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Human Skeletal Muscle-Specific α-Actinin-2 and -3 Isoforms Form Homodimers and Heterodimers *in Vitro* and *in Vivo*

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α-actinins belong to a family of actin-binding and crosslinking proteins and are expressed in many different cell types. Multiple isoforms of α-actinin are found in humans and are encoded by at least four distinct genes. Human skeletal muscle contains two sarcomeric isoforms, α-actinin-2 and -3. Previous studies have shown that the α-actinins function as anti-parallel homodimers but the question of heterodimer formation between two different isoforms expressed in the same cell type has not been explored. To address this issue, we expressed both α-actinin-2 and -3 *in vitro* and were able to detect their interaction by both blot overlay and co-immunoprecipitation methods. We were also able to demonstrate the presence of heterodimers *in vivo* in human skeletal muscle and in COS-1 cells transiently transfected with both isoforms. Our results clearly demonstrate the potential for α-actinin isoforms to form heterodimers which might have unique functional characteristics. © 1998 Academic Press

α-actinin is a major component of the cytoskeleton in many cell types. It belongs to a superfamily of actin-binding and crosslinking proteins, including spectrin and dystrophin (1). Native α-actinin forms an anti-parallel homodimers (2-4) whose major functions include bundling F-actin into parallel arrays and anchoring actin filaments at specific sites within the cell (5). A number of vertebrate α-actinin isoforms have been isolated, including non-muscle (cytoskeletal), skeletal and smooth muscle isoforms (6-9). In addition, invertebrate α-actinins have been characterized in *Dictyostelium discoideum* (10), *Caenorhabditis elegans* (11), and *Drosophila melanogaster* (12). Molecular characterization of these proteins has shown that they all share extensive sequence homology (> 70%) and have similar molecular weights of approximately 100 kD.

There are at least four known α-actinin genes in humans, two skeletal muscle isoforms (α-actinin-2 and -3) and two non-muscle/smooth muscle isoforms (α-actinin-1 and -4) (13-16). The non-muscle isoforms are predominantly localized along the actin-containing microfilament bundles and at adherens type junctions, where they are thought to play a role in anchoring actin (17, 18). In skeletal, cardiac and smooth muscles, α-actinin is localized at the Z-discs and analogous dense bodies where it is involved in constitutively anchoring the myofibrillar actin thin filaments (19). Of the two skeletal muscle-specific isoforms in human, α-actinin-2 is found in both skeletal and cardiac muscle whereas α-actinin-3 is limited to limb skeletal muscle (14).

Structurally, α-actinin can be divided into 3 distinct domains (5). An actin-binding domain at the N-terminus is followed by a rod domain consisting of four internal repeats structurally homologous to those found in spectrin and dystrophin. It has been shown that all four internal repeats are essential for the dimerization of α-actinin (20). At the C-terminus are two EF-hand calcium binding motifs that are only functional in the non-muscle isoforms (21). To date, the calcium sensitivity of α-actinin binding to F-actin is the only known functional difference between the muscle (sarcomeric) and non-muscle (cytoskeletal) isoforms.

Although two distinct α-actinin isoforms are expressed in skeletal muscle, indirect immunofluorescent analysis of adult human skeletal muscle has shown that while all muscle fibers contain α-actinin-2, only a subset of type 2 fast muscle fibers contain α-actinin-3 (22). Given the extensive homology between α-actinin-2 and -3 (80% identity, 90% similarity), we hypothesized that heterodimer formation should be possible when they are co-expressed in the skeletal muscle. Here we present *in vitro* and *in vivo* evidence for α-

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actinin-2/-3 heterodimer formation, demonstrating that these isoforms are functionally, as well as structurally, similar to each other.

