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Differential Assembly of Cytoskeletal and Sarcomeric Actins in Developing Skeletal Muscle Cells *In Vitro*

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ABSTRACT—Monoclonal antibodies (McAb) to actin were prepared to analyze the assembly of actin isoforms in developing muscle cells *in vitro*. One of the antibodies (SkA-06) was specific for α-sarcomeric actin isoforms in skeletal and cardiac muscles, while the others recognized cytoskeletal (β, γ) actin isoforms in smooth muscle and non-muscle tissues as well as the sarcomeric (α) actins. Using SkA-06 and a polyclonal antibody (PcAb) specific for cytoskeletal actins, the subcellular localization of the actin isoforms was examined by immunocytochemical methods. While in developing young myotubes, cytoskeletal and sarcomeric actins were co-localized in nascent myofibrils or stress-fiber-like structures, sarcomeric actins predominated in striated myofibrils in more developed myotubes. When FITC-labeled cytoskeletal and sarcomeric actins were introduced into young myotubes by a microinjection method, the latter became detectable in striated structures sooner than the former but they were finally incorporated into striated myofibrils. These results suggest that α-actin(s) as well as β- and γ-actins can be incorporated into myofibrils, but α-actin(s) is assembled preferentially into myofibrils in developing muscle cells.

INTRODUCTION

Multiple actin isoforms, six in higher vertebrates, are expressed in a tissue-specific manner (Vandekerckhove and Weber, 1978). In adult tissues, cardiac and skeletal muscle α-actins are localized in sarcomeres of myofibrils, while nonmuscle β- and γ-actins are constituents of cytoskeletal actin filaments. Adult skeletal muscle contains only α-skeletal actin isoform, whereas the major actin isoforms in embryonic or cultured skeletal muscle cells are of cardiac α- (Paterson and Eldridge, 1984) and nonmuscle β- and γ-types (Garrels and Gibson, 1976; Shimizu and Obinata, 1980; Schwartz and Rothblum, 1981). The transient expression of α-vascular actin in embryonic skeletal muscle was also reported (Sawtell and Lessard, 1989). During chicken skeletal muscle development, nonmuscle (β and γ) and cardiac (α) actins decline gradually and they are replaced by increased synthesis of the skeletal (α) actin (Hayward and Schwartz, 1986).

In developing muscle cells under culture conditions, actin is detectable as stress-fiber-like structures (SFLS) mostly in the cortical region of the cells in the beginning (Antin *et al.*, 1986), although a considerable amount of actin is pooled as monomer in the cytoplasm (Shimizu and Obinata, 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 particular interest how the cytoskeletal and sarcomeric actins are involved in myofibrillogenesis and whether the cytoskeletal and sarcomeric actins are co-assembled in contractile apparatus in the developing muscle. However, only limited information has been obtained as to the intracellular distribution of the actin isoforms in developing muscle cells, because the antibodies specific for the isoforms are scarce.

Differential location of the actin isoforms has been demonstrated using antibodies specific for either nonmuscle cytoskeletal or sarcomeric actins (Lubit and Schwartz, 1980; Padro *et al.*, 1983; Handel *et al.*, 1989). These studies mostly described that cytoskeletal actins are localized in the structures other than sarcomeres like mitochondria (Pardro *et al.*, 1983), membranous structures (Lubit and Schwartz, 1980) and stress fibers (Handel *et al.*, 1989). Handel *et al.* (1989), however, demonstrated further that cytoskeletal and sarcomeric actins are co-localized in nascent myofibrils. Recently, Arx *et al.* (1995) induced expression of sarcomeric and cytoskeletal actins in adult cardiomyocytes by cDNA transfection, and they observed that sarcomeric actin was mostly assembled into sarcomeres but to a lesser extent into SFLS, while ectopic expression of nonmuscle actin induced morphological

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alteration of sarcomeres in cardiomyocytes. From these resuts, they suggested that sarcomeric and cytoskeletal actins are sorted in the cytoplasm and play different functional roles.

On the other hand, Rubinstein *et al.* (1982) reported that cytoskeletal β- and γ-actins are selectively released into the medium from the cultured quail myoblasts and myotubes, suggesting that the cytoskeletal actins are present as monomers at least in part and compartmentalized from sarcomeric actins within muscle cells. We previously demonstrated that a considerable amount of profilin, a G-actin-binding protein, exists in embryonic muscle and this protein inhibits the polymerization of β- and γ-actins more efficiently, and therefore the assembly of cytoskeletal and sarcomeric actins may be regulated differently by profilin in the developing skeletal muscle, and that the former may not be involved in the myofibril assembly (Ohshima *et al.*, 1989).

In this study, we prepared a monoclonal antibody specific for sarcomeric α-actin. With this and the antibody specific for cytoskeletal β- and γ-actins, localization of sarcomeric and cytoskeletal actins in developing muscle cells *in vitro* was examined by immunocytochemical methods. The results indicate that cytoskeletal actins as well as sarcomeric actins can be incorporated into myofibrils, but sarcomeric actin(s) is assembled preferentially into myofibrils in developing muscle cells.

