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Effects of Exogenous β-Actinin (CapZ) on Actin Filamentous Structures in Cultured Muscle Cells

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ABSTRACT—β-Actinin (CapZ) is a heterodimeric actin-binding protein which is localized in the Z-bands of myofibril. It caps the barbed end of actin filaments and nucleates actin-polymerization in a Ca^{2+}-independent manner. As judged by these properties of β-actinin, it is conceivable that β-actinin is involved in the regulation of actin assembly, especially in the formation of I-Z-I complex during myofibrillogenesis. In this study, to examine the function of β-actinin in myofibrillogenesis, recombinant β-actinin (r-β-actinin) produced in an *E. coli* expression system was introduced into cultured myogenic cells by a microinjection method. Stress fibers in C2 myoblasts were disrupted soon after microinjection of recombinant β-actinin, but nascent as well as well-organized myofibrils were scarcely affected by exogenous β-actinin. Based on these observations, we suggest that in myoblasts where actin filaments are dynamically reorganized, reassembly process of actin filaments may be affected by the exogenous β-actinin, whereas actin filaments become more stable and less sensitive to exogenous β-actinin, when they are organized into myofibrillar structures and anchored to Z-lines in myotubes.

INTRODUCTION

Actin is a major constituent of thin filaments of myofibrils. In myoblasts, actin filaments are detectable as stress fibers mostly in the cortical region of the cells, although a considerable amount of actin is pooled as monomer in the cytoplasm (Shimizu and Obinata, 1986). At the early process of myofibrillogenesis in developing muscle cells under culture conditions, actin is assembled as stress-fiber-like structures (SFLS) (Antin *et al*., 1986). Primitive myofibrils are, then, formed in association with SFLS (Peng *et al*., 1981; Dlugosz *et al*., 1984). As myofibrillogenesis progresses, actin filaments in SFLS may be redistributed into thin filaments of myofibrils and, on the other hand, newly synthesized G-actin is assembled into the thin filaments (Obinata, 1993). It is matter of interest how assembly of monomeric actin and reorganization of filamentous actin are regulated and how actin filaments are anchored to Z-lattice during myofibrillogenesis.

β-Actinin (CapZ) is a heterodimeric actin-binding protein which caps the barbed end of actin filaments and nucleates actin-polymerization in a Ca^{2+}-independent manner (Maruyama *et al*., 1977; Casella *et al*., 1986; Maruyama *et al*., 1990). In myofibrils, it is localized in the Z lines (Casella *et al*., 1987). Spatial and temporal distributions of β-actinin (CapZ) during myofibrillogenesis have been described; β-actinin is localized in a periodic pattern characteristic of mature Z-discs along the non-striated actin filaments, SFLS, prior to the appearance of striated pattern of actin (Schafer *et al*., 1993).

As judged by the properties of β-actinin, it is conceivable that β-actinin is involved in the regulation of actin assembly, especially in the formation of I-Z-I complex of sarcomeres during myofibrillogenesis. The roles of CapZ in developing muscle cells have been examined by means of microinjection of antibodies and cDNA transfection into cultured muscle cells, and it was demonstrated that inhibition of CapZ with antibodies or expression of a mutant form of the subunit of this protein in developing muscle cells alters assembly of actin filaments during myofibrillogenesis (Schafer *et al*., 1995). Thus, β-actinin seems to play an important role in regulating the organization of actin filaments during sarcomere formation in developing muscle cells. However, the detailed behavior of β-actinin in the process of I-Z-I formation remains to be clarified.

Recently, we succeeded in preparing functional recombinant β-actinin (r-β-actinin) in *E. coli* expression system by using a vector containing the cDNAs encoding the two heterosubunits of β-actinin (Soeno *et al*., 1998). Therefore, in the present study, we introduced the r-β-actinin directly into cultured myogenic cells by microinjection method. We found that the exogenous β-actinin disrupted stress fibers in myoblasts, while the myofibrilar structures in cultured muscle cells are resistant to the exogenous protein.