MATERIALS AND METHODS

In Vitro Translation of α-Actinin Isoforms

PCR primers were designed to amplify full length α-actinin-2 and α-actinin-3 from cDNA clones, p18D1 and p7M1 respectively (14) and then subcloned into expression vectors, pMGT-1 and pFHR-1 which have been previously described in detail (23). ^{14}C-leucine labeled peptides were translated *in vitro* using a TNT T7 coupled reticulocyte lysate system according to the manufacturer's protocol (Promega Corp., Madison, WI). Expressed proteins were separated by gradient gels purchased from Novex (San Diego, CA). Electrophoresis was carried out either in the presence of SDS (denaturing condition) or without SDS (native condition). Gels were exposed to a storage phosphor plate and the plate was scanned using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Production of Isoform-Specific Anti-α-Actinin Antisera

Peptides derived from the unique amino termini of α-actinin-2 (NQIEPGVQYNYVYDEDE), α-actinin-3 (QPEGLGAGEGRFAGG) and rod region of α-actinin-2 (NKMEEIARSSIQITGALEDQMNQLKQYEH) were synthesized by the HHMI/Harvard Medical School Biopolymers Facility on an ABI 430A peptide synthesizer using FastMoc chemistry at 0.25 mm scale. Carboxyl-terminal cysteine residues were added to allow coupling to maleimide-activated keyhole limpet hemocyanin using an Imject kit (Pierce, Rockford, IL). Immunogens (100-200 μg total protein) were injected intradermally with Freund's complete adjuvant into New Zealand White rabbits and the animals were boosted at monthly intervals by intramuscular injections using incomplete Freund's adjuvant and bleed following standard procedures (24). Antisera were either used crude at dilutions of 1:500-1000, or were affinity purified using the original peptide conjugated to N-hydroxyl succinimide-activated agarose ProtOn columns (Multiple Peptide Systems, San Diego, CA). Each antigen was injected into two rabbits and the resulting duplicate antisera had indistinguishable patterns of reactivity. The resulting anti-α-actinin-2 antibodies (4A and 4B), anti-α-actinin-3 antibodies (5A and 5B) and anti-α-actinin-2 rod antibodies are herein referred to as α-2, α-3 and 7206 E respectively. Partial characterization of these antibodies has been previously reported (22, 25).

Immunoblot Experiments

Proteins were transferred overnight from SDS gels to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) in Towbin buffer at constant voltage of 20 V at room temperature (26). The membranes were incubated (blocked) in western buffer (20 mM Tris-Hcl, pH 7.4, 150 mM NaCl, 0.1% Tween 20 and 0.5% gelatin) for 2 hours at room temperature and then overnight at 4° with affinity purified primary antibodies at 1:2000 dilution in western buffer. After extensive washing in western buffer, the membranes were incubated at room temperature for one hour with a horseradish peroxidase-conjugated donkey anti-rabbit (or anti-mouse) IgG (H+L) secondary antibody diluted at 1:10,000 in the western buffer (Jackson ImmunoResearch Lab., West Grove, PA). The horseradish peroxidase-conjugated protein complex was detected by the enhanced chemiluminescence method (ECL) according to the manufacturer's protocol (Amersham Corp., Arlington Heights, IL).

Blot Overlay Experiments

In vitro translated α-actinins were separated on 8 % SDS gels and transferred overnight to nitrocellulose membranes as described above. Membranes were then incubated with different ^{35}S-methionine labeled α-actinin isoforms according to methods described previously (27).

Immunoprecipitation Experiments

Cell lysates from COS-1 cells co-transfected with α-actinin isoforms were incubated with antibody directed against one of the two α-actinin isoforms on ice for 4 hours. Immune complexes were captured by addition of 50 μl of 50% protein G-sepharose (Sigma Chem. Co., St. Louis, MO) followed by incubation on ice for one hour. The precipitates were then centrifuged at 10,000 g for 2 minutes at room temperature and the pellets were washed 3 times with 1 ml of TBST buffer (10 mM Tris pH 8.0, 0.1% Tween 20, 150 mM NaCl). The proteins were analyzed by immunoblotting with α-actinin isoform-specific antibodies.

Transfection Experiments

Mammalian expression constructs for α-actinin-2 and -3 were kindly provided by Dr. M. Wyszynski. Briefly, a 3.5 kb cDNA fragment of α-actinin-2 (or a 2.9 kb fragment of α-actinin-3) was subcloned into the mammalian expression vector, pCDNA 3 (Invitrogen, Carlsbad, CA). A total of 20 μg of DNA was transiently transfected into 1.5×10^6 COS-1 cells (American Type Culture Collection, Rockville, MA) using co-precipitation with calcium phosphate (28). Cells were lysed on ice 65 hours post-transfection in 1 ml of lysis buffer containing 50 mM Hepes, pH 7.2, 100 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF and 1 × protease inhibitor cocktail (Boehringer Mannheim, Germany). Cell lysates were centrifuged at 15,000 g at 4° for 5 minutes and the supernatants were stored at −70°.

