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Actin disassembly by cofilin, coronin, and Aip1 occurs in bursts and is inhibited by barbed-end cappers

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Introduction

Actin filaments turn over rapidly in cells, and their assembly through barbed end growth is thought to generate protrusive force. Rapid assembly must be balanced by equally rapid disassembly to replenish the monomer pool and allow remodeling of cell shape. Disassembly is arguably the least understood step in the actin dynamics cycle (Pollard and Borisy, 2003). We need to elucidate its mechanism to understand the morphogenesis of dynamic actin assemblies and answer the fundamental question of how polymerization dynamics transduce the energy of ATP hydrolysis into mechanical work. Physiological disassembly mechanisms must fulfill two requirements: (1) fast kinetics and (2) disassembly in a cytoplasm that contains high concentrations of polymerizable monomers. We here image the disassembly of single actin filaments by coflin, coronin, and actin-interacting protein 1, a purified protein system that reconstitutes rapid, monomer-insensitive disassembly (Brieher, W.M., H.Y. Kueh, B.A. Ballif, and T.J. Mitchison. 2006. J. Cell Biol. 175:315–324). In this three-component system, filaments disassemble in abrupt bursts that initiate preferentially, but not exclusively, from both filament ends. Bursting disassembly generates unstable reaction intermediates with lowered affinity for CapZ at barbed ends. CapZ and cytochalasin D (CytoD), a barbed-end capping drug, strongly inhibit bursting disassembly. CytoD also inhibits actin disassembly in mammalian cells, whereas latrunculin B, a monomer sequestering drug, does not. We propose that bursts of disassembly arise from cooperative separation of the two filament strands near an end. The differential effects of drugs in cells argue for physiological relevance of this new disassembly pathway and potentially explain discordant results previously found with these drugs.
We recently reconstituted a purified protein system consisting of coflin, coronin, and actin-interacting protein 1 (Aip1) that catalyzes the disassembly of *Listeria monocytogenes* comet tails (Brieger et al., 2006), dense actin assemblies that structurally and biochemically resemble the leading edge of motile cells (Cameron et al., 2001). These three purified components together reconstitute disassembly that is both fast (τ < 100 s) and insensitive to polymerizable monomer (τH11601 = 10 μM ATP-actin monomer). Coflin belongs to a family of widely conserved, essential proteins that promote actin disassembly in many, perhaps all, cell types and structures (Maciver and Hussey, 2002). It preferentially binds ADP-actin filaments and presumably helps to couple the energy of ATP hydrolysis to actin turnover (Carlier et al., 1997; Blanchoin and Pollard, 1999). Paradoxically, coflin can also promote actin assembly, presumably by providing new barbed ends to seed polymerization (Ichetovkin et al., 2002; Ghosh et al., 2004). Coronin binds actin filaments and also plays a role in physiological actin turnover (de Hostos et al., 1991; Foger et al., 2006; Cai et al., 2007b). Its mechanism of action is still controversial and may be isoform dependent (Cai et al., 2007a). We used coronin 1a, which was purified to homogeneity from calf thymus (Brieger et al., 2006). Aip1 works with coflin to promote actin disassembly in a variety of organisms (Ono, 2001; Rogers et al., 2003; Ketelaar et al., 2004; Okada et al., 2006). It appears to interact with filament barbed ends (Okada et al., 2002; Balcer et al., 2003) and confer monomer sensitivity to the three-component disassembly reaction (Brieger et al., 2006). Although our previous work began to define roles for each factor in the purified protein system (Brieger et al., 2006), it did not determine how the full purified system promotes disassembly on a single-filament level. We address this question here.

**Results**

Actin filaments disassemble in bursts

We imaged the disassembly of single, fluorescently labeled actin filaments catalyzed by the three-component purified protein system consisting of coflin, coronin, and Aip1. Concentrations of individual components were chosen to be those sufficient for full rate enhancement in *L. monocytogenes* comet tail disassembly (Brieger et al., 2006). All reactions also contained 5 μM of unlabeled actin monomer and 2 mM ATP, which reflects the physiological requirement that disassembly must occur in substantial concentrations of polymerizable monomer. We polymerized fluorescent-labeled actin filaments in a perfusion chamber using the actin cross-linker filamin to immobilize filaments to the coverslip surface. In all assays, filaments were polymerized for <1 min before initiation of disassembly. Filament aging leads to kinetic changes we will detail elsewhere (unpublished data). Newly assembled filaments were then disassembled by perfusion of the three-component system. Immediately after perfusion, a streaming video of actin filaments (acquisition time per image = 400 ms) was acquired by wide-field fluorescence microscopy.

Figure 1. Actin filaments disassemble in bursts in coflin, coronin, and Aip1. (A) Time-lapse wide-field epifluorescence images of fluorescently labeled actin filaments in the presence of 2 μM coflin, 1 μM coronin, 200 nM Aip1, 5 μM of actin monomer, and 2 mM ATP. Filaments shorten and disappear from the field of view. Bar, 3 μm. (B) Successive time-lapse images showing a single actin filament (f1) over time, along with kymographs drawn along the contours of representative filaments (f1–f4). The red lines on the image of f1 at t = 0 denote the contour on which the kymograph was drawn. Time is given on the x axis of the kymograph, whereas the position along the filament contour is given on the y axis. Mean integration time for a single image was 400 ms for f1–f3 and 16 ms for f4. Triangles denote endwise bursting (f1–f3); yellow triangles denote initial burst (f1–f3), red triangles denote successive proximal bursts (f1 and f2), and green triangle denotes a successive distal burst (f3). Same-side bursts occurred more frequently (78%) than opposite-side bursts (22%; P < 0.001, one-tailed z test). The square denotes internal disassembly event counted as a severing event (f3). Bar, 1 μm. (C) Histogram of filament burst size. The mean burst size was 260 subunits. (D) Histogram of waiting times between successive bursts (red), fit to a single exponential (black). Single exponential fit gave characteristic decay time of τ = 14 s.
microscopy (Fig. 1 A and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200801027/DC1).

