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Structure–function analysis of human protein O-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1, POMGnT1

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Abstract

Protein O-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) catalyzes the transfer of GlcNAc to *O*-mannose of glycoproteins. Mutations in the *POMGnT1* gene cause a type of congenital muscular dystrophy called muscle–eye–brain disease (MEB). We evaluated several truncated mutants of POMGnT1 to determine the minimal catalytic domain. Deletions of 298 amino acids in the N-terminus and 9 amino acids in the C-terminus did not affect POMGnT1 activity, while larger deletions on either end abolished activity. These data indicate that the minimal catalytic domain is at least 353 amino acids. Single amino acid substitutions in the stem domain of POMGnT1 from MEB patients abolished the activity of the membrane-bound form but not the soluble form. This suggests that the stem domain of the soluble form of POMGnT1 is unnecessary for activity, but that some amino acids play a crucial role in the membrane-bound form.

Keywords: Muscle–eye–brain disease; Muscular dystrophy; Glycosyltransferase; Catalytic domain; Stem domain; Mutation; POMGnT1

Protein O-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) catalyzes the transfer of GlcNAc to *O*-mannose of glycoproteins [1]. Recently, we demonstrated that POMGnT1 is responsible for muscle-eye-brain disease (MEB), which is an autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormalities, and brain malformation (type II lissencephaly) [2]. *O*-Mannosyl glycan is a rare type of glycan in mammals and is present in a limited number of glycoproteins of brain, nerve, and skeletal muscle [3,4]. One of the best known *O*-mannosyl-modified glycoproteins is α-dystroglycan, which is a component of the dystrophin–glycoprotein complex (DGC) in skeletal muscle. DGC has a crucial role in linking the extracellular basal lamina to the cytoskeletal proteins for stabilization of sarcolemma [5]. *O*-Mannosyl glycan on α-dystroglycan is a laminin-binding ligand [6] and defects of *O*-mannosyl glycan in MEB patients greatly reduced affinities for α-dystroglycan with laminin [7,8].

Human POMGnT1 is a 660-amino acid type II membrane protein [1]. Its amino acid sequence is similar to that of human UDP-GlcNAc: α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT-I). Based on the structure of GnT-I, POMGnT1 is thought to have four domains: an N-terminal cytoplasmic tail (Met1–Arg37), a transmembrane domain (Phe38–Ile58), a stem domain (Leu59–Leu300), and a catalytic domain (Asn301–Thr660) (Fig. 1) [1]. The putative stem domain of POMGnT1 has low homology with the stem domain of human GnT-I, while the putative catalytic domain of POMGnT1 is highly homologous with the catalytic domain of human GnT-I. However, the precise stem and catalytic domains of POMGnT1 have not been determined.

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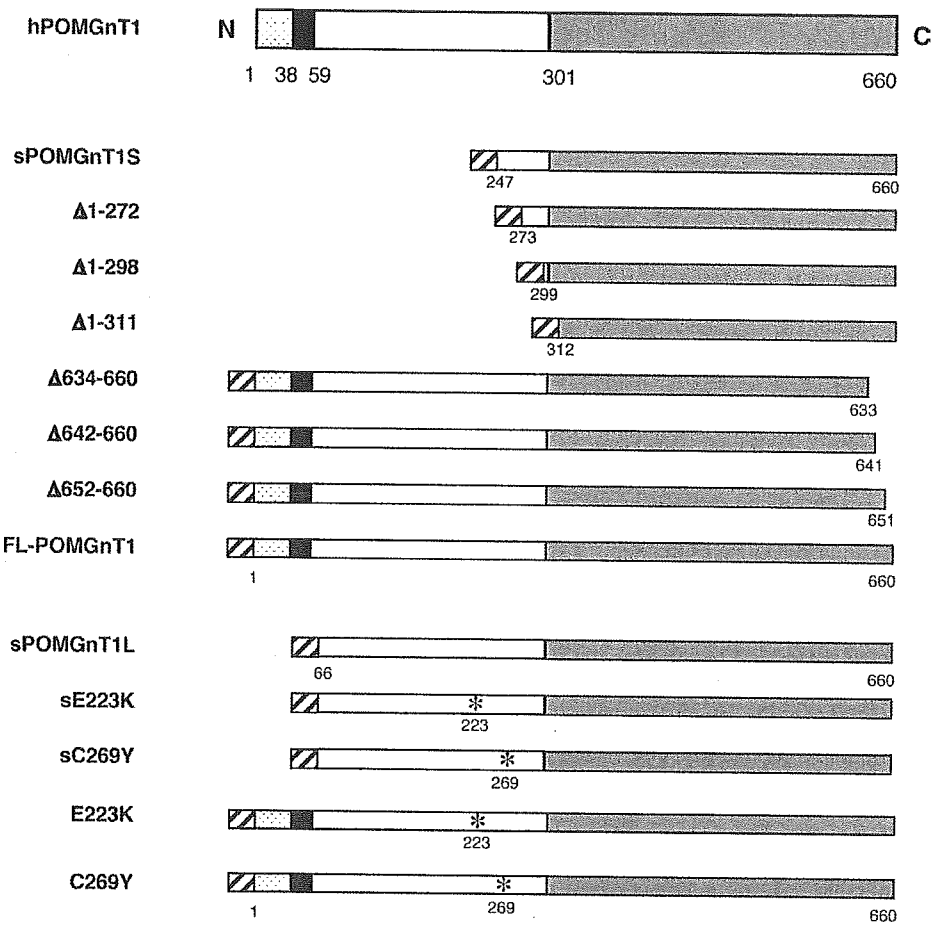

Fig. 1. Schematic representation of human POMGnT1 and truncated mutants. Shaded box, Xpress tag; dotted box, cytoplasmic domain; filled box, transmembrane domain; open box, stem domain; and gray box, catalytic domain. Asterisks show single amino acid substitution sites in MEB patients. The numbers below the boxes indicate the amino acid residue numbers of human POMGnT1 and each mutant.

We previously demonstrated that 13 mutations in the *POMGnT1* gene in MEB patients caused loss of enzymatic activity and resulted in failure of synthesis of *O*-mannosyl glycan [1,9]. All mutations were dispersed in the putative stem domain and catalytic domain [10]. Among the 13 mutations, two had amino acid substitutions in the stem domain.

In this study, in order to delineate the catalytic domain of human POMGnT1, we expressed mutants with truncations in the N- and C-termini and assayed for POMGnT1 activity. Furthermore, to understand the effect of the stem domain on enzymatic activity, we expressed mutants with amino acid substitutions in the stem domain. We found that the effect of single amino acid substitutions in the stem domain on enzymatic activity was different between the soluble and membrane-bound forms.

Materials and methods

Construction of truncated POMGnT1. To make the C-terminal truncated forms (Δ634–660, Δ642–660, and Δ652–660), human *POMGnT1* cDNA was used as the template for PCR amplification with the gene-specific primers listed below. The template encodes a full-length *POMGnT1* (*FL-POMGnT1*) cDNA tagged with the Xpress epitope, and a His-tag at the N-terminus, and was cloned into pcDNA3.1 Zeo (+) (Invitrogen) as described previously [1,9]. PCR products were sequenced and digested, and introduced into the *Xho*I and *Pst*I sites of the template plasmid. The N-terminal truncated forms (Δ1–272, Δ1–298, and Δ1–311) were prepared from secretory POMGnT1S (sPOMGnT1S) [1]. *sPOMGnT1S* encodes amino acid residues 247–660, the Xpress epitope and a His-tag at the N-terminus, and was cloned into pcDNA3.1 Zeo (+). Truncated N-terminus fragments were obtained from PCR and introduced into the *Bam*HI and *Xho*I sites of sPOMGnT1S/pcDNA 3.1 Zeo (+). The reverse primers for the C-terminal truncated forms were Δ634–660 (5′-CTCGAGTCACACTGAG TAGGGGGAAGCCGG-3′), Δ642–660 (5′-CTCGAGTCATGGGG TGACTGAGGGTGGCTT-3′), and Δ652–660 (5′-CTCGAGTCACT CCTCCTTTGGGGGTGGCTC-3′). The forward primer was 5′-GC TTCTGCAGCAAAGTTGAGG-3′ for all the C-terminal truncated forms. The forward primers for N-terminal truncated forms were Δ1–272 (5′-GGATCCGAGGGCTATGGAAGTGTATGC-3′), Δ1–298 (5′-GGATCCGTCCTCAATGTGCCTGTGGCT-3′), and Δ1–311 (5′-GGATCCCCCAATTACCTGTACAGGATG-3′). The reverse primer was 5′-AACGGGCCCTCTAGACTCGAGG-3′ for all the N-terminal truncated forms.

Construction of POMGnT1 mutants. Expression vectors encoding two *sPOMGnT1* mutants (sE223K and sC269Y) were generated using

the QuickChange site-directed mutagenesis system following the protocol of the manufacturer (Invitrogen). The primers used to make the mutants were: E223K, 5′-GGAGGTCCTGTCTTCGGGAAGAAAC ATTCTAAATC-3′ and 5′-GATTTAGAATGTTTCTTCCCGAAG ACAGGACCTCC-3′; C269Y, 5′-GCCGGCGCTTCTACAGCAAA GTTGAGGG-3′ and 5′-CCCTCAACTTTGCTGTAGAAGCGCC GGC-3′. Secretory *POMGnT1L* (*sPOMGnT1L*) comprising amino acid residues 66–660 of POMGnT1, the Xpress epitope, and a His-tag at the N-terminus was used as a template. Each plasmid was sequenced to confirm the presence of the mutation.

