

Filamin 2 (FLN2): A Muscle-specific Sarcoglycan Interacting Protein

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Abstract. Mutations in genes encoding for the sarcoglycans, a subset of proteins within the dystrophin-glycoprotein complex, produce a limb-girdle muscular dystrophy phenotype; however, the precise role of this group of proteins in the skeletal muscle is not known. To understand the role of the sarcoglycan complex, we looked for sarcoglycan interacting proteins with the hope of finding novel members of the dystrophin-glycoprotein complex. Using the yeast two-hybrid method, we have identified a skeletal muscle-specific form of filamin, which we term filamin 2 (FLN2), as a γ - and δ -sarcoglycan interacting protein. In addition, we demonstrate that FLN2 protein localization in limb-girdle muscular dystrophy and Duchenne muscular dystrophy

patients and mice is altered when compared with unaffected individuals. Previous studies of filamin family members have determined that these proteins are involved in actin reorganization and signal transduction cascades associated with cell migration, adhesion, differentiation, force transduction, and survival. Specifically, filamin proteins have been found essential in maintaining membrane integrity during force application. The finding that FLN2 interacts with the sarcoglycans introduces new implications for the pathogenesis of muscular dystrophy.

Key words: dystrophin • muscular dystrophy • filamin C (FLNC) • cytoskeleton • cell signaling

Introduction

The muscular dystrophies are a genetically heterogeneous group of disorders that affect muscle membrane integrity, resulting in a loss of muscle function. The most common form of muscular dystrophy is caused by abnormalities in dystrophin, the protein product of the Duchenne muscular dystrophy (DMD)¹ gene (Hoffman et al., 1987). Dystrophin is a large subsarcolemmal, actin-binding protein associated with a complex of sarcolemmal and cytoskeletal proteins known as the dystrophin-glycoprotein complex (DGC) (Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991). DGC can be separated into three subcomplexes: (1) dystroglycan, (2) dystrobrevin-syntrophin, and (3) sarcoglycan complexes, which together are believed to confer structural support by providing a link between the extra-

cellular matrix (ECM) and the actin cytoskeleton (Campbell and Kahl, 1989; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1993; Yoshida et al., 1994).

The pathogenic mechanism by which the muscular dystrophy phenotype arises remains poorly understood. As new genes associated with this group of disorders are discovered, the complexity of proposed mechanisms increases. This phenomenon is best seen in the various limb-girdle muscular dystrophies (LGMDs). Here, positional cloning and candidate gene approaches have shown that at least 14 different genetically defined disorders are responsible for the LGMD phenotype. This list includes the following: sarcoglycans (α , β , γ , and δ), $\alpha 7$ integrin, the calcium-dependent protease calpain-3, caveolin-3, and dysferlin (Roberds et al., 1994; Bonnemann et al., 1995; Lim et al., 1995; Noguchi et al., 1995; Richard et al., 1995; Nigro et al., 1996; Mayer et al., 1997; Bashir et al., 1998; Hayashi et al., 1998; Liu et al., 1998; McNally et al., 1998; Minetti et al., 1998). Originally, it was believed that dystrophin and the dystroglycan complex had merely a structural role in skeletal muscle by anchoring muscle cells to the ECM, which would involve a more stationary process. Although some proteins involved in

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¹Abbreviations used in this paper: CoIP, coimmunoprecipitate; DGC, dystrophin-glycoprotein complex; DMD, Duchenne muscular dystrophy; ECM, extracellular matrix; EST, expressed sequence tag; IMF, immunofluorescence; FLN2, filamin 2; GP, glycoprotein; LGMD, limb-girdle muscular dystrophy.

the dystrophic process do provide structural support, the majority of proteins associated with the phenotype are involved in signal transduction cascades. For example, the $\alpha 7$ integrin subunit belongs to a family of transmembrane receptor proteins known to modulate gene expression specific for cell migration, adhesion, or prevention of apoptosis (Yamada and Miyamoto, 1995; Giancotti and Ruoslahti, 1999). M-calpain has been implicated in regulating actin-myosin filament formation, and calpains, in general, are thought to regulate transcription factors' entry into the nucleus by cleavage (Kwak et al., 1993b; Sorimachi et al., 1997; Baghdiguian et al., 1999). The caveolins (types 1, 2, and 3) form vesicular invaginations termed caveolae within the plasma membrane on which preassembled signaling complexes are organized (Okamoto et al., 1998). With the cloning of these genes, it has become evident that the process of maintaining muscle fiber integrity is dynamic and not simply a static, structural process. The remaining proteins, including the sarcoglycans, have no known function and intensive research is being done to unravel their roles in the maintenance of muscle fiber integrity.

Some clues as to the function of the sarcoglycans can be gained through biochemical analysis. Coimmunoprecipitation and chemical cross-linking studies in the skeletal muscle have demonstrated the close physical association between members of the sarcoglycan complex by showing that β -, γ -, δ -, and to a lesser extent, α -sarcoglycan are tightly connected with one another (Chan et al., 1998). Further evidence for this association is seen through immunofluorescence (IMF) studies using skeletal muscle sections from sarcoglycanopathy patients and staining for various sarcoglycans. These experiments reveal a reduction or complete absence of all four sarcoglycan proteins at the sarcolemmal membrane, suggesting that a mutation in any one sarcoglycan can create a secondary instability of the entire sarcoglycan complex. Interestingly, although mutations in dystrophin and the sarcoglycans produce a similar phenotype, dystrophin staining in the sarcoglycanopathies is either normal or only slightly reduced, indicating that the selective loss of the sarcoglycan complex is sufficient to cause muscular dystrophy (McNally et al., 1996; Hack et al., 1998; Araishi et al., 1999).

To further study the sarcoglycan complex and the role it plays in maintaining muscle fiber integrity, we looked for interacting proteins with the hope of finding additional clues as to the function of the sarcoglycan complex. Using the yeast two-hybrid system, we tested the intracellular domains of α -, β -, γ - and δ -sarcoglycans. From these library screens, we identified a skeletal and cardiac muscle filamin (filamin 2; FLN2) as a γ -sarcoglycan interacting protein, and confirmed this interaction using *in vivo* coimmunoprecipitation experiments and *in vitro* protein assays. In addition to binding γ -sarcoglycan, we determined that this protein also binds δ -sarcoglycan but not α - or β -sarcoglycans. Through EM studies, FLN2 appears to be located both at the Z-line and the sarcolemmal membrane in skeletal muscle, indicating that a subpopulation of this protein colocalizes with the DGC. Patients with mutations in γ -sarcoglycan, δ -sarcoglycan, or dystrophin show an increase of FLN2 content at the plasma membrane compared with normal individuals and, interestingly, pa-

tients with general myopathy appear to have normal FLN2 staining.

Filamin proteins have been implicated as signal transducers in several systems and, therefore, might aid in the maintenance of skeletal muscle by regulating and/or protecting the muscle during the process of muscle contraction. The identification of filamin as a sarcoglycan interacting protein strengthens the idea that the DGC does not merely function as structural support, but rather that this group of proteins has an active role (i.e., signaling role) in maintaining the skeletal muscle.

Materials and Methods

Two-Hybrid Library Screening

The cytoplasmic tails of α - (amino acids 312–387), β - (amino acids 1–62), γ - (amino acids 1–38), and δ -sarcoglycan (amino acids 1–35) were amplified by reverse transcriptase-PCR from human skeletal muscle total RNA with primers containing EcoRI linkers, and inserted downstream of the Gal4 DNA-binding domain in the (bait) vector pGBT9 (CLONTECH Laboratories). The resulting clones were sequenced and found to be in-frame with the yeast GAL4 DNA-binding domain.

A CLONTECH yeast two-hybrid cDNA library derived from the human skeletal muscle, inserted downstream of the Gal4 activation domain in the vector pGAD, was screened using the yeast two-hybrid protocol (MATCHMAKER-1 system; CLONTECH Laboratories). In brief, *Saccharomyces cerevisiae* strain HF7C was cotransformed with either library DNA plus a sarcoglycan bait vector or sequentially transformed with bait vector followed by library DNA. The HF7C strain contains two reporter genes, *HIS3* and *LacZ*, which allow for the selection of library cDNAs encoding proteins that interact with the intracellular portion of the sarcoglycans. Transformants were plated onto SD/Trp⁻/Leu⁻/His⁻ plates and incubated at 30°C until colonies appeared (~5 d). Colonies able to grow on minimal plates were screened for β -galactosidase activity using a filter-lift assay. Yeast DNA isolated from colonies positive for β -galactosidase activity was used to electroporate *Escherichia coli* to recover the interacting cDNA. To eliminate false positive colonies, plasmid DNA from the β -galactosidase-positive clones was reintroduced into yeast with either the sarcoglycan/pGBT9 bait vector, pGBT9 alone, or a control vector p53/pGBT9.

