Membrane Targeting and Stabilization of Sarcospan Is Mediated by the Sarcoglycan Subcomplex

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Abstract. The dystrophin–glycoprotein complex (DGC) is a multisubunit complex that spans the muscle plasma membrane and forms a link between the F-actin cytoskeleton and the extracellular matrix. The proteins of the DGC are structurally organized into distinct subcomplexes, and genetic mutations in many individual components are manifested as muscular dystrophy. We recently identified a unique tetraspan-like dystrophin-associated protein, which we have named sarcospan (SPN) for its multiple sarcolemma spanning domains (Crosbie, R.H., J. Heighway, D.P. Venzke, J.C. Lee, and K.P. Campbell. 1997. *J. Biol. Chem*. 272:31221–31224). To probe molecular associations of SPN within the DGC, we investigated SPN expression in normal muscle as a baseline for comparison to SPN's expression in animal models of muscular dystrophy. We show that, in addition to its sarcolemma localization, SPN is enriched at the myotendinous junction (MTJ) and neuromuscular junction (NMJ), where it is a component of both the dystrophin– and utrophin–glycoprotein complexes. We demonstrate that SPN is preferentially associated with the sarcoglycan (SG) subcomplex, and this interaction is critical for stable localization of SPN to the sarcolemma, NMJ, and MTJ. Our experiments indicate that assembly of the SG subcomplex is a prerequisite for targeting SPN to the sarcolemma. In addition, the SG–SPN subcomplex functions to stabilize α-dystroglycan to the muscle plasma membrane. Taken together, our data provide important information about assembly and function of the SG–SPN subcomplex.

Key words: sarcospan • dystrophin • sarcoglycans • tetraspans • muscular dystrophy

THE dystrophin–glycoprotein complex (DGC[1]; Campbell and Kahl, 1989; Ervasti et al., 1990, 1991; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991) is comprised of peripheral and integral membrane proteins and provides a structural linkage between the extracellular matrix and the intracellular cytoskeleton of muscle cells. Several forms of muscular dystrophy arise from primary mutations in genes encoding dystrophin-associated proteins (for review see Campbell, 1995; Straub and Campbell, 1997). Patients with mutations in the dystrophin gene develop either Duchenne or Becker muscular dystrophy, which is characterized by progressive wasting of skeletal muscles. Likewise, a nonsense mutation in the murine dystrophin gene (*mdx*) eliminates expression of dystrophin and, consequently, the DGC proteins are reduced at the sarcolemma. While the function of the DGC is obviously essential for normal muscle physiology, its precise role in muscle function is unclear. It has been hypothesized that this transmembrane protein complex provides mechanical support to the plasma membrane during myofiber contraction (Weller et al., 1990; Petrof et al., 1993). More recently, data from several laboratories have suggested that the DGC may also play a role in cellular communication, as highlighted by the association of this complex with known signaling molecules (Brenman et al., 1995; Yang et al., 1995; Chang et al., 1996).

The proteins that comprise the DGC are structurally organized into three distinct subcomplexes. These are the cytoskeletal proteins, dystrophin and syntrophins; the dystroglycans (DGs; α and β subunits); and the sarcoglycans

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1. *Abbreviations used in this paper:* DG, dystroglycan; DGC, dystrophin–glycoprotein complex; EOM, extraocular muscle; LGMD, limb-girdle muscular dystrophy; *mdx*, murine dystrophin gene; MTJ, myotendinous junction; NMJ, neuromuscular junction; SG, sarcoglycan; Sgca-null, α-SG deficient mice; SPN, sarcospan; utrn$^{-/-}$, utrophin deficient; *mdx*:utrn$^{-/-}$, utrophin–dystrophin deficient; wt, wild-type.

(SGs; α, β, γ, and δ subunits). Exactly how these proteins are arranged with respect to one another is uncertain, but interactions between subcomplexes are clearly important for targeting to the sarcolemma, as well as for membrane stabilization. Recent reports have demonstrated that the NH_2 terminus of dystrophin interacts directly with F-actin in an extended, lateral fashion, similar to many actin side-binding proteins (Rybakova et al., 1996; Rybakova and Ervasti, 1997; Amann et al., 1998). Dystrophin connects with the other DGC subcomplexes through its COOH-terminal domain, which binds directly to the COOH terminus of β-DG, an integral membrane protein with a single transmembrane helix (Jung et al., 1995). β-DG, in turn, binds α-DG, anchoring it to the extracellular surface of the sarcolemma. α-DG serves as a receptor for laminin 2, thereby completing the physical connection between the actin cytoskeleton and the extracellular matrix (Ervasti and Campbell, 1993).

The SG subcomplex is composed of four distinct single-pass transmembrane glycoproteins, referred to as α-, β-, γ-, and δ-SG (for review see Lim and Campbell, 1998). The SGs, in conjunction with β-DG, mediate attachment of α-DG to the muscle plasma membrane. A defect in any one of the SGs results in specific loss of the SG subcomplex, destabilization of α-DG, and sarcolemma damage (Holt et al., 1998). Autosomal recessive limb-girdle muscular dystrophy (LGMD) types 2D, 2E, 2C, and 2F are caused by mutations in α-, β-, γ-, and δ-SG, respectively (Roberds et al., 1994; Bönnemann et al., 1995; Lim et al., 1995; Noguchi et al., 1995; Piccolo et al., 1995; Jung et al., 1996; Nigro et al., 1996a,b; Passos-Bueno et al., 1996). Likewise, the BIO 14.6 hamster (Iwata et al., 1993; Roberds et al., 1993), which serves as an animal model for LGMD2F, has a large deletion in the δ-SG gene (Nigro et al., 1997). BIO 14.6 hamsters display both cardiomyopathic and myopathic features. Successful intervention of disease progression has been achieved by introduction of a recombinant δ-SG adenovirus into skeletal muscle of the BIO 14.6 hamster (Holt et al., 1998). Targeted deletions of the α- (Duclos et al., 1998b) and γ-SG (Hack et al., 1998) genes in mice result in dystrophic muscle phenotypes and have provided additional animal models for LGMD.

We have recently characterized a novel 25-kD dystrophin-associated protein and have shown that it is an integral member of the DGC (Crosbie et al., 1997, 1998). We have named this protein sarcospan (SPN) for its multiple sarcolemma spanning helices, which are predicted based on hydropathy analysis (Crosbie et al., 1997). Dendrogram analysis shows that SPN is a member of the transmembrane four or tetraspan superfamily of proteins (Crosbie et al., 1997). Each possess four transmembrane domains, a large extracellular loop, and are thought to play important roles in mediating transmembrane protein interactions (Wright and Tomlinson, 1994; Maecker et al., 1997). These characteristics make SPN unique among other dystrophin-associated proteins. Furthermore, given the propriety of tetraspan proteins for mediating protein–protein interactions, SPN is structurally poised to be an important player in facilitating interactions between subcomplexes of the DGC. In the present study, we examine SPN expression in several animal models of muscular dystrophy as a means of assessing the molecular associations of SPN with subcomplexes of the DGC. We find that SPN interacts with the SGs, forming an SG–SPN protein complex.

Materials and Methods

cDNA Isolation and Sequencing

SPN cDNA clones were isolated by hybridization screening of a CLONTECH rabbit skeletal muscle cDNA library with a PCR-derived SPN cDNA probe encoding exons 1–3. Sequence analysis of the clones was performed using dye terminator cycling and analyzed on a 373 stretch fluorescent automated sequencer (PE Applied Biosystems). The nucleotide and deduced amino acid sequences of rabbit SPN have been deposited in the GenBank/EMBL/DDBJ data bank with the accession number AF120276. Multiple sequence alignment was performed using the DNAsis sequence analysis software (Hitachi Software Engineering, Inc.). Also, we have isolated SPN cDNA clones independently from a mouse skeletal muscle library and found clones identical to those found by Scott et al. (1994).

Northern Blotting

Adult mouse multiple tissue Northern blots (CLONTECH Laboratories, Inc.) containing 2 μg of poly $(A)^+$ RNA per lane were probed with an expressed sequence tag corresponding to the 3′ untranslated region of mouse SPN (GenBank accession number W83284). Identical results were obtained when blots were hybridized with PCR-amplified probes representing the entire coding region (GenBank accession number U02487) of mouse SPN.

