Low Molecular-weight G-actin Binding Proteins Involved in the Regulation of Actin Assembly during Myofibrillogenesis

Takashi Obinata1,*, Rie Nagaoka-Yasuda1,*, Shoichiro Ono1,**, Kenichi Kusano2, Kurato Mohri1, Yoshiharu Ohtaka1, Sawako Yamashiro1, Kyoko Okada1, and Hiroshi Abe1

1Department of Biology, Faculty of Science, Chiba University, Yayoi-cho, Chiba, 263 and 2Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

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ABSTRACT. We previously demonstrated that small G-actin binding proteins, cofilin, ADF and profilin, are involved in the actin dynamics during myofibrillogenesis (OBINATA, T. (1993). Int. Rev. Cytol., 143: 153-189.). To better understand how they are responsible for the regulation of actin assembly, the amounts of the actin-binding proteins were quantified by means of quantitative immunoblotting and compared with that of G-actin pool. The sum of the amounts of cofilin, ADF and profilin was insufficient at early developmental stages but sufficient at later stages to account for the pool of G-actin in muscle cells. We detected expression of thymosin β4 at a considerable level in young embryonic but not in adult skeletal muscles. We, therefore, conclude that the G-actin pool in young embryonic skeletal muscle is mainly due to cofilin, ADF, profilin and thymosin β4. Switching from a non-muscle-type (NM-) cofilin to a muscle-type (M-) cofilin was observed during muscle development of mammals. In order to clarify cofilin-dependent regulation of actin assembly in muscle cells, cofilin tagged with fluorescence dyes was introduced into C2 myoblasts by a micro injection method. The exogeneous cofilin, but not ADF, caused quick disassembly of actin filaments and accumulated in furrow region of dividing cells. The analogs of the unphosphorylated form (A3-cofilin) and the phosphorylated form (D3-cofilin) were prepared by converting Ser3, a regulatory phosphorylation site, to Ala or Asp. When A3-cofilin and D3-cofilin were injected into living cells, the former was concentrated at the membrane ruffles and cleavage furrow, while the latter showed only diffuse distribution in the cytoplasm. These results suggest that the subcellular distribution of cofilin as well as its interaction with actin in vivo is regulated by its phosphorylation and dephosphorylation.

In young embryonic skeletal muscle, about 40-50% of the total actin is pooled in a monomeric form in the cytoplasm (31). Three G-actin-binding proteins, cofilin, ADF and profilin, have been isolated from chicken embryonic skeletal muscle as a complex with G-actin. Therefore, they have been postulated as being inhibitors for actin polymerization in developing muscle (1, 2, 25). However, it has not yet been clarified whether the amounts of the three actin-binding proteins in embryonic skeletal muscle are sufficient to account for the G-actin pool. Recently, thymosin β4, a G-actin-sequestering protein, has been detected in a variety of tissues (12, 27, 35), and this protein is now regarded as a primary candidate for buffering G-actin in platelets and other non-muscle cells because of its abundance and its high affinity for actin monomer (27, 35). Therefore, thymosin β4 might also be responsible for the G-actin pool in developing muscle cells, but the presence of this protein in developing skeletal muscle has scarcely been investigated.

Among the actin-binding proteins, cofilin is of special interest as a regulator for reorganization of actin in developing and degenerating muscles, since this protein is expressed at a high level in embryonic (1), denervated (32), and dystrophic (13) skeletal muscles. In mammals, a cofilin characteristic of muscle (M-cofilin) (26) has been discovered in addition to a non-muscle-type cofilin (NM-cofilin) (17). The activity of cofilin can be regulated in cytoplasm by several ways. Cofilin binds to both G- and F-actin and regulates actin assembly in a pH dependent manner (23, 36). Phosphoinositides, especially PIP2 have been considered as a regulator for cofilin activity (20, 37). Phosphorylation is another major way to modulate the activity of cofilin and ADF, a homologue of cofilin. In the oocytes of Xenopus laevis, almost 100% of Xenopus ADF/cofilin (XAC) is phosphorylated, but more than 60% of the protein is dephosphorylated within 30 min after fertilization (6). The phosphorylated form of cofilin is shown to be free from
actin filament in vivo, and lacking binding activity to purified actin in vitro (4, 18). Biochemical analyses revealed that phosphorylated ADF neither binds to G-actin nor affects the rate or extent of actin assembly, but removal of phosphate from phosphorylated ADF restores full activity to depolymerize F-actin (7). Recently, it has been demonstrated that the phosphorylation site of both ADF and coflin is Ser #3 in their amino acid sequences (7, 18). However, it remained to be clarified how phosphorylated and dephosphorylated coflin behave in the cytoplasm in developing muscle cells.