In the presence of coflin, coronin, and Aip1, filaments shortened and disappeared from the field of view within the first 100 s (Fig. 1, A and B; and Video 1). We tracked the locations of filament ends using kymographs drawn along the contours of individual actin filaments (Fig. 1, B and C, filaments f1–f4). Kymographs showed that filaments did not shrink smoothly from an end; instead, they disassembled in infrequent bursts, with filaments abruptly losing mass from an end (Fig. 1 B, triangles). Most disassembly events occurred at filament ends, but events were occasionally initiated in the middle of the filament; these were scored as severing events (Fig. 1 B, blue square; see Fig. 2 for a detailed comparison of this reaction to a coflin-mediated severing reaction). In an attempt to resolve molecular events catalyzed by a three-component system to the coflin-alone reaction, we scored disassembly events under both conditions and counted them as either bursting or severing events. For scoring purposes, a severing event was one in which both daughter filaments remained attached to the surface of the coverslip for at least two frames after breakage. Such an analysis revealed that, although filaments in the three-component system underwent occasional internal disassembly events that were scored as severing events (Fig. 1 B, f3, square), bursting from ends was the predominant mode of disassembly (Fig. 2 B). In contrast, severing was the predominant mode of disassembly catalyzed by coflin alone (Fig. 2 B), which is consistent with previous studies (Maciver et al., 1991; Andrianantoandro and Pollard, 2006). Our coflin-alone data reinforce our conclusion that bursting disassembly catalyzed by coflin, coronin, and Aip1 is a novel mechanism of filament disassembly.

Actin filaments are structurally polarized, so we next tested whether the bursts of disassembly occur preferentially from a particular end of the filament. To separately probe disassembly near barbed ends and pointed ends, we imaged disassembly of filaments elongated from fragments of Limulus polyphemus, acrosomal processes, which are highly bundled arrays of filaments all oriented in the same direction (Bonder and Moosker, 1983). Filaments grown off the barbed end of acrosomal bundles have free barbed ends but pointed ends that are connected to the acrosome. The reverse applies at the pointed end. We polymerized bundles of filaments off the barbed ends and pointed ends of L. polyphemus acrosomes using 5 μM of fluorescently labeled actin monomer and 2 mM ATP and then perfused them with coflin, coronin, and Aip1, as well as unlabeled actin monomer and ATP. To minimize filament aging effects, filaments were perfused within 2 min after initiation of polymerization. Interestingly, filament bundles grown from either barbed ends (Fig. 3 A, b) or pointed ends (Fig. 3 A, p) of acrosomal processes disassembled at comparable rates (Fig. 3 A). Most of the polymer mass in the filament bundles disappeared within the first 2 min; the remainder, which manifest as a short
similar rates. Bursting disassembly generates unstable filament intermediates not only at their tips but also along their lengths (Fig. 4 B, left), which is consistent with binding of CapZ to barbed ends of severed filaments. To quantify the fraction of capped filaments before and after cofilin treatment, we measured for individual filament bundles the CapZ/filament ratio, which reports on the proportion of capped filaments (see Materials and methods). This analysis showed that cofilin-treated filament bundles showed no significant decrease in the fraction of capped filaments compared with untreated bundles (Fig. 4, A and B, right; 1-tailed \( t \) test, \( t = 1.6, \) degrees of freedom \( df = 14, P > 0.01 \)). We conclude that filaments severed by cofilin retain the ability to bind CapZ at their barbed ends, as expected.

When filament bundles were partially disassembled with cofilin, coronin, and Aip1 before CapZ incubation, their fluorescence also tapered due to disassembly (Fig. 4 C, left). However, unlike cofilin-treated filament bundles, these bundles recruited almost no CapZ (Fig. 4 C, middle). The CapZ/filament ratio for bundles treated with cofilin, coronin, and Aip1 was much lower than that for untreated bundles (Fig. 4, A and C, right; 1-tailed \( t \) test, \( t = 11.1, \) df = 22, \( P < 0.01 \)) and not significantly different from zero (Fig. 4 C, right; \( t \) test, \( t = 2.3, \) df = 15, \( P > 0.01 \)). A similar decrease in CapZ recruitment was also seen when CapZ was incubated concurrently with cofilin, coronin, and Aip1 (unpublished data). These results suggest that, unlike a pure severing reaction, bursting disassembly by the three-component system generates as reaction intermediates barbed ends that are not recognized by CapZ. To determine whether these barbed ends were stable in buffer, we partially disassembled filament bundles with cofilin, coronin, and Aip1, then incubated them in buffer for 10 s before incubation with CapZ. These filament bundles recruited significantly more CapZ than bundles treated directly with CapZ (Fig. 4, C and D; 1-tailed \( t \) test, \( t = 4.8, \) df = 22, \( P < 10^{-4} \)), which suggests that, in the absence of disassembly factors, nonstandard barbed ends rapidly revert to a standard CapZ-recognizable form. Consistent with this interpretation, others have observed that cofilin and Aip1 do not inhibit filament elongation from barbed ends when washed out (Ono et al., 2004). These data suggest that bursting disassembly generates unstable filament intermediates with nonstandard barbed ends not recognized by CapZ.

