Antagonistic Effects of Cofilin, Beryllium Fluoride Complex, and Phalloidin on Subdomain 2 and Nucleotide-Binding Cleft in F-Actin

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ABSTRACT Cofilin/ADF, beryllium fluoride complex (BeFx), and phalloidin have opposing effects on actin filament structure and dynamics. Cofilin/ADF decreases the stability of F-actin by enhancing disorder in subdomain 2, and by severing and accelerating the depolymerization of the filament. BeFx and phalloidin stabilize the subdomain 2 structure and decrease the critical concentration of actin, slowing the dissociation of monomers. Yeast cofilin, unlike some other members of the cofilin/ADF family, binds to F-actin in the presence of BeFx; however, the rate of its binding is strongly inhibited by BeFx and decreases with increasing pH. The inhibition of the cofilin binding rate increases with the time of BeFx incubation with F-actin, indicating the existence of two BeFx-F-actin complexes. Cofilin dissociates BeFx from the filament, while BeFx does not bind to F-actin saturated with cofilin, presumably because of the cofilin-induced changes in the nucleotide-binding cleft of F-actin. These changes are apparent from the increase in the fluorescence intensity of F-actin bound ε-ADP upon cofilin binding and a decrease in its accessibility to collisional quenchers. BeFx also affects the nucleotide-binding cleft of F-actin, as indicated by an increase in the fluorescence intensity of ε-ADP-F-actin. Phalloidin and cofilin inhibit, but do not exclude each other binding to their complexes with F-actin. Phalloidin promotes the dissociation of cofilin from F-actin and slowly reverses the cofilin-induced disorder in the DNase I binding loop of subdomain 2.

INTRODUCTION

Actin has a central role in biological motility as an essential constituent of cytoskeleton and a partner of all myosin-based motor systems. In all eukaryotic cells actin exists in the rapidly interconverting monomer (G-actin) and polymer (filaments, F-actin) forms. The actin-based systems are highly dynamic and strongly regulated by a number of factors, including several actin-binding proteins. These factors can be subdivided into two antagonistic groups, which either stabilize or destabilize the structure of actin filaments.

Phalloidin, and inorganic phosphate (Pi) and its analogs—beryllium fluoride (BeFx) and aluminum fluoride (AlF_4)—belong to the group of F-actin stabilizing factors. These small molecules stabilize F-actin by reducing the critical concentration for polymerization and introducing conformational changes into the filament structure.

The complexes of beryllium and aluminum with fluoride were found to be good structural analogs of Pi (1) and are widely used in studying the activity of various nucleotide binding proteins, including G-proteins, Na^+, K^+-ATPase, tubulin, and others (2). Combeau and Carlier found that BeFx (BeFx stands for the BeF_3^- and $BeF_2(OH)^-$ complexes) and AlF_4 bind strongly to F-actin (3). BeFx and AlF_4 bind to F-actin with orders of magnitude higher affinity ($K_d = 2\ \mu M$ and 25 μM, respectively) than Pi ($K_d = 1.5$ mM). BeFx competes with Pi for binding to the nucleotide-binding cleft of ADP-F-actin protomers at the place of the γ-phosphate of ATP (3). It stabilizes strongly F-actin by decreasing the rate of protomer dissociation (~150-fold) and the critical concentration for polymerization (~100-fold) (3). BeFx stabilizes in particular the structure of subdomain 2, as indicated by strong and cooperative inhibition of its cleavage by subtilisin (in the DNase I binding-loop (D-loop)) and trypsin (in the 60–69 loop) (4), and by electron microscopy studies (5). These effects of BeFx on F-actin are similar to those of Pi (3,4), but BeFx is more effective at much smaller concentrations. BeFx-induced changes in the C-terminus region were also detected by fluorescence (3) and proteolysis (4) methods.

Phalloidin has the strongest stabilizing effect on actin filaments. It decreases the critical concentration of actin polymerization, reduces the rate of monomer dissociation from both filament ends (6), and inhibits phosphate release from the nucleotide binding cleft after ATP hydrolysis. Phalloidin binds at the interface of three actin monomers (7–9) and stabilizes lateral interactions between the two filament strands.

Among actin destabilizing factors, the actin depolymerizing factor/cofilin (ADF)/cofilin) family of proteins, or AC proteins (10), have attracted much attention because of their important role in regulating actin dynamics in cells. These proteins change the twist of actin filaments (11), and destabilize, sever (12), and depolymerize (13) them by weakening longitudinal (14,15) and lateral (16,17) interprotomer contacts in F-actin. Extensive, cofilin-induced conformational changes in subdomain 2 of F-actin are readily monitored via quenching of the fluorescence of tetramethyl rhodamine cadaverine (TRC) (attached to Gln-41 on the D-loop (18)),

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and a strong acceleration of subtilisin (between Met-47 and Gly-48) and tryptic cleavage (after Arg-62 and Lys-68) in subdomain 2 (18).

The antagonistic structural effects of AC proteins and BeFx (and phalloidin) on F-actin appear consistent with their reciprocal inhibition of binding to F-actin (13) and the reported blocking of *Acanthamoeba* actophorin (AC protein) binding by BeFx (19) and human cofilin binding by phalloidin (20) to F-actin. On the other hand, we found that yeast cofilin removes rhodamine-phalloidin from F-actin (17), and obtained preliminary evidence (21) that BeFx inhibits the rate but not the extent of yeast cofilin binding to muscle F-actin. Following these observations, we used here the binding of yeast cofilin to BeFx-F-actin as a tool to study the structural effects of BeFx on actin filaments. Cofilin binding to F-actin was monitored via changes in the susceptibility of subdomain 2 to subtilisin and trypsin, and via changes in the fluorescence intensity of the TRC probe attached Gln-41 (18) and of 1,N^6-ethenoadenosine diphosphate (ε-ADP) bound in the nucleotide-binding cleft of F-actin. In addition, we also monitored the effect of cofilin on the release of BeFx from F-actin by ^{19}F-NMR. We found that BeFx strongly inhibits the rate, but not the extent of cofilin binding, while cofilin greatly facilitates the dissociation of BeFx from F-actin, and affects the structure of subdomain 2 and the nucleotide binding cleft of F-actin. Our results indicate the existence of two types of BeFx-F-actin complexes, with different conformations and different rates of cofilin binding.

MATERIALS AND METHODS

Reagents

Tetramethyl rhodamine cadaverine (TRC) was obtained from Molecular Probes (Eugene, OR). ATP, 1,N^6-ethenoadenosine triphosphate (ε-ATP) trypsin, soybean trypsin inhibitor, subtilisin (Carlsberg), phalloidin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical (St Louis, MO). Bacterial transglutaminase was a generous gift from Dr. K. Seguro (Ajimoto, Kawasaki, Japan).

