The actin-severing activity of cofilin is exerted by the interplay of three distinct sites on cofilin and essential for cell viability

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Cofilin/actin-depolymerizing factor is an essential and conserved modulator of actin dynamics. Cofilin binds to actin in either monomeric or filamentous form, severs and depolymerizes actin filaments, and speeds up their treadmilling. A high turnover rate of F-actin in actin-based motility seems driven largely by cofilin-mediated acceleration of directional subunit release, but little by fragmentation of the filaments. On the other hand, the filament-severing function of cofilin seems relevant for the healthy growth of cells. In this study, we have characterized three mutants of porcine cofilin to elucidate the molecular mechanism that underlies the filament-severing activity of cofilin. The first mutant could neither associate with actin filaments nor sever them, whereas it effectively accelerated their treadmilling and directional subunit release. The second mutant bound to actin filaments, but failed to sever them and to interfere with phalloidin binding to the filament. The third mutant could associate with actin filaments and sever them, although with a very reduced efficacy. Of these mutant proteins, only the last one was able to rescue Δ*cof1* yeast cells and to induce thick actin bundles in mammalian cells upon overexpression. Therefore, the actin-severing activity of cofilin is an essential element in its vital function and suggested to be exerted by co-operation of at least three distinct sites of cofilin.

Key words: actin-binding protein, cell motility, cell survival, cytoskeleton, destrin.

INTRODUCTION

Cofilin/actin-depolymerizing factor (ADF) is a key regulator of actin dynamics and indispensable for viability in all eukaryotic organisms examined so far [1–5]. Many pieces of evidence have substantiated crucial roles of cofilin in cell division [5,6], cell motility [7–9], endocytosis [10] and muscle development [3]. Cofilin is also implicated in cellular stress responses, as it forms actin/cofilin rods upon administration of kinds of environmental stress to cultured cells [11]. Biochemically, cofilin binds to actin in a 1:1 molar ratio, in either monomeric (G-actin) or filamentous (F-actin) form [12]. Cofilin severs actin filaments and depolymerizes them in a pH-dependent manner [13]. These effects of cofilin are inhibited reversibly by phosphorylation in higher eukaryotes [14,15], as well as by phosphoinositide binding [16].

A couple of intriguing activities of cofilin have recently become the foci of arguments. Cofilin dramatically accelerates the treadmilling (or turnover) of actin filaments, a driving force behind actin-based motility [8,9]. The other unique property of cofilin is induction of a large structural change in the actin filament accompanying its lateral association [17,18], which seems responsible for cofilin-induced severing of the filament. However, the mechanism of cofilin-mediated acceleration of the directional subunit release is still unknown. It is also unclear how cofilin changes the structure of the actin filament, such as increasing the filament twist. In addition, the causal relationship between these two effects has not yet been resolved conclusively. We have demonstrated previously that the two activities of cofilin are differentially contributed to by the unusual consecutive hydrogen bonds within the longest helix of porcine cofilin [19]. In this study, we have analysed the other two mutants of porcine cofilin, which harbour characteristic functional defects, and compared them with a previously reported mutant, which possesses enormously reduced filament-severing activity (A120-cofilin). Based on extensive biochemical analyses, we propose a novel mechanism of cofilin action, which finally leads to fragmentation of the actin filament.

Another subject of this study was to determine whether the naturally weak activity of cofilin to sever actin filaments is dispensable for cell viability or not. One of the most important aspects of cofilin is that this protein is essential for viability in *Saccharomyces cerevisiae* [1,2], *Dictyostelium discoideum* [4], *Drosophila melanogaster* [5] and *Caenorhabditis elegans* [3], and it must be true for all eukaryotes, whereas the functional loss of other actin-binding proteins caused relatively milder phenotypes. It is, however, little known why cofilin is essential for cell survival. Actin-based motility depends on cofilin-mediated acceleration of the directional subunit release, and it seems that little, if any, contribution to this process is made by the naturally weak filament-severing activity of cofilin [8]. This may be true, but cofilin is involved in various kinds of cellular event, as mentioned above. We have shown previously that a cofilin mutant defective in filament severing still supported cell growth in *S. cerevisiae*, despite conferring very slow growth and temperature-sensitivity [19]. In this study, we found that this mutant of cofilin can still sever actin filaments, although far less effectively than wild-type cofilin. Further comparative works with the three cofilin mutants have led us to the conclusion that this activity of cofilin is in fact strictly required for cell growth. Acceleration of filament turnover or association with F-actin is, by itself, not sufficient for cofilin to support cell survival.

Abbreviations used: ADF, actin-depolymerizing factor; pyrene-actin, *N*-(1-pyrene)iodoacetamide-labelled actin; ε ATP, 1,N^6-ethenoadenosine 5′-triphosphate.

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EXPERIMENTAL

Plasmid construction and protein expression

pQE-cof.His and pQEH-cof were used for the bacterial production of porcine cofilin carrying a hexahistidine tag (His tag) at the C- and the N-terminus, respectively [15]. The N-terminal deletion mutant of cofilin (ΔN5-cofilin) was created using the oligonucleotide 5′-C TCC tct aga GCT GTC TCT GAC GGG GTC-3′ and recombined at the *Xba*I site (represented by the lower-case letters). The resulting ΔN5 proteins had the N-terminal sequences MASHHHHHHSRA (N-terminally His-tagged cofilin) and MARA (C-terminally His-tagged cofilin; the final A corresponds to Ala-6 of the original cofilin sequence). Other site-specific mutants were created previously [15,19]. Mutant cofilins were expressed in *Escherichia coli* BL21(Rep4) and purified as described in [15].