**Bursting disassembly generates barbed ends not recognized by CapZ**

The disassembly pathway catalyzed by cofilin, coronin, and Aip1 may involve reaction intermediates with structurally altered filament ends. To test this hypothesis, we probed the barbed ends of filaments using fluorescently labeled CapZ, a protein that caps standard filament barbed ends with fast kinetics and high affinity (Caldwell et al., 1989; Schafer et al., 1996). We polymerized filament bundles from *L. polyphemus* acrosomal fragments, treated them under different depolymerizing conditions, and then perfused them with 3 μM Alexa 488 CapZ for 10 s. This CapZ incubation was sufficient to completely cap all standard barbed ends (unpublished data), which is consistent with previous kinetic studies (kcat \( \approx 3 \) μM \(^{-1} \) s\(^{-1} \) from Schafer et al., 1996). When filament bundles were incubated with CapZ in the absence of disassembly, they recruited CapZ at their tips, which is consistent with all filaments recruiting CapZ to their barbed ends (Fig. 4 A, middle). When filament bundles were partially disassembled with cofilin alone before CapZ incubation, their fluorescence intensity tapered toward the end of the bundle, which reflects shortening of filaments due to severing (Fig. 4 B, left). Cofilin-treated filament bundles recruited CapZ not only at their tips but also along their lengths (Fig. 4 B, middle), which is consistent with binding of CapZ to barbed ends of severed filaments. To quantify the fraction of capped filaments before and after cofilin treatment, we measured for individual filament bundles the CapZ/filament ratio, which reports on the proportion of capped filaments (see Materials and methods). This analysis showed that cofilin-treated filament bundles showed no significant decrease in the fraction of capped filaments compared with untreated bundles (Fig. 4, A and B, right; 1-tailed \( t \) test, \( t = 1.6, \) degrees of freedom \( df = 14, P > 0.01 \)). We conclude that filaments severed by cofilin retain the ability to bind CapZ at their barbed ends, as expected.

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Barbed end-capping agents inhibit actin disassembly in vitro

Our experiments with fluorescent CapZ argue against a disassembly mechanism where depolymerization factors form a stable cap with filament barbed ends to prevent monomer addition and filament annealing. They instead suggest that bursting disassembly may involve active disruption of filament internal structure, leading to ragged barbed ends that cannot recruit CapZ. To test these ideas further, we imaged single filaments after perfusion with cofilin, coronin, and actin monomer with or without Aip1, and in the presence of CapZ or cytochalasin D (CytoD), a well-characterized barbed end-capping drug.

Omission of Aip1 from the three-component system stopped disassembly (Fig. 5 A, green) and greatly reduced the frequency of both bursts and scored severing events (Fig. 5 B, green). Omission of either cofilin or coronin had the same effect (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200801027/DC1), which is consistent with a requirement for all three factors for rapid monomer-insensitive disassembly in L. monocytogenes comet tails (Brieger et al., 2006). CapZ could not substitute for Aip1 in the three-component reaction (Fig. 5, A and B, yellow), which suggests a role for Aip1 in disassembly that is distinct from barbed-end capping. When added to the full three-component system, both CytoD and CapZ strongly inhibited filament disassembly (Fig. 5 A, black and blue), blocking both endwise bursting and internal severing modes of filament disassembly (Fig. 5 B, black and blue). To separately test the effect of CytoD and CapZ on disassembly from filament barbed ends and pointed ends, we imaged acrosomal filament bundles in the presence of the full three-component system and either CytoD or CapZ (Fig. 5 C). Remarkably, disassembly of filaments grown off barbed ends and pointed ends were inhibited with equal efficacy, which suggests that filament segments near both ends disassemble through the same CytoD/CapZ-sensitive mechanism (Fig. 5 C). CytoD inhibited filament bundle disassembly with an IC₅₀ of 90 nM (measured using total polymer mass from all filament bundles in a field of view), which is similar to its IC₅₀ for inhibition of polymerization off acrosomal fragments (30 nM; Fig. 5 D). In contrast, although CapZ also inhibited polymerization off acrosomal fragments with a low IC₅₀ (30 nM; Fig. 5 E), its inhibitory effects on disassembly did not saturate at 10 μM (Fig. 5 E). The difference in IC₅₀ between CytoD- and CapZ-mediated inhibition of disassembly suggests differences in the detailed mechanism between these two barbed end-capping agents. For example, barbed ends generated during disassembly may have a conformation that is recognized by CytoD but not by CapZ. Regardless of the detailed mechanism, their common inhibitory effects suggest an important role for barbed end-capping agents in controlling filament stability in the three-component system, even when disassembly occurs by bursting from the pointed end. In contrast, CytoD did not inhibit filament severing in high concentrations of cofilin alone (Fig. 5 F), which is consistent with others’ results (Ono et al., 2004).

Barbed end-capping agents inhibit actin disassembly in cells

To determine whether physiological actin disassembly also occurs by a mechanism that is inhibited by barbed end-capping agents, we treated mammalian tissue culture cells with CytoD and measured disassembly by several different assays. As a control, we used latrunculin B (LatB), a drug that also inhibits polymerization but does so by sequestering a free monomer. We first used a simple bulk assay for total cellular F-actin. Human HeLa S3 cells grown in suspension were treated with either 10 μM CytoD or LatB, fixed at various time points after treatment, and then stained with TRITC-phalloidin to quantify bulk F-actin levels. Before drug treatment, F-actin was present throughout the entire cell and was enriched at the cell cortex (Fig. 6 A, top). Treatment of the cells with CytoD did not reduce the amount of F-actin per cell (Fig. 6 A, middle; and Fig. 6 B, blue). The TRITC-phalloidin signal remained high for >30 min after CytoD treatment. The drug was active in these cells as it induced extensive blebs after a few minutes (unpublished data). In contrast, treatment of cells with LatB led to a rapid drop in the amount of F-actin per cell (Fig. 6 A, bottom; and Fig. 6 B, red).
kabiramide C (KabC), a barbed end–capping drug structurally unrelated to CytoD (Tanaka et al., 2003). We recorded time-lapse fluorescence videos of L. monocytogenes actin comet tails inside mammalian tissue culture cells expressing GFP-actin. Before drug addition, actin comet tail assembly propelled L. monocytogenes forward in the cytoplasm. Comet tail actin disassembled within a minute after assembly (Fig. 6 C, control; and Fig. 6 D, green; Theriot et al., 1992), causing a decrease in filament density away from the bacterium. Both CytoD and KabC stopped comet tail assembly and L. monocytogenes motility within a few seconds after addition (Fig. 6 C), which is consistent with the drugs capping barbed ends to inhibit actin polymerization. Both CytoD and KabC also inhibited disassembly of comet tails.

