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Disruption of the β-Sarcoglycan Gene Reveals Pathogenetic Complexity of Limb-Girdle Muscular Dystrophy Type 2E

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Summary

Limb-girdle muscular dystrophy type 2E (LGMD 2E) is caused by mutations in the β-sarcoglycan gene, which is expressed in skeletal, cardiac, and smooth muscle. β-sarcoglycan-deficient (*Sgcb*-null) mice developed severe muscular dystrophy and cardiomyopathy with focal areas of necrosis. The sarcoglycan–sarcospan and dystroglycan complexes were disrupted in skeletal, cardiac, and smooth muscle membranes. ε-sarcoglycan was also reduced in membrane preparations of striated and smooth muscle. Loss of the sarcoglycan–sarcospan complex in vascular smooth muscle resulted in vascular irregularities in heart, diaphragm, and kidneys. Further biochemical characterization suggested the presence of a distinct ε-sarcoglycan complex in skeletal muscle that was disrupted in *Sgcb*-null mice. Thus, perturbation of vascular function together with disruption of the ε-sarcoglycan-containing complex represents a novel mechanism in the pathogenesis of LGMD 2E.

Introduction

The sarcoglycan complex is a group of single pass transmembrane proteins (α-, β-, δ-, and γ-sarcoglycan) that is tightly associated with sarcospan to form a subcomplex within the dystrophin–glycoprotein complex (DGC) in skeletal and cardiac muscle (Lim and Campbell, 1998; Crosbie et al., 1999). The DGC is further comprised of dystrophin, the dystroglycan complex, and the syntrophins (Hoffman et al., 1987; Froehner et al., 1997; Durbeej et al., 1998). The expression of the sarcoglycan–sarcospan complex is necessary for the stabilization of dystroglycan at the sarcolemma (Duclos et al., 1998a, 1998b; Holt et al., 1998; Straub et al., 1998), which in turn confers a link between the extracellular matrix and the F-actin cytoskeleton (Ervasti and Campbell, 1993). Thus, the DGC is thought to protect muscle cells from contraction-induced damage (Petrof et al., 1993). In agreement with this hypothesis, mutations in the genes for the sarcoglycans, dystrophin and laminin α2 chain are responsible for limb-girdle muscular dystrophy, Duchenne or Becker muscular dystrophy, and congenital muscular dystrophy, respectively (Straub and Campbell, 1997; Lim and Campbell, 1998). Clinical evidence of cardiomyopathy is variably present in these muscular dystrophies (Towbin, 1998), but a correlation between the primary mutation of the sarcoglycan genes and cardiomyopathy is yet to be established (Melacini et al., 1999). Several components of the DGC are also expressed in smooth muscle (Houzelstein et al., 1992; North et al., 1993; Ozawa et al., 1995; Durbeej et al., 1998). Interestingly, potential smooth muscle dysfunction has been described in patients with Duchenne muscular dystrophy (Bahron et al., 1988; Jaffe et al., 1990). However, smooth muscle dysfunction has not been reported in patients with limb-girdle muscular dystrophy.

Recently, a fifth sarcoglycan, ε-sarcoglycan, was cloned and shown to be highly homologous to α-sarcoglycan (Ettinger et al., 1997; McNally et al., 1998). ε-sarcoglycan is expressed in skeletal and cardiac muscle, and also in several nonmuscle tissues. Whether ε-sarcoglycan is associated with the other sarcoglycans in striated muscle is yet to be determined. At the immunofluorescence level, however, it has been shown that ε-sarcoglycan is still present in skeletal muscle of α-sarcoglycan-deficient mice (*Sgca*-null mice) although the other sarcoglycans are greatly reduced (Duclos et al., 1998b). This indicates that ε-sarcoglycan is not an additional member of the known tetrameric complex of α-, β-, γ-, and δ-sarcoglycan in skeletal muscle, but may be part of a distinct complex at the sarcolemma.

For the present study, we engineered *Sgcb*-null mice, to analyze the biological role of β-sarcoglycan in the pathogenesis of limb-girdle muscular dystrophy, type 2E (LGMD 2E). *Sgcb*-null mice exhibited severe muscular dystrophy and cardiomyopathy with extensive regions of necrosis as the main morphological feature, which is similar to morphological changes detected in tissue infarcts. Moreover, analysis of the DGC components at the molecular level revealed that the sarcoglycan and dystroglycan complexes were disrupted in skeletal, cardiac, and smooth muscle. Interestingly, in vivo perfusion of coronary arteries with Microfil unveiled vascular constrictions in both skeletal and cardiac muscle, which were detected prior to the onset of necrotic changes. Also, vascular constrictions were detected in *Sgcb*-null kidneys. However, no vascular constrictions were detected in diaphragms of *Sgca*-null mice, despite overt signs of dystrophic alterations. Together these data support the hypothesis that the vascular phenotype is independent of the muscular dystrophy phenotype. Additionally, ε-sarcoglycan was greatly reduced at the muscle membranes in *Sgcb*-null mice. Further biochemical characterization suggested the presence of a distinct large ε-sarcoglycan complex that was disrupted in skeletal muscle of *Sgcb*-null mice, but not in *Sgca*-null mice. Upon adenovirus-mediated gene transfer of β-sarcoglycan, ε-sarcoglycan expression was restored, suggesting an association between β- and ε-sarcoglycan.

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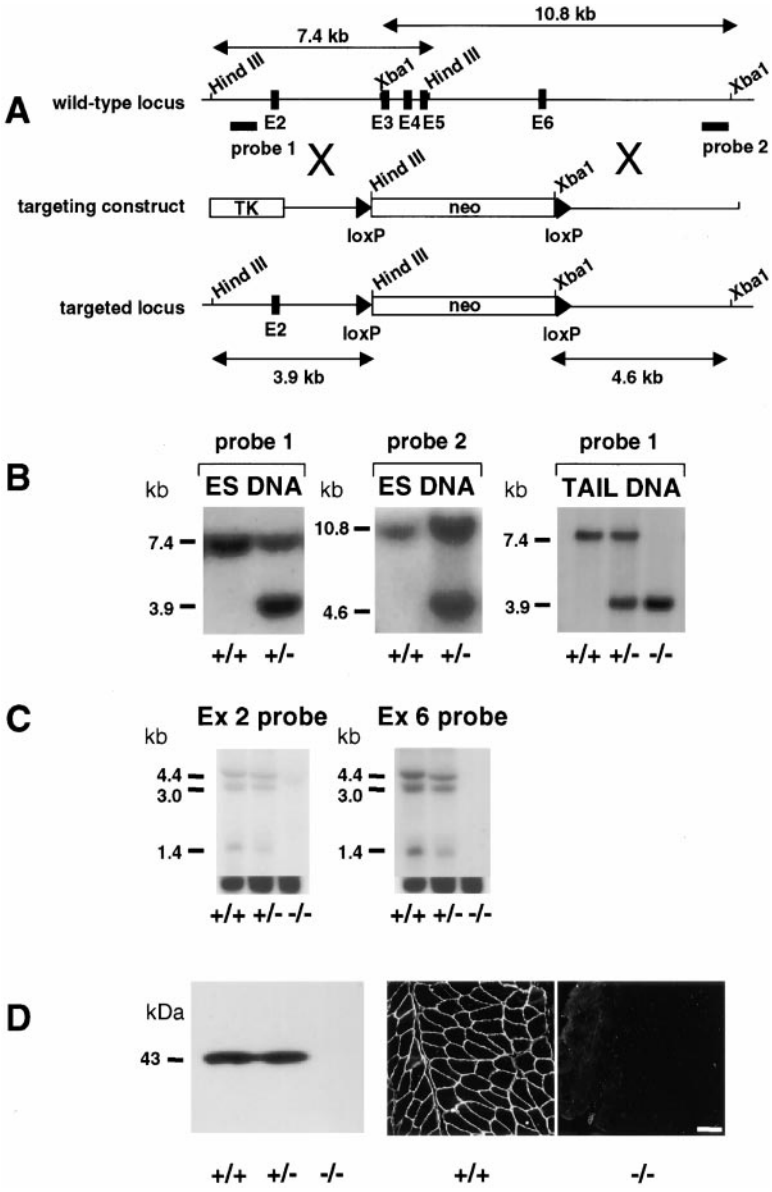

Figure 1. Generation of *Sgcb*-null Mutant Mice

(A) Restriction map of the wild-type *Sgcb*-locus, the targeting construct, and the targeted locus. Exons 3, 4, 5, and 6 were replaced by phosphoglycerate kinase neomycin cassette (neo).

