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Differential requirement for individual sarcoglycans and dystrophin in the assembly and function of the dystrophin-glycoprotein complex

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SUMMARY

Sarcoglycan is a multimeric, integral membrane glycoprotein complex that associates with dystrophin. Mutations in individual sarcoglycan subunits have been identified in inherited forms of muscular dystrophy. To evaluate the contributions of sarcoglycan and dystrophin to muscle membrane stability and muscular dystrophy, we compared muscle lacking specific sarcoglycans or dystrophin. Here we report that mice lacking δ-sarcoglycan developed muscular dystrophy and cardiomyopathy similar to mice lacking γ-sarcoglycan. However, unlike muscle lacking γ-sarcoglycan, δ-sarcoglycan-deficient muscle was sensitive to eccentric contraction-induced disruption of the plasma membrane. In the absence of δ-sarcoglycan, α-, β- and γ-sarcoglycan were undetectable, while dystrophin was expressed at normal levels. In contrast, without γ-sarcoglycan, reduced levels of α-, β- and δ-sarcoglycan were expressed, glycosylated and formed a complex with each other. Thus, the elimination of γ- and δ-sarcoglycan had different molecular consequences for the assembly and function of the dystrophin-glycoprotein complex. Furthermore, these molecular differences were associated with different mechanical consequences for the muscle plasma membrane. Through this in vivo analysis, a model for sarcoglycan assembly is proposed.

Key words: Sarcoglycan, Dystrophin, Muscle, Extracellular matrix

INTRODUCTION

Components of the dystrophin-glycoprotein complex (DGC) have been implicated in muscular dystrophy and cardiomyopathy (reviewed by Culligan et al., 1998; Ferlini et al., 1999; Hack et al., 2000; Ozawa et al., 1998). The absence of one or more DGC components causes progressive muscle degeneration and leads to skeletal and cardiac muscle weakness. Specifically, mutations in human α-, β-, γ- and δ-sarcoglycan cause limb-girdle muscular dystrophy while dystrophin mutations cause Duchenne and Becker muscular dystrophy (reviewed by Bonnemann et al., 1996; Campbell, 1995). The DGC is a large multi-protein complex expressed at the plasma membrane of cardiac and skeletal myocytes. Sarcoglycan is an independent subcomplex within the larger dystrophin-glycoprotein complex (Yoshida et al., 1994) and is composed of at least four sarcoglycan subunits, α, β, γ and δ (Jung et al., 1996; Yoshida et al., 1994). γ- and δ-sarcoglycan are highly related, sharing 67% amino acid similarity (Jung et al., 1996; Nigro et al., 1996; Noguchi et al., 1995). The role of ε-sarcoglycan in the striated muscle sarcoglycan complex is less clear (Ettinger et al., 1997; McNally et al., 1998). Sarcoglycan complexes exist in multiple tissues including striated muscle, smooth muscle and non-muscle tissues (Durbeej and Campbell, 1999; Straub et al., 1999). At the muscle plasma membrane, sarcoglycan associates with dystroglycan, a second subcomplex within the DGC, and dystrophin (reviewed by Hemler, 1999). Because dystrophin and dystroglycan interact with actin and laminin, respectively, this molecular linkage directly connects the cytoskeleton and the extracellular matrix (Ervasti and Campbell, 1993), where it is thought to provide support for the plasma membrane against the forces generated during muscle contraction.

Although the biochemical properties of sarcoglycan in normal striated muscle have been studied, less is known about the molecular consequences of sarcoglycan deficiency for the assembly and function of the dystrophin-glycoprotein complex. Studies of patient muscle biopsies have demonstrated that the absence of one sarcoglycan has important, but variable consequences for the stability of the other remaining sarcoglycan components at the plasma membrane (reviewed by Bushby, 1999). Likewise, mice with null mutations in specific members of the sarcoglycan complex have shown variation in the pattern of sarcoglycan complex disruption depending on the specific sarcoglycan mutated (Araishi et al., 1999; Coral-Vazquez et al., 1999; Duclos et al., 1998; Hack et al., 1998; Liu and Engvall, 1999). Based on results obtained in a heterologous, in vitro system it has been proposed that the

absence of any single sarcoglycan may be sufficient to completely prevent the assembly of the sarcoglycan complex (Holt and Campbell, 1998). This is in contrast to results obtained from patient studies and may reflect the heterologous cell type used for the in vitro experiments or the specific sarcoglycan mutants studied. Furthermore, the existence of non-tetrameric sarcoglycan complexes in tissues other than skeletal muscle (Durbeej and Campbell, 1999; Straub et al., 1999) suggests that sarcoglycan subcomplexes can be stable with fewer than four members.

To examine the cellular and molecular consequences of multiple mutations in the sarcoglycan complex, we have generated a targeted mutation in the murine δ-sarcoglycan gene. The absence of δ-sarcoglycan caused skeletal and cardiac muscle degeneration and resembled the dystrophic phenotype of mice lacking γ-saroclgycan (Hack et al., 1998) or dystrophin (Bulfield et al., 1984). Isolated muscle mechanics revealed that δ-sarcoglycan-deficient muscle differed from muscle lacking γ-sarcoglycan in its increased sensitivity to eccentric contraction-induced injury. In order to further investigate the molecular basis for this difference in vivo we studied the assembly of the sarcoglycan complex in muscle lacking either γ-sarcoglycan, δ-sarcoglycan or dystrophin. δ-sarcoglycan-deficiency resulted in the secondary elimination of α-, β-, γ- and ε-sarcoglycans in all parts of the secretory pathway. In contrast, comparative analysis of muscle lacking γ-sarcoglycan (*gsg*$^{-/-}$) or dystrophin (*mdx*) revealed diminished, but continued, expression of the remaining sarcoglycans. Moreover, these residual sarcoglycans were glycosylated and formed a multimeric complex in the absence of dystrophin or γ-sarcoglycan. These findings suggest that residual sarcoglycan in *gsg*$^{-/-}$ muscle may be competent to prevent the mechanical defects seen in *mdx* or δ-sarcoglycan-deficient muscle. Based on our findings, we propose a model for the assembly, trafficking and function of sarcoglycan within the dystrophin-glycoprotein complex.

MATERIALS AND METHODS

Mice lacking γ-sarcoglycan (*gsg*$^{-/-}$) or dystrophin (*mdx*)

We have previously reported the generation of mice with a null mutation of the γ-sarcoglycan gene (Hack et al., 1998). Animals were genotyped by PCR as described previously (Hack et al., 1998). Mice lacking dystrophin (*mdx*, The Jackson Laboratory, Bar Harbor, ME) were maintained as a colony of homozygous mutant animals. All animals used in this study were housed and treated in accordance with standards set by the University of Chicago Animal Care and Use Committee.

Generation of mice lacking δ-sarcoglycan (*dsg*$^{-/-}$)

Genomic phage containing exon 2 of δ-sarcoglycan were isolated from a murine 129SvJ Library (Stratagene, La Jolla, CA). A 7.5 kb fragment 5′ of exon two and a 2.0 kb fragment 3′ of exon two were generated with restriction sites by long range PCR (TaKaRa, Panvera, Madison, WI) and cloned into pPNT (Tybulewicz, 1991). Homologously recombined ES cell clones were selected as described previously (Hack et al., 1998). Recombinant ES cell clones were injected into C57Bl/6J blastocysts, and chimeric males were identified by coat color. Once germline contribution was confirmed by Southern blotting, chimeric males were mated to 129SvEms-+^{Ter}/J females (The Jackson Laboratory, Bar Harbor, ME) to generate heterozygous offspring on a mixed 129SvJ/129SvEms-+^{Ter}/J background. A colony of animals of carrying the δ-sarcoglycan null allele was maintained by heterozygote interbreeding.

