Review

Mammalian O-mannosyl glycans: Biochemistry and glycopathology

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Abstract: Glycosylation is an important posttranslational modification in mammals. The glycans of glycoproteins are classified into two groups, namely, N-glycans and O-glycans, according to their glycan-peptide linkage regions. Recently, O-mannosyl glycan, an O-glycan, has been shown to be important in muscle and brain development. A clear relationship between O-mannosyl glycans and the pathomechanisms of some congenital muscular dystrophies has been established in humans. Ribitol-5-phosphate is a newly identified glycan component in mammals, and its biosynthetic pathway has been elucidated. The discovery of new glycan structures and the identification of highly regulated mechanisms of glycan processing will help researchers to understand glycan functions and develop therapeutic strategies.

Keywords: O-mannosylation, congenital muscular dystrophy, dystroglycan, ribitol-5-phosphate

Introduction

The major glycans of glycoproteins are classified into two groups according to their glycan-peptide linkages. Glycans linked to asparagine (Asn) residues of proteins are termed N-glycans, whereas glycans linked to serine (Ser) or threonine (Thr) residues are called O-glycans. In N-glycans, the reducing terminal N-acetylglucosamine (GlcNAc) is linked to the amide group of Asn via an aspartylglycosylamine linkage. In O-glycans, the reducing terminal N-acetylgalactosamine (GalNAc) is attached to the hydroxyl group of Ser and Thr residues. In addition to the abundant O-GalNAc forms, several unique types of protein O-glycosylation have been identified, such as O-linked fucose (Fuc), glucose (Glc), GlcNAc, and mannose (Man), which have been shown to mediate diverse physiological functions. We and other researchers have shown that O-Man glycan is important in muscle and brain development, and its deficiency leads to a group of congenital muscular dystrophies known as α-dystroglycanopathies. In 2004, a review of human genetic diseases characterized by altered glycosylation was published in this journal, including our pioneering findings of muscular dystrophy and glycosylation. Since then, many biochemists, molecular biologists, pediatricians, neurologists, and geneticists have entered this new research field. This review will describe recent progress in establishing the biochemistry and glycopathology of O-Man glycans in mammals.

Structure

O-Mannosylation is known as a yeast-type modification, and all O-Man glycan structures that have been elucidated in yeast are neutral linear structures consisting of only Man residues. O-Mannosylation of proteins is essential for viability in yeast, and its absence is thought to affect the cell wall structure and rigidity. On the other hand, mammalian O-Man glycan is a unique type of protein modification that is present in a limited number of glycoproteins in the brain, nerves, and skeletal muscle. One of the best known O-Man-modified glycoproteins is α-dystroglycan (α-DG), which is a central component of the dystrophin-glycoprotein complex (DGC) isolated from skeletal muscle membranes. α-DG is heavily glycosylated, and its glycans have an important role in binding to proteins such as laminin, neurexin, perlecan, pikachurin, and agrin, which contain laminin G (LG) domains. Recently,
the binding mode of the LG4 and LG5 domains of laminin-α2 with the GlcA/β1-3Xyl disaccharide repeat was resolved using X-ray crystallography.\textsuperscript{5}

(1) Core M1 and core M2. We first identified a sialylated O-Man glycan, Siaα2-3Gal/β1-4GlcNAc/β1-2Man, in α-DG present in bovine peripheral nerves\textsuperscript{6} and then in rabbit skeletal muscle.\textsuperscript{7} Subsequently, many studies of the O-Man glycan structure have been performed and various O-Man glycan structures have been elucidated. Currently, these glycan structures are classified into three core O-Man structures based on the linkage of GlcNAc to the Man residue: core M1 (GlcNAc/β1-2Man), core M2 [GlcNAc/β1-6(GlcNAc/β1-2)Man], and core M3 (GalNAc/β1-3GlcNAc/β1-4Man).\textsuperscript{2} Furthermore, in addition to Siaα2-3Gal/β1-4GlcNAc (sialyl glycan), Gal/β1-4(Fucα1-3)GlcNAc (Lewis X glycan) and HSO\textsubscript{3}-3GlcA/β1-3Gal/β1-4GlcNAc (HNK-1 epitope) are exclusively attached to core M1 and core M2. Notably, the core M2 structure is present in the brain. HNK-1 and Lewis X glycans on core M1 and core M2 are thought to play important roles in brain development.\textsuperscript{8,9}

(2) Core M3. The extended complete core M3 structure is novel and has recently been revealed (bottom structure in Fig. 1).\textsuperscript{10} Characteristic features include 1) the phosphorylation of the 6-position of Man; 2) a tandem ribitol-5-phosphate (Rbo5P) structure; 3) a (-3GlcA/β1-3Xylα1-) repeat; and 4) a single GlcA/β1-4Xyl/β1-4 unit.

