A novel form of recessive limb girdle muscular dystrophy with mental retardation and abnormal expression of α-dystroglycan

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Abstract

The limb girdle muscular dystrophies are a heterogeneous group of conditions characterized by proximal muscle weakness and disease onset ranging from infancy to adulthood. We report here eight patients from seven unrelated families affected by a novel and relatively mild form of autosomal recessive limb girdle muscular dystrophy (LGMD2) with onset in the first decade of life and characterized by severe mental retardation but normal brain imaging. Immunocytochemical studies revealed a significant selective reduction of α-dystroglycan expression in the muscle biopsies. Linkage analysis excluded known loci for both limb girdle muscular dystrophy and congenital muscular dystrophies in the consanguineous families. We consider that this represents a novel form of muscular dystrophy with associated brain involvement. The biochemical studies suggest that it may belong to the growing number of muscular dystrophies with abnormal expression of α-dystroglycan.
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1. Introduction

Autosomal recessive limb girdle muscular dystrophy (LGMD2) is a heterogeneous group of myopathies characterized by progressive muscle weakness involving the proximal muscles of the shoulder and pelvic girdles and a variable progression with symptoms ranging from Duchenne-like to milder, later onset forms [1,2]. Ten LGMD2 loci and their respective genes have been identified: these include the enzymatic protein calpain 3 (CAPN3, responsible for LGMD2A, on 15q) [3], the sarcolemmal proteins dysferlin (LGMD2B, 2p) [4,5], α, β, γ and δ sarcoglycans (LGMD2D, 2E, 2C and 2F) [6–10] and LGMD2G mapping to 17q11–q12, the gene product of which is the sarcomeric protein telethonin [11]. The genes for LGMD2H, LGMD2I and LGMD2J have recently been identified: LGMD2H is due to mutations in the tripartite-motif containing gene 32 (TRIM32), an ubiquitin ligase putatively involved in proteasome degradation [12] whilst the gene encoding the giant sarcomeric protein titin is mutated in LGMD2J [13].

The gene responsible for LGMD2I is unusual in that it encodes a putative glycosyltransferase, the fukutin related protein gene (FKRP). This gene has been found to be mutated in patients with phenotypes ranging from severe congenital muscular dystrophy (MDC1C) to a mild form of limb girdle muscular dystrophy [14,15]. Abnormal α-dystroglycan expression is a feature of both MDC1C and LGMD2I, suggesting that *FKRP* might be involved in α-dystroglycan processing.

Dystroglycan is a central component of the dystrophin–glycoprotein complex (DGC), which links dystrophin to

the extracellular matrix. In skeletal muscle it consists of a 156 kDa extracellular subunit (α-dystroglycan) and a 43 kDa transmembrane subunit (β-dystroglycan) [16].

α-Dystroglycan is a heavily glycosylated protein expressed in a wide variety of cell types. In muscle it is a high-affinity receptor for several extracellular matrix molecules containing LG domains, including agrin [17], laminin α chains [18], perlecan [19] and biglycan [20], whilst in brain it also binds neurexin [21].

The interactions between α-dystroglycan and many of its extracellular binding partners are mediated by its carbohydrate side chains. α-dystroglycan contains both N-linked and O-linked carbohydrates. N-Linked glycosylation is less common but is required for the proper cell surface localization of both α- and β-dystroglycan [22]. O-Linked glycosylation is the predominant modification and responsible for the high affinity binding of α-dystroglycan to its ligands [18].

To date, no disease has been found that is due to primary mutations in dystroglycan, although a mild form of muscular dystrophy associated with a secondary β-dystroglycan deficiency has been described [23]. In addition to LGMD2I, abnormal α-dystroglycan expression has been documented in several forms of congenital muscular dystrophy (CMD), with and without central nervous system (CNS) involvement. These include Fukuyama CMD (FCMD) [24], MDC1C [15], muscle–eye–brain disease (MEB) [25] and Walker–Warburg syndrome (WWS) [26].