Phenotypic analysis, histology and Evans blue staining

Animals were observed frequently and weighed weekly. Survival was assessed at the end of each week on a cohort of homozygous mutant (n=8) and normal mice (n=5) (129SvEms-+^{Ter}/J, The Jackson Laboratory, Bar Harbor, ME). Serum creatine kinase levels were determined as described previously (Hack et al., 1998). Tissues for histology were either placed in 10% neutral buffered formalin overnight and embedded in paraffin or rapidly frozen in liquid nitrogen-cooled isopentane for sectioning on a cryostat. Evans blue (Sigma-Aldrich, St Louis, MO) staining of muscle in vivo was performed as described previously (Hack et al., 1998).

Isolated muscle mechanics

Intact extensor digitorum longus (EDL) muscles were isolated from normal (n=4) and homozygous mutant (*dsg*$^{-/-}$) animals (n=7) at 13-15 weeks of age and mechanics were performed as described previously (Hack et al., 1999). All stimulations were performed with the muscle maintained at that length at which maximum twitch force was achieved (L_o). Isolated normal and *dsg*$^{-/-}$ EDL showed similar mean (± s.e.m.) L_o (10.9±0.2 and 12.0±0.1, respectively) and weight (9.4±0.3 and 9.0±0.1, respectively). Eccentric contractions (ECC) were performed in a regimen consisting of five fused tetanic contractions (700 milliseconds) during which the muscle was lengthened by 10% L_o over the final 200 milliseconds. A 4 minute recovery period was allowed between each ECC, during which the muscle was maintained at L_o. The low molecular mass dye, procion orange (0.2% w/v; Sigma-Aldrich, St Louis, MO) was included in the buffer to allow for detection of sarcolemmal disruption during the ECC protocol. After the ECC protocol, muscles were rapidly frozen in liquid nitrogen-cooled isopentane. 10 μm frozen sections were cut on a cryostat (Leica Microsystems, Germany) and mounted on slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Slides were examined under epifluorescence on a Zeiss Axiophot microscope (Carl Zeiss, Germany) and photographed.

Antibodies

The mouse monoclonal antibodies to α-sarcoglycan (NCL-a-sarc), β-sarcoglycan (NCL-b-sarc) and γ-sarcoglycan (NCL-g-sarc) were purchased from Novocastra (Newcastle upon Tyne, UK). Rabbit polyclonal antibodies to dystrophin (AB6-10) and ε-sarcoglycan have been described previously (Lidov et al., 1990; McNally et al., 1998). A rabbit polyclonal antibody to murine δ-sarcoglycan was generated to the peptide PRLPRGSYTPTRQK conjugated to Keyhole limpet hemacyanin (Zymed Laboratories, South San Francisco, CA).

Immunocytochemistry

Skeletal muscle from normal, *mdx*, *gsg*$^{-/-}$ and *dsg*$^{-/-}$ animals was dissected and rapidly frozen in liquid nitrogen-cooled isopentane. Cryosections were cut (7 μm thick) and blocked in phosphate-buffered saline (PBS) with 5% fetal bovine serum (FBS) at room temperature. Primary antibodies (see above) were used in PBS with FBS and incubated with sections overnight at 4°C. Cy3-congugated secondary antibodies were then used in PBS with FBS at room temperature (Jackson Immunoresearch Laboratories, West Grove, PA). Immmunostained sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA), viewed and photographed on a Zeiss Axiophot microscope (Carl Zeiss, Germany).

Microsome preparation and immunoblotting

Heavy microsomes were purified from skeletal muscle (animals ranging in age from 6 weeks to one year old) according to previously described protocols (Ohlendieck et al., 1991) with modifications

(Duclos et al., 1998). Microsomes were prepared from a minimum of three animals, using six distinct muscle groups from each animal. In each case, the entire muscle, including the myotendinous junction, was taken. Microsomal protein content was determined for each sample using the Bio-Rad (Hercules, CA) protein assay with bovine serum albumin as a standard. Protein was subjected to denaturing and reducing conditions, resolved by SDS-PAGE using either 4-12% or 4-20% linear gradient gels (Novex, San Diego, CA), and transferred to Immobilon P membranes (Millipore, Bedford, MA). Equal loading was confirmed by Coommassie blue staining of equivalently loaded lanes. Immunoblotting was performed as described previously (Hack et al., 1998). Detection was performed with ECL-Plus (Amersham Pharmacia Biotech, Piscataway, NJ) and visualized on autoradiographic film (Eastman Kodak, Rochester, NY) for data presentation or using a Molecular Dynamics Storm 860 (Amersham Pharmacia Biotech, Piscataway, NJ) and quantified using ImageQuant software (Amersham Pharmacia Biotech-Molecular Dynamics).

Northern blot analysis

Total RNA was prepared from skeletal muscle using Trizol (Life Technologies, Grand Island, NY). 10 μg of total RNA was resolved on a 1% agarose gel, transferred to Hybond N+ (Amersham-Pharmacia, Piscataway, NJ), and crosslinked to the membrane using a Stratalinker (Stratagene, La Jolla, CA). Membranes were prehybridized and hybridized in ExpressHyb (Clontech, Palo Alto, CA) using radioactively labeled probes. The following probes to murine sarcoglycan sequences were used: α-sarcoglycan cDNA coding region (nucleotides 254 to 1417, GenBank accession number AB024920); β-sarcoglycan partial cDNA coding region (nucleotides 325 through 834, GenBank accession number AB024921); γ-sarcoglycan cDNA from exon 2 through 3′ untranslated region (nucleotides 348 through 1421, GenBank accession number AB024922 and A.A.H. and E.M.M., unpublished data); δ-sarcolgycan cDNA coding region (nucleotides 271 through 1140, GenBank accession number AB024923).

Immunoprecipitation and glycosidase treatment

One hundred and sixty μg of skeletal muscle microsomal protein was incubated with 25 μl of an anti-β-sarcoglycan monoclonal antibody (NCL-b-sarc) at 4°C overnight. Immunoprecipitation buffer conditions were as described previously (Yoshida et al., 1998). For carbohydrate chain analysis, 40 μg of skeletal muscle microsomal protein was denatured and incubated with 5 μl of endoglycosidase H (Endo H) according to the manufacturer's specifications (New England Biolabs, Beverly, MA) in order to remove any immature N-linked carbohydrate chains. For Peptide:N-glycosidase F (PNGase F) treatment, 40 μg of normal microsomal protein was denatured and incubated with 5 μl of PNGase F (New England Biolabs, Beverly, MA) to remove all N-linked glycosylation from each sarcoglycan. Samples were reduced, denatured and resolved by SDS-PAGE as described above. Each experiment was repeated three independent times.

