Stimulus-dependent Disorganization of Actin Filaments Induced by Overexpression of Cofilin in C2 Myoblasts

Shoichiro Ono1, 2*, Hiroshi Abe2, and Takashi Obinata2

1Mitsubishi Kasei Institute of Life Sciences, Minamiooya, Machida, Tokyo 194, Japan and 2Department of Biology, Faculty of Science, Chiba University, Yayoi-cho, Inage-ku, Chiba 263, Japan

Key words: stress fibers/actin rods/myosin/inhibitor/transfection

ABSTRACT. Actin depolymerizing factor (ADF)/cofilin is a widely distributed family of actin-binding proteins which regulate actin polymerization in a pH-dependent manner. In cultured cells, cofilin, as well as ADF, translocates from the cytoplasm into the nucleus together with actin and forms rod-like structures in response to heat shock or dimethylsulfoxide (DMSO) treatment. In order to study in vivo interaction of cofilin with actin, we examined the effects of cofilin overexpression on actin cytoskeleton in C2 myoblasts. Interestingly, no remarkable effect was observed on phalloidin-stained patterns in cells overexpressing cofilin as compared with normal cells. However, upon treatment with DMSO, cytoplasmic actin filaments were disrupted and intranuclear rod structures containing cofilin and actin were apparently larger and thicker in cells overexpressing cofilin than in normal cells. Heat shock also stimulated disruption of microfilaments and formation of both intranuclear and prominent cytoplasmic cofilin-actin rods in cofilin-transfected cells, suggesting that DMSO-treatment or heat shock triggers cofilin-actin interaction. We further found that a myosin ATPase inhibitor (BDM) induced a reduction in cytoplasmic staining with phalloidin in cofilin-transfected cells. The results suggest that myosin activity might be involved in the regulation of cofilin-actin interaction in vivo.

In the process of organizing actin filaments, such as myofibrils, contractile rings and stress fibers, actin polymerization must be regulated so that proper structures are formed. Several families of actin monomer-binding proteins act as modulators of actin polymerization (30). They include β-thymosins, profilin, and actin depolymerizing factor (ADF)/cofilin.

Actin depolymerizing factor (ADF) (7) and cofilin (23) are highly homologous proteins and are regarded to constitute a widely distributed family of actin-binding proteins in eukaryotic cells (for review, 17). ADF/cofilin binds to both G- and F-actin at a stoichiometry of 1:1 and regulates actin polymerization in a pH-dependent manner; actin depolymerizing activity is stronger at slightly alkaline pH (pH 7.3–8.0) than at neutral pH (2, 12, 13, 14, 23, 33).

Interaction of ADF/cofilin with actin must be regulated in vivo in order to achieve reorganization of actin cytoskeleton. For example, ADF/cofilin, together with actin, translocates into the nucleus in response to heat shock and treatment with dimethylsulfoxide (22), and cofilin is localized in contractile rings, which are temporally formed actin bundles during cell division (20).

To date, there are two hypotheses as to the mechanism of regulation of cofilin-actin interaction. The first is regulation by binding of phosphoinositides with ADF/cofilin. Phosphoinositides bind to ADF/cofilin to inhibit its interaction with actin in vitro (32). In cultured cells, disruption of microfilaments by microinjection of cofilin is suppressed by co-injection of phosphatidylinositol 4,5-bisphosphate (21). The second is regulation by phosphorylation. ADF (18) and cofilin (19) are phosphorylated in vivo and the phosphorylated form cannot bind to actin. The phosphorylation site is located at Ser-3 (5, 19) which is conserved in all the proteins of the ADF/cofilin family reported to date. However, the sequence does not fall into any consensus sequences for conventional kinases, and the responsible kinase(s) has not been identified. In addition, dephosphorylation of ADF/cofilin has been observed in various cells, and this accompanies cytoskeletal changes (for review, 17). More recently, Xenopus ADF/cofilin has been shown to be rapidly dephosphorylated upon fertilization of eggs (4). These reports strongly suggest that ADF/cofilin acts as a modulator of actin polymerization which is responsive to a certain signal transduction pathway.

