

Absence of γ -sarcoglycan (35 DAG) in autosomal recessive muscular dystrophy linked to chromosome 13q12

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Abstract We have partially sequenced rabbit skeletal muscle γ -sarcoglycan an integral component of the dystrophin–glycoprotein complex. Specific antibodies were produced against a γ -sarcoglycan peptide and used to examine the expression of γ -sarcoglycan in skeletal muscle of patients with severe childhood autosomal muscular dystrophy linked to chromosome 13q12 (SCARMD). We show by immunofluorescence and Western blotting that in skeletal muscle from these patients γ -sarcoglycan is completely absent and α - and β -sarcoglycan are greatly reduced in abundance, whereas other components of the DGC are preserved. In addition, we show that in normal muscle α -, β -, and γ -sarcoglycan constitute a tightly associated sarcolemma complex which can not be disrupted by SDS treatment.

Key words: Dystrophin-associated glycoprotein, 35 kDa; γ -Sarcoglycan; Severe childhood autosomal muscular dystrophy; Limb-girdle muscular dystrophy; Sarcoglycan complex

1. Introduction

Dystrophin, the product of the Duchenne muscular dystrophy gene [1], binds F-actin through its N-terminal domain [2] and is tightly associated with a large sarcolemma protein complex through its cysteine-rich and C-terminal domains [3–11]. This complex consists of α -dystroglycan, a 156 kDa extracellular laminin-binding glycoprotein [12]; syntrophin, a 59 kDa intracellular protein triplet [13]; adhalin (or α -sarcoglycan), a 50 kDa transmembrane glycoprotein [14]; β -sarcoglycan, a 43 kDa transmembrane glycoprotein [15,16]; β -dystroglycan, a 43 kDa transmembrane glycoprotein [12]; 35 DAG or γ -sarcoglycan [17], a 35 kDa transmembrane glycoprotein; and a 25 kDa transmembrane protein. One function of the dystrophin–glycoprotein complex (DGC) in skeletal muscle is to act

as a molecular link between the extracellular matrix, via laminin 2, and the actin cytoskeleton, via dystrophin [18,19].

The DGC may play an important role in maintaining muscle fiber stability. Defects in DGC components lead to muscle fiber necrosis, which is the major pathologic event in progressive muscular dystrophy. The most severe muscular dystrophy is Duchenne muscular dystrophy (DMD) in which mutations in the dystrophin gene cause the complete absence of dystrophin and a drastic reduction of the dystrophin-associated proteins [20]. Several cases of severe childhood autosomal recessive muscular dystrophy (SCARMD) which closely resembles DMD have been reported [21]. α -Sarcoglycan immunostaining at the sarcolemma of these SCARMD patients is dramatically reduced compared to control [22]. A less severe reduction of the 35 kDa component of the DGC is also observed whereas the other components of the DGC are preserved [22]. These criteria have allowed distinction between SCARMD and DMD. SCARMD was first linked to the pericentromeric region of chromosome 13q12 in several North African families [23–25] and excluded from the 13q locus in non-North African families [26,27]. Therefore, mutations in at least two independent genes may cause SCARMD. The α -sarcoglycan gene has been localized to chromosome 17q21 and mutations in this gene have been demonstrated to be responsible for SCARMD [28–30], also referred to limb-girdle muscular dystrophy type 2D (LGMD2D) [31]. Therefore, SCARMD linked to chromosome 13q12 has been suggested as caused by a defect in the 35 DAG gene although this protein has never been shown to be completely absent in SCARMD patients. Recently, mutations in the β -sarcoglycan gene, located on chromosome 4q12, have been shown to be responsible for LGMD2E [15,16]. Immunofluorescence analysis of skeletal muscle from LGMD2E patients showed a great reduction or absence of β -sarcoglycan concomitant with a reduction of α - and γ -sarcoglycan. SCARMD linked to chromosome 13q12 has been renamed LGMD2C [30] and recently Noguchi et al. [17] reported the identification of the 35 DAG gene on chromosome 13q12. Furthermore, they identified mutations in the γ -sarcoglycan gene in LGMD2C.