Full-length FLN2 cDNA Cloning, DNA Sequencing and Analysis

Sequence from a γ -sarcoglycan interacting clone, 2-14, was used to screen a human skeletal muscle cDNA library (CLONTECH Laboratories) in conjunction with two express sequence tags (ESTs) previously reported to be muscle-specific forms of *FLN1* (GenBank accession numbers X70083 and X70084). The probes for X70083 and X70084 were obtained using primers designed for the GenBank sequences to amplify cDNA from reverse transcribed human skeletal muscle RNA. Of 10⁶ recombinant phage screened, ~200 phage clones hybridized with the presumed *FLN2* probes. 40 randomly picked phage clones were used to plaque purify and obtain DNA. Phage inserts were subcloned into the EcoRI site of the pZERO1.1 vector (Invitrogen Corp.) for further characterization. Nine clones were chosen for sequencing because of their large insert size.

All pGBT9 and pZERO1.1 subclones were sequenced from both DNA strands using vector primers. An additional sequence on larger inserts was obtained by designing primers to the previously obtained sequence. The sequence was analyzed on either an ABI 373 or 377 automated sequencer with fluorescent dye terminator chemistry (Applied Biosystems). Sequences were assembled using Sequencher™ 3.1 software (Genecodes). Subsequent amino acid prediction analysis was performed using MacVector™ software (Oxford Molecular Group) and the GAP program within the GCG database. In addition, the BLAST computer program was used to search the GenBank database for ESTs and sequence-tagged sites containing sequences similar to those in the 2-14 cDNA clone.

Preparation of Cell Lysates and Immunoprecipitation

Whole cell lysates and immunoprecipitation experiments were performed

as described previously (Chan et al., 1998). In brief, cultured mouse myotubes were lysed on ice for 15 min in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 1× protease inhibitor cocktail) and cell debris was pelleted by centrifugation for 10 min. Protein concentration of the resulting lysate was determined by the Bio-Rad DC protein colorimetric assay using BSA as a standard (Bio-Rad Laboratories). For immunoprecipitation experiments, 50–100 µg of cell lysate was precleared using protein G–Sepharose beads (Sigma Chemical Co.). Precleared lysate was incubated with 5–10 µl of antisarcoglycan antibody at 4°C for 4 h, and then with 15 µl of protein G–Sepharose beads for 1 h. The immune complex was washed three times with cold lysis buffer and solubilized in 2× protein sample buffer (Novex). Protein complexes were separated by electrophoresis on 4–20% denaturing gradient gels (Novex) and transferred onto nitrocellulose membrane.

Antibody Production

A 511-residue polypeptide corresponding to the carboxy terminus of FLN2 was fused in-frame to glutathione-S-transferase in the pGEX-4T1 vector using a PCR based cloning method. In brief, PCR primers designed with EcoRI linkers were used to amplify a DNA fragment encoding amino acids 2,128–2,688 of FLN2 from the 2-14 clone and subcloned into an EcoRI-digested pGEX-4T1 vector. The sequence was confirmed using a 373 or 377 ABI automated sequencer. The fusion protein was purified from *E. coli* using B-PER bacterial protein extraction reagent (Pierce Chemical Co.). Protein preparations were used to inject New Zealand white rabbits for polyclonal antibody production (Covance). A second antigen was generated to the unique region of FLN2 because of the overall similarity of FLN2 to FLN1. A peptide corresponding to amino acids 2,160–2,177 (C-SKTRGGGETKREVRVEEST) was synthesized and used to inject New Zealand white rabbits (Research Genetics). The amino terminal cysteine is not part of the FLN2 sequence, and was added for use in affinity purification.

Antibodies were purified from both sets of sera using previously described purification techniques (Harlow and Lane, 1988). For the fusion protein antibody (FLN2-A1), the cDNA fragment encoding for the 511-residue carboxy terminus was subcloned into the pTrcHis2-TOPO TA cloning vector (Invitrogen Corp.). 6× His⁺-tagged fusion protein was purified from *E. coli* using His-Bind resin and buffer kit (Novagen), and was coupled to Affi-gel 10 immunoaffinity matrix as per the manufacturer's instructions (Bio-Rad Laboratories). SulfoLink coupling gel (Pierce Chemical Co.) was used to couple the 15-amino acid peptide (residues 2,160–2,177) for use in the affinity purification of the peptide antibody FLN2-A2. Crude sera was affinity-purified over the appropriate column using standard acid-base elution methods (Harlow and Lane, 1988). Resulting affinity-purified antibodies were dialyzed against PBS, pH 7.4.

Immunoblot Analysis

Proteins were transferred to nitrocellulose membranes at room temperature (Schleicher and Schuell) in Towbin buffer at 20 V overnight (Towbin et al., 1979). Membranes were blocked using (1× PBS, 0.1% Tween 20, and 5% nonfat milk) for 1 h at room temperature to prevent nonspecific proteins from binding to the membranes. Membranes were incubated for 2 h at room temperature with the primary antibody diluted in blocking solution. Membranes were given four, 15-min washes in 1× PBS, 0.1% Tween 20, and then incubated for 1 h with an HRP-conjugated donkey anti-rabbit (or anti-mouse) IgG (H + L) secondary antibody (Jackson ImmunoResearch Laboratories) diluted to 1:10,000 in blocking buffer. Membranes were given three, 15-min washes in 1× PBS, and 0.1% Tween 20. The HRP-conjugated protein complex was detected by chemiluminescence according to the manufacturer's protocol (Renaissance; NEN) and visualized on X-ray film.

In Vitro Transcription/Translation

Portions of FLN2 and the sarcoglycans were subcloned into expression vectors pMGT-1 and pFHR-3, which have been described previously in detail (Ahn and Kunkel, 1995). [³⁵S]Methionine-labeled peptides were translated in vitro using a TNT T7 coupled reticulocyte lysate system according to the manufacturer's protocol (Promega Corp.). Expressed proteins were separated by electrophoresis on SDS gradient gels purchased from Novex. Gels were exposed to a storage phosphor plate that was subsequently scanned using a PhosphorImager (Molecular Dynamics).

Microsome Preparation

Samples of cultured C2C12 myotubes or mouse skeletal muscle were homogenized with a Dounce homogenizer in 1 ml of ice cold buffer I (10 mM Tris, pH 7.5, and 2 mM PMSF with protease inhibitor cocktail; Boehringer Mannheim). After 10 min of incubation on ice, 1 ml of buffer II (10 mM Tris, pH 7.5, 20% sucrose, 0.3 M KCl, 2 mM PMSF, and protease inhibitor cocktail) was added to the solution and centrifuged at 12,000 g at 4°C for 3 min. The remaining cell pellet was rehomogenized with fresh buffer I and II. The supernatant from both rounds of homogenization was subsequently combined and centrifuged at 105,000 g for 1 h using a Beckman SW55Ti rotor. The final microsome pellet and the supernatant (soluble fraction) were used in Western blot analysis.

Immunohistochemistry

Skeletal muscle was obtained under an institutional discarded tissue protocol (Children's Hospital, Boston, MA) for short postmortem interval autopsies of patients without neuromuscular disease. Specimens were frozen in isopentane, cooled in liquid nitrogen, and stored at –80°C until cryosectioning was performed. Mounted sections of frozen muscle (8 µm) were fixed in methanol for 3 min and blocked in 1× PBS with 15% horse serum and 0.1% Triton X-100 for 1 h at 4°C. Incubation with primary anti-FLN2 antibodies, FLN2-A1 or FLN2-A2 (1:1,000), and antisarcoglycan (Novocastra; 1:200) was carried out overnight at 4°C. The slides were washed three times, 20 min each in 1× PBS and 0.25% Triton X-100, re-blocked for 30 min and incubated for 1 h at room temperature with a Cy3- or FITC-conjugated donkey anti-rabbit (or anti-mouse) IgG (H + L) secondary antibody cross-adsorbed for human IgG from Jackson ImmunoResearch Lab (1:300). Slides were subsequently given three 20-min washes in 1× PBS and 0.25% Triton X-100, and the coverslips were mounted using the ProLong antifade kit from Molecular Probes. Analysis was performed using a Zeiss Axioplan II microscope. Resulting slides were washed and the coverslips were mounted for analysis using the Zeiss microscope with a triple filter.