MATERIALS AND METHODS

Antibodies

The monoclonal antibodies (McAb) specific for actin were prepared by using a conjugate of chicken skeletal muscle actin and a synthetic peptide, WAPESAPLKSKM, corresponding to an actin-binding site of cofilin as an immunogen. Both were coupled with 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC). Hybridoma cells were formed between spleen cells of Balb/c mice immunized with the immunogen and nonsecreting myeloma cell line P3x63/Ag8U1 cells using the technique of Galfre *et al.* (1977) as modified by Gefter *et al.* (1977). 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 antibody to gizzard smooth muscle actin which recognizes cytoskeletal β- and γ- actins (Iida *et al.*, 1992) was kindly provided by Dr. Kazuko Iida (Tokyo Metropolitan Institute of Medical Sciences). Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (GAM) and HRP-labeled goat anti-rabbit IgG (GAR) were purchased from Bio-Rad (Richmond, California), and fluorescein (FITC)-labeled GAM and GAR, tetramethylrhodamine (TRITC)-labeled GAM and GAR were from Tago (Burlingame, California), respectively.

Preparation of actin

Actin was prepared from chicken skeletal muscle or gizzard smooth muscle by the method of Spudich and Watt (1971) and purified by gel filtration on a Sephadex G-100 column.

Gel electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 13.5% polyacrylamide gel in a discontinuous Tris-glycine buffer system according to Laemmli (1970). For immunoblotting, proteins were electrophoretically transferred from SDS-polyacrylamide gel to nitrocellulose paper (Towbin *et al.*, 1979). The nitrocellulose paper was treated with 3% gelatin and incubated for 1 hr with the antibodies, followed by treatment with HRP-labeled GAM or GAR for 1 hr. After the immunoreaction, the paper was washed with 0.5 M NaCl-10 mM Tris-HCl, pH 7.5. The HRP-labeled antibodies bound to the paper were detected as the diaminobenzidine reaction product with nickel and cobalt ions (De Blas and Cherwinski, 1983).

ELISA

Polystyrene 96-well plates were incubated with actin of 1.6 - 100 μg/ml in PBS overnight at 4°C and treated with 1% BSA in PBS for 2 hr at 37°C. The plates were, then, reacted with the antibodies to actin for 1 hr at 37°C, and followed by treatment with peroxidase-GAM or peroxidase-GAR for 1 hr at 37°C. After washing with PBS, *o*-phenylenediamine in citrate-phosphate buffer (pH 5.0) with H_2O_2 was added as a substrate. The reaction was measured as absorbance at 490 nm.

Cell culture and microinjection

Chicken mononucleated myogenic cells were dissociated from breast muscles of 12-day-old chicken embryos by means of mechanical dissociation (Ii *et al.*, 1982) 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. Actin was labeled with FITC as described previously (Saitoh *et al.*, 1988) and was filtrated through a Millipore filter (pore size, 0.22 μm) or centrifuged at 100,000 g just before use. Microinjection was performed by the method of Hiramoto (1974) with a micromanipulator (Narishige, Tokyo).

Indirect immunofluorescence microscopy

Pieces of freshly dissected chicken tissues were frozen in liquid nitrogen-cooled isopentane and serial transverse cryosections were cut at 8 μm. The sections were fixed with 4% paraformaldehyde in PBS (0.15 M NaCl and 10 mM sodium phosphate, pH 7.0) for 5 min. Muscle cell cultures grown on collagen-coated coverslips were fixed with 4% paraformaldehyde containing 0.2% Triton X-100 in PBS for 10 min at room temperature and then with acetone at -20°C for 5 min. The specimens were washed with PBS containing 10 mM glycine, and treated with 1% bovine serum albumin (BSA) in PBS. They were then exposed to anti-actin antibodies, followed by staining with FITC-labeled GAM or TRITC-labeled GAR. For double labeling, the cells were first reacted for 1 hr with the mixture of polyclonal and monoclonal anti-actin antibodies. After washing with PBS, they were reacted with a mixture of second antibodies, FITC-GAM and TRITC-GAR. Washing with PBS was carried out after the respective antibody treatments. 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 microscopy.

RESULTS

Characterization of monoclonal antibodies

Two hybridoma clones, SkA-04 and SkA-06, were established which produce antibodies to actin. When the total lysate of chicken skeletal muscle was examined by immunoblotting combined with SDS-PAGE, only a single actin band was recognized by both the antibodies, indicating that they are specific for actin (Fig. 1A). The antibody produced by SkA-06, however, reacted strongly with skeletal muscle actin but only faintly with gizzard smooth muscle actin (Fig. 1B). The antibody produced by SkA-04 interacted similarly with both skeletal and smooth muscle actins. When non-denaturing actin molecules from skeletal and smooth muscles were dot-

