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Effects of cofilin on actin filamentous structures in cultured muscle cells

Intracellular regulation of cofilin action

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SUMMARY

The previous investigation (Abe et al. (1989) *J. Biochem.* 106, 696-702) suggested that cofilin is deeply involved in the regulation of actin assembly in developing skeletal muscle. In this study, to examine further the function of cofilin in living myogenic cells in culture, recombinant cofilin having extra Cys residues at the N terminus was produced in *Escherichia coli* and was labeled with tetramethylrhodamine-iodoacetamide (IATMR). When the cofilin labeled with IATMR (IATMR-cofilin) was introduced into myogenic cells, actin filaments in the cytoplasm or nascent myofibrils were promptly disrupted, and many cytoplasmic rods which contained both IATMR-cofilin and actin were generated. Sarcomeric myofibrillar structures were not disrupted but tropomyosin was dissociated from the structures by the exogenous cofilin, and the IATMR-cofilin became localized in I-band regions. 24 hours after the injection, however, the actin-cofilin rods disappeared completely and the IATMR-cofilin became diffused in the cytoplasm as endogenous cofilin. Concomitantly, actin filaments were recovered and tropomyosin was re-associated with sarcomeric I-bands. At this point, the IATMR-cofilin in the cells still retained the functional activity to form intranuclear actin-cofilin rods in response to stimulation by DMSO just as endogenous cofilin. FITC-labeled actin introduced into myogenic cells at first failed to assemble into filamentous structures in the presence of the exogenous cofilin, but was gradually incorporated into myofibrils with time. The drastic effects of the exogenous cofilin on actin assembly were suppressed by phosphatidylinositol 4,5-bisphosphate (PIP_2). These results indicate that the exogenous cofilin is active and alters actin dynamics remarkably in muscle cells, but its activity in the cytoplasm gradually becomes regulated by the action of some factors including PIP_2-binding.

Key words: cofilin, actin binding protein, actin, myofibrillogenesis, muscle cell, phosphatidylinositol 4,5-bisphosphate (PIP_2), microinjection

INTRODUCTION

Multiple actin-regulatory proteins have been characterized as being profoundly involved in various cell functions by regulating the assembly and disassembly of actin (for review see Pollard and Cooper, 1986; Vandekerckhove, 1990; Way and Weeds, 1990). During the development of vertebrate striated muscles, actin is assembled into thin filaments of myofibrils and becomes responsible for muscle contraction by interacting with myosin filaments. We previously demonstrated that in embryonic skeletal muscle, a considerable amount of actin exists in an unpolymerized form, although the majority of actin is polymerized to constitute thin filaments of myofibrils in adult muscle (Shimizu and Obinata, 1986). Three actin-regulatory proteins which regulate actin assembly in the developing skeletal muscle have been isolated and characterized; they were identified with profilin (Oshima et al., 1989), actin depolymerizing factor (ADF) (Abe and Obinata, 1989), and cofilin (Abe et al., 1989). Among these proteins, cofilin is of special interest as a regulator for actin assembly in muscle, since this protein is expressed at a high level in embryonic (Abe et al., 1989), denervated (Shinagawa et al., 1993), and dystrophic (Hayakawa et al., 1993) skeletal muscles. In addition, a cofilin isoform characteristic of muscle tissues was recently discovered (Ono et al., 1994). Therefore, it is assumed that cofilin is deeply involved in the regulation of assembly and/or redistribution of actin and in modulating its turnover during myofibrillogenesis and myofibril disorganization, although its functional roles in skeletal muscle have not yet been established.

The entire cDNA sequence of cofilin, which encodes a protein molecule of about 18,700 Da composed of 166 amino acid residues was determined in both chicken (Abe et al., 1990) and mammals (Matsuzaki et al., 1988; Ogawa et al., 1990; Moriyama et al., 1990). Cofilin binds to both G- and F-actin at a 1:1 molar ratio and inhibits actin polymerization in a pH-dependent manner (Yonezawa et al., 1985; Abe et al., 1989). At neutral pH, cofilin interacts with actin monomer units in actin filaments and increases the apparent critical concentration for actin polymerization to a certain extent, whereas it

depolymerizes actin filaments at weak alkaline pH. Binding of tropomyosin to F-actin and actin-myosin interaction are both inhibited in the presence of cofilin (Nishida et al., 1984; Abe et al., 1989). In addition, it was demonstrated that phosphoinositides bind to cofilin and thereby inhibit the interaction between actin and cofilin in an in vitro system (Yonezawa et al., 1990). It has been demonstrated that heat-shock or DMSO treatment causes disruption of stress fibers in the cytoplasm, and instead induces formation of intranuclear actin rods in both fibroblasts (Osborn and Weber, 1980) and myoblasts in culture (Ono et al., 1993). In myotubes, intranuclear rods were formed by DMSO-treatment without disruption of myofibrils (Abe et al., 1993). These rods are composed of actin and cofilin, as revealed by immunofluorescence microscopy (Nishida et al., 1987). A signal sequence responsible for nuclear localization has been identified in the cofilin molecule, although the molecular mechanism(s) of nuclear transportation has not yet been clarified (Iida et al., 1992; Abe et al., 1993). On the other hand, Iida and Yahara (1986) showed that alteration of ionic environments surrounding cells induces the formation of actin-cofilin rods in the cytoplasm but not in the nuclei, and that the rod induction is accompanied by a decrease in cell volume. These observations suggested that the activity of cofilin in the cells is modulated by physical and/or chemical stimuli and that an increase in the concentration of the actin-cofilin complex causes the formation of the cytoplasmic rods. Recently, cofilin homologue has been found in yeast, *Saccharomyces cerevisiae*, and the cofilin in this organism is associated with cortical actin filament networks and is essential for cell growth (Moon et al., 1993; Iida et al., 1993).

In this study, we introduced fluorescent dye-conjugated cofilin into myocytes with or without actin by a microinjection technique and then examined its behavior as well as the localization of actin by fluorescence microscopy. We showed that the exogenous cofilin drastically reorganized actin filaments and induced formation of the cytoplasmic actin-cofilin rods in myoblasts and prematured myotubes, whereas well-organized myofibrils were less sensitive to the exogenous protein.

MATERIALS AND METHODS

Construction of a cofilin expression vector

In order to label cofilin with a fluorescent dye, we prepared a cofilin molecule having additional cysteine residues at the N terminus, termed cys-cofilin, by manipulating cofilin cDNA. Two oligonucleotides encoding 5′-CGCCATGGCTTGTGGATGTAA and 5′-CATGTTACATCCACAAGCCATGGCGT designated as cys#1 and cys#2, respectively, were synthesized. Both nucleotides were phosphorylated with T4 polynucleotidyl kinase and then they were annealed to each other. Chicken cofilin cDNA (pCMC-16; Abe et al., 1990) was digested with restriction enzymes *Nco*I and *Eco*RI. The resultant fragment, which begins with the initiation codon ATG located at the *Nco*I site and encodes an entire open reading frame for cofilin, was ligated with the double-stranded synthetic oligonucleotides. The ligated products were amplified by polymerase chain reaction (PCR) by using cys#1 and another synthetic reverse primer 5′-TTTATAAGGGTTTTCCTTCAAG, which contains a stop codon of the cofilin sequence. The PCR products were blunted with T4 DNA polymerase and inserted into a pBluescript II KS+ vector which was predigested with *Eco*RV and dephosphorylated with calf intestine alkaline phosphatase. Integrity of the construct was confirmed by DNA sequencing, and it was excised from the vector plasmid by digesting with *Nco*I (only present in the additional sequence) and *Hinc*II (only present in the vector). The *E. coli* expression vector pET-3d was digested with *Bam*HI, then blunted by T4 DNA polymerase, and further digested with *Nco*I. This vector was treated with phosphatase and ligated with the construct as above. By using this expression plasmid, the cofilin molecule containing an additional sequence, MACGCA, at the N terminus (designated as cys-cofilin), was produced in an *E. coli* expression system.