Confocal Microscopy

Flash frozen needle biopsies of normal adult skeletal muscle were prepared as described in the immunohistochemistry section above. Cryosections (7–8 µm) were cut at –20°C using a Microm cryostat. Frozen sections were mounted on gelatin-coated slides blocked with 5% goat serum in PBS containing 5% BSA, and 0.15% glycine (buffer A) for 40 min. Sections were washed three times in buffer A, and then incubated for 1 h with FLN2-A2 antibody (1:1,000; see Antibody Production). Sections were washed three times in buffer A, and incubated for 1 h with an Alexa 488 conjugated secondary antibody (1:500; Molecular Probes). After three further wash steps, coverslips were mounted using gelvatol (Monsanto). Sections were observed using a confocal laser scanning microscope (TCS NT; Leica). Parameters, including pinhole size, PMT settings, laser intensities, and magnifications (60×) were kept constant for all the imaging analysis conducted. Randomly sampled areas were selected, and the abundance and distribution of proteins were examined.

Immunoelectron Microscopy

Biopsies were fixed in 2% paraformaldehyde, and 0.01% glutaraldehyde, diced into 1-mm cubes, and cryoprotected overnight in a solution containing 1× PBS and 2.3 M sucrose. Subsequently, the tissue was mounted on cutting stubs in a specific (longitudinal or cross-sectional) orientation, shock frozen, and stored in liquid nitrogen. Thin sections (70–100 nm) were cut using a Reichert ultracut S ultramicrotome with an FC4S cryoattachment, lifted in a small drop of sucrose, and mounted on Formvar-coated carbon grids. Sections were washed three times in PBS containing 0.5% BSA and 0.15% glycine, pH 7.4 (buffer B), followed by a 30-min incubation with purified goat IgG (50 µg/ml) at 25°C and three additional washes with buffer B. All the preceding steps were designed to ensure minimal nonspecific reaction to the antibodies used. Sections were incubated for 60 min with primary antibody, followed by three washes in buffer B and a 60-min incubation in gold-labeled secondary antibody (a 5-nm goat anti-rabbit gold conjugate at 1–2 µg/ml). The sections were given six 5-min washes in buffer B, followed by three changes in 1× PBS, and then fixed briefly in 2.5% glutaraldehyde in 1× PBS to ensure that the antibodies didn't dissociate. After fixation, the sections were washed three times in 1× PBS, followed by five washes in water and counterstain-

ing with uranyl acetate. The sections were embedded in 1.25% methylcellulose and analyzed using a JEOL 1210 electron microscope.

Results

Yeast Two-Hybrid Analysis

To identify novel proteins that interact with the sarcoglycan complex *in vivo*, we screened a human skeletal muscle yeast two-hybrid library with the intracellular domains of α -, β -, γ -, and δ -sarcoglycans. Each sarcoglycan intracellular domain was subcloned into the pGBT9 bait vector using a PCR based method. To ensure that the pGBT9/sarcoglycan clones alone were not able to initiate transcription of the yeast reporter genes alone, each construct was cotransformed into the yeast with the library vector alone and tested for β -galactosidase activity. None of the sarcoglycan clones self-activated and were, therefore, used in library screens. Each sarcoglycan subclone was transformed into yeast in conjunction with a skeletal muscle cDNA library present in the prey vector pGAD to screen for interacting clones. Approximately 4×10^6 colonies were screened for α - and β -sarcoglycan intracellular domains and $\sim 2 \times 10^6$ were screened for γ - and δ -sarcoglycan. The γ -sarcoglycan library screen produced a single clone that was HIS^+ and positive for β -galactosidase activity; however, the other sarcoglycan screens were negative. This interacting clone, 2-14, contained a 2.5-kb insert. To confirm the interaction between γ -sarcoglycan and 2-14, we reintroduced purified 2-14 plasmid DNA into yeast with either pGBT9/ γ -sarcoglycan bait vector, pGBT9 vector, or pGBT9/p53 control vector. Transactivation of $HIS3$ and LacZ was only detected with the bait vector containing γ -sarcoglycan.

Although the δ -sarcoglycan library screen did not identify any interacting clones, the γ - and δ -sarcoglycan proteins share $\sim 60\%$ sequence identity and, in fact, contain a stretch of 15 residues in their intracellular tails of which 14 are identical (Fig. 1 A). Therefore, we tested whether δ -sarcoglycan could also interact with 2-14 in the yeast two-hybrid system. The δ -sarcoglycan intracellular domain produced colonies on the HIS^- plates, and were subsequently tested for β -galactosidase activity. The HIS^- colonies produced a blue color within 2 h, indicating an interaction between δ -sarcoglycan and 2-14. In addition, we tested the other sarcoglycans, the carboxyl terminus of dystrophin (exons 68–78) and α syntrophin for their ability to interact with 2-14 in yeast. No colonies grew when the dystrophin, syntrophin, α - or β -sarcoglycan constructs were coexpressed with 2-14, suggesting there was no interaction (Table I).

The 2-14 clone was sequenced using pGAD forward and reverse vector primers, and the resulting sequence was used to search the GenBank database BLASTP computer program for similar sequences. Several ESTs were found with significant protein similarity to 2-14. The greatest homology was between 2-14 and the 3' portion of human nonmuscle filamin FLN1 (E value = 6×10^{-49}), β -filamin (E value = 2×10^{-42}), and chicken smooth muscle filamin (E value = 8×10^{-15}), with slightly lower homology to other actin-binding proteins (thyroid autoantigen; E value = 2×10^{-6}). These similarities indicate that 2-14 is a member of

Table I. Yeast Two-Hybrid Analysis

Construct	Growth on His ⁺ plates	β -Galactosidase activity
α -Sarcoglycan	–	N/A
β -Sarcoglycan	–	N/A
γ -Sarcoglycan	+	+
δ -Sarcoglycan	+	+
Dystrophin (C-term)	–	N/A
α -Syntrophin	–	N/A

the actin-binding protein family and, more specifically, a filamin protein family member. Because of the significant homology with the other filamin family members, we believe that this clone corresponds to the previously described skeletal muscle filamin, FLN2 (Wang et al., 1975; Maestrini et al., 1993).

Comparison of FLN2 with Other Filamin Family Members

The FLN2 clone 2-14 was used in combination with two previously reported muscle-specific filamin-like ESTs (GenBank/EMBL/DDBJ accession numbers X70083 and X70084) to isolate overlapping cDNA clones for the entire transcript. Sequence analysis determined that FLN2 has considerable similarity and identity to the human nonmuscle filamins (FLN1 and β -filamin) over their entire length, being 74/71% identical and 84/83% similar to human FLN1 and β -filamin, respectively (data not shown). The amino terminus actin-binding domain of FLN2 is nearly 100% identical to the actin-binding domain of both FLN1 and β -filamin, which share considerable identity to other actin-binding proteins such as dystrophin, spectrin, and α -actinin. Like FLN1 and β -filamin, FLN2 contains 24 repeating units averaging 96 amino acids in length; however, these repeats are disrupted by two hinge regions in FLN1 and β -filamin and only one hinge region in skeletal muscle FLN2. All three filamins contain the second hinge region between repeats 23 and 24 but, unlike FLN1 and β -filamin, skeletal muscle FLN2 does not appear to contain the first hinge region that separates repeats 15 and 16. The FLN2 carboxy terminus is highly similar to FLN1 and β -filamin, which contain the binding site for several receptor proteins including tissue factor receptor, $\beta 1$ integrin, $\beta 2$ integrin, and the glycoprotein (GP) Ib-IX complex (Andrews and Fox, 1991, 1992; Sharma et al., 1995; Loo et al., 1998; Ott et al., 1998; Takafuta et al., 1998). This is consistent with our data in which γ - and δ -sarcoglycan bind to FLN2 in the carboxyl-terminal region between residues 2,177 and 2,688. Interestingly, FLN2 contains an 82-amino acid unique region between repeats 19 and 20 not found in either FLN1 or β -filamin (Fig. 1 B). In addition, β -filamin appears to be missing eight amino acids just 5' to the first hinge region that are present in both FLN1 and FLN2.