In Vivo Crosslinking Experiments

Following 65 hours post-transfection, COS-1 cells were washed 3 times with PBS and treated with 1 mM DSP (dithiobis (succinimidyl-propionate)) (Pierce, Rockford, IL) in Hanks' balanced salt solution, pH 7.2 (Sigma, St. Louis, MO). The DSP was diluted 1:50 from a 20 mg per ml stock solution in DMSO (freshly prepared each time). After a 10 minute incubation at 37°, the cells were washed 3 times with PBS and lysed on ice in 1 ml of lysis buffer. DSP is a reversible crosslinker which can be cleaved by adding 10 mM β-mercaptoethanol and boiling for 5 minutes.

RESULTS

Interaction between α-Actinin-2 and -3 in Blot Overlay Assay

α-actinins-2 and -3 were expressed by an *in vitro* translation system and each gave a single polypeptide of predicted size without major degradation (<1%) as judged by SDS gel separation (data not shown). By western analyses, each isoform was only recognized by the corresponding isoform-specific antibody that it was directed against (data not shown). When *in vitro* translated α-actinin isoforms were immobilized on nitrocellulose membrane and incubated with different ^{35}S-methionine labeled α-actinin probes, binding was observed for both α-actinin-2 and α-actinin-3 with themselves (Fig. 1, lane 3 and 5) and to each other (lane 2 and 6). These interactions appear to be specific as neither of the α-actinin isoforms bound to *in vitro* expressed α-syntrophin or dystrophin fragments even though dystrophin also contains spectrin-like repeat

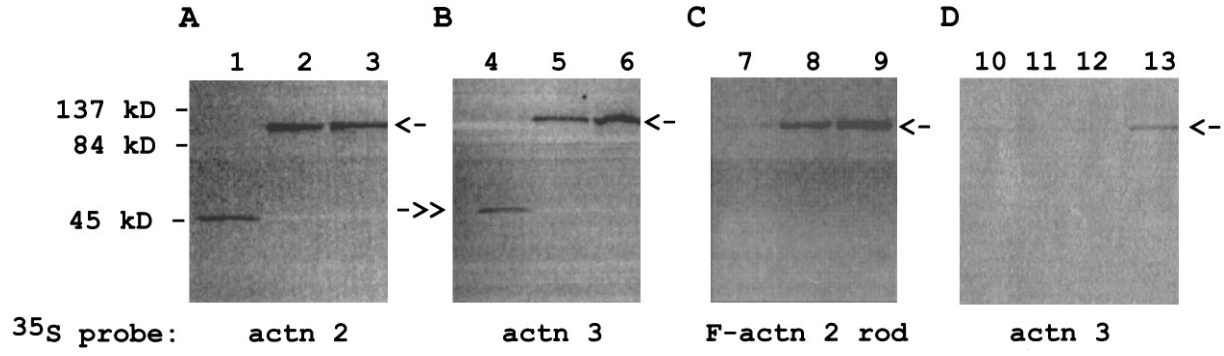

FIG. 1. Interaction between α-actinin-2 and -3 in blot overlay assay. *In vitro* translated α-actinins and other polypeptides were transferred to nitrocellulose membrane. Lane 1, 4, and 7, F-actn-2 rod; lane 2, 5, and 8, actn-3; lane 3, 6, 9 and 13, actn-2; lane 10, α-syntrophin; lane 11, C-terminal dystrophin fragment C2979; lane 12 N-terminal dystrophin fragment N828. The membrane was then overlaid with *in vitro* translated ^{35}S-methionine labeled α-actinin-2 in A; α-actinin-3 in B and D; and F-actin-2 rod in C. The size of full length α-actinin and the truncated version of α-actinin-2 was indicated by a single and a double arrow, respectively.

units similar to α-actinin (lane 10-12). Interestingly, the truncated F-actn-2 rod probe interacted with both α-actinin isoforms (lane 8 and 9) but not with itself (lane 7). This finding is consistent with observations of others that removal of either the first or fourth repeat dramatically diminished the ability of α-actinin to dimerize (20, 29). In addition, both α-actinin isoforms were equally able to bind to native α-actinin immunopurified from human skeletal muscle homogenates (data not shown).