Subunit-exchange assay

Preparation of *N*-(1-pyrene)iodoacetamide-labelled actin (pyrene-actin) and the method of fluorescence measurement were described previously [19,20]. Mg^{2+}-bound actin (4.2 μM) was polymerized in the presence or absence of 12 nM gelsolin. Fully polymerized actin (30 μl) was removed and added to 30 μl of 10 mM Hepes/KOH/16 mM Tris/HCl/2 mM $MgCl_2$/100 mM KCl/0.5 mM EGTA/0.1 mM dithiothreitol (pH 7.2) containing mutant cofilin. Then it was mixed with 60 μl of 0.4 μM pyrene-actin (10 % labelled) that had been converted into its MgATP-bound form just before mixing. The incorporation of pyrene-actin into unlabelled F-actin was monitored by a PerkinElmer LS-50B Luminescence spectrophotometer.

Observation of actin-turnover kinetics

The rate of actin-filament turnover (treadmilling) was assessed using 1,N^6-ethenoadenosine 5′-triphosphate (ϵATP)-labelled F-actin as described previously [20]. ϵATP-bound actin (6 μM) was converted into its Mg^{2+}-bound form and polymerized in the presence of mutant cofilin in 7 mM Hepes/KOH/10.5 mM Tris/HCl/2 mM $MgCl_2$/100 mM KCl/10 μM ϵATP/0.5 mM EGTA/0.1 mM dithiothreitol (pH 7.2). After a steady state (apparent equilibrium) was reached, ATP was added at a final concentration of 0.25 mM to chase the bound ϵATP, and the decreasing fluorescence was recorded by measuring emission at 410 nm and excitation at 360 nm.

Dilution-induced depolymerization assay

Mg^{2+}-bound actin (4 μM; 5 % pyrene-labelled) was polymerized for 12 h at 20 °C. Depolymerization was initiated by a 12-fold dilution in 10 mM Hepes/KOH/16 mM Tris/HCl/2 mM $MgCl_2$/100 mM KCl/0.5 mM EGTA/0.1 mM dithiothreitol (pH 7.2) containing mutant cofilin. The decline in fluorescence was monitored immediately.

Other methods

Vitamin-D-binding-protein-induced depolymerization of gelsolin-capped actin filaments, co-sedimentation with F-actin, gel densitometry and falling-ball viscometry were performed as described previously [19]. Binding of rhodamine-labelled phalloidin to F-actin was examined using the method of Nishida et al. [11]. The methods used for yeast plasmid shuffling and immunofluorescent cell staining were described previously [15].

RESULTS

Mutational dissection of biochemical activity of cofilin

In a previous study with the aim of identifying the phosphorylation site of porcine cofilin [15], we introduced a series of point mutations that separately converted individual serine residues of cofilin into alanines or aspartic acids. We then identified Ser-3 as the unique phosphorylation site of cofilin using these mutations [15]. Among them, Ala-3, Asp-3, Asp-94, Asp-119, Ala-120 and Asp-120 substitutions were found to affect biochemical and/or cellular functions of cofilin ('A3-cofilin' and 'D3-cofilin' represent the mutant cofilins in which Ser-3 was replaced with alanine and aspartic acid, respectively, and other mutant proteins are designated in the same manner). The properties of A3- and D3-cofilins were reported in [15], and D119-cofilin and D120-cofilin were found to exhibit close biochemical similarities to D3-cofilin and A120-cofilin, respectively (results not shown). We report here on the other mutants, the D94- and A120-cofilins. In addition, another mutant cofilin, lacking the N-terminal five residues (ΔN5-cofilin), is also described.

First, association of each mutant cofilin with F-actin was examined (Figures 1 and 2). Figure 1 illustrates profiles of co-sedimentation of the N-terminally His-tagged cofilin mutants with polymerized actin as well as demonstrating purity of these

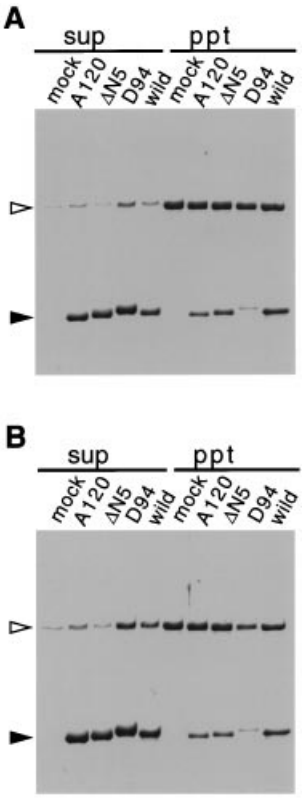

Figure 1 Profiles of co-sedimentation of His_6-tagged cofilin mutants with polymerized actin

Actin (4.5 μM) was polymerized for 60 min and reacted in the absence (mock) or presence of 10 μM wild-type (wild) or mutant cofilin carrying a His tag at the N-terminus at pH 7.0 (**A**) or 8.3 (**B**). After 75 min, F-actin and bound cofilin were sedimented by centrifugation, and supernatant (sup) and sediment (ppt) were resolved by SDS/PAGE. Electrophoretic positions of actin (open arrowheads) and cofilin (closed arrowheads) are shown.

