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Expression and localization of the phosphoglucomutase-related cytoskeletal protein, aciculin, in skeletal muscle

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SUMMARY

Recently, a 60/63 kDa cytoskeletal protein, highly homologous to the glycolytic enzyme phosphoglucomutase (PGM 1), was isolated from smooth muscle tissue and shown to localize in various adherens-type junctions of muscle and some nonmuscle cells. Since this protein, tentatively named 'aciculin', was enriched in muscle tissues and cells, we have attempted to study its expression and localization during myodifferentiation. C2C12 mouse myoblasts did not express any aciculin before cell fusion in culture. Immediately after cell fusion aciculin became detectable and its content continued to rise during myotube maturation. In early myotubes aciculin appeared first at cell tips and was predominantly localized to focal adhesions of immature myotubes. As myotubes matured in culture, aciculin became associated with growing myofibrils, and finally was found redistributed in striations, corresponding to sarcomere Z-discs. Immunoblotting showed that aciculin content in chicken breast skeletal muscle remained very low until day 11 of embryogenesis, but significantly increased in late prenatal and early postnatal development. By immunofluorescence, aciculin was not revealed in thigh skeletal muscle of day 11 chicken embryos, but was prominently localized at myotendinous junctions in thigh muscle of day 16 embryos. Myotendinous junctions appeared to be major sites of aciculin accumulation in developing and mature skeletal muscle fibers in vivo, suggesting some role for this protein in thin filament-membrane interactions and, potentially, in force transmission at these cell-matrix contacts. In adult skeletal muscle faint aciculin staining appeared at the sarcolemma and as striations in register with Z-discs. Since the protein was not identified in glycerinated myofibrils but was localized to striations in C2C12 myotubes and within the limited areas on skeletal muscle tissue sections, we conclude that aciculin is a component of skeletal muscle costameres. In cultured C2C12 myotubes we found some codistribution of aciculin with clusters of acetylcholine receptors, suggesting its presence at neuromuscular junctions. However, we did not detect any significant concentration of aciculin at neuromuscular junctions in both embryonic and adult skeletal muscle. Taken together, our data show that aciculin expression in skeletal muscle is differentiation-dependent and upregulated during muscle development, and that this novel cytoskeletal protein is a component of various cell-matrix adherens junctions in muscle cells.

Key words: aciculin, phosphoglucomutase, cytoskeleton, adherens junction, skeletal muscle, myodifferentiation

INTRODUCTION

Adherens junctions are a specialized type of contact structure, characterized by a local association of actin filament bundles with the cytoplasmic face of the plasma membrane. Both cell-matrix and cell-cell adherens junctions are widely represented in vivo and in cultured cells, playing an important role in various aspects of cell behavior, including cell adhesion, motility, signal transduction, cell division, differentiation and oncogenic transformation (Geiger et al., 1987; Burridge et al., 1988; Geiger and Ginsberg, 1991; Luna and Hitt, 1992). In striated muscle, various junctional structures, i.e. myotendinous junctions, costameres and neuromuscular junctions, provide anchoring of thin filaments to the sarcolemma. Myotendinous junctions are cell-matrix contact structures where myofibrils are linked to the extensively folded membrane by both end-on and lateral associations at the tips of myofibers (Trotter et al., 1985; Tidball and Law, 1991; Reedy and Beall, 1993). They represent major force transmission sites in skeletal muscle fibers (Tidball, 1991). Costameres are subsarcolemmal junctional structures, providing lateral actin-membrane associations in the Z-disc register (Craig and Pardo, 1983). These cell-matrix contacts serve to stabilize the sarcolemma during muscle contraction (Pardo et al., 1983; Shear and Bloch, 1985), allowing for the transmission of force laterally to the extracellular matrix, adjacent myofibers and, subsequently, to the tendon (Danowski et al., 1992). The highly specialized postsynaptic membrane at neuromuscular junctions is organized by a peripheral membrane cytoskeleton, interacting with actin filaments. A variety of actin-associated cytoskeletal proteins were localized to junctional folds of the cholinergic postsynaptic membrane of skeletal muscle fibers, suggesting their potential role in the organization of these contact sites and/or

clustering of acetylcholine receptors (Bloch and Hall, 1983; Sealock et al., 1986; Froehner et al., 1987; Bloch and Morrow, 1989; Rochlin et al., 1989; Sealock et al., 1991; Turner et al., 1991; Bockholt et al., 1992).

Molecular organization of the cytoskeletal domain of these junctional structures in muscle fibers was shown to resemble that of well characterized microfilament-membrane attachment sites in cultured cells, named focal contacts (focal adhesions, adhesion plaques). Many known cytoskeletal proteins, like α-actinin, vinculin, talin, tensin and paxillin, initially identified in focal contacts of cultured cells (Lazarides and Burridge, 1975; Geiger, 1979; Burridge and Connell, 1983; Wilkins et al., 1986; Turner et al., 1990), were also shown to accumulate at junctional regions of muscle fibers (Bloch and Hall, 1983; Pardo et al., 1983; Shear and Block, 1985; Sealock et al., 1986; Tidball et al., 1986; Belkin et al., 1986; Turner et al., 1991; Bockholt et al., 1992). However, various types of adherens junctions in muscle cells contain some specific molecular components at their cytoskeletal and membrane domains, therefore providing a structural basis for their functional diversity. Thus, some muscle-specific cytoskeletal proteins, such as dystrophin, and the 58 kDa dystrophin-associated protein (syntrophin) were shown to concentrate specifically at various junctional sites within the skeletal muscle fibers (Froehner et al., 1987; Chen et al., 1990; Kramarcy and Sealock, 1990; Sealock et al., 1991; Masuda et al., 1992; Porter et al., 1992; Straub et al., 1992). Potentially, some muscle-specific isoforms of widely distributed cytoskeletal proteins, such as metavinculin, may also contribute to a distinct molecular composition and function of adherens junctions in muscle cells (Saga et al., 1985; Belkin et al., 1988; Gimona et al., 1988). On the contrary, α-actinin, a major cytoskeletal component of adherens junctions in nonmuscle cells, was shown to be absent from the submembranous densities of myotendinous junctions (Tidball, 1987), suggesting that some other cytoskeletal proteins link thin filaments to the membrane at these sites. These data, as well as continuing identification of new adherens junction proteins, show that the molecular composition and structure of junctional complexes in muscle cells remain incompletely understood.

Recently we identified a novel cytoskeletal component of focal contacts in muscle and some nonmuscle cells, sharing significant homology with the glycolytic enzyme phosphoglucomutase type 1 (PGM1) (Belkin et al., 1994). This 60/63 kDa protein was shown to be clearly distinct from PGM1 by several criteria and did not display any detectable enzymatic activity. The 60/63 kDa PGM1-related protein was particularly enriched in muscle cells and tissues and localized at various junctional structures, including smooth muscle dense plaques, cardiomyocyte intercalated discs and myotendinous junctions of striated muscle. In view of the distinct properties of the 60/63 kDa cytoskeletal protein compared to PGM1, and based on the localization of this cytoskeletal protein in a needle-like pattern in cultured smooth muscle and some nonmuscle cells (Belkin et al., 1994), we have suggested the name 'aciculin' for this novel adherens junction component (derived from the Latin 'acicula', meaning a small needle or spine). In the present work we have analyzed aciculin expression and localization during myodifferentiation, using cultured mouse C2C12 myogenic cell line and chicken skeletal muscle tissue samples, taken at different developmental stages.