Animal Models

Wild-type (wt; C57BL/10) and *mdx* (C57BL/10ScSn) mice, obtained from Jackson ImmunoResearch Laboratories, Inc. were maintained at the University of Iowa Animal Care Unit in accordance with animal usage guidelines. The dystrophin transgenic mice have been described previously (Cox et al., 1994; Rafael et al., 1994, 1996; Phelps et al., 1995). Male F1B and BIO 14.6 cardiomyopathic hamsters were obtained from BioBreeders. We have previously reported the generation and initial characterization of the α-SG deficient (Sgca-null) mice (Duclos et al., 1998b). The targeted disruption of the α-SG gene was accomplished by replacement of exons 2 and 3, and flanking intronic sequences with the neomycin resistance gene through homologous recombination (Duclos et al., 1998b). Utrophin deficient ($utrn^{-/-}$) and utrophin–dystrophin deficient mice (*mdx*:$utrn^{-/-}$) have been described previously (Grady et al., 1997a,b). $Utrn^{-/-}$ and *mdx*:$utrn^{-/-}$ mice were maintained at Washington University (St. Louis, MO).

Antibodies

mAbs against α- (20A6), β- (5B1), and γ-SG (21B5), as well as mAbs against β-DG (8D5) were generated in collaboration with Dr. Louise V.B. Anderson (Newcastle General Hospital, Newcastle upon Tyne, UK). mAb against α-DG (IIH6) have been described by Ervasti and Campbell (1991). Antibodies against the laminin α2 chain (Allamand et al., 1997) and the NH_2 terminus of rabbit SPN (Rabbit 216; Crosbie et al., 1997) have been described previously. For generating antibodies against mouse SPN, two New Zealand White rabbits (rabbits 235 and 236; Knapp Creek Farms) were injected at intramuscular and subcutaneous sites with a COOH-terminal SPN–glutathione S transferase fusion protein (amino acids 186–216 of mouse SPN; CFVMWKHRYQVFYVGVGLRSLMASDGQLPKA). Affinity purification of SPN antibodies was accomplished using Immobilon-P (Millipore Corp.) strips containing the COOH-terminal SPN–maltose-binding fusion protein. Antibody specificity was verified for both immunofluorescence and immunoblotting by competition experiments using the COOH-terminal SPN fusion protein and peptides synthesized to the COOH-terminal region of mouse SPN (data not shown).

Immunofluorescence

Transverse muscle cryosections (7 μm) were analyzed by immunofluorescence as described in Crosbie et al. (1997). For extraocular muscle (EOM) studies, rectus muscles (global layer) were examined. Affinity purified rabbit 235 SPN antibody was incubated at a dilution of 1:50 and 1:10 with

mouse and hamster sections, respectively. After washing with TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.4), the sections were incubated with Cy3-conjugated secondary antibodies at a dilution of 1:250 (Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. For staining of neuromuscular junctions (NMJs), samples were simultaneously incubated with fluorescein-conjugated α-bungarotoxin (1:1,000; Molecular Probes, Inc.). After washing with TBS, the slides were mounted with Vectashield mounting medium (Vector Labs Inc.) and observed under a BioRad MRC-600 laser scanning confocal microscope. Digitized images were captured under identical conditions.

Recombinant Adenovirus Injections

The human δ-SG cDNA sequence was subcloned into the pAdRSVpA adenovirus vector through standard methods of homologous recombination with Ad5 backbone dl309 by the University of Iowa Gene Transfer Vector Core. Preparation of the recombinant adenovirus and the intramuscular injections were performed as previously described (Holt et al., 1998). In brief, 10^9 viral particles in 100 μl of normal saline were injected into the quadriceps femoris of 3-wk-old BIO 14.6 hamsters after the animals were anesthetized by intraperitoneal injection of sodium pentobartital (Nembutal; Abbott Laboratories) at a calculated dose of 75 mg/kg. Quadriceps muscle was collected 2 wk after the injection.

Preparation of Skeletal Muscle Membranes

KCl washed membranes from wt, *mdx*, and Sgca-null mice were prepared from skeletal muscle as described previously (Duclos et al., 1998b).

Isolation of the SG–SPN Subcomplex by pH 11 Treatment

Purified DGC (Campbell and Kahl, 1989; Ervasti et al., 1990, 1991) from rabbit skeletal muscle membranes was titrated to pH 11 using 1 M NaOH and incubated for 1 h at room temperature with gentle mixing (Ervasti et al., 1991) in a buffer consisting of 50 mM Tris, 0.1% digitonin, 175 mM NaCl, 0.1 mM PMSF, 0.75 mM benzamidine. The alkaline treated DGC was concentrated fourfold using Centricon-10 filters (Amicon Corp.). The samples were loaded onto 5–30% linear sucrose gradients in a buffer of 50 mM Tris-HCL, 500 mM NaCl, 0.1% digitonin, 0.1 mM PMSF, 0.75 mM benzamidine, pH 11. The gradients were centrifuged at 4°C in a Beckman Vti 65.1 vertical rotor for 2.5 h at 200,000 *g*. 16 0.8-ml fractions were collected from the top of the gradient using an Isco model 640 density gradient fractionator. The protein samples (60 μl) were separated by 3–15% SDS-PAGE and immunoblotted, as described (vide infra).

Sucrose Gradient Separation of WGA Enriched Proteins from mdx Muscle

Quadriceps femoris muscle was dissected from *mdx* mice and snap frozen in liquid nitrogen. Frozen tissue (1 g) was pulverized into small pieces with a pestal and mortar filled with liquid nitrogen. The tissue was solubilized by dounce homogenization in 10 ml of cold buffer A (50 mM Tris-HCl, pH 7.8, 500 mM NaCl, 1.0% digitonin) with a cocktail of protease inhibitors (0.6 μg/ml pepstatin A, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.1 mM PMSF, 0.75 mM benzamidine, 5 μm calpain I inhibitor, and 5 μM calpeptin). The samples were spun at 142,400 *g* for 37 min at 4°C. The pellets were resolublized with 5 ml of buffer A, rotated at 4°C for 1 h, and centrifuged as before. The two supernatants were combined and incubated overnight at 4°C with 1 ml of WGA–Sepharose (Vector Labs, Inc.). The WGA–Sepharose was washed extensively (50 mM Tris-HCl, pH 7.8, 0.1% digitonin, 500 mM NaCl) and proteins were eluted with 0.3 M N-acetyl glucosamine (Sigma Chemical Co.). Samples were concentrated to 500 μl using a Centricon-30 filter and applied to a 5–30% sucrose gradient at pH 7.8, as described previously (Ervasti et al., 1991).

Immunoblotting

Protein samples were resolved under reducing conditions by 3–15% SDS-PAGE and transferred to PVDF (Immobilon-P) membranes (Millipore Corp.). PVDF membranes were probed with anti-SG mAbs, as described previously (Holt et al., 1998). For mouse SPN immunoblotting, the membranes were probed with affinity purified rabbit 235 antibody at a dilution of 1:50. Note that for mouse SPN immunoblotting, proteins were resolved on 3–15% SDS-PAGE under nonreducing conditions and transferred to PVDF (Immobilon-P). For rabbit SPN staining, nitrocellulose blots were probed with affinity purified rabbit 216 antibody as described (Crosbie et al., 1997). For α- and β-DG staining, SDS-polyacrylamide gels were transferred to nitrocellulose (Immobilon-NC) and probed with IIH6 (1:3 dilution) and 20A6 (1:100 dilution). Following incubation with primary antibodies, blots were probed with the appropriate HRP-conjugated secondary antibodies (1:5,000; Boehringer Mannheim Corp.) and developed using enhanced chemiluminescence (SuperSignal; Pierce Chemical Co.).

In Vivo Reconstitution Experiments

A human SPN expression construct was prepared by PCR amplification of cDNA using primers containing appropriate restriction sites for subcloning into pcDNA3 (Pharmacia Biotech, Inc.). The SPN construct was engineered to encode a myc-tag at the COOH terminus. All constructs were verified by direct DNA sequence analysis performed by the DNA Core Facility at the University of Iowa (Iowa City, IA). Full-length myc-tagged α-, β-, γ-, and δ-SG pcDNA3 (Pharmacia Biotech Inc.) expression constructs have been previously described (Holt and Campbell, 1998). Construction and design of the Grb2 cDNA expression vector has been described (Holt et al., 1996). CHO cells were electroporated with SG and SPN expression constructs (~5 μg of each plasmid DNA) at 340 V at 950 μF using a BioRad electroporator, as previously described (Holt and Campbell, 1998). 30 h after transfection, cells were analyzed for protein expression by SDS-PAGE and immunoblotting. Membrane surface proteins were biotinylated using membrane impermeant sulfo-NHS-biotin (Pierce Chemical Co.) as described previously for the SGs expressed in CHO cells (Holt and Campbell, 1998). Immunoprecipitation using a β-SG mAb (5B1) and analysis of protein samples by SDS-PAGE and immunoblotting with an anti-myc mAb (9E10) were performed as documented in Holt and Campbell (1998).

