully explained by the simple relationship: rate = k_+ (ends) (free actin monomer) – k_- (ends). However, the relationship is made complicated by the following: the two ends of filaments differ in kinetic properties; nucleotide bound to actin monomers (ADP or ATP) or subunits in actin filaments (ATP, ADP–P_i or ADP) influence reaction rates and interactions with regulatory proteins; at least at the barbed end, nucleotide in the medium appears to exchange with nucleotide on terminal subunits; regulatory proteins (profilin, thymosin, ADF/cofilin) bound to monomers influence their reactions with nucleotides and the ends of filaments; and capping/uncapping, severing/annealing and branching/de-branching all influence the concentrations of ends. Even in the minimal system required to reconstitute assembly of actin comet tails by bacteria [27], five different proteins participate: actin, Arp2/3 complex, profilin, ADF/cofilin and capping protein. Each has an optimal concentration and their inter-relationships are extensive and sometimes subtle (reviewed in [28]). For example, to cap barbed ends effectively, capping protein requires profilin to suppress its tendency to nucleate new pointed ends, because, if unchecked, this side reaction consumes all of the capping protein [4].

The present study of depolymerization reactions yielded four main findings. First, dissociation of γ-phosphate from actin filament branches weakens the interaction of the pointed end of the branch with Arp2/3 complex. It also causes the branch to dissociate and, by preventing recapping, probably contributes to making depolymerization of ADP–actin filament subunits from pointed ends processive. Second, actophorin promotes de-branching by accelerating the rate-limiting release of phosphate from ADP–P_i–actin filament subunits. Third, if their barbed ends are not capped, dissociated branches rapidly anneal, perhaps contributing to the pool of long unbranched filaments observed in cells. Fourth, provided that most barbed ends are capped, actophorin and profilin dissociate ADP–actin filament subunits from free pointed ends and recycle them back to the profilin–ATP–actin pool, ready to elongate new barbed ends created by Arp2/3 complex. Although this work is a step towards understanding disassembly, we are impressed by the complexity of the reactions and expect that surprises lie ahead. Nevertheless, our findings do explain how cells achieve endwise disassembly of actin filaments in the presence of high concentrations of proteins that cap both barbed ends (capping protein, gelsolin) and pointed ends (Arp2/3 complex, tropomodulin) of actin filaments and inhibit dissociation of subunits. Our findings also explain why filament ends created by ADF/cofilin shorten rather than anneal or become capped, and what makes the shortening processive.

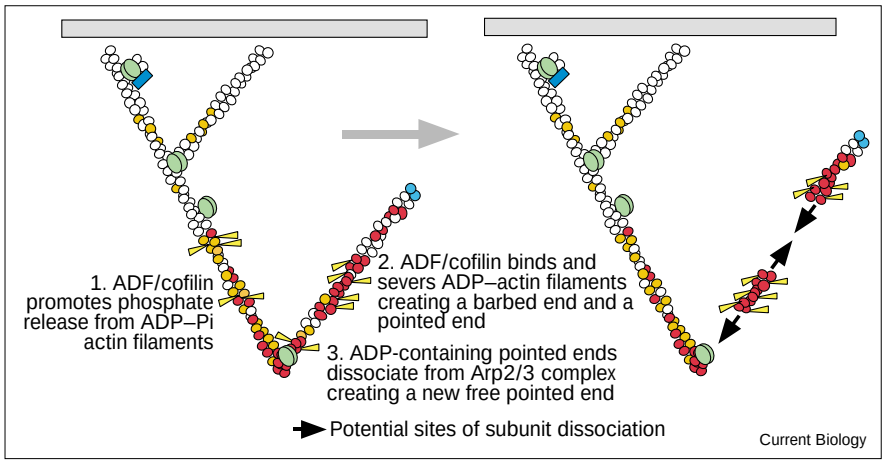
De-branching mechanism

Our central finding is that Arp2/3 complex binds the pointed end of ATP–actin filaments with much higher affinity (K_d ~40 nM) than ADP–pointed ends (K_d ~1 μM). In ATP, saturating concentrations of the amoeba Arp2/3 complex inhibited pointed-end elongation by 95% on the basis of the absence of visible growth at the pointed end of most filaments and slow or intermittent growth on the remainder. Pointed-end capping by Arp2/3 complex at branches was absolute. In ADP, the lower affinity of Arp2/3 complex for pointed ends allowed growth of 30% of gelsolin-capped actin seeds in the presence of 1.1 μM complex.