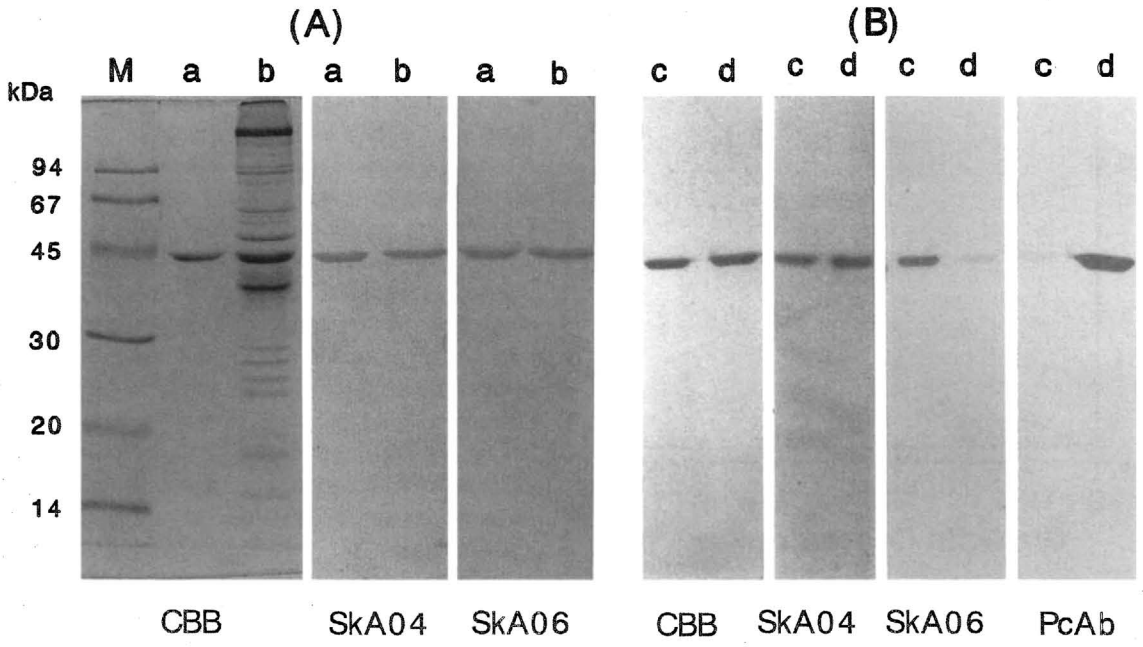

Fig. 1. Specificity of the monoclonal antibodies to actin as examined by immunoblotting. In (A), purified skeletal muscle actin (a) and the whole protein extract from chicken pectoralis muscle (b), and in (B), purified skeletal muscle actin (c) and purified gizzard smooth muscle actin (d) were electrophoresed on SDS-polyacrylamide gels. One lane of each specimen, marked CBB, was stained with Coomassie Brilliant Blue to see the protein bands. The others were electrophoretically transferred to nitrocellulose paper, and treated with the monoclonal antibodies (SkA-04 or SkA-06) or the polyclonal antibody to smooth muscle actin (PcAb), followed by the treatment with peroxidase-conjugated GAM or peroxidase-conjugated GAR. SkA-04 and SkA-06 reacted with the purified skeletal muscle actin or the crude muscle extract to give only one band corresponding to actin. SkA-04 also reacted with gizzard actin, but SkA-06 did only faintly with gizzard actin.

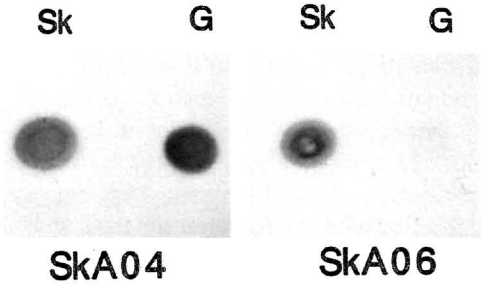

Fig. 2. Immunoreaction of non-denaturing actin with SkA-04 and SkA-06. Five microgram of purified skeletal muscle (Sk) and gizzard (G) actins without SDS-treatment were blotted on nitrocellulose membrane and treated with SkA-04 or SkA-06, as in Fig. 1.

blotted on nitrocellulose paper and treated with the antibodies, SkA-04 showed positive reaction with both skeletal muscle and smooth muscle actins but SkA-06 failed to react with smooth muscle actin (Fig. 2). Similar results were obtained by an ELISA method (Fig. 3); SkA-06 showed positive reaction with skeletal muscle actin but not with smooth muscle actin under non-denaturing conditions, while SkA-04 reacted similarly with both actins. These results indicated that SkA-06 recognizes skeletal muscle actin specifically under non-denaturing conditions.

To confirm the specificity of SkA-04 and SkA-06 further, immunoreaction of SkA-04 and SkA-06 with the cryosections of various chicken muscle and non-muscle tissues was examined by indirect immunofluorescence methods (Fig. 4). SkA-04 reacted positively with all the tissues examined, while SkA-06 reacted clearly with both skeletal and cardiac muscles which contain sarcomeric α-actins and very faintly with aorta which contains α-smooth muscle actin, but with neither gizzard smooth muscle nor brain at all which contains only β- and γ-actins. Immunofluorescence staining of skeletal muscle myofibrils with SkA-04 and SkA-06 was detected in a pattern characteristic of actin; the staining was obvious at the I-bands except the Z-lines and faint at the A-bands adjacent to the I-bands in the region of the thin and thick filament overlap (Data not shown). From these results, we conclude that SkA-06 is specific for the α-sarcomeric actins of skeletal muscle- and cardiac muscle-types and can interact with neither smooth muscle nor cytoplasmic β- and γ-actins under non-denaturing conditions, while SkA-04 recognizes both sarcomeric and cytoskeletal actin isoforms.

