[11] Defect in Glycosylation that Causes Muscular Dystrophy

By TAMAO ENDO and HIROSHI MANYA

Abstract

Muscular dystrophies are a diverse group of inherited disorders characterized by progressive muscle weakness and wasting. The dystrophin–glycoprotein complex is composed of α-, β-dystroglycan (DG), dystrophin and some other molecules. α- and β-DG stabilize the sarcolemma by acting as an axis through which the extracellular matrix is tightly linked to the cytoskeleton. The relative molecular weights of α-DG differ in different tissues as a result of differential glycosylation. New findings indicate that disrupted glycosylation of α-DG results in a loss of ligand binding, giving rise to both progressive muscle degeneration and abnormal neuronal migration in the brain. This article discusses methods, including purification of α-DG and glycosyltransferase assays involved in α-DG glycosylation.

Overview

Muscular dystrophies are genetic diseases that cause progressive muscle weakness and wasting (Emery, 2002). The causative genes of several muscular dystrophies have been identified in the past 15 years. Recent data suggest that the aberrant protein glycosylation of a specific glycoprotein, α-DG, is the primary cause of some forms of congenital muscular dystrophy (Endo and Toda, 2003; Michele and Campbell, 2003).

Dystroglycan is encoded by a single gene and is cleaved into two proteins, α-DG and β-DG, by posttranslational processing. α-DG is an extracellular peripheral membrane glycoprotein anchored to the cell membrane by binding to a transmembrane glycoprotein, β-DG. The α-DG–β-DG 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 cytoskeleton. α-DG is heavily glycosylated, and its sugars have a role in binding to laminin, neurexin, and agrin (Michele and Campbell, 2003; Montanaro and Carbonetto, 2003). The relative molecular weights of α-DG differ in different tissues as a result of differential glycosylation. We previously demonstrated that a sialyl *O*-mannosyl glycan, Siaα2-3Galβ1-4GlcNAcβ1-2Man, is a laminin-binding ligand of α-DG (Chiba *et al.*, 1997). Mammalian *O*-mannosylation

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is a rare type of protein modification that is observed in a limited number of glycoproteins of brain, nerve, and skeletal muscle (Endo, 2004).

We have identified and characterized glycosyltransferases, protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase (POMGnT1) (Yoshida *et al.*, 2001), and protein *O*-mannosyltransferase 1 (POMT1) and POMT2 (Manya *et al.*, 2004), involved in the biosynthesis of *O*-mannosyl glycans (Fig. 1). We subsequently found that loss of function of the *POMGnT1* gene is responsible for muscle-eye-brain disease (MEB) (Yoshida *et al.*, 2001). It has also been reported that the *POMT1* gene and the *POMT2* gene are responsible for Walker–Warburg syndrome (WWS) (Beltran-Valero de Bernabe *et al.*, 2002; van Reeuwijk *et al.*, 2005). MEB and WWS are autosomal recessive disorders characterized by congenital muscular dystrophies with abnormal neuronal migration. Like MEB and WWS, some muscular dystrophies have been suggested to be caused by abnormal glycosylation of α-DG (e.g., Fukuyama-type congenital muscular dystrophy [FCMD], congenital muscular dystrophy type 1C [MDC1C], limb-girdle muscular dystrophy2I [LGMD2I], congenital muscular dystrophy type 1D [MDC1D], and the myodystrophy [LARGE^{myd}] mouse) (Table I). Highly glycosylated α-DG was found to be selectively deficient in the skeletal muscle of these patients, and the gene products were suggested to be glycosyltransferases (Endo and Toda, 2003; Michele and Campbell, 2003). Therefore, the ability to assay enzyme activities of *O*-mannosylation would facilitate progress in the identification of other *O*-mannosylated proteins, the explanation of their functional roles, and the understanding of muscular dystrophies. This chapter describes the biochemical protocols used to analyze α-DG glycosylation.

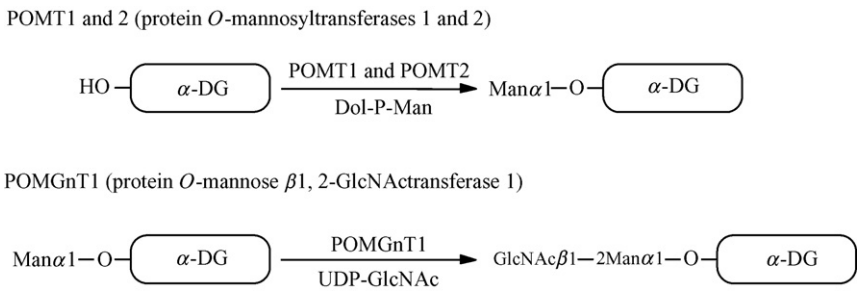

FIG. 1. Biosynthetic pathway of *O*-mannosylglycan of α-DG. Two homologs, POMT1 and POMT2, are responsible for protein *O*-mannosylation, and POMGnT1 is responsible for GlcNAcβ1-2Man linkage of *O*-mannosylglycan.