Protein Expression of FLN2

Previous reports of FLN2 gene expression describe a skeletal and cardiac muscle-specific staining pattern that we also found (data not shown) (Maestrini et al., 1993). To determine the protein expression pattern of FLN2 and compare it to the expression pattern of FLN1, tissue ly-

A

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γ IC      M V R E Q Y T T A T E G I C T E R P E N Q Y V Y K I G I Y G W R K R C L Y L F
δ IC      M P Q E Q Y T T H H R S T M P G S V G P Q V Y K V G I Y G W R K R C L Y F F
C. elegans Q A Y P S Q T T V I H S T T K P - V A D A D I Y R V G I Y G W R K R F L Y T F
  
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B

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FLN 1  Q E Y D F E E E E K T H I Q D H R D G E Y T W A Y V F E V Y E Y Y T L I E Y G G D R E F F A E Y F V R A V F F E D A A K C I T Y T V S I G E E L Q 1657
beta FLN  Q E Y D F E E E E K T H I Q D H R D G E Y T W A Y V F E V Y E Y Y T L I E Y G G D R E F F A E Y F V R A V F F E D A A K C I T Y T V S I G E E L Q 1621
FLN 2  Q E Y D F E E E E K T H I Q D H R D G E Y T W A Y V F E V Y E Y Y T L I E Y G G D R E F F A E Y F V R A V F F E D A A K C I T Y T V S I G E E L Q 1650

FLN 1  A G E F T L Q I G E R R W E I E V S T A A A G E E Y F C T F C E F F E G I E Y D E V Y E E E E D G T F E E E R A D Q F E E Y I C W E E E E E V 1732
beta FLN  A G E F T L Q I G E R R W E I E V S T A A A G E E Y F C T F C E F F E G I E Y D E V Y E E E E D G T F E E E R A D Q F E E Y I C W E E E E E V 1763
FLN 2  A G E F T L Q I G E R R W E I E V S T A A A G E E Y F C T F C E F F E G I E Y D E V Y E E E E D G T F E E E R A D Q F E E Y I C W E E E E E V 1725

FLN 1  M F F F Q V T A L A G D P F R R Q Q L A P Q Y T F A C G G Q Q T W A P F S F L V G N H G D V Y W L R F F D L V F F F T I K E E E E E 1807
beta FLN  M F F F Q V T A L A G D P F R R Q Q L A P Q Y T F A C G G Q Q T W A P F S F L V G N H G D V Y W L R F F D L V F F F T I K E E E E E 1763
FLN 2  M F F F Q V T A L A G D P F R R Q Q L A P Q Y T F A C G G Q Q T W A P F S F L V G N H G D V Y W L R F F D L V F F F T I K E E E E E 1767

FLN 1  R R R R R R V A C T Y T I R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 1882
beta FLN  R R R R R R V A C T Y T I R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 1838
FLN 2  R R R R R R V A C T Y T I R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 1842

FLN 1  F A F F F F T R D A G G G E L A F E E F E A Z I E C T H N G R R E T G S F L F L F G D Y I L V E E E E Q V F E F F F A R V Y D D F 1957
beta FLN  F A F F F F T R D A G G G E L A F E E F E A Z I E C T H N G R R E T G S F L F L F G D Y I L V E E E E Q V F E F F F A R V Y D D F 1912
FLN 2  F A F F F F T R D A G G G E L A F E E F E A Z I E C T H N G R R E T G S F L F L F G D Y I L V E E E E Q V F E F F F A R V Y D D F 1917

FLN 1  R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 2032
beta FLN  R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 1987
FLN 2  R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 1992

FLN 1  R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 2107
beta FLN  R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 2062
FLN 2  R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 2067

FLN 1  R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 2167
beta FLN  R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 2122
FLN 2  R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 2142

FLN 1  R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 2175
beta FLN  R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 2130
FLN 2  R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R 2217

FLN 1  A Q V Y E E E E E Y H A A R E E E E H N T Y C I E F F A A K G G I E T V Y K K K G Q H V Y G E E E E C E F Y G L E E G A A E V E A R E E E E 2250
beta FLN  A Q V Y E E E E E Y H A A R E E E E H N T Y C I E F F A A K G G I E T V Y K K K G Q H V Y G E E E E C E F Y G L E E G A A E V E A R E E E E 2205
FLN 2  A Q V Y E E E E E Y H A A R E E E E H N T Y C I E F F A A K G G I E T V Y K K K G Q H V Y G E E E E C E F Y G L E E G A A E V E A R E E E E 2292

FLN 1  A R A Q V Y A E F F I W E R A G A G E L L A T A Y E F R E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E 2325
beta FLN  A R A Q V Y A E F F I W E R A G A G E L L A T A Y E F R E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E 2280
FLN 2  A R A Q V Y A E F F I W E R A G A G E L L A T A Y E F R E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E 2367

FLN 1  A R R F F D A R R L L Y V H I Q R R Q L K V F A F A F V S I G M A R R G A D P A R E E E E E G A L X C C V V T I D P G E Y A V E F F T F R E E E V Y 2400
beta FLN  A R R F F D A R R L L Y V H I Q R R Q L K V F A F A F V S I G M A R R G A D P A R E E E E E G A L X C C V V T I D P G E Y A V E F F T F R E E E V Y 2355
FLN 2  A R R F F D A R R L L Y V H I Q R R Q L K V F A F A F V S I G M A R R G A D P A R E E E E E G A L X C C V V T I D P G E Y A V E F F T F R E E E V Y 2442

FLN 1  L I D V Y F F H G T I R E E E E E I E Y V E E E E Q D G D F V V A F A G E E E E V I R H N A R E V V H E E E E E G A A E A L E V E E E E E E E E E 2475
beta FLN  L I D V Y F F H G T I R E E E E E I E Y V E E E E Q D G D F V V A F A G E E E E V I R H N A R E V V H E E E E E G A A E A L E V E E E E E E E E E 2430
FLN 2  L I D V Y F F H G T I R E E E E E I E Y V E E E E Q D G D F V V A F A G E E E E V I R H N A R E V V H E E E E E G A A E A L E V E E E E E E E E E 2516

FLN 1  C Q C F E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E 2548
beta FLN  C Q C F E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E 2503
FLN 2  C Q C F E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E 2589

FLN 1  A P G P F A D A R R V Y A K A L G E E R A Y V G Q E E E E E Y D C R A A G H N M L L Y V G E E E E E T T C E E I L V E E V G E L R L Y V F L I E D E 2623
beta FLN  A P G P F A D A R R V Y A K A L G E E R A Y V G Q E E E E E Y D C R A A G H N M L L Y V G E E E E T T C E E I L V E E V G E L R L Y V F L I E D E 2578
FLN 2  A P G P F A D A R R V Y A K A L G E E R A Y V G Q E E E E E Y D C R A A G H N M L L Y V G E E E E T T C E E I L V E E V G E L R L Y V F L I E D E 2664

FLN 1  G E T I L V Y X X G H E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E 2647
beta FLN  G E T I L V Y X X G H E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E 2602
FLN 2  G E T I L V Y X X G H E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E E 2688
  
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Figure 1. Protein sequence comparisons between γ - and δ -sarcoglycans. (A) The alignment of γ - and δ -sarcoglycans with a predicted open reading frame from the *Caenorhabditis elegans* cosmid F07H5.2 using the GRAIL exon prediction program. The protein sequence from the intracellular tails of γ - and δ -sarcoglycans (consisting of the first 39 and 41 amino acids, respectively) and a predicted open reading frame from a *C. elegans* were aligned using the MacVector™ software. (B) Protein sequence comparison of the three filamin family members. The carboxy terminus of the filamins were aligned using MacVector™ software. The two hinge regions are underlined. The peptide sequence used to raise the FLN2-A2 antibody is boxed.

sates from nine different tissues were prepared and used in Western blot analysis. To detect FLN2 protein, we generated a polyclonal antibody using a GST/FLN2 fusion protein containing FLN2 amino acids 2,128–2,688 (see Materials and Methods). The affinity-purified FLN2-A1 antibody detected a single band at ~280 kD, and this band was present only in the heart and skeletal muscles (Fig. 2), which is consistent with Northern blot analysis results. In contrast, FLN1 antibodies identified FLN1 protein in kidney, lung, ovary, testis, and weakly in heart tissues but not skeletal muscle. In addition, the FLN1 mAb detected several smaller bands in addition to the predicted 280-kD band (Fig. 2). FLN1 antibodies have been reported previously to detect cleavage products in some tissues (Kwak et al., 1993a). All of the nonmuscle tissues that contain FLN1 correspond to the previously published RNA ex-

pression pattern for both nonmuscle filamins by Northern analysis (Takafuta et al., 1998). Interestingly, heart and skeletal muscles do not have the abundance of FLN1 protein that corresponds to Northern data provided by other groups for FLN1. It is possible that the cDNA probes used to identify the FLN1 transcript in these tissues cross-hybridize to the FLN2 message. Overall, FLN1 and FLN2 antibodies reveal distinct protein expression patterns, suggesting that the FLN2 antibodies do not cross-react with other filamin family members.