Results

SPN is the most recently identified dystrophin-associated protein, and therefore the least characterized. Hydropathy analysis of the primary amino acid sequences of human (Heighway et al., 1996; Crosbie et al., 1997) and murine (Scott et al., 1994) SPN predicts a protein with intracellular NH_2 and COOH termini, and four transmembrane domains. We report determination of the primary structure of rabbit SPN, as deduced from a rabbit skeletal muscle cDNA (Fig. 1 a). Multiple sequence alignment demonstrates that amino acid sequences derived from rabbit, mouse, and human SPN are ≥75% identical (Fig. 1 a). Human and rabbit SPN contain a short insertion at the NH_2 terminus, which is absent in mouse SPN. The four predicted transmembrane domains are extremely well conserved. SPN's membrane topology is strikingly different from other dystrophin-associated proteins, which only have a single pass transmembrane domain, and is reminiscent of the tetraspan superfamily of proteins (Wright and Tomlinson, 1994; Maecker et al., 1997). Using phylogenetic analysis, we previously demonstrated that SPN is closely related to the divergent family members Rom-1, peripherin, and uroplakin (Crosbie et al., 1997). The tetraspans are thought to play important roles in mediating interactions between transmembrane proteins as mechanisms to control cell growth and adhesion. We speculate that SPN, a novel dystrophin-associated tetraspan, may be facilitating interactions among proteins of the DGC and perhaps mediating interactions of the DGC components with other sarcolemma proteins.

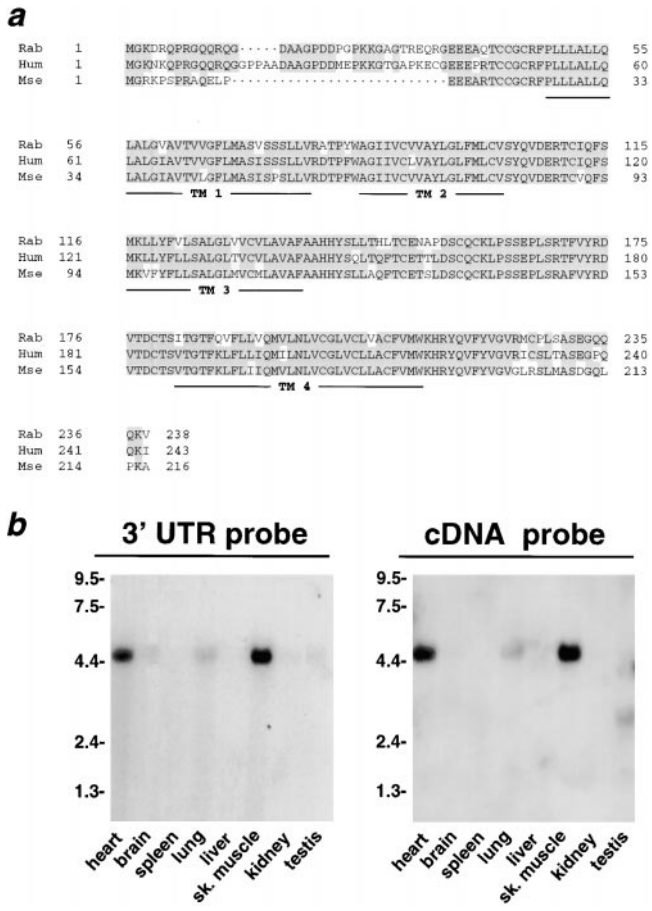

Figure 1. SPN is a novel tetraspan expressed predominantly in muscle. (a) Multiple amino acid sequence alignment of SPN isolated from rabbit, human, and mouse skeletal muscle. Residues identical in two or more species are shaded with gray boxes. Predicted transmembrane domains are indicated with underlining. These sequence data are available from GenBank/EMBL/DDBJ under accession numbers AF016028 for human, U02487 for mouse, and AF120276 for rabbit. Mouse SPN cDNAs isolated from Y1 adrenal carcinoma cells (Scott et al., 1994) and skeletal muscle (this study) are identical. (b) Northern blots containing 2 μg of poly $(A)^+$ RNA from the indicated mouse tissues were hybridized with probes representing the 3′ untranslated region (UTR) and the coding region of mouse SPN. Molecular size markers are indicated on the left (kb).

SPN Is a Unique Tetraspan Predominantly Expressed in Muscle

To examine the distribution of SPN in mouse tissue, we performed RNA hybridization analysis. Hybridization of mouse multiple tissue Northern blots with probes representing either the coding region or the 3′ untranslated region of SPN gave identical results (Fig. 1 b). As shown in Fig. 1, a 4.4-kb transcript is predominant in skeletal and cardiac tissues, with minor transcript levels present in lung, brain, and testis. Human skeletal and cardiac muscles express two SPN transcripts (4.4 and 6.5 kb; Crosbie et al., 1997). The additional 6.5-kb transcript in humans is likely the result of alternate splicing, since the 6.5-kb transcript does not hybridize to SPN exons 2 and 3 (Heighway et al., 1996).

SPN Localization to the Sarcolemma Is Dependent on the DGC

We have previously shown that SPN is expressed throughout the sarcolemma of normal human skeletal muscle (Crosbie et al., 1997, 1998). The limitations of patient biopsies with known mutations prompted our use of murine models as a method to investigate the interactions of SPN with the DGC. First, we examined expression of SPN in quadriceps femoris (thigh), diaphragm, and cardiac muscles from *mdx* mice, a model for Duchenne muscular dystrophy. The *mdx* phenotype is inherited as an X-linked recessive trait, which stems from a premature stop codon in exon 23 of the dystrophin gene, leading to absence of dystrophin protein (Bulfield et al., 1984; Hoffman et al., 1987; Chamberlain et al., 1988). As a consequence, the dystrophin-associated proteins are nearly ablated from the sarcolemma (Ohlendieck and Campbell, 1991). Using indirect immunofluorescence on muscle cross sections, we show that SPN is dramatically reduced in skeletal muscle of *mdx* mice (Fig. 2). Our data also demonstrate that SPN is expressed in normal cardiac tissue, which is consistent with the presence of SPN transcript in Northern blots (Fig. 1 b). As shown in Fig. 2, SPN is significantly reduced in *mdx* cardiac tissue. The diaphragm muscle, which is the most severely affected muscle in *mdx* mice, also lacks normal sarcolemma expression of SPN. In addition to its expression in skeletal muscle, recent experiments from our group demonstrate that SPN is also present in smooth muscle (Straub, V., and K.P. Campbell, personal communication). Lastly, we observed positive SPN staining in muscle from the laminin α2 deficient *dy* (Arahata et al., 1993; Sunada et al., 1994; Xu et al., 1994a) and dy^{2J} (Xu et al., 1994b; Sunada et al., 1995) mice (data not shown), which are naturally occurring animal models of congenital muscular dystrophy.

Association of SPN with Dystrophin Isoforms

To further explore the association of SPN with the DGC, we examined SPN expression in a collection of dystrophin transgenic mice. The transgenes, which were individually expressed on an *mdx* background, encode truncated dystrophin products. Muscle from the transgenic mice was previously evaluated for its morphology and for the ability of the transgene to restore sarcolemma localization of the DGC (Cox et al., 1994; Greenberg et al., 1994; Rafael et al., 1994, 1996; Phelps et al., 1995). We detected SPN expression in skeletal muscle cross sections by indirect immunofluorescence staining with SPN antibodies, and found normal levels of SPN expression in muscle from Δ17–48 (Phelps et al., 1995), Δ1–62 (Cox et al., 1994; Greenberg et al., 1994), Δ71–74 (Rafael et al., 1994, 1996), and Δ75–78 (Rafael et al., 1996) transgenic mice (Fig. 3). Despite large deletions, these transgenes are able to restore SPN to the sarcolemma and, with the exception of the Δ1–62 transgene, alleviate muscular dystrophy. The Δ71–74 transgene represents an alternately spliced dystrophin isoform that is predominantly expressed in brain, and the Δ1–62 (Dp71) transgene mimics a form of dystrophin present in lung, spleen, testis, and retina.