The TRITC-phalloidin signal dropped by nearly threefold over the first 100 s, which implies a timescale of disassembly consistent with fluorescence recovery after photobleaching studies of cortical actin turnover (Murthy and Wadsworth, 2005) and our data with the purified protein system. The differential responses of cellular F-actin levels to CytoD and LatB treatment provides evidence that CytoD also actively inhibits actin filament disassembly in vivo, as it also does in our purified protein system in vitro.

To probe disassembly on a well-characterized actin array in vivo, we imaged actin comet tails formed by the intracellular pathogen L. monocytogenes (Sanger et al., 1992; Theriot et al., 1992) while perturbing cells with CytoD or LatB. To control for secondary effects of CytoD, we also used in this assay a barbed end–capping drug structurally unrelated to CytoD (Tanaka et al., 2003). We recorded time-lapse fluorescence videos of L. monocytogenes actin comet tails inside mammalian tissue culture cells expressing GFP-actin. Before drug addition, actin comet tail assembly propelled L. monocytogenes forward in the cytoplasm. Comet tail actin disassembled within a minute after assembly (Fig. 6 C, control; and Fig. 6 D, green; Theriot et al., 1992), causing a decrease in filament density away from the bacterium. Both CytoD and KabC stopped comet tail assembly and L. monocytogenes motility within a few seconds after addition (Fig. 6 C), which is consistent with the drugs capping barbed ends to inhibit actin polymerization. Both CytoD and KabC also inhibited disassembly of comet tails.
Lamellipodia and other dynamic cellular actin assemblies contain dense dendritic arrays resembling those seen in L. monocytogenes comet tails (Sechi et al., 1997; Cameron et al., 2001). To test if these arrays show similar sensitivities to CytoD versus LatB with respect to disassembly, we imaged the ruffling edge of BSC-1 cells stably expressing a fusion of actin to monomeric RFP and photoactivatable GFP (mRFP-PAGFP-actin; Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200801027/DC1). PAGFP allows localized illumination of specific actin subpopulations, and the in-tandem mRFP permits simultaneous viewing of the bulk actin population. Unlike GFP-actin, this probe localized to actin-rich cellular structures like the ruffling cell edge and stress fibers (Fig. 7A). During the experiments, we imaged both red and green fluorescence and tails, which showed no visible decrease in fluorescence for up to a minute after drug addition (Fig. 6, C and D, blue; and Videos 3 and 4, available at http://www.jcb.org/cgi/content/full/jcb.200801027/DC1). Cell rounding induced by both drugs hindered longer observation; however, tails were still visible in rounded-up cells up to 10 min after CytoD addition, which implies a reduction in the disassembly rate by at least 20-fold. Although LatB also rapidly stopped tail assembly and L. monocytogenes movement, it did not inhibit disassembly. Comet tails disappeared after drug addition at a rate similar to that in untreated cells (Fig. 6, C and D, red; and Video 5). Others have previously noted that comet tails are stable in CytoD-treated cells (Sanger et al., 1992) but did not attribute this observation to inhibition of disassembly.

Figure 6. Barbed end-capping drugs inhibit actin disassembly in mammalian tissue-culture cells. [A] Images of suspension HeLa S3 cells showing F-actin stained with TRITC-phalloidin (top) and DNA stained with Hoechst (bottom). Images show untreated cells (left), cells treated with 10 μM CytoD for 480 s (middle), and cells treated with 10 μM LatB for 480 s (right). All images used identical acquisition settings and contrast levels. Bar, 10 μm.[B] The level of F-actin per cell at different times after treatment with either CytoD (blue) or LatB (red). F-actin levels remained high after CytoD treatment. In contrast, F-actin levels fell rapidly upon LatB treatment. Each point represents the mean and SD of at least 10 fields of cells. [C] Images of actin comet tails in L. monocytogenes-infected BSC-1 cells taken at successive times after drug treatment. In the absence of any drugs (control), comet tails disassembled within 50 s. Upon LatB treatment, comet tails also disassembled within 50 s, which suggests that there is no effect of LatB on comet tail disassembly kinetics. In contrast, comet tails did not disassemble in CytoD- and KabC-treated cells for at least 50 s after drug addition, which implies that CytoD and KabC inhibited comet tail disassembly. All drugs were added at time 0. Bar, 5 μm. [D] Curves showing decay of actin polymer mass from L. monocytogenes comet tails. In the absence of drugs, actin disassembled at an initial rate of $k_{control} = (2.3 \pm 0.2) \times 10^{-2}$ s$^{-1}$. LatB did not change the disassembly rate, with $k_{LatB} = (2.6 \pm 0.2) \times 10^{-2}$. In contrast, CytoD reduced the L. monocytogenes comet tail decay rate to $k_{CytoD} = (1.9 \pm 6) \times 10^{-4}$ s$^{-1}$. Data represent the mean of polymer mass in multiple tails (control, 27 tails; CytoD treatment, 40 tails; LatB treatment, 27 tails). Disassembly rates were obtained by fitting the initial slope of the decay curve to a straight line.
This study aimed to elucidate the mechanism of rapid monomer-insensitive actin disassembly by the three-component system consisting of coflin, coronin, and Aip1. By directly imaging filament disassembly, we found that this three-component system disassembled filaments in abrupt bursts, where 100-subunit-long segments near both filament ends were lost very rapidly. Bursting disassembly generated filament barbed ends not recognized by CapZ, a protein that binds tightly to standard barbed ends. When added to the three-component system, CapZ and CytoD inhibited bursting disassembly, and did so from both ends with equal efficacy. CytoD and KabC, a structurally unrelated barbed-end capper, also inhibited actin disassembly in mammalian cells, which suggests that the three-component system reconstitutes a prevalent physiological disassembly pathway.

