

# The Presence of Two Skeletal Muscle $\alpha$ -Actinins Correlates with Troponin–Tropomyosin Expression and Z-line Width

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**ABSTRACT** Two species of  $\alpha$ -actinin from rabbit fast skeletal muscles were identified with a monospecific antisera. Designated  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub>, their distribution in muscles does not correlate with histochemically defined fast fiber type. Rather, the presence of each correlates with Z-line width and with the expression of different thin filament Ca<sup>2+</sup>-regulatory complexes.  $\alpha$ -Actinin<sub>1f</sub> is expressed with troponin T<sub>1f</sub>- $\alpha\beta$  tropomyosin, and  $\alpha$ -actinin<sub>2f</sub> with troponin T<sub>2f</sub>- $\alpha_2$  tropomyosin. CNBr peptide maps show that the fast  $\alpha$ -actinin species differ in primary structure. In contrast, the slow  $\alpha$ -actinin is indistinguishable from  $\alpha$ -actinin<sub>1f</sub>. Further evidence for the similarity of  $\alpha$ -actinin<sub>1f</sub> and slow  $\alpha$ -actinin comes from electron microscopic studies which show that fibers that express these species exhibit thick Z-lines. So, unlike other contractile proteins, the multiple forms of  $\alpha$ -actinin do not reflect the distinction between fast- and slow-twitch muscles.

Multiple forms of most of the major skeletal muscle contractile proteins have been identified in mammalian muscles. In the case of myosin (11), troponin T (7),  $\alpha$ -tropomyosin subunits (4), and C-protein (6), these homologous species are thought to be part of the molecular basis responsible for the differences between fast- and slow-twitch muscle. Similarly, Suzuki et al. (33, 34) described two porcine skeletal muscle forms of  $\alpha$ -actinin: one found in fast muscle and the other in slow muscle. In the studies described here, two forms of  $\alpha$ -actinin in fast skeletal muscles of rabbit are characterized. Identified with a monospecific antisera on immunoblots, they differ extensively in primary structure and have been designated  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub>.

$\alpha$ -Actinin is a major component of the Z-line in skeletal muscle (5). Because differences in Z-line structure have been associated with the two histologically and ultrastructurally defined types of fast fibers (9, 10, 12), the relationship between Z-line width, histochemical fiber type, and the expression of these two fast  $\alpha$ -actinin species was investigated. No simple correlation between histochemically defined fast fiber type and the expression of either fast  $\alpha$ -actinin species was found. Instead, Z-line width appears to be at least partly determined by the  $\alpha$ -actinin species expressed. In addition to this morphological correlate, comparison of the pattern of fast  $\alpha$ -actinin expression with that of the two major forms of the fast skeletal thin filament protein troponin T (described by Briggs et al. [3]) shows that the presence of each  $\alpha$ -actinin is

linked to the presence of a different fast troponin T.

An investigation of the relationship between the two forms of fast  $\alpha$ -actinin and slow  $\alpha$ -actinin by CNBr peptide mapping reveals no differences in the CNBr fragments of  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>s</sub>. Because there is commonly more homology among fast myofibrillar proteins than between fast and slow homologs, the differences between the peptide maps of the two fast  $\alpha$ -actinin species, coupled with the similarity of one of the fast  $\alpha$ -actinin species and slow  $\alpha$ -actinin, was unexpected. It suggests that the selective pressures that resulted in the multiple forms of  $\alpha$ -actinin differed from those that led to the multiplicity of other myofibrillar proteins.

## MATERIALS AND METHODS

**Reagents:** The monospecific antisera to  $\alpha$ -actinin was a gift from Dr. Keith Burrige and was characterized as previously described (18). Second antibodies for immunoblot reactions were obtained from either Tago Inc. (Burlingame, CA) or Vector Laboratories, Inc. (Burlingame, CA). Trasylol was purchased from Mobay Chemical Corp. (Pittsburg, PA) and Tris-HCl and Tris base were from Sigma Chemical Company (St. Louis, MO). Pepstatin A, leupeptin, antipain, and chymostatin were purchased from the Peptide Institute (Osaka, Japan). Glutaraldehyde was purchased from Tousimis (Rockville, MD). All other chemicals were reagent grade. Nitrocellulose paper was purchased from Schleicher & Schuell, Inc. (Keene, NH).

**Myofibril and Single Fiber Preparations:** Dissection of muscles and single muscle fibers and preparation of myofibrils has been described previously (4, 30, 31).

**Gel Electrophoresis:** Polyacrylamide gel electrophoresis in the pres-

ence of sodium dodecyl sulfate (SDS PAGE) was performed as described by Laemmli (17) with the minor modifications described in Schachat et al. (31) for the gels of myofibrils. An acrylamide to bis-acrylamide ratio of 50:1 was used in the 8% gels. Coomassie Brilliant Blue and silver staining were as described in Schachat et al. (30, 31). Densitometric analysis was performed as described in Schachat et al. (31).

**Fiber Typing:** Fiber typing was performed by histochemical staining of serial cross-sections as described in Bronson and Schachat (4). Using the nomenclature of Peter et al. (24), fast-twitch glycolytic (FG)<sup>1</sup> fibers were dark after the alkaline ATPase staining and pale after the succinate dehydrogenase stain. Fast-twitch oxidative-glycolytic (FOG) fibers were dark after either stain protocol. Slow-twitch oxidative (SO) fibers were pale after the ATPase incubation and dark after the succinate dehydrogenase staining. At least 300 fibers were scored from each muscle.

**Immunotransfer Analysis:** Transfer from SDS polyacrylamide gels was performed as described by Towbin et al. (35) using peroxidase-coupled second antibodies obtained from Tago Inc. or by using Vectastain ABC reagents (Vector Laboratories, Inc.) and the procedure recommended by them.

**Time-course Degradation Analysis:** To demonstrate the stability of  $\alpha$ -actinin species, a time-course degradation experiment was performed as described for troponin T species in Briggs et al. (3) and Schachat et al. (31). Muscles were removed within 10 min from an animal killed by intracardiac injection of Nembutal. They were immediately homogenized at 4°C using a Brinkmann polytron (Brinkmann Instruments Co., Westbury, NY) in 100 mM NaCl, 20 mM Tris, 2 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride 5 mM  $\beta$ -mercaptoethanol, pH 8.1. In a procedure requiring <90 s, crude myofibril preparations were made by adding 0.5-ml aliquots of the homogenate to 1 ml of the homogenization buffer supplemented with 1.25% Triton X-100 and 5  $\mu$ g/ml each of leupeptin, pepstatin A, antipain, and chymostatin, centrifuged in an Eppendorf microfuge for 30 s, and then prepared for SDS PAGE.

