

Progressive Muscular Dystrophy in α -Sarcoglycan-deficient Mice

Franck Duclos,* Volker Straub,* Steven A. Moore,‡ David P. Venzke,* Ron F. Hrstka,§
Rachelle H. Crosbie,* Madeleine Durbeej,* Connie S. Lebakken,* Audrey J. Ettinger,||
Jack van der Meulen,** Kathleen H. Holt,* Leland E. Lim,* Joshua R. Sanes,|| Beverly L. Davidson,¶
John A. Faulkner,** Roger Williamson,§ and Kevin P. Campbell*

*Howard Hughes Medical Institute, Department of Physiology and Biophysics and Department of Neurology, University of Iowa College of Medicine, Iowa City, Iowa 52242-1101; ‡Department of Pathology and §Department of Obstetrics and Gynecology, University of Iowa College of Medicine, Iowa City, Iowa 52242; ||Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110; ¶Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, Iowa 52242; and **Institute of Gerontology, University of Michigan, Ann Arbor, Michigan 48109

Abstract. Limb-girdle muscular dystrophy type 2D (LGMD 2D) is an autosomal recessive disorder caused by mutations in the α -sarcoglycan gene. To determine how α -sarcoglycan deficiency leads to muscle fiber degeneration, we generated and analyzed α -sarcoglycan-deficient mice. *Sgca*-null mice developed progressive muscular dystrophy and, in contrast to other animal models for muscular dystrophy, showed ongoing muscle necrosis with age, a hallmark of the human disease. *Sgca*-null mice also revealed loss of sarcolemmal integrity, elevated serum levels of muscle enzymes, increased muscle masses, and changes in the generation of absolute force. Molecular analysis of *Sgca*-null mice demonstrated that the absence of α -sarcoglycan resulted in the complete loss of the sarcoglycan complex, sarcospan, and a disruption of α -dystroglycan associa-

tion with membranes. In contrast, no change in the expression of ϵ -sarcoglycan (α -sarcoglycan homologue) was observed. Recombinant α -sarcoglycan adenovirus injection into *Sgca*-deficient muscles restored the sarcoglycan complex and sarcospan to the membrane. We propose that the sarcoglycan-sarcospan complex is requisite for stable association of α -dystroglycan with the sarcolemma. The *Sgca*-deficient mice will be a valuable model for elucidating the pathogenesis of sarcoglycan deficient limb-girdle muscular dystrophies and for the development of therapeutic strategies for this disease.

Key words: gene targeting • muscular dystrophy • sarcoglycan • dystroglycan • sarcospan

IN skeletal and cardiac muscle, dystrophin is associated with a large complex of sarcolemmal and cytoskeletal proteins (for reviews see Henry and Campbell, 1996; Straub and Campbell, 1997; Ozawa et al., 1998). The dystrophin-glycoprotein complex (DGC)¹ includes α - and

β -dystroglycan, the syntrophins, and the sarcoglycans. The sarcoglycans are a group of single pass transmembrane glycoproteins, which form a complex within the DGC, consisting of α -, β -, γ -, and δ -sarcoglycan. Recently the 25-kD component of the DGC was characterized and named sarcospan (Crosbie et al., 1997). The DGC confers a structural link between laminin 2 in the extracellular matrix and the F-actin cytoskeleton (Ervasti and Campbell, 1993), and is thought to protect muscle cells from contraction-induced damage (Weller et al., 1990; Petrof et al., 1993).

Primary mutations in the genes encoding several components of the DGC have been associated with muscular dystrophy (Straub and Campbell, 1997; Ozawa et al., 1998). In autosomal recessive limb-girdle muscular dystrophy (LGMD), mutations in any of the sarcoglycan genes (Roberds et al., 1994; Bönnemann et al., 1995; Lim et al., 1995; Noguchi et al., 1995; Nigro et al., 1996) lead to the

Address all correspondence to Kevin P. Campbell, Howard Hughes Medical Institute, University of Iowa College of Medicine, 400 EMRB, Iowa City, IA 52242. Tel.: (319) 335-7867. Fax: (319) 335-6957. E-mail: kevin-campbell@uiowa.edu WWW site: <http://www-camlab.physiology.uiowa.edu/>

1. *Abbreviations used in this paper:* CK, creatine kinase; DGC, dystrophin-glycoprotein complex; EBD, Evans blue dye; EDL, extensor digitorum longus; H & E, haematoxylin and eosin; LGMD, limb-girdle muscular dystrophy; P_o , maximum isometric tetanic force; PK, pyruvate kinase; SG, sarcoglycan.

concomitant loss or reduction of all four sarcoglycans (α , β , γ , and δ) from the sarcolemma. Thus far, the BIO 14.6 hamster has served as an animal model for sarcoglycan-deficient muscular dystrophy (Roberds et al., 1993b). The hamster has a genomic deletion in the δ -sarcoglycan gene (Nigro et al., 1997; Sakamoto et al., 1997) that results in reduced sarcoglycan levels in striated muscles (Nigro et al., 1997; Sakamoto et al., 1997). However, mutations in the human δ -sarcoglycan gene (LGMD 2F) seem to be very rare compared with the prevalence of α -sarcoglycan gene mutations (LGMD 2D) (Duggan et al., 1997). Furthermore, the BIO14.6 hamster reveals a comparatively mild skeletal muscle pathology and develops a hypertrophic cardiomyopathy (Homburger, 1979) generally not seen in patients with primary sarcoglycan deficiency. Generation of a phenotypically more accurate model of LGMD is therefore critical for developing effective therapeutic strategies as well as for elucidating the pathogenesis of the disease.

In the present study, we have developed *Sgca*-null mice in order to analyze the biological role of the sarcoglycans in the pathophysiology of LGMD. The null mutant represents the first engineered animal model for autosomal recessive muscular dystrophy with a primary sarcoglycan gene defect. The mice developed histopathological features of muscular dystrophy shortly after birth and showed ongoing fiber degeneration until nine months of age. Biochemical analysis revealed loss of the entire sarcoglycan complex along with a complete loss of sarcospan. Our data indicate that sarcospan is an integral component of the sarcoglycan complex. In addition to sarcoglycan/sarcospan deficiency we observed a reduction of all other DGC components. The disruption of the DGC in *Sgca*-null mutant mice resulted in increased masses of both extensor digitorum longus (EDL) and soleus muscles and a decrease in specific force developed by the EDL. We found no alteration in the expression level of ϵ -sarcoglycan, the recently identified homologue of α -sarcoglycan (Ettinger et al., 1997; McNally et al., 1998). Intramuscular injection of a recombinant α -sarcoglycan adenovirus in homozygous mutants restored expression of the DGC at the sarcolemma, demonstrating the feasibility of gene transfer for sarcoglycan-deficient LGMD. Our results suggest that the absence of the sarcoglycan-sarcospan complex due to a null mutation in the *Sgca* gene causes dissociation of the DGC and contributes to progressive muscle degeneration in LGMD 2D.