Proteins

G-actin was prepared from back and leg muscles of rabbit by the method of Spudich and Watt (22) and stored in G-buffer containing 5.0 mM TrisHCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM dithiotreitol, pH 8.0. F-actin was prepared from G-actin by polymerizing it with 2.0 mM $MgCl_2$. Recombinant yeast cofilin was prepared as described before (23) with minor modifications (15). The concentrations of cofilin and unlabeled skeletal muscle α-actin were determined spectrophotometrically by using the extinction coefficients $E^{1\%}_{280} = 9.2$ and $E^{1\%}_{290} = 11.5\ \mathrm{cm}^{-1}$, respectively. (The optical density of actin was measured in the presence of 0.5 M NaOH, which shifts the maximum of absorbance from 280 to 290 nm). Molecular masses were assumed to be 42 and 15.9 kDa for skeletal actin and yeast cofilin, respectively.

Proteolysis

Labeled or unlabeled F-actin (10 μM) was digested in the presence and absence of cofilin in pH 8.0 F-buffer (20.0 mM TrisHCl, 2.0 mM $MgCl_2$, 0.2 mM ATP, 0.5 mM dithiotreitol), and pH 6.5 F-buffer (20.0 mM PIPES, 2.0 mM $MgCl_2$, 0.2 mM ATP, 0.5 mM dithiotreitol), with 25 μg/ml subtilisin or 800 μg/ml trypsin, respectively. The products of digestion were run on SDS-PAGE. Protein bands on SDS gels were analyzed by densitometry.

Chemical modification

Actin labeled with TRC at Gln-41 (TRC-actin) was prepared by incubating 50 μM skeletal G-actin with 100 μM TRC and 0.18 mg/ml bacterial transglutaminase in G-buffer pH 8.0, at 22°C for 2 h. Reagent excess was removed by filtering actin through a PD-10 column equilibrated with G-buffer. The extent of actin labeling for TRC was estimated using extinction coefficient of $E_{554} = 78{,}000\ \mathrm{cm}^{-1}\mathrm{M}^{-1}$. The concentration of the labeled actin was measured by the Bradford protein assay (24), using native actin as a standard.

Preparation of ε-ADP-F-actin

This was done essentially as described previously (25). Briefly, skeletal muscle G-actin was passed through a desalting column (Amersham, PD10, Piscataway, NJ) of Sephadex G-25 equilibrated with ATP-free G-buffer. The eluted actin was supplemented with a 20-fold molar excess of ε-ATP and was incubated for 1 h on ice. Excess ε-ATP was removed from G-actin by passing it through another PD10 column. Actin was polymerized by addition of 2.0 mM $MgCl_2$ and during the polymerization the actin-bound ε-ATP was hydrolyzed to ε-ADP.

Fluorescence measurements

Fluorescence measurements were carried out at 22°C with a PTI spectrofluorometer (Photon Technology Industries, South Brunswick, NJ) in pH 8.0 and pH 6.5 F-buffer. For TRC and ε-ADP, the excitation wavelength was set at 544 and 350 nm and the emission at 580 and 412 nm, respectively. Emission spectrum of ε-ADP-F-actin was recorded between 370 and 550 nm wavelengths.

^{19}F NMR measurements

Spectra were obtained on the Varian *Inova* 500 instrument at 470.215 MHz, in a 5-mm probe. Spectrum width was 50,000 Hz, delay time 1 s, and acquisition time 0.7 s. Line broadening function was set to 20 Hz. In all experiments temperature was kept at 20°C and the solutions were supplemented with 15% D_2O.

RESULTS

Binding of cofilin to BeFx-F-actin

We showed before that labeling of Gln-41 on actin with tetramethyl rhodamine cadaverine (TRC) offers a convenient tool for monitoring the binding of cofilin to F-actin. The fluorescence intensity of TRC-F-actin is decreased by >70% upon cofilin binding, while the fluorescence intensity of G-actin changes little with the addition of cofilin (18). Here, we monitored the decrease in TRC fluorescence (Fig. 1) upon addition of 12 μM cofilin to 10 μM TRC-F-actin in the presence and absence of 60 μM BeFx at pH 8.0 and 6.5 (BeFx alone did not affect the fluorescence of TRC-F-actin). In the absence of BeFx, the decrease in the fluorescence of TRC-F-actin by cofilin was essentially completed within the mixing time of the solutions, in agreement with our earlier

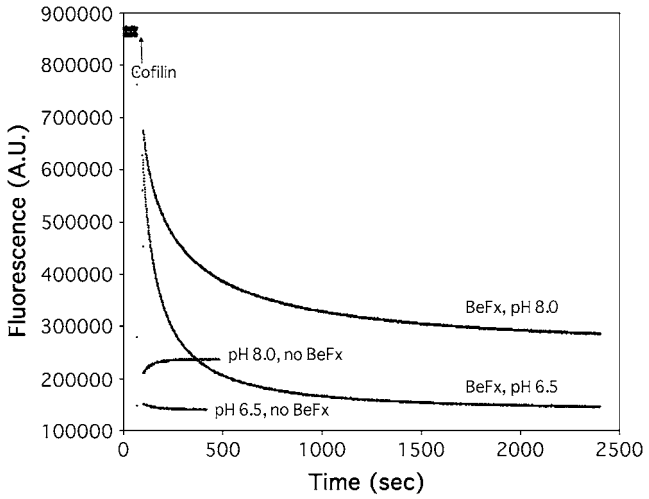

FIGURE 1 Effect of cofilin on the fluorescence of BeFx-TRC-F-actin at pH 8.0 and 6.5. TRC-F-actin, 10 μM, was incubated with 60 μM $BeCl_2$ and 5 mM NaF in a pH 8.0 or pH 6.5 F-buffer, overnight, on ice. Cofilin (12 μM) was added to the sample (*arrow*) after the initial reading, and the time course of fluorescence intensity change was monitored at 22°C as described in Materials and Methods.

observations (18). Yeast cofilin also binds to TRC-F-actin in the presence of BeFx at both pH-s, but at a much slower rate than in its absence. Fig. 1 shows that BeFx inhibits the rate, but not the extent of cofilin binding and that this binding is faster at pH 6.5 than at pH 8.0. The time course of fluorescence changes could be well fitted to a two-exponential expression, yielding the apparent first order fast (k_f) and slow (k_s) cofilin binding rates at pH 6.5 and 8.0, from which the second order association rate constants were calculated by taking into account their dependence on cofilin concentration (Fig. 2 and Table 1).

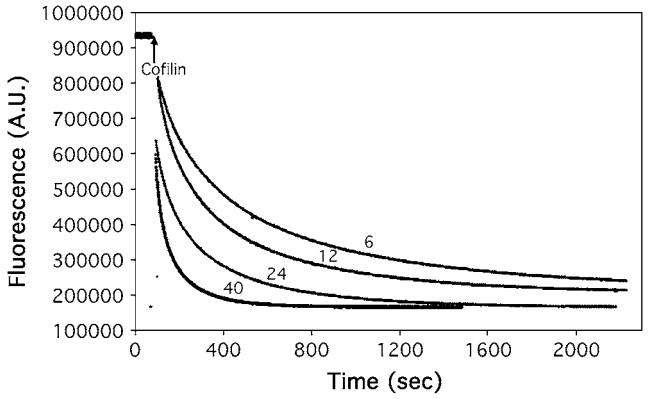

FIGURE 2 Effect of cofilin concentration on the rate of cofilin binding to BeFx-TRC-F-actin. TRC-F-actin, 4 μM, was incubated with 120 μM $BeCl_2$ and 5 mM NaF in a pH 8.0 F-buffer, overnight, on ice. Cofilin (6–40 μM), as indicated on the figure, was added to the sample (*arrow*) after the initial reading, and the time course of fluorescence intensity change was monitored at 22°C as described in Materials and Methods. Cofilin concentration in μM is given on each curve.

