

Detection of a Specific Isoform of Alpha-Actinin with Antisera Directed Against Dystrophin

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Abstract. We have characterized a protein immunologically related to dystrophin, the protein product of the Duchenne muscular dystrophy gene. We identify this related protein as a fast-twitch glycolytic isoform (mouse extensor digitorum longus-specific) of myofibrillar alpha-actinin. This specific isoform of alpha-actinin exhibits a more restricted pattern of expression in skeletal muscle than fast-twitch-specific

isoforms of both myosin and Ca^{2+} -ATPase. Our results provide evidence that dystrophin and myofibrillar alpha-actinin are related proteins, reinforcing the previous data concerning the sequence homologies noted between nonmuscle cytoskeletal alpha-actinin and dystrophin. In addition, we describe the first antisera directed against a specific myofibrillar skeletal muscle isoform of alpha-actinin.

DUCHENNE muscular dystrophy is a common human hereditary disease which causes the progressive loss of muscle tissue and results in early death. Recently the pathobiochemical basis for Duchenne muscular dystrophy was identified as the deficiency of a large, membrane-associated, cytoskeletal protein called "dystrophin" (427 kD) (3, 9, 10, 15, 27, 32, 35). Based on amino acid sequence analysis, dystrophin appears to be related to two other cytoskeletal proteins, spectrin (heterodimer of ~200- and ~250-kD subunits) and nonmuscle alpha-actinin (homodimer of ~95-kD subunit) both of which contain an extensive central domain hypothesized to be composed of antiparallel triple helical-coiled coils which potentially form a dimeric rod structure (1, 6, 15). All three of these proteins contain nonrodlike carboxyl- and amino-terminal domains which are believed to form noncovalent interactions with other proteins, thus forming a linkage between bound proteins at each end of the central rod domain (15, 17, 32). Dystrophin appears more closely related to the cytoskeletal (nonmuscle) alpha-actinins than it does to spectrin; there is considerable sequence homology shared between the nonrodlike terminal domains of cytoskeletal alpha-actinin and dystrophin, while the analogous domains of spectrin appear unrelated to either of these proteins (6, 8, 15).

Alpha-actinins have been defined in a large number of species and cell types by their characteristic molecular mass, size, and molecular structure, and also by their ability to bind F-actin (1, 2, 18, 23, 24, 29). Despite the structural homogeneity of the alpha-actinins, there are two classes of alpha-actinins which appear to have very different functions. Cytoskeletal alpha-actinins appear to be dynamic, Ca^{2+} -sensitive components of intracellular actin filament networks (5, 16, 26), while myofibrillar alpha-actinins are static, Ca^{2+} -

insensitive major components of the myofibrillar Z-line (18, 19, 29, 30). Both classes of alpha-actinin bind F-actin via their amino-terminal domain (21, 22). Indeed, the actin-binding domain has been shown to be very highly conserved at the amino acid level between *Dictyostelium* cytoskeletal alpha-actinin and chicken cytoskeletal alpha-actinin (23). It is not clear, however, whether this domain is similarly conserved with the myofibrillar alpha-actinins at the primary sequence level, as amino acid sequence data is not yet available for these isoforms. Despite the obvious functional differences between myofibrillar alpha-actinins and cytoskeletal (nonmuscle) alpha-actinins, they are highly immunologically related. The only reported antibodies specific for alpha-actinin isoforms were produced via immunoabsorption of nonspecific antisera against skeletal muscle and smooth muscle alpha-actinins (7). No antibody specific for a single skeletal muscle (myofibrillar) alpha-actinin isoform has, to our knowledge, been described.

During our previous work on the characterization of dystrophin we noted that an antibody preparation raised against a portion of the rod domain of dystrophin cross-reacted strongly with a Triton-insoluble protein of ~90 kD molecular mass (9). This cross-reactive protein continued to be strongly recognized by the anti-dystrophin antiserum despite affinity purification of antibodies specifically directed against dystrophin (9, 32). We now report the characterization of this cross-reactive protein as a fast-twitch glycolytic isoform of alpha-actinin. We thereby provide the first evidence for the structural homology of myofibrillar alpha-actinin and the dystrophin rod domain. In addition, we describe the first antibody preparation directed against a specific myofibrillar isoform of alpha-actinin.

Materials and Methods

Antisera

The 30-kD dystrophin peptide used for antisera production was produced in bacteria via protein expression vectors by splicing a segment of the mouse cardiac dystrophin cDNA into the indolacrylic acid-inducible *pATH2* expression vector as previously described (9). The resulting antigen contained a 207 amino acid segment of dystrophin derived from the center of the rod domain of dystrophin, corresponding to amino acid numbers 1,181–1,388 of the human sequence (15). Antisera were raised against purified antigen in a soluble native form in rabbits, and against SDS-solubilized, denatured antigen in polyacrylamide gel slices in sheep (9). Dystrophin-specific antibodies were affinity purified from sheep antisera as described (9), and from the rabbit serum as described (32).

