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ARTICLE

Congenital muscular dystrophy type 1D (MDC1D) due to a large intragenic insertion/deletion, involving intron 10 of the *LARGE* gene

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Mutation of the *LARGE* gene is the rarest of the six known genetic causes of α-dystroglycanopathy. We report further a family with MDC1D due to a complex genomic rearrangement that was not apparent on standard sequencing of *LARGE*. Two sisters in a consanguineous family had moderate mental retardation and cerebellar malformations, together with dystrophic changes and markedly reduced α-dystroglycan glycosylation staining on muscle biopsy. There was homozygous linkage to the *LARGE* locus but sequencing of *LARGE* coding regions was normal. Analysis of *LARGE* cDNA showed an abnormal sequence inserted between exons 10 and 11, in most of the transcripts, predicted to introduce a premature stop codon. The abnormal sequence mapped to a spliced EST (DA935254) of unknown function, normally located at 100 kb centromeric of *LARGE* on chromosome 22q12.3. Quantitative PCR analysis of the EST and adjacent regions showed twice the normal copy number in patients' genomic DNA samples, consistent with a large intra-chromosomal duplication inserted into intron 10 of *LARGE* in a homozygous state. This insertion was associated with deletion of a central region of intron 10, but the exact break points of the deletion/duplication were not found, suggesting that an even more complex rearrangement may have occurred. The exact function of LARGE, a golgi protein, remains uncertain. POMT and POMGnT enzyme activities were normal in patients' lymphoblast cells, suggesting that defects in LARGE do not affect the initiation of *O*-mannosyl glycans.
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INTRODUCTION

Alpha-dystroglycan (αDG) is a highly glycosylated cell surface protein that has important roles in neuronal cell migration and cell-to-extracellular matrix interactions in muscle. Abnormality in the glycosylation of αDG is the hallmark histological abnormality and the likely pathogenic mechanism for a group of congenital muscular dystrophies (CMD), collectively called the 'α-dystroglycanopathies'.[1] At present, six genes have been linked to this common clinicopathological presentation (*POMT1, POMT2, POMGNT1, FKTN, FKRP* and *LARGE*). All of these encode confirmed or putative enzymes that are thought to function in the *O*-mannosyl glycosylation pathway. Abnormal αDG glycosylation correlates with an increasingly severe pattern of tissue effects that extend from an adult-onset limb-girdle muscular dystrophy with normal intelligence (LGMD type 2I) to severe congenital brain and eye malformations (Walker–Warburg syndrome, WWS).[1] The *LARGE* gene became a candidate for human CMD after the discovery of the myodystrophic (myd) mouse that has a spontaneous null mutation in *Large* because of a genomic deletion involving exons 5 to 7.[2–4] Another spontaneous murine mutant, named veils (vls) also results from an intragenic deletion leading to loss of exons 3 to 5 and premature protein termination.[5] The two strains share typical cerebral, ocular and muscular changes of an α-dystroglycanopathy. MDC1D due to mutations in *LARGE* is the rarest of the α-dystroglycanopathies identified to date, with only three confirmed families previously described.[6–8] Affected children have had typical neurological and muscle abnormalities associated with the α-dystroglycanopathies, but with very different severities; one family had mild muscle-eye-brain disease (MEB) and the other two had typical WWS. Another WWS patient has been reported with a single heterozygous nonsense mutation in *LARGE*.[9] Confirmation that this patient has CMD due to *LARGE* awaits the discovery of a second mutation. In this paper, we describe the fourth confirmed family with MDC1D due to a homozygous *LARGE* mutation, in which two sisters from consanguineous parents have moderate mental retardation, cerebellar and

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pontine hypoplasia, and persistent white matter abnormalities on brain magnetic resonance imaging (MRI), features consistent with mild MEB. We identified an unusual genetic mechanism for *LARGE* gene disruption, namely the splicing of an abnormal sequence into the *LARGE* mRNA transcript likely due to a duplication/deletion event involving intron 10. This report extends our knowledge of the clinical phenotype associated with *LARGE*, and is the second family in which disease results from a large-scale gene rearrangement.

The *POMT1*, *POMT2* and *POMGNT1* genes encode enzymes that together mediate the first two steps in the *O*-mannosyl glycosylation pathway. A recent study suggests that *LARGE* participates in post-phosphoryl glycosylation of a novel phosphorylated *O*-mannosyl glycan on the mucin-like domain of recombinant αDG, which is required for laminin binding.[10] Evidence from cell culture and a *fukutin* transgenic mouse suggests that fukutin physically interacts with POMGnT1 and can cause reduced POMGnT1 activity when abnormal.[11] To investigate whether LARGE may also function by influencing the first steps of the *O*-mannosyl pathway, we assessed the activity levels of POMT and POMGnT1 in cultured lymphoblasts from one of our patients.

MATERIALS AND METHODS

Case reports

The two affected girls are the only children of first-cousin parents from Lebanon. There is no other family history of neuromuscular disease. Both children were born full-term after uneventful pregnancies with normal foetal movements and deliveries. Birth weights, lengths and head circumferences were within normal limits.

Patient 1: The older sibling first presented to medical attention at an age of 2.5 months with hypotonia and regurgitation. Acquisition of all motor skills was delayed. She sat at an age of 9 months, stood unsupported at an age of 2.5 years and at an age of 8 years, she could climb stairs with difficulty but could not run. Her full-scale IQ was 61. On examination at an age of 8 years, she weighed 24 kg (50th centile), her height was 122.5 cm (35th centile) and the occipitofrontal circumference (OFC) was 53 cm (75th centile). Her speech was restricted to dysarthric simple words, and she followed simple commands. She had a myopathic face with an open-mouth posture and mild tongue hypertrophy. There was generalised muscle hypertrophy, most prominent in the calves, thighs and the shoulder girdle. This contrasted with moderate proximal lower limb weakness that manifested as a waddling gait and a positive Gowers' manoeuvre. Upper limb strength was within normal limits. There were no joint contractures, except for mild tightness of the Achilles' tendons, associated with toe walking. The only abnormalities found on eye assessment were mild myopia and strabismus. There was no history of seizures. Cardiac and respiratory examinations were normal. Brain MRI performed at ages 3 ½ and 6 years showed marked cerebellar atrophy (particularly affecting the vermis) and cerebellar cysts (Figure 1). There was mild generalised cerebral and pontine atrophy, dilatation of all ventricular spaces and pachygyria of the frontal lobes (Figure 1). Diffuse symmetrical high signal abnormalities were seen within the periventricular and deep white matter of both cerebral hemispheres, with sparing of the subcortical U fibres. These hyperintensities were unchanged at the age of 6 years. A generalised skeletal X-ray survey, abdominal ultrasound, echocardiogram, electroencephalogram and auditory-evoked potentials with brainstem responses were all normal. Creatine kinase (CK) levels were 30 times higher than the upper limit of normal.

Patient 2: The younger sister followed a clinical course similar to her sister, but was less delayed in her motor and cognitive skills. She stood unsupported at an age of 16 months and walked independently at an age of 2 years. On examination at an age of 4 ½ years, her weight was 15 kg (25th centile), height was 106 cm (75th centile) and OFC was 51 cm (50th centile). There was generalised hypotonia, firm hypertrophic calves and a mild equinovarus foot position. Mild myopia was the only abnormality observed on eye examination. Myopathic features were noted on EMG analysis, whereas nerve conduction studies were normal. Brain MRI performed at 2 years showed diffuse high signal white matter abnormalities, mainly in the frontal and parieto–occipital regions, dilated ventricular spaces and marked cerebellar hypoplasia. CK levels were 60 times above the upper limit of normal.

Muscle biopsy findings

Muscle and skin biopsy samples were taken from Patient 1 from the right gastrocnemius muscle. Standard histochemical analysis revealed a dystrophic pattern characterised by fibre size variation, markedly increased connective tissue, internalised nuclei and scattered necrotic fibres. Immunohistochemistry (IHC) for αDG showed markedly reduced staining using VIA4-1 (Millipore, Billerica, MA, USA), an antibody that recognises a glycosylated epitope. Laminin-α2 expression was mildly reduced by IHC using antibodies that recognize both the 80 kDa (clone 5H2; Millipore) and the 300 kDa (NCL-merosin; Novocastra Labs, Newcastle, UK) fragments. Staining for β-dystroglycan (βDG), dystrophin, collagen VI and sarcoglycans (α and γ) was normal.

WESTERN BLOT ANALYSIS

Western blotting was used to investigate the degree of abnormal αDG glycosylation using a skin fibroblast cell line obtained from Patient 1, as frozen muscle was not available. Fibroblasts were maintained at 37°C and 5% CO_2 in DMEM medium plus 20% foetal bovine serum and 0.5% penicillin-streptomycin (Gibco, Invitrogen Corp., Carlsbad, CA, USA). Cultured cells were lysed, and glycoproteins from cell homogenates were enriched with WGA lectin as previously described.[11] We performed immunoblots (for αDG and βDG) and laminin ligand overlay assays using polyvinylidene difluoride membranes, and blots were developed by horseradish peroxidase-enhanced chemiluminescence (Pierce, Thermo Fisher Scientific, Rockford, IL, USA).[12] The monoclonal antibodies IIH-6 and VIA4-1, which are specific for functionally glycosylated αDG,[13] showed no detectable binding affinity to

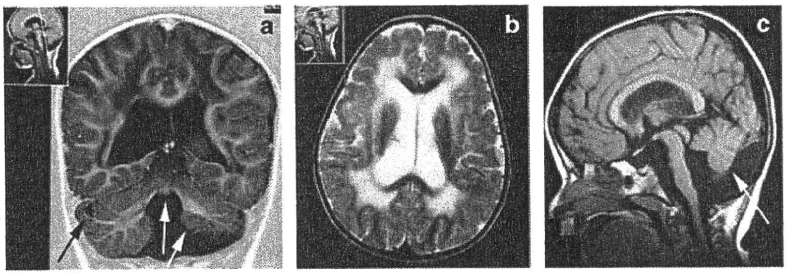

Figure 1 Brain MRI images obtained from Patient 1 performed at an age of 3.5 years. (**a**) Coronal T1-weighted image showing cerebellar and vermis hypoplasia (white arrows), cerebellar cysts (black arrow), generalised white matter atrophy and increased CSF spaces. (**b**) Transverse T2-weighted image showing abnormal high white matter signal and dilated lateral ventricles. (**c**) Sagittal T1-weighted image showing cerebellar (white arrow) and pontine hypoplasia.

