

## $\epsilon$ -Sarcoglycan Replaces $\alpha$ -Sarcoglycan in Smooth Muscle to Form a Unique Dystrophin-Glycoprotein Complex\*

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The sarcoglycan complex has been well characterized in striated muscle, and defects in its components are associated with muscular dystrophy and cardiomyopathy. Here, we have characterized the smooth muscle sarcoglycan complex. By examination of embryonic muscle lineages and biochemical fractionation studies, we demonstrated that  $\epsilon$ -sarcoglycan is an integral component of the smooth muscle sarcoglycan complex along with  $\beta$ - and  $\delta$ -sarcoglycan. Analysis of genetically defined animal models for muscular dystrophy supported this conclusion. The  $\delta$ -sarcoglycan-deficient cardiomyopathic hamster and mice deficient in both dystrophin and utrophin showed loss of the smooth muscle sarcoglycan complex, whereas the complex was unaffected in  $\alpha$ -sarcoglycan null mice in agreement with the finding that  $\alpha$ -sarcoglycan is not expressed in smooth muscle cells. In the cardiomyopathic hamster, the smooth muscle sarcoglycan complex, containing  $\epsilon$ -sarcoglycan, was fully restored following intramuscular injection of recombinant  $\delta$ -sarcoglycan adenovirus. Together, these results demonstrate a tissue-dependent variation in the sarcoglycan complex and show that  $\epsilon$ -sarcoglycan replaces  $\alpha$ -sarcoglycan as an integral component of the smooth muscle dystrophin-glycoprotein complex. Our results also suggest a molecular basis for possible differential smooth muscle dysfunction in sarcoglycan-deficient patients.

A large complex of membrane-associated proteins, the dystrophin-glycoprotein complex (DGC),<sup>1</sup> is critical for the integrity of skeletal muscle fibers (1). This complex consists of dystrophin (2, 3), the dystroglycans ( $\alpha$  and  $\beta$ ) (4), the sarcoglycans ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) (5), the syntrophins ( $\alpha 1$ ,  $\beta 1$ , and  $\beta 2$ ) (6), and

sarcospan (7). Dystrophin binds to cytoskeletal actin and to a transmembrane protein,  $\beta$ -dystroglycan; the extracellular domain of  $\beta$ -dystroglycan binds to the peripheral membrane protein,  $\alpha$ -dystroglycan; and  $\alpha$ -dystroglycan binds to laminin  $\alpha 2$  in the basal lamina (8). In this way, the DGC serves as a link between the extracellular matrix and the subsarcolemmal cytoskeleton. The essential role of the DGC is emphasized by findings that mutations of genes encoding dystrophin, all four sarcoglycans, and the laminin  $\alpha 2$  chain underlie muscular dystrophies in recently described experimental animals (9–11) and in humans (12). Consistent with the presence of the DGC in cardiac muscle, cardiomyopathies are associated with some of these mutations (13).

Several components of the DGC are also found in smooth muscle (2, 14). Interestingly, dysphagia, vomiting, chronic constipation, increased gastric emptying time, and acute digestive dilatations, all potentially due to malfunctions of digestive smooth muscle, have been reported in patients with progressive muscular dystrophy (15–18). Furthermore, fibrosis and atrophy of the gastrointestinal smooth muscle has been repeatedly described in autopsies of dystrophin-deficient humans (15–17). Thus, clinical observations raise the possibility that the DGC plays an important role in smooth muscle. It has been infeasible to investigate this possibility, however, because the presence of a DGC in smooth muscle has not yet been directly demonstrated. Accordingly, we have used several approaches to identify and analyze DGC components of smooth muscle.

The starting point for our study was the recent identification of  $\epsilon$ -sarcoglycan, a transmembrane glycoprotein showing 43% amino acid identity with  $\alpha$ -sarcoglycan (19, 20). In striated (skeletal and cardiac) muscle fibers,  $\alpha$ -sarcoglycan is associated with three other sarcoglycans ( $\beta$ ,  $\gamma$ , and  $\delta$ ), forming a subcomplex within the DGC (21, 22). The sarcoglycan subcomplex may strengthen the binding of dystroglycan and dystrophin to the sarcolemma, thereby stabilizing the link between the inside and the outside of the cell (23). However, whereas the expression of  $\alpha$ -sarcoglycan is restricted to striated muscle cells (24, 25),  $\epsilon$ -sarcoglycan is broadly expressed (19, 20). We therefore hypothesized that  $\epsilon$ -sarcoglycan might replace  $\alpha$ -sarcoglycan in smooth muscle.

Here, we have used biochemical and genetic methods to test that hypothesis. We show that a DGC can be purified from smooth muscle and that  $\epsilon$ -sarcoglycan is an integral component of the smooth muscle DGC. Together with  $\beta$ - and  $\delta$ -sarcoglycans, it is enriched in purified smooth muscle DGC. We further show that  $\alpha$ - and  $\gamma$ -sarcoglycans are not integral components of the smooth muscle DGC. Moreover, mice deficient in both dystrophin and its autosomal homologue, utrophin, and hamsters deficient in  $\delta$ -sarcoglycan showed loss of the smooth muscle sarcoglycan complex. In contrast, the smooth muscle complex

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<sup>1</sup> The abbreviations used are: DGC, dystrophin-glycoprotein complex; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; E, embryonal day; P, postnatal day; WGA, wheat germ agglutinin; DEAE, diethylaminoethyl; LGMD, limb-girdle muscular dystrophy.

was unaffected in  $\alpha$ -sarcoglycan null mice. In summary, these results demonstrate that smooth muscle cells are similar to striated muscle fibers in that they contain a DGC, but differ in the composition of the complex. Our results also provide molecular bases for functional studies of the smooth muscle DGC and for clinical investigations of smooth muscle malfunctions in diverse muscular dystrophies.

#### EXPERIMENTAL PROCEDURES

**Animals**—Control and the original *mdx* mutant mice were bred at the University of Iowa from stocks originally obtained from the Jackson Laboratories (Jackson Laboratories, Bar Harbor, ME). Utrophin<sup>-/-</sup> and utrophin/dystrophin double mutants were generated as described previously (26, 27) and maintained at Washington University. Male F1B control and BIO14.6 cardiomyopathic hamsters were obtained from BioBreeders (Fitchburg, MA). A colony of  $\delta$ -sarcoglycan-deficient hamsters (28) and *Sgca*-deficient mice (10) was established at the University of Iowa. All animal studies were authorized by the Animal Care Use and Review Committee of the University of Iowa.

