Cofilin, a Protein in Porcine Brain That Binds to Actin Filaments and Inhibits Their Interactions with Myosin and Tropomyosin†

Eisuke Nishida,* Shohei Maekawa,‡ and Hikoichi Sakai

ABSTRACT: Cofilin, a 21 000 molecular weight protein of porcine brain, reacts stoichiometrically with actin in a 1:1 molar ratio. Upon binding of cofilin, the fluorescence of pyrene-labeled actin under polymerizing conditions is changed into the monomer form, irrespective of whether cofilin is added to actin before or after polymerization. Cofilin decreases the viscosity of actin filaments but increases the light-scattering intensity of the filaments. The centrifugation assay and the DNase I inhibition assay demonstrate that cofilin binds to actin filaments in a 1:1 molar ratio of cofilin to actin monomer in the filament and that cofilin increases the monomeric actin to a limited extent (up to 1.1–1.5 μM monomer) in the presence of physiological concentrations of Mg^{2+} and KCl. Cofilin is also able to bind to monomeric actin, as demonstrated by gel filtration. Electron microscopy showed that actin filaments are shortened and slightly thickened in the presence of cofilin. No bundle formation was observed in the presence of various concentrations of cofilin. The gel point assay using an actin cross-linking protein and the nucleation assay also suggested that cofilin shortens the actin filaments and hence increases the filament number. Cofilin blocks the binding of tropomyosin to actin filaments. Tropomyosin is dissociated from actin filaments by the binding of cofilin to actin filaments. Cofilin was found to inhibit the superprecipitation of actin–myosin mixtures as well as the actin-activated myosin ATPase. All these results suggest that cofilin is a new type of actin-associated protein.

Actin is a major constituent of cytoskeletons in various types of nonmuscle cells and exhibits a variety of structural forms. Since the mode of self-assembly of actin alone does not exhibit such a diversity, it seems highly probable that the state of actin in cells is regulated by many types of actin-binding proteins. Actually, many new proteins have been isolated and identified as actin-regulatory proteins. These proteins have been classified into essentially three functional groups: actin cross-linking (gelation and/or bundling) proteins, actin capping and/or severing proteins, and actin depolymerizing proteins (Weeds, 1982; Craig & Pollard, 1982).

Recently we have purified several G-actin-binding proteins from porcine brain using DNase I–agarose affinity chromatography (Maekawa et al., 1984). These include 26K, 21K, and 19K proteins and profilin. Characterization of the action of the 19K protein on actin has revealed that it has the cutting as well as depolymerizing activities and therefore depolymerizes actin filaments very quickly (Nishida et al., 1984b). On the other hand, profilin (both *Acanthamoeba* and brain profilins) has been demonstrated to induce slow depolymerization of actin filaments through binding to monomeric actin only (Tseng & Pollard, 1982; Tobacman & Korn, 1982; Nishida et al., 1984a). On the basis of these results, we proposed that depolymerizing proteins should be further classified into the following two subclasses: cutting and depolymerizing proteins such as the brain 19K protein and an echinodermatous actin depolymerizing protein (Nishida et al., 1984b; Mabuchi, 1983); monomer sequestering proteins such as profilin and vitamin D binding protein (Baelen et al., 1980).

In the present study, we characterize the interaction of brain 21K protein with actin. The protein regulates the state of actin polymerization by binding to both G- and F-actins. Moreover, the binding of the 21K protein to F-actin blocks the binding of tropomyosin to F-actin and inhibits actin–myosin interactions. We call this protein cofilin, which stands for "cofilamentous protein".

Experimental Procedures

Preparation of Proteins. Cofilin and brain actin were purified from porcine brain as previously described (Maekawa et al., 1984). Rabbit skeletal muscle actin was prepared by the method of Spudich & Watt (1971). Both brain and muscle actins were further purified by gel filtration on Sephadex G-100 equilibrated with a buffer solution containing 0.1 mM $CaCl_2$, 0.2 mM ATP, 0.05 mM dithiothreitol (DTT),[1] 0.01% NaN_3, and 2 mM HEPES, pH 7.8. Fodrin was prepared from porcine braine by the method of Glenney et al. (1982). Tropomyosin was isolated from rabbit skeletal muscle as described previously (Wakabayashi et al., 1975). Rabbit skeletal muscle myosin was prepared according to the method of Perry (1955). Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Actin concentration was determined by UV absorption measurement based on $A^{1\%}_{290nm} = 6.5$.

Assays for Actin Polymerization. Light scattering at 500 nm was measured in a temperature-controlled cuvette chamber by using a Hitachi 650-10S fluorescence spectrophotometer. (Wegner & Engel, 1975; Nishida et al., 1984b). High shear viscosity was assayed with an Ostwald-type viscometer as previously described (Nishida et al., 1984b). The absorbance change at 237 nm which represents the G–F transformation of actin (Higashi & Oosawa, 1965; Nishida & Sakai, 1983) was measured with a Gilford 260 spectrophotometer in a temperature-controlled cuvette chamber. The concentration of monomeric actin was determined by the method of the DNase I inhibition assay as described by Blikstad et al. (1978).

Fluorescence Assay. The fluorescence assay using *N*-pyrenyliodoacetamide-labeled actin was carried out by a modified

† From the Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan. *Received January 31, 1984.* This work was supported in part by Grants-in-Aid 57440004, 57380016, and 57780157 from the Ministry of Education, Science and Culture of Japan.