In this study, we have isolated and cleaved the 35 kDa component of the DGC into different peptides which have been sequenced after HPLC purification. Eight different pep-

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Abbreviations: DGC, dystrophin-glycoprotein complex; LGMD, limb-girdle muscular dystrophy; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence.

tide sequences representing one-third of the apparent molecular mass of γ -sarcoglycan were obtained. Specific polyclonal antibodies were raised against one of these peptides and used to examine the tissue distribution of γ -sarcoglycan. The status of γ -sarcoglycan was also determined by immunofluorescence and Western blotting of skeletal muscle from patients with LGMD2C. We observed a specific absence of γ -sarcoglycan together with a drastic, but not complete reduction of α - and β -sarcoglycan. In addition, we show that in normal muscle α -, β -, and γ -sarcoglycan constitute a tightly associated sarcolemma complex which cannot be disrupted by SDS treatment.

2. Materials and methods

Tryptic peptides were prepared for acquisition of internal sequence information by SDS-PAGE of purified DGC [3] through 10% polyacrylamide gels, electrotransferred to PVDF membranes. The protein band corresponding to the 35 kDa protein was excised. The immobilized protein was then digested with trypsin as previously described [32]. Peptides were purified by reverse phase HPLC using Applied Biosystems model 130A HPLC system equipped with 2.1×100 mm RP-300 column. The purified tryptic peptides were sequenced by Automated Edman degradation using an Applied Biosystems model 470A Sequencer equipped with an on-line model 120A phenylthiohydantoin derivative analyzer using the manufacturer's standard programming and chemicals.

Peptides corresponding to the rabbit skeletal muscle γ -sarcoglycan sequences were chemically synthesized with an additional cysteine at

the N-terminus. These synthetic peptides were conjugated through the N-terminal cysteine to keyhole limpet hemocyanin (Pierce Chemical Co) using *m*-maleimidobenzoic acid/*N*-hydroxysuccinimide ester (Pierce Chemical Co.) as described [33], mixed with Freund's adjuvant and injected to sheep. Polyclonal antibodies against each individual peptide were affinity purified from crude sera using BSA-conjugated peptide as previously described [34].

Microsomes from various rabbit tissues, crude rabbit skeletal muscle sarcolemma, and purified DGC were prepared as previously described [3,35]. Proteins were resolved on 3–12% SDS-PAGE and electrotransferred to nitrocellulose membranes. Nitrocellulose membranes were blocked in Blotto (50 mM sodium phosphate, pH 7.4, 150 mM sodium chloride, 5% non-fat dry milk) and subsequently incubated overnight with primary antibody. The primary antibodies used were affinity purified anti- γ -sarcoglycan peptide from sheep, affinity purified anti- β -sarcoglycan from rabbit [15] and affinity purified anti-dystrophin, anti- α/β -dystroglycan, anti-syntrophins and anti- α -sarcoglycan from sheep [22]. Immunoblots were then washed with Blotto and incubated for 1 h with peroxidase-conjugated secondary antibody (Boehringer-Mannheim) at a dilution of 1:1,000. After washing the nitrocellulose blots with Blotto, they were developed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl using 4-chloro-1-naphthol as substrate or by ECL (Amersham). For immunofluorescence, 7 μ m transverse cryosections were prepared from rabbit, mouse and human muscle or from LGMD2C patient muscle. Sections were treated with avidin/biotin blocking solutions (Vector), blocked with 5% BSA in PBS for 30 min, and then incubated for 90 min with primary antibodies described above. After extensive washing with PBS, sections were incubated with biotinylated secondary antibodies for 30 min, washed with PBS, and then incubated with FITC-conjugated streptavidin (Jackson immunoresearch laboratories) for 30 min. After wash-