**Antibodies**—Monoclonal antibody IIIH6 against  $\alpha$ -dystroglycan (29), and rabbit polyclonal antibodies against  $\alpha$ -sarcoglycan (rabbit 98) (24),  $\gamma$ - and  $\delta$ -sarcoglycan (rabbits 208 and 215) (23), and sarcospan (rabbits 216 and 235) (7, 10) were described previously. Two rabbit polyclonal antibodies against  $\epsilon$ -sarcoglycan were used and both were previously characterized (10, 20). An affinity-purified rabbit antibody (rabbit 245) was produced against a COOH-terminal fusion protein of  $\gamma$ -sarcoglycan containing amino acids 167–291. Monoclonal antibodies Ad1/20A6 against  $\alpha$ -sarcoglycan,  $\beta$ Sarc1/5B1 against  $\beta$ -sarcoglycan, 35DAG/21B5 against  $\gamma$ -sarcoglycan, and  $\delta$ sarc3/12C1 against  $\delta$ -sarcoglycan were generated in collaboration with Louise V. B. Anderson (Newcastle General Hospital, Newcastle upon Tyne, United Kingdom). Monoclonal antibody 43DAG/8D5 against  $\beta$ -dystroglycan was generated by Louise V. B. Anderson. Polyclonal antibodies against dystroglycan fusion protein D and fusion protein B (30) were affinity-purified from goat 20 (30) and sheep OR12, respectively. Sheep OR12 was injected with fusion protein B and boosted with fusion protein D.

**Immunofluorescence Analysis**—For smooth muscle staining, tissues were embedded in Tissue-Tek O.C.T. mounting medium (Miles, Inc., Elkhart, IN), and frozen in liquid nitrogen-cooled isopentane. Immunofluorescence microscopy of 7- $\mu$ m cryosections was performed as described (10). Briefly, sections were blocked with 5% BSA in PBS for 30 min, and then incubated with the primary antibodies for 90 min in 1% BSA/PBS. After washing with 1% BSA/PBS, sections were incubated with CY3-, fluorescein-, rhodamine-, or biotin-conjugated secondary antibodies for 30 min, then washed with 1% BSA/PBS. If necessary, slides were then incubated with fluorescein isothiocyanate-conjugated streptavidin (1:1000) for 30 min. 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).

**Immunoblot Analysis of Skeletal and Smooth Muscle DGC**—KCl-washed membranes from skeletal muscle were prepared as described previously (31). Rabbit skeletal muscle DGC was extracted with 1% digitonin from KCl-washed membranes and then purified by succinyl wheat germ agglutinin (WGA) affinity chromatography followed by ion exchange on a DEAE column, as described (32). Smooth muscle DGC was extracted from rabbit visceral smooth muscle, as described below, with 1% digitonin and then purified by WGA affinity chromatography followed by ion exchange on a DEAE column. Proteins from the DEAE eluate were separated by SDS-PAGE on 3–15% linear gradient gels and transferred to nitrocellulose membranes. Immunoblot staining was performed as described previously (33).

**Sucrose Gradient Fractionation Analysis of Smooth Muscle Dystroglycan-Glycoprotein Complex**—The muscularis propria was prepared from rabbit pylorus muscle. Smooth muscle DGC was extracted in 50 mM Tris-HCl, pH 7.4, 500 mM NaCl containing 1% digitonin (Sigma) with a mixture of protease inhibitors. The extracted proteins were circulated overnight on a WGA-agarose column (Vector Laboratories). The columns 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, pH 7.4, 500 mM NaCl containing 0.1% digitonin. The WGA eluate was incubated with Protein G-agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 1 h, to remove contamination of immunoglobulins. The supernatant after Protein G incubation was diluted to 100 mM NaCl with 50 mM Tris-HCl, pH 7.4, containing 0.1% digitonin and applied to a DEAE-cellulose column and washed with 50

mM Tris-HCl, pH 7.4, 100 mM NaCl containing 0.1% digitonin. The column was eluted with a gradient of 100 mM–300 mM NaCl buffer containing 50 mM Tris-HCl, pH 7.4, 0.1% digitonin. The fractions containing DGC were pooled and concentrated to 800  $\mu$ l. The samples were applied to a 5–30% sucrose gradient and centrifuged with a Beckman VTi65.1 vertical rotor (Beckman Instruments Inc., Fullerton, CA) at 200,000  $\times g$  for 150 min at 4  $^{\circ}$ C. The gradient was fractionated into 1-ml fractions, which were blotted as described previously (33).

**$\delta$ -Sarcoglycan Recombinant Adenovirus Injection**—A human  $\delta$ -sarcoglycan expression construct was prepared as previously described (23). Hamsters were anesthetized by intraperitoneal injection of sodium pentobarbital (Abbott Laboratories, Abbott Park, IL) at a calculated dose of 75 mg/kg. The abdominal wall overlying the bladder was disinfected, and a 1-cm vertical incision was made. The bladder was exposed, and  $10^9$  viral particles in 100  $\mu$ l of normal saline were injected into the bladder wall of 3-month-old BIO 14.6 hamsters. The incision was closed with 3–4 sutures. Hamsters recovered with continual supervision and were housed postoperatively at the University of Iowa Animal Care Facility. Five, 10, and 14 days after injection, hamsters were killed by CO<sub>2</sub> asphyxiation. Injected bladders were removed by dissection, embedded in Tissue-Tek O.C.T. compound (Miles Inc.), and quickly frozen in liquid N<sub>2</sub>-cooled isopentane.

#### RESULTS

**Early Embryonic Appearance of  $\epsilon$ -Sarcoglycan in All Muscle Lineages**—A common feature of all sarcoglycans, including  $\epsilon$ -sarcoglycan, is their sarcolemmal expression. Previous studies have demonstrated differential expression of sarcoglycans in non-muscle tissues:  $\alpha$ - and  $\gamma$ -sarcoglycan are expressed exclusively in skeletal and cardiac muscle (25);  $\beta$ - and  $\delta$ -sarcoglycan are selectively expressed in skeletal and cardiac muscle, but are also detectable in other tissues (21, 22, 34, 35); and  $\epsilon$ -sarcoglycan is widely expressed (19, 20). We began the present study by asking how these differences arise, focusing on the three muscle lineages: skeletal, cardiac, and smooth. To this end, we stained sections from staged embryonic and postnatal mice with antibodies specific for three sarcoglycans that are differentially distributed in adults:  $\alpha$ -,  $\beta$ -, and  $\epsilon$ -sarcoglycan.