Expression and purification of cys-cofilin

E. coli strain BL21(DE3) pLysS was transformed by the construct, pET-cys-cofilin. Cells were grown overnight at 37°C in LB medium containing 50 μg/ml ampicillin and diluted 50-fold with the medium containing 5 μg/ml ampicillin. After incubating at 37°C for 3 hours, 1 mM isopropyl-β-thiogalactoside (IPTG) was added to the medium to induce expression of cys-cofilin and the culture was continued for an additional 3 hours. Cells were harvested by centrifugation and then suspended in the lysis buffer composed of 25% sucrose, 20 mM EDTA, 1 mM PMSF, 1 mM DTT, 2 mg/ml lysozyme and 50 mM Tris-HCl, pH 8.0. The suspension was incubated for 15 minutes at room temperature and then completely lysed by adding 5% Triton X-100 followed by sonication. The lysate was centrifuged for 30 minutes at 10,000 ***g***, and the supernatant was fractionated by ammonium sulfate at 60% saturation. After stirring for 1 hour, the soluble fraction was obtained by centrifugation at 10,000 ***g*** for 30 minutes and dialyzed against 0.2 M KCl, 0.1 mM DTT and 20 mM Tris-HCl, pH 7.5. The dialyzed sample was then applied to a hydroxyapatite column preequilibrated with the dialysis buffer. The column was washed with 0.6 M KCl, 0.1 mM DTT and 20 mM Tris-HCl, pH 7.5, and then cys-cofilin was eluted from the column with the washing buffer containing 10 mM potassium phosphate, pH 7.5. The cys-cofilin-containing fraction was concentrated by ultrafiltration with YM5 membrane and further purified by gel filtration on Sephadex G-75 equilibrated with 0.1 M KCl, 0.1 mM DTT, 0.05% NaN_3 and 20 mM Tris-HCl, pH 7.5. The purified cys-cofilin was stored on ice until use.

Preparation of actin and cofilin

Actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt (1971) and purified by gel filtration on a Sephadex G-100 column. Chicken cofilin was purified from chicken embryonic skeletal muscle as described elsewhere (Abe et al., 1989).

Preparation of fluorescent conjugates

Actin was labeled with FITC as described previously (Saitoh et al., 1988). Cys-cofilin was labeled with IATMR, a rhodamine derivative reactive to protein thiol groups; IATMR, dissolved in DMSO, was added to cys-cofilin in 0.1 M KCl and 20 mM Tris-HCl (pH 8.3) at a molar ratio of 10:1. The labeling reaction was continued for 6 hours on ice and terminated by adding DTT to a final concentration of 10 mM. The labeled cys-cofilin (IATMR-cofilin) was separated from free dye by gel filtration on a Sephadex G-25 column which was equilibrated with an injection buffer composed of 0.1 mM DTT, 0.05 mM $MgCl_2$, and 0.5 mM Pipes-KOH, pH 7.0.

Antibodies

The monoclonal antibody specific for cofilin (MAB-22) was prepared as described (Abe et al., 1989). Anti-actin antibody used for staining of C2 myoblasts was kindly provided by Dr Kazuko Iida (Tokyo Metropolitan Institute of Medical Sciences). Anti-actin antibody used for myotube staining was raised against skeletal muscle actin (Kuroda and Masaki, 1980). The monoclonal antibody against PIP_2 was a generous gift from Dr Tadaomi Takenawa (Fukami et al., 1988). The polyclonal antibody specific for tropomyosin was prepared from rabbit antiserum as described (Matsuda et al., 1981). Fluorescence-labeled goat antibody anti-mouse IgG (GAM) and goat antibody anti-rabbit IgG (GAR), and rhodamine-labeled GAM and GAR were

obtained from TAGO, Inc. (Burlingame, CA). Peroxidase-conjugated GAM and GAR were purchased from Bio-Rad Laboratories (Richmond, CA).

Gel electrophoresis and immunoblotting

SDS-PAGE was carried out using a 13.5% polyacrylamide gel in a discontinuous Tris-glycine buffer system according to the method of Laemmli (1970). For immunoblotting, proteins were electrophoretically transferred from the SDS-polyacrylamide gel to nitrocellulose paper (Towbin et al., 1979). The immunoreaction was performed as described (Abe et al., 1993).

Co-sedimentation assay

The binding of cofilin to F-actin was examined by ultracentrifugation. Cofilin (9.4 μM) and F-actin (4.7 μM) were incubated in 0.1 M KCl, 2 mM $MgCl_2$ and 20 mM Tris-HCl (pH 8.3) or 20 mM Pipes-KOH (pH 7.0) for 2 hours at room temperature with or without PIP_2 (400 μM). The mixtures were then centrifuged at 100,000 ***g*** for 20 minutes and the resultant supernatant and pellet were examined by SDS-PAGE. PIP_2 purchased from Sigma was dissolved in water at a final concentration of 4 mg/ml and stored at –80°C. The lipid solution was thawed and sonicated three times for 10 seconds just before use.

Cell culture and microinjection

C2 cells (Yaffe and Saxel, 1977) were propagated in DMEM supplemented with 10% FBS. For microinjection, cells were replated on glass coverslips and maintained for 36 to 48 hours after plating. Chicken mononucleated myogenic cells were dissociated from breast muscles of 12-day-old chicken embryos by means of mechanical dissociation and were plated on glass coverslips coated with collagen in 60-mm tissue culture dishes at a density of 5×10^5 cells. The culture medium consisted of 81% MEM (Nissui, Tokyo) supplemented with 2 mM L-glutamine, 15% horse serum, and 4% chick embryo extract. Cultures were maintained in a humidified atmosphere with 5% CO_2 and 95% air at 37°C. For treatment with DMSO, the culture medium was replaced with prewarmed medium containing 10% DMSO and then the cells were incubated for 1 hour. For treatment with an NaCl buffer (150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Mes-Tris, pH 6.5), cells were washed once with the buffer and incubated for 90 minutes. Microinjection was performed by the method of Hiramoto (1974) with a micromanipulator (Narishige, Tokyo). Samples were filtered through a Millipore filter (pore size, 0.22 μm) or centrifuged at 100,000 ***g*** just before use.

Fluorescence microscopy

Cells cultured on glass coverslips were fixed with 4% paraformaldehyde in PBS (0.15 M NaCl and 10 mM sodium phosphate, pH 7.0) for 20 minutes at room temperature. They were then fixed and permeabilized for an additional 5 minutes with 100% methanol at –20°C. In the case of staining with anti-PIP_2 antibody, 100% acetone was used instead of methanol. The cultures were washed thoroughly with PBS and blocked with 1% BSA in PBS for 1 hour, followed by sequential reactions with MAB-22 or anti-PIP_2 antibody and FITC-GAM, or anti-actin antibody or anti-tropomyosin antibody and FITC-GAR for 1 hour at room temperature in a humid chamber. Washing with PBS was carried out after the respective antibody treatments. For double labeling, the cells were first reacted for 1 hour with MAB-22 together with anti-actin antibody. After washing with PBS, they were reacted with a mixture of second antibodies, FITC-GAM and TRITC-GAR. After the final wash, the cells were mounted with 50% glycerol containing 75 mM KCl, 10 mg/ml paraphenylenediamine and 50 mM sodium carbonate buffer, pH 8.0. The specimens were examined under a Zeiss epifluorescence microscope.

RESULTS

Preparation of dye-conjugated recombinant cofilin

In the cofilin sequence, functional domains responsible for actin binding and nuclear localization have been identified (Yonezawa et al., 1991; Iida et al., 1992; Abe et al., 1993). Since these domains contain a cluster of lysine residues, the use of fluorescent dye reactive to the amino group seemed to be unsuitable for labeling of this protein. In fact, treatment of cofilin with FITC caused a loss of biological activities of cofilin. On the other hand, an attempt to label two cysteine residues in a cofilin molecule with IATMR was unsuccessful; the native cofilin was scarcely labeled with this dye, unless it was denatured by urea. Therefore, we constructed the cofilin cDNA which had an additional sequence, MACGCA, at the N terminus of the intrinsic cofilin sequence, and this cDNA was expressed in *E. coli* (Fig. 1A). The recombinant protein, designated as cys-cofilin, was purified from the *E. coli* lysate by a combination of ammonium sulfate fractionation, hydroxyapatite column chromatography, and gel filtration with Sephadex G-75. When the cys-cofilin was reacted with IATMR, a considerable amount was successfully labeled. The labeling efficiency was estimated at about 50%. As shown in Fig. 1B, the labeled protein (IATMR-cofilin) exhibited lower mobility on an SDS-polyacrylamide gel than the unlabeled protein.