**Peptide Mapping:** CNBr fragments were generated from  $\alpha$ -actinin in gel slices excised from 8% polyacrylamide SDS gels using the procedure of Pepinski (23). The fragments were then resolved on 16% polyacrylamide SDS gels.

**Electron Microscopic Analysis:** Two 3–4 kg adult female, New Zealand White rabbits were killed by anaesthetizing as described in Bronson and Schachat (4). The following muscles were taken from each rabbit: soleus, psoas, diaphragm, and tongue. The muscles were exposed and flushed with Ringer's solution (120 mM NaCl/1.6 mM KCl/4.3 mM NaCO<sub>3</sub>/1.4 mM KH<sub>2</sub>PO<sub>4</sub>/1.5 mM MgSO<sub>4</sub>/15 mM morpholine propane sulfonic acid/5 mM glucose, pH 7.0). When possible, sarcomere length was controlled by holding the limb in a flexed or extended position during primary fixation. Fixation was performed in situ; the primary fixative (3% glutaraldehyde, 0.2% tannic acid, 1% paraformaldehyde, in Ringer's) was injected into the muscle mass and applied to the surface of the muscle for a period of 10 min. Muscles were then dissected tendon-to-tendon, mounted on sticks to maintain sarcomere length, and immersed in the primary fixative at 4°C for 2–12 h. After primary fixation, small muscle fiber bundles were dissected, rinsed three times in Ringer's, and once in 100 mM potassium phosphate, pH 6.0. Secondary fixation was performed as described in Reedy et al. (25) using 1% OsO<sub>4</sub> in 100 mM potassium phosphate, 10 mM MgCl<sub>2</sub>, pH 6.0 for 2 h at 0°C. The samples were then rinsed in deionized water, block stained overnight in 2% uranyl acetate in deionized water, and dehydrated in a graded (50–100%) ethanol series and infiltrated with Araldite 506. Samples were then trimmed down to two to five fibers from each bundle, and fibers from the different muscles were co-embedded in rafts according to Reedy et al. (25). Blocks were allowed to polymerize for 48 h at 65°C.

Sections with gold to grey interference colors were cut with a Diatome diamond knife (Diatome-US Co., Fort Washington, PA) on a Reichert OMU-3 ultramicrotome (Reichert Scientific Instruments, Buffalo, NY), spread with xylene vapors, and picked up on thin carbon films on 200-mesh copper grids (Mason and Norton, Ltd., Herts, England). Sections were stained with 2% KMnO<sub>4</sub> for 10–20 min according to Reedy (26), followed by 1 min in Sato's lead stain (29). Electron micrographs were taken at original magnifications of 6, 10, and 12,000 on a Siemens 101 (Siemens-Allis Inc., Cherry Hill, NJ), operated at 80 kV with a 50- $\mu$ m objective aperture, on Kodak S0163 EM film. Magnification calibration was performed with a grating replica (2,146 lines/mm) (Ladd Research Industries, Inc., Burlington, VT). Lens currents were normalized and objective current was monitored so that within each series, all micrographs were taken at the same objective current. The fixation procedure

<sup>1</sup> Abbreviations used in this paper: EDL, extensor digitorum longus; FG, fast-twitch glycolytic; FOG, fast-twitch oxidative-glycolytic; SO, slow-twitch oxidative.

used has been shown to result in <4% lattice shrinkage in X-ray diffraction-monitored bundles of insect flight muscle (25).

Co-embedding fibers from each of the four muscles in the same block allowed accurate comparison of structural features to be made from micrographs, since these could be recorded from the same section, so all fibers were identically fixed, processed, of the same thickness, and subjected to the same stain conditions. In addition, different fibers in the section could be photographed without changing the magnification or even the focus. In the case of diaphragm, intact blocks composed of several fibers were also made to allow comparison of the different fiber types.

Measurements of Z-line thickness were performed on the 12,000 $\times$  negatives using a Scherr-Tumico (New York, NY) optical comparator with a 10 $\times$  objective. The Z-line was defined as extending to its fuzziest border, that is, the position where its density decreased sharply. For soleus, 25 Z-lines from five fibers were measured; for psoas, 28 Z-lines from five fibers were measured; for tongue, 21 Z-lines from three fibers; and, for diaphragm, 31 Z-lines from eight fibers.

## RESULTS

### $\alpha$ -Actinin Heterogeneity in Rabbit Fast Skeletal Muscles

When myofibril preparations from rabbit fast skeletal muscles are analyzed by SDS PAGE (Fig. 1, lanes *a–h*), two protein bands with  $M_r$ s characteristic of  $\alpha$ -actinin (~100,000) are found. Exhibiting  $M_r$ s of 100,000 and 104,000, they are labeled  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub> in Fig. 1, where the subscripts 1 and 2 indicate order of mobility, and the subscript *f* indicates they are from fast muscles. Their identity as  $\alpha$ -actinin species was confirmed by immunochemical studies described below.

It is clear in Fig. 1 that the proportion of these two species varies in the fast muscles studied. Psoas, adductor magnus, and rectus femoris (lanes *a*, *b*, and *c*, respectively) exhibit  $\alpha$ -actinin<sub>2f</sub> as their predominant  $\alpha$ -actinin species. In contrast, the intrinsic musculature of the tongue overwhelmingly exhibits  $\alpha$ -actinin<sub>1f</sub> (lane *h*). And several muscles from the hindlimb, gracilis, tibialis anterior, extensor digitorum longus (EDL), and plantaris express both species.

The identification of  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub> was confirmed by immunoblot analysis of the same myofibril samples from psoas and tongue used in Fig. 1. Fig. 2 shows that both  $\alpha$ -actinin<sub>2f</sub> and  $\alpha$ -actinin<sub>1f</sub> are recognized as  $\alpha$ -actinin by a monospecific sera raised against chicken gizzard  $\alpha$ -actinin (lanes *a* and *b*, respectively).

Because the proportions of  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub> varied in the fast muscles and  $\alpha$ -actinin is known to be susceptible to proteolysis (26), two studies were undertaken to determine whether  $\alpha$ -actinin<sub>2f</sub> is a proteolytic fragment of  $\alpha$ -actinin<sub>1f</sub>. First, a time-course degradation study was performed, and second, peptide maps of the two proteins were compared.