MATERIALS AND METHODS

All reagents were reagent grade and purchased from Sigma Chemical Co. (St Louis, MO) unless stated otherwise. Fertile chicken eggs were purchased from Sanford poultry (Sanford, NC).

Antibodies

Monoclonal antibody XIVF8, which reacts with aciculin but does not recognize PGM1 enzyme, and polyclonal anti-aciculin antibodies, reacting with both these proteins were previously described (Belkin et al., 1994). Monoclonal antibody VIIF9 against human vinculin and metavinculin was characterized by Glukhova et al. (1990). Polyclonal antibodies against chicken vinculin and bovine heart α-actinin were prepared using chicken gizzard vinculin and bovine heart α-actinin, purified as described by Feramisco and Burridge (1980).

Cell culture

C2C12 mouse myogenic cell line, initially described by Blau et al. (1983), a subclone of mouse skeletal muscle C2 cells (Yaffe and Saxel, 1977), was obtained from the American Type Culture Collection (Rockville, MD). For biochemical experiments, cells were plated on 100 mm plastic dishes, precoated with 0.1% gelatin in PBS. C2C12 cells were cultured in DMEM medium, containing 10% fetal bovine serum, 2 mM glutamine and antibiotics (Gibco), until they formed a confluent monolayer. After reaching confluence, usually on the third day after plating, the culture medium was switched to DMEM plus 10% horse serum. Depletion of growth factors triggered cell fusion and myogenic differentiation in C2C12 cultures. Differentiating C2C12 cells were maintained in this medium for an additional 10-12 days with fresh medium added every second day. For immunofluorescence staining, C2C12 cells were plated on laminin-coated glass coverslips and cultured as described above. Approximately 80-90% of the nuclei were present in myotubes 3-4 days after the shift to the differentiation medium.

Skeletal muscle tissue samples

Freshly isolated samples of adult chicken skeletal muscle (breast and thigh muscle) or chicken embryonic skeletal muscle (breast and thigh muscle), taken at different developmental stages, starting from day 7 of embryonic development, were frozen by immersion into freezing isopentane and 5 μM longitudinal or transverse cryostat sections of skeletal muscle tissue were prepared at –20°C. Otherwise, freshly isolated skeletal muscle tissue samples were boiled in SDS-PAGE sample buffer for electrophoresis.

Fluorescence microscopy

Cultured C2C12 cells, taken at different stages of myogenic differentiation, were fixed in ice-cold absolute methanol for 5 minutes, rinsed in 50 mM Tris-HCl, 150 mM NaCl, 0.1% NaN_3, pH 7.6 (TBS), and blocked in 0.5% BSA/TBS for 30 minutes before the immunostaining. For aciculin detection, coverslips were incubated for 1 hour with XIVF8 mouse mAb. Donkey anti-mouse affinity-purified IgG, conjugated with rhodamine (Chemicon Int. Inc., Temecula, CA), was used as a secondary antibody. Chicken skeletal muscle tissue sections were fixed in ice-cold absolute methanol for 5 minutes and postfixed with acetone for 10 minutes at room temperature and blocked in 0.5% BSA/TBS before the immunofluorescent staining. Chicken breast muscle myofibrils, prepared as described by Knight and Trinick (1982) were dried on coverslips, fixed in ice-cold absolute methanol, blocked and processed for immunofluorescence as described above. In some experiments, biotinylated goat anti-mouse IgG and rhodamine-streptavidin (Miles Scientific Division, Naperville, IL) were used to enhance the staining intensity. For the visualization of acetylcholine receptor clusters in muscle cultures and on muscle tissue sections, fluorescein-labelled α-bungarotoxin (Molecular Probes, Inc., Eugene, OR) was used. For double-immunostaining experiments, XIVF8 anti-aciculin mAb was used in combination

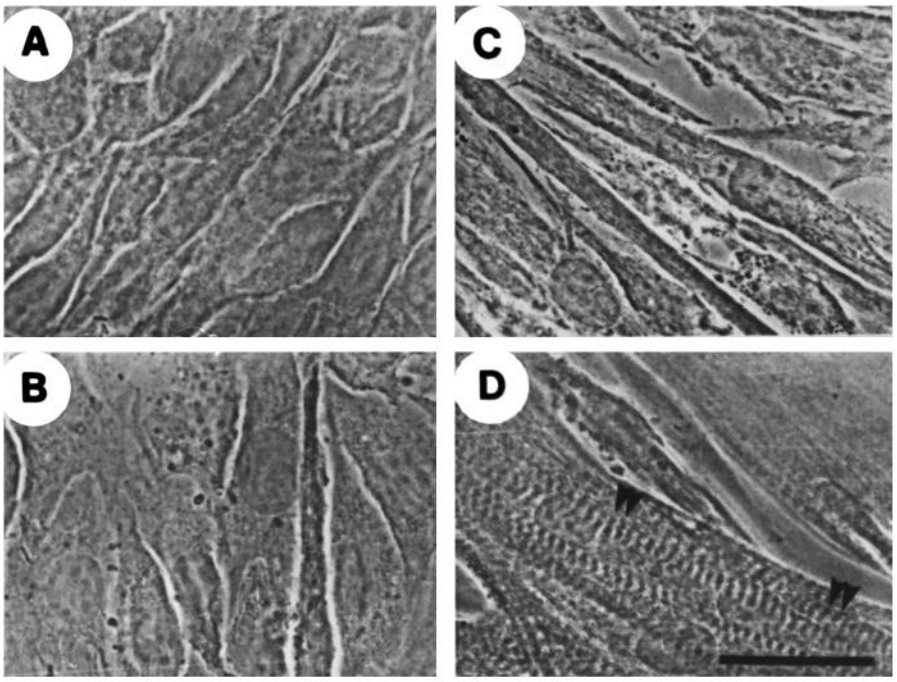

Fig. 1. Phase micrographs of C2C12 mouse myocytes, differentiating in culture. (A) C2C12 myoblasts reach confluency on day 3; (B) cell fusion starts on day 5 in culture and some early myotubes appear; (C) on day 8, a number of differentiating multinucleated myotubes are present in culture; (D) mature contractile myotubes, possessing well-developed sarcomeric organization (arrowheads), are visible on day 12 in culture. Bar, 20 μm.