In this study, we investigated the effects of overexpres-
sion of cofilin on actin cytoskeleton in cultured cells in order to examine how cofilin interacts with actin in vivo and how the interaction is regulated. We showed that interaction of overexpressed cofilin with actin was suppressed, while heat shock and treatment with DMSO triggered their interaction. We also found evidence that myosin was involved in the regulation of cofilin-actin interaction.

MATERIALS AND METHODS

Construction of a vector for cofilin expression. An Eco RI-Kpn I fragment (1.1 kb) of chicken cofilin cDNA (pCMC-16, ref. 3) containing the entire coding region was cloned into pcDL-SRα 296 (31), a mammalian expression vector.

Cell culture and transfection of cells with cofilin cDNA. Mouse skeletal muscle cells (C2) were grown in DMEM supplemented with 10% fetal calf serum. CDNA transfection was performed as described (9). Briefly, cells were plated onto coverslips at a density of 5 x 10⁴ cells per 60 mm dish. The next day, coprecipitates of the plasmid (10 μg) with calcium phosphate were added to the media and incubated for 12–15 hr. The cells were washed with phosphate-buffered saline (PBS), and fresh media were added. They were then cultured for 2 days and used for experiments.

Heat shock and treatment with dimethylsulfoxide and other drugs. For heat shock, the culture medium was replaced with HEPES-buffered DMEM containing fetal calf serum, and the dishes were incubated in a water bath at 42°C for 1 hr.

Dimethylsulfoxide (spectroscopic grade, Dojindo Laboratory, Kumamoto, Japan) was added to the medium at a concentration of 10% and the dishes were incubated for 1 hr. A myosin ATPase inhibitor, 2,3-butanedione 2-monoxime (BDM; Sigma Chemical Co., St. Louis, MO), was used at 10 mM.

Immunofluorescence microscopy. The cells on the coverslips were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and then permeabilized with cold acetone (−20°C) for 5 min. They were blocked with 1% bovine serum albumin (BSA) and incubated sequentially with anti-cofilin monoclonal antibody (MAB-22) [Abe et al., 1989] and with fluorescein-labelled goat anti-mouse IgG. For double staining, the cells were further reacted with rhodamine-la-

![Image 1](https://example.com/image1.jpg)

**Fig. 1.** Localization of cofilin and actin in C2 myoblasts which overexpressed cofilin. C2 myoblasts were transfected with cofilin cDNA and double-stained with anti-cofilin antibody (MAB-22) (a and c) and either phalloidin (b) or anti-actin antibody (d). Overexpressed cofilin and actin was colocalized at the leading edges (c and d, arrowheads). Note that filamentous actin structures appear almost intact in cells overexpressing cofilin. Bar = 20 μm.
belled phalloidin, or with anti-actin antibody (16) (a kind gift from Dr. Ichiro Yahara, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), followed by rhodamine-la-
belled goat anti-rabbit IgG. Each treatment was performed
for 60 min at room temperature and followed by washing with
PBS. Specimens were mounted with 10 mg/ml p-phenylenedi-
amine, and 90% glycerol, in PBS.