In Vitro Binding of FLN2 and the Sarcoglycans

To substantiate the yeast two-hybrid interaction between FLN2 and the sarcoglycans, full-length γ - and δ -sarcoglycans were translated in an in vitro transcription/translation

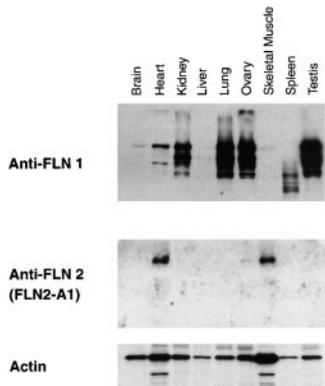


Figure 2. Western blot analysis of FLN1 and FLN2. Approximately 30 μ g of total protein lysate from several tissues were separated by electrophoresis on a 4–20% gradient gel and transferred to nitrocellulose membranes. Anti-FLN1 and anti-FLN2 (FLN2-A1) antibodies were used to detect each protein in the tissue lysates. For FLN1, a prominent band at \sim 280 kD with several smaller bands were detected

in most nonmuscle tissues and in heart. Hydrolytic fragments have been reported previously for FLN1. The FLN2 antibody detected FLN2 protein in only skeletal and cardiac muscles and not in the nonmuscle tissues, which is consistent with the Northern blot results (data not shown). As a control for loading, anti-actin antibodies were also used.

assay system described previously (Ahn and Kunkel, 1995) and combined with GST/FLN2 fusion protein. Fusion protein was mixed with [35 S]methionine-labeled translated γ - and δ -sarcoglycans, and then precipitated using glutathione beads. The results indicate that both γ - and δ -sarcoglycan are precipitated using glutathione beads only when FLN2 is present (Fig. 3 A, lanes 1 and 3). Although

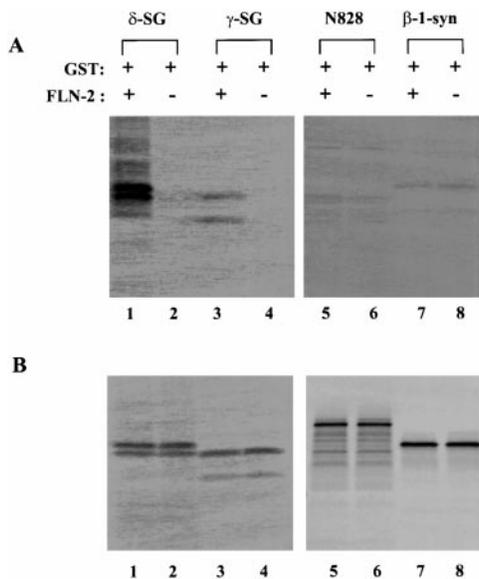


Figure 3. In vitro translated γ - and δ -sarcoglycan interaction with a GST/FLN2 fusion protein. (A) 35 S-labeled in vitro translated products were mixed with a GST/FLN2 fusion protein containing FLN2 amino acids 2,128–2,688. Lanes 1 and 2 contain translated δ -sarcoglycan protein mixed with fusion protein (lane 1) or GST protein alone (lane 2). Lanes 3 and 4 contain translated γ -sarcoglycan mixed with fusion protein (lane 3) or GST protein alone (lane 4). Lanes 5–8 contain either the carboxyl terminus of dystrophin (lanes 5 and 6) or syntrophin (lanes 7 and 8) combined with FLN2 fusion protein (lanes 5 and 7) or GST protein alone (lanes 6 and 8). (B) Supernatants of the above reactions corresponding to the respective lanes in A (lanes 1–8), showing that translated product was present in all tubes.

there is some labeled protein found in the reactions absent for fusion protein (lanes 2 and 4), there is considerably more protein found in the lanes that include fusion protein, suggesting that the FLN2 fusion protein can precipitate with both γ - and δ -sarcoglycans in this system. As controls, labeled dystrophin (carboxy terminus) and β 1-syntrophin were also mixed with the FLN2 fusion protein; however, FLN2 was not able to precipitate either of these translated proteins (lanes 5–8). The supernatant from each of the above reaction tubes shows that translated protein was present in each tube in equal amounts (Fig. 3 B, lanes 1–8).

To confirm this interaction in another in vitro system, we mixed in vitro translated full-length γ - and δ -sarcoglycans with in vitro translated FLN2 (clone 2-14). Translated FLN2 was combined with each sarcoglycan individually and these lysates were used to coimmunoprecipitate (CoIP) either sarcoglycan or FLN2 protein using antibodies directed toward either the FLN2 flag epitope (see Materials and Methods) or the respective sarcoglycan. After the isolation of immune complexes using protein G-Sepharose and an antibody specific to either FLN2 or a sarcoglycan, the samples were subjected to SDS-PAGE. Antibodies specific for δ -sarcoglycan were able to CoIP FLN2 and, conversely, FLN2 antibodies coimmunoprecipitated δ -sarcoglycan (Fig. 4, lanes 1 and 4, respectively), which is consistent with previous in vivo and in vitro binding data. In contrast, antibodies directed toward γ -sarcoglycan were not able to CoIP FLN2 and, likewise, FLN2 antibodies were not able to CoIP γ -sarcoglycan (data not shown). Specificity was shown by withholding either antibody (lane 2) or labeled protein (lane 6). Lanes 3 and 5 (Fig. 4)

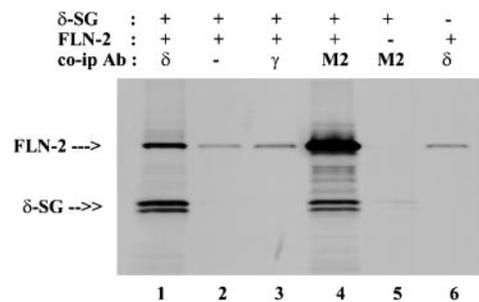


Figure 4. In vitro analysis of the δ -sarcoglycan interaction with FLN2. FLN2 clone 2-14 was inserted into the transcription/translation vector pFHR3 that contains a seven amino acid flag-tag. δ -sarcoglycan was inserted into the transcription/translation vector pMGT1, which does not contain the flag-tag. 35 S-labeled in vitro translated products were incubated together and precipitated with a specific antibody. After incubation with protein G-Sepharose, the samples were washed three times with lysis buffer (see Materials and Methods) and electrophoresed on a 4–20% SDS-PAGE gel. After electrophoresed, the gels were exposed to a storage phosphor plate and the plate was scanned using a PhosphorImager. (lanes 1–4) Coimmunoprecipitation of translated labeled FLN2 with labeled δ -sarcoglycan using a δ -sarcoglycan antibody (lane 1); no antibody (lane 2); γ -sarcoglycan antibody (lane 3); M2 antibody (directed toward the flag-tag) (lane 4). Lane 5 corresponds to translated labeled δ -sarcoglycan alone with M2 antibody. Lane 6 corresponds to translated labeled FLN2 alone with δ -sarcoglycan antibody.

show that nonspecific antibodies are unable to CoIP the δ -sarcoglycan or FLN2 proteins, respectively. Therefore, in this system, FLN2 binds to δ -sarcoglycan, and although the γ -sarcoglycan translated product was not coimmunoprecipitated with FLN2, the possibility remains that the binding between FLN2 and γ -sarcoglycan is slightly weaker than this method's detection limits.

In Vivo Association of γ - and δ -Sarcoglycans with FLN2

To further explore the association between FLN2 and the sarcoglycans, anti- γ - and anti- δ -sarcoglycan antibodies were used to CoIP γ - and δ -sarcoglycan immune complexes from cultured mouse myotube lysates. The γ -sarcoglycan antibodies from Novocastra have been shown previously to immunoprecipitate only γ -sarcoglycan, and the δ -sarcoglycan antibodies have been shown to immunoprecipitate only δ - and β -sarcoglycans from mouse muscle cell lysates (Chan et al., 1998). Antibodies raised against FLN2, dystrophin, α -actinin 2, and α -, β -, γ -, and δ -sarcoglycans were used to determine the presence or absence of these proteins in the complexes. In the γ -sarcoglycan antibody immune complexes, only FLN2 and γ -sarcoglycan were detected (Fig. 5 A, lane 2). Dystrophin, α -actinin 2, and the other sarcoglycans were not detected in these complexes. Immune complexes generated using δ -sarcoglycan antibodies revealed the presence of FLN2 and β - and δ -sarcoglycans (Fig. 5 B). The finding of β -sarcoglycan in δ -sarcoglycan immune complexes is not unexpected as previous studies have shown β - and δ -sarcoglycan to be a tightly associated core complex (Chan et al., 1998). These complexes did not contain dystrophin, α -actinin 2, or α - or γ -sarcoglycan. To test whether FLN2 protein could be immunoprecipitated with just sarcoglycan antibody, anti- γ -sarcoglycan antibodies were used to immunoprecipitate a cytoplasmic FLN2-rich soluble fraction in which no γ -sarcoglycan protein was present (see Fig. 6). The anti- γ -sarcoglycan antibodies were not able to precipitate FLN2 protein; however, using the FLN2 antibody as a control to CoIP FLN2, FLN2 protein was immunoprecipitated from this fraction, indicating that the sarcoglycan antibodies do not nonspecifically immunoprecipitate FLN2 protein (Fig. 5 C, lanes 1 and 2). In the absence of sarcoglycan antibodies, no FLN2 protein was detected, suggesting that the FLN2 protein did not adhere directly to protein G-Sepharose (Fig. 5 C, lane 4). In addition, when the nonspecific antibody M2 was used to CoIP from whole cell lysates, no FLN2 protein was detected (Fig. 5 C, lane 6). Taken together, these data suggest that FLN2 can be coimmunoprecipitated from mouse myotube lysates using γ - and δ -sarcoglycan antibodies, and that this interaction is specific for FLN2 with the respective sarcoglycan proteins.