TABLE I
MUSCULAR DYSTROPHIES POSSIBLY CAUSED BY ABNORMAL GLYCOSYLATION

Condition	Gene	Protein function	Gene locus
Muscle-eye-brain disease (MEB)	*POMGnT1*	GlcNActransferase	1p33
Fukuyama congenital muscular dystrophy (FCMD)	*Fukutin*	Putative glycosyltransferase	9q31
Walker-Warburg syndrome (WWS)	*POMT1* *POMT2*	*O*-Mannosyltransferase	9q34.1 14q24.3
MDC1C Limb-girdle muscular dystrophy 2I (LGMD2I)	*FKRP* (Fukutin-related protein)	Putative glycosyltransferase	19q13.3
MDC1D	*LARGE*	Putative glycosyltransferase	22q12.3
Myodystrophy ($LARGE^{myd}$) mouse	*large*		8 (mouse)

Purification of α-DG

Reagents

Protease inhibitor cocktail (Complete, EDTA-free, Roche Diagnostics, Basel, Switzerland)
Silver Stain ''DAIICHI'' reagent kit (Daiichi Pure Chemicals, Tokyo, Japan)

Solutions

Buffer A (50 m*M* Tris-HCl, pH 7.4, 0.75 m*M* benzamidine, and 0.1 m*M* PMSF)
Buffer B (buffer A containing 300 m*M* GlcNAc)
Buffer C (50 m*M* Tris-HCl, pH 7.4, 140 m*M* NaCl, 1 m*M* $CaCl_2$, 0.75 m*M* benzamidine, 0.1 m*M* PMSF, and 0.1% Triton X-100)
Buffer D (50 m*M* Tris-HCl, pH 7.4, 140 m*M* NaCl, 2 m*M* EGTA, 0.75 m*M* benzamidine, 0.1 m*M* PMSF, and 0.1% Triton X-100)

Materials

Wheat germ agglutinin (WGA)-Sepharose (GE Healthcare Bio-Sciences Corp., Piscataway, NJ)

Laminin-Sepharose (Mouse Engelbreth-Holm-Swarm [EHS] laminin [Biomedical Technologies Inc., Stoughton, MA]) was coupled to CNBr-activated Sepharose 4B (GE Healthcare)]
Centricon 30 and Microcon-100 (Millipore Corp., Billerica, MA)

Method for Purification of α-DG

1. Crude rabbit skeletal muscle membranes were suspended at a protein concentration of 5 mg/ml in 50 m*M* Tris-HCl, pH 7.4, containing 0.5 *M* NaCl and a protease inhibitor cocktail.
2. The suspension was titrated to pH 12 by slowly adding 1 *N* NaOH, extracted for 1 h, and then centrifuged at 140,000*g* for 30 min at 25°.
3. The supernatant was titrated to pH 7.4 and centrifuged at 140,000*g* for 30 min at 4°.
4. The supernatant was circulated over the WGA-Sepharose overnight at 4°. After extensive washing with buffer A, the proteins bound to the WGA-Sepharose were eluted with the buffer B.
5. The eluate was added to 10 m*M* $CaCl_2$ and 10% Triton X-100 (final concentrations of 1 m*M* and 0.1%, respectively), then circulated over laminin-Sepharose overnight at 4° in the presence of 1 m*M* $CaCl_2$. After extensive washing with the buffer C, the proteins bound to the laminin–Sepharose were eluted with the buffer D. The eluate was collected and concentrated using Centricon 30 down to 200 μl.
6. This concentrated sample was further separated by 5–30% sucrose gradient centrifugation. This sample was concentrated and desalted by using Microcon-100.

The purity of the sample was determined by SDS-PAGE with 7.5% gel followed by silver staining using the Silver Stain ''DAIICHI'' reagent kit. To increase the sensitivity of glycoprotein detection, the periodic acid-silver stain method (Dubray and Bezard, 1982) was used with a modification. The gel was treated with 1% periodic acid for 10 min at room temperature before fixation instead of with 0.2% periodic acid treatment for 1 h at 4° after fixation in the original method. By this staining method, rabbit skeletal muscle α-DG was detected as a single prominent broad band around 150 kDa. Bovine peripheral nerve α-DG was also purified by the similar procedures by the sequential WGA-Sepharose and laminin-Sepharose columns without sucrose gradient centrifugation. Thus, obtained bovine peripheral nerve α-DG migrated at approximately 116 kDa.

