Microscopic Evidence That Actin-interacting Protein 1 Actively Disassembles Actin-depolymerizing Factor/Cofilin-bound Actin Filaments*

The rearrangement of the actin cytoskeleton is controlled by dynamic assembly and disassembly of actin filaments. Regulated disassembly as well as assembly is important for maintaining constant turnover of actin filaments. Actin-depolymerizing factor (ADF)/cofilin and gelsolin are the two major classes of actin-regulatory proteins that accelerate filament disassembly (1). ADF/cofilin weakly severs filaments without capping ends and also enhances monomer dissociation from the pointed ends (reviewed in Refs. 2–5). In contrast, gelsolin strongly severs filaments and caps the barbed ends in a calcium-dependent manner (reviewed in Refs. 6 and 7). Recently, actin-interacting protein 1 (AIP1) has been shown to selectively fragment ADF/cofilin-bound filaments (reviewed in Ref. 8). Xenopus AIP1 caps the barbed ends of ADF/cofilin-bound filaments (9). Although this capping activity is proposed to contribute to enhanced fragmentation by preventing re-annealing of ADF/cofilin-severed filaments (9, 10), a direct link between capping and fragmentation has not been demonstrated. In addition, the combination of ADF/cofilin and AIP1 induces strong severing and capping of filaments, which are very similar to the effects of gelsolin. However, direct comparison of their effects has not been reported.

The functions of actin-regulatory proteins have been studied extensively by a variety of spectroscopic methods. However, the results of these experiments are sometimes difficult to interpret or misleading. ADF/cofilin is especially problematic for several spectroscopic assays. Upon binding of ADF/cofilin to F-actin, it increases light scattering of the filaments (11) and quenches the fluorescence of pyrene that is attached to Cys-374 of actin (12, 13), which are often used to monitor the extents of polymerization and depolymerization. To overcome this problem, direct observation of the effects of actin-binding proteins on fluorescently labeled actin filaments has been used to demonstrate filament severing by gelsolin (14) or ADF/cofilin (15–17), filament branching by Arp2/3 (18–21), and enhancement of Arp2/3-mediated filament branching by cofolin (22). Thus, direct observation has been a powerful method to understand the functions of actin-binding proteins. Here, we used the direct observation assay to investigate the mechanism of filament disassembly by ADF/cofilin and AIP1 in comparison to the functions of gelsolin. Our results suggest that AIP1 has an active role in filament disassembly and that ADF/cofilin and AIP1 have distinct roles from gelsolin.

**Experimental Procedures**

Proteins and Reagents—Rabbit muscle actin was purified as described previously (23). Bacterially expressed recombinant UNC-60B (24) and UNC-78 (25) were purified as described previously. Gelsolin was purified from bovine newborn calf serum (Invitrogen) as described (26). Gelsolin-actin complex was prepared by incubating 6 μM F-actin and 2 μM gelsolin in F-buffer (0.1 M KCl, 2 mM MgCl2, 20 mM HEPES-KOH, pH 7.5) plus 0.1 mM CaCl2 for 2 h at room temperature and diluted to 0.1 μM in an assay buffer that contains EGTA (see below) immediately before use. Cytochalasin D was purchased from Sigma.