We examined by co-sedimentation assay whether the IATMR-cofilin retains the actin binding activity. As Fig. 2 shows, IATMR-cofilin was cosedimented with F-actin and modulated the actin polymerization in a pH-dependent manner; at pH 8.3, a large amount of actin was present in the supernatant together with IATMR-cofilin after ultracentrifugation,

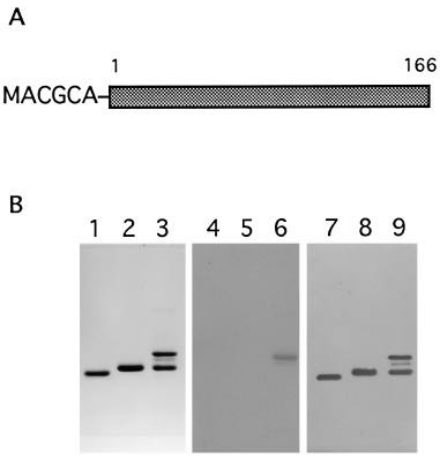


Fig. 1. Preparation of recombinant cofilin conjufluorescent dye. (A) Schematic demonstration of the sequence of the recombinant cofilin, termed cys-cofilin, having an additional hexapeptide (MACGCA) at the N terminus of the chicken cofilin sequence indicated by the stippled box. (B) SDS-PAGE patterns of cofilin from chicken embryonic skeletal muscle (lanes 1, 4 and 7), recombinant cys-cofilin (lanes 2, 5 and 8), and cys-cofilin conjugated with iodoacetamide tetramethylrhodamine (IATMR) (lanes 3, 6 and 9). Lanes 4-9 were blotted on nitrocellulose paper. Lanes 1-3, staining with CBB; lanes 4-6, detection of IATMR under visible light illumination; lanes 7-9, immunoblotting with anti-cofilin antibody (MAB22). The labeling efficiency was estimated at 50% by comparing the amount of labeled and non-labeled proteins by densitometry.

while actin was mostly cosedimented with IATMR-cofilin at pH 7.0. These properties were comparable to those of the authentic cofilin isolated from chick embryo (Fig. 2, lanes 5-8 and 13-16). From these results, we conclude that the IATMR-cofilin possesses basically the same biological activities as the authentic cofilin isolated from muscle cells. In addition, this conjugate interacted with FITC-labeled actin just as with intact actin in a pH dependent manner (Fig. 2, lanes 9-12).

Injection of cofilin into C2 myoblasts

When the cellular localization of cofilin and actin in C2 myoblasts was examined by immunofluorescence microscopy, cofilin was diffusely distributed in both the cytoplasm and the nuclei without associating with actin stress fibers, except for colocalization in the ruffled areas of the cell periphery (Fig. 3a and b). In these cells, microinjection of IATMR-cofilin caused immediate and drastic reorganization of the actin cytoskeleton. Within 10 minutes after the injection, thin actin-cofilin rods began to be formed in the cytoplasm, and they increased in number, width and length by 30 minutes (Fig. 3c). This rod formation progressed for an additional 2-6 hours, gradually then decreasing to become undetectable at 24 hours post-injection (Fig. 3e and g). Staining with anti-actin antibody revealed that the formation of the cytoplasmic rods was accompanied by the disruption of actin stress fibers. By 2 hours, the injected cells showed a great reduction in the number of stress fibers as compared with the neighboring uninjected cells, while the actin filament bundles along the cell periphery appeared to be unaffected by the injection of IATMR-cofilin (Fig. 3d and f). During this process, microtubules remained relatively unaffected (data not shown). The same results were also obtained by injecting unlabeled cys-cofilin or the authentic cofilin purified from chick embryos instead of the IATMR-cofilin. Injection of the buffer solution without cofilin induced neither disruption of actin filaments nor the rod formation (data not shown).

The effects of the exogenous cofilin on the reorganization of the actin cytoskeleton gradually diminished with time. As shown in Fig. 3g and h, at 24 hours after the injection, the distribution patterns of cofilin and actin filaments in the injected cells became hardly distinguishable from those in uninjected cells (Fig. 3a and b). Some modifications or degradation of the exogenous cofilin can be assumed to have taken place in the living cells in culture that led to the change in the distribution pattern. In this regard, we examined the activity of the injected exogenous cofilin by treating the cells with 10% DMSO. When the cells having IATMR-cofilin were maintained for 24 hours and then incubated in a medium containing 10% DMSO for 1 hour, the exogenous IATMR-cofilin was translocated into the nuclei to form intranuclear actin-cofilin rods (Fig. 4c, d, e and f), as the endogenous cofilin in the uninjected control cells (Fig. 4a and b). This result strongly suggests that the exogenous cofilin was present in the cells without being degraded for at least 24 hours. Some regulatory mechanisms other than degradation of exogenous cofilin must be considered as being responsible for the gradual restoration of the distribution patterns of cofilin and actin as seen in the intact cells after injection.

One possibility for this would be the formation of a complex between cofilin and phosphoinositides, since it was demonstrated that, in a test tube, PIP_2 binds to cofilin and inhibits its association with actin molecules (Yonezawa et al., 1990). We confirmed that the activity of the IATMR-cofilin was also inhibited by PIP_2 as that of native cofilin; as shown in Fig. 5A, in the presence of PIP_2, the IATMR-cofilin was not co-precipitated with F-actin at neutral pH and was unable to disassemble F-actin at alkaline pH, although the same protein could interact with actin in the absence of PIP_2 (see Fig. 2). We then injected IATMR-cofilin pre-treated with PIP_2 at a different molar ratio (Table 1). When the cofilin treated with PIP_2 at a 1:1 molar ratio was introduced into the cells, the ability of cofilin to reorganize cytoplasmic actin filaments into actin-cofilin rods was apparently reduced (Fig. 5B-a and Table 1). Only in about 20% of the cells, small and thin rods were observed in this case. At a lower concentration of PIP_2, the inhibitory action of PIP_2 became weaker. It is to be noted that a much higher concentration of PIP_2 was required to inhibit actin-cofilin interaction in a test tube as examined by co-sedimentation assay (See Fig. 5A and Yonezawa et al., 1990). The present results indicate that PIP_2 inhibited the cofilin activity more efficiently in living cells than in a test tube.

We observed that the cofilin inactivated by the PIP_2 treatment became active in terms of rod formation just as the cofilin without PIP_2-treatment, when the cells were incubated in a medium containing 150 mM NaCl (Fig. 5B-c and Table 2). The induction of the cytoplasmic rods by the NaCl stimulation has previously been described (Iida and Yahara, 1986). The location of PIP_2 was then examined during the change in the localization pattern of cofilin by immunocytochemical methods using anti-PIP_2 antibody. As shown in Fig. 5B-a and b, cofilin and PIP_2 showed similar localization patterns when both were injected as a complex. However, when the cofilin rod structures were induced by the NaCl stimulation, PIP_2 was not detected in the rods, suggesting that cofilin became free from PIP_2 (Fig. 5B-c and d). PIP_2 was also undetectable in the cofilin rods which were formed by the injection of exogenous IATMR-cofilin alone (Fig. 5C-a and b), while both cofilin and PIP_2 were similarly diffused, when the rods disappeared in 24 hours after the injection (Fig. 5C-c and d). These results

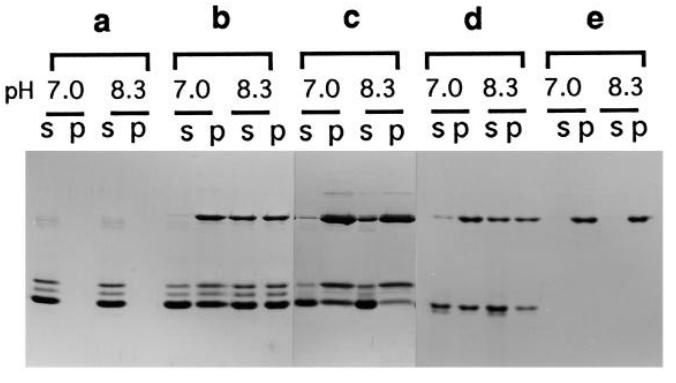


Fig. 2. Interaction of IATMR-cofilin with actin. Actin or FITC-conjugated actin at 4.7 μM was incubated with either IATMR-labeled recombinant cofilin or chicken cofilin at 9.4 μM in 0.1 M KCl, 2 mM $MgCl_2$ and 20 mM Pipes (pH 7.0) or 20 mM Tris-HCl (pH 8.3) for 2 hours. After centrifugation at 100,000 ***g***, both supernatants (s) and pellets (p) were suspended in an equal amount of SDS-sample buffer and subjected to SDS-PAGE. a, IATMR-cofilin alone; b, IATMR-cofilin plus actin; c, IATMR-cofilin plus FITC-actin; d, chicken cofilin plus actin; e, actin alone.

suggests that PIP_2 is involved in the regulation of cofilin-actin interaction in the cells.