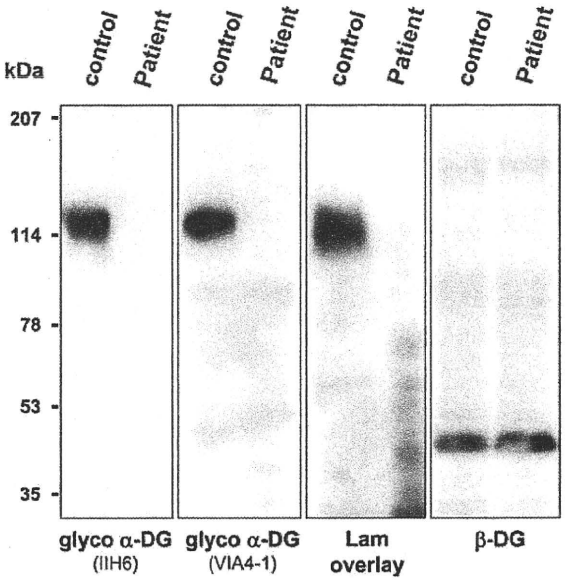

Figure 2 Western blot analysis of α-dystroglycan. Immunoblot analysis of WGA-enriched homogenates from fibroblasts obtained from Patient 1 and a healthy individual (control). Absent staining of antibodies IIH6 and VIA4 that bind to glycosyl epitopes on α-dystroglycan (α-DG) and a laminin-1 (Lam) overlay assay show abnormalities in αDG glycosylation in patient fibroblasts that is associated with loss of laminin-1 protein binding. Beta-dystroglycan (β-DG) is used as a loading control.

αDG obtained from patient fibroblast cells, even though βDG (monoclonal antibody 7D11) was normally present, consistent with a glycosylation defect in αDG (Figure 2). This defect in αDG glycosylation also resulted in loss of laminin-1 ligand binding (Figure 2).

GENETIC ANALYSIS

We performed linkage analysis using microsatellite markers to the five α-dystroglycanopathy loci known at the time (*POMGNT1*, *FKRP*, *POMT1*, *POMT2* and *FKTN*) and the *DAG1* gene that encodes DG, but we identified no homozygous linkage. A genome-wide linkage study using 400 microsatellite markers showed potential linkage to the newly identified *LARGE* locus and analysis of further markers supported homozygous linkage (Figure 3). We sequenced the coding regions of *LARGE* bi-directionally by standard techniques. Oligonucleotide primers, at least 80 base pairs (bp) outside the exons, were chosen for PCR amplification and sequencing on ABI 3730 capillary electrophoresis system (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA), but no abnormalities were found. Subsequently, mRNA was extracted from cultured EBV-transformed lymphoblasts obtained from Patient 1 (Trizol method), cDNA was generated using oligodT Superscript II First-Strand Synthesis (Invitrogen, Carlsbad, CA, USA) and the *LARGE* gene transcript was sequenced using 14 overlapping primer pairs. Two abnormally large PCR products, together with a faint product of the expected size, were obtained for primers that spanned between exons 9 and 12 (Figure 4a). Sequencing revealed that most mRNA transcripts contained an abnormal 197 bp sequence inserted between exons 10 and 11. Low levels of a 171 bp insertion (identical to the 197 bp sequence except lacking 26 bases from the 3′ end) and a normally spliced transcript were also obtained. Identical splicing abnormalities were also seen in mRNA from cultured patient fibroblast cells, except a greater proportion of transcripts showed normal splicing

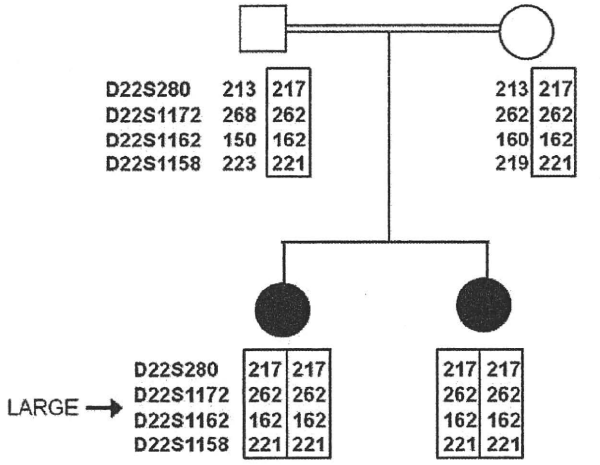

Figure 3 Genotyping for the *LARGE* locus. Family tree showing analysis of microsatellite markers in close proximity to the *LARGE* locus (22q12.3). Both of the affected children are homozygous for four contiguous markers, consistent with homozygosity by descent for this chromosomal region.

(Figure 4a). The abnormal sequences mapped to a part of a spliced EST (GenBank accession no: DA935254) of unknown function, originally identified in a human splenic cDNA library,[14] normally located at 100 kb centromeric to the *LARGE* gene on chromosome 22q12.3 (Figure 5). The presence of the two most abundant abnormally inserted sequences are predicted to immediately introduce a premature stop codon in the *LARGE* mRNA transcript, resulting in truncation of the LARGE protein mid-way through translation (Figure 4b). Analysis of the inserted EST sequence identified potential splice acceptor and donor sites for both the larger and smaller insertions that likely mediate the abnormal splicing (Supplementary E, Figure 1). Archived frozen muscle was no longer available to verify the abnormalities in *LARGE* splicing in muscle.

To investigate whether the abnormal *LARGE* splicing arose because of a chromosomal duplication, we performed quantitative PCR (qPCR) analysis of the EST sequence obtained from patients' genomic DNA (gDNA) using the LightCycler 480 (Roche Diagnostics, Basel, Switzerland) and the SYBR Green I detection system (Roche Applied Science, Indianapolis, IN, USA). We normalised gDNA concentrations between individual samples using PCR primers for a distant gene (*PTPLA* on chromosome 10). Supplementary E, Table 1 lists the key primer pairs used. The EST (DA935254) was present in double the expected copy number in both the patients compared with two healthy unrelated individuals (Figure 4c), consistent with a homozygous duplication of the EST sequence in both patients (ie, they have four copies instead of two). To investigate the size of the putative duplication, we repeated qPCR analysis for genomic regions at variable distances up- and downstream of the EST sequence (Supplementary E, Table 1). These studies indicate that between 40825 and 42937 bp of gDNA, surrounding the EST sequence, is present at twice the normal copy number in gDNA in both patients (Figure 4c). Both parents had 1.5 times the normal copy number, consistent with each having one copy of the chromosome-22 duplication and a normal allele. The break point at the 3′-end is likely located in a 1313 bp region located between regions 3b and 3c (Chr 22:33548218–33549530; GRCh37/hg19 assembly) and the 5′-break point in a 799 bp region located between regions 5b and 5c (Chr 22:33590356–33591154).

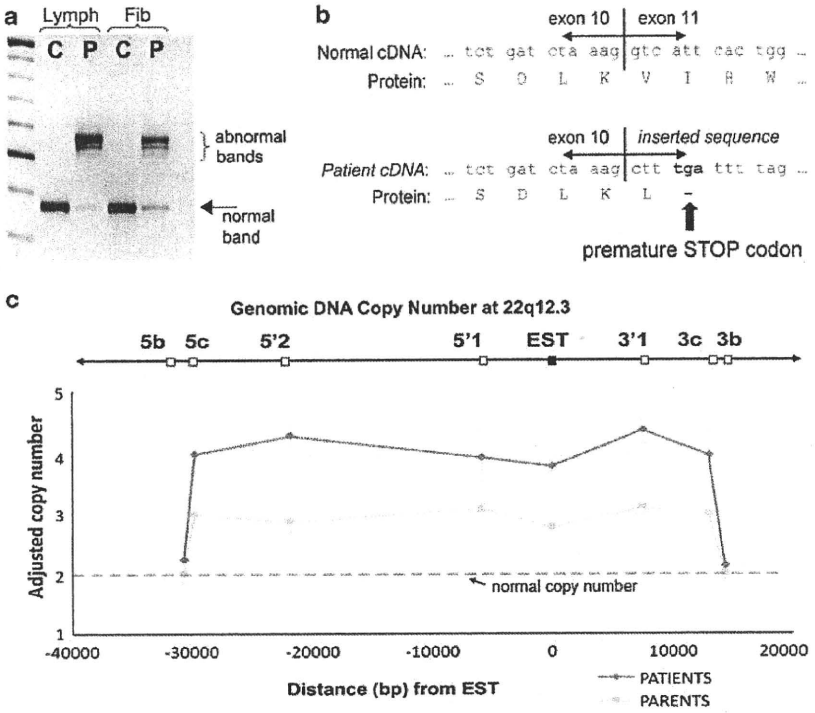

Figure 4 Analysis of the *LARGE* gene insertion in genomic and cDNA. (**a**) Agarose gel showing the PCR products from patient (P) and control (C) cDNA generated from cultured lymphoblasts (Lymph) and fibroblasts (Fib) using primers that span from exons 9 to 12 of *LARGE* (Supplementary E, Table 1). In patient cDNA sample, two abnormally high bands predominate that correspond to insertions of abnormal DNA sequences between exons 10 and 11. A faint band of the normal size is also seen (arrow). (**b**) Diagram showing the amino acid sequence that is coded by normal and patient cDNA around the exon 10/11 boundary. The abnormal insertion found in patient cDNA results in the introduction of a premature STOP codon, predicted to cause protein termination mid-way through the translation of the LARGE protein. (**c**) Results from qPCR analysis of genomic DNA (gDNA) for EST DA935254 and the surrounding chromosomal region. gDNA concentrations between samples were adjusted using qPCR results from a distant gene (*PTPLA*) and results were scaled so that control samples had a mean copy number of 2 at each point (data not shown). In patients' gDNA sample, qPCR analysis of the EST sequence and for five regions, located at variable distances upstream and downstream, shows twice the expected copy number consistent with homozygous duplication of this region. Genomic copy number returns to normal for markers 5b and 3b in the patient gDNA samples, and therefore these markers define the maximum size of the duplication. Analysis of gDNA from both parents shows that both had three copies of the duplicated region, consistent with each having the duplication in a heterozygous state. All results are shown relative to the reverse gDNA strand.