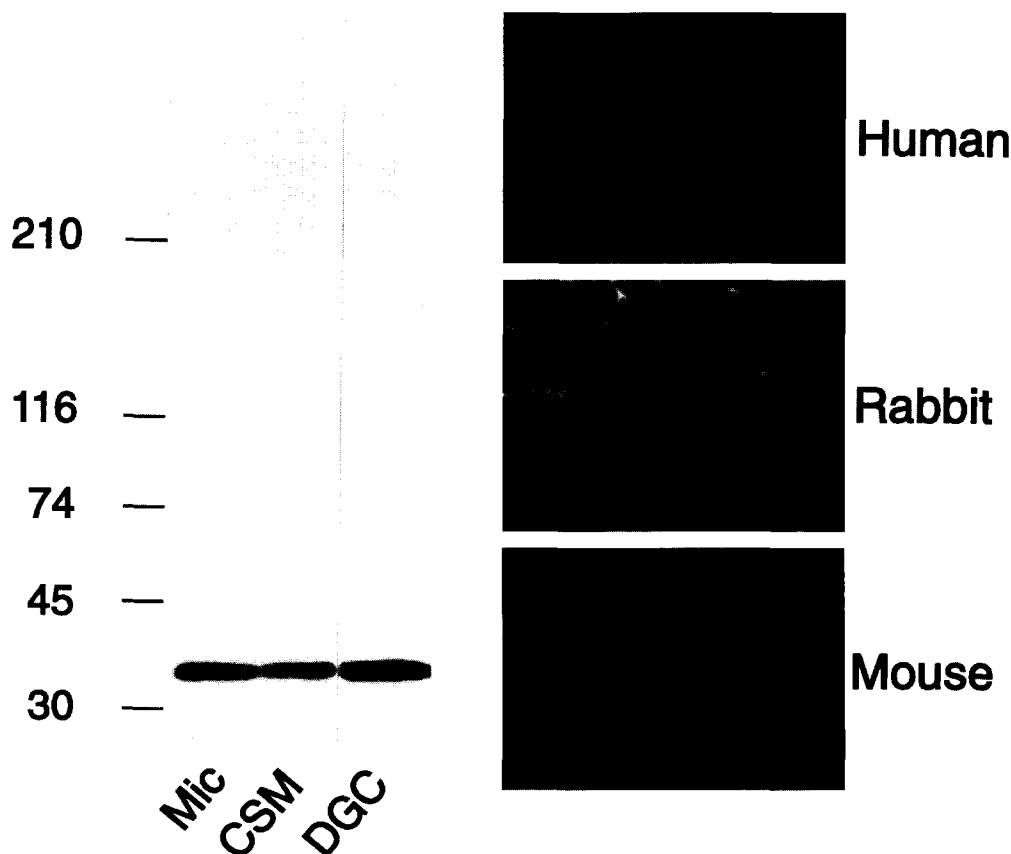


Fig. 1. Characterization of γ -sarcoglycan peptide antibodies. (Left panel) Rabbit skeletal muscle total membranes (Mic), crude sarcolemmal membranes (CSM) and purified dystrophin-glycoprotein complex (DGC) were resolved on SDS-PAGE and electrotransferred to nitrocellulose membrane. The nitrocellulose membranes were immunoreacted with affinity purified anti γ -sarcoglycan peptide antibodies and developed with 4-chloro-1-naphthol as substrate. Molecular mass standards (10^3) are indicated on the left. (Right panel) Immunofluorescence of human, rabbit and mouse quadriceps muscles stained with affinity purified anti γ -sarcoglycan peptide antibodies. All photographs were taken at the same exposure and magnification.

ing with PBS, sections were mounted with FITC-guard (Testog) and observed under a Zeiss Axioplan fluorescence microscope. Photographs were taken under identical conditions with the same exposure time.

