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Expression of γ-Sarcoglycan in Smooth Muscle and Its Interaction with the Smooth Muscle Sarcoglycan-Sarcospan Complex*

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The sarcoglycan complex in striated muscle is a heterotetrameric unit integrally associated with sarcospan in the dystrophin-glycoprotein complex. The sarcoglycans, α, β, γ, and δ, are mutually dependent with regard to their localization at the sarcolemma, and mutations in any of the sarcoglycan genes lead to limb-girdle muscular dystrophies type 2C–2F. In smooth muscle β- and δ-sarcoglycans are associated with ε-sarcoglycan, a glycoprotein homologous to α-sarcoglycan. Here, we demonstrate that γ-sarcoglycan is also a component of the sarcoglycan complex in the smooth muscle. First, we show the presence of γ-sarcoglycan in a number of smooth muscle-containing organs, and we verify the existence of identical transcripts in skeletal and smooth muscle. The specificity of the expression of γ-sarcoglycan in smooth muscle was confirmed by analysis of smooth muscle cells in culture. Next, we provide evidence for the association of γ-sarcoglycan with the sarcoglycan-sarcospan complex by biochemical analysis and comparison among animal models for muscular dystrophy. Moreover, we find disruption of the sarcoglycan complex in the vascular smooth muscle of a patient with γ-sarcoglycanopathy. Taken together, our results prove that the sarcoglycan complex in vascular and visceral smooth muscle consists of ε-, β-, γ-, and δ-sarcoglycans and is associated with sarcospan.

The sarcoglycan-sarcospan complex (SGC)[1] is part of the dystrophin-glycoprotein complex (DGC), a group of proteins well characterized in skeletal and cardiac muscle. The DGC also includes dystrophin, dystroglycan (α- and β-), and syntrophins (for reviews see Refs. 1–4). Recently, other proteins such as dystrobrevin (5–7) and neuronal nitric-oxide synthase (8–10) have been shown to correlate with the DGC at the sarcolemma. The interaction of the DGC with components of the extracellular matrix (11–13) may have an important role in force transmission and in sarcolemmal protection (14–16). The importance of the SGC in maintaining the sarcolemmal stability is evident from the various forms of limb-girdle muscular dystrophy (LGMD) caused by mutations in any of the genes coding for the SGs (17–21). In addition, a functional role for the SGC in signaling has been hypothesized (22, 23).

Several mouse models of muscular dystrophy have been engineered in which α- (*Sgca*-null) (24, 25), β- (*Sgcb*-null) (26, 27), γ- (*Sgcg*-null) (28), or δ-SG (*Sgcd*-null) (29, 30) genes have been disrupted. In addition to the skeletal muscle pathology, all but the *Sgca*-null mice show a cardiomyopathic phenotype. Furthermore, a spontaneous mutant, the δ-SG-deficient BIO14.6 hamster, has been studied as an animal model for dilated and hypertrophic cardiomyopathy (31, 32). Correspondingly, patients with defects in β-, γ-, and δ-SGs, but not α-SG, are occasionally affected by dilated cardiomyopathy (33–35).

In skeletal and cardiac muscle, the SGC is a heterotetrameric unit composed of the transmembrane glycoproteins α-, β-, γ-, and δ-SGs. The synthesis of all four of the proteins is required in order to ensure the proper localization of the complex to the cell surface membrane (36); thus, the occurrence of a single mutated SG causes the loss or the reduction of the other SGs at the plasma membrane.

Whereas the expression of α-SG is restricted to striated muscle cells (37), a recently identified homologous protein, ε-sarcoglycan, is also expressed in several other tissues (38, 39). In skeletal muscle ε-SG interacts with β-, γ-, and δ-SGs constituting a second SGC that co-exists with the conventional one (25), whereas in smooth muscle it associates with β- and δ-SGs and sarcospan (11, 40). Since most studies indicate the presence of four SGs as a requirement for the functionality of the complex, it is expected that the smooth muscle SGC may either contain a yet unidentified sarcoglycan, a homologue of γ-SG, or γ-SG itself.

In previous studies the γ-SG transcript and protein expression have been considered confined to striated muscle (18, 41). However, recent reports showed the presence of the γ-SG transcript in lung (11). Nevertheless, attempts to immunolocalize γ-SG in smooth muscle have failed, and the question whether γ-SG or a smooth muscle isoform existed was unanswered.

Addressing this issue, the present study demonstrates that γ-SG itself is a component of the DGC complex in smooth muscle. By using immunoblot analysis of several smooth muscle-containing organs from wild-type mice, a panel of antibod-

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number AF282901.