Cell culture and expression of POMGnT1 mutants. Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO_2. The expression vectors were transfected into HEK293T cells using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. To obtain the transmembrane forms of POMGnT1, cells were harvested 3 days after transfection and homogenized in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 250 mM sucrose. After centrifugation at 600*g* for 10 min, the supernatant was subjected to ultracentrifugation at 100,000*g* for 1 h. The precipitate was used as the microsomal membrane fraction. Protein concentration was determined by the BCA assay. To obtain the secretory forms of POMGnT1, media were replaced with 5 ml fresh FBS-free DMEM 2 days after transfection and incubated for 3 more days. The microsomal membrane fraction and culture supernatants were subjected to Western blot analysis and assayed for POMGnT1 activity.

Western blot analysis. The proteins were separated by SDS–PAGE (10% gel) and electrophoretically transferred to a PVDF membrane. The membrane was blocked in PBS containing 5% skim milk and 0.5% Tween 20, incubated with anti-Xpress monoclonal antibody (Invitrogen), and subsequently treated with anti-mouse IgG conjugated with horseradish peroxidase (Amersham Biosciences). Blots were developed using an ECL kit or an ECL Plus kit (Amersham Biosciences) and exposed to Hyperfilm-ECL (Amersham Biosciences). Positope protein (Invitrogen) was used as a mass standard of Xpress-tagged protein to determine the amount of each protein. Optical density measurement of the bands (NIH Image 1.61/ppc software) produced a standard curve, which was used to calculate the concentration of each mutant protein.

Assay for POMGnT1 activity. POMGnT1 activity was obtained from the amount of [^{3}H]GlcNAc transferred to a mannosylpeptide [1,9]. The reaction buffer containing 140 mM Mes buffer (pH 7.0), 200 mM UDP-[^{3}H]GlcNAc (228,000 dpm/mol), 400 mM mannosyl nanopeptide (Ac-Ala-Ala-Pro-Thr(Man)-Pro-Val-Ala-Ala-Pro-NH_2), 10 mM $MnCl_2$, 2% Triton X-100, 5 mM AMP, 200 mM GlcNAc, 10% glycerol, and enzyme solution was incubated at 37 °C for 1 h. After boiling for 3 min, the mixture was analyzed by reversed phase HPLC with a Wakopak 5C18-200 column (4.6 × 250 mm). 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.

Results and discussion

In this study, to elucidate the part of human POMGnT1 that is indispensable for enzymatic activity, a series of POMGnT1 cDNAs encoding various N- and C-terminal truncated forms were constructed, transfected into HEK293T, and then assayed for POMGnT1 activity.

Amino acid residues 301 to 660 of POMGnT1 are highly homologous with the catalytic domain of GnT-I [1] and thus appear to be the catalytic domain of POMGnT1. Secretory POMGnT1 (sPOMGnT1S in Fig. 1), corresponding to amino acid residues 247–660, was previously found to have enzymatic activity [1]. To narrow the catalytic region, other N-terminal truncated forms than sPOMGnT1S were produced and assayed for POMGnT1 activity. These truncated forms lacked amino acid residues 1–272 (Δ1–272), 1–298 (Δ1–298), and 1–311 (Δ1–311) (Fig. 1). The activities of each truncated form relative to the activity of sPOMGnT1S were assessed. As shown in Fig. 2B, both Δ1–272 and Δ1–298 had enzymatic activities although the activities were lower than the activity of sPOMGnT1S. Furthermore, expression of Δ1–311 in the culture supernatant was low and required a longer exposure time to be detected (Fig. 2A), and the produced proteins did not have enzymatic activity. These results indicate that amino acid residues 299–311 are important for both protein expression and enzymatic activity.

As reported previously, the C-terminal portion of the POMGnT1 protein was necessary for enzymatic activity because an MEB mutant protein carrying the same amino acid sequence up to position 626 did not show enzymatic activity [9]. To determine to what extent the C-terminus of the catalytic domain is involved in enzymatic activity, three additional truncated forms were prepared. The three forms had deletions of residues 634–660 (Δ634–660), 642–660 (Δ642–660), and 652–660 (Δ652–660) (Fig. 1). Δ652–660 retained POMGnT1 activity whereas the two shorter forms (Δ634–660 and Δ642–660) were almost inactive (Fig. 2D). These results indicate that the 9 amino acids in the C-terminus are not necessary for POMGnT1 activity but the loss of an additional 10 amino acids abolishes all activity. Based on these results, we concluded that the minimal catalytic domain of POMGnT1 is located between positions 299 and 651 (353 amino acids).

The amino acid sequence of POMGnT1 is homologous to GnT-I. Part of the catalytic domain of POMGnT1 (approximately positions 301–530) is highly homologous with the corresponding region of GnT-I. This region probably includes the UDP-GlcNAc and Mn^{2+}-binding domain. This assumption is supported by the X-ray crystal structure of GnT-I [11]. On the other hand, the region around positions 531–562 of POMGnT1, which has low sequence homology with GnT-I, may be the acceptor-specific domain that recognizes the mannose-peptide for the GlcNAc transfer. In contrast to human POMGnT1, whose activity was not reduced by removal of the 9 amino acids in the C-terminus, GnT-I lost over 60% of its catalytic activity after removal of 7 amino acids from the C-terminus [12]. For several glycosyltransferases, the C-terminal region is known to be essential for enzymatic activity [12–15], but

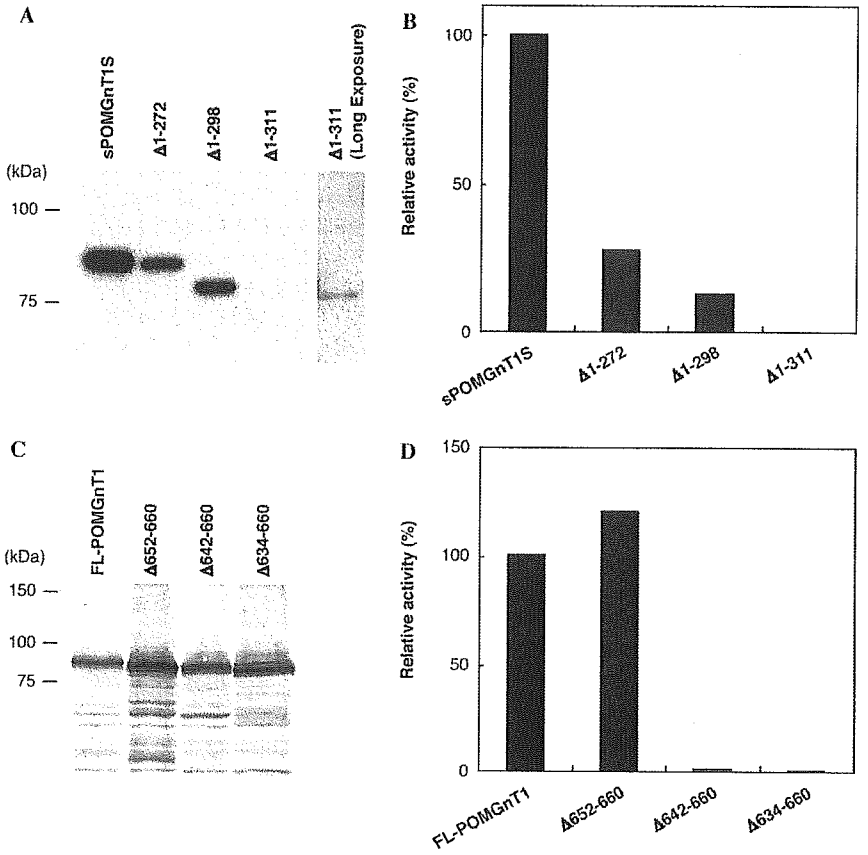

Fig. 2. Expression and activity of truncated POMGnT1 proteins. (A,C) Western blot analysis of Xpress-tagged POMGnT1 proteins. N-terminal truncated mutants (A) and C-terminal mutants (C) were expressed in HEK293T cells, fractionated by SDS–PAGE, transferred to PVDF membrane, and detected by anti-Xpress antibody. In (A), four lanes were exposed for 15 s, except for Δ1–311 (long exposure) which was for 10 min. (B,D) Comparative activities of the truncated mutants. POMGnT1 activity was based on the rate of GlcNAc transfer to a mannosyl-peptide. N-terminal truncated mutants (B) and C-terminal mutants (D).

the numbers of amino acids that are essential for activity are different. Because these enzymes have no amino acid sequence similarity, the conformation of the C-terminal region may be important for catalytic activity. Further X-ray crystal structure studies of POMGnT1 are needed to better understand the functions of each domain.