Monoclonal IgG IVD3₁ against α -sarcoglycan [22], was purified with protein G Sepharose (Pharmacia) using standard procedures. 10 mg of purified IgG were oxidized with NaIO₄ and coupled to 5 ml of Avidchrom hydrazide resin (Unisyn Technologies) using the manufacturer's standard procedure. The column was stored at 4°C in 50 mM Tris pH 7.4, 500 mM NaCl and 0.01% NaN₃.

Rabbit skeletal muscle KCl washed microsomes (1 g) were resuspended in 50 ml of Buffer A (50 mM Tris-HCl pH 7.4, 500 mM NaCl, 1% SDS, 0.1 mM PMSF, 0.75 mM benzamidin, 2.5 μ g/ml aprotinin, 2.5 μ g/ml leupeptin, and 0.5 μ g/ml pepstatin A) and incubated at room temperature for 20 min. After incubation, solubilized microsomes were centrifuged at room temperature for 30 min at 100,000 \times g and the supernatant (SDS-extract) was decanted from the membrane pellet (SDS-pellet). The SDS-extract was supplemented with Triton X-100 to a final concentration of 5% and incubated overnight at 4°C with the anti- α -sarcoglycan affinity column. The affinity column was extensively washed with buffer A containing 5% Triton X-100 and eluted with 20 ml of 50 mM glycine pH 2.5. The eluate was neutralized with 1 ml of 2 M Tris pH 8 and concentrated to 1 ml. SDS-pellet, SDS-extract, voids and concentrated eluates were resolved by 3–12% gradient SDS-PAGE, electrotransferred to nitrocellulose membranes and subjected to Western blotting with DGC antibodies.

Biopsies of deltoid, quadriceps or gastrocnemius muscles from 9 patients were studied either by immunofluorescence or immunoblotting. In 5 of these patients (3 Algerian and 2 Europeans) belonging to 5 different family linkages to chromosome 13q12 were demonstrated. In the 4 other patients (1 Algerian, 2 Europeans and 1 Algerian and European) it was not possible to obtain significant results from linkage studies, but mutations on adhalin gene were excluded. Ages of onset of the clinical manifestations in the 9 patients were comprised between 3 and 8 years and the muscle biopsies were carried out between the ages of 6 and 25 years. The clinical pathological picture of all patients correspond to that of SCARMD as reported by Ben Hamida et al. [21]. It resembled that of Duchenne or Becker muscular dystrophies with raised serum creatine kinase, particularly in early stages of the disease and a histological necrotic regenerating pattern without dystrophin deficiency.

3. Results

To characterize the 35 kDa component of the DGC, purified rabbit skeletal muscle DGC was separated by SDS-PAGE and transferred to PVDF membrane. The region of the membrane containing the 35 kDa protein was excised and the immobilized protein was digested with trypsin. Peptides were purified by reverse-phase HPLC and subjected to amino acid sequencing by Edman degradation. Eight different peptide sequences were obtained and during the process of preparing this manuscript for publication, Noguchi et al. [17] reported the primary structure of γ -sarcoglycan. All the peptide sequences that we obtained were found in the reported amino acid sequence of γ -sarcoglycan. Together, the peptide sequences contain 105 amino acids, which represent more than one third of the total protein sequence and contains 292 amino acids.

In order to obtain specific antibodies to γ -sarcoglycan, several peptide sequences that we obtained by protein sequencing, were synthesized and conjugated to KLH before injection into sheep. Polyclonal antibodies against each individual peptide were affinity purified with BSA-conjugated peptide. The affinity-purified antibodies were then tested on Western blot for reactivity to γ -sarcoglycan. As shown in Fig. 1, an affinity purified polyclonal antibody raised against a peptide (VTGPEGALFEHS) which corresponds to amino acids 167–178 of the reported γ -sarcoglycan sequence specifically reacts

on immunoblotting with a single protein band at 35 kDa in crude rabbit skeletal muscle microsomes, sarcolemmal membranes and purified DGC. In immunofluorescence studies, affinity-purified anti-peptide antibodies selectively stained the sarcolemma of rabbit, mouse, and human skeletal muscle (Fig. 1) and colocalized with all other components of the DGC (data not shown). These data demonstrate the specificity of the γ -sarcoglycan polyclonal antibodies.