RESULTS

Targeted disruption of δ-sarcoglycan

Homologous recombination between the genomic locus and the targeting vector replaced exon 2 of δ-sarcoglycan with a neomycin resistance cDNA (Fig. 1A). Exon 2 encodes the initiator methionine, the entire cytoplasmic domain and the transmembrane domain of δ-sarcoglycan. A similar approach produced a null allele of the highly-homologous γ-sarcoglycan (Hack et al., 1998). After germ-line transmission of the mutant allele, homozygous mutant (*dsg*$^{-/-}$) animals were generated by interbreeding heterozygotes (Fig. 1B). All genotypes were

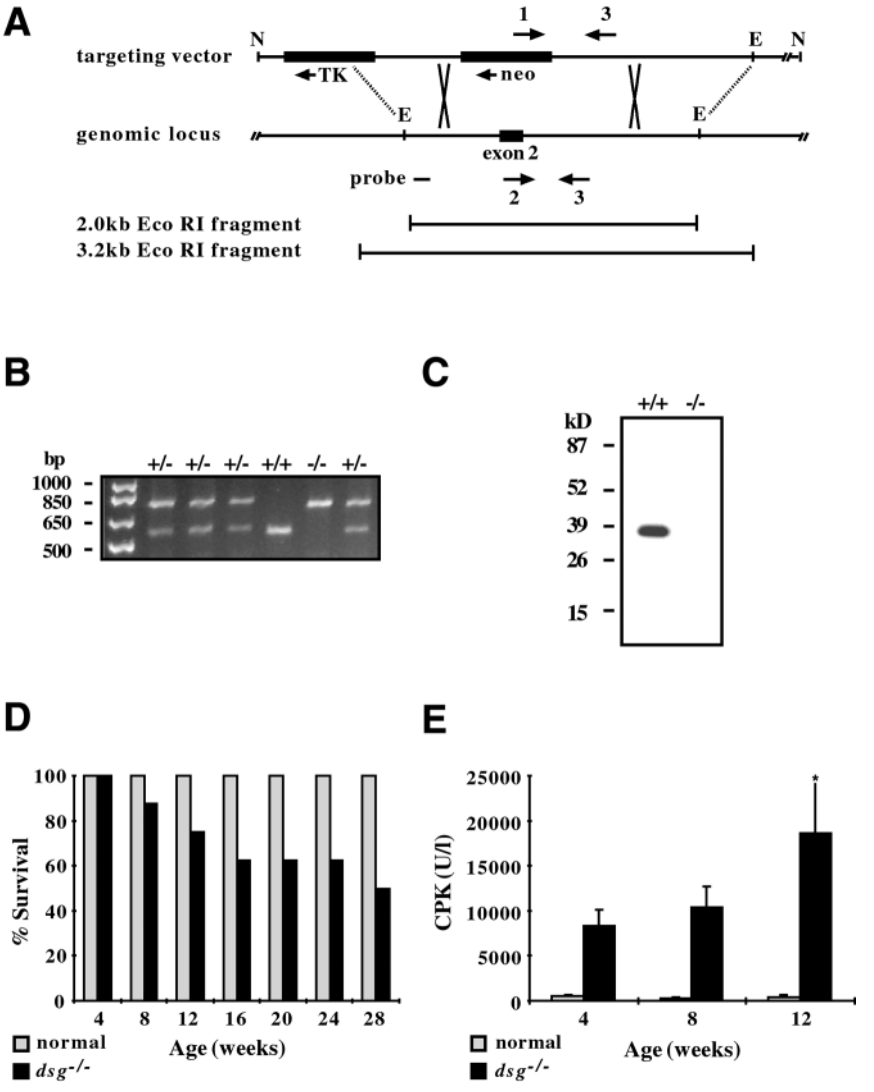
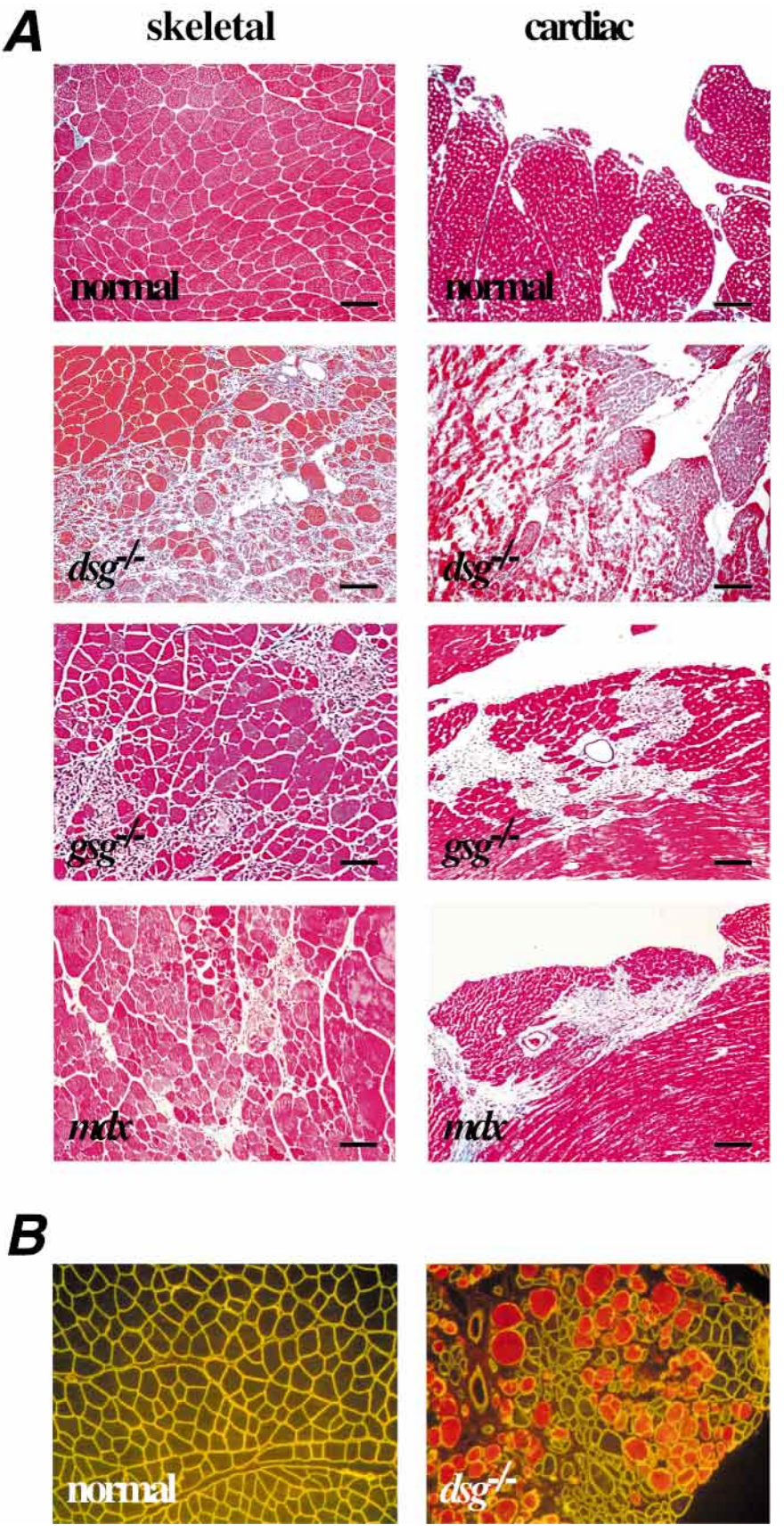