Next, we examined the involvement of the stem domain in POMGnT1 activity. Previously, we demonstrated that two mutations in the stem domain (E223K and C269Y in Fig. 1) in patients with MEB resulted in almost complete loss of POMGnT1 activity [9]. Amino acid substitutions (missense mutations) outside of the catalytic domain rarely diminish glycosyltransferase activity. Several human genetic disorders are caused by missense mutations of glycosyltransferases, but, to our knowledge, all mutations are in the catalytic domain. For example, a human disease caused by mutations in the *GnT-II* (UDP-GlcNAc: α-6-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase II) gene is known as congenital disorder of glycosylation type IIa (CDG-IIa) [16]. The gene responsible is GnT-II, which is an enzyme that forms the GlcNAcβ1-2Man linkage in *N*-glycans. Four missense mutations that have been identified to date resulted in loss of GnT-II activity, and all of these were found in the catalytic domain [17]. No human diseases having defects in *GnT-I* have been reported, suggesting that such defects are embryonically lethal and that GnT-I is essential for normal human development [4]. However, the Lec1 Chinese hamster ovary mutant is unable to synthesize complex and hybrid *N*-glycans due to the lack of GnT-I activity, but shows essentially normal cell growth [18]. Three missense mutations have been identified in the catalytic domain of GnT-I [19]. Progeroid type Ehlers–Danlos syndrome was reported to be caused by defects in galactosyltransferase I (XGalT-1), which is involved in the synthesis of common linkage regions of proteoglycans [20], and two missense mutations in the catalytic domain of the *XGalT-1* gene were found [21]. If POMGnT1, unlike many other glycosyltransferases, loses transferase activity as a result of an amino acid substitution in the stem domain, it may be because POMGnT1 has a larger

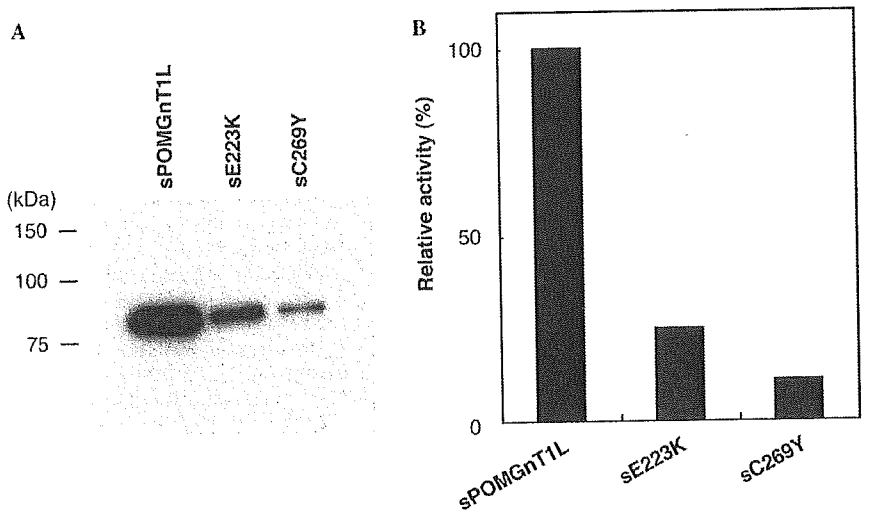

Fig. 3. Expression and activity of point-mutated POMGnT1 proteins. (A) Western blot analysis of Xpress-tagged POMGnT1 proteins. POMGnT1 protein in the culture supernatant (A) was detected by anti-Xpress antibody similar to Fig. 2. (B) POMGnT1 activities were compared to the activity of sPOMGnT1L.

number of amino acids in the stem domain than other glycosyltransferases.

E223K and C269Y mutations were single amino acid substitutions and the mutated sites are close to the catalytic domain. It is possible that these mutations trigger a conformational change in the protein, which inhibits the accessibility of donor substrate (UDP-GlcNAc) and/or acceptor (mannosyl peptide) to the active center of the enzyme, leading to a loss of enzymatic activity. To examine this possibility, we constructed a soluble POMGnT1 form lacking 65 amino acids in the N-terminus (sPOMGnT1L in Fig. 1). Then an E223K or C269Y mutation was inserted in the sPOMGnT1L (sE223K and sC269Y in Fig. 1). Surprisingly, both soluble mutant proteins showed enzymatic activity; sE223K and sC269Y had 30% and 10% of the activity of sPOMGnT1L, respectively (Fig. 3B). Thus, these two amino acid substitutions in the stem domain had different effects on the membrane-bound and soluble forms. These results suggest that the enzymatic activities of the truncated form and full-length form might be different. The majority of glycosyltransferases are type II membrane proteins, and enzymatic activities are often studied using a soluble form after removal of the cytoplasmic tail and transmembrane domain. Many genes in the human genome are thought to encode glycosyltransferases on the basis of sequence homologies with previously characterized glycosyltransferases [22,23]. To determine whether the products of these genes are actually glycosyltransferases, it may be necessary to express the proteins in both their full-length membrane-bound and soluble forms.

Finally, it is of interest that conversion of the membrane-bound form to the soluble form by cleavage of the stem domain induced POMGnT1 activity. Recent studies have shown that the stem domain of α2,6-sialyltransferase was cleaved by β-amyloid-converting enzyme 1 (BACE1) and that the membrane-bound enzyme was changed to the soluble enzyme [24,25]. If a specific protease cleaved the stem domain of the mutant POMGnTI in the same way, then the inactive membrane-bound POMGnT1 may be changed to the active soluble enzyme, which can transfer GlcNAc to *O*-mannose of α-dystroglycan. Finding such a specific protease and elucidating its activating mechanism may lead to a novel therapeutic strategy for treating MEB patients who have mutations in the stem domain of POMGnT1.

Acknowledgments

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Mutations of the *POMT1* gene found in patients with Walker–Warburg syndrome lead to a defect of protein O-mannosylation

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Abstract

Walker–Warburg syndrome (WWS) is an autosomal recessive developmental disorder characterized by congenital muscular dystrophy, brain malformation, and structural eye abnormalities. WWS is due to defects in protein *O*-mannosyltransferase 1 (POMT1), which catalyzes the transfer of mannose to protein to form *O*-mannosyl glycans. POMT1 has been shown to require co-expression of another homologue, POMT2, to have activity. In the present study, mutations in *POMT1* genes observed in patients with WWS were duplicated by site-directed mutagenesis. The mutant genes were co-expressed with POMT2 in Sf9 cells and assayed for protein *O*-mannosyltransferase activity. Expression of all mutant proteins was confirmed by Western blot, but the recombinant proteins did not show any protein *O*-mannosyltransferase activity. The results indicate that mutations in the *POMT1* gene result in a defect of protein O-mannosylation in WWS patients. This may cause failure of binding between α-dystroglycan and laminin or other molecules in the extracellular matrix and interrupt normal muscular function and migration of neurons in developing brain.

Keywords: Walker–Warburg syndrome; Muscular dystrophy; Glycosyltransferase; Mutation; POMT1

Muscular dystrophies are genetic diseases that cause progressive muscle weakness and wasting [1,2]. The pathogenic mechanism of muscular dystrophies may involve the dystrophin–glycoprotein complex (DGC). DGC is composed of α-, β-dystroglycan (α-, β-DG), dystrophin, and some other molecules [3]. DGC is thought to act as a transmembrane linker between the extracellular matrix and intracellular cytoskeleton. This is because α-DG binds to laminin, and the intracellular domain of β-DG interacts with dystrophin in skeletal muscle [3,4]. α-DG is heavily glycosylated, and its sugars have a role in binding to laminin, neurexin, and agrin [3,5,6]. We previously found that the glycans of α-DG include *O*-mannosyl oligosaccharides, and that a sialyl *O*-mannosyl glycan, Siaα2–3Galβ1–4GlcNAcβ1–2Man, is a laminin-binding ligand of α-DG [7].

Protein O-mannosylation has important roles in both lower and higher eukaryotes. It is catalyzed by protein *O*-mannosyltransferases (POMTs). In *Saccharomyces cerevisiae*, O-mannosylation is needed to maintain cell shape and cell wall integrity [8–10]. In *Drosophila melanogaster*, mutations in *rotated abdomen* (*rt*) gene resulted in defects in embryonic muscle development [11]. We found that the *rt* gene product, dPOMT1, has protein *O*-mannosyltransferase activity, but it required co-expression of another *Drosophila* homologue, dPOMT2 [12].