Myofibrils from psoas and the intrinsic musculature of the tongue were chosen as sources of  $\alpha$ -actinin<sub>2f</sub> and  $\alpha$ -actinin<sub>1f</sub>, respectively, for the time-course degradation study. Samples of fresh muscle were dissected and homogenized in myofibril preparation buffer within 10 min of killing an animal. Crude myofibrils were prepared from aliquots taken immediately after homogenization (*t* = 0) and at 15 and 45 min. This preparation was completed and samples placed in SDS diluting buffer within 90 s. The results of SDS PAGE analysis of those samples is shown in Fig. 3. It is apparent in the psoas samples (lanes *a*, *d*, and *g*) that  $\alpha$ -actinin<sub>2f</sub> is present at the earliest time and is not lost when incubated in the myofibril preparation buffer. Similarly,  $\alpha$ -actinin<sub>1f</sub> is present in the tongue preparation at the earliest time and shows no evidence

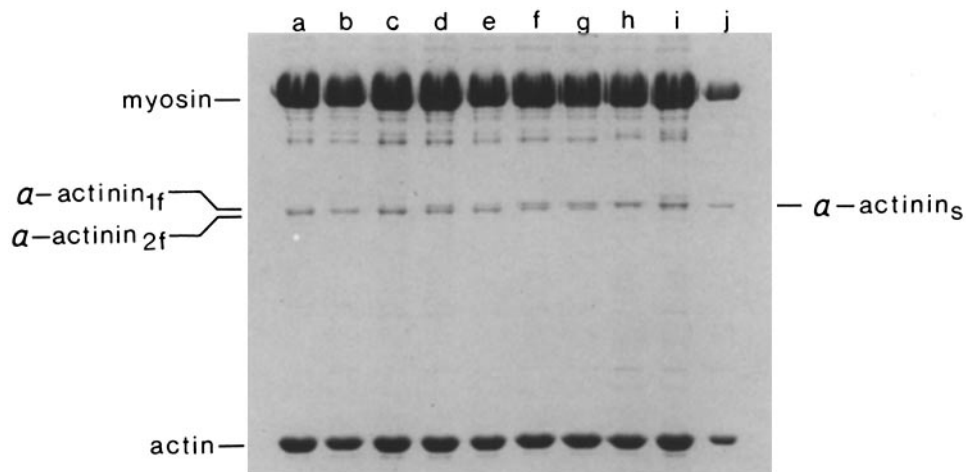


FIGURE 1 Identification of  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub> by SDS PAGE in myofibrils from several fast rabbit skeletal muscles: psoas (a); adductor magnus (b); rectus femoris (c); gracilis (d); tibialis anterior (e); EDL (f); plantaris (g); intrinsic musculature of the tongue (h); and, the mixed muscle diaphragm (i). The two fast forms of  $\alpha$ -actinin are resolved by SDS PAGE on an 8.5% polyacrylamide gel. They are labeled  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub>, and their  $M_r$ s were determined using myosin heavy chain ( $M_r$  210,000), the  $\beta$ ,  $\beta'$ , and  $\alpha$  subunits of RNA polymerase ( $M_r$ s 165,000, 155,000, 39,000, respectively), glycogen phosphorylase ( $M_r$  97,600), bovine serum albumin ( $M_r$  68,000), and ovalbumin ( $M_r$  43,000) as standards. An  $\alpha$ -actinin species labeled  $\alpha$ -actinin<sub>s</sub>, that co-migrates with  $\alpha$ -actinin<sub>1f</sub> is shown in myofibrils from the slow muscle soleus (j).

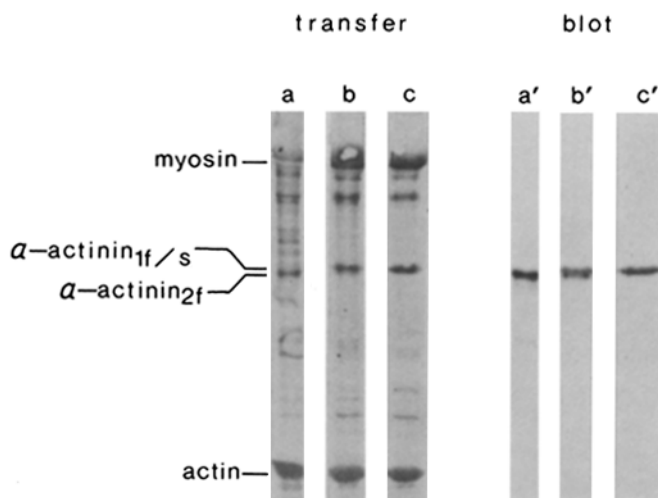


FIGURE 2 Immunoblot reaction of the different  $\alpha$ -actinin species with a monospecific anti- $\alpha$ -actinin serum. Duplicate sets of myofibril samples were transferred to nitrocellulose. One transfer was stained with amido black, and the other reacted with the monospecific anti- $\alpha$ -actinin serum. Stained and immunoblot reactions are shown: lanes a and a' are from psoas (>85%  $\alpha$ -actinin<sub>2f</sub>); lanes b and b' are from the intrinsic musculature of the tongue (>85%  $\alpha$ -actinin<sub>1f</sub>); and, lanes c and c' are from the slow muscle soleus ( $\alpha$ -actinin<sub>s</sub>). SDS PAGE was performed on an 8.0% polyacrylamide gel.

of degradation to either  $\alpha$ -actinin<sub>2f</sub> or any other species during the incubation (lanes b, e, and h). From these observations, we conclude that both fast  $\alpha$ -actinin species are stable and do not interconvert by any proteolytic or posttranslational event occurring in the preparation of myofibrils.

Additional evidence that the two fast  $\alpha$ -actinin species are different proteins is provided by CNBr peptide maps. SDS PAGE gel bands containing the different species were excised and treated with CNBr as described by Pepinski (23). When the CNBr fragment maps are compared (Fig. 4), none of the 23 fragments generated from  $\alpha$ -actinin<sub>2f</sub> (lane a) co-migrate with those of  $\alpha$ -actinin<sub>1f</sub> (lane b). Because CNBr cleavage