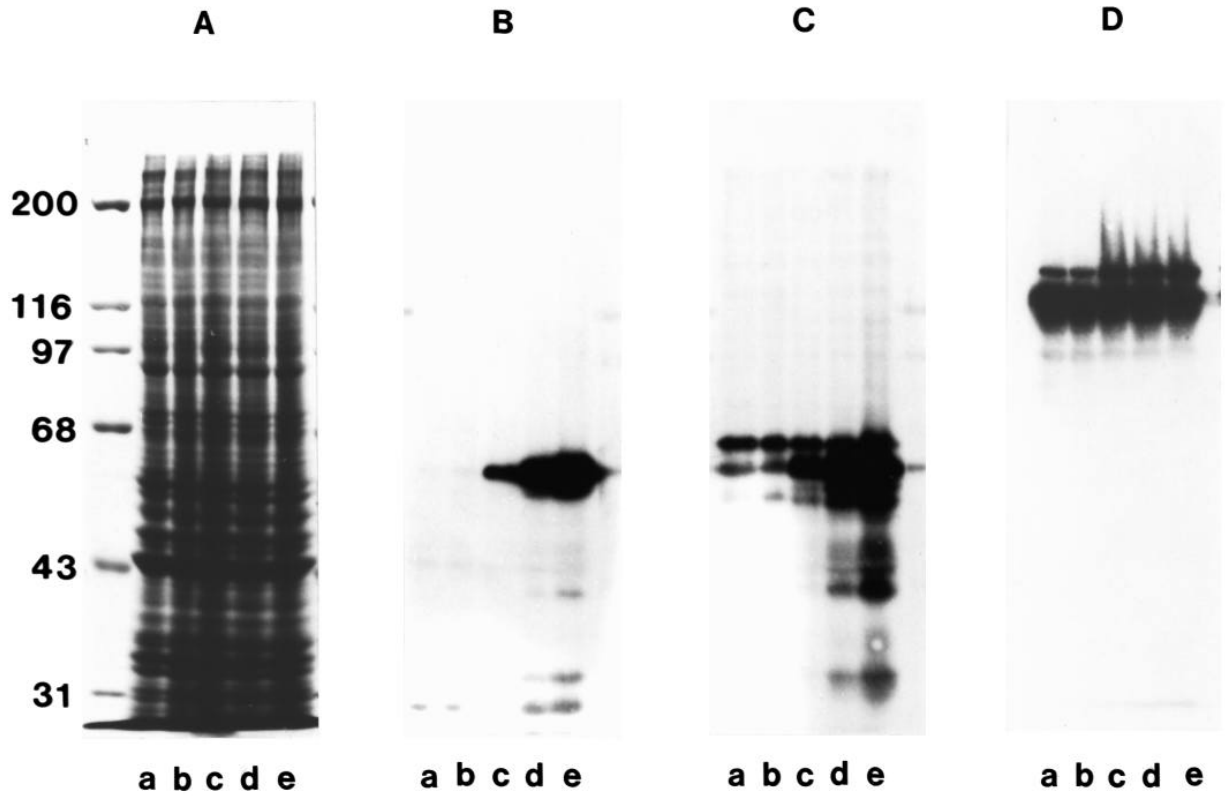

Fig. 2. Aciculin expression during myodifferentiation of C2C12 cells. Cell lysates of C2C12 myocytes, taken on day 2 (a), day 4 (b), day 6 (c), day 9 (d) and day 12 (e), were electrophoresed on 10% polyacrylamide gels and either stained with Coomassie Blue (A) or transferred to nitrocellulose and blotted with XIVF8 aciculin-specific mAb (B), polyclonal antibodies to aciculin, reacting also with PGM 1 (C), or mAb VIIF9 against vinculin and metavinculin (D). Iodinated rabbit anti-mouse or goat anti-rabbit antibodies and autoradiography was used for signal detection. The molecular masses of marker proteins are indicated in kilodaltons to the left of the gel.

with rabbit polyclonal anti-vinculin or rabbit polyclonal anti-α-actinin. A mixture of rhodamine-labeled donkey anti-mouse IgG and fluorescein-labeled donkey anti-rabbit IgG (Chemicon Int. Inc., Temecula, CA) was used for simultaneous detection of both antigens. The stained specimens were mounted with Mowiol and examined on a Zeiss Axiophot microscope equipped for epifluorescence. Fluorescence micrographs were taken on T-max 400 film (Eastman Kodak Co., Rochester, NY).

Gel electrophoresis and immunoblotting

Cell or skeletal muscle tissue samples were immediately boiled for 3 minutes in SDS-PAGE sample buffer and the DNA sheared by passing several times through a 26 gauge needle. Protein concentration in different cell and tissue samples was determined and equalized using A_{230} measurements or Coomassie Protein Assay Reagent (Pierce Co., Rockford, IL). Each electrophoretic sample contained 150±10 μg of total protein. Protein samples were electrophoresed on 10% SDS-polyacrylamide gels (Laemmli, 1970), with a bisacrylamide concentration of 0.13%. After electrophoresis, gels were either stained with Coomassie Blue or transferred to nitrocellulose (Towbin et al., 1979). After extensive blocking, blots were incubated with either XIVF8 mAb, specific to aciculin; polyclonal antibodies, reacting with both aciculin and PGM1; or VIIF9 mAb against vinculin and metavinculin. Iodinated rabbit anti-mouse or goat anti-rabbit affinity-purified antibodies (5×10^6 cpm/μg; $2.5\text{-}5\times10^6$ cpm/ml) were used as secondary antibodies. Autoradiography with X-ray film

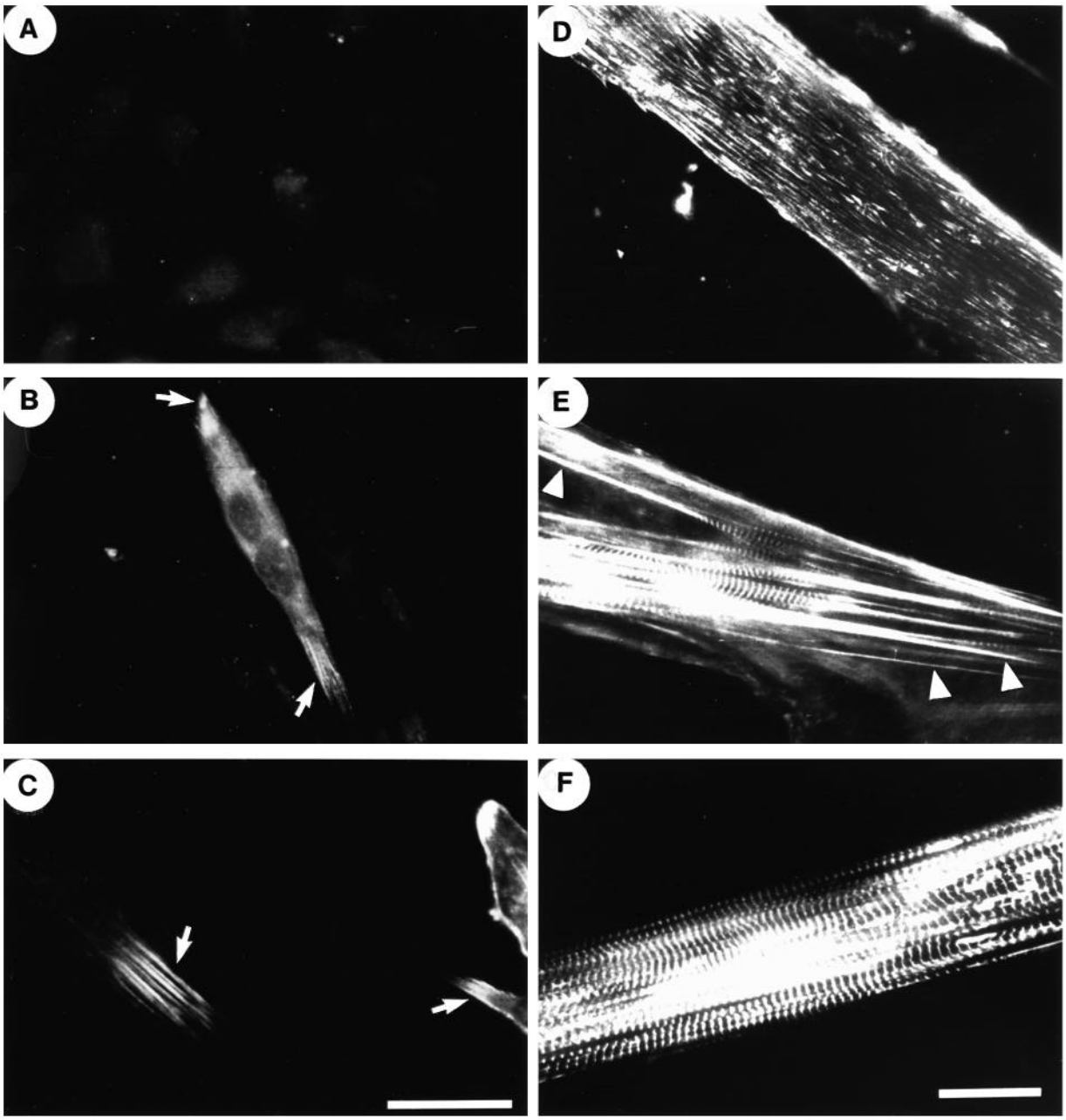

Fig. 3. Aciculin localization in differentiating C2C12 myocytes. Cultured C2C12 cells, taken on day 3 (A), day 5 (B), day 6 (C), day 8 (D), day 10 (E), day 12 (F), were stained with XIVF8 mAb to aciculin. Arrows point to aciculin localization in a streak-like pattern at cell tips (B,C). Arrowheads indicate transient aciculin localization at stress fiber-like structures of differentiating myotubes (E). Bar, 20 μm.