In mammals, *O*-mannosyl glycans are present in a limited number of glycoproteins of brain, nerve, and skeletal muscle [7,13–17]. We recently found that protein *O*-mannosyltransferase 1 (POMT1) forms an enzyme

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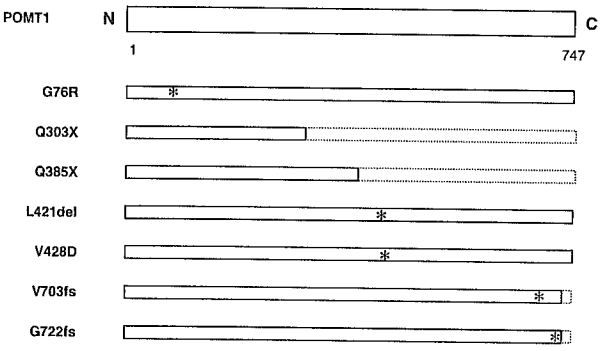
complex with POMT2 and is responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis [18]. Mutations in the *POMT1* gene are considered to be the cause of Walker–Warburg syndrome (WWS: OMIM 236670) [19], an autosomal recessive developmental disorder associated with congenital muscular dystrophy, neuronal migration defects, and ocular abnormalities [20]. Previously, we found that muscle–eye–brain disease (MEB: OMIM 253280), another congenital muscular dystrophy that has a clinical profile similar to that of WWS [21], was caused by failure in the formation of GlcNAcβ1–2Man linkage of *O*-mannosyl glycans on α-DG [22–24]. Thus, *O*-mannosyl glycans are indispensable for normal structure and function of α-DG in muscle and brain in human.

Seven mutations in the *POMT1* gene have been identified in patients with WWS (Fig. 1) [19,25], but no mutations have been found in the *POMT2* gene. These mutations are two single amino acid substitutions (G76R and V428D), two stop mutations (Q303X and Q385X), two frameshifts (V703fs and G722fs), and an amino acid deletion (L421del) (Fig. 1). WWS patients have either homozygous or compound heterozygous mutations in the gene. Two of the mutations in *POMT1* (Q303X and Q385X) cause severe truncations in the protein and are thus assumed to be nonfunctional. It is not clear whether the other five mutations of the *POMT1* gene lead to defects of protein O-mannosylation. Therefore, we examined the effect of these five mutations on protein *O*-mannosyltranferase activity. We co-expressed the mutated POMT1s with wild-type POMT2 in Sf9 cells, and we found that none of them had any protein *O*-mannosyltranferase activity. These results indicate that WWS is due to a loss-of-function of POMT1.



	Mutations	Effects
1	226 G > A	Gly76 Arg Missense (G76R)
2	907 C > T	Gln303 Nonsense (Q303X)
3	1153 C > T	Gln385 Nonsense (Q385X)
4	1260 to 1262 del CCT	Leu421 deletion (L421del)
5	1283 T > A	Val428 Asp Missense (V428D)
6	2110 ins G	Val703 frameshift 729 Stop (V703fs)
7	2167 ins G	Gly722 frameshift 729 Stop (G722fs)

Fig. 1. Schematic representation of human mutated POMT1, predicted products corresponding to each mutation, and a summary of mutations of WWS patients. Asterisks show mutation sites in WWS patients. The numbers below the box indicate the amino acid residue numbers of human POMT1. del, deletion; ins, insertion; and fs, frameshift.

Materials and methods

Vector construction of POMT1 mutants and POMT2. A cDNA containing the most common splicing variant of human *POMT1* (which lacks bases 700–765, corresponding to amino acids 234–255) was selected for site-directed mutagenesis and cloned into pcDNA 3.1 Zeo (−) (Invitrogen), as described previously [18].

For each of the five mutations examined in this study, the *POMT1* gene was modified with a QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The primer pairs used to make the five mutants were: G76R, 5′-GGTGCTGGCC TTGAGAGGTTATTTAGGAGG-3′ and 5′-CCTCCTAAATAACC TCTCAAGGCCAGCACC-3′; V428D, 5′-CCCATTCACAGGAGG ACTCCTGCTACATTG-3′ and 5′-CAATGTAGCAGGAGTCCTCC TGTGAATGGG-3′; V703fs, 5′-CGCCCTGGTGGTGGGCCTGGT ACTCCTCCG-3′ and 5′-CGGAGGAGTACCAGGCCCACCACC AGGGCG-3′; G722fs, 5′-CACTCACCTACGGGGGACAAGTCA CTCTCG-3′ and 5′-CGAGAGTGACTTGTCCCCCGTAGGTGAG TG-3′; and L421del, 5′-CCATGCCCGCCCAGAACTGGAGACTG GAAATTGTG-3′ and 5′-CACAATTTCCAGTCTCCAGTTCTGG GCGGGCATGG-3′. All mutant clones were sequenced to confirm the presence of the mutations.

Wild-type and mutated *POMT1* fragments were digested by restriction enzymes, and were introduced into the *Not*I and *Xba*I sites of pFastBac 1 (Invitrogen). *POMT2* fragment was digested and introduced into the *Xba*I and *Xho*I sites of pFastBac 1.

Cell culture and expression of POMT1 mutants with POMT2. Sf9 cells were maintained in Sf900-II medium (Invitrogen) supplemented with 50 μg/ml kanamycin at 27 °C. Recombinant baculoviruses carrying the expression plasmids were transfected into Sf9 cells using Cellfectin reagent (Invitrogen) according to the manufacturer's instructions. Cells were incubated for 5 days at 27 °C to produce recombinant viruses. The culture supernatant including recombinant viruses was harvested and Sf9 cells were infected with each virus at a multiplicity of infection of 2.5 and incubated for 96 h to express POMT1 and POMT2 proteins.

Western blot analysis. The cells were homogenized in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, and 1 mM DTT, with a protease inhibitor mixture (3 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 mM benzamidine–HCl, and 1 mM PMSF). After centrifugation at 900*g* for 10 min, the supernatant was subjected to ultracentrifugation at 100,000*g* for 1 h. Protein concentration was determined by BCA assay. The proteins in the microsomal fraction (20 μg) were separated by SDS–PAGE (7.5% gel) and transferred to a PVDF membrane. The membrane was blocked in PBS containing 5% skim milk and 0.05% Tween 20, incubated with anti-POMT1 or anti-POMT2 polyclonal antibody [18], and treated with anti-rabbit IgG conjugated with alkaline phosphatase (AP) (Bio-Rad). Rabbit antiserum to the human POMT1 was produced using a synthetic peptide corresponding to residues 348–362 (YPMI YENGRGSSH) of POMT1. A cysteine residue was added to the C-terminus of the POMT1 synthetic peptide so that the antigenic peptides could be conjugated to keyhole limpet hemocyanin (KLH). Rabbits were immunized with the antigenic peptide–KLH conjugates. Proteins that bound to the antibody were visualized with an Alkaline Phosphatase Conjugate Substrate kit (Bio-Rad).

Assay for protein O-mannosyltransferase activity. The protein *O*-mannosyltransferase activity was based on the amount of [^{3}H]mannose

transferred to a glutathione-*S*-transferase fusion α-dystroglycan (GST-αDG) as described previously [18]. Briefly, the reaction mixture contained 20 mM Tris–HCl (pH 8.0), 100 nM [^{3}H]mannosylphosphoryldolichol (Dol-P-Man, 125,000 dpm/pmol) (American Radiolabeled Chemical), 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside, 10 μg GST-αDG, 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 (Nacalai Tesque), and the reaction mixture was centrifuged at 10,000*g* for 10 min. The supernatant was removed, mixed with 400 μl PBS containing 1% Triton X-100 and 10 μl of glutathione–Sepharose 4B beads (Amersham Biosciences), rotated at 4 °C for 1 h, and washed three times with 20 mM Tris–HCl (pH 7.4) containing 0.5% Triton X-100. The radioactivity adsorbed to the beads was measured using a liquid scintillation counter.

Results and discussion

We initially expressed the mutant *POMT1* genes in human embryonic kidney 293T (HEK293T) cells, as we had done previously [18]. However, HEK293T cells have relatively high endogenous protein *O*-mannosyltransferase activity, which makes it difficult to detect changes in protein *O*-mannosyltransferase activity. We then switched to insect Sf9 cells because they have relatively low endogenous protein *O*-mannosyltransferase activity and more clearly show the effects of mutations in *POMT1*.

Protein *O*-mannosyltransferase activity was significantly increased in Sf9 cells that co-expressed POMT1 and POMT2, but was not increased in the cells that expressed POMT1 or POMT2 alone (Fig. 2). Similar results were obtained with HEK293T cells [18]. These results indicate that a similar mechanism of protein O-mannosylation is present in both vertebrates and invertebrates, and that human POMT1 and POMT2 gene products function in insect cells.

Nonsense mutations, 907C>T and 1153C>T, give rise to stop codons at Q303 and Q385, and shorten the POMT1 protein significantly (Fig. 1). Because these shorter products (Q303X and Q385X) probably result in a loss-of-function, we did not perform further studies and examined the effect of the remaining five mutations on enzyme activity.

