Overexpression of Cofilin Stimulates Bundling of Actin Filaments, Membrane Ruffling, and Cell Movement in Dictyostelium

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Abstract. Cofilin is a low molecular weight actin-modulating protein whose structure and function are conserved among eucaryotes. Cofilin exhibits in vitro both a monomeric actin-sequestering activity and a filamentous actin-severing activity. To investigate in vivo functions of cofilin, cofilin was overexpressed in Dictyostelium discoideum cells. An increase in the content of D. discoideum cofilin (d-cofilin) by sevenfold induced a co-overproduction of actin by threefold. In cells overexpressing d-cofilin, the amount of filamentous actin but not that of monomeric actin was increased. Overexpressed d-cofilin co-sedimented with actin filaments, suggesting that the sequestering activity of d-cofilin is weak in vivo. The overexpression of d-cofilin increased actin bundles just beneath ruffling membranes where d-cofilin was co-localized. The overexpression of d-cofilin also stimulated cell movement as well as membrane ruffling. We have demonstrated in vitro that d-cofilin transformed latticework of actin filaments cross-linked by α-actinin into bundles probably by severing the filaments. D. discoideum cofilin may sever actin filaments in vivo and induce bundling of the filaments in the presence of cross-linking proteins so as to generate contractile systems involved in membrane ruffling and cell movement.

Materials and Methods

Cell Lines and Vectors

Overexpression plasmid of d-cofilin, pCOF, was constructed as follows. The d-cofilin cDNA was amplified by PCR using a synthetic oligonucleotide (AAAAATGCATCTrCAGGTATrGCIAGc-'r) and a T7 primer as

1. Abbreviation used in this paper: d-cofilin, D. discoideum cofilin.
primers from the template plasmid, pDCOF2 (Aizawa et al., 1995). The amplified cDNA was sequenced to verify its integrity. Klenow large fragment, and XhoI, and ligated into pBES (Sutoh, 1993) which had been pre-treated sequentially by BamHI, nuclease, and XhoI. The resultant plasmid contains an artificial d-cofilin gene consisting of actin 15 promoter, initiation methionine codon followed by in-frame d-cofilin cDNA, and actin 8 terminator in pUC19. The plasmid was digested by EcoRI and XhoI and self-ligated after treatment with klenow large fragment in order to eliminate an internal HindIII site. The artificial d-cofilin gene was then excised from the plasmid by sequential treatments of HindIII, klenow large fragment, and XhoI, and inserted into the D. discoideum extrachromosomal shuttle vector, pBIG (Uyeda et al., 1994; a generous gift from Dr. J. Spudich, Stanford University of Medicine, Stanford, CA), which had been pre-treated sequentially by BamHI, klenow large fragment, and XhoI. The resultant plasmids, pCOF and pBIG, were separately introduced into D. discoideum Ax2 cells by electroporation and transformed cells were selected in HLS medium containing 10 μg/ml neomycin as described before (Aizawa et al., 1995).

**Protein Compositions**

Transformed cells (1 x 10^7 cells on a 9 cm dish) were harvested and grown in 80 ml of HLS medium with 10 μg/ml neomycin as liquid culture shaking at 22°C to a density at 1 x 10^7 cells/ml. Cells were harvested by centrifugation at 250 g for 5 min at 4°C, and the packed cells (0.6 g) were suspended with 6 ml of MEM buffer (20 mM Mes, 2 mM EGTA, 1 mM MgCl2, pH 6.85) containing protease inhibitors (1 mM PMSF, 50 μg/ml leupeptin, and 0.5% [vol/vol] aprotinin) and sonicated to prepare total protein fraction. The sonicated fraction was centrifuged at 400,000 g for 30 min. The supernatant fraction (6 ml) was adjusted to 40% saturation of ammonium sulfate, and centrifuged at 10,000 g for 15 min, and the precipitates were collected and resuspended in 1 ml of MEM buffer as the 0–40% ammonium sulfate fraction. The supernatant was further sequentially fractionated into the 40–60% ammonium sulfate and the 60–80% ammonium sulfate fraction. After dialysis against 50 mM Tris (pH 7.5), the 60–80% ammonium sulfate fraction was charged onto 1 ml of DEAE-cellulose column, and the flow through fraction was collected. Protein composition of each fraction was analyzed by SDS-PAGE (Laemmli, 1970) with 10–20% gradient gel. Molecular weight markers for SDS-PAGE (Bio-Rad Laboratories, Hercules, CA) contain myosin (200 kD), β-galactosidase (116 kD), phosphorylase b (97 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD). Cells were stained with Coomassie brilliant blue R-250. Western blotting was performed as previously reported (Aizawa et al., 1995) using a rabbit antiserum against d-cofilin (Aizawa et al., 1995) at 2,000× dilution and a mouse anti-actin monoclonal antibody clone C4 (ICN Biomedicals Inc., Costa Mesa, CA) at 500× dilution in blocking solution (5% skim milk in PBS) reacting with other antigens. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and horse radish peroxidase-conjugated goat anti-mouse IgG were used as second antibodies at 2,000× dilution. Reacted proteins were visualized by adding nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate for staining d-cofilin, and H2O2, 4-chloronapthol, and o-dianisidine for staining actin, as substrates.

