J Mol Med
DOI 10.1007/s00109-007-0163-8

RAPID COMMUNICATION

A new evidence for the maintenance of the sarcoglycan complex in muscle sarcolemma in spite of the primary absence of δ-SG protein

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Received: 18 July 2006 / Revised: 17 November 2006 / Accepted: 29 November 2006

Abstract δ-Sarcoglycan (δ-SG) is one of the first proteins of the sarcoglycan complex (SGC) to be expressed during muscle development, and it has been considered fundamental for the assembling and insertion of the SGC in the sarcolemma. Studies using heterologous cell systems and co-precipitation have demonstrated that SGC assembly was dependent on the simultaneous synthesis of all four sarcoglycan proteins. Mutations in any one of sarcoglycan genes, including the common disease causing mutation c.656delC in the δ-SG gene, block complex formation and its insertion in the plasma membrane. Failure in complex assembly in patients with this mutation would be therefore expected. In this study, we provide evidence for the possibility of preservation of part of the SG complex in the sarcolemma, even in the absence of δ-SG. This is based on the study of one mildly affected patient with limb-girdle muscular dystrophy type 2F (LGMD2F) due to the homozygous c.656delC mutation in the δ-SG gene. Protein analysis in his muscle biopsy presented a significant deficiency of only δ-SG with retention of the other three SG proteins in the sarcolemma. RNA expression analysis showed that ζ-SG, a functionally homologous to δ-SG, is not atypically upregulated in his muscle and would not

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replace the absent δ-SG, retaining the complex α−β−γ−ζ. The patient started clinical manifestation at age 25, with frequent falls, but he is currently able to walk unassisted at age 42. His clinical course is significantly milder when compared to several other affected patients carrying the same mutation associated with a total deficiency of the four SG proteins in the muscle studied by our group and confirmed in other patients. Therefore, our results add a new in vivo evidence that α-, β-, and γ-SG proteins can be maintained in the sarcolemma without δ-SG. Additionally, LGMD2F, with retention of the part of the SGC, might be associated to a milder clinical course, which has important implications for clinical prognosis and genetic counseling of the family.

Keywords Sarcoglycanopathies · Limb-girdle muscular dystrophies · DGC

Introduction

Limb-girdle muscular dystrophies (LGMDs) include a heterogeneous group of progressive disorders mainly affecting the pelvic and shoulder girdle musculature, ranging from severe forms with onset in the first decade of life and rapid progression, to milder forms with later onset and slower progression [1–3]. Inheritance may be autosomal dominant (LGMD1) or recessive (LGMD2). During the last decade, 20 LGMD genes, 7 autosomal dominant, and 13 autosomal recessive have been mapped ([4–12], and WMS congress 2006, Bruges).

The more severe forms are caused by mutations in four genes mapped at 17q21, 4q12, 13q12, and 5q33, coding, respectively, for α-sarcoglycan (α-SG), β-SG, γ-SG, and δ-SG, glycoproteins of the SG subcomplex of the dystrophin–glycoprotein complex (DGC) [13–15]. Mutations in these genes cause, respectively, LGMD2D, 2E, 2C, and 2F and constitute a distinct subgroup of LGMDs, the sarcoglycanopathies (SGpathies) [16].

The DGC is a multifunctional protein complex, located on the muscle cell membrane, and is thought to provide a mechanical link between the cell cytoskeleton and the extracellular matrix [13]. The four SG proteins form a subcomplex of the DGC, and its components are interdependent in varying degrees. In most patients, mutations in any one of the genes encoding these four subunits result in the complete loss or marked decrease in the whole complex in muscle fibers [17–19].

δ-SG is one of the first proteins to be expressed during muscle development, and it has been considered fundamental for the assembling and insertion of the SG complex in the sarcolemma [20–23]. Co-immunoprecipitation assays have demonstrated that β- and δ-SG interact with α-SG and these two subunits must be co-expressed for export from the endoplasmatic reticulum [24]. Studies using a heterologous cell system have demonstrated that SG complex assembly was dependent on the simultaneous synthesis of all four SG proteins and mutations in any one of SG genes, including the common c.656del C mutation in the δ-SG gene, block complex formation and insertion on the plasma membrane [25]. Therefore, failure in complex assembly in patients with this mutation would be expected. In this study, we described one LGMD2F patient, homozygous for the c.656delC mutation, in whom protein analysis in muscle biopsy showed a significant deficiency of only δ-SG with retention of other three SG proteins at the sarcolemma. These findings provide a strong evidence for the possibility of the organization of the SG complex in the sarcolemma, even in the absence of δ-SG.