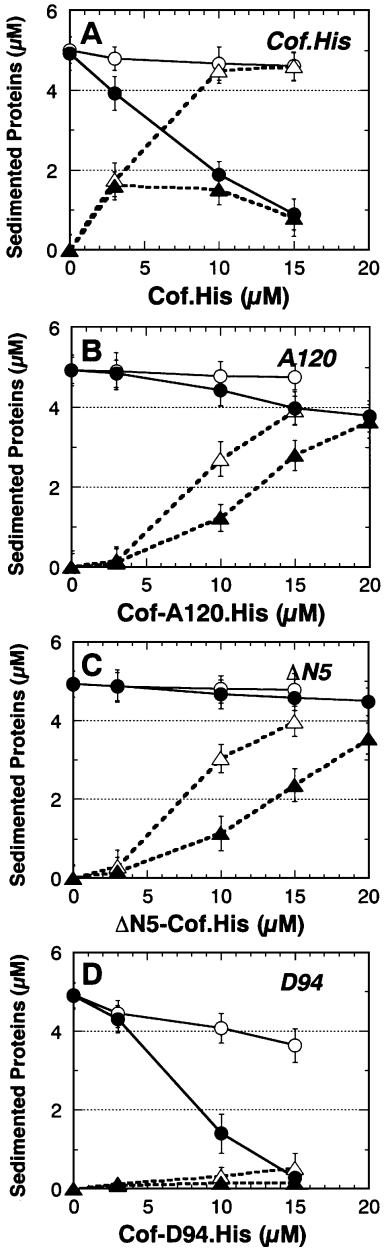

Figure 2 Ability of cofilin mutants to associate with and depolymerize F-actin

Actin (5 μM) was polymerized and reacted with wild-type (**A**), A120 (**B**), ΔN5 (**C**) and D94 (**D**) mutant cofilins carrying a His tag at the C-terminus. The reaction mixture was processed as described for Figure 1. The amount of actin and cofilin in the sediment was quantified by scanning the SDS gels with a densitometer, and converted into concentration in the original volume. The concentrations of sedimented actin (●, ○) and cofilin (▲, △) were plotted as a function of total cofilin concentration. The open symbols represent the data obtained at pH 7.0, and closed symbols show those at pH 8.3. The error bars indicate the maximum deviations from the representative data from three separate experiments.

proteins. Mutant cofilins carrying a His tag at their C-termini, instead of the N-termini, were also subjected to the co-sedimentation assay, and the results obtained are displayed as more quantitative plots in Figure 2. The A120- and ΔN5-mutant cofilins bound to F-actin with comparable efficacy, but less efficiently than the corresponding wild-type cofilin (Figures 1 and 2A–C, triangles). In clear contrast, D94-cofilins bound poorly with F-actin (Figures 1 and 2D, triangles). The data from these experiments also illustrate the ability of the mutant proteins to decrease the steady-state amount of polymerized actin. Actin depolymerization by cofilin is pH-dependent and more evident at higher pH values [13]. This is true for the porcine cofilin carrying a His tag [15,19] and it was also verified here (Figures 1 and 2A, circles). The actin-depolymerizing effects of A120- and ΔN5-cofilins were found to be much weaker than those of wild-type cofilins, especially at a higher pH values (Figures 1B and 2A–C, ●). Interestingly, D94-cofilin depolymerized F-actin no less than wild-type cofilin (Figures 1B and 2D, circles), despite lacking significant association with F-actin (Figures 1B and 2D, triangles). The mutant cofilins used in Figure 1 and those in Figure 2 had the His tag at the opposite terminus of cofilin, and every corresponding pair of mutant cofilins exhibited similar activity, no matter which terminus the His tag was attached to, although the N-terminal tag might slightly diminish the actin-depolymerizing efficiency. Thus addition of the His tag did not appear to influence their activity significantly.

Next, these mutant cofilins were tested for the activity to induce fragmentation of actin filaments (Figure 3). ΔN5- and D94-cofilins did not reduce the viscosity of the F-actin solution (Figure 3A), indicating their inability to sever actin filaments. A120-cofilin was shown previously to have little actin-severing activity [19]. When the amount of this mutant cofilin was much increased, however, it actually reduced the viscosity of the F-actin solution (Figure 3A). Such a drop in viscosity does not necessarily reflect fragmentation of the filament. It may result from efficient nucleation of actin polymerization, considerable depolymerization of F-actin or capping of the filament end(s). In order to clarify whether A120-cofilin actually severs the filament, we observed the subunit exchange between the gelsolin-capped actin filaments (unlabelled) and the pyrene-labelled G-actin pool (Figure 3B). If the filament is severed, new barbed ends are generated and the subunit exchange is stimulated, resulting in more rapid incorporation of the labelled G-actin into the filament. The wild-type cofilin remarkably increased the maximal rate of incorporation of pyrene-actin (Figure 3B). A120-cofilin also promoted the subunit exchange, although less efficiently than wild-type cofilin (Figure 3B). Thus A120-cofilin preserves the ability to sever actin filaments. ΔN5-cofilin exhibited little, if any, stimulation of the subunit exchange (Figure 3B), and D94-cofilin rather suppressed it (Figure 3B). These results indicate that ΔN5- and D94-cofilins have little ability to sever actin filaments, consistent with the above results from the viscometry (Figure 3A).

The observed differences between A120- and ΔN5-cofilins in Figure 3 suggest distinct modes of binding to actin filaments, in spite of their similar affinities for F-actin, as seen in Figures 1 and 2. Cofilin is known to prevent several actin-binding proteins, such as tropomyosin and myosin, from binding to actin filaments [12]. Cofilin-decorated fixed filaments are also resistant to association of phalloidin [11], which is considered to result from one or more of the structural changes in the filament [17,21,22]. We then examined whether A120- and ΔN5-cofilins could interfere with phalloidin in binding to F-actin. A120-cofilin prevented the association of phalloidin with the filament, although not so effectively as the wild-type cofilin (Figure 3C). In contrast, phalloidin co-sedimented efficiently with the ΔN5-cofilin-decorated actin filaments (Figure 3C). Thus the mode of binding of ΔN5-cofilin to the side of the actin filament is clearly distinct from those of wild-type cofilin and A120-cofilin. These results suggest strongly that the N-terminal few residues of