To see whether cofilin binds to BeFx containing F-actin protomers or the binding is limited by the dissociation of BeFx from the protomers, we measured the effect of increasing cofilin concentration on the rate of binding (Fig. 2). We monitored the binding of 6–40 μM cofilin to 4 μM TRC-F-actin in the presence of 120 μM BeFx. Relatively high concentration of BeFx was used to slow down the reaction. The binding rates were found to increase significantly with cofilin concentration, indicating that the binding is not limited by the dissociation of BeFx. All binding curves could be well fitted to a two-exponential expression. In the presence of 40 μM cofilin and 4 μM TRC-F-actin the k_f and k_s (apparent first-order rate constants) were 0.0515 and 0.0105 s^{-1}, respectively. At the lowest cofilin concentration (6 μM) the k_f and k_s rates were 0.0071 and 0.0012 s^{-1}, respectively. The calculated second-order association rate constants were presented in Table 1. Because some AC proteins, such as ADF1 and actophorin, apparently do not bind to BeFx-F-actin (13,19), we have confirmed the binding of yeast cofilin to BeFx-TRC-F-actin also by their cosedimentation at pH 8.0 (Fig. 3). Similar results were obtained also with unlabeled actin, both at pH 6.5 and 8.0 (data not presented), showing that yeast cofilin binds to F-actin in the presence of BeFx.

The binding of BeFx to the nucleotide binding cleft of F-actin stabilizes strongly the structure of subdomain 2, which is manifested in its resistance to subtilisin and trypsin cleavage (4). Cofilin binding has the opposite effect, as it increases dramatically the proteolysis of the D- and 60–69-loops by subtilisin and trypsin (18). We show in Fig. 4 *A* that cofilin increases the rate and extent of subtilisin cut in the D-loop of BeFx-F-actin at pH 6.5 and pH 8.0, with a bigger effect noted at the lower pH. This is consistent with the TRC-F-actin fluorescence results (Fig. 1). The rate and the extent of subtilisin cleavage increased with the time of cofilin incubation with BeFx-F-actin (Fig. 4 *B*). Similar results were obtained also for the tryptic cleavage of the 60–69 loop (after Arg-62 and Lys-68), which became faster and more extensive with cofilin incubation (Fig. 4 *C*). It should be noted, however, that even after 20 min incubation with cofilin both the subtilisin and trypsin digestions were less extensive than in the presence of cofilin without BeFx. The results of proteolysis experiments confirm cofilin binding to BeFx-F-actin.

Effects of cofilin and BeFx on the conformation of the nucleotide-binding cleft of F-actin

To test whether cofilin removes the bound BeFx from the nucleotide-binding cleft of F-actin or binds to BeFx-actin without releasing this phosphate analog, we used ^{19}F NMR. The free fluoride and beryllium were removed from BeFx-F-actin by extensive dialysis, after which the bound BeFx was released by denaturing actin with perchloric acid. Actin denaturation was needed because the actin bound fluoride does not give the ^{19}F signal. The bound fluoride (0.6 mol per

TABLE 1 Second-order association rate constants of cofilin binding to TRC-F-actin in the absence and presence of BeFx at pH 8.0 and 6.5

TRC-F-actin	Fast cofilin binding k_f, s^{-1} mM^{-1}	Slow cofilin binding k_s, s^{-1} mM^{-1}	Rate in % of the binding rate in the absence of BeFx
*TRC-F-actin at pH 8.0	43.7 ± 2.15‡	–	100
*TRC-F-actin at pH 6.5	207.0 ± 9.16‡	–	100
†BeFx-TRC-F-actin at pH 8.0	1.38 ± 0.19‡	0.225 ± 0.031‡	3.15
†BeFx-TRC-F-actin at pH 6.5	1.71 ± 0.24‡	0.325 ± 0.053‡	0.83

*Data were taken from stopped flow measurements (unpublished results).
†Data were taken from the experiments shown in Figs. 1 and 2.
‡Mean ± SD.

mol actin) was calculated from the ^{19}F NMR spectrum (Fig. 5). We added cofilin to another aliquot of the dialyzed F-actin and calculated the dissociation of the bound BeFx from actin from the recorded ^{19}F NMR spectrum (Fig. 5). According to these measurements, ~80% of the bound fluoride dissociates from F-actin upon 30 min incubation with cofilin. These results indicate that the binding of cofilin induces conformational changes in the nucleotide-binding cleft of F-actin, and decreases BeFx affinity to actin.

In the light of the above findings we tested the effect of BeFx and cofilin on the nucleotide-binding cleft in F-actin by substituting the actin-bound ADP with its fluorescent ε-ADP analog. We found that upon addition of BeFx to ε-ADP-F-actin the fluorescence intensity of the bound ε-ADP increases by ~11% (Fig. 6 *A*). The time course of the binding of BeFx to ε-ADP-F-actin (Fig. 6 *B*), which was fitted to a single exponential expression, is rather slow. The calculated second order association rate constant of this reaction is $3.67 \pm 0.57 \times 10^{-5}\ s^{-1}\ \mu M^{-1}$. BeFx does not affect the quenching of the fluorescence intensity of actin bound ε-ADP by nitromethane (Fig. 6 *C*), (K_{SV} values at pH 8.0 in the absence and presence of BeFx are $1.87 \pm 0.1\ M^{-1}$ and $1.86 \pm 0.1\ M^{-1}$, respectively). On the other hand cofilin significantly reduces the accessibility of nitromethane to ε-ADP in the nucleotide-binding cleft of F-actin ($K_{SV} = 0.926 \pm 0.04\ M^{-1}$) (see also Muhlrad et al. (25)). Addition of cofilin to ε-ADP-F-actin also increases the fluorescence intensity of the bound ε-ADP by ~50% (25), which is significantly more than the BeFx induced increase (Fig. 6 *A*). The fluorescence change observed upon adding cofilin in the absence of BeFx to ε-ADP-F-actin is very fast while it is slow in the presence of BeFx (Fig. 6 *B*), which is consistent with TRC-F-actin fluorescence results (Fig. 1). When cofilin was added to BeFx-ε-ADP-F-actin the overall fluorescence increase was smaller (30%) than that with cofilin and BeFx-free ε-ADP-F-actin (50%) (Fig. 6).

Two types of BeFx-F-actin complexes

The time course of cofilin binding to BeFx-TRC-F-actin revealed two reaction steps, with fast and slow rate constants (Fig. 1 and Table 1). Because the binding of cofilin is accompanied by BeFx release from F-actin, as shown by NMR results, our data suggest the presence of at least two forms of BeFx-F-actin, tightly and weakly bound, which would account for the fast and slow cofilin binding. We tested this idea by varying the BeFx concentration (10–100 μM) and its incubation time with F-actin (1 and 24 h), and by using ~1:2 mol ratio of cofilin/actin. Predictably, both the relative extent and rate of the fast and slow fluorescence decrease upon cofilin binding to TRC-F-actin depended on BeFx concentration (up to 60 μM), as did also the tryptic digestion of such actin (data not shown).

