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Molecular and genetic characterization of sarcospan: insights into sarcoglycan–sarcospan interactions

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Autosomal recessive limb girdle muscular dystrophies 2C–2F represent a family of diseases caused by primary mutations in the sarcoglycan genes. We show that sarcospan, a novel tetraspan-like protein, is also lost in patients with either a complete or partial loss of the sarcoglycans. In particular, sarcospan was absent in a γ-sarcoglycanopathy patient with normal levels of α-, β- and δ-sarcoglycan. Thus, it is likely that assembly of the complete, tetrameric sarcoglycan complex is a prerequisite for membrane targeting and localization of sarcospan. Based on our findings that sarcospan is integrally associated with the sarcoglycans, we screened >50 autosomal recessive muscular dystrophy cases for mutations in sarcospan. Although we identified three intragenic polymorphisms, we did not find any cases of muscular dystrophy associated with primary mutations in the sarcospan gene. Finally, we have identified an important case of limb girdle muscular dystrophy and cardiomyopathy with normal expression of sarcospan. This patient has a primary mutation in the γ-sarcoglycan gene, which causes premature truncation of γ-sarcoglycan without affecting assembly of the mutant γ-sarcoglycan into a complex with α-, β- and δ-sarcoglycan and sarcospan. This is the first demonstration that membrane expression of a mutant sarcoglycan–sarcospan complex is insufficient in preventing muscular dystrophy and cardiomyopathy and that the C-terminus of γ-sarcoglycan is critical for the functioning of the entire sarcoglycan–sarcospan complex. These findings are important as they contribute to a greater understanding of the structural determinants required for proper sarcoglycan–sarcospan expression and function.

INTRODUCTION

The dystrophin–glycoprotein complex (DGC) is comprised of peripheral and integral membrane proteins (1–4) and provides a structural linkage between the extracellular matrix and the intracellular cytoskeleton of muscle cells (5). The components of the DGC are structurally organized into three distinct 'subcomplexes'. These subcomplexes are: (i) the cytoplasmic proteins dystrophin and syntrophin; (ii) α- and β-dystroglycan; and (iii) the sarcoglycan–sarcospan subcomplex (α-, β-, γ- and δ-sarcoglycan and sarcospan) (for a review see ref. 6). Many other proteins have recently been shown to associate with the DGC at the sarcolemma. These include dystrobrevin (7,8), nNOS (9,10), ε-sarcoglycan (11,12) and caveolin-3 (13,14). The presence of these proteins is significant as it implies a signaling role for the DGC as a whole. Several forms of autosomal recessive proximal muscular dystrophy are caused by mutations in genes encoding the sarcoglycans (for reviews see refs 15,16). Mutations in α-, β-, γ- or δ-sarcoglycan genes are responsible for limb girdle muscular dystrophy (LGMD) type 2D (17–19), 2E (20,21), 2C (22,23) and 2F (24,25), respectively. Sarcoglycanopathies primarily involve wasting of the shoulder and girdle muscles along with calf hypertrophy. Although the precise function of the sarcoglycans is unknown, the complex is clearly important for normal muscle physiology since their absence results in muscular dystrophy and cardiomyopathy (17,20,21,25,26).

We have recently characterized a novel 25 kDa dystrophin-associated protein and have named this protein sarcospan for its multiple sarcolemma spanning helices, which are predicted based on hydropathy analysis (14,27). Dendrogram analysis shows that sarcospan is structurally related to the transmembrane four or 'tetraspan' superfamily of proteins (27), which each possess four transmembrane domains and a large extracellular loop and are thought to play important roles in mediating transmembrane protein interactions. In skeletal muscle fibers, sarcospan is an integral component of the dystrophin–glycoprotein complex (14,28). We have previously shown that

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sarcospan is absent in Duchenne muscular dystrophy patients (27).

In the current report, we show that sarcospan is lost in patients with complete or partial loss of the sarcoglycan complex, even in a case where α-, β- and δ-sarcoglycan are present at the sarcolemma. We also report the identification of a novel and important case of LGMD where membrane expression of a mutant sarcoglycan–sarcospan complex is insufficient in preventing muscular dystrophy and cardiomyopathy. These data indicate that the C-terminus of γ-sarcoglycan is a critical determinant for the functioning of the entire sarcoglycan–sarcospan complex. Taken together, our data provide new information about the importance of protein–protein interactions within the DGC and demonstrate the necessity for thorough screening and identification of autosomal recessive LGMD patients. Given recent works suggesting that the DGC is involved in cellular signaling, future efforts will need to focus on understanding the important interactions between components of the sarcoglycan–sarcospan complex and intracellular signaling molecules.

RESULTS

Loss of sarcospan in sarcoglycan-deficient LGMD

In most cases of sarcoglycan-deficient LGMD, mutations in one sarcoglycan gene lead to the absence or a significant reduction in the tetrameric sarcoglycan complex within the skeletal muscle sarcolemma. We have investigated the membrane localization of the sarcoglycans and sarcospan in >30 autosomal recessive LGMD biopsies (some with defined mutations and others with as yet uncharacterized primary mutations). Skeletal muscle cryosections from these patients were stained with antibodies to each of the four sarcoglycans and sarcospan. In each case, sarcospan was absent or severely reduced from the muscle plasma membrane. In Figure 1 we show two representative cases of autosomal recessive LGMD with complete deficiency of the sarcoglycan–sarcospan complex (cases 21 and 22). Both of these patients have primary mutations in the β-sarcoglycan gene. We have examined a collection of α-, β- and γ-sarcoglyanopathies with severely reduced staining of the sarcoglycans and, again, we found only weak staining of sarcospan at the sarcolemma (Table 1, cases 1, A623a and A623b and B). Many of the mutations that we found in our sarcoglycan-deficient patients have been previously identified as disease-causing mutations. We identified two novel mutations in the γ-sarcoglycan gene, which result in partial loss of the sarcoglycans (cases 23 and 24). Specific information on sarcoglycan primary mutations and expression levels of the sarcoglycan–sarcospan complex are summarized in Table 1.