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MATERIALS AND METHODS

Cell culture

Chicken mononucleated myogenic cells were dissociated from breast muscles of 12-day-old chicken embryos by mechanical dissociation (Ii *et al*., 1982) and filtered through 10 layers of lens paper. They were then plated on glass coverslips coated with collagen in 60-mm culture dishes at a density of 5×10^5 cells per dish. The culture medium consisted of 81% Eagle's minimum essential medium (MEM: Nissui Co., Tokyo) supplemented with 2 mM L-glutamine, 15% horse serum, and 4% chick embryo extract. C2 cells (Yaffe and Saxel, 1977) were cultured on glass coverslips in DMEM containing 10% fetal bovine serum (FBS). All the cultures were maintained in a humidified atmosphere with 5% CO_2 and 95% air at 37°C.

Microinjection of recombinant β-actinin

Functional recombinant β-actinin was prepared as described previously (Soeno *et al*., 1998). Purified protein was dissolved in 0.1 M KCl, 10 mM Hepes-KOH (pH 7.1). Mixture of recombinant β-actinin (βI'/βII) (2 mg/ml) and rhodamine-labeled dextran was micro-injected into the cells by the method of Hiramoto (1974) with a micromanipulator (Narishige, Tokyo). Samples were centrifuged at 100,000 × g just before use and filtered through a Millipore filter (pore size, 0.22 μm).

Antibodies

The monoclonal antibodies (McAb) (CZ-95) specific for α-actinin were prepared using α-actinin from chicken skeletal muscle as an immunogen by a standard procedure (Harlow and Lane, 1988). Hybridoma cells producing antibody were subcloned twice by a limiting dilution method. The supernatant from subcloned cultures was used as the source of antibodies. The specificity of the antibody is shown in Fig. 1; the antibody reacted with purified α-actinin and recognized only α-actinin to give a single band when the whole lysates of skeletal muscle and gizzard smooth were examined by immunoblotting combined with SDS-PAGE (Fig. 1A). It stained only the Z-lines of isolated myofibrils where α-actinin is localized (Fig. 1B).

Antibodies to actin which recognize sarcomeric α-actin specifically (SkA-06) or both cytoskeletal (β- and γ-) and sarcomeric actins (SkA-04) were described previously (Hayakawa *et al*., 1996). Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (GAM) was purchased from Bio-Rad (Richmond, California, USA), and fluorescein (FITC)-labeled GAM was from Tago (Burlingame, California, USA), respectively.

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 10 min on ice, and further fixed and permeabilized with 100% methanol for additional 5 min at –20°C. They were then exposed to the antibody specific for actin (SkA-06 or SkA-04) or anti-α-actinin antibody (CZ-95), followed by staining with FITC-GAM. The cultures were washed thoroughly with PBS and 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

In order to clarify how β-actinin affects actin filament organization during the process of myofibrillogenesis, the purified recombinant β-actinin (r-β-actinin), a complex of βI' and βII, was introduced into cultured myogenic cells. Previously, we observed that the activity of r-β-actinin was indistinguishable from that of authentic β-actinin purified from chicken breast muscle (Soeno *et al*., 1998). The cells were stained with anti-actin antibody, and the localization patterns of actin were examined by immunofluorescence microscopy.

