Muscular Dystrophy with Ribitol-Phosphate Deficiency: A Novel Post-Translational Mechanism in Dystroglycanopathy

Motoi Kanagawa and Tatsushi Toda

Abstract

Muscular dystrophy is a group of genetic disorders characterized by progressive muscle weakness. In the early 2000s, a new classification of muscular dystrophy, dystroglycanopathy, was established. Dystroglycanopathy often associates with abnormalities in the central nervous system. Currently, at least eighteen genes have been identified that are responsible for dystroglycanopathy, and despite its genetic heterogeneity, its common biochemical feature is abnormal glycosylation of alpha-dystroglycan. Abnormal glycosylation of alpha-dystroglycan reduces its binding activities to ligand proteins, including laminins. In just the last few years, remarkable progress has been made in determining the sugar chain structures and gene functions associated with dystroglycanopathy. The normal sugar chain contains tandem structures of ribitol-phosphate, a pentose alcohol that was previously unknown in humans. The dystroglycanopathy genes fukutin, fukutin-related protein (FKRP), and isoprenoid synthase domain-containing protein (ISPD) encode essential enzymes for the synthesis of this structure: fukutin and FKRP transfer ribitol-phosphate onto sugar chains of alpha-dystroglycan, and ISPD synthesizes CDP-ribitol, a donor substrate for fukutin and FKRP. These findings resolved long-standing questions and established a disease subgroup that is ribitol-phosphate deficient, which describes a large population of dystroglycanopathy patients. Here, we review the history of dystroglycanopathy, the properties of the sugar chain structure of alpha-dystroglycan, dystroglycanopathy gene functions, and therapeutic strategies.

Keywords: Muscular dystrophy, dystroglycanopathy, dystroglycan, glycosylation, ribitol-phosphate, fukutin, fukutin-related protein, isoprenoid synthase domain containing protein

HISTORY OF GLYCOSYLATION-DEFICIENT MUSCULAR DYSTROPHIES

Muscular dystrophies are a heterogeneous group of genetic disorders characterized by the progressive loss of muscle strength and integrity, and are categorized into subgroups based on factors such as the responsible genes, inheritance patterns and clinical presentation. Currently, more than 40 genes have been identified whose mutations cause different types of muscular dystrophies (www.muscleenetabtable.fr). The most common type is Duchenne-type muscular dystrophy (DMD), and the gene responsible for DMD, dystrophin, was identified in 1987 [1]. Dystrophin encodes a large actin-binding cytoskeletal protein located underneath the muscle plasma membrane. After the discovery of dystrophin, several transmembrane glycoproteins that co-purified with dystrophin protein were identified [2]. These proteins form a large protein complex that is called the dystrophin-glycoprotein complex (DGC). The components of the DGC include sarcoglycans (SGs), sarcospan, and dystroglycan (DG) [3]. The major function of the DGC is connecting the basement membrane to dystrophin-actin cytoskeleton across the plasma membrane. In addition to dystrophin, several components of the DGC or basement membrane are associated with muscular dystrophies [4]. Mutations in SGs are associated with limb-girdle type muscular dystrophies (LGMDs), and mutations in the laminin alpha2 chain, a basement membrane protein that is a direct ligand for the DGC, are associated with congenital type muscular dystrophy 1A (MDC1A). Biochemical and pathophysiological studies have further established that the DGC serves as a physical link between the basement membrane and the cytoskeleton, which provides mechanical stability to the muscle plasma membrane.

