

# Differential Expression and Distribution of Chicken Skeletal- and Smooth-Muscle-type $\alpha$ -Actinins during Myogenesis in Culture

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**ABSTRACT** Antibodies to chicken fast skeletal muscle (pectoralis)  $\alpha$ -actinin and to smooth muscle (gizzard)  $\alpha$ -actinin were absorbed with opposite antigens by affinity chromatography, and four antibody fractions were thus obtained: common antibodies reactive with both pectoralis and gizzard  $\alpha$ -actinins ([C]anti-P  $\alpha$ -An and [C]anti-G  $\alpha$ -An), antibody specifically reactive with pectoralis  $\alpha$ -actinin ([S]anti-P  $\alpha$ -An), and antibody specifically reactive with gizzard  $\alpha$ -actinin ([S]anti-G  $\alpha$ -An). In indirect immunofluorescence microscopy, (C)anti-P  $\alpha$ -An, (S)anti-P  $\alpha$ -An, and (C)anti-G  $\alpha$ -An stained Z bands of skeletal muscle myofibrils, whereas (S)anti-G  $\alpha$ -An did not. Although (S)anti-G  $\alpha$ -An and two common antibodies stained smooth muscle cells, (S)anti-P  $\alpha$ -An did not. We used (S)anti-P  $\alpha$ -An and (S)anti-G  $\alpha$ -An for immunofluorescence microscopy to investigate the expression and distribution of skeletal- and smooth-muscle-type  $\alpha$ -actinins during myogenesis of cultured skeletal muscle cells. Skeletal-muscle-type  $\alpha$ -actinin was found to be absent from myogenic cells before fusion but present in them after fusion, restricted to Z bodies or Z bands. Smooth-muscle-type  $\alpha$ -actinin was present diffusely in the cytoplasm and on membrane-associated structures of mononucleated and fused myoblasts, and then confined to membrane-associated structures of myotubes. Immunoblotting and peptide mapping by limited proteolysis support the above results that skeletal-muscle-type  $\alpha$ -actinin appears at the onset of fusion and that smooth-muscle-type  $\alpha$ -actinin persists throughout the myogenesis. These results indicate (a) that the timing of expression of skeletal-muscle-type  $\alpha$ -actinin is under regulation coordinate with other major skeletal muscle proteins; (b) that, with respect to expression and distribution, skeletal-muscle-type  $\alpha$ -actinin is closely related to  $\alpha$ -actin, whereas smooth-muscle-type  $\alpha$ -actinin is to  $\gamma$ - and  $\beta$ -actins; and (c) that skeletal- and smooth-muscle-type  $\alpha$ -actinins have complementary distribution and do not co-exist in situ.

$\alpha$ -Actinin, which was discovered by Ebashi and Ebashi (12), is a structural protein of Z bands in skeletal muscle myofibrils (33). It is also located near the fascia adherens of intercalated disks as well as in Z bands in cardiac muscle (44). In smooth muscle, it is present in cytoplasmic dense bodies and membrane-associated dense plaques (21, 41). Proteins immunologically and biochemically related to  $\alpha$ -actinin have been isolated from several nonmuscle tissues or cells: bovine brain (42), Ehrlich tumor cells (36, 37), plasma membranes of

sarcoma 180 ascites cells (46), HeLa cells (6), human platelets (40), rat liver (29), and porcine kidney (28). Many of these proteins, if not all, are  $\text{Ca}^{2+}$  sensitive, i.e., are inhibited from cross-linking actin filaments by  $\text{Ca}^{2+}$ , different from any of the muscle  $\alpha$ -actinins. Immunofluorescence microscopy, immunoelectron microscopy, and microinjection using fluorescence-labeled  $\alpha$ -actinin have revealed that  $\alpha$ -actinin or proteins immunologically related to  $\alpha$ -actinin are also present in cultured fibroblasts (17, 18, 31), intestinal epithelial cells (4,

10, 21, 23), lymphocytes (22, 25), branched microvilli isolated from ascites adenocarcinoma cells (7), secretory vesicle membranes from chromaffin cells of adrenal medulla (26), and so on. These observations suggest that  $\alpha$ -actinin is involved in the organization of microfilament bundles, in the attachment of microfilaments to the cell membrane, and in the assembly of microfilaments in areas of cell-to-cell contact.

Peptide maps and antigenicity are identical or very similar among  $\alpha$ -actinins from chicken striated muscles (fast skeletal, slow skeletal, and cardiac muscles) (14). In contrast,  $\alpha$ -actinins isolated from striated muscles and smooth muscle show considerably different peptide maps and no cross-reaction on double immunodiffusion (3, 14). The immunological cross-reaction, however, can be detected by more sensitive immunofluorescence microscopy. Antibody to skeletal muscle  $\alpha$ -actinin stains smooth muscle cells (14) and antibody to smooth muscle  $\alpha$ -actinin also stains Z bands of skeletal and cardiac muscle myofibrils (3, 14). Moreover, both antibodies stain nonmuscle cells in a similar manner (31, 41). These facts imply that striated muscle, smooth muscle, and nonmuscle  $\alpha$ -actinins share epitope(s), despite their having far different primary structures.

During the course of myogenesis of cultured skeletal muscle cells, the synthesis of many of the major structural proteins of myofibrils—myosin,  $\alpha$ -actin, tropomyosin, troponin components, and desmin—is first detected coordinately at about the time that myoblast fusion normally takes place and then rapidly increases (11, 19). Allen et al. (1) have indicated that  $\alpha$ -actinin, as well as myosin, actin, and tropomyosin, accumulates shortly after fusion. In contrast, Jockusch and Jockusch (27) and Gomer and Lazarides (24) have shown by immunofluorescence microscopy that  $\alpha$ -actinin is also present in myoblasts before and during fusion. This discrepancy may be ascribable to the differential expression of  $\alpha$ -actinin isoforms (polymorphic forms) during myogenesis.

In this study, we prepared antibodies specifically reactive with pectoralis  $\alpha$ -actinin and with gizzard  $\alpha$ -actinin by absorbing affinity-purified original antibodies with opposite antigens coupled to Sepharose columns. We used these antibodies to investigate the expression and distribution of skeletal and smooth-muscle-type  $\alpha$ -actinin isoforms during myogenesis of cultured skeletal muscle cells.

## MATERIALS AND METHODS

**Preparation of  $\alpha$ -Actinins and the Antibodies:**  $\alpha$ -Actinins were prepared from adult chicken fast skeletal muscle (pectoralis) and smooth muscle (gizzard) as described previously (14).

Antibodies to pectoralis  $\alpha$ -actinin and to gizzard  $\alpha$ -actinin were prepared as described (14). They were purified by affinity chromatography using CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) coupled with each immunogen. Affinity-purified anti-pectoralis  $\alpha$ -actinin (anti-P  $\alpha$ -An) was applied to a column of gizzard  $\alpha$ -actinin-coupled Sepharose. The fraction that did not bind to the column should be an antibody specific for pectoralis  $\alpha$ -actinin that reacts with pectoralis  $\alpha$ -actinin but not with gizzard  $\alpha$ -actinin and is designated as "(S)anti-P  $\alpha$ -An." The fraction weakly bound to the column was eluted with 1 M MgCl<sub>2</sub> in PBS, and then the antibody specifically bound to the column was eluted with 4 M MgCl<sub>2</sub> or 3 M KSCN in PBS. This should be an antibody common to pectoralis and gizzard  $\alpha$ -actinins that reacts with both  $\alpha$ -actinins and is referred to as "(C)anti-P  $\alpha$ -An." Affinity-purified anti-gizzard  $\alpha$ -actinin (anti-G  $\alpha$ -An) was applied to a column of pectoralis  $\alpha$ -actinin-coupled Sepharose and separated by procedures similar to those stated above into two fractions, "(S)anti-G  $\alpha$ -An," which reacts specifically with gizzard  $\alpha$ -actinin, and "(C)anti-G  $\alpha$ -An," which reacts with both gizzard and pectoralis  $\alpha$ -actinins.