RESULTS

To investigate the intracellular effects of cofilin on ac-
tin cytoskeleton, C2 myoblasts were transiently trans-
fected with cofilin cDNA under the control of SRα pro-
moter that is able to induce constitutive expression of
cloned genes. Cells which overexpressed cofilin were
easily detected by staining with anti-cofilin antibody
(MAB-22) (Fig. 1). Although surrounding cells which
were not transfected contained endogenous cofilin, the
staining was much weaker than that of the cDNA-trans-
fected cells. Therefore, it did not shown up on the pho-
tographs, and thereby quantitative analysis of the level
of overexpression was difficult to perform from the
micrographs. Thus, in each case, cells which overex-
pressed cofilin were observed in over fifty cells in at least
two separate experiments. Overexpressed cofilin was
diffused in the cytoplasm and partly diffused in the nu-
clei, in a similar distribution pattern to that of endoge-
nous cofilin. Staining with phalloidin and anti-actin an-
tibody revealed that the stress fibers and peripheral ac-
tin filaments in these cells were hardly affected by over-
expression of cofilin (Fig. 1). Although colocalization
of cofilin and actin was observed at cell leading edges
(Fig. 1c and d, arrowheads), most of overexpressed cofi-
lin did not localize in filamentous structures (Fig. 1a
and c). In addition, the localization of tropomyosin was
intact in stress fibers in these cells (data not shown). Pre-
nvious in vitro studies have shown that cofilin binds to
both G- and F-actin to regulate its polymerization and
inhibits binding of tropomyosin to actin. These results
suggest that overexpressed cofilin does not interact with
actin filaments in stress fibers.

Cofilin has been reported to accumulate in the nucle-
us together with actin in response to stresses, includ-
ing heat shock and treatment with dimethylsulfoxide
(DMSO) in fibroblasts (22) and myogenic cells (25). In
order to clarify the role of cofilin in these responses, we
first examined the effects of heat shock on the actin cyto-
skeleton of cofilin-transfected cells. Upon heat shock

Fig. 2. Induction of formation of cofilin-actin rods in cofilin-transfected cells by heat shock. C2 myoblasts were transfected with cofilin cDNA,
incubated at 42°C for 1 hr, and double-stained with anti-cofilin antibody (MAB-22) (a and c) and either phalloidin (b) or anti-actin antibody (d).
Rod structures containing cofilin were formed in cofilin-transfected cells (a and c). These rods were stained by anti-actin antibody (d), but not by
phalloidin (b). Bar = 20 μm.
(42°C, 1 hr), the localization of overexpressed cofilin changed markedly in nearly all the transfected cells, forming large cytoplasmic rod structures as well as intranuclear rods (Fig. 2a). The cytoplasmic rods were stained with anti-actin antibody (Fig. 2d) but not with phalloidin (Fig. 2b). This is characteristic of cofilin-actin rods, as phalloidin is unable to associate with cofilin-bound actin filaments (22). In normal C2 cells, upon heat shock, intranuclear rods which were formed by endogenous cofilin and actin were observed in approximately 10% of the cells, whereas no large cytoplasmic cofilin-actin rods were detected (data not shown). The results suggest that heat shock triggered interaction of overexpressed cofilin with actin and caused the formation of cytoplasmic rods.

Since treatment of cells with dimethylsulfoxide (DMSO) also induces formation of intranuclear cofilin-actin rods (22), we examined the effects of DMSO-treatment on microfilaments of cofilin-transfected cells. As shown in Fig. 3, overexpressed cofilin appeared to disassemble actin filaments in response to DMSO-treatment, so that the stress fibers in such cells almost completely disappeared as revealed by phalloidin-staining (Fig. 3b, arrowheads), and cofilin was translocated into the nucleus to form rod structures (Fig. 3a). Because phalloidin does not recognize monomeric actin or filamentous actin associated with cofilin, localization of total actin was revealed by staining with anti-actin antibody (Fig. 4). The intranuclear rods were also stained with anti-actin antibody (Fig. 4c and d). Cytoplasmic staining of actin in transfected cells appeared weaker and the number of intranuclear rods was greater than that in non-transfected cells (Fig. 4d). In addition, almost all of the transfected cells had nuclear cofilin-actin rods, compared to approximately 30% of nontransfected cells (data not shown). Unlike heat shock, DMSO did not induce the formation of cytoplasmic cofilin-actin rods. The results suggest that cofilin has an important role in mediating reorganization and nuclear translocation of actin in response to DMSO-treatment, and that DMSO induces cofilin to interact with actin.