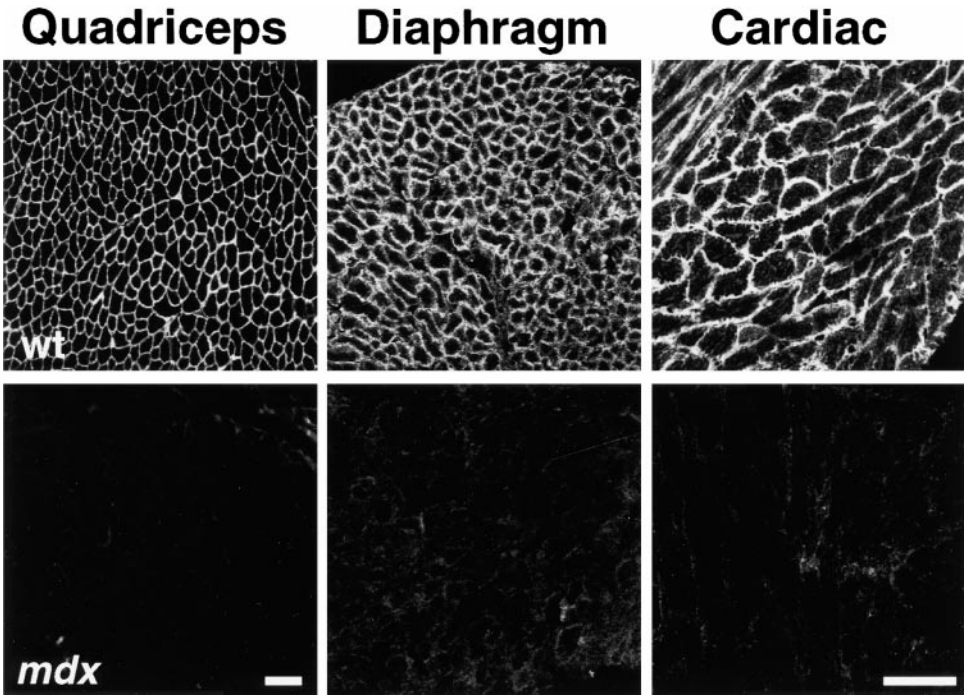

Figure 2. SPN is absent in dystrophin-deficient muscle. Quadriceps, diaphragm, and cardiac muscle cryosections from wt and *mdx* mice were stained with antibodies to SPN by indirect immunofluorescence. SPN staining is dramatically reduced in the dystrophin-deficient *mdx* muscles. Bars, 100 μm.

SPN Enrichment at the NMJ Is Mediated by Dystrophin and Utrophin

To determine if SPN is associated with the utrophin–glycoprotein complex, and if replacement of dystrophin with utrophin would affect SPN's localization to the sarcolemma, we examined NMJs from dystrophin, utrophin (Grady et al., 1997a), and dystrophin–utrophin (Deconinck et al., 1997; Grady et al., 1997b) deficient muscle. The NMJs were identified by staining cross sections of quadriceps femoris with fluorescein α-bungarotoxin, which selectively binds to acetylcholine receptors. By indirect immunofluorescence, we show that SPN is enriched at the NMJ of innervated muscle (Fig. 4). This enrichment is maintained even after denervation, demonstrating that SPN is associated with the postsynaptic membrane (data not shown). At the NMJ, dystrophin is replaced by the structurally and functionally similar protein, utrophin (Khurana et al., 1991; Nguyen et al., 1991; Ohlendieck et al., 1991; Pons et al., 1991; Matsumura et al., 1992; Karpati et al., 1993). Enrichment of SPN at the NMJ is not altered by the absence of dystrophin, as seen by positive NMJ staining in the *mdx* muscle (Fig. 4). In this case, SPN's localization to the NMJ is mediated by utrophin. Conversely, NMJ localization of SPN is preserved by dystrophin in utrn$^{-/-}$ muscle, as demonstrated by SPN NMJ staining in these mice. Loss of SPN staining from the NMJ occurs only in the absence of both utrophin and dystrophin, as in the *mdx*:utrn$^{-/-}$ double mutant mice.

In *mdx* mice, muscles with the greatest upregulation of utrophin exhibit the least pathological changes (Porter et al., 1998). For instance, the EOM are spared the pathological effects in *mdx* mice and Duchenne muscular dystrophy patients, likely from the upregulation of utrophin (Matsumura et al., 1992; Porter et al., 1998). In support of this, Tinsley et al. (1996, 1998) demonstrate that expression of utrophin attenuates the dystrophic pathology in *mdx* mice, suggesting that utrophin can functionally replace dystrophin within the complex. We examined the EOMs from wt, *mdx*, and *mdx*:utrn$^{-/-}$ (Deconinck et al., 1997; Grady et al., 1997b) mice for SPN expression as another method to demonstrate that SPN is part of the utrophin–glycoprotein complex. We show that SPN is located at the sarcolemma of wt EOM and is maintained in the EOM of *mdx* mice, despite absence of dystrophin (Fig. 5). The continued expression of SPN in the *mdx* EOM likely is mediated through SPN's association with the utrophin–glycoprotein complex. Consistent with this idea, SPN expression is lost in the EOM of mice lacking both dystrophin and utrophin (*mdx*:utrn$^{-/-}$; Fig. 5). These data are important as they demonstrate that upregulation of utrophin retains SPN to the sarcolemma and validates this as a reasonable therapy for Duchenne muscular dystrophy.

SPN's Localization to the Sarcolemma Is Dependent on the SGs

The SGs (consisting of α, β, γ, and δ subunits) form a tight subcomplex of four transmembrane glycoproteins within the DGC (Ervasti et al., 1991; Yoshida et al., 1994; Jung et al., 1996). The integrity of this complex is maintained despite harsh treatments with SDS (Jung et al., 1996) and *n*-octyl β-D-glucoside (Yoshida et al., 1994). Absence of any one of the SGs results in absence of the entire SG subcomplex and destabilization of α-DG from the sarcolemma (Roberds et al., 1993; Duclos et al., 1998a; Holt et al., 1998). Furthermore, this subcomplex is critical for protecting the sarcolemma from contraction induced damage.

We wanted to determine if SPN depends on the SG subcomplex for proper membrane targeting by examining

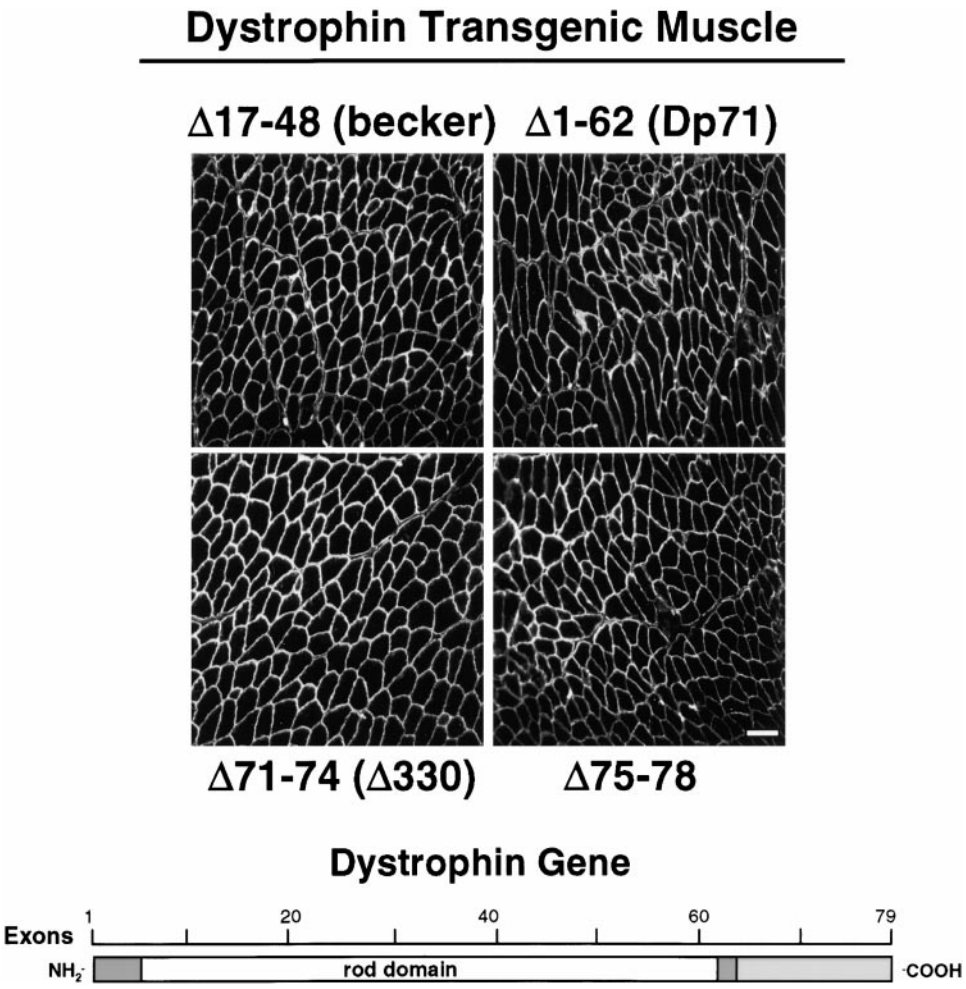

Figure 3. Restoration of SPN by dystrophin transgenic products. Various truncated and internally deleted dystrophin transgenes were expressed on an *mdx* background and previously analyzed for their ability to rescue the *mdx* phenotype. Skeletal muscle cryosections from Δ17–48 (becker), Δ1–62 (Dp71), Δ71–74 (Δ330), and Δ75–78 mice were stained with SPN antibodies and visualized by indirect immunofluorescence. The localization of the deleted exons is represented in the schematic diagram of the dystrophin transgene. Binding regions for F-actin have been identified in the NH_2 terminus, as well as in the mid-rod domain of dystrophin (Rybakova et al., 1996; Rybakova and Ervasti, 1997; Amann et al., 1998). Syntrophin and β-DG binding sites are located at the dystrophin COOH terminus. Bar, 100 μm.