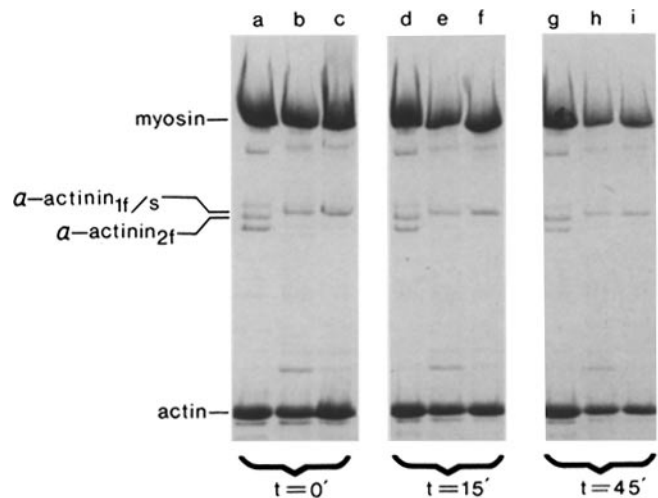


FIGURE 3 Time-course degradation studies on the fast  $\alpha$ -actinin species. Psoas, the inferior longitudinal muscle of the tongue, and soleus were removed within 10 min of death and were homogenized in a Brinkmann polytron as described in Materials and Methods. At the times indicated after homogenization, crude myofibril samples were prepared which were then analyzed by SDS PAGE on 8% polyacrylamide gels: psoas samples are in lanes a, d, and g; tongue samples in lanes b, e, and h; and, soleus samples in lanes c, f, and i.

occurs at Met residues, the differences in these maps indicate that extensive differences exist in the primary structure of the two fast  $\alpha$ -actinin species.

#### Slow $\alpha$ -Actinin Is Not Resolved from $\alpha$ -Actinin<sub>1f</sub> by SDS PAGE

The same procedures used to analyze fast  $\alpha$ -actinin above were also applied to the slow muscle soleus. As can be seen in Fig. 1, lane j, the  $\alpha$ -actinin species present in slow myofibrils, labeled  $\alpha$ -actinin<sub>s</sub>, co-migrates with  $\alpha$ -actinin<sub>1f</sub>. Its identity was confirmed by reaction with the monospecific antisera

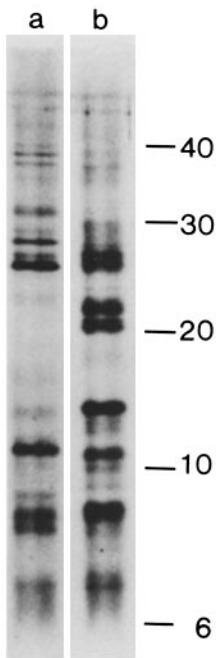


FIGURE 4 CNBr-peptide maps of  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub>. The CNBr peptides of  $\alpha$ -actinin<sub>1f</sub> from tongue (lane a) and  $\alpha$ -actinin<sub>2f</sub> from erector spinae (lane b) are compared by SDS PAGE on a 16% polyacrylamide gel. The CNBr peptides of troponin T<sub>2f</sub> (described in Briggs et al. [3]) were used as  $M_r$  standards. Numbers on the right refer to kilodaltons.

TABLE I. Tabulation of Fiber Type,  $\alpha$ -Actinin, and Z-line Width Distributions in Several Muscles of the Rabbit

Muscle	FG	FOG	SO*	$\alpha$ -Actinin <sub>1f</sub> /total $\alpha$ -actinin	Ratio for fast fibers <sup>‡</sup>	Z-line width $\bar{A} \pm SD$
Erector spinae	79	11	10	0.07	0.01	ND <sup>§</sup>
Psoas	90	4	6	0.14	0.09	666 $\pm$ 140
Plantaris	76	17	8	0.60	0.57	ND
Diaphragm	45	29	26	0.76	0.67	994 $\pm$ 120
Soleus	<0.3	1	99	NA <sup>¶</sup>	NA	1,360 $\pm$ 200
Tongue	<0.3	99	1	0.89	0.89	1,110 $\pm$ 130

\* Histochemical tabulations are as described in Materials and Methods.

<sup>‡</sup> In order to correct for the co-migration of  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>s</sub>, the fraction in the preceding column was corrected for the fraction of SO fibers using the equation (fraction of  $\alpha$ -actinin<sub>1f</sub> - fraction of SO fibers)/(1 - fraction of SO fibers). SO fiber content was also estimated by determining the ratio of the fast and slow 5,5'-dithiobis(2-nitrobenzoic acid) myosin light chains in each muscle. Those values were consistent with the histological determination of the fraction of SO fibers.

<sup>§</sup> ND, not determined.

<sup>¶</sup> NA, not applicable.

(Fig. 2, lane c), and on the basis of time-course degradation studies, it does not arise by a proteolytic process (Fig. 3, lanes c, f, and i).

#### $\alpha$ -Actinin<sub>1f</sub> and $\alpha$ -Actinin<sub>2f</sub> Are Not Specific to Either Fast Fiber Type

Two types of fast-twitch fibers, fast-twitch glycolytic (FG) and FOG, have been distinguished by histochemical, physiological, metabolic, and ultrastructural criteria. The results of histochemical analyses presented in Table I show that psoas, in which  $\alpha$ -actinin<sub>2f</sub> is predominant, is 90% FG in fiber composition. In contrast, the intrinsic musculature of the tongue, in which  $\alpha$ -actinin<sub>1f</sub> is predominant, is almost exclusively FOG in fiber type. Although these two observations suggest a relationship between fiber type and  $\alpha$ -actinin expression, the presence of significant quantities of  $\alpha$ -actinin<sub>1f</sub> in

tibialis anterior, plantaris, and EDL, other predominantly FG muscles, shows that both fast  $\alpha$ -actinin species can be found in FG muscles. Specifically, in plantaris, whose fiber type distribution is virtually indistinguishable from that of psoas (Table I),  $\alpha$ -actinin<sub>1f</sub> composes 57% of the  $\alpha$ -actinin in fast fibers (after correcting for SO fiber content [Table I]), confirming the presence of  $\alpha$ -actinin<sub>1f</sub> in FG fibers. So,  $\alpha$ -actinin<sub>1f</sub> may be the predominant species in either FG or FOG muscle fibers, while  $\alpha$ -actinin<sub>2f</sub> appears to be prevalent only in a restricted set of FG muscles. The absence of a one-to-one correlation between fiber type and  $\alpha$ -actinin expression is further demonstrated by the presence of both  $\alpha$ -actinin species in individual fibers (Fig. 5).