The difference in the affinity of Arp2/3 complex for ATP– and ADP–pointed ends provides a mechanism for de-branching, and explains the effects of phalloidin, BeF_3

Figure 6

Model. Weak binding of actophorin to ADP–P_i–actin subunits accelerates phosphate release and promotes further actophorin binding. The low affinity of Arp2/3 for ADP–pointed ends results in de-branching of actin filaments, creates free ends for depolymerization and makes depolymerization processive by limiting re-capping of ADP–pointed ends. Severing by actophorin generates additional pointed ends, which depolymerize provided barbed ends are capped and profilin is present to sequester monomers.



and actophorin on the rate of de-branching and why pointed-end depolymerization is processive. All of the data are consistent with phosphate dissociation from the pointed end of the branch being the key event in changing the affinity and with the de-branching reaction being dissociation of the pointed end from Arp2/3 complex, which may or may not remain associated with the mother filament. Nevertheless, more detailed mechanistic studies are desirable in the future. In principle, the nucleotide state of the mother filament and of Arp2/3 complex might also influence the stability of the branch point. However, the frequency of branching from ADP–filaments is approximately the same as from newly polymerized filaments (ATP–filaments and ADP–P_i–filaments) [4], suggesting that the lateral interaction with the mother filament is less sensitive to the nucleotide in the filament.

As irreversible ATP hydrolysis [29] followed by slow dissociation of the γ-phosphate [30] are inevitable following polymerization of ATP–actin, de-branching may also be inevitable, although external factors may influence the rate of these reactions. The first example is the ability of ADF/cofilin to accelerate de-branching. The ability of actophorin to promote phosphate dissociation from ADP–P_i–actin filaments is sufficient to explain its effect on de-branching, but actophorin may influence de-branching and disassembly in other ways. Binding of actophorin to Arp2/3 complex, although weak, may reduce the affinity of Arp2/3 complex for pointed ends. In addition, the conformational change induced when ADF/cofilins bind actin filaments [31] may dissociate Arp2/3 complex from filament ends or sides. This conformational change may also influence binding of capping protein to barbed ends. On the other hand, WASp/Scar proteins may stabilize branches by interacting with both Arp2/3 complex and actin. All of these possibilities deserve more detailed investigation.

Response of filaments and branched networks to actophorin and profilin

Much previous work considered how individual actin-binding proteins interact with actin monomers and filaments, but it is now clear that understanding actin filament turnover in cells requires insights about ensembles of regulatory proteins. This task is built on knowledge of the individual reactions, but light microscopy has revealed that it is much more complex than one might have anticipated. We have found that the responses of actin filaments and branched actin filament networks to actophorin and profilin involve not only effects on subunit dissociation at filament ends [15] and severing [14,23,32,33], but also effects of ADF/cofilin on the rate of phosphate dissociation, de-branching, capping and annealing. Capping is particularly important. Barbed-end capping is required for profilin to sequester ATP–actin monomers [34], but it also prevents annealing of filaments [35] created by severing or de-branching, allowing these free pointed ends to disassemble. Thus barbed-end capping is essential for effective disassembly of pointed ends. Addition of actophorin to uncapped filaments results in severing, but the mean length of the filaments reaches a plateau with time [23,32]. We postulated that this steady state results from a balance between the rates of severing and annealing. The light micrographs in this paper confirmed annealing, but its rate and extent need further quantitative studies. Given the impressive annealing observed by light microscopy, we expect that previous solution studies underestimated the extent of severing. Light microscopy with immobilized filaments [14,33] may have given a more accurate account of severing. Addition of profilin to uncapped filaments has no effect on actin monomer or polymer concentrations, but most of the actin monomer binds profilin. Addition of actophorin and profilin to uncapped filaments promotes subunit flux from filaments to ADP–actin–actophorin to ATP–actin–profilin and back to free barbed ends [15,16,23].

Capping barbed ends influences all of these reactions. Capping blocks both association and dissociation of subunits at the barbed end, and as actin–profilin does not associate with pointed ends, the only reaction allowed is dissociation at pointed ends, eventually depolymerizing the filaments (Figure 1a). The combination of actophorin and profilin depolymerizes capped filaments faster because of severing and possibly enhanced ADP–actin dissociation from pointed ends (Figure 1b).