Materials and methods

Patients

Nine unrelated patients in whom we identified mutations in one of the four SG genes were included in the present report. They were ascertained in two independent centers: Human Genome Research Center in São Paulo (two patients) and Sarah Kubitcheck Hospital in Belo Horizonte, Brazil (seven patients), where the first screening, with the analysis of at least two SG proteins, was done for diagnostic purpose.

Screening for mutations in the four SG genes was carried out using a multiplex methodology introduced in our laboratory [26]. This method allowed the simultaneous identification of the most common mutations in the four SG gene in the Brazilian population. Each mutation was confirmed through automatic sequencing of the altered amplicon.

Diagnosis was suspected based on clinical and laboratorial findings, and/or a family history compatible with the diagnosis of LGMD2, according to Bushby and Beckmann [27]. Patients were classified as mild LGMD, when the onset of the disease occurred during the first or second decade and loss of ambulation after age 16, and severe form, when onset occurred in the first decade and patients were wheelchair-bound before age 16, or when the clinical course was as severe as Xp21 Duchenne muscular dystrophy (DMD). Intermediate classification was used in patients who lost ambulation between the ages of 10 and 16 years or when the clinical picture was less severe than DMD in patients between 10 and 15 of age.

Protein analysis in muscle biopsies

Muscle samples were obtained through open biceps biopsies under local anesthesia, frozen in liquid nitrogen

Table 1 Clinical and molecular data of the nine SGpathies patients

Number	Age	Gene	Mutation	Clinical Classification	Immunohistochemical analysis[a]				Dystrophin
					α	β	γ	δ	
1	9	α-SG	c.229C>T/c.229C>T	I—ambulant	−	±	±	±	++++
2	13	α-SG	c.229C>T/c.229C>T	I—ambulant	Deficient[b]				
3	9	α-SG	c.229C>T/c.665G>A	I—ambulant	−	−	++	−	++++
4	12	γ-SG	c.521delT/c.521delT	I—ambulant	±	−	−	+	++
5	10	γ-SG	c.521delT/c.521delT	S—wheelchair	−	n.d.	−	n.d.	+++
6	29	γ-SG	c.521delT/c.521delT	I—wheelchair	++	+++	−	+++	++++
7	9	γ-SG	c.521delT/c.521delT	S—wheelchair	−	−	−	−	++++
8	42	δ-SG	c.656delC/c.656delC	M—ambulant	++	++	+++	−	++++
9	12	δ-SG	c.656delC/c.656delC	S—wheelchair	−	n.d.	−	n.d.	+++

M Mild, *S* severe, *I* intermediate (see "Materials and methods"), *n.d.* not done—because not enough tissue was available for the analysis

[a] Immunohistochemical analysis repeated in our center when tissue material was still available. Classification from ++++ (total positive sarcolemmal labeling, as observed in normal control) to − (negative, no sarcolemmal labeling)

[b] Analysis done in other center, for diagnostic purpose.

immediately after removal, and stored at −70°C until use. In three patients, diagnosed in SKH Center, sufficient material was not available to repeat the reaction for all four SG proteins.

Muscle sections of 5–6 μm thick were cut in a cryostat microtome and mounted in slides coated with polylysine. Slides were allowed to air dry at room temperature for 1 h.

The analysis of the presence and distribution of the four SG proteins was made through single or double immunofluorescence (IF) staining of frozen sections [28] in triplicate. Monoclonal antibodies against the four SG were developed by Louise V. B. Anderson, from Newcastle (UK), and are commercially available in Novocastra (Newcastle, UK). One additional polyclonal rabbit antibody against δ-SG protein, developed by Dr. Vincenzo Nigro, was also used for confirming the deficiency in patient 8 [29]. Double labeling reactions were done with mouse monoclonal anti-α-SG antibody Ad1/20A6 and rabbit polyclonal anti-dystrophin 303-8 antibody (kindly provided by Dr. Jeff Chamberlain). Subsequent IF analysis for β-SG, γ-SG, and δ-SG was made using the respective antibodies.

IF pattern was compared with normal positive control in the same reaction and classified from totally positive (++++), if all fibers were equally stained, up to totally negative (−), if no staining was detected at all (Table 1).