Expression of α-, β- and δ-sarcoglycan is not sufficient for membrane stabilization of sarcospan nor for prevention of muscular dystrophy

By immunostaining muscle biopsies in our patient database with antibodies to each of the four sarcoglycans, we were able to identify novel LGMD cases with partial sarcoglycan complex deficiency. These cases of partial sarcoglycan deficiency represent situations where one, two or three of the sarcoglycans are present at the muscle plasma membrane. Our goal was to determine whether the presence of a partial sarcoglycan complex at the sarcolemma would be sufficient for membrane localization of sarcospan. We examined α-sarcoglycanopathy patients with primary defects in α-sarcoglycan, including missense mutations, premature stop codons and aberrant splicing (Table 1, cases 16–20 and 23-24). In each of these patients, sarcospan was reduced or completely absent from the sarcolemma, as illustrated in cases 17–18 and 23–24 (Fig. 1). We found that membrane expression of either β-sarcoglycan or δ-sarcoglycan alone was not sufficient to restore sarcospan localization to the sarcolemma (Fig. 1, cases 17 and 18). We extended our analysis of patients with partial sarcoglycan loss by examining LGMD type 2C. Interestingly, normal expression of β- and δ-sarcoglycan at the sarcolemma, as found in case 23, was not sufficient to stabilize sarcospan at the muscle plasma membrane (Fig. 1). Furthermore, we identified another interesting γ-sarcoglycanopathy case with near normal expression of α-, β- and δ-sarcoglycan at the sarcolemma (case 24). Despite the presence of three sarcoglycans, sarcospan was not detected at the muscle plasma membrane (Fig. 1). Both of these patients (cases 23 and 24) represent novel mutations in the γ-sarcoglycan gene that have not been previously reported.

Analysis of the sarcospan gene in autosomal recessive muscular dystrophy

We have demonstrated that structural perturbations in the sarcoglycan–sarcospan complex are the cause of certain forms of autosomal recessive LGMD. Based on this finding, we reasoned that mutations in the sarcospan gene might be found in autosomal recessive LGMD muscular dystrophy cases that do not have identified primary mutations in the sarcoglycan genes (or in any other gene). We collected >50 such cases and used a PCR-based approach to screen these patients for possible abnormalities in the sarcospan gene. Using mostly intronic primers, we developed a PCR amplification strategy to separately amplify each of the three exons of the human sarcospan gene. As shown in Figure 2A, PCR amplification of exons 1, 2 and 3 of sarcospan from human genomic DNA resulted in the expected sizes of 300, 200 and 500 bp, respectively. PCR products, which contained sequence from each exon as well as the flanking intron–exon boundaries, were directly sequenced. Patient DNA chromatograms were compared with those from 100 CEPH controls and >150 unrelated disease controls. Although we did not find disease-associated mutations in sarcospan in any of these, we identified several polymorphic sites within the sarcospan coding region after analysis of >600 sarcospan alleles. The first polymorphic site is located in exon 2 (nucleotide 286T→C), and the second and third polymorphisms (nucleotides 557G→A and 682G→A) are located in exon 3 (Fig. 2B–D). Individuals with either one or two polymorphic alleles were detected by sequence analysis, as shown in Figure 2B–D. The exon 3 polymorphisms, separated by only 125 bp, were always found together and occurred in 5.4% of the control population. The exon 2 nucleotide 286T→C polymorphism was identified at a frequency of 7.6% in the control population. Each of these polymorphisms was identified in both control and affected populations at the same rate, demonstrating that these missense changes were not linked with disease. The exon 3 polymorphic site (nucleotide 557G→A) was confirmed by restriction analysis with *Hga*I and *Mae*II (data not shown; see Materials and Methods).

