β-*N*-acetylhexosaminidase at 37°C for 48 h. After incubation, the enzyme is inactivated by boiling at 100°C for 3 min. The enzyme-digested sample is rechromatographed as described in **step 3**. (2) β-Elimination is performed as follows: the product is dissolved in 500 µL of 0.05 *N* NaOH and 1 *M* of $NaBH_4$, and incubated for 18 h at 45°C. After adjusting the pH to 5.0 by adding 4 *N* of 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 (*see* **ref. *14***).

4. Notes

1. Do not dry completely; approx 10 µL of solvent should remain.
2. Synthesis of mannosylpeptide substrate: Mannosylpeptide (Ac-Ala-Ala-Pro-Thr(Man)-Pro-Val-Ala-Ala-Pro-NH_2) is synthesized in a solid-phase manner using 9-fluorenyloxymethylcarbonyl (Fmoc) chemistry. Fmoc-Thr(Man)-OH is synthesized as follows: the reaction of phenyl 2,3,4,6-tetra-*O*-benzyl-1-thio-D-mannopyranoside and *N*-benzyloxycarbonyl-L-threonine benzyl ester (Z-Thr-OBzl) in the presence of *N*-iodosuccinimide and trifluoromethanesulfonic acid give the desired protected mannosyl threonine derivative (Z-Thr(Man$(OBzl)_4$)-OBzl) with a 77% yield. After deprotection of all benzyl groups and the Z group by catalytic hydrogenation, Fmoc-OSu is reacted with the residue to give the desired Fmoc-Thr(Man)-OH with a 75% yield. The product is easily purified by solid-phase extraction using a polymeric adsorbent, such as Dianion HP-20 (Nippon Rensui Co., Tokyo, Japan) or Amberlite XAD-2 (Organo, Tokyo, Japan).

   After the final deprotection from the glycopeptide resin, the crude mannosyl peptide is purified on a C18-preparative reversed-phase column (Inertsil ODS-3, 20X 250 mm, GL Sciences Inc., Tokyo, Japan) eluted by mixing solvent A (0.1% TFA in water) with solvent B (0.1% TFA in acetonitrile) at 45°C at a flow rate of 10 mL/min as follows: 25 min at 5% solvent B, linear gradient to 10 min at 35% solvent B. The glycopeptide separation is monitored continuously by measuring the absorbance at 214 nm. The structure of the product is identified by ^{1}H-NMR, amino acid analysis (6 *M* HCl, 110°C, 24 h), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Distilled water is referred to as water in this text.
3. Cell pellets can be stored at –80°C after removal of PBS.
4. Typical sonication conditions to reach semitranslucent cell suspensions are: 10 cycles of 0.6-s pulse with 0.4-s intervals, and these procedures are repeated again.
5. The precipitate can be stored at –80°C after removal of supernatant.
6. Semitranslucent cell suspensions are obtained by 3-s sonication with 3-s intervals for 5–10 min.
7. POMT activity is inactivated in the presence of Triton X-100.