Dose-dependent effect of cofilin on rod formation

In order to address the possibility of whether a rapid increase of cofilin concentration by the introduction of exogenous cofilin into cells induces the disassembly of actin filaments and cytoplasmic rod formation, we injected cys-cofilin at various concentrations. The cells were fixed 2 hours after the injection, stained with MAB-22, and the number of cells forming the cytoplasmic rods were counted. To distinguish the injected cells from others, FITC-labeled BSA was included in the injection buffer. As shown in Fig. 6, the number of cells containing the cytoplasmic rods increased linearly when cofilin was injected at the concentrations of 25 to 300 μM, suggesting that the formation of the cytoplasmic rods is dependent on the amount of the exogenous cofilin. At concentrations of more than 200 μM, about 90% of the cells formed many large rods

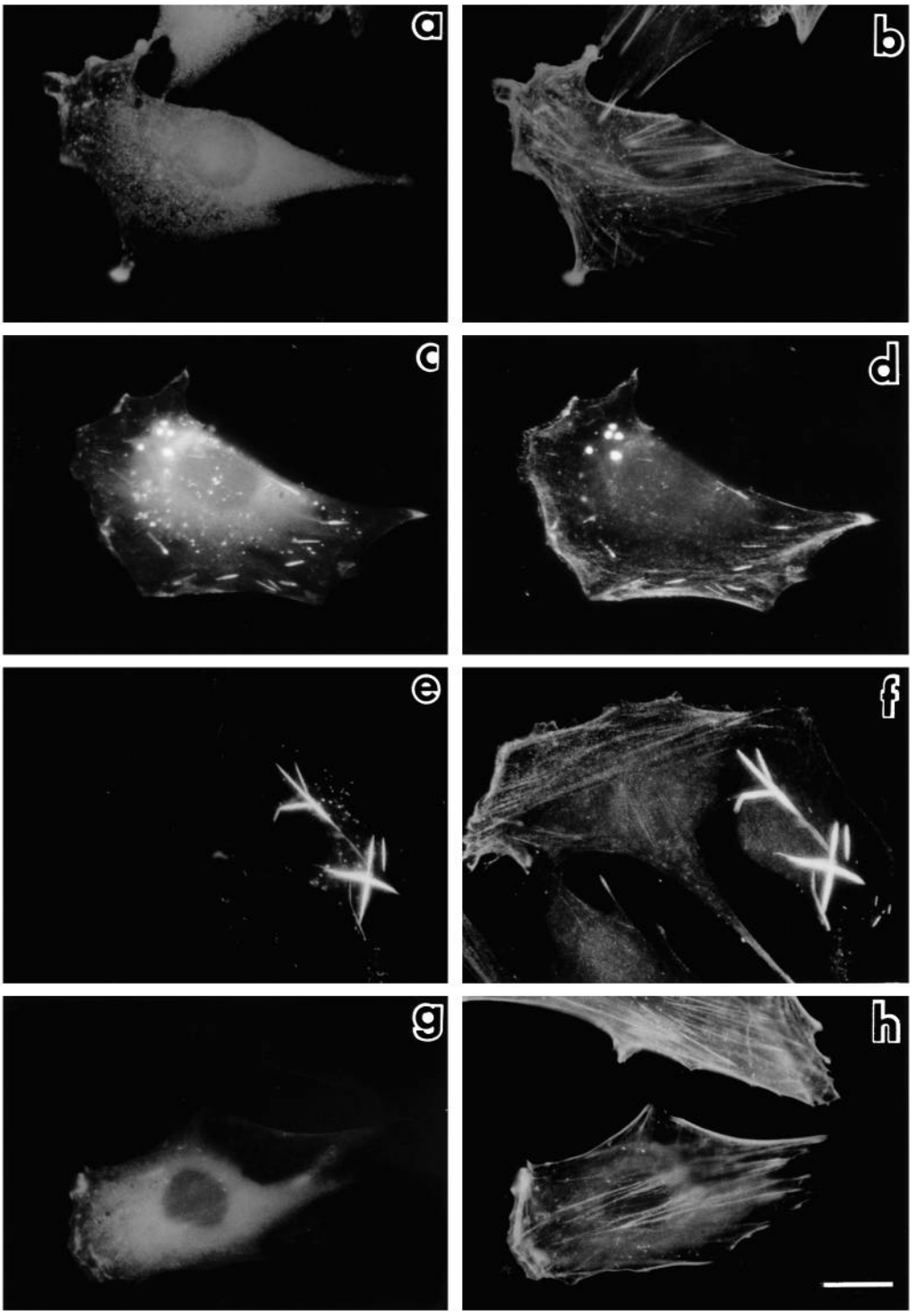


Fig. 3. Effects of cofilin microinjection on actin filaments in C2 myoblasts. IATMR-cofilin at 4 mg/ml (214 μM) was injected into C2 myoblasts (c-h) and the cells were fixed and stained with anti-actin antibody at 30 minutes (c and d), 2 hours (e and f) and 24 hours (g and h) after the injection. Control cells without injection (a and b) were dually stained with anti-cofilin (MAB-22) (a) and anti-actin (b) antibodies, followed by treatment with rhodamine-GAM and FITC-GAR. The location of endogenous (a) and exogenous (c,e and g) cofilin (rhodamine channel) and the location of actin (b,d,f and h) (fluorescein channel) were compared in the same cells under an epifluorescence microscope. Bar, 20 μm.

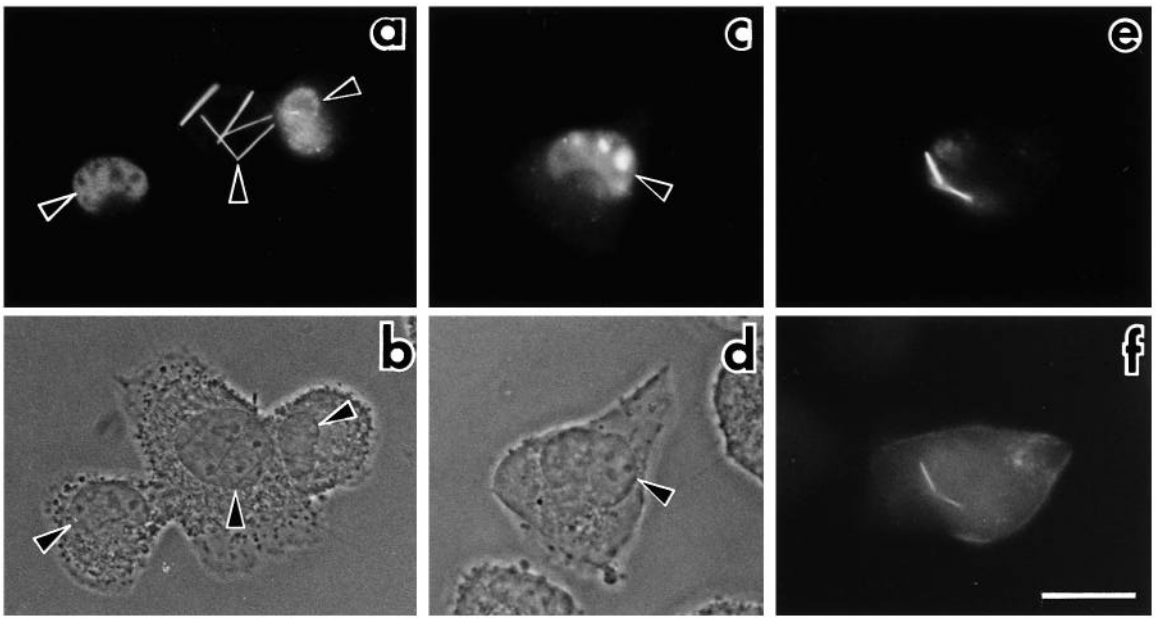


Fig. 4. Nuclear transportation of IATMR-cofilin and FITC-actin introduced into C2 myoblasts by DMSO-treatment. IATMR-cofilin and FITC-actin were co-injected into C2 myoblasts (c-f), and 24 hours later when the IATMR-cofilin became diffused in the cytoplasm (See Fig. 3g), the cells were treated with 10% DMSO. Control cells without injection (a and b) were similarly treated with DMSO and stained with MAB-22 followed by treatment with rhodamine-GAM. The location of endogenous (a) or exogenous (c and e) cofilin (rhodamine channel) and the location of actin (f) (fluorescein channel) were observed under a fluorescence microscope. (a,b and c,d) Fluorescence images and corresponding phase-contrast images were paired. (e,f) The locations of IATMR-cofilin and FITC-actin were compared in the same cell. Arrowheads indicate nuclei. Bar, 20 μm.

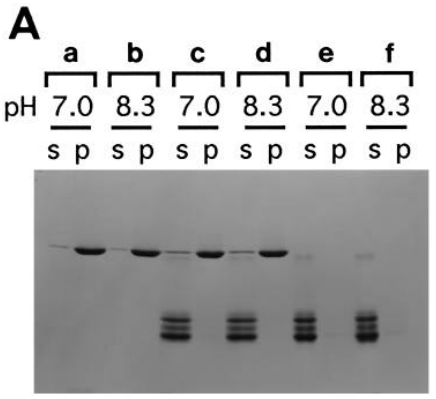
in the cytoplasm. When 25 to 50 μM was injected, about 30% of the cells formed thin rods near the cell periphery. The disruption of actin filaments seemed to occur in parallel with the formation of the cytoplasmic rods as judged by staining with anti-actin antibody (data not shown), however, it was impossible to determine the extent of actin disassembly in individual cells. The rod formation could be a reliable marker for actin reorganization induced by cofilin injection.