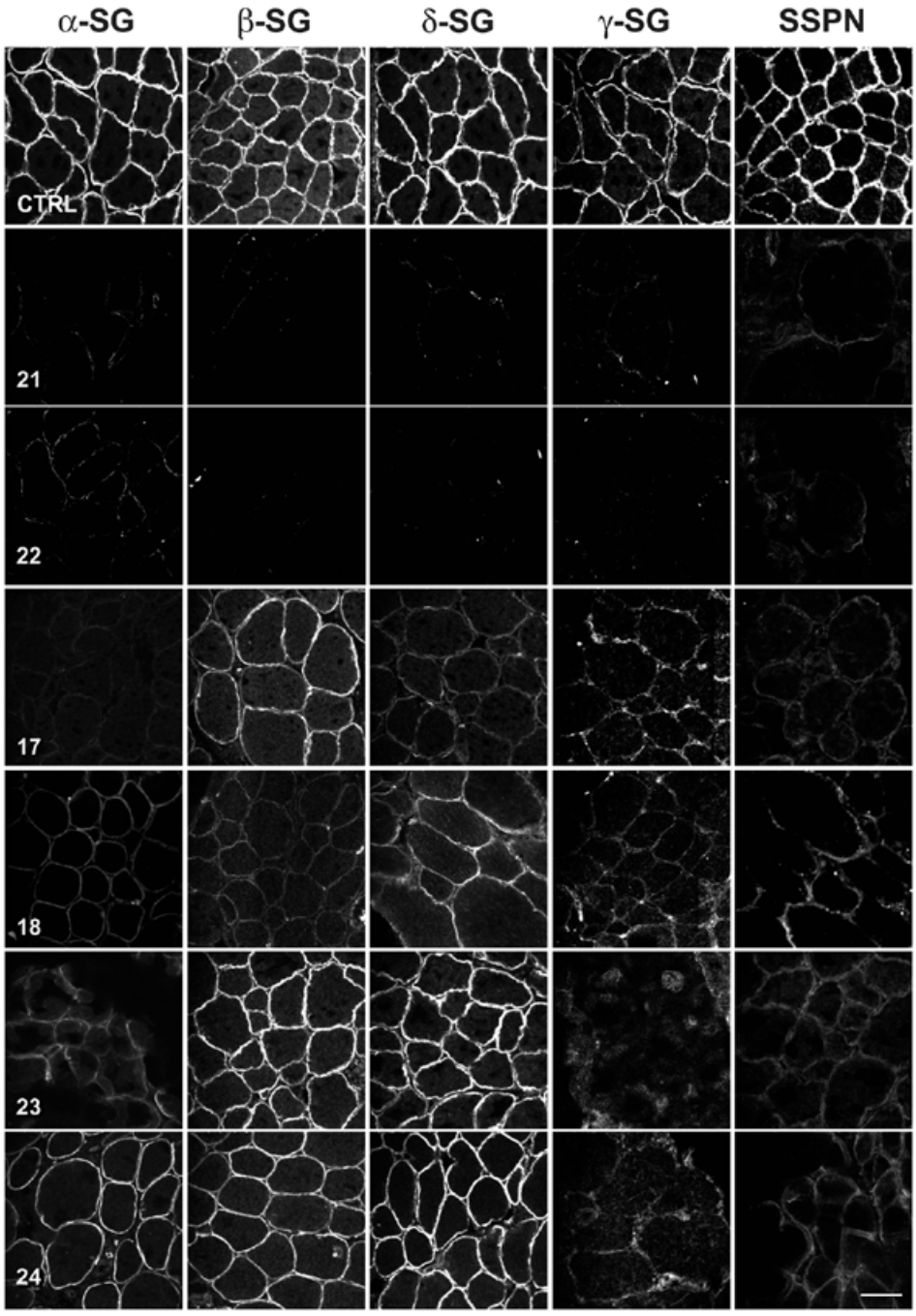

Figure 1. Localization of the sarcoglycan–sarcospan complex in limb girdle muscular dystrophy. Skeletal muscle cryosections from normal and sarcoglycan-deficient patients were stained with antibodies to α-, β-, γ- and δ-sarcoglycan (α-, β-, γ- and δ-SG, respectively) as well as sarcospan (SSPN). Each of these patients has a primary mutation in one of the sarcoglycan genes, as detailed in Table 1. Staining levels of the sarcoglycans and sarcospan in normal skeletal muscle (CTRL) are shown for comparison. Laminin-α2 and dystrophin staining were normal in each case (data not shown). Expression of the sarcoglycan–sarcospan complex is normal in patients with LGMD 2A linked to chromosome 15 (calpain deficiency), as well as myotonic, oculopharyngeal muscular, Emery–Dreifuss and congenital muscular dystrophy (data not shown). This demonstrates that loss of the sarcoglycan–sarcospan complex is not a general result of muscle fiber pathology. Bar, 50 μm.

LGMD and cardiomyopathy caused by membrane localization of a mutant sarcoglycan–sarcospan complex

We now report a novel and important case of γ-sarcoglycanopathy with cardiomyopathy caused by membrane expression of a mutant sarcoglycan–sarcospan complex. This is the first example of a mutant sarcoglycan associating in a complex with the other sarcoglycans and sarcospan. These two cases of γ-sarcoglycanopathy (patients S.D. and R.D.) also reveal interesting complexities in the pathogenesis of LGMD and provide insights into functional domains of the sarcoglycans. Patients S.D. and R.D. are siblings of Middle Eastern descent with

Table 1. Expression of the sarcoglycan–sarcospan complex in sarcoglycanopathies

Case no.	Primary gene	Mutation in protein	SG levels	SSPN levels[a]	Reference
LGMD 2D					
1	α-SG	Arg(77)Cys/Gly(68)Gln	Weak	Weak	35
5	α-SG	Tyr(62)His/Val(242)Ala	Weak	Weak	35
8a	α-SG	Val(247)Met/nd	Weak	Weak	35
10	α-SG	Arg(77)Cys/Arg(34)His	Weak	Weak	35
15a	α-SG	Splice intron 6/splice intron 6	Weak	Weak	35
1a	α-SG	G(410)A/(408)ins15	Weak	Weak	53
16	α-SG	Arg(77)Cys/Arg(77)Cys	Partial loss	Weak	This study
17	α-SG	Arg(77)Cys/FS(118) to X(212)	Partial loss	Weak	This study
18	α-SG	Arg(77)Cys/Val(247)Met	Partial loss	Weak	This study
19	α-SG	Arg(77)Cys/FS(323) to X(368)	Partial loss	Weak	This study
LGMD 2E					
A623a	β-SG	Thr(151)Arg/Thr(151)Arg	Weak	Weak	20
A623b	β-SG	Thr(151)Arg/Thr(151)Arg	Weak	Weak	20
20	β-SG	Ser(114)Phe/Ser(114)Phe	Partial loss	Weak	This study
21	β-SG	Ser(114)Phe/Ser(114)Phe	Absent	Absent	This study
22	β-SG	Ser(114)Phe/exon 3 splice donor	Absent	Absent	This study
LGMD 2C					
B	γ-SG	Cys(283)Tyr/Cys(283)Tyr	Weak	Absent	23
23	γ-SG	Δ bp 423–630/Δ bp 702–825	Partial loss	Weak	This study
R.D.	γ-SG	Phe(175)Leu to X(194)/Phe(175)Leu to X(194)	Normal	Normal	This study
S.D.	γ-SG	Phe(175)Leu to X(194)/Phe(175)Leu to X(194)	Normal	Normal	This study
24	γ-SG	Glu(176)X/Glu(176)X	Partial loss	Weak	This study

