Indications for a Novel Muscular Dystrophy Pathway: γ-Filamin, the Muscle-specific Filamin Isoform, Interacts with Myotilin

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Abstract. γ-Filamin, also called ABP-L, is a filamin isoform that is specifically expressed in striated muscles, where it is predominantly localized in myofibrillar Z-discs. A minor fraction of the protein shows subsarcolemmal localization. Although γ-filamin has the same overall structure as the two other known isoforms, it is the only isoform that carries a unique insertion in its immunoglobulin (Ig)-like domain 20. Sequencing of the genomic region encoding this part of the molecule shows that this insert is encoded by an extra exon. Transient transfections of the insert-bearing domain in skeletal muscle cells and cardiomyocytes show that this single domain is sufficient for targeting to developing and mature Z-discs. The yeast two-hybrid method was used to identify possible binding partners for the insert-bearing Ig-like domain 20 of γ-filamin. The two Ig-like domains of the recently described α-actinin–binding Z-disc protein myotilin were found to interact directly with this filamin domain, indicating that the amino-terminal end of γ-filamin may be indirectly anchored to α-actinin in the Z-disc via myotilin. Since defects in the myotilin gene were recently reported to cause a form of autosomal dominant limb-girdle muscular dystrophy, our findings provide a further contribution to the molecular understanding of this disease.

Key words: filamin • myotilin • limb-girdle muscular dystrophy • myofibrils • Z-disc proteins

Introduction

The three-dimensional structure of actin-based macromolecular assemblages largely depends on the activity of a plethora of actin-binding proteins. Thus, microfilaments can be as short as 13 actin monomers in the submembraneous cytoskeleton of erythrocytes, while they can reach several micrometers in length, for instance in the stress-fibers of cultured cells. Strictly bundled filaments in microvilli or stress fibers oppose to loose networks with very little apparent regularity, as found in the terminal web of epithelial cells or the leading edge of motile cultured cells. Mostly, these distinct structures can be attributed to the specific activity of individual actin filament-binding proteins or protein families (Ayscough, 1998). The various isoforms or individual family members often exhibit strikingly different expression patterns and/or subcellular localizations. The molecular basis for the distinct functionality of highly similar but distinct variants of a protein is, however, little understood. One example for such versatile

proteins that should allow us to analyze isoform-specific cellular functions is the filamin family (Hock, 1999). These ubiquitously distributed actin–cross-linking proteins were described to cause bundling of actin filaments (Wang et al., 1975; Wang and Singer, 1977; Brotschi et al., 1978), a situation compatible with filamin's stress-fiber localization (Mittal et al., 1987) and with its suggested role in organizing the contractile units of smooth muscle cells (Small et al., 1986). Conversely, a number of studies emphasized filamin's role in organizing the cortical actin filaments of, for instance, blood platelets and cultured cells (Cunningham et al., 1992). This latter function is exemplified further by the documentation of interactions between filamins and several transmembrane proteins: the cytoplasmic portion of the glycoprotein Ib IX complex, the receptor for von Willebrand factor (Fox, 1985; Okita et al., 1985; Meyer et al., 1997; Takafuta et al., 1998; Xu et al., 1998), β1-integrin (Pfaff et al., 1998; Loo et al., 1998), β2-integrin (Sharma et al., 1995), caveolin-1 (Stahlhut and van Deurs, 2000), and γ- and δ-sarcoglycan (Thompson et al., 2000) were all identified as ligands for filamins. Intracellular proteins that bind to filamin include furin (Liu et al., 1997), preseni-

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lin-1 (Zhang et al., 1998) and SEK-1, the activator of SAPK (Marti et al., 1997). This leaves us with the need to define distinct functions of certain filamin variants in certain cell types. Until now, three human filamin gene paralogues have been identified: *FLNA* encodes α-filamin (also called ABP-280) and maps to the chromosomal position Xq28 (Gorlin et al., 1990, 1993; Maestrini et al., 1993). *FLNB* codes for β-filamin (also called ABP-278) and was located at chromosome 3p14.3 (Takafuta et al., 1998; Xu et al., 1998; Bröcker et al., 1999). *FLNC* encodes γ-filamin (also called ABP-L or FLN2) and was mapped to chromosome 7q32-q35 (Gariboldi et al., 1994; Xie et al., 1998). Variability, however, goes even further because differential splicing has already been demonstrated for all three genes (Maestrini et al., 1993; Xie et al., 1998; Xu et al., 1998). The exact knowledge of the full complement of filamin isoforms expressed will have to await the detailed characterization of the entire gene family.

Cross-striated muscles offer an excellent system to address several questions regarding filamin isoform-specific cellular functions. The γ-filamin isoform harboring a unique insertion of 78 amino acids was shown to be expressed specifically in muscular tissues (Maestrini et al., 1993; Xie et al., 1998; van der Ven et al., 2000). Its upregulation during the initial stages of myocyte differentiation and its localization predominantly at the periphery of Z-discs implied an important role of this filamin variant during myofibrillogenesis (van der Ven et al., 2000). In addition, the recently documented binding to sarcoglycans (Thompson et al., 2000) and the assignment of a subtype of limb-girdle muscular dystrophy (LGMD)[1] to the same region of chromosome 7 where the *FLNC* gene was mapped (Speer et al., 1999) made γ-filamin an interesting candidate for a muscular disease gene. Our analysis that focused on a differentially spliced muscle-specific region of this polypeptide demonstrated myofibrillar targeting by this portion. At the same time, we identified myotilin (Salmikangas et al., 1999) as a novel filamin ligand. This report therefore sheds new light on the molecular basis of LGMD.

Materials and Methods

Antibodies

The following antibodies were used in this study: (a) RR90, a mouse mAb (immunoglobulin A, IgA) recognizing the aminoterminal region of γ- and α-filamin (van der Ven et al., 2000; this report). (b) mAb 1680, a mouse mAb (IgG1) raised against the carboxy terminal 90-kD fragment of platelet filamin (Wang et al., 1996), purchased from Chemicon International. In this report, we show that this antibody is specific for α-filamin (see below). (c) BM-75.2, a mouse mAb (IgM) recognizing all isoforms of α-actinin (Abd-el-Basset et al., 1991), purchased from Sigma-Aldrich. (d) T7 Tag antibody, a mouse mAb (IgG2b) purchased from Novagen, specific for the aminoterminal end of the T7 major capsid protein. This antibody was used to detect epitope-tagged proteins expressed by the pCMV5-T7 and pET23-T7 vectors, respectively. (e) T12, a mouse mAb (IgG1) directed against domains I2–I4 just carboxy-terminal from the inextensible Z-disc region of titin (Fürst et al., 1988). (f) EA53, a monoclonal mouse antibody (IgG1) specific for sarcomeric α-actinin (Goncharova et al., 1992), purchased from Sigma-Aldrich. (g) YL1/2, a rat mAb directed against the carboxy terminus of tyrosinated tubulin (Wehland et al., 1983). This antibody (a kind gift of Dr. J. Wehland, Gesellschaft fur Biotechnologische Forschung, Braunschweig, Germany) was used to detect epitope-tagged pro-

teins expressed using the pET23-EEF vector described below. (h) 12CA5, a mouse mAb directed against the influenza virus hemagglutinin epitope (HA-tag) was purchased from Roche Diagnostics, and was used to detect the GAL4 AD-HA-epitope fusion proteins upon expression in yeast cells. (i) LexA mouse mAb (CLONTECH Laboratories, Inc.) was used to detect LexA-fusion proteins in yeast extracts. (j) Rabbit polyclonal antibody 948, affinity purified from a rabbit antiserum raised against a peptide encompassing residues 353–369 of myotilin (Salmikangas et al., 1999).

Cell Cultures

Human skeletal muscle cells were isolated and cultured essentially as described before (van der Ven et al., 1992, 1993). In brief, 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 DME supplemented with 20% FCS, 2% Ultroser G, 100 U/ml penicillin and 1 μg/ml streptomycin (all from Life Technologies). Cells were grown until near confluency. Differentiation of the cells was induced by changing the high nutrition medium to differentiation medium (DME, 0.4% Ultroser G and antibiotics as above).

C2C12 cells (ECACC # CB2438) were cultured in DME supplemented with 15% FCS and antibiotics. Differentiation was induced as described for human skeletal muscle cells.

Neonatal rat cardiomyocytes were prepared exactly as described before (Sen et al., 1988; Komiyama et al., 1996). Cells were plated in 35-mm culture dishes in 68% DME/17% medium M199 supplemented with 10% horse serum, 5% FCS, 4 mM L-glutamine, and antibiotics as above.

Ptk2 cells (ECACC # CB2059) were cultured in DME supplemented with 10% FCS, nonessential amino acids, and antibiotics as above.