In the present study, we report seven families with identical clinical features, characterized by relatively mild and static proximal muscle weakness, mild muscle hypertrophy, elevated serum creatine kinase (CK), microcephaly and mental retardation but normal brain imaging. The expression of α-dystroglycan was found to be reduced in their skeletal muscle biopsies. Linkage analysis excluded all the mapped forms of muscular dystrophies in consanguineous families, suggesting these patients represent a new variant of muscular dystrophy.

2. Patients and methods

2.1. Patients

Over the past decade, we have evaluated a large series of autosomal recessive LGMD families, the majority of which have been reported in two consecutive papers which combine clinical and genetic features [27,28]. Nine of the families studied did not map to any of the known LGMD loci and were referred to as 'unlinked'. Among these, there were six Turkish families sharing an almost identical phenotype who form the basis of the present study. More recently, a British family was seen at the Hammersmith Hospital (London, UK) with very similar clinical features. A summary of the clinical features of all families in this study are given in Table 1. Typically, all patients presented in the first decade of life with fatigability, difficulty in climbing stairs and in running. They acquired early motor milestones at a normal age, excluding a congenital muscular dystrophy. All were characterized by only mild muscle pseudohypertrophy and a slow disease evolution. All had mild to severe mental retardation, i.e. IQ levels being on average around 50–55. Serum CK was invariably grossly elevated (9–20 fold increase).

For illustrative purposes the case histories of two patients are presented below.

2.1.1. Case 1

This is a 22-year-old male coming from a first-degree consanguineous marriage who walked at 3 years. His early motor milestones were otherwise normal; symptoms started around 6 years of age with difficulty in gait. His intellectual

Table 1
Clinical characteristics

	Current age/onset (years)	Sex	Consang-uinity	Family history	Age walked (years)	Maximum motor capacity	Hypertrophy	Joint contractures	H.C. %	IQ	CK	CT/MRI	Evolution
Case 1	22 (6)	M	(+)	(+)	3	Walks alone	Calves	Elbow, Achilles	3–10p	50	×9	N	Mild
Case 2	16 (3)	F	(+)	(−)	3	Walks alone	Calves	Achilles	3–10p	55	×20	N	Mild
Case 3	6 (2.5)	F	(+)	(−)	3	Walks alone	Thigh, calves	–	10p	65	×28	N	Mild
Case 4	10 (1)	M	(+)	(−)	3	Walks alone	(−)	(−)	3p	50		N	Mild
Case 5	9 (3)	M	(+)	(−)	3	Walks alone	(−)	(−)	10–25p	50	×40	N	Mild
Case 6	10 (3)	M	(−)	(−)	3	Walks alone	Thigh, calves, upper arms	(−)	50p	55	×25	N	Mild
Case 7[a]	12 (5)	M	(+)	[a]	1.5	Walks alone	Thigh, calves	Achilles	3p	76	×22	N	Mild
Case 8[a]	15 (4)	F	(+)	[a]	2	Walks alone	Thigh, calves, hamstring, upper arm	Achilles	3p	57	×24	N	Mild

H.C., head circumference; CK, creatine kinase; CT/MRI, computed tomography/magnetic resonance imaging; p, centile; N, normal.
[a] Siblings.

milestones were, however, clearly delayed; he said his first few words at 3 years, but later on failed to learn to read and write. When examined at 11 years of age, Gowers sign was negative, he raised himself from the floor in less than 3 s and his proximal muscle strength was MRC $4^+/5$. Serum CK at the time was 2327 U/l (normal < 190). At the age of 16 years, his proximal weakness had increased and he now had a positive Gowers manoeuvre in 4–5 s. A repeated CK was 1931 U/l. When re-examined at 22 years, his head circumference was 53 cm (3–10 centile); muscle weakness had remained virtually static, with muscle power MRC 4/5 proximally. His calves were mildly enlarged. He had increased lumbar lordosis with mild elbow and ankle contractures (Fig. 1). Gowers sign was positive with 7–8 s. He could walk for long distances without significant difficulties. There was no prominent facial weakness or scapular winging. With regard to his intellectual function, he was unable to count money or take a bus ride in the city alone, and formal testing disclosed an IQ of 55. Out of nine siblings, his 7-year younger brother is similarly affected.