(Eastman Kodak Co., Rochester, NY) for 30-96 hours at −70°C was used for detection of immunoreactive bands.

RESULTS

Expression and localization of aciculin in differentiating C2C12 cells

Myogenic differentiation in confluent C2C12 cultures was induced by depletion of growth factors in the culture medium. C2C12 myoblasts in confluent culture (Fig. 1A) started to fuse on the second day after replacing DMEM-10%FBS with DMEM, containing 10% horse serum. Spindle-shaped multinucleated myotubes appeared in culture on day 5 after plating (Fig. 1B). Elongation and enlargement of myotubes (Fig. 1C) was accompanied by myofibrillogenesis and, finally, terminally differentiated contractile C2C12 myotubes, possessing well-developed sarcomeric organization were seen in culture on days 11-13 (Fig. 1D). Thereafter, some mature myotubes were reduced in size and detached from the substrate due to their intense contractile activity.

To study the time-course of aciculin expression during the myodifferentiation, we took cultured C2C12 myogenic cells at certain stages of differentiation and subjected them to electrophoresis and subsequent immunoblotting. As shown in Fig. 2B, immunoblotting with anti-aciculin-specific XIVF8 mAb did not reveal any protein band in C2C12 cultures, taken on day 2 or day 4. Immediately after extensive cell fusion in culture, a single aciculin immunoreactive band with a molecular mass of ~60 kDa appeared (Fig. 2Bc). Relative aciculin content continued to rise during myotube maturation in culture, reaching its highest level in terminally differentiated contractile myotubes (Fig. 2Bd,e). Immunoblotting with antibodies, reacting with both aciculin and PGM1, gave similar patterns of aciculin expression during the myodifferentiation (lower band in Fig. 2C), except some aciculin was detected in postmitotic cultures at earlier time points with this antibody (Fig. 2Ca,b). C2C12 myoblasts expressed a significant amount of PGM1 enzyme with a molecular mass of ~65 kDa before the cell fusion in culture occurred, and the PGM1 expression level remained relatively constant throughout the entire differentiation process (upper band in Fig. 2C). Several low molecular mass bands detected in differentiated myotubes with anti-aciculin monoclonal and polyclonal antibodies are probably aciculin proteolytic fragments (Fig. 2Bd,e and Cd,e). Expression of the ubiquitous adherens junction marker, vinculin, appeared to be uniform in differentiating C2C12 cells. At the same time, metavinculin content was elevated after the cell fusion occurred in culture (Fig. 2D).

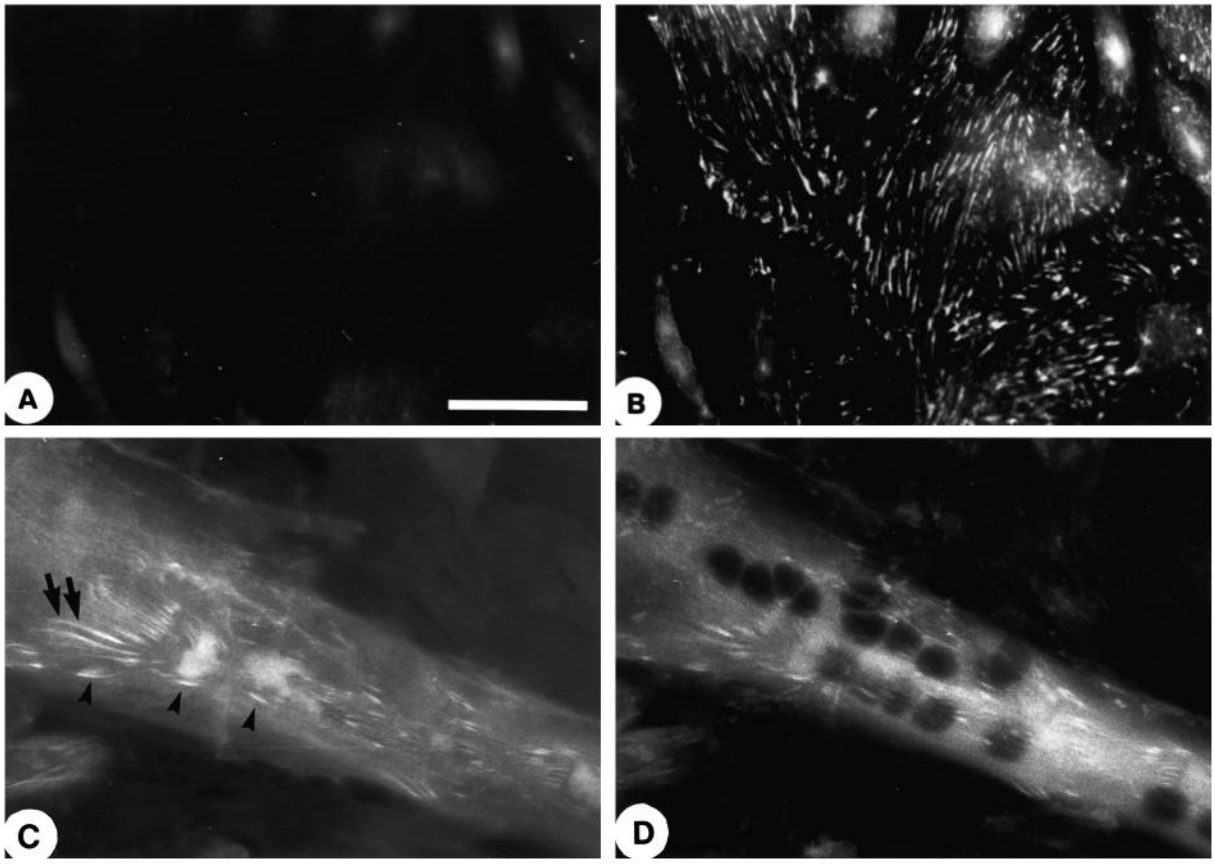

Fig. 4. Aciculin is a focal contact component in myotubes, but is not localized in focal contacts of myoblasts. C2C12 cells, taken on day 8 after plating, were double-stained with XIVF8 mAb to aciculin (A,C) and polyclonal antibodies to vinculin (B,D). Shown are postmitotic prefusion myoblasts (A,B) or immature multinucleated myotubes (C,D), present in the same culture. Arrows in (C) indicate a needle-like pattern of aciculin localization beyond the areas of vinculin-positive focal adhesions. Arrowheads in (C) mark codistribution of aciculin and vinculin at focal adhesions of immature myotubes. Bar, 20 μm.

