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Association of Aciculin with Dystrophin and Utrophin*

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Aciculin is a recently identified 60-kDa cytoskeletal protein, highly homologous to the glycolytic enzyme phosphoglucomutase type 1, (Belkin, A. M., Klimanskaya, I. V., Lukashev, M. E., Lilley, K., Critchley, D., and Koteliansky, V. E. (1994) *J. Cell Sci.* 107, 159–173). Aciculin expression in skeletal muscle is developmentally regulated, and this protein is particularly enriched at cell-matrix adherens junctions of muscle cells (Belkin, A. M., and Burridge, K. (1994) *J. Cell Sci.* 107, 1993–2003). The purpose of our study was to identify cytoskeletal protein(s) interacting with aciculin in various cell types. Using immunoprecipitation from cell lysates of metabolically labeled differentiating C2C12 muscle cells with anti-aciculin-specific antibodies, we detected a high molecular weight band (M_r ~ 400,000), consistently coprecipitating with aciculin. We showed that this 400 kDa band comigrated with dystrophin and immunoblotted with anti-dystrophin antibodies. The association between aciculin and dystrophin in C2C12 cells was shown to resist Triton X-100 extraction and the majority of the complex could be extracted only in the presence of ionic detergents. In the reverse immunoprecipitation experiments, aciculin was detected in the precipitates with different anti-dystrophin antibodies. Immunodepletion experiments with lysates of metabolically labeled C2C12 myotubes showed that aciculin is a major dystrophin-associated protein in cultured skeletal muscle cells. Double immunostaining of differentiating and mature C2C12 myotubes with antibodies against aciculin and dystrophin revealed precise colocalization of these two cytoskeletal proteins throughout the process of myodifferentiation in culture. In skeletal muscle tissue, both proteins are concentrated at the sarcolemma and at myotendinous junctions. In contrast, utrophin, an autosomal homologue of dystrophin, was not codistributed with aciculin in muscle cell cultures and in skeletal muscle tissues. Analytical gel filtration experiments with purified aciculin and dystrophin showed interaction of these proteins *in vitro*, indicating that their association in skeletal muscle is due to direct binding. Whereas dystrophin was shown to be a major aciculin-associated protein in skeletal muscle, immunoblotting of anti-aciculin immunoprecipitates with antibodies against utrophin showed that aciculin is associated with utrophin in cultured A7r5 smooth muscle cells and REF52 fibroblasts. Immunodepletion experiments performed with lysates of metabolically labeled A7r5 cells demonstrated that aciculin is a major utrophin-binding protein in this cell type. Taken together, our data show that aciculin is a novel dystrophin- and utrophin-binding protein. Association of aciculin with dystrophin (utrophin) in various cell types might provide an additional cytoskeletal-matrix transmembrane link at sites where actin filaments terminate at the plasma membrane.

Dystrophin is the largest (~427 kDa) member of the α-actinin/spectrin/dystrophin superfamily of cytoskeletal proteins, originally identified as the gene product absent in Duchenne's (or altered in Becker's) muscular dystrophy (1, 2). Dystrophin is a flexible elongated protein, which consists of four distinct domains, including: 1) an N-terminal actin-binding domain with homology to α-actinin, spectrin, and filamin, 2) a large central rod domain, containing 24 spectrin-like repeats, 3) a cysteine-rich domain, homologous to the α-actinin C terminus, and 4) a C-terminal domain of 420 amino acids without any significant homology outside the dystrophin subfamily (3, 4). Utrophin, an autosomal homologue of dystrophin, is a high molecular mass (~395 kDa) cytoskeletal protein, consisting of the same four domains, as dystrophin (5–7). The N-terminal actin-binding domain, cysteine-rich and C-terminal domains are particularly highly conserved between these two proteins, whereas the sequence homology in the central rod domain, containing spectrin-like repeats, is relatively low (5–7).

Recent data have demonstrated the existence of multiple dystrophin-like proteins in different tissues, including skeletal muscle (5–11). Full-length forms of the dystrophin subfamily include dystrophin itself (3), which is synthesized in all three muscle types and in brain (1, 12–14), and the ubiquitously expressed utrophin (formerly referred to as dystrophin-related protein), encoded by a separate gene (5–7, 15, 16). Also, there are several shorter forms in the dystrophin subfamily, such as the 87-kDa phosphoprotein from Torpedo electric tissue (17), partially homologous to dystrophin (11) and a 71–75-kDa short form(s), that arises by use of an alternative internal promoter, lying between exons 62 and 63 of the dystrophin gene (8, 9, 18, 19). Finally, numerous alternatively spliced transcripts of the extremely large, 65 exon-containing dystrophin gene yield multiple dystrophin isoforms with different C-terminal domains (20–22).

Dystrophin as well as utrophin are particularly enriched at neuromuscular junctions of skeletal muscle (23–26). Unlike utrophin, dystrophin is also localized at the sarcolemma of normal muscle (27–30), more specifically, in a costamere-like subsarcolemmal lattice (31, 32) and is enriched at myotendinous junctions (33). In cultured differentiating muscle cells, dystrophin is transiently localized to focal adhesions (34). In contrast with the tissue-specific expression of dystrophin, utrophin was found in all tissues examined so far (5–7, 15, 16, 35). Smooth muscle tissues and cells, particularly vascular smooth

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muscle cells, produce the largest amounts of utrophin (16). Among the various types of cultured cells, utrophin was identified in primary cultures of neurons and glia (16), proliferating brain cell lines, HeLa cells, smooth muscle-like BC_3H1 cells, COS cells, $P388D_1$ monocyte-macrophage cells, and in cultures of untransformed human skin fibroblasts (16, 36).

Dystrophin interacts with F-actin at its N terminus (3, 4, 37–40). The C-terminal tail of the molecule, which is particularly conservative (12, 41, 42), represents a site of membrane attachment (43, 44). In skeletal and cardiac muscles, dystrophin is associated with several proteins, including a 59-kDa peripheral membrane protein(s), three sarcolemmal glycoproteins with molecular masses 50, 43, and 35 kDa, a 25-kDa transmembrane protein, and a 156-kDa laminin-binding extracellular proteoglycan, termed α-dystroglycan. All together the complex provides a transmembrane cytoskeletal-matrix linkage in muscle tissues (39, 43, 45–47). In skeletal muscle, utrophin is also associated with a complex of sarcolemmal proteins, antigenically indistinguishable from the components of the dystrophin-glycoprotein complex (48). All the major dystrophin-associated proteins are drastically decreased in dystrophic muscle (49, 50), indicating that dystrophin stabilizes the entire transmembrane complex (51). Among these dystrophin-associated proteins, the 59-kDa protein is thought to bind dystrophin directly, presumably near its C terminus (43, 44, 46). The 59-kDa cytoplasmic dystrophin-associated component of the dystrophin-glycoprotein complex is represented by a closely disposed triplet (43, 46, 47). This is composed of a lower band (named 59–1 DAP)[1] that is structurally and functionally homologous to a previously described dystrophin-associated, postsynaptic 58-kDa protein (syntrophin). In addition, two unrelated proteins make up the higher bands of the triplet (52–56). Two different mRNAs encode two structurally distinct syntrophin forms, having drastically different tissue expression patterns (54). Other members of the dystrophin subfamily, including utrophin and the short C-terminal forms of dystrophin also interact with syntrophin (55). Even though the interaction between different dystrophin forms and the 59-kDa peripheral membrane protein(s) is apparently playing a key role in their membrane association in skeletal muscle (50, 51, 55), some other dystrophin-binding proteins associated with various dystrophin forms may exist. This prediction is based mainly on the fact that multiple dystrophin variants, differing in their C-terminal domains, arise by a selective removal of exons at three splice junctions from dystrophin pre-mRNA (20). Notably, these alternatively spliced transcripts of the dystrophin gene were detected in several tissues, including skeletal muscle itself (12, 20, 23). This important observation points to a potential existence of several different dystrophin-binding proteins, interacting with the C-terminal domain of various alternatively spliced forms of dystrophin (20). Nevertheless, until now, the 59-kDa DAP (syntrophin) remains the only characterized dystrophin-binding protein, interacting with the dystrophin C-terminal domain (43, 47, 52–56).