*Electrophoresis, Peptide Mapping, and Immunoblot-*

*ting:* SDS PAGE was performed according to Laemmli (30). "SDS extracts" from muscular tissues were prepared as follows: small pieces of fresh muscular tissues of pectoralis and gizzard were excised from adult chicken and homogenized in the same volume of PBS containing 0.2 mM diisopropyl fluorophosphate (DIFP)<sup>1</sup> with a VirTis 23 Microhomogenizer (VirTis Co., Inc., Gardiner, NY). They were suspended in 3 vol of SDS sample buffer (30) and immediately boiled for 4 min. DIFP was added to the samples to a final concentration of 4 mM. Insoluble materials were removed by centrifugation at 100,000 g for 1 h, and thus obtained supernatant solutions are referred to as SDS extracts from muscular tissues. "SDS extracts" from cultured pectoralis muscle cells were similarly prepared: cultured cells grown on 100-mm plastic dishes were washed three times with PBS and harvested with a rubber policeman. After being washed twice with PBS containing 0.2 mM DIFP by centrifugation, the cells were solubilized with 3 vol of SDS sample buffer by being boiled for 4 min. More DIFP was added and SDS extracts were collected as described above.

Peptide mapping by limited proteolysis during SDS PAGE was carried out by the method of Cleveland et al. (9). Samples were applied to SDS PAGE (7.5% acrylamide) and  $\alpha$ -actinin bands were cut from the gel. The gel slices were loaded on a second SDS polyacrylamide (13.5%) gel with chymotrypsin A<sub>4</sub> (Boehringer GmbH, Mannheim, Federal Republic of Germany). After electrophoresis, the digested peptides were detected by silver staining according to Merrill et al. (35).

Immunoblotting was performed as described elsewhere (15) essentially according to Towbin et al. (43). Protein samples were first subjected to SDS PAGE (7.5% acrylamide) using minislab apparatus (~45 × 85 × 1 mm of separating gel). The proteins were electrophoretically transferred to nitrocellulose sheets in 5 mM Tris, 38 mM glycine, and 20% ethanol for 15 min at 80 mA and then for 25 min at 100 mA. The nitrocellulose sheets were washed with PBS and incubated with 3% BSA or gelatin in PBS for 30 min. They were incubated with antibodies for 30 min at room temperature and washed with four changes of PBS containing 0.5% Tween 80 for 1 h with shaking. After incubation with horseradish peroxidase-conjugated sheep anti-rabbit IgG (Cap-pel Laboratories, Inc., Cochranville, PA) for 30 min at room temperature, the sheets were washed as stated above. For the color reaction, they were soaked in 0.5 mM 3,3'-diaminobenzidine tetrahydrochloride, 0.005% H<sub>2</sub>O<sub>2</sub>, and 50 mM Tris-HCl, pH 7.8. The reaction was terminated by rinsing the sheets in water.

**Cell Culture:** Pectoralis muscle was excised from 11-d-old chick embryos, minced with scissors, and suspended in 0.5 ml per embryo of 0.1% collagenase (from *Clostridium histolyticum*, Boehringer GmbH) in Puck's saline solution (Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free). The suspended tissue was maintained for 15 min at 37°C and subjected to pipetting to disperse the cells. The same volume of cold growth medium (Eagle's minimum essential medium supplemented with 10% horse serum and 5% chick embryo extract) was added to the cell suspension and it was filtered through eight layers of lens cleaning paper, followed by centrifugation for 5 min at 1,200 rpm. The pelleted cells were dispersed by pipetting in growth medium and plated on a 100-mm plastic dish. After 45-min incubation at 37°C, unattached cells were collected and replated at a density of 2 × 10<sup>6</sup> cells per 100-mm collagen-coated dish or 3 × 10<sup>5</sup> cells per 60-mm dish containing three collagen-coated coverslips. To prevent the growth of fibroblasts, cells were exposed for 24–48 h to 10  $\mu$ M D-arabinofuranosylcytosine in growth medium 2–3 d after plating.

**Immunofluorescence Microscopy:** Cells grown on collagen-coated coverslips were fixed with 3.5% formaldehyde in PBS for 15 min at room temperature and permeabilized by treatment with 0.1% Triton X-100 in PBS for 5 min at room temperature. After washing with PBS, they were incubated with primary antibodies for 40 min at room temperature and washed thoroughly with at least four changes of PBS for 40–60 min. The cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Miles-Yeda, Miles Laboratories, Elkart, IN) for 40 min and then washed thoroughly with PBS. Coverslips were mounted in 10% glycerol in PBS.

For cryosections, muscles of pectoralis and gizzard were dissected from adult chicken. They were fixed in a stretched state with 3.5% formaldehyde in PBS for 6–12 h at 4°C and then infused with 10, 15, and 20% sucrose in PBS for 4–6 h each. The blocks were frozen in isopentane chilled in liquid nitrogen and cut into 5  $\mu$ m sections in a cryostat. The sections were treated for immunofluorescence by the same procedure as that used for cultured cells. Myofibrils of pectoralis were prepared and processed for immunofluorescence as described previously (14).

All specimens were observed with a Zeiss universal microscope equipped with phase-contrast and epifluorescence optics using a Zeiss 40× or a 100× Neofluar lens. Micrographs were recorded on Kodak Tri-X pan film and developed in Kodak HC-110 developer (dilution B) for 7.5 min at 20°C.

<sup>1</sup> *Abbreviations used in this paper:* DIFP, diisopropyl fluorophosphate; FITC, fluorescein isothiocyanate.

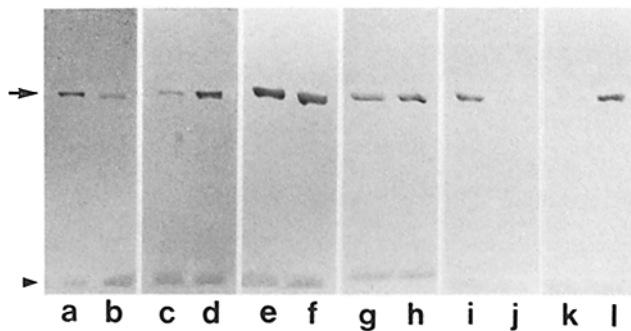


FIGURE 1 Immunoblots characterizing the antibodies. Lanes: (a) SDS extract from chicken pectoralis was transferred from an SDS polyacrylamide gel to a nitrocellulose sheet and incubated with anti-P  $\alpha$ -An and then with peroxidase-conjugated sheep anti-rabbit IgG (pectoralis SDS extract + anti-P  $\alpha$ -An); (b) gizzard SDS extract + anti-P  $\alpha$ -An; (c) pectoralis SDS extract + anti-G  $\alpha$ -An; (d) gizzard SDS extract + anti-G  $\alpha$ -An; (e) pectoralis  $\alpha$ -actinin + (C)anti-P  $\alpha$ -An; (f) gizzard  $\alpha$ -actinin + (C)anti-P  $\alpha$ -An; (g) pectoralis  $\alpha$ -actinin + (C)anti-G  $\alpha$ -An; (h) gizzard  $\alpha$ -actinin + (C)anti-G  $\alpha$ -An; (i) pectoralis  $\alpha$ -actinin + (S)anti-P  $\alpha$ -An; (j) gizzard  $\alpha$ -actinin + (S)anti-P  $\alpha$ -An; (k) pectoralis  $\alpha$ -actinin + (S)anti-G  $\alpha$ -An; (l) gizzard  $\alpha$ -actinin + (S)anti-G  $\alpha$ -An. An arrow indicates the position of  $\alpha$ -actinin (molecular weight = 100,000). An arrowhead denotes the position of tracking dye (malachite green) transferred from SDS polyacrylamide gels to nitrocellulose sheets.