In accordance with the immunoblotting data, immunofluorescence staining of confluent C2C12 myoblast cultures did not reveal any detectable aciculin in culture on day 3 (Fig. 3A). Immediately after cell fusion, we observed positive aciculin staining in cells having two nuclei (Fig. 3B). In early myotubes, initial and predominant sites of aciculin accumulation appeared to be cell tips (Fig. 3B,C, arrows). Bright aciculin streaks, corresponding to the terminal parts of actin bundles, emanating from the edges of early myotubes, were the most typical aciculin localization pattern in early myotubes (Fig. 3C, arrows). Terminal parts of elongated myotubes, containing major cell-matrix attachment sites, analogous to the myotendinous junctions in tissue, displayed distinct staining for aciculin throughout all the stages of myotube maturation in culture (not shown).

As the myotubes continued to enlarge and elongate in culture, prominent aciculin staining appeared at the ventral surface of the myotubes, apparently at the myotubes' cell-matrix contact structures (Fig. 3D). On day 9-10, in more mature, usually branching and multinucleated myotubes, aciculin became redistributed in a fibrillar pattern along strands running parallel to the long axis of the cells (Fig. 3E, arrowheads). This pattern was very similar to the fibrillar staining observed earlier for F-actin, α-actinin, tropomyosin and CapZ, and associated with stress fiber-like structures in developing muscle cells (Dlugosz et al., 1984; Antin et al., 1986; Lin and Lin, 1986; Schafer et al., 1993). Anti-aciculin immunolabeling of stress fiber-like structures was, in general, uniformly distributed along the fibrils without any periodic or punctate pattern. In contrast, no staining of stress fiber-like structures was seen with antibodies to vinculin or talin (not shown). Fibrillar structures, having aciculin localized uniformly along their length were often contiguous with regions along the fibrils where aciculin was observed in the periodic pattern (Fig. 3E). Finally, in mature contractile myotubes possessing well-developed sarcomeric organization, aciculin staining appeared in a periodic pattern along the fibrils at distinct structures resembling Z-discs (Fig. 3F). The spacing of these structures was approximately 2 μm, close to the distance between Z-discs in mature sarcomeres. Previously, a similar pattern of aciculin localization was demonstrated in cardiomyocytes, suggesting its presence at costameres (Belkin et al., 1994).

To confirm the presence of aciculin in cell-matrix contacts at the ventral membrane of differentiating myotubes, we double-stained immature C2C12 myotubes with antibodies against aciculin and vinculin. As shown in Fig. 4C and D, aciculin was colocalized with vinculin at focal contacts of cultured C2C12 myotubes on day 8 (arrowheads in 4C). Sometimes, a more elongated pattern of aciculin staining, exceeding the area of vinculin-positive contacts, was observed (arrows in 4C). A similar needle-like pattern of aciculin localization was demonstrated earlier in cultured smooth muscle cells and fibroblasts (Belkin et al., 1994). However, no focal contact or any other staining was seen with anti-aciculin anti-

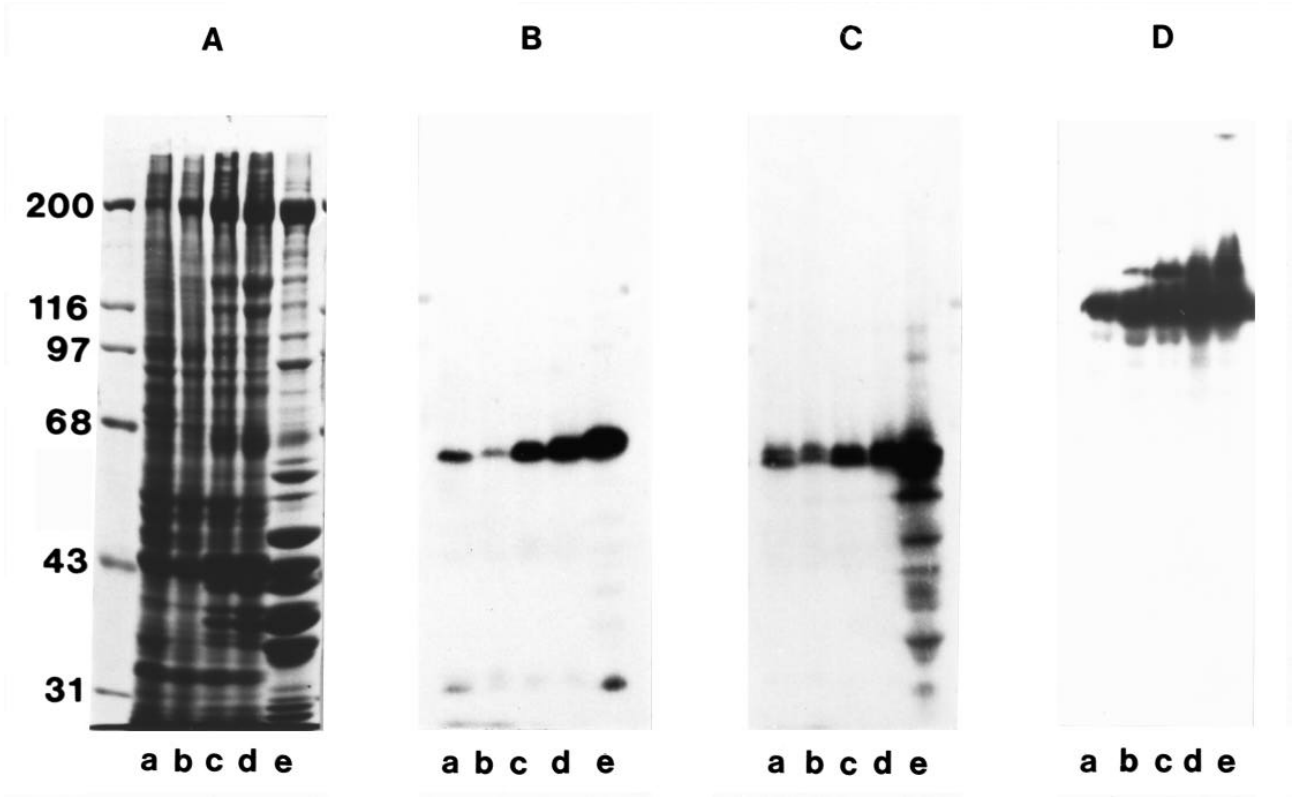

Fig. 5. Aciculin expression during skeletal muscle development. Samples of chicken breast skeletal muscle, taken on day 7 (a), day 11 (b), day 16 (c) of embryogenesis, from hatched (d) and adult (e) animals, were immediately boiled in electrophoresis sample buffer, run on 10% polyacrylamide gels and either stained with Coomassie Blue (A) or blotted with XIVF8 aciculin-specific mAb (B), polyclonal antibodies to aciculin, reacting also with PGM 1 (C), or mAb VIIF9 against vinculin and metavinculin (D). Iodinated rabbit anti-mouse or goat anti-rabbit antibodies and autoradiography were used for signal detection. The molecular masses of marker proteins are indicated in kilodaltons to the left of the gel.

bodies in postmitotic nonfused myoblasts, present in the same culture (Fig. 4A,B).