8. When using a screw-cap tube, a packing seal is required to prevent the leakage of radioactivity.

Acknowledgments

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References

1. Chiba, A., Matsumura, K., Yamada, H., et al. (1997) Structures of sialylated *O*-linked oligosaccharides of bovine peripheral nerve α-dystroglycan. The role of a novel *O*-mannosyl-type oligosaccharide in the binding of α-dystroglycan with laminin. *J. Biol. Chem.* **272,** 2156–2162.
2. Yuen, C. T., Chai, W., Loveless, R. W., Lawson, A. M., Margolis, R. U., and Feizi, T. (1997) Brain contains HNK-1 immunoreactive *O*-glycans of the sulfoglucuronyl lactosamine series that terminate in 2-linked or 2,6-linked hexose (mannose). *J. Biol. Chem.* **272,** 8924–8931.
3. Sasaki, T., Yamada, H., Matsumura, K., Shimizu, T., Kobata, A., and Endo, T. (1998) Detection of *O*-mannosyl glycans in rabbit skeletal muscle α-dystroglycan. *Biochim. Biophys. Acta* **1425,** 599–606.
4. Smalheiser, N. R., Haslam, S. M., Sutton-Smith, M., Morris, H. R., and Dell, A. (1998) Structural analysis of sequences *O*-linked to mannose reveals a novel Lewis *X* structure in cranin (dystroglycan) purified from sheep brain. *J. Biol. Chem.* **273,** 23,698–23,703.
5. Chai, W., Yuen, C. T., Kogelberg, H., et al. (1999) High prevalence of 2-mono- and 2,6-di-substituted manol-terminating sequences among O-glycans released from brain glycopeptides by reductive alkaline hydrolysis. *Eur. J. Biochem.* **263,** 879–888.
6. Endo, T. (1999) *O*-mannosyl glycans in mammals. *Biochim. Biophys. Acta* **1473,** 237–246.
7. Michele, D. E., Barresi, R., Kanagawa, M., et al. (2002) Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. *Nature* **418,** 417–422.
8. Michele, D. E. and Campbell, K. P. (2003) Dystrophin-glycoprotein complex: post-translational processing and dystroglycan function. *J. Biol. Chem.* **278,** 15,457–15,460.
9. Montanaro, F. and Carbonetto, S. (2003) Targeting dystroglycan in the brain. *Neuron* **37,** 193–196.
10. Endo, T. (2004) Structure, function and pathology of *O*-mannosyl glycans. *Glycoconj. J.* **21,** 3–7.
11. Yoshida, A., Kobayashi, K., Manya, H., et al. (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. *Dev. Cell* **1,** 717–724.

12. Manya, H., Chiba, A., Yoshida, A., et al. (2004) Demonstration of mammalian protein *O*-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity. *Proc. Natl. Acad. Sci. USA* **101,** 500–505.
13. Beltran-Valero De Bernabe, D., Currier, S., Steinbrecher, A., et al. (2002) Mutations in the *O*-mannosyltransferase gene *POMT1* give rise to the severe neuronal migration disorder Walker-Warburg syndrome. *Am. J. Hum. Genet.* **71,** 1033–1043.
14. Takahashi, S., Sasaki, T., Manya, H., et al. (2001) A new β-1,2-*N*-acetylglucosaminyltransferase that may play a role in the biosynthesis of mammalian *O*-mannosyl glycans. *Glycobiology* **11,** 37–45.
15. Strahl-Bolsinger, S., Gentzsch, M., and Tanner, W. (1999) Protein *O*-mannosylation. *Biochim. Biophys. Acta* **1426,** 297–307.
16. Jurado, L. A., Coloma, A., and Cruces, J. (1999) Identification of a human homolog of the *Drosophila* rotated abdomen gene *(POMT1)* encoding a putative protein *O*-mannosyl-transferase, and assignment to human chromosome 9q34.1. *Genomics* **58,** 171–180.
17. Willer, T., Amselgruber, W., Deutzmann, R., and Strahl, S. (2002) Characterization of POMT2, a novel member of the *PMT* protein *O*-mannosyltransferase family specifically localized to the acrosome of mammalian spermatids. *Glycobiology* **12,** 771–783.
18. Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Sernett, S. W., and Campbell, K. P. (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* **355,** 696–702.
19. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
20. Zhang, W., Vajsar, J., Cao, P., et al. (2003) Enzymatic diagnostic test for Muscle-Eye-Brain type congenital muscular dystrophy using commercially available reagents. *Clin. Biochem.* **36,** 339–344.

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Molecular interaction between fukutin and POMGnT1 in the glycosylation pathway of α-dystroglycan