The addition of a phosphate to the monosaccharide of glycans is a glycan modification whose significance is partially understood. For example, Man 6-phosphate acts as a recognition marker of lysosomal enzymes.\textsuperscript{11} In mammalian cells, newly synthesized lysosomal enzymes are modified with a phosphate and acquire the Man 6-phosphate marker. These enzymes bind to the luminal domains of sorting receptors (Man 6-phosphate receptors) through their Man 6-phosphate recognition markers in the trans-Golgi network and are targeted to acidified endosomes and lysosomes. Another case is xylose (Xyl) 2-phosphate of glycosaminoglycan as a common linkage tetrasaccharide. Phosphorylation and de-phosphorylation of the 2-position of the Xyl residue are an important modification that regulates the formation of the linkage region and glycosaminoglycan biosynthesis.\textsuperscript{12} This transient phosphorylation is performed by FAM20B,\textsuperscript{12} and its deletion in mice results in embryonic lethality.\textsuperscript{13} As will be described later, the 6-phosphate of Man attached to core M3 is required for the glycan chain elongation of core M3, and its defect causes α-dystroglycanopathy.

Ribitol (Rbo) is a sugar alcohol, and the usage of Rbo or Rbo5P as a glycan component has not been reported in mammals. However, Rbo5P is used as a component of the teichoic acids present in the cell walls of gram-positive bacteria.\textsuperscript{14} Rbo5P was first detected as a glycan component in the extended structure of core M3 by our group.\textsuperscript{15} Shortly thereafter, several groups independently reported that Rbo5P is a component of mammalian glycans.\textsuperscript{16–18}

Before a detailed glycan structure was determined, importance of the glycan moiety of α-DG was well recognized by an antibody, IIIH6, because the IIIH6 antibody recognizes glycosylated α-DG and functionally competes with DG-laminin binding. Thus, IIIH6 was considered to recognize laminin-binding epitopes on sugar chains. The IIIH6 epitope has been proposed to attach to core M3 on α-DG via the phosphodiester linkage because hydrogen fluoride (HF) treatment, which cleaves the phosphodiester bond, ablates the laminin-binding activity.\textsuperscript{19} Originally the IIIH6 epitope glycan was thought to be linked to the 6-position of Man in core M3 but the correct binding to the 3-position of GalNAc in core M3 was subsequently reported.\textsuperscript{15}

The glycosaminoglycan-like (-3GlcA/β1-3Xylα1-) (GlcA-Xyl) repeat is unique. The GlcA-Xyl repeat was identified to be assembled as a result of the enzymatic activity of LARGE (like-acetylglucosaminyltransferase).\textsuperscript{20} LARGE overexpression drastically enhances IIIH6 reactivity and the laminin-binding activity of α-DG, whereas HF treatment induces the loss of IIIH6 reactivity and laminin-binding activity of α-DG, suggesting that the IIIH6 epitope is probably the same as the GlcA-Xyl repeat structure. However, extensive data suggested that the GlcA-Xyl repeat is not directly linked to the 6-position of Man in core M3. For example, mutations in FKTN (fukutin) and FKRP (fukutin-related protein) are responsible for α-dystroglycanopathy, and these patients show a dramatic reduction in the reactivity of IIIH6, suggesting the presence of an unknown “scaffold moiety” between the phosphate and GlcA-Xyl repeat. We used small recombinant α-DG containing the first 20 amino acids of the mucin-like domain to determine the scaffold structure up to the GlcA-Xyl repeat. Finally, we proposed that the scaffold glycan was “GlcA-Xyl-Rbo5P-Rbo5P”.\textsuperscript{15} Rbo is a pentose alcohol (pentitol) and has never been reported in mammalian glycans.
In addition to α-DG, several proteins have been shown to carry the core M1 and/or core M2 structures, such as IgG2, phosphacan, CD24, neurofascin, and lecticans. On the other hand, α-DG is currently the only known core M3-modified protein.

**Biosynthesis**

A series of O-Man glycans with heterogeneous Man-core and peripheral structures is present in mammals. The identification and characterization of enzymes involved in the biosynthesis of mammalian O-Man glycans are necessary to elucidate the function and regulation of the glycans.