Materials and Methods

Isolation of α -Sarcoglycan and Vector Construction

The α -sarcoglycan gene was isolated from a λ FIXII 129/sv genomic library by homology screening using a radiolabeled human α -sarcoglycan cDNA. A 16.2-kb NotI fragment was characterized by restriction mapping and limited sequencing (GenBank/EMBL/DDBJ accession number AF064081). Two StyI fragments of 0.16 and 0.9 kb carrying exon 1 and a portion of 5' and 3' contiguous intron were coligated into the XbaI site of pPNT. Orientation of the two inserted fragments was checked by PCR and sequencing. A 9-kb fragment containing a portion of intron 3, and exon 4-exon 9 was amplified using a high-fidelity PCR reaction (Takara, Shiga, Japan) with intron 3 forward primer from: CCCCTCGAGCCGTTCCTCAGACTTTTATTC and exon 9 reverse primer from: AATGCGGCCGCTCCTGTACGAACAT. The PCR fragment was restric-

tion digested by NotI and XhoI and inserted into a XhoI-NotI cut plasmid. Correct targeting replaced exon 2 (containing part of the signal sequence), exon 3, 571 bp of intron 1, and 65 bp of intron 3 with a neomycin resistance gene in opposite transcriptional orientation (see Fig. 1).

Generation of *Sgca*-deficient Mice

The NotI linearized construct was introduced into 2×10^7 R1 ES cells by electroporation (240 V, 500 μ F; Bio-Rad Gene Pulser; Hercules, CA). The ES cells were maintained on feeder layers and passaged clonally. Targeting fidelity was determined by Southern analysis. Cells from three correctly targeted clones were microinjected into C57BL/6J blastocysts and transferred into pseudopregnant recipients. After germ-line transmission, genotypes were determined by PCR on DNA from tail biopsies (see Fig. 1). The following primers and PCR conditions were used: (a) INT1 in intron 1: CAGGGCTGGGAGCTGGGTCTG; (b) EX2 in intron 3 (deleted in the null allele): CCCAGGGCCTTGATGCCT; and (c) NEOTR: GCTATCAGGACATAGCGTTGGCTA: first denaturation at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 64°C, 2 min 30 s at 72°C, and 7 min last extension at 72°C. All three primers were used in the same PCR reaction. Wild-type and null alleles corresponded to PCR fragments of 1,061 and 618 bp, respectively.

Northern Blot Analysis

Total RNA from control, heterozygous, and homozygous-null mutant skeletal and cardiac tissues was extracted using RNazol (Tel-Test, Friendswood, TX) according to manufacturer specifications. 30 μ g of total RNA was run on a 1.25% agarose gel containing 5% formaldehyde and transferred to Hybond N membrane (Amersham Corp., Arlington Heights, IL). RNA was cross-linked to the membrane using a Stratagene UV cross-linker (La Jolla, CA). Membranes were then prehybridized and hybridized using standard methods. Washes were carried out at 65°C in $1 \times$ SSC/1% SDS initially, then $0.1 \times$ SSC/0.1% SDS. Blots were exposed for autoradiography.

Evans Blue Dye Injection and Microscopic Evaluation

Evans blue dye (EBD) (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS (10 mg/ml) and sterilized by passage through membrane filters with a pore size of 0.2 μ m. Mice were injected intravenously with 0.25 μ l per 10 g of body weight of the dye solution through the tail vein. Animals were killed 6 h after injection by cervical dislocation. During the time period between injection and cervical dislocation, animals were kept in standard laboratory cages. All mice were skinned and inspected for dye uptake in the skeletal muscles, indicated by blue coloration. Muscle sections for microscopic Evans blue detection were incubated in ice-cold acetone at -20° C for 10 min, and after a rinse with PBS, sections were mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Sections were observed under a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) or a MRC-600 laser scanning confocal microscope (Bio-Rad Laboratories, Hercules, CA).

Serum Levels of Muscle Enzymes

Activities of muscle specific pyruvate kinase (PK) isozyme found in the blood serum were measured as previously documented (Edwards and Watts, 1981). Quantitative, kinetic determination of creatine kinase activity in serum of control and *Sgca*-deficient mice was measured using creatine kinase (CK) reagent (Sigma Chemical Co.) according to the manufacturer's instructions. Blood was collected from the retroorbital sinus of 2-18-wk-old mice and the serum was stored at -80° C before measurements.

Antibodies

Monoclonal antibodies IIH6 against α -dystroglycan (Ervasti and Campbell, 1991) and 8D5 against β -dystroglycan (Lim et al., 1995) were previously characterized. mAbs 20A6 against α -sarcoglycan, 5B1 against β -sarcoglycan, and 21B5 against γ -sarcoglycan were generated in collaboration with L.V.B. Anderson (Newcastle General Hospital, Newcastle upon Tyne, UK). We used a mAb against caveolin-3 (Transduction Laboratories, Lexington, KY). Rabbit polyclonal antibodies against α -sarcoglycan (Roberds et al., 1993a), dystrophin, and utrophin (Ohlendieck et al., 1991a), neuronal nitric oxide synthase (Crosbie et al., 1998), the α_1 subunit of the dihydropyridine receptor (Ohlendieck et al., 1991b), and

the laminin α 2-chain (Allamand et al., 1997) were described previously. Two affinity-purified rabbit antibodies (rabbit 208 and 215) were produced against a full-length COOH-terminal fusion protein of γ -sarcoglycan, and against an NH₂-terminal peptide (MMPQEQYTHHRSTMP-GAA) of δ -sarcoglycan, respectively. An affinity-purified goat antibody (goat 26) was produced against a NH₂-terminal fusion protein of β -sarcoglycan containing amino acids 1–65. Polyclonal antibodies against α -dystroglycan fusion protein D were affinity-purified from goat 20 (Ibraghimov-Beskrovnyaya et al., 1992). An affinity-purified rabbit antibody (rabbit 235) was produced against a COOH-terminal fusion protein of sarcospan (CFVMWKHRYQVFYVGVGLRSLMASDGLPKA). Two polyclonal antibodies against ϵ -sarcoglycan were used. One was previously characterized (Ettinger et al., 1997) and the other (rabbit 232) was generated against a COOH-terminal peptide of ϵ -sarcoglycan (PHQT-QIPQQTTGKWYP).

Immunofluorescence Analysis

For immunofluorescence analysis, 7- μ m transverse cryosections were prepared from control and *Sgca*-null mutant skeletal and cardiac muscle. All procedures were performed at room temperature. Sections were blocked with 5% BSA in PBS for 1 h and then incubated with the primary antibodies for 90 min. After washing with 1% BSA/PBS, sections were incubated with Cy3-conjugated secondary antibodies (1:250) for 1 h and then washed with 1% BSA/PBS. After a rinse with PBS, sections were mounted with Vectashield mounting medium (Vector Laboratories, Inc.) and observed under a Zeiss Axioplan fluorescence microscope (Carl Zeiss Inc.).

Immunoblot Analysis of Membrane Preparations

KCl-washed membranes from skeletal and cardiac muscle were prepared as described previously (Ohlendieck et al., 1991b) with the addition of two protease inhibitors, calpeptin and calpain inhibitor 1 (Calbiochem-Novabiochem Corp., San Diego, CA). Both inhibitors were used in the buffers at a concentration of 2 nM. Membranes were resolved by SDS-PAGE (Laemmli, 1970) on 3–15% linear gradient gels and transferred to nitrocellulose membranes (Towbin et al., 1979). Immunoblot staining was performed as previously described (Ohlendieck et al., 1991b).