Aciculin is a recently identified component of the peripheral membrane cytoskeleton in muscle and some nonmuscle cells (57). This 60/63-kDa phosphoglucomutase type-1 (PGM1)-related cytoskeletal protein is enriched in all three muscle types, where its expression is developmentally regulated and differentiation-dependent (57, 58). In skeletal muscle, aciculin is concentrated at different types of cell-matrix adherens junctions, such as myotendinous junctions and costameres, and is localized to focal adhesions of differentiating myotubes in culture (58). Here we present data showing that this newly described cytoskeletal protein interacts with dystrophin in skeletal muscle and is associated with utrophin in smooth muscle cells and fibroblasts.

EXPERIMENTAL PROCEDURES

Materials—Except where otherwise stated, materials were purchased from Sigma.

Antibodies—Anti-aciculin-specific mAb XIVF8 and polyclonal antibodies against aciculin, cross-reacting with PGM1, were described previously (57, 58). Anti-dystrophin mAbs NCL-Dys1, recognizing the central rod domain of dystrophin and NCL-Dys2, raised against the last 17 amino acids of dystrophin and anti-utrophin mAb NCL-DRP1, reacting with the last 11 amino acids at the C terminus of utrophin, were from Novocastra Laboratories (Newcastle upon Tyne, United Kingdom). Anti-dystrophin mAb 1808 against Torpedo dystrophin, cross-reacting with mammalian dystrophin, anti-dystrophin mAb 1958, reacting with avian dystrophin, and mAb 1351 against the 58-kDa post-synaptic dystrophin-binding protein (syntrophin) were gifts from Drs. S. Froehner and R. Sealock (24). Rabbit polyclonal antibody raised against a dystrophin fusion protein, representing the 60-kDa N-terminal segment of the molecule, was a gift from Dr. E. Hoffmann (2). Anti-vinculin mAb VIIF9 was previously shown to react with mouse vinculin and metavinculin (57, 58). For preadsorbtion experiments and immunoaffinity purification of corresponding antigens anti-aciculin mAb XIVF8 and anti-dystrophin mAb 1958 were coupled to cyanogen bromide-activated Sepharose 4B (10 mg/ml of resin) by conventional methods.

Cell Cultures—C2C12 mouse skeletal muscle myocytes were purchased from the American Type Culture Collection (Rockville, MD) and cultured as described earlier (58). Cells were cultured either on gelatin-coated plastic dishes for subsequent biochemical experiments or on laminin-coated coverslips for immunofluorescent staining. A7r5 rat embryonic thoracic aorta smooth muscle cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Rat embryonic fibroblasts (REF52) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Metabolic Labeling and Immunoprecipitation—Cultured C2C12 mouse myocytes, taken at different stages of myodifferentiation, were metabolically labeled with 0.1 mCi/ml of Translabel (a mixture of [^{35}S]methionine and [^{35}S]cysteine, ICN Biomedicals, Irvine, CA) in methionine-, cysteine-free medium for 4 h at 37 °C. After the labeling, cells were washed four times with PBS (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5) and lysed on ice for 3 min with RIPA buffer (50 mM TrisCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.5, containing protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). To equalize the total amounts of *de novo* synthesized proteins in different cell samples, 2-μl aliquots of each sample were counted in an LS5000CE scintillation counter (Beckman, Palo Alto, CA) and equal amounts of total incorporated ^{35}S radioactivity (2×10^8 counts/min/sample) were taken for subsequent immunoprecipitations.

Cell lysates were precleared by spinning at 15,000 × *g* for 15 min at 4 °C, and all the subsequent incubations were done at 4 °C on a rotator. ^{35}S-Labeled cell lysates were preincubated for 1 h with 100 μl of 20% suspension of Protein A-Sepharose beads, and supernatants were incubated for 2 h with primary anti-aciculin antibodies (XIVF8 mAb or polyclonal anti-aciculin antibody) and then for 1 h with 100 μl of 20% bead suspension of Protein A-Sepharose. Immune complexes were extensively washed with the ice-cold RIPA buffer, then with PBS (once), and finally boiled in 30 μl of SDS electrophoretic sample buffer for 5 min.

To estimate protein partitioning between Triton-soluble (not associated with cytoskeleton) and Triton-insoluble ("cytoskeletal") fractions of cultured C2C12 cells, cultures of differentiated myotubes were sequentially extracted on ice for 3 min, first with 0.5% Triton X-100 in 100 mM potassium-PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.0, containing protease inhibitors, and then with RIPA buffer supplemented with protease inhibitors (as above).

For the comigration experiment with aciculin and dystrophin, we immunoprecipitated dystrophin from ^{35}S-labeled C2C12 myotubes under denaturing conditions. To do this, we precipitated both Triton X-100 -soluble and Triton X-100-insoluble fractions of C2C12 myotubes with ice-cold acetone. Protein precipitates were spun down, dried, redis-

[1] The abbreviations used are: DAP, dystrophin-associated proteins; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; PBS, phosphate-buffered saline; RIPA buffer, radioimmunoprecipitation buffer; PGM1, phosphoglucomutase type 1; PIPES, 1,4-piperazinediethanesulfonic acid.

solved in 100 μl of 1% SDS, and boiled for 3 min. Then, both ^{35}S-labeled cell lysates were reconstituted up to 1 ml volume (final SDS concentration 0.1%) with 1% Triton X-100 in 50 mM TrisCl, 150 mM NaCl, pH 7.5, and subsequently used for immunoprecipitation with anti-dystrophin antibodies.

To analyze the potential association of aciculin with dystrophin (utrophin) in C2C12 cells, Triton X-100-soluble and -insoluble fractions of unlabeled differentiated C2C12 myotubes were subjected to immunoprecipitation with antibodies against aciculin (XIVF8 mAb), dystrophin (NCL-Dys1 mAb), or utrophin (NCL-DRP1 mAb), as described above, except that rabbit anti-mouse IgG (Chemicon, Temecula, CA) conjugated to Protein A-Sepharose was used for immunoprecipitation with anti-dystrophin and anti-utrophin mAbs. 3 × 100-mm dishes of differentiated C2C12 myotubes were taken for immunoprecipitation with each antibody. Corresponding immunoprecipitates were washed, boiled in SDS, and run on SDS-polyacrylamide gels. Protein bands were transferred to Immobilon membranes (Millipore, Bedford, MA) and probed with antibodies against aciculin or dystrophin.

To detect whether aciculin interacts with utrophin in some other cell types outside skeletal muscle, cultures of A7r5 smooth muscle cells, REF52 fibroblasts as well as C2C12 myotubes were lysed in RIPA buffer containing protease inhibitors (as above) and subjected to immunoprecipitation with XIVF8 anti-aciculin mAb. Immunoprecipitates were washed several times with RIPA buffer, then with PBS, boiled in SDS sample buffer, and subjected to electrophoresis and subsequent immunoblotting (59, 60) with antibodies against aciculin, dystrophin, and utrophin. An extract of 2 g of bovine heart muscle tissue in 20 ml of 1% Triton X-100, 20 mM TrisCl, 0.5 M NaCl, pH 7.4, containing protease inhibitors, was used for immunoprecipitation of aciculin, dystrophin, and utrophin as a positive control for the presence of these proteins in various cell cultures.

Immunodepletion Experiments—To examine whether aciculin is a major dystrophin-associated protein in cultured C2C12 cells, immunodepletion experiments with RIPA extracts of ^{35}S-labeled C2C12 myotubes were performed. To remove the majority of aciculin before the subsequent immunoprecipitation, anti-aciculin XIVF8 mAb, coupled to Sepharose 4B, was used for preincubation with an ^{35}S-labeled cell lysate. A precleared RIPA extract of 10 × 60-mm dishes of differentiated C2C12 myotubes was divided into two equal parts. Half was preadsorbed (2 h at 4 °C on the rotator) with 2 mg of XIVF8 mAb, coupled to Sepharose resin, and the other half with unconjugated Sepharose 4B. The resulting supernatants were immunoprecipitated in parallel with XIVF8 anti-aciculin mAb, NCL-Dys1 anti-dystrophin mAb, NCL-DRP1 anti-utrophin mAb, mAb 1351 against 58-kDa protein (syntrophin), and VIIF9 anti-vinculin mAb, following rabbit anti-mouse IgG/Protein A-Sepharose. The resulting immune complexes were washed, boiled in SDS, and subjected to electrophoresis.