Interaction between α-actinin-2 and -3 in Co-Immunoprecipitation Assay

We were also able to immunoprecipitate both α-actinin-2 (Fig. 2A, lane 3) and α-actinin-3 (Fig. 2B, lane 3) from an *in vitro* co-translation mixture of α-actinin-2 and a flag-tagged α-actinin-3 using an anti-flag antibody M2 (Kodak, Rochester, NY). This antibody did not precipitate α-actinin-2 when incubated with α-actinin-2 alone (Fig. 2A, lane 4). Identical results were also obtained when antibody α-3 was used (data not shown).

Interestingly, the formation of α-actinin-2/-3 heterodimers was only possible when they were co-translated in the same test tube simultaneously. When each isoform was translated separately and subsequently mixed together, antibody M2 could only precipitate the flag-tagged α-actinin-3 (Fig. 2B, lane 1) but not α-actinin-2 (Fig. 2A, lane 1). This is probably due to the rapid homodimerization when each isoform was translated separately and the extreme stability of these homodimers (2, 3). However, this stable interaction could be disrupted by SDS. When both α-actinin-2 and -3 were incubated together with SDS, antibody α-3 was able to co-immunoprecipitate both isoforms after the denaturing agent was removed (data not shown).

Presence of Three Different Conformations of α-Actinin in Vitro and in Vivo

Three different types of α-actinin dimer are possible, that is two homodimers from each isoform and a heterodimer from both isoform. To show whether or not each type of α-actinin dimer has a different conformation, we explored their migration pattern on gradient gel electrophoresis under native conditions. When either α-actinin-2 or α-actinin-3 was translated alone and labeled with ^{14}C-leucine, each one showed a distinctive

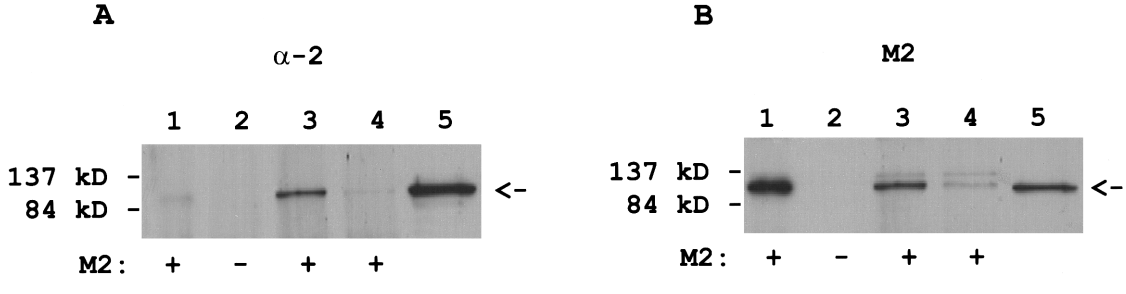

FIG. 2. Co-immunoprecipitation of α-actinin-2 and -3 *in vitro.* Lane 1, actn-2 and F-actn-3 were translated separately and then mixed together. Lane 2 and 3, actn-2 and F-actn-3 were co-translated simultaneously. Lane 4, actn-2 was translated alone. Lane 5, markers, *In vitro* translated actn-2 (A) and F-actn-3 (B). They were subsequently immunoprecipitated with (+) or without (−) an anti-flag antibody, M2. The immunoprecipitated complex was then immunoblotted with antibody α-2 in A, or M2 in B.