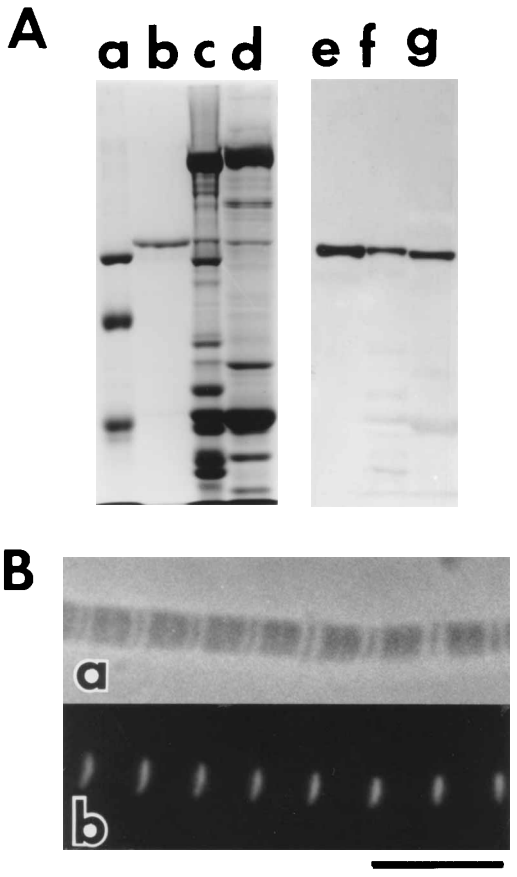

Fig. 1. Specificity of the monoclonal antibody to α-actinin (CZ-95) as examined by immunoblotting (**A**) and immunofluorescence staining (**B**). In (**A**), purified skeletal muscle α-actinin (b, e), the whole lysate of chicken pectoralis muscle (c, f), and the whole lysate of chicken gizzard muscle (d, g) were electrophoresed on SDS-polyacrylamide gels. One lane of each specimen (b, c, d) was stained with Coomassie Brilliant Blue to see the protein bands. The others (e, f, g) were electrophoretically transferred to nitrocellulose paper, and treated with the monoclonal antibody (CZ-95), followed by the treatment with peroxidase-conjugated GAM. Molecular weight markers, 94 kDa, 67 kDa and 43 kDa in the order from the top to the bottom, are shown in lane a. (**B**) Glycerinated myofibrils from chicken pectoralis muscle were reacted with CZ-95, followed by treatment with FITC-labeled GAM. Photographs were paired as phase-contrast (a) and fluorescence (b) images. Bar, 5 μm.

As shown in Fig. 2, when β-actinin was introduced into C2 myoblasts, soon after the microinjection, stress fibers were drastically disrupted and majority of actin was diffused in the cytoplasm (Fig. 2a). However, in 6 hr after the injection, actin filaments were significantly recovered in the cytoplasm (Fig. 2c). The activity of the exogenous β-actinin may be suppressed in the cytoplasm by some unknown mechanism. Such phenomena have also been observed in the case of other actin-binding protein, cofilin. It has been suggested that the activity of cofilin is regulated by binding of inositolphosphates in the cytoplasm (Nagaoka *et al*., 1995). β-Actinin could be regulated