δ-SG-deficient BIO 14.6 hamsters (Homburger et al., 1962; Okazaki et al., 1996) for SPN expression. A large deletion in the δ-SG gene (Nigro et al., 1997; Sakamoto et al., 1997) causes selective loss of the entire SG subcomplex from BIO 14.6 skeletal muscle without affecting β-DG (Roberds et al., 1993; Mizuno et al., 1995; Duclos et al., 1998b). We now demonstrate that SPN expression is absent from the sarcolemma (Fig. 6), as well as the NMJ (data not shown) of the BIO 14.6 hamster. Furthermore, we show that SPN expression is restored to normal levels after delivery of an adenovirus encoding δ-SG into BIO 14.6 muscle (Fig. 6). Control injections of α-SG did not restore proper localization of SPN or the SGs (Fig. 6). Recent experiments from our laboratory have shown that injection of δ-SG into muscle of the BIO 14.6 hamster rescues expression of the entire SG subcomplex (Holt et al., 1998). Muscle fibers expressing the restored SG–SPN subcomplex are spared the pathological features of muscular dystrophy (i.e., sarcolemma damage and central nucleation) and have stable expression of α-DG at the plasma membrane (Holt et al., 1998). Thus, SPN and the SGs are required for normal muscle physiology and prevention of dystrophic features.

In addition to this naturally occurring hamster model for LGMD, our laboratory has created α-SG null mice by a targeted disruption of the murine α-SG gene (Duclos et al., 1998b). Like the BIO 14.6 hamsters, Sgca-null mice specifically lack the SG subcomplex (Duclos et al., 1998b). We now demonstrate that Sgca-null muscle is completely devoid of SPN (Fig. 7 a). The NMJ and myotendinous junction (MTJ), which we show are normally enriched for SPN expression, also lack SPN in the Sgca-null mice (Fig. 7 a). As further demonstration of the tight association of SPN with the SGs, we immunoblotted KCl washed membranes prepared from skeletal muscle of wt, *mdx*, and Sgca-null mice. SPN is dramatically reduced in *mdx* membranes (~90% compared with wt), but SPN was not detected in the Sgca-null membranes (Fig. 7 b).

Isolation of the SG–SPN Subcomplex

To demonstrate that SPN is tightly associated with the SGs, we isolated the SG–SPN subcomplex from skeletal muscle. We prepared purified DGC from rabbit skeletal muscle microsomes and titrated the complex to pH 11 to dissociate pH-sensitive protein–protein interactions. Alkaline-treated DGC was centrifuged through a 5–30% linear sucrose gradient. Proteins from the sucrose gradient fractions were separated by SDS-PAGE and immunoblotted with anti-DGC antibodies. As shown in Fig. 8 a, sucrose gradient sedimentation of alkaline-treated DGC separates

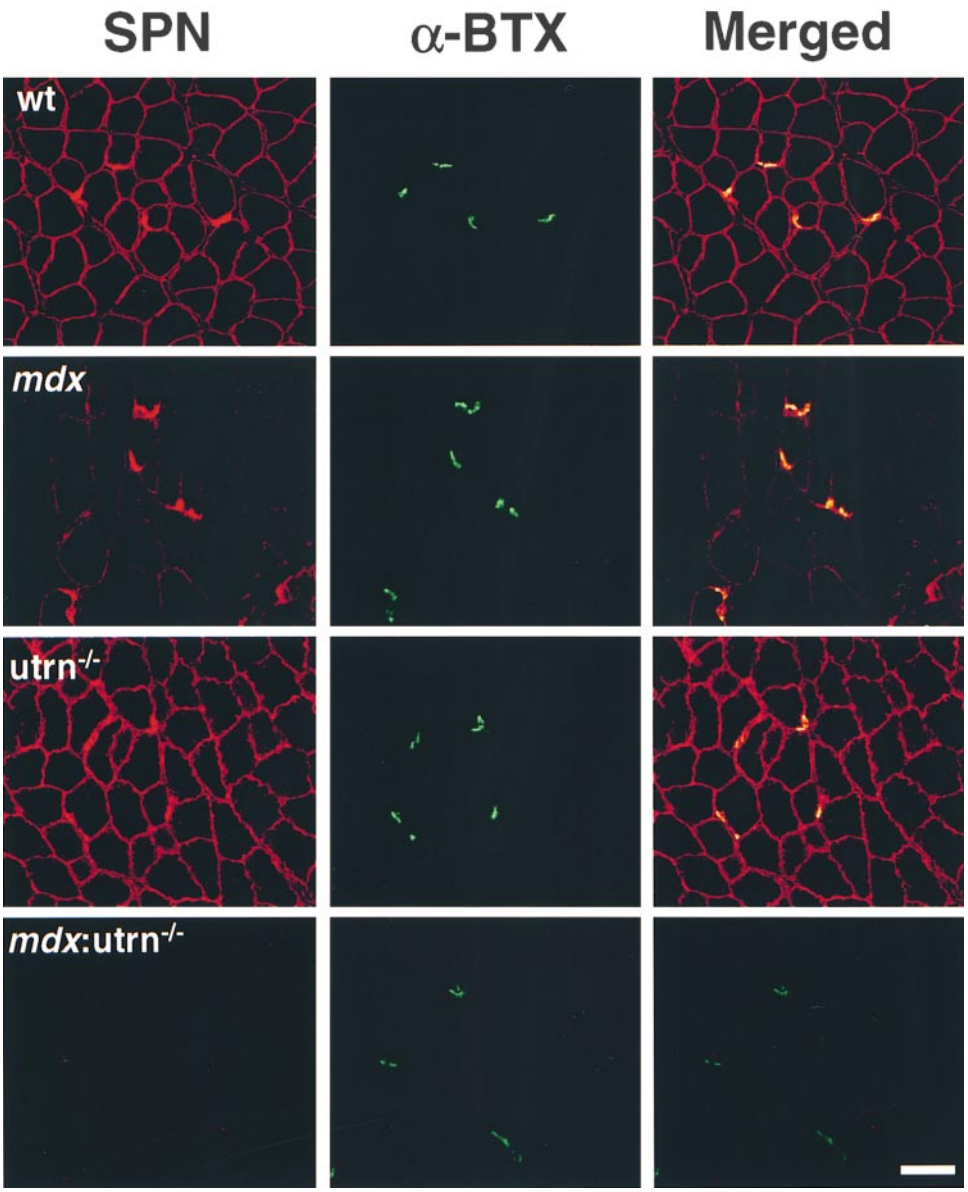

Figure 4. Enrichment of SPN at the NMJ is mediated by dystrophin and utrophin. Sections from quadriceps femoris muscles of adult mice were doubly stained with α-bungarotoxin (α-BTX) and SPN antibodies. SPN expression at the NMJ was examined for wt, *mdx*, utrn$^{-/-}$, and *mdx*:utrn$^{-/-}$ mice. SPN staining was visualized by indirect immunofluorescence with Cy3-conjugated secondary antibodies (red) and synaptic sites were identified by fluorescein–α-bungarotoxin staining (green). Merged images (yellow) are shown in the right panels.

the DG (fractions 6–9) and SG (fractions 9–12) subcomplexes from one another. SPN displays a sedimentation pattern similar to that of the SG subcomplex, indicating a preferential association of SPN with the SGs.

In addition to chemically disrupting the DGC, we analyzed the dissociation of SG and DG subcomplexes resulting from the absence of dystrophin. The dystrophin-associated proteins are present in the extrajunctional sarcolemma of *mdx* muscle, although at significantly reduced levels. Figs. 2 and 7 illustrate that ~10% of SPN expression is maintained at the *mdx* sarcolemma. We prepared glycoproteins by WGA–Sepharose chromatography of digitonin-solubilized *mdx* skeletal muscle. Without dystrophin, the SG and DG subcomplexes are no longer associated and can be separated by sucrose gradient centrifugation. The subcomplexes peak in separate fractions and the relative separations between the SG and DG containing fractions are similar for both *mdx* and pH 11 treated samples. As shown in Fig. 8 b, SPN migrates exclusively with the SG containing fractions.

