Regular Article

POMGNT1 Is Glycosylated by Mucin-Type O-Glycans

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Protein O-linked mannose β 1,2-*N*-acetylglucosaminyltransferase 1 (POMGNT1) is a Golgi glycosyltransferase that catalyzes the formation of the *N*-acetylglucosamine (GlcNAc) β 1→2Man linkage of O-mannosyl glycan. POMGNT1 is not modified by N-glycans because there are no potential N-glycosylation sites; however, it is not clear whether POMGNT1 is modified by O-glycans. To determine whether POMGNT1 is O-glycosylated, we prepared recombinant human POMGNT1 from HEK293T cells. The recombinant POMGNT1 was recognized by *Sambucus sieboldiana* lectin (SSA), and sialidase digestion of POMGNT1 decreased SSA reactivity and enhanced the reactivity of *Arachis hypogaea* lectin (PNA). These results suggest that POMGNT1 is modified by a sialylated core-1 O-glycan. Next, we analyzed the structures of the O-glycans on POMGNT1 by β -elimination and pyrazolone-labeling methods in combination with mass spectrometry. We identified several mucin-type O-glycans containing (NeuAc)₁(Hex)₁(HexNAc)₁, (NeuAc)₂(Hex)₁(HexNAc)₁, and (NeuAc)₂(Hex)₂(HexNAc)₂. To examine whether the O-glycans affect the functions and properties of POMGNT1, we compared glycosylated and non-glycosylated forms of recombinant sPOMGNT1 for their activity and surface hydrophobicity using the hydrophobic probe 1-anilino-8-naphthalene sulfonate (ANS). POMGNT1 activity and surface hydrophobicity were not affected by the presence or absence of O-glycans.

Key words O-glycosylation; mucin-type O-glycan; glycan structure; protein O-linked mannose β 1,2-*N*-acetylglucosaminyltransferase 1

Protein O-linked mannose β 1,2-*N*-acetylglucosaminyltransferase 1 (POMGNT1) catalyzes the formation of the *N*-acetylglucosamine (GlcNAc) β 1→2Man linkage by transferring GlcNAc from a uridine 5'-diphosphate (UDP)-GlcNAc to an O-mannose of glycoproteins.^{1,2)} POMGNT1 is a typical type II membrane protein and is localized in the Golgi apparatus.³⁾ Human POMGNT1 is composed of 660 amino acids and the following four domains: an N-terminal cytoplasmic tail, a transmembrane domain, a stem domain, and a catalytic domain⁴⁾ (Fig. 1A). The gene encoding *POMGNT1* is responsible for muscle-eye-brain disease (MEB),¹⁾ which is a type of α -dystroglycanopathy and an autosomal recessive disorder characterized by congenital muscular dystrophy with neuronal migration disorder.²⁾ All known mutations in the *POMGNT1* gene in MEB patients cause a loss of enzyme activity, indicating that loss-of-function of the *POMGNT1* gene induces defective O-mannosylation.^{1,5)} More recently, a selective deficiency in glycosylated α -dystroglycan (α -DG) in MEB patients has been found, suggesting that hypoglycosylation of α -DG may be the pathomechanism of MEB.²⁾ In spite of the progress in identifying the disease-causing gene (*POMGNT1*) and understanding its pathomechanism, effective therapies for MEB have not yet been established. There are several therapeutic strategies, for example, gene replacement therapy and enzyme supplement therapy. For the latter strategy, it is important to

characterize the properties of the POMGNT1 protein in detail.

Protein glycosylation is a major post-translational modification.⁶⁾ The major glycans of glycoproteins can be classified into two groups according to their glycan-peptide linkage regions. Those that are linked to asparagine (Asn) residues of polypeptides are termed N-glycans, while those that are linked to serine (Ser) or threonine (Thr) residues are called O-glycans. It is well known that these glycans affect various biological and physiological properties of proteins, such as their stabilities, conformations, interactions, cellular localizations, trafficking and quality control.^{7–12)} Therefore, it is possible that the POMGNT1 activity may be affected by changes in its glycosylation, in addition to genetic mutations of POMGNT1 itself. The purpose of this study was to reveal whether POMGNT1 is modified by glycosylation. Since POMGNT1 is not modified by N-glycans because there are no potential N-glycosylation sites,¹⁾ we focused on POMGNT1 O-glycosylation.