More revealing were the experiments in which we examined the effect of F-actin preincubation with BeFx on the binding of cofilin (Fig. 7 *A*). TRC-F-actin (10 μM) was incubated with 60 μM BeFx for 1 and 24 h, respectively, and then mixed with cofilin (5.6 μM). The initial, fast fluorescence decrease was faster and greater for the sample incubated with BeFx for 1 h than for 24 h. Similarly, the rate and the extent of subtilisin digestion of BeFx-F-actin in the presence of cofilin were greater after 1 h than 24 h incubation with BeFx (Fig. 7 *B*). These results indicate that the binding of cofilin to BeFx-F-actin depends on the incubation time of F-actin with this phosphate analog, i.e., most likely, on the ratio of the strongly to the weakly bound BeFx-F-actin complex, which increases with the time of incubation. Interestingly, in the absence of cofilin no difference was observed in the inhibition of subdomain 2 proteolysis between samples incubated for 1 h and 24 h with BeFx. In fact, full protection against proteolysis of F-actin by subtilisin appears already after 5 min incubation with 60 μM BeFx (Fig. 7 *C*). This result is consistent with the time course of the fluorescence intensity increase observed upon adding BeFx to ε-ADP-F-actin (Fig. 6 *B*). Similar results

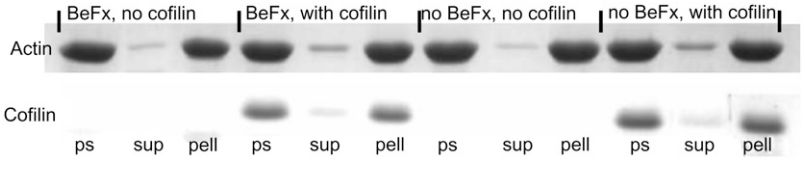

FIGURE 3 Cosedimentation of TRC-F-actin with cofilin in the presence and absence of BeFx at pH 8.0. TRC-F-actin, 10 μM, was incubated with 60 μM $BeCl_2$ and 5 mM NaF in a pH 8.0 F-buffer, for 2 h, on ice. Cofilin (12 μM)was added to actin samples, which were incubated for 20 min and then centrifuged at 80 K at 20°C for 30 min. Prespin (*ps*), supernatant (*sup*), and pellet (*pell*) samples of these solutions were analyzed by SDS-PAGE.

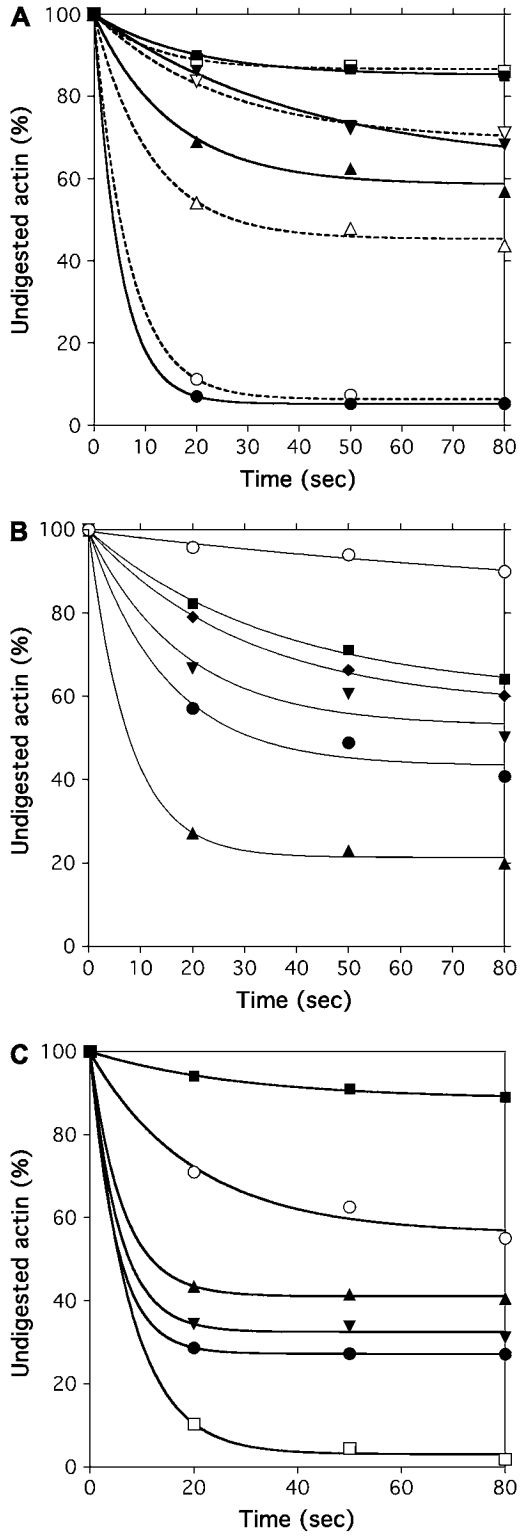

FIGURE 4 Effect of cofilin on the limited proteolysis of BeFx-F-actin at pH 8.0 and 6.5. F-actin (33 μM) was incubated with 100 μM $BeCl_2$ and 5 mM NaF in the pH 8.0 or pH 6.5 F-buffer, overnight, on ice. (*A*) After 1 min incubation of 10 μM F-actin or BeFx-F-actin with 12 μM cofilin at 22°C, actin was digested by 25 μg/ml subtilisin for 20, 50, and 80 s. The samples were run on SDS-PAGE and analyzed by densitometry. (*Solid symbols* and *solid line*) pH 8.0; (*open symbols* and *dotted line*) pH 6.5; (■) BeFx only; (▲) BeFx and cofilin; (▼) no addition; (●) cofilin only. (*B*) After 0.5, 5, and 20 min incubation of 10 μM BeFx-F-actin with 10 μM cofilin at 22°C, actin was digested with 18 μg/ml subtilisin at pH 8.0 for 20, 50, and 80 s. (○) BeFx only; (■) no BeFx and cofilin; (♦) 0.5 min incubation with cofilin; (▼) 5 min incubation with cofilin; (●) 20 min incubation with cofilin; (▲) cofilin only, no BeFx. (*C*) After 0.5, 5, and 20 min incubation of 10 μM BeFx-F-actin with 10 μM cofilin at 22°C it was digested by 0.8 mg/ml trypsin at pH 8.0 for 20, 50, and 80 s. (■) BeFx only; (○) no addition; (▲) 0.5 min incubation with cofilin; (▼) 5 min incubation with cofilin; (●) 20 min incubation with cofilin; (□) cofilin only, no BeFx.