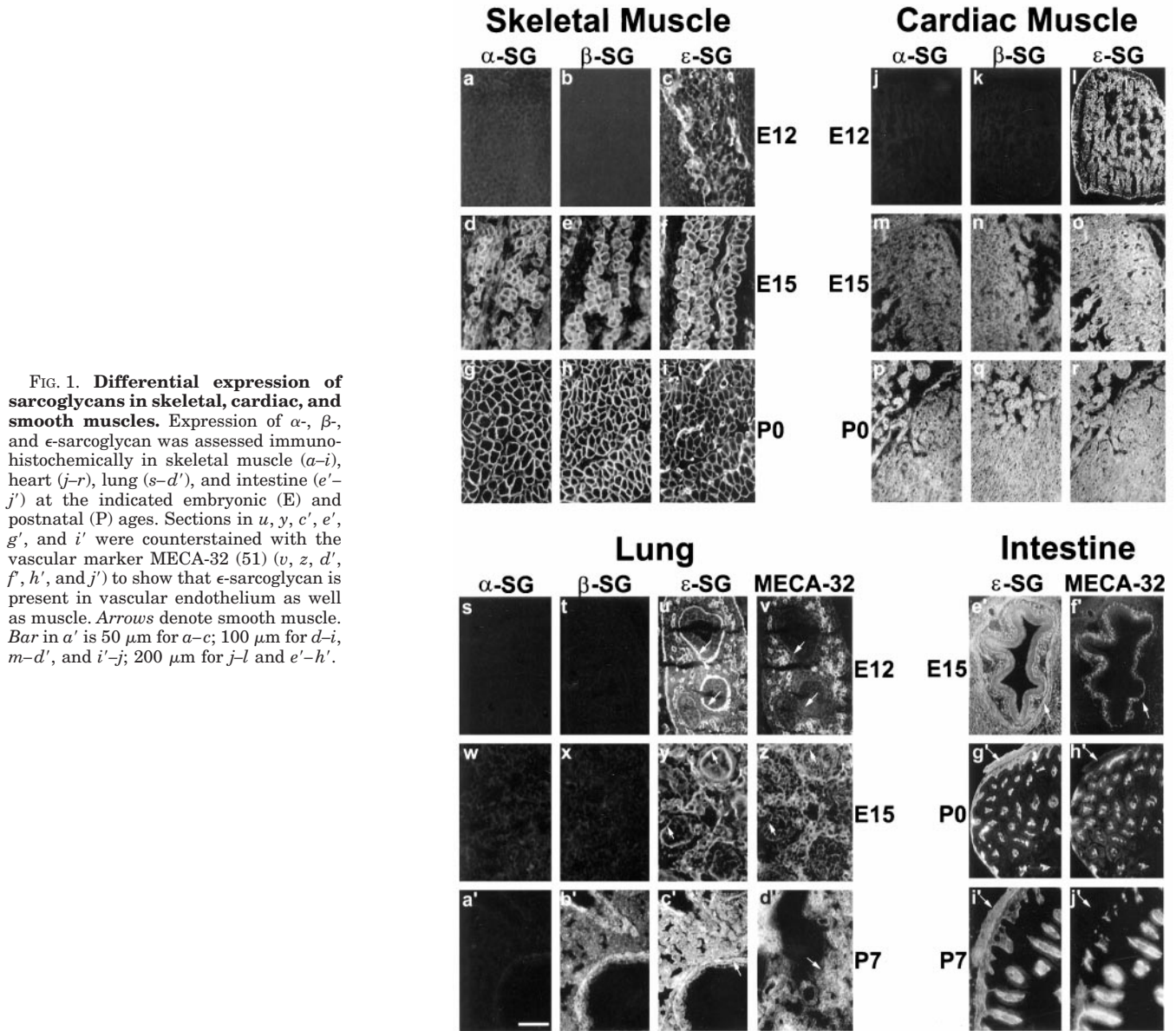
$\epsilon$ -Sarcoglycan was detectable in embryos by E8.5; the earliest age examined (data not shown) and was broadly distributed by E12.  $\epsilon$ -Sarcoglycan-positive cells included myoblasts or myotubes in forming axial muscle, myocytes in heart, prospective smooth muscle surrounding bronchi in lung, and vascular endothelium throughout the embryo (Fig. 1, *c*, *l*, *u*, and *v*). In contrast, neither  $\alpha$ - nor  $\beta$ -sarcoglycan was detectable in any tissue at E8.5–E12 (Fig. 1, *a*, *b*, *j*, *k*, *s*, and *t*). Thus, expression of  $\epsilon$ -sarcoglycan precedes that of  $\alpha$ - and  $\beta$ -sarcoglycans.

$\epsilon$ -Sarcoglycan remained widely distributed at E15. It was abundant in smooth muscle of the lung and bronchus, in skeletal myotubes and cardiac myocytes, and in a variety of endodermal and ectodermal derivatives (Fig. 1, *f*, *o*, *y*, *z*, *e'*, and *f'*).  $\alpha$ - and  $\beta$ -sarcoglycan were also present at this stage, but their distribution was sharply restricted to skeletal and cardiac muscle (Fig. 1, *d*, *e*, *m*, *n*, *w*, and *x*; data not shown). At this stage, therefore, muscles of different sublineages become distinguishable by the complement of sarcoglycans they express.

This difference between striated muscle and other tissues is maintained and accentuated as development proceeds; levels of  $\alpha$ - and  $\beta$ -sarcoglycan increase in skeletal and cardiac muscle, but neither sarcoglycan is detectable in smooth muscle or non-muscle tissues (Fig. 1, *g*, *h*, *p*, and *q*; data not shown). In contrast, levels of  $\epsilon$ -sarcoglycan remain high in smooth muscle and vascular endothelium but decline perceptibly in skeletal and cardiac muscle (Fig. 1, *i*, *r*, *g'*, and *h'*). Thus, by birth,  $\alpha$ - and  $\beta$ -sarcoglycan appear to be more abundant than  $\epsilon$ -sarcoglycan in skeletal muscle, and at least as abundant as  $\epsilon$ -sarcoglycan in cardiac muscle.

Patterns on sarcoglycan expression do not change appreciably in skeletal or cardiac muscle postnatally (see below). In smooth muscles, however, an additional change in the comple-