Because this pattern of  $\alpha$ -actinin expression was analogous to that previously described for  $\alpha_2$  and  $\alpha\beta$  tropomyosin (4) and for troponin T<sub>2f</sub> and troponin T<sub>1f</sub> (2, 3, 31), the ratio of the different fast  $\alpha$ -actinin species was compared with that of the two major fast troponin T species to determine if their expression was linked. Three sets of myofibril samples, each from a different animal, were analyzed using 8% SDS PAGE to separate the  $\alpha$ -actinin species and 11% SDS PAGE to resolve TnT<sub>1f</sub> and TnT<sub>2f</sub>, as shown in Fig. 6. Fig. 7 shows the densitometric ratio of the fast  $\alpha$ -actinin species plotted against the ratio of fast TnT species from these preparations. At the boundaries, the correlation between the expression of TnT<sub>1f</sub> and  $\alpha$ -actinin<sub>1f</sub> and that of TnT<sub>2f</sub> and  $\alpha$ -actinin<sub>2f</sub> is strong (for example, in tongue and psoas, respectively). At intermediate values the relationship is monotonic. The variation in the proportion of the  $\alpha$ -actinin species in tibialis anterior, EDL, and plantaris from animal to animal is evident in Fig. 6. This is not due to proteolysis or differential solubility in SDS, but, as shown for the troponin T species, is an inherent aspect of contractile protein expression in these muscles (31).

#### Peptide Maps of $\alpha$ -Actinin<sub>1f</sub> and $\alpha$ -Actinin<sub>s</sub> Are Indistinguishable

Because SDS PAGE did not resolve  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>s</sub>, CNBr fragments from these proteins were compared to determine how similar they are. Fragments were generated as described above for the two fast  $\alpha$ -actinins and then compared by SDS PAGE. Fig. 8 shows that the CNBr peptides of  $\alpha$ -actinin<sub>1f</sub> (lane a) and  $\alpha$ -actinin<sub>s</sub> (lane b) co-migrate. There-

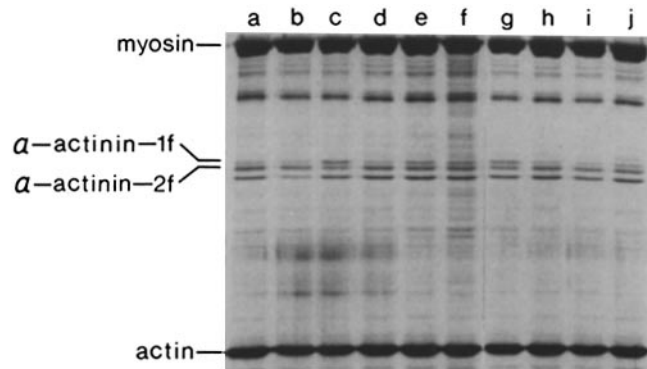


FIGURE 5 Electrophoretic evidence that both  $\alpha$ -actinin species are expressed in the same single fiber. Several single fibers from plantaris (lanes a-j) dissected and prepared for electrophoresis as described in Schachat et al. (30, 31) were analyzed by SDS PAGE on 8.5% polyacrylamide gels. As is evident, both  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub> are present in individual single fibers from this muscle.

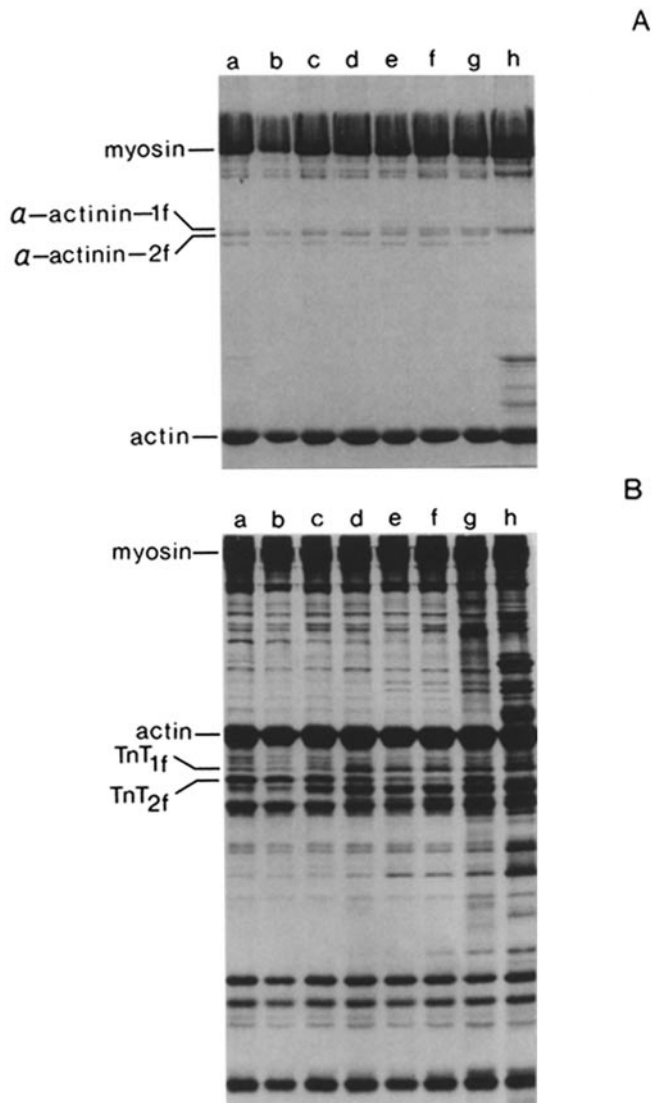


FIGURE 6 Determination of the ratios of the fast  $\alpha$ -actinin and troponin T species in several rabbit fast muscles by SDS PAGE. SDS PAGE on an 8.5% (A) and 11% (B) polyacrylamide gel of myofibril samples from psoas (lane a), adductor magnus (lane b), rectus femoris (lane c), gracilis (lane d), tibialis anterior (lane e), EDL (lane f), plantaris (lane g), and the intrinsic musculature of the tongue (lane h).

fore, we find no evidence for a distinct slow  $\alpha$ -actinin in the rabbit, as it cannot be distinguished from  $\alpha$ -actinin<sub>1f</sub> by either SDS PAGE or CNBr fragment maps.

### Effect of the Different $\alpha$ -Actinin Species on Sarcomere Morphology

$\alpha$ -Actinin is a major component of the skeletal muscle Z-line (5, 27). Gauthier (12, 13) proposed that differences in Z-line width correlated with the difference between fast- and slow-twitch fibers. In guinea pig muscles, she found that FG fibers had thin Z-lines, whereas SO fibers exhibited thick Z-lines, and, in rat diaphragm, FOG fibers could have either thin or thick Z-lines. Because this relationship is similar to that described for the distribution of  $\alpha$ -actinin species and histochemical fiber type, electron microscopic studies were performed on fibers from rabbit muscles that expressed  $\alpha$ -actinin<sub>2f</sub> and  $\alpha$ -actinin<sub>1f/s</sub>.