Reconstitution of the SG–SPN Subcomplex

Using an in vivo cell expression system, we demonstrate that SPN and the SGs are associated in a complex at the plasma membrane. Myc-tagged human cDNA constructs of the SGs (α, β, γ, and δ) and SPN were transiently introduced into CHO cells by electroporation. Immunoblots of cellular protein lysates with anti-myc antibodies demonstrate that each of the SGs and SPN, as well as the Grb2 negative control, are expressed at relatively equal quantities (Fig. 9). We confirm that these proteins are targeted to the plasma membrane by treatment of cells with sulfo-NHS-biotin, which forms a covalent bond with free amines of proteins at the cell surface. Clarified lysates from transfected CHO cells were incubated with avidin–Sepharose

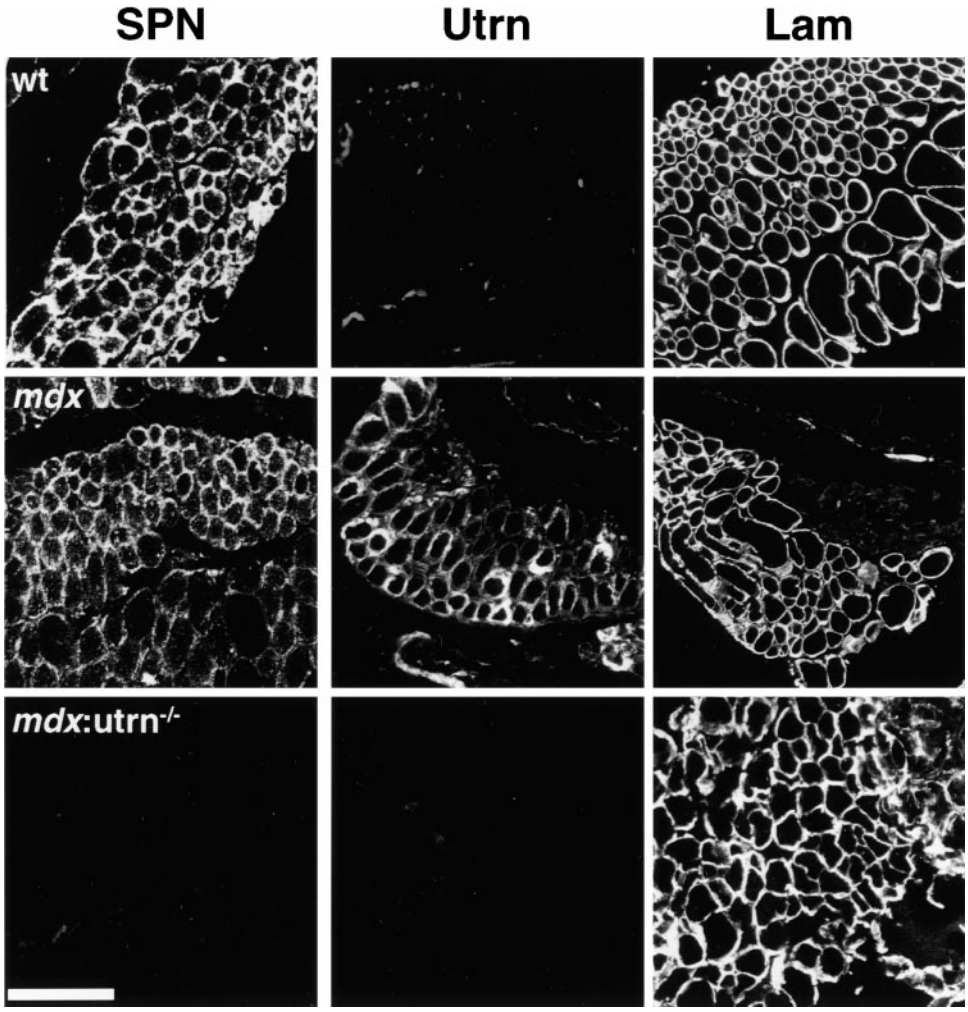

Figure 5. SPN staining is preserved in the EOMs of *mdx* mice by utrophin upregulation. Cryosections from the EOMs of wt, *mdx*, and *mdx*:utrn$^{-/-}$ were stained with antibodies to SPN, utrophin (Utrn), and laminin α2 chain (Lam). The *mdx* EOM are among the few muscles that are spared the pathological consequences of muscular dystrophy, likely due to its sarcolemma expression of utrophin. Shown are EOM rectus muscles. SPN expression is maintained in the *mdx* EOM. Bar, 50 μm.

to precipitate plasma membrane–associated proteins. As shown in Fig. 9, SPN and the SGs are properly localized to the plasma membrane.

To demonstrate that the SGs and SPN are assembled into a stable molecular complex, we performed immunoprecipitation experiments. CHO cells transfected with the SGs plus SPN were immunoprecipitated using mAbs to β-SG. SPN coimmunoprecipitates along with the SGs from CHO cells. To demonstrate the specificity of this association, control immunoprecipitation experiments from cells expressing the SGs and myc-tagged Grb2 were performed. Grb2 serves as a negative control since it is a soluble protein that is not expected to associate with the SGs at the plasma membrane. The SGs and Grb2 were cotransfected into CHO cells and cellular lysates were immunoprecipitated with the β-SG mAb. Grb2 is not found in the immune complex with the SGs (Fig. 9). These data provide strong evidence that the simultaneous expression of the SGs and SPN in CHO cells results in the formation of a tight molecular complex.

Discussion

The DGC spans the sarcolemma and links the intracellular actin cytoskeleton of muscle cells to the extracellular matrix. Current evidence indicates that the DGC confers structural stability to the muscle plasma membrane, thus protecting it from stresses that develop during muscle fiber contraction. In support of this theory, perturbations in the dystrophin-associated components lead to loss of membrane integrity. This is evidenced by increased permeability of muscle fibers to intravenously administered Evans blue dye (Straub et al., 1997, 1998; Holt et al., 1998) as well as leakage of muscle-specific enzymes into the serum. This loss of membrane integrity eventually manifests itself as fiber degeneration. Thus, understanding the structural organization of the DGC is critical for understanding the function of this complex.

Our findings represent the first account of SPN's localization in normal muscle, the expression of SPN in mutant mice, and the molecular associations of SPN within the DGC. We report that SPN, found at the sarcolemma of skeletal, cardiac, and diaphragm muscles, is also expressed at many specialized muscle membrane interfaces, including the NMJ (Fig. 4) and MTJ (Fig. 7), as well as at muscle spindles (data not shown). SPN is also expressed in smooth muscle, where it is part of a unique smooth muscle SG–SPN complex (Straub, V., and K.P. Campbell, personal communication). Although SPN seems to be predominantly expressed in muscle, we detect SPN transcripts

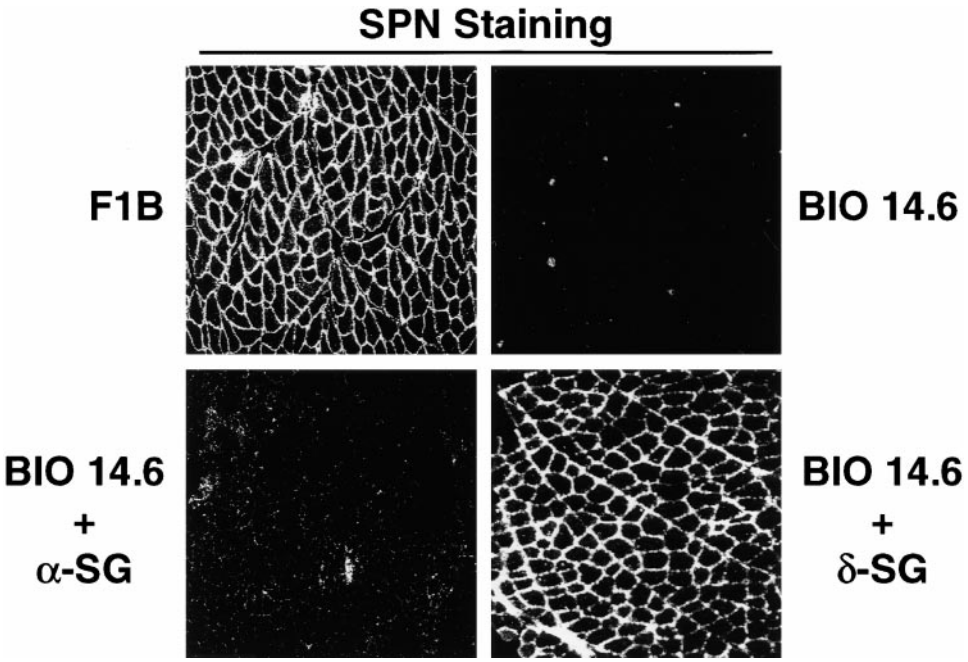

Figure 6. SPN's absence from the sarcolemma of δ-SG-deficient BIO 14.6 hamster can be restored with recombinant δ-SG adenovirus. Quadriceps muscle of the BIO 14.6 hamster was injected with 10^9 particles of δ-SG adenovirus or α-SG adenovirus particles. Tissue was harvested 7 d after injection. Muscle cryosections from these injected animals, as well as from the F1B and BIO 14.6 (uninjected) hamsters were examined for SPN expression by indirect immunofluorescence with SPN antibodies.

in many nonmuscle tissues (Fig. 1 b; Crosbie et al., 1997). Consistent with this, our examination of dystrophin transgenic mice indicates that SPN may be associated with nonmuscle isoforms of dystrophin, such as Dp71 (Fig. 3). Further experimentation is necessary to determine whether SPN protein is present in these nonmuscle tissues. The discovery that a subset of dystrophin-associated proteins (i.e., dystrophin, DG, and ϵ-SG) is present in a broad array of cell types is a provocative finding since all tissues are not subjected to the same shear stresses as muscle. This suggests that the DGC may serve a more fundamental role in the cell, in addition to the structural one ascribed to the DGC in muscle.

