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Characterization of Muscle Filamin Isoforms Suggests a Possible Role of γ-Filamin/ABP-L in Sarcomeric Z-Disc Formation

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Filamin, also called actin binding protein-280, is a dimeric protein that cross-links actin filaments in the cortical cytoplasm. In addition to this ubiquitously expressed isoform (FLN1), a second isoform (ABP-L/γ-filamin) was recently identified that is highly expressed in mammalian striated muscles. A monoclonal antibody was developed, that enabled us to identify filamin as a Z-disc protein in mammalian striated muscles by immunocytochemistry and immunoelectron microscopy. In addition, filamin was identified as a component of intercalated discs in mammalian cardiac muscle and of myotendinous junctions in skeletal muscle. Northern and Western blots showed that both, ABP-L/γ-filamin mRNA and protein, are absent from proliferating cultured human skeletal muscle cells. This muscle specific filamin isoform is, however, up-regulated immediately after the induction of differentiation. In cultured myotubes, ABP-L/γ-filamin localises in Z-discs already at the first stages of Z-disc formation, suggesting that ABP-L/γ-filamin might play a role in Z-disc assembly.Cell Motil. Cytoskeleton 45:149–162, 2000.

Key words: ABP-280; filamin; ABPL; sarcomere; Z-disc; myofibrillogenesis; monoclonal antibody

INTRODUCTION

The functional complexity of actin in living cells is largely determined by a vast number of actin-binding proteins. Actin-crosslinking proteins, for instance, create either stable bundles of parallelly arranged actin filaments or stabilize two- and three-dimensional networks of considerable elastic properties [Janmey, 1991]. A very versatile member of this group of actin-crosslinking proteins is filamin [Wang et al., 1975; also called actin binding protein ABP-280, Brotschi et al., 1978]. Filamin seems to be capable of stabilizing both actin filament bundles and networks: on the one hand, filamin from smooth muscle was described to cause excessive bundling of actin filaments in in vitro experiments [Wang and Singer, 1977; Brotschi et al., 1978]. This might help to explain the organization of cytoskeletal and contractile

Abbreviations used: DMEM, Dulbecco«s modified Eagles medium; DTT, dithiothreitol; EDTA, ethylendiaminotetraacetat;EGTA, ethylenglycol-bis-(2-aminoethylether)-N, N, N′,N′-tetraacetate; FCS, fetal calf serum; kbp, kilo base pairs; kDa, kilo Dalton; LSB, low salt buffer; mAb, monoclonal antibody; NEAA, non-essential amino acids; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonylfluoride; SDS, sodiumdodecylsulphate,

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domains that were identified in smooth muscle [Small et al., 1986; Tachikawa et al., 1997]. On the other hand, the prominent cortical network of actin filaments found, for instance, in blood platelets seems to be crosslinked by filamin. In addition, this network is anchored to the plasma membrane by the interaction of filamin with a transmembrane complex of glycoproteins, which acts as the receptor for the von Willebrand factor [Fox, 1985; Okita et al., 1985; Meyer et al., 1997]. More recently, a series of other ligands for filamin was identified, such as SEK-1 [Marti et al., 1997], β1-integrin [Loo et al., 1998], β2-integrin [Sharma et al., 1995], furin [Liu et al., 1997], and presenilin-1 [Zhang et al., 1998] strongly suggesting that filamin plays a key regulatory role in the organization of the actin cytoskeleton.

Even greater complexity is added by the fact that three human filamin isoforms have been described until now: The first one is the ubiquitously distributed filamin, also called ABP-280 [Wang et al., 1975; Brotschi et al., 1978; Gorlin et al., 1990]. Its gene (FLN1) was mapped to the chromosomal position Xq28 [Gorlin et al., 1993; Gariboldi et al., 1994]. A second isoform was identified by PCR analysis and called ABP-L [Maestrini et al., 1993]. Its gene (FLN2) was localized on chromosome 7q32-q35 [Gariboldi et al., 1994]. The complete cDNA sequence was established recently and was named γ-filamin [Xie et al., 1998]. Since both ABP-L and γ-filamin describe the same cDNA/protein, both names can be considered synonyms. Almost at the same time, a third isoform, named β-filamin was identified as encoding a ligand of glycoprotein Ib alpha by yeast two hybrid screening [Takafuta et al., 1998; Xu et al., 1998]. All three filamin isoforms show an essentially identical molecular building principle: an aminoterminal actin binding domain is followed by 24 repeats, all of which show an immunoglobulin-like fold [Hock, 1999]. The carboxy-terminally situated domain is involved in filamin dimer formation [Davies et al., 1980; Gorlin et al., 1990].

While filamin was detected in avian muscle samples by some antibodies [Koteliansky et al., 1981; Price et al, 1994], it has thus far escaped antibody detection in mammalian muscles [see e.g. Brown and Binder, 1993]. Since the human filamin/ABP-280 gene was mapped to distal Xq28 [Gorlin et al., 1993], it was included in a search for candidate genes for muscle diseases that mapped to Xq28 in genetic linkage analysis. This search revealed not only alternative splicing in the filamin mRNA, but also identified by PCR analysis two small fragments of a second obviously muscle-specific isoform that was called actin-binding protein-like protein [ABP-L; Maestrini et al., 1993] or γ-filamin [Takafuta et al, 1998] and that was preliminarily mapped to chromosome 7q32-q35 [Gariboldi et al., 1994].

Here we have independently identified and characterized the ABP-L/γ-filamin isoform and established that it is highly expressed in mammalian striated muscles. The protein was purified to homogeneity from bovine skeletal muscle. With a newly developed mAb that recognizes both the mammalian non-muscle and muscle filamin isoforms, we show that the N-terminus of ABP-L/γ-filamin is localized at the periphery of the Z-disc. Analysis of filamin expression by immunoblotting as well as immunofluorescence microscopy, shows that ABP-L/γ-filamin is expressed during the very first stages of myocyte differentiation in vitro. Since ABP-L/γ-filamin localizes to developing Z-discs at a very early stage, we conclude that this filamin isoform might be involved in the formation of sarcomeric Z-discs.

MATERIALS AND METHODS

Antibodies

Our mAb RR90 resulted from a fusion using Balb/c mice that were immunized with a recombinant fragment from the M band portion of human cardiac titin following standard protocols [see Fürst et al., 1988; Obermann et al., 1996]. In the primary screen using immunofluorescence microscopy on frozen sections of rat psoas muscle, the supernatant of this hybridoma exhibited a cross-striated pattern, and in Western blots it exclusively recognized a polypeptide of ~300 kDa molecular mass. Hybridomas were made monoclonal by limiting dilution.

The monoclonal α-actinin antibody BM-75.2, recognising all α-actinin isoforms [Abd-el-Basset et al., 1991], was purchased from Sigma (Deisenhofen, Germany). The polyclonal desmin antiserum [Ramaekers et al., 1985] was a kind gift of Dr. G. Schaart (Maastricht, The Netherlands). The anti-skeletal muscle α-actinin rabbit serum (RbaA653) was raised against purified porcine skeletal muscle α-actinin (a kind gift of M. Gimona, Institute for Molecular Biology, Salzburg, Austria) using standard procedures. A summary of the further characterization of the two new antibodies used in this study (RR90 and RbaA653) is given in Table I.