**Subcellular Fractionation**

Transformed cells (2 x 10^7 cells) were grown on a 9 cm dish. After starvation for 1 h at 22°C in MCM buffer (20 mM Mes, 0.2 mM CaCl2, 2 mM MgCl2, pH 6.85), the cells were washed twice on the plate by lysis buffer (20 mM Mes, 15 mM KCl, 2 mM MgCl2, 5 mM EGTA, 1 mM DTT, 20 μg/ml protease inhibitors, pH 6.85) at 22°C. After a complete removal of the lysis buffer, 0.5 ml of lysis buffer containing 0.5% NP-40 was added gently onto the plate, and was incubated at 22°C for 5 min to lyse all the cells on the plate completely. The complete lysis was checked under microscopy. The lysate was collected by cell scrapers as the total fraction. The lysate and 1-vol packed Ax2 cell pellet is 10 mg/ml.

**Cell Staining**

Cells grown on a glass cover slip were starved in MCM buffer for 1 h, and then fixed in MCM buffer containing 3.7% formaldehyde. For single staining of actin filaments, the fixed cells were incubated in PBS containing 1 U/ml rhodamine phalloidin (Molecular Probe, Eugene, OR) for 1 h at 25°C. For double staining of actin filaments and plasma membrane, cells were fixed and stained by rhodamine phalloidin as above, and then incubated with 800× diluted DII stock solution (3 mg/ml in ETOH) in PBS for 30 min at 25°C. For double staining of d-cofilin and actin filaments, the fixed cells were further permeabilized by incubation in ETOH containing 1% formaldehyde at −15°C for 5 min. Then the cells were incubated subsequently in the blocking solution (10% goat serum in PBS) for 20 min at 25°C, 100× diluted anti-d-cofilin antiserum for 16 h at 4°C, 100× diluted fluorescein-conjugated goat anti-rabbit IgG antibody for 2 h at 25°C, and 1 U/ml rhodamine phalloidin in PBS for 1 h at 25°C. The stained cells were observed and recorded under a confocal laser scanning microscopy (MRC600; Bio-Rad Laboratories, Tokyo, Japan) equipped with an Ar ion (2 × 106 W) and Helium/Neon (0.3 mW) dual laser system, Nikon epifluorescence (2,048× oil immersion objective [Nikon Co., Tokyo, Japan], and a Zeiss Axiovert 135 equipped with an Plan-Apochromat 63× oil immersion objective (Carl Zeiss, Oberkochen, Germany).

**Protein Purification**

Rabbit muscle actin was prepared as described (Spudich and Watt, 1971), and further purified as described before (Aizawa et al., 1995). D. discoideum cofilin was purified from Ax2 cells as described before (Aizawa et al., 1995). D. discoideum α-actinin was purified from Ax2 cells as described (Condecella et al., 1982) except for the use of HILoadTM 16/60 Superdex TM 2000 preparation grade gel filtration column (Pharmacia LKB Biotechnology, Uppsala, Sweden) eluted with 10 mM Mes, 1 mM EGTA, pH 6.9 instead of Bio-Rad A15M gel filtration open column.