In this study, first of all, we measured the concentrations of coflin, ADF and profilin in developing chicken skeletal muscle by a quantitative immunoblot assay with specific antibodies, and compared them with that of G-actin to clarify how they are responsible for the pool of G-actin in developing muscle. Next, we examined the switching of the expression of coflin isoforms, NM-cofilin to M-cofilin, during muscle development. Then, finally, to better understand the function of coflin and the regulation of its activity by phosphorylation in the cytoplasm of myogenic cells, a wild-type coflin as well as functional analogs of the unphosphorylated and phosphorylated forms, A3-cofilin and D3-cofilin respectively, were generated in an E. coli expression system. They were introduced into living cells to examine their function and their cellular distribution.

**MATERIALS AND METHODS**

**Antibodies.** Rabbit anti-serum to mouse M-cofilin was prepared with the purified recombinant M-cofilin which was produced in E. coli as an immunogen. The serum was extensively absorbed with NM-cofilin until the serum became negative with NM-cofilin. The specific immunoglobulin was isolated by affinity chromatography using Sepharose 4B (Phar- macia) coupled to M-cofilin. The polyclonal antibody to thymosin β4 was a generous gift from Dr. Helen Yin (University of Texas) (38). The other antibodies used were a polyclonal antibody to chicken coflin (MAB-22) (1), a monoclonal antibody to chicken ADF (ADF-1) (13), and a monoclonal antibody to actin (SkA- 04) (14).

**Northern blotting.** The cDNA probes used were the 400 bp fragment of the cDNA of rat thymosin b4 including the entire coding region (38) and 0.5 kb fragments containing the coding region of mouse NM-cofilin or M-cofilin (26). Total RNA was prepared by the rapid one-step method (8). 10–15 µg of RNA for each lane was separated on 1% agarose-formaldehyde gel and transferred to nitrocellulose membranes. Hybridization was performed by the procedure of Thomas (34), with probes labeled with 32P by the method of Feinberg and Vogelstein (10). The filters were finally washed in 2 x SSC or 5 x SSC (1 x SSC = 150 mM NaCl, 25 mM trisodium citrate) containing 0.1% SDS at 55°C.

**RESULTS AND DISCUSSION**

**Quantitative analysis of low molecular weight G-actin-binding proteins involved in the G-actin pool in embryonic chicken skeletal muscle.** The concentrations of coflin, ADF and profilin in developing chicken skeletal muscle were measured by quantitative immunoblot assay with specific antibodies to the respective proteins (21). Purified proteins were used as standards for assay. As shown in Fig. 1, in early stages of muscle development (10- to 15-days-old embryo), profilin was most abundant (0.38-0.55% of total protein) among the three G-actin-binding proteins. ADF was next in abundance (0.37%) in 10-days-old embryo, but declined remarkably as muscle developed, and became undetectable in the muscle at 1-day posthatch. Cofilin was also detected in a considerable amount (0.20%) in developing
skeletal muscle. As judged by the sensitivity of the method used, the level of ADF was estimated as below 0.01% of total protein at 1-day posthatch and below 0.007% of total protein at 6-months posthatch. Cofilin and profilin also diminished as muscle developed, but they could be detected even in adult muscle; cofilin and profilin constituted approximately 0.028 and 0.019% of the total protein, respectively. Their concentrations in 6-months-old chicken were calculated as being about 3.5 and 3.0 μM, respectively.