Expression and localization of aciculin in developing skeletal muscle

To analyze the expression of aciculin during skeletal muscle development in vivo, chicken skeletal muscle tissue samples, taken at different developmentl stages, were extracted with SDS and processed for western blots. As shown in Fig. 5Ba,b, a relatively weak positive signal was observed in the breast skeletal muscle of day 7 and day 11 chicken embryos with aciculin-specific antibodies. The slight drop in aciculin level detected at day 11 compared with day 7 is probably due to a higher amount of protein loaded for the day 7 sample or due to the day 7 sample containing more contaminating smooth muscle. Aciculin expression in skeletal muscle drastically increased by day 16 of embryogenesis (Fig. 5Bc) and continued to rise during late prenatal and postnatal development (Fig. 5Bd,e). Immunoblotting with antibodies against aciculin and PGM1 showed very similar patterns of aciculin expression during skeletal muscle development. Appearance of a closely disposed doublet on the blots with polyclonal anti-aciculin antibodies (Fig. 5Ca,b) probably reflects the existence of a posttranslationally modified form of aciculin, which is not recognized by monoclonal antibody XIVF8. No detectable PGM1 bands were observed on these blots, suggesting that this glycolytic enzyme is not abundantly expressed throughout all the prenatal and postnatal development in this particular skeletal muscle type (Fig. 5C). High level of uniform PGM1 enzyme expression during development was observed in chicken thigh skeletal muscle, characterized by a significant level of glycolytic activity in this tissue (not shown). Again, the relative amount of vinculin in skeletal muscle remained more or less constant at different stages of development, whereas metavinculin content progressively elevated starting from late embryogenesis (Fig. 5D).

By immunofluorescence, no aciculin accumulation was revealed in thigh skeletal muscle of day 11 chicken embryos (Fig. 6A). Later, on day 16 of embryogenesis, bright aciculin staining appeared at muscle-tendon interfaces (Fig. 6B). Starting from this stage of embryonic development, aciculin staining was highly concentrated at myotendinous junctions and these structures appeared to be major sites of aciculin accumulation in adult skeletal muscle fibers (data not shown). However, no distinct staining of sarcolemma or other elements of skeletal muscle fibers was observed at this time-point with aciculin-specific antibodies (Fig. 6B). In adult chicken thigh skeletal muscle, some weak staining of sarcolemma appeared on both transverse and longitudinal sections of skeletal muscle tissue (Fig. 6C,D).

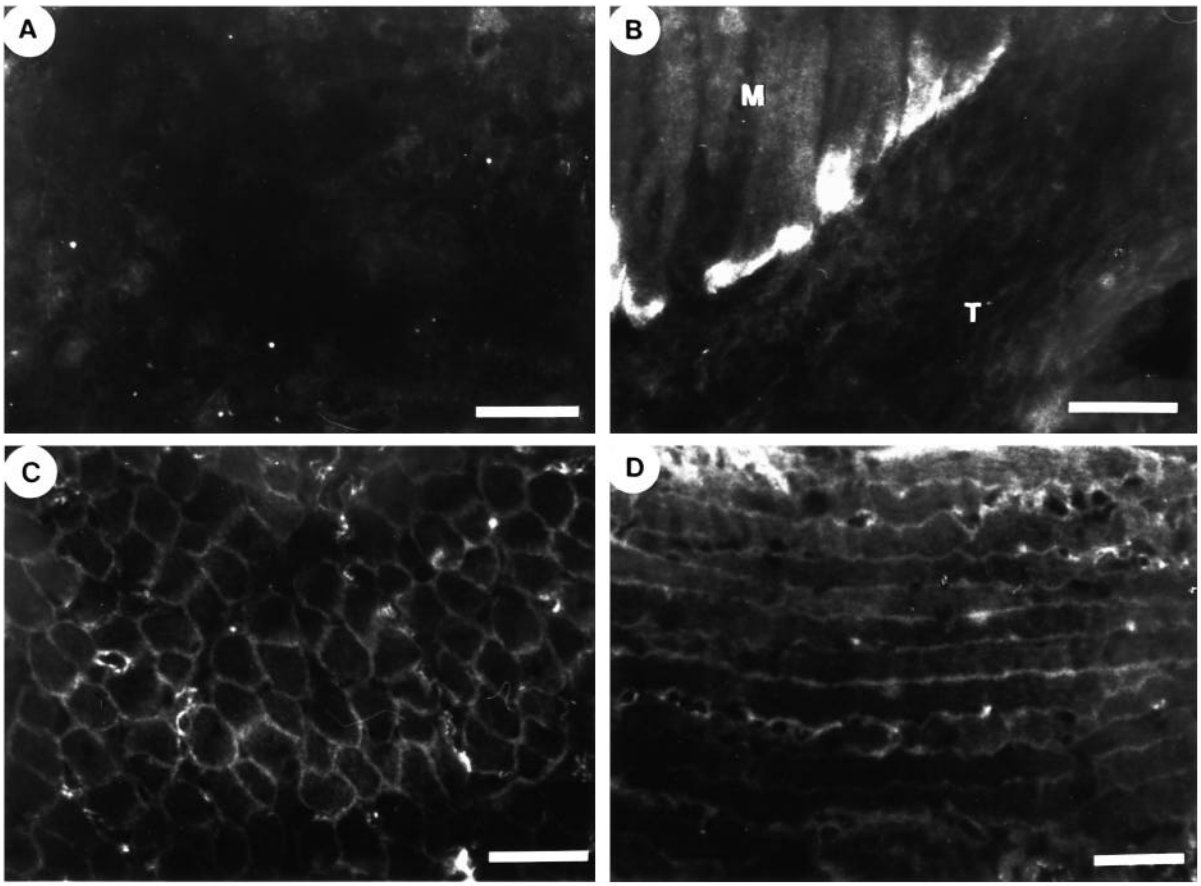

Fig. 6. Aciculin localization in embryonic and adult skeletal muscle. Cryostat sections (5 μm) of 11-day chicken embryonic thigh skeletal muscle (A), 16-day chicken embryonic thigh skeletal muscle (B), and transverse (C), or longitudinal (D) sections of adult chicken thigh skeletal muscle were stained with XIVF8 mAb, specific to aciculin. M, muscle; T, tendon. Bars: 20 μm (A,B); 50 μm (C,D).