In the present study, POMGNT1 expressed in HEK293T cells was subjected to lectin blotting to detect O-glycosylation and mass spectrometry (MS) analysis to elucidate O-glycan structures. We also examined the effect of O-glycosylation on the enzymatic activity and the surface hydrophobicity of the POMGNT1 protein.

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Fig. 1. Preparation of sPOMGNT1

(A) Schematic representation of human POMGNT1 and recombinant sPOMGNT1. Hatched box, cytoplasmic domain; filled box, transmembrane domain; open box, stem domain; gray box, catalytic domain; checked box, PreScission protease cleavage site; crossed box, His-tag. The numbers above the boxes indicate the amino acid residue numbers of human POMGNT1. (B) For anion-exchange chromatography, the proteins were eluted with a linear gradient of 1–300 mM NaCl (gray line) and monitored by UV absorbance 280 nm (black line). The sPOMGNT1 was fractionated into two peaks that were identified as a non-glycosylated form (peak a) and a glycosylated form (peak b) by FT-ICR-MS analysis. (C) The two purified forms of sPOMGNT1 (0.5 μ g) were stained with the Coomassie brilliant blue.

MATERIALS AND METHODS

Preparation of POMGNT1 The secreted form of human POMGNT1 (92–660) (sPOMGNT1, Fig. 1A) was cloned into the pSecTag2/Hygro plasmid (Life Technologies Japan, Tokyo, Japan) with a C-terminal HRV3C protease recognition sequence and a His-tag. sPOMGNT1 plasmid was transfected into HEK293T with Lipofectamine Plus (Life Technologies) to establish stable cell lines. Recombinant proteins were purified from the culture supernatants. sPOMGNT1 was precipitated with 50% saturated ammonium sulfate and resuspended in phosphate buffered saline (PBS) with 10 mM imidazole. The protein suspension was loaded onto a Ni-NTA resin (QIAGEN, Limburg, the Netherlands). The bound sPOMGNT1 eluted from the column after digestion with PreScission protease (GE Healthcare, Buckinghamshire, England). The sample was further purified by anion-exchange chromatography using a Mono Q column (GE Healthcare). The buffer containing the purified sPOMGNT1 was replaced with 20 mM Tris-HCl (pH 7.5), 150 mM sodium chloride (NaCl), and 1 mM dithiothreitol (DTT) after purification with a Superdex200 column (GE Healthcare).

Preparation of Anti-POMGNT1 Antibody Rabbit anti-serum specific to the C-terminus of POMGNT1 was produced using a synthetic peptide corresponding to residues 648–660 (KEEGAPGAPEQT) of human POMGNT1. A cysteine residue was added to the N-terminus of the synthetic peptide so

that the antigenic peptide could be conjugated to keyhole limpet hemocyanin (KLH). Rabbits were immunized with the antigenic peptide–KLH conjugate.

Lectin and Western Blot Analysis Purified sPOMGNT1 (2 μ g) was treated with sialidase (5 mU) from *Arthrobacter ureafaciens* (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) in 0.1 M ammonium acetate buffer, pH 5.0 for 18 h at 37°C. Heat-inactivated sialidase (100°C for 3 min) was used as a negative control. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% gel) and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk in 137 mM NaCl, 2.7 mM potassium chloride (KCl), 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ and 0.05% Tween 20 (PBS-T) for Western blotting or 3% bovine serum albumin (BSA) in 10 mM Tris-HCl (pH 7.4) containing 140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂ and 0.05% Tween 20 (TBS-T) for lectin blotting, incubated with anti-POMGNT1 polyclonal antibody in 5% skim milk PBS-T or biotin-conjugated lectin in 1% BSA TBS-T, respectively, and treated with anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) (GE Healthcare) in 5% skim milk PBS-T or the Vectastain ABC kit (Vector, Burlingame, CA, U.S.A.) in 1% BSA TBS-T, respectively. Proteins that bound to the antibody or lectins were visualized with an ECL kit (GE Healthcare). The biotin-conjugated lectins, *Sambucus sieboldiana* lectin (SSA) and *Arachis hypogaea* lectin (PNA)

were purchased from J-OIL MILLS (Tokyo, Japan).