To establish the tissue-specific expression of γ -sarcoglycan, immunoblot analysis was performed. Blots containing membrane preparations of various rabbit tissues were probed with the affinity purified anti-peptide antibodies (Fig. 2). γ -Sarcoglycan was detected specifically in skeletal, cardiac and diaphragm muscles. A protein band of approximately 65 kDa is also specifically detected in brain. Noguchi et al. [17] did not detect γ -sarcoglycan mRNA in brain by Northern blot analysis. However, it is possible that similar to the case with β -sarcoglycan, the level of brain γ -sarcoglycan mRNA is low and prolonged exposure is required for detection of the messenger in this tissue. Therefore, it is likely that the 65 kDa protein detected in brain with the specific γ -sarcoglycan antibodies may correspond to a brain specific γ -sarcoglycan isoform. However, we do not exclude the possibility that this protein contains the antibody epitope and is unrelated to γ -sarcoglycan.

In order to determine the status of γ -sarcoglycan in patients with LGMD2C, linkage analysis was performed on several unrelated families with SCARMD. Using polymorphic markers, the genetic defect was linked to chromosome 13q12 in several families (data not shown). Skeletal muscle biopsy specimens from these patients with LGMD2C were examined by immunofluorescence. Serial frozen sections were stained with antibodies to γ -sarcoglycan or with antibodies to other components of the DGC. Fig. 3A shows the results of an immunofluorescence study from one patient. Dystrophin is present at comparable levels with control muscle; however, the im-

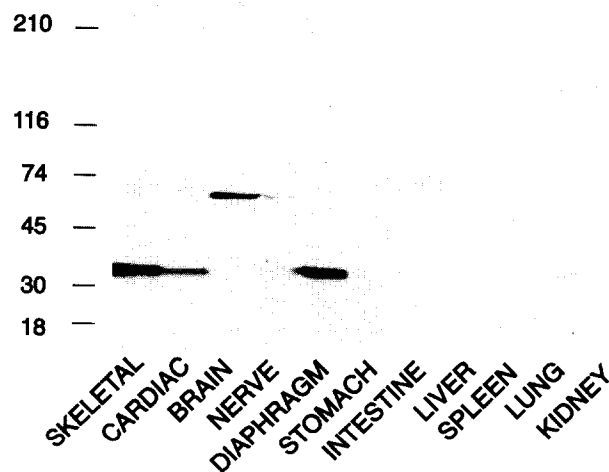


Fig. 2. Tissue distribution of γ -sarcoglycan. Total membranes from various rabbit tissues were resolved by SDS-PAGE and electrotransferred to nitrocellulose membrane. The nitrocellulose membrane was immunoreacted with affinity purified anti γ -sarcoglycan antibodies and developed with 4-chloro-1-naphthol as substrate. Molecular mass standards (10³) are indicated on the left.

munostaining of γ -sarcoglycan is absent with a concomitant reduction of α - and β -sarcoglycan (Fig. 3A). α/β -Dystroglycan and syntrophin staining are comparable to control muscle (data not shown). Similar immunostaining results were obtained for all the chromosome 13q12 linked patients (data not shown), indicating that one characteristic of LGMD2C is a primary deficiency in γ -sarcoglycan. To further characterize the γ -sarcoglycan defect in LGMD2 patient, total skeletal

muscle extracts were subjected to immunoblot analysis with the specific affinity purified γ -sarcoglycan antibodies. γ -Sarcoglycan was clearly detected in control muscle but we were unable to detect γ -sarcoglycan in skeletal muscle from one chromosome 13 linked patient as well as in two patients without linkage data (Fig. 3B), suggesting that these two patients may be linked to chromosome 13q12. In another chromosome 13 linked patient, γ -sarcoglycan appears to be greatly reduced. Taken together, our results demonstrate that the genetic defect in LGMD2C leads to either a great reduction or a complete absence of γ -sarcoglycan in skeletal muscle. It also appears that the absence of γ -sarcoglycan leads to a reduction of α - and β -sarcoglycan suggesting a close association between these three proteins in muscle sarcolemma.