## RESULTS

### Characterization of Antibodies

The specificity of antibodies was confirmed by immunoblotting. Fig. 1, lanes *a-d*, shows that anti-P  $\alpha$ -An and anti-G  $\alpha$ -An reacted with both pectoralis and gizzard  $\alpha$ -actinins in SDS extracts from muscular tissues, and that  $\alpha$ -actinin was the only polypeptide that reacted with the antibodies. The reaction of the antibodies with their own antigens (Fig. 1, lanes *a* and *d*) was stronger than that with the opposite antigens (Fig. 1, lanes *b* and *c*). (C)Anti-P  $\alpha$ -An and (C)anti-G  $\alpha$ -An reacted with both  $\alpha$ -actinins with similar intensities (Fig. 1, lanes *e-h*). (S)Anti-P  $\alpha$ -An reacted only with pectoralis  $\alpha$ -actinin and not with gizzard  $\alpha$ -actinin (Fig. 1, lanes *i* and *j*), whereas (S)anti-G  $\alpha$ -An reacted only with gizzard  $\alpha$ -actinin and not with pectoralis  $\alpha$ -actinin (Fig. 1, lanes *k* and *l*).

By indirect immunofluorescence microscopy, we have shown previously that both anti-P  $\alpha$ -An and anti-G  $\alpha$ -An stain Z bands of pectoralis myofibrils and gizzard muscle (14). (C)Anti-P  $\alpha$ -An, (C)anti-G  $\alpha$ -An, and (S)anti-P  $\alpha$ -An stained only Z bands of pectoralis myofibrils (Fig. 2, *a-f*), whereas (S)anti-G  $\alpha$ -An did not stain any structures of the myofibrils (Fig. 2, *g* and *h*). Cryosections of adult gizzard were found to be fluorescently stained with (C)anti-P  $\alpha$ -An, (C)anti-G  $\alpha$ -An, and (S)anti-G  $\alpha$ -An, but not with (S)anti-P  $\alpha$ -An (Fig. 2, *i-l*). (S)Anti-G  $\alpha$ -An decorated cell membrane-associated structures (Fig. 2*l*, arrowhead) and intracellular structures (Fig. 2*l*, arrow) of the gizzard muscle cells. These structures seem to correspond to dense plaques and dense bodies, respectively, because the two structures have been reported to contain  $\alpha$ -actinin (21, 41). (C)Anti-P  $\alpha$ -An and (C)anti-G  $\alpha$ -An stained the cells similarly to each other and they do not display such clear decoration of membrane-associated structures as did (S)anti-G  $\alpha$ -An (Fig. 2, *i* and *j*).

The results of immunoblotting and immunofluorescence indicate that these antibodies are highly specific for  $\alpha$ -actinin

and that (S)anti-P  $\alpha$ -An and (S)anti-G  $\alpha$ -An recognize only their own antigen, whereas (C)anti-P  $\alpha$ -An and (C)anti-G  $\alpha$ -An recognize both antigens. For convenience we will refer to the protein that reacts with (S)anti-P  $\alpha$ -An as skeletal-muscle-type  $\alpha$ -actinin and to the one that reacts with (S)anti-G  $\alpha$ -An as smooth-muscle-type  $\alpha$ -actinin.

### Expression and Distribution of $\alpha$ -Actinin Isoforms Examined by Immunofluorescence

To examine the expression and detailed distribution of each type of  $\alpha$ -actinin during the course of differentiation of skeletal muscle cells, we used (S)anti-P  $\alpha$ -An and (S)anti-G  $\alpha$ -An for immunofluorescence microscopy on cultured embryonic chicken pectoralis cells. Skeletal-muscle-type  $\alpha$ -actinin was absent from mononucleated myoblasts and fibroblasts 8 h after plating (Fig. 3, *a* and *b*). However, smooth-muscle-type  $\alpha$ -actinin was present in both myoblasts and fibroblasts at this early stage (Fig. 3, *c* and *d*). Fluorescence micrographs at higher magnification show that smooth-muscle-type  $\alpha$ -actinin was present diffusely throughout the cytoplasm, especially concentrated on the membrane-associated structures and at the end of pseudopodial processes of spindle-shaped myoblasts (Fig. 4*a*). In fibroblasts, the  $\alpha$ -actinin was localized along microfilament bundles at periodic intervals and at the ruffling membranes (Fig. 4*b*). This localization in fibroblasts is consistent with the previous reports using antibodies to skeletal muscle  $\alpha$ -actinin and to smooth muscle  $\alpha$ -actinin and fluorescence-labeled smooth muscle  $\alpha$ -actinin (17, 18, 31).

Within 36 h after plating, a large number of myoblasts fused and several streaks of myofilament bundles<sup>2</sup> were seen in their cytoplasm. Skeletal-muscle-type  $\alpha$ -actinin appeared on the myofilament bundles with a punctated pattern (Fig. 4, *c* and *d*) but it was still absent from myogenic cells before fusion of fibroblasts (Fig. 3, *e* and *f*). Although it is infrequent under our culture conditions, mononucleated myoblasts before fusion have myofilament bundles, on which skeletal-muscle-type  $\alpha$ -actinin is located punctatedly (T. Endo and T. Masaki, manuscript in preparation). The  $\alpha$ -actinin seems to be detected on the myofilament bundles almost at the same time that the filament bundles are formed in myogenic cells, which are usually fused. In other words, the protein could not be detected in early myogenic cells lacking myofilament bundles on the level of immunofluorescence. Smooth-muscle-type  $\alpha$ -actinin remained in both the cytoplasm and membrane-associated structures including the end of pseudopodial processes of fused myogenic cells (Figs. 3, *g* and *h* and 4, *e* and *f*).

By 5 d after plating, multinucleated myotubes containing well-spread myofilament bundles were dominant. Skeletal-muscle-type  $\alpha$ -actinin was located on these myofilament bundles with a periodicity, probably on Z bodies, precursors of Z bands (Fig. 5, *a* and *b*). The average length of the periodicity was  $\sim 2.8 \mu\text{m}$ , which is close to that of Z bands in sarcomeric myotubes (Fig. 5, *e* and *f*) or to that in fused myoblasts (Fig. 4, *c* and *d*). However, it is two to three times larger than that

<sup>2</sup> In this paper, we will refer to the filament bundles in the fused myogenic cells or the myotubes as myofilament bundles instead of microfilament bundles because they are thought to be the precursors of thin and thick myofilaments in myofibrils and are distinguished from microfilament bundles in nonmuscle cells by the isoform types of actin (cf. references 5, 20, and 45) and  $\alpha$ -actinin (see Discussion) which are constituents of the filament bundles.