**Cell Movement**

Transformed cells were grown on a 35 mm dish at a density of 1 x 10^7 cells/dish for overnight in HL5 medium containing 10 μg/ml neomycin. After removal of the medium, the cells were suspended in 1 ml of MCM buffer by gentle pipetting. An aliquot (50 μl) of the suspension was added to a Falcon 35 mm tissue culture dish (Becton Dickinson Co., Mountain View, CA) containing 2 ml of MCM buffer and the dish was settled at 25°C for 30 min so that the cells attached to the substrate. The movement of the cells was observed under Axiovert 135 equipped with Argus 50 image analyzing computer system and SIT camera C2400-08. For high-magnification observation by Nomarski DIC system, we used Plan-Apochromat 63× oil immersion object and 35 mm glass bottom microwell dishes coated with poly-L-lysine (MatTek Corp., Ashland, MA). Original images were enhanced, subtracted with the background image in real time by Argus system and recorded sequentially at every 10-s intervals on its RAM disk. To regulate temperature of the buffer at 22°C, we placed the observation system in a room adjusted at 22°C, and the power of light for observation was settled under the range 4. For low-magnification observation under light microscopy, we used PlanNeofluar 20× object and a Falcon tissue culture dish uncoted or coated with various materials. Original microscopic images were enhanced, subtracted with the background image, sliced, and negatively polarized in real time, and recorded sequentially on a time-lapse 8-mm video recorder (Mitsubishi, Tokyo, Japan).

**Results**

**Preparation and Characterization of Cells Overexpressing d-Cofilin**

To overexpress d-cofilin in D. discoideum Ax2 cells, we used an extrachromosomal plasmid pBIG as a shuttle vector, whose copy number is ~150 in a single cell (Firtel et al., 1985). Since the pBIG contains a neomycin resistant gene, cells carrying the plasmid were selectively cloned in HL5 medium containing 10 μg/ml neomycin (Uyeda et al., 1994). The d-cofilin gene, DCOFI, was first introduced together with actin 8 terminator into pBIG. Transfectants
carrying the plasmid did not overproduce d-cofilin, however (data not shown). Next, we introduced actin 15 promoter, d-cofilin cDNA, and actin 8 terminator into pBIG. The resultant plasmid, pCOF, successfully overexpressed d-cofilin in Ax2 cells (Fig. 1). When 10 μg plasmid was introduced into vegetatively growing Ax2 cells (1 × 10^7 cells) by electroporation, more than 100 colonies were reproducibly formed after neomycin selection. 2 wk after electroporation, all colonies were harvested and used in further experiments. The content of d-cofilin in cells carrying pCOF was seven times higher than that in cells carrying the control plasmid pBIG (Fig. 1, A and C). We found that the content of a 42-kD protein in cells carrying pCOF also increased threefold compared to that in the control cells (Fig. 1 A). The 42-kD protein was identified as actin on the basis of immunoreactivity with a monoclonal anti-actin antibody (Fig. 1 B) and binding to DNase-I column (data not shown). The contents of proteins other than d-cofilin and actin did not differ (Fig. 1 A, lanes 3–12). The expression level of d-cofilin in the above transformants continued high for 3 wk after electroporation, and gradually decreased thereafter to the control level (data not shown). The expression of actin in cells carrying pCOF decreased during passages for several weeks as that of d-cofilin decreased. Thus, we performed all the experiments in this study within 3 wk after electroporation.

Starvation of the overexpressing cells on a filter induced

**Figure 1.** Protein composition of control and overexpressor cells. (A) SDS-PAGE of fractions prepared from control and overexpressor cells. (B) Western blotting of fractions using anti-actin antibody. (C) Western blotting of fractions using anti-d-cofilin antiserum. Lanes 1, 3, 5, 7, 9, and 11; fractions prepared from control cells. Lanes 2, 4, 6, 8, 10, and 12; fractions prepared from overexpressor. Lanes 1 and 2, total protein; lanes 3 and 4, ultracentrifuged supernatant; lanes 5 and 6, 0–40% ammonium sulfate fraction; lanes 7 and 8, 40–60% ammonium sulfate fraction; lanes 9 and 10, 60–80% ammonium sulfate fraction; lanes 11 and 12, DEAE flow-through fraction of 60–80% ammonium sulfate fraction. M, marker proteins.

**Figure 2.** Quantitative analysis of d-cofilin and actin in control and overexpressor cells. Total protein fraction was prepared from control and overexpressor cells by lysis buffer containing 0.5% NP-40 as described in Materials and Methods. The fraction was further fractionated by ultracentrifugation into precipitates and supernatant. The amounts of d-cofilin (A) and actin (B) in each fraction were determined by SDS-PAGE followed by densitometric analysis. The concentrations of d-cofilin and actin which were produced by pCOF were also calculated (C). The mean value of three experiments were presented.
normal differentiation into aggregation stage and mature fruiting body at 5.5 and 22 h, respectively. Starved control cells differentiated into the above two stages at 6 and 24 h, respectively. The content of d-cofilin and that of actin in the overexpressing cells decreased to 30% and 10% of the vegetative levels at 6 and 16 h, respectively, after the induction of differentiation. For this reason, we performed all the experiments using cells in MCM buffer solution within 3 h. No significant difference was detected between doubling times of the overexpressing cells (17 ± 0.5 h) and control cells (18 ± 0.5 h) when they grew on a dish in HL5 medium containing 10 μg/ml neomycin. Overexpressing cells and control cells did not differ in phagocytic activity with microbeads (data not shown).