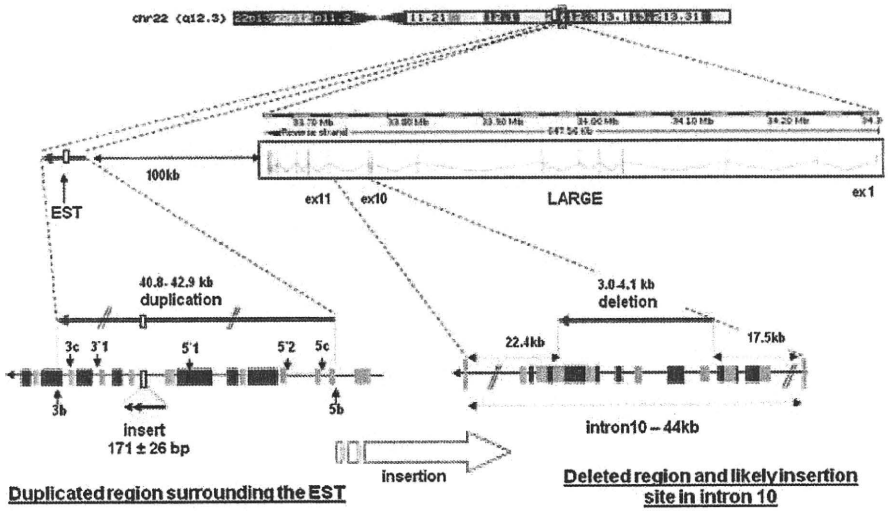

Figure 5 Diagram of the insertion/deletion mutation involving intron 10 of the *LARGE* gene. The EST DA935254, normally located at 100 kb centromeric of *LARGE*, is duplicated together with ~40 kb of flanking DNA and likely inserted into intron 10 of *LARGE*. Numerous repetitive elements, which may have contributed to the large intra-chromosomal rearrangement, are present in both intron 10 and flanking the EST, including SINEs (green), LINEs (red) and LTRs (blue). bp=base pairs.

Together, these results indicate that the patients are homozygous for a duplication of gDNA around EST (DA935254), and the most likely site of insertion for the duplicated DNA is intron 10 of *LARGE*, leading to the abnormal splicing of the EST into the *LARGE* mRNA transcript between exons 10 and 11.

To identify the site of the insertion in intron 10 of LARGE, we designed twelve pairs of overlapping primers (Supplementary E, Figure 2) to cover the whole length of the intron (44117 bp). With two primer pairs (5aF-5aR and 6aF-6aR), we amplified the expected bands in controls and parents but not in the two patients, indicating that the insertion should be in this region (Supplementary E, Figure 2). We repeated the same strategy with several other primer pairs chosen inside this region and in the bordering regions to finally determine a region from 3012 to 4175 bp that could never be amplified in the patients, which likely corresponds to a deleted region associated with the insertion. By qPCR analysis using primer pairs L5F-L6R and L6F-L7R (Supplementary E, Table 1), both parents had reduced amplification compared with controls, consistent with them being heterozygous for a deletion in this region. We were not able to amplify any product with primers chosen on the borders of the insertion and on the non-deleted sides of the intron, and thus did not identify the exact break points for the insertion/deletion rearrangement.

As repetitive sequences are implicated in the pathogenesis of recurrent chromosomal deletion or duplication syndromes (eg, CMT1A),[15] we analysed intron 10 and the duplicated region around EST DA935254 for homologous sequences. Both regions shared multiple SINE, LINE and LTR repeats that may have had roles in the genesis of the mutation, but no extended regions of homology were found.

POMT AND POMGNT1 ACTIVITY IN PATIENTS' LYMPHOBLAST CELLS

The POMT and POMGnT1 enzymes mediate the first and second steps, respectively, of the *O*-mannosyl glycosylation of αDG. To investigate whether *LARGE* mutations may disrupt the glycosylation of αDG by influencing the activity of either POMTs or POMGnT1, we assessed the enzymatic activity of these enzymes in cultured patients' lymphoblast cells using previously validated assays.[16] The POMT activity was based on the amount of [^{3}H]mannose transferred from [^{3}H]mannosylphosphoryldolichol to a glutathione-*S*-transferase fusion αDG, and the POMGnT1 activity was based on the amount of [^{3}H]GlcNAc transferred from UDP-[^{3}H]GlcNAc to a mannosyl-peptide. In brief, we incubated microsomal membrane fractions prepared from patients' and control EBV-immortalised B lymphocytes, with an acceptor substrate and radiolabelled sugar donor substrate. We then assessed the incorporation of radioactivity into each acceptor substrate. We normalised control and patient samples using the activity level of GnT1, a golgi enzyme involved in the *N*-glycosylation pathway, which functions independently of *O*-glycosylation. Results showed no difference between the patient and eight control samples for POMT/GnT1 activity (patient 0.283 *vs* control 0.287 ± 0.067 SD) or for POMGnT1/GnT1 activity (patient 0.068 *vs* control 0.071 ± 0.019 SD).

DISCUSSION

In this paper, we report the fourth confirmed family with MDC1D due to mutations in *LARGE*. The clinical abnormalities are typical for the α-dystroglycanopathy group of disorders. Both sisters have typical features of an α-dystroglycanopathy, such as raised CK levels, dystrophic changes on muscle histology associated with reduced glycosylated α-dystroglycan staining, intellectual disability and brain malformations. In terms of severity of neurological involvement, the two sisters are best classified as having mild MEB disease, even though there were only minor ocular abnormalities. The findings were similar to the first family identified with mutations in *LARGE* with mild/moderate developmental abnormalities of the cerebellum, pons, brain stem and cerebrum, although our patients are the first ones to be reported with cerebellar cysts.[6]

Even though standard sequencing of the coding regions of *LARGE* was normal in the proband of this family, we persisted with analysis of *LARGE* because there was homozygous linkage to the gene locus in the context of consanguinity. Analysis of cDNA, generated from mRNA derived from patients' lymphoblast cells, showed an abnormally large product for a PCR reaction that spanned from exons 9 to 12. Sequencing of this product showed that an abnormal sequence of either 197 or 171 bases was spliced between exons 10 and 11 in almost all transcripts. The abnormal sequence is normally situated on chromosome 22 centromeric to the 3′ end of *LARGE*. In this position, it would not usually be incorporated into the *LARGE* transcript, even though the sequence contains the necessary splice donor and acceptor sites. To explain its incorporation into the *LARGE* mRNA, we hypothesised that the EST sequence was translocated into intron 10 of *LARGE* in our family, possibly through duplication of this genomic region. We assessed the copy number of the EST sequence by qPCR relative to other genes in patients' gDNA samples and showed twice the expected levels, consistent with a genomic duplication in a homozygous state. Both parents had a 1.5-fold increase in copy number (an increase from two to three copies of the sequence) that was consistent with both of them being asymptomatic heterozygous carriers of the duplication. To map the approximate size of the duplication, we repeated qPCR analysis up- and downstream of the EST sequence and showed the duplication spans between 40 and 43 kb of gDNA. Even though we identified the likely site of insertion in the central part of intron 10 and delimited a large intronic region that cannot be amplified in the patients, mapping the precise break points of the proposed duplication/insertion was not feasible, possibly because of the presence of a more complex rearrangement than anticipated.

Analysis of *LARGE* mRNA transcripts from patients' lymphoblast and fibroblast cells suggest that most *LARGE* mRNA transcripts contain an abnormal sequence. Both versions of the abnormal insertion are predicted to immediately introduce a premature stop codon mid-way through protein translation, just before the second catalytic domain, and result in a non-functional protein (Figure 4b). Small amounts of normally spliced transcript were also seen and the levels appeared slightly higher in fibroblasts than in lymphoblasts, suggesting this may vary in different tissues. Unfortunately, it was not possible to assess levels of normally spliced *LARGE* mRNA or protein function either in muscle or in brain, the two main tissues that show pathology in the α-dystroglycanopathies. We observed no functionally glycosylated αDG by western blot analysis in patient fibroblast cells, but it is likely that low levels of functional αDG are present in patient tissues at levels below the threshold of detection by western blot for several reasons. Residual glycosylated αDG staining was present in the muscle biopsy, and both sisters have less severe cognitive deficits and brain malformations than a patient with homozygous null mutations in *LARGE* who had a WWS phenotype.[7] We also detected low levels of a normally spliced *LARGE* gene mRNA transcript in both lymphoblasts and fibroblasts, which should allow normal LARGE protein to be produced at low levels.