DG, which serves as the central component of the DGC, is encoded by a single mRNA and is post-translationally cleaved into two subunits, α- and β-DG. α-DG is a highly glycosylated extracellular subunit and serves as a receptor protein for laminin in the basement membrane. Glycosylation is required for ligand binding activities of α-DG. β-DG is a transmembrane subunit and anchors α-DG on the plasma membrane and intracellularly binds to dystrophin. As mentioned, mutations in SGs or laminin alpha 2 were identified as causes of muscular dystrophies in the 1990s, whereas no mutation in the DG gene, DAG1, was identified until 2011 [5]. However, in 2001 Arahata’s group reported abnormal glycosylation of α-DG in patients suffering from Fukuyama type congenital muscular dystrophy (FCMD) [6]. Subsequently, Campbell’s group showed direct evidence for abnormal glycosylation of α-DG with a severe reduction in ligand binding activities in patients with muscle-eye-brain disease (MEB) and Walker-Warburg syndrome (WWWS) in addition to FCMD [7]. Abnormal glycosylation can be determined by immunostainings of patients’ biopsies with IIH6 antibody [8], which recognizes functionally glycosylated and ligand-binding forms of α-DG. Because the common biochemical feature of these diseases is abnormal glycosylation of α-DG, these conditions have been collectively called dystroglycanopathy (DGpathy) [9, 10]. By the early 2000s, six genes were known as causative genes for DGpathy (fukutin, POM121, POMT1, POMT2, fukutin-related protein, and LARGE) [11–17]. Now, eighteen genes are known to be associated with DGpathy, including the DG gene DAG1 itself. Currently, DGpathies can be classified as primary (caused by mutations in the DG gene itself), secondary (caused by mutations in genes encoding proteins that directly modify α-DG), or tertiary (caused by mutations in genes encoding proteins that indirectly modify α-DG but affect α-DG function). The clinical phenotype of DGpathy shows a wide spectrum. The most severe end of the spectrum is characterized by CMD with extensive structural abnormalities in the brain and eye, which typically results in early infantile death. Patients at the mildest end of the spectrum might present in adult life with LGMD without brain or eye involvement. New Online Mendelian

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5701763/
Inheritance in Man (OMIM) entries have created a simplified classification scheme for DGPathy (MDDG; muscular dystrophy dystroglycanopathy) by combining three broad phenotypic groups and gene defects [18]. In this classification scheme, DGPathy is divided into three groups: (A) CMD with brain/eye abnormalities, (B) CMD with milder brain structural abnormalities, and (C) LGMD. In addition, the causative gene is indicated numerically as shown in Table 1. According to this classification, for example, typical FCMD is “MDDG type A4.”

<table>
<thead>
<tr>
<th>α-DGgene Number</th>
<th>α-DG Gene</th>
<th>Function</th>
<th>DMDG Number</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>GalNAcβ1-4GlcNacβ1,2-Man-O</td>
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disaccharide straddles a Ca\(^{2+}\) ion in the LG4 domain of the laminin α2-chain [35]. This chelating binding mode explains the high affinity of this protein-carbohydrate interaction. Multiple GlcA-Xyl repeats are thought to increase the apparent affinity for the LG domains present in ligand proteins.

For the matrilycan modification, in addition to the LARGE enzyme activities, physical protein-protein interactions between LARGE and the α-DG N-terminal domain are required [31]. During the process of α-DG maturation, the N-terminal domain functions as a recognition target by LARGE, which may ensure the LARGE-dependent modification of GlcA-Xyl repeats. In 2011, a mutation in the N-terminal domain of α-DG was identified in a patient with LGMD with cognitive impairment; the mutation impairs the LARGE-DG interaction and consequently reduces matrilycan modification [5]. This was the first case in which a mutation in the DG gene (DAG1) itself was known to cause muscular dystrophy ("primary" DGpathy).