Mechanism of actin filament bursting disassembly
What is the mechanism underlying bursting filament disassembly? We propose the following model (Fig. 8 A): during a burst of disassembly, cooperative weakening of lateral interactions between the two strands of the actin filament causes the strands to transiently separate near an end (Fig. 8 A, left). Coflin has been shown to disrupt lateral contacts between subunits in adjacent strands (Bobkov et al., 2004) as well as induce splaying or unwinding of strands observed by electron microscopy (Bremer et al., 1991; McGough et al., 1997). Aip1, which is
believed to bind filaments at barbed ends and along their lengths (Okada et al., 2002), may also catalyze coflin-induced strand separation. Consistent with this idea, Aip1 interacts with the F-actin subunit near surfaces that form lateral interactions with adjacent filament strands in the Holmes model (Holmes et al., 1990; Amberg et al., 1995; Rodal et al., 1999; Clark and Amberg, 2007). As separated strands lack stabilizing lateral interactions, they are highly unstable and rapidly disassemble into constituent subunits. Rapid disassembly of separated filament strands is plausible, as single-stranded polymers are not limited by dissociation of individual subunits from ends (Oosawa and Asakura, 1975). This burst leaves behind a nonstandard filament end (Fig. 4), which can then initiate another burst at a significantly faster rate, giving rise to a successive series of bursts from the same filament end (Fig. 1 B, f1 and f2). Occasionally, strand separation also occurs in the middle of the filament, giving rise to internal bursts of disassembly scored as severing events in our assay (Fig. 1 B, f3; and Fig. 8 A, right). However, most disassembly occurs as bursts from either filament end (Fig. 2 B), as strands separate more easily near ends.

An alternative interpretation of our observations is that bursting disassembly simply involves filament severing, where a spatially localized disruption of the filament causes it to break at the site of disruption (Fig. 8 B). Both severed daughter filaments are completely intact but one daughter filament immediately diffuses away and cannot be detected. However, we favor cooperative strand separation over severing for several reasons: (1) strand separation accounts for preferential bursting from an end that has already undergone a burst (Fig. 1), whereas severing does not; (2) strand separation accounts for preferential initiation of disassembly near filament ends (Fig. 1), whereas severing does not (moreover, the classical severing we observed using coflin alone was not biased toward ends [Fig. 2], which argues that nothing in our experimental setup, such as filament immobilization, is causing an artificial bias toward ends); (3) we were able to score severing by single filament imaging (Fig. 2), which argues that we would have seen severing if it contributed to bursting; (4) strand separation accounts for disassembly intermediates with lowered affinity for CapZ (Fig. 4); it is also possible that barbed ends are structurally intact but capped by Aip1 after a burst and thus unable to bind CapZ; however, this putative Aip1–barbed end interaction would be quite unstable ($t < 10$ s; Fig. 4 D); and (5) strand separation plausibly accounts for inhibition of disassembly by capping factors (Figs. 5–7), whereas severing does not (Fig. 5 F). In our view, severing better describes the behavior of filaments treated with high concentrations of coflin alone (Fig. 2). Future efforts to distinguish between these alternative models will require electron microscopy of intermediates or development of spectroscopic methods that report on filament internal structure.

The mechanism of inhibition of disassembly by barbed end–capping agents presents a paradox we have yet to fully resolve. Mutual antagonism between barbed end capping and bursting disassembly suggests a possible means of inhibition. Bursting disassembly may inhibit binding of CytoD/CapZ by separating filament strands; conversely, CytoD/CapZ may inhibit bursting disassembly by keeping strands together near barbed ends. However, filaments do not disassemble preferentially from barbed ends but instead disassemble near both ends with similar kinetics by a similar capping-sensitive mechanism. We considered several possible explanations. One possibility is that barbed end factors control the stability of the whole filament by binding only to the free barbed end. However, the effect of barbed-end binding would have to propagate through the L. polyphemus acrosomal bundle, which itself is highly stabilized. Alternatively, barbed end factors may act in solution as opposed to on the polymer to inhibit disassembly. For instance, CytoD may interact with an actin monomer to perturb the activity of the purified system (Goddette and Frieden, 1986). However, we failed to detect any possible indirect effects of CytoD in solution (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200801027/DC1). A third possibility is that barbed end factors may bind along the filament length to inhibit strand separation. Consistent with this idea, in vitro studies point to low-affinity cytochalasin B binding sites along the filament length (Hartwig and Stossel, 1979).

Implications for actin dynamics in cells

What are the implications of this new disassembly reaction for dynamic organization and morphogenesis of actin assemblies in cells, assuming that it is physiologically relevant? Actin cytoskeletal networks in cells must disassemble through a mechanism that is both fast and insensitive to monomers. Monomer insensitivity is a basic thermodynamic requirement for non-equilibrium polymer turnover in the cell, and speed is important to allow fast remodeling of the actin cytoskeleton. The three-component reaction may achieve speed by disassembling entire filaments in cells in a single burst, as many actin filaments do not exceed hundreds of subunits in length in cells (Sechi et al., 1997; Cameron et al., 2001). Moreover, the three-component reaction may achieve monomer insensitivity by generating non-standard filament ends (Fig. 4 C) that cannot readily add subunits, which is akin to the low affinity of shrinking microtubule (+) ends for tubulin. We have not visualized these nonstandard ends and speculate that the two filament strands may be unequal in length or partially separated (Fig. 8 A).