We also investigated whether LARGE may exert some of its effects on αDG glycosylation through interactions with either the POMT or the POMGnT1 enzymes. Both POMT and POMGnT1 activities were normal in patients' lymphoblast cells, strongly suggesting that the two initial steps of αDG glycosylation occur independently of LARGE.

At present, it appears that *LARGE* is a rare causative of CMD, as the coding regions of the gene have been screened in large cohorts of patients with α-dystroglycanopathy of unknown cause.[6,8] Although experience is very limited at present, it is notable that two of the four confirmed families with CMD, caused by *LARGE* mutation, have had large gene rearrangements, either a deletion involving several exons[7] or a likely intragenic insertion/deletion (our family). In another family, a heterozygous nonsense mutation (p.Trp516X) was identified on standard gene sequencing, leaving open the possibility that a large deletion/duplication or rearrangement on the other allele was overlooked.[9] In addition, consanguineous families are described that link to the *LARGE* locus but no mutation has been found by exon sequencing.[7,17] LARGE was so named for the enormous genomic region it occupies on chromosome 22q12.3 (647 kb) because of the presence of very large introns enriched in low-copy repeats (LCRs). The structure of the *LARGE* gene may predispose to mutations that involve duplication or deletion of large regions of DNA because of non-allelic homologous recombination between LCRs, the proposed mechanism for many genomic disorders.[18] Sequencing of coding regions from gDNA will not identify many large gene rearrangements. Therefore, sequencing LARGE from cDNA and the application of CGH, qPCR, and other methods for detecting rearrangement mutations should be considered routinely.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Martin PT: Mechanisms of disease: congenital muscular dystrophies-glycosylation takes center stage. *Nat Clin Pract Neurol* 2006; **2**: 222–230.
2 Lane PW, Beamer TC, Myers DD: Myodystrophy, a new myopathy on chromosome 8 of the mouse. *J Hered* 1976; **67**: 135–138.
3 Mathews KD, Rapisarda D, Bailey HL, Murray JC, Schelper RL, Smith R: Phenotypic and pathologic evaluation of the myd mouse. A candidate model for facioscapulohumeral dystrophy. *J Neuropathol Exp Neurol* 1995; **54**: 601–606.
4 Grewal PK, Holzfeind PJ, Bittner RE, Hewitt JE: Mutant glycosyltransferase and altered glycosylation of alpha-dystroglycan in the myodystrophy mouse. *Nat Genet* 2001; **28**: 151–154.
5 Lee Y, Kameya S, Cox GA *et al*: Ocular abnormalities in Large(myd) and Large(vls) mice, spontaneous models for muscle, eye, and brain diseases. *Mol Cell Neurosci* 2005; **30**: 160–172.
6 Longman C, Brockington M, Torelli S *et al*: Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of alpha-dystroglycan. *Hum Mol Genet* 2003; **12**: 2853–2861.
7 van Reeuwijk J, Grewal PK, Salih MA *et al*: Intragenic deletion in the LARGE gene causes Walker-Warburg syndrome. *Hum Genet* 2007; **121**: 685–690.
8 Mercuri E, Messina S, Bruno C *et al*: Congenital muscular dystrophies with defective glycosylation of dystroglycan: a population study. *Neurology* 2009; **72**: 1802–1809.
9 Godfrey C, Clement E, Mein R *et al*: Refining genotype phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan. *Brain* 2007; **130**: 2725–2735.
10 Yoshida-Moriguchi T, Yu L, Stalnaker SH *et al*: *O*-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. *Science* 2010; **327**: 88–92.
11 Xiong H, Kobayashi K, Tachikawa M *et al*: Molecular interaction between fukutin and POMGnT1 in the glycosylation pathway of alpha-dystroglycan. *Biochem Biophys Res Commun* 2006; **350**: 935–941.
12 Michele DE, Barresi R, Kanagawa M *et al*: Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. *Nature* 2002; **418**: 417–422.
13 Ervasti JM, Campbell KP: Membrane organization of the dystrophin-glycoprotein complex. *Cell* 1991; **66**: 1121–1131.
14 Kimura K, Wakamatsu A, Suzuki Y *et al*: Diversification of transcriptional modulation: large-scale identification and characterization of putative alternative promoters of human genes. *Genome Res* 2006; **16**: 55–65.
15 Pentao L, Wise CA, Chinault AC, Patel PI, Lupski JR: Charcot-Marie-Tooth type 1A duplication appears to arise from recombination at repeat sequences flanking the 1.5 Mb monomer unit. *Nat Genet* 1992; **2**: 292–300.
16 Manya H, Bouchet C, Yanagisawa A *et al*: Protein *O*-mannosyltransferase activities in lymphoblasts from patients with alpha-dystroglycanopathies. *Neuromuscul Disord* 2008; **18**: 45–51.
17 Manzini MC, Gleason D, Chang BS *et al*: Ethnically diverse causes of Walker-Warburg syndrome (WWS): FCMD mutations are a more common cause of WWS outside of the Middle East. *Hum Mutat* 2008; **29**: E231–E241.
18 Inoue K, Lupski JR: Molecular mechanisms for genomic disorders. *Annu Rev Genomics Hum Genet* 2002; **3**: 199–242.

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CHAPTER NINETEEN

POMGnT1, POMT1, and POMT2 Mutations in Congenital Muscular Dystrophies

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Contents

Abstract

α-Dystroglycanopathies are a group of rare inherited neuromuscular disorders characterized by reduced glycosylation of α-dystroglycan (α-DG). Mutations in six genes (*POMT1*, *POMT2*, *POMGNT1*, *FKTN*, *FKRP*, and *LARGE*) have been identified in patients with α-dystroglycanopathies. Due to an extremely broad clinical spectrum and relatively poor phenotype–genotype correlation, diagnosis of α-dystroglycanopathies is difficult and requires searching for mutations gene by gene. At present, of the six proteins involved on α-dystroglycanopathies, the function of the gene products is only known for POMT1, POMT2, and POMGnT1, all responsible for the *O*-mannosylglycan biosynthesis. This chapter describes the assay protocols to diagnose patients with α-dystroglycanopathy by measuring glycosyltransferase activity.

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1. Overview

Recent studies indicate that *O*-mannosylation of α-dystroglycan (α-DG), a highly glycosylated surface membrane protein, plays an important role in muscle and brain development. Defects in glycosylation of α-DG cause several forms of autosomal recessive muscular dystrophy, also called α-dystroglycanopathies, that share common features such as high serum creatine kinase and muscle hypertrophy. Six genes (*POMT1*, *POMT2*, *POMGNT1*, *FKTN*, *FKRP*, and *LARGE*) are responsible for these diseases with overlapping phenotypes (Michele and Campbell, 2003; Muntoni *et al.*, 2008).

Muscle–eye–brain disease (MEB) is an autosomal recessive disorder characterized by congenital muscular dystrophy (CMD), ocular abnormalities, and brain malformation (type II or cobblestone lissencephaly). Mutations in the *POMGNT1* gene were first identified in patients with MEB (Yoshida *et al.*, 2001). A selective deficiency of glycosylated α-DG in MEB patient muscle biopsies was found, suggesting that hypoglycosylation of α-DG may be the pathomechanism of MEB. Walker–Warburg syndrome (WWS) is the most severe form characterized by CMD, major structural brain defects and eye malformations. The first mutations in *POMT1* and *POMT2* were reported in patients with WWS (Beltran-Valero De Bernabe *et al.*, 2002; van Reeuwijk *et al.*, 2005). The gene *POMGNT1* encodes the protein *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) which forms GlcNAcβ1-2Man linkage of *O*-mannosyl glycans (Yoshida *et al.*, 2001), and the protein products of *POMT1* and *POMT2*, protein *O*-mannosyltransferase 1 and 2, are responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis on α-DG (Manya *et al.*, 2004). *POMT1* and *POMT2* are both required for protein *O*-mannosyltransferase activity (Akasaka-Manya *et al.*, 2006; Manya *et al.*, 2004). In WWS patients, as in MEB patients, the highly glycosylated α-DG was selectively deficient in skeletal muscle and brain. WWS and MEB are similar disorders, but the clinical spectrum associated with both diseases is broad (Biancheri *et al.*, 2006; D'Amico *et al.*, 2006).

Other forms of muscular dystrophies have been suggested to be caused by abnormal glycosylation of α-DG, for example, Fukuyama-type congenital muscular dystrophy (FCMD), CMD type 1C (MDC1C), limb-girdle muscular dystrophy 2I (LGMD2I), and CMD type 1D (MDC1D), since highly glycosylated α-DG was also found to be selectively deficient in the skeletal muscle of these patients. These gene products are thus thought to have glycosyltransferase activity or be involved in glycan stability.

FCMD is the second most common form of muscular dystrophy in Japan, and characterized by central nervous system involvement. Severe mental retardation and epilepsy are characteristic clinical features of FCMD, with brain showing polymicrogyria/pachygyria caused by altered neuronal migration. Kobayashi *et al.* (1998) identified the gene responsible for FCMD, which

encodes a protein named fukutin. Sequence analysis of fukutin predicts it to be an enzyme that could modify glycoconjugates. In addition, mutations in a homologue of fukutin, the fukutin-related protein (*FKRP*), were found in MDC1C patients (Brockington *et al.*, 2001a). MDC1C is characterized by a rapidly progressive muscle disease leading to a complete loss of muscle function and lethal respiratory insufficiency during the second decade (Quijano-Roy *et al.*, 2002). Mental retardation and cerebellar cysts have been observed in some patients. In contrast, allelic mutations in the *FKRP* gene cause a milder and more common form of myopathy, named LGMD2I, with a variable onset ranging from adolescence to adulthood (Brockington *et al.*, 2001b). Patients with *FKRP* mutations have reduced expression of glycosylated α-DG, broadly correlating with disease severity (Brown *et al.*, 2004). Finally, the gene *LARGE* encodes a putative glycosyltransferase (Grewal *et al.*, 2001). However, its biochemical activity has not yet been confirmed. Mutations in the *LARGE* gene cause MDC1D, a novel form of CMD also with a variable degree of mental retardation and brain abnormalities (Longman *et al.*, 2003; van Reeuwijk *et al.*, 2007).

