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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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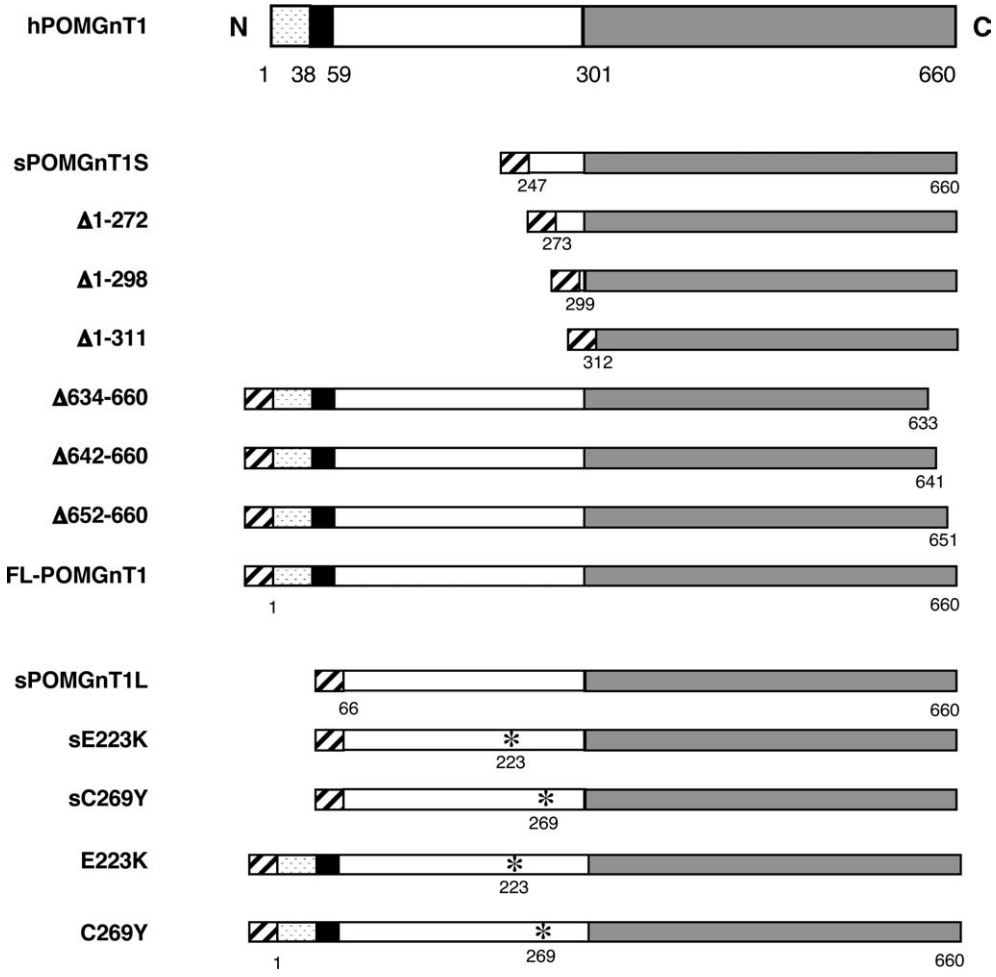

Fig. 1. Schematic representation of human POMGnT1 and truncated mutants. Shaded box, Xpress tag; dotted box, cytoplasmic domain; filled box, transmembrane domain; open box, stem domain; and gray box, catalytic domain. Asterisks show single amino acid substitution sites in MEB patients. The numbers below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