Cellular Location of FLN2

FLN1 has been found to exist in two intracellular pools in nonmuscle cells: one associated with the plasma membrane and the other within the actin cytoskeletal network (Gorlin et al., 1990; Sharma et al., 1995; Liu et al., 1997; Meyer et al., 1997; Ott et al., 1998). Given the tremendous amount of FLN2 in the crude lysate, the relatively small

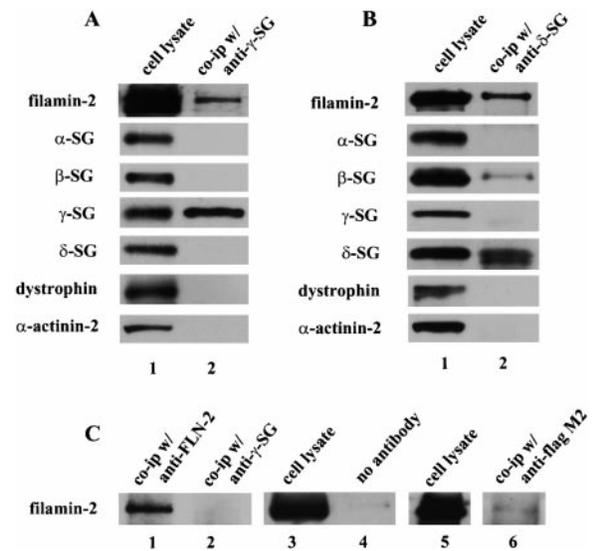


Figure 5. In vivo interaction of FLN2 with γ - and δ -sarcoglycan. (A) Cultured mouse myotube lysates were used to coimmunoprecipitate protein complexes using anti- γ -sarcoglycan antibodies. Approximately 25 μ g of protein from total cell lysates was separated by electrophoresis and blotted onto nitrocellulose membranes (lane 1). γ -Sarcoglycan antibody immune complexes were washed three times with lysis buffer (see Materials and Methods), separated by electrophoresis, and blotted onto nitrocellulose membranes (lane 2). Antibodies raised against various DGC members were used to determine the presence or absence of their respective proteins in each reaction. (B) In a similar experiment as stated above, protein complexes were immunoprecipitated using anti- δ -sarcoglycan serum. The doublet detected for δ -sarcoglycan is present in all experiments using the anti- δ -sarcoglycan antibodies presumably because this antibody was not affinity-purified, and is recognizing the immunoglobulin chains. (C) Controls for the coimmunoprecipitation experiments. Lanes 1 and 2 contain supernatant from a microsome preparation precipitated using either anti-FLN2 (lane 1) or anti- γ -sarcoglycan (lane 2) antibodies. Lanes 3–6 contain immune complexes from cell lysates using either no antibody (lane 4) or a nonspecific antibody (lane 6).

amount of FLN2 associated with the sarcoglycans at the membrane suggests that FLN2 might also be found in two intracellular pools: a small fraction associated with the sarcolemmal membrane and a larger intracellular portion. To quantify the amount of FLN2 at the membrane, we prepared fractionated microsomes from cultured mouse myotubes and tested for the presence of FLN2 protein. After several centrifugations, the final microsome pellet and the resulting supernatant were separated by electrophoresis on a 4–20% SDS-PAGE gel, and subsequently blotted onto nitrocellulose membrane. Lane 1 of Fig. 6 represents 12.5% of total microsome protein, and lane 2 is 1.25% of the protein content in the total soluble cytoplasmic fraction. Using antibodies raised against various DGC members, FLN2 and hsp70 (an intracellular protein), the composition of each fraction was determined. Densitometry analysis was performed using the ImageQuant v1.1 software (Molecular Dynamics) to determine approximate percentages for each protein in the two fractions. Approximately 3% of the total cellular FLN2 was found associ-

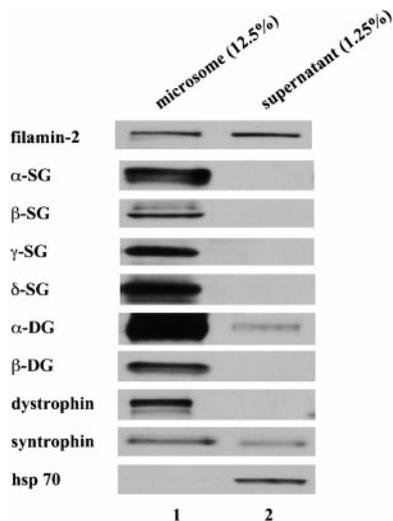


Figure 6. Quantification of membrane-associated FLN2. Microsomes were prepared from cultured mouse myotubes, and both the soluble and microsome fractions were separated by electrophoresis and subsequently blotted onto nitrocellulose membranes. Lane 1 represents 12.5% of the total protein content of the microsome, and lane 2 represents 1.25% of the total protein content of the soluble fraction. Antibodies for each protein were used to determine the protein content for FLN2, α -, β -, γ -, and δ -sarcoglycans, α - and β -dystroglycans, dystrophin, syntrophin, and hsp70. The relative amount of FLN2 associated with the membrane was determined to be $\sim 3\%$ using the ImageQuant v1.1 software.

ated with the membrane fraction and 97% with soluble fraction. Comparing the FLN2 cell lysate signal in Fig. 5 A with the microsome fraction in Fig. 6, it can be seen that FLN2 is much more abundant in the total cell lysate, supporting the quantitative analysis. Most of the other DGC members were found associated with the microsome fraction with the exception of syntrophin and α -dystroglycan, which were also found in the soluble fraction. As a negative control, hsp70 was added to the analysis to ensure for purity of microsome preparations, and as expected, was found exclusively in the soluble fraction.

To further explore the subcellular location of FLN2, we performed immunofluorescence microscopy, confocal microscopy, and immunoelectron microscopy studies. The initial immunofluorescence microscopy studies suggested that there were indeed two intracellular pools for FLN2 like that found for FLN1. A distinct banding pattern was observed on longitudinal sections of normal adult skeletal muscle that is characteristic for intermediate filament proteins (Fig. 7 A). Cross-sections displayed a punctate pattern throughout each myofiber representing the banded pattern seen on longitudinal sections in addition to a clear sarcolemmal staining (Fig. 7 B). Confocal microscopy demonstrated staining predominantly within the contractile apparatus (presumably the Z-line) and at the sarcolemmal membrane although the plasma membrane staining is more diffuse than what has been previously shown for other DGC members such as dystrophin (Fig. 8). Furthermore, the Z-line labeling appears to extend beyond the lateral limits of this structure. This finding may

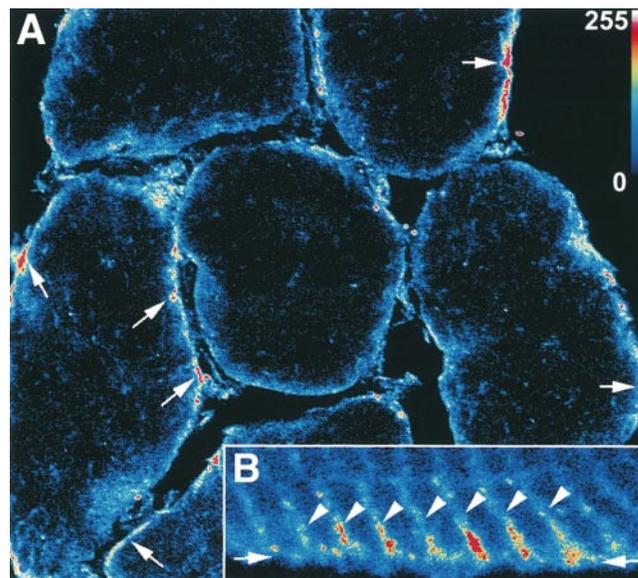


Figure 7. Confocal analysis of FLM2-A2 shows a clear localization within skeletal muscle. In cross-section, label appears strongly associated with the plasma membrane of the muscle fiber. While continuous, this label has a variable intensity (A) along the perimeter of the fibers. For example, regions of high intensity are highlighted (arrows). In longitudinal sections (B), a strong association with the Z-line is apparent. A similar, though slightly less intense label is also apparent at the plasma membrane.