Fig. 1. Targeted mutation in δ-sarcoglycan. (A) The targeting strategy used to eliminate exon 2 of δ-sarcoglycan. (B) PCR determination of germline transmission of the mutated allele is shown. The location of primers used to genotype animals is indicated by the arrows. (C) Immunoblotting for δ-sarcoglycan in normal and homozygous mutant (*dsg*$^{-/-}$) microsomes revealed that no δ-sarcoglycan protein was synthesized and that a null allele had been generated. (D) Monthly survival of normal and *dsg*$^{-/-}$ mice over a 28-week period, and (E) mean (± s.e.m.) serum levels of creatine kinase over a 12-week period are shown. (*) The s.e.m. for the 12-week CPK levels in *dsg*$^{-/-}$ was 10,711 U/l. Abbreviations used: neo, neomycin resistance cDNA; TK, thymidine kinase cDNA; E, *Eco*RI restriction site; N, *Not*I restriction site; bp, base pairs; kb, kilobase pairs; CPK, serum creatine phosphokinase.



recovered in the expected Mendelian ratios. Immunoblotting with anti-δ-sarcoglycan antibodies directed at retained residues encoded by exon 8 revealed that no δ-sarcoglycan protein was produced in homozygous mutant skeletal muscle microsomes (Fig. 1C). Beginning at 8 weeks, *dsg*$^{-/-}$ mice began to die suddenly, with no apparent cause. In the cohort studied, premature mortality was also noted in *dsg*$^{-/-}$ mice at 12, 16 and 28 weeks of age (Fig. 1D) with a 50% survival rate at 28 weeks of age compared to a 100% survival rate for normal controls. Examination of serum creatine kinase levels at 4, 8 and 12 weeks showed a dramatic elevation in *dsg*$^{-/-}$ mice consistent with a degenerative condition of striated muscle (Fig. 1E). Mortality and serum CK levels were similar between *dsg*$^{-/-}$ and *gsg*$^{-/-}$ mice (Hack et al., 1998).

Muscle degeneration and membrane permeability changes in mice lacking δ-sarcoglycan

All *dsg*$^{-/-}$ skeletal muscles examined showed dystrophic changes consistent with an intrinsic muscle degenerative disorder (Fig. 2A). Regional degeneration and regeneration of muscle fibers were common and accompanied by an inflammatory infiltrate. Calcification was also frequently noted. Skeletal muscle histopathology was similar to that seen in humans with limb girdle muscular dystrophy (Ben Hamida et al., 1983). At 4 and 8 weeks of age, hearts lacking δ-sarcoglycan showed no histologic signs of cardiac muscle degeneration (data not shown). Beginning at 12 weeks, areas of cell death and inflammatory infiltrate were seen (Fig. 2A), consistent with the sudden

Fig. 2. Striated muscle degeneration and membrane permeability changes in the absence of sarcoglycans or dystrophin. (A) Masson trichrome stained histologic sections of normal, *dsg*$^{-/-}$, *gsg*$^{-/-}$ and *mdx* 8 to 12-week-old skeletal (quadriceps) and cardiac muscle (ventricle) are shown. Note frank degeneration in both types of striated muscle. All three mutant genotypes showed similar histologic features including cell death, regeneration, inflammatory infiltrate and fibrosis, but such findings were more extensive in *dsg*$^{-/-}$ and *gsg*$^{-/-}$ than in *mdx*. Skeletal muscle histopathology is accompanied by regeneration. Bar, 100 μm. (B) Evans blue injection of normal and *dsg*$^{-/-}$ muscles revealed altered membrane permeability (red cytoplasmic staining) as a result of the loss of δ-sarcoglycan. The sarcolemma was demonstrated by immunostaining with an anti-dystrophin antibody (yellow).

mortality observed in this age group (Fig. 1D). The histologic appearance of skeletal and cardiac muscle from *dsg*$^{-/-}$ mice was identical to that seen in *gsg*$^{-/-}$ mice, including the frequent occurrence of perivascular fibrosis (Hack et al., 1998). Skeletal muscle histopathology in *dsg*$^{-/-}$ was more severe than in age matched *mdx*. Cardiac histopathology was also more severe in *dsg*$^{-/-}$ and *gsg*$^{-/-}$ compared to *mdx* (Fig. 2A).

Alterations in membrane permeability were studied using Evans blue dye (EBD). Normal muscle is impermeable to EBD (Fig. 2B, left panel), while some forms of muscular dystrophy cause changes that permit EBD to pass into the myofiber cytoplasm (Matsuda et al., 1995; Straub et al., 1997). The specific membrane defect represented by EBD uptake is not known, although EBD uptake is a feature of some, but not all, mutations in the DGC (Araishi et al., 1999; Coral-Vazquez et al., 1999; Duclos et al., 1998; Hack et al., 1998; Matsuda et al., 1995; Straub et al., 1997). Mice lacking δ-sarcoglycan showed pronounced EBD uptake in all muscles examined (Fig. 2B, right panel). EBD uptake was confined to the myofiber cytoplasm and tended to be clustered. No specific regional distribution pattern was consistently observed within any of the muscle groups examined.

Sarcoglycan mutations have different functional consequences

We previously examined the mechanical properties of muscle lacking dystrophin or γ-sarcoglycan using an eccentric contraction protocol (ECC) to test the mechanical integrity of the sarcolemma. Muscle lacking γ-sarcoglycan showed a normal pattern of mechanical resistance to contraction-induced injury (Hack et al., 1999). In contrast, dystrophin-deficient muscle exhibited a significant decrement in force-generation over five eccentric contractions (Petrof et al., 1993). Like *gsg*$^{-/-}$ muscle, *dsg*$^{-/-}$ muscle showed normal twitch and tetanic force generation (Fig. 3A) indicating that the sarcomere and contractile apparatus were intact in the absence of the sarcoglycan complex. However, unlike *gsg*$^{-/-}$ muscle, *dsg*$^{-/-}$ muscle showed a substantial drop (42±9%) in force generation over the five eccentric contraction protocol (Fig. 3B). This percentage drop in force-generation was near that seen for *mdx* muscle (51±5%) that lacks dystrophin, and is indicative of mechanically-induced cell damage (Hack et al., 1999; Petrof et al., 1993). To demonstrate mechanical disruption of the sarcolemma as a result of eccentric contraction, procion orange dye was added to the bath during the ECC protocol. Substantial procion orange uptake was observed in sections of *dsg*$^{-/-}$ EDL muscles and was absent in normal controls (Fig. 3C). Therefore, the drop in force production that occurs with ECC in *dsg*$^{-/-}$ was likely due to heightened sensitivity to sarcolemmal disruption in the absence of the sarcoglycan complex.