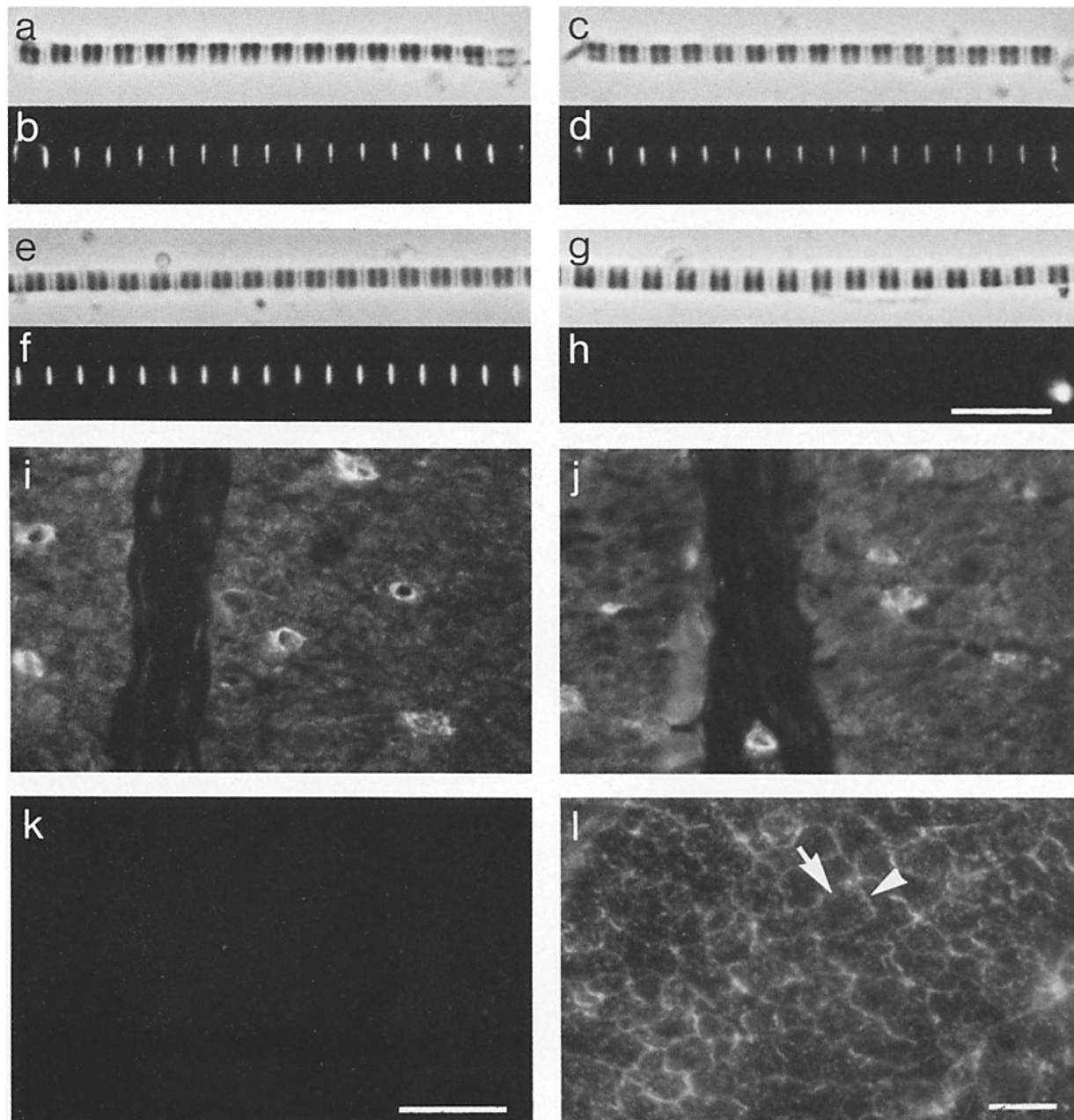


FIGURE 2 Indirect immunofluorescence micrographs of pectoralis myofibrils and gizzard cryosections stained with antibodies to  $\alpha$ -actinins. Pectoralis myofibrils (a–h) and cryosections of gizzard (i–l) were treated with (C)anti-P  $\alpha$ -An (a, b, and i), (C)anti-G  $\alpha$ -An (c, d, and j), (S)anti-P  $\alpha$ -An (e, f, and k), or (S)anti-G  $\alpha$ -An (g, h, and l), and then with FITC-conjugated goat anti-rabbit IgG. Fluorescence micrographs (b, d, f, and h) are paired with phase-contrast micrographs (a, c, e, and g). For gizzard cryosections, only fluorescence micrographs (i–l) are shown. In l, an arrowhead indicates the cell membrane-associated structures and an arrow designates the intracellular structures. (a–h) Bar, 10  $\mu$ m;  $\times$  1,500. (i–k) Bar, 40  $\mu$ m;  $\times$  400. (l) Bar, 10  $\mu$ m;  $\times$  1,000.

in fibroblasts (Fig. 4b). Smooth-muscle-type  $\alpha$ -actinin became confined to the membrane-associated structures of the myotubes (Fig. 5, c and d).

By 8 d after plating, numerous myofibrils containing Z bands were visible in myotubes. Skeletal-muscle-type  $\alpha$ -actinin was restricted to the Z-bands of the myofibrils at this stage (Fig. 5, e and f), whereas smooth-muscle-type  $\alpha$ -actinin remained confined to the membrane-associated structures (Fig. 5, g and h). (S)Anti-G  $\alpha$ -An preabsorbed with gizzard  $\alpha$ -actinin did not stain the cells (Fig. 5i). When the cells were not permeabilized without treatment with Triton X-100, (S)anti-G  $\alpha$ -An did not decorate any structures of the cells (Fig. 5j). In addition, no staining could be seen with nonim-

munized rabbit IgG or FITC-labeled secondary antibody. These results indicate that the  $\alpha$ -actinin is associated with the cytoplasmic aspect of cell membrane.

Immunofluorescence on cryosections of adult pectoralis muscle fibers also showed that skeletal- and smooth-muscle-type  $\alpha$ -actinins were restricted to Z bands and membrane-associated structures, respectively (Fig. 6, a–d).

#### Expression of $\alpha$ -Actinin Isoforms Examined by Immunoblotting and Peptide Mapping

Immunoblotting was applied to ensure the differential expression of skeletal- and smooth-muscle-type  $\alpha$ -actinins