be due to the ultimate limited diffraction resolution of the confocal microscope (0.2 microns). To investigate this finding further as well as confirm sarcolemmal membrane staining, we performed EM analysis. By EM, the plasma-membral labeling appeared to exist in two populations: one population adjacent to the membrane and another more diffuse (Fig. 9). In Fig. 9 A, labeling can be seen associated with the cytoplasm at the intracellular aspect of the plasma membrane (arrowheads), which is also seen in Fig. 9 B (arrow). The labeling of the Z-line within the muscle fiber is clearly shown in Fig. 9 C where there is a sarcomere away from the cell surface. No label is seen within the A band, though considerable label is associated with the lateral edges of the Z-line within the I band (arrows). An occasional label is seen within the Z-line (arrowheads).

Analysis of FLN2 in Patients and *gsg*^{-/-}, *dgs*^{-/-}, and *mdx* Mice

Often a secondary instability of DGC members occurs when there is a primary mutation in one of the proteins. This is frequently seen with the sarcoglycan complex and sarcospan when one sarcoglycan is altered (Bonnemann et al., 1996; Duclos et al., 1998; Araishi et al., 1999). Since FLN2 interacts with members of the sarcoglycan complex, we reasoned that FLN2 might also be altered within the muscle fiber. To assess FLN2 in LGMD2C, DMD patients and an undetermined myopathy patient with an increased CPK level and normal DGC member staining, we performed IMF on muscle biopsies. All biopsies were previ-

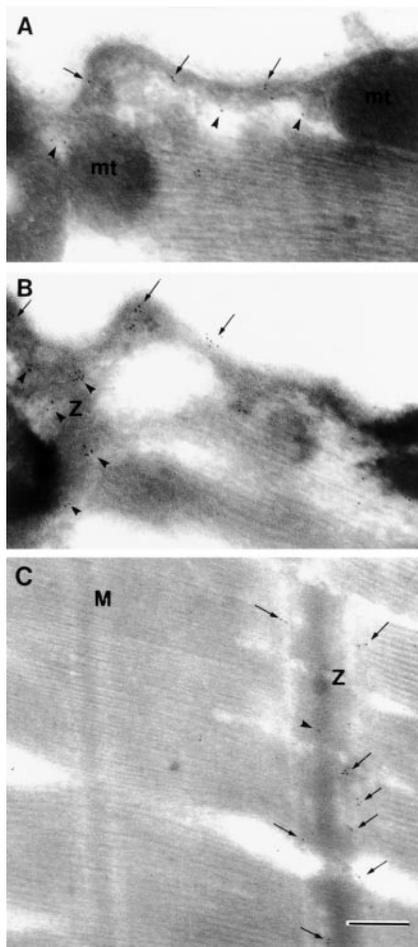


Figure 8. Immunoelectron microscopic analyses show two apparent pools of label at the plasma membrane of muscle fibers (A) labeling associated with the electron dense plasmalemmal cytoskeleton is seen (arrows) as well as within the cytoplasm adjacent to the plasmalemma (arrowheads). The plasmalemmal labeling is also apparent at the Z-line (B, arrow). However, in this image, it is also clear that the label associated with the Z-line persists right up to the plasma membrane (arrowhead). Labeling of the Z-line is also detected within fibers away from the plasma membrane, as shown in C. In this figure, the label can be seen to be associated with the lateral aspect of the Z-line (arrows) although occasional label within the Z-line is also apparent (arrowhead).

ously screened for the sarcoglycans and dystrophin. Strikingly, both LGMD patients tested and the DMD patient had an observable FLN2 increase at the membrane when compared with the normal (Fig. 10 A). In contrast, the patient with the unknown myopathy had no observable increase of FLN2 at the muscle membrane. All of the sarcoglycans and dystrophin were found to be normal in this patient, suggesting that the FLN2 increase in LGMD2C and DMD patients is specific for the sarcoglycans and dystrophin and not a general finding of myopathies.

To determine whether this phenomenon could also be seen in the null mice, we analyzed membrane preparations from *gsg*^{-/-}, *dsg*^{-/-}, and *mdx* mice by Western blot

analysis. In all cases, the membrane bound FLN2 increased to at least 25% (Fig. 10 B). In *mdx* membranes, FLN2 was increased as much as 45%. These results support the patient results found by IMF.

Discussion

Although new genes and proteins are continuously identified that are involved in the dystrophic phenotype, the one question that remains unanswered is the mechanism by which this phenotype occurs. Several animal model systems are being developed to explore this question and, through these models, it has become clear that the sarcoglycans help in the maintenance of skeletal muscle, although how this is accomplished remains elusive. Interestingly, although mutations in dystrophin and the sarcoglycans produce a similar phenotype, dystrophin staining in the sarcoglycanopathies is either normal or slightly reduced, indicating that the selective loss of the sarcoglycan complex is sufficient to cause muscular dystrophy.

Here, we describe the novel interaction between members of the sarcoglycan complex and FLN2, a muscle-specific relative of filamin 1 (FLN1). We demonstrate that both γ - and δ -sarcoglycan interact with FLN2 specifically through in vivo and in vitro studies. In addition, we show that FLN2 is located in two intracellular pools, with $\sim 3\%$ of the cellular content at the sarcolemmal membrane and the remaining $\sim 97\%$ contained within the cytoplasm, presumably at the Z-line of the contractile apparatus as seen by EM. Strikingly, in LGMD patients, DMD patients, and knockout mice lacking either γ - or δ -sarcoglycan, the membrane component of FLN2 increases to $\sim 30\%$. In contrast, in a patient with an undetermined myopathy, FLN2 expression at the membrane appeared normal.

Filamin (ABP-280) was first identified in chicken gizzard and rabbit peripheral blood as a protein capable of inducing actin polymerization (Hartwig and Stossel, 1975; Wang et al., 1975). Subsequent biochemical and molecular studies have shown that three different types of filamin proteins exist in mammals (*FLN1*, β -*FLN*, and a third muscle-specific form, here named *FLN2*). Virtually all cells express at least one filamin form at some point in development, often changing forms as the cell undergoes terminal differentiation (Stossel and Hartwig, 1975; Shizuta et al., 1976; Maestrini et al., 1993; Takafuta et al., 1998). All three filamins are encoded by separate genes: *FLN1* is located on chromosome Xq28; β -filamin is located on chromosome 3p14.3-p21.1; and an EST, shown here to be part of the *FLN2* transcript, is located on chromosome 7q32-q35 (Gorlin et al., 1993; Maestrini et al., 1993).

Most reports of filamin function include a role in actin polymerization, a process that is critical for the regulation of the contractile apparatus in skeletal muscle as well as cell structure, organization of membrane receptors with signaling molecules, and mechanoprotection in other tissues. These processes can regulate cell behavior by providing the cell with the information necessary for making decisions regarding cell shape, adhesion and migration, growth and differentiation, and apoptosis and survival. An example of one of these processes is found in platelets, where the transient binding of FLN1 to the GP Ib-IX re-