To analyze further aciculin-utrophin association in cultured A7r5 smooth muscle cells, aciculin immunodepletion experiment was also performed with RIPA extracts of ^{35}S-labeled A7r5 cells, using the method described above. In this case aciculin-depleted and control ^{35}S-labeled cell lysates were used in parallels for immunoprecipitation with XIVF8 anti-aciculin mAb, NCL-DRP1 anti-utrophin mAb, and anti-syntrophin mAb 1351.

Immunoaffinity Purification of Dystrophin—Stripped membranes obtained from 100 g of chicken gizzard smooth muscle were prepared as described earlier by Kelly *et al.* (61). The final pellet, containing mostly integral membrane and membrane-associated proteins, and lacking most of the actin, actin-binding cytoskeletal proteins, and myosin, was extracted for 4 h at 4 °C with 600 ml of 1% Triton X-100, 20 mM TrisCl, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, pH 7.4, containing protease inhibitors 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. The detergent extract of smooth muscle tissue was spun down at 10,000 × *g* for 30 min and prefiltered through a Sepharose 4B column. The resulting precleared supernatant was applied to a column containing anti-dystrophin 1958 mAb immobilized on Sepharose 4B. The column was sequentially washed with 10 column volumes of 0.1% Triton X-100 in 1.5 M NaCl, 50 mM TrisCl, 1 mM EDTA, 1 mM EGTA, pH 7.4, then with 10 volumes of 0.1% Triton X-100 in 100 mM NaCl, 50 mM TrisCl, 1 mM EDTA, 1 mM EGTA, pH 9.5, and finally eluted with 2.5 column volumes of 0.1% Triton X-100 in 50 mM triethanolamine, pH 11.5. All column buffers contained 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 25 μg/ml leupeptin, and 25 μg/ml pepstatin as protease inhibitors. The pH in the final pH 11.5 eluate was immediately readjusted to 7.5 with 1 M HCl, and the eluate was extensively dialyzed against 0.1% Triton X-100 in 10 mM TrisCl, 10 mM NaCl, pH 7.5, at 4 °C. The pH 11.5 eluate from the anti-dystrophin immunoaffinity column, containing ~70–80% pure dystrophin and some minor bands, representing copurifying proteins and/or dystrophin proteolytic fragments, was concentrated in Centricon microconcentrators (Amicon), applied onto a HR 10/30 Superose 6 fast protein liquid chromatography gel-filtration column (Pharmacia, Sweden), and eluted with 10 mM TrisCl, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 7.5, containing protease inhibitors. Dystrophin was mostly separated from low molecular weight contaminants on the column, and fractions containing ~90% pure dystrophin were concentrated in microconcentrators and used for analytical gel-filtration experiments with aciculin.

Aciculin Purification—Aciculin was purified from human uterus smooth muscle as described previously (57), except that the hydroxylapatite column chromatography step was omitted and gel filtration on a 2.5 × 100-cm Sephacryl S-300 (Pharmacia) column was used as a second chromatography step and an fast protein liquid chromatography monoQ anion-exchanger column (Pharmacia) was used as a final purification step.

Analytical Gel Filtration—Gel filtration binding studies were performed using a high resolution analytical Superose 6 10/30 column (Pharmacia). Electrophoresis and subsequent immunoblotting of column fractions was performed as described by Crawford *et al.* (62). Purified aciculin and dystrophin were dialyzed against 10 mM TrisCl, 50 mM NaCl, 1 mM EDTA, and 1 mM EGTA, pH 7.5, with protease inhibitors and precleared by centrifugation in a Microfuge (Beckman) for 15 min at 12,000 revolutions/min. Aciculin was mixed with dystrophin and incubated 1 h on ice before the column run. Sequential gel filtration runs were aciculin alone (20 μg in 200 μl of buffer), a mixture of 20 μg of aciculin and 10 μg of dystrophin, and a mixture of 20 μg of aciculin and 50 μg of dystrophin. 300-μl fractions were collected for these three runs, and 30-μl aliquots of the column fractions were mixed with SDS sample buffer, boiled for 5 min, and run on 10% polyacrylamide gels. Proteins were transferred onto Immobilon membranes and probed with anti-aciculin mAb XIVF8 and anti-dystrophin 1958 mAb.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—For electrophoresis of ^{35}S-labeled proteins, 10% polyacrylamide gels, containing 0.13% bisacrylamide, were used (59). After electrophoresis, gels were fixed in 25% methanol, 10% acetic acid, treated with Amplify (Amersham Corp.) for 30 min, dried, and exposed to x-ray film (Eastman Kodak Co.) for 36–72 h at −70 °C to detect ^{35}S-labeled protein bands. Unlabeled proteins were visualized in gels by Coomassie Blue staining. To enhance the electrotransfer of high molecular weight proteins, 5-12% acrylamide (40:1 ratio of acrylamide/bisacrylamide) gels were used for immunoblots with anti-dystrophin or anti-utrophin antibodies. Proteins were transferred onto Immobilon membranes in 25 mM TrisCl, 192 mM glycine, 20% methanol, pH 8.3, for 40 h at 0.5 A and then 3 h at 1 A (60). Blots were blocked with 2% bovine serum albumin, 2% cold fish gelatin, 0.1% Tween 20 in TBS (50 mM TrisCl, 150 mM NaCl, pH 7.5) for 1 h. Then blots were incubated with anti-aciculin XIVF8 mAb, anti-utrophin NCL-DRP1 mAB, or a mixture of NCL-Dys1, NCL-Dys2, and 1808 mAb for detection of dystrophin. Rabbit anti-mouse affinity-purified IgG, conjugated with peroxidase (Jackson, West Grove, PA), diluted 1:10,000, was used as secondary antibody. Blots were extensively washed in TBS plus 0.1% Tween 20, then with TBS and finally developed using ECL reagents (Amersham).

Immunofluorescence—For immunostaining of C2C12 cells, muscle cells growing on laminin-coated glass coverslips, taken at various stages of myogenic differentiation, were fixed for 5 min with ice-cold absolute methanol. For simultaneous detection of aciculin and dystrophin in cultured muscle cells, coverslips were incubated with rabbit polyclonal anti-aciculin antibody and a mixture of anti-dystrophin mAbs NCL-Dys1, NCL-Dys2, and 1808, followed by incubation with fluorescein-labeled goat anti-rabbit IgG and rhodamine-labeled donkey anti-mouse IgG (Chemicon).

For immunofluorescent staining of muscle tissue sections, 5–8-μm cryosections of rabbit adult or chicken 18-day embryonic thigh skeletal muscle tissues were fixed for 5 min in ice-cold methanol and then postfixed with acetone. Cryosections were treated with XIVF8 anti-aciculin mAb, NCL-DRP1 anti-utrophin mAb, 1958 anti-dystrophin mAb for chicken tissue or mixture of NCL-Dys1, NCL-Dys2, and 1808 anti-dystrophin mAbs for rabbit tissues. Stained C2C12-cultured muscle cells and muscle tissue sections were mounted in Mowiol medium and examined on a Zeiss Axiophot microscope equipped with epifluorescence. Fluorescence micrographs were taken on T-max 400 film (Eastman Kodak).