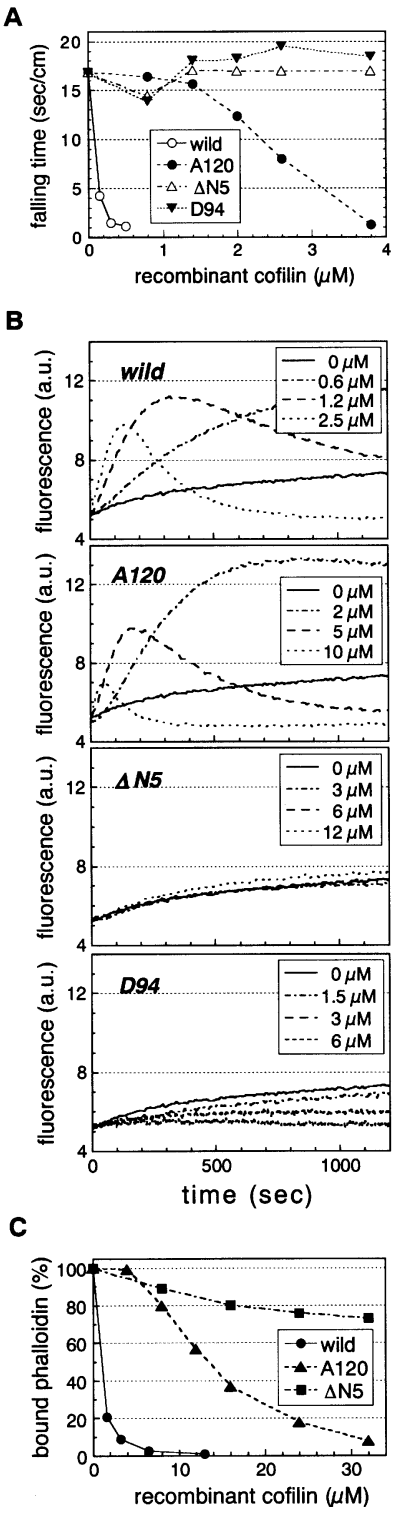

Figure 3 Ability of cofilin mutants to sever actin filaments

(**A**) Effect of mutant cofilin on viscosity of F-actin solution. Low-shear viscosity of the solution was assessed using a miniature falling-ball apparatus. Actin (3.4 μM) was polymerized for 4 h in 50 μl capillary tubes in the presence of various concentrations of wild-type or mutant cofilin carrying a C-terminal His tag at pH 7.0. Viscosity was measured as the mean time required for the ball to fall by 1 cm. (**B**) Subunit-exchange assay using gelsolin-capped F-actin seeds. Actin (4.2 μM) was polymerized in the presence of 12 nM gelsolin and mixed with wild-type or mutant cofilin, then an equal volume of 0.4 μM Mg^{2+}-bound pyrene-actin (10% labelled) was added immediately. The incorporation of pyrene-actin into unlabelled actin filaments was monitored as the change in fluorescence. Pyrene-labelled actin is more fluorescent when it is in F-actin than it is in its monomeric state [35]. The keys in each panel show the cofilin concentrations in μM. In the presence of cofilin, the intensity of fluorescence peaked after an initial increase, then gradually decreased because cofilin promoted subunit release from the pointed end as well as partially quenching the pyrene fluorescence of the bound ADP-actin in the filaments. (**C**) Effect of mutant cofilin on phalloidin binding to F-actin. Actin (3.2 μM) was polymerized, mixed with the indicated amount of each mutant cofilin and fixed with glutaraldehyde. Then the solution was supplemented with rhodamine-conjugated phalloidin (0.2 μM) and centrifuged to sediment the fixed F-actin and the bound phalloidin. The amount of bound phalloidin was calculated from the residual fluorescence of the supernatant and plotted assuming the amount of bound phalloidin in the absence of cofilin as 100%. a.u., arbitrary units.

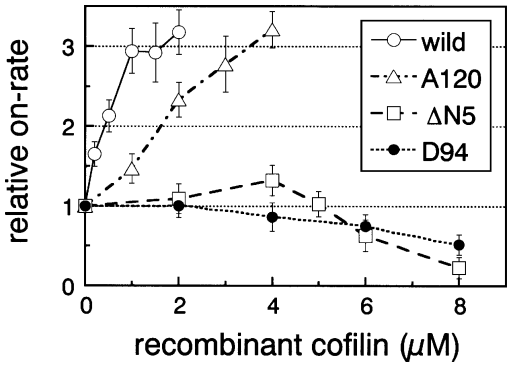

Figure 4 Subunit-exchange assay using F-actin seeds with free barbed ends

Mg^{2+}-bound actin (4.2 μM) was polymerized and mixed with wild-type or mutant cofilin carrying a His tag at the C-terminus, then an equal volume of 0.4 μM Mg^{2+}-bound pyrene-actin (10% labelled) was immediately added. The incorporation of pyrene-actin into unlabelled filaments was monitored as the time-dependent increase in fluorescence, as was done in Figure 3(B). The maximal rates of fluorescence increase were calculated and plotted as relative values. The error bars indicate the maximum deviations from the representative data from three repeated measurements.

cofilin are required to induce a unique structural change in actin filaments prior to their fragmentation.

The results in Figure 3(B) suggest that D94-cofilin inhibits subunit addition to the pointed end of the actin filament. We next examined whether D94-cofilin sequesters actin monomers so strongly as to prevent them from re-assembly on to free barbed ends of actin filaments. When non-capped F-actin seeds were used for measuring the rate of subunit incorporation (Figure 4), inhibition by this mutant cofilin was much weaker than when the barbed end-capped seeds were used. Thus D94-cofilin functions as a buffering factor for actin monomers, but not as a monomer-sequestering factor. Wild-type and A120-cofilins, as expected from the results of Figure 3, promoted subunit incorporation into actin filaments through fragmentation of the filaments (Figure 4). In contrast, the activity of ΔN5-cofilin was somewhat peculiar. A moderate amount of ΔN5-cofilin exhibited only little effect on the subunit-exchange rate, but a large amount of it markedly decreased the rate of subunit exchange (Figure 4). Such an inhibition may reflect the potential barbed-end capping by ΔN5-cofilin rather than the result of monomer buffering, because a comparable amount of it did not inhibit subunit addition to gelsolin-capped filaments (Figure 3B). In addition, ΔN5-cofilin reduced the initial rate of the dilution-induced depolymerization of non-capped actin filaments (Figure 5B), also suggesting the presence of a barbed-end-directed effect of ΔN5-cofilin.