Sarcoglycan mutations have different molecular consequences

Since the mechanical properties of γ-sarcoglycan and δ-sarcoglycan mutations differed, we explored the molecular consequences of these mutations. We purified microsomes

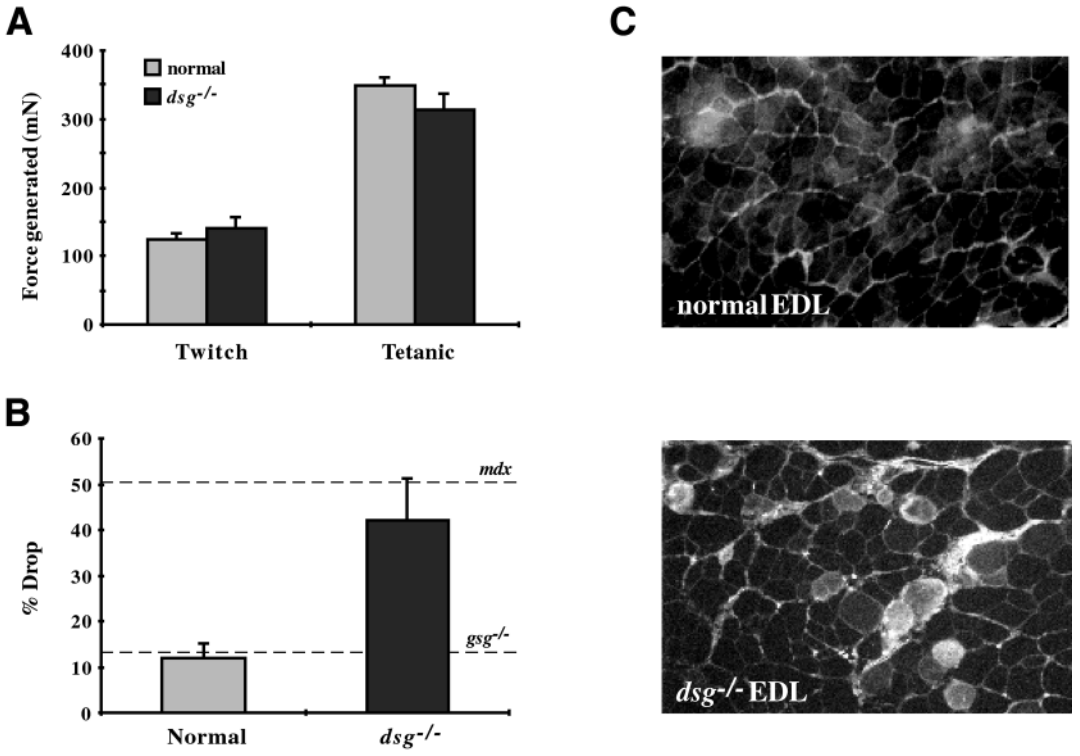


Fig. 3. Mechanical consequences of a complete absence of sarcoglycan. (A) Twitch and tetanic force measurements for normal and *dsg*$^{-/-}$ extensor digitorum longus (EDL) muscles from 13- to 15-week-old mice. The contractile apparatus functioned normally and force generation was not impaired in the absence of the sarcoglycan complex. (B) Force drop as a result of an eccentric contraction (ECC) regimen in normal and *dsg*$^{-/-}$ EDL muscles. The absence of the entire sarcoglycan complex compromised the ability of *dsg*$^{-/-}$ muscles to resist damage caused by the five ECC protocol, likely as a result of a disrupted dystrophin-dystroglycan-laminin axis. Previously published values for percentage drop in γ-sarcoglycan-deficient (*gsg*$^{-/-}$) and dystrophin-deficient (*mdx*) EDL are shown (Hack et al., 1999). (C) 10 μm sections of EDL subjected to the five ECC protocol were examined for procion orange uptake under epifluorescent microscopy. Normal control EDL (top panel) showed little dye uptake. EDL from *dsg*$^{-/-}$ mice showed substantial dye uptake (bottom panel), indicative of disruption of the plasma membrane during the ECC protocol. Abbreviations used: twitch, twitch force generation; tetanic, tetanic force generation;% drop, percentage drop in force generation after five ECCs; EDL, extensor digitorum longus.