(B) Southern blot analysis. One correctly targeted clone is shown as an example. DNA from ES cells surviving double selection was digested with *Hin*dIII or *Xba*1 and probed by Southern blot with probe 1 and 2 respectively. The replacement of exons 3–6 by the neo cassette yields a new 3.9 kb *Hin*dIII fragment with probe 1 in addition to the 7.4 kb wild type *Hin*dIII fragment. As a consequence of the *Xba*1 site introduced by the neo cassette, probe 2 hybridizes with a 4.6 kb fragment and the 10.8 kb wild-type fragment. Also tail DNA from wild type (+/+), heterozygous (+/−) and *Sgcb*-null (−/−) mice was genotyped using Southern blot analysis.

(C) Northern blot analysis of RNA extracted from skeletal muscle of wild-type, heterozygous, and *Sgcb*-null mice. A cDNA probe against exon 2 detected the previously described β-sarcoglycan transcripts of 4.4, 3.0, and 1.4 kb in both wild-type and heterozygous mice. In contrast, none of the known transcripts were detected in *Sgcb*-null mice. A faint transcript of 4 kb, however, was detected in *Sgcb*-null mice. This transcript could represent a transcript containing exons 1 and 2 and the neocassette. However, our attempts to amplify such a transcript with RT-PCR have failed. Using an exon 6 probe we did not detect any β-sarcoglycan transcripts in *Sgcb*-null animals but they were present in wild-type and heterozygous animals.

(D) Western blot and immunofluorescence analysis. Using monoclonal (see Figure 1D) and polyclonal (data not shown) antibodies directed against the N terminus of β-sarcoglycan (epitopes between amino acids 1–65, encoded from exon 1 and parts of exon 2), Western blot analysis on membrane-enriched preparations revealed the presence of β-sarcoglycan in wild-type and heterozygous mice but not in *Sgcb*-null mice. Likewise, using immunofluorescence analysis with polyclonal antibodies, β-sarcoglycan was visualized at the sarcolemma in wild-type skeletal muscle, whereas no protein could be detected at the sarcolemma of *Sgcb*-null mice. Bar, 50 μm.

Together, our findings suggest additional pathogenetic mechanisms for the development of muscular dystrophy and cardiomyopathy. Absence of the sarcoglycans in smooth muscle leads to vascular irregularities that may aggravate muscular dystrophy and initiate cardiomyopathy. In addition, absence of ε-sarcoglycan in skeletal, cardiac, and smooth muscle may be crucial in the pathogenesis of a severe muscular dystrophy and cardiomyopathy. Thus, our data suggest a novel complex mechanism for the development of limb-girdle muscular dystrophy type 2E.

Results

Generation of *Sgcb*-null Mice

To design a targeting vector for the generation of *Sgcb*-null mice, we characterized a P1 clone containing the murine β-sarcoglycan gene. Murine and human β-sarcoglycan are highly homologous at the amino acid level, and the structural organization of the gene into six exons is shared by both species (GenBank/EMBL/DDBJ accession number AF169288). Given that most human mutations have been found in exons 3, 4, 5, and 6, which encode part of the transmembrane domain and the extracellular portion of β-sarcoglycan (Bönnemann et al., 1995; Lim et al., 1995; Bönnemann et al., 1996, 1998; Duclos et al., 1998a), we targeted these exons to create a mutant allele of *Sgcb* representative of human mutations. Homologous recombination replaced exons 3 to 6 with the phosphoglycerate kinase promoter/neomycin phosphotransferase cDNA (Figure 1A). A total of 361 colonies surviving G418 and gancyclovir selection were analyzed by Southern blotting for the presence of homologous recombination and 15 correctly targeted clones

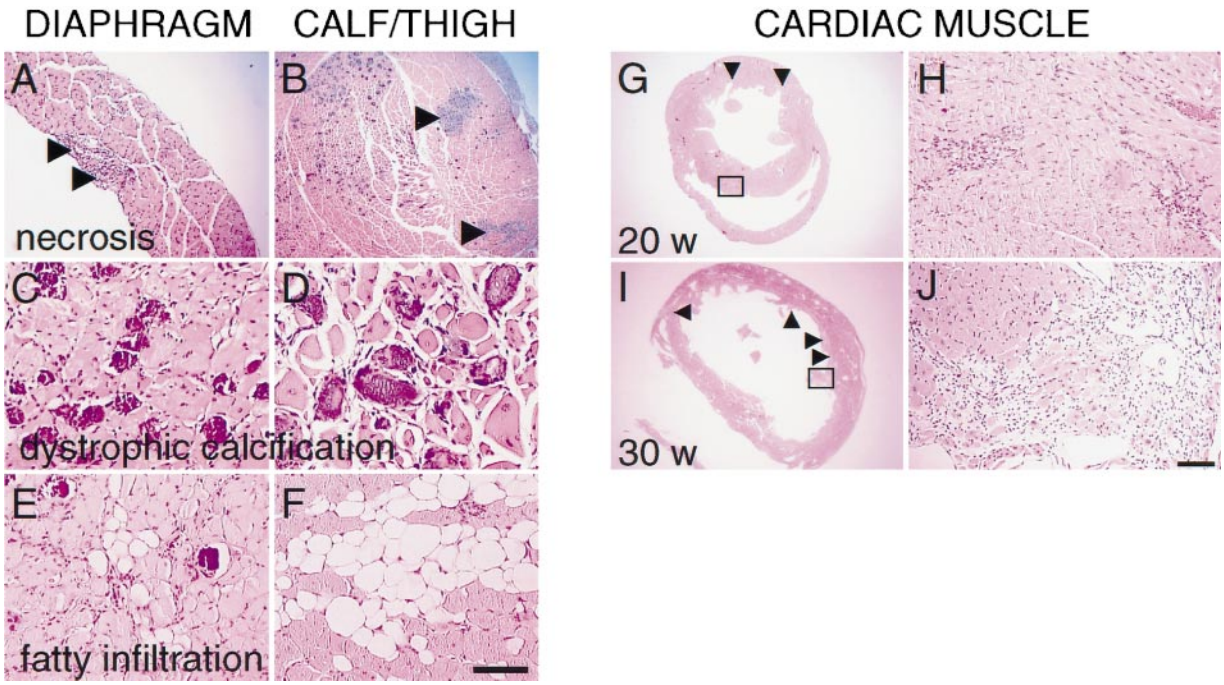

Figure 2. Histological Analysis of Diaphragm, Calf/Thigh, and Cardiac Muscles from *Sgcb*-null Mice

Severe dystrophic changes including necrosis (arrows) (A and B), dystrophic calcification (C and D), fatty infiltration (E and F), central nucleation, atrophy and hypertrophy were detected in diaphragm and calf/thigh of *Sgcb*-null mice (A–F). Some of these changes had already occurred in 4-week-old animals (A) and accumulated with age. In 20-week-old hearts, several regions of focal myocardial necrosis were observed (G). Two such lesions are present within the boxed area in (G), and shown by higher magnification in (H). In 30-week-old heart, large areas of fibrosis were detected (I). One such area is present within the boxed area in I, and shown at higher magnification in (J). Arrows denote additional necrotic and fibrotic areas. All sections were stained with hematoxylin and eosin (H&E). Bar: (A and B) 400 μm; (C–F) 50μm; (G and I) 1.5 mm; (H and J) 150 μm.

were identified (Figure 1B). Three were injected and yielded chimeras producing germline transmission. So far, we have analyzed mice from two clones. Heterozygous mice appeared normal, and homozygous mice were produced in expected numbers in accordance with Mendelian inheritance. Southern blot analysis confirmed the disruption of the β-sarcoglycan gene (Figure 1B). The effect of the mutation on β-sarcoglycan RNA was assessed by Northern blot analysis. A probe specific for exon 6 revealed that none of the known β-sarcoglycan transcripts of 1.4, 3.0, and 4.4 kb were present in skeletal muscle of *Sgcb*-null mice (Figure 1C). An additional hybridization with a probe specific for exon 2 (still present in the genome, along with exon 1) revealed small amounts of a shorter transcript of 4 kb (Figure 1C). However, the absence of β-sarcoglycan in KCl-washed microsomes prepared from skeletal (Figures 1D and 5), cardiac, and lung tissue (Figure 5) of homozygous mice was demonstrated using monoclonal and polyclonal N-terminal antibodies directed to amino acids 1–65 (encoded by exon 1 and parts of exon 2) of β-sarcoglycan. Additionally, immunofluorescence analysis confirmed the absence of β-sarcoglycan in skeletal (Figures 1D and 3), cardiac (data not shown), and smooth muscle (Figure 4).