2.1.2. Case 2

This is a 16-year-old girl coming from a second-degree cousins marriage with no other relevant family history. The onset was around 3 years of age, shortly after she started to walk. Her initial difficulties were an unsteady gait and difficulties climbing stairs. This later progressed to a waddling gait with a Gowers manoeuvre of 10 s at the age of 14 years. At the last visit at the age of 16 her head circumference was 53 cm (3–10 centile). Currently, she has mild enlargement of the calves and ankle contractures as well as increased lumbar lordosis. There is no facial weakness nor muscle atrophy. Her proximal muscle strength is MRC $3^+/5$. She is independently ambulant, but only for short distances, 25–30 m. Her intellectual development was

Fig. 1. Case 1: can easily raise arms above head, mild pseudohypertrophy, IQ 55.

slow: she said her first few words at 7 years and now, aged 16, she only uses two-word sentences. Her IQ is 50. She is also unable to count money and perform independent activities. The CK at the age of 15 is 4133 U/l (normal < 209). Her cranial magnetic resonance image (MRI) is normal. This case represents the most severely affected patient in our series.

2.2. Genotyping

Genomic DNA was extracted from whole blood by standard methods after obtaining informed consent. Highly polymorphic markers of chromosomes 2p13–p16, 4q12, 5q33–q34, 13q12, 15q15.1–q15.3, 17q12–q21.33 were analysed for these six LGMD2 families in the previous study [28]. The markers used in this study were the following: LGMD2G (D17S1851, D17S1818, D17S1814); LGMD2H (D9S1811, D9S195, D9S1872, D9S1850); LGMD2I (MDC1C) (D19S412, D19S606, D19S596, D19S879). In addition, the following loci responsible for the following CMD syndromes were excluded: LAMA2 with markers D6S407, D6S1705, and D6S1620 [29–31]; RSMD1 with markers D1S458, D1S2674, D1S234, D1S2885, and D1S511 [32]; MEB with markers D1S211, D1S2677, D1S427, D1S2652, and D1S200 [33]; FCMD with markers D9S2105 and D9S2107 [34]; and MDC1B (D1S213, D1S2833, D1S2709, D1S459 [14]). In addition, the FKRP gene was sequenced in all families as already described [15].

(CA)n microsatellite markers were provided from the Genethon human genetic linkage map [35]. Haplotypes were constructed by minimizing the number of recombination events. Linkage was ascertained by homozygosity by descent.

2.3. Immunocytochemistry

Unfixed frozen 8-μm sections were incubated with monoclonal antibodies to β-spectrin, dystrophin, α-, β-, γ-, and δ-sarcoglycan, laminin $\alpha2$, β-dystroglycan (all available from Novocastra Laboratories), and α-dystroglycan (VIA4-1, Upstate Biotechnology) for 1 h. Then, a biotin-conjugated secondary antibody (Amersham) and Texas Red-conjugated streptavidin (Amersham) were applied to all sections for 45 and 30 min, respectively. All dilutions and washings were made in phosphate buffered saline. Sections were examined by a Leica Aristoplan microscope.

2.4. Immunoblotting

Immunoblot analyses were performed in two patients (cases 1 and 2) as described by Piccolo et al. [36]. The primary antibodies used were against, respectively, dystrophin (DYS1 and DYS2, Novocastra 1:100), α-, β-, γ- and δ-sarcoglycan (Novocastra 1:50).