The polyclonal antibody raised against chicken gizzard actin (Iida *et al.*, 1992) was specific for smooth muscle and/or cytoplasmic β- and γ-actins, when it was absorbed with the acetone-dried powder of skeletal muscle. It did not interact with skeletal muscle actin either under denaturing conditions in immunoblotting combined with SDS-PAGE (Fig. 1B) or under non-denaturing conditions in ELISA assay (Table 1). The antibody stained the cryosections of gizzard smooth muscle but not the sections of skeletal muscle (Fig. 5). It should be noted that this antibody reacted also with actin in non-

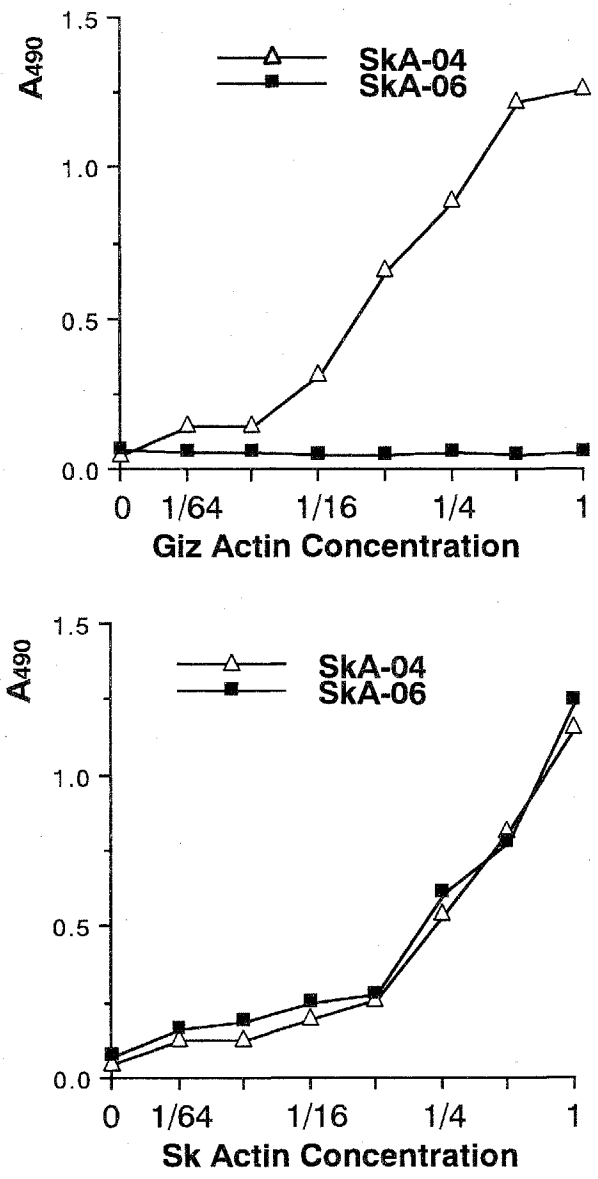

Fig. 3. Specificity of the monoclonal anti-actin antibodies, SkA-04 and SkA-06, as examined by ELISA assay. The solid phase of polystylene-plates was coated with actin either from chicken gizzard (Giz)(Top) or skeletal(Sk)(Bottom) muscles by incubating with the serially diluted (2×; starting from 0.1 mg/ml) proteins and treated with the monoclonal antibodies, SkA-04 (-△-) or SkA-06 (-■-), followed by treatment with peroxidase-labeled GAM. Binding of the antibodies to the plates was detected as the product of *o*-phenylenediamine reaction by measurement of absorbance at 490 μm.

muscle tissues but not with actin in cardiac muscle (Data not shown).

Subcellular localization of cytoskeletal and sarcomeric actins in cultured skeletal muscle cells

Localization of cytoskeletal (β- and γ-) and sarcomeric (α-) actins in developing myotubes in culture was examined by staining the cells dually with the antibodies specific for cytoskeletal (PcAb) and sarcomeric actins (SkA-06). As shown in Fig. 6, in young myotubes without striated myofibrils, cytoskeletal and sarcomeric actins were colocalized in the same stress-fiber-like structures (marked by arrowheads). The PcAb to cytoskeletal actins stained not only the filamentous structures but also the overall cytoplasm. In contrast, SkA-06 stained the filamentous structures more brightly. This suggest that a considerable amount of cytoskeletal actins may be present in unpolymerized forms in the cytoplasm, while sarcomeric actins are assembled more efficiently into filamentous forms. In agreement with this view, it was previously demonstrated by biochemical experiments that unpolymerized actin in young muscle cells is constituted by cytoskeletal actins rather than sarcomeric actins (Shimizu and Obinata, 1987). It should be noted that SkA-06 stained almost all of multi-nucleated myotubes but very few mono-nucleated cells, while the PcAb to gizzard actin stained multi-nucleated myotubes as well as mono-nucleated cells including myoblasts and fibroblasts.

When more developed myotubes having cross-striated myofibrils were treated with the antibodies, SkA-06 clearly stained the striated structures (Fig. 7b). However, the PcAb stained the cells mostly in a diffused pattern and gave only a faint striated pattern at limited area (Fig. 7c). These observations indicate that sarcomeric actins are preferentially assembled into sarcomeres.

Assembly of actin isoforms introduced into myotubes

In order to examine how cytoskeletal and sarcomeric actins are incorporated into myofibrillar structures in developing muscle cells, FITC-labeled smooth muscle (β- and γ-) and skeletal muscle (α-) actins in a monomeric form were introduced into myotubes by a micro-injection method. As shown in Fig. 8b, the FITC-actin from skeletal muscle was assembled into cross-striated structures from the beginning, 2 hr after injection. Although the width of the striations having the FITC-actin was narrower than that of I-bands of sarcomeres, the periodicity seemed to be identical with that of sarcomeres. We assume that the exogenous actins were predominantly incorporated into the end of actin filaments of sarcomeres in the beginning. The width became wider when the cells were maintained for about 24 hr (data not shown).

When the FITC-actin from smooth muscle was introduced into myotubes, however, the actin was assembled only into stress-fiber-like structures in the beginning, although not so remarkable (Fig. 8d), and later, it was clearly incorporated into cross-striated structures (Fig. 8f). These results indicate that not only the α-actin but also the β- and/or γ-actins can be assembled into sarcomeres, but skeletal muscle actin is incorporated into myofibrils faster than smooth muscle actin. Polymerization of smooth muscle actin in myotubes seems to be less efficient than skeletal muscle actin.