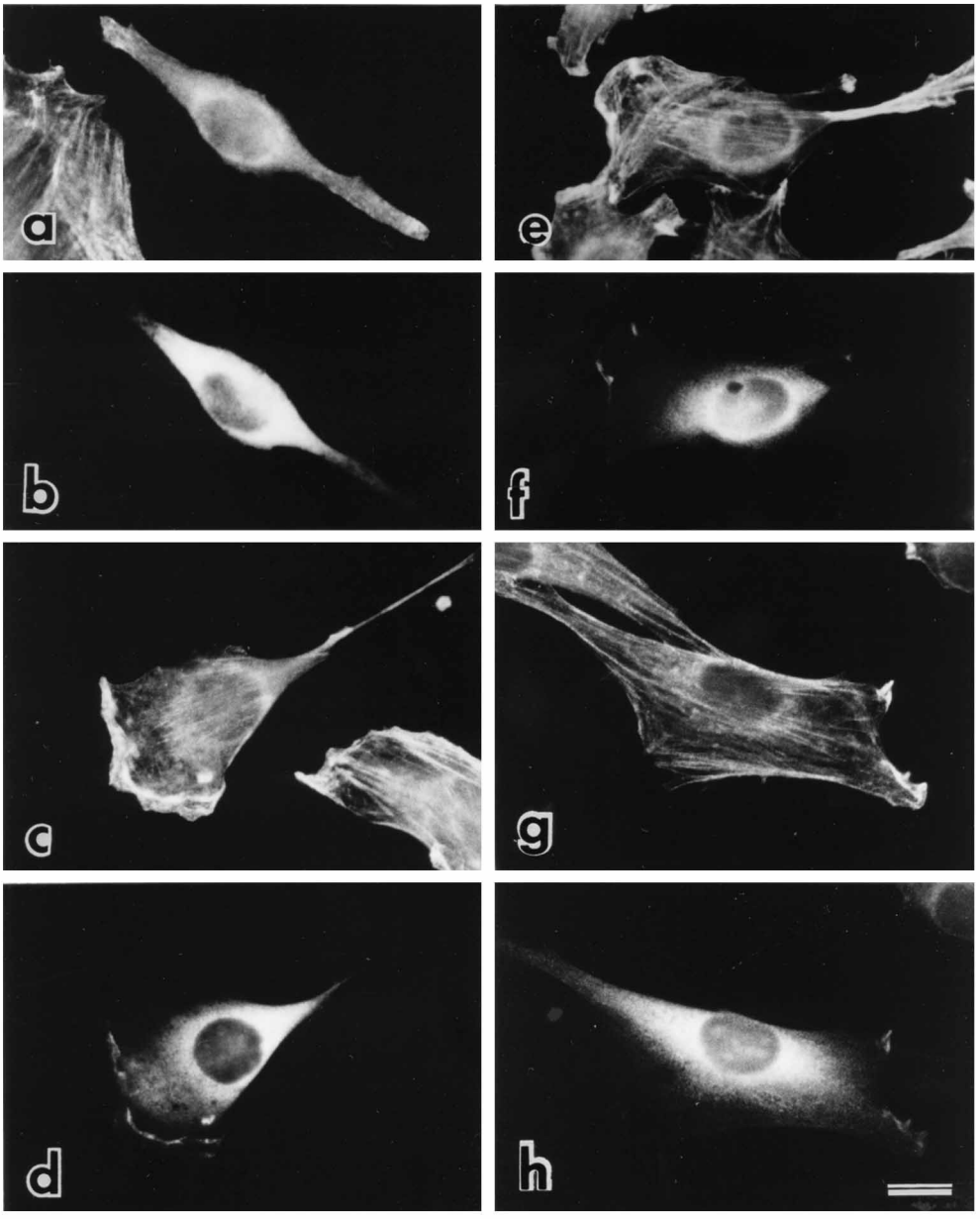

Fig. 2. Effects of r-β-actinin on actin filaments in C2 myoblasts in culture. r-β-Actinin at 2 mg/ml (**a**–**d**) or BSA at 2 mg/ml (**e**–**h**) was injected into C2 myoblasts together with rhodamine-labeled dextran. One hour (**a**, **b**, **e**, **f**) or 6 hr (**c**, **d**, **g**, **h**) after injection, cells were fixed and stained with anti-actin antibody (SkA-04) followed by treatment with fluorescein-labeled anti-mouse IgG. The cells injected with the exogenous proteins were identified under a rhodamine channel (**b**, **d**, **f**, **h**), and localization patterns of actin in the cells containing exogenous r-β-actinin (**a**, **c**) or BSA (**e**, **g**) were detected under a fluorescein channel. Bar, 20 μm.

in a similar way, since this protein also binds inositolphosphates (Heiss and Cooper, 1991; Schafer *et al*., 1996). In the control cells where bovine serum albumin was introduced, stress fibers were scarcely affected (Fig. 2e, g).

Effect of β-actinin on the process of myofibrillogenesis was examined by introducing r-β-actinin into young myotubes at 4-day of culture. As shown in Fig. 3a, stress-fiber-like structures (SFLS) which are regarded as actin filament bundles at the earliest process of myofibrillogenesis in the myotubes were scarcely affected by the exogenous β-actinin. Thus, SFLS seems to be less sensitive to the exogenous β-actinin than stress fibers, although SFLS and stress fibers appear morphologically similar under a fluorescence microscope.

r-β-Actinin was also introduced into more developed myotubes with cross-striated structures to examine whether sarcomeric structures and Z-lines are affected by the exogenous protein. As shown in Fig. 4, 1 hr after microinjection of r-β-actinin, localization of actin and α-actinin, a major component of Z-line, in myofibrils were scarcely affected as judged by the staining with SkA-06 (Fig. 4a) and CZ-95 (Fig. 4c). We further examined the effects for several hours after the introduction of r-β-actinin, but significant changes in myofibrillar