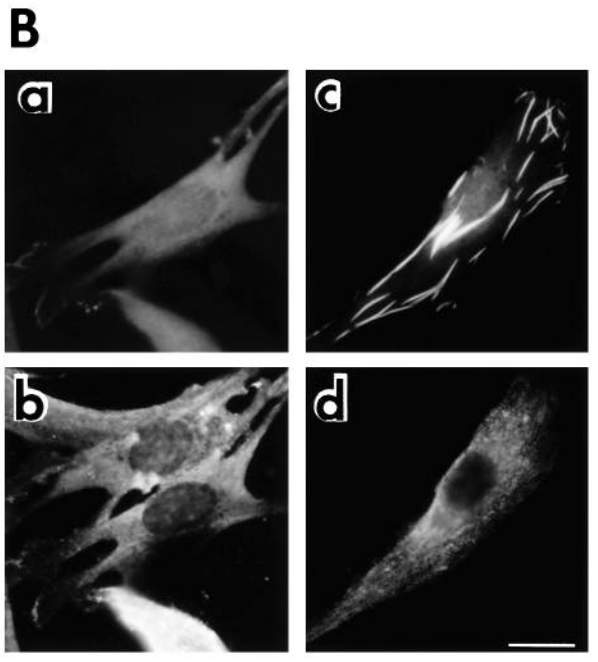
We assume that the volume of the cofilin solution injected might be about one tenth of the cell volume, since it has been generally accepted that the maximum volume to be injected without damaging the cells is 8 to 10% of cell volume. The injected cofilin might be diluted approximately 10 times in the cytoplasm; in other words, the concentrations of the exogenous cofilin in the cytoplasm might become 2.5 to 30 μM. We roughly estimated the amount of the endogenous cofilin in C2 myoblasts as around 5 μM by analyzing the serially diluted cell extracts by immunoblotting with MAB-22 using purified cofilin as a standard. Therefore, cofilin concentration in the cytoplasm might be increased up to 7 times by injecting cys-cofilin. It has been estimated that the actin concentration in mammalian cultured cells, for example BHK cells, is about 70 μM (Koffer et al., 1983). Assuming that actin concentration in myoblasts is similar to that of such non-muscle cells, the molar ratio of actin:cofilin in C2 myoblasts might become 3:1, when 200 μM of cofilin was injected.

Dose-dependency was also observed in the rod-formation induced by DMSO. Cofilin-injected cells were treated with 10% DMSO at 24 hours after the injection when the exogenous cofilin became diffused in the cytoplasm (see Fig. 3). As shown in Fig. 6, increase in cofilin concentration in the cytoplasm enhanced nuclear rod formation remarkably; when the molar ratio of actin:cofilin was about 2:1, approximately 90% of the cells contained nuclear actin-cofilin rods, while in the control cells without the exogenous cofilin, nuclear actin-cofilin rods were formed only in about 30% of cells. This results also supports that the increase in cofilin concentration in the cells directly induced actin disassembly and actin-cofilin rod formation.

Injection of cofilin into nascent myotubes

In order to examine the effects of cofilin on myofibril assembly, the IATMR-cofilin was introduced into chicken myotubes of primary culture. Myotubes at 3-4 days of culture under the present culture conditions did not have well developed sarcomeric structures. In these myotubes, endogenous cofilin was diffusely distributed in the cytoplasm, and the association of cofilin with nascent myofibrils or stress fiber-like structures was apparently not present, as was shown by dual staining with MAB-22 and anti-actin antibody (Fig. 7a and b). When IATMR-cofilin was introduced into these myotubes, however, many actin-cofilin rods were induced in the cytoplasm, as observed in the case of C2 myoblasts (Fig. 7c and d). In addition to the formation of the cytoplasmic rods, the exogenous cofilin was localized at the I-bands of the nascent myofibrils. Staining with anti-actin antibody revealed that in the regions where the exogenous cofilin was densely present, the I-bands were somewhat disrupted and rod-like structures were often formed. The exogenous cofilin was incorporated into the I-bands only transiently, disappearing within 2 hours after the injection, whereas the cytoplasmic rods retained a stable presence during this period (Fig. 7e and f). As can be expected from the results





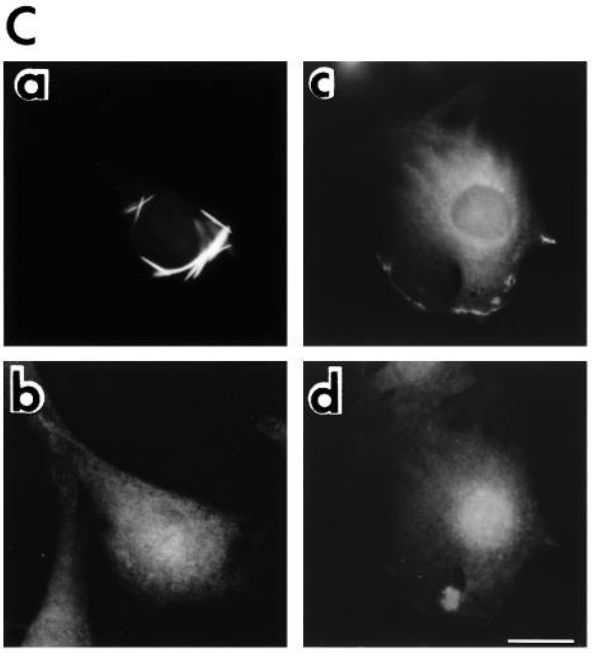


Table 1. Suppression of cofilin activity by PIP_2

Sample injected	Molar ratio (cofilin:PIP_2)	*Cellular distribution (%) In actin cofilin rods	Diffused in cytoplasm	Number of cells injected
IATMR-cofilin	1:0	83.3	16.7	96
		80.8	19.2	52
IATMR-cofilin	1:0.125	66.7	33.3	84
+PIP_2		68.5	31.5	92
IATMR-cofilin	1:0.25	65.3	34.7	49
+PIP_2		73.8	26.2	42
IATMR-cofilin	1:0.5	62.3	37.7	85
+PIP_2		66.7	33.3	69
IATMR-cofilin	1:1	23.9	76.1	46
+PIP_2		15.4	84.6	13
PIP_2	0:1	0	100	80
		0	100	100

IATMR-cofilin (214 μM) was incubated with PIP_2 at different molar ratio as indicated, and injected into the cytoplasm of C2 myoblasts. 2 hours after the injection, cells were fixed and the localization of IATMR-cofilin was determined by fluorescence microscopy. The results of two independent experiments are demonstrated.

*Percentage of cells showing the indicated distribution.

in C2 myoblasts, by 24 hours post-injection, the cytoplasmic rods were disassembled and the exogenous cofilin showed diffuse distribution throughout the cytoplasm, while the myofibril formation appeared to become normal (Fig. 7g and h), as in control uninjected cells. Thus, it is unlikely that the inhibitory effect of exogenous cofilin on myofibril formation, if any, persists for a long period in the cells.

Injection into matured myotubes

When IATMR-cofilin was injected into 7-8 day-cultured myotubes, which had well-organized myofibrils in the whole cytoplasm and exhibited spontaneous contraction, the injected cofilin was mostly associated with the I-bands of myofibrils without forming cytoplasmic rods by 30 minutes after injection (Fig. 8a and b). Anti-actin antibody uniformly stained the whole I-bands and they showed no apparent disruptions, suggesting that the structure of well-organized sarcomeres is resistant to the exogenous cofilin.

Fig. 5. Functional assay of IATMR-cofilin pre-treated with PIP_2. (A) Co-sedimentation assay using PIP_2-loaded IATMR-cofilin and F-actin. Actin (4.7 μM) was incubated with IATMR-cofilin (9.4 μM) and PIP_2 (400 μM) in 0.1 M KCl, 0.1 mM $CaCl_2$, 20 mM Pipes (pH 7.0) or 20 mM Tris (pH 8.3) for 2 hours. After centrifugation at 100,000 ***g***, both supernatant (s) and pellet (p) were dissolved in SDS-sample buffer and subjected to SDS-PAGE. a and b, actin and PIP_2; c and d, actin, IATMR-cofilin and PIP_2; e and f, IATMR-cofilin and PIP_2. (B) IATMR-cofilin (214 μM) was incubated with PIP_2 (214 μM) for 10 minutes at room temperature and injected into C2 myoblasts. The cells were fixed at 150 minutes after the injection (a and b), or at 60 minutes after the injection, they were incubated with an NaCl buffer (150 mM NaCl, 1 mM $CaCl_2$, $MgCl_2$ and 10 mM Mes-Tris, pH 6.5) for 90 minutes (c and d). The cells were then stained with anti-PIP_2 antibody. The localization patterns of IATMR-cofilin (a and c) and PIP_2 (b and d) were observed under an epifluorescence microscope. Bar, 20 μm. (C) The cells having injected IATMR-cofilin (214 μM) were fixed 2 hours (a and b) and 24 hours (c and d) after the injection, and stained with anti-PIP_2 antibody. The localization patterns of IATMR-cofilin (a and c) and endogenous PIP_2 (b and d) were observed by epifluorescence microscopy. Bar, 20 μm.