Sgcb-null Mice Develop a Severe Muscular Dystrophy and Cardiomyopathy

To evaluate the consequences of β-sarcoglycan deficiency, we examined hematoxylin and eosin (H&E) stained sections of the calf, thigh, and diaphragm muscle in wild-type, heterozygous, and *Sgcb*-null mice. Histopathological features of muscular dystrophy were never observed in wild-type or heterozygous animals. In *Sgcb*-null mice, however, pronounced morphological changes were detected. Large areas of necrosis were observed in calf, thigh, and diaphragm muscles of mice at all ages (Figure 2). Other dystrophic changes included internally placed nuclei of nonregenerating fibers (based on the evaluation of 200–1100 myofibers per muscle, 80%–100% of nonregenerating myocytes contained internally placed nuclei at the age of 2 months), fiber splitting and hypertrophy, extensive dystrophic calcification, endomysial fibrosis, and massive fatty infiltration (Figure 2). The *Sgcb*-null mice from both correctly targeted cell lines demonstrated an identical dystrophic phenotype. Our laboratory has previously generated *Sgca*-null mice (Duclos et al., 1998b), and we note that the skeletal muscle pathology was much more severe in *Sgcb*-null mice. Large areas of necrosis and fatty infiltration are not detected in *Sgca*-null mice.

Consistent with the severe dystrophic pattern, 13- to 16-week-old *Sgcb*-null mice displayed elevated serum creatine kinase activity, compared with age-matched wild-type and heterozygous mice (data not shown). Evans blue dye (EBD) is a small molecular mass tracer that tightly complexes with serum albumin. Normally, this is a membrane impermeable molecule, but if the sarcolemma integrity is compromised this dye is found in the cytoplasm of muscle fibers (Matsuda et al., 1995; Straub

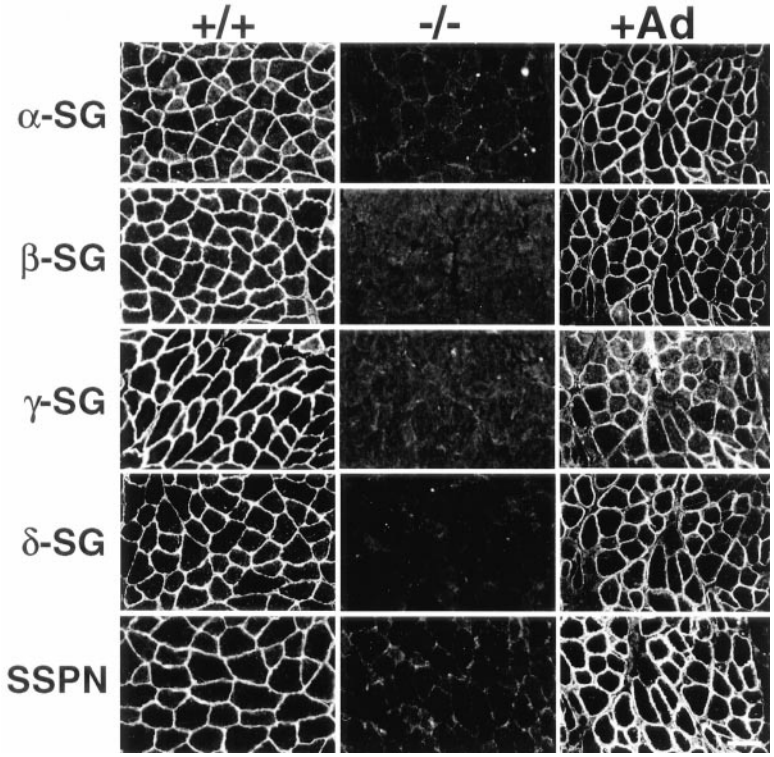

Figure 3. Restoration of the Sarcoglycan–Sarcospan Complex in Skeletal Muscle upon Renewed Expression of β-Sarcoglycan

Skeletal muscle cryosections from wild-type, *Sgcb*-null mice and *Sgcb*-null mice injected with recombinant β-sarcoglycan adenovirus were stained with antibodies against α-sarcoglycan (α-SG), β-sarcoglycan (β-SG), γ-sarcoglycan (γ-SG), δ-sarcoglycan (δ-SG) and sarcospan (SSPN). Upon injection with recombinant β-sarcoglycan adenovirus containing the human sequence under the control of the viral CMV promoter, the entire sarcoglycan–sarcospan complex was restored. Bar, 50 μm.

et al., 1997). EBD injections into 9-week-old *Sgcb*-null mice revealed uptake of the blue dye in various skeletal muscles, whereas EBD staining was never detected in control mice (data not shown).

Dystrophin defects, including Duchenne and Becker muscular dystrophies, are also manifested at the cardiac level (Towbin, 1998). Less is known about the heart involvement in muscular dystrophies caused by sarcoglycan mutations. To evaluate the consequences of β-sarcoglycan deficiency in the heart we performed H&E staining of transverse sections of hearts from wild-type, heterozygous, and *Sgcb*-null mice. We did not see any cardiac abnormalities in control mice of any age. In sharp contrast, we detected small necrotic areas already in 9-week-old hearts from *Sgcb*-null mice (data not shown). The hearts of 20-week-old *Sgcb*-null animals showed more extensive alterations. Prominent necrotic areas, resembling ischemic-like lesions, were present throughout the right and left ventricles (Figure 2). In 30-week-old animals, active myocardial necrosis was less evident, and instead widespread areas of fibrosis were detected (Figure 2).

β-Sarcoglycan Deficiency Causes Loss of the Sarcoglycan–Sarcospan Complex, the Dystroglycan Complex, and ε-Sarcoglycan in Skeletal, Cardiac, and Smooth Muscle

To analyze consequences of β-sarcoglycan deficiency at the molecular level in skeletal, cardiac, and smooth muscle, we performed immunofluorescence and Western blot analysis for each component of the DGC. Immunofluorescence analysis revealed that β-sarcoglycan was absent from the sarcolemma of skeletal muscle fibers in *Sgcb*-null mice (Figures 1 and 3). Also, α-, γ-, and δ-sarcoglycan were concomitantly reduced, along with sarcospan (Figure 3). Dystrophin, utrophin, α- and β-dystroglycan, α-dystrobrevin, and laminin α2 chain staining appeared normal in skeletal muscle of *Sgcb*-null mice (data not shown). Also, the sarcoglycan–sarcospan complex was reduced in cardiac muscle of *Sgcb*-null mice, whereas dystrophin, utrophin, α- and β-dystroglycan, and laminin α2 chain were normally expressed (data not shown).

To test that the observed phenotypes in *Sgcb*-null mice were due to the primary loss of β-sarcoglycan, recombinant β-sarcoglycan adenovirus was injected into the quadriceps femoris muscle of 3-day-old *Sgcb*-null mice, which was assessed for β-sarcoglycan expression 3 weeks later. Approximately 90% of the fibers of vastus lateralis and vastus medialis were converted to β-sarcoglycan positive, and a part of vastus lateralis is shown in Figure 3. Renewed expression of β-sarcoglycan at the sarcolemma was coincident with the rescue of the entire sarcoglycan–sarcospan complex (Figure 3). No β-sarcoglycan positive fibers could be detected in the rectus femoris.