Actophorin has a special effect on filaments with Arp2/3 complex branches, but otherwise the effects of profilin and/or actophorin on branched actin filament networks closely parallel their effects on unbranched actin filaments. By promoting phosphate dissociation (and possibly by other mechanisms considered above), actophorin accelerates debranching, creating short filaments with free pointed ends. If free barbed ends are available, the short dissociated branches anneal rapidly to form long unbranched filaments. This side reaction will affect the observation of the early time course of branch dissociation. Consequently, we probably underestimated the extent of debranching, but ADF/cofilin clearly promoted debranching (Figure 4g). If capping protein is present, capping and annealing will compete. Although all filaments near the leading edge are branched, annealing of dissociated branches may contribute to the formation of longer unbranched filaments found deeper in the cytoplasm. De-branching and annealing may also contribute to formation of bundles of long actin filaments in the cortex and filopodia. If the rate of barbed-end capping exceeds the rate of annealing, free pointed ends will be available for disassembly by actophorin and profilin, recycling actin back to the profilin–actin pool.

Although clear in broad outline, essentially every reaction on these pathways needs more work. For example, having established the existence of both severing by actophorin and annealing of the fragments, the rates of these reactions need to be determined. With that understood, it should be possible to evaluate more rigorously exactly how fast branches dissociate, how fast actophorin–ADP–actin dissociates from free pointed ends and to learn if the combination of severing and enhanced dissociation account fully for the turnover of filaments in the presence of actophorin (and profilin). As most of the components in the system appear to be interchangeable across the phylogenetic tree, we expect that the basic mechanisms have been conserved, as *Acanthamoeba* branched from the main line of eukaryotes more than 1 billion years ago. Nevertheless, comparative studies are needed to confirm that higher organisms use the same mechanisms as amoeba.

Conclusions

The effect of bound nucleotide on capping of pointed ends allows Arp2/3 complex and ATP–actin monomers to form a stable, rigid, but temporary branch on the side of another actin filament (Figure 6). Assembly of the branched network is favored near the plasma membrane where prenylated GTP-bound Cdc42 and phosphatidyl-inositol 4,5-bisphosphate (PIP_2) activate WASp to stimulate nucleation by Arp2/3 complex. Deeper in the cytoplasm, inevitable hydrolysis of bound ATP and dissociation of phosphate lead to de-branching and processive depolymerization of the pointed end of the ADP–filament, provided that most barbed ends are capped (to prevent annealing and to maintain the profilin–actin pool) and that profilin is available to sequester dissociated subunits. Thus phosphate dissociation from polymerized ADP–P_i–actin is a key event in giving a direction to the whole cycle of actin assembly and recycling. ATP hydrolysis is the only irreversible step, but as actin filaments containing ATP or ADP–P_i are identical in every aspect measured, it is the subsequent dissociation of phosphate that initiates disassembly. Actophorin promotes filament turnover by dissociating phosphate from ADP–P_i–filaments (enhancing actophorin binding and dissociating Arp2/3 complex from pointed ends). Actophorin also contributes through severing, which makes more ADP–pointed ends, and by dissociating ADP–subunits.

Materials and methods

Reagents

DTT, EDTA, Tris, sodium azide, DMSO, hexokinase, ATP, ADP, phalloidin, and Sephadex G-25 medium were from Sigma; Tris-(2-carboxyethyl)phosphine, tetramethylrhodamine maleimide 5′ isomer (TCEP), and Alexa green–phalloidin were from Molecular Probes; 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were from Pierce; DEAE–cellulose DE-52 was from Whatman; and rhodamine–phalloidin was from Fluka.