DISCUSSION

In this study, we used chicken skeletal muscle actin coupled with a synthetic peptide including actin-binding

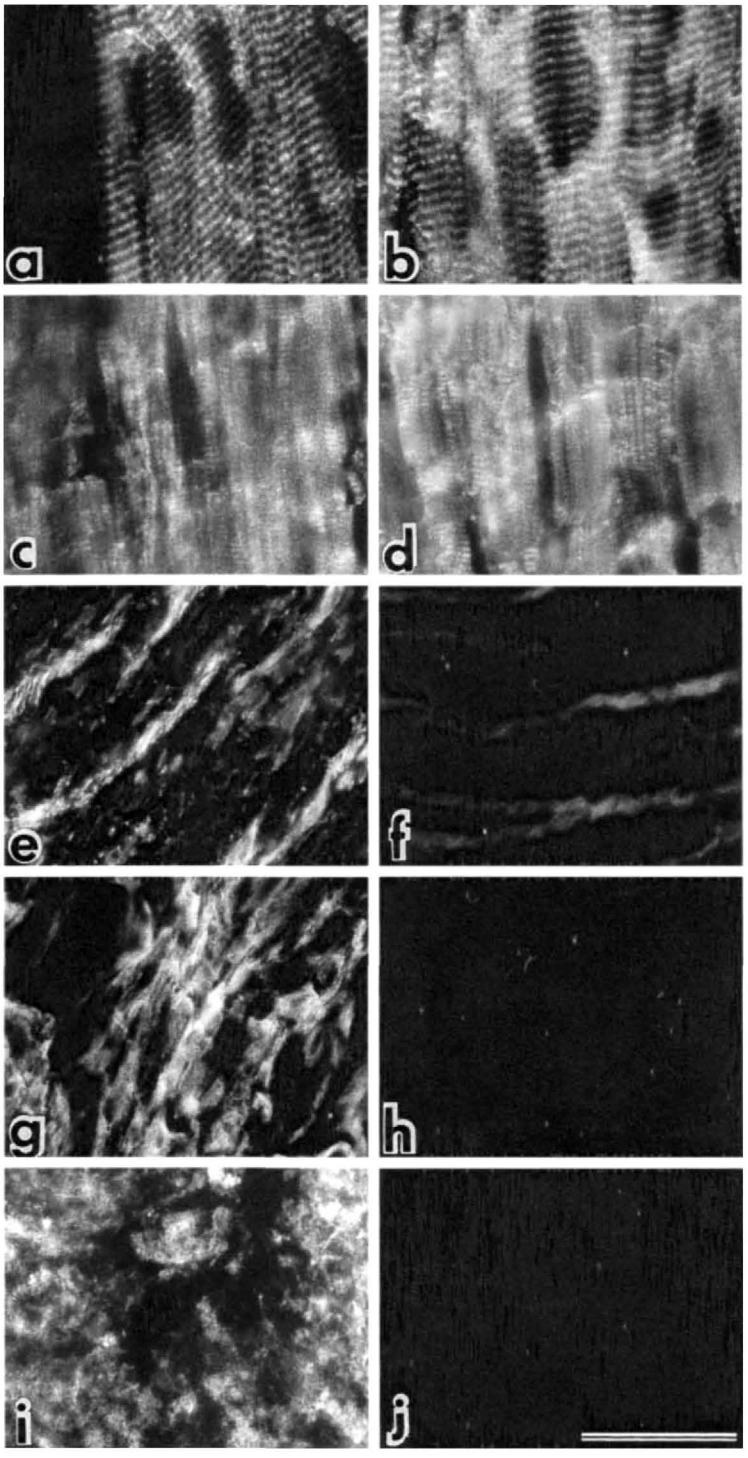

Fig. 4. Reactivity of the anti-actin antibodies, SkA-04 and SkA-06, with the cryosections of various chicken tissues. Frozen sections (8 μm) of chicken skeletal (pectoralis) muscle (a, b), heart (c, d), aorta (e, f), gizzard (g, h), and brain (i, j) were treated with either SkA-04 (a, c, e, g, and i) or SkA-06 (b, d, f, h, and j), followed by treatment with FITC-GAM. Bar, 50 μm.

sequence of cofilin as an immunogen to prepare the antibody to actin as well as the antibody to the actin-binding site of cofilin. Although actin is known to be weak in antigenicity because of the highly conserved sequence beyond various animal species, the complex we used seemed to have increased the antigenicity of actin, but we failed to prepare the antibody to the cofilin sequence with this complex. We obtained several hybridoma clones which produced anti-actin antibodies. Only one of them (SkA-06) was specific for sarcomeric α-actins, and the others including SkA-04 recognized both cytoskeletal and sarcomeric actin isoforms. Since isoform-specific anti-actin antibodies are scarce, SkA-06 should be useful for the studies of localization and assembly of α-sarcomeric actins in the cells. Handel *et al.* (1989) reported an antibody to distinguish α-sarcomeric actins from nonmuscle actins, but this antibody also recognizes smooth muscle γ-actin.