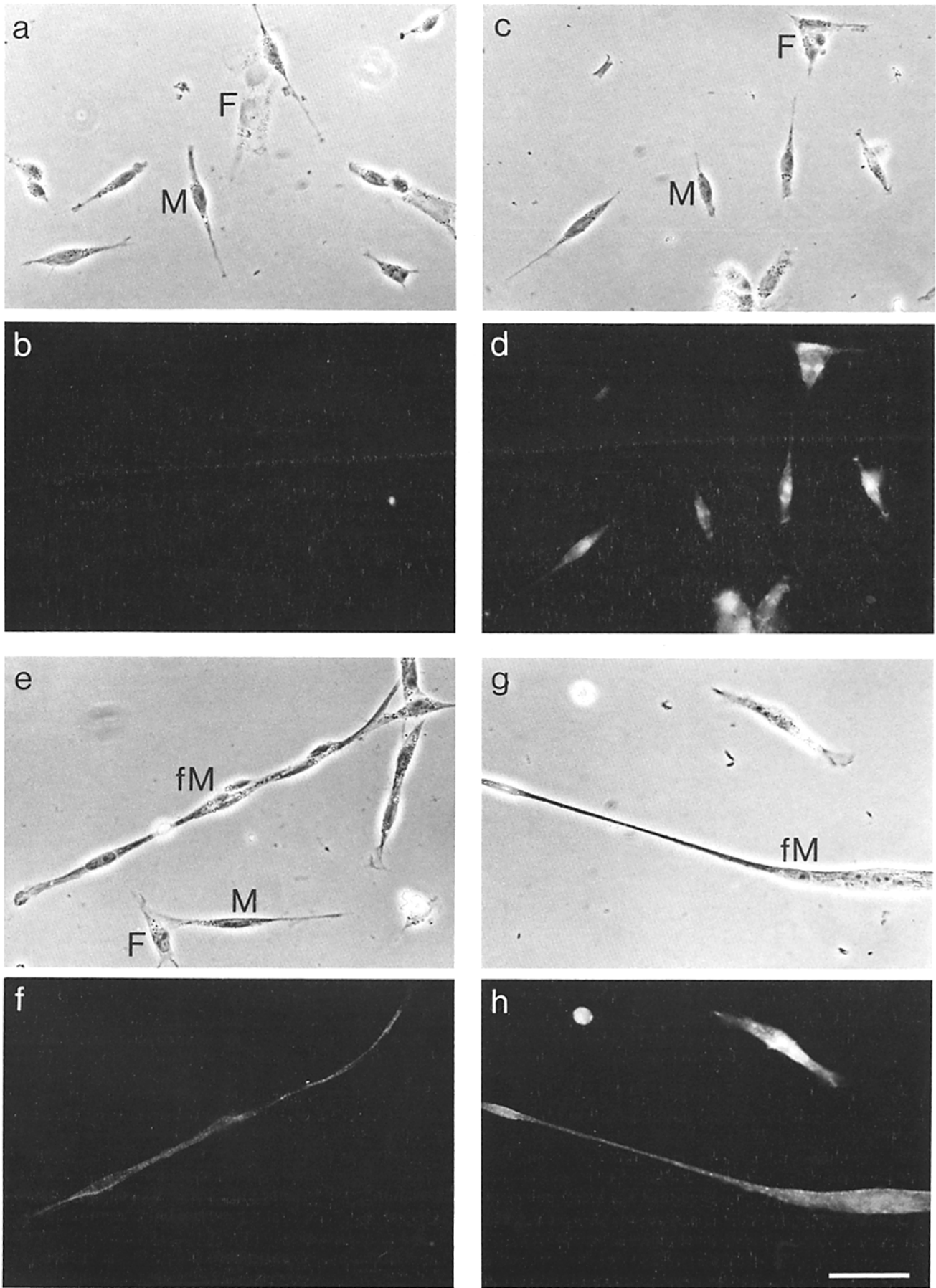


FIGURE 3 Phase-contrast and immunofluorescence micrographs of cultured embryonic pectoralis cells stained with (S)anti-P  $\alpha$ -An or (S)anti-G  $\alpha$ -An. Cells 8 h after plating (a–d) and 36 h after plating (e–h) were treated with (S)anti-P  $\alpha$ -An (a, b, e, and f) or (S)anti-G  $\alpha$ -An (c, d, g, and h) and then with FITC-conjugated goat anti-rabbit IgG. Fluorescence micrographs (b, d, f, and h) are paired with phase-contrast micrographs (a, c, e, and g). (S)Anti-P  $\alpha$ -An does not stain mononucleated myoblasts (M) and fibroblasts (F), but stains fused myoblasts (fM) (a, b, e, and f). (S)Anti-G  $\alpha$ -An stains mononucleated myoblasts, fused myoblasts, and fibroblasts (c, d, g, and h). Bar, 50  $\mu$ m.  $\times$  300.

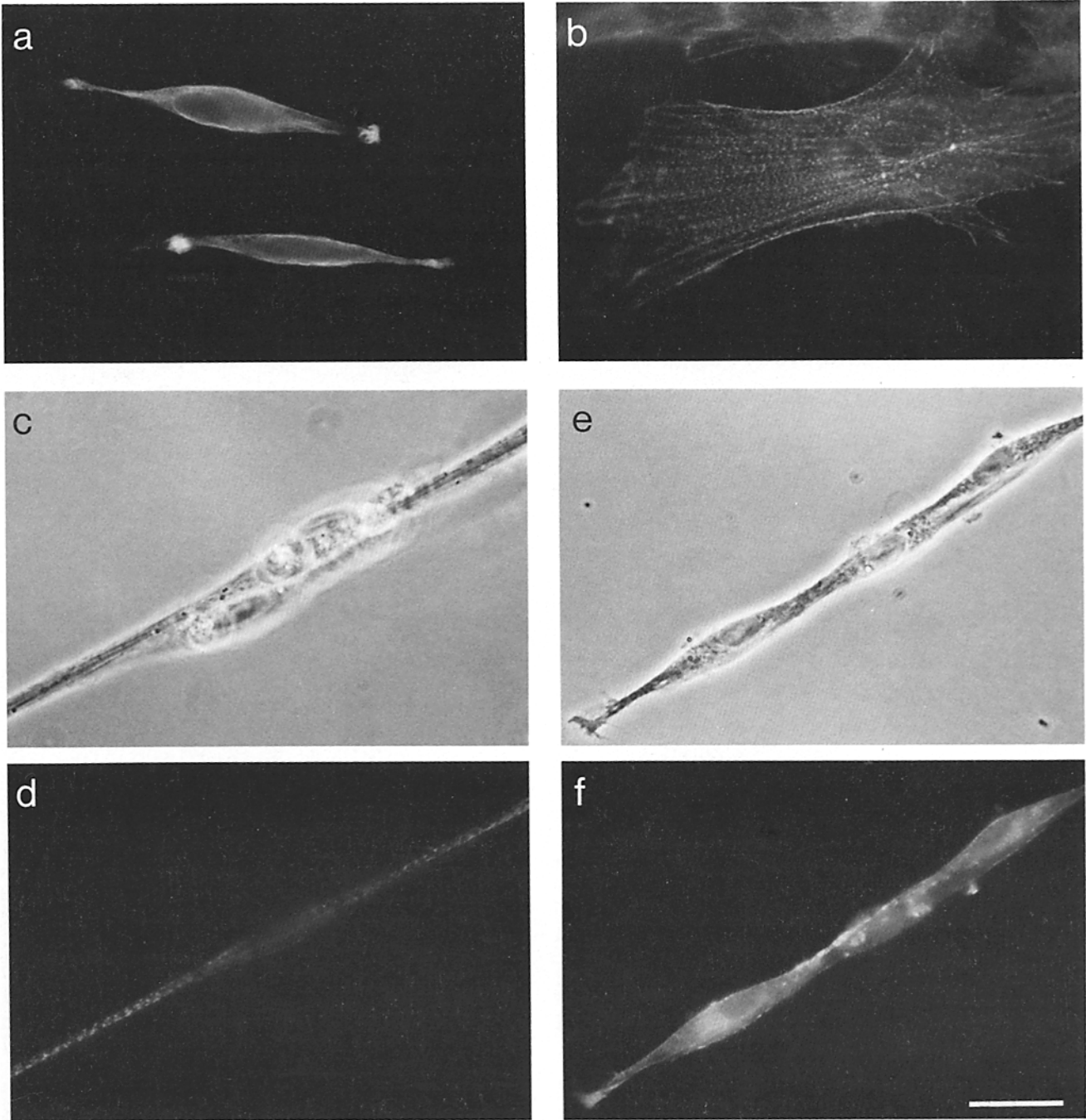


FIGURE 4 Immunofluorescence micrographs at higher magnification of cultured myoblasts and fibroblasts stained with (S)anti-P  $\alpha$ -An or (S)anti-G  $\alpha$ -An. (a) Mononucleated myoblasts 8 h after plating stained with (S)anti-G  $\alpha$ -An; (b) fibroblasts stained with (S)anti-G  $\alpha$ -An; (c, d, e, and f) fused myoblasts 36 h after plating stained with (S)anti-P  $\alpha$ -An (c and d) or (S)anti-G  $\alpha$ -An (e and f). Fluorescence micrographs (d and f) are paired with phase-contrast micrographs (c and e). (S)Anti-P  $\alpha$ -An stains myofilament bundles in fused myoblasts periodically (c and d). (S)Anti-G  $\alpha$ -An stains the cytoplasm of mononucleated and fused myoblasts and, above all, the membrane-associated structures and the end of pseudopodial processes (a, e, and f). It stains microfilament bundles periodically and ruffling membranes in fibroblasts (b). Bar, 20  $\mu$ m.  $\times$  750.

during myogenesis which has been shown by immunofluorescence microscopy. Fig. 7a shows the SDS PAGE pattern of SDS extracts from cultured pectoralis muscle cells harvested at 8 h, 36 h, 5 d, and 8 d after plating. The amounts of  $\alpha$ -actinin and actin were almost equal throughout the myogenesis, but that of myosin heavy chain increased as myogenesis proceeds. The identical gels were transferred to nitrocellulose sheets and incubated with (S)anti-P  $\alpha$ -An or (S)anti-G  $\alpha$ -An.