β -Elimination and Labeling of *O*-Glycans with Pyrazolone Analogue A mixture containing sPOMGNT1 (50 μ L, 30 μ g) was concentrated using an Amicon Ultra Centrifugal Filter (3K) (Merck Millipore, Darmstadt, Germany). Concentrated sPOMGNT1 (10 μ L, 30 μ g) was treated with 20 μ L each of sodium hydroxide (0.4M) and 0.5M methanolic 1-phenyl-3-methyl-5-pyrazolone (PMP, Tokyo Chemical Industry, Tokyo, Japan) solution, followed by heating at 85°C for 16 h as described previously.¹³⁾ Ethanol precipitation was performed by the addition of a 4-fold volume of cold ethanol and incubation at -30°C for 3 h. Supernatants and precipitated proteins were separated by centrifugation at 15000rpm for 10 min at 4°C. Supernatants were neutralized with 1.0M hydrochloric acid and shaken vigorously with chloroform. The chloroform layer was discarded to remove the excess reagents, and the resultant aqueous layer was subjected to purification by passage through an Iatrobeds silica gel column.

Mass Spectrometry Eluted PMP-labeled *O*-glycans were directly mixed with 2,5-dihydroxybenzoic acid (10 mg/mL in 30% acetonitrile) at a 1:1 dilution, and an aliquot (1 μ L) was deposited onto a stainless steel target plate. Matrix assisted laser desorption/ionization-time-of-flight (MALDI-TOF) data were obtained on an Ultraflex II time-of-flight mass spectrometer (Bruker Daltonics) equipped with a LIFT-TOF/TOF facility controlled by Flex-Control 3.0 software according to the general procedure reported previously. All spectra were obtained in reflectron mode with an acceleration voltage of 25 kV and a reflector voltage of 26.3 kV in the positive-ion mode. The spectra were the sum of 1000 laser shots. All the peaks were selected by FlexAnalysis 3.0 and the sophisticated numerical annotation procedure (SNAP) algorithm, which fits isotopic patterns to the corresponding experimental data. The algorithm provides the monoisotopic mass, intensity, area under the envelope of the isotopic cluster, and resolution of the peaks in the cluster. *O*-Linked-type oligosaccharide structures were estimated from inputs of peak masses into the UniCarbKB database (<http://www.unicarbkb.org/query>).

Assay for POMGNT1 Activity POMGNT1 activity was obtained from the amount of [³H]GlcNAc transferred to a mannosylpeptide as described previously.¹⁴⁾ Briefly, the reaction buffer containing 140 mM maximal electroschock seizure or 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 7.0), 1 mM UDP-[³H]GlcNAc (225000 dpm/nmol), 1 mM mannosylpeptide, 10 mM MnCl₂, 2% Triton X-100, 5 mM AMP, 200 mM GlcNAc, 10% glycerol and enzyme solution was incubated at 37°C for 30 min. The mixture was separated by reversed-phase HPLC with a Wakopak 5C18-200 column (4.6×250 mm) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The peptide separation was monitored continuously at 214 nm, and the radioactivity of each fraction was measured using a liquid scintillation counter.

Measurements of 1-Anilino-8-naphthalene Sulfonate (ANS) Fluorescence Spectra The sPOMGNT1 protein was dissolved in 10 mM potassium phosphate (pH 6.8) with 100 mM KCl to a final concentration of 4 μ M, mixed with 140 μ M ANS (Tokyo Chemical Industry) and then incubated for 1 h at 37°C. The excitation wavelength was 390 nm and emission spectra were recorded at 1-nm intervals from 400 to 600 nm. Fluorescence spectra of ANS were measured with an EnVision multi-label plate reader (PerkinElmer, Inc., Waltham, MA, U.S.A.).