We now show that SPN's localization to the sarcolemma is compromised in dystrophin and utrophin double null mice. SPN's enrichment at the NMJ is achieved by its association with utrophin. It has been suggested that upregulation of utrophin compensates for loss of dystrophin (Mat-

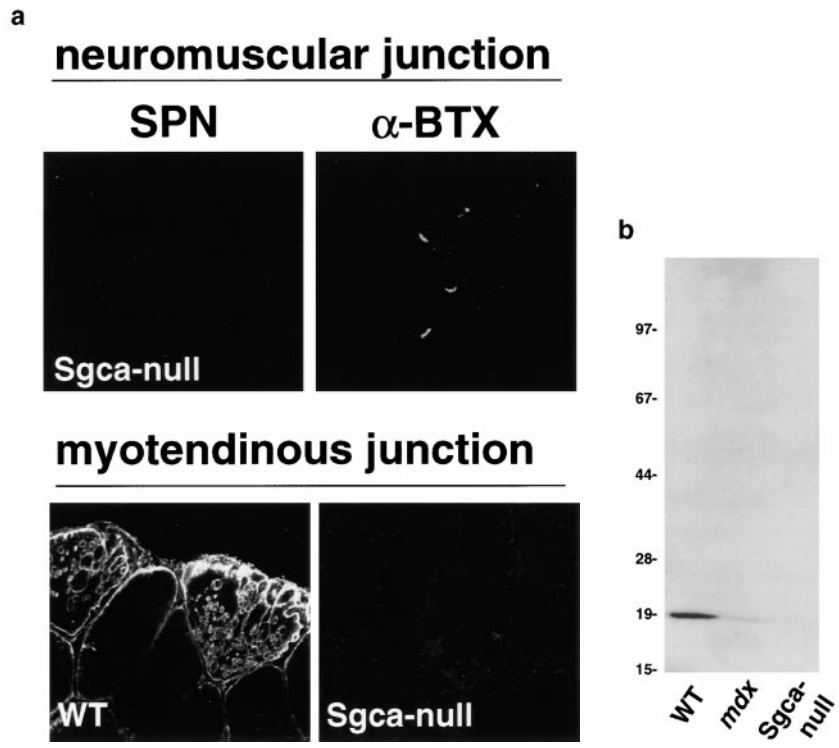

Figure 7. SPN is absent from the NMJ and MTJ of Sgca-null mice. (a) Cryosections from Sgca-null muscle were analyzed for SPN expression at the sarcolemma, NMJ, and MTJ. SPN is completely absent in the SG deficient muscle. (b) Skeletal muscle membranes from wt, *mdx*, and Sgca-null mice were analyzed by 3–15% SDS-PAGE and immunoblotted using antibodies against SPN. SPN isolated from mouse skeletal muscle membranes migrates at 20 kD. The level of SPN expression is dramatically reduced in the *mdx* membranes and is completely absent from the Sgca-null muscle. Molecular weights are indicated ($\times 10^3$ D).

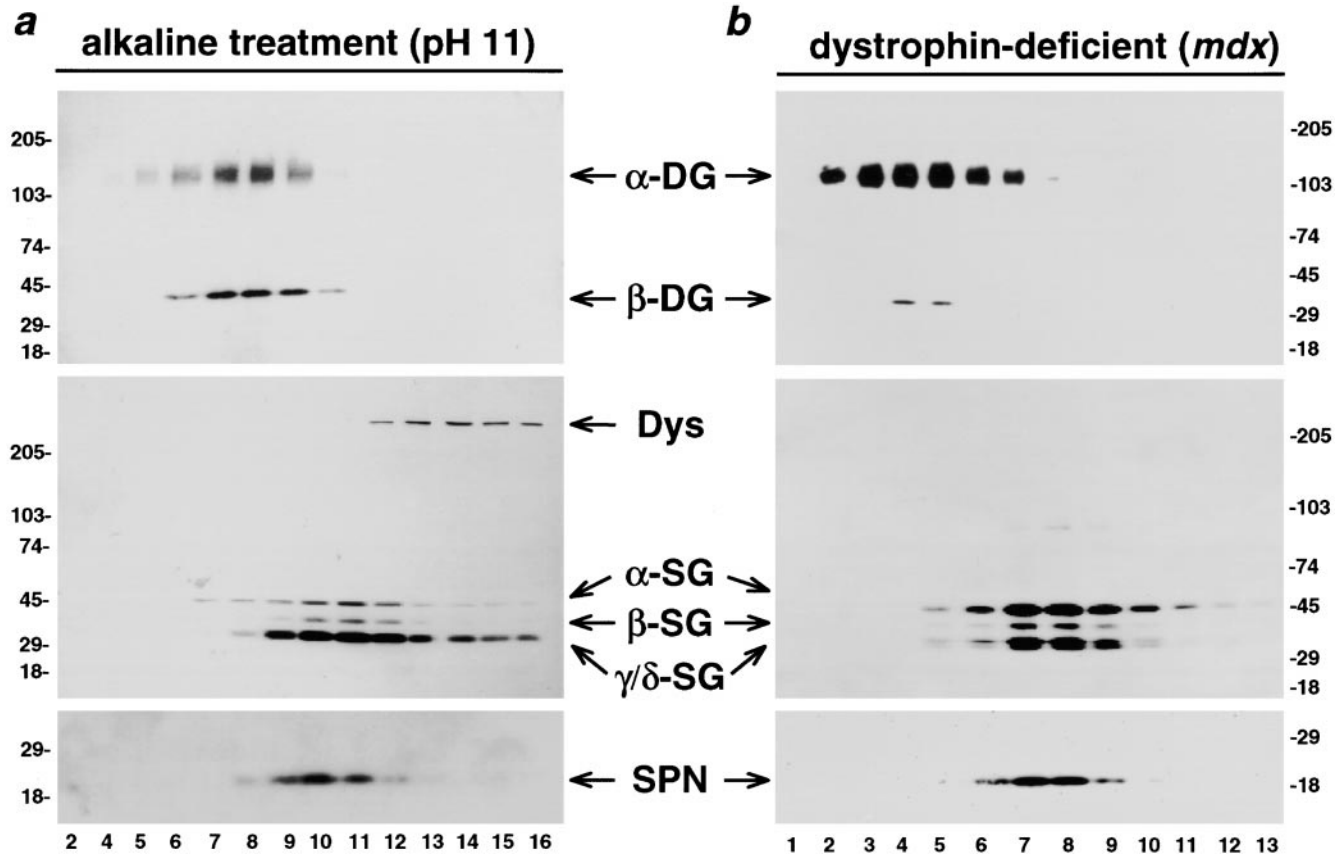

Figure 8. Isolation of the SG–SPN subcomplex from skeletal muscle. (a) The DGC, purified from rabbit skeletal muscle membranes, was titrated to pH 11 and centrifuged through 5–30% linear sucrose gradients. Fractions from the gradients were separated by SDS-PAGE and separately immunoblotted with antibodies to α- and β-DG, SGs, dystrophin, and SPN. Alkaline treatment dissociates the SG subcomplex from the DG subcomplex. SPN comigrates with the SG subcomplex. (b) Glycoproteins from skeletal muscle of dystrophin-deficient *mdx* mice were prepared and centrifuged through 5–30% linear sucrose gradients. Protein fractions were immunoblotted with the indicated antibodies. Without dystrophin, the DG and SG subcomplexes are no longer associated, and migrate separately during sucrose gradient centrifugation. SPN preferentially associates with the SG subcomplex. Molecular size standards are indicated on each panel ($\times 10^3$ D).

sumura et al., 1992). Indeed, we have now demonstrated that the EOMs of *mdx* mice, which are spared from the pathological features of muscular dystrophy, express utrophin (Porter et al., 1998) and SPN throughout the sarcolemma. If utrophin can functionally replace dystrophin, then it may be possible to upregulate utrophin expression in Duchenne muscular dystrophy patients (Matsumura et al., 1992; Tinsley et al., 1996, 1998). Our current data lend credence to the proposed theory that sarcolemma expression of utrophin would completely restore the dystrophin-associated proteins to the muscle plasma membrane.