2.5. Immunoprecipitation

Co-immunoprecipitation was carried out using a previously reported protocol by Kessler et al. [37] with the following modifications. Muscle tissue (0.25 cm^3) was homogenized by Dounce homogenizer in 100 μl RIPA (1% NP-40,1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 100 mM sodium phosphate, pH 7.2) containing 1× protease cocktail (Roche Biochemicals) and centrifuged. The supernatant was retained, and incubated overnight at 4 °C with 25 μl protein-G-Sepharose (Pharmacia) coupled with antibodies specific to anti-α-dystroglycan (polyclonal antibody, courtesy of Dr Stephan Kröger, 1:100) [38] antibodies. Subsequently, the beads were washed three times with RIPA, boiled in Laemmli buffer and applied into 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis. They were then transferred onto nitrocellulose membranes and blotted with anti-β-dystroglycan antibodies (Novocastra 1:250).
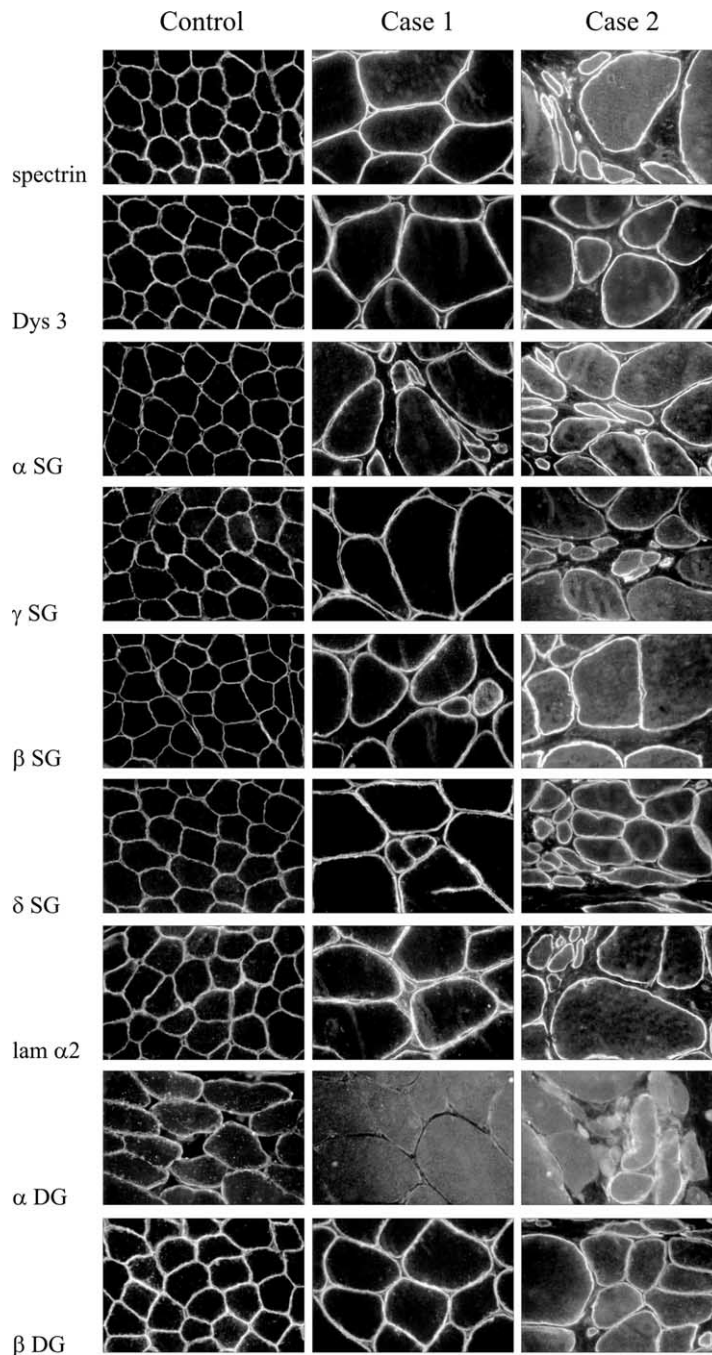