Direct Observation of Actin Filament Severing and Capping—Observation of actin filament severing by fluorescence microscopy was performed as described previously (17) with slight modifications. In all of these experiments, we did not use phalloidin because it inhibits the binding of ADF/cofilin to actin (27). Unlabeled actin (1.4 μM), Alexa488-labeled actin (Molecular Probes) (0.4 μM), and biotin-labeled actin (Cytokeleton Inc.) (0.2 μM) were co-polymerized at room temperature for 2 h in ISAP buffer (50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1 mM ATP, 1 mM dithiothreitol, and 20 mM HEPES-KOH, pH 7.2). Coverslips (35 mm, number 1, Fisher Scientific) were coated with 0.1% nitrocellulose in methanol and allowed to air-dry. A small coverslip (18 mm square, number 1, Corning) was mounted on the coated coverslip with two pieces of Scotch double-coated tape to make a perfusion chamber. Anti-biotin monoclonal antibody (Molecular Probes) was diluted to 30 μg/ml with ISAP buffer containing 0.5 mg/ml bovine serum albumin and perfused into the chamber to coat the surface. After washing twice with ISAP plus bovine serum albumin, Alexa488/biotin-labeled actin was diluted 30-fold with anti-bleaching buffer (ISAP plus 5 mg/ml...
bovine serum albumin, 0.036 mg/ml catalase, 0.2 mg/ml glucose oxidase, 6 mg/ml glucose, and 100 mM dithiothreitol), perfused into the chamber, and incubated for 5 min. The chamber was washed twice with anti-bleaching buffer and mounted onto the stage of a Nikon Eclipse TE2000 inverted microscope. The proteins were diluted in anti-bleaching buffer and perfused into the chamber. For assays with gelsolin, anti-bleaching buffer containing CaCl₂ instead of EGTA was used to dilute the proteins. Filaments were observed by epifluorescence with a ×60 Plan Apo objective (oil, numerical aperture of 1.4), and the images were captured by a SPOT RT Monochrome CCD camera (Diagnostic Instruments) and processed by IPLab (Scanalytics Inc.) and Adobe Photoshop 6.0 or 7.0. Quantitative analysis of the images was performed using IPLab.

Barbed end capping was examined by the ability of the Alexa488-labeled actin filaments to incorporate rhodamine-actin. Alexa488/biotin-labeled actin filaments were treated with UNC-60B, UNC-78, gelsolin, cytochalasin D, or gelsolin-actin complex in a perfusion chamber as described above for 30 s. The chamber was washed once with anti-bleaching buffer, perfused with 0.4 mM rhodamine-labeled G-actin (Cytoskeleton Inc.) in anti-bleaching buffer, and incubated for 3 min. Unincorporated actin was washed with anti-bleaching buffer containing 0.2 mM cytochalasin D. Inclusion of cytochalasin D prevented loss of rhodamine-actin from the barbed ends and stabilized the filaments during observation.

**Assay for Barbed End Elongation**—A spectroscopic assay to examine actin elongation from filament ends was performed essentially as described previously (28) with the exception that rabbit muscle actin was used as seeds in this study.

**RESULTS**

**Direct Observation of Actin Filament Disassembly and Severing**—To visualize actin filament disassembly and severing by ADF/cofilin and AIP1, we used the perfusion assay developed by Ichetovkin et al. (17). Actin filaments were partially labeled with Alexa488 and biotin and attached to a coverslip that had been coated by anti-biotin antibody. The morphology of the same filaments then was observed by fluorescence microscopy before and after perfusion with various actin-severing or depolymerizing proteins. All of the experiments were performed without phalloidin. In the control experiment, actin filaments were stable for 3 min with only minor morphological changes after perfusion with buffer alone (compare Fig. 1, A and B). The filament morphology changed dramatically after the incubation with either UNC-60B (C, D, K, and L), UNC-78 (E, F, M, and N), or a mixture of both (O and P).

**Fig. 1. Direct observation of actin filament severing and disassembly.** Alexa488/biotin-labeled actin filaments were tethered to a coverslip and treated with buffer alone (A, B, I, and J), 2 μM UNC-60B (C, D, K, and L), 2 μM UNC-78 (E, F, M, and N), 2 nM UNC-78 (G, H, O, and P) in the absence (A–H) or presence of 5 nM gelsolin (I–P). The filaments were observed before (A, C, E, G, I, K, M, and O) and 3 min after the incubation (B, D, F, H, J, L, N, and P) by fluorescence microscopy, and the same fields are shown for each treatment. Bar, 20 μm.