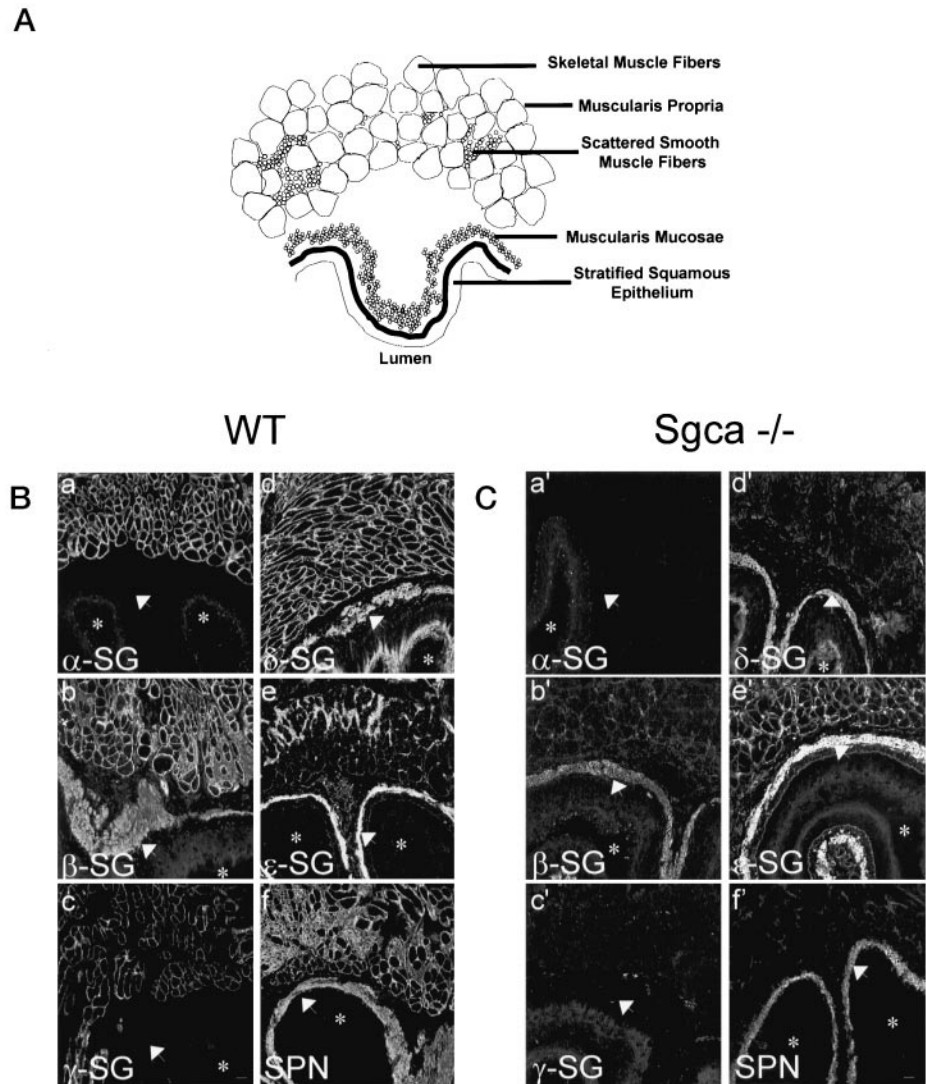
ment of sarcoglycans occurs after birth; although  $\epsilon$ -sarcoglycan is abundant in and  $\alpha$ -sarcoglycan is absent from smooth muscles throughout development (Fig. 1, a', c', d', i', and j'),  $\beta$ -sarcoglycan appears in this muscle lineage during the postnatal period. In bronchiolar smooth muscle, for example,  $\beta$ -sarcoglycan appears during the first postnatal week (Fig. 1b').

*$\epsilon$ -Sarcoglycan Is an Integral Component of the Smooth Muscle Sarcoglycan Complex*—To determine whether  $\epsilon$ -sarcoglycan replaces  $\alpha$ -sarcoglycan in smooth muscle cells and is associated with the sarcoglycan complex, we investigated the expression of  $\epsilon$ -sarcoglycan and the sarcoglycans in several adult smooth muscle-containing tissues, including the esophagus. In contrast to humans, the muscularis propria of the esophagus in mice consists of skeletal muscle fibers throughout the entire length of the tube and only contains scattered smooth muscle cells in its distal part (36). On transverse sections of the esophagus, one can therefore study protein expression in smooth muscle cells of the muscularis mucosa and skeletal muscle fibers of the muscularis propria side by side. Immunofluorescence staining of  $\epsilon$ -sarcoglycan was stronger at the plasma membrane of smooth muscle cells than in striated muscle fibers (Fig. 2B). In control animals,  $\epsilon$ -sarcoglycan was expressed in

smooth muscle cells of the gastrointestinal tract (Fig. 2B), the bladder, the lung, and the uterus (data not shown). Testing antibodies against the sarcoglycan complex in smooth muscle cells, we found coexpression of  $\beta$ - and  $\delta$ -sarcoglycan with  $\epsilon$ -sarcoglycan in all investigated tissues (Fig. 2B and data not shown). In contrast to striated muscle,  $\alpha$ - and  $\gamma$ -sarcoglycan were not expressed at the plasma membrane of smooth muscle cells (Fig. 2B). Using antibodies against other components of the DGC, we found expression of sarcospan, dystrophin, and  $\alpha$ - and  $\beta$ -dystroglycan in smooth muscle cells of control animals (Figs. 2-6).

Next, we tested whether  $\epsilon$ -sarcoglycan is a component of the isolated sarcoglycan complex from smooth muscle cells. The DGC was extracted from rabbit visceral smooth muscle by digitonin and further purified by WGA affinity chromatography followed by ion exchange on a DEAE-column. Proteins from the DEAE eluate were separated by SDS-PAGE, and the resultant polyacrylamide gels were immunoblotted with anti-sarcoglycan antibodies. As shown in Fig. 3,  $\epsilon$ -sarcoglycan copurified with the smooth muscle DGC.  $\beta$ - and  $\delta$ -sarcoglycan were enriched in smooth muscle DGC to a similar level as  $\epsilon$ -sarcoglycan, but immunoblots of smooth muscle DGC did not

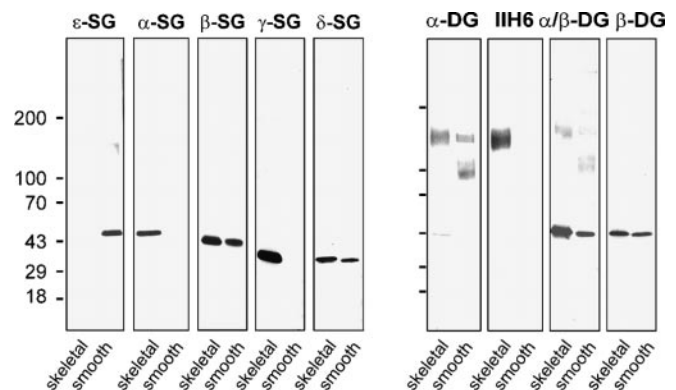
**FIG. 2. Smooth muscle cells express a sarcoglycan complex different from the one in striated muscle cells.** *A*, the schematic diagram shows part of a transverse section through the esophagus. In rodents, the muscularis propria of the esophagus consists of skeletal muscle fibers throughout the entire length of the tube and only contains scattered smooth muscle cells in its distal part. On transverse sections of the esophagus, one can therefore study protein expression in smooth muscle cells of the muscularis mucosa and skeletal muscle fibers of the muscularis propria side by side. *B*, the panels show 7- $\mu\text{m}$  transverse sections of the esophagus of control mice. Sections were stained with antibodies against  $\alpha$ - (*a*),  $\beta$ - (*b*),  $\gamma$ - (*c*),  $\delta$ - (*d*), and  $\epsilon$ -sarcoglycan (*e*) and sarcospan (*f*). All antibodies showed membrane staining of skeletal muscle fibers in the muscularis propria. In addition, antibodies against  $\beta$ -,  $\delta$ -, and  $\epsilon$ -sarcoglycan and sarcospan stained smooth muscle cells in the muscularis mucosa and within the muscularis propria. The  $\epsilon$ -sarcoglycan signal was stronger in smooth muscle cells than in skeletal muscle fibers (*e*). The  $\delta$ -sarcoglycan antibody showed unspecific staining of the keratinized stratified squamous epithelium. *C*, the panels show 7- $\mu\text{m}$  transverse sections of the esophagus of *Sgca*-deficient mice. Sections were stained with antibodies against  $\alpha$ - (*a*),  $\beta$ - (*b*),  $\gamma$ - (*c*),  $\delta$ - (*d*), and  $\epsilon$ -sarcoglycan (*e*) and sarcospan (*f*). The loss of  $\alpha$ -sarcoglycan causes a concomitant loss of  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan and sarcospan in skeletal muscle fibers. No effect of the  $\alpha$ -sarcoglycan gene mutation was detected on sarcoglycan expression in smooth muscle cells. *Arrows* denote smooth muscle cells in the muscularis mucosa and asterisks denote lumen. *Bar* is 50  $\mu\text{m}$ .