We also quantitatively examined the changes in the expression of cofilin, ADF and profilin in denervated and dystrophic chicken muscles in which myofibrillar disorganization is induced. The content of cofilin in the denervated breast muscle increased up to about 10-fold of that in the control muscle, and we also found that the profilin content was increased in the denervated muscle by 7-fold (Fig. 1). The increase in cofilin as well as profilin expressions was detected in the dystrophic chicken muscle; however, the alteration of their expressions was relatively unremarkable in comparison to that in the denervated muscle. In contrast, we could not detect any increase in ADF expression in denervated or dystrophic muscles.

In order to determine how the G-actin-binding proteins contribute to the G-actin pool in developing muscle, the molar concentration of G-actin was compared with those of cofilin, ADF and profilin. The concentration of total actin was determined by quantitative immunoblotting, and the G-actin concentrations were calculated using the values of the monomer-polymer ratio which was previously demonstrated (31). The total actin in the skeletal muscle was about 57 μM in 10-days-old embryo and 620 μM in adult, values consistent with those in the previous report (31). The concentration of G-actin in 10-days-old embryo was calculated as 22 μM (approximately 40% of the total actin). In the same age of embryo, the total concentration of the three G-actin-binding proteins (19.5 μM) was a little lower than that of G-actin, but in later developmental stages, it became somewhat higher than that of G-actin.

Since cofilin and ADF can bind not only to G-actin but also to F-actin, we estimated the proportion of the actin-binding proteins which is present in the actin filament fraction and in the soluble G-actin fraction of muscle homogenate of 10-days-old chicken embryo. A considerable amount of cofilin, approximately 20% of total cofilin, was detected in the filament fraction, while in the case of ADF and profilin, less amounts appeared in the same fraction (21). The amounts of cofilin, ADF and profilin in the soluble fraction were estimated as 2.5 μM, 5.0 μM, 9.4 μM, respectively. Since about 20% of ADF in muscle may be inactivated by phosphorylation at this age (16), the concentration of functional

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**Fig. 2.** Western and Northern blot analysis of thymosin β4 in chicken skeletal muscles. Left: Protein extracts from chicken breast muscle obtained from 10-days-old embryo (10dE), 15-days-old embryo (15dE), 1-day posthatch (1dPH) and 180-day posthatch (adult) were subjected to SDS-PAGE. Immunoblotting was performed using anti-thymosin β4 antibody (38). In one lane (Tβ4), 24 ng of purified rat thymosin β4 was applied. The position of rat thymosin β4 is indicated by an arrow. Right: Total RNA was isolated from skeletal muscle, brain and spleen of adult mouse, skeletal muscle, brain and spleen of adult chicken, and skeletal muscle, brain and liver of chicken embryo. RNA (15 μg) was electrophoresed, blotted and hybridized with the 400 bp fragment of mouse thymosin β4 cDNA coding region. After hybridization, the membranes were washed with 5× SSC at 55°C. The positions of 18 S ribosomal RNAs (small arrows) and thymosin β4 mRNA (large arrows) are indicated (taken from 21).
ADF may be 4 μM. Taking together, we assume that the sum of the amounts of cofilin, ADF and profilin which may be responsible for the G-actin pool is about 16 μM. The affinities (Kd) of the G-actin-binding proteins for G-actin has been regarded as around 0.2 μM (33). Most of the barbed ends of thin filaments at early phase of myofibrillogenesis may be capped by CapZ and/or α-actinin (28, 29, 30), while the pointed ends may be uncapped (12). In such case, the total amount of the three G-actin-binding proteins required for creating the 22 μM of G-actin pool present in the muscle of 10-days-old embryo can be calculated as about 31 μM, as described by Fechheimer and Zigmond (9). Therefore, the amounts of cofilin, ADF and profilin are not sufficient to account for the G-actin pool in young embryonic skeletal muscle.