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[1] The abbreviations used are: SGC, sarcoglycan complex; DGC, dystrophin-glycoprotein complex; LGMD, limb-girdle muscular dystrophy; *mdx*, dystrophin-deficient mice; SG, sarcoglycan; *Sgca*-null, α-SG-deficient mice; *Sgcd*-null, δ-SG-deficient mice; wt, wild-type; RT-PCR, reverse transcriptase-polymerase chain reaction; RACE, rapid amplification of cDNA ends; PAGE, polyacrylamide gel electrophoresis.

ies directed against different portions of γ-SG is able to detect a protein of the same size as in the skeletal muscle (35 kDa). To exclude the presence of an isoform of γ-SG, we performed RT-PCR and sequenced two identical full-length cDNAs in skeletal and smooth muscle. Through 5′-RACE PCR, we were able to identify two alternatively spliced mRNAs in skeletal muscle but only one form in smooth muscle. By analysis of cultures of human coronary artery smooth muscle cells, we demonstrated that not only the expression of γ-SG actually occurs in smooth muscle but also the biosynthesis of the SGs in the smooth muscle is dependent on differentiation. In addition, we demonstrated by sucrose gradient fractionation and co-immunoprecipitation of smooth muscle DGC that γ-SG is part of the DGC and, along with the other SGs, is absent in the smooth muscle of *Sgcd*-null mice. Furthermore, we observed that the expression of the SGC was perturbed in the smooth muscle layer of the vasculature in a patient with a mutation in the γ-SG gene. In summary, these results demonstrate that the SGC in smooth muscle cells is identical to the less abundant SGC isoform of striated muscle fibers.

EXPERIMENTAL PROCEDURES

Animal Models—Wild-type C57BL/10J and *mdx* mice were obtained from Jackson ImmunoResearch Laboratories, Inc. Colonies of *Sgca*- (24) and *Sgcd*-null mice (29) were established and maintained at the University of Iowa Animal Care Unit according to the animal care guidelines.

Antibodies—Mouse monoclonal antibodies Ad1/20A6 against α-sarcoglycan, βSarc1/5B1 against β-sarcoglycan, and 35DAG/21B5 against γ-sarcoglycan were generated in collaboration with Louise V. B. Anderson (Newcastle General Hospital, Newcastle upon Tyne, UK). Monoclonal antibody 43DAG/8D5 against β-dystroglycan was generated by L. V. B. Anderson. Polyclonal antibodies directed against α-sarcoglycan (Rabbit 98), β-sarcoglycan (Goat 26), γ-sarcoglycan (Rabbit 204, Rabbit 245, Rabbit 208, and Sheep 25), δ-sarcoglycan (Rabbit 229 and Rabbit 215), ϵ-sarcoglycan (Rabbit 232), sarcospan (Rabbit 217 and Rabbit 256), β-dystroglycan (Rabbit 83), dystrophin (Rabbit 31), and utrophin (Rabbit 56) were described previously (24, 37, 40, 42–45). A commercial monoclonal antibody (clone 1A4, Sigma) was used to detect smooth muscle α-actin.

Immunoblot Analysis of Total Homogenate—Tissue samples obtained from wild-type, *Sgca*-null, *Sgcd*-null, and *mdx* mice were dissected and snap-frozen in liquid nitrogen and stored at −80 °C until use. Frozen tissues were processed as described (46). Briefly, the samples were solubilized in a SDS extraction buffer containing 80 mM Tris-HCl, pH 6.8, 10% SDS, 0.115 M sucrose, 1% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 mM EDTA. The protein concentration in each sample was assessed with D_CProtein Assay (Bio-Rad). Cell cultures were washed in phosphate-buffered saline, pH 7.4, and directly solubilized in buffer. 150 μg of proteins were then analyzed by SDS-polyacrylamide gel electrophoresis. The gels were blotted onto nitrocellulose membranes (Immobilon-NC). Membranes were incubated with primary antibodies and detected by SuperSignal chemiluminescence (Pierce) after incubation with the appropriate horseradish peroxidase-conjugated secondary antibody.

RT-PCR and 5′-RACE PCR—Total RNA from control C57BL/10 skeletal muscle, heart, uterus, and aorta was extracted using RNAzol B (Tel-Test) according to the manufacturer's instructions. For RT-PCR, 2 μg of RNA from various tissues was reverse-transcribed with Moloney murine leukemia virus (Stratagene). We used three sets of overlapping primers spanning the sequence of exons 2–8 of the mouse γ-sarcoglycan cDNA (GenBank™ AB024922) as follows: nucleotides 277–297 (5′-CCACGGTCACCGAGGGCACTC-3′) and nucleotides 538–555 (5′-CACTCTGGAGCGTATTTC-3′); nucleotides 453–471 (5′-AATAGGAATGGGTCACTTG-3′) and nucleotides 819–836 (5′-CTAAGGTCTTGAAATGGG-3′); and nucleotides 762–780 (5′-GGCCAGAAGGAGCTCTTTT-3′) and nucleotides 1240–1261 (5′-CCCCTGCATGCTTCTAAGTGTT-3′). Two sets of primers were used to amplify exons 1 and 2 as follows: A-sense, nucleotides 38–56 (5′-CCCTCATCGGCAATCAAGT-3′); A-antisense, nucleotides 381–398 (5′-CGAGGATGGCGAGCAGGA-3′); B-sense, nucleotides 91–108 (5′-CTATTGCTTCAGACTTTG-3′); B-antisense, nucleotides 423–440 (5′-ACATCACTTTCAGAATCC-3′). 40 PCR cycles were performed under the following conditions: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The resulting PCR products were run on an agarose gel; the band was excised, and the DNA was cloned into pGEM®-T Easy vector (Promega) and sequenced using universal primers.