Two of the mutations with a base substitution (226G>A and 1283T>A; Fig. 1) result in single amino acid substitutions (G76R and V428D, respectively). The products expressed by these two mutants did not show any enzyme activity (Fig. 2). Two mutations with base insertions (2110insG and 2167insG) create frameshift mutations at V703 and G722, respectively. The frameshift at V703 is predicted to cause a replacement of 44 C-terminal amino acids by 26 irrelevant ones after V703. The frameshift at G722 is predicted to remove the 25 amino acids following G722 and replace seven amino

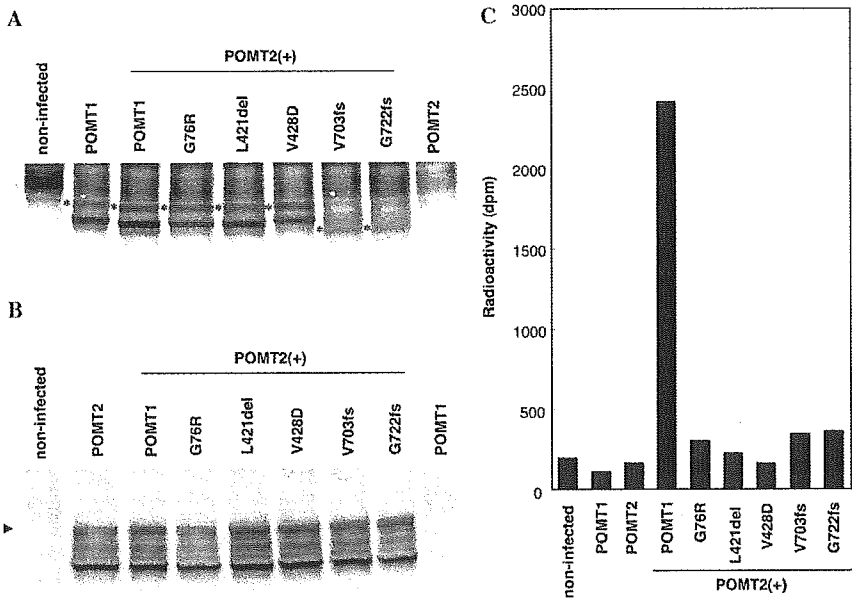


Fig. 2. Expression and activity of mutated POMT1 proteins. (A,B) Western blot analyses of recombinant POMT1 and POMT2 proteins detected by anti-POMT1 antibody (A) and anti-POMT2 antibody (B). The proteins were expressed in Sf9 cells by baculovirus, fractionated by SDS–PAGE (7.5% gel), transferred to PVDF membrane, and stained with antibodies. Asterisks in (A) indicate the migration positions of each POMT1 protein. Arrowhead in (B) shows the estimated migration position of POMT2 protein. Partial degradation of recombinant POMT2 occurred. (C) Protein *O*-mannosyltransferase activities of the POMT1 mutants co-expressed with wild-type POMT2. Protein *O*-mannosyltransferase activity was based on the rate of mannose transfer to a GST-αDG. Lanes indicated as POMT2(+) show proteins from cells that co-expressed POMT2 and either wild-type or mutant POMT1. POMT1 lanes, cells that expressed POMT1 only; POMT2 lanes, cells that expressed POMT2 only; and noninfected lanes, untransformed Sf9 cells.

acids with irrelevant ones. The replacement of 26 amino acids at the C-terminus of 2110insG and the removal of 25 amino acids at the C-terminus of 2167insG caused inactivation of POMT1 (Fig. 2). The L421del mutant is caused by a deletion of 3 bases (CCT) and results in the deletion of a single amino acid (leucine). The deletion of a leucine in L421del also caused a loss of activity (Fig. 2).

POMT1 is a 747-amino acid protein with 30.5% identity and 54% similarity to yeast protein *O*-mannosyltransferases (Pmts) on average [26]. The hydropathy profile suggests that POMT1 protein contains seven to 12 potential transmembrane helices. A seven-transmembrane helical model for the yeast Pmt1 and Pmt4 proteins has been proposed [27–29]. Based on this model, the N-terminus of Pmt1 protein faces the cytoplasm, whereas its C-terminus faces the lumen of endoplasmic reticulum (ER). A large hydrophilic domain (loop 5) of Pmt1 protein is oriented toward the ER lumen and is essential for protein *O*-mannosyltransferase activity [28]. If POMT1 has a similar seven-transmembrane structure like yeast Pmt1 protein, the Q303X, Q385X, L421del, and V428D mutations would be located in loop 5. The fact that the L421del and V428D mutations resulted in loss of enzymatic activity suggests that loop 5 is the catalytic domain.

The G76R mutation (a glycine to arginine substitution) appears to be located in loop 1, which is necessary for enzyme activity in yeast [28]. The protein *O*-mannosyltransferases activity in G76R suggests that loop 1 of POMT1 is important for enzymatic activity.

The V703fs mutation, which results in a 44-amino acid deletion and a 26-amino acid replacement in the C-terminus, and the G722fs mutation, which results in a 25-amino acid deletion in the C-terminus and a 7-amino acid replacement in the C-terminus caused a loss of activity. In the yeast Pmt1 protein, however, an 86-amino acid deletion in the C terminus did not affect enzyme activity [28]. Thus, the C-terminal region of POMT1, unlike that of Pmt1, appears to be important for enzymatic activity.

In the present study, we found that all five mutations in the *POMT1* gene that we examined resulted in a defect of protein *O*-mannosylation. In WWS patients, this may cause failure of binding between α-DG and laminin or other molecules in the extracellular matrix and interrupt normal muscular function and migration of neurons in developing brain.

Following our report that MEB is caused by a defect of protein *O*-mannosyl glycans [22], abnormal glycosylation of α-DG has been suggested as the cause of some muscular dystrophies, e.g., WWS, Fukuyama-type congenital muscular dystrophy (FCMD: OMIM 253800), congenital muscular dystrophy type 1C (MDC1C: OMIM 606612), congenital muscular dystrophy type 1D (MDC1D), and the myodystrophy (*myd*) mouse [3,30]. As mentioned above, WWS is caused by a defect of protein O-mannosylation. Highly glycosylated α-DG was found to be selectively deficient in the skeletal muscle of the patients with FCMD, MDC1C, and MDC1D, and the causative genes of these diseases are thought to encode putative glycosyltransferases [31]. However, it is unclear whether these diseases are due to defects of *O*-mannosyl glycans. Identification of these defects may provide new clues to the glycopathomechanism of muscular dystrophy.

O-mannosylation is an uncommon protein modification in mammals, but it is important in muscle and brain development. Since a few *O*-mannosylated proteins have been identified, further studies are needed to clarify the distribution of *O*-mannosyl glycans in various tissues. Future studies may also reveal that presently uncharacterized forms of muscular dystrophy are caused by defects in other glycosyltransferases. 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 neuronal migration disorder.

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Review

Human genetic deficits in glycan formation

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Abstract: Glycans are associated with most proteins found in secretions and on the surface of mammalian cells. Glycans of secreted glycoproteins affect many protein properties such as solubility, stability, protease sensitivity, and polarity, while glycans on cell surface glycoproteins are involved in various cellular functions including cell-cell and cell-matrix interactions during embryogenesis, immune reactions, and tumor development. Recent advances in human genomic research together with newly developed and sensitive methods for the analysis of glycan structures have elucidated the etiology of a growing number of human genetic diseases with aberrant glycan formation. Among these diseases, defects of protein *N*-glycosylation and *O*-mannosylation are reviewed here. The former is relatively common and the latter is rather uncommon. Both types of defects lead to severe abnormalities, which indicate the importance of glycosylation. Sequencing of the human genome is essentially complete and now glycobiology becomes an important area of postgenomic research. Glycobiology is expected to produce remarkable advances in the understanding and treatment of certain genetic diseases.

Key words: *O*-mannosylation; muscular dystrophy; glycosyltransferase; congenital disorder of glycosylation; *N*-glycosylation.

Introduction. Recent advances in glycobiology have revealed the importance of sugar chains as biosignals for multi-cellular organisms including cell-cell adhesion, cell-matrix adhesion, extracellular receptor-ligand interactions, quality control of proteins, and sorting of proteins within cells, and regulation of intracellular signal transduction processes.[1] These studies have elucidated the regulation of various fundamental biological processes, including cell migration, cell fate determination, and morphogenesis, and mechanisms that modulate development. Because over 60% of the proteins produced by the human body are thought to contain sugar chains, a large number of important physiological events are possibly related to the research field of glycobiology. The major sugar chains of glycoproteins can be classified into two groups according to their sugar-peptide linkages. Those linked to asparagine (Asn) residues of proteins are termed *N*-glycans, while those 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 found, such as *O*-linked fucose, glucose, GlcNAc, and mannose, which have been shown to mediate diverse physiological functions. For example, *O*-fucose has been identified on epidermal growth factor-like repeats for Notch, and elongation of *O*-fucose has been implicated in the modulation of Notch signaling by Fringe.[2)-5)]

The biosynthesis of sugar chains is not controlled by the intervention of a template, and the sugar chains are formed as secondary gene products by the concerted action of glycosyltransferases. There is growing evidence that these enzymes are involved in cellular differ-

Table I. Congenital disorders of glycosylation (CDGs)