Purification and Proteolytic Fragmentation of Filamin From Bovine Skeletal Muscle

Bovine skeletal muscle (*M. iliacus*) was removed immediately after slaughter, chopped into small pieces, and frozen in liquid nitrogen. This material was kept at −80°C until use. Four hundred grams of tissue were homogenized at 4°C in 3 l of low salt buffer (LSB: 100 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 1 mM NaN_3, 10 mM Tris-maleate, pH 6.8) containing 2 mM $Na_4P_20_7$. This and all subsequent buffers were supplemented with the following protease inhibitors: 0.5 mM PMSF, 10 mg/l Trypsin inhibitor (Sigma, T 9253), 5 μM

TABLE I. Characterization of the New Antibodies Used in This Study

Name (species, Ig type)	RR90 (mouse IgA)	RaA653 (rabbit polyclonal)
Reactivity		
Human skeletal muscle	+++	+++
Rat skeletal muscle	+++	±
Rat cardiac muscle	++	±
Mouse skeletal muscle	++	±
Bovine skeletal muscle	++	n.d.
Porcine cardiac muscle	n.d.	+++[1]
Chicken skeletal muscle	−	−
HSkM[2], proliferating	+++	−
HSkM, differentiated	+++	+++
HeLa	+++	−
NIH:3T3	++	−
Ptk2	++	−

[1]Schaart et al., [1997]
[2]Cultured primary human skeletal muscle cells

E64. Myofibrillar material was collected by centrifugation (3,000*g*, 15 min) and washed three times with 2 l of LSB. The final pellet was resuspended in 2 l extraction solution (600 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 1 mM NaN_3, 50 mM Tris-HCl, pH 7.9) and stirred for 45 min. After centrifugation (20,000*g*, 60 min), the supernatant was dialyzed overnight against two changes of hydroxyl apatite buffer 1 (HA1 buffer: 40 mM K-phosphate pH 7.0, 100 mM NaCl, 1 mM EGTA, 1 mM DTT) and then applied to a 6 × 6 cm hydroxyl apatite column (BioRad) equilibrated in the same buffer. After washing with buffer HA1, the protein was eluted with hydroxyl apatite buffer 2 (150 mM K-phosphate pH 7.0, 300 mM NaCl, 1 mM EGTA, 1 mM DTT). Filamin-containing fractions (detected by SDS gel electrophoresis) were pooled, dialyzed against buffer Q (50 mM Tris-HCl pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT), and applied to a 1.6 × 25 cm Q-Sepharose Fast Flow column (Pharmacia, Freiburg, Germany) connected to an FPLC system (Pharmacia). Elution was done with a gradient from 50 to 500 mM KCl in 500 ml buffer Q. Fractions containing filamin were pooled, dialyzed against buffer S (50 mM Tris-HCl pH 7.9, 1 mM EDTA, 1 mM EGTA, 1 mM DTT), and loaded onto a 1 × 10 cm S-Sepharose Fast Flow column (Pharmacia). After washing with buffer S, filamin was eluted with a linear salt gradient from 0 to 500 mM KCl in 80 ml buffer S. Finally, filamin was purified to homogeneity by gel filtration on a Superose 6 HR10/30 column (Pharmacia) equilibrated in buffer Q. All purification steps were carried out at 4°C. Approximately 1 mg of pure filamin was obtained from 200 g of frozen muscle.

For proteolytic fragmentation, filamin was dialyzed against 150 mM K-phosphate buffer pH 7.0 containing 300 mM NaCl, and incubated at RT for 8 h with endoproteinase Asp-N (molar ratio 1:100). Resulting fragments were characterized by SDS-PAGE, immunoblotting [Fürst et al., 1988, 1989], and determination of their aminoterminal sequences. An Applied Biosystems sequenator (model A470) and a Knauer Sequenator (model 810), both equipped with on-line PTH amino acid analyzers were used for automated sequencing. All methods employed were described previously [Bauw et al., 1987; Fürst et al., 1992; Obermann et al., 1995].

Cell Culture and Immunofluorescence Miscroscopy

Human skeletal muscle cells were isolated and cultured essentially as described before [van der Ven et al., 1992, 1993]. Briefly, enzymatically isolated satellite cells from normal human skeletal muscle biopsies, passaged two to five times and frozen in liquid nitrogen, were quickly thawed and plated on glass coverslips in DMEM supplemented with 20% FCS, 2% Ultroser G, and 100 U/ml penicillin and 100 µg/ml streptomycin (all from LifeTechnologies, Eggenstein, Germany). Cells were grown until near confluency. Differentiation of the cells was induced by changing from this high nutrition medium to a low nutrition medium (DMEM, 0.4% Ultroser G, and antibiotics).

Ptk2 cells (obtained from the ECACC, Salisbury, UK) were grown in DMEM supplemented with 10% FCS, non-essential aminoacids (NEAA), 200 mM L-glutamine, antibiotics as above. NIH:3T3 fibrolblasts were cultured in the same medium, except that NEAA were omitted.

For immunofluorescence assays, all cells were fixed in methanol for approximately 5 min and subsequently in acetone for 30 sec, both at −20°C. After air drying, cells were stained immediately or frozen at −80°C until use.

For the localization of cytoskeletal proteins by immunofluorescence microscopy, cells and 5-µm-thick cryosections were stained using standard procedures. Secondary antibodies, directed against respective species and appropriate Ig-subtype and conjugated with fluorescein isothiocyanate or Texas Red, were purchased from Southern Biotechnology Associates (Birmingham, AL) and diluted in PBST according to the recommendations of the manufacturer.

Immunoelectron Microscopy

Preparation of fiber bundles from rat psoas muscle and antibody labeling was performed as described [Fürst et al., 1988]. An immunogold conjugate (5 nm Gold) of goat anti mouse IgG (British Bio Cell International) was used undiluted as second antibody. Electron micrographs were taken on a Philips electron microscope CM12 at an accelerating voltage of 80 kV.

Miscellaneous Procedures

SDS-PAGE and immunoblotting were performed as described [Fürst et al., 1988]. Northern blotting was done as described [Steiner et al., 1998]. As probe we used a ~300 bp sequence, which is specific for the γ-filamin/ABP-L isoform. The region situated beween bp 6,406 and 6,648 of the sequence described in the EMBL database under accession number AJ012737 was amplified by PCR [Saiki et al., 1985]. The PCR product was purified by agarose gel electrophoresis and radiolabeled as described [Steiner et al., 1998].

RESULTS

Characterization of Two Novel Antibodies

A fusion with lymphocytes from Balb/c mice that were immunized with a recombinant titin fragment also yielded a hybridoma (called RR90) that produced antibodies staining sections of skeletal muscle in a cross-striated pattern in immunofluorescence microscopy, and labeling exclusively a ~300 kDa polypeptide in immunoblots. Reactivity was found in a variety of muscle and non-muscle tissues of mammalian but not of avian origin (summarized in Table I). The lack of crossreactivity with titin implied that the hybridoma resulted most likely from an autoantibody of the mouse that was used for immunization. This explanation is also supported by the unusual finding that RR90 is of the IgA immunoglobulin subtype that is usually secreted from mucosal tissues.