5′-RACE reactions were performed using the First Choice™ RLM-RACE Kit (Ambion Inc.). A random-primed reverse transcription reaction and nested PCR was then carried out to amplify the 5′ end of the γ-SG transcript. Two sense primers corresponding to the adapter sequence were provided. Two antisense primers were used against nucleotides 474–497 (5′-CAGGCGAAGTCCATCTGCTGTAAC-3′), and 380–412 (5′-GCGAGATTCACAACGAGGATGGCGAGCAGGAGA-3′) of the mouse γ-SG sequence. The PCR conditions were as follows: hot start; 35 cycles, 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C; 7 min at 72 °C in the last cycle. The pool of the resulting PCR products was cloned and sequenced as described above.

Smooth Muscle Cell Culture—Human coronary artery smooth muscle cells, media, and supplements were purchased from Cascade Biologics, Inc. The cells were grown at 37 °C, 5% CO_2 in Medium 231 supplemented with smooth muscle growth supplement. Proliferating cultures at 70% of confluence were switched to differentiation medium (M231 and smooth muscle differentiation supplement) and processed for immunoblot as described above.

DGC Isolation and Sucrose Gradient Fractionation—Mouse skeletal muscle and lung tissues (5 g) were solubilized in 50 mM Tris-HCl, pH 7.4, 500 mM NaCl containing 1% digitonin in the presence of protease inhibitors (24). The solubilized material was incubated overnight at 4 °C with pre-equilibrated wheat germ agglutinin-agarose beads (Vector Laboratories). The beads were washed with 50 mM Tris-HCl, pH 7.4, 500 mM NaCl containing 0.1% digitonin and eluted with 0.3 M *N*-acetylglucosamine in 50 mM Tris-HCl, 500 mM NaCl containing 0.1% digitonin. The eluate was concentrated and loaded onto 5–30% sucrose gradient and centrifuged with a Beckman VTi65.1 vertical rotor at 200,000 × *g* for 2 h at 4 °C. Fractions were collected and analyzed by immunoblot as described (47).

Co-immunoprecipitation Assay—100 μg of DGC isolated from skeletal muscle and lung were diluted to 1 ml in immunoprecipitation buffer as described (25). Sepharose-bound protein G beads (Amersham Pharmacia Biotech) were equilibrated in immunoprecipitation buffer, and 50 μl were incubated for 30 min at 4 °C with each sample. The beads were then spun down, and the supernatant was incubated with the anti-β-SG affinity purified antibody (Goat 26) for 4 h at 4 °C. 50 μl of Sepharose-bound protein G beads were added and incubated for an additional 2 h. After four washes in lysis buffer, the beads were incubated for 10 min at 65 °C in loading buffer. The bound proteins were then resolved by a 10% SDS-PAGE and analyzed by immunoblot.

Immunofluorescence Analysis—An 11-year-old male with clinical signs and symptoms of LGMD was biopsied for diagnostic testing. Samples from patients with myopathies unrelated to muscular dystrophy were used as controls. Seven-μm cryosections of these skeletal muscle biopsies were analyzed by immunofluorescence as described previously (24). Sections were observed under a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Inc.). Digitized images were captured under identical conditions using PAX-it software (Midwest Information Systems, Inc.).

RESULTS

Immunoblot Analysis Demonstrates the Presence of γ-Sarcoglycan in Smooth Muscle—Previous reports have clearly demonstrated the expression of ϵ-, β-, and δ-SG in smooth muscle both by immunofluorescence and immunoblotting (27, 29, 40). In contrast, because the immunostaining remains elusive, the presence of γ-SG in smooth muscle has been controversial.

In order to verify whether the γ-SG in smooth muscle is the same form as the striated muscle one, or a tissue-specific isoform, we analyzed several smooth muscle-containing organs from wild-type mice. Total homogenate from uterus, lung, bladder, and aorta, along with skeletal muscle from quadriceps femoris, was tested by immunoblot for the presence of the 35-kDa γ-SG band. A panel of five antibodies directed against different portions of the protein was used (Fig. 1*a*). All the samples were found positive, and the result was reproducible with all the antibodies (Fig. 1*b* and data not shown). Although the amount of protein loaded was the same for each lane, the antibodies against γ-SG consistently showed bands of different intensity in the examined organs. This might be related to the