The immunoblots demonstrate that skeletal-muscle-type  $\alpha$ -actinin was absent from 8-h culture but present in 36-h, 5-d, and 8-d cultures (Fig. 7b) and that smooth-muscle-type  $\alpha$ -actinin continued to exist throughout the myogenesis (Fig. 7c). The  $\alpha$ -actinins that reacted with the antibodies were found to have identical molecular weight of 100,000 on the immunoblots. Consequently we can exclude the possibility that the entirety of  $\alpha$ -actinin that reacted with (S)anti-G  $\alpha$ -An

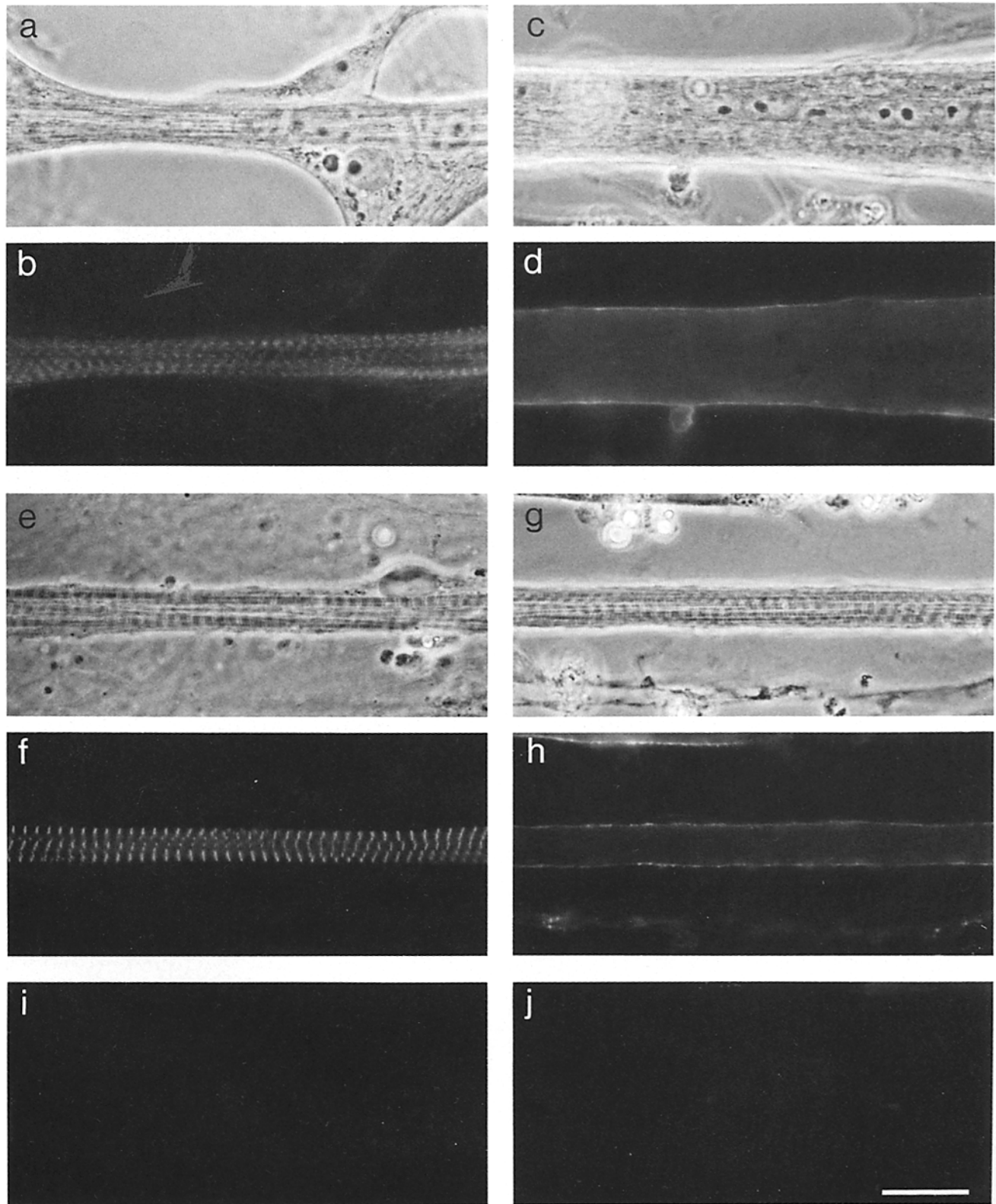


FIGURE 5 Phase-contrast and immunofluorescence micrographs of cultured embryonic pectoralis cells stained with (S)anti-P  $\alpha$ -An or (S)anti-G  $\alpha$ -An. Cells 5 d after plating (a-d) and 8 d after plating (e-j) were treated with (S)anti-P  $\alpha$ -An (a, b, e, and f), (S)anti-G  $\alpha$ -An (c, d, g, h, and j), or (S)anti-G  $\alpha$ -An preabsorbed with gizzard  $\alpha$ -actinin (i), and then with FITC-conjugated goat anti-rabbit IgG. In j, cells were not permeabilized without treatment with Triton X-100. Fluorescence micrographs (b, d, f, and h) are paired with phase-contrast micrographs (a, c, e, and g). (S)Anti-P  $\alpha$ -An stains Z bodies in immature myotubes (a and b) and Z bands in mature sarcomeric myotubes (e and f). (S)Anti-G  $\alpha$ -An stains membrane-associated structures in both myotubes (c, d, g, and h). Note the absence of staining in fluorescence micrographs (i and j). Bar, 20  $\mu$ m.  $\times$  750.

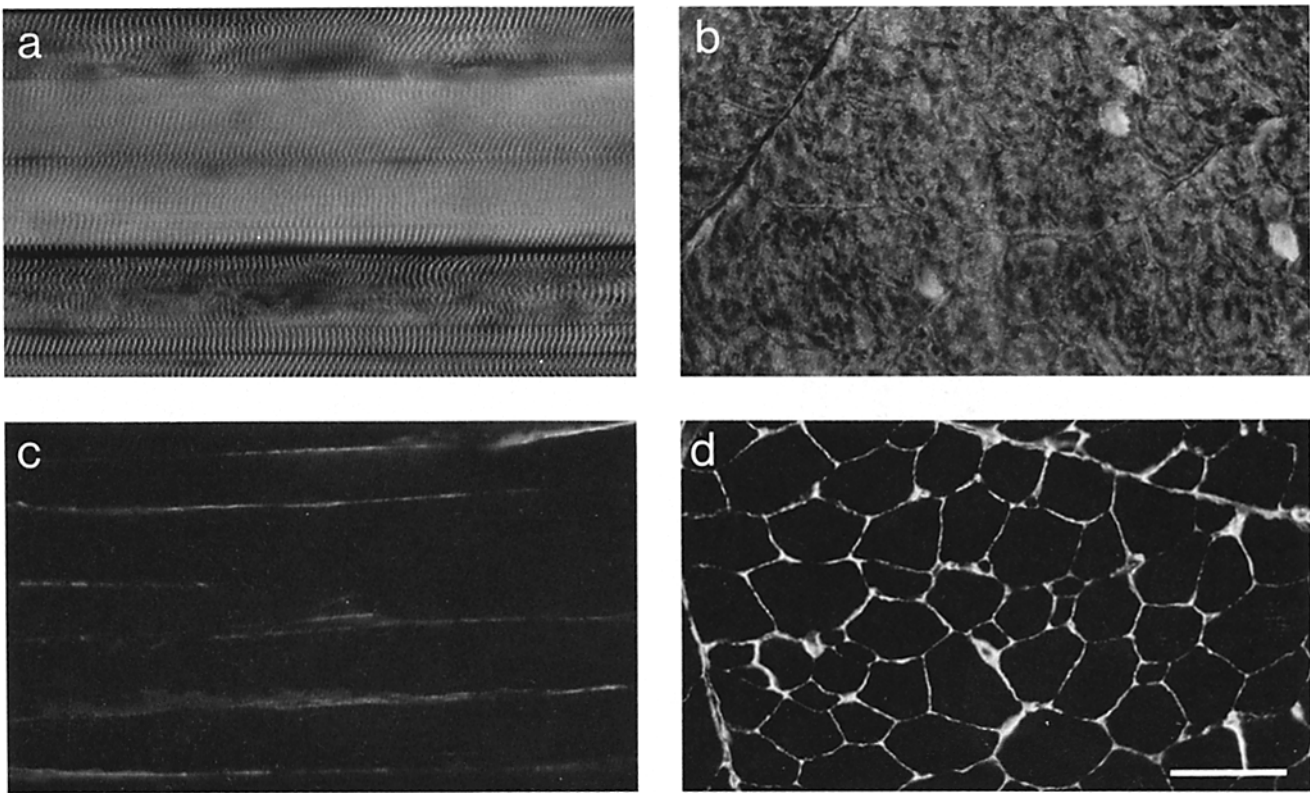


FIGURE 6 Immunofluorescence micrographs of cryosections of adult pectoralis muscle. (a) Longitudinal section stained with (S)anti-P  $\alpha$ -An; (b) transverse section stained with (S)anti-P  $\alpha$ -An; (c) longitudinal section stained with (S)anti-G  $\alpha$ -An; (d) transverse section stained with (S)anti-G  $\alpha$ -An. (S)Anti-P  $\alpha$ -An stains only Z bands, whereas (S)anti-G  $\alpha$ -An stains only membrane-associated structures. Bar, 50  $\mu$ m.  $\times$  300.