The reactivity in immunoblots with a band of molecular mass close to 300 kDa suggested that the respective protein might be filamin. This idea was confirmed by purifying the reactive polypeptide to homogeneity and by subsequent peptide sequencing of its proteolytic fragments. The purification from bovine skeletal muscle is summarized in Figure 1. Our protocol involved the following major steps: high salt extraction of washed myofibrils, dialysis against a low salt buffer to remove myosin and most of the titin, hydroxylapatite chromatography to remove H-protein and phosphorylase, Q-Sepharose ion exchange chromatography to remove C-protein, S-Sepharose ion exchange chromatography to remove most of the M-protein and to concentrate the immunoreactive protein. The final gel filtration step removed residual M-protein and some lower molecular mass contaminants. Digestion of a crude filamin preparation with endoproteinase Asp-N yielded two major cleavage products with molecular masses ~180 and 45 kDa, respectively (Fig. 2a, lane 2). Direct sequencing of the 180 kDa band gave the N-terminal sequence DANKVSARGPGLEPVGNVANVANXXXYF. Since the amino-terminus of the 45 kDa fragment was blocked, the fragment was subjected to a digestion with BrCN. As a result, we obtained the peptide sequence TYLSQFP. A

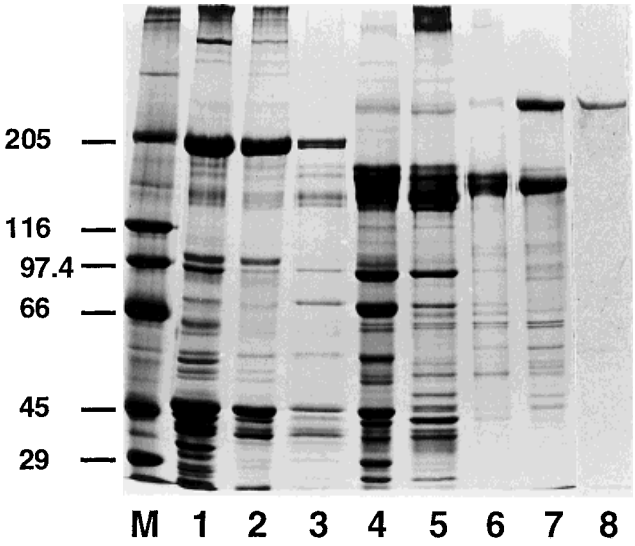

Fig. 1. Purification of filamin from a mammalian cross-striated muscle. The gel (4 to 15 % acrylamide) monitors the purification of bovine skeletal muscle filamin: bovine *M. iliacus* whole muscle tissue (**lane 1**), washed myofibrils (**lane 2**), 0.6 M KCl extract (**lane 3**), supernatant after dialysis against a low salt buffer (**lane 4**), pooled fractions from hydroxyl apatite column (**lane 5**), fraction from Q-Sepharose column (**lane 6**), crude filamin after MonoS column (**lane 7**), and purified filamin after gel filtration (**lane 8**). M_r standards ($\times 10^3$) are indicated in **lane M**.

search of EMBL and SWISS-PROT databases revealed that both sequences exhibited homology exclusively with the translated cDNA sequences of human actin binding protein ABP-280 [non-muscle filamin; Gorlin et al., 1990], human β-filamin [Takafuta et al., 1998], and chicken filamin [Barry et al., 1993] and the protein sequence of ABP-L/γ-filamin [Xie et al., 1998]. This implied that the purified protein was indeed filamin. The alignment of both sequences with the complete filamin sequences from the database allowed the proteolytic bands to be correlated with the domain structure of filamin. Since the short 7 residue sequence starts at residue 259 of ABP-280 [Gorlin et al., 1990], the 45 kDa band essentially comprises the amino-terminally located actin-binding domain plus the first of the 24 repeat domains of filamin. The longer 28 residue sequence aligns with the sequence of filamin starting at position 380. Thus, the 185 kDa band of the digest covers most of the extended rod portion following the actin-binding site (Fig. 2b).

In immunoblots, RR90 reacted specifically with purified filamin or with the 45 kDa fragment of the endoproteinase Asp-N fragment (Fig. 2). The characterization of proteolytic bands described above therefore allowed us to restrict the epitope of RR90 to the region spanning the actin-binding domain plus the first of the 24 filamin repeats.

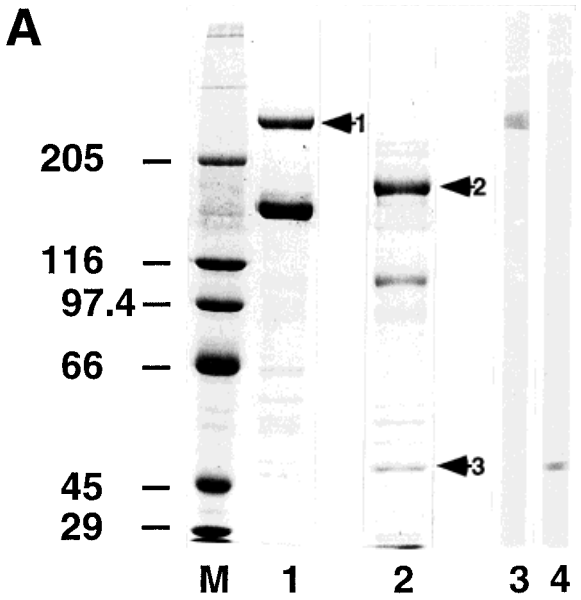

Fig. 2. Proteolytic cleavage of filamin and alignment of peptide sequences. **A:** Crude skeletal muscle filamin (band marked by *arrowhead* 1 in **lane 1**) was treated with endoproteinase Asp-N. The limited digest was separarated by SDS-PAGE (**lane 2**) and transferred to nitrocellulose as described in Materials and Methods. Immunoreactivity of these samples with mAb RR90 is shown in **lanes 3** and **4**, respectively. Note that filamin is cleaved by endoproteinase Asp-N into two main fragments (marked by *arrowheads* 2 and 3 in lane 2) and that only the smaller, 45 kDa fragment is recognized by the antibody (see lane 4). **B:** Alignment of peptide sequences derived from the proteolytic cleavage of bovine muscle filamin (upper lines) with published translated cDNA sequences: human ABP-280 [Gorlin et al., 1990; second lines], human β-filamin [Takafuta et al., 1998; third lines], and chicken filamin [Barry et al., 1993; bottom lines].

To characterize the suitability of this new hybridoma for localization studies, we analyzed the subcellular distribution of filamin in the non-muscle cell lines Ptk2 and NIH:3T3 by immunofluorescence microscopy. In these cells, RR90 predominantly stained stress fibers in a spotty to continuous pattern (Fig. 3A,C,E). Furthermore, a weak but specific staining of adhesion plaques and leading edges of the cells was observed. Invariably, filamin was found to be localized in conjunction with α-actinin (Fig. 3).

The rabbit α-actinin antiserum RbaA653 was shown to be specific for the striated muscle isoforms of α-actinin by Western blotting and by immunofluorescence microscopy. Western blots using extracts from proliferating and differentiated human skeletal muscle cells were incubated with this novel α-actinin serum and with mAb BM75–2, which is known to recognize all α-actinin isoforms [Abd-el-Basset et al., 1981]. The latter antibody strongly stained an approximately 100 kDa protein in both cell extracts, whereas RbaA653 stained a protein of equal molecular mass exclusively in extracts from differentiated muscle cells (Fig. 4). The specificity of the rabbit antiserum was confirmed by immunofluorescence assays on frozen sections of rat muscles and on fixed cultured human skeletal muscle cells. Thus, RbaA653 stained Z-discs of all muscle fibers, irrespective of fiber type (not shown; summarized in Table I). In developing muscle cells, this antiserum was only positive after the induction of differentiation. In contrast, mAb BM75–2 also reacted with undifferentiated cells (Fig. 5).

Localization of Filamin in Adult Striated Muscle Tissue

Immunolabelling of frozen sections of rat cardiac muscle with mAb RR90 revealed that filamin expression was strongest in the smooth muscle cells of blood vessels and in the intercalated discs of cardiomyocytes (Fig. 6A). The cross-striated staining of myofibrils in cardiomyocytes was evident at higher magnifications (Fig. 6B). Double immunofluorescence using mAb RR90 in combination with a desmin antiserum confirmed that the filamin label occured at the Z-disc (Fig. 6B,C). Striking was also the colocalization of filamin and desmin in intercalated discs, resulting in intense labelling at the cytoplasmic face of the sarcolemma at these sites (Fig. 6B,C).