Fig. 2. Detection of Sialylated *O*-Glycans by Lectin and Western Blotting

Purified sPOMGNT1 (peak b in Fig. 1) was treated with sialidase and then subjected to SDS-PAGE. The proteins were transferred to PVDF membranes and stained with (A) anti-POMGNT1 antibody, (B) SSA and (C) PNA. sPOMGNT1 has a molecular weight of approximately 66 kDa. +, sialidase treatment; -, heat-inactivated sialidase treatment.

RESULTS

Preparation of Recombinant Human POMGNT1

The soluble form of the recombinant human POMGNT1 (sPOMGNT1) used in the present study lacked the cytoplasmic tail and transmembrane domain and included a His-tag at the C-terminus (Fig. 1A). To remove the His-tag after anion-exchange chromatography, a protease digestion sequence (PreScission protease cleavage site) was inserted in front of the His-tag region. The sPOMGNT1 protein was produced by HEK293T cells and purified from the culture medium as described in Materials and Methods. In the anion-exchange chromatography, the sPOMGNT1 was eluted as two peaks (Fig. 1B). After cleavage of the His-tag, the two forms of sPOMGNT1 showed different molecular weights; the most abundant isotopic molecular weight in peak a was 65.7 kDa and peak b's molecular weight was 66.7 kDa, when both were analyzed by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). The 65.7 kDa protein corresponds to the predicted molecular weight of sPOMGNT1. This result indicated that the sPOMGNT1 in peak b was modified by the addition of approximately 1 kDa in mass. The purity of peaks a and b is shown in Fig. 1C.

Lectin Blot Analysis and Sialidase Digestion Protein glycosylation is the frequent post-translational modification. Therefore, we first checked whether sPOMGNT1 was *O*-glycosylated because there are no consensus sequences for *N*-glycosylation (N-X-T or N-X-S) in the amino acid sequence of human POMGNT1. To identify the *O*-glycans on sPOMGNT1, sPOMGNT1 in peak b was subjected to sialidase treatment and analyzed with lectin blotting using SSA and PNA. As shown in Fig. 2A, the migration distance of sPOMGNT1 in peak b was not altered by sialidase digestion. The binding of SSA to sPOMGNT1 in peak b could be detected, but this binding of SSA was decreased by sialidase digestion (Fig. 2B). By contrast, PNA binding was enhanced after sialidase digestion (Fig. 2C). From these results, it was concluded that sPOMGNT1 was modified with sialylated core 1 *O*-glycan (Gal β 1 $\rightarrow$ 3GalNAc) because it is known that PNA specifically binds with the Gal β 1 $\rightarrow$ 3GalNAc group but not with the sialylated Gal β 1 $\rightarrow$ 3GalNAc group.¹⁵⁾ Hereafter, sPOMGNT1 in peak b and sPOMGNT1 in peak a are named as glycosylated sPOMGNT1 and non-glycosylated sPOMGNT1, respectively.

Structural Analysis of *O*-Glycans on sPOMGNT1 The

Fig. 3. MALDI-TOF and TOF/TOF MS Spectra of *O*-Glycans on sPOMGNT1

(A) MALDI-TOF MS spectrum showing sPOMGNT1 *O*-glycans. sPOMGNT1 was subjected to β -elimination in 0.4 M NaOH and 0.5 M methanolic PMP at 85°C for 16 h. (B–D) LIFT TOF/TOF MS spectra of sPOMGNT1 *O*-glycans. (B) LIFT with selection of the ion at m/z 1005.66 resulted in the detection of ions at m/z 714.344 and m/z 552.237. (C) LIFT with selection of the ion at m/z 1296.84 resulted in the detection of ions at m/z 1005.506, m/z 843.339, m/z 714.220, and m/z 552.131. (D) LIFT with selection of the ion at m/z 1662.05 resulted in the detection of ions at m/z 1496.732, m/z 1208.434, m/z 1005.180, m/z 917.160, m/z 714.040, and m/z 551.992.