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Abstract

The recent identification of mutations in genes encoding demonstrated or putative glycosyltransferases has revealed a novel mechanism for congenital muscular dystrophy. Hypoglycosylated α-dystroglycan (α-DG) is commonly seen in Fukuyama-type congenital muscular dystrophy (FCMD), muscle–eye–brain disease (MEB), Walker–Warburg syndrome (WWS), and Large^{myd} mice. POMGnT1 and POMTs, the gene products responsible for MEB and WWS, respectively, synthesize unique *O*-mannose sugar chains on α-DG. The function of fukutin, the gene product responsible for FCMD, remains undetermined. Here we show that fukutin co-localizes with POMGnT1 in the Golgi apparatus. Direct interaction between fukutin and POMGnT1 was confirmed by co-immunoprecipitation and two-hybrid analyses. The transmembrane region of fukutin mediates its localization to the Golgi and participates in the interaction with POMGnT1. Y371C, a missense mutation found in FCMD, retains fukutin in the ER and also redirects POMGnT1 to the ER. Finally, we demonstrate reduced POMGnT1 enzymatic activity in transgenic knock-in mice carrying the retrotransposal insertion in the *fukutin* gene, the prevalent mutation in FCMD. From these findings, we propose that fukutin forms a complex with POMGnT1 and may modulate its enzymatic activity.

Keywords: Fukutin; POMGnT1; Fukuyama-type congenital muscular dystrophy; Muscle–eye–brain disease; α-Dystroglycan

Fukuyama-type congenital muscular dystrophy (FCMD: MIM 253800) is the second most common muscular dystrophy and is among the most prevalent autosomal recessive disorders in Japan. Its clinical symptoms include congenital muscular dystrophy associated with brain malformation and eye disorders [1]. FCMD also is characterized by increased serum creatine kinase levels and prominent necrosis and regeneration in muscle tissue. Magnetic resonance imaging examination reveals pachygyria and transient T2-weighted high intensity. The responsible gene for FCMD, *fukutin*, was identified at 9q31 by linkage analysis and positional cloning [2,3]. Most FCMD patients have a 3-kb retrotransposal insertion in the 3′ noncoding region of *fukutin*. Point mutations in *fukutin* also have been identified, and the severe phenotype generated by two point mutations precludes survival [4]. Fukutin is a 461-amino-acid protein with a predicted molecular weight of 53.7 kDa. It is a type II membrane protein, but its precise function is undetermined.

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Muscle–eye–brain disease (MEB: MIM 253280), Walker–Warburg syndrome (WWS: MIM 236670), congenital muscular dystrophy 1C (MDC1C: MIM 606612), and congenital muscular dystrophy 1D (MDC1D: MIM 608840) are autosomal recessive congenital muscular dystrophies (CMDs) that share similar symptoms to FCMD. Common characteristics include severe muscular dystrophy, neuronal migration defects including lissencephaly type II (cobblestone complex), pachygyria, cerebellar, and brainstem abnormalities, and various ocular anomalies. MEB was first described in Finland, where it is most prevalent. It has since been demonstrated that MEB exists outside of Finland and has a broader clinical spectrum than originally thought [5,6]. MEB presents with more severe ocular abnormalities, including severe congenital myopia, congenital glaucoma, pallor of the optic discs, and retinal hypoplasia. WWS generally presents with the most severe brain involvement and is lethal either prenatally or within the first year of life.

Responsible genes for these disorders have been identified: MEB is causally associated with mutations in protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1); WWS with protein *O*-mannosyltransferase 1 and 2 (POMT1 and POMT2); MDC1C with fukutin-related protein (FKRP); and MDC1D with like-glycosyltransferase (LARGE) [5,7]. Hypoglycosylation of α-dystroglycan (α-DG) is commonly seen in all four disorders. α-DG is a highly glycosylated protein that serves as the laminin-receptor unit in the dystrophin–glycoprotein complex (DGC), linking extracellular laminin to the actin cytoskeleton across the skeletal muscle plasma membrane [7]. Glycosyltransferase activity has been demonstrated only for POMGnT1 and POMT1/POMT2, however [5,8]. POMGnT1, a 660 amino-acid protein with a predicted molecular weight of 75 kDa, adds *N*-acetylglucosamine to *O*-mannose protein. The POMT1/POMT2 complex adds a mannose directly to the polypeptide backbone of α-DG [8,9]. A recent study revealed that LARGE can functionally bypass α-DG glycosylation defects in cells from FCMD, MEB, and WWS [10]. No glycosyltransferase activity has been reported for fukutin; however, as in MEB, WWS, and Large^{myd} mice, FCMD also shows a ~60 kDa reduction in the relative molecular weight of α-DG [11].