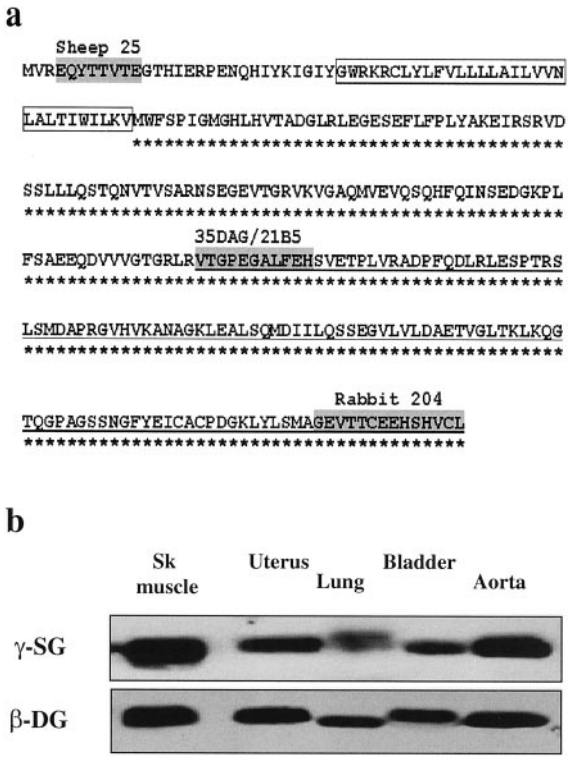

FIG. 1. *a*, a panel of five antibodies directed against γ-sarcoglycan was used for immunoblot analysis. γ−SG is a transmembrane protein with an extracellular carboxyl terminus and a single transmembrane domain (*boxed*). The mouse γ−SG protein sequence is *shaded* on the regions recognized by our antibodies. The amino acid sequences corresponding to the fusion proteins against which Rabbit 208 and Rabbit 245 were produced are indicated by *asterisks* and underlined, respectively. *b*, total homogenates from various mouse organs were electrophoresed on 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. Identical membranes were probed with antibodies against γ-sarcoglycan 35DAG/21B5 (*γ-SG*) and β-dystroglycan Rabbit 83 (*β-DG*). Variations in the size of the proteins are artifacts due to the viscosity of the samples.

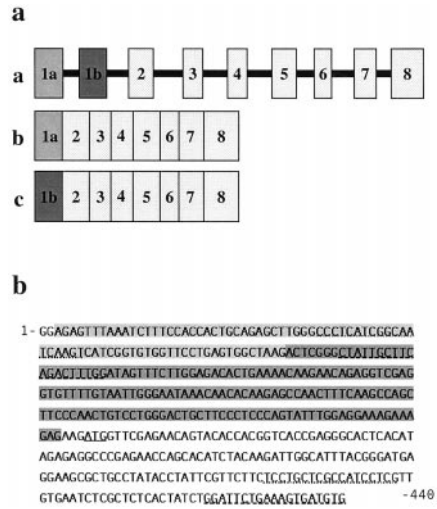

FIG. 2. *a*, *line a,* proposed organization for the γ-sarcoglycan gene. The structures of the transcripts are schematized respectively in *lines b* and *c*. Transcript *b* is expressed in skeletal and smooth muscle, whereas transcript *c* is weakly detected only in skeletal muscle with the set of primer B. *b*, partial sequence of the γ-sarcoglycan mRNA. The putative sequences for exons 1a and 1b are *highlighted* in *light gray* and *dark gray*, respectively. The *dashed line* indicates primers A; the *dotted line* indicates primers B. The start codon is *underlined* and corresponds to the beginning of exon 2. These sequence data are available from GenBank™/EMBL/DDBJ under accession numbers AB024922, NM_011892, and AF282901.

different composition of these organs in smooth muscle and other tissues such as endothelium, epithelia, connective tissues, and blood. For example, after microdissection of aortic tissue to eliminate blood residue and the surrounding connective tissue, a thick wall of smooth muscle and a single thin layer of endothelial cells were isolated, and the sample appeared to be more enriched in γ-SG than the other non-skeletal muscle samples. In contrast, γ-SG seemed to be particularly low in the lung, which contains a significant amount of epithelium, vascular endothelium, and blood. An identical nitrocellulose membrane was immunoblotted against β-dystroglycan, a protein present in skeletal and smooth muscle as well as in epithelial cells (Fig. 1*b*). In this case the intensity of the bands was comparable in all samples but still relatively low in the lung. This suggests that the amount of blood in this organ contributes significantly to the total amount of protein present in the sample.

For the following experiments we chose to use the monoclonal antibody 35DAG/21B5. In view of the fact that γ- and δ-SG proteins (both 35 kDa) share about 70% amino acid similarity (48), we performed peptide competition experiments, demonstrating that this antibody does not cross-react with δ-SG (not shown).

Only One of the Two Alternatively Spliced γ-Sarcoglycan mRNAs Is Present in Smooth Muscle—All the antibodies we successfully used on immunoblot to detect γ-SG failed in localizing the protein by immunofluorescence. Although our five antibodies cover the majority of the sequence of γ-SG, regions such as the transmembrane domain and part of the extracellular domain have not been analyzed. A smooth muscle isoform presenting variations in the amino acid composition might show a different structure and unique interactions with the other components of the complex. Consequently, the epitopes may become undetectable *in situ* but still detectable when the protein is unfolded in a gel under reducing conditions.

In order to establish whether the protein sequences are the same in skeletal and smooth muscle, we isolated total RNA from uterus, lung, bladder, aorta, and skeletal muscle from wild-type mice. RT-PCR was performed using primers specific for γ-SG. The sequence of the PCR products was identical in skeletal and smooth muscle samples.

The set of primers A and B was designed to detect the presence of the two alternatively spliced exons 1 (GenBank™ AB024922 and NM_011892). Whereas the PCR performed with the primers A amplified the expected product in skeletal and smooth muscle samples, the reaction performed with the set of primers B revealed a faint product only in skeletal muscle (not shown).