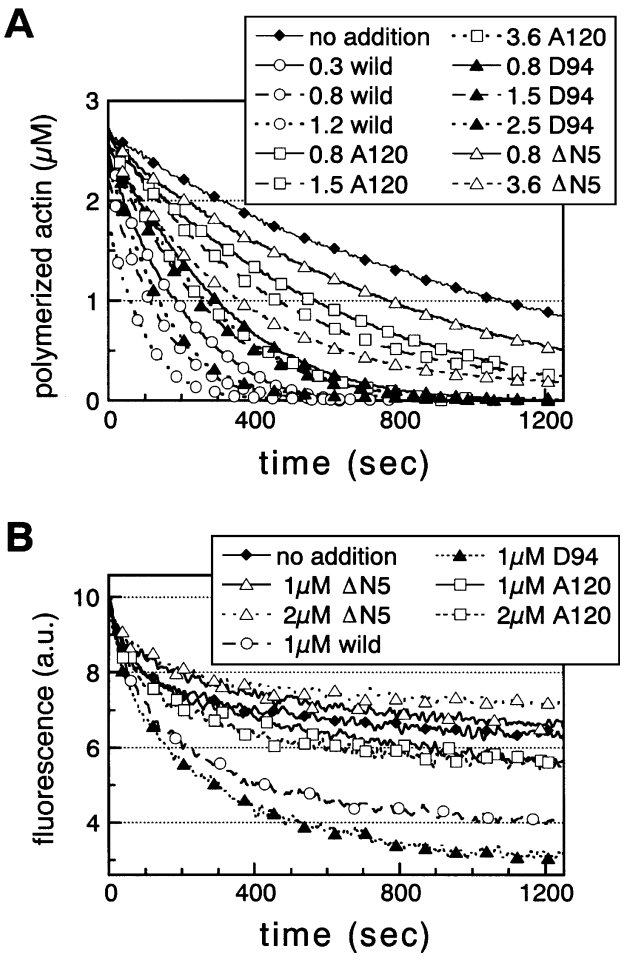

Figure 5 Effect of cofilin mutants on the depolymerization rate of F-actin

(**A**) Pyrene-actin (3.3 μM; 5% pyrene-labelled) was polymerized in the presence of 24.5 nM gelsolin. After incubation with wild-type or mutant cofilin carrying a C-terminal His tag for 10 min in the presence of gelsolin–actin dimers (20 nM), depolymerization was initiated by adding vitamin-D-binding protein, a potent actin-monomer-sequestering protein. The depolymerization was monitored by the gradual decrease in fluorescence and the trace was drawn after normalization as described in Moriyama and Yahara [19]. The key states the cofilin concentrations in μM. (**B**) Non-capped actin filaments (5% pyrene-labelled) were diluted 12-fold into the solution containing wild-type or mutant cofilin carrying a His tag at the C-terminus. The fluorescence was recorded to monitor the gradual depolymerization.

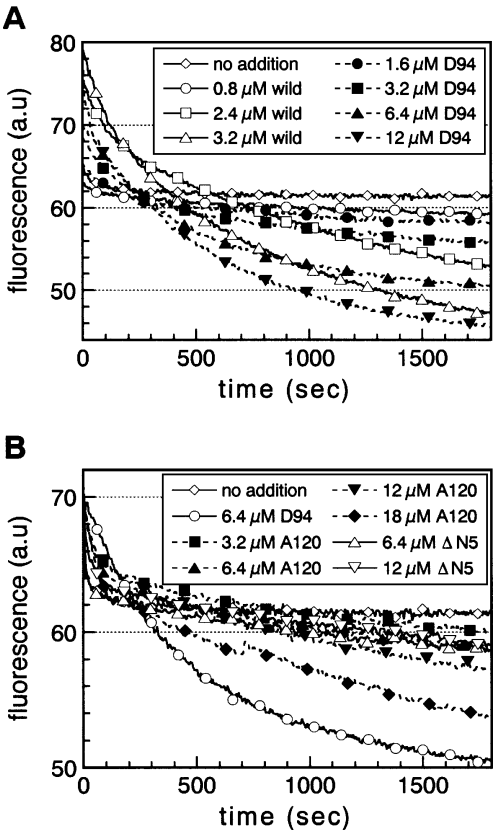

Figure 6 Effect of cofilin mutants on the turnover rate of F-actin

ϵATP-bound actin (6 μM) was polymerized in the presence of each mutant cofilin carrying a His tag at the C-terminus. The intensity of ϵATP fluorescence is higher when it binds to actin [36]. After a steady state was reached, unlabelled ATP was added to chase the bound ϵATP and the decline in fluorescence was recorded. Although binding of cofilin to ϵATP-bound actin was observed to increase the basal fluorescence intensity, it was possible to semi-quantitatively evaluate the accelerating activity. (**A**) The activity of D94-cofilin was compared with that of wild-type cofilin. (**B**) Comparison of the effects of D94-, A120- and ΔN5-cofilins.

A higher turnover rate of F-actin in living cells seems primarily due to cofilin-mediated acceleration of subunit release from the pointed ends of actin filaments. The three mutant cofilins indeed speeded up the depolymerization at the pointed end with different efficiencies (Figure 5A). In order of efficiency they ranked as wild-type > D94 > A120 > ΔN5. Whether these mutant cofilins promote the turnover of F-actin is another critical issue to be addressed. We then we observed the turnover kinetics of ϵATP-bound actin by chasing the fluorescent ϵATP with excess ATP (Figure 6). D94-cofilin increased the turnover rate of F-actin much more efficiently than A120- or ΔN5-cofilin, although its effect was approx. 3-fold weaker than that of wild-type cofilin (Figure 6). This result also supports the above notion that D94-cofilin is not an actin-monomer-sequestering factor. ΔN5-cofilin exhibited the weakest ability to promote the turnover of F-actin, and higher concentrations of this mutant protein did not further increase the turnover rate (Figure 6B and results not shown).