X, premature stop; FS, frameshift; Δ bp, deletion of DNA bases; ins, insertion of DNA bases. Amino acids are represented by three letter code and DNA bases are represented by single letter code.

[a]The levels of staining were determined by immunofluorescence where normal represents the level of staining observed in normal human skeletal muscle, weak represents weak staining and partial loss represents partial loss of the sarcoglycans. Two new mutations (cases 23 and 24) are presented. Images of selected patients are shown in Figures 1 and 2.

weakness predominantly in the shoulder and pelvic girdle muscles. Patient R.D. also presented with symptoms of dilated cardiomyopathy. Histochemical analysis of biopsies from patients R.D. and S.D. demonstrated the classic hallmarks of muscular dystrophy: fiber size variation, central nucleation and endomysial fibrosis, as well as the presence of rimmed vacuoles (Fig. 3A).

Analysis of the γ-sarcoglycan gene in patients R.D. and S.D. revealed a homozygous Δ525T mutation, which results in a frameshift at codon 175 and an amino acid change at this position from phenylalanine to leucine. An additional 18 missense amino acids are produced until premature termination at codon 194. This results in a significantly truncated protein product, in which more than half of the extracellular domain is missing. Immunofluorescence analysis of muscle from both patients shows that dystrophin, laminin-α2 and α- and β-dystroglycan were normal (Fig. 3B). Interestingly, γ-sarcoglycan staining was completely absent from the sarcolemma of both patients, whereas expression of α-, β- and δ-sarcoglycan as well as sarcospan was completely normal (Fig. 3B). We could not detect γ-sarcoglycan in these patients by immunostaining with two C-terminal γ-sarcoglycan antibodies (rabbit 245 and 35DAG/21B5). Truncation of the C-terminus of γ-sarcoglycan eliminates the epitopes recognized by both of these γ-sarcoglycan antibodies. The polyclonal rabbit 245 antibody was produced against a fusion protein construct comprising the distal half of γ-sarcoglycan and thus would not bind the truncated protein. The monoclonal antibody 35DAG/21B5 recognizes amino acids 167–178 and the frameshift mutation results in alteration of the last four amino acids of this epitope. Since the missense mutation in γ-sarcoglycan may produce a protein truncated at the C-terminus, we immunostained with an N-terminal γ-sarcoglycan antibody (rabbit 202). We found that this antibody stained γ-sarcoglycan in patient R.D., indicating that the truncated γ-sarcoglycan was assembled into a complex with the other sarcoglycans and sarcospan and localized to the sarcolemma (Fig. 3B). The presence of a mutant γ-sarcoglycan is sufficient for proper localization of the sarcoglycan–sarcospan complex to the sarcolemma, but not for normal function or prevention of muscular dystrophy (Fig. 4). The C-terminal region of γ-sarcoglycan is therefore an important functional determinant within the sarcoglycan–sarcospan complex.

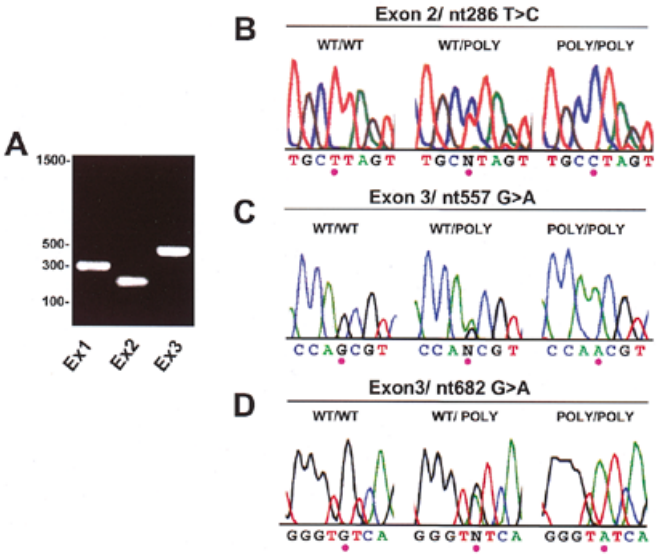

Figure 2. Identification of three polymorphic sites in the human sarcospan gene. (**A**) Exons 1–3 of sarcospan were PCR amplified from human genomic DNA using sequence-specific primers (PCR conditions are given in Materials and Methods). The resultant PCR products are shown after separation on an ethidium bromide stained agarose gel: exon 1 at 300 bp (lane 1), exon 2 at 200 bp (lane 2) and exon 3 at 500 bp (lane 3). DNA standards (bp) are indicated to the left of the panel. For each PCR reaction set, no DNA template controls were performed (data not shown). (**B**–**D**) Direct sequencing of PCR products amplified from genomic DNA. DNA chromatograms of unaffected control individuals with polymorphisms located in exon 2 (nucleotide 286T→C) and in exon 3 (nucleotides 557G→A and 682G→A) are shown. We found individuals with one wild-type and one polymorphic allele and individuals with two polymorphic alleles (WT/POLY and POLY/POLY, respectively). Also shown for comparison are sequences from controls with two wild-type alleles (WT/WT). Interestingly, the polymorphisms in exon 3 co-migrate in all samples analyzed and occur in 5.4% of control alleles. The altered nucleotides are highlighted with a pink dot.