Additional antisera used included a mouse monoclonal antibody directed against a fast-twitch isoform of the chicken Ca^{2+} - Mg^{2+} -ATPase (5D2; reference 12); a monoclonal antibody against a fast-twitch isoform of chicken myosin (F59; reference 20); a polyclonal rabbit anti-chicken gizzard (smooth muscle) alpha-actinin antisera kindly provided by Dr. David Critchley of the University of Leicester, Leicester, UK (B8); a polyclonal rabbit anti-chicken gizzard alpha-actinin antibody purchased from Sigma Chemical Co., St. Louis, MO (A2543); and a monoclonal alpha-actinin antibody purchased from Amersham Corp., Arlington Heights, IL.

Immunoblotting

Specific muscle groups were quickly dissected from C57/B6 mice which had been killed by cervical dislocation. Muscles were stored frozen at -80°C until needed, whereupon the muscles were pulverized into a frozen powder, the powder weighed, and then solubilized in 20 vol of sample buffer (10% SDS, 0.1 M Tris, pH 8.0, 5 mM EDTA, 50 mM DTT). Samples were boiled for 2 min, centrifuged to remove insoluble proteins, and then loaded onto 0.8-mm-thick, 3.5–12.5% polyacrylamide-SDS gradient gels with a 3.0% stacking gel. After electrophoresis, fractionated proteins were electroblotted onto nitrocellulose (31) and the filters allowed to completely air dry. Filters were then processed for immunodetection of specific proteins as previously described (9).

Immunoprecipitation of alpha-actinin was performed by first extensively sonicating an extensor digitorum longus (EDL)¹ and soleus muscle from a single 5-mo male C57B10 mouse in 1 ml of cold buffer (1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 10 mM Phosphate, pH 6.8, 0.15 M NaCl, 2 mM EDTA, supplemented with Trasylol [Boehringer Mannheim Diagnostics, Inc., Houston, TX]). Insoluble proteins were discarded after centrifugation, and 20 μl of crude rabbit alpha-actinin antiserum (B8) added to the supernatant. After a 3-h incubation on ice, immune complexes were collected by incubation with formalin-fixed *Staphylococcus aureus* followed by centrifugation. The *S. aureus*/antibody/alpha-actinin complexes were washed five times in 1 ml of cold buffer, then the complex disassociated by boiling in sample buffer. One fifth of the solubilized protein was electrophoretically fractionated and blotted as above.

Immunofluorescent Microscopy of Thick Sections

The indicated muscles were frozen in -80°C isopentane and then sectioned (8 μm) onto subbed slides using a cryostat set for -20°C . Sections were processed for immunofluorescent microscopy as described below for thin sections.

Immunofluorescent Microscopy of Thin Sections and Immunoelectron Microscopy

The EDL and soleus muscles were carefully dissected from normal C57/BL6 mice, pinned to dental wax, and lightly fixed (0.01% glutaraldehyde, 2% formaldehyde) for 1 h at 4°C . Immunolabeling was found to be quenched when fixation stronger than that described was used. After washing in 0.15 M PBS, pH 7.4, the muscle was cut into small 1-mm³ cubes perfused in 2.3 M sucrose overnight, mounted on cutting stubs in either a transverse or longitudinal orientation, and shock-frozen in liquid nitrogen. Sections 70–100 nm thick were cut using an Ultracut microtome (Reichert Scientific Instruments, Buffalo, NY) fitted with an FC4 cryoattachment, mounted on

Formvar carbon-coated grids, and immunolabeled as described elsewhere (33) using either the rabbit antidystrophin antibody described above or a monoclonal antibody to alpha-actinin purchased from Amersham Corp., air dried, and then examined using a Philips Electronic Instruments, Inc. (Mahwah, NJ) EM300 electron microscope.

For semi-thin immunofluorescence, sections (250 nm) were cut as above and mounted on glass slides subbed in 0.2% gelatin. Nonspecific immunoreactivity was blocked with purified goat IgG, and the sections then washed in PBS, and incubated in the primary antibody as above which was revealed with either a rhodamine- or fluorescein-labeled specific antibody (Cappel Laboratories, Cochranville, PA). All antibody incubations were at room temperature for 1 h. After thorough washes in PBS, the slides were then mounted in Gelvatol (Monsanto Co., Dayton, OH) and examined using a Nikon microphot fluorescence microscope.