Cell-biological effects of mutant cofilins

Overexpression of cofilin induces the formation of thick bundles of actin filaments in cultured cells [15]. It has been assumed that their formation was initiated by fragmentation of the actin-filament latticework, followed by its transition to bundles [7,23]. We then examined whether mutant cofilins could induce such actin bundles when overproduced in human HEK-293 cells. A3-cofilin, a phosphorylation-resistant form, was observed previously to be most effective in generating cytoplasmic actin bundles [15] and it was also true here (Figures 7a and 7b). When Ser-120 of A3-cofilin was replaced by alanine, this effect was still observed, although less frequently (Figures 7c and 7d). In contrast, such actin bundles were not induced when either the D94- or ΔN5-variants of A3-cofilin were overexpressed (Figures 7e–7h), whereas A3D94-cofilin was often localized at actin-rich regions of the cell periphery (Figures 7e and 7f). Even simultaneous expression of both A3D94- and ΔN5-cofilins failed to generate any intracellular actin bundle, and analogous results were obtained when we used mouse C3H-2K cells instead of HEK-293 cells (results not shown).

Cofilin is indispensable for the survival of the budding yeast *S. cerevisiae* [1,2]. This yeast is, however, allowed to grow by heterologous expression of porcine cofilin even in the complete

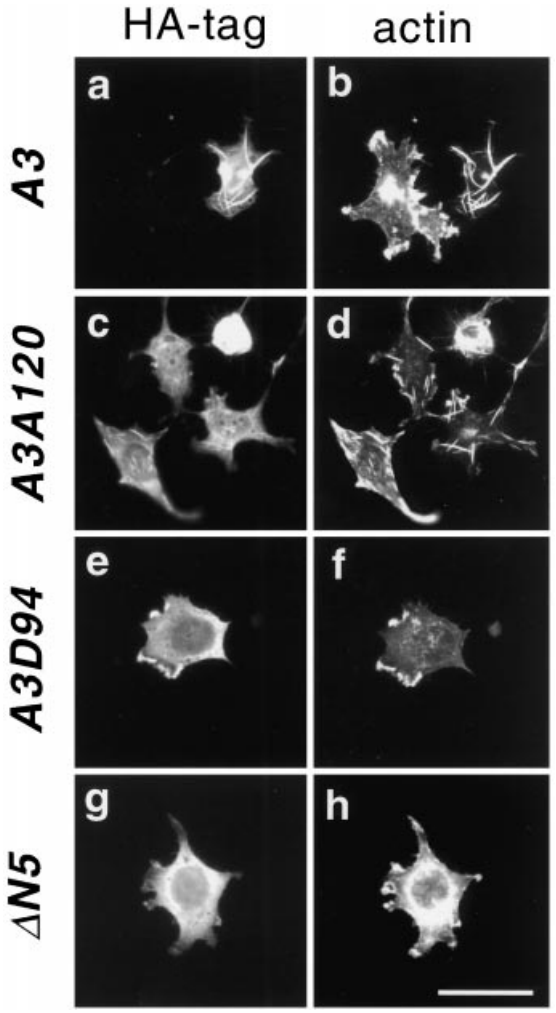

Figure 7 Ability of cofilin mutants to induce cytoplasmic bundles of actin filaments upon overexpression

HEK-293 cells were transfected with a plasmid designed to express A3-cofilin (**a** and **b**), A3A120-cofilin (**c** and **d**), A3D94-cofilin (**e** and **f**) or ΔN5-cofilin (**g** and **h**). The expressed cofilins had a His tag at the N-terminus and a haemagglutinin 1 (HA) epitope tag at the C-terminus. After 6 h, cells were washed and cultured in fresh medium for a further 40 h. Cells were fixed, incubated with a rabbit anti-actin antibody and a 12CA5 monoclonal antibody that reacts with the haemagglutinin 1 epitope tag. The staining with these antibodies was visualized using fluorescein-conjugated (**b**, **d**, **f** and **h**) and rhodamine-conjugated (**a**, **c**, **e** and **g**) secondary antibodies. Scale bar, 20 μm.

absence of the intrinsic *COF1* gene [1,15]. Adapting this phenomenon, we examined whether the above mutant cofilins preserved the ability to support the growth of yeast cells. Prior to this test, we verified that all three mutant proteins were produced as soluble forms at approximately similar amounts when expressed in wild-type yeast cells (results not shown). The A120 mutant was viable below 30 °C, but it was unable to form colonies at 37 °C (Table 1), consistent with our previous report [19]. In addition, it grew more slowly than cells expressing the wild-type protein, even at permissive temperatures. Notably, both D94- and ΔN5-mutants failed to form any colony in the absence of the functional *COF1* gene at all temperatures tested (Table 1). These results indicate that the latter two mutants lack sufficient activity for the essential vital function of cofilin, whereas A120-cofilin retains such activity despite that activity being reduced compared with the wild type.

Table 1 Ability of cofilin mutants to rescue *COF1*-deficient budding yeast

Whether mutant cofilins can functionally replace authentic cofilin in *S. cerevisiae* was determined by a plasmid-shuffling method as described in [15]. Strain HE8 (*MAT***a** *leu2 ura3 trp1 his3 cof1*Δ::*HIS3* + YCpYCS/*COF1*/*URA3*/*CEN*) was transformed with a YEplac181-based plasmid designed for expression of each mutant cofilin from the *COF1* promoter. At this stage, the expression levels of all mutant cofilins were almost the same. Then, the transformants were forced to lose the YCpYCS plasmid by streaking them on to selective agar plates containing 5-fluoro-orotic acid. These plates were incubated at 18 or 25 °C for 2 weeks and examined for colony formation. For wild-type and A120-cofilins, colonies that appeared on the 25 °C plates were re-streaked on SC (synthetic complete medium) plates and tested for colony formation at 37 °C. ++, Colony size similar to that of the original strain; +, smaller colony size; −, no visible colony formed.