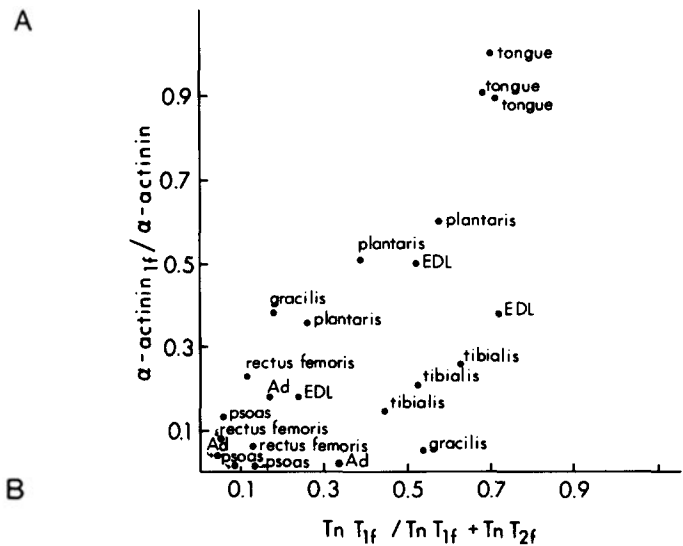


FIGURE 7 Comparison of the ratio of the two fast  $\alpha$ -actinin species with that of the two major fast troponin T species in several fast muscles of the rabbit. The ratios of  $\alpha$ -actinin<sub>1f</sub> to total  $\alpha$ -actinin are plotted against  $Tn T_{1f} / (Tn T_{1f} + Tn T_{2f})$  from several fast muscles. The ratios were determined by densitometry and planimetry of SDS polyacrylamide gels including those shown in Fig. 5. Each point represents a determination on myofibrils from a single muscle. Ad, adductor magnus; EDL, extensor digitorum longus.

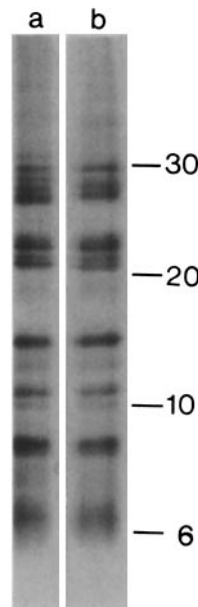


FIGURE 8 Comparison of the CNBr-peptide maps of  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub>. The CNBr peptides of  $\alpha$ -actinin<sub>1f</sub> from tongue (lane a) and  $\alpha$ -actinin<sub>2f</sub> from soleus (lane b) are compared by SDS PAGE on a 16% polyacrylamide gel. The CNBr peptides of troponin T<sub>2f</sub> were used as  $M_r$  standards as in Fig. 4. Numbers on the right refer to kilodaltons.

$\alpha$ -Actinin<sub>1f/s</sub> is present in SO fibers in soleus, in FOG fibers from tongue and diaphragm, and also in FG fibers in diaphragm, whereas  $\alpha$ -actinin<sub>2f</sub> is found in the FG fibers of psoas. Electron microscopic analysis on fibers from these muscles indicates that there may be a simple relationship between the expression of  $\alpha$ -actinin<sub>2f</sub> and thin Z-lines and  $\alpha$ -actinin<sub>1f/s</sub> and thicker Z-lines. The relative Z-line widths of fibers from these muscles from the same animal were determined in fixed fiber bundles that were co-embedded to equalize the effects of sectioning, staining, and variation in magnification. Inspection of Fig. 9 and the tabulated data in Table I shows that the width of Z-lines in SO fibers from soleus (Fig. 9A) is twice that of FG fibers from psoas (Fig. 9B). Fibers from the FOG intrinsic musculature of the tongue also exhibit thick Z-lines