Thymosin β is known to be primarily important for generating G-actin pool in non-muscle cells (12, 26, 29). However, thymosin β4 protein is not remarkable in muscle tissues, although the mRNA for thymosin β4 has been detected in heart and skeletal muscles (11). Thus, the role of β-thymosins in skeletal muscle has remained to be clarified. The expression of thymosin β4 in embryonic chicken skeletal muscle was detected by Northern blotting using rat thymosin β4 cDNA as a probe. As shown in Figure 2 (right), mRNA of about 0.8 kb was detected clearly in brain and spleen of both chicken and mouse, as previously reported (11), but the message was scarce in adult skeletal muscle. However, a clear signal was detectable in chicken embryonic skeletal muscle, when hybridization was carried out at low stringency. If we washed the membrane at higher stringency, the message became much weaker.

We also performed immunoblotting using anti-thymosin β4 antibody in order to examine whether thymosin β4 protein is present in developing skeletal muscle. Rat thymosin β4 was used as a standard. In the muscle extract from 10-days-old and 15-days-old chick embryo, a peptide band with the same mobility as rat thymosin β4 was detected by the antibody (Fig. 2, left). A protein band with lower mobility was also recognized by the antibody. In the adult skeletal muscle, protein bands were scarcely recognized by the antibody. We assumed that the peptide with the same mobility as rat thymosin β4 is chicken thymosin β4, and the concentration of the protein was roughly estimated as about 20-30 μM in the embryonic skeletal muscle. Since the Kd of thymosin β4 for G-actin is known to be 0.4-0.7 μM (29), this amount of thymosin β4 may sequester 8-16

![Fig. 3. Change in the expression of M- and NM-cofilins during development of mouse thigh muscle. Northern blot analysis of total RNA (15 μg) from the muscle was performed using the cDNA probes specific for M- and NM-cofilins and the relative proportion of the messages for two cofilin isoforms were estimated by densitometry.](image)

![Fig. 4. Immunofluorescence staining of mouse C2 cells with the antibody specific for M-cofilin (a) and anti-cofilin monoclonal antibody (MAB-22) which recognizes both M- and NM-cofilin (b). Myoblasts (marked by arrowheads) were scarcely stained with the anti M-cofilin antibody. Bar: 10 μm.](image)
$\mu$M of G-actin. From such estimation, we suggest that the combination of thymosin $\beta 4$, cofilin, ADF and profilin is sufficient to account for the G-actin pool in the embryonic muscle.

Thymosin $\beta 4$ has been regarded as an ideal factor for generating the cytoplasmic G-actin pool in non-muscle cells. However, for the regulation of rather complicated actin dynamics in developing and degenerating muscle cells, ADF, cofilin and profilin might be preferable to thymosin $\beta 4$, because they are not merely G-actin sequestering factors, but also possess the ability to promote actin assembly under certain conditions. Profilin-actin complex can bind to the barbed end of a filament, and the subsequent rapid dissociation of profilin leads to elongation of the filament. ADF and cofilin can increase the free barbed end by severing the filaments. While this effect of ADF and cofilin may lead to further depolymerization of filaments, profilin-induced polymerization can also be enhanced and the resultant fragments of the filaments may serve as nuclei for the polymerization of actin.

**Cofilin isoform expression during muscle development.** We previously demonstrated that two cofilin isoforms, muscle-type (M-cofilin) and non-muscle-type (NM-cofilin), are present in mammals (24), while a single isoform exists in chicken. As judged by the sequence homology, mouse M-cofilin is more like chicken cofilin; M-cofilin exhibits 96% identity in the amino acid sequence with chicken cofilin, while the identity between M- and NM-cofilins is only 81%. Among adult mouse tissues, the expression of the M-cofilin was restricted to skeletal muscle, heart and testis, although the mRNA for the NM-type was detected in a variety of non-muscle tissues.