To evaluate if the absence of β-sarcoglycan also affected the expression of the other sarcoglycans and sarcospan in smooth muscle, we performed immunofluorescence analysis on pulmonary arteries. β-, δ-, and ε-sarcoglycan and sarcospan were all expressed in vascular smooth muscle of the pulmonary arteries in wild-type mice, whereas α- and γ-sarcoglycan were not detected (Figure 4). Absence of β-sarcoglycan in vascular smooth muscle also affected the expression of the smooth muscle sarcoglycans, along with sarcospan in *Sgcb*-null mice (Figure 4).

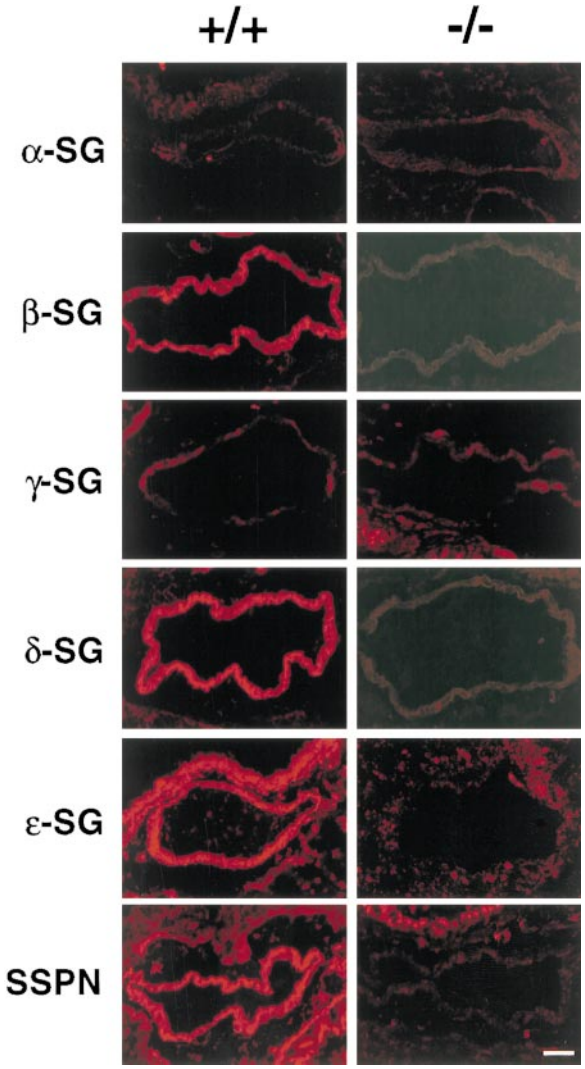

Figure 4. Loss of the Smooth Muscle Sarcoglycan–Sarcospan Complex in *Sgcb*-null Mice

Lung cryosections from wild-type and *Sgcb*-null mice (4 weeks old) were stained with antibodies against α-sarcoglycan (α-SG), β-sarcoglycan (β-SG), γ-sarcoglycan (γ-SG), δ-sarcoglycan (δ-SG), ε-sarcoglycan (ε-SG) and sarcospan (SSPN). Note that the smooth muscle sarcoglycan–sarcospan complex is greatly reduced in smooth muscle of pulmonary arteries in *Sgcb*-null mice. Bar, 50 μm.

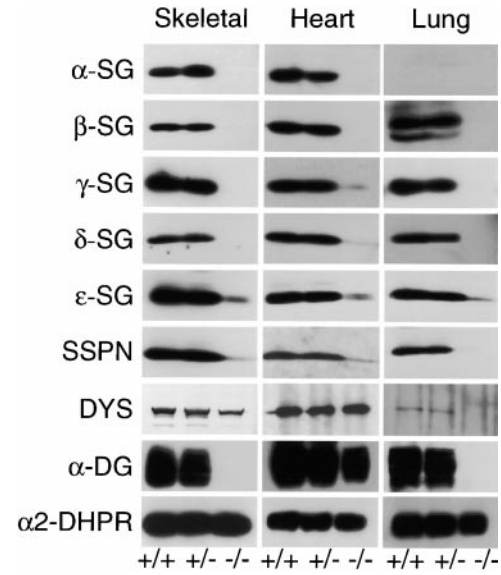

Figure 5. Immunoblot Analysis of Skeletal, Cardiac, and Lung Membranes

Skeletal, cardiac, and lung KCl-washed microsomes from wild-type, heterozygous, and *Sgcb*-null mice were analyzed by 3%–12% SDS-PAGE and immunoblotted with antibodies against the sarcoglycans (α-, β-, γ-, δ- and ε-SG), sarcospan (SSPN), dystrophin (DYS), and α-dystroglycan (α-DG). To demonstrate equal loading of protein samples we used antibodies against the α2 subunit of the dihydropyridine receptor (α2-DHPR).

To further examine the expression of the DGC components, immunoblot analysis was performed on isolated membrane preparations from wild-type, heterozygous, and homozygous mutant skeletal, cardiac, and smooth muscle. In accordance with the immunofluorescence analysis, β-sarcoglycan was absent in skeletal and cardiac muscle membranes of *Sgcb*-null mice (Figure 5). Heterozygous mice expressed control levels of β-sarcoglycan (Figure 5). Furthermore, α-, γ-, and δ-sarcoglycan were concomitantly reduced in both skeletal and cardiac muscle membranes from *Sgcb*-null mice (Figure 5). We used lung tissue as a source for smooth muscle membranes, since the major cell types in the lung are smooth muscle (vascular and airway) and epithelial cells and β-sarcoglycan is, along with δ-sarcoglycan and sarcospan, strictly confined to smooth muscle in lung (Durbeej and Campbell, 1999). As expected, β-sarcoglycan was deficient in lung membranes of *Sgcb*-null mice (Figure 5). As noted above, α-sarcoglycan is not expressed in smooth muscle (Figure 5). Although we did not detect γ-sarcoglycan in any cell type in the lung by immunofluorescence, γ-sarcoglycan was detected by Western blot analysis of wild-type and heterozygous lung membrane preparations (Figure 5). In addition, γ-sarcoglycan was greatly reduced in *Sgcb*-null lungs. Also, δ-sarcoglycan was significantly decreased in smooth muscle membranes of *Sgcb*-null mice (Figure 5). Interestingly, ε-sarcoglycan was greatly reduced in all three muscle lineages of *Sgcb*-null mice (Figure 5), and so was sarcospan (Figure 5). Also, α-dystroglycan was greatly reduced in skeletal muscle membranes of *Sgcb*-null mice. In the supernatant from *Sgcb*-deficient skeletal muscle membrane preparations, α-dystroglycan was enriched and fully glycosylated (data not shown), but obviously failed to be stably anchored to the membrane without the sarcoglycans. In cardiac muscle membranes of *Sgcb*-null mice α-dystroglycan was moderately reduced (Figure 5). Dystrophin was moderately reduced in skeletal muscle membranes of *Sgcb*-null mice, not altered in cardiac muscle membranes, but reduced in smooth muscle membranes. Together, these data indicate that β-sarcoglycan deficiency causes loss of the sarcoglycan–sarcospan complex, the dystroglycan complex, and ε-sarcoglycan in skeletal, cardiac, and smooth muscle.