‡ Present address: Department of Cell Biology and Anatomy, The Medical School, Northwestern University, Chicago, IL 60611.

[1] Abbreviations: DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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method (Brenner & Korn, 1983) of Kouyama & Mihashi (1981) as described previously (Nishida et al., 1984b). The excitation and emission wavelengths were 365 and 407 nm, respectively.

Pelleting Assay. Binding of cofilin and tropomyosin to F-actin was examined by the pelleting assay. Aliquots of sample solutions were centrifuged at 100000*g* for 50 min. The supernatants were saved, and the pellets were suspended in 1% $NaDodSO_4$. Then, both series of the pellet and supernatant fractions were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970) with 12% polyacrylamide slab gels. Gels were stained with Coomassie blue, and the intensity of the stained band was determined by scanning the gels with a densitometer.

Electron Microscopy. Samples were mounted on glow-discharged Formvar-coated grids and negatively stained with 1.5% uranyl acetate. Specimens were observed with a Hitachi 11UB electron microscope at an accelerating voltage of 75 kV.

Falling Ball Assay. Low shear viscosity was assayed in a falling ball device (MacLean-Fletcher & Pollard, 1980).

Actin-Activated Myosin ATPase. The effect of cofilin on actin-activated myosin ATPase was examined as follows. Various amounts of cofilin were added to muscle F-actin solutions (final concentration 150 μg/mL) and incubated at 25 °C for about 5 min. Then, the reactions were allowed to start by the addition of myosin (final concentration 60 μg/mL) and stopped after 10 min at 25 °C with 5% trichloroacetic acid. The assay conditions were 1 mM ATP, 2.4 mM $MgCl_2$, 40 mM KCl, and 7 mM PIPES, pH 6.9. Inorganic phosphate liberated was determined by the method of Chen et al. (1956).

Superprecipitation. The superprecipitation of actin–myosin mixtures was followed by measuring the increase in the turbidity at 660 nm. The reactions were started by the addition of myosin. The assay medium contained 160 μg/mL muscle F-actin, 240 μg/mL myosin, 0.28 mM ATP, 0.55 mM $MgCl_2$, 110 mM KCl, 0.06 mM DTT, 0.02 mM $CaCl_2$, and 8 mM PIPES, pH 6.9 at 25 °C. When the effect of cofilin on the superprecipitation was examined, F-actin solutions were preincubated at 25 °C for about 5 min with various concentrations of cofilin before the addition of myosin.

Results

Interaction of Cofilin with Actin. Our previous study demonstrated that cofilin reduced the viscosity of muscle actin solutions in a concentration-dependent manner irrespective of whether cofilin was added to monomeric actin or to preformed actin filaments (Maekawa et al., 1984). To investigate the action of cofilin in detail, we used a variety of assay methods in this study.

(*1*) *Light-Scattering and Viscosity Assays.* Figure 1A shows the effect of increasing amounts of cofilin on brain actin polymerization kinetics in 2 mM $MgCl_2$–80 mM KCl, measured by the light-scattering assay. Cofilin prolonged in a concentration-dependent manner the duration of the lag phase preceding a detectable increase in the light-scattering intensity, as shown in Figure 1A. When actin was polymerized in the absence of Mg^{2+} to slow down the nucleation rate, cofilin was found to also prolong the duration of the lag phase (not shown). The steady-state light-scattering intensity, however, was not reduced by cofilin. Rather, it was increased slightly. The extent of the increase appears to be saturable (Figure 1B). The maximal level of intensity was attained when about an equimolar amount of cofilin was contained. It was 55% more

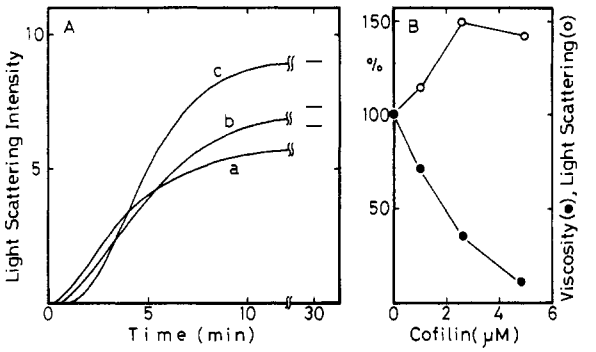

FIGURE 1: (A) Effect of cofilin on the time course of actin polymerization, measured by the light-scattering assay. Brain actin (3.93 μM) was polymerized at 30 °C in a solution of 2 mM $MgCl_2$, 80 mM KCl, 0.01 mM $CaCl_2$, 0.08 mM DTT, 0.02 mM ATP, and 8 mM PIPES, pH 7.3, in the absence (a) or presence [1.0 (b) and 4.85 μM (c)] of cofilin. The increase in the light-scattering intensity was monitored. The units for light-scattering intensity are arbitrary. (B) Steady-state light-scattering intensity (○) and the high shear viscosity (●) of actin filament solution as a function of cofilin concentration. The viscosity was measured with an Ostwald-type viscometer after the light-scattering intensity reached the steady-state level.

than the light-scattering intensity of F-actin alone (an average value from three independent experiments ranged from 40% to 70%, using 3.8–4.0 μM actin). The increase in the light-scattering intensity of actin filaments by cofilin was also observed when cofilin was mixed with preformed actin filaments (in this experiment, F-actin–cofilin mixtures were centrifuged for 3 min at 10000*g* to remove any amorphous aggregates formed before measurements were done). Figure 1B shows the steady-state light-scattering intensity and viscosity values of actin solutions as a function of cofilin concentration added, which clearly indicates that the reductions in the viscosity do not result from the net depolymerization of actin. It should be noted that essentially the same results were obtained when muscle actin was used and that whether Ca^{2+} was present or not did not affect the results.

