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Assembly of the Sarcoglycan Complex

INSIGHTS FOR MUSCULAR DYSTROPHY*

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Four unique transmembrane glycoproteins comprise the sarcoglycan complex in striated muscle. The sarcoglycan complex contributes to maintenance of sarcolemma integrity. A shared feature of four types of autosomal recessive limb girdle muscular dystrophy (LGMD) is that mutations in a single sarcoglycan gene result in the loss of all sarcoglycans at the sarcolemma. The mechanism of destabilization is unknown. We report here our findings of sarcoglycan complex biosynthesis in a heterologous cell system. We demonstrate that the sarcoglycans are glycosylated and assemble into a complex that resides in the plasma membrane. Complex assembly was dependent on the simultaneous synthesis of all four sarcoglycans. Mutant sarcoglycans block complex formation and insertion of the sarcoglycans into the plasma membrane. This constitutes the first biochemical evidence to support the idea that the molecular defect in sarcoglycan-deficient LGMD is because of aberrant sarcoglycan complex assembly and trafficking, which leads to the absence of the complex from the sarcolemma.

The dystrophin-glycoprotein complex (DGC)[1] is a group of integral and membrane-associated proteins located in the sarcolemma of cardiac and skeletal muscle fibers (for review see Refs. 1 and 2). The DGC consists of dystrophin, the syntrophins, α- and β-dystroglycan, and sarcoglycan, which is composed of four distinct transmembrane glycoproteins named α-, β-, γ-, and δ-sarcoglycan (SG) (for review see Refs. 1 and 2). Sarcospan (25 kDa), a recently identified member of the DGC, is unique in that it is predicted to contain four transmembrane domains (3). The DGC spans the sarcolemma and forms a functional link between the extracellular matrix and the cytoskeleton via the laminin-binding protein α-dystroglycan and the actin-binding protein dystrophin. The DGC is thought to stabilize the membrane against contraction-induced damage (4).

Mutations in α-, β-, γ-, and δ-SG cause autosomal recessive limb girdle muscular dystrophy (LGMD) types LGMD2D (5), LGMD2E (6, 7), LGMD2C (8), and LGMD2F (9), respectively. In each of these diseases a mutation in any one sarcoglycan gene results in the deficiency of the entire sarcoglycan complex. Genetic studies identified the BIO14.6 hamster as an animal model for sarcoglycan-deficient LGMD2F because of a deletion in the δ-SG gene (10). In this animal, dystrophic features are evident in skeletal and cardiac muscle (11), and the sarcoglycan complex is missing (12, 13). We reported the first example of sarcoglycan gene transfer and successful intervention of disease progression in the BIO14.6 hamster, employing a recombinant δ-SG adenovirus as a vehicle for gene transfer (14). Our work demonstrated that the sarcoglycan complex is a requisite component of the DGC for stable anchorage of α-dystroglycan to the extracellular face of the sarcolemma, as well as maintenance of an intact sarcolemma.

Despite rapid advances regarding the genetic defects that cause LGMD, little is known about the molecular defect underlying the disease. Furthermore, the sarcoglycan-deficient limb girdle muscular dystrophies are a heterogeneous group of diseases (15), and it will be important to understand how individual mutations abrogate sarcoglycan complex assembly. Mutations may result in the complete absence of messenger RNA for that gene, or alternatively an aberrant protein may be synthesized. In either case, protein trafficking miscues may result that block sarcoglycan complex biosynthesis.

Maturation and trafficking of another integral membrane protein, the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel, have been extensively studied. Cystic fibrosis (CF) is an autosomal recessive disorder characterized by defects in epithelial ion transport, often because of the loss of functional CFTR from the cell surface (16). The majority of CF mutations affect maturation of the CFTR by altering the secondary structure and normal processing. The misfolded protein is targeted for degradation via the ubiquitin-proteasome pathway (17–19). A similar defect in sarcoglycan biosynthesis and trafficking may underlie LGMD, although no studies have been performed to date to address this issue.