Method for Glycoprotein Enrichment and Western Blot Analysis (Laminin Overlay Assay)

Functional glycosylation of α-DG (purified α-DG or enrichment of α-DG from tissues) was examined by laminin binding and by dystroglycan antibodies.

Reagents

Anti-α-DG antibodies from mouse (VIA4-1 and IIH6, Upstate Biotechnology, Lake Placid, NY)
Anti-laminin antibody from rabbit (Sigma-Aldrich Corp., St. Louis, MO)
Horseradish peroxidase (HRP)–conjugated anti-mouse or rabbit Ig secondary antibodies (anti-mouse Ig-HRP, anti-rabbit Ig-HRP, GE Healthcare)
Enhanced chemiluminescent reagent kit (ECL Plus, GE Healthcare)

Solutions

Laminin-binding buffer (LBB: 10 m*M* triethanolamine, 140 m*M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, pH 7.6)

Materials

Polystyrene ELISA microplates (Corning Inc., Corning, NY)

1. For enrichment of α-DG from skeletal muscle, skeletal muscle was disrupted with a polytron followed by Dounce homogenization and incubation in 50 m*M* Tris-HCl, pH 7.4, 500 m*M* NaCl, 1% Triton X-100 and protease inhibitors.
2. The solubilized fraction was incubated with WGA-Sepharose beads for 16 h. Pellets formed from the beads, and these were washed three times in TBS containing 0.1% Triton X-100 and protease inhibitors.
3. The beads were then either directly boiled for 2 min in SDS-PAGE loading buffer (Western blotting and laminin overlay assay) or eluted with TBS containing 0.1% Triton X-100, protease inhibitors and 300 m*M* GlcNAc (solid-phase binding assay).
4. Proteins were separated by 3–15% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes and probed with dystroglycan antibodies (VIA4-1 and IIH6), and then incubated with anti-mouse Ig-HRP.

Method for Laminin Overlay Assay

Laminin overlay assay was performed on PVDF membranes using EHS laminin. PVDF membranes were blocked in LBB containing 5% nonfat dry milk followed by incubation with anti-laminin antibody followed by anti-rabbit Ig-HRP. Blots were developed by enhanced chemiluminescence.

Solid-Phase Binding Assay

WGA eluate was diluted 1:50 in TBS and coated on polystyrene ELISA microplates for 16 h at 4°. Plates were washed in LBB and blocked for 2 h in 3% BSA in LBB. EHS laminin was diluted in LBB and applied for 2 h. Wells were washed with 3% BSA in LBB, incubated for 30 min with 1:10,000 anti-laminin antibody followed by anti-rabbit HRP. Plates were developed with *o*-phenylenediamine dihydrochloride and H_2O_2, reactions were stopped with 2 *N* H_2SO_4, and values were obtained on a microplate reader at 492 nm.

Assay for POMT and POMGnT1 Activities

Preparation of Enzyme Sources from HEK293T Cells

Reagents

pcDNA3.1-POMGnT1, *pcDNA3.1-POMT1* and *pcDNA3.1-POMT2* expression plasmids: Human cDNAs encoding *POMGnT1* (Yoshida *et al.*, 2001), *POMT1* (Jurado *et al.*, 1999) (the most common splicing variant of human *POMT1,* which lacks bases 700–765, corresponding to amino acids 234–255) and *POMT2* (Willer *et al.*, 2002) are inserted into mammalian expression vectors, pcDNA3.1/Zeo or pcDNA3.1/Hygro (Invitrogen Corp., Carlsbad, CA).

Lipofectamine transfection reagent and Plus reagent (Invitrogen).

Antibodies: Rabbit antisera specific to the human POMT1, POMT2, and POMGnT1 are produced by using synthetic peptides corresponding to residues 348–362 (YPMIYENGRGSSH) of POMT1, 390–403 (HNTN SDPLDPSFPV) of POMT2 and 649–660 (KEEGAPGAPEQT) of POMGnT1, respectively.

Anti-rabbit IgG conjugated with HRP (GE Healthcare).

Enhanced chemiluminescent reagent kit (ECL, GE Healthcare)

Amplify fluorographic reagent (GE Healthcare)

Kodak BioMax MS X-ray film (GE Healthcare)

Solutions

Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, pH 7.4, and store at 4°.