**Fig. 2. Concentration-dependent effects of UNC-60B and UNC-78 on actin filament severing and disassembly.** Alexa488/biotin-labeled actin filaments were treated with buffer alone (A and B) or various concentrations of UNC-60B (U60B) (C–H) or UNC-60B and UNC-78 (U78) (I–P) as indicated in the figure. The filaments were observed before (A, C, E, G, I, K, M, and O) and 3 min after the incubation (B, D, F, H, J, L, N, and P) by fluorescence microscopy, and the same fields are shown for each treatment. Bar, 20 μm.
length of the filaments before perfusion (4.2 ± 2.6 μm, n = 180) was not significantly different from that after perfusion with buffer alone (4.1 ± 2.6 μm, n = 166). UNC-60B, a muscle-specific Caenorhabditis elegans ADF/cofilin isoform (28), caused filament severing (compare Fig. 1, C and D) and decreased the length to 0.79 ± 0.57 μm (n = 245). There was only a 7–20% increase in the number of filaments after perfusion with UNC-60B (three separate experiments), suggesting that many filaments became too short to visualize or were disassembled. Perfusion with both UNC-60B and UNC-78, a C. elegans AIP1 protein (29), drastically reduced the number of visible filaments (compare Fig. 1, E and F), and only dot-like residual filaments remained, suggesting that filaments were disassembled or became shorter than the optical resolution (~200 nm). The length of the residual filaments was 0.49 ± 0.27 μm (n = 11). However, perfusion with UNC-78 alone did not alter stability of the filaments (compare Fig. 1, G and H) or the length of the filaments (4.4 ± 2.4 μm, n = 134).

UNC-60B caused significant severing at >0.5 μM (Fig. 2, C–F), but at 0.1 μM, it caused only minimal severing (Fig. 2, G and H). In contrast, UNC-78 induced extensive disassembly at >2 nM when UNC-60B was present at 2 μM (Fig. 2, I and J) but at 0.2 nM it did not enhance disassembly (compare Fig. 2, D and L). In addition, in the presence of 0.5 μM UNC-60B, enhanced disassembly by UNC-78 was not very extensive (compare Fig. 2, F and N). At 0.1 μM UNC-60B, 2 nM UNC-78 did not enhance disassembly (Fig. 2, O and P). These results suggest that UNC-78 is a very strong disassembly factor and functions in a non-stoichiometric manner to UNC-60B.

Previously, Okada et al. (9) demonstrated that Xenopus AIP1 caps the barbed ends of ADF/cofilin-bound filaments and proposed that this capping activity of AIP1 enhances fragmentation by preventing re-annealing of ADF/cofilin-severed filaments. However, our results in Fig. 1, A–H, indicated that UNC-78/AIP1 was able to enhance disassembly of physically tethered filaments where re-annealing of filaments was not likely to occur. If barbed end capping is sufficient for enhancing disassembly of ADF/cofilin-severed filaments, other barbed end-capping reagents could enhance disassembly in the presence of ADF/cofilin and mimic the action of AIP1 in the perfusion assay. To test this hypothesis, we examined the effects of gelsolin, a capping/severing protein, and cytochalasin D, a barbed end-capping agent, on severing by UNC-60B (Figs. 1, I–P, and 3). Gelsolin alone severed filaments and shortened the length to 1.7 ± 1.1 μm (n = 402) (compare Fig. 1, I and J). In the presence of UNC-60B, however, gelsolin did not enhance disassembly (compare Fig. 1, K and L) and the length of the filaments (0.80 ± 0.48 μm, n = 718) was similar to that after the treatment with UNC-60B alone (Fig. 1, C and D). Interestingly, there was a 60–84% increase in the number of filaments...
after the treatment with gelsolin and UNC-60B (three separate experiments) as compared with 7–20% with UNC-60B alone. This could be explained that barbed end capping by gelsolin prevented subunit loss from the barbed ends where depolymerization is faster, thereby slowing filament shortening and disassembly. Gelsolin did not prevent extensive disassembly by UNC-60B and UNC-78 (compare Fig. 1, M and N), and the length of the residual filaments were 0.51 ± 0.23 μm (n = 53), suggesting that the extensive disassembly was not the result of depolymerization from the barbed ends. Gelsolin-severed filaments were not disassembled by UNC-78 (compare Fig. 1, O and P), and the length of the filaments (2.2 ± 1.6 μm, n = 566) was similar to that by the treatment with gelsolin alone (Fig. 1, I and J).