Protein purification

Acanthamoeba actin was purified from DEAE column fractions by polymerization–depolymerization and gel filtration [36] and stored in Buffer G (2 mM Tris-Cl pH 8, 0.2 mM ATP, 0.5 mM DTT, 0.1 mM $CaCl_2$, 1 mM azide). Actin was labeled on Cys374 to a stoichiometry of 0.8–1.0 with pyrene iodoacetamide ([37] as modified by [36]) and diluted with nine parts of unlabeled actin for polymerization assays. Mg–ATP–actin monomers were prepared on ice by addition of 0.2 mM EGTA and 11-fold molar excess of $MgCl_2$ over actin and used within hours. Mg–ADP–actin monomers were prepared by treatment of Mg–ATP–actin monomers with soluble hexokinase and glucose [36]. ADP–BeF_3 actin filaments were prepared by polymerizing 20 μM Mg–ATP–actin in 100 mM KCl, 2 mM $MgCl_2$, 5 mM NaF, 150 μM $BeCl_2$ at room temperature for 4 h [24]. Wild-type actophorin and the S88C mutant of actophorin [16] in plasmid vector pMW172 were expressed in *Escherichia coli* strain BL21 (DE3) pLysS and purified [38] with 2 mM DTT in all buffers to avoid cysteine oxidation. Purified actophorins were stored in 10 mM Tris-Cl pH 7.5, 1 mM EDTA, 2 mM DTT, 1 mM NaN_3. Actophorin S88C was labeled with tetramethylrhodamine maleimide 5′ isomer and purified [16]. Actophorin was labeled with ^{35}S according to Gao *et al.* [39]. Arp2/3 complex was purified from *Acanthamoeba* by ion exchange on DEAE followed by poly-L-proline affinity chromatography [40], or from bovine thymus [5]. Recombinant plasma gelsolin was purified according to Yu *et al.* [41]. *Acanthamoeba* profilin-I was purified by poly-L-proline affinity chromatography [42]. *Acanthamoeba* capping protein was purified according to Cooper *et al.* [43]. Recombinant WASp-WA domain was purified from *E. coli* [5].

Polymerization assays

Polymerization buffer contained 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole pH 7.0 and, except where noted, actin buffer G. We

formed gelsolin-capped actin seeds as described by Mullins *et al.* [3]. Briefly, we incubated purified recombinant plasma gelsolin with a twofold molar excess of *Acanthamoeba* or muscle actin (Buffer: 200–400 μM $CaCl_2$, 0.5 mM DTT, 0.2 mM ATP, 2 mM Tris pH 8.0) for 2 h at room temperature followed by an overnight incubation at 4°C (this step is essential as gelsolin-capped actin dimers form quite slowly). We warmed these gelsolin-capped actin dimers to room temperature, added a fivefold excess of actin and then adjusted the buffer to 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM Imidazole pH 7.0 by adding 0.1 volume of a 10× stock solution. We quantified the number of free pointed ends by elongation assays using known concentrations of pyrenyl–actin. We verified that seeds prepared in this manner contained no free barbed ends by adding profilin to the elongation reaction. When the percentage of pyrene-labeled actin was low, 1–5% excess profilin completely inhibited elongation from our gelsolin-capped actin seeds. In our experience, gelsolin-capped seeds formed this way and stored on ice are good for up to 5 days.

Light microscopy assay

Actin at 4 μM was polymerized under the conditions specified in the figure legends, labeled with rhodamine–phalloidin during polymerization or at an indicated time point thereafter and diluted for observation to a final concentration of 10 nM in fluorescence buffer containing 10 mM imidazole pH 7.0, 50 mM KCl, 1 mM $MgCl_2$, 100 mM DTT, 100 μg/ml glucose oxidase, 3 mg/ml glucose, 20 μg/ml catalase, 0.5% methylcellulose [4]. A dilute sample of 2 μl was applied to a 22 × 22 mm coverslip coated with 0.1% nitrocellulose in amyl acetate. Actin filaments were observed by epi-fluorescence illumination with an Olympus IX-70 microscope and digital images were collected with a Hamamatsu ORCA CCD camera using Metamorph software. Analysis of the images was done using Metamorph software.

Filament turnover assay

Dissociation of subunits from filaments labeled with fluorescent nucleotide (εATP) was measured in single turnover experiments [9,10]. Dissociation of fluorescent subunits from the ends of filaments did not change the fluorescence, but in the presence of an excess of unlabeled ATP in the buffer, εATP exchanged for ATP with an irreversible reduction in fluorescence regardless of subsequent reactions.

Chemical cross-linking

Stock solutions of 100 mM EDC and NHS were made fresh in dry DMSO immediately before use. The final concentration of DMSO in all reactions was 10%. Reactions were carried out for 1 h at room temperature and quenched by addition of 100 mM glycine, 10 mM Tris pH 7.5.

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