Homogenization buffer: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 250 m*M* sucrose (SET buffer) with protease inhibitor cocktail (Roche Diagnostics). SET buffer is stored at 4°. Addition of protease inhibitor cocktail is before use.

Materials

Silicone blade cell scraper (SUMILON, SUMITOMO BAKELITE Co., Tokyo, Japan)

Method for Preparation of Membrane Fraction from HEK293T Cells

HEK293T cells are maintained in DMEM supplemented with 10% FBS, 2 m*M* L-glutamine, and 100 units/ml penicillin–50 μg/ml streptomycin at 37° with 5% CO_2. The expression plasmids of human *pcDNA3.1-POMT1* and *pcDNA3.1-POMT2* are transfected into HEK293T cells using Lipofectamine PLUS reagent (Fig. 2A).

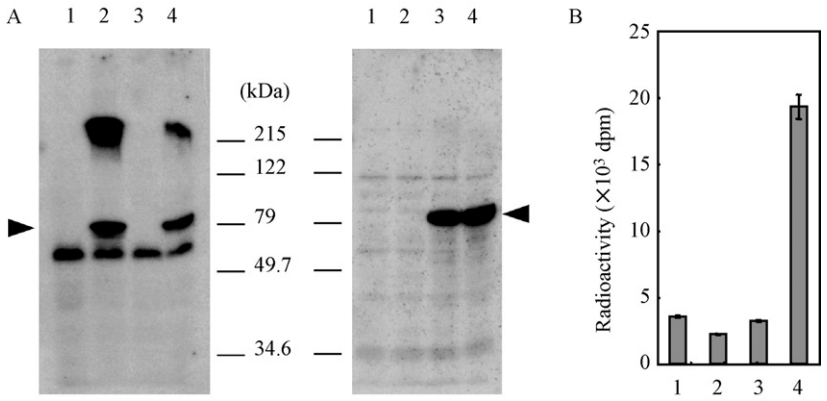

FIG. 2. Western blot analysis of POMT1 and POMT2 (A) and POMT activity of POMT1 and POMT2 (B) expressed in HEK293T cells. Lanes 1, cells transfected with vector alone; lanes 2, cells transfected with human *POMT1*; lanes 3, cells transfected with human *POMT2*; lanes 4, cells co-transfected with *POMT1* and *POMT2*. The proteins (20 μg of membrane fraction) were subjected to SDS-PAGE (10% gel), and the separated proteins were transferred to a PVDF membrane. The PVDF membrane was stained with anti-POMT1 (left panel of A) or anti-POMT2 (right panel of A). Arrowheads indicate the positions of the corresponding molecules. Molecular weight standards are shown in the middle. Reprinted with permission from (Manya *et al.*, 2004). Copyright 2004 National Academy of Sciences, USA.

1. For transient expression of POMT1 and POMT2, subconfluent HEK293T cells are plated on 100-mm culture dish with antibiotic-free 10% FBS-DMEM 1 day before transfection. Cells are transfected at 60–70% confluence. Avoid antibiotics during transfection.
2. Dilute 4 μg of DNA with 750 μl of serum-free DMEM, add the 20 μl of Plus reagent and let stand at room temperature for 15 min (reagent A). In another tube, dilute 30 μl of Lipofectamine reagent with 750 μl of serum-free DMEM (reagent B).
3. Mix reagent A with reagent B and let stand at room temperature for 15 min (reagent C).
4. During step 3, replace medium on the cells with 5 ml of serum-free DMEM.
5. Add reagent C to the cells (from step 4) and incubate at 37° with 5% CO_2 for 3 h.
6. Add 5 ml of 20% FBS-DMEM to the cells (from step 5) and culture for 2–3 days.
7. The culture supernatants are aspirated and the cells are rinsed gently with cold-PBS. Then, 5 ml of cold-PBS is added, and the cells are harvested with a cell scraper and then are collected by centrifugation at 1000*g* for 10 min at 4°.
8. The cell pellet is broken with a tip type sonicator in 500 μl of homogenization buffer (Typical sonication conditions to reach semi-translucent cell suspensions are: 10 cycles of 0.6-sec pulse with 0.4-sec interval, and these procedures are repeated again.). After centrifugation at 900*g* for 10 min, the supernatant is dispensed in halves and subjected to ultracentrifugation at 100,000*g* for 1 h. The precipitates thus obtained are used as microsomal membrane fraction.
9. One-half is used to determine protein concentration and is subjected to Western blotting, and the remaining is used to assess the enzymatic activity.
10. Western blot is performed for detection of products. The microsomal fraction (20 μg) is separated by SDS-PAGE (10% gel), and proteins are transferred to a PVDF membrane. The membrane, after blocking in PBS containing 5% skim milk and 0.5% Tween 20, is incubated with each antibody and then the membrane is treated with anti-rabbit IgG conjugated with HRP. Proteins bound to antibody are visualized with ECL.