Results

Results of mutation analysis in the nine patients are summarized in Table 1.

The analysis of the four SG proteins in the muscle was possible in six of them due to material limitation. In four patients, the whole SG complex was highly deficient, whereas, in two patients, only one of the four SG was deficient: In patient 6, with γ-SG mutation, total deficiency of γ-SG was accompanied by only a mild reduction in α-, β-, and δ-SG. Patient 8, who carries a mutation in the δ-SG

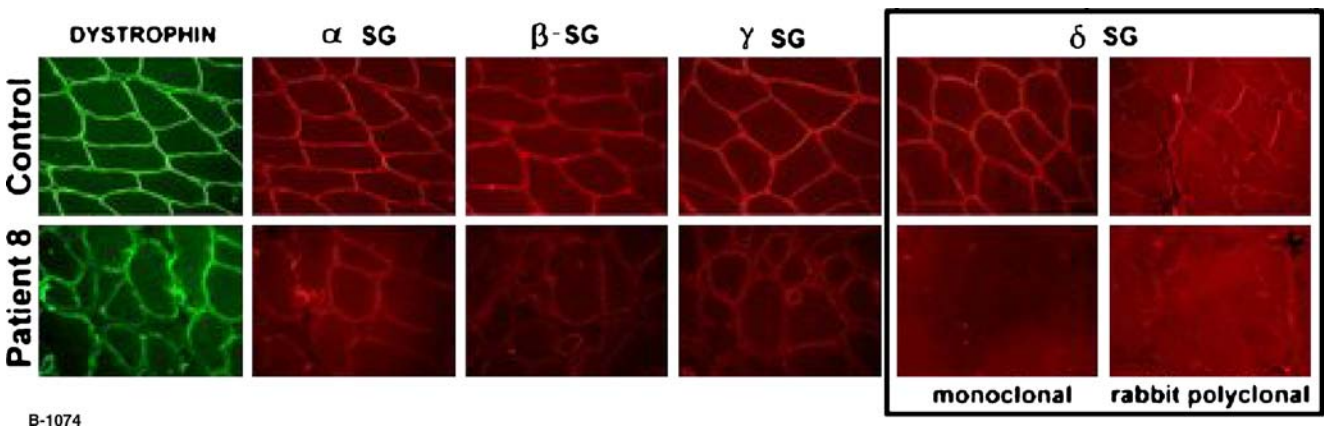

Fig. 1 Immunohistochemical analysis for the four SG and dystrophin on muscle section from controls and patient 8 showing the total deficiency of δ-SG, confirmed with two different antibodies, and partial retention of the other three SG proteins. Magnification ×200

gene, called our attention. In this 42-year-old man, we observed, for the first time, the drastic reduction in only the δ-SG protein, with the retention of the other three proteins from the DGC complex, confirmed with two different antibodies for δ-SG protein (Fig. 1).

Clinical description of patient 8: Start of symptoms began at age 25, with frequent falls and difficulty to walk long distances. Now, at 42 years of age, he is showing a slow progression, with a global decrease in muscle strength, mainly in the lower girdle, lordosis, and a significant calf hypertrophy. He is able to walk unassisted and shows mild difficulties to climb stairs. The reflexes are hipo-active, mainly in the lower limbs. Creatine kinase is six to eight times elevated, and the electromyography presents a myopathic pattern.

Discussion

In most SGpathy patients, a primary loss or deficiency of anyone of the four SG proteins in the muscle (β-SG and δ-SG in particular) leads to a secondary deficiency of the whole subcomplex [1, 17–19, 30]. In fact, the majority of the patients here analyzed showed deficiencies of one or more proteins of the SG complex, and primary SGpathy was confirmed through our DNA multiplex test.

Exceptions may occur, such as partial deficiency of only α-SG with the retention of the other three in LGMD2D [31] or deficiency of γ-SG with a partial preservation of the other three SG in LGMD2C [32, 33]. In this study, we identified one additional LGMD2C patient, homozygous for the c.521delT mutation in the γ-SG gene, who had an isolated loss of γ-SG protein in the muscle (patient 6), suggesting that this type of secondary deficiency is not so rare in LGMD2C.