Fig. 2. Immunocytochemical analysis of dystrophin, sarcoglycans (α, β, γ, and δ), laminin α-2, and dystroglycans (α-dystroglycan (VIA4-1 antibody) and β-dystroglycan) in cases 1 and 2. Note significantly reduced labelling for only α-dystroglycan.
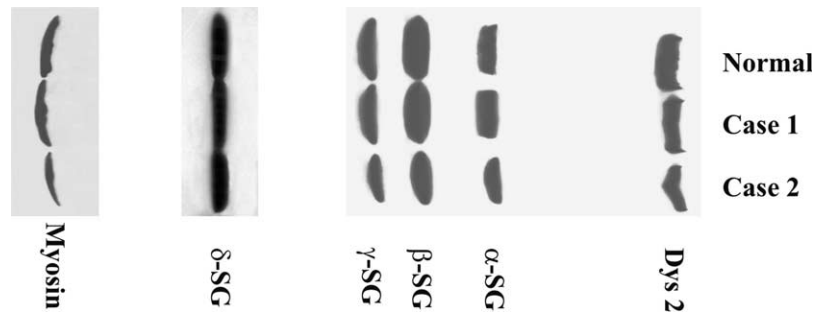
Fig. 3. Western blot analysis of dys2, α-, β-, γ- and δ-sarcoglycans in cases 1 and 2.

3. Results

3.1. Genotyping

All families were haplotyped with microsatellite markers spanning the loci of autosomal recessive LGMD genes (LGMD2A, LGMD2B, LGMD2C, LGMD2D, LGMD2E, LGMD2F, LGMD2G, LGMD2H, LGMD2I) and five CMD forms (MDC1A, MDC1B, MDC1C, FCMD and MEB). No family showed haplotypes consistent with linkage to any of these loci.

3.2. Immunocytochemistry and Immunoblotting

The histological changes on muscle biopsies were compatible with muscular dystrophy. There was mild fibrosis with many regenerating and few necrotic fibres. Variation in fibre size was remarkable; many fibres were hypertrophic with multiple central nuclei and there were atrophic fibres most of which were type II. Some angular fibres were also present.

The expression of all proteins we regularly screen in the biopsies of patients with muscular dystrophy (dystrophin, laminin α2, α-, β-, γ- and δ-sarcoglycans) were normal in all patients in whom muscle biopsy was available for immunofluorescence (cases 1, 2, 3, 5, 6, and 7) (Fig. 2: data shown for cases 1 and 2) and immunoblotting (cases 1 and 2) (Fig. 3). With monoclonal VIA4-1 antibody directed

towards a glycosylated epitope, significantly reduced labelling for α-dystroglycan was observed in all biopsies examined (Figs. 2 and 4: data shown for cases 1, 2, and 7). The muscle biopsies of cases 2 and 7 were also labelled using the antibody to the core dystroglycan protein and showed a profound reduction in agreement with that seen with V1A4-1 (Fig. 4). β-Dystroglycan immunolabelling was within normal limits in all patients (Fig. 2: data shown for cases 1 and 2).

3.3. Demonstration of α- and β-dystroglycan complex

We tested the presence of α- and β-dystroglycan complex formation by co-immunoprecipitation in cases 1 and 2. Co-immunoprecipitation using anti-α-dystroglycan and immunoblotting using anti-β-dystroglycan demonstrated that α- and β-dystroglycan were able to form a complex in both patients (Fig. 5).