Results

Muscle-type Distribution of Dystrophin and the 90-kD Protein

Dystrophin has been shown to be equally distributed in all skeletal muscle groups, cardiac muscle, and smooth muscle (3, 11). In addition, dystrophin is present in embryonic, newborn, and adult muscle at apparently equal levels (11). To determine the muscle group specificity of the 90-kD cross-reactive protein, adult mouse soleus (50% fast-twitch oxidative glycolytic, 50% slow-twitch oxidative [34]), EDL (100% fast-twitch glycolytic [34]), heart, and smooth muscle (gravid uterus) were solubilized, and the constituent proteins subjected to immunoblotting. As shown in Fig. 1 A, the 90-kD protein is recognized solely in the fast-twitch glycolytic EDL muscle. The tissue distribution of this protein is much more limited than that of other, well-characterized fast-twitch-specific protein isoforms (Fig. 1, C and D). A monoclonal antibody specific for a fast-twitch isoform of myosin recognizes such a protein in soleus, heart, and EDL (but not smooth muscle) (Fig. 1 D), while a monoclonal antibody specific for a fast-twitch isoform of the Ca^{2+} - Mg^{2+} -ATPase recognizes this isoform in both soleus and EDL (but not heart or smooth muscle) (Fig. 1 C). The myosin and ATPase isoforms recognized by these antibodies have been shown to have a more limited distribution in chicken muscle (11, 12, 20), though the analogous muscles of the mouse appear to be significantly faster in their metabolism than in the chicken and other higher vertebrates. Thus, the 90-kD protein recognized by the affinity-purified dystrophin antibodies shows a muscle group distribution suggesting that it is limited to fast-twitch glycolytic skeletal muscle fibers.

Though the recognition of the 90-kD protein by the rabbit antinative dystrophin serum appears highly specific and of high affinity, affinity-purified sheep antidenatured dystrophin raised against the same dystrophin antigen fails to detect the 90-kD protein in any muscle type (Fig. 1 B). This data suggests that the antigenic sites held in common between dystrophin and the 90-kD protein are dependent on secondary or tertiary structure. However, another explanation is that the common antigenic sites are not immunogenic in sheep.

Localization of the 90-kD Protein to the Myofibrillar Matrix

To localize the 90-kD protein within a subcellular compartment, immunofluorescence of semi-thin (250 nm), lightly fixed cross-sections of C57B6 (normal; dystrophin-positive) mouse EDL and soleus, and *mdx* (dystrophin-negative) EDL

1. Abbreviation used in this paper: EDL, extensor digitorum longus.

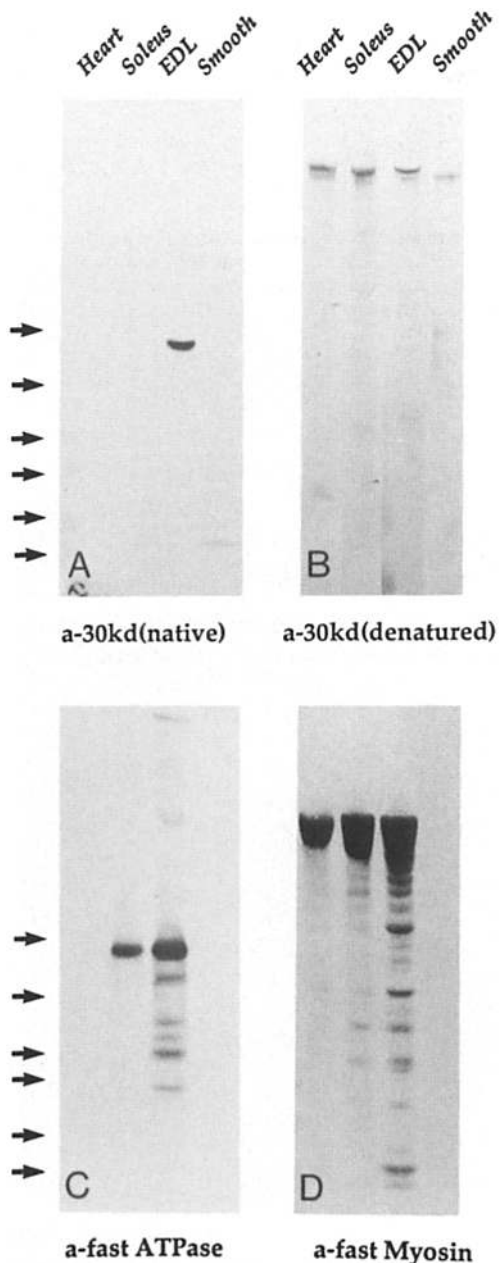


Figure 1. Specificity of antisera used and muscle-group distribution of the recognized proteins in the mouse. The indicated mouse muscle groups were solubilized, and constituent proteins (50 μ g) subjected to immunoblotting. Identical filters were incubated with the antisera indicated. Arrows indicate the position of prestained molecular mass markers (from top, 116, 84, 58, 48.5, 36.5, and 26.6 kD). *A* was incubated with affinity-purified rabbit antidystrophin raised against the 30-kD dystrophin antigen in an SDS-free, soluble, native form (9). The major protein species recognized by this antiserum is a cross-reactive (nondystrophin), EDL-specific protein of \sim 90 kD. The 90-kD protein is \sim 1,000-fold more abundant than dystrophin, which is not visualized in this particular blot. Dystrophin is, however, detected with this same antibody preparation (see references 9 and 32; and Fig. 3). *B* was incubated with affinity-purified sheep antidystrophin raised against the identical antigen used in *A*, but in an SDS-solubilized, denatured form. Dystrophin (427 kD; reference 15) and its smaller smooth muscle isoform (11) is recognized with high sensitivity in all muscle types, while the 90-kD protein seen in *A* is not evident. *C* was incubated with a monoclonal antibody against a fast-twitch isoform of the 105-kD