Our goal was to develop a cell culture system for the analysis of sarcoglycan complex biosynthesis. We reasoned that a heterologous cell system that lacks muscle-specific proteins would be ideal for studying both normal and mutant sarcoglycans. We report here expression of α-, β-, γ-, and δ-SG in Chinese hamster ovary (CHO) cells utilizing a high efficiency transfection protocol. Biochemical studies demonstrate that the sarcoglycans are glycosylated and assemble into a tight complex that resides in the plasma membrane. Individually expressed sarcoglycans were likewise glycosylated; however, they were located in internal membrane pools. Strikingly, mutant sarcoglycan constructs engineered to recapitulate known human mutations were found to abrogate sarcoglycan complex assembly and targeting to the plasma membrane. These results strongly suggest that the molecular defect in sarcoglycan-deficient LGMD results from the aberrant assembly and trafficking of the sarcoglycan complex to its final destination, the

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[1] The abbreviations used are: DGC, dystrophin-glycoprotein complex; SG, sarcoglycan; LGMD, limb girdle muscular dystrophy; CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.

sarcolemma. Furthermore, these results provide compelling support for the idea that sarcoglycan complex assembly requires concomitant synthesis of all four sarcoglycans.

EXPERIMENTAL PROCEDURES

cDNA Expression Constructs—The Myc-tagged human α-, β-, γ-, δ-, and δ^{Δ} SG pcDNA3 (Amersham Pharmacia Biotech) expression constructs have been described (14). The Grb2myc expression construct has been described (20). For the β^{Δ}-SG and γ^{Δ}-SG deletion mutants, stop codons were introduced immediately following amino acids 150 and 173, respectively. All constructs were sequenced by the DNA Core Facility at the University of Iowa.

Cell Culture and Transient Transfection by Electroporation—CHO cells were maintained in α-minimal Eagle's medium supplemented with nucleosides and 10% fetal bovine serum. Electroporation was performed as described previously (20). Optimal expression was achieved using approximately 5 μg of each plasmid DNA.

Surface Biotinylation, Immunoprecipitation, and Immunoblotting—Surface molecules were biotinylated using NHS-biotin (Pierce). Confluent cell monolayers were incubated with NHS-biotin in PBS (0.5 mg/ml) for 30 min at room temperature. Unreacted NHS-biotin was inactivated and removed by washing with Tris-buffered saline. Lysates were prepared by solubilization in lysis buffer (50 mM HEPES, pH 7.8, 300 mM NaCl, 1% digitonin, 0.1% Nonidet P-40, 2.5 mM EDTA, 100 mM sodium vanadate, 2 mM pepstatin, 0.5 trypsin inhibitory units of aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μM leupeptin). Clarified lysates were incubated with 20 μl of avidin-Sepharose (Pierce) at 4 °C with rotation overnight. The avidin-Sepharose complexes were washed three times with lysis buffer and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with the monoclonal 9E10 antibody. For co-immunoprecipitations, lysates were prepared as above and incubated overnight with monoclonal antibodies raised against α-SG (Ad1/20A6, Ref. 14) or β-SG (βSarc1/5B1, Ref. 14). Immune complexes were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting using the 9E10 monoclonal antibody. For immunoblotting with the 9E10 monoclonal antibody, proteins were visualized using enhanced chemiluminescence (Pierce).

Cytosolic versus Particulate Fractionation and Sucrose Gradient Sedimentation—Detergent-free cell extracts were separated into soluble and particulate fractions as described previously (20). Cell lysates were subjected to 5–30% linear sucrose gradient sedimentation (14).

Enzymatic Deglycosylation—Cell lysates (20 μg) were treated as recommended by the manufacturer (Oxford Glycosystems) and analyzed by immunoblotting with 9E10 antibody.

Immunocytochemical Staining and Fluorescence Microscopy—Cells were fixed in 2% paraformaldehyde, 0.2% Triton X-100 in PBS for 5 min at room temperature. The cells were blocked with 2% bovine serum albumin in PBS and then incubated with primary 9E10 antibody. Primary antibody was detected with fluorescently tagged secondary antibody (Jackson Immunoresearch). Cells were imaged using a Bio-Rad MRC-1024ES laser scanning confocal microscope.

RESULTS AND DISCUSSION

We engineered cytomegalovirus expression vectors encoding full-length human α-, β-, γ-, and δ-SG, as well as sarcoglycan deletion constructs that recapitulate mutations found in LGMD patients. All constructs contain a Myc epitope tag at the intracellular tail. A schematic representation of these proteins is shown in Fig. 1. Overall, the sarcoglycans share a similar topology with a single transmembrane domain. The sarcoglycans are all glycosylated and possess clusters of cysteine residues in their extracellular domains.