Preparation of Membrane Fraction for Enzyme Sources from Brain

1. Brain is harvested from newborn rat (F344/N, Nihon SLC, Shizuoka, Japan) and rinsed with cold-PBS. Immediately, 9 ml of homogenization

buffer for every gram of brain is added and homogenized on ice using a potter's homogenizer (800 rpm, 8 strokes).
2. Nuclei, cellular debris, and connective tissues are removed by centrifugation at 900*g* for 10 min.
3. For preparation of microsomal membranes, the postnuclear supernatant is subjected to ultracentrifugation at 100,000*g* for 1 h. The pellet fraction is aliquoted and stored at −80° until used.

Preparation of GST-α-DG

Reagents and Solutions

LB broth (Invitrogen) supplemented with 50 μg/ml ampicillin.
LB agar plate (1.5 w/v% agar) supplemented with 50 μg/ml ampicillin (Nacalai Tesque, Kyoto, Japan).
Isopropyl-D-thiogalactopyranoside (IPTG) (Invitrogen): prepare 1 *M* stock solution in water, sterilize by filtration, and store at −20°.
50 m*M* $(NH_4)HCO_3$, pH 7.0.

Materials

pGEX-GST-α-DG: The region corresponding to amino acids 313–483 sites of α-DG (Ibraghimov-Beskrovnaya *et al.*, 1992) was amplified from mouse brain total RNA by RT-PCR using the primer set 5′-GGGAATTCCACGCCACACCTACAC-3′ (sense) and 5′-GGG TCTAGAACTGGTGGTAGTACGGATTCG-3′ (antisense), and subcloned it into pGEX-4T-3 vector to express the peptide as a glutathione-S-transferase (GST)-fusion protein (GE Healthcare).
Glutathione-Sepharose column (GSTrap, 1 ml) (GE Healthcare).

Method for Preparation of GST-α-DG

1. BL21(DE3) *E. coli* cells are transformed with *pGEX-GST-α-DG*. Cultures are prepared by growing single colony overnight in LB broth at 37°. The overnight culture is then used to inoculate a fresh 50-ml culture, which is grown at 37° to $A620 = 0.5$. At this point, 1 m*M* IPTG is added to the culture to induce GST-α-DG expression. The induced cells are grown for an additional 4 h and harvested by centrifugation at 6000*g* for 15 min at 4°.
2. The cell pellet is suspended in 10 ml of PBS, pH 7.4, and broken with a tip type sonicator (Semi-translucent cell suspensions are obtained by 3-sec sonication with 3-sec intervals for 5–10 min.). The cell supernatant is recovered by ultracentrifugation at 100,000*g* for 1 h.

3. Recombinant GST-α-DG proteins are purified from the supernatant with a GSTrap column as follows. Pre-equilibrate the GSTrap column with 10 ml of PBS. Load the supernatant onto the column and wash with PBS at a flow rate 0.2 ml/min. The absorbed recombinant GST-α-DG proteins are eluted with 10 ml of 10 m*M* reduced glutathione in PBS at a flow rate 1 ml/min.
4. The purified GST-α-DG is dialyzed with 50 m*M* $(NH_4)HCO_3$, pH 7.0.
5. Protein concentration is determined by BCA assay (PIERCE, Rockford, IL), and the purity of GST-α-DG is checked by SDS-PAGE.
6. The GST-α-DG aliquots are dispensed by 10 μg in microcentrifugal tubes, dried up with a centrifugal evaporator and kept at -80°.

POMT Assay

The POMT activity is based on the amount of [^{3}H]-Man transferred from Dol-P-Man to GST-α-DG (Manya *et al.*, 2004). The reaction product is purified with a glutathione-Sepharose column and radioactivity of mannosyl GST-α-DG is measured by a liquid scintillation counter. Although whole cells instead of membrane fractions may be used as an enzyme source, we recommend using membrane fractions, because mammalian tissues and cells have a low specific activity (Akasaka-Manya *et al.*, 2004).