muscle was performed with the affinity-purified antidystrophin antisera. As has been shown previously (3, 27, 35), dystrophin immunostaining is seen as a faint ring surrounding all normal (C57B6) myofibers in both EDL (Fig. 2 *A*) and soleus (Fig. 2 *B*) which is absent in *mdx* myofibers (Fig. 2 *C*). The immunostaining corresponding to the cross-reactive 90-kD protein, on the other hand, is seen as a 'zebra' type of pattern in the EDL muscles of both normal (Fig. 2 *A*) and *mdx* (Fig. 2 *C*) muscle fibers. As expected from the immunoblot data (Fig. 1), little or no immunostaining of the 90-kD protein is observed in the soleus muscle fibers (Fig. 2 *B*). The zebra pattern of immunostaining of the 90-kD protein in semi-thin cross sections implied a myofibrillar localization of this protein. This subcellular localization was reinforced by immunofluorescence of longitudinal thick (8- μ m) sections of *mdx* semimembranous myofibers. As shown in Fig. 2 *D*, the 90-kD protein appears in a striated pattern, again suggesting that the 90-kD protein is a component of the myofibrillar matrix.

Identification of the 90-kD Protein as an Alpha-Actinin

Dystrophin has been shown to exhibit primary amino acid sequence homology to cytoskeletal (nonmuscle) alpha-actinin, a protein of \sim 90 kD (6, 8, 15). To determine if the EDL-specific 90-kD myofibrillar protein was indeed an isoform of myofibrillar (muscle) alpha-actinin, total mouse EDL muscle protein was solubilized and subjected to electrophoresis using a single large lane on a polyacrylamide gradient gel. The proteins were blotted onto nitrocellulose, with the nitrocellulose then being sliced into parallel sections. Adjacent nitrocellulose slices were incubated with the rabbit affinity-purified antidystrophin antisera, and with two different polyclonal antibodies raised against chicken gizzard (smooth muscle) alpha-actinin. As shown in Fig. 3, all three antisera recognize a common polypeptide of \sim 90 kD which appear to comigrate, suggesting that the protein recognized by the dystrophin antisera is an alpha-actinin.

To more directly show that antigenic determinants are shared by dystrophin and alpha-actinin, alpha-actinin was immunoprecipitated from both EDL and soleus muscles with polyclonal anti-alpha-actinin (B8). The immunoprecipitated proteins were then fractionated and immunoblotted as above, using both affinity-purified antidystrophin antibodies, and the same anti-alpha-actinin antisera used for the immunoprecipitation. As shown in Fig. 4, the antidystrophin antibodies recognize the immunoprecipitated EDL alpha-actinin, but not that precipitated from the soleus. The alpha-actinin antiserum, on the other hand, recognizes the immunoprecipitated alpha-actinin in both the EDL and soleus. As the rabbit immunoglobulins were also subjected to electrophoresis in this experiment, the reduced, monomeric IgG heavy chain is detected by the enzyme-linked second antibody (most antibodies are directed against the constant region of the heavy chain in anti-immunoglobulin preparations).

Ca²⁺-Mg²⁺ ATPase (12). *D* was incubated with a monoclonal antibody directed against a fast-twitch isoform of 205-kD myosin (20).