Transient Transfection Experiments

C2C12 cells were seeded the day before transfection and grown on glass coverslips placed in a 35-mm culture dish to ∼80% confluency. The cells were transfected using lipofectamine according to the manufacturer's instructions (Life Technologies), except that 5 μg plasmid DNA and 10 μl lipofectamine were used per culture dish. Ptk2 cells were transfected using Fugene6, according to the manufacturer's instructions (Roche Diagnostics). Transfected C2C12 cells were induced to differentiate by changing the culture medium to differentiation medium the day after transfection, and cells were allowed to differentiate for 2–6 d. Transfected Ptk2 were analyzed for expression of the recombinant protein 24–48 h after transfection. Cells were fixed and stained as described below.

Transfections of neonatal rat cardiomyocytes were performed using a modified calcium-phosphate protocol as described (Komiyama et al., 1996). In brief, 4×10^6 cardiomyocytes were seeded per 35-mm culture dish and were allowed to grow for ∼24 h at 10% CO_2. 1–2 μg of DNA was diluted with H_2O to a total volume of 135 μl and, after vortexing, 15 μl of a 2.5 M $CaCl_2$ solution was added. While vortexing, 150 μl of 2× BES (*N,N*-bis[2-hydroxyethyl]-2 aminoethane sulfonic acid)-buffered saline (50 mM BES, 1.5 mM Na_2HPO_4, 280 mM NaCl, pH 6.7) was added dropwise, and the mixture was incubated for 12 min at room temperature. Subsequently, the mixture was added dropwise to culture dishes containing 2 ml medium and cells were incubated at 37°C, 3% CO_2 for 5 h. After transfection, cells were washed twice with Tris-buffered saline, and the medium was changed to a low nutrition medium consisting of 78% DME/20% medium 199 supplemented with 1% horse serum, 0.1 mM phenylephrine (Sigma-Aldrich), 4 mM L-glutamine and antibiotics as above, and the cells were kept in this medium for 72 h, at 10% CO_2 until they were fixed and stained.

Immunofluorescence Microscopy

At different time points after the switch of media, human skeletal muscle cells and C2C12 cells were fixed for ∼5 min in methanol and subsequently for 30 s in acetone, both at −20°C. Ptk2 cells were fixed identically. After air drying, cells were immediately used or frozen at −80°C. Cardiomyocytes were fixed with 3% paraformaldehyde in PBS for 15 min. Subsequently, they were washed with PBS, treated with 0.1 M glycine in PBS for 3 min, and permeabilized with 0.2% Triton X-100 in PBS for 15 min. Before application of the antibodies (diluted in 1% BSA in PBS), cardiomyocytes were blocked with 5% normal goat serum, 1% BSA in PBS for 20 min. Double immunofluorescence assays were performed using mixtures of the primary antibodies followed by a mixture of the matching secondary antibodies (Southern Biotechnology Associates, Inc.; Jackson ImmunoResearch Laboratories; Cappel) using standard procedures (van der Ven et al., 1993). The cells were embedded in Mowiol or in Tris-buffered

glycerol containing 50 mg/ml *N*-propyl gallate (Sigma-Aldrich). The stained cells were analyzed using an Axiophot microscope (Carl Zeiss, Inc.) equipped with a cooled CCD camera, or with a confocal microscope (Leica) as described (Auerbach et al., 1999).

Construction of Expression Constructs

The prokaryotic expression vector pET-23a (Novagen) was modified by the introduction of an immunotag directly following the His6 tag, resulting in the vector called pET23-EEF. This immunotag consists of merely three amino acids (glutamic acid–glutamic acid–phenylalanine, EEF) and is recognized by the rat mAb YL1/2 (Wehland et al., 1983). Expressed proteins therefore carry a carboxyterminal His6 tag, allowing purification on Ni-agarose (QIAGEN) columns, while at the same time the EEF tag allows for immunodetection with mAb YL1/2. Another variant of the original pET-23a vector (pET23-T7) resulted in an amino-terminal T7 tag added to the expressed protein. Thus, expressed proteins carry an amino-terminal T7 tag enabling immunodetection with the T7 tag antibody (Novagen), and a carboxyterminal His6 tag.

cDNA fragments covering different parts of α- and γ-filamin (see Fig. 1) were obtained by reverse transcription–PCR using total RNA purified from nondifferentiated or differentiated cultured human skeletal muscle cells, respectively, as a template using the "Expand long template PCR system" according to the manufacturer's instructions (Roche Diagnostics). These fragments were used to amplify and subclone truncated variants of both proteins in the prokaryotic expression vector pET23-EEF and/or the eukaryotic expression vector pCMV5-T7 (Obermann et al., 1998) using forward primers containing an MluI site, and reverse primers containing a SalI site adjacent to the sequence homologous to the desired filamin sequence. In the case of β-filamin, a full-length cloned cDNA (kindly provided by Drs. S. Shapiro and T. Takafuta, Cardeza Foundation for Hematologic Research, Department of Medicine, Jefferson Medical College, Philadelphia, PA) was used as a template to amplify cDNA fragments (see Fig. 1). Double digested PCR fragments were ligated into the correspondingly cut vectors. Similarly, truncated myotilin fragments (see Fig. 1) were cloned in pET23-T7 for bacterial expression, or in pCMV5-T7 or pcDNA3.1/CT-GFP (Invitrogen) to allow the expression of T7- or green fluorescent protein (GFP)–tagged myotilin fragments in eukaryotic

cells, respectively. The human nonmuscle α-actinin cDNA cloned in the pEGFP-N3 vector (CLONTECH Laboratories, Inc.) was a kind gift of Dr. C. Otey (University of North Carolina, Chapel Hill, NC). Constructs were transformed to and propagated in *Escherichia coli* JM 109 bacteria. DNAs were purified using the Plasmid Kit (QIAGEN). Constructs in both modified pET vectors were transformed to *E. coli* BL21(DE3)pLysS cells. Expression and purification of recombinant proteins were carried out essentially as described before (Obermann et al., 1997, 1998; see also below).

Genomic PCR

Genomic fragments encoding specific parts of the *FLNC* gene were amplified by PCR (Saiki et al., 1985) using the Expand long template PCR system with DNA isolated from cultured normal human myoblasts using the blood and cell culture DNA Kit (QIAGEN) as a template. Amplified fragments were cloned in the pCR Vector using a TA cloning kit (Invitrogen) and sequenced.

Yeast Two-Hybrid Library Screening

Portions of the γ-filamin cDNA comprising the Ig-like domain 20 (including the unique insertion) with one or two of the flanking domains (Fig. 1) were amplified by PCR and cloned into a modified pLexA vector containing a new multicloning site (Stenmark et al., 1995; Young et al., 1998). The resulting plasmids were pretransformed into the *Saccharomyces cerevisiae* L40 reporter strain (Vojtek et al., 1993) using a modified lithium acetate protocol (see protocol 01525; Elsevier's Trends Journals Technical Tips Online, available at http://tto.trends.com). Subsequently, this strain was cotransformed with a human skeletal muscle cDNA library in the vector pACT2 (Matchmaker cDNA library HL4047AH; CLONTECH Laboratories, Inc.). Selection for HIS3 reporter gene activation was performed on agar plates without histidine, leucine, or tryptophane (SD-LWH agar) according to the Matchmaker protocol. Colonies growing after ~5 d at 30°C were screened for activity of the LacZ reporter gene in filter assays. Plasmids from positive clones were purified and transferred to *E. coli* JM109 or DH5α by electroporation (Matchmaker protocol). For submapping the interaction between filamin and myotilin, constructs encoding distinct portions of both proteins were cloned into pGAD10AD and
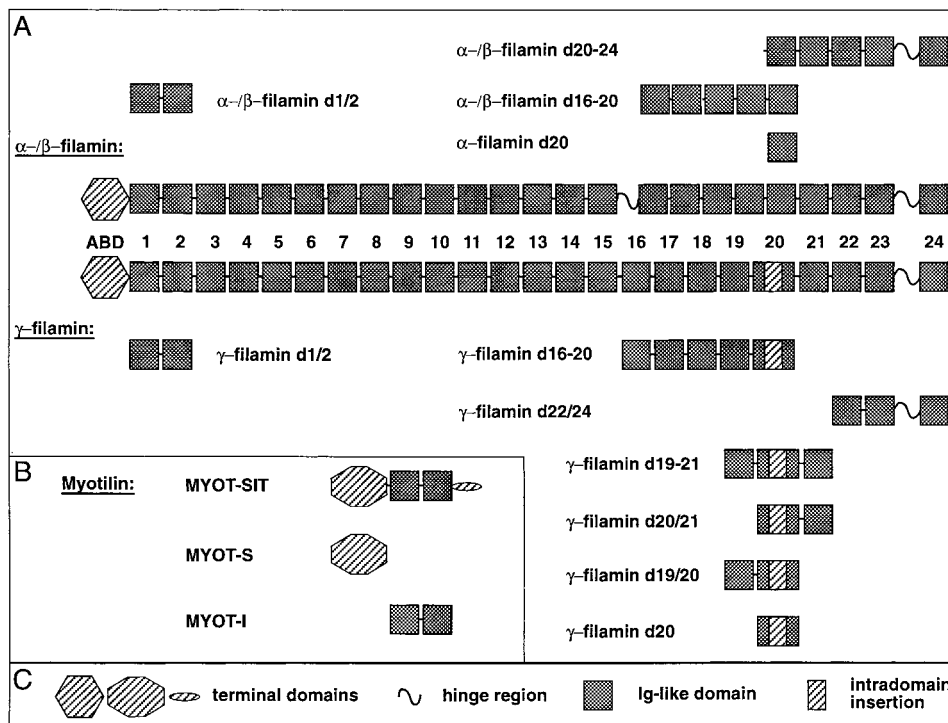