Figure 3. Intracellular distribution of actin filaments in flattened control and overexpressor cells. Cells were placed on a glass coverslip in MCM buffer for 1 h before fixation. The fixed cells were stained with rhodamine phalloidin to visualize actin filaments. Optically sectioned images were recorded by laser scanning confocal microscopy at 0.5-μm intervals along the vertical axis. Reconstituted figures by projecting all the Z-series of two typical flattened cells were represented here for control (A and B) and overexpressor (C and D), respectively. Bars: (B) 5 μm (D) 10 μm.

Figure 4. Double-staining of overexpressor for actin filaments and plasma membrane. Overexpressor cells were grown and fixed as in Fig. 3. The cells were doubly stained by rhodamine phalloidin and DiI to visualize actin filaments and plasma membrane, respectively. Observation was performed as in Fig. 3. Reconstituted figures from optical sections for actin filaments (A), plasma membrane (B), and merger of A and B (C) are represented. Bar, 5 μm.
Figure 5. Intracellular distribution of actin filaments in actively moving control and overexpressor cells. Control cells (A and B) and overexpressor (C and D) were grown, fixed, and stained with rhodamine phalloidin as in Fig. 3. Observation was performed using Nomarski differential microscopy (A and C) and fluorescent microscopy (B and D). Asterisks indicate actin latticework on contact sites with the substratum. Arrows indicate actin bundles under plasma membrane. Bar, 20 μm.

**Effect of d-Cofilin Overexpression on Actin Assembly in Cells**

The total concentration of d-cofilin and that of actin in cells carrying pCOF were both approximately 600 μM higher than those in control cells (Fig. 2). Cell lysates were subjected to centrifugation so as to recover polymerized actin and unpolymerized actin as precipitated and supernatant fractions, respectively. Fig. 2 C showed that 83% of overexpressed d-cofilin and 90% of co-overproduced actin were recovered in precipitated fractions, indicating that the overexpression of d-cofilin co-overproduced actin filaments but not actin monomers in *D. discoideum* cells.

**Effect of d-Cofilin Overexpression on Actin Architectures in Cells**

Actin architectures of cells were visualized with rhodamine phalloidin by fluorescence microscopy. In control cells, actin filaments were observed predominantly in the regions of peripheral ruffling membranes (Fig. 3, A and B). In cells overexpressing d-cofilin and actin filaments, numerous bundles of actin filaments just under the dorsal plasma membranes were observed (Fig. 3, C and D). Double staining of actin filaments with rhodamine phalloidin and plasma membranes with diI in a single flattened cell clearly revealed that the overexpression of d-cofilin significantly stimulated membrane ruffling in dorsal cell surfaces which appeared to be associated with bundles of actin filaments (Fig. 4).

Next, we examined actin architectures in cells with elongated cell shapes which were actively moving. In control cells, we observed at least three types of phalloidin staining; first, relatively strong staining at the ventral adhesion

Figure 6. Double staining of overexpressor for actin filaments and d-cofilin. Overexpressor cells were grown and fixed as in Fig. 3. The cells were doubly stained by rhodamine phalloidin and anti-d-cofilin antibodies to visualize actin filaments (A, C, E, and G) and d-cofilin (B, D, F, and H), respectively. Observations were performed as in Fig. 3. Reconstituted figures by projecting all the Z series are shown at the top (A and B). Sectioned images at 3.0 μm (C and D), 1.5 μm (E and F), 0 μm (G and H) from the glass substrate are also shown. Bar, 20 μm.
The latticework was not brightly stained with rhodamine by d-Cofilin In Vitro

Enhancement of Actin Filament Bundling

We performed an analogous experiment using d-cofilin as a possible severing factor (Fig. 7). Actin latticework was reconstituted from 10 \( \mu \)M actin and 0.5 \( \mu \)M \( D. \) discoideum \( \alpha \)-actinin (Fig. 7 A). The latticework was not brightly stained with rhodamine phalloidin because each actin filament was thin and, therefore, stained under a threshold level to be detected. When the latticework was treated with 1 \( \mu \)M d-cofilin, the bundles of actin filaments brightly stained with rhodamine phalloidin were induced (Fig. 7 B).