stain with antibodies against  $\alpha$ - and  $\gamma$ -sarcoglycan (Fig. 3). The molecular weight of  $\delta$ -sarcoglycan and  $\beta$ -dystroglycan also seemed to be slightly lower in smooth muscle cells compared with skeletal muscle fibers (Fig. 3).

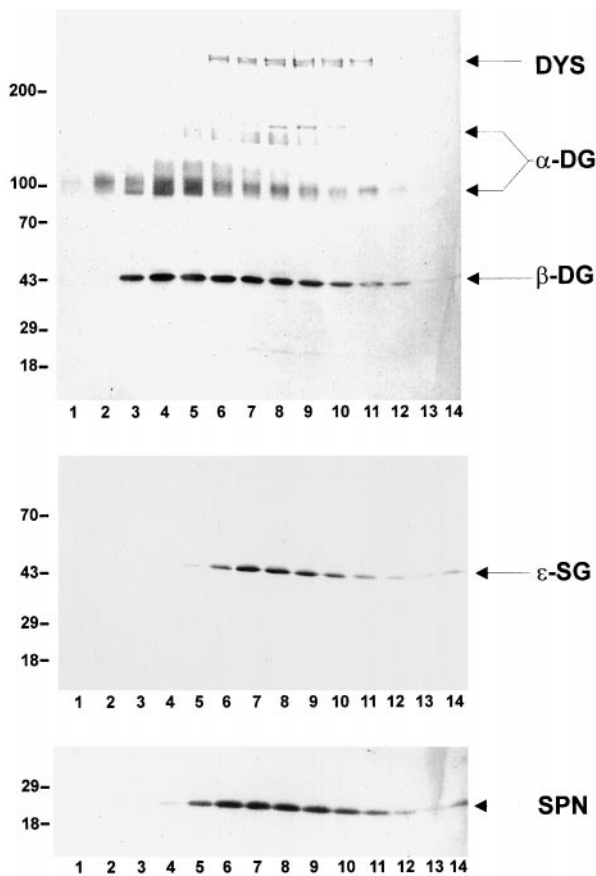
To further study the composition of smooth muscle DGC, we stained immunoblots with different anti-dystroglycan antibodies. In contrast to the single 156-kDa form of  $\alpha$ -dystroglycan in skeletal muscle, we detected two bands of about 156 and 100 kDa with antibodies against  $\alpha$ -dystroglycan (goat 20 and sheep OR12) in the smooth muscle DGC (Fig. 3). The anti- $\alpha$ -dystroglycan antibody IIH6, which shows carbohydrate-dependent staining (8), did not react with the smooth muscle form of  $\alpha$ -dystroglycan (Fig. 3), indicating different glycosylation patterns of skeletal and smooth muscle  $\alpha$ -dystroglycan.

The association of  $\epsilon$ -sarcoglycan with smooth muscle DGC was further illustrated by centrifugation of smooth muscle DGC through sucrose gradients. Proteins from the sucrose gradient fractions were separated by SDS-PAGE. Immunoblotting with antisera against DGC components revealed that the peak of smooth muscle DGC migrates in fractions 6–10 (Fig. 4), indicated by the 427-kDa dystrophin band. Western blotting of the same fractions with antibodies against  $\epsilon$ -sarcoglycan and  $\beta$ -sarcoglycan demonstrated that  $\epsilon$ -sarcoglycan co-migrates in the same fractions as the DGC during sedimentation through sucrose gradients (Fig. 4, and data not shown). This result confirmed that  $\epsilon$ -sarcoglycan is an integral component of the



**FIG. 3. Enrichment of  $\epsilon$ -sarcoglycan in smooth muscle DGC.** Skeletal muscle and smooth muscle DGC were electrophoretically separated on 3–15% SDS-polyacrylamide gels and transferred to nitrocellulose. Nitrocellulose transfers were separately stained with antibodies against all sarcoglycans (*SG*). Enrichment in skeletal muscle DGC was found for  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan. Enrichment in smooth muscle DGC was found for  $\epsilon$ -,  $\beta$ -, and  $\delta$ -SG. Nitrocellulose transfers of skeletal and smooth muscle DGC were separately stained with several antibodies against dystroglycan, including an affinity-purified polyclonal anti  $\alpha$ -dystroglycan antibody from goat 20; mouse monoclonal antibody IIH6 against  $\alpha$ -dystroglycan; affinity-purified polyclonal antibodies from sheep OR12 reacting with both  $\alpha$ - and  $\beta$ -dystroglycan; and an affinity-purified polyclonal anti  $\beta$ -dystroglycan antibody from sheep 005. Molecular size standards are indicated on the *left* of each panel ( $\times 10^3$  Da).