In order to fully characterize the 5′ end of the γ-SG transcript, we performed 5′-RACE PCR on mouse skeletal muscle RNA samples. We successfully amplified a major product that corresponds to the exon 1 found by RT-PCR with primers A. None of the clones sequenced contained the sequence amplified with primers B, indicating that this transcript is either less stable or relatively rare in skeletal muscle. Our findings suggest the presence of at least two exons 1 in the γ-SG gene, as schematized in Fig. 2*a*. Exon 1a is mainly spliced with exon 2, generating a major transcript of 1119 base pairs, which is also the only transcript present in smooth muscle. The genomic analysis of this region was not extensively performed in the present study. Since the protein coding sequence of γ-SG starts from the second exon, and exon 1a and 1b correspond to an untranslated region, the alternative splicing of the mRNA is not expected to affect the final sequence of the protein.

The Sarcoglycans Are Developmentally Expressed in Smooth Muscle—In view of the fact that the smooth muscle organs

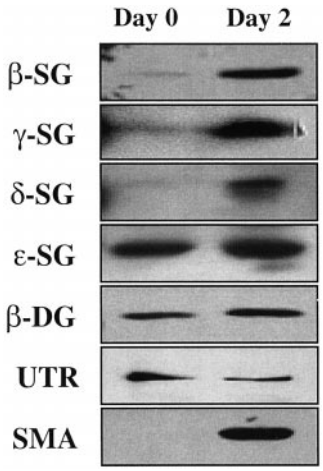

FIG. 3. **Lysates of human coronary artery smooth muscle cells were electrophoresed and transferred to nitrocellulose membranes.** The membranes were probed with antibodies against the sarcoglycans (*β-, γ-, δ-,* and *ε-SG*), *β*-dystroglycan (*β-DG*), and utrophin (*UTR*). A smooth muscle actin antibody (*SMA*) was used as a marker for differentiation. *Lane 1,* undifferentiated cells. *Lane 2,* differentiated cells at day 2.

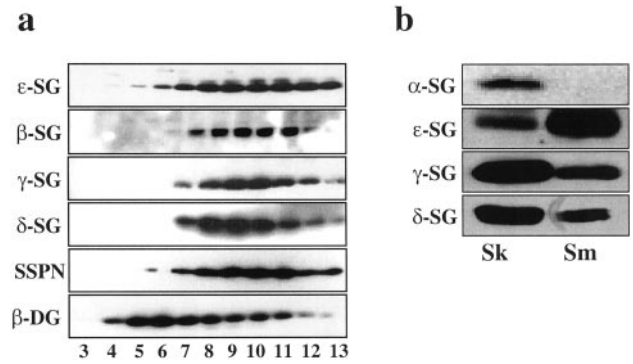

FIG. 4. *a*, fractions 3–13 of the sucrose gradient separation of mouse lung DGC were resolved by 3–12% SDS-PAGE. After transfer the membranes were incubated with antibodies directed against *β*-sarcoglycan (*β-SG*), γ-sarcoglycan (*γ-SG*), δ-sarcoglycan (*δ-SG*), ε-sarcoglycan (*ε-SG*), sarcospan (*SSPN*), and *β*-dystroglycan (*β-DG*). The peak of the SG-sarcospan complex co-migrates in fractions 8–11 with the smooth muscle *β*-dystroglycan. *b,* DGC preparations from mouse lung and skeletal muscle were co-immunoprecipitated with an anti-*β*-sarcoglycan antibody (Goat 26) and electrophoretically separated on 10% SDS-polyacrylamide gels. Nitrocellulose transfers were probed with antibodies against *α*-sarcoglycan (*α-SG*), γ-sarcoglycan (*γ-SG*), δ-sarcoglycan (*δ-SG*), and ε-sarcoglycan (*ε-SG*). In skeletal (*Sk*) and smooth muscle (*Sm*) γ-SG co-immunoprecipitates with the other SGs.

analyzed contain different cell types, we investigated the specificity of the expression of the εβγδ-SGC in smooth muscle by biochemical analysis of cultured human coronary artery smooth muscle cells. The cells were lysed and loaded on 10% polyacrylamide gels at days 0, 2, 4, 6, and 8 after switch to differentiation medium. Fig. 3 represents the immunoblot analysis of the samples at day 0 and 2, as no differences have been observed among the lysates after longer periods of differentiation. Although ε-SG and *β*-dystroglycan have the same strong level of expression before and after differentiation, the synthesis of *β*-, γ-, and δ-SGs dramatically increases in differentiating cells.

γ-Sarcoglycan Is an Integral Component of the SGC in Smooth Muscle—In order to demonstrate that γ-SG is part of a smooth muscle SGC that is similar to skeletal muscle, we performed sucrose gradient fractionation of smooth muscle DGC isolated from mouse lung, the largest source of mouse smooth muscle tissue available in our laboratory. Proteins from the sucrose gradient fractions were separated by electrophoresis on polyacrylamide gel. Immunoblotting with antibodies against DGC components showed that the peak of the four smooth muscle SGs and sarcospan migrates in fractions 8–11 (Fig. 4*a*). As none of the smooth muscle SGs, with the exception of ε-SG, is expressed in epithelial cells (11), we were able to analyze the smooth muscle SGC. This result confirmed that γ-SG is an integral part of the SGC in smooth muscle tissue. Furthermore, as already shown (11), *β*-dystroglycan migrates in fractions 5–7 as epithelial complex, and with the SG-sarcospan complex in fractions 8–11.