Figure 1. Domain organization of the filamins and of myotilin, and recombinant constructs used for transfection assays. A emphasizes the general modular organization of filamins: the actin binding head domain (ABD) is followed by 24 Ig-like modules (identified by numbers between the boxes). Stretches of unique sequence, so-called hinge regions, are found between Ig-like domains 15 and 16, as well as between domains 23 and 24. Muscle γ-filamin, however, lacks this insertion, which might make the molecule less flexible. A conspicuous difference between γ-filamin and the other members of the filamin family is a unique insertion in the middle of Ig-like domain 20. Constructs derived from α- or γ-filamin, which were used for the experiments of this work, are shown schematically above and below the respective mother molecules. For yeast two-hybrid screens and for prokaryotic expression experiments, identical inserts were cloned into the respective vectors (see Materials and Methods). (B) A layout of the structure of myotilin is given. The molecule consists of a serine-rich amino terminal portion (S), followed by two immunoglobulin-like domains (I) and a carboxyterminal "tail" sequence (T). Inserts were cloned into the respective vectors for yeast two-hybrid experiments and for prokaryotic expression. (C) Legend to the domain symbols used in A and B.
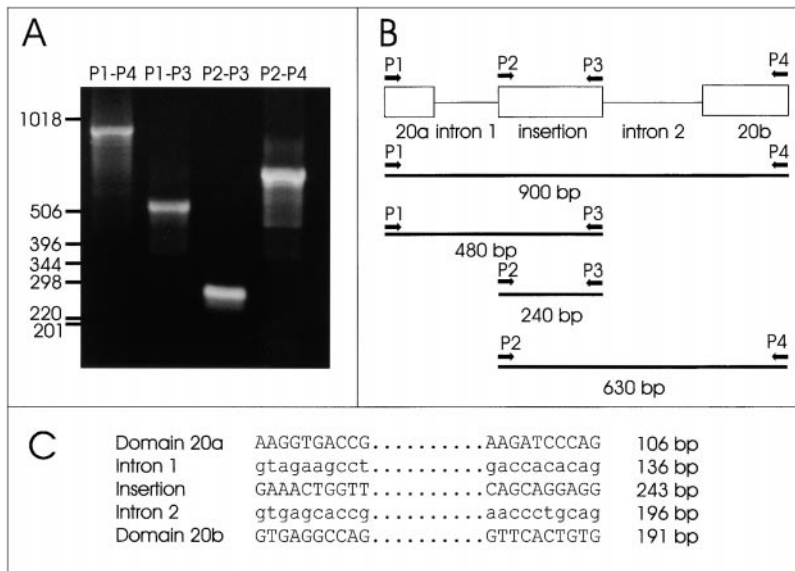
Figure 2. Analysis of the structure of the *FLNC* gene encoding domain 20. (A) PCR products after amplification of human genomic DNA with primers P1 and P4 (P1–P4), P1 and P3 (P1–P3), P2 and P3 (P2–P3), and P2 and P4 (P2–P4). (B) Horizontal lines represent the four PCR products depicted in A, and the derived exon/intron structure of domain 20. Note that the primers used for amplification (P1: GTCACCAACAGCCCCT-TCAAG; P2: AAGGTGACCGGCGAGGGC-CGC; P3: CACAGTGAACTGAAAGGGGCT; P4: AGCGTCATCCGAGAGGGAGG) all had a tail of nine additional basepairs to allow restriction enzyme–based cloning. Therefore, the actual length of the products was reduced by subtraction of 18 basepairs. (C) Splice junctions and exon and intron sizes of the part of the *FLNC* gene that encodes domain 20 are shown.

pLexA vectors, respectively, and cotransformed into L40 yeast cells. Growth on SD-LWH agar plates and activity of β-galactosidase were assayed as described above.

Immunoprecipitation

For coimmunoprecipitation experiments, a mixture of 0.5 mg of each recombinant protein was diluted in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% BSA. After the addition of either 1 μg anti–EEF antibody (YL1/2) or anti–T7 antibody, the mixture was incubated at 4°C for 60 min with gentle shaking. Subsequently, 5 μl GammaBind G Sepharose (Amersham Pharmacia Biotech) was added, and the mixture was further incubated for 30 min with gentle shaking. The sepharose beads were washed three times with 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1% Triton X-100, and once with 20 mM Tris-HCl, pH 7.4, 500 mM NaCl. Sepharose bound immune complexes were sedimented twice through a sucrose cushion (1 M sucrose in 20 mM Tris-HCl, pH 7.4, 500 mM NaCl), and the beads were washed twice with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100. Finally, beads were boiled in SDS-sample buffer, and bead-associated proteins were separated by SDS-PAGE (Laemmli, 1970). Proteins were blotted to polyvinyl difluoride membrane using a semidry blot apparatus (Bio-Rad Laboratories), and the precipitated proteins were identified by immunodetection. The following combinations of antibodies were used: (a) anti–T7 antibody (Novagen) and peroxidase-conjugated goat anti–mouse secondary antibody (Jackson ImmunoResearch Laboratories); (b) anti–EEF antibody YL 1/2 and peroxidase-conjugated goat anti–rat secondary antibody (American Qualex). Reactions were detected by enhanced chemiluminescence using "SuperSignal West Pico Chemiluminescent Substrate" (Pierce Chemical Co.).

Coimmunoprecipitations from yeast cells expressing the HA-tagged myotilin fragment fused to the GAL 4AD domain and the LexA-γ-filamin fusion protein were performed as previously described (Grönholm et al., 1999). The anti–HA-tag antibody (12CA5) as well as the polyclonal anti–myotilin antiserum (948) were used in a 1:500 dilution.

Results

The γ-Filamin–specific Insertion in Domain 20 Is Encoded by an Individual Exon

To examine the genomic organization of the region encoding the unique insertion within the Ig-like domain 20 of γ-filamin, defined portions of the human *FLNC* gene were amplified. Analysis of the length of the PCR products, and comparison with the theoretical length of the products derived from the cDNA sequence, showed that the product representing the insertion of domain 20 alone did not differ

from the expected length, whereas all three other products, which included sequences from the insert-harboring domain, were considerably longer (Fig. 2, A and B). This indicated that at least one intron should be present between the 5′ end of domain 20 and the insertion, and another intron must be localized between the 3′ end of domain 20 and the insertion. To analyze the precise structure of this part of the *FLNC* gene, the ~900-bp-long PCR product yielded by amplification of the complete domain 20 was cloned and sequenced. This revealed that the insertion within domain 20 is encoded by a single exon with a length of 243 bp. This exon is separated from the parts of the gene that encode the 5′ and 3′ parts of domain 20 by introns with a length of 136 and 196 bp, respectively (Fig. 2 C).
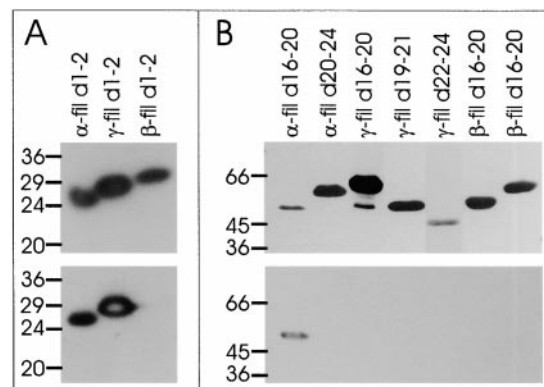