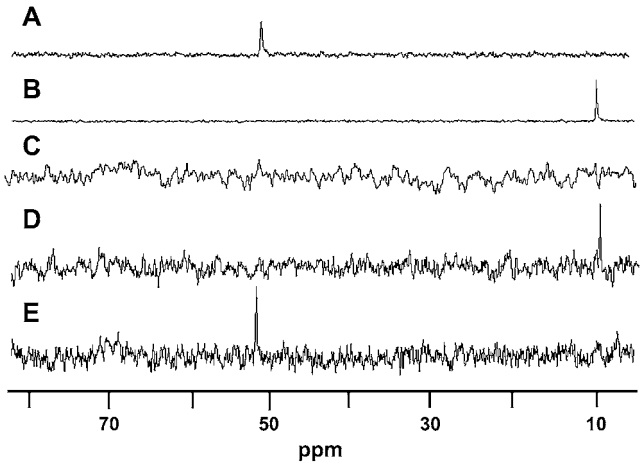

FIGURE 5 Removal of BeFx from BeFx-F-actin. $BeCl_2$ (150 μM) and 5 mM NaF were added to 168 μM F-actin and incubated overnight, on ice. This was followed by dialysis (four changes) against 100-fold volume of F-buffer, pH 8.0, for 2 days. ^{19}F NMR measurements were carried out at 20°C as described in Materials and Methods. (*A*) 0.5 mM fluoride in F-buffer; (*B*) 0.5 mM fluoride in 6% perchloric acid; (*C*) 104.0 μM BeFx-F-actin in F-buffer; (*D*) 104.0 μM BeFx-F-actin in 6% perchloric acid was centrifuged and the supernatant was measured; (*E*) 90.2 μM cofilin was added to 104.0 μM BeFx-F-actin in F-buffer.

were obtained at pH 6.5 with subtilisin and at pH 8.0 with trypsin digestion (data not shown). Thus, although both the weakly and strongly bound BeFx-F-actin complexes are equally well protected against proteolysis in the absence of cofilin, they appear to present different binding environments to cofilin.

Reversal of cofilin effect on F-actin by BeFx

Because cofilin displaces BeFx from F-actin, we tested also the reverse case, i.e., cofilin displacement by BeFx. To this end, cofilin (4.0, 8.0, and 11.0 μM) was added first to TRC-F-actin (10 μM) at pH 6.5 or 8.0, and then mixed with 5 mM NaF and 100 μM $BeCl_2$. As shown in Fig. 8, there was an immediate, cofilin concentration-dependent drop in TRC-F-actin fluorescence (Table 2), reflecting the formation of a complex. This fluorescence decrease was reversed by BeFx slowly, and only to a small extent, at substoichiometric ratios of cofilin/actin (Table 2). The fluorescence intensity recovery decreased with increasing cofilin concentration. At pH 6.5 no

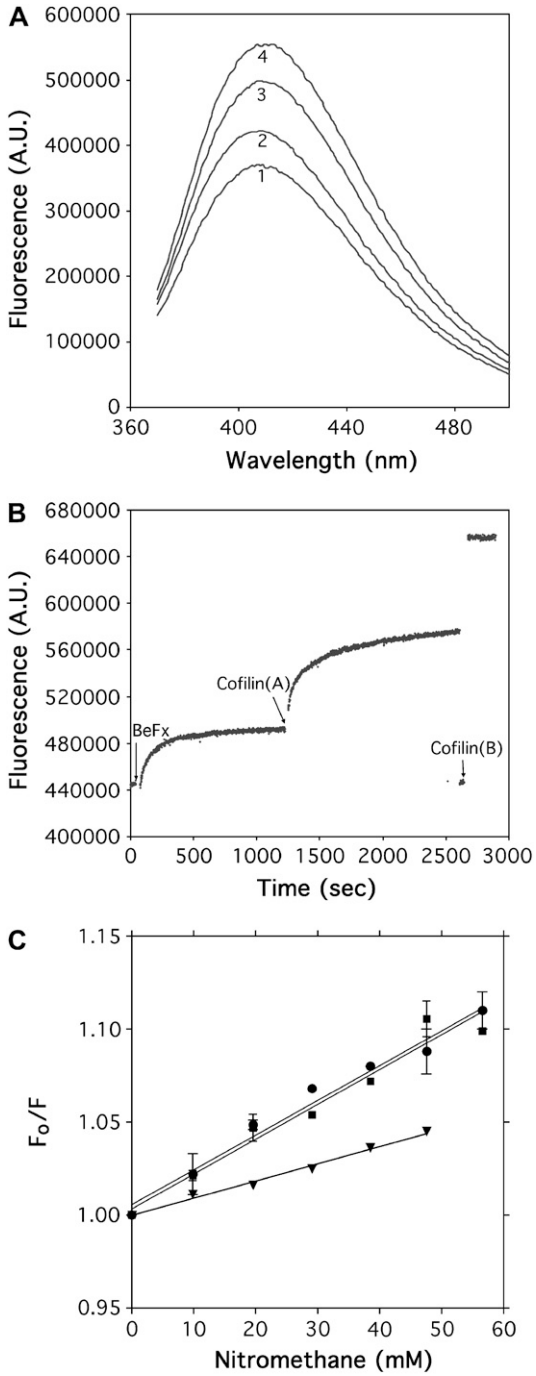

FIGURE 6 Effect of BeFx and cofilin on the fluorescence of ε-ADP-F-actin. To 8 μM ε-ADP-F-actin in pH 8.0 F-buffer 0.1 mM $BeCl_2$ and 5 mM NaF was added, followed by the addition of 10 μM cofilin. (*A*) Spectrum, (*1*) before addition of BeFx; (*2*) 35 min after addition of BeFx; (*3*) 2 h after addition of cofilin to BeFx-ε-ADP-F-actin; (*4*) cofilin added to ε-ADP-F-actin in the absence of BeFx. (*B*) Time course of fluorescence change following BeFx and cofilin additions. BeFx addition is shown by the first arrow; cofilin additions to BeFx-ε-ADP-F-actin and BeFx free ε-ADP-F-actin are indicated by the second (Cofilin A) and third arrow (Cofilin B), respectively. (*C*) Nitromethane was added to 8 μM ε-ADP-F-actin in 10 mM increments in the presence of 10 μM cofilin (▼); in the presence of 0.1 mM BeFx (■); and in the absence of cofilin or BeFx (●). The fluorescence intensity change was monitored at 22°C as described in Materials and Methods.

fluorescence intensity recovery due to BeFx was observed at a saturating cofilin concentration (11 μM; Table 2). Similar conclusion was reached from subtilisin digestion experiments; BeFx did not affect the digestion of F-actin saturated with cofilin (data not shown). These results indicate that BeFx reverses only partially the effect of substoichiometric cofilin on F-actin structure, but not when F-actin is saturated with cofilin. It appears that the binding of BeFx in the nucleotide binding cleft is inhibited in those actin protomers to which cofilin is attached.