The biosynthesis of O-Man glycans begins with the transfer of a Man residue from dolichol-phosphate-mannose (Dol-P-Man) to Ser/Thr residues of
certain proteins in the endoplasmic reticulum (ER) (Fig. 1). O-Mannosylation is essential for normal development in *Drosophila melanogaster*, zebrafish, and mice. Of note, both components, POMT1 (protein O-mannosyltransferase 1) and POMT2 (protein O-mannosyltransferase 2), are necessary for O-mannosyltransferase activity. Recently, the presence of another protein in the O-mannosylation machinery was suggested in addition to the POMT1/POMT2 system. An O-mannosylation pathway that selectively modifies cadherins and protocadherins has been reported. According to proteomics data, the initiation of the O-Man glycosylation of cadherins and protocadherins does not depend on the evolutionarily conserved POMT1/POMT2 enzymes that initiate O-Man glycosylation on α-DG. Four TMTC (transmembrane and tetra- tricopeptide repeat containing) genes are predicted to encode distinct O-mannosyltransferases that cooperatively mannosylate the common extracellular cadherin domains of cadherins and protocadherins, suggesting the existence of another as yet undiscovered O-Man glycosylation pathway. It is important to determine whether or not the TMTC products actually exhibit enzymatic activity towards the cadherin family.

(1) Core M1 and core M2. After O-mannosylation by POMT1/POMT2, POMGNT1 (protein O-linked mannose β-1,2-N-acetylglucosaminyltransferase 1) forms the GlcNAcβ1-2Man (core M1) using UDP-GlcNAc as a donor substrate in the Golgi (Fig. 1). The core M2 structure [GlcNAc β1-6(GlcNAc β1-2Man)] is formed sequentially through the actions of POMGNT1 and GNT-IX(VNT-VB) [β-1,6-N-acetylglucosaminyltransferase IX(VB)], an enzyme that catalyzes the formation of the GlcNAcβ1-6Man linkage. Because GNT-IX(GNT-VB) is specifically expressed in the brain, O-Man glycans with the core M2 structure are exclusively detected in the brain. Notably, the presence of the HNK-1 glycan on the core M2 of phosphacan/RPTPβ is an important regulator of re-myelination in the brain. Peripheral structures on core M1 and core M2 are synthesized by a series of glycosyltransferases, such as galactosyltransferase, sialyltransferase, glucuronyltransferase, sulfotransferase, and α1,3-fucosyltransferase in the Golgi.

(2) Core M3. On the other hand, the core M3 structure is synthesized in the ER (Fig. 1). After the initial O-Man transfer is catalyzed by the POMT1/POMT2 complex, the GlcNAc/β1-4Man linkage is catalyzed by POMGNT2 (protein O-linked mannose β-1,4-N-acetylglucosaminyltransferase 2), which transfers GlcNAc from a UDP-GlcNAc to the O-Man residue of α-DG. B3GALNT2 (β-1,3-N-acetylgalactosaminyltransferase 2) forms GalNAcβ1-3GlcNAc by transferring GalNAc from UDP-GalNAc to the GlcNAc residue of the POMGNT2 product. Then, the core M3 structure is phosphorylated at the 6-position of Man by POMK (protein O-mannose kinase) in an ATP-dependent manner and forms the phospho-core M3 structure GalNAcβ1-3GlcNAcβ1-4(phospho-6)Man in the ER. POMK is regarded as a pseudokinase because it lacks the functional motifs present in typical kinases, but recent crystal structures have revealed the detailed mechanisms underlying POMK catalysis and substrate recognition. Notably, POMGNT2 functions in the ER before glycosylation by POMGNT1 in the Golgi, and thus the site of α-DG occupied by core M3 should be determined by the substrate specificity of POMGNT2. A better understanding of the determinants of the site requires information about the mechanism by which POMGNT2 recognizes the peptide sequence and/or conformation near the target O-Man. However, the details currently remain unclear and information about the POMGNT2 structure will improve our understanding of the site-recognition mechanism.