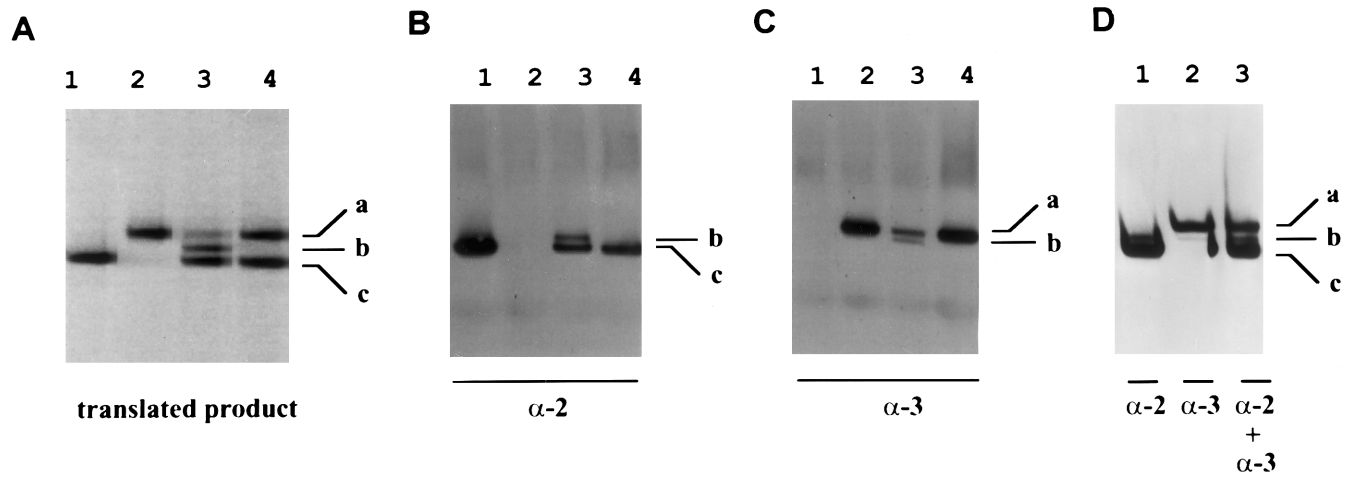

FIG. 3. Presence of three different conformations of α-actinin dimers. A. *In vitro* translated products were labeled with L-(U-^{14}C) leucine and separated by 4-12% gradient gel under native condition. Lane 1, α-actinin-2; lane 2, α-actinin-3; lane 3, α-actinin-2 and -3 were co-translated together; lane 4, α-actinin-2 and -3 were translated separately and then mixed together subsequently. Note that the presence of a middle band (denoted by b) only when both isoforms were translated together (lane 3). B and C. The *in vitro* translated products from A were analyzed by immunoblotting with antibody α-2 in B and α-3 in C. Note that the middle band in lane 3 (denoted by b) was recognized by both isoform-specific antibodies. D. Native α-actinins were extracted from human skeletal muscle according to method described elsewhere (32). They were separated on a 4-12% gel under native condition and immunoblotted with antibody α-2 in lane 1, α-3 in lane 2, α-2 and α-3 in lane 3. α-actinin-3 homodimer was denoted by a, α-actinin-2/-3 heterodimer denoted by b and α-actinin-2 homodimer denoted by c.

band upon autoradiography (Fig. 3A, lane 1 and 2). Co-translation of both isoforms resulted in an additional band between the top and bottom bands (lane 3, indicated by b). When both α-actinin-2 and -3 were translated separately and mixed together afterwards, only the top and the bottom band appeared on the autoradiogram (lane 4).

The identity of each band was determined by immunoblotting with different α-actinin isoform-specific antibodies. Both α-actinin-2 and α-actinin-3 were recognized by their isoform-specific antibodies and appeared as the bottom band (Fig. 3B, lane 1) and the top band (Fig. 3C, lane 2) respectively on a native 4-12% gradient gel. When both isoforms were co-translated at the same time, the additional middle band was recognized by both α-2 and α-3 antibodies (Fig. 3B and 3C, lane 3). Thus, the top band was presumably the α-actinin-3 homodimer, the bottom band was the α-actinin-2 homodimer and the middle one was the α-actinin-2/-3 heterodimer.

Similarly, native α-actinin purified from human skeletal muscle and immunoblotted with antibody α-2 showed the presence of a bottom (indicated by c) and middle band (indicated by b) (Fig. 3D, lane 1). On the other hand, immunoblots stained with antibody α-3 revealed a top band (indicated by a) and the same middle band (indicated by b) (lane 2). Likewise, all three bands were present when both antibodies α-2 and α-3 were used (lane 3). This observation was essentially identical to the patterns seen in the *in vitro* co-translation of α-actinin-2 and -3 described above.