In contrast to bursting disassembly by the three-component system, pure severing by coflin alone nucleates assembly by generating filament ends that can grow (Ichetovkin et al., 2002; Andrianantoandro and Pollard, 2006). The opposite effects of these two reactions may help resolve the paradoxical role of coflin in cells, where it promotes disassembly in some cases.
(Kiuchi et al., 2007; Okreglak and Drubin, 2007) and assembly in others (Ichetovkin et al., 2002; Ghosh et al., 2004). Local activity of Aip1 and/or coronin may be important in determining whether coflin catalyzes assembly or disassembly in cells.

The inhibitory effects of barbed-end cappers in our experiments suggest a role for barbed-end-associated factors such as CapZ, formin, or gelsolin in modulating not only filament assembly but also disassembly. Conversely, bursting disassembly may antagonize barbed-end-capping agents in cells, possibly accounting for the coflin-dependent fast dissociation of CapZ from dynamic actin assemblies in cells (Miyoshi et al., 2006). Similarly, the side-binding factors (Siripala and Welch, 2007) may also modulate filament stability in cells. We note that the rate of bursting disassembly in our in vitro assays decreased when filaments were immobilized with N-ethylmaleimide–inactivated myosin instead of filamin (unpublished data). It will be a challenge to examine how filaments disassemble in the full repertoire of factors that interact with actin filaments in cells.

Differential drug effects
We finally note that Cytod and LatB are widely used research tools and are often used interchangeably. Previous studies demonstrated, but did not explain, differences between these drugs with respect to actin polymerization (Higashida et al., 2004), gross morphology (Spector et al., 1989), cell mechanical properties (Wakatsuki et al., 2001), and other cellular processes (Forer and Pickett-Heaps, 1998; Morales et al., 2000; Omata et al., 2000). The dramatic difference in their effects on disassembly (Figs. 6 and 7) has not been recognized before and may explain these different effects on cell physiology. In the future, differential effects of these two drugs can be used to test the importance of disassembly in different actin-dependent cellular processes, a question that has largely been overlooked.

Materials and methods

Protein purification
Actin was prepared from rabbit skeletal muscle according to the method of Pardee and Spudich (1982). The F-actin was then depolymerized by dialysis into G buffer (2 mM Tris-Cl, 0.2 mM CaCl₂, 0.2 mM ATP, 0.1% β-mercaptoethanol, and 0.05% NaN₃, pH 8.0) and further purified by an additional cycle of polymerization/dem polymerization as described by Pardee and Spudich (1982). Actin was then repolymerized and labeled on lysines using approximately stoichiometric amounts of Alexa 488 and Alexa 647 N-hydroxysuccinimide–ester (Invitrogen) according to the procedure of Kellogg et al. (1998). The labeled actin was subject to two additional cycles of polymerization and depolymerization and then frozen in aliquots at −80°C. Cofflin, coronin, and Aip1 were purified as described by Brieher et al. (2006).

Construction of perfusion chambers
Perfusion chambers were assembled using glass coverslips (48366–227; VWR International), glass slides (3050; Gold Seal), and thin paraffilm strips. Glass coverslips were cleaned for imaging by sonication for 30 min in 1% Triton X-100, rinsed extensively using 10 or more changes of distilled water, and 30 min in ethanol. During each solution change, coverslips were rinsed extensively using 10 or more changes of distilled water. Cleaned coverslips were then stored in ethanol until the day of the experiment.

To assemble the perfusion chamber, two paraffilm strips were placed on surface of the glass slide. The coverslips were then removed from ethanol, dried using filtered air, and pressed onto the paraffilm strips to create a fluid channel. The chamber was then baked at 85°C for 20 s to create a seal between the paraffilm and the glass. Assembled chambers were stored in a box and used within 2 d.

Imaging of single actin filaments
Alexa 488– or Alexa 647–labeled actin was treated with 1 mM EGA and 0.2 mM MgCl₂ on ice for 5 min to convert calcium-ATP-actin to magnesium-ATP-actin. To remove residual filaments, the actin was then diluted in G buffer (0.2 mM CaCl₂, 0.2 mM ATP, and 2 mM Tris-Cl, pH 8), left on ice for 20 min, and centrifuged at 436,000 g with a k-factor of 7 for 20 min. The supernatant, which contained actin monomer, was retained and kept on ice for future experiments. F-actin, an actin cross-linking protein, was diluted into assay buffer (50 mM KCl, 2 mM MgCl₂, 2 mM ATP, and 100 mM K-Hepes, pH 7.8) to a final concentration of 10–50 μg/ml and adsorbed onto coverslips of perfusion chambers using a 10-min incubation. Chambers were then washed with blocking solution (5 mg/ml casein, 0.2% Tween 20, and 0.1% Pluronic F-127 in assay buffer) for 5 min. Immediately before polymerization, Alexa 488 and Alexa 647 actin monomer were mixed with a 1:1 ratio and diluted into assay buffer (500 mM KCl, 20 mM MgCl₂, 20 mM ATP, and 1 M K-Hepes, pH 7.8) and then added, and the mixture was allowed to polymerize in the perfusion chamber for 30–60 s. The chamber was then perfused with 6–7 chamber volumes of assay buffer supplemented with an oxygen scavenging system (4.5 mg/ml glucose, 0.2 mg/ml glucose oxidase, and 35 μg/ml catalase) and 10% blocking solution for imaging. Actin filaments in the perfusion chamber were imaged immediately at room temperature using an upright wide-field epifluorescence microscope (E90i) with a 60× 1.4 NA objective (both from Nikon). To distinguish newly polymerized filaments from filaments already present before salt addition, an image of filaments in the field of view was first taken in both the far-red channel (Alexa 647) and the green channel (Alexa 488). The disassembly buffers were diluted into imaging buffer and then perfused into the chamber, and a streaming video of filament disassembly was taken in the far-red channel using either an intensifying charge-coupled device (CCD) camera (ORCA; Hamamatsu) or an electron microscopy CCD camera (iXon; Andor Technology PLC) with image acquisition software (Metamorph 6.0; Invitrogen).