Since multiple genes are known to cause α-dystroglycanopathies, with an extremely broad clinical spectrum and relatively poor phenotype–genotype correlation (Mercuri *et al.*, 2009), at present molecular diagnosis of α-dystroglycanopathy patients is difficult and often requires the analysis of several genes, which is expensive and time consuming. At present, of the six known α-dystroglycanopathy genes, the functions of the protein products are clear only for POMT1, POMT2, and POMGnT1. To assess the pathogenicity of several mutations, we demonstrated by a specific enzymatic assay that mutations in *POMGNT1* and *POMT1* lead to defects in respective enzymatic activities using mutant constructs transfected into cell lines (Akasaka-Manya *et al.*, 2004; Manya *et al.*, 2003). Another group reported POMGnT1 enzymatic assay in lymphoblasts and muscle biopsies (Vajsar *et al.*, 2006; Zhang *et al.*, 2003). Recent established mouse models for α-dystroglycanopathy will help our understanding between glycosylation and pathophysiology of these diseases (Kanagawa *et al.*, 2009; Liu *et al.*, 2006; Miyagoe-Suzuki *et al.*, 2009).

This chapter describes the assay protocols to diagnose patients with α-dystroglycanopathy by measuring glycosyltransferase activity in lymphoblast microsomal preparations (Manya *et al.*, 2008).

2. Methods

2.1. Cell Culture and Preparation of Microsomal Membrane Fraction

Blood from five healthy subjects and seven patients with CMD, with mental retardation, or hypoglycosylation of α-DG, or both, was collected for B lymphoblasts immortalization and DNA extraction after informed consent

Table 19.1 Summary of patients examined in the present study

Patient	Clinical diagnosis	Molecular diagnosis	
1	MEB	*POMGNT1*	IVS17+1G>A homozygous
2	MEB	*POMGNT1*	p.Arg442His homozygous
3	CMD-MR	*POMT1*	p.Gly65Arg + Trp582Cys heterozygous
4	LGMD-MR	*POMT1*	p.Ala200Pro homozygous
5	MEB	Uncharacterized	
6	CMD-MR	Uncharacterized	
7	MEB	Uncharacterized	

CMD, congenital muscular dystrophy; LGMD, limb-girdle muscular dystrophy; MR, mental retardation.

from the parents. Four patients had already been genetically characterized (patients 1 and 2 for *POMGNT1* and patients 3 and 4 for *POMT1*; Table 19.1). Three other patients (patients 5–7) were genetically uncharacterized.

B lymphoblasts were obtained after immortalization by Epstein–Barr virus and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) according to standard protocols to obtain 100 $\times$ 10^6 cells. After centrifugation at 800$\times g$ for 5 min, the pellets were rinsed twice with 50 ml then with 12 ml of PBS buffer. The final pellets were frozen at –80 °C. The cells ($\sim$7.5 $\times$ 10^6 cells) were homogenized in 10 m*M* Tris–HCl, pH 7.4, 1 m*M* EDTA, 250 m*M* sucrose, 1 m*M* dithiothreitol, with a protease inhibitor cocktail (3 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 m*M* benzamidine–HCl, and 1 m*M* PMSF). After centrifugation at 900$\times g$ for 10 min, the supernatant was subjected to ultracentrifugation at 100,000$\times g$ for 1 h. The precipitate was used as the microsomal membrane fraction (enzyme source). Protein concentration was determined by BCA assay (PIERCE, Rockford, IL). About 40 μg proteins of microsomal membranes were obtained from 1 $\times$ 10^6 cells.

2.2. Assay for glycosyltransferase activity

Since GnT1 (UDP-GlcNAc: α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase 1, EC 2.4.1.101) is not involved in *O*-mannosylglycan biosynthesis, it is not affected in α-dystroglycanopathies and represents a suitable control to normalize samples for baseline microsomal activity.

2.3. GnT1 activity

The GnT1 activity was performed in a total volume of 20 μl reaction mixture containing 100 m*M* MES buffer, 10 μ*M* pyridylaminated $Man_5GlcNAc_2$ (M5-PA, Takara Bio, Inc., Otsu, Japan), 2 m*M* UDP-GlcNAc, 5 m*M*

AMP, 0.5% Triton X-100, 0.2% BSA, 20 m*M* $MnCl_2$, and enzyme source (100 μg of microsomal membrane fraction) at 37 °C for 2 h. The samples were then analyzed by reversed phase HPLC with a COSMOSIL 5C18-AR-II column (4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan). The solvent used was a 100 m*M*, pH 6.0, ammonium acetate buffer containing 0.15% 1-butanol, and the substrate and the product were isocratically separated. Fluorescence was detected with a fluorescence detector (RF-10AXL, Shimadzu Corp., Kyoto, Japan) at excitation and emission wavelengths of 320 and 400 nm, respectively. The GnT1 activity mean (±standard deviation) of all samples was 0.53 (±0.06) nmol/h/mg total proteins with high constancy.

2.4. POMGnT1 activity

The POMGnT1 activity was based on the amount of [^{3}H]GlcNAc transferred to a mannosylpeptide (Ac-Ala-Ala-Pro-Thr(Man)-Pro-Val-Ala-Ala-Pro-NH_2) as described in a previous chapter of this series (Endo and Manya, 2006). The mannosylpeptide is not commercially available but it is possible to use Benzyl-Man, which is commercially available, as a substitute as described previously (Endo and Manya, 2006). Therefore, the procedures are described briefly here. The reaction buffer containing 140 m*M* MES buffer (pH 7.0), 1 m*M* UDP-[^{3}H]GlcNAc (225,000 dpm/nmol) (PerkinElmer, Inc., Waltham, MA), 1 m*M* mannosyl nanopeptide, 10 m*M* $MnCl_2$, 2% Triton X-100, 5 m*M* AMP, 200 m*M* GlcNAc, 10% glycerol, and enzyme source (100 μg of microsomal membrane fraction) in 20 μl total volume was incubated at 37 °C for 4 h. After boiling for 3 min, the mixture was analyzed by reversed phase HPLC with a Wakopak 5C18-200 column (4.6 × 250 mm, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Solvent A was 0.1% trifluoroacetic acid in distilled water and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The peptide was eluted at a flow rate of 1 ml/min using a linear gradient of 1–25% solvent B. The peptide separation was monitored continuously at 214 nm, and the radioactivity of each fraction was measured using a liquid scintillation counter. The average POMGnT1 activity measured in lymphoblasts of control patients was 0.163 (±0.042) nmol/h/mg total proteins.

2.5. POMT activity

The POMT activity was based on the amount of [^{3}H]-mannose transferred to a glutathione-*S*-transferase fusion α-DG (GST-αDG) as described also in a previous chapter of this series (Endo and Manya, 2006). Therefore, the procedures are described briefly here. The reaction mixture contained 20 m*M* Tris–HCl (pH 8.0), 100 n*M* of [^{3}H]-mannosylphosphoryldolichol (Dol-P-Man, 125,000 dpm/pmol) (American Radiolabeled Chemical, Inc., St. Louis, MO), 2 m*M* 2-mercaptoethanol, 10 m*M* EDTA, 0.5% *n*-octyl-β-D-thioglucoside (Dojindo Laboratories, Kumamoto, Japan), 10 μg

GST-α-DG, and enzyme source (80 μg of microsomal membrane fraction) in 20 μl total volume. After 1 h incubation at 22 °C, the reaction was stopped by adding 150 μl PBS containing 1% Triton X-100, and the reaction mixture was centrifuged at 10,000×*g* for 10 min. The supernatant was removed, mixed with 400 μl of PBS containing 1% Triton X-100 and 10 μl of Glutathione Sepharose 4B beads (GE Healthcare Bio-Sciences Corp., NJ), rotated at 4 °C for 1 h, and washed three times with 20 m*M* Tris–HCl (pH 7.4) containing 0.5% Triton X-100. The radioactivity adsorbed to the beads was measured using a liquid scintillation counter. The average POMT activity in lymphoblasts of control subjects was 0.041 (±0.013) pmol/h/mg proteins.

2.6. Mutation analysis

Genomic DNA was extracted from lymphoblasts using standard methods. Primer pairs were designed to amplify all coding exons and flanking intronic sequences of *POMT1* (9q34.1), *POMT2* (14q24), and *POMGNT1* (1p34.1). The primer sequences and PCR conditions are available upon request. The generated amplicons were purified and directly sequenced with the BigDye terminator kit (PerkinElmer Applied Biosystems, Wellesley, MA). Sequences were analyzed on an ABI PRISM 31130 capillary sequencer (Applera, CA). For patient 7, to find the second mutation, total RNA extracts from lymphoblasts were reversed transcribed and *POMT2* cDNA was amplified by nested PCR as previously reported (Yanagisawa *et al.*, 2009).

3. Procedures for Enzymatic Activity and Mutation Search

When we assessed the POMGnT1 activity in lymphoblasts from patients 1 and 2, enzymatic activity in these lymphoblasts was much lower than in the control subjects (Fig. 19.1). Those had previously been genetically confirmed with mutations in the *POMGNT1* gene (Table 19.1). Patient 1 carried the mutation c.1539+1 G>A in the homozygous state, and patient 2 harbored the mutation p.Arg442His, also in homozygous state.