PMP-labeled *O*-glycans were obtained from glycosylated sPOMGNT1 by β -elimination in the presence of PMP and were subjected to normal phase chromatography, MALDI-TOF MS, and MALDI LIFT-TOF/TOF MS (MALDI-TOF-MS/MS employing a potential lift to accelerate fragment ions, LIFT) analyses. As shown in Fig. 3A, three m/z values for the $[M+H]^+$, 1662.05, 1296.84, and 1005.66, were observed by the MALDI-TOF MS spectrum. Because the *O*-glycans were labeled with bis-PMP,¹³⁾ the predicted components of these three peaks correspond to (NeuAc)₂(Hex)₂(HexNac)₂-bis-PMP (m/z 1662.05), (NeuAc)₂(Hex)₁(HexNac)₁-bis-PMP (m/z 1296.84) and (NeuAc)₁(Hex)₁(HexNac)₁-bis-PMP (m/z 1005.66), based on the molecular weights of each component, with PMP=175, HexNac=221, Hex=180, NeuAc=309 and H₂O=18. The differences between these three peaks corresponded to (NeuAc)₁(Hex)₁ (m/z 1662.05–1296.84) and (NeuAc)₁ (m/z 1296.84–1005.66).

To confirm the components of these three peaks, each

peak was subjected to LIFT analysis. The LIFT of the ion at m/z 1005.66 (Fig. 3B) resulted in the detection of ions at m/z 714.344 [(Hex)₁(HexNac)₁-bis-PMP], m/z 552.237 [(HexNac)₁-bis-PMP], m/z 378.130 [(HexNac)₁-PMP], and m/z 175.082 [PMP]. This LIFT spectrum suggested that the structure at m/z 1005.66 was NeuAc-Hex-HexNac-bis-PMP.

The LIFT of the ion at m/z 1296.84 (Fig. 3C) resulted in the detection of ions at m/z 1005.506 [(NeuAc)₁(Hex)₁(HexNac)₁-bis-PMP], m/z 843.339 [(NeuAc)₁(HexNac)₁-bis-PMP], m/z 552.131 [(HexNac)₁-bis-PMP], m/z 378.034 [(HexNac)₁-PMP], and m/z 175.005 [PMP]. This LIFT spectrum suggested that the structure at m/z 1296.84 was NeuAc-Hex-(NeuAc)-HexNac-bis-PMP.

The LIFT of the ion at m/z 1662.05 (Fig. 3D) resulted in the detection of ions at m/z 1496.732 [(NeuAc)₁(Hex)₂(HexNac)₂-bis-PMP], m/z 1208.434 [(NeuAc)₁(Hex)₁(HexNac)₂-bis-PMP], m/z 1005.180 [(NeuAc)₁(Hex)₁(HexNac)₁-bis-PMP], m/z 917.160 [(Hex)₁(HexNac)₂-bis-PMP], m/z 714.040

Fig. 4. Effect of *O*-Glycosylation on sPOMGNT1 Enzyme Activity and Hydrophobicity

(A) Enzyme activities of glycosylated and non-glycosylated sPOMGNT1. POMGNT1 activity was based on the rate of [^3H]GlcNAc transfer to a mannosyl-peptide. (B) Hydrophobicity of glycosylated and non-glycosylated sPOMGNT1. Fluorescence spectra of ANS alone (gray dotted line), non-glycosylated (black broken line) or glycosylated sPOMGNT1 (black line). Protein and ANS concentrations were 4 and 140 μM , respectively. The excitation wavelength was set at 390 nm.

[(Hex)₁(HexNAc)₁-bis-PMP], m/z 551.992 [(HexNAc)₁-bis-PMP] and m/z 377.919 [(HexNAc)₁-PMP]. This LIFT spectrum suggested that the structure at m/z 1662.05 was NeuAc-Hex-(NeuAc-Hex-HexNAc)-HexNAc-bis-PMP.

Taken together with the PNA reactivity, these results suggested that POMGNT1 was modified by a series of mucin-type *O*-glycans such as Sia-Gal-GalNAc-Ser/Thr, Sia-Gal-(Sia)-GalNAc-Ser/Thr and Sia-Gal-(Sia-Gal-HexNAc)-GalNAc-Ser/Thr.