Intranuclear actin rods have been reported to be induced by treatment with several pharmacological reagents, including a calmodulin antagonist (27) and activators of A-kinase (28). Since actin rods induced by these drugs appear quite similar to those induced by heat shock or DMSO-treatment, ADF/cofilin is probably involved in their formation. However, we observed

Fig. 3. Disruption of actin filaments in cofilin-transfected cells by DMSO-treatment. C2 myoblasts were transfected with cofilin cDNA, incubated with 10% DMSO for 1 hr, and double-stained with anti-cofilin antibody (MAB-22) (a) and phalloidin (b). (c): phase-contrast image. Intranuclear rods are formed in cofilin-transfected cells (a). Phalloidin-staining in such cells greatly decreased (arrowheads; b and c). Bar = 20 μm.
Fig. 4. DMSO-induced formation of actin rods in cofilin-transfected cells revealed by staining with anti-actin antibody. C2 myoblasts were transfected with cofilin cDNA, incubated with 10% DMSO for 1 hr, and double-stained with anti-cofilin antibody (MAB-22) (a and b) and anti-actin antibody (c and d). The phase-contrast images (e and f) are shown. Intranuclear rods were stained by both anti-cofilin (a) and anti-actin (c) antibodies. Photograph of (b) is overexposed to show the distribution of cofilin in nontransfected cells (arrowheads). The number of actin rods in cofilin-transfected cells was much greater than that in nontransfected cells. Bar = 20 μm.
that 1 mM dibutyryl-cyclic AMP, a membrane-permeable analogue of cAMP, and 50 μM W7, a calmodulin antagonist, did not induce intranuclear actin rods in either cofilin-transfected or normal C2 myoblasts (data not shown). Instead, when cells were treated with W7, cofilin-transfected cells showed much less staining with phalloidin than surrounding non-transfected cells (data not shown). The result strongly suggests that calmodulin-dependent process is involved in the regulation of cofilin-actin interaction.

In addition, we found that disassembly of stress fibers in cofilin-transfected cells was induced by treatment with 2,3-butanedione monoxime (BDM), an inhibitor of myosin ATPase (Fig. 5). Upon treatment with 10 mM BDM, filamentous staining with phalloidin almost disappeared (Fig. 5c and d) or was much de-
creased in cofilin-transfected cells (Fig. 5e and f), while stress fibers in surrounding normal cells were preserved. However, rod-shaped structures containing cofilin were not observed. Because treatment with BDM induces dissociation of myosin from stress fibers in cultured cells (11), interaction of actin and myosin might prevent cofilin from depolymerizing actin filaments.

**DISCUSSION**

In the present study, we examined the intracellular behavior of cofilin which was overexpressed in C2 myoblasts by cDNA transfection. We showed that overexpressed cofilin did not appear to interact with actin, whereas several treatments of cells induced drastic reorganization of actin filaments, specifically in cells which overexpressed cofilin. These findings suggest that the intracellular activity of cofilin is somehow being attenuated and that heat shock or DMSO-treatment releases cofilin from this "attenuation" so that it interacts with actin.

Although exactly what mediates the signals from heat shock or DMSO-treatment to cofilin is not known, this study demonstrated the possible involvement of myosin. Our result that BDM, an inhibitor of myosin ATPase (11, 15), induced disassembly of actin filaments in cofilin-transfected cells (Fig. 5), strongly suggests that myosin-actin interaction inhibits cofilin from interacting with actin. Previously, we have shown that actin severing activity of ADF is weakened by skeletal muscle myosin (1), supporting our present idea. Cramer and Mitchison (11) have reported that treatment of PtK2 cells with BDM induces dissociation of myosin from stress fibers, while actin filaments are unaffected, suggesting that dissociation of myosin from actin filaments is not sufficient to disorganize stress fibers. Localization of myosin in cofilin-transfected cells needs to be clarified to reveal the role of myosin in these cells. Recently, BDM has been shown to block increased phosphorylation of myosin light chain stimulated by lysophosphatidic acid (10), suggesting that BDM also affects phosphorylation-dependent activation of myosin ATPase. Therefore, this study indicates that cofilin may depolymerize actin filaments during reorganization of actin cytoskeleton associated with myosin inactivation. The relationship between cofilin and myosin in actin-binding needs to be examined in *vitro*.