CDG type	Gene	Protein function	Gene locus
Ia	*PMM2*	Phosphomannonutase 2	16p13.3-p13.2
Ib	*MPI*	Phosphomannose isomerase	15q22-qter
Ic	*ALG6*	Dol-P-Glc: $Man_9GlcNAc_2$-PP-Dol α1,3glucosyltransferase	1p22.3
Id	*ALG3*	Dol-P-Man: $Man_5GlcNAc_2$-PP-Dol α1,3mannosyltransferase	3q27
Ie	*DPM1*	Dol-P-Man synthase 1	–
If	*MPDU1*	Dol-P-Man utilization defect 1	–
Ig	*ALG12*	Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol α1,2mannosyltransferase	22
Ih	*ALG8*	Dol-P-Glc: $Glc_1Man_9GlcNAc_2$-PP-Dol α1,3glucosyltransferase	–
Ii	*ALG2*	GDP-Man: $Man_1GlcNAc_2$-PP-Dol α1,3mannosyltransferase	9q22
Ij	*DPAGT1*	UDP-GlcNAc: Dol-P-GlcNAc phosphotransferase	11q23.3
IIa	*MGAT2*	UDP-GlcNAc: α-6-mannoside β1,2 *N*-acetylglucosaminyltransferase (GnT-II)	14q21
IIb	*GCS1*	α1,2glucosidase I	2p13-p12
IIc	*FUCT1*	GDP-fucose transporter	11
IId	*B4GALT1*	UDP-Gal: *N*-acetylglucosamine β1,4galactosyltransferase (GalT-1)	9q13

entiation and development, and disease processes. The removal of glycosyltransferase genes in knockout mice indicates that some glycosyltransferases are essential for development, and their defects lead to abnormalities.[6] The importance of sugar chains is further highlighted by congenital disorders of glycosylation (CDGs, which are caused by defects in *N*-glycans) that result in hypotonia, psychomotor retardation, coagulopathies, and gastrointestinal signs and symptoms. Sugar chains other than those of the *N*-linked pathway are also important. This is demonstrated by the finding that aberrant *O*-mannosylation is the primary cause of some forms of congenital muscular dystrophy with abnormal neuronal migration. Protein *N*-glycosylation is a common modification, while *O*-mannosylation is an unusual type of protein modification.[7] Defects of both glycosylation lead to severe abnormalities, indicating that glycosylation is important. This article reviews new insights into glycobiology of human glycan abnormality.

_N_-Glycan formation and congenital disorders of glycosylation (CDG). *O*-Glycans are formed by stepwise addition of monosaccharides to the Ser and Thr residues of polypeptides from nucleotide sugars. In contrast, *N*-glycans are formed by a series of complex pathways including lipid-linked intermediates. First, GlcNAc-1-P is transferred from UDP-GlcNAc to a polyisoprenol monophosphate: dolichyl phosphate (Dol-P). The GlcNAc residue of the GlcNAc-PP-Dol is the starting point of *N*-glycans. To this GlcNAc residue, another GlcNAc and five mannose residues are transferred from UDP-GlcNAc and GDP-Man, respectively. The lipid-bound heptasaccharide is converted to $Glc_3Man_9GlcNAc_2$-PP-Dol by the further addition of four mannose residues from Dol-P-Man and three glucose residues from Dol-P-Glc. The tetradecasaccharide of the lipid derivative is then transferred *en bloc* to the Asn residue of the polypeptide chain, which is translated in the rough endoplasmic reticulum, by the catalytic action of a Dol-P-oligosaccharide: polypeptide oligosaccharyltransferase. Only the Asn residue in the sequence of Asn-X-Ser/Thr, where X can be any amino acid other than proline, is glycosylated. Then the completely translated protein with the tetradecasaccharide is transported to the Golgi apparatus. After the three glucose residues and four mannose residues are removed, a set of glycosyltransferases work sequentially and a variety of outer chain

modifications occur.

N-Glycans are associated with most proteins found on the surface of mammalian cells and in secretions. *N*-Glycans bound to secreted glycoproteins have been shown to affect a wide range of protein properties such as solubility, stability, polarity, and protease sensitivity, while *N*-glycans on cell surface glycoproteins are involved in various cellular functions including cell-cell and cell-matrix interactions during embryogenesis, immune reactions, and tumor development. Therefore, defects in *N*-glycans may cause severe damage to the body. CDGs that are responsible for human diseases were initially identified in 1980. Since then, fourteen distinct CDGs have been identified.[8)-11)] Each is autosomal recessive and caused by mutations in different genes involved in *N*-glycosylation (Table I). Most of the disorders were discovered quite recently in one or at most a few patients. Many more types of CDG will probably be found because the ~50 genes are required for *N*-glycan synthesis. The CDGs are a group of inherited multisystemic disorders, which are commonly associated with severe psychomotor and mental retardation. CDG type I is caused by defects of the assembly of lipid-linked oligosaccharides, whereas CDG type II is caused by all defects of trimming and elongating of *N*-glycans. *N*-Glycosylation defects are routinely detected by isoelectric focusing of serum transferrin, which normally carries two-sialylated bianternary *N*-glycans. The hyposialylated transferrin from CDG patients shows a cathodic shift, which in CDG-I is due to the loss of either one or both *N*-glycans, and in CDG-II is due to the incomplete processing of protein-bound *N*-glycans. Because CDG-I results from defects in *N*-glycans that are linked to Asn residues on nascent proteins, the reduction or loss of catalytic action of a Dol-P-oligosaccharide: polypeptide oligosaccharyltransferase has been thought to be the cause of CDG-I. However, no such CDG types have been found. The molecular nature of ten CDG-I types and four CDG-II types have been identified (Table I), and several will be described here briefly.

By far the most common type of CDG, CDG-Ia [OMIM 212065, OMIM = Online Mendelian Inheritance in Man (http://www.ncbi.nih.gov/)], is caused by mutations in the *PMM2* gene. This gene encodes a phosphomannomutase that converts Man-6-P to Man-1-P. The patients were identified at first over 20 years ago based on their clinical features before the genetic basis was known. In the meantime, more than 300 patients with similar but not identical symptoms were identified. Mutations reduce the size of the GDP-Man pool and produce insufficient amount of the lipid-linked oligosaccharide for complete glycosylation. Other type-I CDGs are caused by defects in different steps of lipid-linked oligosaccharide biosynthesis. On the other hand, type-II CDGs are caused by alterations in the processing of *N*-glycans on proteins. A human disease caused by mutations in the *GnT-II* (UDP-GlcNAc: α-6-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase II) gene is known as CDG-IIa (OMIM 212066). Patients with CDG-IIa show hypotonia, severe psychomotor retardation, frequent infections, and widely spaced nipples.[12)] CDG-IIc (OMIM 266265), which is caused by mutations in the Golgi GDP-fucose transporter, results in fucosylation defects in the whole body, profound mental retardation, failure to thrive, recurrent infections, and leukocytosis.[13),14)] CDG-IId has been observed in only one patient, who showed brain malformation, mental retardation, myopathy, and blood clotting defects. This patient was found to have a 1bp insertion in the *β4GalT1* gene and a reduced activity of β1,4galactosyltransferase.[15)] Taken together, the CDG studies indicate that correct *N*-glycosylation of proteins is essential for normal development.

***O*-Mannosyl glycan and dystroglycan.** *O*-Mannosylated glycoproteins are abundant in the yeast cell wall, and all *O*-mannosyl glycan structures elucidated so far are neutral linear glycans consisting of 1 to 7 mannose residues.[16)] *O*-Mannosylation of proteins has been shown to be vital in yeast, and its absence may affect cell wall structure and rigidity. Additionally, a deficiency in protein *O*-mannosylation in the fungal pathogen, *Candida albicans*, leads to defects in multiple cellular functions including expression of virulence.[17)] In addition to fungi and yeast, clam worm has an *O*-mannosyl glycan (a glucuronylα1-6mannosyl disaccharide) in skin collagen.[18)] Mammalian *O*-mannosylation is an unusual type of protein modification that was first identified in chondroitin sulfate proteoglycans of brain[19)-21)] and is present in a limited number of glycoproteins of brain, nerve, and skeletal muscle.[7)] In brief, we previously found that the glycans of α-dystroglycan include *O*-mannosyl oligosaccharides, and that a sialyl *O*-mannosyl glycan, Siaα2-3Galβ1-4GlcNAcβ1-2Man, is a laminin-binding ligand of α-dystroglycan.[22)] Interestingly, we found the same *O*-mannosyl glycan in rabbit skeletal muscle α-dystroglycan.[23)] After our reports of the sialylated *O*-mannosyl glycan, an HNK-1 epitope (sulfoglucuronyl lactosamine) carrying *O*-mannosyl glycan (HSO_3-3GlcAβ1-3Galβ1-4GlcNAcβ1-2Man) was detected in total brain glycopeptides.[24)] It is noteworthy that