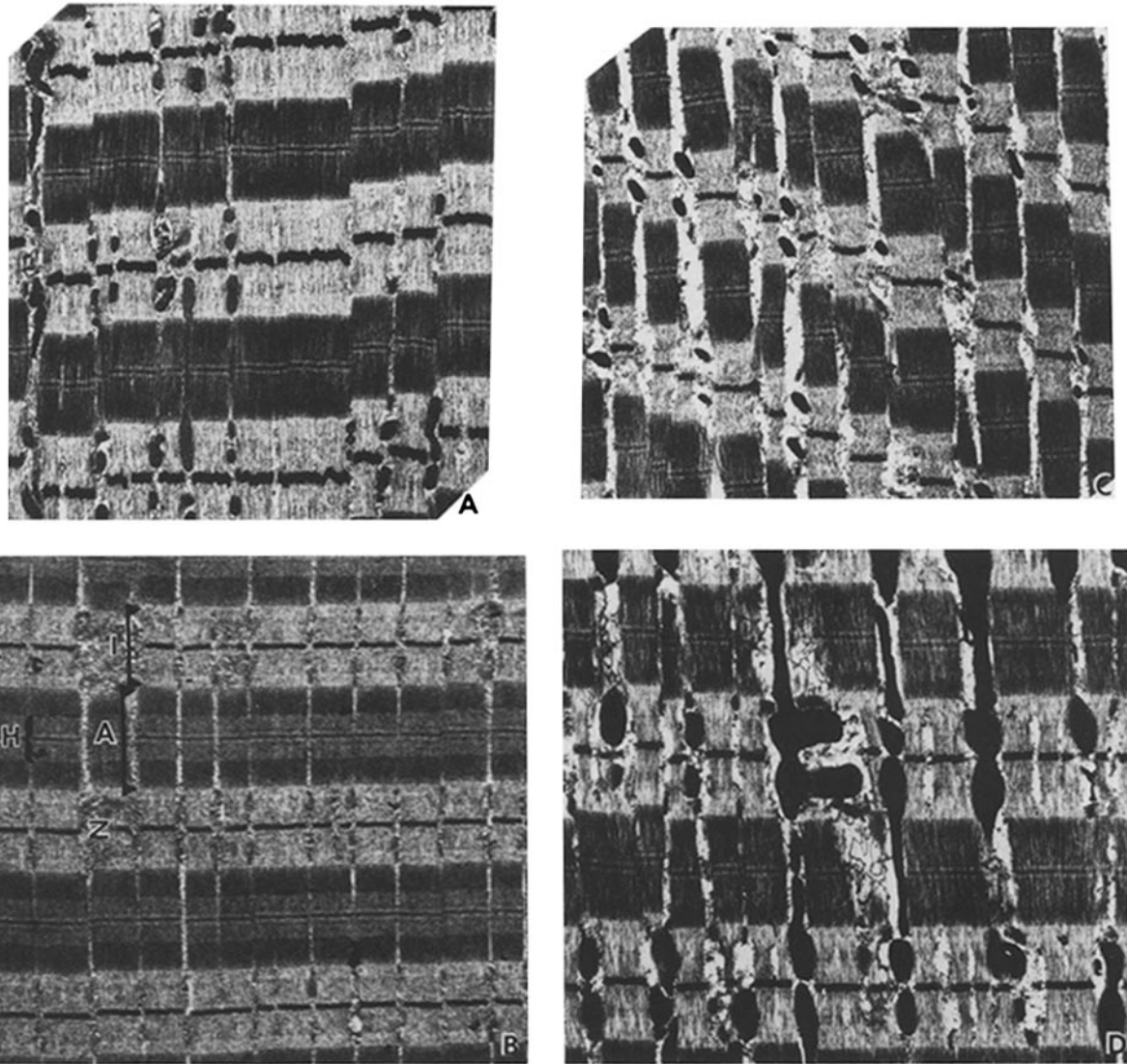


FIGURE 9 Electron microscopic observations on fibers from psoas, tongue, diaphragm, and soleus. These fibers were co-embedded before sectioning, and all micrographs were taken from the same section as described in Materials and Methods. Differences in the relative widths of Z-lines are apparent: the Z-lines in the FG fiber from psoas (B) are thinner than those in the SO fiber from soleus (A), the FOG fiber from the intrinsic musculature of the tongue (C), and the oxidative fiber from diaphragm (D). The position of the Z-line (Z), I-band (I), A-band (A), and H-zone (H) are indicated in the fiber from psoas (B).  $\times 9,600$ .

(Fig. 9 C). However, in contrast with the proposal that thick Z-lines are related to fiber type, all fibers from diaphragm, regardless of type, exhibit Z-lines thicker than those of psoas.

In the case of psoas, soleus, and tongue, fiber type assignments were made statistically, based on the purity of fiber type composition (each muscle is at least 90% a single fiber type) and the uniformity of ultrastructural features in the many fibers studied from each muscle. Because diaphragm is composed of a mixture of fiber types (Table I), both statistical and ultrastructural parameters must be used to show Z-line width is independent of fiber type. The tightness of the distribution of Z-line width, calculated by dividing the variance by the mean, is as sharp in diaphragm, where all fiber types are included, as in any of the muscles of a single fiber type. This argues that there are not several groups of fibers with different mean Z-line widths in diaphragm, but rather a population of fibers with a distribution of Z-line widths about a single mean. In addition, although ultrastructural parameters cannot be used precisely to identify FG fibers (10, 11), as

recognized by Gauthier (12) and quantitated by Eisenberg and co-workers (9, 10), FG fibers differ from SO and FOG fibers in the size, number, and shape of their mitochondria. This is a reflection of the difference in their capacity for oxidative phosphorylation (24). In Fig. 9 this difference is apparent. The FG fiber from psoas (Fig. 9 B) exhibits smaller and fewer mitochondria than the SO fiber from soleus or the FOG fiber from tongue (Fig. 9, A and C, respectively). In Fig. 10 these same criteria can be used to distinguish two fibers from diaphragm. The fiber in A is FG, and that in B is oxidative. Both have virtually identical Z-line widths. So, on the basis of both direct measurements and statistical arguments, FG fibers in diaphragm have thick Z-lines, and there is a relationship between Z-line width and the  $\alpha$ -actinin species present, not histochemical fiber type.

## DISCUSSION

The recognition of extensive heterogeneity in the major skeletal muscle contractile proteins has led to difficulty with two



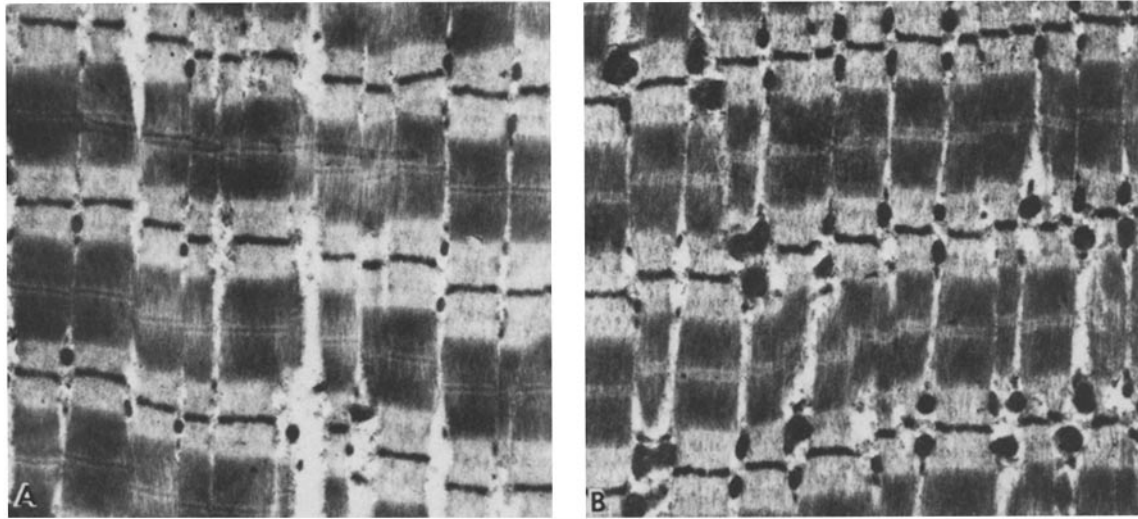


FIGURE 10 Comparison of an FG and an FOG fiber from diaphragm by electron microscopy. On the basis of mitochondrial size, shape, and number, an FG fiber (A) and an adjacent FOG fiber (B) from diaphragm are shown. Measurements of the relative Z-line widths of the two fibers showed that in diaphragm all fiber types exhibit Z-lines of similar width.  $\times 9,600$ .

concepts that have dominated thinking about muscle: the existence of discrete fiber types with defined histochemical, biochemical, molecular, morphological, and physiological properties, and the coordinate regulation and synthesis of myofibrillar proteins. In previous work, we suggested that the proposal that there are discrete fiber types at the molecular level was based on extrapolation from a small set of well-analyzed muscles and that a more extensive analysis of heterogeneity was not consistent with that idea. Bronson and Schachat (4) found that all three dimeric forms of tropomyosin are expressed in fast muscles and that their distribution is not a function of histochemically determined fiber type. The two major adult forms of fast troponin T described by Briggs et al. (3) also are not distributed in a manner consistent with either being fiber-type specific. Instead, quantitative analysis of the patterns of troponin T and tropomyosin expression in single skeletal muscle fibers indicates that there are several different combinations of these  $\text{Ca}^{2+}$ -regulatory proteins that are expressed in different proportions in fibers from different muscles in the rabbit (22, 31). A similar link in the expression of different troponins and tropomyosins has been observed in chicken pectoralis muscle transiently during development (20) and in response to denervation (21).