The sarcoglycan expression constructs were introduced into CHO cells by electroporation (estimated transfection efficiency of 90%). To evaluate the extent of glycosylation, cell extracts were subjected to enzymatic deglycosylation with PNGaseF and analyzed by immunoblotting with monoclonal antibody 9E10 (Fig. 2*A*). All of the sarcoglycans are glycosylated, as evidenced by a decrease in molecular weight following PNGaseF treatment. Based on this crude estimation, the extent of glycosylation appears similar whether the proteins are expressed individually or all four together (Fig. 2*A*). These results indicate that the proteins are properly folded and competent to move through the glycolytic pathways. Next, to determine

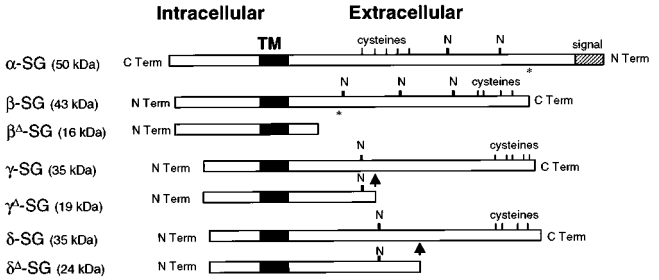

FIG. 1. **Human sarcoglycan expression constructs.** A schematic of the sarcoglycan expression constructs is shown. Transmembrane domains (*TM*), cysteine clusters (*cysteines*), consensus sites for asparagine-linked glycosylation (*N*), and the signal sequence (*signal*) are indicated. Expected molecular masses are in *parentheses*. All constructs contain a Myc epitope tag on the intracellular tail. Disease-causing missense mutations in α-SG (R77C (23, 24), denoted by *asterisk*) and β-SG (T151R (7), denoted by *asterisk*), as well as frameshift mutations that result in truncations in γ-SG (deletion of nucleotide 645 (8), denoted by *arrow*) and δ-SG (deletion of nucleotide 656 (9), denoted by *arrow*) are shown.

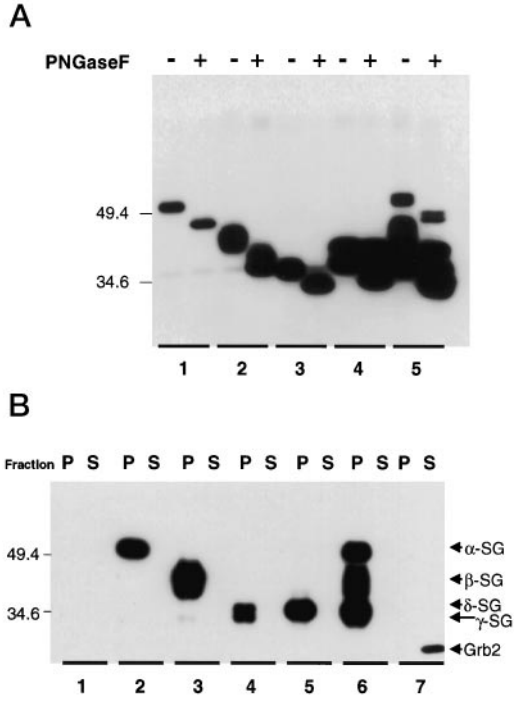

FIG. 2. **The sarcoglycans are glycosylated and are located in membrane fractions in CHO cells.** *A*, cells expressing α-SG (*lanes 1*), β-SG (*lanes 2*), γ-SG (*lanes 3*), δ-SG (*lanes 4*), or all four sarcoglycans α-, β-, γ-, and δ-SG (*lanes 5*) were examined for their state of glycosylation using PNGaseF and 9E10 immunoblotting. *B*, cell lysates from cells expressing empty vector (*lanes 1*), α-SG (*lanes 2*), β-SG (*lanes 3*), γ-SG (*lanes 4*), δ-SG (*lanes 5*), all four sarcoglycans α-, β-, γ-, and δ-SG (*lanes 6*), or the cytosolic protein Grb2myc (*lanes 7*) were separated into soluble (*S*) and particulate (*P*) membrane fractions as indicated and analyzed by 9E10 immunoblotting.

where the sarcoglycans reside within the cell, we examined the cytosol *versus* membrane distribution of the proteins. The sarcoglycans were found exclusively in the membrane fractions (Fig. 2*B*, *lanes 2–6*). As a control, the cytosolic adapter protein Grb2 was found in the soluble fraction (Fig. 2*B*, *lane 7*).