Segmentation of single filaments by automated image analysis
Single actin filaments in the time-lapse images were segmented using a custom image analysis algorithm written in MATLAB (Mathworks). For each image in a time-lapse sequence, background correction was performed by subtracting a grayscale morphological opening of the image from its parent. The contrast of the image was then adjusted. The low contrast value was set to: $C_{\text{low}} = I_{\text{mode}} - n \times I_{\text{max}}$, where $I_{\text{mode}}$ is the grayscale value at the peak of the image intensity histogram, $I_{\text{max}}$ is the width of the peak of the intensity histogram, and $n$ is an adjustable parameter. The high contrast value was set to: $C_{\text{high}} = I_{\text{mode}} + n \times I_{\text{max}}$, where $I_{\text{max}}$ is the highest grayscale value in the image and $0 < n < 1$ is the degree of saturation. The image was then segmented and the segmentation was iteratively refined using the following procedure: the contrast-adjusted image was passed through a Canny edge detector, which generated a binary image with closed contours around the actin filaments. Single-pixel gaps in the contours in the binary images were bridged, and the regions enclosed in the contours were filled. Unfilled open contours were removed by morphological opening, and the filled objects were thinned to lines. The shape of each object was automatically inspected, and only objects without branch points (corresponding to linear segments) were retained. The grayscale values from pixels corresponding to objects in the segmentation were then averaged to obtain mean filament intensity $I_{\text{f}}$ from this estimate, and the new high contrast value of the image was then set to: $C_{\text{high}} = I_{\text{f}} + n \times I_{\text{max}}$. The procedure was then repeated until $I_{\text{f}}$ converged to a constant value. A typical image would require approximately three or four iterations before convergence. This iterative procedure achieves automated segmentation robust to fluctuations in fluorescence intensity, variations in filament density, and the presence of bright outliers. It also does not require input parameters that explicitly depend on microscope acquisition settings.

Once segmentation was achieved, lengths of individual linear contours were estimated by the number of pixels occupied by the contours in the binary image. The lengths from all linear objects were then averaged to give the mean filament length.

Imaging of actin filament bundles grown off L. polyphemus acrosomal processes
L. polyphemus acrosomal actin bundles (a gift from G. Waller and P. Matsudaira, Massachusetts Institute of Technology, Cambridge, MA) were absorbed to coverslips of perfusion chambers for 5 min. Perfusion chambers were then incubated with blocking solution (5 mg/ml casein, 0.2% Tween 20,
and 0.1% Pluronic F-127 in assay buffer) for 5 min. Filaments were elongated off acrosomal bundles by incubation with 3 μM Alexa 647-labeled actin monomer in assay buffer for 2 min and then washed. For time-lapse imaging experiments, filament bundles were perfused with disassembly factors and 100 μg/ml filamin, which held bundles together for imaging. For experiments with Alexa 488 CapZ, filament bundles were treated under different depolymerizing conditions, incubated with 3 μM Alexa 488 CapZ for 10 s, washed again, bundled with 200 μg/ml filamin, and then imaged. Unless otherwise stated, all perfusion steps were performed using assay buffer supplemented with oxygen scavenging system and 10% blocking solution. Imaging was performed using the fluorescence microscope described for single filaments.

Measurement of polymer mass decay in acrosome filament bundles

To quantify polymer mass in fluorescent filament bundles, we wrote an automated image analysis routine in MATLAB (Mathworks). The routine measures integrated intensity of a bright object over a dark background. The grayscale value for the background in each image was estimated by finding the mode of the image histogram, assuming that the objects of interest were small in the field of view. The pixel intensities were background-subtracted using the obtained value and then summed to obtain the integrated fluorescence intensity. This procedure was repeated for all images in the time-lapse sequence. The polymer mass decay curves of all filament bundles were obtained by running this routine for the entire field of view (Fig. S1 A, left). Polymer mass decays for long bundles and short bundles were obtained by running the routine on the appropriate regions of interest (Fig. S1 A, middle and right). The polymer mass exhibited a rapidly decaying component followed by a decay with significantly slower kinetics. We then subtracted from the polymer mass decay curve the slow-varying component, which was obtained by fitting the last segment of the curve to a straight line and extrapolating to time 0 (Fig. S1 B, red and orange lines). The resultant decay curve was then normalized to unity at time 0, and the characteristic rate of polymer mass decay was then obtained by fitting the first few points of the normalized decay curve to a straight line (Fig. S1 C).

For experiments involving Alexa 488 CapZ (capping protein was a generous gift of A. Lebensohn, Harvard Medical School, Boston, MA), the integrated CapZ fluorescence in a single filament bundle was measured in a region of interest surrounding the filament bundle. Background was measured in a neighboring region of interest where no filament bundles were present. The number of actin filaments in the bundle was estimated by taking the maximal fluorescence intensity along the filament bundle length. The integrated CapZ fluorescence was divided by estimated actin filament number to obtain the CapZ/actin filament ratio (Fig. 3 B), which measures the proportion of CapZ-bound filaments in the filament bundle.