Contractile Properties

For the measurement of contractile properties of the EDL or soleus muscles of control or *Sgca*-deficient mice in vitro, mice were anesthetized with sodium pentobarbital (30–50 mg/kg for control or *Sgca*-deficient mice, respectively) (Nembutal; Abbott Laboratories, Chicago, IL). Contractile properties were measured on 28 muscles, seven EDL and five soleus muscles from heterozygous littermates of *Sgca*-deficient mice and 10 EDL and six soleus muscles from eight *Sgca*-null mutant mice. Muscles were isolated and removed carefully from the anesthetized mice and immersed in an oxygenated (95% O₂ and 5% CO₂) bath containing a buffered mammalian Ringer's solution, pH 7.4, which included curare. The solution was maintained at 25°C. The tendons were tied securely to a servomotor and a force transducer. Muscles were stimulated directly by the current flow between two large platinum electrodes (Brooks and Faulkner, 1988). The voltage of the stimulator was set to provide maximum twitch force and the muscle length was set at optimum length for force development. With the muscle at optimum length, the frequency of stimulation was increased until force plateaued at maximum isometric tetanic force (P_o). After the measurement of P_o , the length of the muscle was set at 90% of the fiber length and with the muscle passive the muscle was stretched to 110% of fiber length at 1 fiber length(s) and then returned at the same velocity to 90% of fiber length. The peak force at the end of the stretch was used as a measure of the resistance to stretch of the passive muscle. The muscle was removed from the bath, blotted, and weighed to obtain the muscle mass. Based on the direct measurements of muscle mass, muscle length, fiber length, and P_o , total fiber cross-sectional area and specific P_o were calculated. The total fiber cross-sectional area (mm²) was calculated by dividing the muscle mass (mg) by the fiber length (mm) and then by 1.06 (g/cm²) to correct for the muscle density (Brooks and Faulkner, 1988). The force (kN) was divided by the total fiber cross-sectional area (m²) to obtain an estimate of the specific force (kN/m²) of the EDL and soleus muscles. Each data set was analyzed by a two-way analysis of variance (ANOVA) in a general linear model algorithm appropriate for unequal sample sizes (Statistical Analysis System, Gary, NC). In circumstances where the overall F-ratio for the ANOVA was significant, the differences

between individual group means were determined by post hoc pairwise t-comparisons of least square means with appropriate correction of the significance level to account for multiple comparisons. Significant differences between data on control and *Sgca*-deficient mice are indicated in Fig. 6 by *asterisks*. Significance was set a priori at $P < 0.05$.

Recombinant Adenovirus Injections

The human α -sarcoglycan 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. Lysates from the infected cells were collected and tested for the expression of α -sarcoglycan using a polyclonal antibody. Recombinant viruses were plaque purified three times, amplified, and then concentrated using established methods (Graham and Eb, 1973; Davidson et al., 1994). Recombinant adenovirus injections were performed as previously described (Holt et al., 1998). For 2-d-old *Sgca*-null mutant mice, the adenovirus was injected directly through the skin into the hamstring. 3-wk-old mice were anesthetized by intraperitoneal injection of sodium pentobarbital at a calculated dose of 50 mg/kg. A human δ -sarcoglycan adenovirus (Holt et al., 1998) was used as control.

Results

Generation of *Sgca*-null Mutant Mice

To design a targeting vector to generate *Sgca*-null mice, we cloned the murine homologue of the human *SGCA* gene from a mouse genomic library. Murine and human α -sarcoglycan are highly related at the amino acid level and their expression pattern at the mRNA level is similar (Roberds et al., 1994; Liu et al., 1997). The structural organization of the gene into 10 exons is shared by both species (GenBank/EMBL/DBJ accession number AF064081).

Targeted inactivation of one of the *Sgca* alleles was accomplished by replacement of exons 2 and 3 and flanking intronic sequences with the neomycin resistance gene (Fig. 1 a). The targeting construct was designed to create a mutant allele of *Sgca* representative of certain human mutations. One-third of α -sarcoglycan mutations characterized to date affect exons 2 and 3 of the *SGCA* gene (Piccolo et al., 1995; Carrie et al., 1997). A total of 1,023 colonies surviving G418 and gancyclovir selection were analyzed by Southern-blotting for the presence of homologous recombination (Fig. 1 b). Two clones yielded chimeras producing germ-line transmission. Transmission of the mutant allele followed normal Mendelian segregation ratios for an autosomal recessive gene in mice derived from both clones. Homozygous mutant and heterozygous newborn pups appeared healthy, showing no gross developmental abnormalities compared with control littermates.

To determine if the targeting approach produced a null allele, we evaluated tissues from homozygous mutants and heterozygous mice, and compared them to wild-type mice. Northern blot analysis using a probe against the full-length coding sequence revealed the absence of α -sarcoglycan transcript in *Sgca*-deficient skeletal and heart tissue from the two independently derived lines (Fig. 1 d and data not shown). In contrast to control and heterozygous animals, no α -sarcoglycan expression was detected in the homozygous mutant mice (Fig. 1 e). Reverse transcription (RT)-PCR revealed the presence of a minor transcript in skeletal muscle RNA resulting from the use of cryptic splice sites in the neomycin cassette in homozygous mutants and heterozygous mice. Sequencing of the RT-PCR product

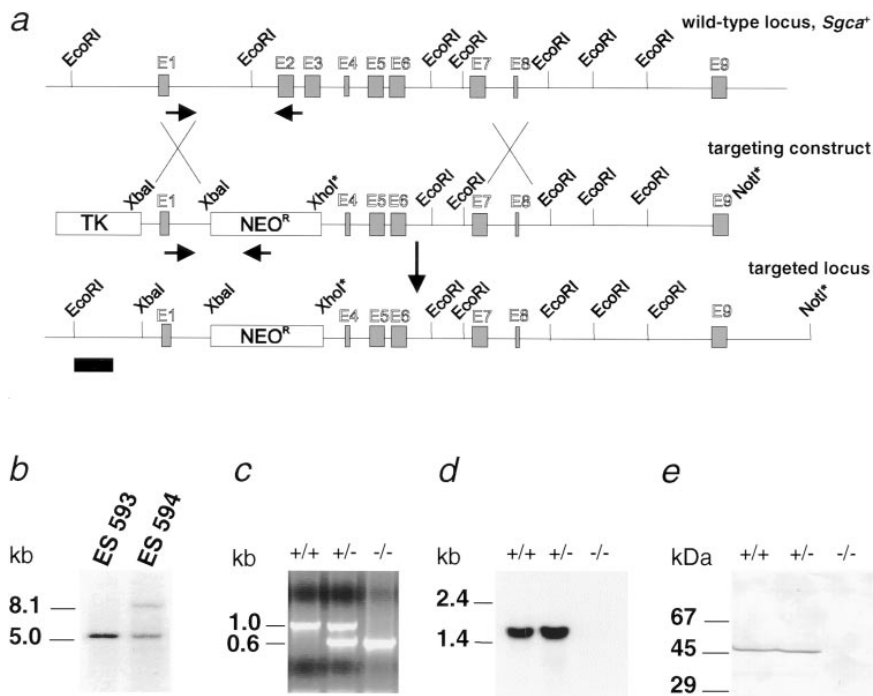


Figure 1. Generation of *Sgca*-null mutant mice. (a) Restriction map of the wild-type *Sgca* locus (*Sgca*⁺), the targeting construct, and the targeted locus. A region of 902 bp including exons 2 and 3 (*E2* and *E3*) was deleted and replaced by a phosphoglycerate kinase-neomycin cassette (*NEO*^R). (b) Southern blot analysis. Using the probe shown (black box), the targeted locus contains an *Eco*RI fragment of 8.8 kb, whereas the intact allele shows a 5.5-kb band; clone 594 is correctly targeted. (c) Genotyping by PCR. Primer sites are shown in a; using primers INT1 and EX2 the wild-type allele (+/+) corresponds to a 1,061-bp fragment; using primers INT1 and NEOTR, the null allele is 618 bp. (d) Northern blotting. An α -sarcoglycan cDNA probe reveals the correct sized transcript in wild type (+/+) and heterozygotes (+/-) from 30 μ g total RNA extracted from skeletal muscle; homozygous mutant tissue shows no α -sarcoglycan transcript. (e) Western blot analysis. Using an affinity-purified polyclonal antibody against a COOH-terminal peptide of α -sarcoglycan, membrane-enriched preparations of skeletal muscle reveal the protein in (+/+), and (+/-), but not in (-/-).