Gelsolin itself has strong filament severing activity, which makes it difficult to conclude that gelsolin simply functioned as a barbed end-capping reagent as shown in Fig. 1. Therefore, we tested the effects of short gelsolin-actin seeds on actin filament severing and disassembly by UNC-60B and UNC-78 (Fig. 3, A–J). The gelsolin-actin (GA) complex anneals with uncapped barbed ends and caps the barbed ends without severing the filaments. GA alone did not affect the stability of the filaments (Fig. 3, C and D). Still, GA did not enhance disassembly in the presence of UNC-60B (compare Fig. 3, E and F with K and L), suggesting that barbed end capping does not enhance fragmentation by UNC-60B. Also, similarly to gelsolin, GA did not prevent extensive disassembly by UNC-60B and UNC-78 (Fig. 3, G and H) and UNC-78 did not disassemble filaments in the presence of GA (Fig. 3, I and J).

Similar results were obtained by other barbed end-capping agent, cytochalasin D. Cytochalasin D itself did not change the stability of actin filaments (Fig. 3, M and N) and did not enhance disassembly in the presence of UNC-60B (Fig. 3, O and P). Cytochalasin D did not prevent extensive disassembly by UNC-60B and UNC-78 (Fig. 3, Q and R), and UNC-78 did not disassemble filaments in the presence of cytochalasin D (Fig. 3, S and T). These results using capping reagents strongly suggest that barbed end capping does not enhance filament disassembly and that AIP1 might have an active role in severing or depolymerization of ADF/cofilin-bound filaments.

Filament Binding by UNC-60B is Required for Disassembly by UNC-78—To further characterize the functional relationship between ADF/cofilin and AIP1, we performed sequential perfusions of each protein and examined the effects on actin filaments. When actin filaments were preincubated with UNC-60B for 2 min and washed by the buffer, subsequent perfusion with UNC-78 effectively enhanced disassembly (Fig. 4, A–C), whereas the second perfusion with buffer alone did not induce extensive disassembly (Fig. 4, D–F). UNC-78 did not disassemble filaments that had been preincubated with the buffer alone (Fig. 4, G–I). These results suggest that UNC-60B is still bound to the filaments after washing and mediated disassembly by UNC-78. In contrast, when actin filaments were preincubated with UNC-78, subsequent perfusion with UNC-60B severed the filaments (Fig. 4, J–L), but the effect was indistinguishable...
from the filaments that had been preincubated with buffer alone (compare Fig. 4, F and L). Thus, these results suggest that the binding of UNC-60B (ADF/cofilin) to actin filaments is required for extensive disassembly by UNC-78 (AIP1).

**Direct Observation of Barbed End Capping by ADF/Cofilin, AIP1, and Gelsolin**—Gelsolin severs filaments and tightly caps the barbed ends, whereas ADF/cofilin severs filaments without capping ends. Okada et al. (9) demonstrated that Xenopus AIP1 inhibits barbed end elongation of ADF/cofilin-bound filaments, concluding that AIP1 caps the barbed ends. Thus, the combination of ADF/cofilin and AIP1 might have a gelsolin-like function. In the presence of UNC-60B, UNC-78 slows down the rate of spontaneous actin polymerization (25). In addition, using the actin elongation assay that was used to demonstrate the capping activity of Xenopus AIP1 (9), we found that UNC-78 partially inhibited actin elongation only in the presence of UNC-60B (Fig. 5). This finding suggests that UNC-78 and UNC-60B cap the barbed end in a manner similar to Xenopus AIP1 and ADF/cofilin, although complete depolymerization of a subset of the filaments might occur in the presence of AIP1 and reduce the apparent elongating activity.