POMT1 localizes to the endoplasmic reticulum (ER) and requires the formation of a protein complex with POMT2 to show enzymatic activity [9]. Fukutin is reported to localize to the Golgi apparatus [3,12]; therefore we suspected an interaction between fukutin and Golgi-resident glycosyltransferases that ultimately results in the transfer of sugars to α-DG. Here we demonstrate an interaction between fukutin and POMGnT1 that is mediated by the transmembrane region of fukutin. Decreased POMGnT activity in fukutin-deficient tissues suggests a role for fukutin in the α-DG *O*-mannosylation pathway. These findings demonstrate a function for fukutin for the first time, contributing to a greater understanding of the α-DG modification pathway and the pathomechanism of glycosylation-defect congenital muscular disorders.

Materials and methods

Molecular constructs and mice. Expression vectors were constructed by cloning human *POMGnT1* or *LARGE* into pEF1/V5-HisA (Invitrogen, Carlisbad, CA). Human *fukutin* and its mutated versions with FLAG epitope were cloned into pcDNA3.1+ (Invitrogen), and the fukutin transmembrane region was cloned into pEGFP-N1 (Clonetech, Palo Alto, CA). The transgenic knock-in mice carrying the human 3-kb retrotransposal insertion in the 3′ noncoding region of the *fukutin* gene (fukutin knock-in mice) were generated by a site-directed DNA integration technique [13]. Briefly, lox71 and loxP sites were inserted 5′ and 3′ to exon 10 of mouse *fukutin*, and the exon 10 was excised by Cre expression in mouse embryonic stem (ES) cells. Subsequently, a targeting construct containing exon 10 of human *fukutin* with a retrotransposal insertion put between lox66 and loxP sites was transfected into the ES cells with Cre, and recombination was confirmed by Southern blotting. Mice were maintained in accordance with the animal care guidelines of Otsuka Pharmaceutical Co. Ltd. and Osaka University.

Antibodies. Antibodies used in this study were obtained elsewhere; monoclonal anti-V5 and anti-GFP (Invitrogen), polyclonal anti-V5 and anti-GFP (MBL, Nagoya, Japan), monoclonal and polyclonal anti-FLAG (Sigma, St. Louis, MO), monoclonal anti-β-DG clone 8D5 (Novocastra Laboratories, Newcastle, UK), monoclonal anti-GM130 (BD Biosciences, San Jose, CA), and monoclonal anti-KDEL antibodies (Stressgen, Victoria, Canada). Monoclonal anti-fukutin antibodies were produced as described previously [14]. Affinity-purified sheep anti-α-DG core protein antibody was kindly provided by Dr. Kevin P. Campbell [10].

Cell culture and transfection. Cos-7 and C2C12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfection of cell lines was carried out using Trans IT-LT1 transfection reagent (Mirus, Madison, WI). Transfected cells were grown at 37 °C and harvested 48 h after transfection.

Immunofluorescence analysis. Transfected cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. After blocking with 5% BSA, the cells were incubated with antibodies. Next, the cells were washed and incubated with fluorescent secondary antibodies. After the final wash, cells were observed using fluorescence microscopy.

Binding assay. Transfected cells were lysed with the lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1.0% CHAPS) containing protease inhibitor mixture (Nacalai, Kyoto, Japan). Lysates were precleared with Protein G Sepharose (Amersham Bioscience, Piscataway, NJ), and the supernatants were incubated with antibodies and then mixed with Protein G Sepharose preblocked with 2% BSA. Immunocomplexes were pelleted and washed five times with lysis buffer. Cell lysates and immunocomplexes were analyzed by Western blotting.

Assay for POMGnT1 enzymatic activity. The enzymatic activity assay was performed as described previously [15].