Up to now, all patients with LGMD2F and the c.656delC mutation reported in the literature, including four Brazilian cases, presented total deficiency of the four SG proteins in the muscle and a severe phenotype [17, 34–36]. Patients with other mutations in the δ-SG gene also were described as presenting DMD-like phenotype [37–39], which classify LGMD2F among the very severe neuromuscular diseases. In this study, we present two additional cases carrying the c.656delC mutation. Patient 9 is very similar to the other LGMD2F patients described with the same mutation. He is presenting severe phenotype and total deficiency of at least α- and δ-SG in his muscle, which suggests deficiency of the whole complex in the sarcolemma, in accordance to previously reported cases (the other proteins could not be analyzed due to the small amount of muscle tissue available for analysis). However, patient 8 showed a significant deficiency of only δ-SG, a finding that was apparently not reported before. Interestingly, his unusually mild course came to our attention, because at age 42, he is able to walk and climb stairs unassisted.

This case is an in vivo important evidence that, even in the absence of δ-SG, which is not produced in his muscle

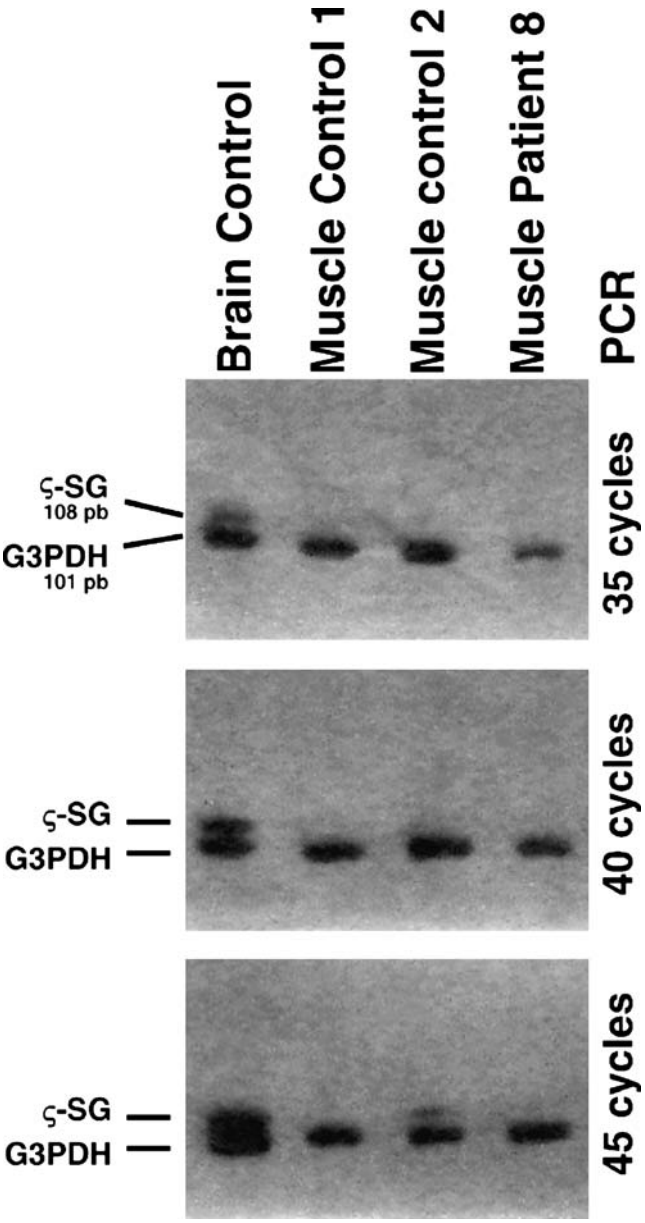

Fig. 2 Semiquantitative duplex-PCR analysis of ς-SG (108 pb) and G3PDH (101 pb) expression in acrylamide gel 10.5% (silver staining). RNA brain tissue was used as positive control for ς-SG expression. ς-SG is a rare transcript in muscle tissue, as shown in two normal muscles (1 and 2). Increased number of PCR cycles were used (35, 40, and 45 cycles), with ς-SG only being detected in one of the normal muscles (control 2), and at 45 cycles. No expression of ς-SG was detected under none of the conditions in the muscle from patient 8. The internal housekeeping control G3PDH was detected in both tissues and in all conditions. These results highly suggest no over expression of ς-SG replacing δ-SG in our patient 8. Primers sequences: ς-SG forward, 5′ TGGGAAATCTGAGAGTCACCAAG 3′; ς-SG reverse 5′ CCAGCGGACTATCCTTTCGAG 3′; G3PDH forward, 5′ CAGGGCTGCTTTTAACTCTGGTA 3′; G3PDH reverse, 5'GGGTGGAATCATATTGGAACATG 3′