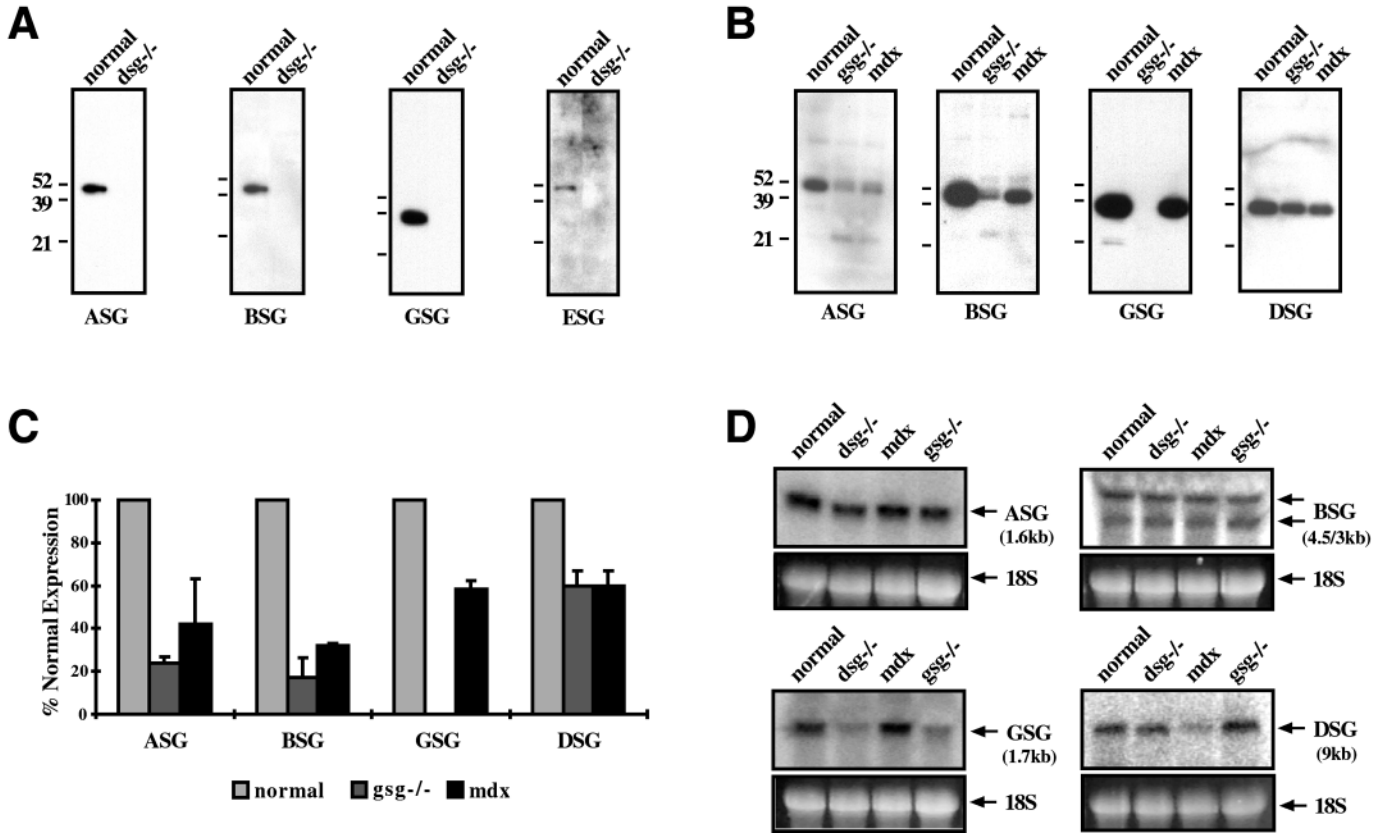


Fig. 4. Dystrophin-glycoprotein complex mutations have different molecular consequences that occur post-translation. (A) Immunoblotting for sarcoglycan complex members in normal and *dsg*$^{-/-}$ microsomes. δ-sarcoglycan was absolutely required for the stability of all four sarcoglycans. The same membrane was analyzed with each antibody. (B) Immunoblotting for sarcoglycan complex members in normal, *gsg*$^{-/-}$ and *mdx* microsomes. In contrast to the findings for *dsg*$^{-/-}$ muscle, residual sarcoglycans are present in the absence of γ-sarcoglycan and in the absence of dystrophin, suggesting that these components are not required for the initial assembly of the sarcoglycan complex. (A and B) Molecular mass standards are shown to the left of each panel. (C) Quantitation of residual sarcoglycan complex members in *gsg*$^{-/-}$ and *mdx* microsomes revealed differences in their stability as a result of each genetic mutation. (D) Northern blot analysis demonstrated that all four (α, β, γ, δ) sarcoglycan genes are transcribed in the absence of γ-sarcoglycan (*gsg*$^{-/-}$), δ-sarcoglycan (*dsg*$^{-/-}$) or dystrophin (*mdx*). The size of each RNA species is noted on each panel. Equal loading was assessed by comparing the amount of ribosomal RNA (18S) in each lane. Abbreviations used: ASG, α-sarcoglycan; BSG, β-sarcoglycan; GSG, γ-sarcoglycan; DSG, δ-sarcoglycan.

from skeletal muscle with a primary absence of δ-sarcoglycan (*dsg*$^{-/-}$), γ-sarcoglycan (*gsg*$^{-/-}$) or dystrophin (*mdx*). Each microsome preparation represents six different muscles from three individual animals analyzed in aggregate; each experiment was repeated a minimum of three independent times. In *dsg*$^{-/-}$ muscle, no α-, β- or γ-sarcoglycan protein was detectable in microsome preparations under any conditions (Fig. 4A). In contrast, *gsg*$^{-/-}$ microsomes showed persistent expression of reduced levels of α-, β- and δ-sarcoglycans (Fig. 4B), at 24%, 17% and 60% of their normal levels, respectively (Fig. 4C). ε-sarcoglycan was also absent in *dsg*$^{-/-}$ muscle microsomes (Fig. 4A). We previously showed that ε-sarcoglycan was not altered by the loss of γ-sarcoglycan (Hack et al., 1998). Similarly, in *mdx* muscle, the greatest retention was seen for δ-sarcoglycan (60%) and γ-sarcoglycan (59%) (Fig. 4C). Northern blot analysis demonstrated that α-, β-, γ- and δ-sarcoglycan mRNAs were synthesized in normal, *dsg*$^{-/-}$, *gsg*$^{-/-}$ and *mdx* muscle, consistent with post-transcriptional regulation of sarcoglycan stability (Fig. 4D). Both δ-sarcoglycan mRNA and γ-sarcoglycan mRNA migrated slightly faster in *dsg*$^{-/-}$ and *gsg*$^{-/-}$, respectively. This is consistent with the loss of 195bp (γ-sarcoglycan) or 201bp (δ-sarcoglycan) from each mRNA as a result of the targeted elimination of exon 2 of each gene. Equivalent loading of RNA was demonstrated by visualization of the 18S (shown) and 28S ribosomal RNAs.

To assess the localization of DGC components in mutant muscles, we performed immunostaining with polyclonal antiserum specific for dystrophin or a single sarcoglycan. Although sarcoglycan proteins are synthesized in muscle lacking either γ-sarcoglycan or dystrophin, they are greatly reduced or absent at the plasma membrane. Dystrophin expression at the sarcolemma was normal in *gsg*$^{-/-}$ and *dsg*$^{-/-}$ but absent in *mdx* (Fig. 5). Thus, while γ- and δ-sarcoglycan mutations had different consequences for the sarcoglycan complex, neither mutation appeared to affect the expression and localization of other components of the DGC such as dystrophin. Residual sarcoglycan expression in *gsg*$^{-/-}$ muscle, coupled with continued expression of dystrophin, appears sufficient to protect against the mechanical defect seen in the absence of dystrophin (*mdx*) or the entire sarcoglycan complex (*dsg*$^{-/-}$).

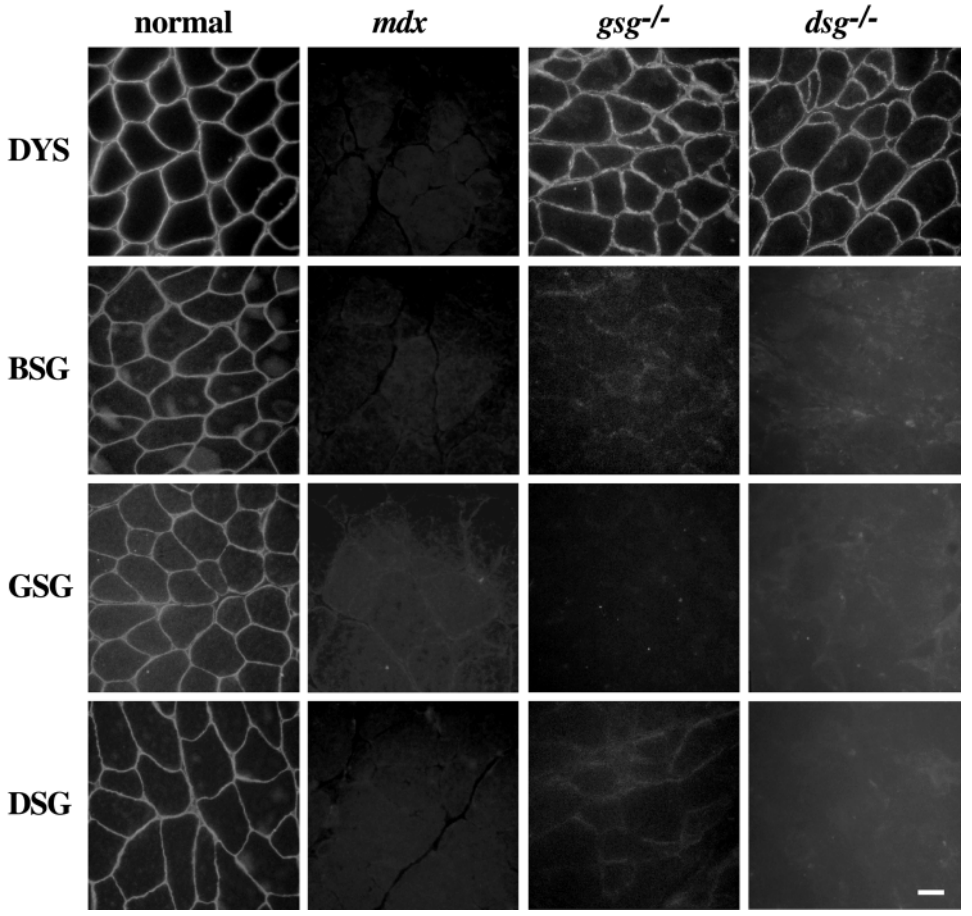


Fig. 5. Immmunofluoresence analysis of dystrophin-glycoprotein complex members in *mdx*, *gsg*$^{-/-}$ and *dsg*$^{-/-}$ skeletal muscle. Staining of 7 μm cryosections was performed using polyclonal antibodies to dystrophin, β-sarcoglycan, γ-sarcoglycan and δ-sarcoglycan. A small amount of residual sarcolemmal staining for β-sarcoglycan and δ-sarcoglycan was seen in *gsg*$^{-/-}$. Dystrophin expression at the sarcolemma was normal in *gsg*$^{-/-}$ and *dsg*$^{-/-}$, but absent in *mdx*. Bar, 25 μm. Abbreviations used: DYS, anti-dystrophin antibody; BSG, anti-β-sarcoglycan antibody; GSG, anti-γ-sarcoglycan antibody; DSG, anti-δ-sarcoglycan antibody.