Reagents

n-Octyl-β-D-thioglucoside and CHAPS (DOJINDO LABORATORIES, Kumamoto, Japan): prepare 10% (w/v) stock solution in water and store at -20°.

Mannosylphosphoryldolichol$_{95}$: [Mannose-6-^{3}H] Dol-P-Man (1.48-2.22 TBq/mmol, American Radiolabeled Chemical, Inc., St. Louis, MO). 1.85 MBq of solution in chloroform and methanol is transferred into screw-cap centrifugal tube and evaporated with centrifugal evaporator (Do not dry completely, $\sim$10 μl solvent should remain.). Add 1 ml of 20 m*M* Tris-HCl (pH 8.0), 0.5% CHAPS and dissolve by sonication with bath type sonicator in ice-cold water (10 cycles of 15-sec pulse with 30-sec interval). Measure radioactivity and then adjust to 40,000 cpm/μl with 20 m*M* Tris-HCl (pH 8.0), 0.5% CHAPS. Aliquot and store at -80°.

Jack bean-α-mannosidase (Seikagaku Corp., Tokyo, Japan): 0.8 U enzyme is dissolved in 50 μl of 0.1 *M* ammonium acetate buffer (pH 4.5). The enzyme solution is dried up with centrifugal evaporator and stored at -20°. The dried enzyme is dissolved with 50 μl of 1 m*M* $ZnCl_2$ before use (Li and Li, 1972).

Solutions

POMT reaction buffer: 10 m*M* Tris-HCl, pH 8.0, 2 m*M* 2-mercaptoethanol, 10 m*M* EDTA, 0.5% *n*-octyl-β-D-thioglucoside. Store at -20°.
PBS containing 1% Triton X-100 (1% Triton-PBS): Store at 4°.
0.5% Triton-Tris buffer: 20 m*M* Tris-HCl (pH 7.4) containing 0.5% Triton X-100. Store at 4°.

Materials

Glutathione-Sepharose 4B (GE Healthcare): Prepare 25% slurry working suspension as follows. Suspension (1 ml, equivalent to 0.75-ml beads) is put in centrifugal tube. Water (9 ml) is added to the suspension and vortexed. After centrifugation at 1000*g* for 1 min, the supernatant is removed by aspiration. The beads are rinsed with 10 ml of PBS and collected by centrifugation. 1% Triton-PBS (2.25 ml) is added and stored at 4°.

Method for POMT Assay

1. The POMT reaction buffer is added to the microsomal membrane fraction at a protein concentration of 4 mg/ml. The fraction is suspended by moderate pipetting and solubilized for 30 min on ice with mild stirring occasionally.
2. 20 μl of solubilized fraction and 2 μl of Dol-P-Man solution are added to the dried GST-α-DG, vortexed, and spun down gently. Immediately, the reaction mixture is incubated at 25° for 1 h. The reaction is stopped by adding 200 μl of 1% Triton-PBS (POMT activity is inactivated in the presence of Triton X-100.).
3. The reaction mixture is centrifuged at 10,000*g* for 10 min. The supernatant is transferred into a screw-cap tube with a packing seal. 400 μl of 1% Triton-PBS and 40 μl of 25% slurry glutathione-Sepharose beads are mixed with the supernatant, and rotated with rotary mixer at 4° for 1 h.
4. After centrifugation at 1000*g* for 1 min, the supernatant is removed by aspiration, and the beads are washed three times with 0.5% Triton-Tris buffer. 2% SDS is added to the beads and boiled at 100° for 3 min. The suspension is cooled down to room temperature and mixed with liquid scintillation cocktail. The radioactivity adsorbed to the beads is measured using a liquid scintillation counter.
5. The incorporation of radioactive mannose into GST-α-DG can be detected by SDS-PAGE and subsequent autoradiography as follows. Instead of 2% SDS in step 4, 20 μl of 2$\times$ loading buffer is added to

the beads followed by boiling at 100° for 3 min. After centrifugation at 1000*g* for 1 min, the supernatant is subjected to SDS-PAGE. Gel is stained with CBB to visualize GST-α-DG, soaked in Amplify fluorographic reagent for 30 min to enhance detection efficiency of tritium, dried with vacuum gel dryer, and exposed to x-ray film.

6. The linkage of the mannosyl residue to peptide is determined as follows. Instead of 2% SDS in step 4, 50 μl of jack bean-α-mannosidase (0.8 U) is added to the beads and incubated with at 37°. α-Mannosidase (0.8 U) is added freshly every 24 h and is incubated for up to 60 h. Inactivated α-mannosidase, prepared by heating the enzyme (100° for 5 min), is used as a control. After incubation, the radioactivities of the supernatant and the beads are measured using a liquid scintillation counter.