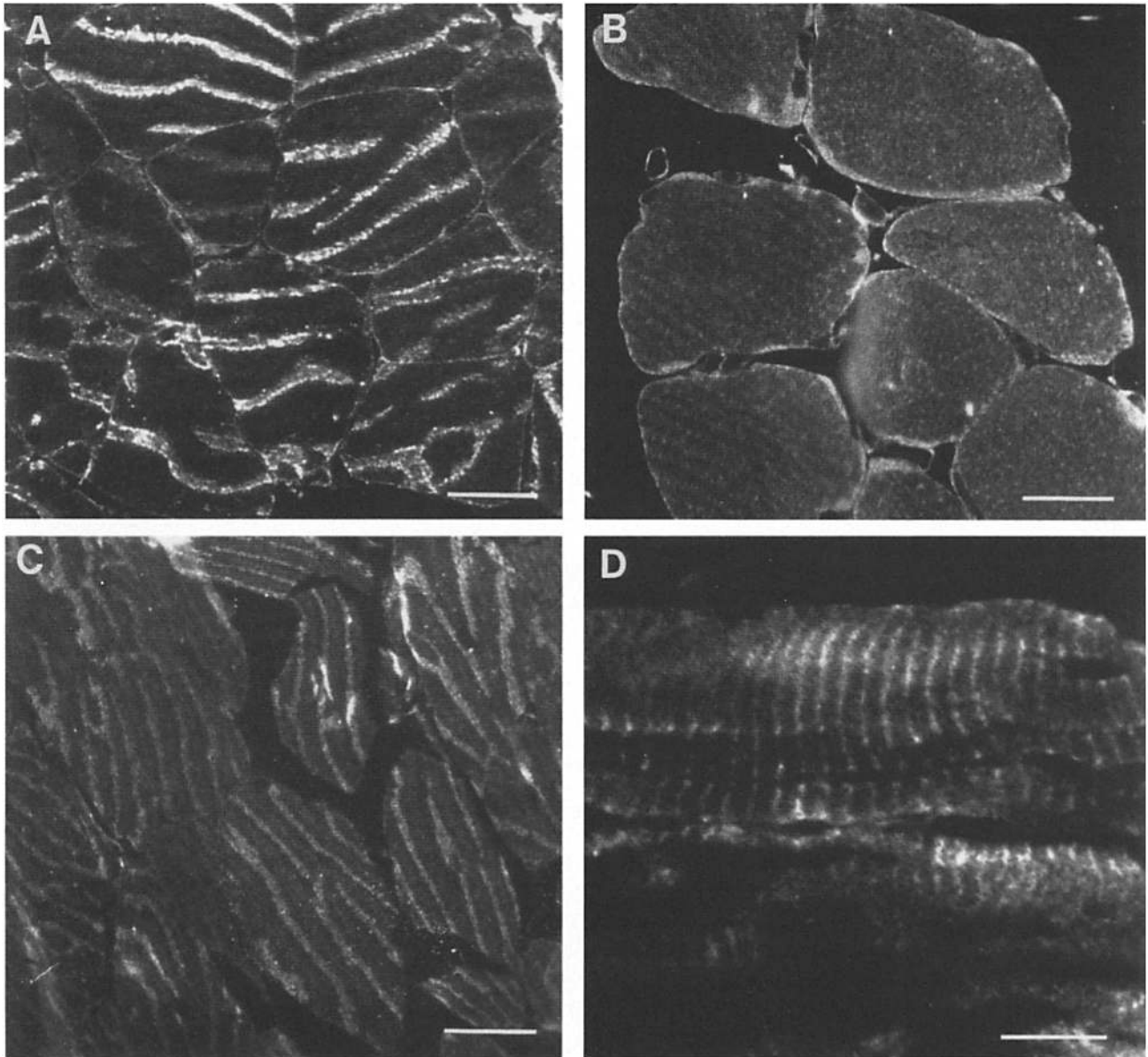


Figure 2. Immunofluorescent localization of the 90-kD protein. *A*, *B*, and *C* show thin (250-nm) cryosections of lightly fixed adult C57B6 (normal) mouse EDL muscle (*A*), C57B6 soleus (*B*), and *mdx* EDL (*C*) in slightly oblique cross-sections. The faint plasma membrane-staining characteristic of dystrophin localization is seen as a ring around the normal mouse fibers (*A* and *B*), which is absent in the dystrophin deficient *mdx* myofibers (*C*) (references 3 and 27). The 90-kD cross-reactive protein, on the other hand, is localized in a zebra-like pattern (arrows) in the EDL muscles of both C57B6 and *mdx* mice (*A* and *C*), but not in soleus (*B*). The immunostaining pattern of the 90-kD protein is suggestive of EDL-specific myofibrillar localization. *D* shows longitudinally sectioned (8 μ m) unfixed myofibers from an adult *mdx* mouse semimembranous muscle (predominantly fast-twitch) incubated with affinity-purified rabbit antidystrophin antisera to visualize the localization of the 90-kD cross-reactive protein. A striated pattern of immunostaining is evident. Bars, 20 μ m.

Immunolocalization of the 90-kD Protein to the Z-line

To further reinforce the identification of the 90-kD protein as a specific isoform of myofibrillar alpha-actinin, immunoelectron microscopy of ultra-thin cryosections was performed. As shown in Fig. 5 *A*, the 90-kD protein is clearly localized within the Z-line of myofibrils of the EDL muscle. Immunostaining with the same antibody was completely ab-

sent in soleus myofibrils (Fig. 5 *B*), verifying the immunoblot analysis above. Parallel immunostaining of myofibrils with a monoclonal anti-alpha-actinin antibody revealed a very similar pattern of myofibrillar localization (Fig. 5 *C*), as has been previously described (30). Dystrophin has been immunolocalized on the cytoplasmic face of the plasma membrane and possibly the transverse tubules (32), and is not evident in the figures shown.