Figure 3. Characterization of the filamin antibodies. Total protein extracts from bacteria expressing the carboxyterminally immunotagged polypeptides indicated above each lane were separated on a polyacrylamide gel and transferred to nitrocellulose. Subsequently, the filters were incubated with YL1/2 (A and B, top), RR90 (A, bottom) or mAb 1680 (B, bottom). The reactivity with the anti–EEF tag antibody indicates that the polypeptides are intact and in the correct reading frame. Note that RR90 recognizes an epitope in the first two immunoglobulin-like domains of α- and γ-filamins, but not in β-filamin. mAb 1680 recognizes an epitope in the polypeptide α-filamin d16–20, and not in β- or γ-filamin, indicating that it is specific for this isoform.
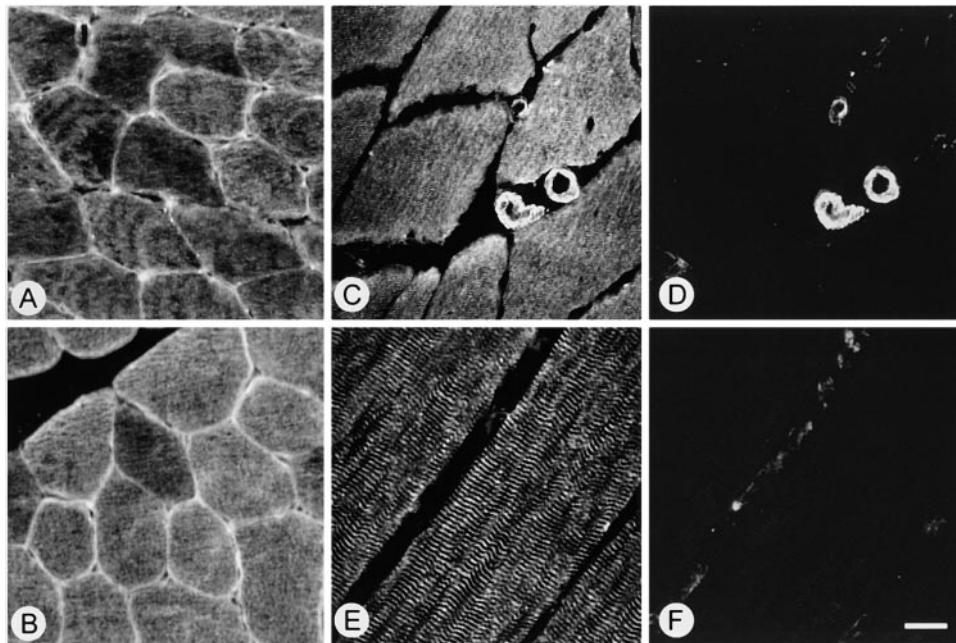
γ-Filamin Is the only Filamin Isoform Expressed in Differentiating and Adult Human Skeletal Muscle Cells

In a previous report, we characterized the expression of filamins in striated muscle tissues and in cultured cells with the murine antibody mAb RR90. Here, we characterized the epitope and isoform specificity of RR90 and of mab 1680 (raised against platelet filamins; Fig. 3, A and B). Thus, the epitope of RR90 is confined to Ig-like domains 1 and 2 of both, α- and γ-filamin. In contrast, the corresponding β-filamin was not recognized (Fig. 3 A). Similarly, mAb 1680 was established to react solely with Ig-like domains 16–20 of α-filamin (Fig. 3 B).

This enabled a comparison of the staining patterns of an antibody specific for human α-filamin and RR90, to characterize more precisely which filamin isoforms are expressed in developing and mature human skeletal muscle cells, both with respect to temporal expression patterns and differential spatial distribution. In cross sections of adult skeletal muscle, both the sarcolemma and the sarcoplasm of muscle fibers as well as blood vessels were strongly stained by mAb RR90 (Fig. 4, A–C), whereas the α-filamin–specific antibody exclusively stained blood vessels (Fig. 4 D). This clearly indicates that the filamin isoform detected by mAb RR90 in muscle fibers solely represents γ-filamin. In longitudinal sections, cytoplasmic γ-filamin was localized in a distinct cross-striated pattern, due to its localization in Z-discs (Fig. 4 E). Again, no staining of muscle fibers was observed with the α-filamin antibody (Fig. 4 F), firmly establishing that γ-filamin is the only filamin isoform specifically expressed in myofibers.

In a previous study, Northern and Western blotting demonstrated that in proliferating cultured human skeletal muscle cells, only nonmuscle filamin is expressed and that within a few hours after the induction of differentiation, the muscle-specific γ-filamin isoform can be detected (van der Ven et al., 2000). These findings were now complemented by immunofluorescence microscopy using an antibody specific for α-filamin. Thus, α-filamin was revealed solely in proliferating myoblasts and only residual staining of stress fibre-like structures was observed in early myotubes 2 d after the induction of differentiation (Fig. 5, A–D). At later stages, the α-filamin–specific antibody did not detect any protein in the myotubes, while RR90 mainly gave a strong staining of Z-discs (Fig. 5, E–H). These results make it clear that γ-filamin is the filamin isoform that is involved in myofibril development.

Ig-like Domain 20 of γ-Filamin Including the Muscle-specific Insertion Is Sufficient for Proper Z-Disc Targeting

γ-Filamin is the only filamin isoform that bears this unique insertion in its Ig-like domain 20. Its size, 78 amino acids, must change the three-dimensional structure of this otherwise ~90-residues domain dramatically. Both findings are indicative of an important and specific function of this insertion in striated muscle. To test for such a function in myofibril assembly or myofibrillar targeting, the cDNA encoding Ig-like domain 20 of γ-filamin was cloned either alone or with one or both of the neighboring Ig-like domains in the eukaryotic expression vector pCMV5-T7 (for positions of clones, see Fig. 1). For comparison, the corresponding Ig-like domain 20 of α-filamin (which is devoid of this unique insertion) was also cloned in this vector. Transient transfections were carried out in two myogenic
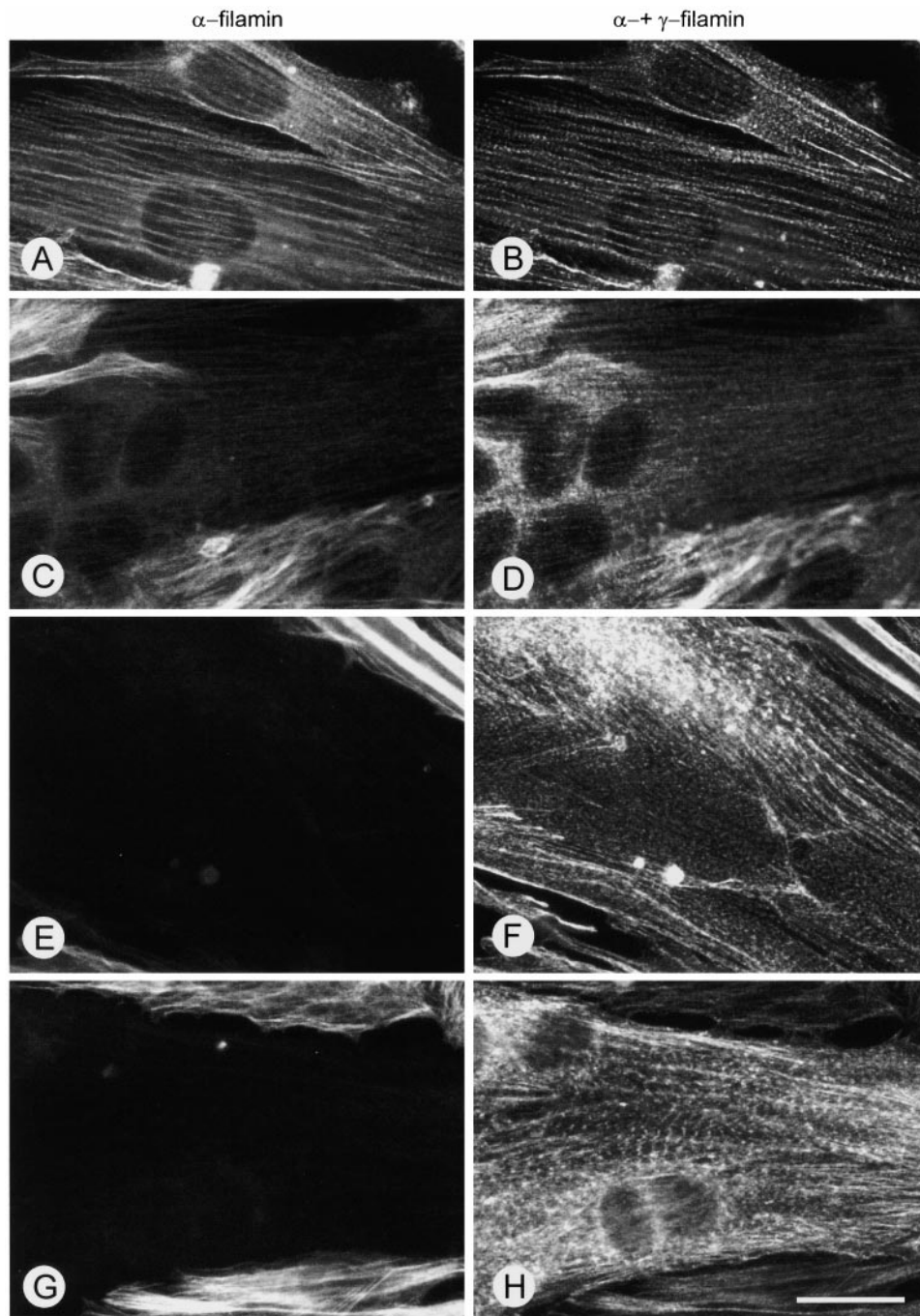
Figure 5. Immunofluorescence localization of filamin isoforms in developing human skeletal muscle cells. The micrographs show cultured human skeletal muscle cells differentiated for 0 (A and B), 2 (C and D), 4 (E and F) or 6 (G and H) d, double stained with an antibody specific for α-filamin (left) and mAb RR90 (right). Note that only in nondifferentiated cells a high level of α-filamin is detected by both antibodies (A and B). After 2 d of differentiation, RR90 strongly stains the young myotubes (D), while the level of α-filamin has strongly decreased (C), indicating a clear predominance of the muscle-specific filamin isoform. In further developed myotubes, α-filamin is not detectable (E and G), and γ-filamin seems to be the only filamin isoform that is expressed at these developmental stages (F and H). Note that, after 6 d of differentiation, γ-filamin is localized in a cross-striated pattern due to its localization in Z-discs. Bar, 20 μm.