Expression and Enzymatic Activity of POMT1 Mutants

An expression vector encoding each mutant of *POMT1* was prepared by site-directed mutagenesis (Akasaka-Manya *et al.*, 2004). For each mutation that found patients with WWS, the *POMT1* gene was modified with a QuickChange Site-Directed Mutagenesis Kit (Stratagene Corp., La Jolla, CA) according to the manufacturer's instructions. The expression plasmids of POMT1 mutants and wild-type POMT2 were co-transfected into HEK293T cells, and the transfected cells were harvested and homogenized after being cultured for 2 days. POMT activity was assayed as described.

POMGnT1 Assay

The POMGnT1 activity is based on the amount of [^{3}H]-GlcNAc transferred from UDP-GlcNAc to benzyl-α-mannose (Benzyl-Man) (Zhang *et al.*, 2003) or mannosylpeptide (Ac-Ala-Ala-Pro-Thr(Man)-Pro-Val-Ala-Ala-Pro-NH_2) (Takahashi *et al.*, 2001). The reaction product is purified with a reverse-phased HPLC, and radioactivity is measured. The mannosylpeptide is not commercially available, but it is possible to use Benzyl-Man, which is commercially available, as a substitute. Although whole cells instead of membrane fractions may be used as an enzyme source, we recommend using membrane fractions, because mammalian tissues and cells have a low specific activity.

Membrane fraction from transiently transfected HEK293T cells (Fig. 3A) and brain were prepared similar to POMT1 and POMT2 as described previously.

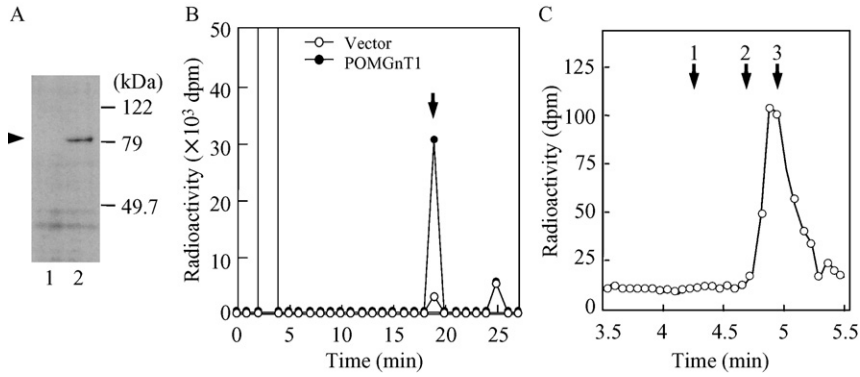

FIG. 3. Western blot analysis of POMGnT1 (A) expressed in HEK293T cells and POMGnT activity (B) expressed in HEK293T cells. (A), Lane 1, cells transfected with vector alone; lane 2, cells transfected with human *POMGnT1*. The proteins (20 μg of membrane fraction) were subjected to SDS-PAGE (10% gel), and the separated proteins were transferred to a PVDF membrane. The PVDF membrane was stained with anti-POMGnT1 antibody. Arrowhead indicates the positions of the corresponding molecule. Molecular weight standards are shown on the right. (B) UDP-[^{3}H]GlcNAc and mannosylpeptide were reacted with membrane fraction in POMGnT1 reaction buffer and then subjected to reversed-phase HPLC. Arrow indicates the elution position of the mannosylpeptide. Vector (open circle), cells transfected with vector alone; POMGnT1 (closed circle), cells transfected with human *POMGnT1*. (C), Analysis of the β-eliminated product by HPAEC-PAD. The radioactive component in B was coinjected with standard disaccharides into a CarboPac PA-1 column. Arrows 1, 2, and 3 indicate the elution positions of authentic standard disaccharide alditols: 1, GlcNAcβ1-6Man$_{OH}$; 2, GlcNAcβ1-4Man$_{OH}$, and GlcNAcβ1-3Man$_{OH}$; 3, GlcNAcβ1-2Man$_{OH}$.

Reagents

UDP-GlcNAc (Sigma-Aldrich): Prepare 1 m*M* stock solution in water and store at -20°.

UDP-GlcNAc [glucosamine-6-^{3}H(N)] (UDP-[^{3}H]-GlcNAc, 0.74–1.66 TBq/mmol, PerkinElmer, Inc., Wellesley, MA): Store at -20°.