revealed that this mutant transcript encoded exon 1 and a stretch of 516 bp from the inverted neo cassette spliced with exon 4 of the *Sgca* gene (data not shown). Unexpectedly, this aberrant splicing event inserted 172 amino acids from the noncoding strand of the neo cassette, maintaining the frame with exon 4. Translation of the altered transcript would produce a protein lacking the 91 amino acids encoded by exons 2 and 3, including part of the signal sequence. Using a COOH-terminal peptide antibody and mAb 20A6 against α -sarcoglycan, this mutant protein could not be detected in *Sgca*-deficient skeletal and cardiac tissues by immunoblot or immunofluorescence analysis (Fig. 1 e and see Figs. 4 and 5).

Sgca-null Mutant Mice Display a Progressive Muscular Dystrophy

Sgca-null mutant mice did not show any overt signs of a myopathy, and were in this respect similar to dystrophin deficient *mdx* mice. To examine the progression of the muscular dystrophy in these mutant mice, haematoxylin and eosin (H & E)-stained frozen sections of the sural triceps and the diaphragm muscles were evaluated between the ages of 8 d and 9 mo. Pathology characteristic of muscular dystrophy was observed in every *Sgca*-deficient mouse but never in control animals. The earliest changes consisted of widely scattered clusters of necrotic myocytes or regenerating myocytes with internally placed nuclei (Fig. 2). These clusters increased in both number and size as the mice increased in age (Fig. 2). Based on the evaluation of 200–1,100 myocytes per muscle, the number of nonregenerating myocytes with internally placed nuclei

also increased with age. At 8 d, between 1 and 2.5% of the sural triceps and diaphragm myocytes already showed centrally placed nuclei, respectively. These numbers continuously increased and at 8–16 wk of age more than 70% and as high as 99% of the *Sgca*-deficient myocytes contained centrally placed nuclei (Fig. 2). In wild-type mice on the other hand, the numbers of centrally placed nuclei never exceeded 1%.

In addition to necrosis, regeneration, and central nucleation, a broad spectrum of other dystrophic changes was also noted in *Sgca*-deficient muscle (Fig. 2). The most prominent of these included atrophy, hypertrophy, fiber splitting, and endomysial fibrosis. In some *Sgca*-deficient mice 8 wk of age or older, dystrophic calcification was noted in association with myocyte necrosis (Fig. 2). Fatty infiltration was present in some of the muscles from 16-wk-old mice. A qualitative comparison of fiber type distribution assessed with ATPase staining as well as staining characteristics with NADH and Gomori trichrome stains suggested no substantial additional differences between null mutant and wild type mice at any age (data not shown). The homozygotes from both correctly targeted cell lines demonstrated an identical dystrophic phenotype.

Sarcolemmal Integrity in α -Sarcoglycan-deficient Muscle

To test whether the mutation of the α -sarcoglycan gene leads to damage of the plasma membrane, we intravenously injected *Sgca*-deficient mice with Evans blue dye (EBD), a normally membrane impermeant molecule. This dye penetrates into the cytoplasm of fibers with compromised sarcolemmal integrity (Matsuda et al., 1995; Straub

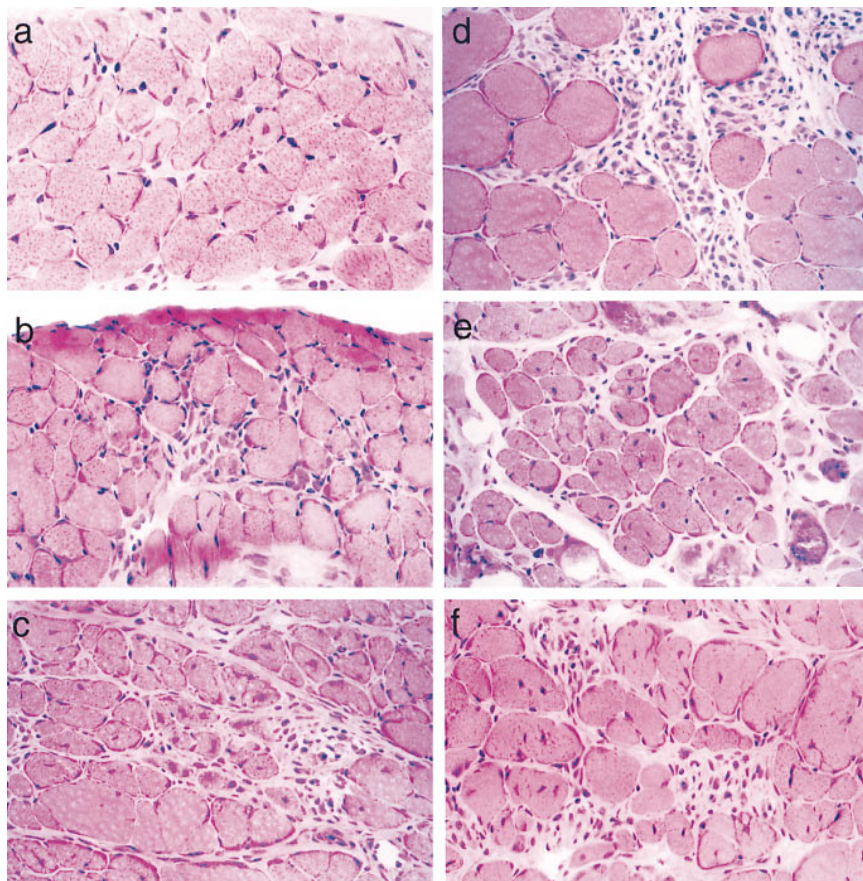


Figure 2. Histological analysis of *Sgca*-deficient diaphragm muscle. *Sgca*-null mutant mice started to develop a progressive muscular dystrophy at 1 wk of age and, in contrast to *mdx* mice, showed ongoing muscle necrosis with increasing age. Examples of muscle pathology in the diaphragm of different aged mice are demonstrated. (a) Myocyte atrophy (small fibers scattered throughout the micrograph) and central nucleation (see fibers near the center and in upper left quadrant) after regeneration in an 8-d-old mouse. (b) A small focus of myocyte necrosis (center) with surrounding regenerating and atrophic fibers in an 18-d-old mouse. (c) Skeletal muscle of a 4-wk-old mouse with ongoing necrosis (right of center), regeneration, central nucleation, endomysial fibrosis (increase in tissue between muscle fibers), atrophy, hypertrophy (large fibers in left lower quadrant), and fiber splitting. (d) The edge of a large, confluent area of acute myocyte necrosis in an 8-wk-old mouse. (e) More severe endomysial fibrosis with atrophy, central nucleation, fiber splitting, and dystrophic calcification (dark structures in lower right) in an 8-wk-old mouse. (f) Small foci of myocyte necrosis surrounded by atrophic and hypertrophic fibers, central nucleation, and fiber splitting are shown in a 16-wk-old mouse. All panels show 7- μ m frozen sections of diaphragm muscle stained with H & E.