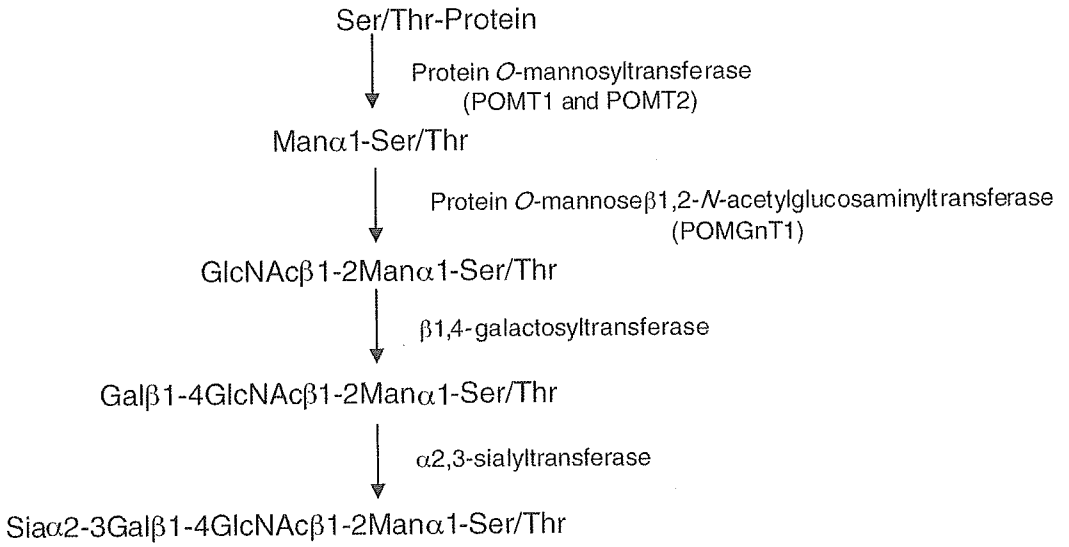

Fig. 1. Biosynthetic pathway of mammalian *O*-mannosyl glycan.

these oligosaccharides have not only 2-substituted mannose but also 2,6-disubstituted mannose.[25] Very recently a gene encoding this 6-branching enzyme (GnT-IX) has been cloned.[26] Further, dystroglycan from sheep brain has a Galβ1-4(Fucα1-3)GlcNAcβ1-2Man structure[27] and mouse J1/tenascin, which is involved in neuron-astrocyte adhesion, contains the *O*-mannosyl glycans.[28] Therefore, it is likely that a series of *O*-mannosyl glycans, with heterogeneity of mannose-branching and peripheral structures, is present in mammals. Further studies are needed to clarify the distribution of such *O*-mannosyl glycans in various tissues and to examine their changes during development and under pathological conditions.

Identification and characterization of the enzymes involved in the biosynthesis of mammalian type *O*-mannosyl glycans will help to elucidate the function and regulation of these glycans (Fig. 1). A key difference between mammalian and yeast-type *O*-mannosyl glycans is that those in mammals have the GlcNAc β1-2Man linkage. This linkage is assumed to be catalyzed by a glycosyltransferase, UDP-GlcNAc: protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase (POMGnT1). POMGnT1 catalyzes the transfer of GlcNAc from UDP-GlcNAc to *O*-mannosyl glycoproteins. We developed an enzyme assay for POMGnT1, and found its activity in several mammalian brains.[29] It should be noted that GlcNAcβ1-2Man linkages are also found in *N*-glycans, where they are catalyzed by two enzymes, UDP-GlcNAc: α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT-I) and GnT-II. However, we found that recombinant GnT-I and GnT-II had no ability to catalyze the GlcNAc β1-2Man linkage in *O*-mannosyl glycans,[29] suggesting that a new enzyme must be responsible for the formation of this linkage. Thus, we cloned the human *POMGnT1* gene.[30] The nucleotide sequence indicated that human POMGnT1 is a 660 amino acid protein and is a type II membrane protein.

Careful examination of substrate specificity of POMGnT1 indicated that POMGnT1 did not have either GnT-I or GnT-II activity.[30] As described above, GnT-I and GnT-II did not have any POMGnT1 activity. Taken together, these results suggest that loss-of-function of POMGnT1 is not compensated by GnT-I and GnT-II. Mammals are known to have an absolute requirement for GnT-I during early embryogenesis. Mouse embryos lacking the functional *GnT-I* gene die prenatally at E9.5 with multisystemic abnormalities.[31),32] On the other hand, over 60% of mouse embryos with null mutations in the *GnT-II* gene survive to term, but 99% of newborns die during the first week of postnatal development with multisystemic abnormalities.[33] Furthermore, a human disease caused by mutations in the *GnT-II* gene is known as CDG-IIa (Table I). No human diseases having defects in *GnT-I* have been reported, suggesting that such defects result in embryonic lethality and that GnT-I is essential for normal human development.

As mentioned above, we found *O*-mannosyl glycan

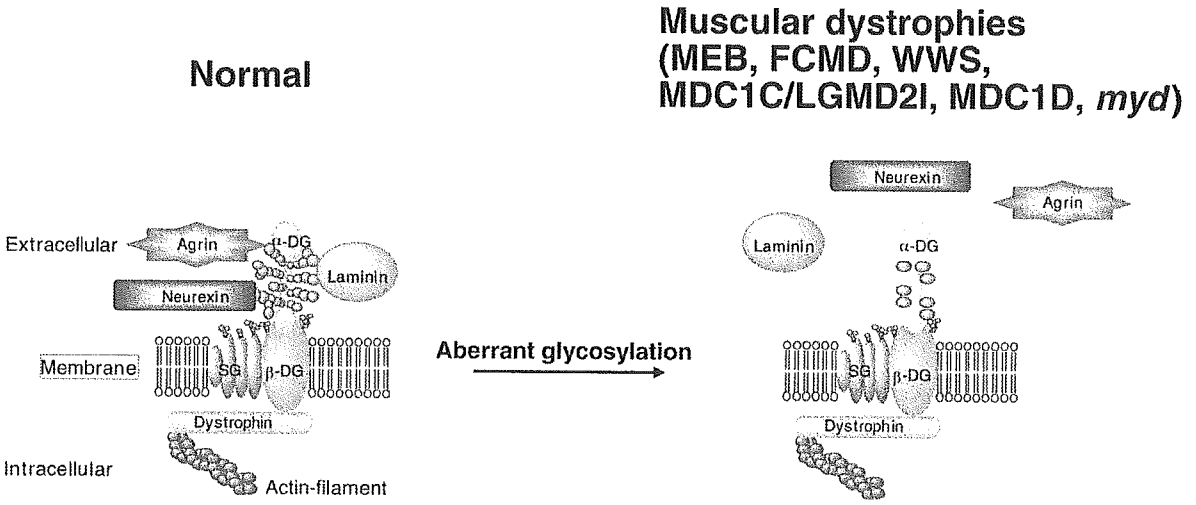

Fig. 2. Dystrophin-glycoprotein complex (DGC) and linkage between the extracellular matrix and the subsarcolemmal cytoskeleton. Left, α-Dystroglycan is a key component of the DGC and is modified by *O*-mannosyl glycan and binds to laminin via its glycan. α-Dystroglycan is also known to bind to other extracellular matrix proteins containing laminin G-domains (LamG), such as neurexin and agrin. On the other hand, inside the cell, β-dystroglycan is known to bind to dystrophin and several components directly or indirectly. Right, Disruption of linkage between the extracellular components and α-dystroglycan due to defects of *O*-mannosyl glycan is thought to cause several muscular dystrophies (MEB, FCMD, WWS, MDC1C, LGMD2I, MDC1D and *myd* in Table III). α-DG, α-dystroglycan; β-DG, β-dystroglycan; SG, sarcoglycan.

during structural analysis of dystroglycan glycans. Dystroglycan is encoded by a single gene (*DAG1*) and is cleaved into two proteins, α-dystroglycan and β-dystroglycan, by posttranslational processing.[34),35)] In skeletal muscle, dystroglycan is a central component of the dystrophin-glycoprotein complex (DGC)(Fig. 2, left). α-Dystroglycan is a heavily glycosylated extracellular peripheral membrane glycoprotein that anchors to the cell membrane by binding to a transmembrane glycoprotein, β-dystroglycan. The α-dystroglycan-β-dystroglycan complex is expressed in a broad array of tissues and is thought to stabilize the plasma membrane by acting as an axis through which the extracellular matrix is tightly linked to the cytoskeleton. This is because α-dystroglycan strongly binds to extracellular matrix proteins containing laminin G (LamG) domains, such as laminin, neurexin, and agrin in a calcium-dependent manner.[36)] On the other hand, the cytoplasmic domain of β-dystroglycan contains a PPXY motif that interacts with dystrophin, which in turn binds to the actin cytoskeleton.[37)] Based on this molecular organization, the DGC is thought to contribute to the structural stability of the muscle cell membrane during cycles of contraction and relaxation. In human, mutations in dystrophin cause Duchenne and Becker muscular dystrophy, mutations in sarcoglycan (SG in Fig. 2) cause limb-girdle muscular dystrophy, and mutations in laminin α2 chain cause congenital muscular dystrophy.[38)]

The function of dystroglycan in the body has been examined by targeting the *DAG1* gene in mice. However, disruption of this gene in mice results in embryonic lethality.[39)] To allow the embryo to develop, chimeric mice generated from targeted embryonic stem cells have been produced. Dystroglycan-null chimeric mice showed muscular dystrophy, although muscle basement membrane formation was normal.[40)] The function of dystroglycan in specific tissues was examined with the Cre/LoxP system. Targeting the dystroglycan gene specifically in differentiated skeletal muscle did not affect muscle basement membrane formation but resulted in a mild dystrophic phenotype.[41)] Targeting the dystroglycan gene in brain resulted in abnormal cerebral cortical layering resembling human cobblestone lissencephaly, and in abnormal cerebellar granule cell migration.[42)] Targeting the dystroglycan gene in peripheral nerves caused defects in both myelination and nodal architecture.[43)] These results indicate that dystroglycan is essential for normal development. As described below, not only dystroglycan itself but also the attached sugars are important.