One remarkable feature of the sarcoglycan complex isolated from native tissue is that the complex is resistant to disassembly under extreme conditions of detergent or alkaline pH (21, 22). To that end, we performed biochemical experiments to determine whether the sarcoglycans expressed in CHO cells assemble into a stable molecular complex. Cells transfected with individual sarcoglycans or with all four sarcoglycans were

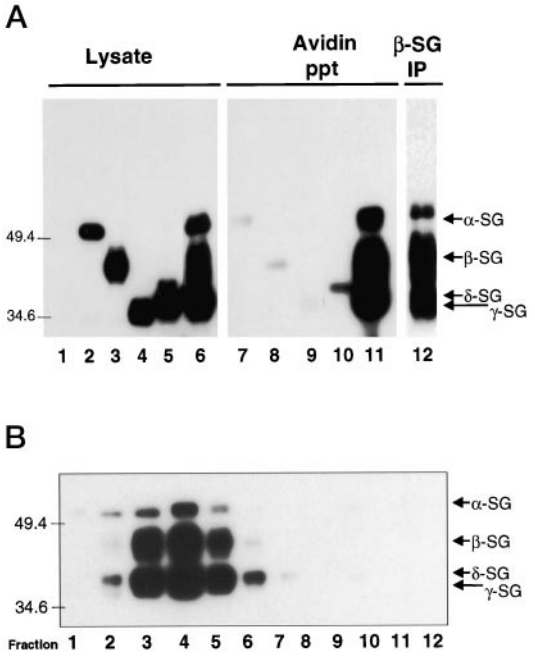

FIG. 3. **α-, β-, γ-, and δ-SG assemble into a molecular complex that assumes residence in the plasma membrane.** *A*, CHO cells were transfected with empty vector (*lane 1*) or expression vectors encoding α-SG (*lanes 2* and *7*), β-SG (*lanes 3* and *8*), γ-SG (*lanes 4* and *9*), δ-SG (*lanes 5* and *10*), or all four sarcoglycans α-, β-, γ-, and δ-SG (*lanes 6*, *11*, and *12*). Lysates are shown (*lanes 1–6*). Surface molecules were biotinylated and recovered from detergent extracts as avidin precipitates (*ppt*) (*lanes 7–11*). Immunoprecipitation (*IP*) of β-SG from cells expressing all four sarcoglycans results in the co-precipitation of α-, γ-, and δ-SG (*lane 12*). Proteins were visualized by 9E10 immunoblotting. *B*, cell lysates were prepared from cells transfected with α-, β-, γ-, and δ-SG and fractionated on a linear 5–30% sucrose gradient. Fractions collected from the top were analyzed by 9E10 immunoblotting.