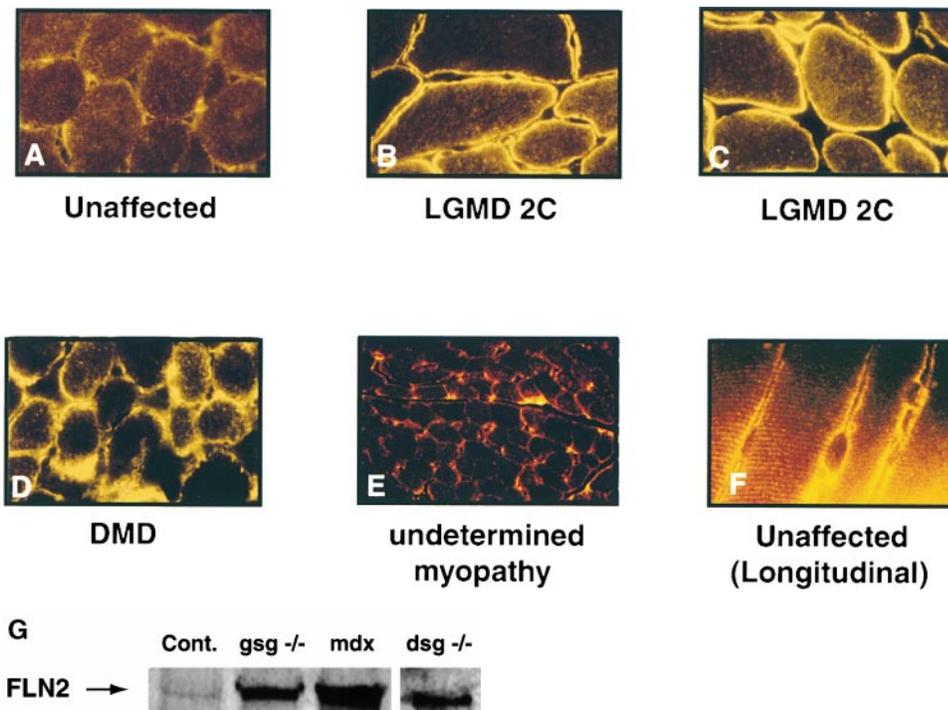


Figure 9. FLN2 analysis of patients and null mice. (A) A–E are cross-sections of skeletal muscle biopsies. B–D are short exposure times because of the intense staining at the membrane. F is a longitudinal section of normal skeletal muscle exposed for a longer period to bring out Z-line pattern. All panels are at a magnification of 40 except panel E, which is at 25. B and C represent two different LGMD 2C patients. (G) Approximately 40 μ g of protein from null mouse microsomes was loaded in each lane for Western analysis. The designation *gsg*^{-/-} and *dsg*^{-/-} refers to the γ -sarcoglycan and δ -sarcoglycan knockout mice, respectively. Membranes were probed with FLN2 antibodies (1:2,500). Coomassie brilliant blue staining was used to normalize protein loading (data not shown). FLN2 protein amounts relative to the control animal were analyzed using UN-SCAN-IT™ software (Silk Scientific Corporation).

ceptor complex has been shown to be important in platelet activation by inducing changes in cellular shape, adhesion, and membrane organization. Interestingly, the binding of both of these proteins to the GP Ib-IX complex is dependent on the stress-induced conformational change of the receptor complex (Andrews and Fox, 1991; Andrews and Berndt, 1998).

In addition to establishing cell structure and reorganizing receptor/signaling molecules, FLN1 also has been shown to be crucial in protecting cells from external stress, a process commonly referred to as mechanoprotection. This phenomenon has been chiefly studied in fibroblasts where FLN1 is recruited to integrin receptor complexes in response to the stress-induced binding of ECM proteins.

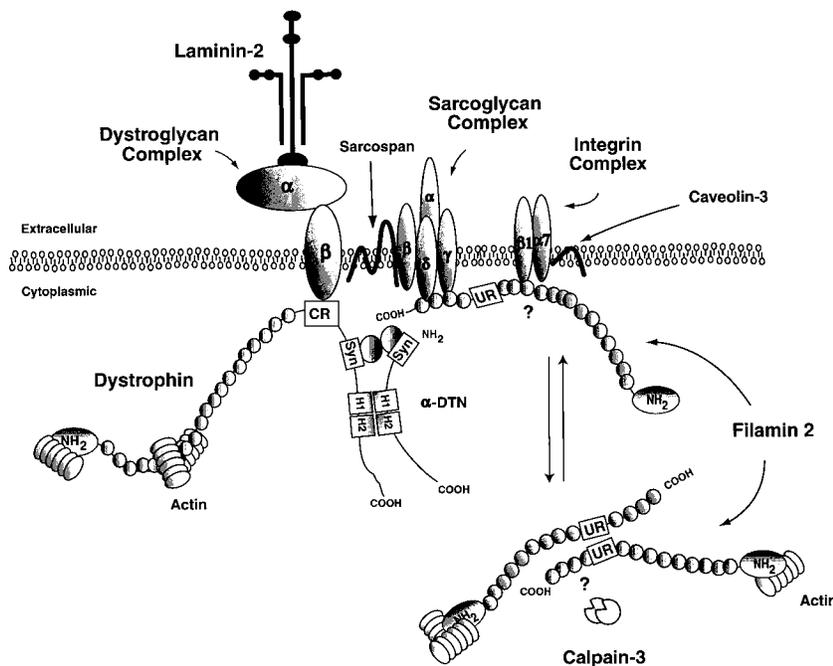


Figure 10. LGMD model. Shown is a schematic representing some proteins involved in muscular dystrophy and how they might interact with FLN2. Depending on the phosphorylation state of FLN2, it may be present at the membrane or interacting with the actin cytoskeleton. At the membrane, FLN2 interacts with γ - and δ -sarcoglycan and possibly with β 1 integrin. One set of receptor signals may recruit FLN2 to the membrane, whereas another set of signals allows FLN2 to translocate back to the actin cytoskeleton. Also depicted in this diagram is calpain-3, which may help to regulate FLN2 levels within the cell.

This signal triggers the rearrangement of the actin cytoskeleton to accommodate for the sensed tension. Remarkably, after force application, cells deficient for FLN1 show membrane disruption and a >90% increase in cell death compared with FLN1 containing cells (Glogauer et al., 1998). Therefore, in the absence of FLN1, these cells are unable to maintain membrane integrity when exposed to external forces, ultimately leading to cell death.

With the identification of FLN2 as a sarcoglycan interacting protein, the sarcoglycans join the list of proteins that are both involved in signal transduction and the dystrophic phenotype. Moreover, this interaction connects other proteins involved in dystrophy with the sarcoglycans in a signaling process that has been extensively studied in other systems, and is now unfolding in skeletal muscle. Mutations in the integrin $\alpha 7$ subunit, a muscle-specific integrin, result in congenital myopathy (Vachon et al., 1997; Hayashi et al., 1998), whereas mutations in calpain-3, caveolin-3, and the sarcoglycans result in an LGMD phenotype. Family members of all three of these proteins have been found to contribute in various signaling cascades. Moreover, the integrins are also involved in mechanoprotection pathways in fibroblasts where they bind to FLN1 (Glogauer et al., 1998). Although these studies focus on the $\alpha 2$ subunit, it has been established that both FLN1 and β -FLN bind $\beta 1$ integrin in other cells.

Given the data presented here and the known functions of FLN1, a mechanistic model for some forms of muscular dystrophy can be explored. The FLN2 membrane increase in LGMD and DMD patients suggests that FLN2 is binding other membrane bound proteins other than the sarcoglycans. A logical candidate for this second interacting protein would be $\beta 1$ integrin given that both of the other filamin family members bind to this subunit in other cells. If this is true, it is possible that the sarcoglycans and the integrins both bind FLN2 at the membrane (Fig. 10). FLN1 has been shown to bind a variety of receptors, validating this possibility. Signals received by the integrins might recruit FLN2 to the membrane until a signal via the sarcoglycans is received, thereby allowing FLN2 to translocate back to the actin cytoskeleton. If selected components of the DGC are missing from the membrane, integrin bound FLN2 remains at the membrane failing to translocate back to bind F-actin. Data from patient and mouse studies support this scenario in which membrane bound FLN2 goes from 3% (normal) to 20–40% in individuals lacking either γ -sarcoglycan, δ -sarcoglycan, or dystrophin. Another important connection between filamin proteins and muscular dystrophy are the calpains. FLN1 is cleaved by nonmuscle calpain to regulate actin-myosin filament formation (Kwak et al., 1993a). Thus, the overall regulation of FLN2 levels could be accomplished by the muscle-specific calpain, calpain-3. To date, no substrate has been identified for calpain-3, leaving FLN2 as an attractive candidate.

In summary, the identification of the interaction between FLN2 and the sarcoglycans should help in establishing a more definitive function for the sarcoglycan complex in skeletal muscle. It was originally believed that the DGC had simply a structural role in the maintenance of skeletal muscle by anchoring muscle cells to the extracellular matrix (Campbell, 1995). Examination of FLN2 and several

proteins involved in the dystrophic phenotype reveals that it is more likely a dynamic process such as signal transduction. Furthermore, as a new member of the dystrophin-glycoprotein complex, FLN2 becomes a candidate disease gene in LGMD patients who do not have mutations in any of the known LGMD genes. Recently, two LGMD families were reported to be genetically linked to chromosome 7q to a region that may include FLN2 (Speer et al., 1999). Thus, it will be interesting to determine if these patients have disease causing mutations in FLN2.

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