Table II. Summary of mutations found in patients with MEB

	Mutation	Effect
1	281 C > T	Arg63Stop Nonsense
2	541 del T	Phe149 frameshift 167Stop
3	761 G > A	Glu223 Lys Missense
4	900 G > A	Cys269 Tyr Missense
5	1077 ins G	Val328 frameshift 338Stop
6	1106 ins T	Asp338 frameshift 338Stop
7	1572 C > G	Pro493 Arg Missense
8	IVS17+1 G > A	Glu514 read-through 526Stop and Leu472-His513 del
9	IVS17+1 G > T	
10	1743 G > A	Ser536-Ser550 del
11	1813 del C	His573 frameshift 633Stop
12	1926 del T	Leu611 frameshift 633Stop
13	1970 del G	Val626 frameshift 633Stop

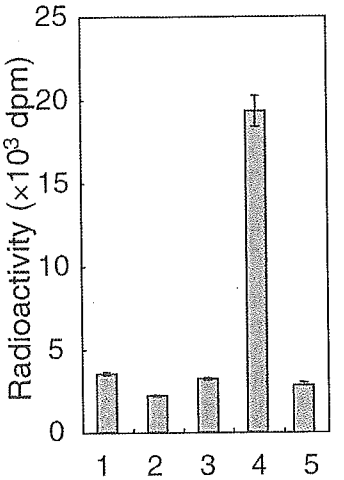

Fig. 3. Protein *O*-mannosyltransferase activity of human POMT1 and POMT2. Protein *O*-mannosyltransferase activity of membrane fractions from HEK293T cells transfected with vector alone (1), cells transfected with human POMT1 (2), cells transfected with human POMT2 (3), cells cotransfected with POMT1 and POMT2 (4), and a mixture of the membrane fractions from *POMT1*-transfected cells and *POMT2*-transfected cells (5). Reprinted with permission from ref. 51). Copyright (2004) National Academy of Sciences, U.S.A.

Muscle-eye-brain disease (MEB). The human *POMGnT1* gene is located at 1p33, within the small candidate interval for muscle-eye-brain disease (MEB: OMIM 253280). MEB is an autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormalities, and brain malformation (type II lissencephaly).[44] Patients with MEB show severe cerebral and ocular anomalies, but some patients reach adulthood. MEB has been observed mainly in Finland.

After we screened the entire coding region and the exon/intron flanking sequences of the *POMGnT1* gene for mutations in patients with MEB, we identified 13 independent disease-causing mutations in these patients (Table II).[30),45)] We have not detected these 13 substitutions in any of 300 normal individuals, indicating that the mutations are pathogenic and that the *POMGnT1* gene is responsible for MEB. To confirm that the mutations observed in patients with MEB are responsible for the defects in the synthesis of *O*-mannosyl glycan, we expressed all of the mutant proteins and found that none of them had enzymatic activity.[30),46)] These findings indicate that MEB is inherited as a loss-of-function of the *POMGnT1* gene. If POMGnT1 does not function, no peripheral structure (Neu5Acα2-3Galβ1-4GlcNAc, Galβ1-4(Fucα1-3)GlcNAc, and HSO_3-3GlcAβ1-3Galβ1-4GlcNAc) can be formed on *O*-mannose residues. Because these structures are involved in adhesive processes, a defect of *O*-mannosyl glycan may severely affect cell migration and cell adhesion. Additionally, we found a selective deficiency of α-dystroglycan in MEB patients.[47] This finding suggests that α-dystroglycan is a potential target of POMGnT1 and that hypoglycosylation of α-dystroglycan may be a pathomechanism of MEB. MEB muscle and brain phenotypes can be explained by a loss-of-function of α-dystroglycan due to abnormal *O*-mannosylation.

After our report that MEB is caused by a defect of *O*-mannosylation,[30] some muscular dystrophies have been suggested to be caused by abnormal glycosylation of α-dystroglycan, e.g., Fukuyama-type congenital muscular dystrophy (FCMD: OMIM 253800), congenital muscular dystrophy type 1C (MDC1C: OMIM 606612), Walker-Warburg syndrome (WWS: OMIM 236670), congenital muscular dystrophy type 1D (MDC1D), and the myodystrophy (*myd*) mouse (Table III).

Walker-Warburg syndrome (WWS). WWS is another form of congenital muscular dystrophy that is characterized by severe brain malformation (type II lissencephaly) and eye anomalies.[48] Patients with WWS are severely affected from birth and usually die within their first year. WWS has a worldwide distribution. Recently, 20% of WWS patients (6 of 30 unrelated WWS cases) have been found to have mutations in protein *O*-mannosyltransferase 1 (*POMT1*), a putative *O*-mannosyltransferase that catalyzes the transfer of mannose to a Ser or Thr residue on the basis of homology with seven yeast protein *O*-mannosyltransferases.[49] *POMT1* is highly expressed in fetal brain, testis, and skeletal muscle, which are the affected tissues in WWS. It

Table III. Possible muscular dystrophies caused by abnormal glycosylation of α-dystroglycan

Condition	Gene	Protein function	Gene locus
Muscle-eye-brain disease (MEB)	*POMGnT1*	GlcNActransferase	1p33
Fukuyama-type congenital muscular dystrophy (FCMD)	*fukutin*	Putative glycosyltransferase	9q31
Walker-Warburg syndrome (WWS) (20%)	*POMT1*	*O*-Mannosyltransferase	9q34.1
MDC1C and limb-girdle muscular dystrophy 2I(LGMD2I)	*FKRP* (fukutin-related protein)	Putative glycosyltransferase	19q13.3
Myodystrophy (*myd*) mouse	*large*	Putative glycosyltransferase	8 (mouse)
MDC1D	*LARGE*		22q12.3-13.1

is noteworthy that none of the 30 cases studied had mutations in another homologue, *POMT2*, which is 33% identical to *POMT1*. However, it was unclear whether the POMT1 and POMT2 proteins actually catalyze protein *O*-mannosylation,[49),50)] and attempts to detect protein *O*-mannosyltransferase activity of POMTs in vertebrates have not been successful. Recently, we developed a new method to detect the enzymatic activity of protein *O*-mannosyltransferase in mammalian cells and tissues. Using this new method, we demonstrated that human POMT1 and POMT2 have protein *O*-mannosyltransferase activity, but only when they are co-expressed (Fig. 3).[51)] This suggests that POMT1 and POMT2 form a hetero-complex to express enzymatic activity. *POMT1* and *POMT2* are expressed in all human tissues, but *POMT1* is highly expressed in fetal brain, testis, and skeletal muscle, and *POMT2* is predominantly expressed in testis.[50),52)] *O*-Mannosylation seems to be uncommon in mammals and only a few *O*-mannosylated proteins have been identified.[7)] It will be of interest to determine the regulatory mechanisms for protein *O*-mannosylation in each tissue. In view of the potential importance of this form of glycosylation for a number of developmental and neurobiological processes, the ability to assay vertebrate *O*-mannosyltransferase activity and knowledge of the requirement of a heterodimeric complex for enzyme activity should greatly facilitate progress in the identification and localization of *O*-mannosylated proteins and the elucidation of their functional roles.

Recently, 6 of 30 WWS patients were found to have mutations in *POMT1*, while none had mutations in *POMT2*.[49)] A possible explanation for the absence of *POMT2* mutations in human subjects is that *POMT2* may be essential for normal development, i.e., *POMT2* mutations may result in embryonic lethality. Another possibility is that patients with *POMT2* mutations were simply not included in the 30 WWS patients. A worldwide survey of the occurrence of *POMT2* mutations is needed to determine whether WWS is caused by *POMT* mutations.

In WWS patients, as in MEB patients, the glycosylated α-dystroglycan was selectively deficient in skeletal muscle. WWS and MEB are clinically similar autosomal recessive disorders that are characterized by congenital muscular dystrophy, lissencephaly, and eye anomalies, but WWS is a more severe syndrome than MEB.[48),53)] Patients with WWS are severely affected from birth (brain malformation is particularly common), and few live beyond infancy. In MEB, the cerebral and ocular anomalies are also severe, but some patients reach adulthood.[44),53)] The difference of severity between the two diseases may be explained as follows: If POMGnT1, which is responsible for the formation of the GlcNAcβ1-2Man linkage of *O*-mannosyl glycans,[30)] is non-functional, only *O*-mannose residues may be present on α-dystroglycan in MEB. On the other hand, *POMT1* mutations cause complete loss of *O*-mannosyl glycans in WWS. It is possible that attachment of a single mannose residue on α-dystroglycan is responsible for the difference in clinical severity of WWS and MEB.

Interestingly, the *Drosophila rt* mutant exhibiting defects of myogenesis was found to be due to a mutation in a homologue of *POMT1*.[50),54)] The mutation also causes reduced fertility and reduced viability. Although the *rt* gene product is not known to be a component involved in the initial step of *O*-mannosyl glycan biosynthesis, *O*-mannosylation is an evolutionarily conserved protein modification,[7)] and may be essential for muscle develop-