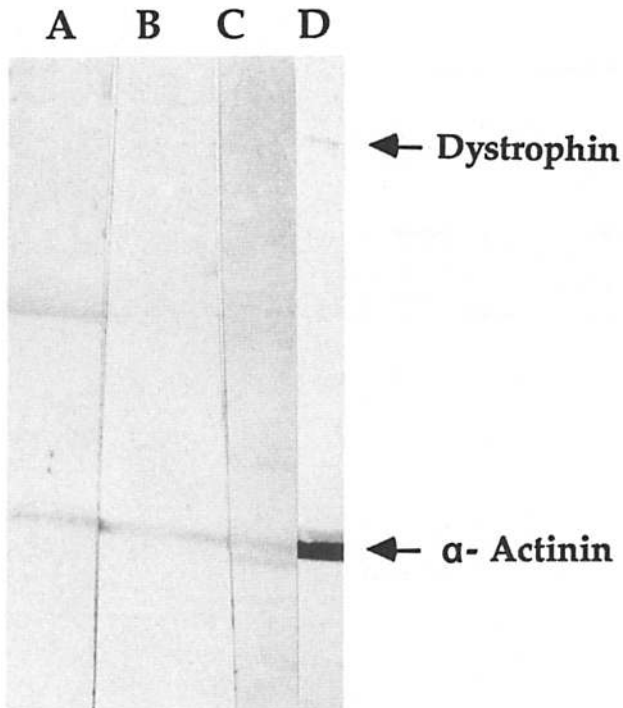


Figure 3. The 90-kD dystrophin-related protein comigrates with alpha-actinin. Total protein was solubilized from mouse EDL muscle and subjected to electrophoresis as a single lane on a 3.5–12.5% gradient SDS–polyacrylamide gel. The proteins were transferred to a nitrocellulose filter, and the filter cut into parallel segments. Lane *A* was incubated with affinity-purified rabbit antidystrophin antisera to visualize the cross-reactive 90-kD protein. Lanes *B* and *C* were incubated with crude alpha-actinin antisera from Sigma Chemical Co. and Dr. David Critchley of the University of Leicester, respectively, to visualize alpha-actinin. The 90-kD cross-reactive protein recognized by the dystrophin antisera clearly comigrates with the alpha-actinin band recognized by the other two alpha-actinin antisera. To show that dystrophin is also recognized by the dystrophin antisera, a similar blot was overdeveloped to visualize the very low abundance dystrophin protein (lane *D*).

Discussion

We have demonstrated that dystrophin, the product of the Duchenne muscular dystrophy gene, is immunologically related to myofibrillar alpha-actinin. Contamination of the original dystrophin antigen preparation by alpha-actinin antigenic determinants is not possible given the methods used for dystrophin antigen production (bacterial dystrophin cDNA expression) (9). Thus dystrophin and myofibrillar (muscle) alpha-actinin must share antigenic determinants. Previous primary amino acid sequence analysis has indicated a similarity between dystrophin and cytoskeletal (nonmuscle) alpha-actinin (6, 8, 15). This paper, however, presents the first evidence suggesting that dystrophin is also related to myofibrillar alpha-actinin. Indeed, the antibodies used in this study were raised against the central rod domain of dystrophin, a domain which exhibits very little direct sequence homology to cytoskeletal alpha-actinin (15). The central domain of dystrophin has been hypothesized to be structurally homologous to alpha-actinin, however, despite the lack of extensive sequence homology (6, 15). In agreement with the structural nature of the homology, the shared antigenic deter-