Many aspects of the heterogeneity of fast  $\alpha$ -actinin reported here are similar to the observations on fast troponin and tropomyosin. Two species of  $\alpha$ -actinin in fast muscles,  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub>, were identified. They differ in  $M_r$ , and the absence of any co-migrating CNBr fragment implies they are the products of different mRNAs. As is true for the fast troponin T and tropomyosin species, their distribution in fast muscles is not consistent with either  $\alpha$ -actinin species being FG or FOG type specific.  $\alpha$ -Actinin<sub>1f</sub> can be the predominant  $\alpha$ -actinin in either FG or FOG fibers, whereas  $\alpha$ -actinin<sub>2f</sub> is prevalent only in several FG muscles. Additional evidence for the absence of fiber-type specificity for the fast  $\alpha$ -actinin species comes from the fact that both  $\alpha$ -actinin species can be expressed in the same fiber.

Based on the expression of troponin T<sub>1f</sub> with  $\alpha\beta$  tropomyosin and troponin T<sub>2f</sub> with  $\alpha_2$  tropomyosin in fast muscle fibers, we proposed that, at the molecular level, there is a continuum of fast fibers due to the expression of these two

troponin T-tropomyosin combinations in different ratios in individual muscle fibers (31). The expression of both fast  $\alpha$ -actinin species in the same fiber adds to the evidence for the continuous, rather than discrete, nature of skeletal muscle fiber types. More specifically, with regard to the continuous nature of fast fiber diversity, it is possible that the continuum of Z-line widths observed in fast fibers by Eisenberg and Kuda (10) is a consequence, at least in part, of the expression of both  $\alpha$ -actinin species in different ratios in individual fast fibers.

The contemporaneous expression of  $\alpha$ -actinin<sub>1f</sub> with troponin T<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub> with troponin T<sub>2f</sub> in adult fast muscles indicates that the presence of each  $\alpha$ -actinin correlates with the presence of a different thin filament  $\text{Ca}^{2+}$ -regulatory complex:  $\alpha$ -actinin<sub>1f</sub> with troponin T<sub>1f</sub>- $\alpha\beta$  tropomyosin and  $\alpha$ -actinin<sub>2f</sub> with troponin T<sub>2f</sub>- $\alpha_2$  tropomyosin. The role of troponin-tropomyosin in the regulation of skeletal muscle contraction (8) suggests a physiological consequence of the expression of different fast troponin-tropomyosin combinations: fine control of the actomyosin ATPase. Experimental evidence for this proposal comes from the studies of Brandt et al. (1) where differences in the  $\text{Ca}^{2+}$ -dependence of force generation in fast fibers correlates with different patterns of troponin-tropomyosin expression. It is possible that this variation may be the molecular basis of the continuum of contractile properties found in fast motor units by Henneman and his colleagues (14). The absence of any defined role of  $\alpha$ -actinin in regulating contraction, however, means that the expression of each  $\alpha$ -actinin with a different troponin-tropomyosin combination is most likely not related to the  $\text{Ca}^{2+}$ -regulation of the actomyosin ATPase. For that reason, we would suggest that the contemporaneous expression is a consequence of the fact that the Z-line and thin filaments form a structurally continuous unit.

Most probably the  $\alpha$ -actinin species characterized here correspond to the fast and slow  $\alpha$ -actinin species reported by Suzuki et al. (33, 34) in pig. However, in contrast to their conclusion that there are distinct fast and slow forms of  $\alpha$ -actinin, analysis of a greater variety of skeletal muscles shows that one of the  $\alpha$ -actinin species appears to be expressed in both fast and slow fibers. We were unable to distinguish

between  $\alpha$ -actinin from the slow muscle soleus and  $\alpha$ -actinin<sub>1f</sub> by either SDS PAGE or CNBr fragment mapping. Studies on Z-line morphology also suggested that  $\alpha$ -actinin<sub>1f</sub> is more closely related to  $\alpha$ -actinin<sub>s</sub> than to  $\alpha$ -actinin<sub>2f</sub>. Fibers that express either  $\alpha$ -actinin<sub>1f</sub> or  $\alpha$ -actinin<sub>s</sub> have significantly thicker Z-lines than those that express  $\alpha$ -actinin<sub>2f</sub>. The relationship of  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub> and the two fast-twitch glycolytic species isolated from rabbit by Kobayashi et al. (15, 16) is less clear. Those proteins, I  $\alpha$ -actinin and II  $\alpha$ -actinin, were found in erector spinae and psoas, respectively. They could be distinguished from slow  $\alpha$ -actinin immunologically and by partial proteolytic mapping, and their  $M_r$ s differed by 12,000. These observations are not consistent with either the distribution or properties of  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub>. Using both immunoblot and SDS PAGE analyses of freshly prepared myofibrils from both erector spinae and psoas, we have not found different  $\alpha$ -actinin species in those muscles;  $\alpha$ -actinin<sub>2f</sub> composes more than 85% of the total  $\alpha$ -actinin in both. Also,  $\alpha$ -actinin<sub>1f</sub> is virtually identical with slow  $\alpha$ -actinin, and the difference in  $M_r$  between  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>2f</sub> is only 4,000. In addition, we find no Coomassie Blue-staining band that corresponds to the II  $\alpha$ -actinin species in freshly prepared myofibrils.

The most unexpected observation of this study is the similarity of  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>s</sub>. While the simplest explanation for the peptide mapping data is that  $\alpha$ -actinin<sub>1f</sub> and  $\alpha$ -actinin<sub>s</sub> are identical, we are hesitant to suggest this solely on the basis of peptide maps. Nonetheless, their similarity alone is sufficient to argue that the differences between fast and slow myofibrillar proteins observed in myosin, troponin, tropomyosin, and C-protein are not characteristic of all contractile proteins. Clearly, these observations identify a protein of the myofibrillar lattice whose homologous forms have not evolved along the fast/slow dichotomy. There is evidence to suggest this may be true of other myofibrillar proteins. Reinach et al. (28) have identified an isoform of C-protein that composes part of the C-protein in fast fibers from the chicken posterior latissimus dorsi, and cross-reacts with a monoclonal prepared against adult slow C-protein. An understanding of how such non-neurogenic patterns of expression have come about will likely tell us a great deal about the mechanisms that coordinate synthesis of myofibrillar proteins.

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