The association of γ-SG with smooth muscle SGC was further demonstrated by co-immunoprecipitation of DGC isolated from mouse lung using a polyclonal antibody against *β*-SG (Goat 26). DGC isolated from mouse skeletal muscle was used as a positive control. The immunocomplexes were analyzed by immunoblotting with antibodies directed against components of the SGC as shown in Fig. 4*b*. In the smooth muscle γ-SG co-immunoprecipitated with the other SGs. The absence of *α*-SG in the lung sample ruled out any contamination from skeletal muscle. Furthermore, in skeletal muscle the detection of ε-SG immunoprecipitated with the anti-*β*-SG antibody confirmed the co-existence of the two distinct αβγδ and εβγδ complexes. These results indicate that the SGC complex in smooth muscle is constituted by *β*-, γ-, δ-, and ε-SGs.

γ-Sarcoglycan Is Absent in Smooth Muscle of Sgcd-null Mice—It has been reported that the absence of *β*- and δ-SGs leads to the loss of other components of the sarcoglycan-sarcospan complex in smooth muscle (27, 29, 40). The disruption of the SGC has dramatic consequences for the smooth muscle of the vessels, particularly in the heart where a pattern of constrictions of the coronary arteries is associated to ischemic-like lesions finally leading to severe cardiomyopathy (27, 29). In order to determine if γ-SG is deficient along with the other SGs and sarcospan in the vessels of cardiomyopathic mice, we analyzed aortas from wild-type, *Sgca*-null, *Sgcd*-null, and *mdx* mice by immunoblot for several components of the DGC and utrophin (Fig. 5).

As expected, the slight amount of dystrophin normally present in the aorta was absent in the sample from the *mdx* mouse, whereas utrophin was strongly expressed in all the lanes. Also, predictably we did not observe *α*-SG in any of the samples in this study. The lack of *α*-SG expression demonstrated that there was no contamination from striated muscle in the samples from wild-type, *Sgcd*-null, and *mdx* mice. To microdissect the aorta from the *Sgca*-null mouse, we used our standard method. With this procedure we never detected *α*-SG in any of the samples from other strains of mice. In the aortas from the *mdx* mice, which occasionally show a cardiomyopathic phenotype at old age (49), the sarcoglycan-sarcospan complex was intact. In marked contrast, in the aortas of the *Sgcd*-null mice all the SGs, including γ-SG and sarcospan, were missing, whereas *β*-dystroglycan expression was unaffected. Similar results were obtained with the aortas from *Sgcb*-null mice (not shown).

Sarcoglycans Are Similarly Reduced in Skeletal and Smooth Muscle of a LGMD-2C Patient—In order to characterize further the alterations of the SGC in smooth muscle, particularly in regard to the human sarcoglycanopathies, we tested in parallel skeletal muscle and vascular smooth muscle in the muscle biopsies of several controls and one LGMD patient with a mutation in the γ-SG gene.

Genetic analysis of the LGMD patient revealed mutations IVS2del+4-7 and T581C (L194S) in the γ-SG gene. The IVS2 mutation changes the splice donor site from C(A/g)taagtatcattat to C(A/g)tatcattat. The effect of this mutation is unknown. The skeletal muscle from the thigh had prominent, grouped

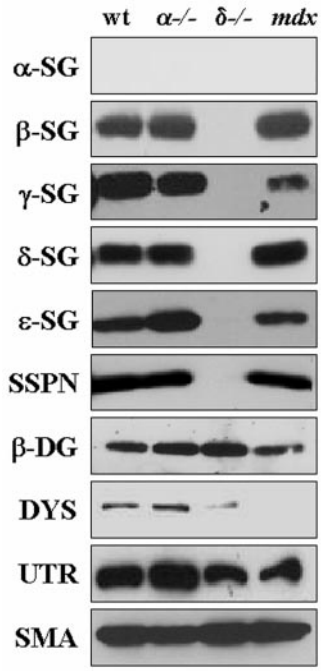

FIG. 5. **Immunoblot analysis of aorta total homogenates.** Samples from wild-type (*wt*), *Sgca*-null (α−/−), *Sgcd*-null (δ−/−), and *mdx* mice were analyzed by 10% SDS-PAGE and immunoblotted using antibodies against the sarcoglycans (*α-, β-, γ-,* δ-, and *ε-SG*), β-dystroglycan (*β-DG*), sarcospan (*SSPN*), dystrophin (*Dys*), and utrophin (*UTR*). In the aortas of the *Sgcd*-null mice all the SGs and sarcospan are absent. To demonstrate equal loading of protein samples, we used a smooth muscle actin antibody (*SMA*).

necrosis and regeneration, variation in fiber size from 10 to 100 μm, and many internally placed nuclei. Endomysial fibrosis and fatty replacement were mild. Immunofluorescence evaluation of skeletal muscle revealed normal expression of dystrophin; however, γ-SG staining was absent, and staining for α-, β-, δ-SG, and sarcospan was variably reduced (Fig. 6*a*).