Sarcoglycan forms a multi-protein complex and is glycosylated without γ-sarcoglycan or without dystrophin

To investigate the assembly and maturation of the DGC in the absence of γ-sarcoglycan or dystrophin, co-immunoprecipitation experiments were performed using skeletal muscle microsomes. Immunoprecipitation with anti-β-sarcoglycan antibodies revealed that sarcoglycan multimers of α-, β- and δ-sarcoglycan were present in the absence of γ-sarcoglycan (Fig. 6A). Likewise, sarcoglycan tetramers of α-, β-, γ- and δ-sarcoglycan were present in *mdx* microsomes immunoprecipitated with anti-β-sarcoglycan antibodies. Therefore, complexes containing the expressed sarcoglycans can mature and are stable in the absence of γ-sarcoglycan or dystrophin. However, based on immunofluorescence studies in intact *gsg*$^{-/-}$ or *mdx* muscle they were either unable to efficiently complete a terminal step in their maturation to the plasma membrane or are reasonably unstable once at the surface, as assessed by immunofluorescence (Fig. 5; Hack et al., 1998; Ohlendieck and Campbell, 1991).

The status of N-linked carbohydrates was used to assess glycosylation of the sarcoglycans in the secretory pathway. In general, resident N-linked carbohydrate chains are remodeled and become resistant to endoglycosidase H (endo H) as glycoproteins mature through the Golgi apparatus. As expected for studies of the secretory pathway, endo H sensitive (immature, lower band) sarcoglycan was abundant in each sample, including normal muscle (Fig. 6B). This immature protein indicates the presence of the entire secretory pathway in the crude microsomal fraction, including the endoplasmic reticulum, Golgi apparatus and plasma membrane. However, endo H digestion of microsomal proteins also revealed endo H resistant (mature) α-sarcoglycan in normal, *gsg*$^{-/-}$ and *mdx* muscle (Fig. 6, upper band). β-sarcoglycan may partially depend on γ-sarcoglycan for fully efficient maturation, but nonetheless was glycosylated in *gsg*$^{-/-}$ and *mdx*. Interestingly, in all samples, γ-sarcoglycan existed exclusively in an endo H-sensitive state, suggesting that its immature N-linked sugars are protected from modification in the Golgi apparatus, possibly as a result of interactions with the other components of the sarcoglycan complex (data not shown). δ-Sarcoglycan showed only a small amount of endoH resistant material in each sample, and like its homologue γ-sarcoglycan, it may not depend on alteration of its carbohydrate chains for maturation (data not shown). Thus, residual sarcoglycans are glycosylated and assemble in to a complex in the absence of either γ-sarcoglycan or dystrophin.

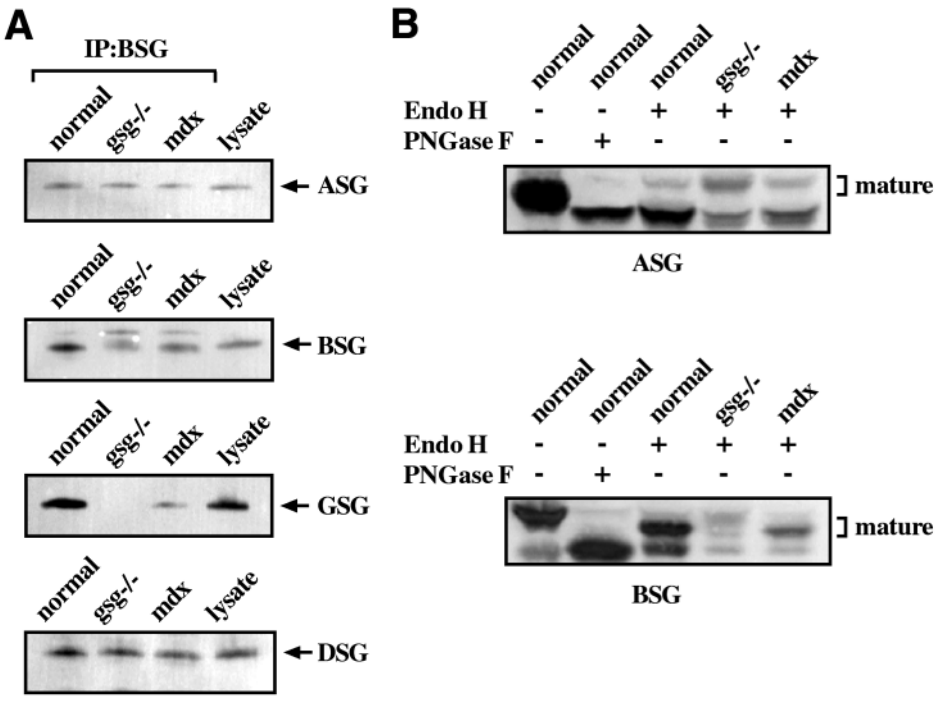


Fig. 6. Genetic analysis of sarcoglycan complex assembly and glycosylation. (A) Co-immunoprecipitation of sarcoglycan complex members with an anti-β-sarcoglycan antibody in normal, *gsg*$^{-/-}$ and *mdx* microsomes. Microsomes that were not subjected to immunoprecipitation were loaded as a control (lysate). In the absence of γ-sarcoglycan or dystrophin, sarcoglycan multi-protein complexes formed properly. (B) Glycosidase analysis of sarcoglycan complex maturation in normal, *gsg*$^{-/-}$ and *mdx* microsome preparations. Both mature and immature forms of each sarcoglycan were detected. The mature (slower migrating) form is highlighted with a bracket. The antibody used to detect individual sarcoglycans is specified below each panel. Abbreviations used: Endo H, endoglycosidase H; PNGase F, Peptide: N-glycosidase F; ASG, anti-α-sarcoglycan antibody; BSG, anti-β-sarcoglycan antibody; DSG, anti-δ-sarcoglycan antibody; ESG, ε-sarcoglycan; IP, immunoprecipitation.