		Ability to rescue *COF1*-deficient yeast		
Cofilin	Growth temperature (°C) ...	18 °C	25 °C	37 °C
Wild-type		++	++	+
A120		+	+	−
D94		−	−	Not determined
ΔN5		−	−	Not determined

DISCUSSION

Several reports have demonstrated that the G-actin-binding activity of cofilin/ADF could be dissociated from its F-actin-binding activity by site-specific mutageneses or a C-terminal truncation [24–26]. Pope et al. [25] characterized the cofilin mutants K96Q and K95QK96Q, in which Lys-96 and/or Lys-95 of human cofilin were replaced by glutamine. These residues are located on the major loop in the three-dimensional structure of cofilin (Figure 8). These 'loop mutants' bound to G-actin, but not to F-actin. Ono et al. [26] found that specific truncations or mutations of the C-terminal tail of UNC-60B, a nematode cofilin/ADF, also selectively eliminated its F-actin-binding activity. Lappalainen et al. [24] had made similar observations through the alanine-scanning mutagenesis of yeast cofilin, Cof1p. The location of their *cof1-16* mutation (R80A, K82A) overlapped with Lys-96 of mammalian cofilin (Figure 8) and its property resembled the loop mutants of Pope et al. [25]. Their *cof1-22* mutation (E134A, R135A, R138A) was located near the C-terminus and it also reduced Cof1p binding to F-actin. As illustrated in Figure 8, the 'loop site' and the C-terminus of cofilin/ADF are spatially very close to each other [27–29]. Thus these sites become recognized as parts of the molecular surface interacting with the actin filament, but not with G-actin [24–26]. Their loop mutants and C-terminal variants all remarkably increased the steady-state amount of unpolymerized actin in F-actin solution, but they only marginally accelerated the depolymerization rate of F-actin, which was far less than wild-type cofilin [24–26]. This means that their mutants prefer the G-actin-bound state, i.e. they sequester G-actin from re-polymerization rather than buffering G-actin and, as a result, cannot effectively promote the turnover of F-actin. At a glance, our D94-cofilin resembled such mutants in its lack of binding to F-actin while retaining actin-depolymerizing activity. When considering the role for cofilin in actin-based motility, however, it is very critical whether a particular mutant cofilin retains the ability to promote the treadmilling of actin filaments. We demonstrated that D94-cofilin accelerated the turnover of F-actin much more effectively than A120-cofilin, although approx. 3-fold less efficiently than

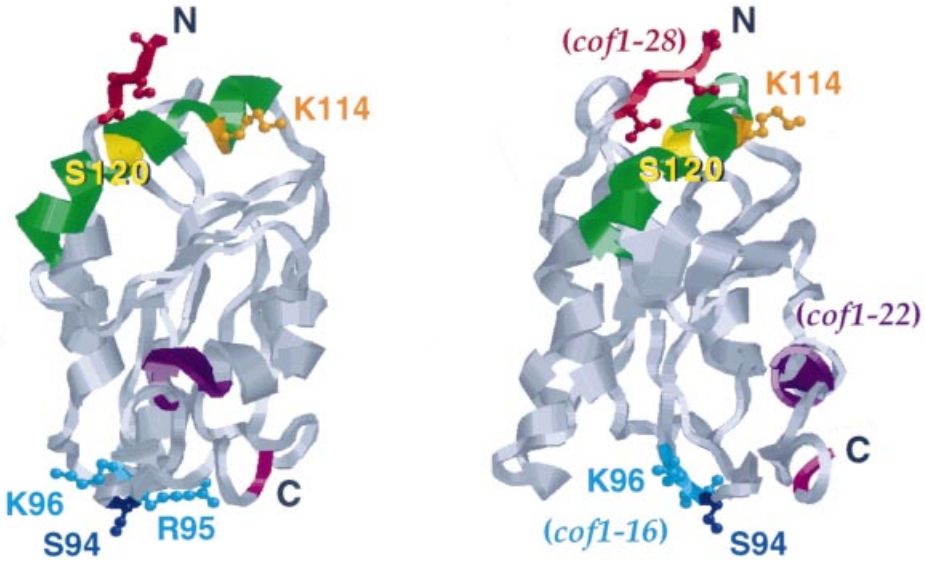

Figure 8 The locations of the cofilin mutations on the tertiary structure

The structure of porcine destrin, an isoprotein of cofilin, is derived from PDB source 1AK6 [27], and presented as two images of a model with the use of a RasMol software (version 2.6). The two views are rotated reciprocally by 60° around a vertical axis. The side chains of some important residues are shown as stick-and-ball structures. The N-terminal segment from Ala-2 to Val-5 is drawn in red and is absent in the ΔN5-mutant. The actin-binding helix is drawn in light green and orange is used to label Lys-114, which is disposed to chemical cross-linking with the N-terminal acidic segment of actin (the front of subdomain 1) [37]. Ser-120 is present near the middle of the kinky helix and drawn in yellow. Ser-94 is shown in blue, and the locations of previously reported loop mutations (Lys-96 and Arg-95) are shown by cyan. This loop and the C-terminus (violet) are close to each other and seem to constitute the second binding site, which is assumed to bind to the rear of subdomain 1 of the lower subunit in the filament. In the right-hand panel, relevant mutations of yeast cofilin are shown in parentheses, and the residues affected by the *cof1-28*, *cof1-16* and *cof1-22* mutations are shown in red, cyan and purple, respectively.

the wild-type cofilin. Thus D94-cofilin does not sequester G-actin from re-assembly on to the barbed ends. A decrease in the steady-state amount of polymerized actin in the presence of D94-cofilin should be achieved primarily by acceleration of subunit release at the pointed ends, but not by monomer sequestration. In other words, the depolymerized actin molecules in the presence of D94-cofilin are more ready for re-polymerization than those in the presence of the other loop mutants or C-terminal variants. Therefore, D94-cofilin is actually distinct from the previous mutants in stimulating treadmilling of actin filaments, and such a unique property further substantiates the notion that either significant side-binding by cofilin or alteration of the filament twist is not required for cofilin-mediated acceleration of F-actin turnover.