The close association of these sarcoglycan proteins has previously been suggested by Yoshida et al. [36]. They have shown that *n*-octyl- β -D-glucoside disrupts the DGC into four groups of proteins: (i) α - and β -dystroglycan; (ii) dystrophin; (iii) the syntrophins; and (iv) α -, β - and γ -sarcoglycan. However, a direct demonstration of an association between the sarcoglycan proteins has never been demonstrated in skeletal muscle sarcolemma. In order to determine whether or not the three sarcoglycan proteins are tightly associated in sarcolemma, crude rabbit skeletal muscle membranes were solubilized with SDS and applied over an affinity column consisting of α -sarcoglycan monoclonal antibody coupled to Avidchrom hydrazide resin. After extensive washing, the antibody affinity column was eluted with pH 2.5 glycine buffer. SDS solubilized membranes, column void and column eluate were analyzed by SDS-PAGE and immunoblotted using specific antibodies to the DGC components. As shown in Fig. 4, all the DGC components except the syntrophins can be removed from the membrane with SDS. Western blot analysis of the antibody affinity column void and eluate reveal that dystrophin and α/β -dystroglycan are exclusively recovered in the void whereas α -, β - and γ -sarcoglycan are coeluted from the column (Fig. 4). Thus, these results clearly demonstrated that the sarcoglycan complex composed of α -, β - and γ -sarcoglycan exists as a unique entity at the sarcolemma. Furthermore, the fact that SDS does not disrupt the sarcoglycan complex suggests that these three proteins are tightly associated.

4. Discussion

In conclusion, we confirmed here that SCARMD linked to chromosome 13q12 or LGMD2C is characterized by a specific absence or great reduction of γ -sarcoglycan in skeletal muscle. This characterization has been made possible by the production of specific and reliable anti- γ -sarcoglycan antibody raised against a γ -sarcoglycan peptide. Diagnosis of LGMD2C may now be possible by immunofluorescence or immunoblotting analysis of muscle biopsies rather than linkage analysis, a procedure which is not always possible due to inadequate family size. It is interesting to notice that in a previous study using antibodies raised against the 35 kDa protein band of the DGC, a reduction of staining at the sarcolemma of patients with LGMD2C was observed [22]. In contrast in this study using the γ -sarcoglycan peptide antibody, all patients with LGMD2C tested displayed a complete absence of staining at the sarcolemma. These divergent results may suggest that the first antibody raised against the 35 kDa protein band of the