(*2*) *Studies with Fluorescently Labeled Actin.* When *N*-(1-pyrenyl)iodoacetamide-labeled actin polymerizes into F-actin, there is a large enhancement in fluorescence intensity. Thus, the G–F transformation of actin can be followed sensitively by measuring the fluorescence intensity. Previous experiments have demonstrated that measurement of pyrene-labeled actin fluorescence is a valid and reliable assay for actin polymerization (Kouyama & Mihashi, 1981; Brenner & Korn, 1983; Harris & Weeds, 1983). We confirmed that the fluorescence assay corresponded very well with the light-scattering assay and that the presence of pyrene-labeled actin, at least up to 6% of the total actin, did not affect significantly the polymerization kinetics which were measured by the light-scattering assay (E. Muneyuki and E. Nishida, unpublished results).

Figure 2 shows the effect of cofilin on the pyrene-labeled actin fluorescence. Cofilin reduces the fluorescence intensity in a concentration-dependent manner, irrespective of whether cofilin is added to actin before polymerization (Figure 2A) or to preformed actin filaments (Figure 2B). The steady-state fluorescence intensity was inversely proportional to the concentration of cofilin added (Figure 2A, inset). It was found that 3.65 μM cofilin was required for totally inhibiting the fluorescence increase of 3.85 μM actin, suggesting a 1:1 stoichiometry between actin and cofilin. When an equimolar amount of cofilin was added to the pyrene-labeled actin under polymerizing conditions, both excitation and emission spectra became indistinguishable from those of G-actin under depo-

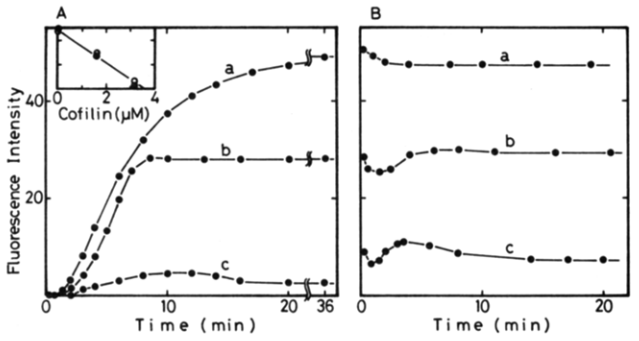

FIGURE 2: Effect of cofilin on the pyrene-labeled actin fluorescence. Cofilin was added to muscle actin (6% pyrene-labeled actin) before (A) or after (B) polymerization. The fluorescence change was monitored at 30 °C. The assay conditions were 3 mM $MgCl_2$, 80 mM KCl, 0.01 mM $CaCl_2$, 0.08 mM DTT, 0.02 mM ATP, and 8 mM PIPES, pH 7.3: (a) actin (3.85 μM) alone; (b) actin + 1.6 μM cofilin; (c) actin + 3.15 μM cofilin. The inset shows the dependence of the steady-state fluorescence intensity on cofilin concentration [(●) from the data shown in (A); (○) from the data shown in (B)].

lymerizing conditions (data not shown). In addition, the spectra of G-actin under depolymerizing conditions were not altered by the presence of cofilin (data not shown). At a glance, these results seemed to suggest that cofilin depolymerizes actin filaments stoichiometrically. However, this was not the case. The conversion of pyrene-labeled actin fluorescence from the F to the G form by cofilin mainly results from binding of cofilin to F-actin (see below).

(*3*) *Pelleting Assay.* The interaction of cofilin with actin was investigated by the pelleting assay. Figure 3A shows the results obtained with the pyrene-labeled actin samples, the fluorescence of which was measured as shown in Figure 2. It was found that cofilin binds to F-actin and reduces the sedimentable actin to some extent. It should be noted that the result does not deped on whether cofilin is added to actin before or after polymerization. Figure 3B shows the result of the pelleting assay using unmodified actin in the presence of a wide range of cofilin concentrations. The amounts of actin and cofilin in both the pellet and supernatant fractions were quantified by $NaDodSO_4$–polyacrylamide gel electrophoresis. It was shown that 1.6 μM cofilin increased the concentration of nonsedimented actin from 0.14 μM (control) to 1.0 μM. Further increases in the concentration of added cofilin did not increase the nonsedimented actin further. For example, in the presence of 6.3 μM cofilin, the actin concentration in the supernatant was still maintained at 1.1 μM. On the other hand, the amount of cofilin bound to F-actin increased with increasing added cofilin concentration. The binding was saturable. At the maximum, 1 mol of cofilin bound to 1 mol of actin monomer on the F-actin (Figure 3B). This indicates again that cofilin reacts with actin in a 1:1 molar ratio.