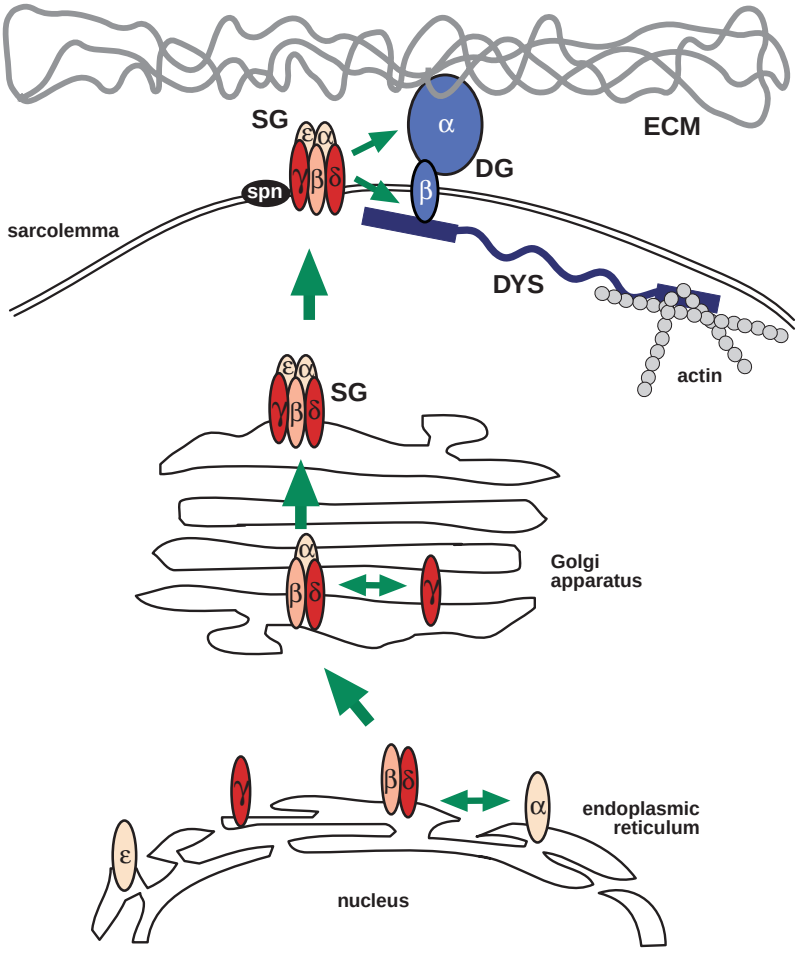


Fig. 7. Model for the assembly, processing and membrane stability of sarcoglycan. The sarcoglycan complex assembles in a stepwise fashion. δ-sarcoglycan is critical for the initial stability of the sarcoglycan complex in the endoplasmic reticulum. γ-Sarcoglycan is required for efficient complex assembly and maturation. Dystrophin is required for a final step of sarcoglycan assembly at the plasma membrane (sarcolemma). Furthermore, the interaction between the sarcoglycan complex and dystrophin or dystroglycan is important for the mechanical function of this linkage. Abbreviations used: SG, sarcoglycan complex; DG, dystroglycan complex; DYS, dystrophin; spn, sarcospan; ECM, extracellular matrix.

DISCUSSION

The muscular dystrophy and cardiomyopathy seen in mice lacking δ-sarcoglycan is identical to that seen in mice lacking γ-sarcoglycan (Hack et al., 1998) and demonstrates non-redundant functions for the highly homologous proteins γ- and δ-sarcoglycan. The phenotypic severity of mice lacking either γ-sarcoglycan or δ-sarcoglycan was greater than that seen in mice lacking dystrophin (*mdx*). Despite phenotypic similarity between $gsg^{-/-}$ and $dsg^{-/-}$ mice, the mechanical consequences of these sarcoglycan mutations differed substantially for individual muscles. Like dystrophin-deficient muscle, $dsg^{-/-}$ muscle showed a significant decrement in force-generation and increase in procion orange uptake as a result of eccentric contraction, indicative of myocyte damage and loss. In contrast, $gsg^{-/-}$ muscle, examined similarly, showed no evidence of the eccentric contraction-induced force drop that characterized $dsg^{-/-}$ and *mdx* muscles. Furthermore, eccentric contraction produced increased procion orange uptake in *mdx* muscle (Petrof et al., 1993) and $dsg^{-/-}$ muscle, while procion orange uptake was minimal in $gsg^{-/-}$ muscle (Hack et al., 1999). Both $gsg^{-/-}$ and $dsg^{-/-}$ muscles showed significant in vivo EBD uptake, suggesting that EBD uptake and procion orange uptake with ECC may represent different alterations in the plasma membrane. Procion orange uptake after ECC may be non-specifically indicative of contraction-induced damage. EBD uptake in vivo may reflect membrane permeability changes specific to DGC disruption that do not require contraction-induced damage. In summary, the lack of contraction-induced damage with γ-sarcoglycan deficiency suggests that the loss of this protein may be targeting 'non-mechanical', possibly signalling, functions of the DGC.

At a molecular level, the loss of δ-sarcoglycan leads to the complete absence of α-, β-, γ- and ε-sarcoglycan, despite normal levels of transcription. In contrast, a mutation in γ-sarcoglycan reduced but did not eliminate expression of α-, β-, δ- and ε-sarcoglycan. The residual sarcoglycan subunits in $gsg^{-/-}$ and *mdx* muscle were glycosylated and assembled with each other. While the residual sarcoglycan found in $gsg^{-/-}$ muscle is inadequate to prevent the phenotype of muscular dystrophy (Hack et al., 1998), it is sufficient to protect against eccentric contraction-induced injury, further underscoring the multifunctional nature of the DGC (Hack et al., 1999).

Another molecular difference that may account for the mechanical differences between $dsg^{-/-}$ and $gsg^{-/-}$ muscle is ε-sarcoglycan. ε-sarcoglycan is undetectable in $dsg^{-/-}$ muscle while it is retained at normal levels in $gsg^{-/-}$ muscle. This observation suggests that ε-sarcoglycan is an integral component of a striated muscle sarcoglycan complex that is stable in the absence of γ-sarcoglycan but not in the absence of δ-sarcoglycan (Fig. 7). Studies in other tissues have suggested that ε is a part of an ε/β/δ-sarcoglycan multimer (Durbeej and Campbell, 1999; Liu and Engvall, 1999; Straub et al., 1999). Based on the data presented here, such an ε/β/δ-sarcoglycan complex may exist in striated muscle and be disrupted by the absence of δ-sarcoglycan. This hypothesis is in agreement with the finding that ε-sarcoglycan is retained at normal levels in α- or γ-sarcoglycan-deficient muscle (Duclos et al., 1998; Hack et al., 1998; Liu and Engvall, 1999). Recent in vitro and human mutation studies have suggested that δ- and β-sarcoglycans may form a core of the sarcoglycan complex (Chan et al., 1998; Higuchi et al., 1998; Noguchi et al., 1999; Vainzof et al., 1999). Based on the in vivo data presented here, we can conclude that δ-sarcoglycan is required for sarcoglycan complex assembly while γ-sarcoglycan is not. Moreover, this model is corroborated by the finding that β-sarcoglycan is also required for expression of the other sarcoglycan proteins (Araishi et al., 1999).

We propose that the sarcoglycan complex assembles early in the secretory pathway around a β/δ-sarcoglycan core (Fig. 7). The presence of γ-sarcoglycan is required later for efficient assembly and processing. γ-sarcoglycan is also required to prevent muscle degeneration. An interaction with dystrophin late in the secretory pathway or at the sarcolemma is required for the sarcoglycan complex to insert in, or be stable at the cell surface where it is multifunctional. Although the full range of sarcoglycan function is not known, it clearly includes both mechanical and non-mechanical roles. Specifically, the absence of contraction-induced damage in $gsg^{-/-}$ muscle suggests a non-mechanical role for γ-sarcoglycan in myocyte survival (Hack et al., 1999). This function may be related to its proposed interaction with integrin or to the potential function of α-sarcoglycan as an ecto ATPase (Betto et al., 1999; Yoshida et al., 1998). In contrast, sarcoglycan is also important for the mechanical function of the DGC, where it may strengthen interactions within the dystrophin-dystroglycan-laminin axis. The multifunctional nature of the sarcoglycan complex, and the entire DGC, highlights the importance of understanding the full role of each component in both muscle function and disease.

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