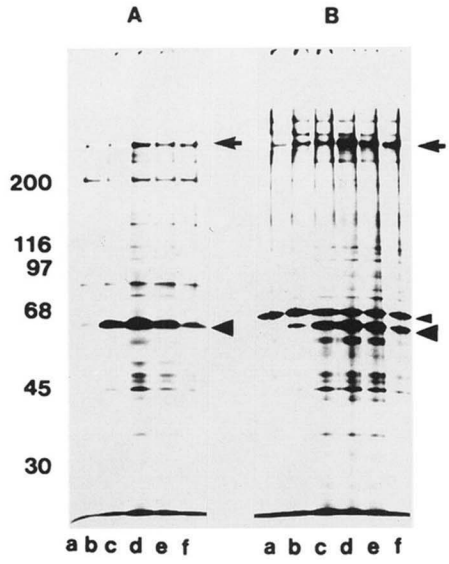

FIG. 1. **Identification of aciculin-binding proteins in differentiating C2C12 cells by metabolic labeling and immunoprecipitation.** C2C12 myogenic cells, cultured for 3 days (*a*), 5 days (*b*), 7 days (*c*), 10 days (*d*), 12 days (*e*), and 14 days (*f*), were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine and ^{35}S-labeled cell lysates in RIPA buffer were used for immunoprecipitation with anti-aciculin mAb XIVF8 (*A*) or polyclonal anti-aciculin antibodies (*B*). Immunoprecipitates were washed and run on 10% SDS-polyacrylamide gel. Positions of molecular mass marker proteins are indicated in kilodaltons to the left of the gel (*A*). Large *arrowheads* indicate aciculin in *A* and *B*, and the *small arrowhead* marks the position of PGM1 in *B*. *Arrows* to the right of both gels point to a high molecular weight protein (M_r ~ 400,000), consistently coprecipitating with aciculin in C2C12 cells.

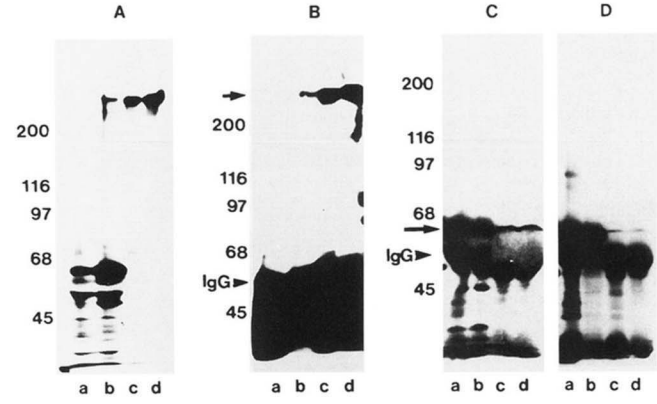

FIG. 2. **Aciculin is associated with dystrophin in cultured C2C12 myotubes.** *A*, comigration experiment with anti-aciculin and anti-dystrophin immunoprecipitates. C2C12 cells, cultured for 10 days, were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine and lysed sequentially in Triton X-100 (*a* and *c*) and then in RIPA buffer (*b* and *d*). Corresponding ^{35}S-labeled lysates were immunoprecipitated with XIVF8 anti-aciculin mAb (*a* and *b*) or with NCL-Dys1 anti-dystrophin mAb (*c* and *d*). Dystrophin immunoprecipitation from both Triton X-100-soluble and -insoluble fractions of C2C12 myotubes (*c* and *d*) was done under denaturing conditions (see "Experimental Procedures"). Immunoprecipitates were washed and run on 10% SDS-polyacrylamide gel. Note comigration of a high molecular weight protein, coprecipitating with aciculin from the Triton-insoluble ("cytoskeletal") fraction of C2C12 myotubes, with dystrophin. *B-D*, association of aciculin with dystrophin (utrophin) in C2C12 myotubes. Triton X-100-soluble (*a* and *c*) and -insoluble (*b* and *d*) fractions of differentiated C2C12 myotubes were subjected to immunoprecipitation with anti-aciculin XIVF8 mAb (*B-D*, *a* and *b*), anti-dystrophin NCL-Dys1 mAb (*B* and *C*, *c* and *d*), or anti-utrophin NCL-DRP1 mAb (*D*, *c* and *d*). Immunoprecipitates were washed extensively and run on 5–12% (*B*) or 10% (*C* and *D*) SDS-polyacrylamide gels. Proteins were transferred to Immobilon membrane, and blots were probed with antibodies to dystrophin (mixture of NCL-Dys1, NCL-Dys2, and 1808 mAbs in *B*) or anti-aciculin XIVF8 mAb (*C* and *D*). Immunoglobulin heavy chains are shown by *arrowhead* to the left of the gels (*B* and *C*). Dystrophin immunoreactive bands are indicated by the *short arrow* in *B*; aciculin bands are indicated by the *long arrow* in *C* and *D*. Positions of molecular mass markers in kilodaltons are given to the left of each gel.

RESULTS

In order to detect any proteins that are potentially associated with aciculin in muscle cells, cultured C2C12 myocytes taken at various stages of myogenic differentiation were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine, lysed in RIPA buffer, and cell lysates were subjected to immunoprecipitation with either mAb XIVF8, specific to aciculin, or polyclonal anti-aciculin antibodies. Besides aciculin itself (a ~60 kDa band in immunoprecipitates with either monoclonal or polyclonal antibodies) and PGM1 (a ~64 kDa band with polyclonal anti-aciculin antibodies), several additional bands were found in both types of immunoprecipitates (Fig. 1). Among these additional bands, a high molecular weight band (M_r ~ 400,000) was consistently present in precipitates with mAb XIVF8 and polyclonal anti-aciculin antibodies (*arrow* in Fig. 1, *A* and *B*). This band was much more prominent in differentiated C2C12 cells, expressing higher amounts of aciculin (Fig. 1*A*, *lanes d-f*; 1*B*, *lanes b-f*), but was barely detectable in early myoblast cultures (Fig. 1*A*, *a-c*; 1*B*, *a*).

In an attempt to identify this high molecular weight aciculin-associated protein, we compared the migration of this band with dystrophin. Aciculin and dystrophin were immunoprecipitated from Triton X-100-soluble and -insoluble fractions of ^{35}S-labeled terminally differentiated C2C12 myotubes (Fig. 2*A*). The high molecular weight aciculin-associated protein, enriched in the Triton X-100-insoluble ("cytoskeletal") fraction of cultured myotubes, comigrated with dystrophin (Fig. 2*A*, *b-d*). Immunoblotting of corresponding unlabeled immunoprecipitates from differentiated C2C12 myotubes with antibodies against dystrophin showed that this protein represents dystrophin (Fig. 2*B*). Notably, the majority of the complex was resistant to the Triton X-100 extraction, showing its association with the cytoskeleton (Fig. 2*B*, *a* and *b*). In the converse experiment, aciculin was detected by immunoblot in anti-dystrophin immunoprecipitates from Triton X-100-soluble and -insoluble fractions of C2C12 myotubes (Fig. 2*C*, *c* and *d*). Similar immunoblotting experiments performed with utrophin immunoprecipitates revealed much less aciculin associated with the dystrophin homologue in differentiated C2C12 (Fig. 2*D*, *c* and *d*). It should be noted, that in some experiments, we were not able to detect any aciculin in utrophin immunoprecipitates from C2C12 cells. Immunoprecipitating other major actin-associated cytoskeletal proteins, such as vinculin or α-actinin, from C2C12 myotubes and subsequent immunoblotting of these immunoprecipitates with anti-aciculin antibodies did not reveal any aciculin in association with these proteins (data not shown), pointing to specificity in the aciculin and dystrophin immunoprecipitates.

To explore further the interaction between aciculin and dystrophin in C2C12 muscle cells, several anti-dystrophin antibodies were used in immunoprecipitation experiments with RIPA extracts of C2C12 myotubes, followed by immunoblot of these immunoprecipitates with XIVF8 anti-aciculin mAb (Fig. 3). Interestingly, polyclonal antibodies against the N-terminal 60-kDa fragment of dystrophin revealed the largest amounts of aciculin in anti-dystrophin immunoprecipitates. The levels of aciculin in these immunoprecipitates were comparable to those found in anti-aciculin immunoprecipitates (Fig. 3, *a* and *e*). Immunoprecipitates with NCL-Dys1 mAb, which recognizes an epitope in the central rod domain of dystrophin, gave a weaker, but still easily detectable reaction with anti-aciculin (Fig. 3*b*). When we used NCL-Dys2 mAb, which is directed against the last 17 amino acids of dystrophin, or NCL-DRP1 mAb, which

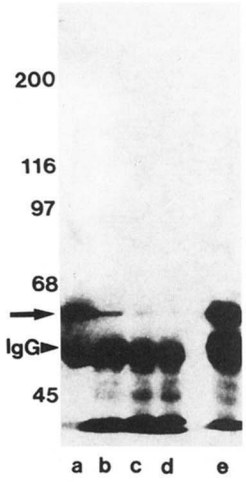

FIG. 3. **Aciculin is present in immunonoprecipitates with different anti-dystrophin antibodies.** C2C12 myotubes were taken on the 10th day of culture, lysed in RIPA buffer, and cell lysates were immunoprecipitated with: polyclonal anti-dystrophin antibody, raised against 60-kDa N-terminal fragment of dystrophin (*a*), mAb NCL-Dys1 (*b*), mAb NCL-Dys2 against dystrophin (*c*), mAb NCL-DRP1 against utrophin (*d*), and mAb XIVF8 to aciculin (*e*). Immunoprecipitates were extensively washed with RIPA buffer, run on 10% SDS-polyacrylamide gel, and blotted with anti-aciculin XIVF8 mAb. *Arrow* indicates the position of aciculin. *Arrowhead* shows immunoglobulin heavy chains. Positions of molecular mass markers are indicated in kilodaltons to the left of the gel.