(*4*) *DNase I Inhibition Assay.* When increasing concentrations of cofilin were incubated with actin under polymerizing conditions, the concentration of monomeric actin, determined by the DNase I inhibition assay, was found to increase to a limited extent (Figure 4). The maximal level was 1.5 μM actin monomer, which was produced by the presence of 3–4 μM cofilin. Even in the presence of 6.7 μM cofilin, actin monomer concentration remained the same. This dependence of monomer concentration on the concentration of cofilin correlated roughly with that of nonsedimented actin described in the previous section (cf. Figure 4 with Figure 3B).

(*5*) *Binding of Cofilin with Monomeric Actin.* The direct evidence for the binding of cofilin with G-actin was obtained by gel filtration. Actin was mixed with about an equimolar amount of cofilin, and then the mixture was gel filtered on

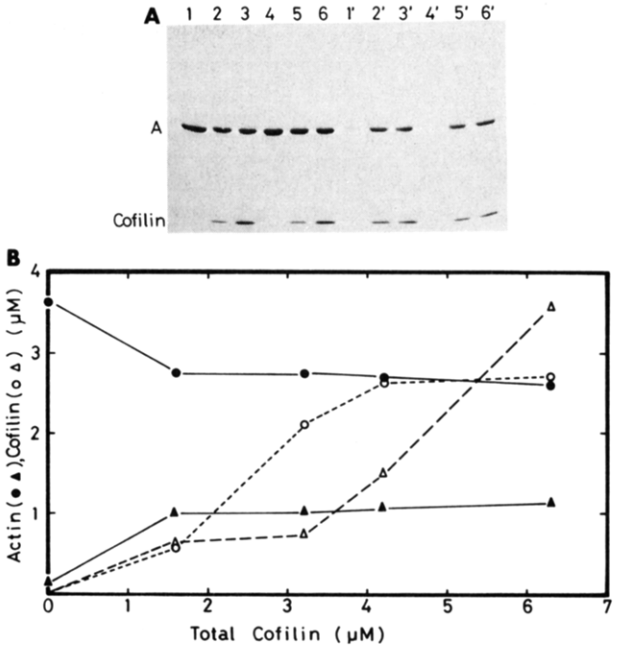

FIGURE 3: Pelleting assay. (A) Actin samples from the experiment shown in Figure 2 were centrifuged, and the pellet (slots 1–6) and supernatant (slots 1′–6′) fractions were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as described under Experimental Procedures. Slots 1 (1′), 2 (2′), and 3 (3′) correspond to samples a, b, and c, respectively in the experiment shown in Figure 2A, and slots 4 (4′), 5 (5′), and 6 (6′) correspond to samples a, b, and c, respectively in Figure 2B. (B) Brain actin (3.8 μM) was polymerized as in Figure 1 in the presence of various amounts of cofilin to the steady state. The pellet and the supernatant fractions after centrifugation were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as described under Experimental Procedures. The concentrations of actin (●, ▲) and cofilin (○, △) in the pellet and the supernatant are plotted against the total cofilin concentration added: (●) actin in pellet; (▲) actin in supenatant; (○) cofilin in pellet; (△) cofilin in supernatant. Cofilin alone did not sediment under the conditions used.

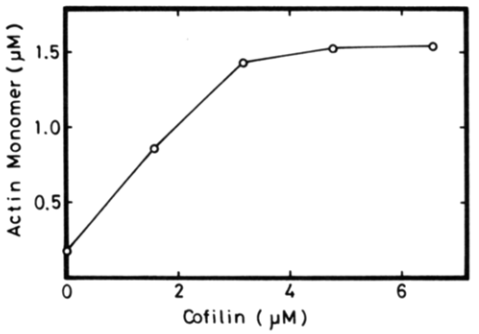

FIGURE 4: Effect of cofilin on the steady-state actin monomer concentration. Muscle actin (4.6 μM) was polymerized as in Figure 1 to the steady state in the presence of increasing amounts of cofilin, and then the actin monomer concentration was determined by the DNase I inhibition assay.

Sephadex G-100 in a buffer solution containing 0.1 mM $CaCl_2$, 0.1 mM DTT, and 4 mM HEPES, pH 7.9. Actin and cofilin were coeluted (Figure 5), demonstrating the complex formation. The elution position was almost the same as that of bovine serum albumin.

(*6*) *Electron Microscopy.* Electron micrographs of negatively stained actin filaments in the presence and absence of cofilin are shown in Figure 6. It is clear that the filaments in the presence of cofilin are shorther than its absence. Under the conditions used (3.8 μM actin in 2 mM $MgCl_2$–80 mM KCl), the number-average filament length was larger than 2 μm in the absence of cofilin, and it decreased to 1.3 and 0.45 μm in the presence of 2.1 and 6.3 μM cofilin, respectively.

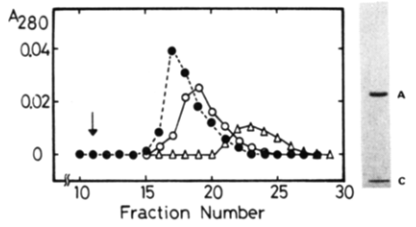

FIGURE 5: Binding of cofilin with monomeric actin. Cofilin (76 μg/mL) and muscle G-actin (140 μg/mL) were mixed and incubated at 0–4 °C for about 15 min, and then a 0.35-mL aliquot of the mixture was gel filtered on a Sephadex G-100 (0.9 × 19.5 cm) column in the medium described in the text. The absorbance at 280 nm of each fraction was measured (●). The $NaDodSO_4$ gel electrophoretic pattern of fraction 17 is also shown: A, actin; C, cofilin. As a control, cofilin alone (△) or G-actin alone (○) was gel filtered as above. The arrow indicates the void volume. Bovine serum albumin eluted at the position of fraction 17.