Each glycosyltransferase responsible for synthesizing the extended complete core M3 structure has been identified (Fig. 1). The first enzymes to be identified that form the extended structure were the enzymes that synthesize the novel glycosaminoglycan-like GlcA-Xyl repeat, which is essential for the binding of α-DG to laminin. In 2012, Inamori et al. found that LARGE exhibits both xylosyltransferase and glucuronyltransferase activities and produces repeating units of GlcA-Xyl using UDP-GlcA and UDP-Xyl as donor substrates. LARGE is an α-dystroglycanopathy [congenital muscular dystrophy 1D (MDC1D)] gene product. The molecular mechanism by which LARGE determines the length of the GlcA-Xyl repeat synthesized remains unclear. LARGE2, a LARGE paralog, also exhibits both enzyme activities and synthesizes GlcA-Xyl repeats on α-DG. Although LARGE is likely specific for α-DG, LARGE2-dependent glycosylation also elongates a glycosaminoglycan chain on proteoglycans such as glypicans.

We assumed that FKTN and FKRP might be candidate enzymes that synthesize the tandem Rbo5P structure because: 1) FKTN and FKRP are responsible for α-dystroglycanopathy; 2) both pro-
teins exhibit similarities to glycosyltransferases containing the DXD motif, which is conserved in many glycosyltransferases; 3) both belong to the nucleotidyltransferase fold protein superfamily, and 4) both have similarities to enzymes involved in phosphor-
ycholine modification or the mannose-phosphorylation of glycans in bacteria and yeast. Actually, as shown in our previous study,\textsuperscript{15} FKTN transfers the first Rbo5P to the 3-position of GalNAc from CDP-
Rbo and FKRP transfers the second Rbo5P to the 1-position of the first Rbo5P (Fig. 1). Previously, the glycan was assumed to extend from the 6-position of the core Man, but it actually extends from the 3-position of GalNAc, as confirmed by an NMR study.\textsuperscript{15} The sequential actions of FKTN and FKRP produce the “Rbo5P-1Rbo5P-3GalNAc/β3-3GlcNAc/β3-
4(phosphate-6)Man” structure. Notably, the 6-phosphate of Man in the phospho-core M3 peptide is required for FKTN activity because the non-phosphorylated core M3 peptide does not serve as an acceptor.\textsuperscript{15} Notably, FKTN does not transfer the second Rbo5P and does not form the tandem Rbo5P structure by itself. On the other hand, FKRP does not transfer the first Rbo5P and does not form a Rbo5P trimer or more. Based on the data, the synthesis of the tandem Rbo5P unit is highly regulated by the strict substrate specificities of FKTN and FKRP, although both enzymes share homology. Information about the FKTN and FKRP structures will facilitate the elucidation of the substrate recognition mechanism.

We and other groups have observed an additional GlcA/β1-4Xyl/β1-4 unit linked to the tandem Rbo5P structure. As described above, this GlcA/β1-
4Xyl/β1-4 unit was not formed by LARGE. Two groups independently reported that the GlcA/β1-4 unit was formed by the actions of B4GAT1 (β1-4-
glucuronosyltransferase 1), which shares homology with the glucuronyltransferase domain of LARGE.\textsuperscript{37,38} As shown in our previous study,\textsuperscript{10} TMEM5 acts as a ribitol β1,4-xylosyltransferase to generate a Xyl/β1-4Rbo5P linkage, in which the second Rbo5P in the tandem structure serves as the acceptor for Xyl. TMEM5 has been proposed to be renamed RXLYLT1 (Rbo5P/β1,4-Xyl transferase). Furthermore, the TMEM5 product serves as an acceptor substrate for B4GAT1, but not for LARGE.\textsuperscript{10} Because the glucuronosyltransferase activity of LARGE is specific for α-linked Xyl and not β-linked Xyl, the formation of GlcA/β1-4Xyl by B4GAT1 is required for the LARGE reaction to form GlcA-Xyl repeats. The different Xyl linkages produced by TMEM5(RXLYLT1) and LARGE may be important for the formation of the functional O-
Man glycan on α-DG.

**α-Dystroglycanopathy**

Muscular dystrophies are genetic diseases that cause progressive muscle weakness and wasting. According to recent data, aberrant O-mannosylation of α-DG is the primary cause of some forms of congenital muscular dystrophy known as α-dystroglycanopathy. To date, eighteen genes have been identified as causative factors in these diseases (Table 1). α-Dystroglycanopathies are classified as primary (caused by mutations in the gene encoding DG, DAG1), secondary (caused by mutations in the genes that directly modify the O-Man glycan of α-
DG), or tertiary (caused by the genes that indirectly modify the O-Man glycan of α-DG).