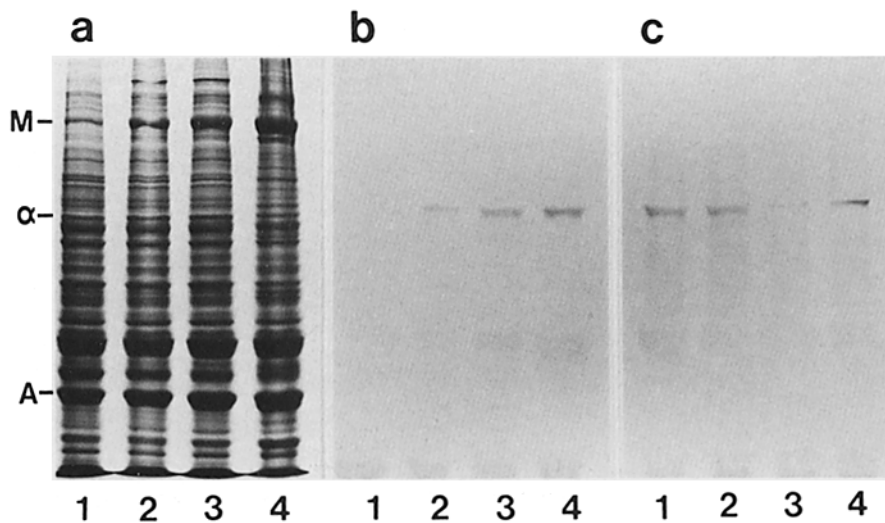


FIGURE 7 Immunoblots of SDS extracts from cultured pectoralis muscle cells stained with (S)anti-P  $\alpha$ -An and (S)anti-G  $\alpha$ -An. (a) SDS PAGE pattern of SDS extracts from 8-h (lane 1), 36-h (lane 2), 5-d (lane 3), and 8-d (lane 4) cultures. M, myosin heavy chain;  $\alpha$ ,  $\alpha$ -actinin; A, actin. (b) Immunoblots correspond to those in a incubated with (S)anti-P  $\alpha$ -An followed by horseradish peroxidase-conjugated sheep anti-rabbit IgG.  $\alpha$ -Actinin cannot be detected in 8-h culture but can in 36-h, 5-d, and 8-d cultures. (c) Immunoblots correspond to those in a incubated with (S)anti-G  $\alpha$ -An followed by horseradish peroxidase-conjugated secondary antibody.  $\alpha$ -Actinin is detected throughout the myogenesis.

was derived from contaminated fibroblasts in culture because nonmuscle  $\alpha$ -actinins have molecular weight slightly different from that of skeletal or smooth muscle  $\alpha$ -actinin (6). The results on the expression of  $\alpha$ -actinin isoforms obtained by immunoblotting are consistent with those by immunofluorescence microscopy.

To characterize further  $\alpha$ -actinin in cultured pectoralis muscle cells, we cut each  $\alpha$ -actinin band from the SDS polyacrylamide gel and examined it on peptide maps by

limited proteolysis with chymotrypsin A<sub>1</sub> (Fig. 8). The overall characteristics changed considerably between 8-h culture and 36-h culture. Peptides specific for adult pectoralis  $\alpha$ -actinin were absent in 8-h culture  $\alpha$ -actinin but present in 36-h, 5-d, and 8-d culture  $\alpha$ -actinins. On the other hand, peptides specific for adult gizzard  $\alpha$ -actinin were present throughout the differentiation. These results are also compatible with those from immunofluorescence microscopy and immunoblotting.

Fig. 9 summarizes the expression and distribution of skel-



etal- and smooth-muscle-type  $\alpha$ -actinins during myogenesis of cultured skeletal muscle cells, in smooth muscle cells, and in cultured fibroblasts.

## DISCUSSION

In the present study, we separated anti-P  $\alpha$ -An and anti-G  $\alpha$ -An into four fractions, (C)anti-P  $\alpha$ -An, (S)anti-P  $\alpha$ -An, (C)anti-G  $\alpha$ -An, and (S)anti-G  $\alpha$ -An. Since immunoblotting and immunofluorescence microscopy demonstrated that

(S)anti-P  $\alpha$ -An and (S)anti-G  $\alpha$ -An reacted specifically with their own antigens, these antibodies are useful tools to study the expression and distribution of the  $\alpha$ -actinin isoforms. Although (S)anti-G  $\alpha$ -An was obtained by absorbing anti-G  $\alpha$ -An with pectoralis  $\alpha$ -actinin, it reacted with a protein on membrane-associated structures of adult pectoralis muscle cells (Fig. 7). The protein on membrane-associated structures may, therefore, either not be extracted with the solution used for extracting pectoralis  $\alpha$ -actinin or be another protein than  $\alpha$ -actinin which has antigenicity in common with  $\alpha$ -actinin. The latter is less probable because anti-G  $\alpha$ -An reacted only with a 100,000-mol-wt polypeptide, probably  $\alpha$ -actinin, in SDS extract from adult pectoralis muscle on immunoblot.

Skeletal-muscle-type  $\alpha$ -actinin was absent from mononucleated myoblasts but appeared on the myofilament bundles in fused myoblasts and persisted on Z bodies or Z bands in myotubes during myogenesis of cultured embryonic pectoralis muscle cells. Although it is infrequent, mononucleated myoblasts before fusion have  $\alpha$ -actinin-containing myofilament bundles under our culture conditions. Fusion-blocked cultures of myoblast by the addition of EGTA or taxol to the culture medium revealed that synthesis and accumulation of several muscle-specific proteins or myofilament assembly begin after withdrawal of myogenic cells from the cell cycle and can occur independent of cell fusion (2, 13, 34). Accordingly, the mononucleated myoblasts containing myofilament bundles in the normal culture medium in our experiment may have been withdrawn from the cell cycle but prevented from fusion for some cause other than low concentration of  $Ca^{2+}$  or the effect of taxol. In most of the myogenic cells, however, myofilament assembly occurred and skeletal-muscle-type  $\alpha$ -actinin appeared at the onset of myoblast fusion.

On the other hand, smooth-muscle-type  $\alpha$ -actinin was present in mononucleated and fused myoblasts both in the cytoplasm and on the membrane-associated structures but was restricted to the membrane-associated structures in immature and mature myotubes. It is not surprising that  $\alpha$ -actinin was found to be located on membrane-associated structures of skeletal muscle cells because the protein (a) is present in the ruffling membranes of cultured fibroblasts (Fig. 4b and references 17, 18, and 31); (b) is associated with the cell membrane and participates in the movement of cell surface receptors of lymphocytes (22, 25); (c) is located in the vicinity of

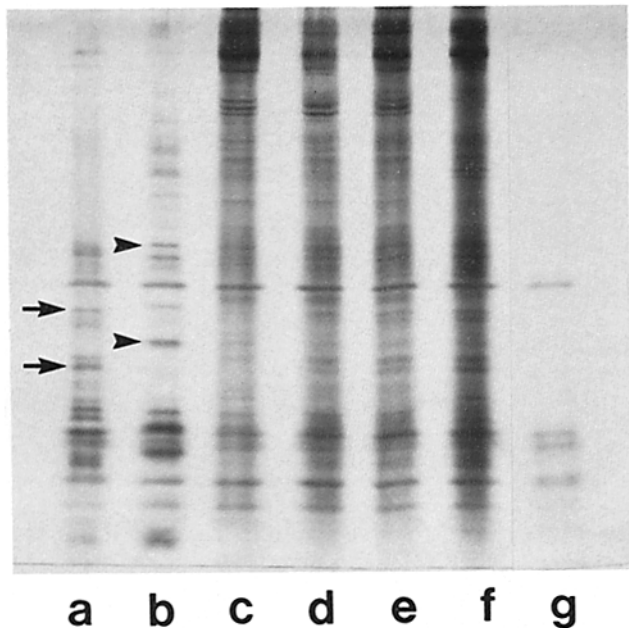


FIGURE 8 Peptide maps of  $\alpha$ -actinins from cultured pectoralis muscle cells by limited proteolysis with chymotrypsin  $A_4$ . Lanes: (a) Adult pectoralis  $\alpha$ -actinin; (b) adult gizzard  $\alpha$ -actinin; (c) 8-h culture  $\alpha$ -actinin; (d) 36-h culture  $\alpha$ -actinin; (e) 5-d culture  $\alpha$ -actinin; (f) 8-d culture  $\alpha$ -actinin; (g) chymotrypsin  $A_4$ .  $\alpha$ -Actinin bands were cut from a first SDS polyacrylamide gel. The gel slices containing proteins were applied to a second SDS polyacrylamide (13.5%) gel and incubated with 300 ng of chymotrypsin  $A_4$  during re-electrophoresis. The gel was stained with silver. Arrows and arrowheads indicate the peptides specific for adult pectoralis  $\alpha$ -actinin and gizzard  $\alpha$ -actinin, respectively.