Expression of α-Actinin-2 and -3 in COS-1 Cells

When COS-1 cells transfected with α-actinin-2 and -3 were crosslinked with 1 mM DSP prior to cell lysis, a major band at approximately 235 kD was observed by western blots using α-2 or α-3 antibody (Fig. 4A). It most likely represented crosslinked α-actinin dimers. In order to determine the composition of the dimers, COS-1 cells transfected with both α-actinin-2 and -3 were immunoprecipitated with α-3 antibody. A shown in figure 4B and 4C, untransfected COS-1 cells expressed only the cytoskeletal (non-muscle) isoforms and were negative for both α-actinin-2 and -3 (lane 1) whereas COS-1 cells 1 co-transfected with α-actinin-2 and -3 were positive for both isoforms (lane 2). Antibody α-3 co-precipitated α-actinin-2 and -3 from COS-1 cells transfected with both isoforms (lane 3). On the other hand, when cell lysates from COS-1 cells transfected with just α-actinin-2 were simply mixed with those transfected with α-actinin-3, only α-actinin-3 was immunoprecipitated by antibody α-3 (lane 6), suggesting that the interaction between α-actinin-2 and -3 occurred *in vivo*. Antibody α-3 did not precipitate α-actinin-2 from COS-1 cells transfected with only α-actinin-2 (data not shown). Neither α-actinin-2 and -3 was detected using, α-4, an antibody directed against a non-muscle isoform of α-actinin (lane 4) or in the absence of antibody (lane 5).

DISCUSSION

In this study, we have demonstrated that two different, but closely related, skeletal muscle-specific α-ac-