This section will briefly describe a history of the identification of the traits of α-dystroglycanopathy. The best known and most common type of muscular

| Gene name | Function |
|-----------|----------|
| **Primary** |          |
| DAG1      | Dystroglycan |
| **Secondary** |          |
| POMT1     | Protein O-mannosyltransferase |
| POMT2     | Protein O-mannosyltransferase |
| POMGNT1   | Protein O-mannose β1, 2-N-acetylglucosaminyltransferase |
| FKTN      | Ribitol-5-phosphate transferase |
| FKRP      | Ribitol-5-phosphate transferase |
| LARGE     | β1, 3-glucuronosyltransferase and α-1, 3-xylosyltransferase |
| POMGNT2   | Protein O-mannose β1, 4-N-acetylglucosaminyltransferase |
| B3GALNT2  | β1, 3-N-acetylgalactosaminyltransferase |
| B4GAT1    | β1-4-glucuronyltransferase |
| POMK      | Protein O-mannose kinase |
| TMEM5     | Ribitol-5-phosphate β1, 4-xylosyltransferase |
| **Tertiary** |          |
| GMPPB     | GDP-mannose pyrophosphorylase |
| DPM1      | Dolichol-phosphate-mannose synthase |
| DPM2      | Dolichol-phosphate-mannose synthase |
| DPM3      | Dolichol-phosphate-mannose synthase |
| DOLK      | Dolichol kinase |
| ISPD      | CDP-ribitol synthetase |

Table 1. Summary of genes responsible for α-dystroglycanopathies and their functions.
dystrophy is Duchenne-type muscular dystrophy, and its causative gene, dystrophin, was identified in 1987. Dystrophin encodes an actin-binding cytoskeletal protein that is present inside the muscle membrane and is similar to spectrin. Later, many proteins, including glycoproteins, form a large complex called DGC. The function of DGC is thought to connect extracellular matrix components and intracellular components with the actin cytoskeleton. Notably, several components of the DGC have been identified as causative agents for different type muscular dystrophies. DG is a component of the DGC that is encoded by a single gene and is cleaved into two proteins, α-DG and β-DG, by posttranslational processing.

(1) **Primary α-dystroglycanopathy.** In a case study of primary α-dystroglycanopathy, a mutation (Thr192Met) in the N-terminal domain of α-DG was identified in a patient with limb-girdle muscular dystrophy and cognitive impairment. The mutation reduces the number of GlcA-Xyl repeats because this amino acid is required for the recognition by LARGE. This study reported the first case in which a mutation in the DAG1 gene itself was shown to cause muscular dystrophy via a defect in O-Man glycan formation. Another patient with different mutations showed similar hypoglycosylation of α-DG.

(2) **Secondary α-dystroglycanopathy.** We identified and characterized the glycosyltransferases POMGNT1 and POMT1/POMT2. Mutations in POMGNT1 are responsible for muscle-eye-brain disease (MEB), and mutations in POMT1/2 are responsible for Walker–Warburg syndrome (WWS). MEB and WWS are congenital muscular dystrophies characterized by brain malformations and structural abnormalities in the eye. At approximately the same time, other groups reported abnormal glycosylation of α-DG with substantial reductions in lamin-binding activity and IIH6 antibody reactivity in patients with MEB and WWS. Because the common biochemical feature in MEB and WWS is abnormal glycosylation of α-DG, we proposed that MEB and WWS are glycan-deficient diseases. By the early 2000s, causative mutations in six genes (POMT1, POMT2, POMGNT1, FKTN, FKRP, and LARGE) have been identified in patients with α-dystroglycanopathy. Fukuyama-type congenital muscular dystrophy (FCMD) results from mutations in FKTN. Congenital muscular dystrophy type 1C (MDC1C) and limb-girdle muscular dystrophy type 2I (LGMD2I) are caused by a defect in fukutin-related protein (FKRP), a homolog of FKTN. FKMD is the predominant form of α-dystroglycanopathy in Japan, and FKRP-deficient MDC1C and LGMD2I are the most frequent forms of α-dystroglycanopathy in the USA and Europe. We revealed that the first three gene products were glycosyltransferases themselves, but the functions of the remaining three gene products were unclear until recently. FKTN and FKRP are Rbo5P transferases and LARGE is a xylosyltransferase and glucuronyltransferase, whose mutations were identified to cause α-dystroglycanopathy. The mutations of all genes led to defects in O-Man glycan formation. Notably, not all patients with WWS carry mutations in POMT1 or POMT2. Furthermore, cohort studies of patients with α-dystroglycanopathy reported that two-thirds of patients had no mutations in these six genes. Clearly, other unidentified causative genes are present in these patients, and a molecular diagnosis of each patient is necessary to improve our understanding of α-dystroglycanopathy because patients with this syndrome exhibit an extremely broad clinical spectrum of symptoms. The most severe form is characterized by muscular dystrophy with structural abnormalities in the brain and eye. The mildest form is limb-girdle muscular dystrophy without brain or eye involvement. Later, many previously unidentified α-dystroglycanopathy causative genes were revealed through genetic analyses. As described in the previous sections, recent studies have finally revealed and characterized these causative gene products involved in processing the entire core M3 glycan. Among them, the pathogenic role of the 6-position of Man should be noted. It is phosphorylated by POMK, and POMK mutations cause α-dystroglycanopathy because the 6-phosphate of Man is required for the FKTN activity.