Table 2. Activation of PIP_2-loaded cofilin by stimulating with an NaCl buffer

Sample injected	NaCl treatment	*Cellular distribution (%) In actin-cofilin rods	*Cellular distribution (%) Diffused in cytoplasm	Number of cells injected
IATMR-cofilin	–	83.3	16.7	96
		80.8	19.2	52
IATMR-cofilin	+	92.1	7.9	38
		91.8	8.2	73
IATMR-cofilin +PIP_2	–	23.9	76.1	46
		15.4	84.6	13
IATMR-cofilin +PIP_2	+	90.2	9.8	41
		92.3	7.7	52
Injection buffer	+	4.1	95.9	148
		9.9	90.1	141

IATMR-cofilin (214 μM) with or without PIP_2 (214 μM) was injected into C2 myoblasts. Cells were incubated either in DMEM containing 10% FBS for 150 minutes, or in DMEM containing 10% FBS for 60 minutes followed by incubation in an NaCl buffer for 90 minutes before fixation, and the cellular distribution of IATMR-cofilin was determined by fluorescence microscopy. The results of two independent experiments are demonstrated.
*Percentage of cells showing the indicated distribution.

It was a matter of interest whether the localization of tropomyosin was affected by the injection of exogenous cofilin, since it is known that cofilin inhibits the binding of tropomyosin to the actin filaments in vitro. We examined the distribution of tropomyosin in myotubes with or without exogenous cofilin by immunocytochemistry with anti-skeletal tropomyosin antibody. As shown in Fig. 8g and h, tropomyosin was localized at the I-bands of myofibrils in normal myotubes, whereas in the myotubes having been injected with cofilin the striated pattern of tropomyosin became unclear, and instead the cytoplasm around the injected region was dotted with large aggregations stained by the antibody (Fig. 8c and d). These effects by exogenous cofilin persisted for 2 to 4 hours and then gradually declined. At 24 hours after the injection, the exogenous cofilin was diffusely distributed in the cytoplasm and the localization of tropomyosin was restored to myofibrils (Fig. 8e and f).

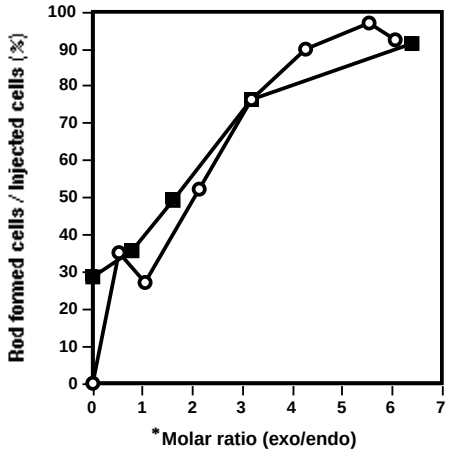


Fig. 6. Dose dependent effects of cofilin on rod formation. Cys-cofilin at various concentrations (25 to 300 μM) was injected into C2 myoblasts together with FITC-BSA as a marker for distinguishing the injected cells from others. 2 hours after the injection, the cells were fixed (○), or 24 hours after the injection, the cells were treated with 10% DMSO for 1 hour as in Fig. 3g and then fixed (■). In each case, the cells were stained with MAB-22. The number of cells having actin-cofilin rods among the cells having FITC-BSA was counted. Approximately, 100 cells were injected at each cofilin concentration. Two independent experiments gave similar results. *Injected cofilin/endogenous cofilin; concentration of endogenous cofilin was roughly estimated as 5 μM by densitometry.

Co-injection of cofilin with actin

When IATMR-cofilin and FITC-labeled monomeric actin (FITC-actin) were co-injected into C2 myoblasts, the cofilin assembled quickly to form rod structures together with the FITC-actin (Fig. 9). Filamentous structures containing FITC-actin were also formed. Endogenous stress fibers were scarcely disrupted under this condition (data not shown). We also co-injected FITC-actin with IATMR-cofilin into myotubes at 6 days of culture to investigate whether cofilin suppresses incorporation of actin molecules into myofibrils. As shown in Fig. 10, although FITC-actin without cofilin was quickly incorporated into myofibrils within 10 minutes (Fig. 10d, e and f), the actin co-injected with cofilin mostly exhibited a diffuse distribution pattern in the cytoplasm until 30 minutes after the injection (Fig 10a, b and c), suggesting that cofilin retards the incorporation of actin into myofibrils. This effect of cofilin disappeared when cells were incubated for more than 30 minutes, and then the localization of FITC-actin in myofibrils became indistinguishable between the two cases, with or without cofilin, by 24 hours after the injection (Fig. 10c and f).

DISCUSSION

Previous investigations suggested that cofilin may play important roles in the regulation of assembly and/or dynamic redistribution of actin and the modulation of its turnover during myofibrillogenesis and myofibrillar disorganization based on the following observations: (1) a considerable amount of monomeric actin was detected as a complex with cofilin in embryonic chicken skeletal muscle when myofibrillogenesis was in progress (Abe et al., 1989); (2) the expression of cofilin was increased remarkably in degenerating, denervated and dystrophic muscle (Shinagawa et al., 1993; Hayakawa et al., 1993); (3) cofilin exhibited various localization patterns in cultured myogenic cells under different culture conditions (Abe et al., 1993); (4) a cofilin isoform characteristic of muscle tissues was discovered (Ono et al., 1994); (5) mutation of the gene which encodes a cofilin homologue in *C. elegans* caused irregular aggregation of actin filaments in the muscle cells and abnormal muscle movement (McKim et al., 1994). However, it remained to be clarified how cofilin actually functions in the cells.

In this study, we demonstrated that exogenous cofilin introduced into living myocytes has dramatic effects on cytoplasmic actin filaments. Stress fibers and the actin filaments in nascent myofibrils were disrupted by the exogenous cofilin, and subsequently actin-cofilin rods were generated. Actin filaments in sarcomeric myofibrils were resistant to the cofilin, but tropomyosin was released from them. Recombinant cofilin with or without fluorescent dye as well as inherent cofilin from embryonic tissues were similarly active when introduced into

the cells. Injection of the buffer alone or another protein like BSA induced neither disruption of actin filaments nor the rod formation. The assembly of FITC-actin introduced into myotubes was obviously modulated by the exogenous cofilin. Thus, cofilin is a potent regulator for the reorganizationn of cytoplasmic actin filaments and actin assembly in developing muscle cells as well as in non-muscle cells.