cell types. To study targeting of the expressed constructs to specific subcellular regions, constructs were expressed in primary neonatal rat cardiomyocytes that upon the time point of transfection already contain fully assembled myofibrils. Thus, the major fraction of the expressed γ-filamin domain 20 was specifically incorporated into myofibrils of transfected cardiomyocytes in a regular, cross-striated pattern (Fig. 6 E). No obvious targeting to the cell membrane was observed. Double immunofluorescence microscopy using an antibody directed against the immuno-tag of the expressed protein and an antibody specific for the sarcomeric isoform of α-actinin (Fig. 6 F) revealed a precise colocalization of both proteins. This indicated that the Ig-like domain 20 of γ-filamin was incorporated into Z-discs

of cardiac myofibrils. Similarly, transfections of the longer constructs γ-filamin d19/20 (Fig. 6 C) and γ-filamin d19-d21 (A), which include the flanking Ig-like domains, also resulted in the incorporation of the expressed polypeptides in Z-discs, as demonstrated by double staining using the same combination of antibodies described above (Fig. 6, A–D). Interestingly, both longer constructs seemed to be targeted to a somewhat narrower region of the Z-disc in comparison to the construct γ-filamin d20, indicating that at least the addition of Ig-like domain 19 could stabilize the interaction of γ-filamin d20 to Z-discs.

The importance of the unique insertion in Ig-like domain 20 of the γ-filamin isoform for the interaction with Z-discs was confirmed by transient expression of α-filamin d20
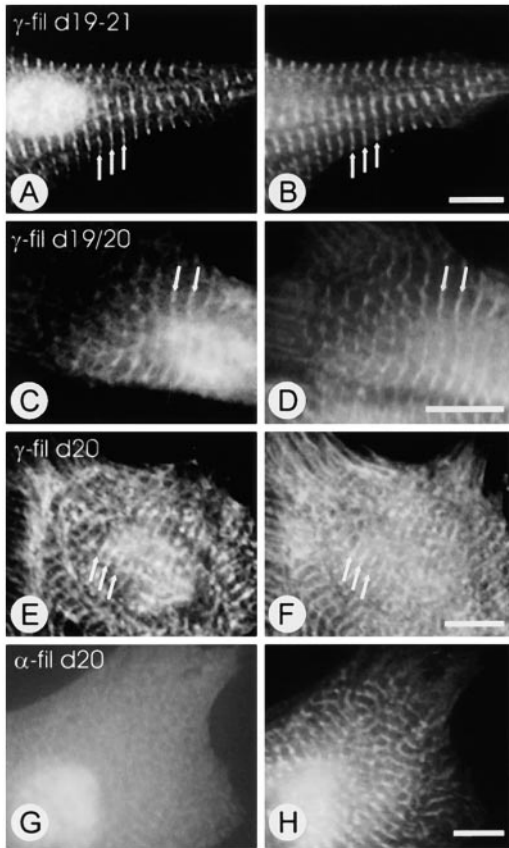
Figure 6. Expression of recombinant filamin fragments in neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes transfected with constructs encoding T7-tagged γ-filamin d19-21 (A and B), γ-filamin d19/20 (C and D), γ-filamin d20 (E and F), or α-filamin d20 (G and H) were fixed and double stained with T7-tag antibody (left) and an antibody specific for sarcomeric α-actinin (right). Note that the recombinant polypeptides γ-filamin d19–21 and γ-filamin d20/21 colocalize precisely with α-actinin in sharp striations, indicating that this part of γ-filamin is targeted to Z-discs (A–D, arrows). The single domain γ-filamin d20 is also targeted to the Z-disc region, although the fluorescent stripes appear less sharp (E and F, arrows). α-filamin d20 is distributed diffusely in the cytoplasm (G) and does not show any specific targeting to Z-discs or myofibrils in general (H). Bars, 10 μm.

Figure 7. Expression of recombinant filamin fragments in differentiating C2C12 mouse myoblasts. C2C12 cells transfected with constructs encoding T7-tagged γ-filamin d20 (A and F), γ-filamin d20/21 (G and H), or α-filamin d20 (J and K) were allowed to differentiate for 2 (A and B) or 4 (C–K) d. Subsequently, they were fixed and double stained with T7-tag antibody (left) and T12 (right), an antibody specific for a Z-disc epitope of titin. Note that γ-filamin d20 exactly colocalizes with Z-disc titin in nascent myofibrils with immature Z-discs (A and B, arrows), as well as in mature myofibrils (C–F) where the recombinant protein and Z-disc epitope of titin are localized in sharp striations (E and F, arrows). Note that, although γ-filamin d20/21 associates with stress fiber–like structures, it is never found at Z-discs (G and H). α-Filamin d20 is distributed diffusely in the cytoplasm (J), and does not show any targeting to immature or mature myofibrils (K). Bars, 20 μm.

(i.e., the corresponding insert-less domain from the α-filamin isoform) in the cardiomyocytes. This construct was distributed diffusely in transfected cardiomyocytes and did not show any specific targeting to Z-discs (Fig. 6, G and H).

Subsequently, murine C2C12 cells were used to study the effects of the expression of the construct γ-filamin d20 on the development of myofibrils. In contrast to the cardiomyocytes described above, these cells were transfected during proliferative stages during which they are devoid of myofibrils. Differentiation of the cells, and thus myofibril assembly, therefore occurred simultaneously to the expression of recombinant polypeptide. The expression of γ-filamin d20 in differentiating C2C12 mouse myoblasts confirmed the observation described above; i.e., that this single domain is sufficient for targeting to Z-discs. This domain clearly colocalized with the amino terminus of titin at Z-discs (Fig. 7, C–F). Interestingly, the recombinant pro-
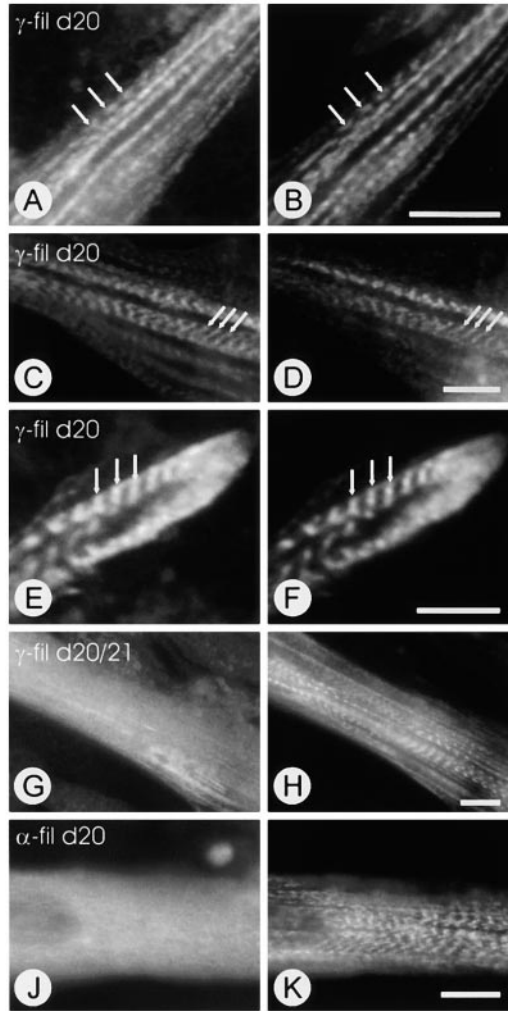
tein was also targeted to nascent myofibrils that did not yet show a mature cross-striated distribution of Z-disc titin (Fig. 7, A and B). In none of the transfected cells did the overexpression of the construct γ-filamin d20 lead to an evident perturbation of myofibril assembly, nor was any targeting to the sarcolemma observed. Surprisingly, the addition of the neighboring Ig-like domain 21 to the construct abolished the Z-disc binding capacity of the recombinant protein. Although some association with premyofibrils was observed, the polypeptide was never observed in Z-discs (Fig. 7, G and H). Similar to the observation in cardiomyo-
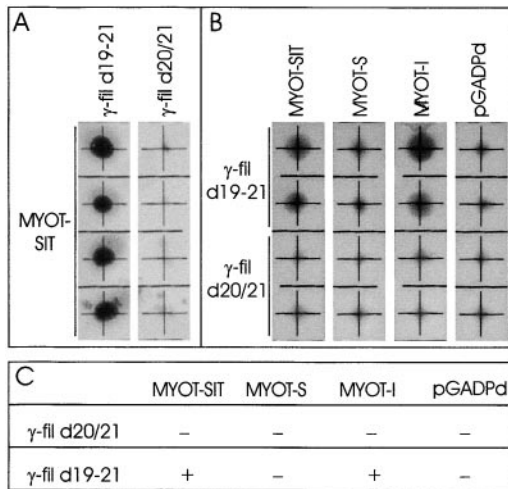
Figure 8. Characterization of the interaction of γ-filamin with myotilin. Yeast cells pretransformed with γ-filamin d20/21 or γ-filamin d19–21 in pLexPd were transformed with the empty prey vector (pGADPd) or with the region of the myotilin cDNA encoding its complete polypeptide (MYOT-SIT), its serine rich domain (MYOT-S), or the immunoglobulin domains (MYOT-I) in pGADPd. (A) In four independent colonies double transformed with γ-filamin d19–21 and MYOT-SIT, β-galactosidase activity was restored, indicating an interaction between both polypeptides. This reactivity was absent in all colonies of yeast cells transformed with γ-filamin d20/21 and MYOT-SIT. (B) Two independent yeast colonies cotransformed with γ-filamin d19–21 or γ-filamin d20/21, and truncated myotilin fragments. Note that only in yeast cells cotransformed with γ-filamin d19–21 and MYOT-I or MYOT-SIT β-galactosidase reactivity was restored. These results are summarized in C.