et al., 1997). No obvious uptake of the blue tracer into skeletal muscles of heterozygous and control mice was detected by macroscopic inspection. In contrast, EBD uptake was consistently observed in skeletal muscle fibers of 4–20-wk-old homozygous-null mutants. The extent of EBD accumulation varied among muscles. Areas of blue staining appeared mainly within the proximal limb muscles and the muscles of the pelvic and the shoulder girdle. Skeletal muscles which macroscopically showed dye uptake always revealed red EBD autofluorescence by fluorescence microscopy analysis (Fig. 3 a). Most EBD-positive fibers showed intense staining, whereas the signal was faint in others. Interestingly, EBD-positive fibers were distributed in clusters throughout the different muscles (Fig. 3 a). Fibers that had taken up the tracer and were assumed to have pathologic plasma membrane permeability often showed characteristic features of degeneration and necrosis by H & E staining. In contrast, dye uptake was not readily visible in cardiac muscle by macroscopic inspection, and microscopic analysis revealed no EBD in cardiomyocytes of control or diseased animals.

We also evaluated membrane damage in *Sgca*-deficient mice by determining the release of muscle enzymes into the circulating blood. Therefore we measured muscle-specific serum PK (Fig. 3 b) activity and serum CK activity. In 7–10-wk-old wild-type and heterozygous mice we found normal serum levels of PK activity. Age-matched homozy-

gous mice, on the other hand, exhibited high serum levels of PK activity similar to that of *mdx* mice, indicating that membrane damage occurred to comparable extents in *Sgca*-null mutants as in *mdx* mice (Fig. 3 b). Similarly, in older animals (up to 18 wk of age) we found no differences in PK activity between *Sgca*-null mice and age-matched *mdx* mice. The serum CK activity was \sim 10 times higher in *Sgca*-deficient mice compared with control animals (data not shown).

Loss of Sarcoglycan and Sarcospan Expression in *Sgca*-null Mutant Mice

Immunofluorescence analysis was performed for each component of the sarcoglycan complex. In *Sgca*-deficient mice, α -sarcoglycan protein was absent from the sarcolemma of skeletal and cardiac muscle fibers. In addition, there was a concomitant drastic reduction of β -, γ -, and δ -sarcoglycan (Fig. 4, a and b). Other components of the DGC were also examined by immunofluorescence microscopy. The laminin- α 2 chain and β -dystroglycan were present at comparable levels with control muscle (Fig. 4, a and b). We observed a slight reduction in the α -dystroglycan staining in *Sgca*-deficient muscle compared with control muscle. However, the sarcolemmal staining for dystrophin was consistently patchy and reduced in *Sgca*-null mutant skeletal muscle, although dystrophin staining in

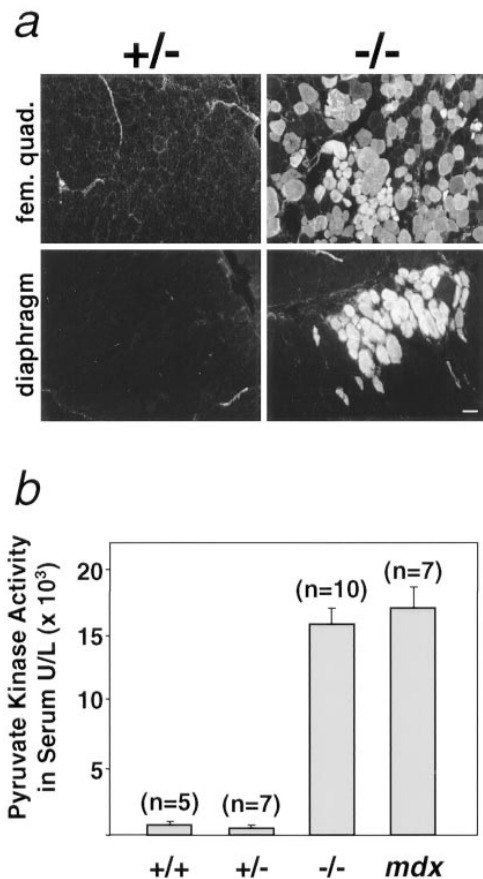


Figure 3. Evaluation of sarcolemma permeability. (a) Heterozygous (+/-) and homozygous-null (-/-) mice were intravenously injected with EBD. The panels show dye uptake into muscle fibers of the femoral quadriceps and diaphragm muscles 6 h after injection. Dye accumulation was only detected in skeletal muscle from *Sgca*-null mutants. Activity of muscle-specific PK in 7–10-wk-old wild-type (+/+), heterozygotes (+/-), homozygotes (-/-), and *mdx* mice (b). Measurement of PK released from the muscle fiber into the circulating blood showed similar high levels of PK activity in (-/-) and *mdx* mice compared with (+/-) and control (+/+). Error bars indicate the standard deviation where *n* equals the number of mice in each set. Bar, 50 μ m.

heart appeared similar to control (Fig. 4, *a* and *b*). We also analyzed expression of the newly identified ϵ -sarcoglycan (Ettinger et al., 1997) and the 25-kD DGC component sarcospan (Crosbie et al., 1997). In contrast to the other sarcoglycans, skeletal and cardiac muscle staining for ϵ -sarcoglycan in *Sgca*-deficient mice was comparable to control levels. Interestingly, sarcospan was absent along with the sarcoglycans at the sarcolemma of homozygous-null mice (Fig. 4, *a* and *b*), indicating that sarcospan may be an integral component of the sarcoglycan complex.

Dissociation of the DGC in *Sgca*-null Mutant Mice

To further examine the expression of DGC components, immunoblot analysis was performed on isolated membrane preparations from control and homozygous mutant skeletal muscle. We observed that *Sgca*-deficient muscle preparations were more susceptible to proteolytic degradation. Use of calpain inhibitor I and calpeptin reduced

degradation in the membrane preparations from *Sgca*-null mice. The ryanodine receptor and dystrophin, for example, were almost completely degraded in skeletal muscle membrane preparation without the use of calpain inhibitor I and calpeptin. Coomassie blue staining (data not shown) and staining for caveolin-3, the α_1 subunit of the dihydropyridine receptor, and the ryanodine receptor (data not shown) showed that equivalent levels of membrane protein were present in control and homozygous mutant preparations (Fig. 5). α -Sarcoglycan was not detected in skeletal and cardiac membrane preparations from *Sgca*-null mice (refer to Fig. 1 *e*, Fig. 5, and data not shown). Heterozygotes expressed control levels of α -sarcoglycan (Fig. 1 *e*). β -, γ -, and δ -sarcoglycan were greatly reduced in α -sarcoglycan-deficient skeletal muscle membranes compared with control muscle (Fig. 5). Dystrophin was slightly reduced in accordance with the patchy staining observed by immunofluorescence. Together with the dystrophin reduction we also found reduced levels of the free radical-producing enzyme neuronal nitric oxide synthase, which is anchored to the sarcolemma by dystrophin (Brennan et al., 1995). Utrophin, the autosomal homologue of dystrophin, was found at higher levels in membrane-enriched preparations from homozygous mutant mice compared with control mice. This observation could be related to the large number of regenerating fibers in *Sgca*-deficient muscle, which would be expected to express higher levels of utrophin (Helliwell et al., 1992). In addition, α - and β -dystroglycan were reduced in membrane preparations of dystrophic mice compared with control mice (Fig. 5), whereas immunofluorescence showed only little dystroglycan reduction at the sarcolemma (Fig. 4). In the supernatant from *Sgca*-deficient membrane preparations, α -dystroglycan was enriched and fully glycosylated but was not tightly associated with membranes (data not shown). Thus, α -dystroglycan is synthesized correctly but is not stably anchored to the sarcolemma in the absence of the sarcoglycan complex. In addition, Western blot analysis confirmed the immunofluorescence analysis in showing that dystrophin was no longer tightly held at the skeletal plasma membrane in the absence of the sarcoglycan complex.