The short N-terminal segment of cofilin/ADF has also been implicated in the interaction with actin. In depactin, an echinoderm ADF, the N-terminal portion of 20 residues was chemically cross-linked with both N- and C-terminal portions (subdomain 1) of G-actin [30]. A *cof1-28* mutation of yeast cofilin eliminated the N-terminal five residues and resulted in gross defect in both G- and F-actin binding [24]. In addition, a molecular-dynamics simulation study predicted strong interactions between the N-termini of actin and yeast cofilin [31]. However, the direct role that the N-terminal segment plays in the mechanistic action of cofilin on actin dynamics has not been addressed, besides the issue of its phosphorylation [14,15]. ΔN5-cofilin behaved similarly to A120-cofilin in our co-sedimentation assays (Figures 1 and 2), but they are clearly different in their ability to sever actin filaments (Figure 3). In addition to the lack of actin severing by ΔN5-cofilin, this mutant failed to prevent phalloidin from binding to actin filaments (Figure 3C). The phalloidin-binding site of F-actin is relatively close to its nucleotide-binding pocket [32,33] and is not masked directly by cofilin in any proposed structural models for cofilin-decorated filaments [17,21,22,27]. Thus inhibition of phalloidin binding is interpreted as a result of cofilin-induced structural change in the actin filament, such as changes in the filament twist, subunit tilt and/or interdomain motion, as proposed previously [17,21,22]. Therefore, failure of ΔN5-cofilin to interfere with phalloidin binding suggests strongly that the N-terminal few residues are essential for inducing a pivotal structural change in the actin filament following the initial landing of cofilin on the filament. Thus the activity of cofilin to associate with F-actin can be dissociated mutationally from the activity to sever and to alter the structure of actin filaments. It remains to be determined whether this structural change is identical with the previously proposed change in the filament twist and/or the tilt of actin subunits [17,21]. ΔN5-cofilin exhibited another distinct effect on the dynamics of the barbed end (Figures 4 and 5). A large amount of it reduced the rates of both subunit addition and release at the barbed end. Gelsolin-like proteins also decrease the subunit-exchange rate at the barbed end by capping this end. However, ΔN5-cofilin manifested its effect only at much higher concentrations than the proteins of a gelsolin family (gelsolin is effective even at nanomolar concentrations). Moreover, it could slightly promote subunit addition to the barbed end at lower concentrations (Figure 4). Thus it is still ambiguous whether the reduction of the barbed-end dynamics by the large amount of ΔN5-cofilin was due to a direct capping of the barbed end or not. It might have resulted from some structural change in the actin filament through the lateral association of ΔN5-cofilin, instead of end capping.

There are several structural models for cofilin binding to the actin filament. As mentioned above, it is now recognized that cofilin would use its empirically defined molecular surface (so-called 'second site') involving the 'loop' containing Ser-94 and the C-terminal region to associate with the actin filament, in

Table 2 Overview of the characteristics of porcine cofilin mutants

++, Efficient; +, moderate; ±, detectable, but very weak; −, not detectable (or almost absent).

	In vitro							
	F-actin binding		Depolymerizing				Cultured cells	Budding yeast
Cofilin	pH 7.0	pH 8.3	pH 7.0	pH 8	Severing	Accelerating actin turnover	Induction of actin bundles	Rescue of Δ*cof1* cells
Wild-type	++	+	±	++	++	++	++	++
A120	+	+	±	±	+	+	+	+*
D94	−	−	+	++	−	++	−	−
ΔN5	+	+	±	±	−	±	−	−

* Temperature sensitive.

addition to the major kinky helix of cofilin [25,26]. In these models, the kinky helix containing Ser-120 of cofilin locates between subdomains 1 and 3 of an upper actin molecule, and the second site makes contact with a lower actin molecule of longitudinally neighbouring subunits in the filament. However, it remains controversial whether the second site of cofilin positions outside (in front of) or inside (to the rear of) subdomain 2 of the lower actin molecule [21,22,26,34]. It is also not clear whether cofilin binding induces the subsequent change in the twist of the actin filament, or whether cofilin binds to the filament in a particular twisted state and stabilizes it [21]. In this study we found the involvement of the N-terminal segment of cofilin in changing the filament structure, because ΔN5-cofilin failed to interfere with phalloidin binding to F-actin and to sever actin filament. If this structural change is identical with the previously recognized change in the twist, the inability of the filament-bound ΔN5-cofilin to induce the structural change suggests that the second site of cofilin initially positions outside or on the top of the subdomain 2 of the lower actin subunit. Subsequently, cofilin may move inward to dispose its second site behind the subdomain 1 of the lower actin subunit in the course of rigid association of both the major helix and the N-terminal portion of cofilin with the upper actin subunit, concomitant with a conformational change in the bound actin. The resulting intercalated association should be responsible for the large change in filament twist, as proposed by Renoult et al. [34]. The unique kink in the major helix may facilitate such a motion of cofilin, because the mutational perturbations of the kink differentially affect its severing and depolymerizing efficacy [19].

The relationships between the biochemical activities and the cellular effects of cofilin mutants are summarized in Table 2. ΔN5- and D94-cofilins both failed to rescue the Δ*cof1* yeast cells and to induce actin bundles in mammalian cultured cells, whereas A120-cofilin exhibited these abilities despite being much less effective than wild-type cofilin. Because D94-cofilin was much more effective than A120-cofilin in promoting the turnover of actin filaments *in vitro*, this result indicates that the acceleration of F-actin turnover by itself cannot suffice the essential role of cofilin for cell survival. Similarly, the F-actin-binding ability of cofilin by itself is not sufficient for playing its vital role. The physiologically indispensable activity of cofilin should be preserved in A120-cofilin that can support the growth of Δ*cof1* cells, but not in either ΔN5- or D94-cofilin. Hence, cofilin must possess enough ability to change the structure of actin filaments or to sever them in order to maintain cell viability. The cytoplasmic actin-bundle-inducing ability of cofilin should also rely on the same activity of cofilin, because this ability of the mutant cofilins is well correlated with their ability to support yeast growth.

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