Previously, several actin-binding proteins were introduced

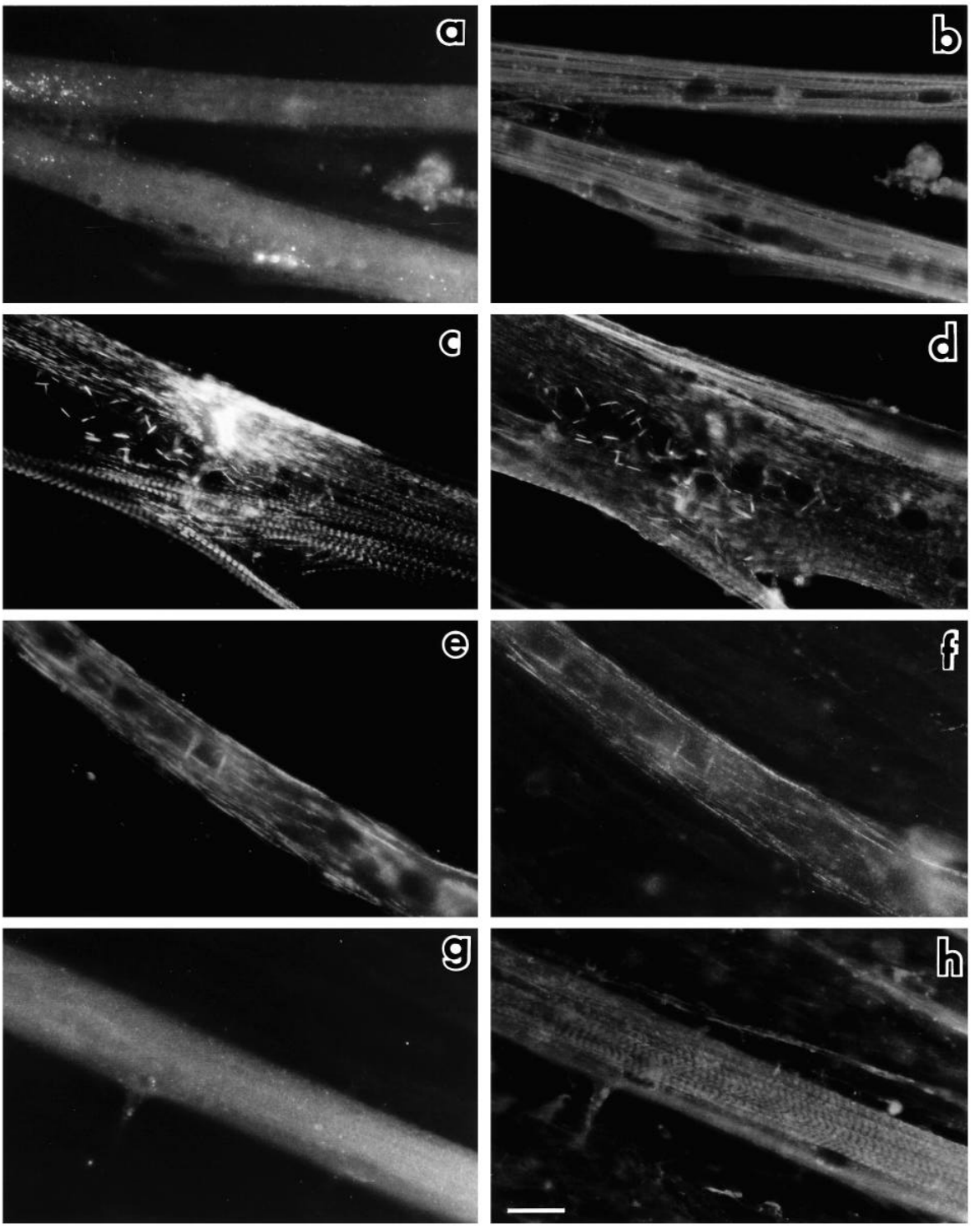


Fig. 7. Injection of IATMR-cofilin into nascent myotubes. IATMR-cofilin at 4 mg/ml (214 μM) was injected into chicken primary myotubes cultured for 3.5 days in vitro. The cells were fixed and stained with anti-actin antibody 30 minutes (c and d), 2 hours (e and f) and 24 hours (g and h) after injection. Control myotubes without injection (a and b) were dually stained with MAB-22 (a) and anti-actin antibody (b), followed by treatment with rhodamine-GAM and and FITC-GAR. The location of endogenous (a) or exogenous (c,e and g) cofilin (rhodamine channel) was compared with the location of actin (b,d,f and h) (fluorescein channel) under an epifluorescence microscope. Bar, 20 μm.

into living cells to determine the effects on actin-containing structures (Burridge and Feramisco, 1980; Fuchtbauer et al., 1983; Cooper et al., 1987; Sanger et al., 1990; Pavalko and Burridge, 1991), but none of them exhibited such a drastic action that led to rod formation as cofilin. Profilin injected into fibroblasts decreased actin filaments and diminished ruffled membranes without affecting the gross cell shape, but actin rods were not produced (Cao et al., 1992).

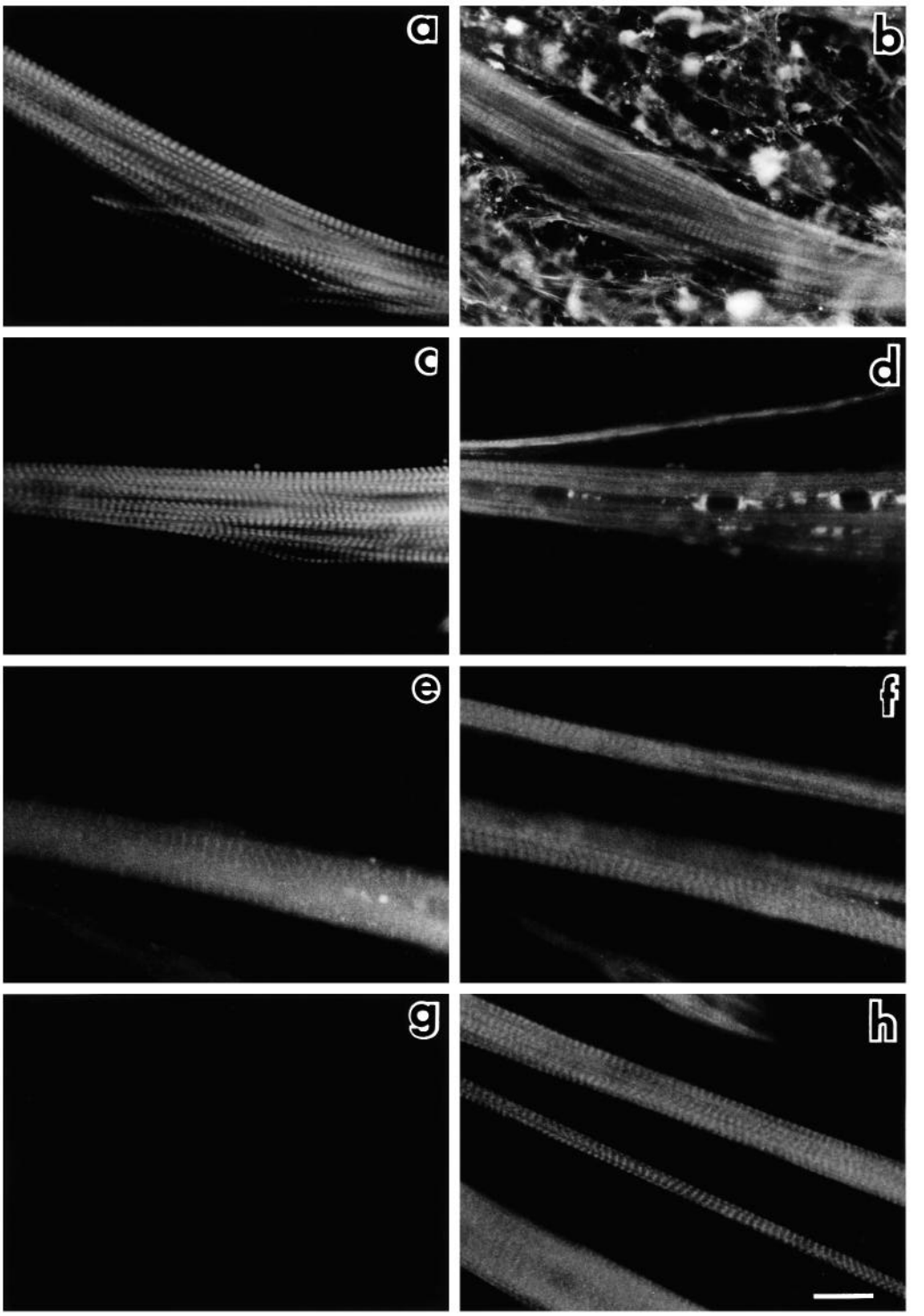


Fig. 8. Injection of IATMR-cofilin into mature myotubes. IATMR-cofilin at 4 mg/ml (214 μM) was injected into chicken myotubes cultured for 8 days (a-f). The cells were fixed at 30 minutes (a,b,c and d), or 24 hours (e and f) after the cofilin injection, and stained with anti-actin antibody (b) or anti-tropomyosin antibody (d and f). Control myotubes without injection (g and h) were stained with anti-tropomyosin antibody, followed by treatment with FITC-GAR. The location of exogenous cofilin (a,d,e and g) (rhodamine channel) was compared with the location of actin (b) or tropomyosin (d,f and h) (fluorescein channel) under a fluorescence microscope. Bar, 20 μm.