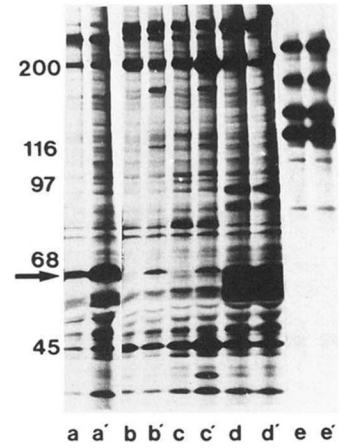

FIG. 4. **Aciculin is a major dystrophin-binding protein in cultured differentiated C2C12 myotubes.** RIPA extract of [^{35}S]methionine/[^{5}S]cysteine-labeled differentiated C2C12 myotubes was preadsorbed with unconjugated Sepharose 4B (*a'-e'*) or with anti-aciculin mAb XIVF8, coupled to Sepharose 4B (*a-e*). Preadsorbed ^{35}S-labeled supernatants were taken for immunoprecipitation with anti-aciculin mAb XIVF8 (*a* and *a'*), anti-dystrophin NCL-Dys1 mAb (*b* and *b'*), anti-utrophin mAb NCL-DRP1 (*c* and *c'*), anti-syntrophin mAb 1351 (*d* and *d'*), and anti-vinculin mAb VIIF9 (*e* and *e'*). Immunoprecipitates were extensively washed with RIPA buffer and run on 10% SDS-polyacrylamide gel. Positions of molecular mass markers are given in kilodaltons to the left of the gel. Note that a major ~60 kDa band, indicated by the *arrow* to the left of the gel, present in anti-dystrophin (*b'*) and anti-utrophin (*c'*) immunoprecipitates, is depleted after preincubation with anti-aciculin antibody (*b* and *c*).

binds the 11 most C-terminal amino acids in utrophin, aciculin was only barely detectable (Fig. 3, *c* and *d*).

To analyze if aciculin is, indeed, a major dystrophin-binding protein in differentiated C2C12 muscle cells, aciculin immunodepletion experiments were performed with RIPA extracts of ^{35}S-labeled C2C12 myotubes. ^{35}S-Labeled cell extracts, preadsorbed with anti-aciculin XIVF8 mAb, coupled to Sepharose 4B, and control extracts, preincubated with unconjugated Sepharose 4B, were used for subsequent immunoprecipitation with antibodies against aciculin, dystrophin, utrophin, 58-kDa protein (syntrophin), and anti-vinculin as a control antibody. Overexposed autoradiographs were used to detect the whole spectrum of proteins, associated with aciculin, dystrophin, and utrophin in cultured C2C12 myotubes (Fig. 4). A majority of aciculin was depleted from C2C12 cell extracts by preadsorbtion with XIVF8 mAb-Sepharose (Fig. 4, *arrow*, *lanes a* and *a'*). Notably, a major ~60 kDa band, present in both anti-dystrophin and anti-utrophin immunoprecipitates (Fig. 4, *b'* and *c'*), was considerably diminished in these immunoprecipitates after preincubation with anti-aciculin-Sepharose (Fig. 4, *b* and *c*). A large cluster of bands around 52–60 kDa was seen in anti-syntrophin immunoprecipitates, but these bands were not depleted by the preincubation with anti-aciculin antibody (Fig. 4, *d* and *d'*). Control immunoprecipitation with anti-vinculin mAb revealed several additional bands, besides vinculin (116 kDa) and metavinculin (150 kDa), but none of them could be identified as aciculin (Fig. 4, *e* and *e'*).

Electrophoresis of ^{35}S-labeled immunoprecipitates in 10% gels allowed resolution of at least 3 protein bands in the range of ~400 kDa in anti-dystrophin, anti-utrophin, and anti-syntrophin immunoprecipitates (Fig. 4, *b*, *b'*, *c*, *c'*, *d*, and *d'*), perhaps pointing to the existence of several dystrophin (utrophin) isoforms in C2C12 myotubes. Interestingly, only two of them, the fastest and slowest migrating dystrophin bands, were found in association with aciculin, whereas the middle band was missing or barely seen in anti-aciculin immunoprecipitates (Fig. 4, *a* and *a'*).

The apparent association of aciculin with dystrophin and, potentially, with utrophin, in cultured C2C12 myocytes, urged us to study whether these cytoskeletal proteins are codistributed in muscle cell cultures. Neither aciculin nor dystrophin were detectable by immunofluorescence in cultured C2C12 myoblasts before cell fusion. Double immunostaining of differentiating C2C12 cells with rabbit polyclonal anti-aciculin antibodies and mouse mAbs against dystrophin revealed a precise colocalization of these two cytoskeletal proteins throughout the various stages of myocyte differentiation and myotube maturation in culture following myoblast fusion (Fig. 5). In early myotubes, both proteins appeared first at the termini of actin bundles near the cell tips (Fig. 5, *A* and *B*). These structures correspond to the major cell-matrix attachment sites of cultured myotubes and are homologous to the myotendinous junctions in tissue. More mature, elongated, and multinucleated myotubes have both aciculin and dystrophin colocalized at focal adhesions (Fig. 5, *C* and *D*). Upon subsequent growth and maturation of C2C12 myotubes, aciculin and dystrophin were found redistributed along the stress fiber-like structures in developing myotubes (data not shown). Finally, in terminally differentiated contractile myotubes, possessing sarcomeric organization, both aciculin and dystrophin appeared in a regular periodic pattern, corresponding to sarcomere Z-discs (Fig. 5, *E* and *F*).

In contrast to the distinct localization of dystrophin at various cell-matrix adherens-type junctions in cultured muscle cells, immunostaining of C2C12 cultures with the anti-utrophin-specific mAb NCL-DRP1 did not reveal any utrophin associated with stress fiber-like structures, focal adhesions, or cell-matrix attachment sites at the edges of myotubes. Occasional irregular bright spots of utrophin immunofluorescence were detected in some maturing C2C12 myotubes and were shown to colocalize with clusters of acetylcholine receptors (data not shown). Taken together, our observations indicate precise colocalization of aciculin and dystrophin in cultured skeletal muscle cells and a lack of any codistribution of aciculin and utrophin in these muscle cultures.

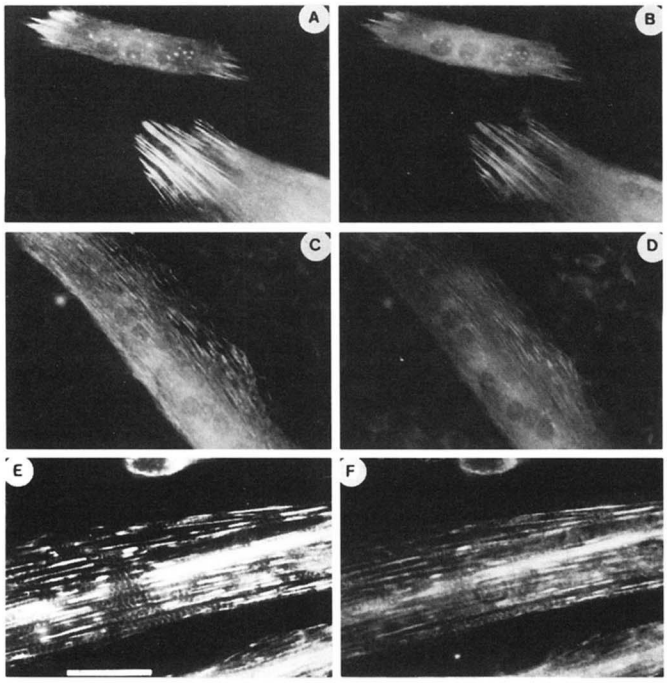

FIG. 5. **Colocalization of aciculin and dystrophin during myodifferentiation of C2C12 cells in culture.** C2C12 cells, taken on day 4 (*A* and *B*), day 6 (*C* and *D*), and day 11 (*E* and *F*) of culture, were costained with rabbit polyclonal antibody against aciculin (*A*, *C*, and *E*) and mixture of mouse mAbs against dystrophin (mAbs NCL-Dys1, NCL-Dys2, and 1808), (*B*, *D*, and *F*). *Bar* indicates 20 μm.