The smooth muscle of the vessels present in the same sections displayed an equivalent pattern of perturbation of the SGC (Fig. 6*b*); δ-SG appeared to be the least reduced, whereas traces of β-SG and sarcospan were detected. The weak fluorescence seen after incubation with the antibody against α-SG is considered specific since it is also visible on slides incubated only with secondary antibody. The analysis of ε-SG expression was not performed because our antibody does not recognize the human protein on immunofluorescence.

DISCUSSION

The objective of this study was to characterize the fourth component of the SGC in smooth muscle. Our results prove that γ-SG is present in smooth muscle as well as in striated muscle and is associated with the previously described εβδ-SGC. We also show that the loss of one of the other sarcoglycans in smooth muscle affects the expression of γ-SG. The reverse is also true; mutated γ-SG protein is responsible for the lack of the remainder of the SGC.

Previous reports anticipated the presence of γ-SG in smooth muscle (11, 27). Our data provide the final identification of this protein as an integral component of the smooth muscle SGC. The fact that different antibodies recognized the protein on immunoblot was not sufficient to exclude the existence of an isoform of γ-SG. However, the sequence of the RT-PCR products clearly revealed identical transcripts in skeletal and smooth muscle. Furthermore, whereas in skeletal muscle two transcripts have been identified, derived from the alternative splicing of exon 1a and 1b, the only transcript in smooth muscle is generated by splicing of exon 1a. Interestingly, the genomic organization of δ-SG, which shows 58% identity to γ-SG at the DNA-level, displays three different untranslated exons 1 alternatively spliced and differentially expressed in cardiac, skeletal, and smooth muscle. A large deletion comprising exon 1b and 1c has been demonstrated to cause cardiomyopathy and muscular dystrophy in the BIO14.6 hamster (50). Although the differential expression of the transcript provides evidence for a tissue regulation in the γ- and δ-SG genes, the physiological significance of this phenomenon is still not clear.

A major objection to the presence of γ-SG in smooth muscle has been raised because of the unsuccessful immunolocalization. We speculate that in conjunction with ε-, β-, and δ-SGs the antigens required for recognition by our antibodies *in situ* may be somehow masked, but they become more easily recognizable when the protein is unfolded on SDS-PAGE.

The expression of the SGC in smooth muscle was explicitly demonstrated by biochemical analysis of smooth muscle cells in culture. As our interest was predominantly directed to vessel dysfunction leading to cardiomyopathy, we chose to analyze smooth muscle cells from human coronary arteries. Here we showed that all the components of the εβγδ-SGC were expressed. Furthermore, the synthesis of β-, γ-, and δ-SGs in smooth muscle cells dramatically increases with differentiation, as already described in skeletal muscle (40).

Once we confirmed the presence of γ-SG in smooth muscle, our intention was to verify its interaction with the other DGC components. To this end, we performed sucrose gradient fractionation and co-immunoprecipitation of DGC isolated from mouse lung, identifying γ-SG in association with the other sarcoglycans and sarcospan.

The animal models for limb-girdle muscular dystrophy types 2E and 2F lack expression of the sarcoglycans and sarcospan at the smooth muscle plasma membrane, whereas the complex is unaffected in LGMD-2D mice (27, 29, 40). In addition, perfusion of the vascular beds of the heart and diaphragm with Microfil, a liquid silicon rubber, showed focal vascular constrictions in *Sgcb*-null and *Sgcd*-null mice. Based on these data, a novel hypothesis has been put forward that relates the cardiomyopathic phenotype to a dysfunction in the smooth muscle of the coronary arteries due to the disruption of the SGC in this tissue (29, 51). Mice deficient in γ-SG also develop cardiomyopathy (28). Our finding that γ-SG is a component of the smooth muscle SGC implies that vascular dysfunction may also play a role in the pathogenesis of cardiomyopathy and muscular dystrophy in these mice.

Further support for this hypothesis was obtained by comparing the expression of the DGC in the aorta of three animal models of muscular dystrophy. Only in the *Sgcd*-null aorta was the SGC missing. Normal levels of SGC proteins in *Sgca*-null and *mdx* arteries are consistent with the mild or late onset of cardiomyopathy displayed in these mice, respectively. To date, no reports have been published with regard to smooth muscle expression of the other DGC components in the *Sgcg*-null mouse. Nevertheless, the occurrence of cardiomyopathy suggests there may be disruption of the SGC in the smooth muscle of this animal model.

The vascular dysfunction may also have an important influence on the severity of muscular dystrophy in human patients. Here we showed that mutations in the γ-SG gene destabilize the SGC in the vessels as well as in the skeletal muscle of an LGMD-2C patient, providing the first direct evidence of the presence of the SGC in human smooth muscle tissue. The disease severity in sarcoglycanopathies can vary from mild to severe. This variability has been correlated with the mutations in the sarcoglycan genes, *e.g.* missense mutations in both alle-