DISCUSSION

Sarcoglycan gene mutations were initially identified as the causes of certain forms of muscular dystrophy and it was discovered that the entire complex could be lost due to a single mutation in one sarcoglycan gene (17,20,21,25,26). This led to the general hypothesis that the assembly, targeting and stability of the sarcoglycan–sarcospan complex is dependent on the integrity of each sarcoglycan subunit (29). With one exception, studies of various animal models of sarcoglycan-deficient LGMD have demonstrated complete loss of the tetrameric sarcoglycan subcomplex as a result of disruption of a single sarcoglycan gene (30–32). McNally and colleagues have generated a γ-sarcoglycan null mouse and their analysis indicates that genetic disruption of the γ-sarcoglycan gene results in partial retention of α- and ε-sarcoglycan (33). These data, along with identification of patients with partial sarcoglycan complex deficiency, suggests that the mechanism of sarcoglycan protein stabilization is more complex than originally thought (34–36). We now show that expression of a mutant sarcoglycan–sarcospan complex is not sufficient for prevention of muscular dystrophy. Thus, the extracellular C-terminal portion of γ-sarcoglycan is a critical structural determinant for functioning of the sarcoglycan–sarcospan complex (Fig. 4).

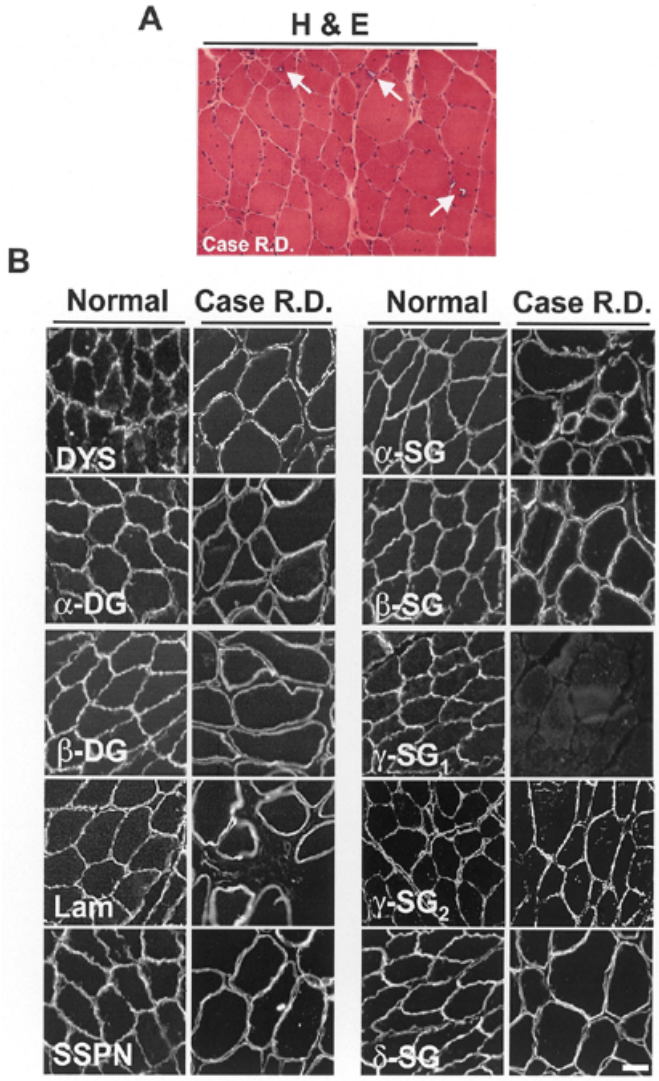

Figure 3. Membrane expression of a mutant sarcoglycan–sarcospan complex results in limb girdle muscular dystrophy. (**A**) Muscle histology of LGMD 2C patient R.D. Hematoxylin and eosin staining (H&E) of skeletal muscle from patient R.D., who has a primary mutation in γ-sarcoglycan (Table 1). Note the presence of central nucleation and the large variation in myofiber size. There are also small groups of atrophic fibers and mild endomysial fibrosis (data not shown). Three rimmed vacuoles are denoted by arrows. (**B**) Expression of the sarcoglycan–sarcospan complex in patient R.D. Transverse cryosections of skeletal muscle biopsies from a human control and patient R.D. were examined by immunofluorescence using antibodies against dystrophin (Dys), α/β-dystroglycan (α- and β-DG), laminin-α2 (Lam), α-, β- and δ-sarcoglycan (α-, β- and δ-SG) and sarcospan (SSPN). γ-SG_1 represents immunostaining with the C-terminal monoclonal antibody (35DAG/21B5) and γ-SG_2 represents immunostaining with an N-terminal polyclonal γ-sarcoglycan antibody (rabbit 202). Patient R.D. did not have reactivity with an additional C-terminal γ-sarcoglycan antibody (rabbit 245; data not shown).