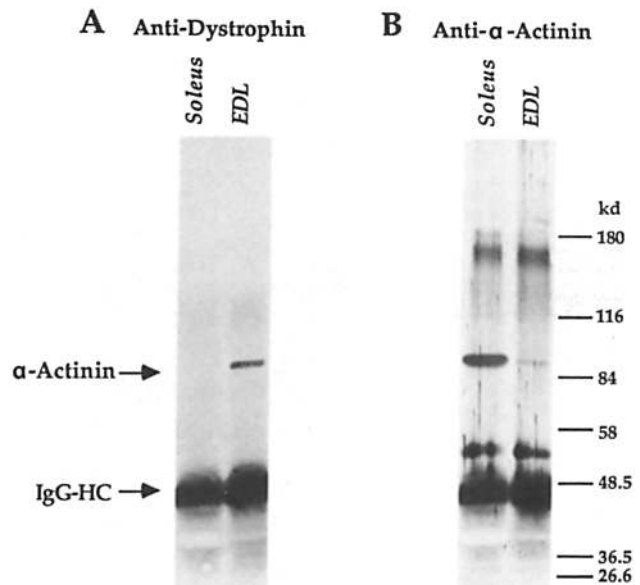
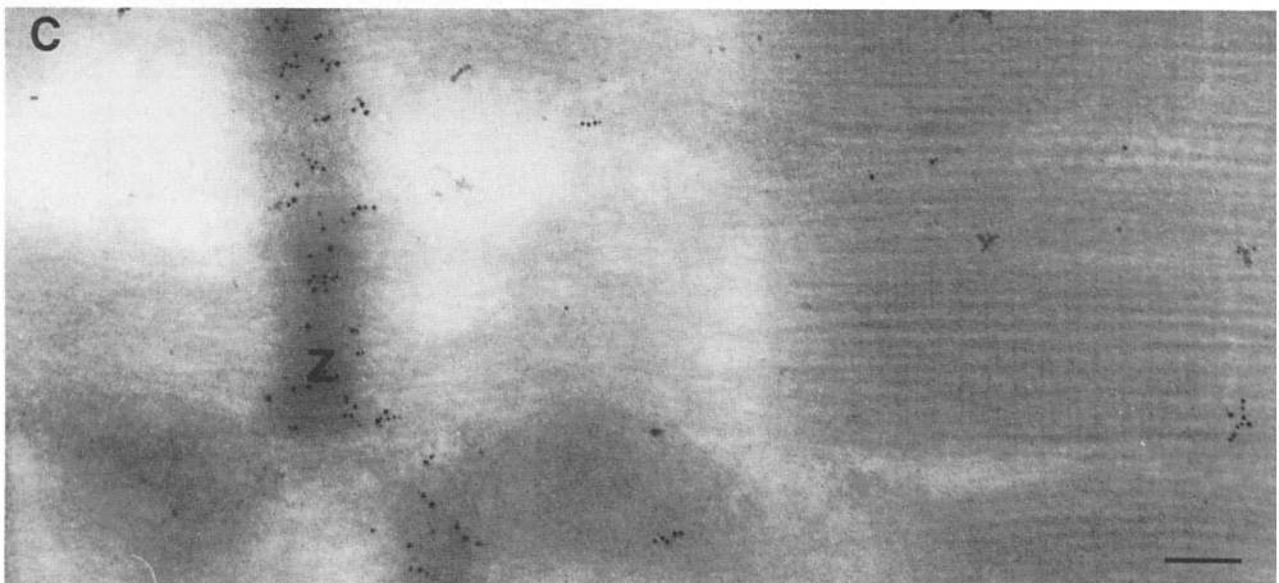
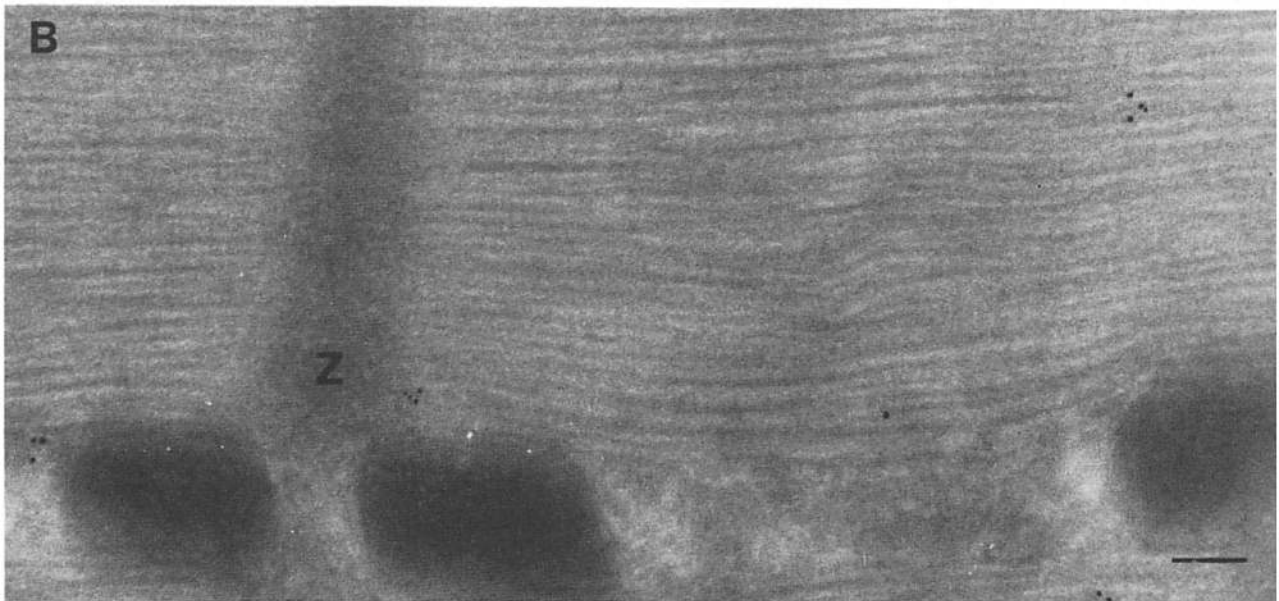
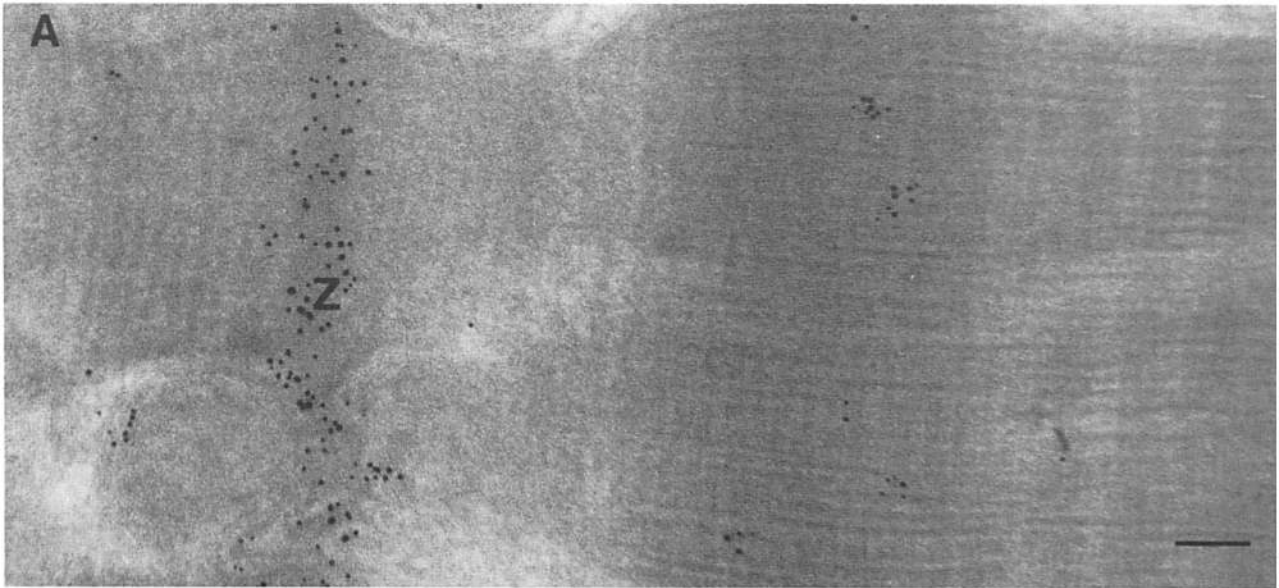