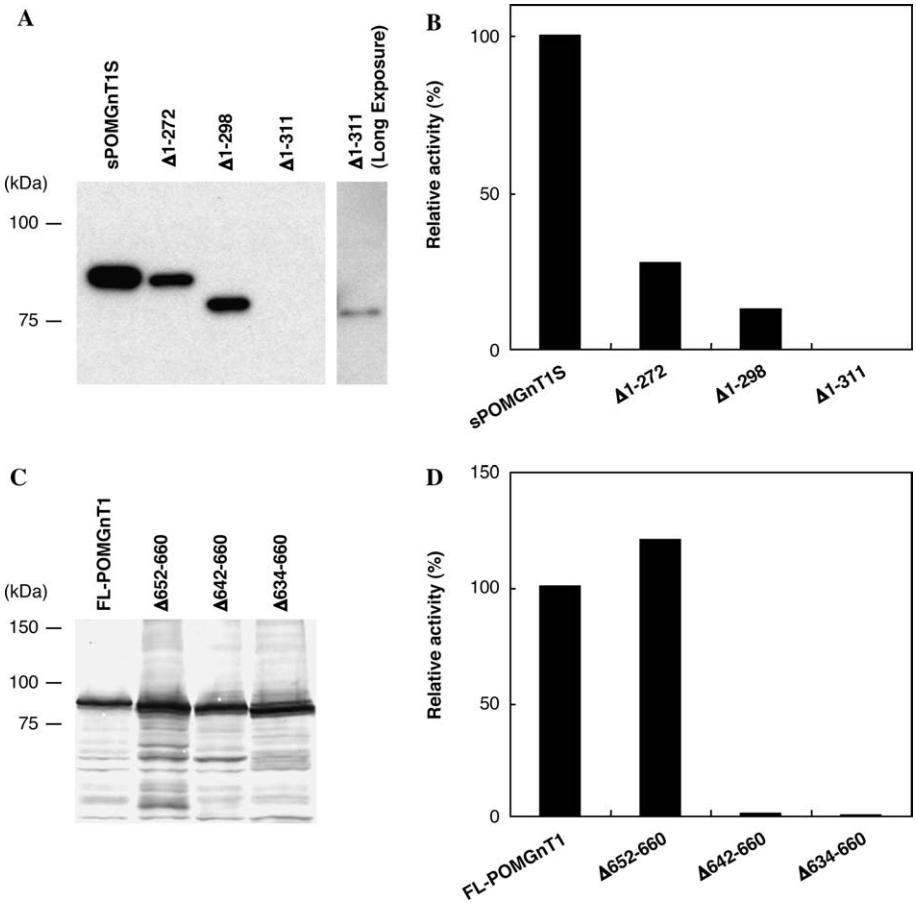

Fig. 2. Expression and activity of truncated POMGnT1 proteins. (A,C) Western blot analysis of Xpress-tagged POMGnT1 proteins. N-terminal truncated mutants (A) and C-terminal mutants (C) were expressed in HEK293T cells, fractionated by SDS–PAGE, transferred to PVDF membrane, and detected by anti-Xpress 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

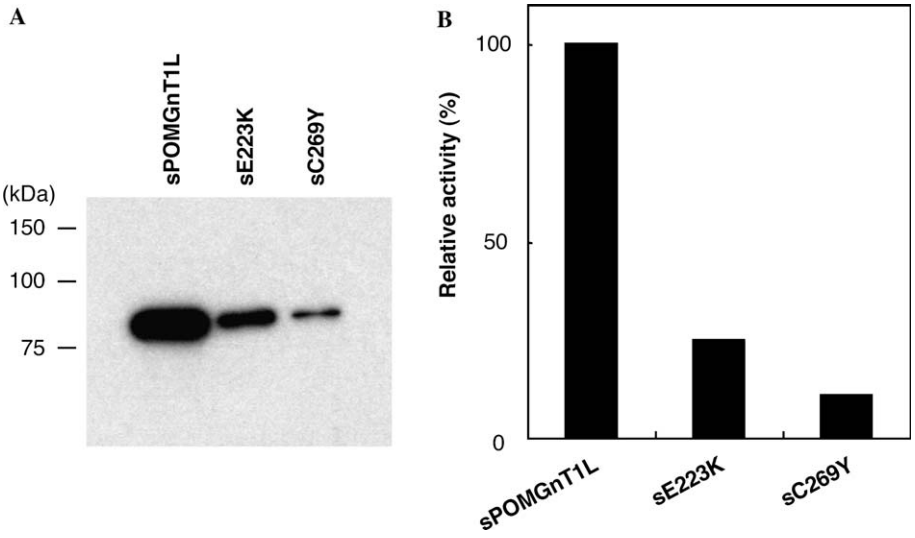

Fig. 3. Expression and activity of point-mutated POMGnT1 proteins. (A) Western blot analysis of Xpress-tagged POMGnT1 proteins. POMGnT1 protein in the culture supernatant (A) was detected by anti-Xpress antibody similar to Fig. 2. (B) POMGnT1 activities were compared to the activity of sPOMGnT1L.

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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