With the antibodies which distinguish the actin isoforms,

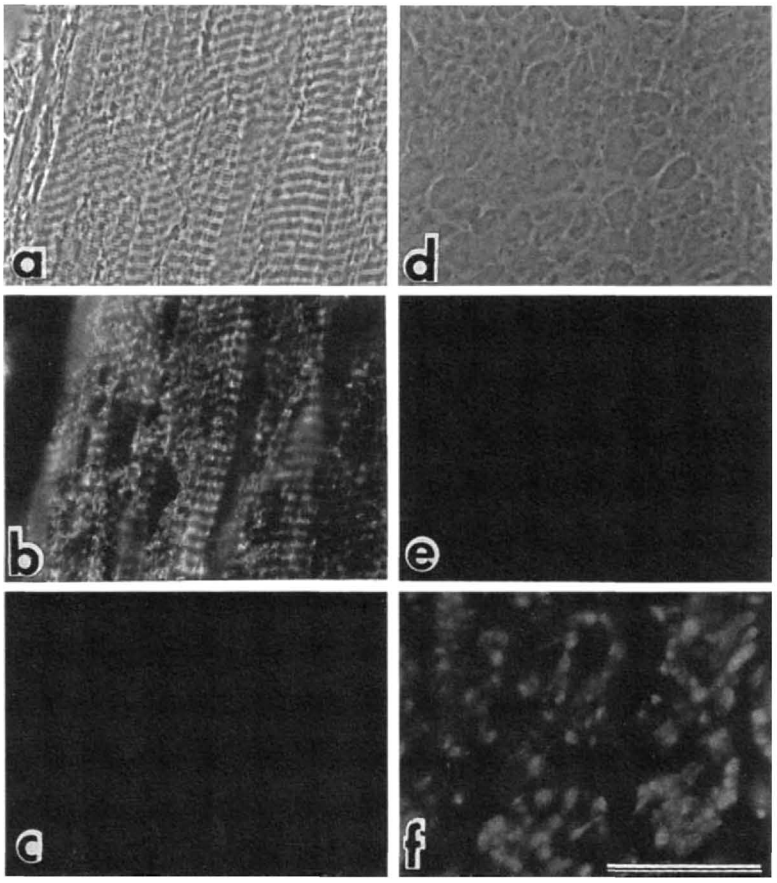

Fig. 5. Specificity of the polyclonal antibody to gizzard actin as examined by immunofluorescence method. Frozen sections (8 μm) of chicken skeletal (pectoralis) (a, b, c) and gizzard smooth (d, e, f) muscles were treated with either SkA-06 (b, e) or polyclonal anti-gizzard actin antibody (PcAb) (c, f), followed by treatment with FITC-GAM or FITC-GAR. Phase-contrast micrographs (a, d) and corresponding immunofluorescence micrographs (b-c, e-f) are shown. Bar, 50 μm.

Table 1. Specificity of the antibody to chicken gizzard actin as examined by ELISA assay.

Antigens	Absorbance at 490 nm
Skeletal muscle actin	0.017
Gizzard muscle actin	0.570

The solid phase of polystylene-plates was coated with either purified chicken skeletal muscle actin or gizzard smooth muscle actin by incubating with the proteins at the concentration of 0.1 mg/ml and reacted with the polyclonal antibody to chicken gizzard actin, followed by treatment with peroxidase-labeled GAR. Binding of the antibodies to the plates was detected as the product of *o*-phenylenediamine reaction by measurement of absorbance at 490 nm.

we have demonstrated that sarcomeric (α) and cytoskeletal (β and γ) actins were co-localized in stress-fiber-like structures in young myotubes, but they became segregated during the formation of sarcomeric structures. We assume that differential localization of the actin isoforms in myotubes may be caused by 1) increased expression of sarcomeric α-actins during muscle development (Shimizu and Obinata, 1980; Hayward and Schwartz, 1986) and 2) differential action of profilin on cytoskeletal and sarcomeric α-actins (Ohshima *et al.*, 1989). Before terminal muscle differentiation, actin cytoskeleton in myogenic cells is constituted mainly by cytoskeletal β- and γ-actins, since sarcomeric α-actin is scarcely expressed. In myotubes, expression of cardiac and skeletal muscle α-actins which constitute sarcomeres of respective muscles is initiated and therefore, both cytoskeletal β- and γ-actins could be constituents of developing myofibrils in the beginning but later replaced by α-actins.

The assembly of actin in young muscle cells is known to be regulated by profilin, ADF and cofilin (Ohshima *et al.*, 1989; Abe and Obinata, 1989; Abe *et al.*, 1989). Among these actin-binding proteins, profilin is most abundant in embryonic muscle cells (Nagaoka *et al.*, 1995). Profilin binds to cytoskeletal β- and γ-actins at higher affinity than to sarcomeric α-actins to inhibit their polymerization (Tobacman and Korn, 1983; Nishida *et al.*, 1984; Larsson and Lindberg, 1988) and it has been observed that profilin makes a complex with monomeric β- and γ-actins rather than with α-actins in developing muscle cells (Ohshima *et al.*, 1989).