The DGC can be broken down into at least three interconnected subcomplexes: dystrophin, the DGs, and the SGs. Using several independent criteria, we demonstrate that SPN's localization to the sarcolemma is dependent on an intact SG subcomplex. SPN is completely absent from the sarcolemma, NMJ, and MTJ of the SG-deficient BIO 14.6 hamster and Sgca-null mouse. The preferential association of SPN with the SGs is demonstrated by biochemical isolation of the SG–SPN subcomplex. Alkaline treatment of purified DGC causes dissociation of the complex into distinct subcomplexes, where SPN preferentially associates with the SG containing fractions (Fig. 8 a). Likewise, in the absence of dystrophin, the remaining extrajunctional dystrophin-associated proteins dissociate into distinct protein complexes, where SPN's specific interactions with the SGs are maintained (Fig. 8 b).

Furthermore, we reconstitute the SG–SPN complex in a recently developed heterologous cell system, which lacks muscle specific proteins (Fig. 9; Holt and Campbell, 1998). Previous work from our group has shown that mutations in an individual SG result in intracellular accumulation of the SG subcomplex (Holt and Campbell, 1998). These experiments suggest that obligatory steps in the biosynthetic pathway for SG subcomplex assembly cannot occur if individual SG proteins are aberrant or missing (Holt et al., 1998; Holt and Campbell, 1998).

Taken together, our in vivo experiments now indicate that assembly of the SG subcomplex is a prerequisite for targeting and stabilization of SPN to the sarcolemma, as illustrated in Fig. 10. We currently do not know the molecular basis of the interaction between the SG–SPN and DG subcomplexes. It is clear, however, that proper structural alignment of these two subcomplexes, along with dystro-

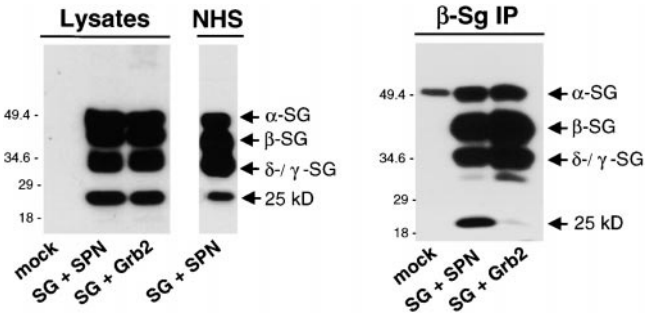

Figure 9. Reconstitution of the SG–SPN subcomplex. The SG–SPN subcomplex was reconstituted in vivo using a heterologous cell expression system. CHO cells were transfected with expression vectors encoding myc-tagged human α-, β-, γ-, and δ-SGs with either SPN (SGs + SPN) or Grb2 (SGs + Grb2). Mock transfected CHO cells are shown. To demonstrate similar expression of proteins, whole cell lysates are shown (lysates). Note that Grb2, which serves as a negative control, and SPN migrate at similar molecular weights. Cells transfected with all four SGs and SPN were treated with NHS-biotin and recovered from detergent extracts as avidin precipitates to demonstrate surface localization of these proteins (NHS). Immunoprecipitation of β-SG from lysates prepared from cells expressing all four SGs and SPN results in coprecipitation of the SG–SPN subcomplex (β-SG IP). Grb2 does not immunoprecipitate with the SGs. Proteins are detected by immunoblotting with an anti-myc mAb, which recognizes the 9E10 tag on each protein construct. The 50-kD protein band in the mock transfected cell represents the β-SG immunoprecipitating antibody. Molecular weights are indicated on each blot ($\times 10^3$ D).

phin, is required for DGC function and prevention of muscular dystrophy. The data presented in the current study are also consistent with our finding that SG-deficient LGMD patients also lack SPN (Crosbie, R.H., and K.P. Campbell, personal communication).

Although SPN is tightly associated with the SGs, SPN bears no structural homology to the SGs. To date, there are five known SGs, including the ubiquitously expressed ε-SG, which exhibits >40% amino acid identity to α-SG (Ettinger et al., 1997; McNally et al., 1998). ε-SG shares all the structural features of the skeletal muscle SGs, but is also expressed in many nonmuscle tissues. β-, γ-, and δ-SG are type II transmembrane proteins, while α- and ε-SG are type I membrane proteins with an NH_2-terminal signal sequence. ε-SG expression is not perturbed by targeted deletion of the α-SG gene, suggesting that ε-SG is not an additional member of the α-, β-, γ-, δ-tetrameric SG subcomplex in skeletal muscle (Duclos et al., 1998b). Each of the SGs have a five cysteine residue motif in its extracellular domain, which is unique to this group of proteins. The SGs also possess one or more consensus sites for glycosylation and treatment with PNGase F has been shown to shift the molecular weight of these proteins. SPN, on the other hand, has many characteristics that distinguish it from the SGs. Most obviously, SPN is predicted to have multiple transmembrane domains and has no consensus sites for N-linked glycosylation. Consistent with this, treatment of purified DGC with PNGase F does not alter SPN's molecular weight (data not shown). Thus, SPN represents the first non-SG protein to be associated with the SG subcomplex of the DGC.

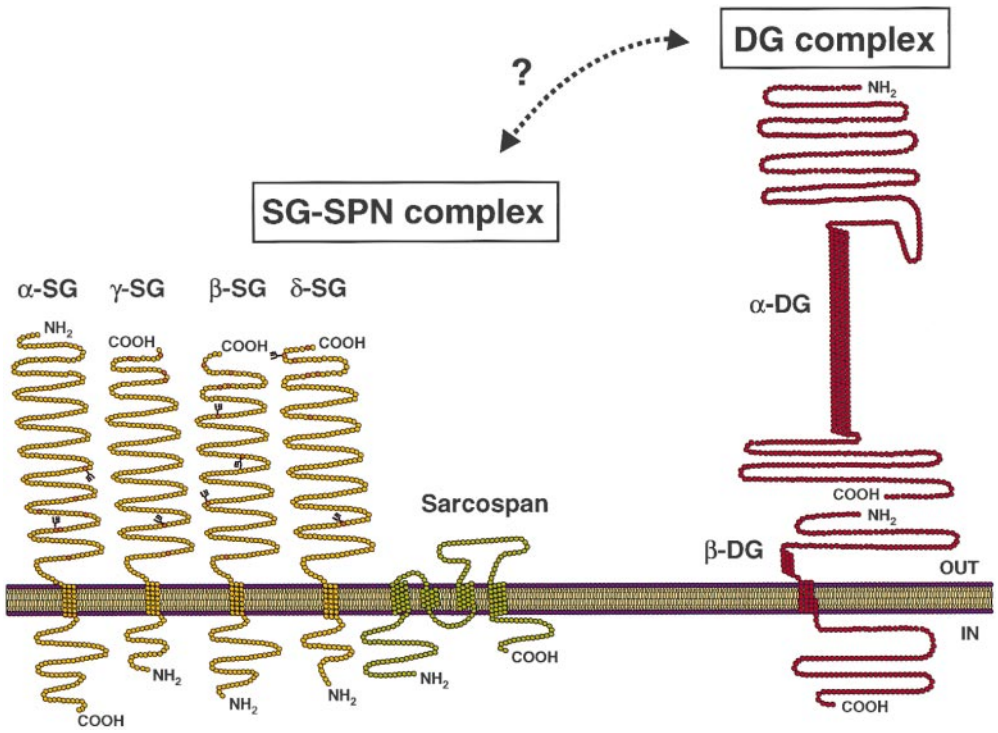

Figure 10. Schematic diagram representing interactions among DGC subcomplexes. Evidence presented in the current report demonstrates that assembly and membrane localization of the SG subcomplex are prerequisites for SPN localization to the sarcolemma, NMJ, and MTJ. This dependence is likely mediated through direct interaction of SPN with the SGs, as suggested by cofractionation and coimmunoprecipitation of SPN with the SG subcomplex. In the absence of the SG–SPN subcomplex, α-DG is not properly anchored to the muscle plasma membrane. This suggests that there may be direct interactions between α-DG and the SG–SPN subcomplex, which stabilize α-DG to the cell membrane (dashed arrows). The molecular basis of the interaction between the SG–SPN and DG subcomplexes is not thoroughly understood. Clearly, proper structural alignment of the two subcomplexes along with dystrophin is required for DGC function and prevention of muscular dystrophy.

The tight association of SPN with the SGs is consistent with SPN's homology to the tetraspan superfamily of proteins. The tetraspans are thought to function as facilitators of transmembrane protein interactions, and we suspect SPN serves to coordinate protein–protein interactions within the DGC. The results of our study provide support for this notion, since we find that SPN is intimately associated with at least one subcomplex of the DGC. Further examination of SPN's interaction with other DGC subcomplexes should provide significant insight into how the DGC is structurally organized, which is critical for understanding the function of this complex.

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