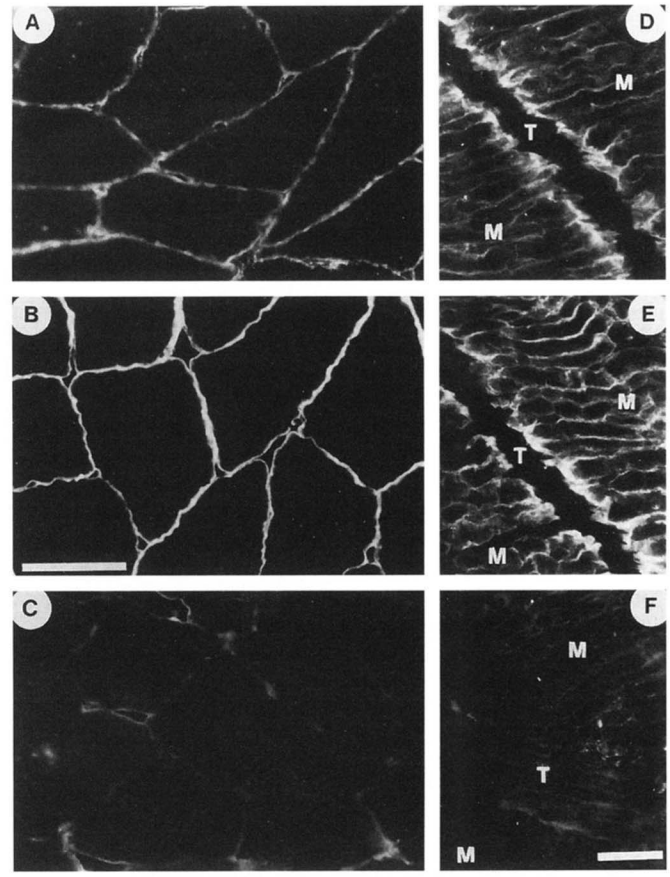

FIG. 6. **Localization of aciculin, dystrophin, and utrophin in skeletal muscle.** 7-μm cryosections of adult rabbit skeletal muscle (*A-C*) or chicken embryonic thigh skeletal muscle (*D-F*) were stained with antibodies against aciculin (*A* and *D*), dystrophin (*B* and *E*), or utrophin (*C* and *F*). *M*, muscle; *T*, tendon. *Bars* represent 50 μm (*A-C*) or 20 μm (*D-F*).

In skeletal muscle tissues, both aciculin and dystrophin were detected at the sarcolemma and at myotendinous junctions (Fig. 6, *A*, *B*, *D*, and *E*), even though aciculin staining at the sarcolemma was considerably weaker than that of dystrophin (Fig. 6, *A*, *B* and Refs. 23, 27–30, 33, 34, 57, and 58). In accordance with earlier observations, utrophin was detected only very weakly at the sarcolemma (Fig. 6*C* and Refs. 26, 63) and at myotendinous junctions (Fig. 6*F*).

To determine, whether the interaction between aciculin and dystrophin is due to their direct binding, or mediated by some other protein(s), we subjected a mixture of purified aciculin and dystrophin to gel filtration on a high resolution Superose 6 analytical column. Column fractions were analyzed by SDS-PAGE and immunoblotting with anti-aciculin and anti-dystrophin antibodies (Fig. 7). When aciculin alone was run through the column, the protein was detected starting from fraction 32 with most of the aciculin present in fractions 35–47 (Fig. 7*A*). Preincubation of 20 μg of aciculin with 10 μg of purified dystrophin before the column run shifted a detectable proportion of aciculin toward much higher molecular weights on the column and considerable amounts of the protein were detected in the dystrophin-containing fractions 25–32 (Fig. 7*B*, *a* and *b*). A gel filtration experiment performed with a larger amount of dystrophin (50 μg) increased the proportion of aciculin in the high molecular weight fractions (Fig. 7*C*, *a* and *b*).

Trying to identify proteins, associated with aciculin in cells and tissues outside skeletal muscle, we probed anti-aciculin immunoprecipitates from cultured A7r5 smooth muscle cells, and REF52 fibroblasts for the presence of utrophin by immunoblotting (Fig. 8). Indeed, utrophin was detected in anti-aciculin immunoprecipitates from both these cell cultures (Fig. 8*C*, *b* and *c*, *arrowhead*). Both dystrophin and utrophin were found in association with aciculin in bovine heart muscle tissue, when anti-aciculin immunoprecipitates were blotted with anti-dystrophin (Fig. 8*B*, *a*) and anti-utrophin (Fig. 8*C*, *a*) antibodies. In contrast, only dystrophin but not utrophin was readily identified in anti-aciculin immunoprecipitates from C2C12-cultured myotubes (Fig. 8, *B* and *C*, *d*). Therefore, our results indicate that utrophin, but not dystrophin, is associated with aciculin in cultured smooth muscle and nonmuscle cells. It should be noted that ~75–80 kDa immunoreactive bands were also detected on immunoblots with anti-dystrophin and anti-utrophin antibodies (Fig. 8, *B* and *C*, *asterisks*), suggesting a cross-reaction of these mAbs with short forms of dystrophin and/or utrophin (8, 9, 18, 19).

We also analyzed whether aciculin is a major utrophin-associated protein in cultured A7r5 smooth muscle cells. An immunodepletion experiment was performed with RIPA extracts of ^{35}S-labeled A7r5 cells (Fig. 9). Preadsorbtion with anti-aciculin antibody removed the majority of aciculin from cell lysates (Fig. 9, *a* and *a'*, *arrow*). A major ~60 kDa protein band, present in anti-utrophin immunoprecipitates, was shown to represent aciculin, since this band was depleted upon preincubation with anti-aciculin mAb XIVF8 (Fig. 9, *b* and *b'*, *arrow*). Moreover, this ~60 kDa band, migrating slightly slower than the syntrophin ~55–58-kDa doublet, was also detected in anti-syntrophin immunoprecipitates and was noticeably depleted after preadsorbtion of a cell lysate with XIVF8 mAb (Fig. 9; compare *c* and *c'*; the *arrowheads* indicate syntrophin). This result might be explained by the association of syntrophin with the utrophin doublet in cultured A7r5 cells (Fig. 9, *c* and *c'* and Ref. 55). This suggests that the association of aciculin and syntrophin with utrophin in cultured cells is not mutually exclusive and that these two utrophin-associated proteins may have non-overlapping binding sites on the utrophin molecule. In both anti-