In frozen sections of rat striated muscles, we observed strong staining of Z-discs that were identified by double staining with an antibody specific for the Z-disc region of titin, in all fibers (Fig. 6E,F). In addition, myotendinous junctions were strongly labelled (Fig. 6D).

Aminoterminus of Filamin Maps to the Ends of Thin Filaments

To determine where the aminoterminal end of filamin is situated at the sarcomere level, we investigated Triton X-100 extracted fiber bundles of rat psoas muscle by immunoelectron microscopy using mAb RR90. This resulted in increased density at the edge of the Z-disc comb leaving the central Z-disc portion unlabelled. Since in previous studies we had obtained antibody decoration closer to the Z-disc centre [Fürst et al., 1988], we concluded that we did not face penetration problems in this experiment. For better visualization, however, we also used gold-coupled secondary antibodies. Digitized electron micrographs were recorded with a CCD camera and analyzed as described in Materials and Methods.

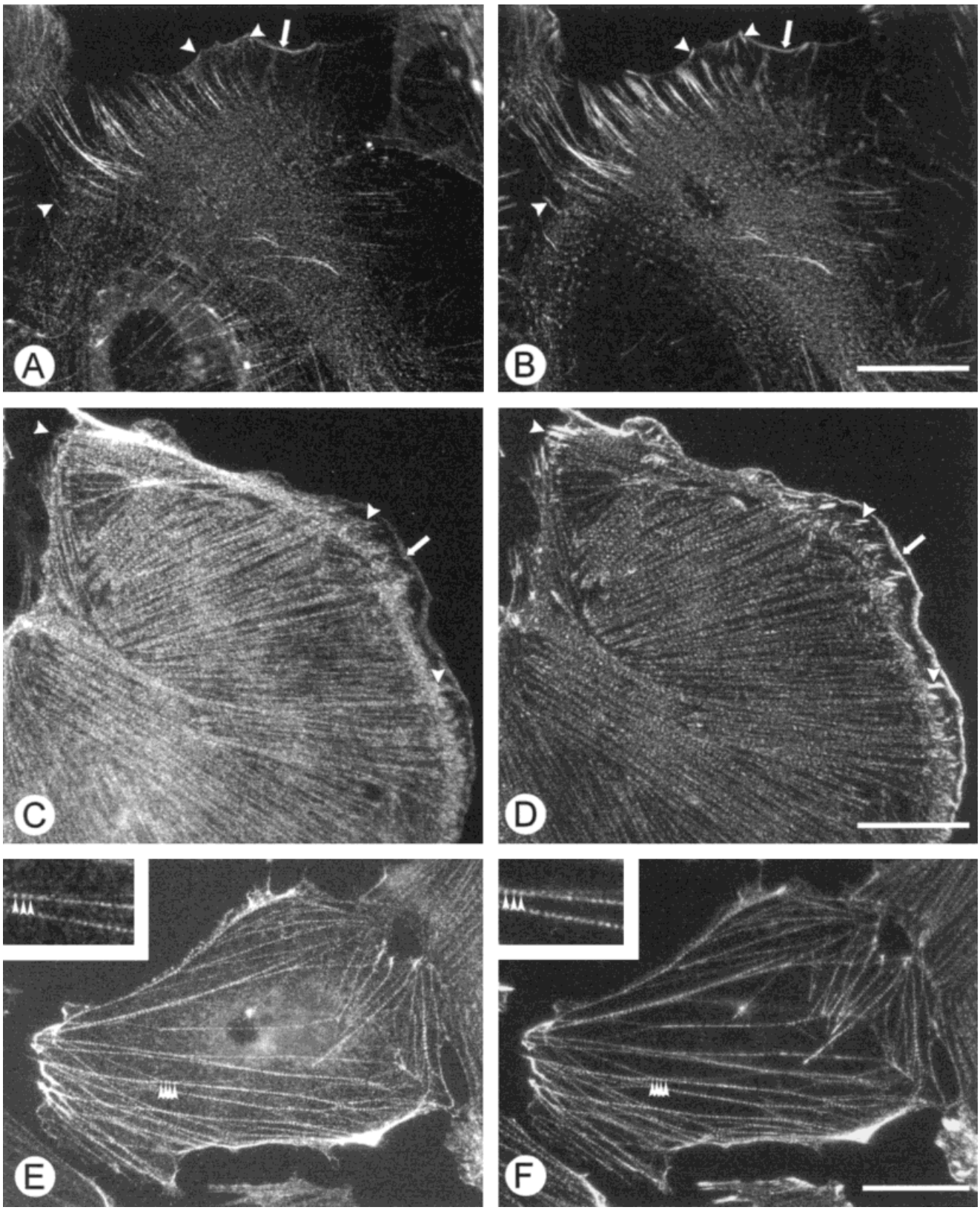

Fig. 3. Immunolocalization of filamin in non-muscle cells. NIH3T3 (**A, B**) or Ptk2 (**C–F**) cells were double stained for filamin (RR90; A, C, E) and α-actinin (BM 75–2; B, D, F). Within the cytoplasm of both cell types RR90 stains stress-fibers discontinuously (A, C, E). At exactly the same positions α-actinin is localized (*arrowheads* in E, F). Furthermore, filamin colocalizes with α-actinin in adhesion plaques (*arrowheads* in A to D) and in leading edges (*arrows* in A–D). Bar = 20 μm.

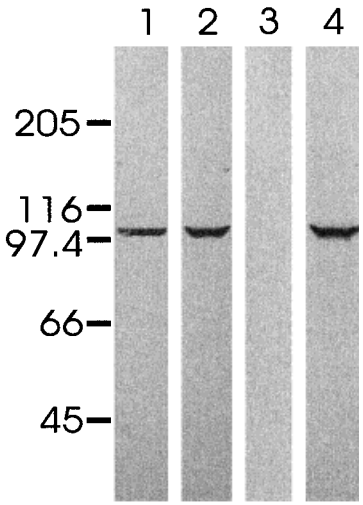

Fig. 4. Characterization of a novel α-actinin antibody (RbaA673) by immunoblotting. Total extracts of proliferating myoblasts (**lanes 1** and **3**) and differentiated myotubes (**lanes 2** and **4**) were separated in 8% SDS-PAGE and transfered to nitrocellulose. Lanes 1 and 2 give the reactivity with antibody BM-75.2 that recognizes all α-actinin isoforms. Lanes 3 and 4 show labeling with rabbit serum RbaA673 that was raised against porcine α-actinin. Note that BM-75.2 reacts with both non-muscle and muscle isoforms of α-actinin, while the reactivity of RbaA673 is limited to sarcomeric α-actinin, which is not expressed in undifferentiated myoblasts.

Figure 7 shows specific decoration at both edges of the Z-disc. The precise position of antibody label was determined by image analysis and the resulting histogram fitted a Gaussian distribution. Thus, the aminoterminal epitope of filamin that is visualized with mAB RR90 and gold tagged antibody, was revealed as two symmetrical peaks in a distance of ~50 nm from the Z-disc center (see histogram in Fig. 7c). Both peaks therefore coincide with the end of the Z-disc comb, which corresponds to the ends of thin filaments from the opposite sarcomere half.