When we assessed POMT activity in lymphoblasts from the patients who were been previously genetically confirmed with mutations in the *POMT1* gene (Table 19.1). Patient 3 was a compound heterozygous carrier of two missense mutations, p.Gly65Arg and p.Trp582Cys (van Reeuwijk *et al.*, 2006). Patient 4 was homozygous for the missense mutation p.Ala200Pro (Balci *et al.*, 2005). The enzyme activity in these patient lymphoblasts was extremely low.

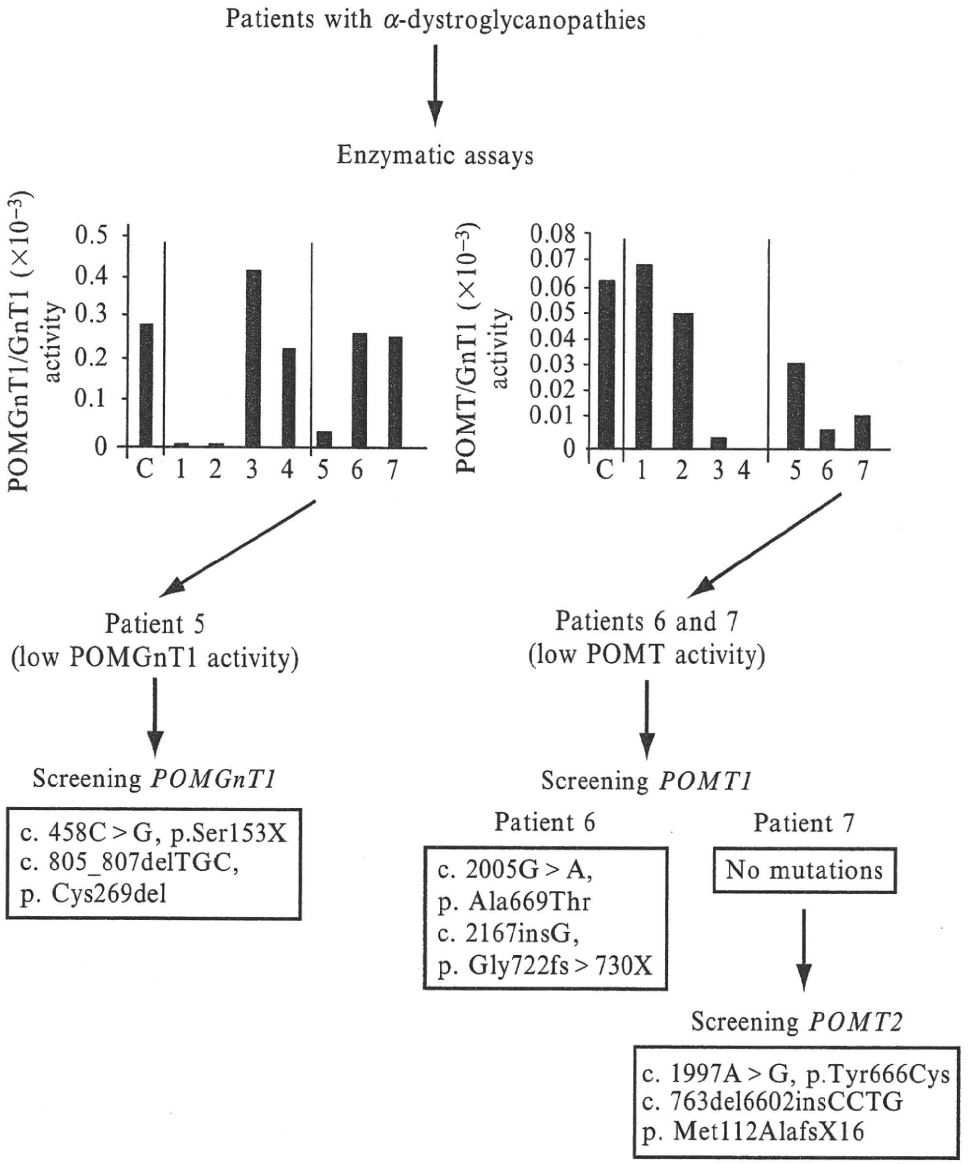

Figure 19.1 Schematic illustration of procedures for enzymatic activity and mutation search. Enzymatic activities in lymphoblasts from uncharacterized patients with α-dystroglycanopathies were measured. If a patient showed low enzymatic activity, the potential responsible gene was screened. Patient 5 showed low POMGnT1 activity, and *POMGNT1* was thus screened. Patients 6 and 7 showed low POMT activity, and thus patients 6 and 7 were screened for *POMT1* at first. However, since no mutations were found in the *POMT1* gene of patient 7, then the *POMT2* gene was further studied.

Among the uncharacterized patients, patient 5 showed low POMGnT1 activity and was thus secondarily screened for *POMGNT1*. The DNA study of this patient revealed two heterozygous mutations: a nonsense mutation, p.Ser153X (c.458C>G), and a deletion of three nucleotides c.805-807delTGC, which is expected to delete one amino acid, cysteine at position 269 (p.Cys269del), localized in the stem domain of the protein (Leu59-Leu300) (Manya *et al.*, 2008).

When we assessed POMT activity in the uncharacterized patients, we observed a markedly reduced activity in patient 6 and patient 7 (Fig. 19.1). Then patient 6 and patient 7 were secondarily screened for *POMT1* at first. We found two heterozygous mutations, in *POMT1* for patient 6: p.Ala669Thr (c.2005G>A), associated with c.2167insG which leads to a premature stop codon in amino acid 730 (Manya *et al.*, 2008). However, no mutation was found in the *POMT1* gene of patient 7. Then we screened *POMT2* for mutations and finally found two heterozygous mutations: a missense mutation, p.Tyr666Cys, and a large deletion 763del6602insCCTG leading to a premature stop codon (Yanagisawa *et al.*, 2009).

In conclusion, the lymphoblast-based enzymatic assay is an accurate and extremely useful method to select the patients harboring *POMT1*, *POMT2*, and *POMGNT1* mutations among those with suspected α-dystroglycanopathies. In other words, the enzymatic assay can be used as a first screening tool for narrowing the responsible gene in α-dystroglycanopathies. Interestingly, the same POMT assay was successfully used in skin fibroblasts from patients (Lommel *et al.*, 2010). The combinatory study of enzyme activity and gene mutation screening will help surveying patients with α-dystroglycanopathies and better understanding the clinical spectrum of theses pathologies.

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REFERENCES

Akasaka-Manya, K., Manya, H., and Endo, T. (2004). Mutations of the POMT1 gene found in patients with Walker–Warburg syndrome lead to a defect of protein O-mannosylation. *Biochem. Biophys. Res. Commun.* **325,** 75–79.

Akasaka-Manya, K., Manya, H., Nakajima, A., Kawakita, M., and Endo, T. (2006). Physical and functional association of human protein O-mannosyltransferases 1 and 2. *J. Biol. Chem.* **281,** 19339–19345.

Balci, B., Uyanik, G., Dincer, P., Gross, C., Willer, T., Talim, B., Haliloglu, G., Kale, G., Hehr, U., Winkler, J., and Topaloglu, H. (2005). An autosomal recessive limb girdle muscular dystrophy (LGMD2) with mild mental retardation is allelic to Walker–Warburg syndrome (WWS) caused by a mutation in the *POMT1* gene. *Neuromuscul. Disord.* **15,** 271–275.

Beltran-Valero De Bernabe, D., Currier, S., Steinbrecher, A., Celli, J., Van Beusekom, E., Van Der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W. B., Cormand, B., Lehesjoki, A. E., *et al.* (2002). Mutations in the O-mannosyltransferase gene *POMT1* give rise to the severe neuronal migration disorder Walker–Warburg syndrome. *Am. J. Hum. Genet.* **71,** 1033–1043.

Biancheri, R., Bertini, E., Falace, A., Pedemonte, M., Rossi, A., D'Amico, A., Scapolan, S., Bergamino, L., Petrini, S., Cassandrini, D., Broda, P., Manfredi, M., *et al.* (2006). POMGnT1 mutations in congenital muscular dystrophy: Genotype–phenotype correlation and expanded clinical spectrum. *Arch. Neurol.* **63,** 1491–1495.

Brockington, M., Blake, D. J., Prandini, P., Brown, S. C., Torelli, S., Benson, M. A., Ponting, C. P., Estournet, B., Romero, N. B., Mercuri, E., Voit, T., Sewry, C. A., *et al.* (2001a). Mutations in the fukutin-related protein gene (*FKRP*) cause a form of congenital muscular dystrophy with secondary laminin α2 deficiency and abnormal glycosylation of α-dystroglycan. *Am. J. Hum. Genet.* **69,** 1198–1209.

Brockington, M., Yuva, Y., Prandini, P., Brown, S. C., Torelli, S., Benson, M. A., Herrmann, R., Anderson, L. V., Bashir, R., Burgunder, J. M., Fallet, S., Romero, N., *et al.* (2001b). Mutations in the fukutin-related protein gene (*FKRP*) identify limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C. *Hum. Mol. Genet.* **10,** 2851–2859.

Brown, S. C., Torelli, S., Brockington, M., Yuva, Y., Jimenez, C., Feng, L., Anderson, L., Ugo, I., Kroger, S., Bushby, K., Voit, T., Sewry, C., *et al.* (2004). Abnormalities in α-dystroglycan expression in MDC1C and LGMD2I muscular dystrophies. *Am. J. Pathol.* **164,** 727–737.

D'Amico, A., Tessa, A., Bruno, C., Petrini, S., Biancheri, R., Pane, M., Pedemonte, M., Ricci, E., Falace, A., Rossi, A., Mercuri, E., Santorelli, F. M., *et al.* (2006). Expanding the clinical spectrum of POMT1 phenotype. *Neurology* **66,** 1564–1567.

Endo, T., and Manya, H. (2006). Defect in glycosylation that causes muscular dystrophy. *Methods Enzymol.* **417,** 137–152.