Furthermore, subtle but significant differences in the filament structure are observed between them. The double-stranded nature of F-actin was clearly seen in the absence of cofilin, but in its presence, this characteristic was somewhat obscured. In addition, F-actin in the presence of cofilin appears to be slightly thicker than in its absence (Figure 6C–E). This corresponds well with the increased light-scattering intensity of F-actin in the presence of cofilin (see Figure 1).

Under the electron microscope, some actin filament bundles were observed in the absence of cofilin (Figure 6A), but they decreased with increasing concentrations of cofilin added (Figure 6B), indicating that cofilin has no ability to bundle actin filaments. Consistent with this result, the low-speed centrifugation assay demonstrated that cofilin does not increase the sedimentability of actin filaments (data not shown). These results rule out the possibility that the reduction in viscosity and the increase in the light-scattering intensity of F-actin by cofilin are due to the bundling of F-actin.

(*7*) *Are There Any Contaminating Proteins in Our Cofilin Preparation*? The foregoing results revealed the two major effects of cofilin on actin: (i) Cofilin binds to F-actin in a 1:1 molar ratio of cofilin to actin monomer in the filament. (ii) Cofilin shortens F-actin and increases the steady-state monomer concentration to a limited extent. There might be an argument that effect ii could be due to the contamination of a small amount of a capping and/or severing protein. However, several lines of evidence indicate this is unlikely. (i) When 10 μg of our cofilin preparation was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis, no protein bands other than cofilin were visible. Since even 0.07 μg of a single protein band is easily detected when stained with Coomassie blue, the amount of a possible contaminating protein is estimated to be less than 0.7% of cofilin. (ii) In a final purification step, cofilin (determined by electrophoresis) eluted as a single symmetrical peak on a Sephadex G-75 column (Maekawa et al., 1984), and the elution of the activity reducing the steady-state actin viscosity was precisely superimposed on that of the cofilin band on a $NaDodSO_4$–polyacrylamide gel. Moreover, the elution of the activity increasing the steady-state monomer concentration (measured by the DNase I inhibition assay) also correlated strongly with the amount of cofilin (not shown). (iii) If a capping protein is contained in our cofilin preparation, it should cosediment with actin when the cofilin preparation is mixed with F-actin and pelleted. However, there were no visible bands other than actin and cofilin when a large amount (20–30 μg) of the pelleted fraction was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. These results suggest that all the effects of our cofilin preparation on actin described in this paper reside in cofilin itself.

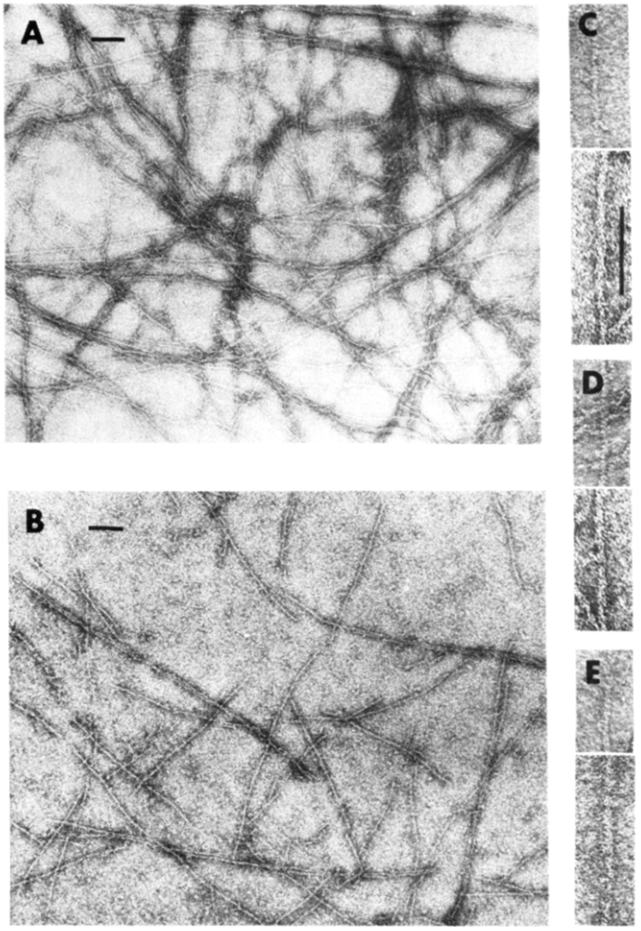

FIGURE 6: Electron micrographs of actin filaments in the presence or absence of cofilin. Brain actin (3.8 μM) was polymerized in the absence (A) or presence (B) of cofilin (6.3 μM) as in Figure 1 for about 3 h at 25 °C and then mounted on the grids. Electron micrographs in (C), (D), and (E) show a few typical examples of actin filaments with (lower panel) or without (upper panel) cofilin at a higher magnification. Note that actin filaments with cofilin are consistently thicker than those without cofilin. Scale bars represent 0.1 μm.