**Tandem ribitol-phosphate**

In 2012, mutations in the **ISPD** gene, which encodes isopenosyl synthase domain containing protein, were identified to be the second most common cause of WWS [36, 37]. Later, a growing number of DGpathy patients who carry mutations in **ISPD** were reported [38]. The ISPD protein belongs to the family of 4-diphosphocytidyl-2C-methyl-D-erythritol (CDP-ME) synthases, also known as 2-C-methyl-D-erythritol 4-phosphate (MEP) cytidylyltransferases. In bacteria, LspD functions as part of the MEP pathway of isopenosyl synthesis, but this pathway is not present in humans. Therefore, the functional role of human ISPD in α-DG glycosylation and DGpathy remained to be elucidated. In 2015, a crystal structural analysis revealed that human ISPD cytidylyltransferase domain superimposes well with a series of closely related prokaryotic cytidylyltransferases and ISPD homologs including bacterial TarI [39]. In fact, the data demonstrated that human ISPD possesses cytidylyltransferase activities toward pentose phosphates, suggesting that human ISPD may be a cytidylyltransferase that produces novel nucleotide sugars essential for functional glycosylation of α-DG; however, the relationship between this and the sugar chain structure was still missing. Shortly thereafter, three groups independently confirmed that human ISPD catalyzes CDP-ribitol synthesis as CDP-ribitol pyrophosphorylase [33, 40, 41]. Meanwhile, a Japanese group found a novel moiety that contains tandemly connected ribitol-phosphates (RboP) between matrilycan and CoreM3 by mass spectrometry [33]. Ribitol is a sugar alcohol that was not known to be used in humans. In bacteria, RboP is used as a component of the teichoic acids present in cell walls, and the RboP polymer is synthesized by enzymes that use CDP-Rbo as a donor substrate. In bacteria, CDP-Rbo is synthesized by the enzyme TarI from cytidine triphosphate (CTP) and RboP. These studies indicated that human ISPD provides CDP-Rbo, which is used to synthesize the tandem RboP structure in the sugar chain of α-DG.

The next question to answer was what gene products catalyze the synthesis of the tandem RboP structure. The fukutin family was predicted to encode a phosphoryl-ligand transferase [42, 43]. **Fukutin-related protein (FKRP)** was originally based on its sequence homology with fukutin [15]. Both fukutin and FKRP proteins contain the putative catalytic DXD motif, which is a conserved motif found in many families of glycosyltransferases. Therefore, both fukutin and FKRP were primary candidates for the tandem RboP biosynthesis. Indeed, the Japanese group revealed that fukutin transfers RboP from a CDP-Rbo to GalNAc in CoreM3, and then FKRP transfers RboP from a CDP-Rbo to the first RboP [33]. Thus, the tandem RboP structure was shown to be synthesized through the sequential enzymatic actions of fukutin and FKRP as RboP transferases. Several lines of evidence have also shown that there is a GlcAβ1-4Xyl unit, which is formed independently of LARGE, between matrilycan and the tandem RboP. This structure serves as a primer for the initiation of matrilycan formation, and is synthesized by B4GAT1, which exhibits [β1-glucuronoyl]transferase activity, and by TMEM5, which exhibits Xyl transferase activity to form a Xylβ1-4Rbo5P linkage [44-48]. It has been proposed that TMEM5 could be renamed RXYLT1 on the basis of its function (Rbo5P β1,4-Xyl transferase) [49, 50].

A recent review article refers to TMEM5 as a ribitol β1,2-xyllosyltransferase [49], which differs from the nomenclature in the original report by Manya et al (ribitol β1,4-xyllosyltransferase) [47]. This difference is based on whether CDP-ribitol is recognized by the researcher as an organic compound (R/S nomenclature) or a sugar derivative (D/L nomenclature). The IUPAC name of CDP-Rbo is [(2R,3S,4R,5R)-5-(4-amino-2-oxopirimidin-1-yl)-3,4-dihydroxyxolan-2-yl]methoxy-hydroxyporphoryli[(2R,3S,4S)-2,3,4,5-tetrahydroxypentyl] hydrogen phosphate. As for ribitol-phosphate, D-ribitol-5-phosphate is the preferred name as per IUPAC nomenclature for the sugar derivative. Based on the IUPAC recommendation for nomenclature of carbohydrates that includes alditols (D/L representation), CDP-ribitol is defined as CDP-D-ribitol in which the C5 position of ribitol is modified with phosphate. According to this nomenclature, the structure of tandem RboP with a Xyl modification is Xylβ1,4-Rbo5P-1Rbo5P. However, CDP-L-ribitol is also found to be named according to R/S nomenclature for CDP-ribitol. Using this nomenclature, the structure can be alternatively referred to as Xylβ1,2-Rbo1P-5Rbo1P and TMEM5 can be referred to as ribitol β1,2-xyllosyltransferase. In this review, we pointed out this inconsistency in nomenclature stemming from the mixed use of R/S and D/L nomenclature, and have used the nomenclatures that were originally reported in research articles.