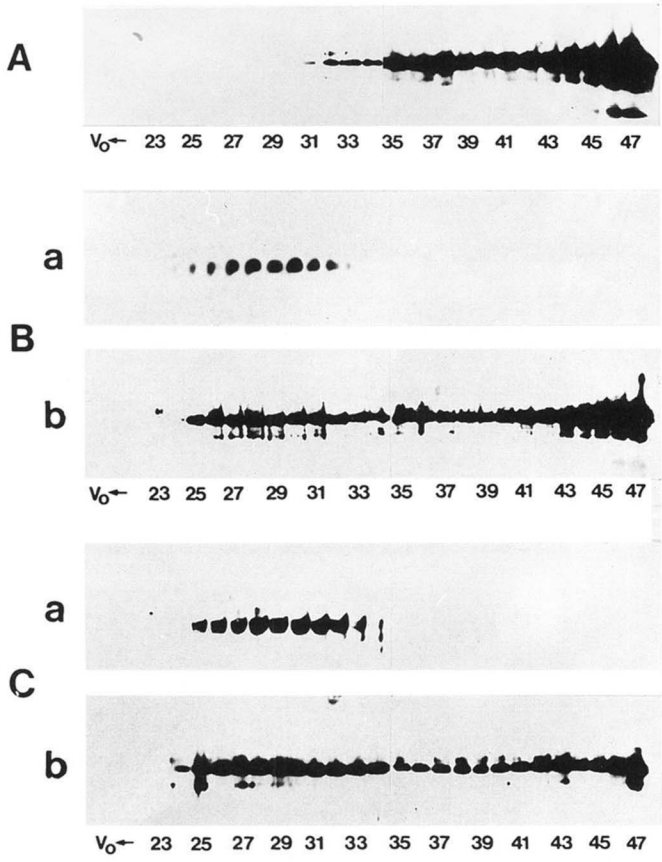

FIG. 7. **A direct interaction between aciculin and dystrophin detected by analytical gel filtration.** Western immunoblots with anti-aciculin mAb XIVF8 show the elution profile of 20 μg of aciculin when chromatographed alone (*A*) or in the presence of 10 μg (*B*, *b*) or 50 μg (*C*, *b*) of purified dystrophin. Dystrophin-containing fractions in *B* and *C* were visualized by immunoblotting with 1958 mAb (*B*, *a*; *C*, *a*). Fraction numbers are given below the corresponding immunoblots.

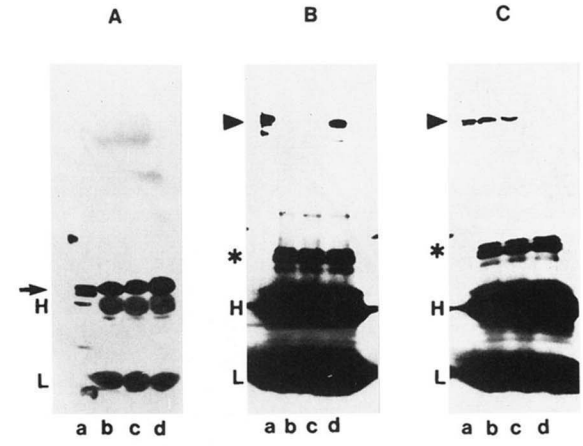

FIG. 8. **Aciculin is associated with utrophin in cultured A7r5 smooth muscle cells and REF52 fibroblasts.** Anti-aciculin immunoprecipitates from bovine heart muscle (*a*), A7r5 smooth muscle cells (*b*), REF52 fibroblasts (*c*), or C2C12 myotubes (*d*) were washed, run on 5–12% SDS-polyacrylamide gel, transferred to Immobilon membrane, and blotted with XIVF8 mAb against aciculin (*A*), a mixture of mAbs NCL-Dys1, NCL-Dys2, and 1808 against dystrophin (*B*) or NCL-DRP1 mAb against utrophin (*C*). *Arrow* in *A* shows the position of aciculin on the immunoblot. *Arrowheads* indicate the position of dystrophin (in *B*) or utrophin (in *C*) on the immunoblots. *H* and *L* designate the immunoglobulin heavy and light chains, respectively. *Asterisks* in *B* and *C* mark immunoreactive bands in the range of 75–80 kDa, cross-reacting with anti-dystrophin (*B*) and anti-utrophin (*C*) antibodies.

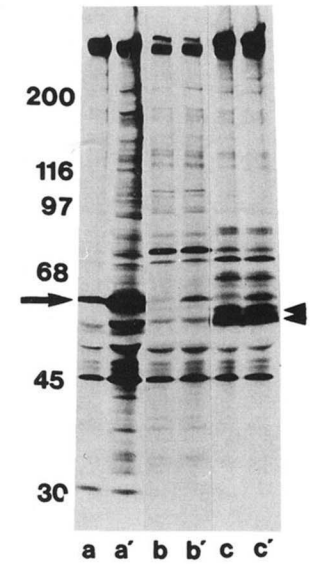

FIG. 9. **Aciculin is a major utrophin-binding protein in cultured A7r5 smooth muscle cells.** RIPA extracts of [^{35}*S*]methionine/[^{35}S]cysteine-labeled A7r5 cells were preadsorbed with either anti-aciculin mAb XIVF8, coupled to Sepharose 4B (*a*-*c*), or plain Sepharose 4B (*a′*-*c′*). Preadsorbed supernatants were taken for immunoprecipitation with anti-aciculin mAb XIVF8 (*a* and *a′*), anti-utrophin mAb NCL-DRP1 (*b* and *b′*), or anti-syntrophin mAb 1351 (*c* and *c′*). Immunoprecipitates were extensively washed with RIPA buffer and run on 10% SDS-polyacrylamide gel. Positions of molecular mass markers are given in kilodaltons to the left of the gel. Note, that a major ~60 kDa band, indicated by *arrow* to the left of the gel, present in anti-utrophin (*b′*) and anti-syntrophin (*c′*) immunoprecipitates, is depleted after preincubation with the anti-aciculin antibody (*b* and *c*). *Arrowheads* point to the syntrophin doublet around ~55–58 kDa.

utrophin and anti-syntrophin immunoprecipitates, we were able to resolve a closely disposed doublet around ~400 kDa (Fig. 9, *b*, *b′*, *c*, and *c′*), indicating the existence of two utrophin isoforms in A7r5 cultures. However, only one of these, representing the slightly faster migrating utrophin variant, was detected in association with aciculin (Fig. 9, *a* and *a′*). Control immunoprecipitation of vinculin or α-actinin from A7r5 cells did not reveal any aciculin bound to these two major actin-associated proteins, indicating specificity for the observed aciculin-utrophin coprecipitation (data not shown). Immunodepletion experiments performed with REF52 cultured cells revealed similar, but less prominent association of utrophin with aciculin in this cell type (not shown).

DISCUSSION

Recent work has shown that aciculin is a cytoskeletal protein with some sequence homology to PGM1 (57). Aciculin is expressed primarily in muscle tissues and is associated with adherens junctions and the actin cytoskeleton (57, 58). Since aciculin is not an actin-binding protein,[2] it is important to determine which proteins are able to interact with aciculin, linking it to microfilaments and to peripheral or integral membrane components of adherens junctions. In the present study, using immunoprecipitation of aciculin from cultured muscle cells, we demonstrated that aciculin is associated with dystrophin in skeletal muscle myotubes. Metabolic labeling of muscle cultures, followed by immunoprecipitation, showed that a ~400-kDa protein, identified as dystrophin on immunoblots, appeared to be a major aciculin-associated protein in cultured muscle cells. The dystrophin-aciculin complex is substantially enriched in the Triton X-100-insoluble fraction, indicating its stable association with the actin cytoskeleton in cultured C2C12 cells. Immunodepletion experiments with differentiated

[2] A. M. Belkin and K. Burridge, unpublished results.

C2C12 cells showed that a major 60 kDa band, coprecipitating with dystrophin and utrophin, is depleted after preincubation with anti-aciculin antibodies, therefore identifying aciculin as one of a few major dystrophin-associated proteins in this cell type. Taken together, these data point to an association of aciculin with dystrophin in cultured skeletal muscle cells.

In skeletal muscle tissue, a large transmembrane dystrophin-glycoprotein complex, containing a 59-kDa triplet of dystrophin-associated cytoplasmic protein(s), was described by Campbell and co-workers (43, 45, 46, 52). Several lines of evidence suggested that this 59-kDa protein(s) interact(s) directly with dystrophin, presumably near its C terminus (43, 44, 55, 56). A 58-kDa postsynaptic protein (syntrophin), interacting with dystrophin, utrophin, and short C-terminal forms of dystrophin (53, 55, 56), was shown to be structurally and functionally homologous to one of the recently sequenced proteins in the 59-kDa triplet, 59–1 DAP (52, 54). However, in spite of the similarity in apparent molecular weight between aciculin and 59–1 DAP (or syntrophin), it should be noted, that the anti-syntrophin mAb 1351, provided by R. Sealock, did not react with aciculin in immunoblots. Reciprocally, anti-aciculin mAbs did not recognize syntrophin.[3] Moreover, none of five anti-aciculin mAbs reacted with the 59-kDa protein triplet of the dystrophin-glycoprotein complex, purified from rabbit skeletal muscle sarcolemma.[4] These facts define aciculin as a new cytoskeletal protein associated with dystrophin, unrelated to previously described dystrophin-associated proteins (43, 47, 52, 55, 56).