Effect of phalloidin on cofilin binding

We found earlier that yeast cofilin, unlike some other AC proteins, binds to phalloidin–F-actin and dissociates phalloidin or rhodamine-phalloidin (17). Here we tested the effect of phalloidin on cofilin binding to F-actin by subtilisin digestion, taking advantage of the strong phalloidin inhibition of the D-loop cleavage (26). Phalloidin, 12 μM, was added to 10 μM F-actin containing 5 or 12 μM cofilin and after 1.5 or 22 h incubation at pH 8.0 the samples were digested with subtilisin. We also reversed the order of additions, adding phalloidin first and cofilin second. The results of such digestions in the presence of 12 μM cofilin are presented in Fig. 9. The digestion pattern clearly shows that the addition of phalloidin to cofilin-F-actin decreases, whereas the addition of cofilin to phalloidin F-actin increases the rate and extent of subtilisin cleavage. However, the extent of actin cleavage after 90 min incubation (Fig. 9 *A*) was greater when phalloidin was added to cofilin-F-actin than in the case of a reversed order of additions, when cofilin was added to phalloidin-F-actin. Such digestion differences disappeared after 22 h incubation (Fig. 9 *B*), showing the slow equilibration of this system. These results indicate that phalloidin, unlike BeFx or inorganic phosphate (25), binds to F-actin also in the presence of cofilin.

DISCUSSION

The structural phosphate analog BeFx (2) binds strongly to F-actin, stabilizing its structure, and according to several reports prevents the binding of some ADF/cofilin proteins (*Acanthamoeba* actophorin (19), human cofilin (20), and plant ADF (13)) to F-actin. We found in this study that in contrast to above-mentioned three members of the AC family, yeast cofilin binds to BeFx-F-actin, albeit at a much slower rate than to F-actin. This feature of yeast cofilin indicates that it has a higher affinity to F-actin than those AC family members that do not bind to F-actin in the presence of BeFx. The high affinity of yeast cofilin to F-actin may have physiological significance. We took advantage of this property of yeast cofilin and examined its binding to BeFx-actin to gain further insight into the changes caused in actin structure by this phosphate analog.

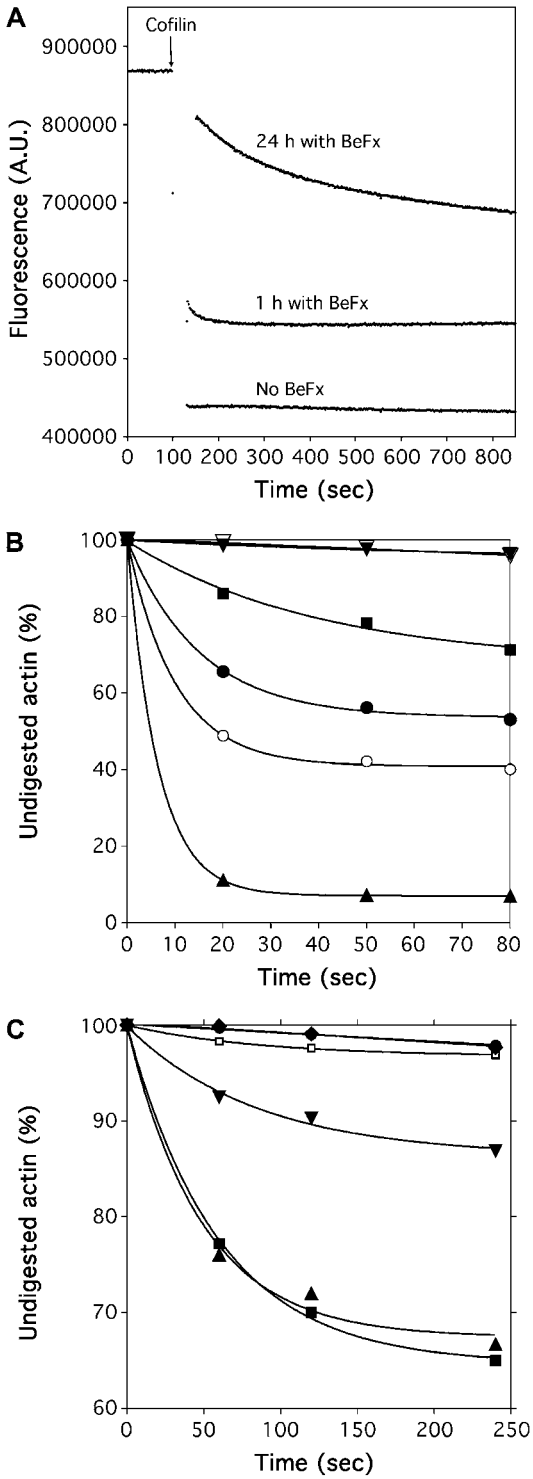

FIGURE 7 Dependence of cofilin binding on the incubation time of TRC-F-actin with BeFx. TRC-F-actin, 10 μM, was incubated with 60 μM $BeCl_2$ and 5 mM NaF in pH 8.0 F-buffer for 1 and 24 h on ice. (*A*) 5.6 μM cofilin was added to 10 μM TRC-F-actin (*arrow*) and the time course of fluorescence intensity change was monitored at 22°C as described in Materials and Methods. (*B*) 10 μM TRC-F-actin, which had been incubated with BeFx, was mixed with 12 μM cofilin and digested by 25 μg/ml subtilisin for 20, 50, and 80 s at 22°C. The samples were run on SDS-PAGE and analyzed by densitometry. (▽) 1-h incubation with BeFx, no cofilin; (▼) 24-h incubation with BeFx, no cofilin; (○) 1-h incubation with BeFx, then cofilin added; (●) 24-h incubation with BeFx, then cofilin added; (■) no addition; (▲) cofilin only, no BeFx. (*C*) Effect of incubation time of BeFx with F-actin on its digestion with subtilisin. F-actin, 10 μM, was incubated with 60 μM BeFx or 5 mM NaF for various time intervals, and then was digested with 50 μg/ml subtilisin for 60, 120, and 240 s at 22°C at pH 8.0. Digestion samples were run on SDS-PAGE and analyzed by densitometry. (■) no BeFx; (▲) 1-h incubation with 5 mM NaF; (▼) 0.5-min incubation with BeFx; (□) 5-min incubation with BeFx; (♦) 1-h incubation with BeFx; (●) 24-h incubation with BeFx.

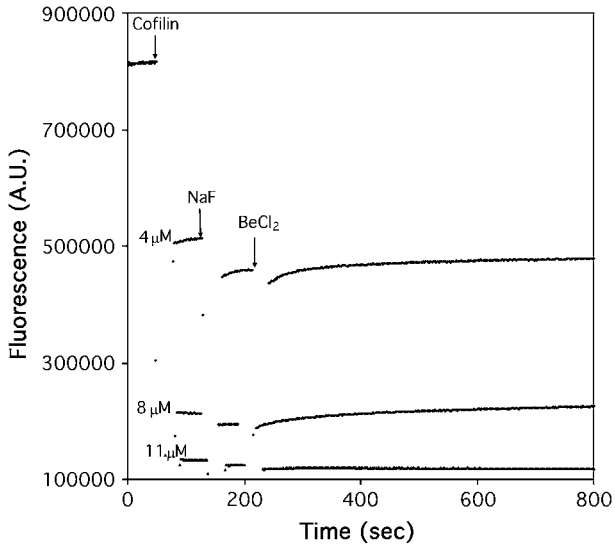

FIGURE 8 Reversal of cofilin effect on the fluorescence of TRC-F-actin by BeFx. To 10 μM TRC-F-actin in pH 6.5 F-buffer 4.0–11.0 μM cofilin was added (*arrow*) after the initial reading. This was followed by addition of 5 mM NaF (*second arrow*) and 100 μM $BeCl_2$ (*third arrow*) and the time course of fluorescence intensity change was monitored at 22°C, as described in Materials and Methods.