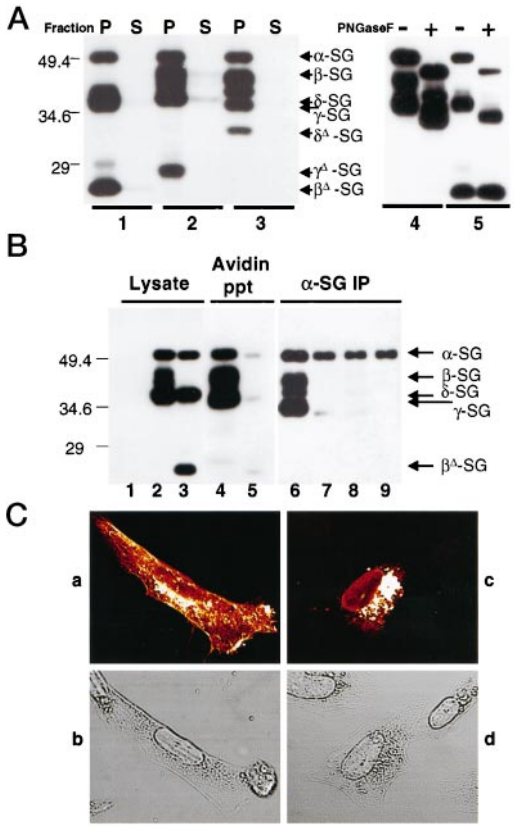

FIG. 4. **LGMD mutants abrogate sarcoglycan complex assembly and trap normal sarcoglycans in internal membranes.** *A*, cells expressing α-, β^{Δ}-, γ-, and δ-SG (*lanes 1*), α-, β-, γ^{Δ}-, and δ-SG (*lanes 2*), or α-, β-, γ-, and δ^{Δ}-SG (*lanes 3*) were separated into soluble (*S*) and particulate (*P*) membrane fractions and analyzed by 9E10 immunoblotting. Cells expressing α-, β-, γ-, and δ-SG (*lanes 4*) or α-, β^{Δ}-, γ-, and δ-SG (*lanes 5*) were examined for their state of glycosylation using PNGaseF and 9E10 immunoblotting. *B*, lysates from mock cells (*lane 1*) or cells expressing α-, β-, γ-, and δ-SG (*lanes 2* and *4*) or α-, β^{Δ}-, γ-, and δ-SG (*lanes 3* and *5*) were analyzed as lysate (*lanes 1–3*) or biotinylated surface molecules precipitated (*ppt*) by avidin-Sepharose (*lanes 4* and *5*) and visualized by 9E10 immunoblotting. Cells expressing α-, β-, γ-, and δ-SG (*lane 6*), α-, β^{Δ}-, γ-, and δ-SG (*lane 7*), α-, β-, γ^{Δ}-, and δ-SG (*lane 8*), or α-, β-, γ-, and δ^{Δ}-SG (*lane 9*) were subjected to α-SG immunoprecipitation (*IP*) and 9E10 immunoblotting. *C*, cells expressing α-, β-, γ-, and δ-SG (*a* and *b*) or α-, β^{Δ}-, γ-, and δ-SG (*c* and *d*) were analyzed by immunofluorescence using 9E10 antibody (a, c) and in the same focal plane using differential interference contrast (*b* and *d*) to image the cell borders.

treated with NHS-biotin, a hydrophilic probe for surface molecules. This probe forms a covalent bond with free amines in surface proteins of living cells, and the biotin moiety then functions as a tag for precipitation with avidin-Sepharose. Fig. 3*A* demonstrates that in cells expressing α-, β-, γ-, and δ-SG, the sarcoglycan complex is resident at the cell surface (*lane 11*). In contrast, in cells expressing any single sarcoglycan, little if any sarcoglycan protein appears at the cell surface, even in this overexposed blot (Fig. 3*A*, *lanes 7–10*). This experiment has been replicated in at least six independent experiments. As a control, lysates were examined by 9E10 immunoblotting to confirm that similar amounts of sarcoglycan proteins were synthesized in all cells (Fig. 3A, *lanes 2–6*).

To demonstrate formation of a stable molecular sarcoglycan complex, we performed co-immunoprecipitations using monoclonal antibodies against individual sarcoglycans. Immune complexes were analyzed by 9E10 immunoblotting, and as shown in Fig. 3*A* (*lane 12*), α-, γ-, and δ-SG co-immunoprecipitate as a complex with β-SG. Specific immunoprecipitation using monoclonal antibodies against α-, γ-, and δ-SG also resulted in the co-immunoprecipitation of all other sarcoglycans (data not shown). Finally, we performed linear sucrose gradient fractionation of lysates from cells expressing α-, β-, γ-, and δ-SG. As shown in Fig. 3*B*, the sarcoglycans migrate as a complex that peaks in fractions 3–5. Together these data provide strong evidence that simultaneous expression of α-, β-, γ-, and δ-SG in CHO cells results in the formation of a tight molecular complex. Furthermore, these results suggest that surface membrane localization of sarcoglycans depends on the coordinate synthesis and assembly of α-, β-, γ-, and δ-SG into a molecular complex.

Most missense and deletion mutations known to cause LGMD are concentrated in the extracellular domains of the sarcoglycan proteins. With this in mind, we engineered sarcoglycan extracellular deletion mutants. The β^{Δ}-SG mutant construct was designed to remove a large portion of the extracellular domain (see Fig. 1). In addition, the γ^{Δ}- and δ^{Δ}-SG constructs recapitulate known mutations causing LGMD2C and LGMD2F, respectively (8, 9). For completeness, an α-SG deletion mutant was also constructed; however, expression was too low for use in this study. Lysates prepared from cells transfected with three normal sarcoglycans and the corresponding single deletion mutant were fractionated into soluble and membrane fractions and analyzed by 9E10 immunoblotting. As seen in Fig. 4*A*, the deletion mutants were expressed at levels comparable with the normal sarcoglycans, and like the normal proteins, the mutants were highly enriched in membranes. This was expected as the deletion mutants retain their transmembrane domains. In the case of the β^{Δ}-SG, the large region removed from this protein includes the potential consensus site for *N*-linked glycosylation. In fact, this protein is

not glycosylated nor does it interfere with glycosylation of the other normal sarcoglycans as shown in Fig. 4*A* (*lanes 4* and 5).