Amoeboid Movement

During the course of selection for cells containing plasmids, pCOF and pBIG, we realized that colonies of cells overexpressing d-cofilin were generally larger than those of the control cells. At day 7 and 11 after plating, the overexpressing cells formed approximately 1.6-times larger colonies in radius than the control cells (Fig. 8). Doubling time of the overexpressing cells and that of the control cells were 17 \( \pm \) 0.5 and 18 \( \pm \) 0.5 h, respectively. It seems that this difference, if any, in doubling time does not account for difference in colony size. Since the radius of a colony is a function of growth rate and cell motility, we speculated that the overexpressing cells move faster than the control cells. To confirm this, we decided to measure cell movement directly by real-time observation using a computer-digitated video-microscopy.

Although there is some deviation in the motility among cells, we found that cells overexpressing d-cofilin moved significantly faster than the control cells. Presented are sequential photographs of examples; a relatively fast moving cell of the control (Fig. 9 A) and one of the overexpressing cells (Fig. 9 B), taken at an interval of 20 s. The overexpressing cells developed a larger lamellipodium in front of the cell (Fig. 9, 20 s) and more rugged dorsal plasma membrane (Fig. 9, 40 s) than the control cell. For statistic treatment of motility data, we used an automatic image analyzing computer system combined with a microscope equipped with a low magnification objective. With the control and overexpressing cell types, we observed about 25 cells in each field at an interval of 3 min for 30 min (Fig. 10, A and B).
Figure 8. Colony formation of control and overexpressor cells. *D. discoideum* Ax2 cells were transformed by electroporation of plasmid vectors pBIG for control and pCOF for overexpression of d-cofilin. After electroporation, cells were grown on a dish in HL5 medium containing 10 μg/ml neomycin in order to screen cells carrying pBIG or pCOF. During the screening of transformed cells, the cells carrying the plasmids proliferated well and formed colonies, while the other Ax2 cells died. We present here photographs of typical colony of cells carrying pBIG (A and C) and pCOF (B and D) at day 7 (A and B) and at day 11 (C and D). (E) Mean radius of control and overexpressor colonies at days 7 and 11. Bar, 500 μm.
Figure 9. Direct observation of single cell movement. Cells were grown in HL5 medium containing 10 µg/ml neomycin. At 1 h before analysis, 10^5 cells were harvested and placed on a 35 mm cell culture dish in MCM solution at a low density (127 cells/mm^2). Cell movement was recorded at every 10-s intervals using a computer video microscopy system. We represent a time series of photographs of control (A) and overexpressor (B) cells, which move the fastest in our hundreds of observed cells, at 20-s intervals for one minute. Bar, (B) 10 µm.

After the automatic calculation of centroid position (Fig. 10, C and D), histograms of cell frequency were made as a function of cell motility after collecting data in four independent experiments (Fig. 10, E and F). The results shown in the histograms clearly indicate that the overexpression of d-cofilin enhanced cell movement. Mean speeds of the control and overexpressing cells were calculated to be 1.09 and 1.86 µm/min, respectively. Even when the surfaces of dishes were coated with poly-L-lysine, gelatin, fibronectin, or collagen, the mean speed of the overexpressing cells (1.62, 1.16, 1.14, and 1.17 µm/min, respectively) was always about two times faster than that of the control cells (0.95, 0.51, 0.60, and 0.74 µm/min, respectively). This indicates that the enhancement of cell movement by the overexpression of d-cofilin is independent of substrate properties which affect cell motility.

Discussion

In this study, to address the question of what is (are) the in vivo function(s) of cofilin, we have analyzed altered properties associated with D. discoideum cells in which d-cofilin was overexpressed. We found that the overexpression of d-cofilin caused (a) an increase in the amount of actin filaments but not in the amount of unpolymerized actin (Fig. 2), (b) an appearance of ruffling membranes associated with cytoplasmic actin bundles (Figs. 3–7), and (c) an enhancement of amoeboid movement (Figs. 8 and 9).