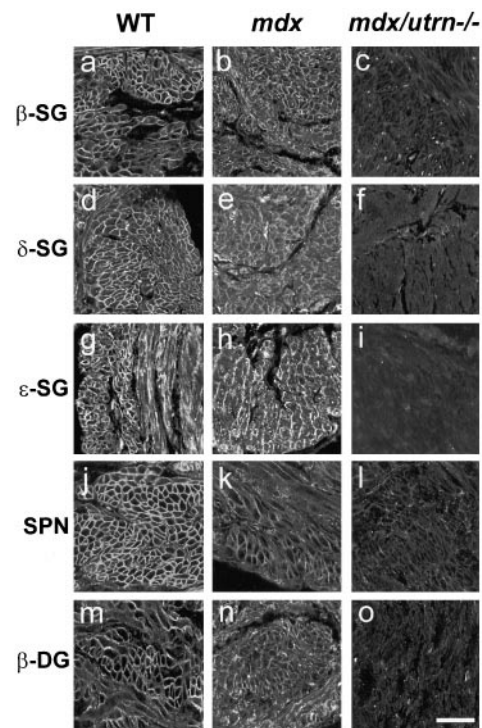


**FIG. 4.  $\epsilon$ -Sarcoglycan co-sediments with the smooth muscle DGC through linear sucrose gradients.** The sucrose gradient fractions from rabbit smooth muscle DGC were resolved by 3–15% SDS-PAGE and transferred to nitrocellulose. Nitrocellulose transfers were separately stained with antibodies against the DGC, recognizing dystrophin (*DYS*),  $\alpha$ - and  $\beta$ -dystroglycan ( $\alpha$ - and  $\beta$ -*DG*),  $\epsilon$ -sarcoglycan ( $\epsilon$ -*SG*), and sarcospan (*SPN*). Molecular size standards are indicated on the left of each panel ( $\times 10^3$  Da).

DGC in smooth muscle tissue. We also investigated if sarcospan, a recently characterized component of the DGC (7), which had not been studied in smooth muscle, is an integral element of the DGC in smooth muscle cells. By staining immunoblots of sucrose gradient fractions with anti-sarcospan antibodies, we illustrated that the peak of sarcospan also migrates in fractions 6–10, supporting the role of sarcospan as a constitutive component of the DGC.

Analysis of sucrose gradient fractions with antibodies against  $\alpha$ - and  $\beta$ -dystroglycan showed an additional protein peak for the dystroglycans in fractions 3–5 (Fig. 4). The 100-kDa form of  $\alpha$ -dystroglycan was particularly enriched in these fractions whereas the 156-kDa form was mainly found in fractions 6–10 together with the other DGC components, including  $\epsilon$ -sarcoglycan. Considering that the vast majority of cells in the muscularis propria of the pylorus are smooth muscle, these findings suggest the existence of two separate dystroglycan complexes in smooth muscle: one dystroglycan complex that co-fractionates with the other DGC components and one dystroglycan complex that does not.

**Loss of  $\epsilon$ -Sarcoglycan in *mdx/utrn*<sup>-/-</sup> Double Knock-out Mice**—We next investigated the distribution of  $\epsilon$ -sarcoglycan in mice deficient in both dystrophin and utrophin (*mdx/utrn*<sup>-/-</sup> mutant mice) (27, 37). If  $\epsilon$ -sarcoglycan is a component of the smooth muscle dystrophin-glycoprotein complex and/or the utrophin-glycoprotein complex, it should be altered in *mdx/utrn*<sup>-/-</sup> mutant mice. Indeed, expression levels of  $\epsilon$ -sarcoglycan in smooth muscle cells of bladder from *mdx/utrn*<sup>-/-</sup> mutant

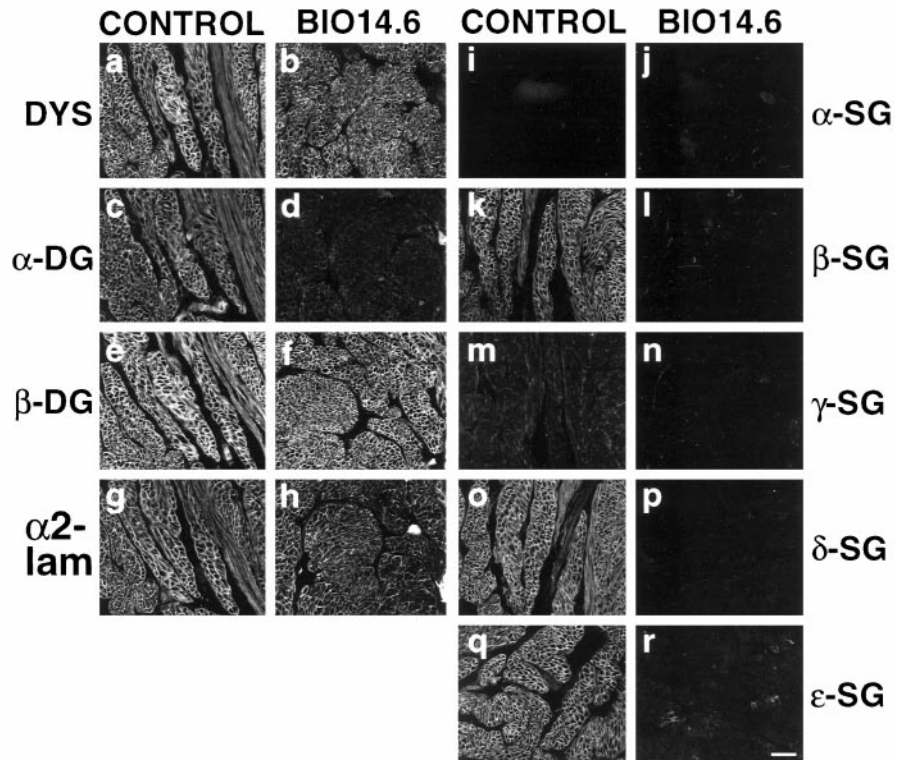


**FIG. 5. Loss of  $\epsilon$ -sarcoglycan in smooth muscle of dystrophin- and utrophin-deficient mice.** Cryosections of control bladder (*a, d, g, j, and m*) *mdx* bladder (*b, e, h, k, and n*), and *mdx/utrn*<sup>-/-</sup> bladder (*c, f, i, l, and o*) were stained with antibodies against  $\beta$ -sarcoglycan ( $\beta$ -*SG*),  $\delta$ -sarcoglycan ( $\delta$ -*SG*),  $\epsilon$ -sarcoglycan ( $\epsilon$ -*SG*), sarcospan (*SPN*), and  $\beta$ -dystroglycan ( $\beta$ -*DG*). The absence of dystrophin and utrophin resulted in the reduction of  $\beta$ -,  $\delta$ -, and  $\epsilon$ -sarcoglycan, sarcospan, and  $\beta$ -dystroglycan in smooth muscle. Bar, 50  $\mu$ m.

mice were greatly reduced (Fig. 5). Likewise, the other smooth muscle DGC components  $\beta$ -sarcoglycan,  $\delta$ -sarcoglycan, sarcospan, and  $\beta$ -dystroglycan were concomitantly reduced (Fig. 5). In smooth muscle cells of bladders from *mdx* mice, the expression levels of  $\beta$ -sarcoglycan,  $\delta$ -sarcoglycan, sarcospan, and  $\beta$ -dystroglycan were slightly reduced compared with wild type controls (Fig. 5).