We next performed biochemical experiments to evaluate what effects mutations in individual sarcoglycan proteins might have on the assembly and localization of the complex as a whole. Living cells expressing normal copies of α-, γ-, and δ-SG, as well as the mutant β^{Δ}-SG, were treated with the membrane-impermeant surface label NHS-biotin. Biotinylated surface molecules were recovered using avidin-Sepharose, and the complexes were analyzed by 9E10 immunoblotting. The sarcoglycan complex is readily detected at the surface of cells expressing α-, β-, γ-, and δ-SG, as demonstrated earlier (Fig. 4*B, lane 4*; Fig. 3*A*, *lane 11*). Dramatically, the sarcoglycan complex is nearly absent from the surface of cells expressing α-, β^{Δ}-, γ-, and δ-SG (Fig. 4*B*, *lane 5*). Lysates from these cells were examined by 9E10 immunoblotting to confirm that similar amounts of each sarcoglycan were in fact synthesized (Fig. 4*B*, *lanes 2* and *3*). This observation, together with the fractionation data, implies that the normal sarcoglycan proteins (α-, γ-, and δ-SG) are trapped in internal membrane pools because of the presence of the β^{Δ}-SG mutant protein.

To assess molecular interactions of sarcoglycans expressed in the presence of a single deletion mutant, we performed co-immunoprecipitation experiments. α-SG immunoprecipitations were performed from cells expressing α-, β-, γ-, and δ-SG (Fig. 4*B*, *lane 6*), α-, β^{Δ}-, γ-, and δ-SG (Fig. 4*B*, *lane 7*), α-, β-, γ^{Δ}-, and δ-SG (Fig. 4*B*, *lane 8*), or α-, β-, γ-, and δ^{Δ}-SG (Fig. 4*B*, *lane 9*), and the immune complexes were examined by 9E10 immunoblotting. β-, γ-, and δ-SG proteins were co-precipitated with α-SG from cells expressing all four normal copies of α-, β-, γ-, and δ-SG (Fig. 4*B*, *lane 6*). In contrast, only α-SG is efficiently immunoprecipitated from cells expressing one mutant sarcoglycan and three normal sarcoglycans (Fig. 4*B*, *lanes 7–9*). In each case, similar amounts of all four sarcoglycans were present (Fig. 4*A*), so changes in protein expression levels because of the co-expression of a mutant construct cannot account for the disruption in sarcoglycan complex assembly. Interestingly, the normal proteins expressed together with β^{Δ}-SG appear fully glycosylated (Fig. 4*A*, *lanes 4* and *5*), suggesting that the block in sarcoglycan complex assembly occurs after glycosylation. This result clearly demonstrates that the assembly of a sarcoglycan complex requires four normal copies of α-, β-, γ-, and δ-SG.

Finally, to substantiate our idea that the mutant sarcoglycans block sarcoglycan complex assembly and trafficking to the plasma membrane, we looked at the localization of these proteins in cells by immunofluorescence. In cells expressing α-, β-, γ-, and δ-SG, the 9E10 antibody detected the sarcoglycan complex at surface membrane structures (Fig. 4*C*, *panel a*). In addition, there was a considerable amount of signal in various intracellular membrane compartments, which we attribute to the high levels of expression. In contrast, in cells expressing α-, β^{Δ}-, γ-, and δ-SG, the 9E10 antibody did not detect sarcoglycans at the surface, and instead the proteins were concentrated in regions around the nucleus (Fig. 4*C*, *panel c*). Our interpretation of this finding is that sarcoglycans that fail to assemble because of the presence of the β^{Δ}-SG are not competent for trafficking to the plasma membrane. The cells were imaged using differential interference contrast optics to clearly define the cell borders (Fig. 4*C, panels b* and *d*).

Despite recent advances in our understanding of the genetic defects in individual sarcoglycan genes that underlie autosomal recessive sarcoglycan-deficient LGMD, essentially nothing is known about the molecular defect that results in loss of the entire sarcoglycan complex from the sarcolemma. We have employed a heterologous cell system to study sarcoglycan complex assembly. The strength of this system is that mutations in individual sarcoglycans can be assessed in terms of their impact on sarcoglycan complex assembly. Additionally, this model system may be useful as a tool to discover pharmacological interventions to overcome the blockade in complex assembly and trafficking. Details about the molecular defect in sarcoglycan biosynthesis gained from this work will help direct efforts aimed at developing therapies for LGMD.

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