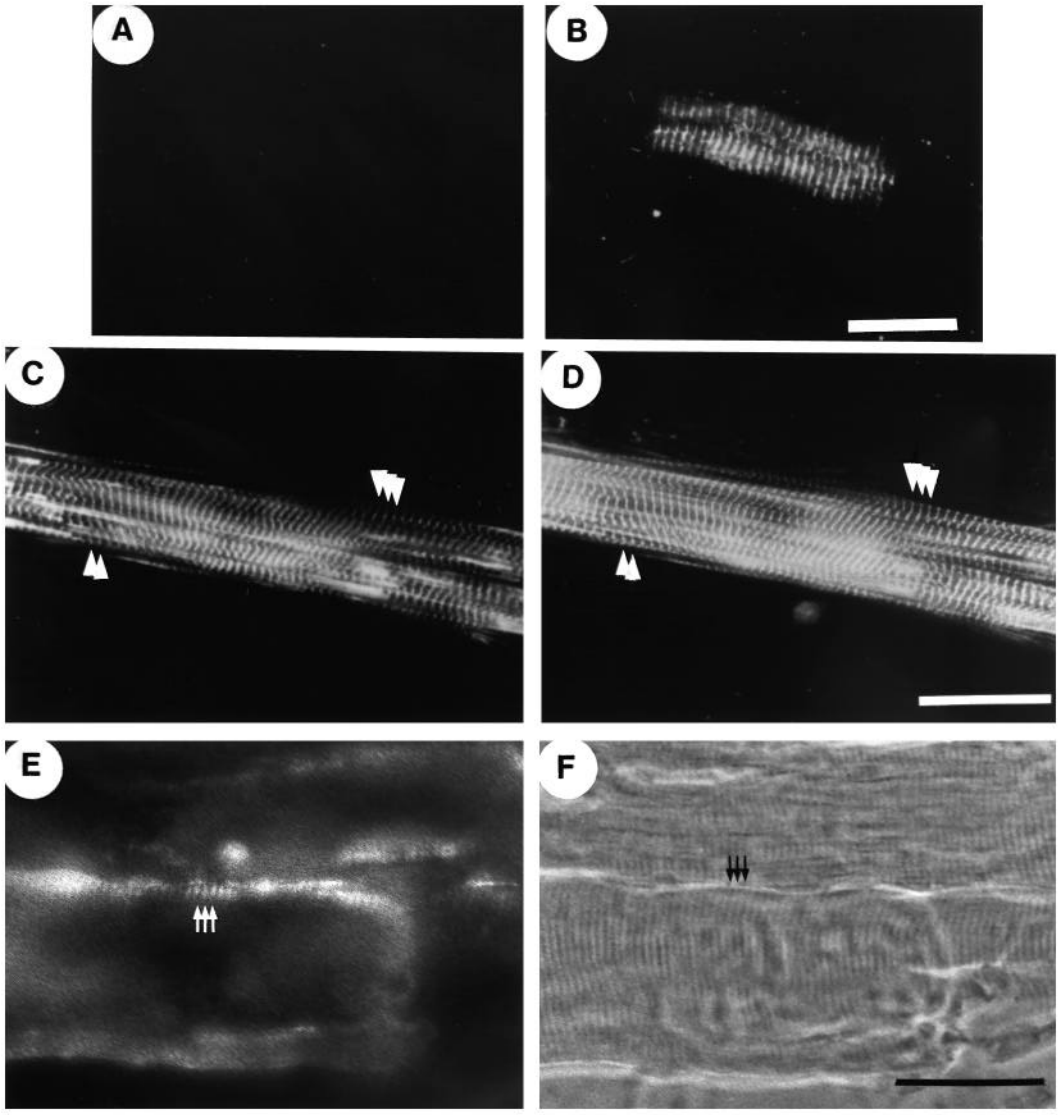

Fig. 7. Aciculin is localized at costameres of skeletal muscle fibers. Adult chicken breast skeletal muscle glycerinated myofibrils (A,B) or mature contractile C2C12 myotubes, taken on day 12 of culture (C,D) were double-stained with XIVF8 aciculin-specific mAb (A,C) and polyclonal antibodies to alpha-actinin (B,D). (E) Staining of 5 μm cryostat sections of adult chicken thigh skeletal muscle with XIVF8 mAb, (F) corresponding phase micrograph. Arrowheads (C,D) show colocalization of aciculin and alpha-actinin staining. Arrows in (E,F) show aciculin localization in striations where areas of sarcolemma are in the plane of section. Bars, 20 μm.

Since we did not observe any extensive aciculin staining of striations on skeletal muscle tissue sections (Fig. 6B,D), but demonstrated prominent aciculin accumulation aligned with Z-discs in cultured C2C12 myotubes (Figs 3E and 7C,D, arrowheads), it suggested that aciculin staining at the level of Z-discs is confined to subsarcolemmal regions of myofibers and could be seen only when the sarcolemmal surface is very close to the plane of section. Indeed, we were able to find some limited areas on tissue sections of adult chicken thigh skeletal muscle, where short aciculin striations were seen near the sarcolemma (Fig. 7E, arrows). However, we did not observe any aciculin staining of entire skeletal muscle Z-discs on these tissue sections (Fig. 7E,F), therefore suggesting the presence of aciculin at costameres, actin filament-membrane attachment sites, flanking sarcomere Z-discs. Double staining of glycerinated chicken skeletal muscle myofibrils with antibodies to aciculin and α-actinin showed that, unlike α-actinin, aciculin is not an internal component of myofibrils (Fig. 7A,B) and, apparently, is accumulated in the vicinity of the sarcolemma within the skeletal muscle fibers, as are some other adherens junction proteins, namely vinculin and talin (Pardo et al., 1983; Belkin et al., 1986).

To analyze the potential localization of aciculin at the postsynaptic membrane of neuromuscular junctions, we double-stained cultured C2C12 myotubes, as well as embryonic and adult skeletal muscle tissue sections, with α-bungarotoxin and antibodies to aciculin. In cultured mouse myotubes, we often observed some codistribution of aciculin with clusters of acetylcholine receptors (arrows in Fig. 8A,B). Nevertheless, we did not see any detectable aciculin accumulation at neuromuscular junctions in either embryonic or adult chicken thigh skeletal muscle (arrowheads in Fig. 8C-F).

DISCUSSION

Aciculin expression in skeletal muscle is differentiation-dependent and upregulated during development

Our previous work showed that aciculin is a novel cytoskeletal protein, highly homologous to the glycolytic enzyme phosphoglucomutase (PGM1), and localized in different types of adherens junctions in muscle and nonmuscle cells. We demonstrated that this PGM1-related cytoskeletal protein is expressed