Abnormal Contractile Properties of *Sgca*-deficient Muscles

The body masses of 8-wk-old *Sgca*-null mutant mice (34 ± 1 g) were not significantly different from their heterozygous littermates (31 ± 1 g) (Fig. 6). For the EDL muscles of the *Sgca*-deficient mice compared with heterozygous littermates, the masses were 40% greater, whereas the absolute forces were not significantly different. As a consequence, the specific P_0 of the EDL muscles of *Sgca*-deficient mice was 31% lower than that of the control mice. The resistance to stretch of the passive muscle in homozygous null mutants was 75% greater than the values for control animals. In contrast to the changes observed in the EDL muscles, the soleus muscles in the *Sgca*-null mutant mice responded in a completely different manner. Compared with soleus muscles in heterozygous littermates, those in the *Sgca*-null mutant mice had a 62% greater mass, a 39% greater absolute P_0 , and no significant difference in either specific P_0 or resistance to stretch.

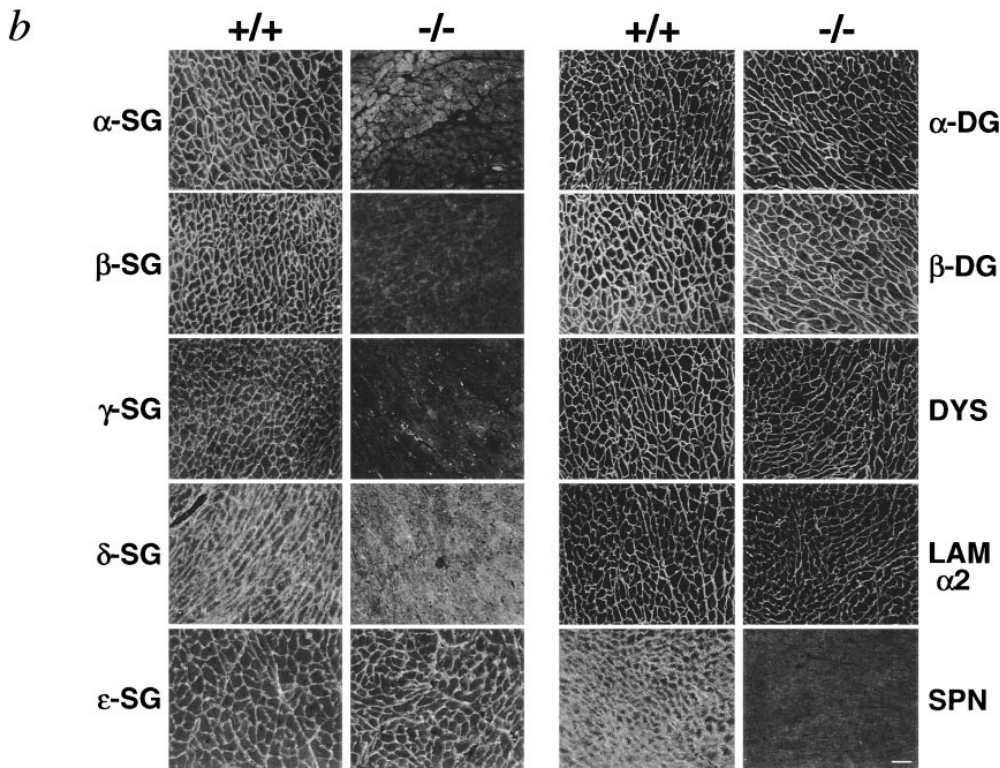
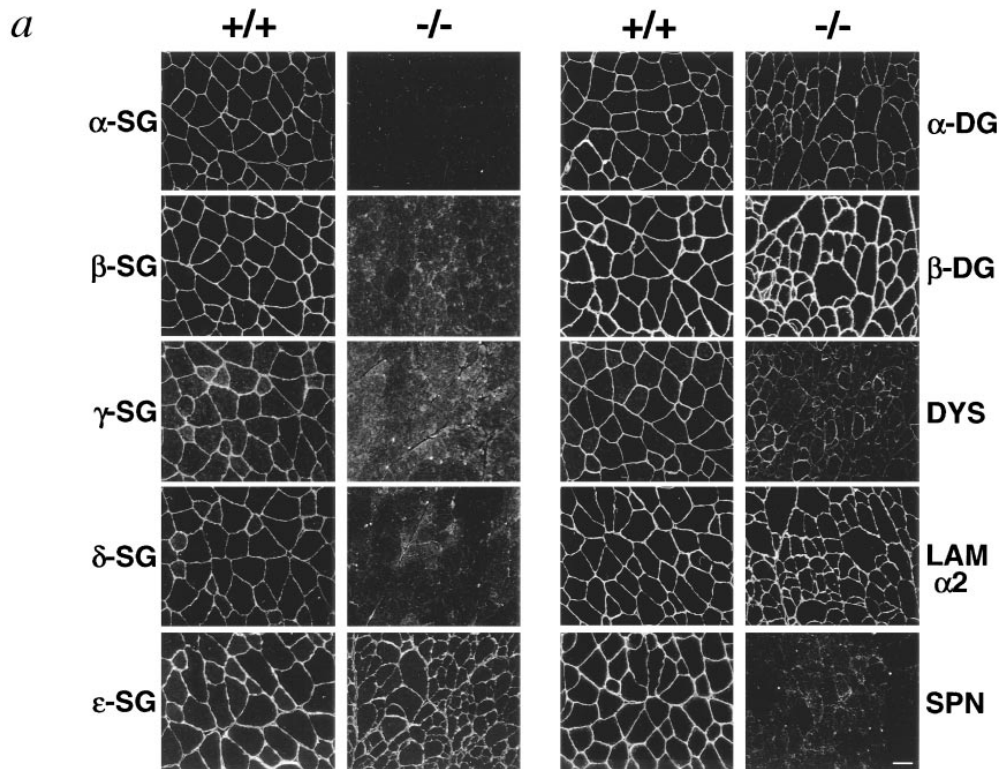


Figure 4. Immunofluorescence analysis of sarcolemma proteins in *Sgca*-deficient skeletal and cardiac muscle. Skeletal (*a*) and cardiac (*b*) muscle cryosections from wild-type (+/+) and *Sgca*-null (-/-) mice were stained with antibodies against dystrophin (*DYS*), α -dystroglycan (α -*DG*), β -dystroglycan (β -*DG*), laminin α 2-chain, (*lam* α 2), α -, β -, γ -, δ -, and ϵ -sarcoglycan (*SG*), and sarcospan (*SPN*). The sarcoglycans and sarcospan were drastically reduced in the *Sgca*-deficient muscle whereas the α -sarcoglycan homologue ϵ -sarcoglycan was present in comparable amount to control. Dystrophin staining is reduced in *Sgca*-null mutant mice from the sarcolemma of skeletal muscle, whereas it is maintained at equal levels to control in cardiomyocytes. Bar, 50 μ m.