**Distribution of  $\epsilon$ -Sarcoglycan in Smooth Muscle of  $\alpha$ - and  $\delta$ -Sarcoglycan-deficient Mutants**—As an additional test of the hypothesis that  $\epsilon$ -sarcoglycan is an integral component of the sarcoglycan complex in smooth muscle cells, we performed immunofluorescence analysis on smooth muscle-containing tissues of the BIO14.6 hamster. The cardiomyopathic hamster is a widely studied animal model for sarcoglycan deficiency that has a primary mutation in the  $\delta$ -sarcoglycan gene (28, 38). If  $\delta$ - and  $\epsilon$ -sarcoglycan are both components of the sarcoglycan complex in smooth muscle cells, the null mutation in the  $\delta$ -sarcoglycan might affect the distribution of  $\epsilon$ -sarcoglycan. Fig. 6 demonstrates the consequences of the  $\delta$ -sarcoglycan gene mutation on DGC expression in smooth muscle cells of the hamster bladder. Loss of  $\delta$ -sarcoglycan in smooth muscle cells of the BIO14.6 hamster resulted in greatly reduced expression levels of  $\epsilon$ -sarcoglycan and  $\beta$ -sarcoglycan (Fig. 6). Whereas  $\delta$ -sarcoglycan was completely deficient, both  $\beta$ - and  $\epsilon$ -sarcoglycan displayed residual staining at the plasma membrane in some fibers (Fig. 6). Applying other antibodies against DGC components, we observed comparable levels of expression as in control animals for dystrophin and  $\beta$ -dystroglycan in BIO14.6 tissues (Fig. 6). However, the expression level of  $\alpha$ -dystroglycan showed a reduction in the diseased animals compared with the control strain (Fig. 6). This result was in accordance with observations of reduced  $\alpha$ -dystroglycan expression in cardiac and skeletal muscle of the BIO14.6 hamsters (23, 28, 38–40)

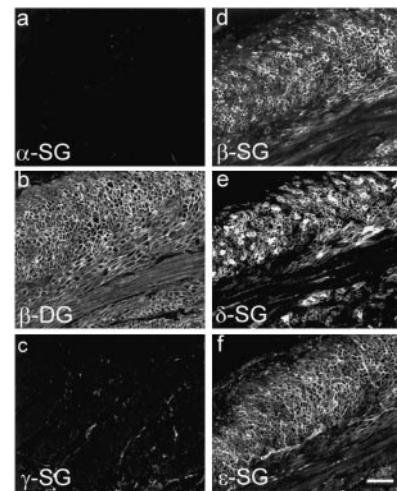
**FIG. 6. Loss of  $\epsilon$ -sarcoglycan in smooth muscle of the  $\delta$ -sarcoglycan-deficient hamster.** Cryosections from control bladder (*a, c, e, g, i, k, m, o, and q*) and BIO 14.6 bladder (*b, d, f, h, j, l, n, p, and r*) were stained with antibodies against dystrophin (*DYS*),  $\alpha$ -dystroglycan ( $\alpha$ -*DG*),  $\beta$ -dystroglycan ( $\beta$ -*DG*), laminin  $\alpha 2$  chain ( $\alpha 2$ -*lam*), and  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -, and  $\epsilon$ -sarcoglycan (*SG*). Antibodies against  $\alpha$ - and  $\gamma$ -sarcoglycan did not reveal membrane staining in smooth muscle cells of control animals. The  $\delta$ -sarcoglycan mutation in the BIO14.6 hamster resulted in the absence of  $\delta$ -sarcoglycan from the muscle fiber plasma membrane and a concomitant reduction of  $\beta$ - and  $\epsilon$ -sarcoglycan and  $\alpha$ -dystroglycan. Bar, 50  $\mu$ m.



and provides further evidence for the close association of the sarcoglycans with  $\alpha$ -dystroglycan. Similar results were observed in intestine and esophagus (data not shown).

We also assessed the distribution of  $\epsilon$ -sarcoglycan in smooth muscle of a recently characterized  $\alpha$ -sarcoglycan null mutant mouse (10). The *Sgca*-deficient mouse showed complete absence of  $\alpha$ -sarcoglycan in skeletal muscles, loss of the entire sarcoglycan complex from the sarcolemma, and progressive muscular dystrophy. Since  $\alpha$ -sarcoglycan is not expressed in smooth muscle cells, a null mutation in the *Sgca* gene should not affect the expression of the other sarcoglycans at the smooth muscle fiber plasma membrane. To test this hypothesis, we performed immunofluorescence analysis on several smooth muscle containing tissues of  $\alpha$ -sarcoglycan-deficient animals. Cryosections were stained with antibodies against the DGC components, including all sarcoglycans and sarcospan, which shows tight association with the sarcoglycan complex in skeletal muscle fibers (10). The tetrameric sarcoglycan complex and sarcospan were completely absent from the skeletal muscle fiber plasma membrane, but expression of  $\beta$ -,  $\delta$ -, and  $\epsilon$ -sarcoglycan and of sarcospan was unaffected in smooth muscle cells (Fig. 2C). These results confirm that the sarcoglycan complex in smooth muscle cells differs in composition from the sarcoglycan complex in skeletal muscle fibers.

**$\delta$ -Sarcoglycan Gene Transfer Restores the Smooth Muscle Sarcoglycan Complex in the BIO14.6 Hamster**—To further study the sarcoglycan complex in smooth muscle tissue, an adenovirus construct encoding human  $\delta$ -sarcoglycan was prepared for injection into hamster bladders. In  $\delta$ -sarcoglycan adenovirus-injected BIO14.6 bladders, many fibers around the injection site became  $\delta$ -sarcoglycan-positive (Fig. 7e). The extent of  $\delta$ -sarcoglycan localization to the plasma membrane was similar to that seen in unaffected control F1B hamster (Fig. 6), and in addition some cytoplasmic staining was evident, due possibly to high levels of expression generated by the cytomegalovirus promoter (Fig. 7e). Renewed expression of  $\delta$ -sarcogly-



**FIG. 7. Reassembly and restoration of the sarcoglycan complex after adenovirus injection.** The panels show 7- $\mu$ m transverse cryosections of a 3-month-old BIO14.6 hamster bladder after the injection of a recombinant  $\delta$ -sarcoglycan adenovirus containing the human  $\delta$ -sarcoglycan coding sequence under the control of a viral cytomegalovirus promoter. Serial sections were stained with antibodies against  $\alpha$ -sarcoglycan (*a*),  $\beta$ -dystroglycan (*b*),  $\gamma$ -sarcoglycan (*c*),  $\beta$ -sarcoglycan (*d*),  $\delta$ -sarcoglycan (*e*), and  $\epsilon$ -sarcoglycan (*f*). The smooth muscle sarcoglycan complex, consisting of  $\beta$ -,  $\delta$ -, and  $\epsilon$ -sarcoglycan, is restored at the smooth muscle plasma membrane 7 days after intramuscular injection of the adenovirus.  $\beta$ -Dystroglycan was serving as a positive control. Bar, 50  $\mu$ m.

can led to rescue of  $\beta$ - and  $\epsilon$ -sarcoglycan to the membrane (Fig. 7, *d* and *f*). Antibodies against  $\alpha$ - and  $\gamma$ -sarcoglycan on the other hand did not reveal any staining in  $\delta$ -sarcoglycan-positive cells (Fig. 7, *a* and *c*). These results confirmed that in smooth muscle the sarcoglycan complex is composed of at least  $\beta$ -,  $\delta$ -, and  $\epsilon$ -sarcoglycan.