Occurrence of Filamins During Skeletal Muscle Cell Differentiation

The bona fide cross-reacitivity of mAb RR90 with at least two of the mammalian filamin isoforms known to date, was used to monitor their temporal expression patterns in human myocyte development. Initially, total protein extracts of adult muscle separated on SDS-polyacrylamide gels, which provide an optimal resolution of high molecular mass polypeptides, were used to screen for filamins in immunoblots using mAb RR90. This consistently revealed a closely spaced doublet migrating at a molecular mass close to 300 kDa. This finding was taken as a starting point to analyze the time course of the expression of these filamin isoforms during the development of human skeletal muscle cells in culture. Thus, in extracts from undifferentiated proliferating myoblasts, mAb RR90 showed reactivity exclusively with a single band comigrating with the higher molecular mass filamin isoform (Fig. 8). We concluded that this reflected reactivity with the ubiquitously distributed non-muscle filamin isoform ABP-280. Subsequently, samples taken at 8-h intervals after the induction of differentiation were analyzed in the same way. This revealed that as early as 8 h after the onset of differentiation, the second isoform of slightly lower molecular mass could be detected. After one day, approximately equal amounts of both isoforms were detected and this ratio remained constant for the rest of the time period examined (Fig. 8).

Since our mAB RR90 recognizes at least two filamin isoforms, one of which is only expressed in differentiating muscle cells, we wanted to provide further evidence for the specific upregulation of the putative muscle-specific γ-filamin/ABP-L isoform in developing myotubes. This was achieved by Northern blotting using the ~300 bp insertion specific for this isoform (see Materials and Methods) as a probe. Figure 9 shows that expression of the corresponding mRNA could be detected as early as 8 h after the induction of differentiation and remained at approximately the same level during subsequent developmental stages.

Filamin Localization Coincides With Z-Disc Formation

The subcellular distribution of filamin was investigated at different time points representative of myotube development in cell culture. We used double immunofluorescence microscopy with a combination of mAb RR90 and BM 75.2, the α-actinin antibody recognizing all isoforms (''pan α-actinin''), for undifferentiated cells, and a combination of mAb RR90 with RbaA653, the α-actinin serum directed against the striated muscle isoforms, to localize these proteins during the various stages of differentiation. In undifferentiated, proliferating myoblasts filamin was detected in stress fibers, adhesion plaques, and leading edges (Fig. 10A). In these locations, filamin was consistently found to be colocalized with α-actinin (Fig. 10B) and both patterns were reminiscent of the distributions of these proteins in non-muscle cells (for comparison see staining patterns in Fig. 3).

Immediately subsequent to the induction of myocyte differentiation, we observed a striking reorganization of both the filamin and α-actinin decoration patterns: The micrographs in Figure 10C and D revealed a colocalization of filamin with skeletal muscle α-actinin, which was confined to stress fiber-like structures (SFLS). In nascent myofibrils, the label occurred with sarcomere periodicity and lateral alignment of these early Z-discs was evident (Fig. 10E and F). Fully developed myotubes finally reflected the situation observed in skeletal muscle sec-

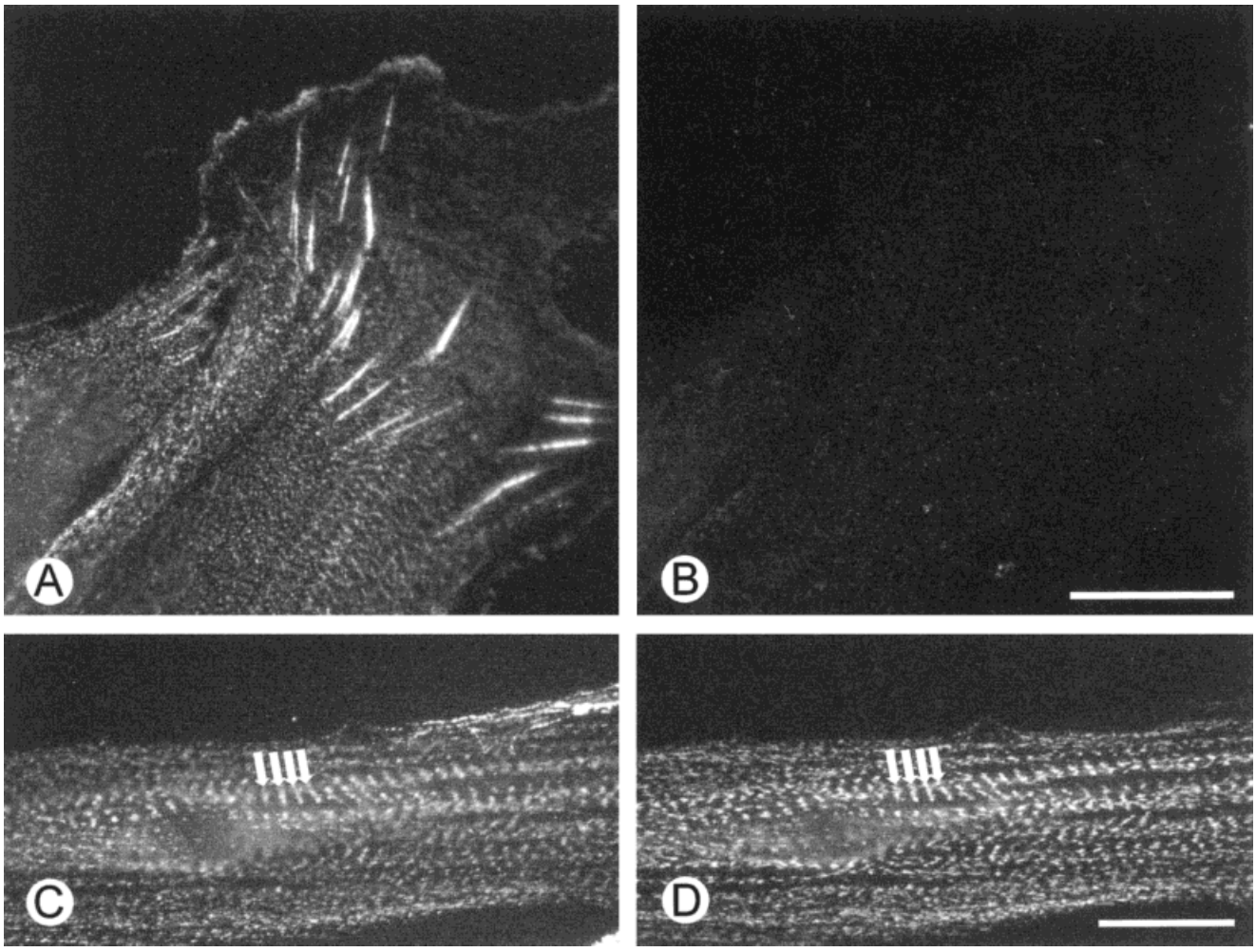

Fig. 5. Characterization of α-actinin antibody RbaA653 by immunofluorescence microscopy. Cultured human skeletal muscle cells were double labeled with α-actinin antibodies BM-75.2 (**A** and **C**) and RbaA653 (**B** and **D**) either in their proliferating, undifferentiated stage (A and B) or as fully differentiated myotubes (C and D). *Arrows* indicate a series of Z-discs in myofibrils which clearly show co-staining with both antibodies. Note that BM-75.2 recognizes α-actinin isoforms being expressed in both developmental stages, while RbaA653 stains α-actinin only in differentiated cells.

tions, i.e., staining of myofibrillar Z-discs coinciding with α-actinin (Fig. 10G and H).