**Dolichol-phosphate-Man synthesis pathway**

The other DGpathy genes involved in the dolichol-phosphate-Man (Dol-P-Man) synthesis pathway [51–55]. Dol-P-Man serves as a donor for Man residues, and the POMT1/POMT2 complex utilizes Dol-P-Man for transferring Man to α-DG core protein. Defects in the Dol-P-Man synthesis pathway result in reduced levels of Dol-P-Man production thus affecting O-mannosyl glycosylation of α-DG. Details of the Dol-P-Man pathway are...
documented elsewhere [56], and here we briefly review genes known to associate with DGiopathy. Dol-P-Man is synthesized from GDP-Man and Dol-P by the DPM synthase complex, which consists of the catalytic component DPM1 and the ER-localized transmembrane proteins DPM2 and DPM3. Dolichol kinase (DOLK) is responsible for the formation of Dol-P. GDP-mannose pyrophosphorylase B (GMPPPB) is the β-subunit of the essential enzyme GDP-mannose pyrophosphorylase, which catalyzes the formation of GDP-Man from Man-1-phosphate and GTP. Mutations in these genes [51–55] and in ISPD [36–38] indirectly affect α-DG function, and thus the diseases caused by these gene mutations are categorized as tertiary DGiopathies. It is notable that in addition to O-mannosylation, Dol-P-Man acts as a Man donor in N-glycosylation and in glycosphatidylinositol-anchor biosynthesis. Therefore, defects in the Dol-P-Man pathway can be associated with human diseases other than DGiopathy. Congenital disorder of glycosylation (CDG) was originally defined as diseases caused by defects in the N-glycosylation process, but now includes O-linked and lipid glycosylation defects. Several cases of combined deficiency of protein N-glycosylation and α-DG O-mannosyl glycosylation in CDG patients with α-DGiopathy have been reported [51, 53, 54].

**MUSCULAR DYSTROPHY WITH RIBITOL-PHOSPHATE DEFECTS**

Recent studies have established that RboP modification is indispensable for functional maturation of α-DG, and have determined the enzyme activities of ISPD, fukutin, and FKR gene that are required for this modification. Fukutin-deficient FCMD is the predominant form of DGiopathy in Japan, and FKR-deficient LGMD2I is the most frequent form of DGiopathy in the U.S. and Europe. The exact number of patients who are ISPD-deficient is not known, but it has been reported that ISPD mutation is the second highest cause of WWS [36, 37]. Together, diseases caused by defects in these three genes can be sub-grouped as DGiopathy with RboP deficiency, and this group accounts for the majority of DGiopathy in the world. In the remainder of this section we will review FCMD, the first identified DGiopathy, and fukutin, its responsible gene.

FCMD, which was first identified by Dr. Yukio Fukuyama, is characterized by severe CMD with central nervous system and eye abnormalities [57]. FCMD is an autosomal recessive muscular dystrophy and is the second most common childhood muscular dystrophy next to DMD in Japan. The disease incidence is ~3/100,000, and one person in approximately 90 is expected to be a heterozygous carrier. FCMD patients manifest muscle weakness and hypotonia by early infancy [58]. Brain malformations characterized by microgyria of the cerebrum and cerebellum, and type II lissencephaly as well as mental retardation are associated with FCMD [59]. Eye abnormalities include myopia, cataracts, abnormal eye movement, pale optic discs, and retinal detachment. The major mutation in FCMD is a SVA retrotransposon insertion in the 3' noncoding region of fukutin, which accounts for ~87% of the FCMD chromosome [11]. It is notable that FCMD is the first human disease identified to be caused by an ancient retrotransposon insertion. This insertion contains a strong splice acceptor site, which induces a rare alternative donor site in the final exon, thus leading to abnormal mRNA splicing (exon-trapping) [60]. The resulting product lacks a portion of the C-terminus and instead acquires extra amino acids derived from the transposon insertion. This aberrant fukutin product loses its function in α-DG modification. Patients heterozygous for the SVA insertion and for other mutations, such as nonsense and missense mutations, develop more severe or milder phenotypes than typical FCMD patients depending on the deleterious effects of the non-transposon mutations.