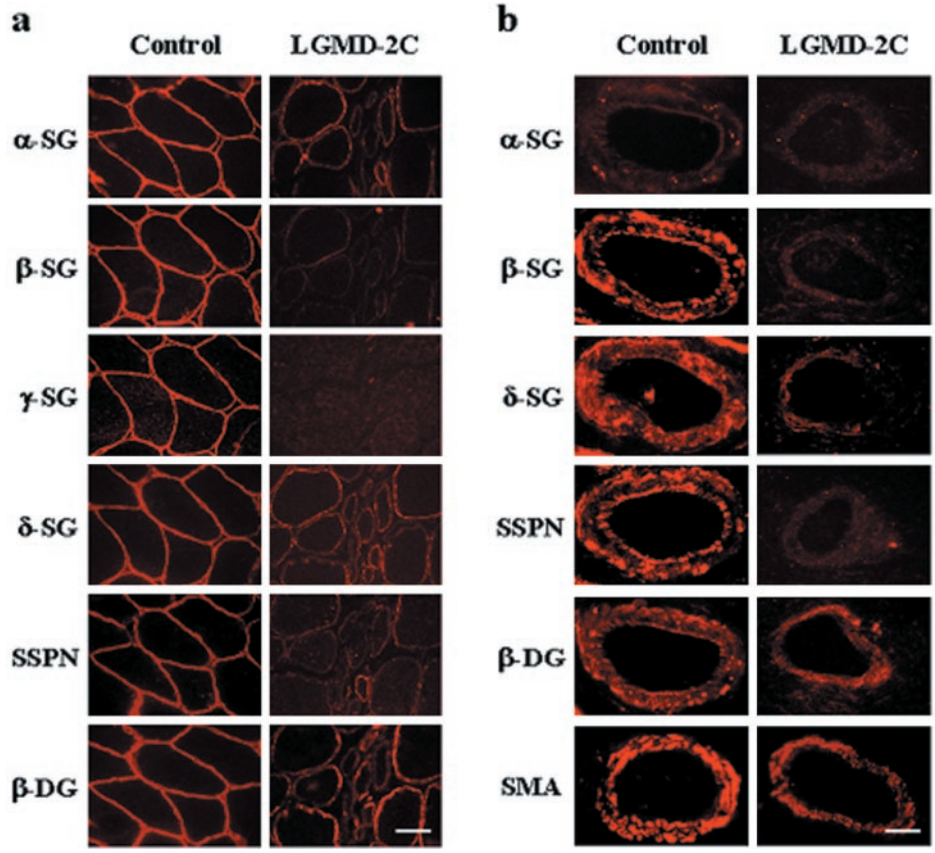

FIG. 6. *a*, cryosections of skeletal muscle biopsies from control and LGMD-2C patients were immunostained using antibodies against α-sarcoglycan (*α-SG*), β-sarcoglycan (*β-SG*), γ-sarcoglycan (*γ-SG*), δ-sarcoglycan (*δ-SG*), sarcospan (*SSPN*), and β-dystroglycan (*β-DG*). γ-Sarcoglycan is absent in the LGMD-2C patient, whereas the other sarcoglycans and sarcospan are variably reduced. *Scale bar* represents 20 μm. *b*, α-sarcoglycan (*α-SG*), β-sarcoglycan (*β-SG*), δ-sarcoglycan (*δ-SG*), sarcospan (*SSPN*), β-dystroglycan (*β-DG*), and smooth muscle actin (*SMA*) immunostaining in vascular smooth muscle of control and LGMD-2C patient. The same pattern of reduction of the SGs and sarcospan is shown in the vascular smooth muscle of the patient. In the vessels, the α-SG antibody staining corresponds to autofluorescent background. γ-Sarcoglycan is not detectable by immunostaining in smooth muscle. *Scale bar* represents 20 μm.

les are associated with a milder phenotype than homozygous null mutations (19, 20, 52). If the expression of the SGC is perturbed not only in the skeletal and cardiac fibers, but also in the smooth muscle of the vasculature of these patients, vascular dysfunction might play a role in the pathogenesis of LGMD. Therefore, the disruption of the SGC in the vessels is an element that may be considered in the genotype-phenotype correlation of the LGMD-2C/E/F patients.

Although the loss of the SGC leads to necrosis of the skeletal muscle, the smooth muscle organs of LGMD2C/F patients and animal models show neither necrosis nor fibrosis. Moreover, in the striated muscle of patients and mice with primary α-SG defects, there is no evidence of up-regulation in the expression of the εβγδ-SGC, which might play a compensatory role. On account of these observations, it is tempting to hypothesize that the functions of the αβγδ-SG and εβγδ-SG complexes may be divergent and to speculate that the εβγδ-SGC may have a role in a still unidentified metabolic or signaling pathway rather than mechanical transduction. In favor of this hypothesis, a number of mouse expressed sequence tag sequences similar to γ-SG have been found in GenBank™ (accession numbers AV378726, AI121415, BB017993, and BB350604); the tissue sources of the libraries were cecum, mammary gland, testis, and cerebellum. Interestingly, the transcript for γ-SG, considered so far to be muscle-specific, seems to be expressed also in non-muscle organs, as has been shown with ε-, β-, and δ-SG mRNAs (38, 39, 47, 53).

The reason why abnormalities in smooth muscle SGC lead in particular to vascular disturbance rather than dysfunction of other smooth muscle organs remains to be elucidated. Endothelial cells release agents that could regulate the function of underlying vascular smooth muscle, thereby controlling vascular tone by modulating the local concentration of vasoactive substances, for instance synthesizing and releasing nitric oxide. The involvement of the εβγδ-SGC in intercellular communication is an intriguing hypothesis that may offer new approaches for the treatment of the sarcoglycanopathies.

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