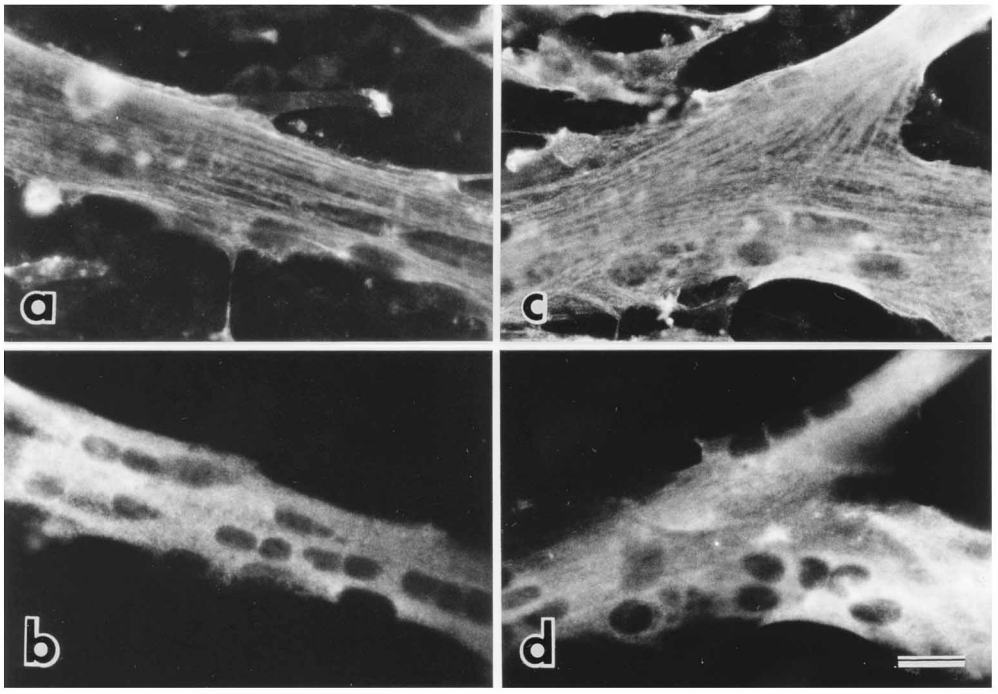

Fig. 3. Effects of r-β-actinin on actin filaments in young myotubes at 4-day in culture. r-β-Actinin at 2 mg/ml (**a**, **b**) or BSA at 2 mg/ml (**c**, **d**) was injected together with rhodamine-labeled dextran. One hour after injection, cells were fixed and stained with the antibody specific for sarcomeric actin (SkA-06). (**a**, **c**) Localization pattern of actin. (**b**, **d**) Detection of rhodamine-dextran. Bar, 20 μm.

structures were not observed. These results indicate that once sarcomeric structures are formed in multinucleated myotubes, the structures are not sensitive to the exogenous β-actinin.

DISCUSSION

We previously established a system to produce r-β-actin in *E. coli* which is functionally active just as the authentic protein in muscle tissues (Soeno *et al*., 1998). Therefore, in this study, we examined the effects of β-actinin (CapZ) on actin filament organization in developing muscle cells by introducing r-β-actinin into myogenic cells in culture. Direct microinjection of recombinant proteins into cells is advantageous to examine the effects of regulatory proteins for cytoskeletons in cytoplasm in comparison with the methodology to generate the proteins by cDNA transfection, since the effects of the exogenous proteins can be observed shortly after the introduction and the amount of the proteins is controlled; when the proteins are produced in the cytoplasm by cDNA transfection, sometimes it takes 2–3 days for production of sufficient amounts of proteins in the cytoplasm and the proteins could be inactivated during this period (Ono *et al*., 1996).

The effects of the exogenous protein may depend profoundly on the amount of protein introduced. We assume that the amount of the exogenous β-actinin introduced was in an excess in comparison with the endogenous protein as discussed below. The concentration of the exogenous β-actinin injected was about 2 mg/ml (roughly 30 μM). Since actin concentration in developing muscle cells may be 5–10 μM (Nagaoka *et al*., 1996), molar ratio of β-actinin to actin at the site of injection might be roughly around 1 : 0.3 in the beginning, and if the injected protein is diluted 10 times in the cytoplasm, the ratio may be around 1 : 3. These values are much higher than the value in mature muscle; the relative proportion of β-actinin and actin in mature muscle may be about 1 : 36 in molar ratio (calculated based on the data given by Ohtsuki *et al*., 1986). Injection of the recombinant protein into cells may lead to down-regulation of the expression of endogenous protein, but we assume that the amount of the endogenous protein may not be changed significantly in the short time range, 1–6 hr, in our present investigation.