Other possible causes of the attenuation in the activity of overexpressed cofilin are phosphorylation of cofilin and its binding to phosphoinositides. Phosphorylation of ADF/cofilin at Ser-3 inhibits its actin-binding ability (5, 19). ADF/cofilin is phosphorylated in *vitro* in part at various rates according to cell types, and its dephosphorylation accompanying reorganization of actin is induced by heat shock (24) and, treatments with DMSO (24, 29) and MLCK inhibitor (8), suggesting that dephosphorylation could be a trigger of ADF/cofilin-actin interaction. Therefore, since phosphorylation appears to be a physiologically significant mechanism that regulates activity of ADF/cofilin, it is quite possible that overexpressed cofilin is inactivated by phosphorylation. Although the amount of overexpressed cofilin phosphorylated has not been quantitated, we have shown that cofilin is phosphorylated in C2 myoblasts at a considerable rate (26), indicating that overexpressed cofilin could be phosphorylated.

Alternatively, phosphoinositides may be regulators of cofilin-actin interaction in *vitro*. Direct binding of phosphoinositides to cofilin inhibits its actin-binding activity in *vitro* (32). Nagaoka et al. (21) have reported that microinjection of cofilin into cultured cells induces rapid disruption of actin filaments within 30 min and formation of cytoplasmic cofilin-actin rods, and that co-injection of phosphatidylinositol 4,5-bisphosphate (PIP2) suppresses such phenomena. In the microinjection experiment, bacterially expressed recombinant cofilin, which could be fully active, was introduced into the cell at a high concentration in a short period. However, since overexpressed cofilin introduced by cDNA transfection could be gradually accumulated in the cell, expressed cofilin may be subjected to binding with PIP2, phosphorylation and/or some unknown mechanisms such that the activity is inhibited. Actually, the activity of microinjected cofilin appears to be diminished in the long term (24 hours after injection) (21).

Recently, Aizawa *et al.* (6) and Moriyama *et al.* (19) have reported that overexpression of cofilin induces formation of cytoplasmic actin bundles in *Dictyostelium* and fibroblasts. One major difference in our results from these reports is the requirement of some stimulation to cause reorganization of actin filaments in cofilin-transfected cells. In our system, overexpressed cofilin did not appear to interact with actin and disorganization of actin filaments was induced only by heat shock and treatment with DMSO and certain drugs. However, in these other reports, actin reorganization occurred just by overexpression of cofilin. This is probably due to the difference in the rate and amount of the overexpressed cofilin and/or the level of intracellular activities that inhibit cofilin-actin interaction among cell types. Although the regulatory mechanism has not totally been elucidated, quantitation of the rate of phosphorylated cofilin and cofilin-bound phosphoinositides needs to be examined. Also, the difference in the level of overexpression may result in morphological variety. Aizawa *et al.* (6) reported that a sevenfold increase in cofilin induced actin-bundle formation in *Dictyostelium*. However, since we used a transient expression system, it was difficult to estimate how much cofilin increased. The study using microinjection to introduce exogenous cofi-
lin showed that the effect of exogenous cofilin was dose-dependent on the rate of cells that contained cofilin-actin rods (21), suggesting that the increase in exogenous cofilin was not sufficient to affect actin organization in our system.

In conclusion, we showed here that intracellular activity of cofilin was regulated by a certain mechanism and activation of cofilin might involve inactivation of myosin. To clarify the role of myosin, the effects of MLCK-phosphorylated and non-phosphorylated myosin on cofilin-actin interaction should be examined.

Acknowledgments. We thank Dr. Ichiro Yahara (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) for providing the anti-actin antibody. This study was supported by grants from the Ministry of Education, Science and Culture of Japan, and the National Center of Neurology and Psychiatry (PCNP) of the Ministry of Health and Welfare of Japan.

REFERENCES


Actin Disorganization by Cofilin


(Received for publication, November 8, 1996
and in revised form, November 30, 1996)