The entire structure of the core M3 glycan and its biosynthetic pathway have been identified. Mutations in enzymes involved in each glycosylation step cause α-dystroglycanopathy. However, a remaining question is that the GlcNAcβ1-2 linkage is lost in the proposed structure (bottom structure in Fig. 1). Approximately two decades ago, we reported that the POMGNT1 gene is responsible for MEB. A selective deficiency in glycosylated α-DG was observed in patients with MEB. MEB is inherited as a loss-of-function of the POMGNT1 gene, and was one of the findings prompting the development of this new research field, glycosylation and muscular dystrophy. The lack of the core M1 structure (GlcNAcβ1-2 elongation) is hypothesized to disturb the processing of the core M3 glycan in
the Golgi. We performed an X-ray crystallographic study of human POMGNT1 to answer this question.46) As shown in our previous study, 27),47) POMGNT1 is composed of a catalytic domain and a stem domain, and the stem domain is unusually longer than the same domain in other glycosyltransferases, but its function was unclear. As a result of the X-ray crystallographic study,46) we unexpectedly identified the presence of a carbohydrate-binding domain (CBD) in the stem domain. After an in-depth investigation of the glycan binding specificity of this domain, we found that it specifically recognizes the GlcNAc\(^{\text{O}1-2}\)Man and GalNAc\(^{\text{O}1-3}\)GlcNAc linkages, corresponding to the core M1 and core M3 structures, respectively. Of note, the CBD did not bind to any other types of glycans. In addition, we reported 10 years ago that FKTN and POMGNT1 formed a complex in the Golgi, but its biological meaning was not determined at the time. 48) Because the POMGNT1 stem domain binds to core M3 via the CBD, FKTN is simultaneously recruited to the reaction site. On the other hand, in the case when the complex binds core M1, FKTN is recruited to the reaction site through a different mechanism. Glycosylated sites modified with core M3 are suggested to be located in close proximity to core M1. First, the FKTN-POMGNT1 complex forms core M1 (GlcNAc\(^{\text{O}1-2}\)Man) on an O-linked Man near core M3 and then the complex binds this newly formed core M1 structure. In this situation, FKTN easily transfers Rbo5P to neighboring core M3 structures. GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; Man, mannose; Rbo, ribitol; P, phosphate; DG, dystroglycan.

![Figure 2](image)

Fig. 2. Proposed mechanisms underlying the efficient recruitment of FKTN to core M3 by the carbohydrate-binding domain (CBD) of POMGNT1. (A) Core M3 (GalNAc\(^{\text{O}1-3}\)GlcNAc\(^{\text{O}1-4}\)Man) binds to the CBD of POMGNT1. Because the POMGNT1 stem domain binds to core M3 via the CBD, FKTN is simultaneously recruited to the reaction site. (B) Core M1 (GlcNAc\(^{\text{O}1-2}\)Man) binding to the CBD of POMGNT1. Glycosylated sites modified with core M3 are suggested to be located in close proximity to core M1. First, the FKTN-POMGNT1 complex forms core M1 (GlcNAc\(^{\text{O}1-2}\)Man) on an O-linked Man near core M3 and then the complex binds this newly formed core M1 structure. In this situation, FKTN easily transfers Rbo5P to neighboring core M3 structures. GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; Man, mannose; Rbo, ribitol; P, phosphate; DG, dystroglycan.