In the two subunits of β-actinin termed βI and βII, several variants have been detected, which are expressed in a tissue-dependent manner (Asami *et al*., 1988b; Hart *et al*., 1997). They are βI (or CapZα2), βI' (or CapZα1), βII (or CapZβ1), βII', and βIII (or CapZβ2) (Funatsu *et al*., 1988; Schafer *et al*., 1994). During development of chicken pectoralis muscle, the amount of βIII subunit gradually decreases, while the amount of βII and βII' subunits gradually increase (Asami *et al*., 1988a). Proportion of actin isoforms also changes during muscle development, but βII and βIII isoforms do not differ in binding ability to different actin isoforms (Schafer *et al*., 1996). No marked difference in function has been detected between varied combinations of subunit isoforms, although difference in the affinity to actin filaments has been reported. Functional recombinant β-actinin has been obtained only in a combination of βI'/βII which we used in this study. This heterodimer exhibited higher affinity for actin, about 4-fold higher, than βI/βII heterodimer, when examined with *in vitro* translation products of RNA transcribed from the cDNAs (Casella and Torres,

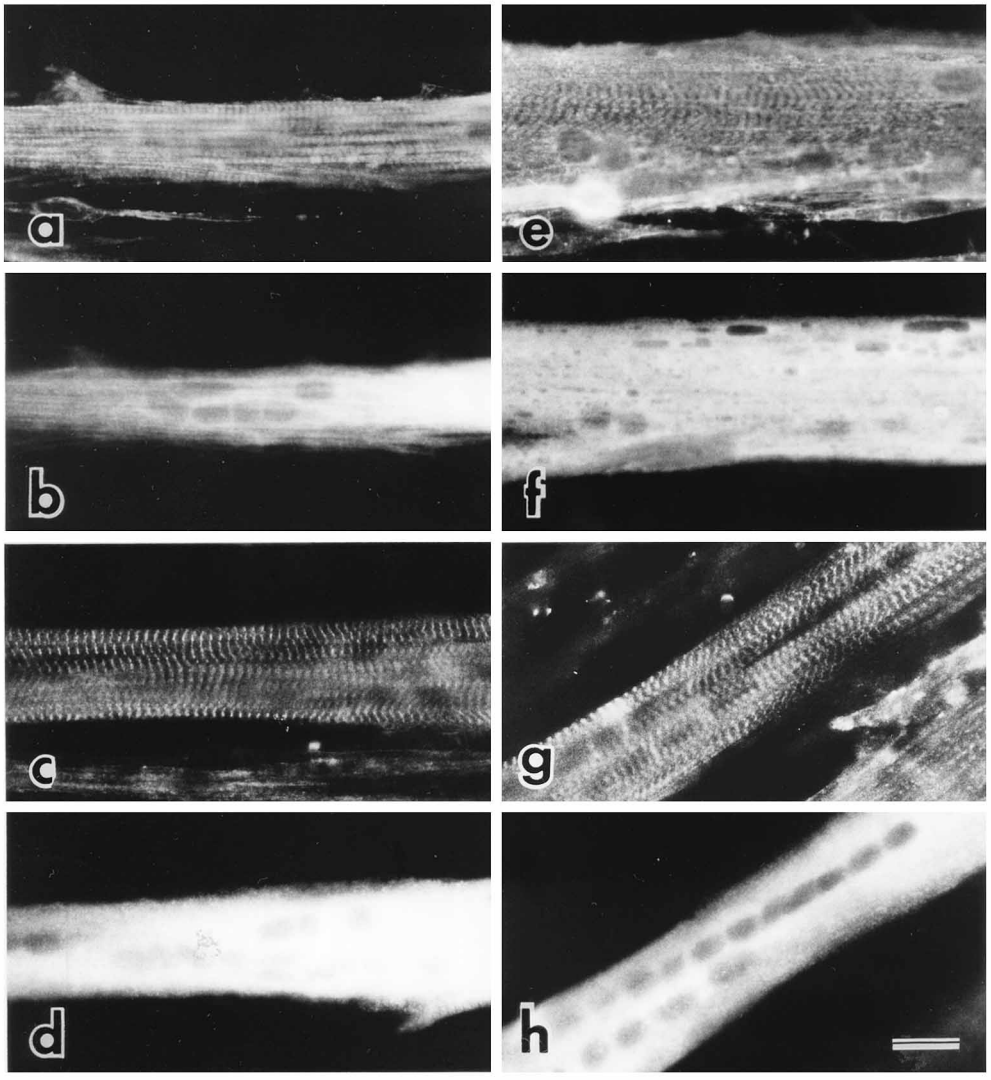

Fig. 4. Effects of r-β-actinin on cross-striated myofibrils. r-β-Actinin at 2 mg/ml (**a**–**d**) or BSA at 2 mg/ml (**e**–**h**) was injected into myotubes at 6-day in culture together with rhodamine-labeled dextran. One hour after injection, cells were fixed and stained with anti-actin antibody (SkA-06) (**a**, **b**, **e**, **f**) or anti-α-actinin antibody (CZ-95) (**c**, **d**, **g**, **h**) followed by treatment with fluorescein-labeled anti-mouse IgG. The cells injected with the exogenous proteins were identified under a rhodamine channel (**b**, **d**, **f**, **h**). Bar, 20 μm.

1994).

We observed that the introduction of r-β-actinin into myoblasts by a microinjection method caused the disruption of stress fibers, but in several hours after the injection, actin filaments were recovered in the cytoplasm (Fig. 2c). Basically the same results were obtained with fibroblasts (data not shown). These results indicate that exogenous β-actinin is functional to disrupt actin filamentous structures in the beginning when introduced, but the activity becomes suppressed gradually in the cytoplasm by the action of some factors. It has been reported that the activity of β-actinin for actin filaments is suppressed by binding of polyphosphoinositides to the protein (Heiss and Cooper, 1991; Schafer *et al*., 1996). The activity of cofilin was also suppressed in the cytoplasm in several hours, when introduced into myogenic cells, and it has been suggested that PIP_2 is involved in the suppression of cofilin activity (Nagaoka *et al*., 1995).