In agreement with Ressad et al. (27), we found that the binding of cofilin is faster at low than at high pH (25), although the depolymerizing effect is stronger at the high pH (28). In the presence of BeFx the binding rates at pH 8.0 and pH 6.5 are much closer (Table 1). It is possible that the nature of the beryllium fluoride complex that binds to F-actin contributes to the change in the rate difference at the two pH values. According to Combeau and Carlier (29), at low pH only BeF_3^- binds, whereas at high pH both BeF_3^- and $BeF_2(OH)^-$ bind to F-actin (only ions with a single negative charge bind to the nucleotide-binding cleft of actin). BeF_3^- may bind stronger to F-actin than $BeF_2(OH)^-$ thereby, inhibits more effectively cofilin binding, which would explain the relatively stronger inhibition of cofilin binding at low pH. Similar conclusion was reached by studying the activation of transducin by the above two beryllofluoride complexes (30).

We monitored the binding of cofilin to F-actin by following the decrease in fluorescence intensity of the TRC group

TABLE 2 Change in fluorescence intensity of 10 μM TRC-F-actin upon addition of cofilin and 0.1 mM BeFx at pH 6.5 and 8.0

Cofilin μM*	pH	Fluorescence decrease (a.u.) upon cofilin addition	Fluorescence increase (a.u.) upon BeFx addition after cofilin	Fluorescence increase by BeFx in % of the cofilin-induced decrease
4	6.5	3.79	0.46	12.1
8	6.5	6.23	0.49	7.9
11	6.5	6.96	0.00	0.0
4	8.0	3.42	0.71	20.8
8	8.0	5.38	0.76	14.1
11	8.0	6.39	0.3	4.7

*Data are taken from the experiment shown in Fig. 8.

attached to Gln-41. The rate of cofilin binding was found to increase with increasing cofilin concentration without reaching a plateau even at 10:1 cofilin/actin molar ratio. This indicates that cofilin binds to BeFx containing F-actin protomers and the BeFx dissociation does not limit the rate of cofilin binding. This conclusion is supported by the results of Combeau and Carlier (3), who found by two methods (rapid dialysis and chasing out ^{7}Be with unlabeled Be) that the rate of dissociation of BeFx from F-actin is extremely slow $\sim 10^{-6}\ s^{-1}$. We suggest that cofilin binding to BeFx containing protomers causes conformational changes at the BeFx binding site in the nucleotide-binding cleft. These changes induce dramatic decrease in the affinity of BeFx to F-actin, leading to its dissociation.

We studied the effects of BeFx and cofilin on the nucleotide-binding cleft of F-actin also by detecting changes in the fluorescence of ε-ADP bound to F-actin. The different effects of these two ligands on ε-ADP fluorescence and collisional quenching indicate that the structural changes induced by the two ligands in the cleft are different. The BeFx induced ε-ADP fluorescence intensity increase is consistent with the proposal of Combeau and Carlier (3,29) that BeFx is located at the place of γ-phosphate of ATP in the nucleotide-binding cleft of actin. The incomplete fluorescence intensity increase by cofilin in the presence of BeFx is presumably due to the residual bound BeFx inhibiting cofilin's effect on the conformation of the nucleotide-binding cleft. This finding is consistent with the effect of cofilin on the extent of proteolysis of the subdomain 2 of F-actin in the presence and absence of BeFx.

The binding of cofilin to F-actin in the presence of BeFx was also monitored by the increase in proteolytic susceptibility of subdomain 2. Since cofilin increases while BeFx decreases proteolytic susceptibility, this method also measures BeFx dissociation. The results of proteolytic experiments show that the dissociation of BeFx is not complete even 20 min after the addition of cofilin (Fig. 4, *B* and *C*), when according to the fluorescence measurements F-actin is fully saturated with cofilin. This indicates that after 20 min incubation with cofilin there are actin protomers to which BeFx and cofilin are simultaneously bound. The F-actin-bound residual BeFx inhibits considerably the proteolysis of subdomain 2 due to its strong cooperative effect on F-actin structure (4).

The binding of BeFx to F-actin is a relatively slow, two-step process (3). The first step is a fast equilibrium binding, which is followed by a slow isomerization step accompanied by a conformational change. We measured the rate of BeFx binding to F-actin by following the increase in the fluorescence intensity of ε-ADP-F-actin and found that it takes $\sim$5 min for the fluorescence to reach the plateau after addition of BeFx (Fig. 6 *B*). About the same time is needed for F-actin to become fully resistant to proteolysis upon addition of BeFx (Fig. 7 *C*). However, cofilin binding experiments revealed that further changes occur in the structure of BeFx-F-actin, even after it became resistant to proteolysis. We showed that the binding of cofilin to BeFx-F-actin is also a two-step process consisting of a fast and a slow step. The amplitude of the fast step decreases with the time of incubation with BeFx; it is much smaller after 24 h than 1 h incubation with BeFx (Fig. 7 *A*). The ratio of the amplitudes of the fast and slow steps also depends on BeFx concentration; with the relative size of the first step decreasing with increasing BeFx concentration. We associate the fast and slow steps with the low and high affinity BeFx-F-actin complex, respectively. Since both complexes are resistant to proteolysis and their ε-ADP fluorescence is increased, we conclude that cofilin binding detects a second isomerization step in the BeFx-F-actin interaction, according to Scheme 1:

$$\text{BeFx} + \text{F-actin} \leftrightarrow \text{BeFx-F-actin} \leftrightarrow \text{BeFx-F-actin}^{*} \leftrightarrow \text{BeFx-F-actin}^{**} \qquad \text{(Scheme 1)}$$

BeFx-F-actin is the fast equilibrium complex and BeFx-F-actin* and BeFx-F-actin** are the low- and high-affinity BeFx-F-actin complexes, respectively. A structural difference between the two complexes is suggested by the different degree of inhibition of cofilin binding. It appears that the same beryllium fluoride species binds to F-actin in the various BeFx-F-actin complexes, because the transformations between these complexes are not pH dependent.