DISCUSSION

Filamin was originally characterized as a protein whose main function seemed to involve cross-linking and membrane anchorage of actin filaments [Hock, 1999]. Recently, however, several reports have raised the possibility of roles for filamin in signal transduction [Marti et al., 1997], in the organization of plasmalemmal receptor molecules [Meyer et al., 1997; Ohta et al., 1991; Sharma et al., 1995] and in the trans-Golgi network [Liu et al., 1997]. It therefore appears that filamin is a very versatile protein with a key function in the transmission of signals to the actin cytoskeleton. Surprisingly little, however, is known about filamin in the tissue with the highest amount of actin, i.e., cross-striated muscle. Previously used antibodies have not detected this protein in mammalian skeletal and cardiac muscles [see Brown and Binder, 1993].

We succeeded in obtaining an antibody that clearly recognizes filamin in mammalian striated muscles and thus allowed for a detailed analysis of this protein during mammalian muscle development. First, the identity of filamin as the antigen for this hybridoma (RR90) was verified by purifying the immunoreacitve protein from bovine *M. iliacus* muscle (Fig. 1). Peptide sequences unequivocally demonstrated that the protein purified was the ABP-L/γ-filamin isoform, which was discovered only

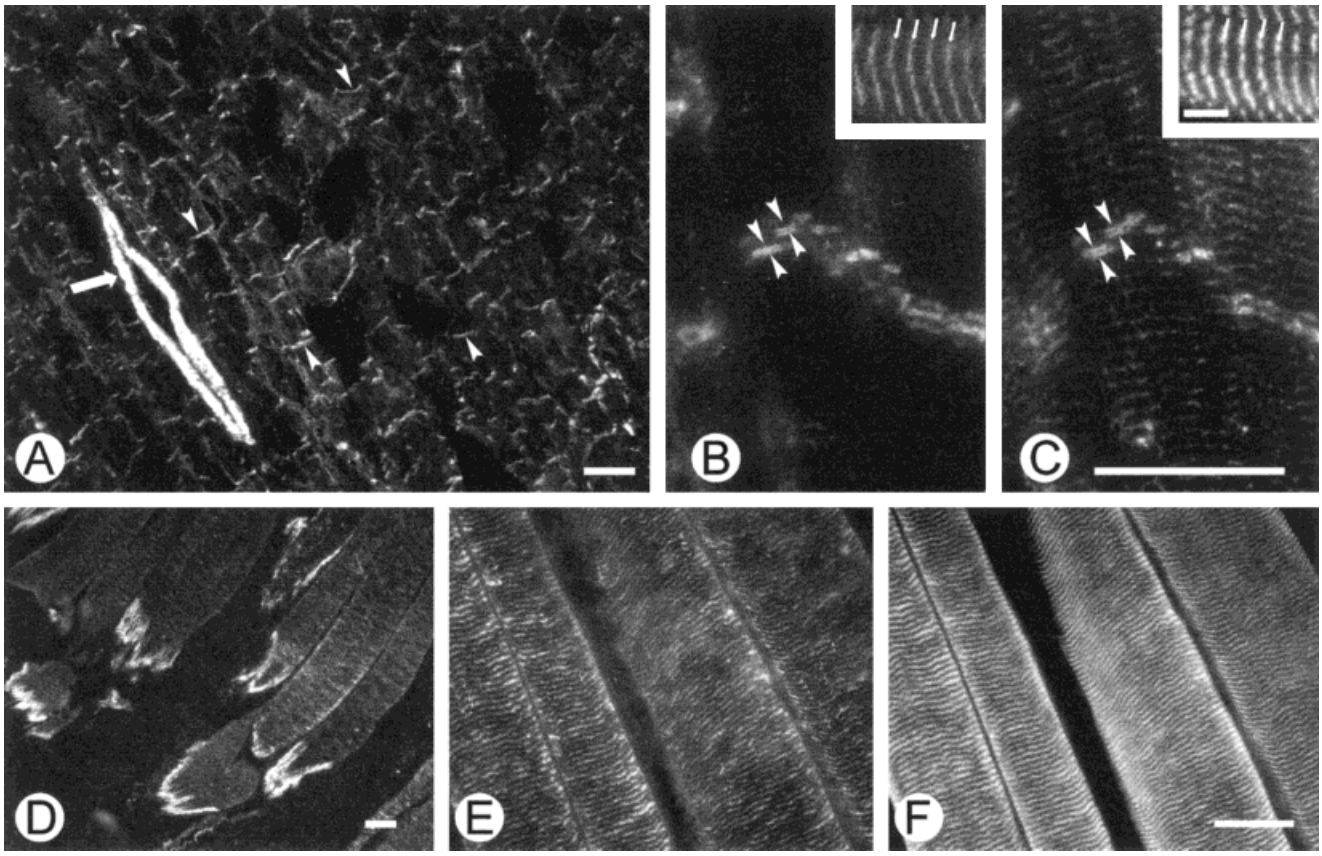

Fig. 6. Immunolocalization of filamin in mammalian striated muscle. Cryosections of adult rat heart (**A–C**) or adult rat M. soleus (**D–F**) were stained with RR90 (A,D), or double stained with RR90 (B,E) and a polyclonal anti-desmin serum (C), or T12 anti-titin (F). In the heart, high levels of filamin were detected in blood vessels (*arrow* in A) and intercalated discs (*arrowheads* in A). At higher magnification, filamin appears to be codistributed with desmin on both sides of the intercalated discs (*arrowheads* in B, C). In addition, staining with RR90 results in a staining of specific regions of the sarcomere that are also stained with the desmin antiserum, identifying these regions as Z-discs (*arrows* in B, C **inset**). In skeletal muscle sections RR90 strongly stains myotendinous junctions (D). Furthermore, staining with RR90 results in a cross-striated staining pattern (E). The same sarcomeric region is stained by anti-Z-disc titin (F). Bar = 25 μm; inset, 5 μm.

recently [Xie et al., 1998]. Immunoblots using proteolytically cleaved protein revealed the epitope of RR90 to be located in the aminoterminally 45 kDa of the 280 kDa polypeptide, i.e., in the actin binding domain and the first one of the 24 repeat domains (Fig. 2).

We used mAb RR90 to determine the location of filamin in mammalian striated muscles by immunofluorescence and immunoelectron microscopy. In both slow and fast skeletal muscle fibers, filamin was found at Z-discs (see Figs. 6 and 7). Striking also was the strong staining of myotendinous junctions (Fig. 6). In cardiac muscle, we also found Z-disc decoration and noted, in addition, very prominent labeling of intercalated discs at the cytoplasmic side of the neighboring cells (Fig. 6). Previous studies on the light microscope level have proposed a desmin-like filamin localization surrounding the myofibrils at the level of Z-discs in avian muscles [Gomer and Lazarides, 1981; Koteliansky et al., 1986]. Two other studies, however, clearly showed decoration of the actual Z-disc [Bechtel, 1979; Koteliansky et al., 1981], identifying filamin as a component of myofibrils. Since the latter high resolution studies agree with the data presented in this report, one can assume that some technical problem involved with cross-sections might have caused the lack of reactivity inside myofibrils in the above-mentioned light microscope work. In addition, our immunoelectron microscopy data now provide increased resolution and, therefore, allow for a more precise interpretation of filamin localization in myofibrils. Antibody decoration occurred ~50 nm from the Z-disc center at the edge of the Z-disc comb. Interestingly, this region corresponds to the end of thin filaments that overlap in the Z-disc and reach approximately 50 nm into the opposite sarcomere half [Vigoreaux, 1994]. These data suggest that the actin binding domain of filamin has a function in cross-linking thin filaments at their ends in myofibrillar Z-discs. In addition, the high concentration of filamin found at myotendinous junctions and intercalated discs implies an