(*8*) *Nucleation Assay*. The nucleating ability of F-actin polymerized in the presence or absence of cofilin was examined (a nucleation assay). This assay enables us to estimate the number of filament ends in actin solutions. This estimate is based on the facts that cofilin alone does not nucleate actin polymerization (Figure 1) and that the rate of the actin elongation induced by the added F-actin solution is proportional to the number of its filament ends (Nishida & Sakai, 1983). As shown in Figure 7, the filaments polymerized in the presence of substoichiometric amounts of cofilin induced a faster polymerization of actin than did the F-actin polymerized in the absence of cofilin (cf. curves b and c with curve a), when added to actin monomers. When preformed F-actin was mixed with cofilin and then added to actin monomers, the F-actin with cofilin also induced a faster elongation than did the F-actin alone (not shown). These results suggest that cofilin increases the number of filaments and hence shortens the length of filaments.

Since it has been demonstrated previously that barbed end-capping factors such as *Acanthamoeba* capping protein (Isenberg et al., 1980), gelsolin (Yin et al., 1981), brain 88K protein–actin complex (Nishida et al., 1983), and cytochalasin B (Nishida et al., 1983) inhibit this type of actin elongation, and since cofilin, even at very low concentrations, did not inhibit the elongation at all, it is suggested that cofilin does not have a barbed end-capping activity.

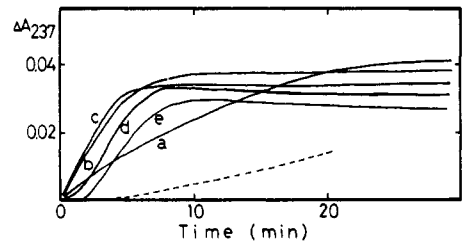

FIGURE 7: Nucleating ability of F-actin polymerized in the presence of cofilin. An aliquot of muscle F-actin (4.6 μM) which had been polymerized in the presence of various concentrations [0 (a), 1.6 (b), 3.2 (c), 4.8 (d), and 6.5 μM (e)] of cofilin as in Figure 1 was added to actin monomers (3.85 μM) to initiate actin polymerization, and the polymerization kinetics were followed by the A_{237} assay, as shown. The assay conditions were 0.4 mM $MgCl_2$, 20 mM KCl, 0.02 mM $CaCl_2$, 0.05 mM DTT, 0.08 mM ATP, 2 mM HEPES, and 3 mM PIPES, pH 7.3 at 30 °C. (---) Actin monomer alone; (a) +F-actin (0.96 μM) without cofilin; (b–e) +F-actin with varying amounts of cofilin.

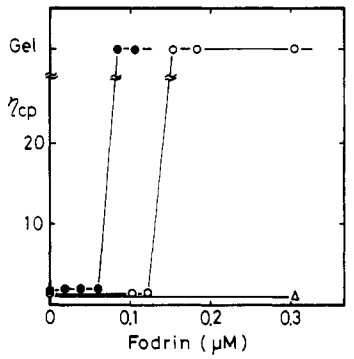

FIGURE 8: Effect of cofilin on the concentration of fodrin required for gelation of F-actin. Brain F-actin was incubated at 25 °C for about 5 min with various amounts of cofilin and then mixed with various amounts of fodrin in a capillary tube in a medium of 0.55 mM $MgCl_2$, 70 mM KCl, 0.01 mM $CaCl_2$, 0.3 mM EGTA, 0.03 mM DTT, 0.04 mM ATP, 7 mM PIPES, and 30 mM MES, pH 6.8. After the mixture stood for 10 min, the low shear viscosity was measured by the falling ball method. "Gel" indicates that the ball did not move at all. (●) F-Actin (3.65 μM) without cofilin; (○) F-actin with 1.0 μM cofilin; (△) F-actin with 3.5 μM cofilin.

However, when the F-actin polymerized in the presence of equimolar or excess amounts of cofilin was added to actin monomers, an immediate elongation did not occur (Figure 7, curves d and e). This suggests that F-actin which is completely saturated with cofilin is incapable of immediately nucleating actin polymerization. However, after a short lag, actin elongated fast, which indicates that the added F-actin comes to have the ability to induce actin elongation with time. One possible explanation for this result is that during the lag period a portion of cofilin is dissociated from the added F-actin to bind to free actin monomers and then F-actin uncomplexed with cofilin appears and induces the elongation. Another is that the cofilin "covering" F-actin can elongate slowly; therefore, with time a portion of F-actin, uncovered with cofilin, which is competent to induce normal elongation is produced.

(9) *Gel Point Assay.* That actin filaments are shortened in the presence of cofilin is further supported by a gel point assay. Fodrin, a spectrin-like protein, was used as a cross-linking protein. As shown in Figure 8, the minimum concentration of fodrin required for gelation of F-actin increased with increasing concentrations of cofilin. Since the binding of fodrin with F-actin was not markedly inhibited by cofilin within the concentrations used in Figure 8 (not shown), the result suggests that cofilin increases the number of filaments, i.e., shortens the filament length.