Our report represents the first description of an LGMD 2C patient with normal expression levels of a mutant sarcoglycan–sarcospan complex. This mutation is believed to be the most frequent mutation in the γ-sarcoglycan gene found in North African populations (26,34,37). It has been reported previously (33–36) that this deletion causes both mild and severe muscular dystrophy with reduction of the sarcoglycan complex. Of particular interest is the presence of dilated cardiomyopathy in patient R.D. Cardiac manifestations are

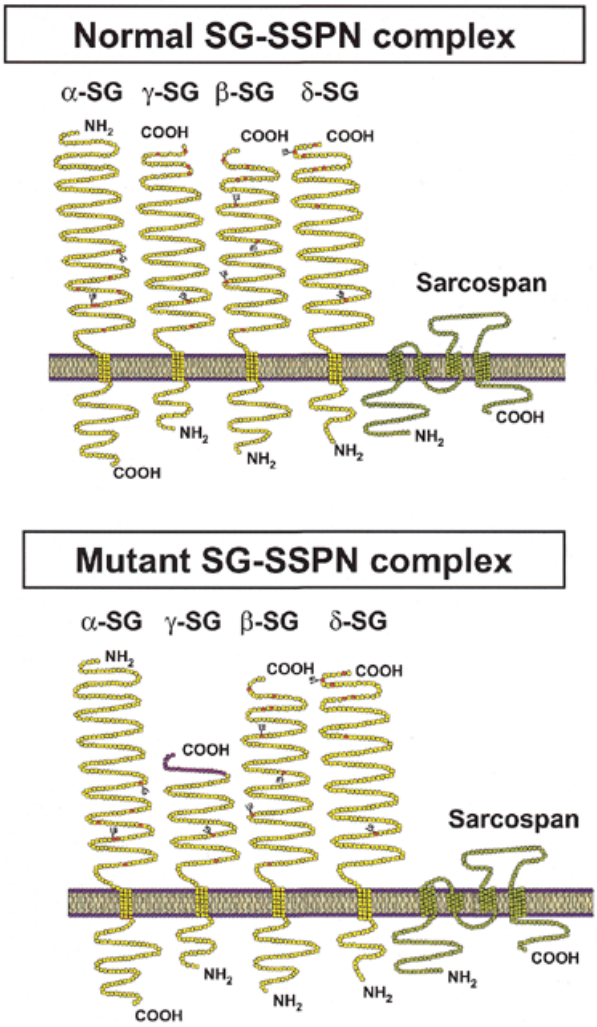

Figure 4. Muscular dystrophy resulting from perturbations in sarcoglycan–sarcospan complex function. Schematic diagram representing the mutant sarcoglycan–sarcospan complex in patient R.D. Analysis of the γ-sarcoglycan gene revealed a homozygous Δ525T mutation in the patient, which changes the amino acid at this position from phenylalanine to leucine and results in a frameshift at codon 175. An additional 18 missense amino acids (purple) are produced until premature termination at codon 194. This truncation removes the conserved cysteine motif in γ-sarcoglycan, which is unique to the sarcoglycan proteins. The premature truncation of γ-sarcoglycan removes an important structural determinant required for sarcoglycan–sarcospan function.

common with dystrophin mutations (38,39) and there have been sporadic cases of LGMD with dilated cardiomyopathy (40-42). Given the predicted similarity of function between skeletal and cardiac sarcoglycan subcomplexes, cardiac disease is not unexpected, though relatively few cases have been reported. In addition, animal models of autosomal recessive LGMD, including the BIO 14.6 hamster and γ- and δ-sarcoglycan knockout mice, show evidence of significant cardiac involvement (33,43). However, the presence of cardiomyopathy in this patient was surprising, since this complication has not been previously reported in patients with the Δ525T mutation. This variability in disease presentation and protein expression highlights the complexity in understanding even a single mutation.

The findings described herein have obvious important and critical diagnostic implications for sarcoglycan-deficient LGMD. Since it was assumed that any sarcoglycan gene disruption could be detected by immunohistochemical analysis of a single sarcoglycan protein, many diagnostic studies of LGMD only include screening for α-sarcoglycan. If α-sarcoglycan were detected, it was generally concluded that the patient in question did not have a sarcoglycan complex deficiency. This assumption formed the basis of several studies that have examined the prevalence of sarcoglycan complex mutations (44–46) and has become the standard for most prospective studies of muscular dystrophy patients. In fact, sarcoglycan complex disruption was initially ruled out in patients S.D. and R.D. based on positive α-sarcoglycan immunostaining. Our study demonstrates that correct diagnosis of sarcoglycan-deficient LGMD necessitates the use of a complete panel of sarcoglycan and sarcospan antibodies, in order to detect the presence of an isolated sarcoglycan protein loss.