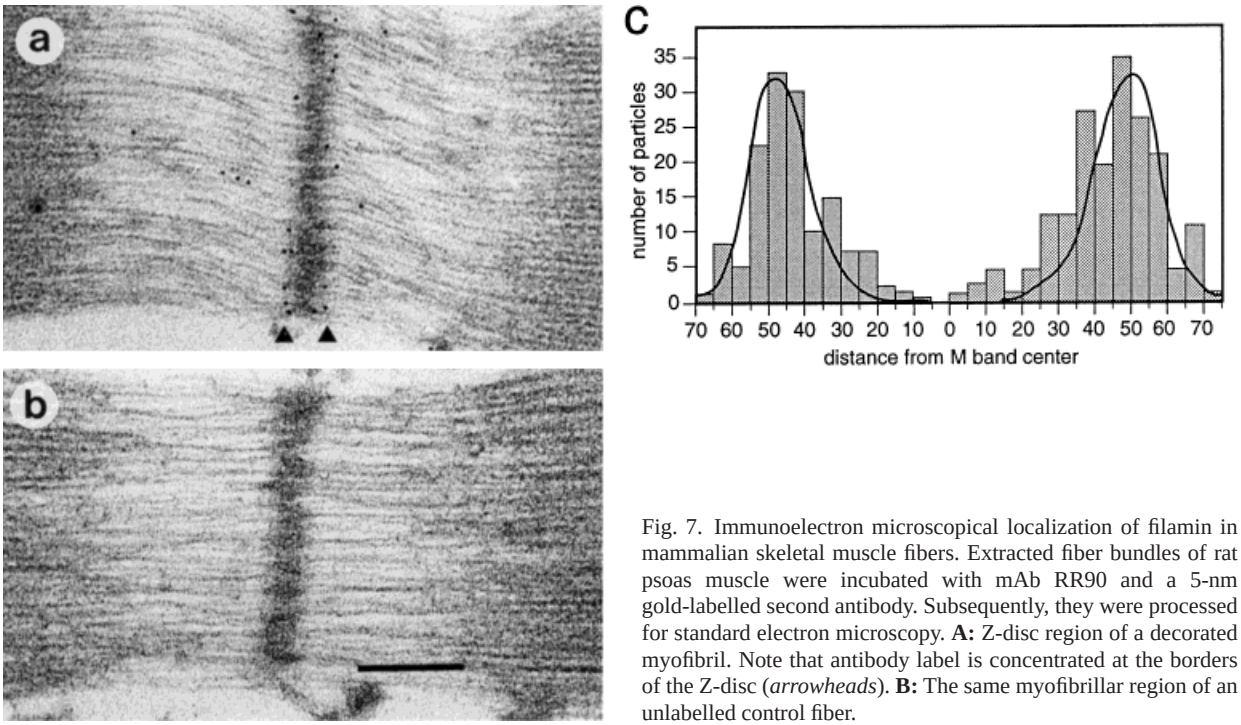

Fig. 7. Immunoelectron microscopical localization of filamin in mammalian skeletal muscle fibers. Extracted fiber bundles of rat psoas muscle were incubated with mAb RR90 and a 5-nm gold-labelled second antibody. Subsequently, they were processed for standard electron microscopy. **A:** Z-disc region of a decorated myofibril. Note that antibody label is concentrated at the borders of the Z-disc (*arrowheads*). **B:** The same myofibrillar region of an unlabelled control fiber.

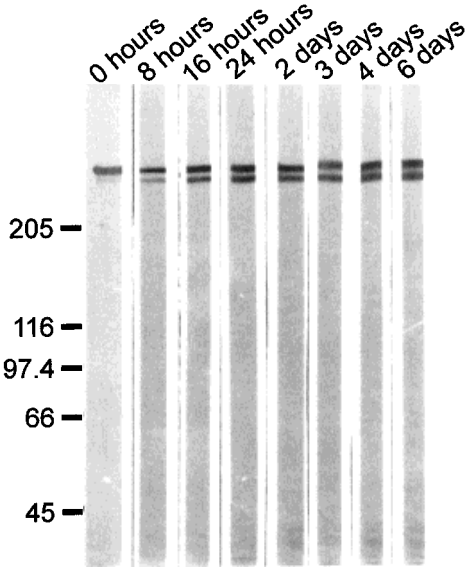

Fig. 8. Immunoblot analysis of filamin isoforms during myogenesis in culture. Human skeletal muscle cells were grown as described in Materials and Methods. Samples for SDS-PAGE and subsequent Western blotting using mAb RR90 were taken from cells in the proliferative stage (**lane 1**) and at different time points after the induction of differentiation: 8 h (**lane 2**), 16 h (**lane 3**), 24 h (**lane 4**), 2 days (**lane 5**), and 4 days (**lane 6**). M = molecular mass markers (numbers indicate $M_r \times 10^3$). Note that in undifferentiated myoblasts, RR90 recognizes only one filamin band, while already 8 h after the induction of differentiation a second band of slightly higher mobility appears. This polypeptide is expressed throughout myotube development.

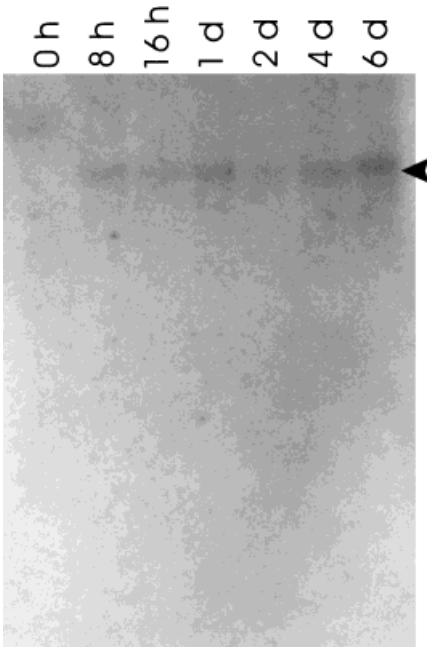

Fig. 9. Northern blot analysis of the expression of γ-filamin/ABPL mRNA during human skeletal muscle cell differentiation. Total RNA was isolated from cultured human skeletal muscle cells, which were differentiated for the time indicated above the respective lane (0 h, undifferentiated proliferating myoblasts; 8 h to 6 d, time after initiation of differentiation). RNAs were separated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with a probe specific for ABP-L/γ-filamin (see Materials and Methods). The *arrowhead* indicates reactivity with a single band migrating at ~9 kb, i.e., in the expected size range. Note that reactivity with the probe is restricted to differentiating myoblasts and that up-regulation of this mRNA accompanies the earliest stages of myofibril differentiation.