To test whether capping by gelsolin is different from that by ADF/cofilin and AIP1, we directly observed the effects of these proteins on barbed end elongation using a modified perfusion assay. Alexa488-labeled actin filaments were incubated with gelsolin, UNC-60B, or UNC-78, and then rhodamine-labeled G-actin was infused at 0.4 μM, which is above the barbed end critical concentration but below the pointed end critical concentration. Therefore, rhodamine-actin is expected to polymerize only from the barbed ends. As expected, 35% of control filaments that were treated with buffer alone (Fig. 6, A and H). Many ends of long filaments were out of focus and thus increased the percentage of the filaments with no rhodamine labels (Rhod only), and rhodamine-labeled small filaments (Rhod only), and the percentages are shown. Total numbers of examined filaments are shown on the right of the graph.
Actin Filament Disassembly by ADF/Cofilin and AIP1

In this study, we demonstrate that UNC-78 (AIP1) has very strong activity to disassemble actin filaments in the presence of UNC-60B (ADF/cofilin). However, barbed end capping by gelsolin or cytochalasin D does not mimic the action of AIP1 in the presence of ADF/cofilin. This finding suggests that the previously proposed model by Okada et al. (9) that AIP1 enhances fragmentation by capping ends of ADF/cofilin-severed filaments and prevents re-annelling is not a probable explanation of the action of AIP1. Okada et al. (9) concluded that AIP1 does not enhance severing or pointed end depolymerization by ADF/cofilin by a spectroscopic assay. However, in their experiment (Fig. 4 of Ref. 9), ADF/cofilin was used at a low concentration (0.036:1 in molar ratio with actin), whereas AIP1 was most active when ADF/cofilin was present at a 1:1 molar ratio with actin (25, 30–32). Therefore, it is possible that the concentration of ADF/cofilin might have been insufficient to activate AIP1.

Rather, our results suggest that AIP1 has an active role in severing or depolymerization of ADF/cofilin-bound filaments. Studies by electron microscopy and spectroscopy show that ADF/cofilins decrease the length of actin filaments to 0.7–1 μm (33, 34). However, AIP1 further decreases the length to ~50 nm in the presence of ADF/cofilin (30) and increases unpelletable actin in a pelleting assay (25, 30–32, 35). Our results by direct observation are consistent with these reports. We also showed that a very low concentration of AIP1 is sufficient for disassembly, suggesting that AIP1 disassembles filaments in a non-stoichiometric manner to actin or ADF/cofilin. We propose that the function of AIP1 is to interact with ADF/cofilin-bound short filaments and further destabilize them into shorter filaments. Nonetheless, our experiments are not definitive to distinguish whether AIP1 disassembles filaments either by severing or depolymerization. A drawback of this assay is that the experimental conditions favor depolymerization from both ends because the G-actin concentrations in the chamber are expected to be far below the critical concentration. An attempt to slow down depolymerization from the barbed ends by adding 0.1 μM G-actin in the assays was not successful (data not shown). As a result, filament disassembly by ADF/cofilin and AIP1 was very rapid and difficult to capture the process by time-lapse microscopy. Therefore, to perform similar experiments with higher concentrations of fluorescently labeled actin at higher resolutions, total internal reflection fluorescence microscopy would be suitable (18). We also found that capping by ADF/cofilin and AIP1 is weak and allows elongation in contrast to tight capping by gelsolin. This finding suggests that ADF/cofilin and AIP1 may not function as an effective capper under physiological conditions where high concentrations of monomeric actin are present.

AIP1 proteins localize to F-actin-rich cellular structures in a variety of cells (reviewed in Ref. 8). UNC-78 is expressed in body wall muscle cells of *C. elegans* and co-localizes with actin to the myofibril (25). However, the utilization of UNC-78 is observed along the entire length of the thin filaments but not limited to the position where the barbed ends are located (25). This finding also suggests that barbed end capping is not a major function of UNC-78 in *in vivo*. Therefore, the role of UNC-78 probably enhances fragmentation of actin filaments by severing or depolymerization and the severe defects in the muscle actin organization in unc-78 mutants (29) might be attributed to lack of this activity. Taken together, we propose that AIP1 is a potent factor to disassemble ADF/cofilin-bound actin filaments.

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