4. Discussion

In this study we present a novel and unique LGMD phenotype. In addition to the classical features of LGMD, all patients invariably had mental retardation with microcephaly and normal brain imaging. All families but one were consanguineous. We propose to name this novel variant recessive limb girdle muscular dystrophy with
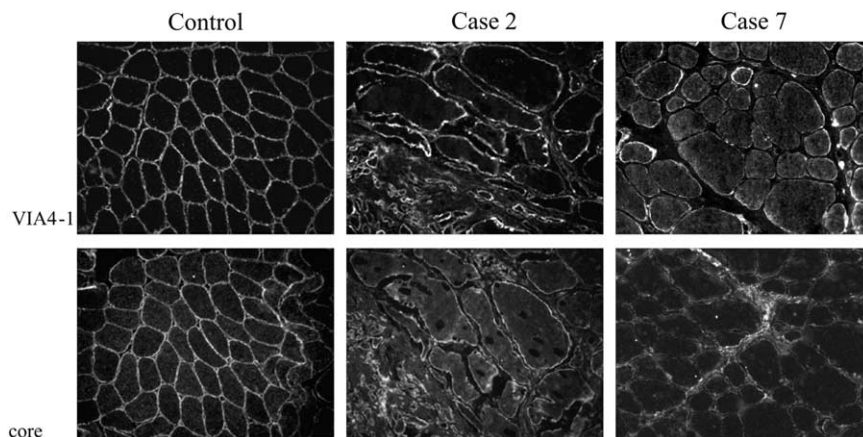

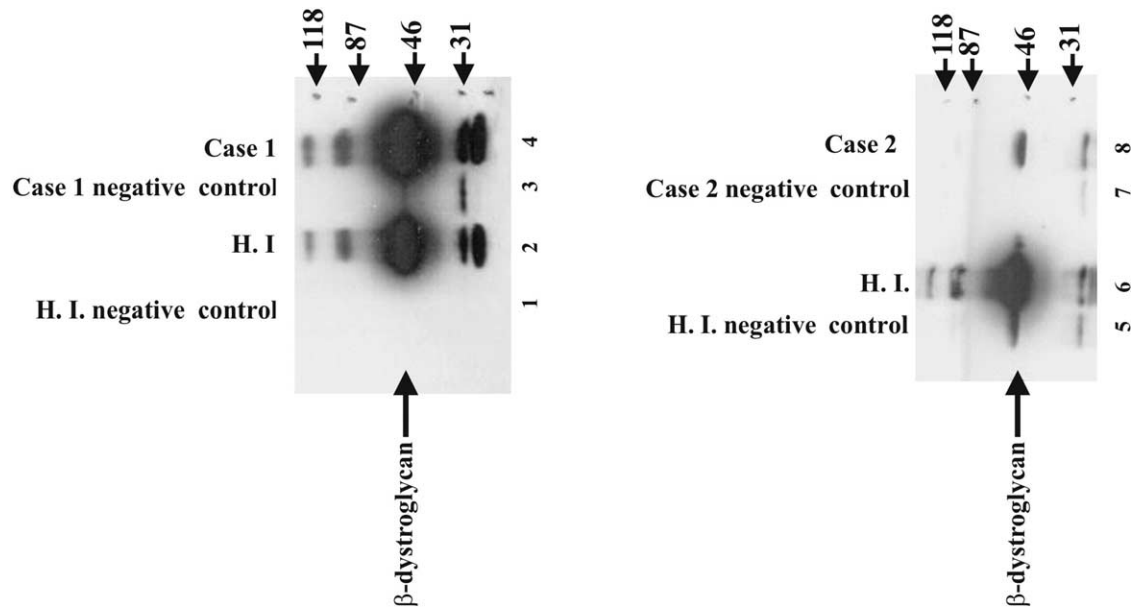Fig. 4. Immunocytochemical analysis of α-dystroglycan by using VIA4-1 and core antibodies in cases 2 and 7.