Vascular Irregularities in *Sgcb*-null Mice

Deficiency of β-sarcoglycan in vascular smooth muscle apparently causes loss of the sarcoglycan–sarcospan

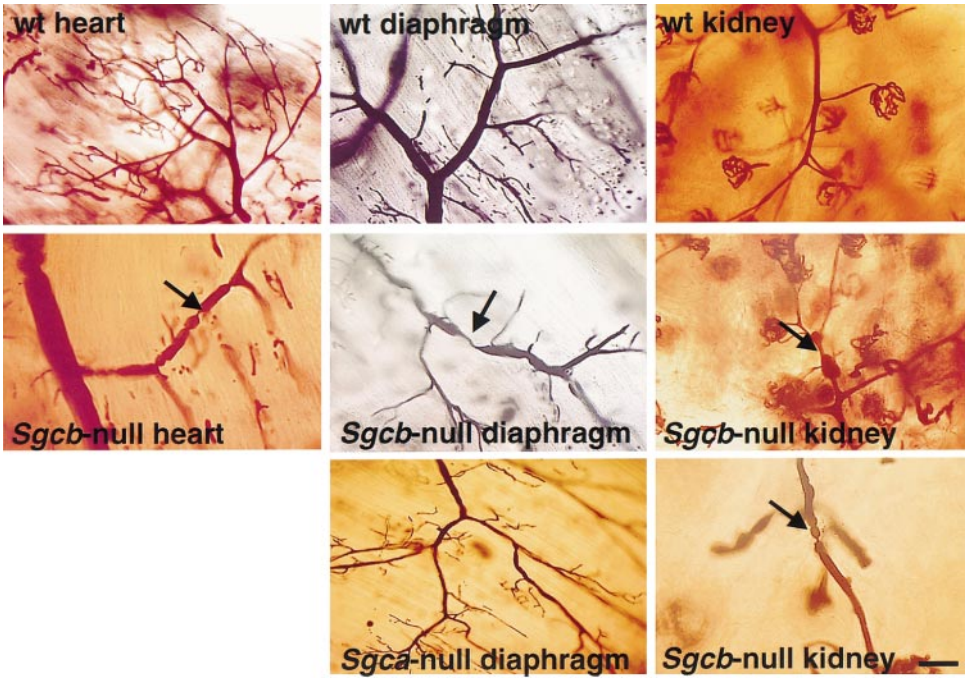

Figure 6. Microfil Perfusion of the Vasculature

Vessels of 4-week-old heart and diaphragm from wild-type mice showed smoothly tapered vessel walls. In contrast, vessels of the heart and diaphragm from *Sgcb*-null mice showed numerous constrictions associated with pre- and poststenotic aneurysms. Also, vessels of 13-week-old kidneys from *Sgcb*-null mice showed constrictions and also aneurysms. Note that vessels of the diaphragm from 1-year-old *Sgca*-null mice did not show any constrictions. Arrows denote constrictions. Bar, 40 μm.

complex in smooth muscle. Moreover, the predominant characteristic feature of the muscular dystrophy and cardiomyopathy was focal areas of necrosis, resembling the pathological observations seen in tissue infarcts occurring in ischemic injury. Therefore, loss of the smooth muscle complex in the vasculature and the presence of necrotic areas prompted us to analyze whether the presence of abnormalities in the vasculature contributed to the pathological changes of skeletal and cardiac muscle. To study the organization of various vascular beds in skeletal and cardiac muscle we used the Microfil perfusion technique in vivo. We perfused wild-type and *Sgcb*-null mice at 4 weeks of age, and cleared sections of the diaphragm and heart were analyzed using transilluminations with low-power magnification. Interestingly, *Sgcb*-null mice exhibited numerous areas of vascular constrictions often associated with pre- and poststenotic aneurysms in the vasculature of both diaphragm and heart, which was never detected in wild-type mice (Figure 6). In addition, the vessels of *Sgcb*-null mice exhibited a serrated contour, rather than the smoothly tapered vessel walls that are seen in wild-type mice. Functional disturbance of the coronary artery microvasculature was detected at an age of 4 weeks, before any overt signs of cardiac morphological alterations were observed. Similarly, vascular irregularities in the diaphragm were observed in 4-week-old *Sgcb*-null mice, at a time when acute necrosis starts to occur in the skeletal muscle. These observations indicate that the disturbance of the vasculature precedes the onset of ischemic-like lesions in *Sgcb*-null mice. To further ascertain that the vascular phenotype is independent of the muscular dystrophy phenotype, we also analyzed Microfil-perfused diaphragms from *Sgca*-null mice. *Sgca*-null mice develop a progessive muscular dystrophy and fibrotic areas are detected in their diaphragms (Duclos et al., 1998b). However, since α-sarcoglycan is not expressed in vascular smooth muscle, *Sgca*-null mice should not display any vascular perturbations, at any age. Indeed, no constrictions were detected in diaphragms of 1-year-old *Sgca*-null mice (Figure 6). Furthermore, we also analyzed Microfil-perfused kidneys from *Sgcb*-null kidneys. β-sarcoglycan is also expressed in kidney vascular smooth muscle (Durbeej and Campbell, 1999) and, interestingly, we also detected vascular constrictions in *Sgcb*-null kidneys, but never in wild-type kidneys (Figure 6). Together these data support our hypothesis that the muscle degeneration does not cause the vascular phenotype.

Presence of a Distinct ε-Sarcoglycan Complex in Skeletal Muscle

Although ε-sarcoglycan is expressed in skeletal muscle (Ettinger et al., 1997; McNally et al., 1998), there are no reports of ε-sarcoglycan being associated with the skeletal muscle sarcoglycans. In membrane preparations of *Sgcb*-null mice, we found that ε-sarcoglycan was greatly reduced, suggesting that ε-sarcoglycan could be part of a skeletal muscle sarcoglycan complex. To test this hypothesis we isolated DGC from skeletal muscle of wild-type, *Sgca*-null, and *Sgcb*-null mice. The skeletal muscle DGC was extracted by digitonin and further purified by WGA affinity chromatography, followed by centrifugation of the skeletal muscle DGC

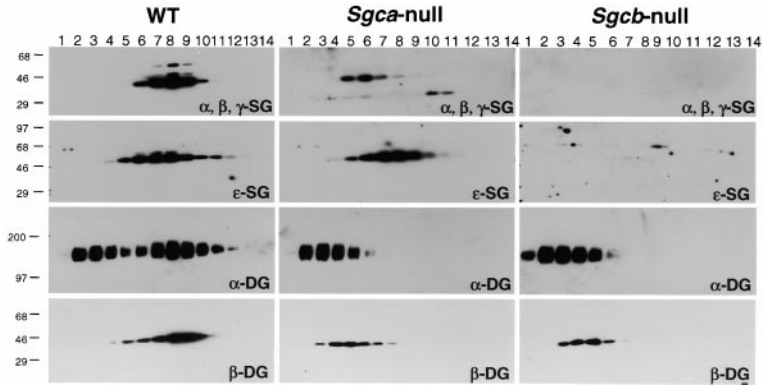

Figure 7. Biochemical Characterization of the Sarcoglycan and Dystroglycan Complexes in Wild-Type, *Sgca*-null, and *Sgcb*-null Mice

The DGC was purified from skeletal muscle of wild-type, *Sgca*-null, and *Sgcb*-null mice and centrifuged through sucrose gradients. Fractions 1–14 from the sucrose gradients were electrophoresed on 3%–12% polyacrylamide gels. Nitrocellulose transfers of identical samples were stained with antibodies against the sarcoglycans (α-, β-, γ-SG) and the dystroglycans (α- and β-DG). The sarcoglycan and dystroglycan complexes were disrupted in both *Sgca*- and *Sgcb*-null mice, whereas ε-sarcoglycan was disrupted only in *Sgcb*-null mice.