Effect of *O*-Glycosylation on the Functions and Properties of POMGNT1 It is well known that the glycosylation affects the functions and physical properties of proteins, such as enzyme activity and conformation.^{16,17)} To examine the effect of *O*-glycans on the function of POMGNT1, the enzyme activity of glycosylated and non-glycosylated sPOMGNT1 was compared. Glycosylated and non-glycosylated sPOMGNT1 showed almost the same activity (Fig. 4A).

Next, we scanned the protein surface for hydrophobicity using ANS. ANS is widely used as a fluorescent probe for the study of protein conformational changes because of its efficient binding to hydrophobic portions of proteins.^{18,19)} As shown in Fig. 4B, although the addition of each sPOMGNT1 to the ANS solution enhanced fluorescent intensity, the fluorescent spectra of the glycosylated and non-glycosylated sPOMGNT1 proteins were not different.

DISCUSSION

In the present study, we expressed recombinant human sPOMGNT1 using HEK293T cells, and we found that this expression produced both glycosylated and non-glycosylated proteins. When the structures of the *O*-glycans from glyco-

sylated sPOMGNT1 were analyzed, a series of mucin-type *O*-glycans were detected. However, the enzyme activity and the surface hydrophobicity were not affected by the presence or absence of the *O*-glycans. The biological significance of *O*-glycans on POMGNT1 at the present is unclear.

Many mutations have been reported in the *POMGNT1* gene in MEB patients, and all of the known mutations caused a loss of enzyme activity.^{1,5)} Amino acid substitution due to the gene mutations may cause the conformational changes in POMGNT1 that lead to the loss of enzymatic activity. *O*-Glycans are also known to cause protein conformational changes.²⁰⁾ However, as reported here, the presence of *O*-glycans on POMGNT1 did not affect the enzyme activity, indicating that the *O*-glycans may not cause conformational changes that affect enzymatic activity. The molecular weight difference between glycosylated and non-glycosylated sPOMGNT1 and the molecular estimation of mucin-type *O*-glycans attached to POMGNT1 is approximately 1 kDa (Fig. 3A), suggesting that a single mucin-type *O*-glycan may be attached in glycosylated sPOMGNT1. In the case of proteins with multiple mucin-type *O*-glycans, the glycans may affect the conformation of the protein moiety. A single mucin-type *O*-glycan may not affect the enzyme activity or the surface hydrophobicity of POMGNT1. However, a detailed structural study of POMGNT1 and activity examination of each glycoform is necessary to reach the conclusions. Furthermore, it is necessary to determine an attachment site of the *O*-glycan on POMGNT1. Such studies will reveal the effect of the *O*-glycan on the conformation of POMGNT1.

Although we found the presence of a mucin-type *O*-glycan, and it did not affect POMGNT1 enzymatic activity, we must consider it carefully, as we have previously observed

the effect of mutations on full-length membrane-bound and soluble forms of POMGNT1.⁴⁾ POMGNT1 with two single amino acid substitutions in the full-length form did not have any enzymatic activity, but the same proteins in the soluble form showed 10–30% activity, suggesting that both forms might have different conformations. Therefore, the effect of glycosylation on the activity of full-length membrane-bound POMGNT1 must be examined in the future. Glycosylation may also affect the localization and stability of POMGNT1 *in vivo*.

So far there are no therapeutic strategies for MEB. One of the therapeutic methods is an enzyme supplement for patients. Because glycan modifications of POMGNT1 are unnecessary for enzyme activity, recombinant POMGNT1 production is not limited to mammalian cells, and bacterial production may be useful for this purpose. It is necessary to examine whether POMGNT1 produced by bacterial systems actually have enzymatic activity. Furthermore, it goes without saying that development of a specific targeting method from recombinant POMGNT1 to the Golgi from outside cell is another important subject that needs to be resolved in the future.

In this study, we found that POMGNT1 has sialylated core-1 *O*-glycol structures. However, the enzyme activity and the surface hydrophobicity were not affected by the presence of *O*-glycans.

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Conflict of Interest The authors declare no conflict of interest.

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