Benzyl-α-D-mannopyranoside (Sigma-Aldrich): Prepare 100 m*M* stock solution in 20% ethanol and store at -20°.

Mannosylpeptide (Ac-Ala-Ala-Pro-Thr(Man)-Pro-Val-Ala-Ala-Pro-NH_2) (Takahashi *et al.*, 2001): Prepare 2 m*M* stock solution in water and store at -20°.

Solutions

POMGnT reaction buffer: 140 m*M* MES (adjust to pH 7.0 with NaOH), 2% Triton X-100, 5 m*M* AMP, 200 m*M* GlcNAc, 10% glycerol, 10 m*M* $MnCl_2$. Store at -20° without $MnCl_2$. $MnCl_2$ is added just before use.

Streptococcal β-*N*-acetylhexosaminidase (HEXaseI, Prozyme, San Leandro, CA): 50 mU of enzyme is dissolved with 50 μl of 0.3 *M* citrate phosphate buffer (pH 5.5) and stored at -20°.
0.05 *N* NaOH, 1 *M* $NaBH_4$ and 4 *N* acetic acid solution in water.
0.1% Trifluoroacetic acid (TFA) in water (Solvent A): Add 1 ml of TFA to 1000 ml of HPLC grade water and degas with aspirator before use.
0.1% TFA in acetonitrile (Solvent B): Add 1 ml of TFA to 1000 ml of HPLC grade acetonitrile and degas by sonication before use.

Materials

Reverse phase column: Wakopak 5C18-200 column (4.6 $\times$ 250 mm, Wako Pure Chemical Industries, Osaka, Japan).
AG-50W-X8 (H^+ form, Bio-Rad Laboratories, Hercules, CA)

POMGnT1 Assay

1. 10 μl of 1 m*M* UDP-GlcNAc, 10 μl of UDP-[^{3}H]GlcNAc (100,000 dpm/nmol) and 10 μl of 2 m*M* mannosylpeptide (or 100 m*M* Benzyl-Man) are mixed in microcentrifugal tube and dried up with a centrifugal evaporator.
2. The POMGnT reaction buffer is added to the microsomal membrane fraction at a protein concentration of 2 mg/ml. The fraction is suspended with a bath type sonicator on ice and solubilized by moderate pipetting until transparent. After centrifugation at 10,000*g* for 10 min, 20 μl of the supernatant is added to dried substrate (prepared in step 1), vortexed gently and incubated at 37° for 2 h.
3. The reaction is stopped by boiling at 100° for 3 min. Water (180 μl) is added to the reaction mixture and filtered with a centrifugal filter device.
4. The filtrate is analyzed by reversed phase HPLC on the condition as follows. The gradient solvents are aqueous 0.1% TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B). The mobile phase consists of (1) 100% A for 5 min, (2) a linear gradient to 75% A, 25% B for 20 min, (3) a linear gradient to 100% B for 1 min, and (4) 100% B for 5 min. The peptide separation is monitored by measuring the absorbance at 214 nm, and the radioactivity of each fraction (1 ml) is measured by liquid scintillation counting (Fig. 3B).

The reaction product is characterized by two different methods as follows.

(1) The product is dried up by an evaporator and then incubated with streptococcal β-*N*-acetylhexosaminidase (50 mU) at 37° for 48 h. After incubation, the enzyme is inactivated by boiling at 100° for 3 min. The enzyme-digested sample is re-chromatographed as described previously.

(2) β-Elimination is performed as described below. The product is dissolved in 500 μl of 0.05 *N* NaOH and 1 *M* $NaBH_4$ and incubated for 18 h at 45°. After adjusting the pH to 5 by adding 4 *N* acetic acid, the solution is applied to a column containing 1 ml of AG-50W-X8 (H^+ form), and the column is then washed with 10 ml of water. The effluent and the washing are combined and evaporated. After the remaining borate is removed by repeated evaporation with methanol, the residue is analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Fig. 3C) (Hardy and Townsend, 1994; Takahashi *et al.*, 2001).

Expression and Enzymatic Activity of POMGnT1 Mutants

An expression vector encoding each mutant of *POMGnT1* was prepared by site-directed mutagenesis. Template cDNA for site-directed mutagenesis encoding full-length *POMGnT1* tagged with the His-tag and Xpress epitope was cloned into pcDNA 3.1 as described previously. Site-directed mutagenesis for mutants found in patients with MEB were performed using a QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The expression plasmids of POMGnT1 mutants were transfected into HEK293T cells, and the transfected cells were harvested and homogenized after being cultured for 2 days. POMGnT1 activity was assayed as described.

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