through sucrose gradients. The migration of the DGC complex during high-speed centrifugation through sucrose gradients has previously been demonstrated (Crosbie et al., 1998). Proteins from the sucrose gradient fractions were separated by SDS-PAGE. Immunoblotting with antibodies against the sarcoglycans revealed that α-, β-, γ-, and δ-sarcoglycan migrated in fractions 7–9 in wild-type mice (Figure 7 and data not shown). Western blotting of the same fractions with antibodies against ε-sarcoglycan demonstrated that ε-sarcoglycan comigrated in the same fractions as the other sarcoglycans, along with α- and β-dystroglycan, although a peak of α-dystroglycan was also seen in earlier fractions (Figure 7). In the *Sgca*-null mice, α-sarcoglycan was absent and β-sarcoglycan was greatly reduced. Some γ- and δ-sarcoglycan remained, but peaked in earlier fractions (5–7 instead of 7–9) (Figure 7 and data not shown). ε-sarcoglycan, however, remained in fractions 7–9. In contrast, α-dystroglycan was absent in fractions 7–9, but was still present in the earlier fractions (Figure 7). β-dystroglycan was also absent from fractions 7–9, but some β-dystroglycan was still present in fractions 4–6, although the remaining β-dystroglycan was not associated with the remaining α-dystroglycan or the remaining γ-sarcoglycan. Together, these results indicate that deficiency of α-sarcoglycan causes dissociation of the sarcoglycan and dystroglycan complexes, without affecting the presence of ε-sarcoglycan. This is in contrast to DGC preparations from *Sgcb*-null mice, in which ε-sarcoglycan was greatly reduced (Figure 7). Also, in DGC preparations from *Sgcb*-null mice all the sarcoglycans were absent (Figure 7). α-dystroglycan was absent from fractions 7–9, but remained in the earlier fractions. Some β-dystroglycan also remained in *Sgcb*-null mice. In summary, loss of β-sarcoglycan causes dissociation of the sarcoglycan and dystroglycan complex and also of ε-sarcoglycan, whereas mice deficient for α-sarcoglycan show normal ε-sarcoglycan expression. These data suggest the presence of an ε-sarcoglycan-containing complex in skeletal muscle. However, it is not associated with the tetrameric unit of α-, β-, γ-, and δ-sarcoglycan, since this complex is very much reduced in *Sgca*-null mice and ε-sarcoglycan is retained in these mice. Nevertheless, the expression of ε-sarcoglycan is obviously affected by the β-sarcoglycan mutation, suggesting that β-sarcoglycan and ε-sarcoglycan may be associated.

To further characterize possible molecular interactions with ε-sarcoglycan, we analyzed isolated DGC from skeletal muscle of dystrophin-deficient mice (*mdx* mice) and mice deficient in both dystrophin and utrophin (*mdx*/*utr*$^{-/-}$) (Grady et al., 1997). ε-sarcoglycan expression remained in both *mdx* and *mdx*/*utr*$^{-/-}$ mice (Figure 8A), indicating that ε-sarcoglycan is not associated with dystrophin or utrophin.

Recombinant Adenovirus-Mediated Gene Transfer of β-Sarcoglycan Restores ε-Sarcoglycan Expression in Skeletal Muscle

To further test the hypothesis that β- and ε-sarcoglycan are associated in skeletal muscle we took an in vivo approach. Recombinant β-sarcoglycan adenovirus was injected into quadriceps femoris muscle of 3-day-old *Sgcb*-null mice to determine if restored β-sarcoglycan also reconstituted ε-sarcoglycan expression. After a 16-d expression period, glycoprotein preparations from the whole quadriceps femoris were examined by immunoblot analysis using antibodies against ε-sarcoglycan and the sarcoglycan–sarcospan complex as indicated in Figure 8. The *Sgcb*-null muscle injected with β-sarcoglycan adenovirus exhibited renewed expression of ε-sarcoglycan and the entire sarcoglycan–sarcospan complex. ε-sarcoglycan and the sarcoglycan–sarcospan complex was restored to about 20%–30% of control levels, which is in accordance with the fact that the vastus lateralis and vastus medialis constitute approximately 20%–30% of the total quadriceps femoris. The finding that ε-sarcoglycan is restored upon adenovirus-mediated gene transfer of β-sarcoglycan strengthens our conclusion that β- and ε-sarcoglycan are associated with one another.

Discussion

We have engineered mice deficient for β-sarcoglycan through homologous recombination in embryonic stem cells. β-sarcoglycan is expressed in cardiac as well as skeletal muscle, but little is known about cardiac involvement in LGMD type 2E (caused by mutations in β-sarcoglycan) (Melacini et al., 1999). Moreover, β-sarcoglycan is also expressed in smooth muscle, and symptoms potentially due to malfunctions of smooth muscle have been reported in patients with Duchenne muscular dystrophy (Bahron et al., 1988; Jaffe et al., 1990). We therefore analyzed skeletal, cardiac, and smooth muscle in mice deficient for β-sarcoglycan. Mice lacking β-sarcoglycan are viable and fertile and have now lived for one year. However, *Sgcb*-null mice develop a progressive

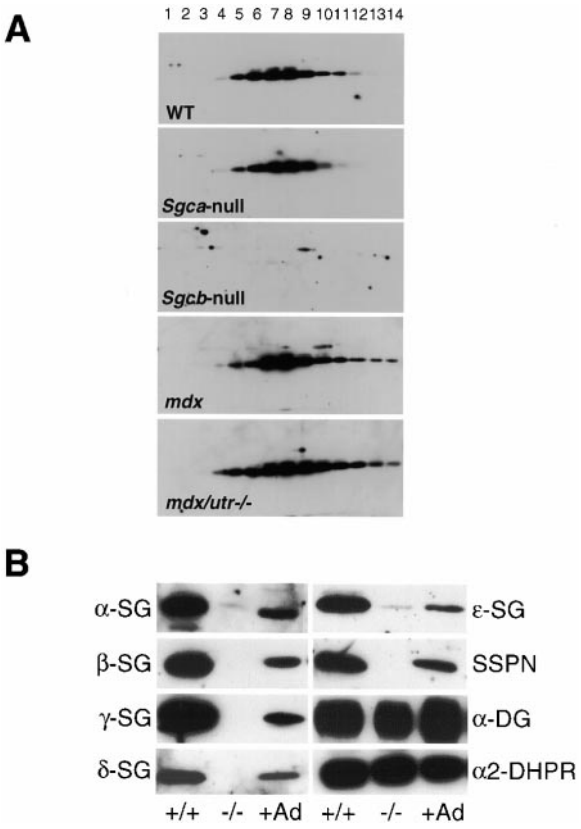

Figure 8. ϵ-Sarcoglycan Is Not Associated with Dystrophin or Utrophin, and ϵ-Sarcoglycan Expression Is Restored in Skeletal Muscle upon Adenovirus-Mediated Gene Transfer of β-Sarcoglycan

(A) The DGC was purified from skeletal muscle of *mdx* and *mdx/utr*$^{-/-}$ mice and centrifuged through sucrose gradients. Fractions 1–14 from the sucrose gradients were electrophoresed on 3%–12% polyacrylamide gels. Nitrocellulose transfers were stained with antibodies against ϵ-sarcoglycan. The blots were compared with the ϵ-sarcoglycan blots from wild-type, *Sgcb*-null, and *Sgca*-null mice shown in Figure 7, demonstrating that the ϵ-sarcoglycan complex is intact in mice lacking dystrophin and utrophin.

(B) Glycoprotein preparations from quadriceps muscle of wild-type, *Sgcb*-null, and *Sgcb*-null mice injected with 2×10^{10} particles of β-sarcoglycan adenovirus were analyzed after 16 days by SDS-PAGE and immunoblotting using antibodies against α-sarcoglycan (α-SG), β-sarcoglycan (β-SG), γ-sarcoglycan (γ-SG), δ-sarcoglycan (δ-SG), ϵ-sarcoglycan (ϵ-SG), sarcospan (SSPN) and α-dystroglycan (α-DG) as indicated. To demonstrate equal loading of protein samples, we used antibodies against the α2 subunit of the dihydropyridine receptor (α2-DHPR).