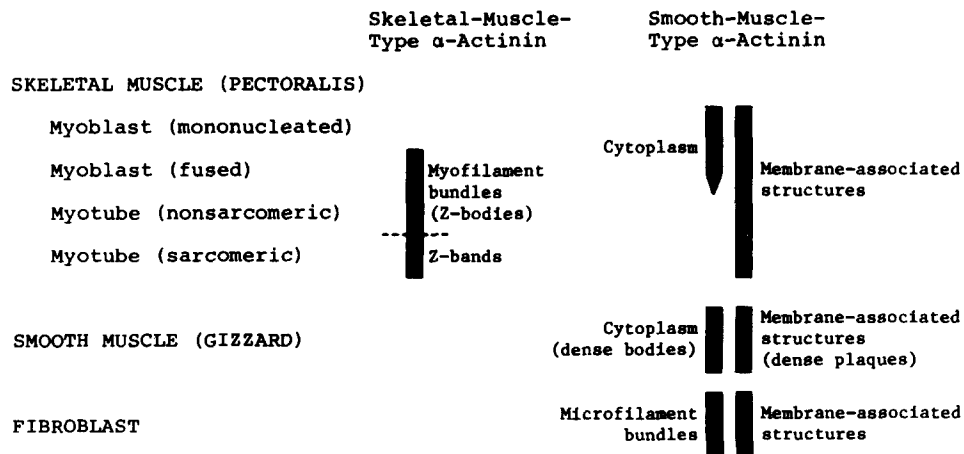


FIGURE 9 Summary of expression and distribution of skeletal- and smooth-muscle-type  $\alpha$ -actinins during myogenesis of cultured skeletal muscle (pectoralis) cells, in smooth muscle (gizzard) cells, and in cultured fibroblasts. Solid bars indicate the presence of  $\alpha$ -actinin.

the belt desmosome (zonula adherens) and near the tight junction (zonula occludens) of intestinal epithelial cells (10, 21, 23); (d) is present in the membrane-associated dense plaques of smooth muscle (21, 41) and near the fascia adherens of the intercalated disks of cardiac muscle (44); and (e) is isolated from cell membrane fraction of sarcoma 180 ascites cells (46). Furthermore,  $\alpha$ -actinin is also found near the locations of vinculin (8, 21, 44). Recent discovery that vinculin is localized at the sarcolemma of skeletal and cardiac muscles (38, 39) may therefore also support the presence of  $\alpha$ -actinin in the sarcolemma or the membrane-associated structures. Jockusch and Jockusch (27) and Gomer and Lazarides (24) have shown by immunofluorescence microscopy that  $\alpha$ -actinin is also present in myoblasts before and during fusion. Their results are incompatible with that of Allen et al. (1) but seem to be ascribable to the fact that their antibodies recognize not only skeletal-muscle-type but also smooth-muscle-type one.

Using pulse-labeling with [<sup>35</sup>S]methionine followed by electrophoresis on two-dimensional gel, Devlin and Emerson (11) have shown that the synthesis of myosin, muscle-specific actin ( $\alpha$ -actin), tropomyosin, and troponin components is not evident in dividing myoblasts in culture but is first detected coordinately at the time when myoblast fusion is initiated and then rapidly increases. Allen et al. (1) have also demonstrated that the accumulation of major myofibrillar proteins—myosin, actin, tropomyosin, and  $\alpha$ -actinin—occurs shortly after myoblast fusion. The synthesis of desmin, an intermediate filament protein located at the periphery of each Z disk, is initiated at the onset of myoblast fusion, whereas another intermediate filament protein, vimentin, is synthesized in dividing myoblasts and the two proteins continue to be synthesized in skeletal muscle culture (19). Immunofluorescent staining with anti desmin and anti vimentin also supports the above facts (19, 47, 48). Both metabolic pulse-labeling with [<sup>35</sup>S]methionine and immunofluorescence have revealed that another peripheral protein of each Z disk, filamin, is present in myoblasts and early fused cells (24). Filamin disappears from the cells ~1 d after cell fusion and reappears several days after the appearance of Z-band striations (24). Consequently, the timing of expression and accumulation of skeletal-muscle-type  $\alpha$ -actinin, and not those of smooth-muscle-type  $\alpha$ -actinin, seem to be under regulation coordinate with muscle structural proteins, myosin,  $\alpha$ -actin, tropomyosin, troponin components, and desmin.

It has been shown the  $\beta$ - and  $\gamma$ -actins and not  $\alpha$ -actin are predominant in cultured skeletal muscle cells before fusion and that  $\alpha$ -actin appears after fusion (5, 20, 45). Moreover, an antibody that recognizes cytoplasmic actin (probably  $\beta$ - and  $\gamma$ -actins) but not myofibrillar actin ( $\alpha$ -actin) stains the cytoplasm of fully differentiated skeletal muscle fibers diffusely and the membrane-associated structures strongly (32). Skeletal-muscle-type  $\alpha$ -actinin is therefore thought to be closely related to  $\alpha$ -actin, and smooth-muscle-type  $\alpha$ -actinin to  $\beta$ - and  $\gamma$ -actins, with respect to timing of expression and distribution. In a previous paper (14), we suggested this relationship between actin and  $\alpha$ -actinin on the level of muscle tissues, i.e., muscles that contain exclusively  $\alpha$ -actin (fast skeletal, slow skeletal, and cardiac muscles) have identical or extremely similar  $\alpha$ -actinins (skeletal-muscle type), whereas muscles containing  $\gamma$ - and  $\beta$ -actins (smooth muscle) have another kind of  $\alpha$ -actinin (smooth-muscle type).

Although both types of  $\alpha$ -actinins are located on the fila-

ment bundles in a similar manner, a punctated periodic pattern, only skeletal-muscle-type  $\alpha$ -actinin is located on the myofilament bundles in fused myoblasts or early myotubes and only smooth-muscle-type  $\alpha$ -actinin on the microfilament bundles in fibroblasts. The latter type of  $\alpha$ -actinin is also present in the ruffling membranes in fibroblasts. The myofilament bundles are composed of  $\alpha$ -actin (5), whereas the microfilament bundles and ruffling membranes are composed of  $\beta$ - and  $\gamma$ -actins. The evidence also supports the above relationship. These filament bundles are similar in morphology and in their constituent proteins (mainly actin, myosin, tropomyosin, and  $\alpha$ -actinin) but are distinct from each other in the types of isoforms of the constituent proteins.

Skeletal- and smooth-muscle-type  $\alpha$ -actinins are present in different subcellular structures in a cell and do not co-exist. Such complementary distribution of isoforms as  $\alpha$ -actinin has been reported on actin (32) and myosin (16). The distinctive distribution between isoforms suggests their separate or at least differential functions. For example, the isoforms of actin, myosin, and  $\alpha$ -actinin may differ in polymerization ability, in susceptibility to regulatory factors for polymerization and depolymerization, or in affinity to the components of some subcellular structures.

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