Fig. 5. Co-immunoprecipitation of β-dystroglycan with α-dystroglycan. β-Dystroglycan protein is co-immunoprecipitated with α-dystroglycan by using form of α-dystroglycan. β-Dystroglycan protein is visualized by immunoblotting using anti-β-dystroglycan antibodies. Healthy individuals' (HI) skeletal muscle tissue is used as positive controls (lanes 1, 2, 5, 6). No α-dystroglycan antibody added to negative controls during co-immunoprecipitation (lanes 1, 3, 5, 7).

mental retardation. From a skeletal muscle point of view, the course is mild despite onset in the first decade of life in all cases and different degrees of reduction in α-dystroglycan expression. All patients remain ambulant, the eldest being 22 years old. Standard cranial MRIs failed to show any evidence of central nervous system malformations or white matter changes. In addition, none of the patients had epilepsy, a common complication of neuronal migration disorders. To our knowledge, such a phenotype has not been previously described. In addition, with the exception of LGMD2I, the abnormal expression of α-dystroglycan has not been reported in other forms of LGMD, further suggesting that this form represents a new clinical entity. α-dystroglycan expression was significantly reduced in skeletal muscle, as judged by using an antibody directed towards a glycosylated epitope and in two patients using the antibody to the core α-dystroglycan protein. Similar findings have been observed in patients with MDC1C, due to mutations in the FKRP gene (Brown et al., submitted for publication). However, mutations in the FKRP gene were excluded in all families. Our experiments also suggest that the residual α-dystroglycan expression is still capable of binding to β-dystroglycan. This is important as α-dystroglycan associates with Grb2 (growth factor receptor bound 2), a protein that participates in signal transduction pathways involving receptor tyrosine kinases [39].

Dystroglycan is a widely expressed and heavily glycosylated protein, but its glycosylation pattern varies in a tissue-specific and developmental pattern. Recently a new pathomechanism responsible for several forms of CMD and for LGMD2I was reported following the identification of mutations in genes with putative or definite glycosyltransferase activity [40]. This includes mutations in fukutin and the fukutin-related-protein gene (FKRP), which are mutated in FCMD and MDC1C, respectively [14,24,41]. While in FCMD there are invariably associated structural brain abnormalities, mutations in the *FKRP* gene do not usually affect the brain. Allelic mutations in the *FKRP* gene were recently shown also to be responsible for LGMD2I, a very common form of LGMD in the Caucasian population [15]. Two other CMD forms have recently been demonstrated to be due to mutations in genes encoding glycosyltransferases, MEB disease and Walker–Warburg syndrome [25,26]. Both these CMD variants result in CNS defects in the form of neuronal migration disorders. These findings unequivocally indicate that altered glycosylation is a common underlying cause of muscular dystrophies. The abnormal expression of α-dystroglycan suggests that this protein is abnormally processed in this novel form, and that its abnormal function might account for both the muscle weakness and the mental retardation.

Dystroglycan is also expressed abundantly in neurons, astrocytes, and in the glial-vascular interface, suggesting a role in maintenance of the blood–brain barrier [42]. This was further confirmed by the recent finding by Moore et al. [43] who generated a mouse with a brain-specific deletion of dystroglycan. This resulted in discontinuities of the pial surface basal lamina (glia limitans) and brain malformation including disarray of cerebral cortical layering, fusion of cerebral hemispheres and cerebellar folia, and aberrant migration of granule cells. This is similar to the abnormalities demonstrated in the brain of patients with MEB disease and FCMD. In addition, Michele et al. [21] recently reported that the abnormal processing of α-dystroglycan in FCMD and MEB disease abolishes its ability to bind laminin, agrin, and neurexin. Both agrin and neurexin are

neuronal dystroglycan receptors in brain, and it has been suggested that neurexins' tightly regulated interaction could mediate cell adhesion between brain cells [44]. We do not have sufficient muscle available to study the binding properties of the α-dystroglycan produced by these patients to neurexin; we could, however, speculate that this interaction could be partially affected (and not totally abolished) in this form of limb girdle muscular dystrophy with mental retardation.

This form of limb girdle muscular dystrophy is likely to represent another form of muscular dystrophy secondary to abnormal glycosylation of α-dystroglycan, adding to the growing number of muscular dystrophies secondary to mutations in genes encoding for putative glycosyltransferases [40].

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