Our results have revealed how the Formation of this complex may ensure the efficient synthesis of rare glycans in mammals. In the case when the FKTN-POMGNT1 complex binds core M3 (Fig. 2A), FKTN is simultaneously recruited to the reaction site. On the other hand, in the case when the complex binds core M1, FKTN is recruited to the reaction site through a different mechanism. Glycosylated sites modified with core M3 in α-DG include Thr317, Thr319, and Thr379, although other precise sites remain unclear. According to the results from mass spectrometry-based analyses, the core M1 structure is located near the core M3 site. 15),18),19) As shown in Fig. 2B, the FKTN-POMGNT1 complex initially synthesizes GlcNAc\(^{\text{O}1-2}\)Man on O-Man near core M3, and then the complex binds this formed core M1. Under this circumstance, FKTN easily transfers Rbo5P to neighboring core M3 structures. Actually, we confirmed that the sugar-binding activity of the CBD of POMGNT1 is necessary for the maturation of glycan on the core M3 structure. 46) Furthermore, the formation of an FKTN, FKRP, and TMEM5 complex was proposed. 18),49) The formation of this multi-component complex may ensure the synthesis of tandem Rbo5P repeats and the subsequent efficient elongation of the core M3 glycan.
(3) Tertiary α-dystroglycanopathy. Glycosylation is determined by the actions of many glycosyltransferases that catalyze the transfer of a monosaccharide moiety from a nucleotide sugar donor substrate to the acceptor substrate. In some cases, glycosyltransferases use a dolichol-pyrophosphate sugar instead of a nucleotide sugar. This mechanism is used for the O-mannosylation of the α-DG protein. POMT1/POMT2 catalyze the transfer of a Man residue from Dol-P-Man to Ser/Thr residues on certain proteins. Therefore, some defects in glycosylation are not only mediated by glycosyltransferases but also by the presentation of nucleotide sugar (or dolichol-sugar). Thus, diseases caused by mutations in these genes are categorized as tertiary α-dystroglycanopathies.

DPM1, DPM2, DPM3, DOLK, and GMPPB encode proteins involved in the synthesis of Dol-P-Man. Dol-P-Man is an essential Man donor that is required for mannosylation, including O-mannosylation, N-glycosylation, and C-mannosylation, as well as the formation of glycosyl-phosphatidylinositol anchors. DOLK (dolichol kinase) encodes the DOLK activity responsible for the formation of Dol-P. A combined N-glycosylation and O-mannosylation deficiency has been observed in patients with a predominant presentation of dilated cardiomyopathy due to DOLK mutations has been observed. GMPPB (GDP-mannose pyrophosphorylase B) catalyzes the synthesis of GDP-Man from GTP and Man-1-P. GDP-Man is required for the synthesis of Dol-P-Man. An O-mannosylation deficiency was observed, but no evidence of abnormal N-glycosylation has been found in patients carrying mutant GMPPB. Dol-P-Man is synthesized from Dol-P and GDP-Man by the DPM (dolichol-phosphate-mannose) synthase complex, which comprises three subunits: DPM1, DPM2, and DPM3. Mutations in each component result in dystroglycanopathy-type muscular dystrophy, but defects in N-glycosylation levels differed. Because the DPM1, DPM2, DPM3, DOLK, and GMPPB proteins are all involved in the synthesis of Dol-P-Man, the amount of available Dol-P-Man affects the O-mannosylation of α-DG.

Rbo5P in a mammalian glycan component is very unique and is incorporated by the sequential actions by FKTN and FKRP using CDP-Rbo, as described above. However, the CDP-Rbo biosynthetic pathway was unknown in mammals. In bacteria, this synthetic pathway has already been elucidated. CDP-Rbo is synthesized from CTP and Rbo5P by the enzyme teichoic acid ribitol I (TarI). Notably, bacterial TarI shares homology with human ISPD (isoprenoid synthase domain-containing), which is known to be responsible for α-dystroglycanopathy. According to data from our previous study, human ISPD is a CDP-Rbo synthetase (CDP-Rbo pyrophosphorylase) that synthesizes CDP-Rbo from CTP and Rbo5P, and CDP-Rbo is required for the biosynthesis of functional O-Man glycans. Shortly thereafter, two other groups independently confirmed that human ISPD catalyzes CDP-Rbo synthesis. Thus, the amount of available CDP-Rbo affects the O-mannosylation of α-DG. Providing further support for this hypothesis, the addition of CDP-Rbo restores the IIIH6 epitope of α-DG in ISPD knockout cells, suggesting that CDP-Rbo supplementation is a possible therapeutic strategy for ISPD-deficient patients. Furthermore, dietary supplementation with Rbo is suggested to be beneficial in mice with not only ISPD mutations but also a FKRP mutation.