cytes, α-filamin d20 was diffusely distributed within the cytoplasm upon expression in C2C12 cells (Fig. 7, J and K).

γ-Filamin Binds to the Z-Disc Protein Myotilin

The yeast two-hybrid system was used to search for binding partners of the region encompassing the unique insertion in Ig-like domain 20 of γ-filamin. Since the single domain construct γ-filamin fused to the activation domain turned out to be autoactivating and therefore was not suitable for screening, we had to use a bait construct that included the flanking Ig-like domains 19 and 21. The yeast two-hybrid screen yielded ~70 HIS3 and β-galactosidase positive clones from ~5 × 10^5 transformants. Plasmids were rescued from 20 colonies and the inserts from 12 were partially sequenced. Three of these were found to contain the full coding region of myotilin, a recently discovered component of the sarcomeric Z-disc (Salmikangas et al., 1999).

To investigate with greater precision which regions of γ-filamin and myotilin are responsible for the interaction of both proteins, different subfragments of their cDNAs were cloned into the yeast two-hybrid bait and pray vectors (as described in Materials and Methods). All constructs were analyzed for autoactivation activity. Interactions were tested by β-galactosidase assays in cotransformed L40 yeast cells. The pair of Ig-like filamin domains

20 and 21 (γ-filamin d20/21) was found to be negative in these assays. Since the bait containing domains 19 and 20 (γ-filamin d19/20) was autoactivating, the original three-domain filamin fragment was the smallest fragment that showed binding to full-length myotilin (Fig. 8). Similarly, myotilin constructs were generated that contained either the unique amino terminal 248 amino acids (MYOT-S) or the subsequent two Ig-like domains (MYOT-I, residues 251–447). In the assay, only the fragment MYOT-I was positive (Fig. 8). Thus, this novel protein–protein interaction could be located to γ-filamin Ig-like domains 19–21 and to the Ig-like domains of myotilin.

Coimmunoprecipitation and Double-Transfection Experiments Confirm the Association of γ-Filamin d19–21 with the Immunoglobulin Domains of Myotilin

The yeast two-hybrid data were confirmed by independent biochemical binding assays. Distinct subfragments of γ-filamin comprising either the insert-bearing domain 20 or this domain with one or both neighboring domains were expressed with a carboxyterminal EEF-tag in *E. coli*. Only two of the recombinant polypeptid*e*s (γ-filamin d19–21 and γ-filamin d20/21) could be purified in a native, soluble form (Fig. 9 A). Likewise, the portion of myotilin that comprises its Ig-like domains was also bacterially expressed, however, with an amino-terminally situated T7 tag (construct MYOT-I; Fig. 9 A). This combination of two differentially tagged constructs allowed us to investigate the binding by immunoprecipitation experiments. In this assay, only the three-domain filamin construct (γ-filamin d19–21) showed binding to the myotilin Ig-domains (Fig. 9, B and C). Immunocomplexes were precipitated both with the anti–T7 tag and the anti–EEF tag antibodies (Fig. 9, B and C, arrows). In contrast, the recombinant polypeptides γ-filamin d20/21 and γ-filamin d24 were not coprecipitated with myotilin (Fig. 9 B).

The validity of the observed association of myotilin with γ-filamin was further strengthened by coimmunoprecipitation experiments from yeast cells that were coexpressing the myotilin immunoglobulin domains fused to GAL 4AD and the HA tag, and γ-filamin d19–21 fused to the LexA protein. Both antibodies specific for the HA tag and antibodies recognizing the immunoglobulin domains of myotilin specifically precipitated protein complexes also containing the expressed γ-filamin fragment (Fig. 9 D). In contrast, the filamin fragment was not coprecipitated with GAL 4AD–HA tag alone, substantiating the specificity of the interaction.

Further evidence for the interaction between myotilin and γ-filamin was achieved by double transfection of nonmuscle cells with GFP-tagged full-length myotilin and T7-tagged γ-filamin d19–21. Whereas myotilin expressed in Ptk2 cells showed an obvious colocalization with α-actinin in stress fibers (Fig. 10, A and B), cells double transfected with the myotilin construct and γ-filamin d19–21 showed a dramatically altered myotilin localization in aggregates together with the truncated γ-filamin polypeptide (C and D). Double transfection of GFP–α-actinin together with γ-filamin d19–21 did not inhibit the targeting of α-actinin to stress fibres (Fig. 10, E and F), indicating that the GFP tag plays no role in the observed association.
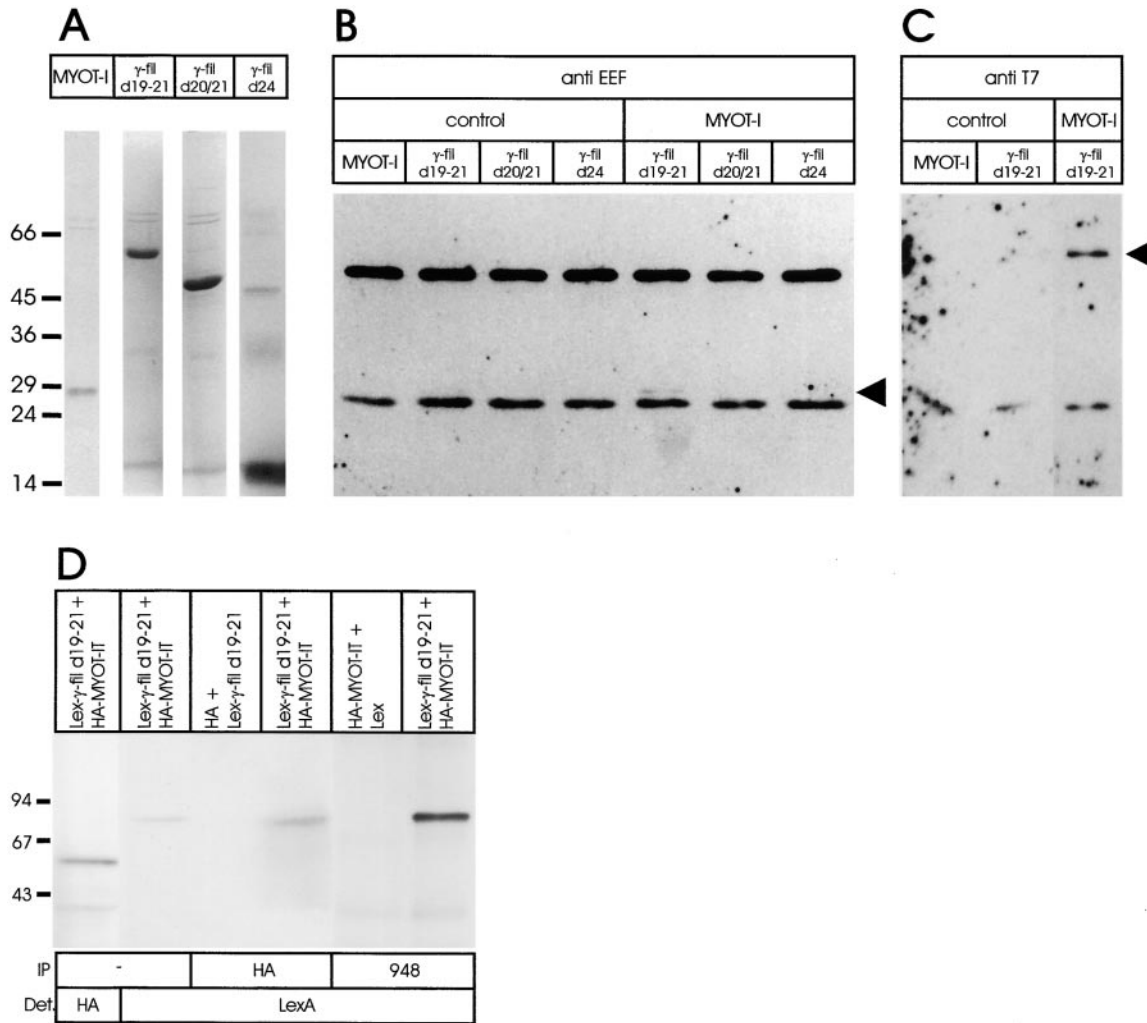
Figure 9. Coimmunoprecipitation of filamin and myotilin fragments. (A) Coomassie blue–stained SDS gel of recombinant protein fragments used for coimmunoprecipitation experiments. The identity of the polypeptides is indicated above each lane. (B and C) T7-tagged MYOT-I and EEF-tagged γ-filamin fragments alone (control) or mixed with MYOT-I (MYOT-I) were incubated with anti–EEF antibody (B) or anti–T7-tag antibody (C), immunoprecipitated as described in *Materials and Methods*, and subjected to SDS-PAGE and Western blotting. For immunodetection of binding partners of the precipitated polypeptide, anti–T7-tag antibody (B) or anti–EEF antibody (C) were used. Arrowheads indicate positions of proteins that are specifically coprecipitated. Note that MYOT-I is coprecipitated with γ-filamin d19–21, but not with γ-filamin d20/21 or γ-filamin d24 (B), and that γ-filamin d19–21 is coprecipitated with MYOT-I (C). (D) Coimmunoprecipitation from yeast cells double transfected with an empty Lex vector (Lex) or the pLex vector containing γ-filamin d19–21, together with an empty pACT2-HA vector (HA) or this vector with MYOT-IT (HA-MYOT-IT), as indicated above the lanes. In the left two lanes, expression of the proteins was confirmed by Western blotting using an anti–HA or anti–LexA antibody. In the middle two lanes, the complexes immunoprecipitated with the anti–HA antibody were separated and stained with the anti–LexA antibody to detect Lex-γ-filamin d19–21. Note that Lex-γ-filamin d19–21 is only coprecipitated with HA-MYOT-I and not with HA alone. In the right two lanes, complexes immunoprecipitated with the anti–myotilin antibody were separated and stained with the anti–LexA antibody to detect Lex-γ-filamin d19–21. Note that Lex-γ-filamin d19–21 is again coprecipitated with HA-MYOT-I. No signal is obtained if the cells are transfected with the empty LexA vector.