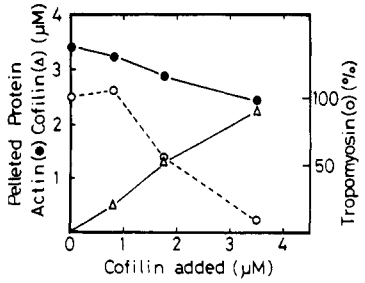

FIGURE 9: Dissociation of tropomyosin from actin filaments by the binding of cofilin. Increasing amounts of cofilin were added to muscle actin filaments polymerized in the presence of tropomyosin (one tropomyosin molecule to four actin monomers on F-actin) and incubated at 5 °C for about 10 min. The medium was the same as described in Figure 1. After the filaments were sedimented by centrifugation, the pellets were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. Concentrations of cofilin added were 0, 0.8, 1.75, and 3.5 μM. Actin concentrations was 3.6 μM. The amount of each protein in the pellet is shown. (●) Actin; (○) tropomyosin; (△) cofilin. The amount of tropomyosin was expressed as the percent of the control value.

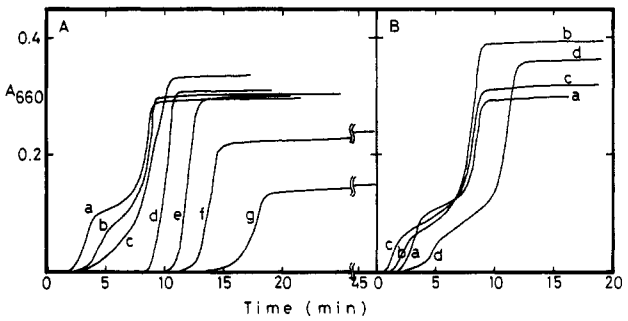

FIGURE 10: (A) Effect of cofilin on the superprecipitation of the actin–myosin mixture. The assays were carried out as described under Experimental Procedures. Molar ratios of cofilin to actin were 0 (a), 0.06 (b), 0.125 (c), 0.25 (d), 0.50 (e), 0.75 (f), and 1.0 (g). (B) Effect of the decrease in the amount of F-actin on the superprecipitation. F-Actin concentrations were 3.81 (a), 2.39 (b), 2.17 (c), and 1.62 μM (d). No cofilin was added.

Inhibition of Tropomyosin Binding to F-Actin by Cofilin. Cofilin inhibits the binding of tropomyosin to actin filaments. When actin and tropomyosin were polymerized in the presence of cofilin, the amount of the tropomyosin bound to F-actin decreased, and F-actin bound cofilin in place of tropomyosin (data not shown). Moreover, the steady-state viscosity value of actin filaments (160 μg/mL) polymerized at 30 °C with tropomyosin (85 μg/mL, in the presence of 2 mM $MgCl_2$ and 70 mM KCl) and reduced by the inclusion of cofilin (55 μg/mL) from 0.57 to 0.19, suggesting that cofilin binds to F-actin more strongly than does tropomyosin.

The effect of cofilin on the preformed F-actin–tropomyosin complex was investigated. In this experiment, increasing amounts of cofilin were added to tropomyosin-containing actin filaments, and then the filaments were pelleted by centrifugation. $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of pelleted fractions showed that the amount of cofilin bound to actin filaments increased, while that of tropomyosin decreased with increasing concentrations of cofilin added. The pelletable actin filaments were slightly decreased by the action of cofilin (Figure 9). This indicates that tropomyosin is forced to be dissociated from actin filaments by the attack of cofilin on actin filaments.

Inhibition of Actin–Myosin Interaction by Cofilin. Figure 10A shows the effect of cofilin on the superprecipitation of the actin–myosin mixture. The superprecipitation process was followed by measuring the increase in turbidity. Cofilin was found to prolong, in a concentration-dependent fashion, the

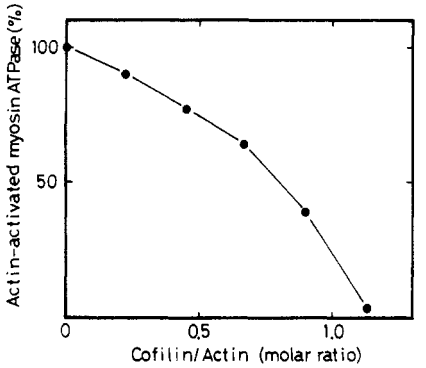

FIGURE 11: Inhibition of the actin-activated myosin ATPase by cofilin. The assays were carried out as described under Experimental Procedures. The data are expressed as the percent activity. The ATPase activity of myosin alone (0.036 μmol of P_i mg^{-1} min^{-1}) was subtracted.

duration of the lag phase preceding a detectable increase in turbidity. In addition, cofilin decreased the final extent of the turbidity increase. At a molar ratio of cofilin to actin of 1:1, the final turbidity was decreased by 53%. These effects do not result from the slight decrease in the amount of F-actin caused by the action of cofilin, since the mere reductions in the amount of included F-actin did not mimic the effects of cofilin on the superprecipitation (Figure 10B). It is likely that the binding of cofilin to F-actin interferes with the actin–myosin interaction.

Figure 11 demonstrates that cofilin inhibits the actin-activated myosin ATPase activity in a concentration-dependent manner. At a molar ratio of cofilin to actin of about 1.1:1, the ATPase activity decreased to a level comparable to that of myosin alone. This result also demonstrates that cofilin inhibits the interaction of actin with myosin.