Our report represents the first demonstration that sarcospan is reduced in sarcoglycan-deficient autosomal recessive LGMD. We show that the presence of three sarcoglycans at the sarcolemma (Fig. 1, case 24) is not sufficient for membrane localization of sarcospan. Previous examination of mice with targeted deletions of the sarcoglycan genes found that sarcospan is absent in these sarcoglycan-deficient mice (28,31,32,43,47). These mouse models do not necessarily reflect the complexities of LGMD, especially given the numerous types of mutation (missense, in-frame/out-of-frame deletions, premature truncation, splicing aberrations, etc.) found in human sarcoglycanopathy patients. The complexities of human LGMD are reflected in the variability of the sarcoglycan proteins at the sarcolemma, a feature that is not reflected in knockout mouse models of LGMD. The differences between human and murine models is especially pertinent in the case of sarcospan, since human skeletal muscle has two sarcospan mRNA transcripts, whereas mouse skeletal muscle has only one, suggesting differences in RNA splicing or regulation between these species (27,28). Furthermore, as we demonstrate here, patients with the same mutation can have different clinical presentations as well as different protein profiles, again underlining the necessity to examine human patients (Fig. 4). There are currently no murine models with partial sarcoglycan deficiency or expression of a mutant sarcoglycan–sarcospan complex. Thus, our studies also emphasize the importance of creating animal models that recapitulate known human missense mutations in the sarcoglycan genes, which could be accomplished by a targeted 'knock-in' of the mutant gene.

Finally, we report the development of a PCR-based approach to analyze the sarcospan gene from human genomic DNA. We identified three missense polymorphisms within the coding region of sarcospan. Recent data have shown that sarcospan-deficient mice have normal expression of the sarcoglycans and maintain muscle function without any signs of muscular dystrophy (48). These experiments suggest that murine muscle can compensate for the loss of sarcospan function. To date, we have not discovered human mutations in sarcospan that are associated with autosomal recessive dystrophy. Human muscle may be capable of functioning in the absence of sarcospan, perhaps by replacement with another tetraspan protein. Alternatively, it is possible that sarcospan may not be required for normal DGC function.

In the current report, we clearly demonstrate that a structural defect in the sarcoglycan–sarcospan complex causes muscular dystrophy. The exact function of the sarcoglycan–sarcospan complex has not been clearly defined. However, recent work (49) suggests a possible biochemical interaction between the sarcoglycan–sarcospan complex and the N-terminus of dystrobrevin. Dystrobrevin, syntrophin and nNOS are associated with the DGC at the subsarcolemma (for a review see ref. 50). The DGC may play an important role in signal transduction, although that precise role has not been identified. Our finding that the extracellular region of γ-sarcoglycan is critical for sarcoglycan–sarcospan complex function may be due to perturbation of protein–protein interactions within the complex or, perhaps, with the binding of as yet unknown extracellular molecules.

MATERIALS AND METHODS

Muscle biopsies and clinical information

Clinical descriptions as well as mutational analysis of several of the α- and γ-sarcoglycan-deficient patients (LGMD 2D and 2C) have been described (23,35). We have also previously characterized the β-sarcoglycan mutation in LGMD 2E patients (20). Dystrophin and laminin-α2 staining in autosomal recessive sarcoglycan-deficient patients was normal in all cases (data not shown). Pathological controls included LGMD 2A linked to chromosome 15 (calpain deficiency), myotonic dystrophy, congenital muscular dystrophy and oculopharyngeal muscular dystrophy, which expressed normal levels of sarcospan.

Patient R.D. is a 25-year-old male of Palestinian origin; his ancestors have lived in Jerusalem for several generations. His parents are not known to be related. At age 23 years, an electrocardiogram revealed evidence of cardiomyopathy. Both patients, R.D. and S.D., had muscle weakness predominantly in the shoulder and pelvic girdle muscles. Serum creatine kinase levels were 500–840 U/l and pulmonary function tests were normal. At age 25 years, he developed congestive heart failure and pulmonary edema with tachyarrhythmia. Patient S.D. is a 32-year-old sister of patient R.D. Birth and early development were normal. Serum creatine kinase levels were 608 U/l (normal <50 U/l). A skeletal muscle biopsy revealed small groups of atrophic fibers, mild endomysial fibrosis, an increase in fibers with central nuclei and rimmed vacuoles, consistent with chronic myopathy. Pulmonary function tests were normal. An electrocardiogram revealed a possible old inferior infarct. The parents of patients R.D. and S.D. are unaffected. Similarly, a brother (26 years old) and four other sisters (17, 24, 26 and 27 years of age) are unaffected.

Antibodies

Monoclonal antibodies. Monoclonal antibodies against α- (Ad1/20A6), β- (Bsarc1/5B1), δ- (dSarc3/12C1) and γ-sarcoglycan (35DAG/21B5), as well as monoclonal antibodies against β-dystroglycan (43DAG/8D5), were generated in collaboration with Dr Louise V.B. Anderson (Newcastle General Hospital, Newcastle upon Tyne, UK). Monoclonal antibody VIA4$_2$ against dystrophin was also used for immunofluorescence.

Polyclonal antibodies. Antibodies to human sarcospan were generated in rabbit (rabbit 216) against an N-terminal sarcospan peptide as previously described (27). Polyclonal antibody VI-IVb recognizes laminin-α2. Polyclonal antibodies against α-dystroglycan (sheep 005) and γ-sarcoglycan (rabbit 245 and goat 020) were affinity purified and used as described. To generate N-terminal anti-γ-sarcoglycan antibodies, a New Zealand White rabbit (rabbit 202) was injected i.m. and s.c. with an N-terminal human γ-sarcoglycan synthetic peptide, MVREQYTTATEGICAAC (peptide 103; Research Genetics, Huntsville, AL) coupled to keyhole limpet hemocyanin in an emulsion of Freund's complete adjuvant (Sigma, St Louis, MO). The amino acids Ala-Ala-Cys were added to the peptide for coupling purposes. Affinity purification of anti-γ-sarcoglycan antibodies was accomplished using Immobilon-P (Millipore, Bedford, MA) strips containing the N-terminal peptide coupled to bovine serum albumin.