**THERAPEUTIC STRATEGIES FOR RIBITOL-PHOSPHATE DEFICIENT-TYPE DGIOPATHY**

Several therapeutic strategies for RboP-deficient type DGiopathy have been proposed, and here we introduce three of them. First, since DGiopathies are single gene disorders, viral vector-mediated gene therapy is the most straightforward strategy. In fact, the effectiveness of fukutin- or FKR-gene delivery has been proven using FCMD and LGMD2I model mice, respectively [61, 62]. Second, for FCMD, an innovative strategy based on the molecular pathomechanism has been proposed. As introduced above, the unique retrotransposon insertion causes abnormal splicing, and the introduction of antisense nucleotides that target the splice acceptor and the splicing enhancer sequences prevents the pathogenic exon trapping. This antisense treatment rescues normal fukutin mRNA expression and protein production, and consequently, α-DG glycosylation is restored in the FCMD patients' cells and in model mice [60]. This strategy can be applied to almost all FCMD patients in Japan. Third, since ISPD deficiency leads to a loss of or severe reduction in cellular CDP-ribitol, the supplementation of CDP-ribitol may be effective. This hypothesis is supported by the addition of CDP-ribitol into the cell culture media of ISPD-deficient cells, which restores α-DG glycosylation [33]. Furthermore, several missense mutations in ISPD protein have been shown to reduce its enzyme activity, but importantly some residual activities are present. In such cases, abundantly supplied RboP may accelerate the synthesis of CDP-RboP by mutant ISPD proteins. Although the cellular origin of RboP or its metabolic pathway are unknown, it has been shown that the supplementation of ribitol in drinking water increases cellular CDP-RboP in wild-type mouse tissues [41]. Thus, ribitol can be also used as a drug. Are such supplementation strategies applicable to fukutin- or FKR-deficient DGiopathies? If missense mutations in these RboP transferases, i.e. fukutin and FKR, reduce the affinity to their donor substrate CDP-ribitol, higher concentrations of cellular CDP-ribitol may enhance their enzyme activities. Further studies are necessary to test these ideas.

Important issues to consider are the timing of intervention and the extent of glycosylation recovery. Systemic gene delivery via the tail vein into adolescent skeletal muscle-selective fukutin conditional knock-out mice (Myf5-fukutin cKO) that exhibit early-stage muscular dystrophy dramatically ameliorated the dystrophic phenotype and restored muscle function to the same level as seen in wild-type mice [61]. The data suggest that even after disease manifestation, gene replacement could ameliorate the disease progression. How much restoration of glycosylation is necessary to prevent the progression of the disease? Several cases have been reported in which patients who show only mild muscular dystrophy without brain malformations
exhibit residual amounts of functionally glycosylated α-DG [63]. A study using a transgenic fukutin knock-in mouse carrying a retrotransposonal insertion showed that even a small amount of functionally glycosylated α-DG is sufficient to maintain skeletal muscle function [64]. These data suggest that effective treatments of DGpathy do not require full recovery of glycosylation.

CONCLUSION

More than a decade has passed since the establishment of the disease concept now known as DGpathy. During this decade, dedicated efforts have been made to understand the sugar chain structure of α-DG and DGpathy gene functions. Advances in just a few years have resolved these mysteries. In this review, we focused on a novel glycan moiety of α-DG, the tandem RboP structure. Fukutin, FKRP, and ISPD are directly involved in the synthesis of the tandem RboP: fukutin and FKRP are RboP transferases, and ISPD is involved in cellular CDP-ribitol synthesis. In Japan, FCMD caused by mutations in fukutin is the dominant form of DGpathy, whereas in the U.S. and Europe, LGMD2I caused by mutations in FKRP is the most prevalent. The number of patients who are diagnosed as carrying ISPD mutations will increase. Muscular dystrophy caused by defects in RboP modification is a subgroup of DGpathy, and many patients fall into this group. Hopefully, therapeutic strategies to correct or enhance RboP modification will be established in the future to overcome these diseases.

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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