Overexpression of the Immunoglobulin Domains of Myotilin, but not of Full-Length Myotilin, Has a Dominant Negative Effect on Myofibril Assembly

To gain insight in the possible function of the interaction between γ-filamin and myotilin, the immunoglobulin domains of myotilin (MYOT-I) and full-length myotilin (MYOT-SIT) were cloned into the pCMV5-T7 vector, and overexpressed in C2C12 cells that were subsequently allowed to differentiate. The effect of the overexpression of both recombinant proteins was studied immunocytochemically by double staining the transfected cells with antibodies against the immunotag and T12, our antibody against Z-disc titin. This allowed us to study Z-disc assembly in the presence of the overexpressed polypeptides. In cells transfected with MYOT-SIT, the recombinant protein was detected in numerous large myotubes, were it colocalized with Z-disc titin during all developmental stages (Fig. 11, G and H). This indicated that MYOT-SIT is targeted to
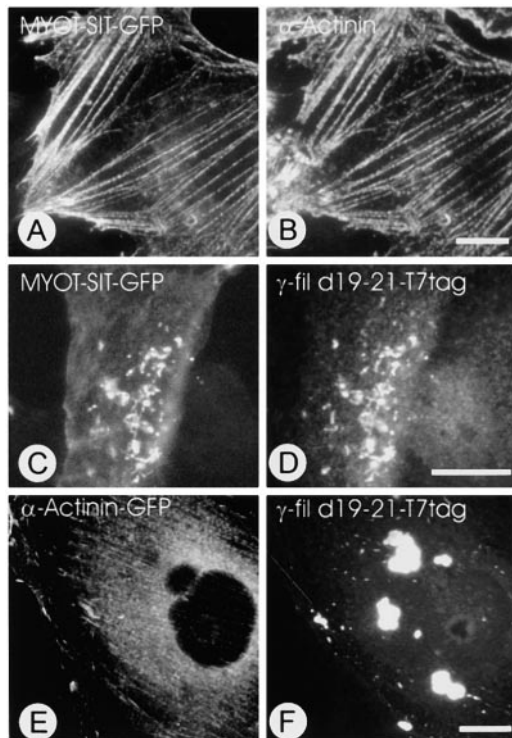
Figure 10. Expression of filamin, myotilin and α-actinin in non-muscle cells. Ptk2 cells were transfected with GFP-tagged MYOT-SIT alone (A and B), GFP-tagged MYOT-SIT together with T7-tagged γ-filamin d19–21 (C and D), or GFP-tagged α-actinin and T7-tagged γ-filamin d19–21 (E and F). The T7-tagged recombinant protein was visualized by staining with the anti–T7-tag antibody (D and F). Endogenous α-actinin was stained with BM-75.2 recognizing all isoforms of α-actinin (B). Full-length myotilin is targeted to stress fibers, where it colocalizes with endogenous α-actinin (A and B). Note that if, in the same cell, γ-filamin d19–21 is expressed, the localization of MYOT-SIT is dramatically changed, and the protein is found in aggregates together with γ-filamin d19–21 (C and D). In contrast, the localization of GFP-tagged α-actinin, which is also targeted to stress fibers, is not changed upon coexpression of γ-filamin d19–21 (E and F), indicating that MYOT-I and not the GFP tag interacts with γ-filamin d19–21. Bars, 10 μm.

Figure 11. Expression of recombinant myotilin fragments in differentiating C2C12 mouse myoblasts. C2C12 cells transfected with constructs encoding T7-tagged immunoglobulin domains of myotilin (MYOT-I; A and F), or full-length myotilin (MYOT-SIT; G and H) were allowed to differentiate for 4 d. Subsequently, they were fixed and double stained with T7-tag antibody (left) and T12 (right), an antibody specific for a Z-disc epitope of titin. Note that MYOT-SIT exactly colocalizes with Z-disc titin in nascent myofibrils with immature Z-discs as well as in mature myofibrils (G and H). MYOT-I associates with stress fiber–like structures (A and B), but it is never found in Z-discs. Moreover, myotubes expressing MYOT-I do not develop myofibrils. Instead, the myofibrillar proteins are colocalized with MYOT-I in filamentous bundles (C and D) or amorphous aggregates (E and F). Bar, 20 μm.

developing and mature Z-discs without obviously disturbing myofibril assembly. Transfection of the MYOT-I construct, however, resulted in a dramatic effect on myofibrillar assembly. Although the recombinant polypeptide was targeted to immature Z-discs (Z-bodies; Fig. 11, A and B), transfected myotubes with fully developed Z-discs were never observed. Instead, irregular filamentous structures (Fig. 11, C and D) or amorphous aggregates (E and F) containing MYOT-I and a major part of titin were present in transfected cells.

Discussion

The development of myofibrils in differentiating muscle cells is one of the most impressive macromolecular assembly processes in nature. It involves the interaction of a great number of different proteins that has to be precisely controlled both in terms of space and time (Franzini-Armstrong and Fischman, 1994; Squire, 1997). Many of these
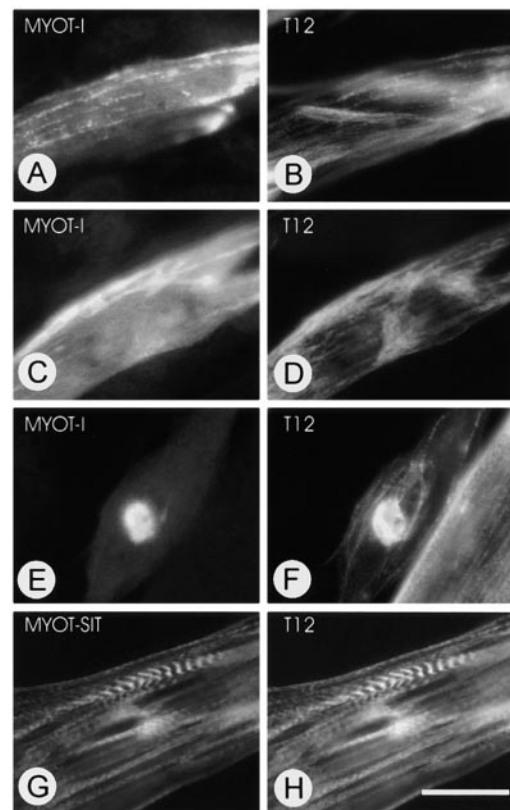
proteins occur in multiple isoforms whose expression patterns are fundamentally different and are therefore thought to fulfill highly specialized roles (Pette and Staron, 1997). The filamins are an example for such a gene family that became evident only rather recently. In the chicken system, the existence of several distinct filamin isoforms was suspected already several years ago, mainly due to differentially reacting antibodies (Gomer and Lazarides, 1981, 1983; Price et al., 1994). Further insights into the differential functions of the avian isoforms has been hindered by the lack of primary sequence information, with only one isoform so far cloned (Barry et al., 1993). Similar studies in mammalian systems relied for a long time on antibodies reacting with only a single polypeptide that on top could not be identified in striated muscles (Brown and Binder, 1993). Molecular genetic studies pointed at the existence of an additional isoform expressed in human skeletal muscle that was called ABP-L (Maestrini et al., 1993). Its complete cDNA was cloned re-