As shown in our recent study, CDP-glycerol inhibits the RboP transfer activities of both FKTN and FKRP, suggesting that CDP-glycerol inhibits the synthesis of the functional O-Man glycan of α-DG by preventing the further elongation of the glycan chain. A remaining question is whether the amount of CDP-glycerol is causative factor for uncharacterized α-dystroglycanopathy.

Glycosylation of Notch receptors regulates ligand-induced Notch signaling in the cell-to-cell communication system that is required for cell-fate decisions during development. Aberrant activation or inactivation of the Notch signaling pathway leads to human diseases, including many different cancers. Notch receptors are known to be modulated by unusual O-glycans: O-Fuc, O-Glc, and O-GlcNAc. A notable glycan is O-Glc, the synthesis of which is catalyzed by protein O-glucosyltransferase1 (POGLUT1). POGLUT1 is an enzyme that transfers Glc from UDP-Glc to a distinct Ser residue in the epidermal growth factor (EGF)-like repeat. Importantly, POGLUT1 is essential for Notch receptor function. Recently, a missense mutation (Asp233Glu) in POGLUT1 was identified in a patient showing an impairment in muscle development and hypoglycosylation of muscle α-DG, but not in fibroblasts. A detailed analysis revealed a clearly different disorder from secondary α-dystroglycanopathy, suggesting that a pathomechanism for this form of muscular dystrophy is Notch signaling-dependent loss of satellite cells. Further studies are necessary.
to determine the detailed mechanism using data from other patients. Similar to 0-Glc, O-Fuc and 0-GlcNAc modifications also occur at specific positions within an EGF repeat if the appropriate O-glycosylation consensus sequence is present,59) suggesting a mechanism for fine-tuning the Notch signaling pathway by O-glycan that is probably related to muscle formation.

The transporters of nucleotide-sugars into the Golgi are critical for the glycosylation of glycoconjugates, and mutations in the transporters may cause a group of genetic disorders named congenital disorders of glycosylation. A missense mutation (Gln101His) in one of these transporters, SLC35A1, was identified in a patient with intellectual disability, seizures, ataxia, macrothrombocytopenia, and bleeding diathesis, and the mutation displayed a reduction in the sialylation of N- and O-glycosylated glycans.60) Of note, SLC35A1-deficient cells showed a lack of α-DG O-mannosylation with a concomitant reduction in sialylation.61) The results indicate a role for SLC35A1 in α-DG O-mannosylation that is distinct from sialic acid metabolism. In addition, SLC35A1-deficient patients present a combined disorder of α-DG O-mannosylation and sialylation, which is a novel variant of the tertiary α-dystroglycanopathies.

**Future directions**

The O-Man glycan moiety including Rbo5P is a novel structure in mammals. However, the findings produce additional questions. For example, are any other protein(s) modified with the core M3 glycan? Do proteins other than α-DG contain the Rbo5P modification? What is the biological meaning of Rbo5P in mammalian glycosylation? How is Rbo5P synthesized and degraded in mammals? Additionally, further studies are needed to clarify the distribution of O-Man glycans in various tissues and to examine their changes during development and in response to pathological conditions. These questions should be addressed in the future. A major challenge will be to integrate the forthcoming structural, cell biological, and genetic information to understand how α-DG O-mannosylation contributes to muscular dystrophy and brain development.

The hypoglycosylation of α-DG in muscle substantially reduces its affinity for a number of extracellular ligands. Based on this finding, the defective glycosylation of α-DG is reasonably explained to cause muscle cell degeneration and abnormal brain structures in patients with α-dystroglycanopathies. In other words, interference with the glycosylation of α-DG may lead to a combination of muscle and brain phenotypes in patients with these diseases. Because α-DG hypoglycosylation is a common feature of α-dystroglycanopathies, α-DG may be a potential target of new glycotherapeutic strategies for these diseases (Fig. 3).
The formation of the interaction between laminin and α-DG induced by glycan supplementation may improve the symptoms of α-dystroglycanopathy in patients who carry mutations in any enzymes of the glycan processing pathway.

Muscular dystrophy is a group of genetic disorders characterized by progressive muscle weakness and degeneration. Unfortunately, an effective treatment for the disease is not available. I hope our findings will provide new opportunities for the development of treatments for these diseases in the future.

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Profile

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