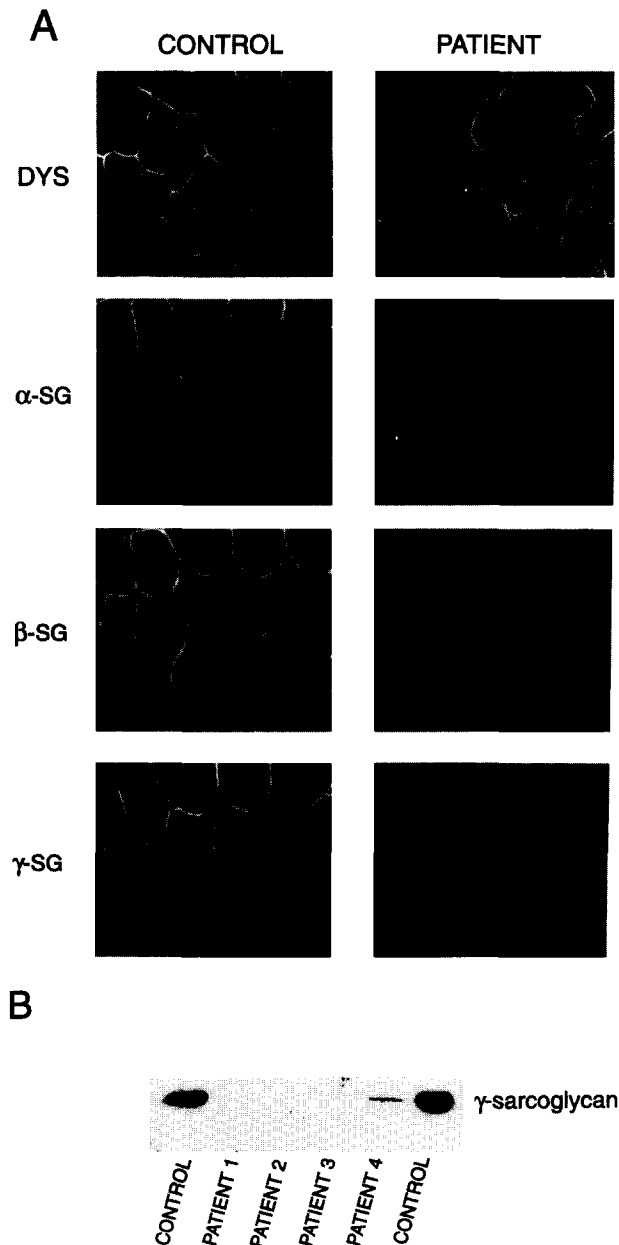


Fig. 3. Specific deficiency of γ -sarcoglycan in LGMD2C. (A) Immunofluorescence of human control (control) and LGMD2C patient (patient) deltoid muscles stained with affinity purified anti-dystrophin (DYS), α -sarcoglycan (α -SG), β -sarcoglycan (β -SG) and γ -sarcoglycan (γ -SG) antibodies. All photographs were taken at the same exposure and magnification ($\times 160$). (B) Total muscle extracts from human control (control) and LGMD2 patients (patient) were resolved on SDS-PAGE and electrotransferred to nitrocellulose membranes. The nitrocellulose membrane was immunoreacted with affinity purified anti- γ -sarcoglycan antibodies and developed by ECL. Patient 1 and 4 are linked to chromosome 13q12 whereas no linkage data were obtained for patient 2 and 3.

DGC recognized another 35 kDa protein in addition to γ -sarcoglycan and this protein corresponds to an uncharacterized protein of the DGC. This could explain the residual staining at the sarcolemma of patients with LGMD2C using the antibodies raised against the 35 kDa protein band of the DGC [22].

Furthermore, we demonstrate the existence of a subcomplex within the DGC, composed of α -, β - and γ -sarcoglycan. In LGMD2C, 2D and 2E, the loss of one sarcoglycan protein leads to a reduction or absence of the two other members, indicating that mutations in one component affect the stability or targeting of the other components. Therefore, it appears that the disruption of the entire sarcoglycan complex causes LGMD2C, -2D and -2E, in opposition to LGMD2A where mutations in calpain 3 gene cause the dystrophic phenotype [37]. The precise function of the sarcoglycan complex has yet to be determined. In Duchenne muscular dystrophy, the absence of dystrophin causes a disruption of the DGC which leads to the loss of the functional link between the extracellular matrix and the cytoskeleton and results in sarcolemma instability. It is likely that the sarcoglycan complex plays a similar role in maintaining the structural and functional integrity of the sarcolemma. Furthermore, β - and γ -sarcoglycan seem to be also expressed in brain whereas α -sarcoglycan is a muscle specific protein, suggesting that β - and γ -sarcoglycan may be part of another subcomplex in this tissue. It will be of interest to determine the structure and cellular localization of these two proteins in brain and to identify their associated proteins.