Grewal, P. K., Holzfeind, P. J., Bittner, R. E., and Hewitt, J. E. (2001). Mutant glycosyltransferase and altered glycosylation of α-dystroglycan in the myodystrophy mouse. *Nat. Genet.* **28,** 151–154.

Kanagawa, M., Nishimoto, A., Chiyonobu, T., Takeda, S., Miyagoe-Suzuki, Y., Wang, F., Fujikake, N., Taniguchi, M., Lu, Z., Tachikawa, M., Nagai, Y., Tashiro, F., *et al.* (2009). Residual laminin-binding activity and enhanced dystroglycan glycosylation by LARGE in novel model mice to dystroglycanopathy. *Hum. Mol. Genet.* **18,** 621–631.

Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M., Hamano, K., Sakakihara, Y., *et al.* (1998). An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. *Nature* **394,** 388–392.

Liu, J., Ball, S. L., Yang, Y., Mei, P., Zhang, L., Shi, H., Kaminski, H. J., Lemmon, V. P., and Hu, H. (2006). A genetic model for muscle-eye-brain disease in mice lacking protein *O*-mannose 1,2-*N*-acetylglucosaminyltransferase (POMGnT1). *Mech. Dev.* **123,** 228–240.

Lommel, M., Cirak, S., Willer, T., Hermann, R., Uyanik, G., van Bokhoven, H., Korner, C., Voit, T., Baric, I., Hehr, U., and Strahl, S. (2010). Correlation of enzyme activity and clinical phenotype in POMT1-associated dystroglycanopathies. *Neurology* **74,** 157–164.

Longman, C., Brockington, M., Torelli, S., Jimenez-Mallebrera, C., Kennedy, C., Khalil, N., Feng, L., Saran, R. K., Voit, T., Merlini, L., Sewry, C. A., Brown, S. C., *et al.* (2003). Mutations in the human *LARGE* gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of α-dystroglycan. *Hum. Mol. Genet.* **12,** 2853–2861.

Manya, H., Sakai, K., Kobayashi, K., Taniguchi, K., Kawakita, M., Toda, T., and Endo, T. (2003). Loss-of-function of an *N*-acetylglucosaminyltransferase, POMGnT1, in muscle-eye-brain disease. *Biochem. Biophys. Res. Commun.* **306,** 93–97.

Manya, H., Chiba, A., Yoshida, A., Wang, X., Chiba, Y., Jigami, Y., Margolis, R. U., and Endo, T. (2004). Demonstration of mammalian protein *O*-mannosyltransferase activity:

Coexpression of POMT1 and POMT2 required for enzymatic activity. *Proc. Natl. Acad. Sci. USA* **101,** 500–505.

Manya, H., Bouchet, C., Yanagisawa, A., Vuillaumier-Barrot, S., Quijano-Roy, S., Suzuki, Y., Maugenre, S., Richard, P., Inazu, T., Merlini, L., Romero, N., Leturcq, F., *et al.* (2008). Protein *O*-mannosyltransferase activities in lymphoblasts from patients with α-dystroglycanopathies. *Neuromuscul. Disord.* **18,** 45–51.

Mercuri, E., Messina, S., Bruno, C., Mora, M., Pegoraro, E., Comi, G. P., D'Amico, A., Aiello, C., Biancheri, R., Berardinelli, A., Boffi, P., Cassandrini, D., *et al.* (2009). Congenital muscular dystrophies with defective glycosylation of dystroglycan: A population study. *Neurology* **72,** 1802–1809.

Michele, D. E., and Campbell, K. P. (2003). Dystrophin-glycoprotein complex: Post-translational processing and dystroglycan function. *J. Biol. Chem.* **278,** 15457–15460.

Miyagoe-Suzuki, Y., Masubuchi, N., Miyamoto, K., Wada, M. R., Yuasa, S., Saito, F., Matsumura, K., Kanesaki, H., Kudo, A., Manya, H., Endo, T., and Takeda, S. (2009). Reduced proliferative activity of primary POMGnT1-null myoblasts *in vitro*. *Mech. Dev.* **126,** 107–116.

Muntoni, F., Torelli, S., and Brockington, M. (2008). Muscular dystrophies due to glycosylation defects. *Neurotherapeutics* **5,** 627–632.

Quijano-Roy, S., Galan, L., Ferreiro, A., Cheliout-Heraut, F., Gray, F., Fardeau, M., Barois, A., Guicheney, P., Romero, N. B., and Estournet, B. (2002). Severe progressive form of congenital muscular dystrophy with calf pseudohypertrophy, macroglossia and respiratory insufficiency. *Neuromuscul. Disord.* **12,** 466–475.

Vajsar, J., Zhang, W., Dobyns, W. B., Biggar, D., Holden, K. R., Hawkins, C., Ray, P., Olney, A. H., Burson, C. M., Srivastava, A. K., and Schachter, H. (2006). Carriers and patients with muscle-eye-brain disease can be rapidly diagnosed by enzymatic analysis of fibroblasts and lymphoblasts. *Neuromuscul. Disord.* **16,** 132–136.

van Reeuwijk, J., Janssen, M., van den Elzen, C., Beltran-Valero de Bernabe, D., Sabatelli, P., Merlini, L., Boon, M., Scheffer, H., Brockington, M., Muntoni, F., Huynen, M. A., Verrips, A., *et al.* (2005). *POMT2* mutations cause α-dystroglycan hypoglycosylation and Walker–Warburg syndrome. *J. Med. Genet.* **42,** 907–912.

van Reeuwijk, J., Maugenre, S., van den Elzen, C., Verrips, A., Bertini, E., Muntoni, F., Merlini, L., Scheffer, H., Brunner, H. G., Guicheney, P., and van Bokhoven, H. (2006). The expanding phenotype of *POMT1* mutations: From Walker–Warburg syndrome to congenital muscular dystrophy, microcephaly, and mental retardation. *Hum. Mutat.* **27,** 453–459.

van Reeuwijk, J., Grewal, P. K., Salih, M. A., Beltran-Valero de Bernabe, D., McLaughlan, J. M., Michielse, C. B., Herrmann, R., Hewitt, J. E., Steinbrecher, A., Seidahmed, M. Z., Shaheed, M. M., Abomelha, A., *et al.* (2007). Intragenic deletion in the *LARGE* gene causes Walker–Warburg syndrome. *Hum. Genet.* **121,** 685–690.

Yanagisawa, A., Bouchet, C., Quijano-Roy, S., Vuillaumier-Barrot, S., Clarke, N., Odent, S., Rodriguez, D., Romero, N. B., Osawa, M., Endo, T., Taratuto, A. L., Seta, N., *et al.* (2009). *POMT2* intragenic deletions and splicing abnormalities causing congenital muscular dystrophy with mental retardation. *Eur. J. Med. Genet.* **52,** 201–206.

Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., *et al.* (2001). Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. *Dev. Cell.* **1,** 717–724.

Zhang, W., Vajsar, J., Cao, P., Breningstall, G., Diesen, C., Dobyns, W., Herrmann, R., Lehesjoki, A. E., Steinbrecher, A., Talim, B., Toda, T., Topaloglu, H., *et al.* (2003). Enzymatic diagnostic test for muscle-eye-brain type congenital muscular dystrophy using commercially available reagents. *Clin. Biochem.* **36,** 339–344.

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Chapter 8

DYSTROGLYCAN AND NEUROMUSCULAR DISEASES: ITS DIVERGING ROLE IN MUSCLE, NERVE AND BRAIN

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ABSTRACT

Dystroglycan is a central component of the dystrophin-glycoprotein complex that links extracellular matrix with cytoskeleton. The dystroglycan complex is composed of two proteins, α- and β-dystroglycan (α-and β-DG). α-DG is a highly glycosylated extracellular peripheral membrane protein and binds to several extracellular matrix proteins. Whereas, the transmembrane protein β-DG anchors α-DG at the extracellular surface of the plasma membrane and intracellularly binds to F-actin via dystrophin. The past decade of researches have revealed that the growing number of muscular dystrophies are linked to the disruption of this molecular axis. Among them, a novel pathogenic mechanism has been identified recently, in which mutations of glycosyltransferases result in defective glycosylation of α-DG, leading to the disruption of its linkage to the extracellular matrix. In addition, progress has been made in our understanding of the functional roles of DG outside the skeletal muscle. In brain, it has been demonstrated that dysfunction of α-DG result in migration defect of neuron and cause type II lissencephaly. In peripheral nerve, the DG linkage has been shown to be crucial to promote myelin formation, Na^+ channel clustering at node of Ranvier and formation of neuromuscular junction. Moreover, hereditary demyelinating polyneuropathy has been identified that is caused by mutation of L-periaxin and involves the intracellular linkage of DG. In this review, we focus on the diverging roles of DG and the molecular pathomechanism of neuromuscular diseases caused by defective DG function.