due to a known nonsense mutation, the other SG were retained in the sarcolemma. This is new information must be interpreted in the context of what has already been described in the literature in vitro systems: (a) δ-SG was found to be one of the first proteins to be expressed during muscle development and has been considered fundamental for the assembling and insertion of the SG complex in the sarcolemma [20–23]; (b) The SG complex is not organized in the absence of δ-SG [23]; (c) The formation of the β-δ-core has been shown to promote the export and deposition of SG subcomplexes on the plasma membrane [24]. Protein findings in the LGMD2F patient here reported provide an evidence in humans that exceptions may occur, as three of the remaining SG proteins were retained in some way in the sarcolemma even in the absence of δ-SG.

The possibility of the organization of SG complex in the absence of δ-SG is, however, still speculative. The SG complex is a structure composed of four SG proteins that is biochemically defined. Although our results showed the presence of three SG on the membrane, it is not predictable whether they are organized into a specified structure, the SG complex, and bind to β-dystroglycan. It is unknown if the three SG can exist in a stable form, organized in a single complex with α-, β-, and γ-SG proteins. However, this hypothesis cannot explain why, in other patients, this trimeric formation would be unstable. A last hypothesis could be the inclusion of some other protein, such as ς-SG within the DGC in the muscle of this patient. Co-transfection studies of six SG (α-, β-, γ-, δ-, ε-, and ζ-SG) into Chinese hamster ovary cells, and immunoprecipitation analysis have shown that ς-SG is a functional homologous of γ-SG rather than δ-SG [40]. However, if and how this protein is expressed in the absence of δ-SG is not known. We tested this hypothesis, analyzing a possible over-expression of ζ-SG in muscle from patient 8, as compared to two normal muscles and normal brain tissue (positive control for ζ-SG expression). No expression at all of this rare muscle transcript was detected in patient 8 (Fig. 2). Therefore, this SG is not atypically upregulated in our patient and would not replace the absent δ-SG retaining the complex α−β−γ−ζ.

A mirror study with LGMD2E patients could also be done using the already described patients with mutation in the β-SG gene. However, patients with LGMD2E are usually severely affected and present deficiency of the whole SG complex in the muscle [17, 36]. Only one exception was found by us in one Brazilian family, in which variability in the clinical course was observed in the two sibs homozygous for the mutation c.2T>C in the β-SG gene. However, both showed an identical immunohistochemical profile for the SG complex, with total deficiency of β-SG and residual amounts of the other three SG proteins [36].

It is tempting to speculate whether and why the retention of a part of the SG complex in this patient would be related to his milder clinical course, mainly because previous studies have shown no correlation between a partial retention of the SG complex in the muscle and the severity of the phenotype in LGMD2C, LGMD2D, and LGMD2E patients [17, 36]. If confirmed in other LGMD2F mildly affected patients, this association would be more specific for primary δ-SG deficiency.

In conclusion, our results add a new in vivo evidence that α-, β-, and γ-SG proteins can be maintained in the sarcolemma without δ-SG. As this is apparently the first reported case in the literature, it is not possible to conclude that the specific absence of only δ-SG in muscle would be responsible for the milder course observed in this patient. Additionally, our findings emphasized the need to test muscle biopsies for all four SG, as many centers around the world, based on the observation of the reduction in all four SG proteins in SGpathies, use only staining for α-SG and/or γ-SG in their diagnostic repertoire.

Acknowledgments The collaboration of the following people is gratefully acknowledged: Dr. Maria Rita Passos-Bueno, Dr. Eloisa S. Moreira, Dr. Ivo Pavanello, Dr. Acary S.B. Oliveira, Dr. Edmar Zanotelli, Dr. Helga Silva, Dr. Lydia U. Yamamoto, Marta Cánovas, Lucas Maia, Dr. Flavia de Paula, Luciana Luchesi, Viviane P. Muniz, Bruno Lima, Alessandra Starling. We would also like to thank the following researchers who kindly provided specific antibodies: J. Chamberlain, L. M. Kunkel, C. Bonnemann, E. E. McNally, G. Faulkner, G. Valle, K. Campbell. This work was supported by FAPESP-CEPID, PRONEX, and CNPq.

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