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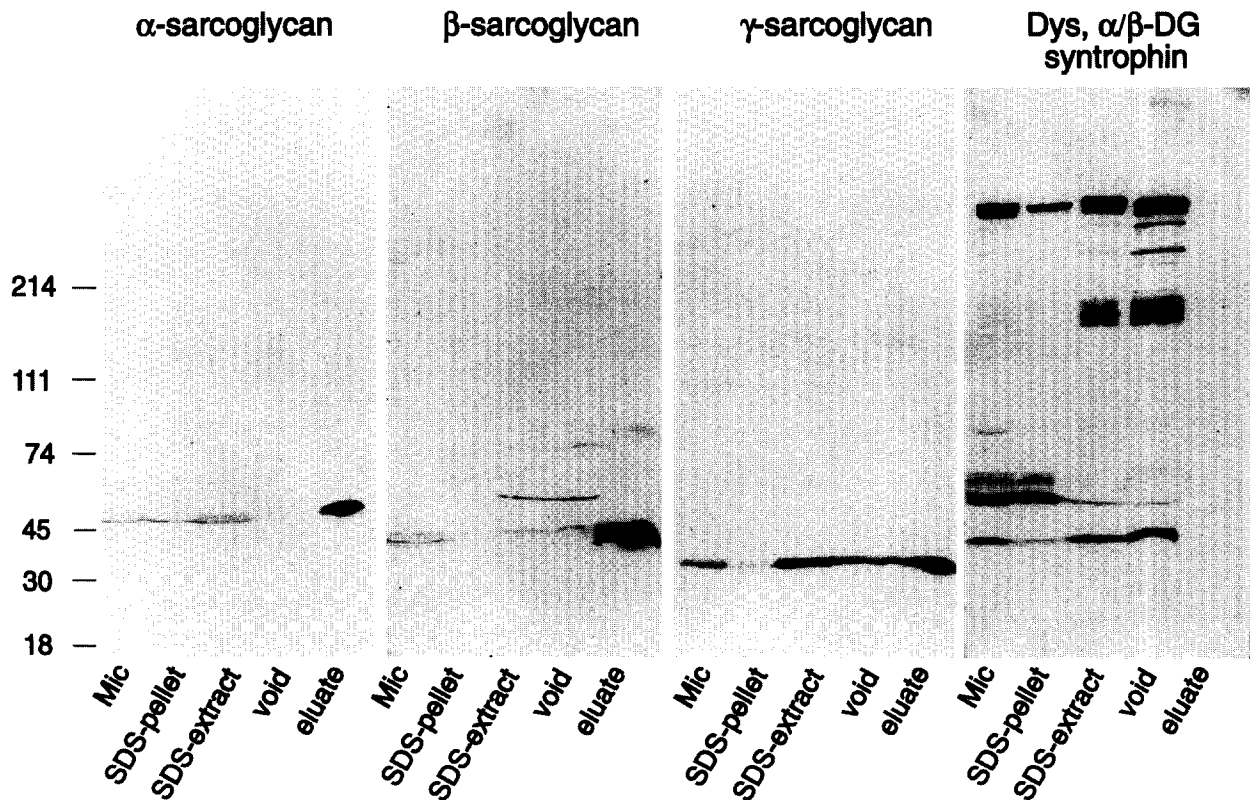


Fig. 4. Purification of rabbit skeletal muscle sarcoglycan complex. SDS solubilized rabbit skeletal muscle membranes were subjected to affinity chromatography on an anti- α -sarcoglycan column. Total membranes (Mic), remaining membranes (SDS-pellet), solubilized membranes (SDS-extract), affinity column void (void) and eluate were resolved on SDS-PAGE and electrotransferred to nitrocellulose membranes. Nitrocellulose membranes were immunoreacted with antibodies to either α -sarcoglycan, β -sarcoglycan, γ -sarcoglycan or a mixture of dystrophin (Dys), α and β dystroglycan (α/β -DG) and syntrophin antibodies. The immunoblots were developed with 4-chloro-1-naphthol as substrate. Molecular mass standards (10^3) are indicated on the left.

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