INTRODUCTION

The dystrophin-glycoprotein complex (DGC) is a multimeric protein complex located at the sarcolemma of muscle fibers (Figure 1) [1]. The DGC is first isolated and characterized in skeletal muscle and consists of dystroglycan (DG) complex, sarcoglycan-sarcospan complex, syntrophin, dystrobrevin and dystrophin [1]. The syntrophin interact with nNOS and mediate its sarcolemmal localization [2]. Recently, it was demonstrated that dystrophin binds ankyrin-B and ankyrin-G through cysteine-rich domain, while β-DG binds ankyrin-G through 10 amino acids located in the cytoplasmic domain adjacent to the membrane-spanning region. The interaction with ankyrin is necessary for dystrophin or DG to localize at costamere or neuromuscular junction [3]. The integrity of this complex is crucial for normal function and viability of muscle cells and mutation in some of its components leads to muscular dystrophy. For example, mutation in dystrophin causes Duchenne/Becker muscular dystrophy (DMD / BMD) and mutation in sarcoglycan lead to limb-girdle muscular dystrophy (LGMD) [4]. In addition, a deficiency of laminin 2, which is an extracellular matrix (ECM) ligand of DG, causes congenital muscular dystrophy 1A (MDC 1A) [5]. Consistent with the fact that constitutive disruption of DG gene results in embryonic lethality in knockout mouse experiment [6], primary mutations of DG has not been reported so far in any human or animal diseases. However emerging genetic and biochemical data have demonstrated that defective glycosylation of DG caused by mutations of known or putative glycosyltransferases leads to muscular dystrophy, brain malformation and ocular abnormalities. Moreover, recent studies have shown that loss of DG function result in defects in myelination of peripheral nerve, formation of neuromuscular junction and clustering of Na^+ channel at node of Ranvier. In this review, we will summarize these diverging roles of DG and its implication in the mechanism of neuromuscular diseases.

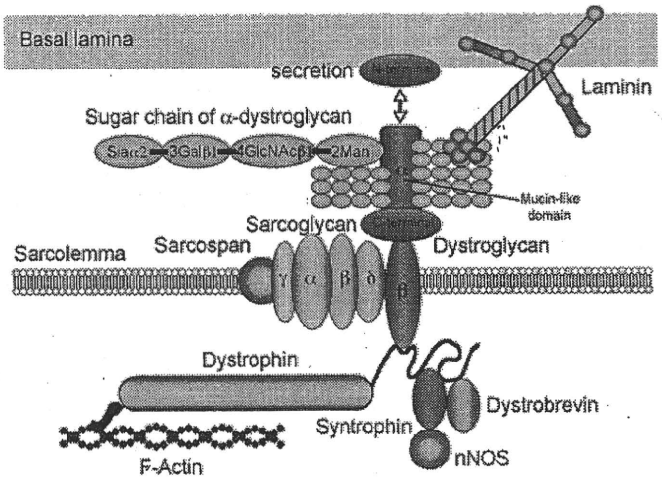

Figure 1. Dystrophin-glycoprotein complex in skeletal muscle.
Peripheral membrane protein α-dystroglycan (α-DG) binds to laminin in basal lamina. Sugar chain structure of α-DG, Siaα2-3Galβ1-4GlcNAcβ1-2Man-Ser/Thr, plays a crucial role in the binding. Transmembrane protein β-dystroglycan (β-DG) binds to α-DG and anchors it at the extracellular surface of plasma membrane. The cytoplasmic domain of β-DG interacts with dystrophin, a large cytoplasmic protein that binds to F-actin. In addition, sarcoglycan and sarcospan bind to DG, whereas syntrophin and dystrobrevin bind to dystrophin.

DOMAIN STRUCTURE OF DYSTROGLYCAN AND ITS FUNCTION

Dystroglycan is encoded by a single gene, *Dag1*, located on human chromosome 3p21 and cleaved into two proteins, α- and β-DG, by post-translational processing [7]. The cleavage occurs within the SEA (sea urchin, enterokinase, agrin) module by autoproteolysis and this cleavage modulates the function of α-DG [8]. The α-DG is a highly glycosylated extracellular peripheral membrane protein with apparent molecular weight of 156kD in skeletal muscle and binds to several ECM proteins including laminin, agrin, perlecan and synaptic proteins such as neurexin, pikachurin via their laminin G (LG) like domain [7, 9, 10, 11, 12, 13]. Small leucine-rich repeat chondroitin sulfate proteoglycan, biglycan, which does not possess the LG like domain, also binds to α-DG [14], but its binding nature is distinct from those of the ECM or synaptic proteins with LG like domains. Transmembrane protein β-DG, with apparent molecular weight of 43kD, non-covalently binds to α-DG and anchors it at the extracellular surface of plasma membrane. The cytoplasmic domain of β-DG interacts with dystrophin, a large cytoplasmic protein that binds to F-actin (Figure 1) [15]. DG plays a central role in the DGC to stabilize the plasma membrane by acting as an axis that tightly links the ECM to cytoskeleton. DG is widely expressed also in non-muscle tissues including brain, peripheral nerve, epithelial cells, endothelial cells, as well as tumor cells. The interaction of DG with ECM proteins is essential for the basement membrane assembly and morphogenesis of these tissues and cell types [16, 17].

α-DG is comprised of distinct three domains, *i.e.* N-terminal, mucin-like and C-terminal domain. The N-terminal and C-terminal domain have a globular shape and the N-terminal domain is cleaved by proprotein convertase, furin, and secreted outside cells (Figure 1) [18, 19, 20]. Putative glycosyltransferase Large binds to the N-terminal domain and this enzyme-substrate interaction is necessary to produce mature and functional α-DG [18]. α-DG is anchored by β-DG through its amino acids 550-585 within the C-terminal domain [21]. The mucin-like domain is highly glycosylated by O-linked oligosaccharides and, indeed, sugar chain moiety constitutes up to two third of the molecule [7]. Chiba *et al.* reported that O-mannosyl glycan, Siaα2-3Galβ1-4GlcNAcβ1-2Man-Ser/Thr, is attached to α-DG as a major sialylated O-linked oligosaccharide structure and this unique glycan is essential for the binding with its ligands (Figure 1) [22]. In vertebrates, the similar O-mannosyl glycan has been reported only in rat brain proteoglycans other than DG [23]. Recently, it was demonstrated that Thr-414 and Thr-351 within the mucin-like domain are prominently modified by this O-mannosyl glycan [24].

DG not only mechanically stabilizes the sarcolemma against contraction-stretch stress but also plays a role in signals transduction. β-DG cytoplasmic domain binds via its C-terminal proline rich region to Grb2, an adaptor protein involved in signal transduction and cytoskeletal organization [25]. Furthermore, *in vitro* experiments revealed that a tyrosine residue in the PPXY motif of the C-tarminus of β-DG is phosphorylated in an adhesion dependent manner and this tyrosine phosphorylation abolishes the binding of dystrophin and its homologue, utrophin, to the PPXY motif [26, 27]. This tyrosine phosphorylation at the C-terminus of β-DG, in turn, recruits SH2 domain containing signaling proteins including c-Src, Fyn, Csk, NCK and SHC [28], raising an intriguing possibility that the tyrosine phosphorylation acts as a switch by which the cytoplasmic linkage of dystroglycan is regulated. It was also shown that disruption of dystroglycan-laminin linkage induces

apoptosis of cultured myotubes due to impaired signaling through phospohoinositide 3-kinase/protein kinase B pathway [29]. β-DG is localized to microvilli structures in a number of cell types, where it associates with the cytoskeletal adaptor ezrin. Through the interaction, DG is able to modulate the actin cytoskeleton and induce peripheral filopodia and microvilli [30]. Recently, it has been reported that the binding of laminin to α-DG causes signaling through DG-syntrophin-Grb2-SOS1-Rac1-PAK1-JNK, which is initiated by Src family kinases and also causes syntrophin tyrosine phosphorylation to initiate signaling [31].

The extracellular domain of β-DG appears to be unfolded [32] and importantly, β-DG is proteolytically cleaved by matrix matalloproteinase activity at this segment [33]. MMP-2 and MMP-9 are supposed to be involved in this process [34]. This proteolytic cleavage gives rise to 30kD fragment of β-DG and this proteolytic fragment was increased in the muscle of DMD patients, sarcoglycan deficient LGMD patients and its model animals [35, 36]. Therefore, it is proposed that the proteolysis of the extracellular domain of β-DG may contribute to skeletal muscle degeneration by disrupting the link between the ECM and cell membrane in these muscular dystrophies. Interestingly, proteolytic cleavage of β-DG by MMP was also observed in brain. β-DG and MMP-9 colocalize in postsynaptic elements in the hippocampus and β-DG is digested by MMP-9 in response to enhanced synaptic activity [37].

Muscular Dystrophy and Defective Glycosylation of α-DG

As mentioned above, recent studies have shown that the dysfunction of α-DG caused by mutations of several known or putative glycosyltransferases underlies the pathogenesis of muscular dystrophies. These disorders are collectively called α-dystroglycanopathy [38]. Congenital muscular dystrophy (CMD) is a heterogeneous group of severe muscular dystrophy and characterized by muscle weakness that start within several months after birth. Many cases of CMD are accompanied by brain anomaly, mental retardation and variable ocular abnormalities. Among the CMD, Fukuyama-type congenital muscular dystrophy (FCMD) is most common in Japanese population and retrotransposon insertion at 3' UTR or point mutation of fukutin gene was identified as causative gene mutations of this type of CMD [39]. Fukutin is predicted to be an enzyme that modifies cell surface glycoprotein or glycolipid, however actual enzyme activity has not been demonstrated yet. It was reported that the immunoreactivity of antibody against sugar chain of α-DG is severely reduced in skeletal muscle of FCMD patients, suggesting defective glycosylation of α-DG [40]. Recently, it was reported that fukutin forms a complex with protein O-mannose β-1, 2-N-acetylglucosaminyltransferase (POMGnT1), which is also involved in α-dystroglycanopathy (see below), thus fukutin may modulate the enzymatic activity of POMGnT1 [41]. A homologue of fukutin, fukutin-related protein (FKRP), was cloned and point mutations in the *FKRP* gene were identified in patients with congenital muscular dystrophy 1C (MDC1C) [42]. MDC1C is a severe form of muscular dystrophy with muscle hypertrophy and marked elevation of serum creatine kinase but lacking brain or ocular anomalies. Interestingly, milder form of limb-girdle muscular dystrophy LGMD 2I, of which onset is late childhood to adult life, is an allelic disorder to MDC1C [43]. These disorders are prevalent in Caucasian populations. Although enzymatic activity of FKRP as a glycosyltransferase has not yet been