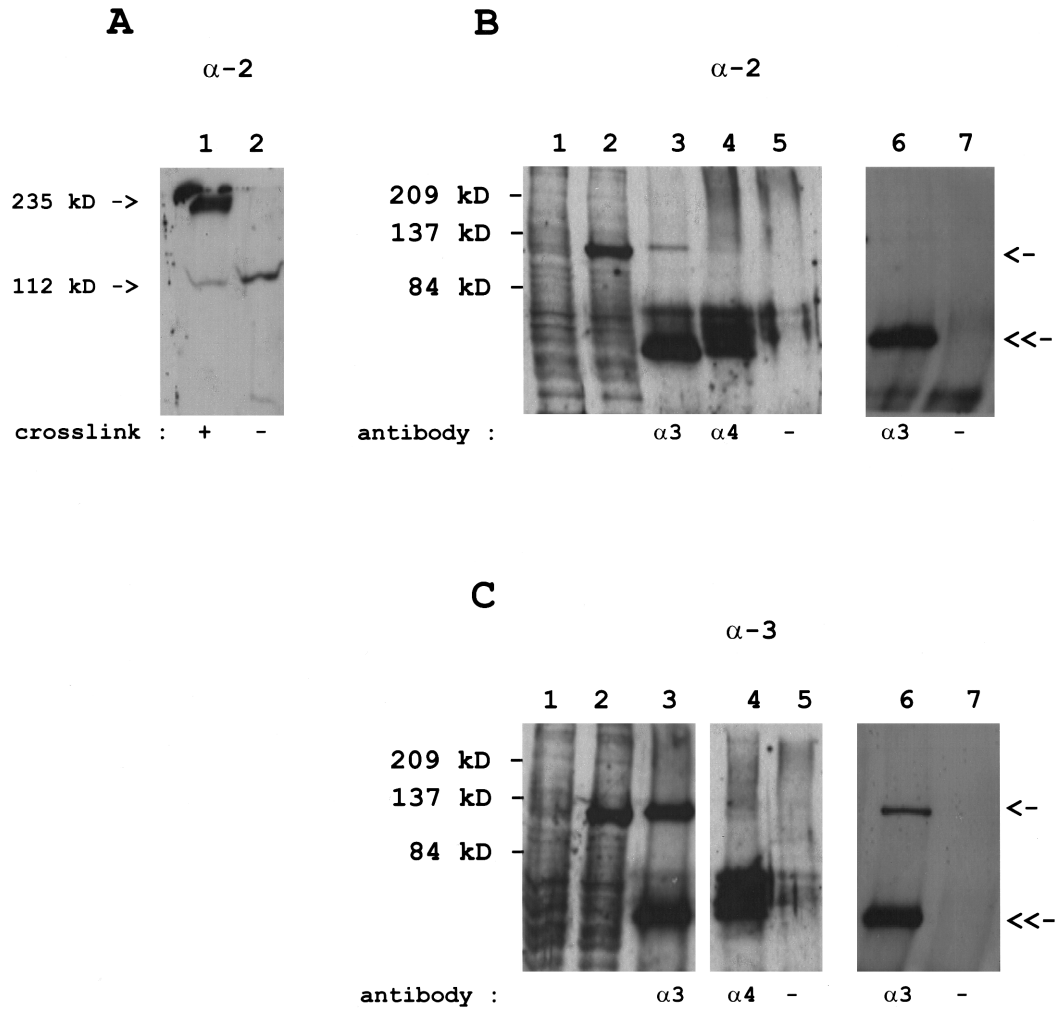

FIG. 4. Interaction between α-actinin-2 and -3 from COS-1 cells co-transfected with both isoforms. A. COS-1 cells co-transfected with α-actinin-2 and -3 were crosslinked with 1 mM DSP (lane 1) or with PBS as negative control (lane 2). The cells were lysed and analyzed on SDS-PAGE gels under non-reducing conditions. B and C. COS-1 cells were transfected with no DNA (lane 1), α-actinin-2 and -3 together (lane 2-5), or α-actinin-2 and -3 separately and then mixed subsequently (lane 6-7). They were immunoprecipitated with antibodies α-3 (lane 3 and 6), α-4 (lane 4) or without (−) antibody (lane 5 and 7). The composition of the immunoprecipitated complex was determined by immunoblotting with antibody α-2 in B or α-3 in C. Note that both α-actinin-2 and -3 were co-precipitated by antibody α-3 only from COS-1 cells co-transfected with both isoforms together (lane 3). The size of α-actinin was indicated by an arrow. The lower band (double arrow) was the heavy chains of the precipitating antibodies.

tinin isoforms interact with each other *in vitro* and *in vivo*. The ability of two similar sequences to form both homdimers and heterodimers have also been observed for other protein isoforms, such as Pho1-type phosphorylase in plant (30) and β-crystallin in lens (31). It is interesting to note that although all three types of α-actinin dimers were present in the skeletal muscle, it seemed there were more homodimers than heterodimers, as one compared the intensity of the three bands corresponding to the three types α-actinin dimers of in figure 3D. Several factors could contribute to this observation. First, heterodimer formation seemed to depend on whether or not α-actinin-2 and -3 were co-expressed simultaneously. Since both α-actinin-2 and -3 are separate gene products, their transcripts could be under different control. Thus, spatial or temporal differences in their expression might play a critical role in the final composition of the α-actinin dimers. Second, differences in the *in vivo* stability of heterodimers and homodimers might affect their relative ratio. Lastly, muscle during different stage of development can have various ratio of different fiber types. Therefore, the level of heterodimer may vary depending on the specific stage of muscle development and the type of muscle being examined.

There are a number of possible reasons why it might be advantageous for skeletal muscle to contain α-actinin-2/-3 heterodimers. The presence of three different

types of α-actinin dimers adds to the functional complexity of the cytoskeletal network. While anti-parallel α-actinin homodimers have no polarity, anti-parallel heterodimers do. Given the anti-parallel nature of the α-actinin dimer, it is possible that the N-terminal actin-binding domains could be differentially affected by the types of isoform in the adjacent neighboring partner. It is possible that different types of α-actinin dimers might possess different affinities towards F-actin. Moreover, heterodimers have the hypothetical advantage of being able to interact with proteins that only associate with one or the other isoform, though such proteins remain to be identified. Alternatively, it may be that heterodimer formation simply reflects the high degree of structural similarity between the isoforms and that there are no significant functional differences between them, as suggested by the low level of heterodimer present *in vivo*. In that case, the role of α-actinin-3 in human skeletal muscles might be redundant since α-actinin-2 is present at the Z-lines of all skeletal muscle fibers. Further studies to identify potential isoform-specific functions of binding partners as well as genetic knock-out experiments will be important to determine which of these hypotheses are correct.

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