The actin filamentous structures in stress fiber-like structures (SFLS) and myofibrils were scarcely affected by the exogenous β-actinin. Actin isoforms which constitute stress fibers and myofibrils are not the same (Hayakawa *et al*., 1996), but this difference may not lead to the difference in sensitivity to β-actinin, since it has been pointed out that β-actinin (CapZ) can interact with different actin isoforms. We suggest that architectural differences of actin filamentous structures in myotubes and myoblasts cause the difference in sensitivity to exogenous r-β-actinin.

Stress fibers are rather unstable structures which can be reorganized in response to cellular circumstances. It has been reported that CapZ (β-actinin) is dissociated from some actin filaments at the ruffling membrane of actively spreading platelets, suggesting that CapZ is involved in dynamic reorganization of stress fibers (Nachmias *et al*., 1996). It has also been reported that CapZ is localized at the periphery of non-muscle

cells and also in the cytoplasm (Schafer *et al.*, 1992). Considering these properties of β-actinin in non-muscle cells, we assume that disruption of stress fibers by the exogenous recombinant β-actinin in the myoblasts may be caused as follows; the exogenous β-actinin may also be localized at the periphery of myoblasts and promotes actin polymerization to generate many short actin filaments. Accordingly, quantity of G-actin as components of stress fibers may be decreased and may lead to disappearance of stress fibers.

In contrast, CapZ is known to be localized along stress fiber-like structures at the early stage of myofibrillogenesis (Schafer *et al.*, 1993). Short actin filaments may be generated by exogenous β-actinin in young myotubes but they may be colocalized along endogenous stress fiber-like structures, so that we could not apparently observe the effect of exogenous β-actinin. In addition, stress fiber-like structures may be stabilized because of the presence of muscle-specific regulatory proteins as well as cytoskeletal proteins like connectin (titin). Organized myofibrils must be more stable structures. Actin filaments are anchored to Z-lines by the action of endogenous β-actinin which is localized in Z-lines together with other Z-line components and therefore, may not be disrupted by the action of the capping protein. The results of this investigation indicate that even developing myofibrils may be substantially stabilized and may not be in the state of dynamic disorganization and reorganization.

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