Restoration of the Sarcoglycan–Sarcospan Complex by Gene Transfer

We used an adenovirus construct encoding human α -sarcoglycan to test the ability of exogenously provided α -sarcoglycan cDNA to restore the sarcoglycan–sarcospan complex in *Sgca*-deficient skeletal muscle. To circumvent

a possible immune response against the neoantigen or adenovirus itself, the α -sarcoglycan adenovirus was injected into the hamstring muscles of 2-d-old *Sgca*-deficient pups. We found high levels of α -sarcoglycan expression at the sarcolemma (Fig. 7) over a time interval between 5 d and 2 mo after injection. In addition, α -sarcoglycan–positive fi-

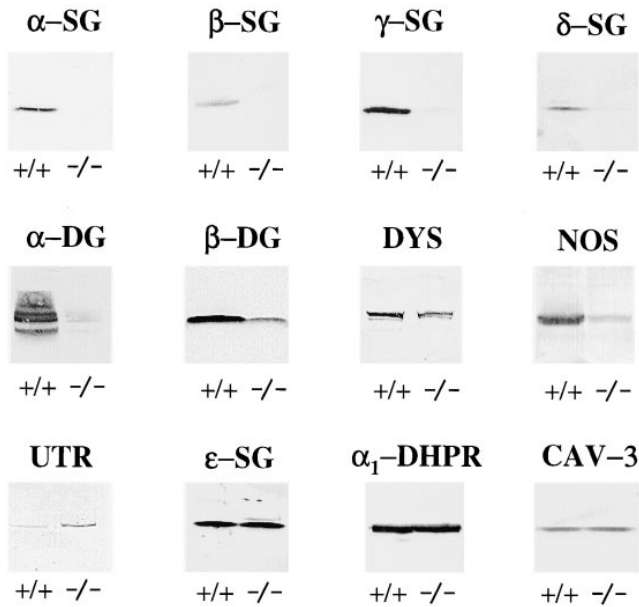


Figure 5. Immunoblot analysis of skeletal muscle membranes. Skeletal muscle microsomes from control (+/+) and *Sgca*-deficient (-/-) mice were analyzed by 3–15% SDS-PAGE and immunoblotting using antibodies against several DGC components. In particular we used antibodies against the sarcoglycans (α -, β -, γ -, δ -, and ϵ -*SG*), dystroglycans (α - and β -*DG*), and dystrophin (*DYS*). In addition we stained blots with antibodies against neuronal nitric oxide synthase (*NOS*), which has been shown to be associated with dystrophin, and the dystrophin homologue utrophin (*UTR*). To demonstrate equal loading of protein samples we used the α_1 subunit of the dihydropyridine receptor (α_1 -*DHPR*) and caveolin-3 (*CAV-3*) as positive markers.

bers revealed expression of the entire sarcoglycan complex, sarcospan and dystrophin by immunofluorescence microscopy (Fig. 7). Immunostaining of the sarcoglycans was found in up to 70% of fibers. Interestingly, central nucleation was reduced in *Sgca*-null mutant muscle after injection of the α -sarcoglycan adenovirus (data not shown).

An adenovirus construct encoding human δ -sarcoglycan was used as a control for the gene transfer studies. In α -sarcoglycan-deficient muscles injected with the δ -sarcoglycan adenovirus, no reconstitution of the complex was detected.

Discussion

Progressive muscle weakness initially in pelvic and shoulder girdle muscles is a hallmark of patients with a primary sarcoglycan gene defect. Mutations in the α -sarcoglycan gene have been well documented and are associated with the deficiency of the entire sarcoglycan complex from the sarcolemma (Roberds et al., 1994; Hayashi et al., 1995; Passos-Bueno et al., 1995; Piccolo et al., 1995; Duggan et al., 1996). To ascertain how the sarcoglycan defect causes muscular dystrophy, we evaluated structural and functional characteristics of skeletal and cardiac muscle in *Sgca*-null mutant mice.

In contrast to the other sarcoglycans, expression of α -sarcoglycan is specifically restricted to striated muscle

fibers (Roberds et al., 1993a; Ettinger et al., 1997; Eymard et al., 1997; Liu et al., 1997). During development, the expression of α -sarcoglycan is coincident with primary myogenesis (Yuan et al., 1990; Liu et al., 1997). In particular, these studies indicated that at 17 d of gestation in rabbit, α -sarcoglycan was already present in all myotubes, where it was confined to the cell periphery (Jorgensen et al., 1990; Yuan et al., 1990). Similarly, it has been reported that α -sarcoglycan in mice was not detected before the onset of myogenesis, whereas it was expressed at the sarcolemma of newly formed fibers at day E14 (Liu et al., 1997).

Targeted disruption of the α -sarcoglycan gene in homozygous mutants resulted in the absence of normal transcript and protein. Analysis of skeletal muscle histology demonstrated that *Sgca*-deficient mice presented with a progressive muscular dystrophy similar to human sarcoglycan deficient LGMD. The histological changes appeared as early as 1 wk of age and extended to most skeletal muscles as evidenced by EBD incorporation and elevated serum PK levels. The presence of extensive central nucleation, connective tissue proliferation, increased variability of muscle fiber diameter, and necrotic fibers was documented during the entire course of the disease studied to date. One striking observation was the persistence of degeneration and regeneration with extensive areas of necrosis in α -sarcoglycan-deficient muscle. The early onset and the severity and persistence of the pathology distinguished the *Sgca*-null mutant mice from *mdx* mice, in which these features decline after a regeneration peak at 3–4 wk of age (McArdle et al., 1995). Dystrophic pathology in the *Sgca*-null mutant mice is more similar to what has been documented in *mdx/utrn*^{-/-} double-knockout mice (Deconinck et al., 1997; Grady et al., 1997). The complete absence of sarcospan in addition to the loss of the sarcoglycan complex may be one reason for the severity of the disease. The complete lack of sarcospan expression in *Sgca*-null mice suggests that sarcospan is an integral com-

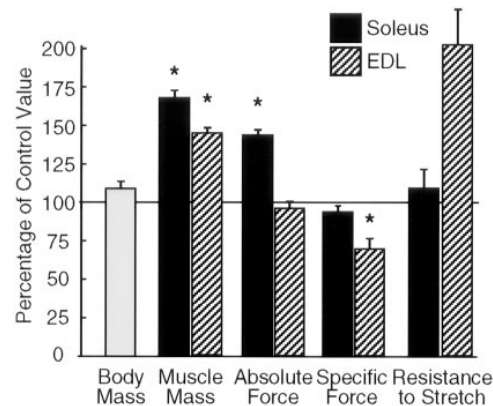


Figure 6. Abnormal contractile properties of *Sgca*-deficient muscle. The data on EDL and soleus muscles of the *Sgca*-null mutant mice are represented as a percentage of the values for muscles of control mice. The diagram shows bar graphs for the data on body mass, muscle mass, absolute maximum isometric tetanic force, specific force, and peak force during resistance to stretch of passive muscles. Asterisk, significant differences between the data obtained in *Sgca*-deficient and control mice. All data are presented as the mean \pm one SEM.