Immunofluorescence

Transverse cryosections (7 μm) were analyzed by immunofluorescence as described (35,51,52) with an MRC-600 laser scanning confocal microscope (Bio-Rad, Hercules, CA). Digitized images were captured under identical conditions. The immunostaining status was graded from 0 (absent) to +++ (normal expression, as observed in biopsy specimens of deltoid muscle from patients without muscle disorders). Dystrophin and laminin-α2 staining in the autosomal recessive sarcoglycan-deficient patients was normal in all cases (data not shown).

Mutation analysis of the sarcoglycan genes

Where indicated, mutational analysis for several patients has been described and reported elsewhere (see refs in Table 1). To identify new patient mutations, genomic DNA was prepared from whole blood leukocytes and the individual exons of each sarcoglycan gene (α-, β-, γ- and δ-SG) were PCR amplified and directly sequenced at the Genetic Diagnostic Laboratory (University of Pennsylvania School of Medicine, Philadelphia, PA). A summary of sarcoglycanopathy primary mutations can be found at the Leiden Muscular Dystrophy Mutation Database (http://www.dmd.nl/dys_mutat.html). Sequence nomenclature is consistent with the human α- (U08895), β- (U63796–U63801) and γ-sarcoglycan (U63388–U63395) gene sequences found in GenBank. We did not perform phenotype–genotype correlations.

PCR amplification of exons 1–3 of the sarcospan gene

All PCR amplifications were performed in 100 μl volumes containing 100 ng of human genomic DNA, 200 ng of each primer, 2.5 mM dNTPs and 10 U of *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). Except for the amplification of exon 1, PCR buffer consisted of 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3. Thermocycling was carried out in an Omnigene Thermocycler (Hybaid, Woodbridge, NJ) with the following cycling conditions: 92°C for 2 min; 35 cycles of 94°C for 1 min, annealing temperature (T_a) for 1 min, 72°C for 1 min; followed by 72°C for 10 min.

Exon 1. In order to amplify the GC-rich region of exon 1, the Advantage-GC genomic PCR kit (Clontech, Palo Alto, CA) was used. The PCR buffer consisted of 40 mM Tris–HCl, pH 9.3, 2 mM $MgCl_2$, 85 mM KOAc, 5% dimethylsulfoxide, 0.5% Triton X-100, 1.1 mM $Mg(OAc)_2$ and 30 µl of GC Melt (Clontech). Forward primer, 5′-GCC ACG CGG CCA GCA GAG GCA GG-3′; reverse primer, 5′-AAC GCG CTT AGG CAA AAC AGA C-3′. The T_a was 57°C. PCR product size was 300 bp.

Exon 2. Forward primer, 5′-gaa cgc aca acg tac cct gat aac-3′; reverse primer, 5′-tgt gat gat aaa aga gaa aga aca-3′. The T_a was 50°C and the resulting PCR product was 200 bp.

Exon 3. Forward primer, 5′-GGC AAA TCA TCA TCC AAT GTT CT-3′; reverse primer, 5′-TGT GCT ACT TTC TCT CGC AAC T-3′. The PCR product size was 500 bp and the T_a was 50°C. The PCR products were purified using a QIAquick PCR purification spin column (Qiagen, Valencia, CA).

Five microliters of the PCR products were analyzed by electrophoresis on 1% Seakem agarose gel stained with ethidium bromide. PCR products (2.5 ng/100 bp) were individually sequenced with their respective forward and reverse primers. DNA sequencing reactions were performed using the dye terminator cycle sequencing chemistry with AmpliTaq DNA polymerase, FS enzyme (PE Applied Biosystems, Foster City, CA). The reactions were run on and analyzed with an Applied Biosystems Model 373A stretch fluorescent automated sequencer at the University of Iowa DNA Core Facility (Iowa City, IA). The DNA chromatograms were analyzed using Sequencher (Gene Codes, Ann Arbor, MI) and the GCG Wisconsin Sequence Analysis software programs (GCG, Madison, WI). The GenBank accession no. for the human sarcospan sequence is AF016028.

Restriction analysis of polymorphic sites within exon 3 of the sarcospan gene

Exon 3 was PCR amplified from human genomic DNA as described (*vide infra*). Aliquots of 30 µl of sarcospan exon 3 PCR product (after QIAquick purification) was digested separately with *Hga*I (4 U) and *Mae*II (3 U) in the buffers provided by the manufacturer (New England Biolabs, Beverly, MA) in a final volume of 50 µl. Restriction digests were performed for 1 h at 37 and 50°C for *Hga*I and *Mae*II, respectively. The normal sarcospan sequence contains an *Hga*I site which is not present with the polymorphic nucleotide substitution. Also, the presence of this polymorphism introduces a *Mae*II restriction site. Cleavage of the normal and polymorphic exon 3 PCR products with *Hga*I resulted in PCR products of 250 and 450 bp, respectively. *Mae*II did not digest the normal exon 3 PCR product (500 bp), but did cleave the polymorphic product into two fragments of 260 and 240 bp.

The exon 3 intragenic polymorphisms are missense mutations that alter the protein sequence of sarcospan. Polymorphism nucleotide 557G→A results in a Ser→Asn change at amino acid 186, which is in the extracellular loop, near transmembrane domain 4. Nucleotide 682G→A causes a Val228→Ile change in the intracellular C-terminus of sarcospan. The exon 2 polymorphic site (nucleotide 286T→C) is a silent mutation and therefore does not alter the amino acid at this position (Leu96).

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