cently and the protein was renamed γ-filamin (Xie et al., 1998). We have succeeded in obtaining an antibody that reacts with both filamin isoforms, which made it feasible to approach the question of the different roles of these proteins in muscle (van der Ven et al., 2000). Here we used this mAb (called RR90) in combination with an antibody that we could show to be specific for α-filamin, with the idea that a subtraction of both antibody labels might yield firm statements about the distribution of the individual filamin isoforms. Thus, in adult striated muscle fibers, γ-filamin is expressed while nonmuscle α-filamin is limited to blood vessels and connective tissue cells (Fig. 4). This still leaves the interesting question unanswered: why, within the myofibers, γ-filamin shows a dual pattern of distribution? In accordance with cell fractionation studies (Thompson et al., 2000), we found the majority of filamin in the myofibrils that correspond to the particulate sarcoplasmic fraction; conversely, a small fraction of γ-filamin was located at the sarcolemma (Fig. 4). We propose that this differential targeting, which at the same time implies distinct functions, could be the result of alternatively spliced γ-filamin variants. This idea is supported by a provisional genetic analysis that revealed short alternatively spliced exons for α-filamin (Maestrini et al., 1993). Definitive proof, however, will require the establishment of the complete genomic structure of the *FLNC* gene and the subsequent elucidation of peptide-specific antibodies. In line with this view, quite distinct staining patterns were observed in developing chicken muscle cells. On the one hand, filamin was first located in stress fiber–like structures, and then disappeared for several days, and finally reappeared at the periphery of Z-discs (Gomer and Lazarides, 1981). On the other hand, filamin was detected in the developing chicken heart only from the 14 somite stage onwards; i.e. at a time when functional myofibrils in the beating heart exist already for ∼4 h (Price et al., 1994). It is very likely that the antibodies used in these studies did not reflect the complete spectrum of filamin isoforms that can be expected to be expressed in avian tissues.

The subtractive antibody labeling approach was also used on developing skeletal muscle cells in culture. This clearly demonstrated not only that γ-filamin is expressed within a few hours subsequent to the induction of muscle differentiation (see also van der Ven et al., 2000), but also that solely this isoform participates in the striking reorganization of the actin cytoskeleton during myofibril formation (Fig. 5).

The specific function of filamin in myofibrils cannot be solely attributed to its well-documented capacity to crosslink actin filaments, particularly since this region shows very little variability between the isoforms. Therefore, different functions must be determined in other, more diverse parts of the molecule. The region that mainly attracted our attention was the unique insertion of 78 amino acids located within Ig-like domain 20. We show here that this insertion, which previously was shown to be specifically expressed in differentiated muscle (Xie et al., 1998; van der Ven et al., 2000), is encoded by a single extra exon that is differentially spliced (Fig. 2). Transfection studies and yeast two-hybrid screenings were used to address the specific function of this molecular region. The transfection studies were aimed at elucidating its role for the subcellular targeting of filamin in muscle cells. Previous studies using either the α- or γ-filamin isoforms have emphasized

their membrane association (see Introduction). Here, we identify for the first time a myofibrillar targeting signal within γ-filamin that is functional in both cardiac and skeletal muscle cells. Since in both cell types myofibrils were assembled normally in the presence of the overexpressed recombinant protein, it does not seem to exert a dominant negative effect on this process, at least at the time scale of our experiments. We also believe it is justified to assume that this signal must reside in the unique domain 20 insertion because α-filamin (which does not contain this insertion) does not show any specific localization upon overexpression in muscle cells.

The strict targeting of Ig-like domain 20 of γ-filamin urged us to search for a myofibrillar protein that might specifically interact with it. Yeast two-hybrid screens identified the recently discovered protein myotilin (Salmikangas et al., 1999) as a binding partner for γ-filamin, and three independent experiments (coimmunoprecipitation experiments using recombinant fragments, coimmunoprecipitation from double-transfected yeast cells, and double transfection of nonmuscle cells) confirmed and further confined this interaction (Figs. 9 and 10). Since myotilin was previously shown to bind α-actinin, we assume that the function of this Z-disc protein is the indirect anchorage of γ-filamin, which is localized at the periphery of sarcomeric Z-discs (Thompson et al., 2000; van der Ven et al., 2000), to α-actinin in the central Z-disc.

In contrast to the overexpression of the myotilin binding part of γ-filamin, the overexpression of the γ-filamin–binding region of myotilin had a dramatic effect on the assembly of myofibrils in differentiating C2C12 myotubes. This implies that the amino terminal part of myotilin that is lacking in this construct and is known to contain an α-actinin binding site (Salmikangas et al., 1999) plays an important role in the control of myofibrillar assembly. Obviously, the interaction γ-filamin–myotilin–α-actinin is disturbed by overexpression of this truncated myotilin fragment that is still able to associate with γ-filamin. This regulatory role is further strengthened by the observation that overexpression of full-length myotilin does not result in abnormal myofibrillogenesis. Thus, it seems that the α-actinin–binding part of the myotilin molecule is indispensable for the proper function of the protein.

The identification of myotilin as a γ-filamin binding partner described in this report, together with earlier observations that γ-filamin binds γ- and δ-sarcoglycan (Thompson et al., 2000) is particularly interesting with regard to recent findings on LGMD. Mutations in the genes encoding all three binding partners cause LGMD (Noguchi et al., 1995; Nigro et al., 1996; Hauser et al., 2000). Furthermore, the *FLNC* gene itself has been mapped to an LGMD candidate region on chromosome 7 (Speer et al., 1999). Interestingly, caveolin-1 was recently identified as a binding partner of α-filamin (Stahlhut and van Deurs, 2000). Considering the high degree of similarity of the isoforms of both proteins, the muscle isoform of caveolin (caveolin-3, which in turn is a further LGMD gene), can be assumed to interact in a similar way with muscle filamin, although definitive proof is still lacking.

Until recently, this genetically heterogeneous disease has been attributed essentially to mutations of the genes encoding sarcolemmal proteins such as α-, β-, γ-, and δ-sarcoglycans, caveolin-3, and dysferlin (reviewed in

Bushby, 1999; Toniolo and Minetti, 1999). In LGMD 2C and LGMD 2F, which are caused by mutations in the γ- and δ-sarcoglycan genes, respectively, the fraction of γ-filamin that is localized to the sarcolemma is highly increased. Considering these observations, the traditional role of filamins in the submembraneous cytoskeleton and the fact that the vast majority of other identified interactors of filamins until now are mainly membrane-associated proteins, an LGMD model was suggested that favors a cyclic translocation of γ-filamin from membrane attachment sites to intracellular locations. In LGMD 2C and 2F and other muscle diseases characterized by abnormalities in both sarcoglycans this cycling was supposed to be disturbed (Thompson et al., 2000).

Our finding that the muscle-specific isoform of filamin specifically binds myotilin, adds an interesting facet to the theme of LGMD: mutations in myotilin, a sarcomeric Z-disc protein that interacts with α-actinin, were suggested to cause LGMD1A (Hauser et al., 2000). Filamin is therefore the first protein identified that can link membrane-related effects to myofibrillar defects. This finding strongly corroborates the notion that, in LGMD myofibers, large amounts of filamin accumulate at the sarcolemma (Thompson et al., 2000). LGMD may therefore be caused by two interdependent molecular events: one, the destabilization of membrane attachment complexes and, the other, the weakening of myofibrils and/or myofibrillar connections particularly at the Z-disc. The recently described LGMD 2G-causing mutations in the Z-disc protein telethonin (Moreira et al., 2000), a protein that has been implied to be involved in signaling events during myofibril development (Mayans et al., 1998; Mues et al., 1998), strongly supports this assumption.

The relationship of γ-filamin with both sarcolemmal and myofibrillar proteins, which are involved in LGMD, is highly indicative of an important function for this protein in signaling processes between the sarcolemma and myofibrils. In line with this idea, patients were identified whose disease phenotype showed genetic linkage to chromosome 7q (Speer et al., 1999), a chromosomal region that also includes the *FLNC* gene (Maestrini et al., 1993; Xie et al., 1998). This increases the probability that defects in the *FLNC* gene itself might cause LGMD in patients who do not have mutations in any of the known LGMD genes. Our work might therefore provide a molecular basis to define a novel signaling pathway from the sarcolemma to the myofibril. Disturbances of this pathway at any point may result in essentially the same clinical manifestation in the form of LGMD.

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