In order to understand how the two types of cofilin isoforms contribute to the regulation of actin assembly during muscle development, the expression of the isoforms during development of mouse skeletal muscle was examined by Northern blotting by using the cDNA probes specific for M-cofilin (26) and NM-cofilin (17). Figure 3 shows the change in the relative proportion of the mRNAs encoding M-cofilin and NM-cofilin in mouse thigh muscle. The messages for both types were detected almost in the same level at the newborn stage, but the proportion of the M-cofilin message gradually increased during postnatal development and in adult,

**Fig. 5.** Effects of cofilin microinjection on actin filaments in C2 myoblasts. IATMR-labeled cofilin was injected into C2 myoblasts at the concentration of 4 mg/ml (c and d) and the cells were fixed and stained with anti-actin antibody at 2 hr after injection. Control cells without injection (a and b) were dually stained with anti-cofilin (a) and anti-actin (b) antibodies, followed by treatment with rhodamine-labeled (a) or fluorescein-labeled (b) second antibody. The location of cofilin, endogenous one in (a) and exogenous one in (b), and the location of actin were compared in the same cells under an epifluorescence microscope. Bar: 20 $\mu$m (Taken from 20).
the message for NM-cofilin became scarcely detectable. When their expression was examined in mouse embryo at earlier stages by in situ hybridization methods, a strong signal for NM-cofilin was detected in the entire embryo, but the message for M-cofilin was restricted to somite area in the beginning and then to muscle regions (data not shown).

During in vitro myogenesis of mouse C2 myogenic cells, expression of M-cofilin is upregulated (22). To further clarify the expression of M-cofilin in individual muscle cells by immunocytochemical methods, we prepared an antibody specific for M-cofilin. C2 myotubes were stained clearly by the anti-M-cofilin antibody, but the majority of myoblasts were scarcely stained by the antibody, while a monoclonal antibody (MAB-22) which recognizes both M- and NM-cofilins stained myoblasts as well as myotubes (Fig. 4).

These results suggest that the expression of M-cofilin is coupled with muscle differentiation and that both NM- and M-cofilins are involved in the regulation of actin assembly in the myotubes at early phase of myofibrillogenesis, while at later stages, M-cofilin becomes essential for muscle cells.

**Intracellular regulation of actin assembly by cofilin.** Cytoplasmic location of cofilin in myogenic cells have been examined by immunocytochemical methods to understand the effects of cofilin on actin filamentous structures in the cytoplasm; we observed that the localization of cofilin drastically changes in response to environmental stress, while it is mostly diffused in the cytoplasm of the resting cells (4, 5, 19, 24). In dividing cells, cofilin was detected in cleavage furrows (19). Heat-shock or DMSO treatment led to nuclear localization of cofilin and in addition, cofilin is concentrated in ruffled membranes (5). It should be noted that cofilin becomes concentrated in the regions where dynamic reorganization of actin occurs.

To further understand how the cofilin activity is regulated in the cytoplasm, we examined the function of cofilin in cytoplasm by microinjecting fluorescence-labeled cofilin into myogenic cells. In order to label cofilin with a fluorescent dye, we prepared a cofilin molecule having additional six amino acid residues including two cysteine at the N-terminus by manipulating chicken cofilin cDNA (3). The recombinant cofilin was labeled with either iodoacetamide tetramethylrhodamine (IATMR...
or 5-iodoacetamide fluorescein (5-IAF). We confirmed that the labeled cofilin is functionally active just as the native protein (20, 22).