Figure 4. Immunoprecipitation of the 90-kD dystrophin-related protein by alpha-actinin antiserum. Alpha-actinin was immunoprecipitated from mouse EDL and soleus muscle protein using polyclonal antiserum, and the immune complexes subjected to immunoblotting as described above. Identical immunoblots were incubated with either affinity-purified antidystrophin antibodies (*A*) or with the same alpha-actinin antisera used for the immunoprecipitation (*B*). A large amount of soleus alpha-actinin, and a smaller amount of EDL alpha-actinin, is precipitated and recognized by the alpha-actinin serum, while only the EDL alpha-actinin is recognized by the antidystrophin antibodies. The IgG heavy chain monomer (*IgG-HC*) is also evident as a consequence of the experimental procedure used. The positions of prestained molecular mass markers (Sigma Chemical Co.) are indicated.

minants appear to be structure dependent, as only antibodies raised against the dystrophin antigen in soluble, native form exhibited the immunological cross-reaction to alpha-actinin (Fig. 1; reference 9).

Perhaps the most intriguing result of the described work is that we have produced an antisera highly specific for a single alpha-actinin isoform. Many groups have described the production of anti-alpha-actinin antisera from various species, tissues, and muscle types (5, 7, 13, 16, 19, 23, 24). From these previous immunological studies, and other biochemical studies using purified alpha-actinin, it has become clear that myofibrillar (muscle) alpha-actinins are distinct from cytoskeletal (nonmuscle) alpha-actinins (4, 5, 16, 17, 21), and that muscle fiber type-specific forms of alpha-actinin exist (5, 13, 14, 25, 28). Despite the well-documented distinctions between the various alpha-actinin isoforms, few groups have been able to successfully distinguish between these isoforms immunologically. Two groups of investigators have reported immunological differences between smooth muscle and skeletal muscle alpha-actinins using double-diffusion analyses (4, 13), however such specificity was either not reproducible using immunofluorescence techniques (4) or was apparently artifactual (14). An additional laboratory has produced affinity-chromatography fractions which are specific for chicken smooth muscle or skeletal muscle (7), however no group has produced a preparation monospecific for a single myofibril-



lar isoform. The alpha-actinin antiserum described in this publication appears specific for a fast-twitch glycolytic isoform of alpha-actinin both by immunoblot and immunocytochemical analyses. Thus, the described antiserum is, to our knowledge, the first reported antisera monospecific for a myofibrillar alpha-actinin isoform. Significantly, this isoform exhibits a distribution in mouse muscle which is more limited with respect to fiber type than any other antibody of which we are aware. The restricted distribution of this alpha-actinin isoform is most apparent in the soleus muscle, where fast-twitch-specific isoforms of both the Ca^{2+} - Mg^{2+} -ATPase and myosin are present (most likely due to the fast-twitch oxidative/glycolytic fibers present in mouse soleus [34]), while the described alpha-actinin isoform is completely absent.

Clearly, myofibrillar alpha-actinin and dystrophin must have distinctly different cellular functions given their very different subcellular localization in the myofiber. Nevertheless, the results presented reinforce the concept of a family of cytoskeletal proteins based on a rodlike central structural domain, which includes dystrophin, spectrin, cytoskeletal (nonmuscle) alpha-actinin, and myofibrillar alpha-actinin. Future studies should identify additional members of this family, and elucidate the sequence and/or functional similarities between these proteins.

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Figure 5. Immunoelectron microscopic colocalization of the 90-kD protein and myofibrillar alpha-actinin. *A* shows lightly fixed, ultra-thin cryosections of normal mouse EDL muscle in longitudinal section. Sections were incubated with affinity-purified antibodies, and with 10-nm gold-conjugated goat anti-rabbit IgG to visualize the immune complexes. The 90-kD protein is seen localized within the Z-line (Z) of the myofibrils. *B* shows adult mouse soleus processed as in *A*, using the same antisera used in *A*. There is an absence of 90-kD protein immunostaining in the Z-line (Z) of the slow-twitch soleus. This is in agreement with the immunoblot data presented in Fig. 1. *C* shows a similar preparation of mouse EDL immunostained with a monoclonal antibody directed against alpha-actinin. The distribution of alpha-actinin staining appears strikingly similar to that of the 90-kD protein shown in *A*. Bar, 0.1 μm .

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