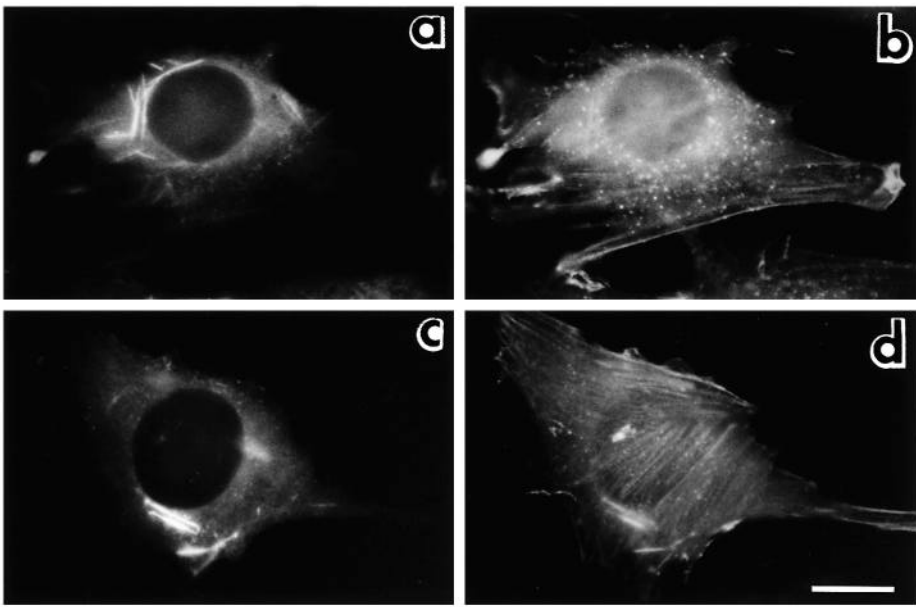


Fig. 9. Co-injection of FITC-actin and IATMR-cofilin into C2 myoblasts. The mixture of actin (47 μM) and IATMR-cofilin (96 μM) was dialyzed against G-buffer overnight, and then they were injected into C2 myoblasts. The cells were fixed at 30 minutes (a and b) or 2 hours (c and d) after the injection. The localization of IATMR-cofilin (a and c) and FITC-actin (b and d) was observed in the same cells (a-b or c-d) under a fluorescence microscope. Bar, 20 μm.

Suppression of actin assembly by cofilin would be a negative factor for the formation of contractile apparatus in developing muscle cells. However, the activity of cofilin is apparently regulated by some mechanism(s) in the cell. A high concentration of cofilin introduced into the cells was active in the beginning, but within about 24 hours after the injection the cytoplasmic actin-cofilin rods disappeared and the cofilin became diffused mostly in the cytoplasm of myoblasts and young myotubes, just as in the control cells under standard culture conditions. Concomitantly, actin filamentous structures as well as myofibrillar structures were recovered. At this time point, however, the exogenous cofilin possessed the ability to translocate into the nuclei to form nuclear actin-cofilin rods in response to DMSO-treatment.

Phosphoinositides, especially PIP_2, have been considered as regulators for cofilin activity. It was demonstrated that cofilin binds to PIP_2 more strongly than to actin filaments (Yonezawa et al., 1990), and that the PIP_2-binding site is the same as the actin-binding site of the cofilin molecule (Yonezawa et al., 1991). Therefore, it is likely that endogenous cofilin is more or less associated with phosphoinositides and therefore exhibits rather weak actin-binding activity. We assume that the

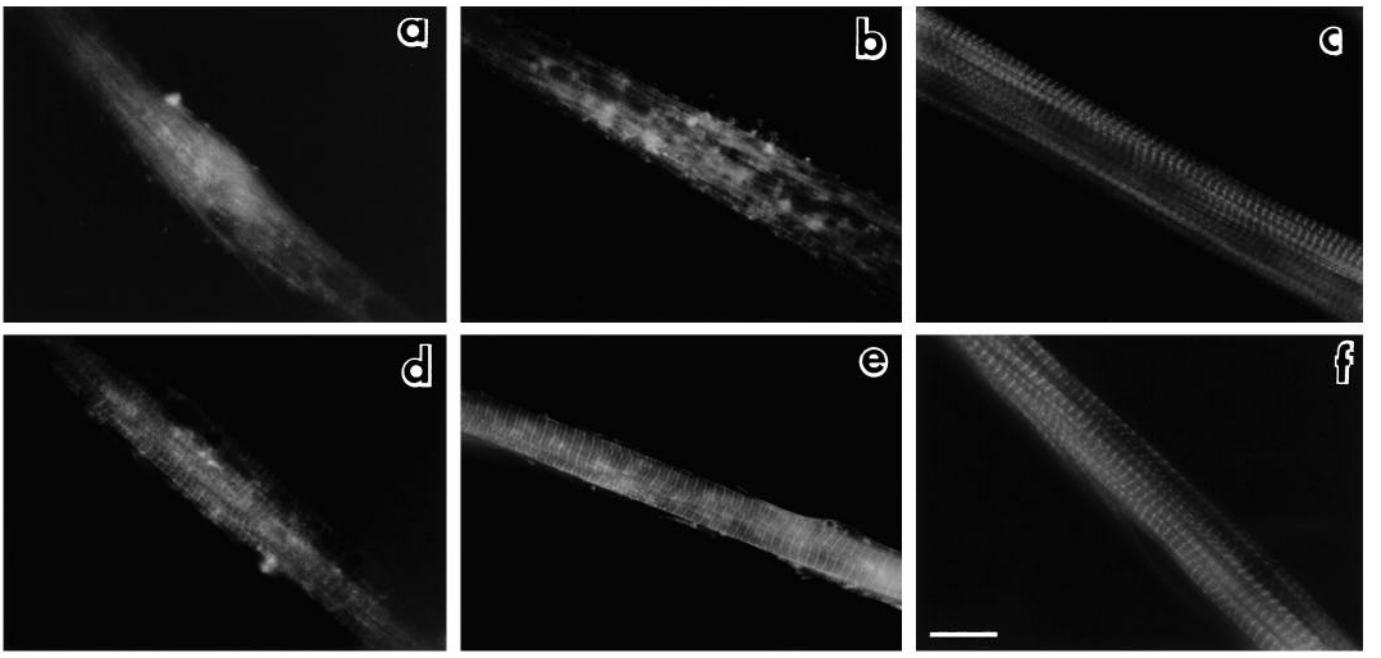


Fig. 10. Co-injection of FITC-actin and IATMR-cofilin into developing myotubes. FITC-actin (47 μM) alone or the mixture of FITC-actin (47 μM) and IATMR-cofilin (96 μM) was dialyzed against G-buffer overnight, and then was injected into chicken myotubes cultured for 6 days. The cells were fixed at 10 minutes (a and d), 30 minutes (b and e) or 24 hours (c and f) after the injection, and the localization of FITC-actin was observed under a fluorescence microscope. (a-c) FITC-actin and IATMR-cofilin were injected; (d-f) FITC-actin alone was injected. Bar, 20 μm.

cofilin introduced into the cells interacts quickly with actin filaments to disassemble them and generates the cytoplasmic actin-cofilin rods without binding to phosphoinositides. This view is supported by our observations that: (1) the number and size of the cytoplasmic rods were significantly decreased when cofilin was pre-incubated with PIP_2 before introducing into the cells; (2) the cofilin treated with PIP_2 became active to form the actin-cofilin rods in the cytoplasm, when the cells were stimulated with DMSO or an NaCl buffer; and (3) in the actin-cofilin rods, PIP_2 was scarcely detected by an immunocytochemical method, while the localization patterns of cofilin appeared similar to that of PIP_2, when cofilin was inactive in terms of the rod formation.

The activity of cofilin might be modulated by phosphorylation of the protein molecules. It has been suggested that cofilin is phosphorylated by serine/threonine kinase(s), and that heat-shock and DMSO-treatment stimulate dephosphorylation of cofilin in fibroblastic cells (Ohta et al., 1989). Actually, the binding ability of cofilin to actin is decreased by phosphorylation (Abe et al., 1992). It was recently demonstrated that in the case of actin depolymerizing factor (ADF), a cofilin homologue, a phosphorylated form of this protein exhibits only weak actin-binding ability, and that the relative proportion of this form increases as muscle develops (Morgan et al., 1993). In contrast, however, the amount of the phosphorylated form of cofilin is very small in cultured myotubes as well as in muscle tissues (Abe et al., 1993). Therefore, it is not yet clear just how important phosphorylation is for regulating the cofilin activity in muscle cells.

During muscle development, the relative proportion of cofilin to actin decreases significantly in muscle cells because the amount of actin increases markedly, although the cofilin expression does remain to some extent. Cofilin may become insufficient to control actin assembly, and its action of cofilin might only persist at local regions of muscle cells. We previously demonstrated that the inhibitory action of cofilin and also ADF on actin polymerization was removed by myosin (Abe et al., 1989; Abe and Obinata., 1989). Myosin filaments may function as an accelerator for actin assembly in developing muscle cells (Hayashi et al., 1977; Shimada and Obinata, 1977) even in the presence of cofilin, although in young immature muscle cells the amount of myosin is small in comparison with that of actin (Obinata, 1993). Actin filaments in myofibrils may become resistant to cofilin because of the presence of neighboring myosin filaments and the other actin-associated proteins, troponin and tropomyosin.

The actin-cofilin rods can be generated in both nuclei and cytoplasm of myoblasts, myotubes (Abe et al., 1993) and fibroblastic cells (Nishida et al., 1987) under various conditions, but the physiological implications of their formation have not yet been clarified. We suggest that the rod formation may be due to the accumulation of the actin-cofilin complex to a certain level, which in turn was caused as a result of the disruption of actin filaments. Actually, the size and number of rods increased in proportion to the amount of cofilin introduced, to wit, to the extent of actin disassembly.

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