When the fluorescence-labeled cofilin was injected into C2 myoblasts, the exogenous cofilin caused drastic reorganization of the actin cytoskeleton (Fig. 5, c and d), although in the control cells without injection, cofilin was diffused in the cytoplasm and actin filament bundles could be seen clearly (Fig. 5, a and b); in the cofilin-injected cells, actin filaments were almost completely disrupted and huge rod structures were formed and in the rod structures, both cofilin and actin were localized (Fig. 5, c and d). However, the effects of the exogenous cofilin on actin filaments were diminished within 24 hours; the rod structures disappeared completely and actin filaments were recovered (20). If we stimulate such cells with chemical agents, like NaCl (150 mM) or 10% DMSO, the actin/cofilin rods were formed again. Similar results were obtained with young developing myotubes of primary muscle cultures (20). These results strongly suggest that the activity of cofilin is controlled by some mechanisms in the cytoplasm.

Interestingly, in contrast to the effects of cofilin, injection of ADF, a homologue of cofilin, into C2 myoblasts did not cause the drastic change in actin cytoskeleton as observed by injection of cofilin. The exogenous ADF was mostly diffused in the cytoplasm and actin rod structures were not formed (Fig. 6). Thus, cofilin and ADF seem to function somewhat differently in the cytoplasm of myogenic cells, although they are very similar in molecular structure and exhibit similar functional properties in a test tube. The expression of the two proteins are also differently regulated (Fig. 1). It remains to be clarified whether ADF and cofilin have different roles in developing and degenerating muscles. The regulatory mechanisms of the possibly differential expressions of ADF and cofilin also represent an interesting and important subject for future studies.

It is matter of particular interest how cofilin activity is controlled in cytoplasm. Although it is known that the activity of cofilin can be regulated by several ways, recently, phosphorylation has been regarded as one of the major ways to modulate the activity of cofilin in cytoplasm and Ser #3 has been identified as the site of phosphorylation to modulate the functional activity (7, 18). Our previous results suggested that inisitol phosphate functions as a regulator for cofilin activity in muscle cytoplasm (18). Therefore, in order to clarify the function of the phosphorylated and unphosphorylated forms of cofilin in myogenic cells, we generated the analogs of the phosphorylated form (D3-cofilin) and the unphosphorylated form (A3-cofilin) by converting the phosphorylation site (Ser #3) to asparatic acid or alanine, respectively. The mutated proteins were produced in an E. coli expression system, and conjugated with fluorescent dyes (22).

In *in vitro* functional assay, A3-cofilin retained the ability to bind to and disassemble F-actin, while labeled D3-cofilin failed to interact with actin (22). We then injected A3-cofilin and D3-cofilin into living C2 cells to examine their cellular distribution. In spreading cells, A3-cofilin was concentrated at the membrane ruffles, but D3-cofilin showed only diffused distribution both in the cytoplasm and nucleus (Fig. 7). In dividing cells, A3-cofilin was concentrated at the cleavage furrow where endogenous cofilin is also known to be enriched, while D3-cofilin was not localized in the furrow region (22). These results suggest that the subcellular distribution of cofilin as well as its interacting with actin in the cells is regulated by its phosphorylation and dephosphorylation.

The present study strongly suggests that cofilin is a po-

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**Fig. 7.** Injection of A3-cofilin or D3-cofilin into C2 myoblasts. IATMR-A3-cofilin (3 mg/ml) (a) or 5-IAF-D3-cofilin (3 mg/ml) (b) was injected. After 9 hr incubation, cells were fixed, and the locations of A3-cofilin (a) (rhodamine channel) and D3-cofilin (b) (FITC channel) in spreading cells were observed under an epifluorescence microscope. Bar: 20 μm.
tent regulator for the reorganization of cytoplasmic actin filaments and actin assembly in developing muscle cells. Cofilin also seems to be involved in actin dynamics in degenerating muscle cells. When an active form of cofilin is accumulated in cytoplasm, it exerts drastic effects on actin filamentous structures. However, cofilin is not always active in the cytoplasm, and it becomes activated depending on physiological demand to reorganize actin filaments. We have shown that phosphorylation and dephosphorylation is involved in the regulation of cofilin activity. The regulatory mechanism(s) of phosphorylation/dephosphorylation of cofilin in the muscle cytoplasm remains for future studies.

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G-actin Binding Proteins in Developing Muscle