muscular dystrophy similar to human sarcoglycan-deficient LGMD type 2E (Bönnemann et al., 1995, 1996, 1998; Lim et al., 1995; Duclos et al., 1998a; Araishi et al., 1999). LGMD type 2E is a genetically and clinically heterogeneous disease; in humans the symptoms can vary from mild to very severe (Bönnemann et al., 1995, 1996, 1998; Lim et al., 1995; Duclos et al., 1998a). *Sgcb*-null mice develop a severe muscular dystrophy and are associated with elevated CK levels, extensive central nucleation, pronounced areas of focal necrosis, and a high degree of dystrophic calcification and fatty infiltration. Moreover, *Sgcb*-null mice develop a marked cardiomyopathy, which is variably detected in patients with LGMD type 2E (Barresi et al., 1999). Focal areas of necrotic cardiomyocytes are evident already in 9-week-old *Sgcb*-null animals, and at 30 weeks of age prominent areas of fibrosis are detected. Absence of β-sarcoglycan results in dissociation of the sarcoglycan and dystroglycan complexes not only in striated muscle but also in smooth muscle. It is therefore interesting to note that *Sgcb*-null mice show pronounced constrictions of numerous blood vessels, indicating that the loss of the sarcoglycan complex in the smooth muscle affects vascular function. Vessel constriction can lead to ischemic injury of the tissue, and the end result is the presence of a localized area of necrosis, very similar to what is seen in both skeletal and cardiac muscle of *Sgcb*-null mice. Thus, our data strongly suggest that loss of the sarcoglycan complex in the vascular smooth muscle provokes vascular perturbation, which in turn exaggerates the muscular dystrophy and initiates cardiomyopathy. Several lines of evidence support our conclusion. First, constrictions of the vessels were detected before the appearance of necrosis. Second, our laboratory recently developed *Sgca*-null mice (deficient in α-sarcoglycan) that develop a less severe muscular dystrophy compared with *Sgcb*-null mice and do not exhibit cardiomyopathy. α-sarcoglycan is specifically restricted to striated muscle (Roberds et al., 1993; Ettinger et al., 1997; Liu et al., 1997), whereas β-sarcoglycan is expressed in both striated and smooth muscle (Bönnemann et al., 1995; Lim et al., 1995; Durbeej and Campbell, 1999; Straub et al., 1999) (see also Figures 3 and 4). The smooth muscle sarcoglycan complex is composed of β-, δ-, and ϵ-sarcoglycan (Durbeej and Campbell, 1999; Straub et al., 1999) (see also Figures 4 and 5). Deficiency of β-sarcoglycan should affect the DGC in smooth muscle of *Sgcb*-null mice, whereas smooth muscle of *Sgca*-null mice should be unaltered. Indeed, the absence of β-sarcoglycan in smooth muscle of vasculature, bladder, and esophagus affects the expression of δ- and ϵ-sarcoglycan, along with sarcospan and α-dystroglycan (Figures 4 and 5 and data not shown), whereas the smooth muscle complex is unaltered in *Sgca*-null mice (Straub et al., 1999). Consequently, perturbation of the vasculature cannot be detected in *Sgca*-null mice. Third, data from our laboratory indicate that the disruption of the sarcoglycan complex in vasculature of δ-sarcoglycan-deficient mice also perturbs vascular function and leads to ischemic inury (Coral-Vazquez et al., 1999). Finally, the cardiomyopathic BIO 14.6 hamster, which has a primary mutation in the δ-sarcoglycan gene (Sakamoto et al., 1997), displays cardiac abnormalities and is associated with microvascular dysfunction (Factor et al., 1982).

Hack et al. (1998) recently presented a mouse deficient for γ-sarcoglycan that displays muscular dystrophy and cardiomyopathy. Using several polyclonal and monoclonal antibodies specific for γ-sarcoglycan, we have not been able to detect γ-sarcoglycan in smooth muscle or in any other cell type of lung by immunofluorescence. Using the same antibodies in Western blot analysis, however, we have detected γ-sarcoglycan in lung preparations. In addition, absence of β-sarcoglycan perturbs expression of γ-sarcoglycan in lung membrane preparations. Thus, we have genetic evidence to suggest that γ-sarcoglycan may be part of a smooth muscle sarcoglycan complex.

The obvious phenotypic differences between the *Sgca*- and *Sgcb*-null mice prompted us to analyze these mice biochemically for the expression of various DGC components on isolated membranes and purified DGC. Interestingly, ε-sarcoglycan is greatly reduced in *Sgcb*-null mice, whereas its expression is preserved in *Sgca*-null mice (see Figure 7). Thus, our data point toward the presence of at least two distinct sarcoglycan complexes in skeletal muscle. One is composed of the well-characterized tetrameric unit of α-, β-, γ-, and δ-sarcoglycan, and the other one is composed of ε-sarcoglycan, β-sarcoglycan, and yet unidentified partners. ε-sarcoglycan is not associated with dystrophin or utrophin, yet the sucrose gradient fractionation indicates that the ε-sarcoglycan complex is as large as the DGC (18S), since the two complexes migrate in the same fractions. Only the DGC including the tetrameric sarcoglycan complex is disrupted in the *Sgca*-null mice, whereas both the DGC and the ε-sarcoglycan complex are disrupted in *Sgcb*-null mice. Also, the β-sarcoglycan gene transfer experiments strengthen our hypothesis that β- and ε-sarcoglycan are associated. Upon renewed expression of β-sarcoglycan, not only is the entire sarcoglycan–sarcospan complex restored, but so is ε-sarcoglycan expression. Taken together, the more severe phenotype seen in the *Sgcb*-null mice compared with *Sgca*-null mice may not only be due to perturbation of vascular function, but also to differential disruption of the ε-sarcoglycan-containing complex. This might also be the case for *Sgcd*-null mice (deficient in δ-sarcoglycan), which show a severe muscular dystrophy and cardiomyopathy due to the presence of coronary vascular constrictions. Indeed, preliminary data show that ε-sarcoglycan is also reduced in striated muscle of *Sgcd*-null mice (unpublished data).

In summary, we have generated and characterized mice deficient in β-sarcoglycan. These mice exhibit a severe muscular dystrophy, cardiomyopathy, and vascular smooth muscle irregularities. Thus, *Sgcb*-null mice should be useful for providing novel insights into the pathogenesis of muscular dystrophy and cardiomyopathy. In addition, they should be useful for studying smooth muscle dysfunction. Moreover, the β-sarcoglycan-deficient mice will also be valuable for evaluation of new therapeutic approaches directed towards skeletal, cardiac, and smooth muscle.

Experimental Procedures

Construction of Targeting Vector

*Hin*dIII fragments of a P1 clone containing the mouse β-sarcoglycan gene (obtained from Genome Systems) were subcloned into pBluescript KS (+) (pBS) and analyzed using restriction mapping and sequencing (GenBank/EMBL/DDBJ accession number AF 169288). The long arm of homology in the targeting vector was a 7.2 kb *Hin*dIII fragment upstream of exon 6, which had been subcloned into pBS and cut with *Xho*1 to generate a 6.5 kb fragment. The short arm was a 1.8 kb Kpn1 fragment carrying approximately half of the intron between exons 2 and 3. These fragments were inserted into cloning sites of pPNT flanking a PGK-neomycin resistance cassette. The vector included a thymidine kinase cassette distal to the short arm. The mutant gene therefore lacked ~7.5 kb, which included exons 3, 4, 5, and 6.

Generation of *Sgcb*-Deficient Mice

The targeting vector was linearized with Not1 and transfected into 2×10^7 R1 ES cells by electroporation (240 V, 500 μF; Bio-Rad Gene Pulser; Hercules, CA). Clones surviving G418 and gancyclovir were isolated. Targeting fidelity was determined by Southern blot analysis. Cells from three correctly targeted clones were microinjected into C57BL/6J blastocysts and transferred into pseudopregnant recipients. Chimeras from the three independently derived ES cells gave rise to heterozygous mice, which in turn were mated to generate homozygous mutants that were genotyped using Southern blot analysis on DNA from tail biopsies. All animals were kept in the animal care unit of the University of Iowa College of Medicine according to the animal care guidelines.

Northern Blot Analysis

Total RNA from control, heterozygous, and homozygous-null mutant skeletal muscle was extracted using RNAzol B (Tel-Test, Friendswood, TX) according to manufacturer specifications. Twenty micrograms 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 with either a 203 bp exon 2 specific probe or a 253 bp exon 6 specific probe. Washes were carried out at 65°C in 1× SSC/1% SDS initially, then 0.1× SSC/1% SDS. Blots were exposed for autoradiography.