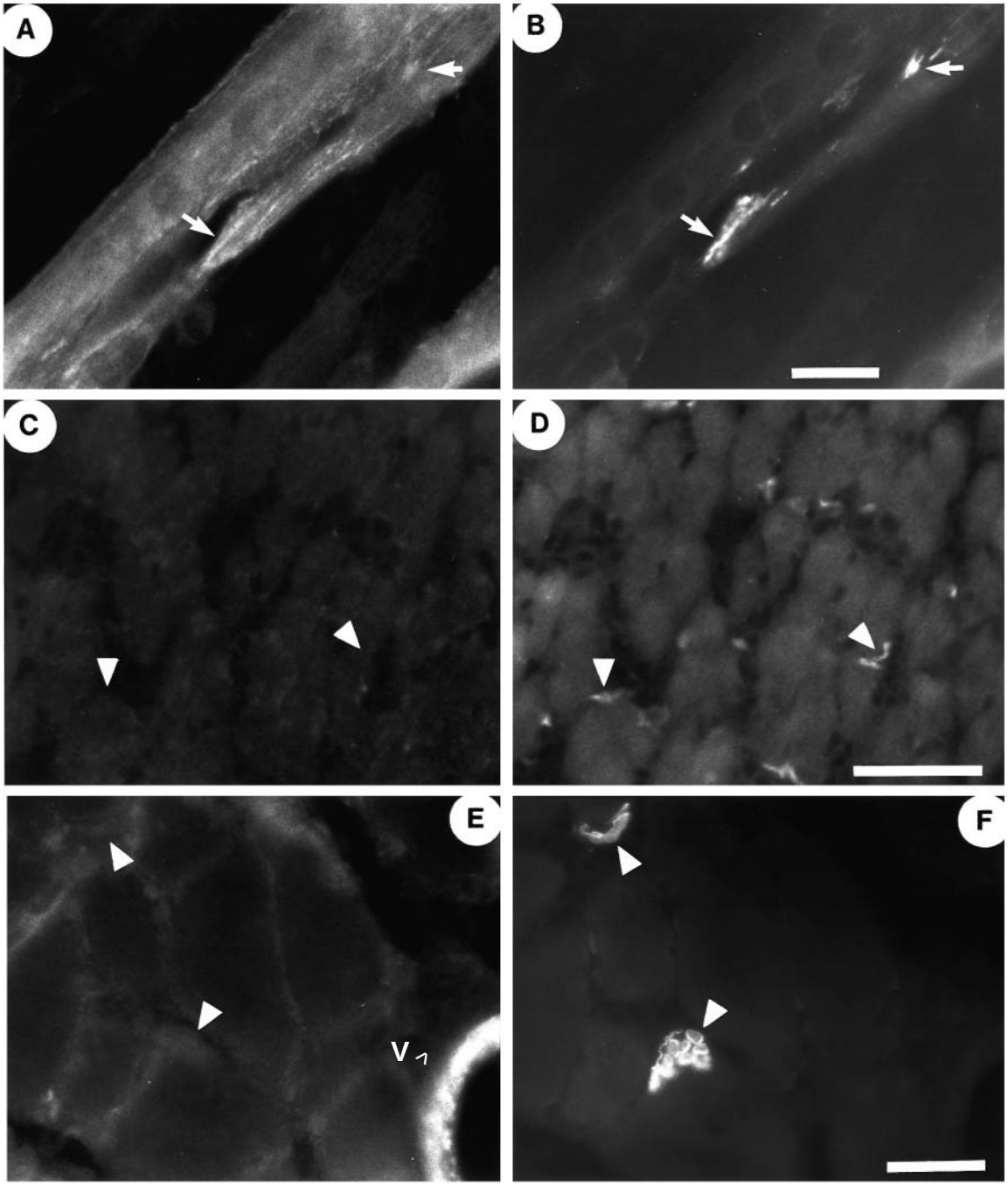

Fig. 8. Absence of aciculin accumulation at neuromuscular junctions. An 8-day culture of C2C12 myotubes (A,B) or 5 μm cryostat sections of 16-day embryonic (C,D) or adult (E,F) chicken thigh skeletal muscle were co-stained with rhodamine aciculin (A,C,E) and fluorescein-conjugated alpha-bungarotoxin (B,D,F). Note partial codistribution of aciculin localization with clusters of acetylcholine receptor in C2C12 muscle cultures (arrows in A and B), but lack of specific aciculin accumulation at neuromuscular junctions of embryonic and adult skeletal muscle (arrowheads in C-F). V, blood vessel. Bars, 20 μm.

most abundantly in smooth muscle tissues and cells and, to a lesser extent, in striated muscle, whereas only some types of nonmuscle cells synthesize detectable amounts of the protein in vivo or in culture conditions (Belkin et al., 1994). In skeletal muscle, both in culture and in vivo, the only prominent aciculin immunoreactive band migrated at approximately 60 kDa on SDS gels. This band corresponded to the upper band of a closely disposed doublet of 60/63 kDa found earlier in smooth and cardiac muscle (Belkin et al., 1994).

Here we report that aciculin expression in skeletal muscle strictly depends on the differentiation stage of skeletal muscle cells both in cultured skeletal muscle and in vivo. By both immunoblotting and immunofluorescence, little detectable expression of the protein could be seen before cell fusion began

in C2C12 cultures. Following fusion, the relative content of aciculin increased progressively in myotubes throughout their subsequent differentiation and maturation. Elevation of aciculin expression during the differentiation and myofibrillogenesis in muscle cell culture was accompanied by the synthesis of various muscle-specific cytoskeletal proteins, including the muscle-specific vinculin isoform, metavinculin (Fig. 2D). However, by immunofluorescence, we were not able to detect any aciculin staining in replicating presumptive myoblasts or postmitotic mononucleated myoblasts, expressing various early muscle-specific cytoskeletal markers, such as desmin, skeletal muscle myosin heavy chain or titin (Hill et al., 1986; Furst et al., 1989; Colley et al., 1990; van der Ven et al., 1993). This observation may indicate that aciculin synthesis starts relatively late in myodifferentiation. However, some aciculin still could be expressed in postmitotic myoblasts in culture, but remained undetected by immunofluorescence because it had not assembled into prominent cytoskeletal structures.

Analysis of skeletal muscle differentiation in vivo showed that a major increase in aciculin expression occurred during late stages of prenatal and early postnatal development. In general, the pattern of aciculin expression during chicken skeletal muscle development in vivo was consistent with that observed in cultured differentiating C2C12 myocytes, except that some aciculin in skeletal muscle was detected at relatively early stages of embryogenesis (day 7 - day 11). We cannot exclude that some of the aciculin in these samples derived from contaminating smooth muscle cells. However, extremely low metavinculin content in these samples makes this possibility unlikely. Taken together, our data show that aciculin expression in skeletal muscle both in culture and in vivo is differentiation-dependent, upregulated during development and similar to that of various muscle-specific cytoskeletal proteins (reviewed by Fischman, 1986).

Aciculin is a novel cytoskeletal component of various cell-matrix adherens-type junctions in skeletal muscle

Both in cultured muscle cells and in vivo, aciculin predominantly accumulates at the tips of differentiating myofibers. These sites within skeletal muscle fibers link the termini of growing myofibrils to the membrane and adjacent extracellular matrix (Tidball, 1991). Appearance of aciculin at these junctional structures was observed relatively early in myogenesis and its localization at the tips of cultured myotubes and the myotendinous junctions in vivo remained very prominent throughout all the subsequent stages of myodifferentiation. This observation points to a potential aciculin involvement in the linking of myofibrils to the sarcolemma at these sites.

By immunofluorescence staining, we showed that aciculin is localized in focal contacts of cultured myotubes, but is not present in myoblasts' focal contacts. This observation, in agreement with our previous data, demonstrates that this newly identified cytoskeletal protein is not a ubiquitous component of cell-matrix adhesions in various cell types (Belkin et al., 1994). At present, it remains unclear why some nonmuscle cells contain aciculin in focal adhesions, whereas some others obviously lack this cytoskeletal protein.

In addition, some aciculin concentration was noticed aligned with Z-discs, apparently at structures linking Z-discs to the sarcolemma. These junctional structures, termed costameres, represent sites of lateral contact between the myofibrils and sarcolemma (Pardo et al., 1983) and, besides aciculin, contain several other cytoskeletal components, including vinculin (Pardo et al., 1983), talin (Belkin et al., 1986), spectrin (Bloch and Morrow, 1989) and dystrophin (Porter et al., 1992; Straub et al., 1992). However, it should be mentioned, that immunofluorescence with anti-aciculin revealed considerably less intense staining of costameres, compared with other contact structures in skeletal muscle, such as myotendinous junctions.

All these cell-matrix adherens-type junctions, including myotendinous junctions, focal contacts and costameres, are known to be sites of force transmission from the contractile apparatus to the extracellular matrix (Burridge et al., 1988; Tidball, 1991; Danowski et al., 1992). The localization of aciculin at these cell-matrix contact structures in skeletal muscle suggest a role for aciculin in the interactions between the thin filaments and the sarcolemma and, potentially, force transmission at these particular sites in skeletal muscle fibers. Notably, aciculin was not detected at the neuromuscular junctions of skeletal muscle, where all the other major cytoskeletal components of adherens junctions were earlier identified (Shear and Bloch, 1985; Sealock et al., 1986; Turner et al., 1991; Bockholt et al., 1992). Neuromuscular junctions represent a functionally distinct type of contact structure in skeletal muscle, mediating the complex intercellular interaction between the nerve and muscle cell. Thus, our data indicate a localization of aciculin at a subset of cell-matrix contacts of skeletal muscle fibers and represent a new example of intracellular sorting of adherens junction proteins among the various junctional structures.

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