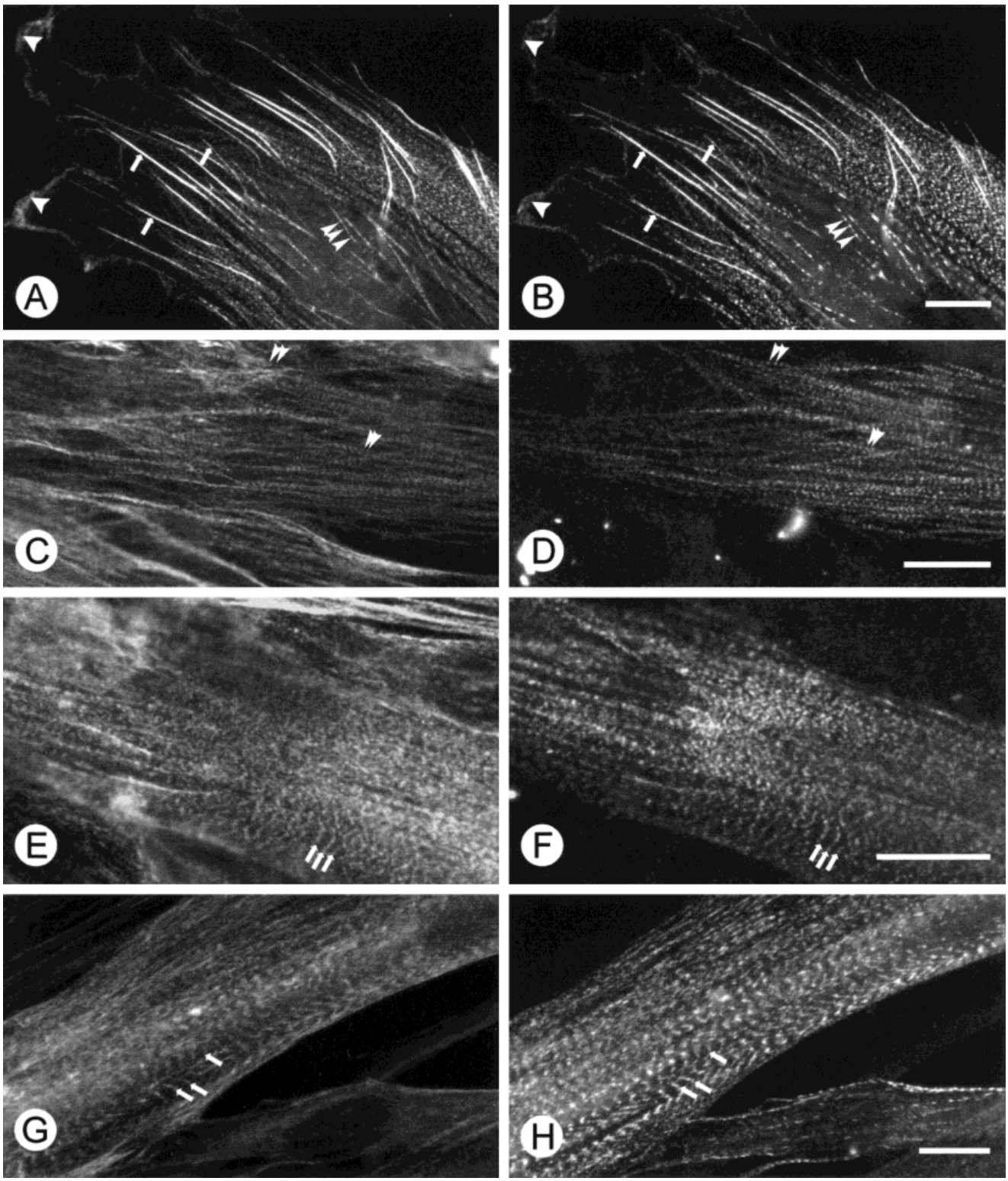

Fig. 10. Immunofluorescence localization of filamin in cultured human skeletal muscle cells. **A,B:** Proliferating myoblasts stained with mAb RR90 or α-actinin (BM 75.2), respectively. Note that filamin colocalizes with α-actinin in dots that are associated with stress-fibers (*small arrowheads*). Furthermore, a colocalization of both proteins is observed in extended focal contacts (*arrows*) and in the leading edge of the cells (*large arrowheads*). **C–H:** Localization of filamin (C, E, G) in differentiating cells is compared with the localization of sarcomeric α-actinin (D, F, H). C,D: Colocalization of both proteins in stress-fiber associated dots (*arrowheads*) in an early myotube 2 days after the induction of differentiation. E,F: First alignment of these dots into nascent Z-discs (*arrows*) is depicted. G,H: Colocalization of filamin and sarcomeric α-actinin in further developed Z-discs (*arrows*) in myotubes after 4 days of differentiation.

important role in the organization of thin filaments at sites that have to resist greater mechanical strain. Thus, filamin in muscle seems to be intimately involved in actin filament organization both in myofibrils and at certain sites of membrane anchorage.

Since the epitope of mAB RR90 is located at or close to the aminoterminus of the filamin polypeptide, our interpretations are limited to one end of this extended and rod-shaped molecule. The lack of antibodies reacting with more carboxyterminal epitopes of ABPL/γ-filamin presently precludes a complete molecular layout to be drawn. The prime candidate is, of course, the ~80 amino acids insertion in domain 20 specific for the ABPL/γ-filamin isoform [Xie et al., 1998]. Unfortunately, this region seems to be only weakly immunogenic leaving our attempts to raise a polyclonal antibody specific for this insertion unsuccesful (our unpublished observation).

Further antibodies will also be needed to discriminate between the three filamin isoforms. Both our Western and Northern blots clearly showed that upon induction of muscle differentiation a second filamin isoform in addition to the ubiquitously expressed ABP-280 is upregulated in our cell cultures (Figs. 8 and 9). Since the Northern blot was reacted with a probe derived from a ~240 bp cDNA fragment encoding the above-mentioned ABP-L/~-filamin isoform specific insertion [Xie et al., 1998], it is highly probable that the second band identified in Western blots corresponds to this isoform. Since ABP280 could never be detected in mammalian skeletal muscle, the persistence of immunoreactivity with ABP-280 most likely arises from cells that did not fuse into myotubes. Similarly, the immunoreactivity with ABP-280 in extracts from striated muscle tissue can be attributed to the presence of several non-muscle cell types and to blood vessels, in which ABP-280 is expressed at high levels (see, e.g., Fig. 6).

Additional complexity is created by differential splicing, which until now has been shown for ABP-280 [Maestrini et al., 1993] and for ABP-L/γ-filamin [Maestrini et al., 1993; Xie et al., 1998]. This mechanism is likely to explain the observation that avian slow and fast muscle fibers contain distinct but highly homologous filamin isoforms with differential distributions [Gomer and Lazarides, 1983]. Two other reports described a biphasic temporal expression pattern for filamin during avian muscle development in vitro [Gomer and Lazarides, 1981; Price et al., 1994]. In light of the complexity of mammalian filamins, a failure to detect all filamin isoforms in the chicken system is the most likely explanation for the described lack of filamin reactivity in intermediate developmental stages not observed in the study described here. In line with this view, strongly divergent species-specific variants were reported, e.g., for myomesin [Vinkemeier et al., 1993; Bantle et al., 1996], and C-protein [MyBP-C; Weber et al., 1993, Yasuda et al., 1995; Gautel et al., 1998].

Our Western and Northern blotting as well as the immunofluorescence results rather imply a role for filamin already at the earliest stages of myofibril assembly. The muscle-specific isoform of ABP-L/γ-filamin clearly is expressed as one of the first structural proteins of the myofibril. Its localization is intimately linked to Z-discs, already from the appearance of Z-patches and Z-bodies along SFLS [Sanger et al., 1984; Rhee et al., 1994] and it persisted throughout all developmental stages. We speculate, therefore, that filamin— together with α-actinin— is involved in the organization of thin filaments in myofibrils. For α-actinin, a direct interaction with titin was demonstrated [Ohtsuka et al., 1997; Sorimachi et al., 1997; Young et al., 1999] and it is most likely this interaction that is the prime cause for its specific Z-disc localization. For filamin in muscle, the targeting mechanism and the according binding partner have not yet been identified. Differentially expressed molecular regions are the best candidates to search for such interactions.

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