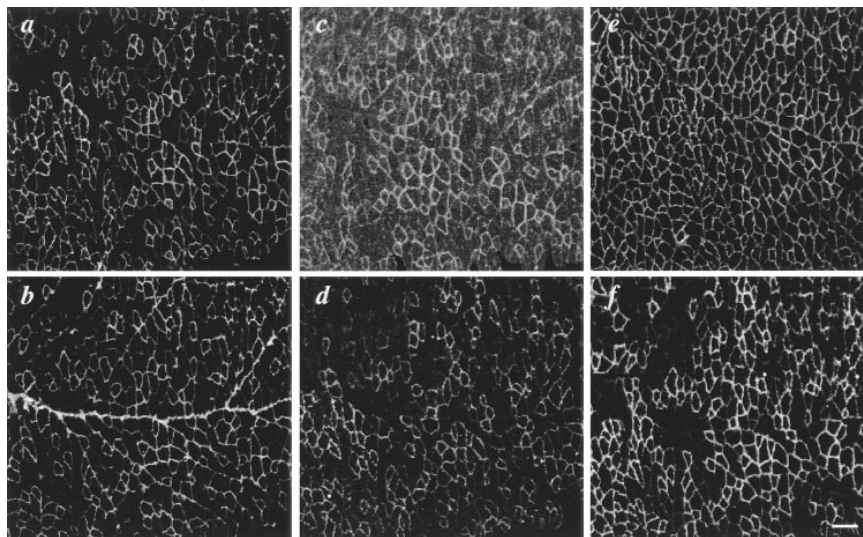


Figure 7. Restoration of sarcoglycan complex after adenovirus injection. Recombinant α -sarcoglycan adenovirus mediates restoration of DGC components to the sarcolemma. 2-d-old *Sgca*-null mutant pups were injected in their hamstring muscle with a recombinant α -sarcoglycan adenovirus containing the human α -sarcoglycan coding sequence under the control of a viral RSV promoter. Serial transverse cryosections of injected muscle after 3 wk, were stained with antibodies against α -sarcoglycan (a), β -sarcoglycan (b), γ -sarcoglycan (c), and δ -sarcoglycan (d), dystrophin (e), and sarcospan (f). Bar, 50 μ m.

ponent of the sarcoglycan complex. Correct assembly and proper transportation of the sarcoglycan-sarcospan complex to the plasma membrane seems to be prevented by the null mutation in the *Sgca*-gene. Investigation of α -sarcoglycan-deficient hearts revealed no gross morphological changes in animals up to 8 mo of age, which is in contrast to what has been observed for the sarcoglycan-deficient BIO 14.6 hamster, which starts to develop hypertrophic cardiomyopathy between 30 and 60 d of age (Homburger, 1979). It will be of interest to observe if cardiac pathology manifests as mice age.

Immunoblot analysis of membrane-enriched preparations demonstrated that α -dystroglycan binding to the sarcolemma was greatly destabilized by loss of the sarcoglycan-sarcospan complex. Our findings provide new clues on the molecular interactions between the components of the DGC. On the one hand, reduction of dystrophin removed a major F-actin binding site from the sarcolemma, whereas on the other hand, unbound α -dystroglycan eliminated the major sarcolemmal laminin-binding site. Free α -dystroglycan could be detected in the supernatant of membrane preparations confirming that it is synthesized, as observed by immunofluorescence, and glycosylated. This result suggests that the sarcoglycan-sarcospan complex anchors α -dystroglycan to the sarcolemma. Furthermore, a direct or mediated interaction of the sarcoglycan-sarcospan complex with dystrophin is implicated by our findings. The interaction of dystrophin with β -dystroglycan does not seem to be sufficient enough to maintain a solid anchorage of dystrophin to the sarcolemma. The decrease of dystrophin is in accordance with reduced levels of neuronal nitric oxide synthase, which has been reported to bind to dystrophin (Brennan et al., 1995). Reduced dystrophin expression has also been reported in patients with sarcoglycan-deficient LGMD (Vainzof et al., 1996).

During membrane preparations, we found that isolated muscle membranes of *Sgca*-null mice were more degraded than control membranes. The degradation process could be prevented by the use of calcium-activated protease inhibitors, calpeptin, and calpain inhibitor I. Our results im-

plicate that compared with control muscle, sarcoglycan-deficient muscle tissue contains an increased amount of proteases, which may be activated during the membrane preparation. Several studies provided indirect evidence consistent with a role for calpains in DMD and *mdx* pathology (Kumamoto et al., 1995; Spencer et al., 1995).

The structural destabilization of the sarcolemma by loss of the entire sarcoglycan complex and sarcospan resulted in dramatically different adaptations in the EDL and soleus muscles. Both muscles showed significant hypertrophy in response to the α -sarcoglycan deficiency. Hypertrophy of skeletal muscles has also been reported in the limb muscles of *mdx* mice (Faulkner et al., 1997), but not of the magnitude observed here. The hypertrophy was functionally less effective in the EDL muscles of the *Sgca*-null mutant mice in which the absolute force was unchanged and specific force decreased compared with control EDL muscles. In contrast, the greater hypertrophy in the weight-bearing soleus muscles produced a considerable gain in absolute force and a value for specific force equivalent to that of soleus muscles in heterozygous litter mates. The mechanisms underlying these major adaptive responses to the α -sarcoglycan deficiency will require further investigation.

In *mdx* mice, pathology is thought to be attenuated because dystrophin is partially replaced by its autosomal homologue utrophin (Deconinck et al., 1997; Grady et al., 1997). To test whether lack of α -sarcoglycan was compensated by a protein homologue, we assessed the expression of ϵ -sarcoglycan in *Sgca*-null mutant animals. ϵ -sarcoglycan shares 44% identity with α -sarcoglycan at the amino acids level (Ettinger et al., 1997), but is only weakly expressed at the sarcolemma (Ettinger et al., 1997). In the *Sgca*-deficient mice, ϵ -sarcoglycan appeared to be normally expressed at the sarcolemma and in the capillaries of skeletal and cardiac tissue. Thus, ϵ -SG does not seem to be an additional member of the known tetrameric complex of α -, β -, γ -, and δ -sarcoglycan in skeletal muscle. Nevertheless, the presence of ϵ -SG may suggest that it could be part of a distinct complex at the sarcolemma.

Intramuscular injection of recombinant adenovirus was performed to test the potential of α -sarcoglycan to restore the sarcoglycan-sarcospan complex in the *Sgca*-deficient mice. Our results demonstrated that in fibers which harbored the α -sarcoglycan recombinant adenovirus, complete assembly and restoration of the sarcoglycan-sarcospan complex to the sarcolemma has occurred. Of particular note, high efficiency gene transfer was achieved when the α -sarcoglycan adenovirus was injected at an early stage of life, preceding the onset of muscle damage and establishment of immunity. These experiments have broad implications for the development of gene therapy for LGMDs with a primary sarcoglycan deficiency. They confirm the feasibility of these procedures which are facilitated by the small size of sarcoglycan coding sequences compared with dystrophin. They also suggest that a genetic intervention should be performed as early as possible to circumvent both the extension of the dystrophic process and the immune response. The *Sgca*-deficient mice will be a valuable model for the development of therapeutic strategies for sarcoglycan deficient LGMD.

Overall, our results suggest that the absence of the sarcoglycans and sarcospan due to a null mutation in the *Sgca* gene causes dissociation of the DGC and contributes to progressive muscle degeneration in LGMD 2D. We propose that the sarcoglycan-sarcospan complex is requisite for the stable association of α -dystroglycan with the sarcolemma.

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