Relative proportion of profilin and actin and that of cytoskeletal actins and sarcomeric actins may account for the assembly pattern of the actin isoforms. If the amount of profilin is sufficient, assembly of β- and γ-actins may be selectively inhibited in the cells. In the present study, when FITC-labeled β- and γ-actins were introduced into myotubes, they were assembled into not only stress-fiber-like structures but also into sarcomeric structures, although the endogenous β- and γ-actins were scarcely detected in striated structures. We assume that the stoichiometry of actin isoforms and profilin may be altered by introducing the exogenous actin. This could be an explanation for that the FITC-labeled β- and γ-actins

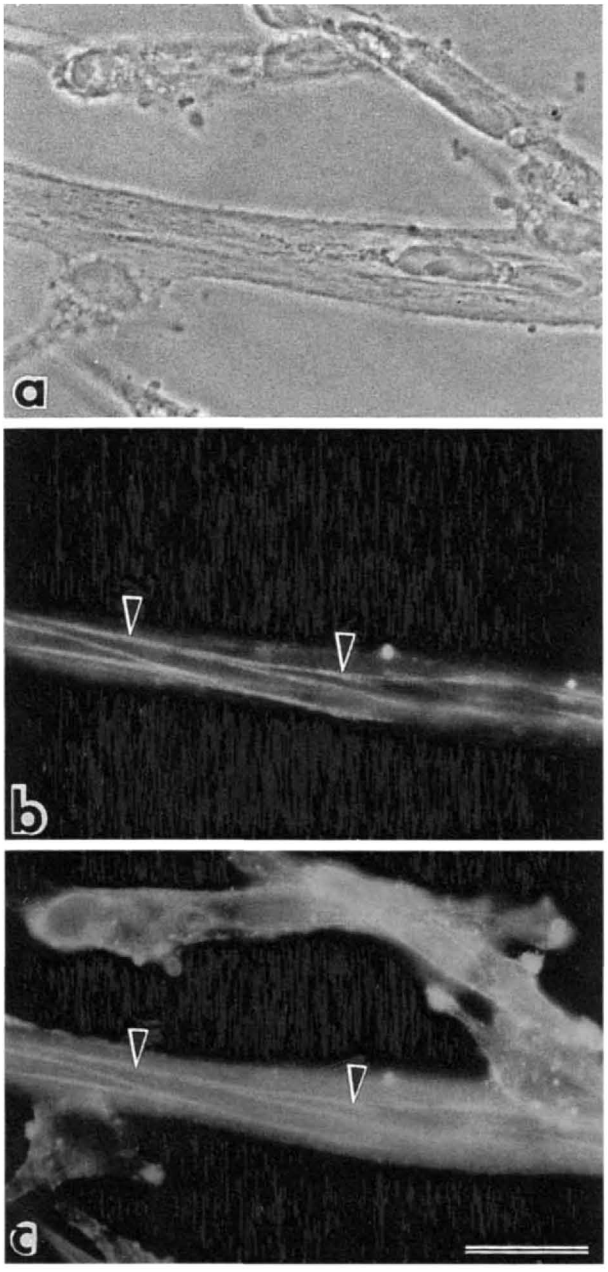

Fig. 6. Double immunofluorescence micrographs of young chicken myotubes in culture which were indirectly labeled with FITC-anti-sarcomeric actin antibody (SkA-06) and rhodamine-anti-cytoskeletal actin antibody. The cells cultured for 3 days in vitro were dually stained with SkA-06 (b) and anti-gizzard actin antibody (c). Stress-fiber-like structures which contained both sarcomeric and cytoskeletal actins were marked by arrowheads. A phase-contrast micrograph (a) and corresponding immunofluorescence micrographs (b, c) are shown. Bar, 20 μm.

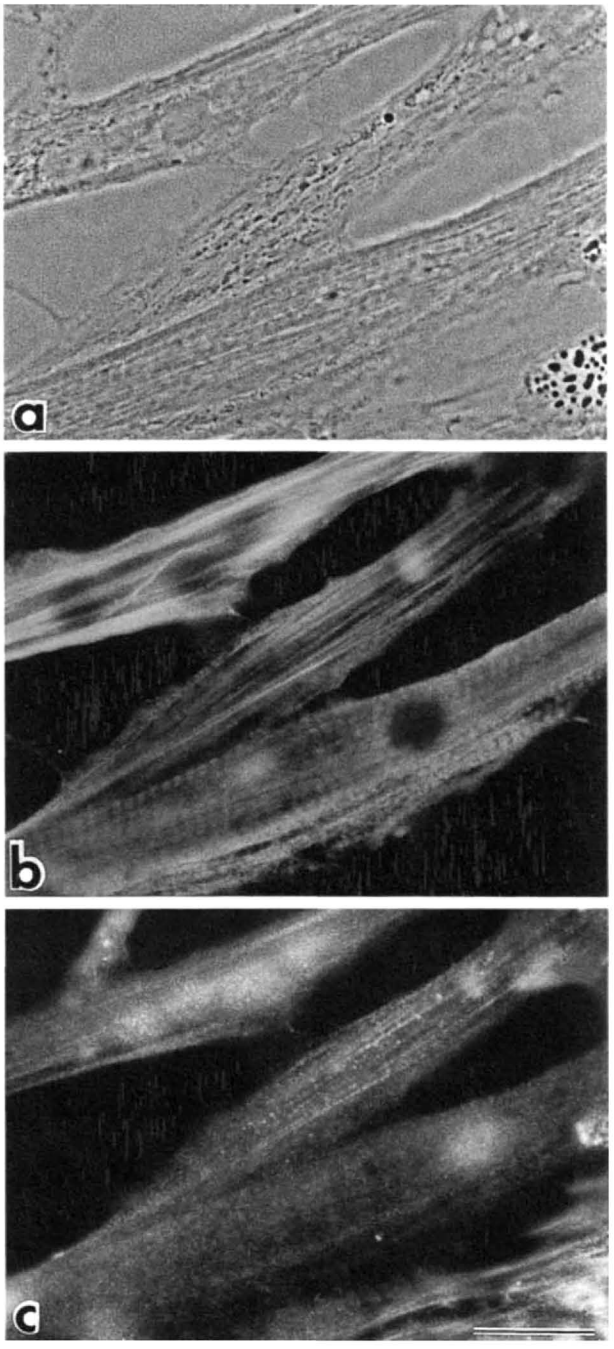

Fig. 7. Double Immunofluorescence micrographs of chicken striated myotubes in culture indirectly labeled with FITC-anti-sarcomeric actin antibody (SkA-06) and rhodamine-anti-cytoskeletal actin antibody. The cells cultured for 5 days *in vitro* were dually stained with SkA-06 (b) and anti-gizzard actin antibody (c). A phase-contrast micrograph (a) and corresponding immunofluorescence micrographs (b, c) are shown. Bar, 20 μm.