## DISCUSSION

The DGC has been well characterized in striated muscle, and defects in its components are associated with muscular dystrophy and cardiomyopathy (12, 13). Although symptoms potentially due to malfunctions of smooth muscle have been reported in patients with muscular dystrophy (15–18), not much is known about the composition of the DGC in smooth muscle. In the present study, we have investigated the expression of the DGC in smooth muscle cells with particular emphasis on the composition of the sarcoglycan complex.

By studying embryonic muscle lineages, we demonstrated that during development striated and smooth muscle cells already become distinguishable by the composition of the sarcoglycans they express. The expression level of the recently characterized  $\epsilon$ -sarcoglycan was high throughout the development of smooth muscle but declined in striated muscle. Its homologue  $\alpha$ -sarcoglycan, on the other hand, was never detected in smooth muscle but showed increasing expression levels in developing striated muscle. This expression pattern was maintained in mature smooth muscle cells of various tissues, where we found expression of  $\beta$ -,  $\delta$ -, and  $\epsilon$ -sarcoglycan instead of the previously described complex of  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan in striated muscle cells (5).

Biochemical fractionation studies of purified smooth muscle DGC further demonstrated the affiliation of  $\beta$ -,  $\delta$ -, and  $\epsilon$ -sarcoglycan. Studies of the  $\delta$ -sarcoglycan-deficient hamster and genetically engineered mice supported these conclusions. The finding that  $\beta$ - and  $\epsilon$ -sarcoglycan were lost from the plasma membrane in smooth muscle cells of the BIO14.6 hamster due to a  $\delta$ -sarcoglycan mutation (38) indicated that all three proteins are tightly associated with each other. Our study showed for the first time that  $\epsilon$ -sarcoglycan is associated with the DGC and an integral component of a unique smooth muscle sarcoglycan complex. Furthermore, we demonstrated that sarcospan is tightly associated with the smooth muscle sarcoglycan complex.

Differences in the composition of smooth and skeletal muscle DGC are not restricted to the sarcoglycan complex. Based on molecular weight and migration in sucrose gradient fractions, our data indicated that there are at least two different forms of  $\alpha$ -dystroglycan in smooth muscle cells. This finding might reflect the plasticity of smooth muscle, which produces divergent smooth muscle cell populations. The ability to synthesize extracellular matrix components and the pharmacological, contractile, and electrophysiological properties of mature smooth muscle cells can vary widely depending on the location and status of a particular cell within the same tissue (41).

What does the composition of the smooth muscle sarcoglycan complex tell us about protein-protein interaction in the DGC? We demonstrated that the expression of  $\alpha$ -dystroglycan in smooth muscle cells was affected by the  $\delta$ -sarcoglycan gene mutation. Similar findings have been reported in skeletal muscle (28). Additionally, a patient homozygous for a mutation in the  $\beta$ -sarcoglycan gene has a reduction of sarcolemmal  $\alpha$ -dystroglycan staining (42). Considering the result that  $\alpha$ - and  $\gamma$ -sarcoglycan are not expressed in smooth muscle cells and  $\epsilon$ -sarcoglycan is not an integral component of the skeletal muscle DGC, the association between the sarcoglycan complex and  $\alpha$ -dystroglycan may either be mediated by  $\beta$ - and  $\delta$ -sarcoglycan or by sarcospan.

What is the potential function for the smooth muscle DGC and its role in the pathogenesis of muscular dystrophies? Previous findings in skeletal muscle supported the idea that one function of the DGC is to provide mechanical reinforcement of the sarcolemma and to maintain membrane integrity during cycles of contraction and relaxation (43–46). It remains specu-

lative whether differences in the composition of the smooth and skeletal muscle DGC are due to differences in contractile properties of skeletal and smooth muscle cells. In contrast to skeletal muscle, which is specialized for relatively forceful contractions of short duration and under fine voluntary control, visceral muscle is specialized for continuous contractions of relatively low force producing diffuse movements, resulting in contraction of the whole muscle mass rather than contraction of individual motor units.

The characterization of a smooth muscle DGC also sheds new light on the vascular hypothesis for muscular dystrophy (47). At least in LGMD2E and -F, caused by mutations in  $\beta$ - and  $\delta$ -sarcoglycan, dysfunction of vascular smooth muscle could contribute to skeletal and cardiac muscle pathology. Likewise, smooth muscle pathology could be the underlying cause for an altered vascular function and aortic contractility seen in the cardiomyopathic hamster (48–50). Together with a higher susceptibility of sarcoglycan-deficient cardiomyocytes, these factors could finally lead to the cardiomyopathy in the hamster and in affected patients.

The presented data also suggest that there might be clinical differences between patients with  $\beta$ - or  $\delta$ -sarcoglycan mutations (LGMD2E and -F) and those with  $\alpha$ - or  $\gamma$ -sarcoglycan mutations (LGMD2D and -C), as the smooth muscle in the latter ones should not be affected by the primary defect. Perturbation of smooth muscle function might contribute to the functional anomalies of the digestive tract reported for patients with muscular dystrophies (15–18). Furthermore, it may be possible to distinguish sarcoglycan-deficient LGMD patients by analyzing sarcoglycan expression in skin biopsies containing arector pili muscles and vascular smooth muscle.

Our findings also have implications for therapeutic approaches in muscular dystrophies. Replacement of dystrophin or sarcoglycans by gene therapy will only succeed when the replaced protein can interact with its appropriate partners and be functional. Therefore, it is of major interest to determine the status and composition of the DGC in the different muscle types and to investigate the role the three muscle lineages play in the pathogenesis of muscular dystrophy. Here, we have provided the first direct evidence that the absence of  $\delta$ -sarcoglycan in smooth muscle cells can be corrected by  $\delta$ -sarcoglycan gene transfer, leading to the reconstitution of the smooth muscle sarcoglycan complex. It remains to be shown whether the sarcoglycan-sarcospan complex in smooth muscle cells only consists of  $\beta$ -,  $\delta$ -, and  $\epsilon$ -sarcoglycan or if another yet to be identified sarcoglycan is part of this complex.

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