Results of analytical gel filtration experiments, obtained in the present study, show that aciculin is able to interact with dystrophin directly. Since *in vitro* experiments have shown dystrophin to be an actin-binding protein (40), aciculin association with actin filaments in muscle cells might be mediated by dystrophin. By analogy with the erythrocyte membrane skeleton, aciculin could stabilize actin-dystrophin interaction at muscle adhesive contacts, playing a role, similar to that described for protein 4.1 in modulating spectrin-actin interaction (64). In future work, it will be important to determine, whether aciculin is able to modulate the affinity of dystrophin for actin *in vitro*. Potentially, some integral membrane proteins in skeletal muscle may interact with aciculin as well, thus providing another actin-membrane link through dystrophin and dystrophin-associated proteins, in skeletal muscle.

At the moment, no precise information concerning the localization of the aciculin-binding site on the dystrophin molecule is available. However, one observation obtained in this study indicated that anti-C-terminal anti-dystrophin and anti-utrophin mAbs may interfere with aciculin binding to dystrophin (and utrophin), perhaps pointing to a putative aciculin-binding site near the dystrophin (and utrophin) C terminus. We did not detect any association of aciculin with α-actinin or spectrin, two actin-binding cytoskeletal proteins, sharing some homology with dystrophin within its N-terminal and central rod domain (3, 4, 37). Therefore, some indirect evidence may potentially indicate a localization of the aciculin-binding site within the cysteine-rich and/or C-terminal domains of dystrophin. Since extensive alternative splicing of dystrophin pre-mRNA produces several dystrophin forms differing in their C-terminal domains (20), and these alternatively spliced dystrophin forms are expressed in skeletal muscle (12, 20, 23), one cannot exclude the possibility that some dystrophin forms may interact with 59–1 DAP (syntrophin), whereas others interact with aciculin.

In the aciculin immunodepletion experiments performed with C2C12 myotube extracts, we were able to resolve several (at least three) high molecular weight bands, likely representing dystrophin and utrophin alternatively spliced variants. All these forms were associated with syntrophin, but only two of them were present in anti-aciculin immunoprecipitates, while one major dystrophin band was not found in association with aciculin. This observation suggests preferential binding of aciculin to some dystrophin isoforms, while some dystrophin variants are unable to interact with aciculin. Similarly, the results of aciculin immunodepletion experiment with A7r5 cell lysate also showed that utrophin is represented by a closely disposed doublet in this cell type. Whereas in A7r5 cells syntrophin was shown to interact with both utrophin forms, having slightly different mobility on SDS-polyacrylamide gels, aciculin was found in association only with the faster migrating form. At present, we do not have any clear explanation for these observations; however, it is reasonable to suggest that alternative splicing of utrophin pre-mRNA or post-translational modification of utrophin may abolish the aciculin-binding site on the molecule, generating some utrophin forms unable to interact with aciculin. Interestingly, our experiments demonstrated the presence of aciculin in anti-syntrophin immunoprecipitates from A7r5 cells, also containing significant amounts of utrophin. This result indicates that dystrophin and utrophin contain two nonoverlapping binding sites for aciculin and syntrophin. Future experiments mapping the aciculin- and syntrophin-binding sites on dystrophin and utrophin might help to clarify this point.

Recently, aciculin localization was reported in adherens junctions of muscle cells during myodifferentiation in culture (58). Even though dystrophin was localized earlier to focal adhesions of cultured *Xenopus laevis* muscle cells (34) and in web-like surface structures of differentiated chicken myotubes (65), to our knowledge, this is a first report showing dystrophin localization at various adherens-type junctions in cultured muscle cells during reorganization of the actin cytoskeleton accompanying myogenesis. Earlier, it was reported that the original C2 mouse muscle cell line expresses only trace, if any, dystrophin (66, 67). On the contrary, we have detected substantial amounts of dystrophin in the C2C12 subclone of C2 cells, therefore, allowing us to localize this protein during myodifferentiation in culture and to search for dystrophin-associated proteins in cultured muscle cells. In C2C12 myocytes, dystrophin was localized at myotube tips, transiently at focal contacts and stress fiber-like structures, and finally at costameres of differentiated myotubes. Double immunolocalization of aciculin and dystrophin in cultured C2C12 showed that these two proteins are precisely colocalized throughout all the stages of actin cytoskeleton reorganization and myofibril assembly, accompanying myodifferentiation in culture, thus making their interaction in cultured muscle cells very likely.

Immunolocalization of both aciculin and dystrophin in skeletal muscle tissues revealed codistribution of these two proteins at myotendinous junctions and at the sarcolemma. With regard to their sarcolemmal localization, both proteins were shown to be present at skeletal muscle costameres (31, 32, 57, 58). Assuming that aciculin and dystrophin interact in cultured muscle cells and colocalize at these two types of cell-matrix adhesive contacts of skeletal muscle fibers, we propose that myotendinous junctions and, potentially, costameres are the major sites where the aciculin-dystrophin interaction in skeletal muscle might occur. Notably, aciculin is not codistribured with dystrophin at neuromuscular junctions (58), where other dystrophin-binding proteins, such as 59–1 DAP (syntrophin), might link dystrophin and utrophin to the membrane (52, 55).

In our experiments we were able to detect some aciculin

[3] A. M. Belkin, K. Burridge, and R. Sealock, unpublished results.
[4] A. M. Belkin, K. Burridge, and K. Campbell, unpublished results.

immunoreactive bands in anti-utrophin immunoprecipitates, both by immunodepletion experiments and by immunoblotting. However, it seems unlikely that utrophin is an interactive partner for aciculin in cultured skeletal muscle myocytes and skeletal muscle tissue *in vivo*. First, aciculin shares differentiation-dependent, developmentally regulated expression in skeletal muscle with dystrophin (14, 58, 68), whereas utrophin is uniformly expressed during myogenesis (68). Then, we observed an obvious lack of utrophin codistribution with aciculin both in muscle cultures and tissues, indicating that the two molecules are not localized at the same intracellular compartment within skeletal muscle fibers. This spatial separation may not allow them to interact in skeletal muscle.

In contrast to the well established dystrophin expression in all three muscle types and brain and its obvious lack or extremely low expression in other tissues (2, 12, 13), aciculin is synthesized in some nonmuscle tissues and nonmuscle cell cultures, even though its expression level is much lower when compared to muscles (57). Looking for aciculin interactive partners in cells and tissues outside skeletal muscle, an obvious candidate is utrophin which is expressed ubiquitously in nonmuscle tissues and cultured cells (5, 6, 15, 16). Indeed, we showed that aciculin is associated with utrophin in some cultured cells, namely in A7r5 smooth muscle cells and REF52 fibroblasts. This is not unexpected given that aciculin associates with dystrophin in skeletal muscle. The prominent structural similarity between dystrophin and utrophin suggests a significant overlapping in their functional properties (2, 6, 7, 50, 55). For instance, all the dystrophin-associated components of the sarcolemmal dystrophin-glycoprotein complex were also found in association with utrophin in skeletal muscle (50). The 58-kDa postsynaptic protein (syntrophin) interacts equally well with dystrophin, utrophin, or both, depending on the tissue type analyzed (53, 55). Therefore, our data on aciculin association with utrophin in cultured cells, combined with the results showing aciculin-dystrophin interaction in skeletal muscle, give another example of functional conservation between these two cytoskeletal proteins. As in the case of syntrophin association with dystrophin and various dystrophin-related proteins in different tissues (55), the association of aciculin with either dystrophin or utrophin appears to be dictated mostly by the expression levels of these proteins in a given cell type.

In conclusion, we have demonstrated that aciculin is associated with dystrophin in skeletal muscle and with utrophin in cultured smooth muscle and fibroblasts. Interaction of aciculin with dystrophin and utrophin in various cell types might provide an additional cytoskeletal-matrix link, strengthening the transmembrane association between the extracellular matrix and the actin cytoskeleton.

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