were incorporated into sarcomeres. Microinjection of exogenous proteins or overexpression of the protein by cDNA transfection in myogenic cells may lead to unusual assembly of the exogenous protein because of imbalance of actin and actin-binding proteins.

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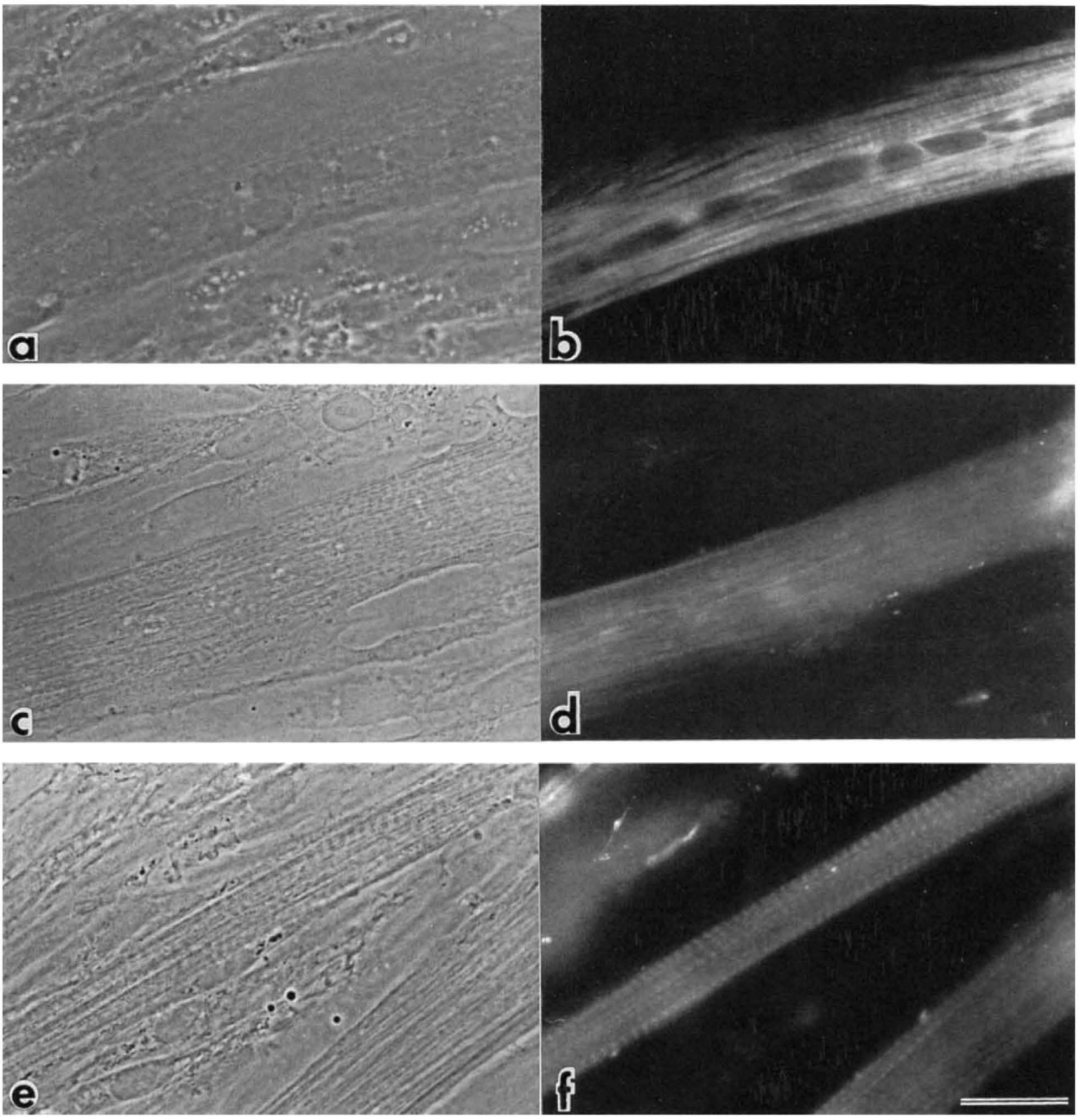

Fig. 8. Assembly of FITC-labeled actin introduced into myotubes by a microinjection method. FITC-labeled skeletal muscle α-actin (a, b) or gizzard smooth muscle β-, γ-actins (c, d, e,f) at 4 mg/ml were injected into chicken myotubes cultured for 4 days *in vitro*. The cells were fixed at 2 hr (a, b), 7 hr (c, d) or 24 hr (e, f) after the actin injection. The localization of the FITC-actin was detected under a fluorescence microscope. Fluorescence images (b, d, f) were paired with the corresponding phase-contrast images (a, c, e). Bar, 20 μm.

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