Histopathology Studies

Wild-type, heterozygous, and *Sgcb*-null mice were anesthetized with Metofane (Schering-Plough, Union, NJ). Subsequently, the animals were perfused with 15 ml of PBS, followed by 15 ml of 10% buffered formalin fixative solution. After embedding the tissue in paraffin, hematoxylin and eosin (H&E) stained sections (4 μm) were prepared to characterize skeletal and cardiac muscle pathology.

Evans Blue Dye Injection and Serum Creatine Kinase Analysis

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 μm. Mice were anesthetized with Metofane and injected in the retro-orbital sinus with 0.05 ml/10 g of body weight of the dye solution. Animals were euthanized 4 hours after injection by cervical dislocation. Muscle cryosections for microscopic Evans blue detection were incubated in acetone at −20°C for 10 minutes and, after a rinse with PBS, sections were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed under a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Thornwood, NY). Quantitative, kinetic determination of creatine kinase activity in serum of wild-type, heterozygous, and *Sgcb*-null mice was measured using a Hitachi 917 on blood drawn from retro-orbital sinus.

Antibodies

Monoclonal antibody IIH6 against α-dystroglycan (Ervasti and Campbell, 1991) was previously characterized. Monoclonal antibodies Ad1/20A6 against α-sarcoglycan, βSarc/5B1 against β-sarcoglycan, and 35DAG/21B5 against γ-sarcoglycan were generated in collaboration with L.V.B. Anderson (Newcastle General Hospital, Newcastle upon Tyne, UK). Monoclonal antibody 43DAG/8D5 against β-dystroglycan was generated by L.V.B. Anderson. Rabbit polyclonal antibodies against α-sarcoglycan (rabbit 98) (Roberds et al., 1993), δ-sarcoglycan (rabbits 215 and 229) (Holt et al., 1998), γ-sarcoglycan (rabbit 245) (Durbeej and Campbell, 1999), ε-sarcoglycan (rabbit 232) (Duclos et al., 1998b), sarcospan (rabbit 235) (Duclos et al., 1998b), dystrophin (rabbit 31) (Ohlendieck and Campbell, 1991), the α2 subunit of dihydropyridine receptor (rabbit 136) (Ohlendieck and Campbell, 1991), and the laminin α2 chain (Allamand et al., 1997) were described previously. The goat polyclonal antibody against β-sarcoglycan (goat 26) was also described previously (Duclos et al., 1998b). An affinity purified rabbit polyclonal antibody (rabbit 256) was produced against an NH_2-terminal fusion protein (amino acids 1–25) of sarcospan. Mark Grady and Joshua Sanes (Washington University School of Medicine) kindly provided rabbit antibody 692 against α-dystrobrevin.

Microfil Perfusion

Wild-type, *Sgcb*-null, and *Sgca*-null mice were anesthetized with 75 mg/kg body weight Phenobarbital and a bilateral sternum incision was performed to expose the left atrium. We perfused 1 ml of Microfil, a silicon rubber (Flow Tech., Carver, MA), into the left atrium. The heart continued beating for about 1 minute. After contraction stopped, the heart, diaphragm, and kidneys were rapidly excised and cured on ice for about 10 minutes. Adequacy of vascular perfusion was judged by the white blush that developed in the ventricular wall, as well as a white filling of other main arteries, including the mesenteric and femoral arteries. The hearts, diaphragms, and kidneys were fixed in 10% formalin for 48–72 hours and cardiac and kidney tissue was sectioned into 2 mm thick transverse cross sections. The diaphragms were taken out as whole tissue and were not further cut. The tissues were subsequently cleared by sequential 24 hour immersions in 25%, 50%, 75%, 95%, and finally 100% ethanol. On day 6, specimens were placed in pure methyl salicylate for 12–24 hours. All steps were carried out at room temperature. Microvascular perfusion was visualized with transillumination and examined under low-power magnification.

Immunofluorescence Analysis

For immunofluorescence analysis 7 μm transverse cryosections were prepared from wild-type, heterozygous, and *Sgcb*-null mutant skeletal muscle, cardiac muscle, lung, bladder and esophagus. The following steps were all performed at room temperature. Sections were blocked with 5% BSA in PBS for 20 minutes and then incubated with the primary antibodies for at least 90 minutes. After washing with PBS, sections were incubated with Cy3-conjugated secondary antibodies (1:200) for 1 hour and then washed in PBS. Subsequently, sections were mounted with Vectashield mounting medium and observed under a Zeiss Axioplan fluorescence microscope or an MRC-600 laser scanning confocal microscope (Bio-Rad Laboratories, Hercules, CA).

Immunoblot Analysis of Membrane Preparations

KCl-washed membranes from skeletal and cardiac muscle and lung were prepared as described previously (Ohlendieck and Campbell, 1991), with the addition of two protease inhibitors, calpeptin and calpain inhibitor I (Duclos et al., 1998b). Membranes were resolved by SDS-PAGE on 3%–12% linear gradient gels and transferred to nitrocellulose membranes. Immunoblot staining was performed as previously described (Ohlendieck and Campbell, 1991). Blots were also developed using enhanced chemiluminescence (SuperSignal, Pierce Chemical Co.).

Sucrose Gradient Fractionation of Skeletal Muscle Dystrophin–Glycoprotein Complex

Skeletal muscle (1.5 g) was dissected from wild-type, *Sgca*-null, *Sgcb*-null, *mdx*, and *mdx/utr*$^{-/-}$ mice and snap frozen in liquid nitrogen. Frozen tissue was pulverized using a mortar and pestle cooled with liquid nitrogen. The tissues were solubilized by dounce homogenization in 10 ml cold buffer A (50 mM Tris–HCl, pH 7.4, 500 mM NaCl, 1% digitonin) with a cocktail of protease inhibitors (0.6 μg/ml pepstatin A, 0.5 μg/ml aprotinin, 0.5 μg/ml leupetin, 0.1 mM PMSF, 0.75 mM benzamidine, 5 μM calpain inhibitor I, and 5 μM calpeptin). The homogenate was rotated at 4°C for 1 hour and subsequently spun at 142,400 *g* for 37 minutes at 4°C. The pellets were resolubilized with 5 ml buffer A, rotated at 4°C for 30 minutes, and centrifuged as before. The two supernatants were pooled and incubated at 4°C with WGA-Agarose (Vector Laboratories). The WGA-Agarose was washed extensively in buffer B (50 mM Tris–HCl, pH 7.4, 500 mM NaCl, 0.1% digitonin with the above-described cocktail of protease inhibitors) and proteins were eluted with 0.3 M *N*-acetyl glucosamine (Sigma Chemical Co.) in buffer B. Samples were concentrated to 0.3 ml, diluted 5-fold in buffer B, again concentrated to 0.3 ml using Centricon 30 filters, and applied to a 5%–30% sucrose gradient at pH 7.4, as described previously (Ervasti et al., 1991).

Recombinant Adenovirus and Adenovirus Injections

The β-sarcoglycan adenovirus was constructed by subcloning the human β-sarcoglycan cDNA into the pAdCMVpA adenovirus shuttle vector. The β-sarcoglycan construct was incorporated into an adenovirus vector through standard methods of homologous recombination with Ad5 backbone dl309 by the University of Iowa Gene Transfer Vector Core. Recombinant viruses were purified using established methods (Graham and van der Eb, 1973; Davidson et al., 1994). Lysates from infected 293 cells were collected and tested for expression of β-sarcoglycan using Western blot analysis. Recombinant virus was plaque purified, amplified, and purified by CsCl gradient centrifugation. For adenovirus injections, 2×10^{10} viral particles in 20 μl of normal saline were injected into the quadriceps femoris muscle of 3-day-old *Sgcb*-null mice. After 16 days, injected quadriceps (along with quadriceps from age-matched wild-type and noninjected *Sgcb*-null mice) were collected and snap frozen in liquid nitrogen. Glycoprotein preparations were prepared as described previously (Holt et al., 1998). For immunofluorescence evaluations, quadriceps were collected after 21 days.

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