It is interesting to compare the BeFx-F-actin complexes with the complexes of Pi-F-actin. According to Combeau and Carlier (3) there are two ADP-Pi-F actin complexes. Pi dissociates slowly from the ADP-Pi-F-actin* complex, which is a product of ATP hydrolysis accompanying actin polymerization, while it dissociates fast from the ADP-Pi-F actin complex, which is produced upon addition of Pi to

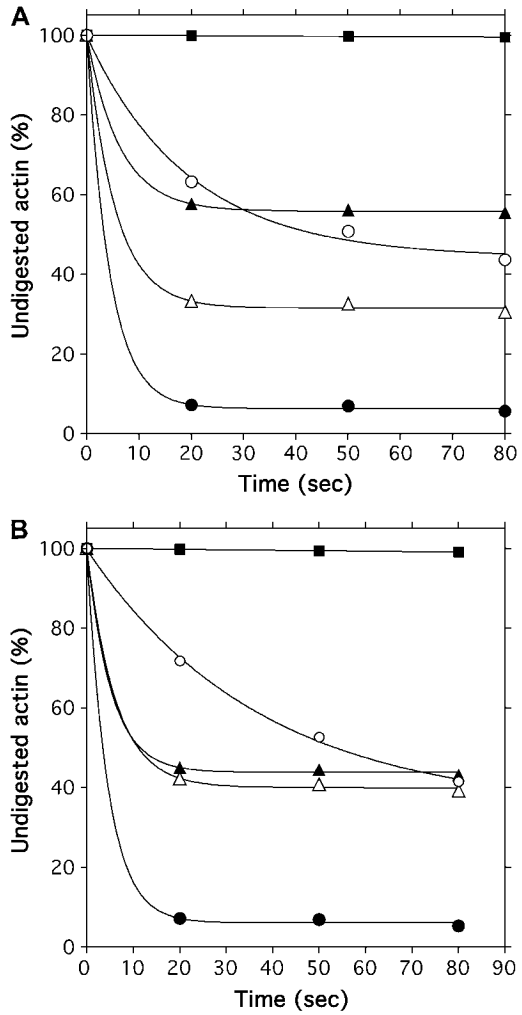

FIGURE 9 Phalloidin reverses the effect of cofilin on the subtilisin digestion of F-actin. F-actin (10 μM) was incubated with cofilin (12 μM) for 5 min in F-buffer, at pH 8.0. Phalloidin (12 μM) was added to that sample, and after 90 min incubation at room temperature (*A*), or 22 h incubation on ice (*B*), the samples were digested with 25 μg/ml subtilisin for 20, 50, and 80 s at 22°C, and then run on SDS-PAGE and analyzed by densitometry. In the reversed order experiment, 12 μM phalloidin was added first to F-actin, and after 5 min this was followed with the addition of 12 μM cofilin. (○) No addition; (■) only phalloidin added; (▲) phalloidin added first then cofilin; (△) cofilin added first then phalloidin; (●) only cofilin added.

ADP-F-actin. The two complexes are connected by an isomerization step, which limits the transformation of ADP-Pi-F-actin* to ADP-Pi-F actin according to Scheme 2.

$$\text{ATP-F-actin} \leftrightarrow \text{ADP-Pi-F-actin}^{*} \leftrightarrow \text{ADP-Pi F-actin} \leftrightarrow \text{ADP-F-actin} + \text{Pi} \qquad \text{(Scheme 2)}$$

The reversal of the isomerization step is extremely slow and thus, essentially only the ADP-Pi-F actin complex is obtained upon adding Pi to ADP-F-actin. We may assume that BeFx-F-actin** could be similar to the ATP-F-actin and BeFx-F-actin* to the ADP-Pi-F-actin* complex. This speculation is supported by the results of Combeau and Carlier (3) on the effect of BeFx on the fluorescence of pyrene-labeled F-actin. However, this hypothesis needs to be corroborated with further evidence.

Cofilin binding induces the dissociation of BeFx from F-actin, similarly to that of phalloidin (17). The removal of BeFx from actin indicates that cofilin causes allosteric conformational changes also in the nucleotide-binding cleft of F-actin where the BeFx is bound. Cofilin-induced conformational changes in the nucleotide cleft were indicated also by decreased phosphate affinity, changed fluorescence emission spectra, and decreased accessibility of F-actin-bound ε-ADP to collisional quenchers (Fig. 6 and Muhlrad et al. (25)).

BeFx cannot bind to F-actin when the filaments are fully saturated with cofilin as shown by the inability of BeFx to reverse the cofilin-induced changes in the fluorescence intensity and proteolytic susceptibility. This indicates lower probability for the initial complex formation (BeFx-F-actin) and its reduced isomerization when the nucleotide-binding cleft of F-actin is allosterically changed by cofilin (Scheme 1). Cofilin probably inhibits the first isomerization step of BeFx, as the proteolytic susceptibility of the F-actin-cofilin complex remains high after the addition of BeFx. The effect of cofilin on BeFx binding is not cooperative; because the binding appears to be prevented only in those protomers of F-actin that are saturated with cofilin.

Phalloidin, unlike BeFx, can bind to cofilin-F-actin, which is fully saturated with cofilin. It competes with cofilin for F-actin and very slowly reverses the cofilin-induced disorder in the D-loop of subdomain 2 of F-actin. The different effect of BeFx and phalloidin on cofilin-F-actin is probably due to their binding to different sites on F-actin (3,7,8), and to the higher affinity of phalloidin to F-actin: ($K_d = 2.1$ nM; (31)) than BeFx ($K_d = 2$ μM; (3)) to F-actin.

In contrast to *Acanthamoeba* actophorin, plant ADF, and human cofilin (19,13,20), yeast cofilin not only binds to F-actin in the presence of phalloidin or BeFx, but also facilitates their dissociation (Fig. 5; (17)). The binding of cofilin to BeFx-F-actin revealed the existence of two types of BeFx-F-actin complexes, which transform to each other. The transformation of the weakly to the strongly bound complex is a very slow process, while the preceding step, of the initial complex formation, depends also on BeFx concentration. It is conceivable that the transformation of the complexes is accompanied by the movement of BeFx in the nucleotide binding cleft, which affects the conformation of the cleft and allosterically induces changes in the stability of the actin filament.

Overall, our studies resulted in several findings. By taking advantage of the high affinity of yeast cofilin to F-actin, which enables this cofilin to bind to F-actin also in the presence of BeFx or phalloidin, the antagonistic effects of the above factors were described quantitatively in this study. The existence of two BeFx-complexes with different stabilities was suggested by the pattern of cofilin binding to BeFx-F-actin.

These complexes mimic different Pi-ADP-F-actin complexes, which exist during the hydrolysis of the F-actin bound ATP and upon the addition of Pi to ADP-F-actin. The study of the two BeFx-F-actin complexes may help to reveal the structure of functionally important Pi-F-actin adducts. Cofilin was found to affect the conformation of the nucleotide binding cleft of F-actin in addition to its influence on the structure of the DNase I binding loop and the 60–69 loop in subdomain 2. This effect of cofilin is manifested in the removal of BeFx from the nucleotide binding cleft, in the increase of F-actin bound ε-ADP fluorescence, a decrease in the accessibility of bound ε-ADP to collisional quencher, and the prevention of the tight BeFx binding. BeFx also increases the fluorescence intensity of the bound ε-ADP, which supports the earlier findings (3,29) that BeFx binds in the nucleotide cleft at the site of γ-phosphate of ATP. The detection of the unique effects of cofilin and the Pi analog, BeFx, on the actin structure will contribute to the understanding of the regulation of the cellular actin dynamics by AC-proteins and inorganic phosphate.

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