Discussion

Previously, we purified a monomeric protein with a molecular weight of 21 000 from porcine brain by using DNase I–agarose affinity chromatography, hydroxyapatite and phosphocellulose chromatographies, and gel filtration on Sephadex G-75 (Maekawa et al., 1984). In the present study, the protein was found to have unique functional properties. One of the most remarkable features of the interaction of the protein with actin is that the protein binds to actin filaments in a 1:1 molar ratio of protein to actin monomer on the filaments. From this cofilamentous nature of the protein, we named it cofilin.

Since it is well-known that tropomyosin also binds to actin filaments stoichiometrically, although the stoichiometry (one muscle tropomyosin per seven actin monomers or one nonmuscle tropomyosin per six actin monomers) is markedly different from that of cofilin, the effect of cofilin on tropomyosin–actin binding was examined. It was found that cofilin blocks the binding of tropomyosin to actin filaments. Moreover, when cofilin was added to tropomyosin-containing actin filaments, tropomyosin was dissociated from the filaments, and the filaments bound cofilin. These results suggest the high affinity of cofilin for actin filaments. It is thought that some portions of actin filaments in nonmuscle cells contain tropomyosin and some other portions cofilin. As mentioned below, there are functional differences between tropomyosin-containing actin filaments and cofilin-containing ones.

Cofilin inhibits the actin-activated ATPase activity of myosin in a concentration-dependent manner. The result suggested that F-actin which binds 1 mol of cofilin per 1 mol of actin monomer on the filament does not have the ability to activate the myosin ATPase. It was also found that cofilin inhibits the superprecipitation of the actin–myosin mixture. These results clearly indicate that cofilin inhibits the actin–myosin interaction by binding to the actin filaments.

A variety of assays demonstrated the following characteristics of cofilin–actin interactions.

(i) The mass concentration of actin filaments at the steady state is decreased to a limited extent by cofilin; that is, the steady-state monomer concentration is increased to a limited extent. This was demonstrated by the DNase I inhibition assay and the pelleting assay.

(ii) Cofilin increases the number of actin filaments and decreases the average length of the filaments. This was clearly seen in the electron micrographs of negatively stained actin filaments and was also supported by the decreased viscosity and the increased nucleating ability of the actin filaments in the presence of cofilin. That the minimum concentration of fodrin required for gelation of F-actin increased in the presence of cofilin also seems to support this.

It is known that severing and/or capping proteins such as gelsolin (Yin et al., 1980) and fragmin (Hasegawa et al., 1980) also shorten the actin filaments. Cofilin, like these proteins, is able to bind to actin monomer. However, unlike these proteins, cofilin does not nucleate actin polymerization. The light-scattering assay revealed that cofilin increases the duration of the lag phase. It was also suggested that cofilin does not cap the actin filaments. These facts indicate that the action of cofilin is different from that of previously identified severing and/or capping proteins. The mechanism by which cofilin changes the steady-state monomer concentration and the length distribution of actin filaments remains to be elucidated in future work.

(iii) The structure of actin filaments in the presence of cofilin is different from that in its absence. First, the filaments are thickened in the presence of cofilin. This was directly observed in the electron microscope and was also supported by the light-scattering assay. The light-scattering intensity of the filaments was increased in the presence of cofilin in spite of the reduced amount of filaments, suggesting that cofilin binds along the entire length of actin filaments. Second, the conformation of the actin molecule in the filament is altered by the binding of cofilin. The fluorescence of pyrene-labeled actin in the filament was decreased to a level of G-actin by the binding of cofilin. The emission and excitation spectra at complete saturation of the filament with cofilin were very similar to those of monomeric actin, suggesting that the local conformation of the actin molecule in the filament was made similar to that of G-actin by the binding of cofilin. It was previously reported that the binding of heavy meromyosin or subfragment 1 to the pyrene-labeled F-actin induced a similar change in the fluorescence (Kouyama & Mihashi, 1981). That F-actin which was completely saturated with cofilin did not nucleate actin polymerization immediately when added to actin monomers also suggests that the structure of actin filaments is altered by the binding of cofilin.

(iv) The electron microscopy and the low-speed centrifugation assay demonstrated the inability of cofilin to bundle actin filaments. This rules out the possibility that the decrease in the viscosity and the increase in the light-scattering intensity result from the bundling of actin filaments.

The fact that cofilin-containing actin filaments could be sedimentable in spite of the marked reduction in the high shear viscosity seems apparently paradoxical. We think that this partly results from the fragility of cofilin-containing actin filaments against shear forces. The decrease in the high shear viscosity corresponds well with the increase in the amount of

bound cofilin, suggesting that cofilin makes the filaments fragile. Thus, the reduction in the viscosity may be attributable to both the shortened actin filaments and the fragility of actin filaments in the presence of cofilin.

These functional properties of cofilin are clearly different from those of the previously identified actin-binding probeins such as actin cross-linking proteins, severing and/or capping proteins, and depolymerizing proteins (Weeds, 1982; Craig & Pollard, 1982). Therefore, we propose that cofilin should be newly classified as "cofilamentous proteins" or "actin coating proteins". Nonmuscle tropomyosin may be included in this group.

Our preliminary experiments have shown the existence of colifin in both porcine kidney and rat liver in addition to porcine brain. Therefore, colifin may play an important role in regulating the state of actin filaments in cells of most mammalian tissues. The localization of cofilin-containing actin filaments and the physiological roles of cofilin will be the subject of future work.

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