Drug treatment of suspension cells

HeLa S3 cells were cultured in suspension in shaking Fernbach flasks in CO₂ independent medium (Invitrogen) plus 10% fetal calf serum. 10 ml of log phase culture (~10⁵ cells/ml) were removed, disaggregated by passing twice through a 27-gauge needle, and kept in a water bath at 37° C with occasional agitation. At time 0, CytoD or LatB was added from a 2,000x stock in DMSO to a final concentration of 10 μM. 0.5 ml aliquots of cells were removed at each time point and fixed by dilution into 1 ml of PBS (155 mM NaCl, 1 mM potassium phosphate, 3 mM sodium phosphate, 1 mM MgCl₂, and 1 mM CaCl₂, pH 7.4) + 2% formaldehyde. After 10–20 min at 25° C, the fixed cells were collected by centrifugation (1,000 g for 5 min) and resuspended in 100 μl of PBS + 0.1% Triton X-100 + 0.5 μM TRITC-phalloidin + 1 μM Hoechst dye. This was a saturating amount of TRITC-phalloidin, as judged by the presence of excess unbound reagent in solution after the incubation. After 30 min at 25° C with occasional agitation, the cells were diluted with 1 ml of PBS + 0.1% Triton X-100, collected by centrifugation, resuspended in 50 μl of PBS + 60% glycerol, and mounted between a slide and coverslip. Random fields were imaged with a 10x objective. Integrated intensity of the TRITC-phalloidin signal in each field of view was measured and used in the same image processing routine described for acrosome filament bundles. The total intensity in the field of view was then divided by the number of cells present to obtain the integrated intensity per cell.

Infection of tissue culture cells with L. monocytogenes

Before infection, BSC-1 cells were cultured in DME media on polylysine-coated glass coverslips. Cells were then infected with an adenovirus expressing GFP-actin and incubated overnight. These GFP-actin-expressing cells were then infected with L. monocytogenes for 3 h, washed with DME containing 50 μg/ml gentamicin, and incubated at 37°C for up to 24 h before live imaging.

Live imaging of L. monocytogenes comet tails in tissue culture cells

Imaging was performed using a 100x 1.4 NA oil objective on an epi-fluorescence microscope (TE300, Nikon). BSC-1 cells expressing GFP-actin were imaged in DME containing 50 μg/ml gentamicin and 20 mM Hepes at pH 7.4 and were maintained at 37°C by a heated metal stage. Infected cells containing visible GFP-actin comet tails were located for time-lapse image acquisition. Time-lapse movies of GFP-actin fluorescence in comet tails were then acquired with a cooled-CCD camera (ORCA-ER; Hamamatsu) using image acquisition software (MetaMorph; MDS Analytical Technologies). For experiments involving drug treatment, the imaging media was exchanged for media containing the drug of interest during the course of image acquisition. Drug concentrations used: 6 μM LatB, 3 μM CytoD, and 0.5 μM KCl (provided by G. Marriot, University of Wisconsin-Madison, WI). In all cases, the concentration of DMSO was <0.5%.

Measurement of polymer mass decay in L. monocytogenes comet tails in cells

Polymer mass in L. monocytogenes actin comet tails was obtained using the same image processing route detailed for analysis of L. polyphemus acrosomal filament bundles. Polymer mass decay curves were obtained in regions of interest around the part of the comet tail that had polymerized immediately before drug addition. Decay curves from multiple cells were then normalized and averaged together.

Preparation of mRFP-PAGFP-actin in a retroviral vector

The mRFP-PAGFP-actin plasmid was based on an EGFP-tagged human β-actin from Clontech Laboratories, Inc. First, EGFP was excised using an NheI–BglII digest and replaced by mRFP (a gift of R. Tsien, University of California, San Diego, La Jolla, CA). Second, PAGFP (a gift from R. Wedlich-Soldner, Max Planck Institute, Martinsried, Germany) was PCR amplified and ligated in the mRFP-actin between XhoI and BglII. In addition, to enhance flexibility of the linking regions, glycine residues were inserted between mRFP and PAGFP (one added) and between PAGFP and actin (three added; Fig. S4). The plasmid sequence was verified by sequencing. For experiments involving Alexa 488 CapZ (capping protein was a generous gift of A. Lebensohn, Harvard Medical School, Boston, MA), the integrated CapZ fluorescence in a single filament bundle was measured in a region of interest surrounding the filament bundle. Background was measured in a neighboring region of interest where no filament bundles were present. The number of actin filaments in the bundle was estimated by taking the maximal fluorescence intensity along the filament bundle length. The integrated CapZ fluorescence was divided by estimated actin filament number to obtain the CapZ/actin filament ratio (Fig. 3 B), which measures the proportion of CapZ-bound filaments in the filament bundle.

Imaging of photoactivated fluorescent actin in tissue culture cells

Imaging was performed using a 100x 1.4 NA oil objective on an inverted microscope (TE2000U, Nikon) with an Ultraview spinning disc confocal unit (PerkinElmer). BSC-1 cells expressing PAGFP-mRFP-actin were imaged in CO₂-independent medium (18045–088; Invitrogen) supplemented with 1-glutamine and were maintained at 37°C by a heated metal stage. Cells were excited with 488- or 568-nm light from an argon-krypton laser (Melles Griot), and images were taken with a cooled-CCD camera (ORCA-ER) using image acquisition software (MetaMorph). During the time lapse, media containing the drug was applied to the cells, and the PAGFP-mRFP-actin was photactivated using a nitrogen pulse laser tuned to 405 nm (Photonic Instruments, Inc.). Both LatB and CytoD were diluted in CO₂-independent media to a final concentration of 5 μM in 0.5% DMSO.

Online supplemental materials

Fig. S1 shows decay rates of L. polyphemus actin bundles with exposed barbed versus pointed ends. Fig. S2 shows that monomer-insensitive disassembly of single filaments requires cofilin, coronin, and Aip1. Fig. S3 tests the effects of CytoD acting in solution on actin disassembly. Fig. S4 is a diagram of the mRFP-PAGFP-actin construct used for the photoactivation experiments in cells. Video 1 shows disassembly of single actin filaments in the presence of cofilin, coronin, Aip1, and G-actin. Video 2
shows disassembly of actin grown off L. polyphemus bundles under the same conditions. Videos 3, 4, and 5 show decay of L. monocytogenes actin comets in the presence of CytoD, KabC, or LatB, respectively. Video 6 shows actin turnover in ruffling BSC-1 cells as a function of CytoD. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801027.DC1

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