How did d-cofilin overexpression cause an increase in the amount of actin filaments? It was demonstrated that a cofilin-related protein protected actin molecule from denaturation by EDTA in vitro (Hayden et al., 1993). Thus, it is possible that the overexpression of d-cofilin stabilizes actin molecules and consequently causes the accumulation of actin in the cells. Since the contribution of d-cofilin to sequestering monomeric actin is small as described below, the amount of polymerized actin is considered to increase as the amount of total actin increases. Another possibility is that severing activity of d-cofilin may cause an increase in the number of free ends of actin filaments where monomeric actin molecules are preferentially polymerized, and that lowering the concentration of free monomeric actin might enhance the synthesis of actin. The latter possibility may be supported by the recent report that the underexpression of capping protein, which increased the number of free ends of actin filaments, resulted in increases of cellular contents of both total and polymerized actin presumably because of the same mechanism as in the case of the overexpression of d-cofilin (Hug et al., 1995).

We previously reported that 9 µM d-cofilin depolymerized and sequestered only 1 µM actin in the presence of 3.2 µM total actin at pH 6.8 in vitro (Aizawa et al., 1995). Several reports have also demonstrated that about a 10 mol excess of cofilin-related proteins is needed to seques-
ter actin monomer at neutral pH (Nishida et al., 1984; Moriyama et al., 1992; Hawkins et al., 1993; Hayden et al., 1993). This weak sequestering activity of cofilin-related proteins well explains the fact that the overexpression of 600 μM d-cofilin by pCOF only slightly increased the concentration of sequestered actin from 120 to 180 μM while it greatly increased the concentration of actin filaments from 180 to 720 μM in D. discoideum cells (Fig. 2). Since the concentration of d-cofilin in A2x cells was estimated to be 100 μM, d-cofilin may sequester ca. 10 μM actin in the cells. Since the total concentration of sequestered actin in A2x cells was calculated to be ca. 120 μM, 110 μM actin should be sequestered by other proteins than d-cofilin such as profilin. We concluded that d-cofilin is not a major actin-sequestering protein in D. discoideum cells.

We showed that the overexpression of d-cofilin enhanced actin bundles in D. discoideum cells (Figs. 3–6). It was also reported that microinjection or transient overexpression of cofilin-related proteins induced actin bundles in mammalian cultured cells (Nagaoka et al., 1995; Moriyama et al., in press). These results suggest that cofilin-related proteins trigger the formation of actin bundles in cells although purified cofilin-related proteins themselves do not have any activity to bundle actin filaments in vitro. Porcine destrin, Acanthamoeba actophorin, and human actin depolymerizing factor, all of which are cofilin-related proteins, have an activity to sever actin filaments in vitro (Nishida et al., 1985; Cooper et al., 1986; Maciver et al., 1991a; Hawkins et al., 1993). It has been shown that actophorin re-organized latticework consisting of actin filaments and a cross-linking protein, into actin bundles by severing actin filaments in vitro (Maciver et al., 1991b). We also observed the lattice-bundle transition of actin filaments by d-cofilin in vitro (Fig. 6). These in vitro results suggest that the formation of actin bundles in the overexpressing cells is attributed to the severing activity of cofilin-related proteins.

Cells overexpressing d-cofilin exhibited enhanced cell motility (Figs. 9 and 10). This indicates that d-cofilin is an upstream positive regulator of cell motility. Very recently, phosphorylation of cofilin-related proteins at their Ser-3 residue has been shown to inactivate the protein in actin binding (Agnew et al., 1995; Moriyama et al., in press). We observed that the overexpressed d-cofilin was not significantly phosphorylated and that the overexpression of d-cofilin mutant containing Glu-3 instead of Ser-3 did not overproduce actin bundles, ruffling membranes, or cell motility in D. discoideum (data not shown). A body of evidence has been accumulated that various cellular activations accompany dephosphorylation of cofilin-related proteins and enhanced cell motilities such as stimulation of ruffling membranes (Davidson and Haslam, 1994) and secretion (Saito et al., 1994; Kanamori et al., 1995). These correlations are consistent with our conclusion that the activation of cofilin-related proteins may cause the enhancement of cell motilities. The enhancement of cell motility may be accounted for by the co-overproduction of actin filaments, since d-cofilin itself is not shown to produce mechanochemical power. The majority of actin filaments in the overexpression cells were organized into bundles under ruffling membranes (Figs. 3–6). Recently it was reported that underexpression of capping protein caused overproduction of actin filaments which were organized into actin arrays just in micromspikes, but did not enhance cell motility (Hug et al., 1993). These results suggest that the enhancement of cell motility induced by the overexpression of d-cofilin is not solely attributed to the overproduction of actin filaments. We suggest that the overexpression of d-cofilin may stimulate the lattice-to-bundle transition of actin architectures, leading to cell locomotion in D. discoideum cells.

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