Results

Golgi localization of fukutin and POMGnT1

The intracellular localization of POMGnT1, LARGE, and fukutin was examined in Cos-7, NIH3T3, and C2C12 cells. Cells were transfected with expression constructs encoding POMGnT1–V5, LARGE–V5, or fukutin–FLAG, then immunostained with a Golgi marker (GM130) and anti-V5 or anti-FLAG antibodies. Fukutin–FLAG co-localized with GM130, indicating localization of fukutin to the Golgi apparatus (Fig. 1A). Similar results were observed in Cos-7 and NIH3T3 cells (data not shown). Monoclonal antibodies specific for fukutin (3C7, 7A2, and 1B5) detected overexpressed fukutin with the same localization pattern in transfected cells (Fig. 1B,

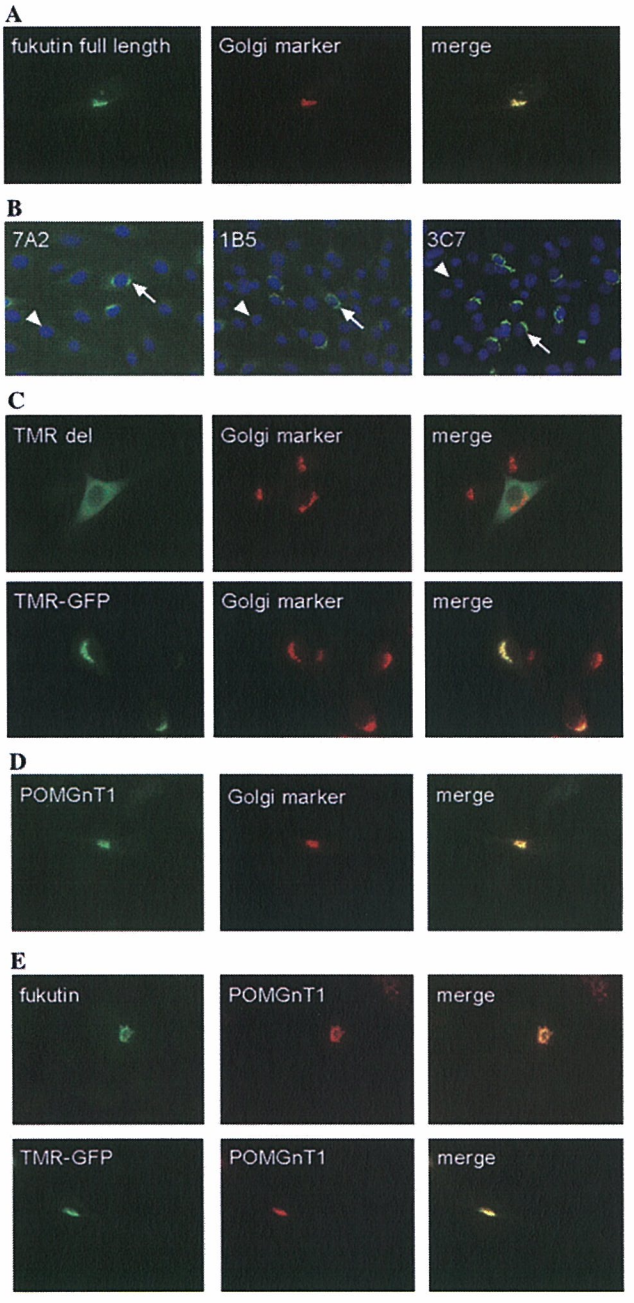

Fig. 1. Co-localization of fukutin and POMGnT1 in the Golgi apparatus. (A) Double labeling of fukutin–FLAG (left panel, green) and the Golgi marker GM130 (middle panel, red) in transfected C2C12 cells. Co-localization can be seen in the merged image (right panel). (B) Labeling of fukutin–FLAG in transfected C2C12 cells with anti-fukutin antibodies (green). The antibodies (7A2, 1B5, and 3C7) were able to detect only overexpressed fukutin in the Golgi (arrows) but could not detect endogenous fukutin (arrowheads). (C) Double labeling of fukutin lacking the transmembrane region (TMR del) or the fukutin transmembrane region (TMR–GFP) (left panels, green) and GM130 (middle panels, red) in transfected C2C12 cells. (D) Double labeling of POMGnT1–V5 (left panel, green) and GM130 (middle panel, red) in transfected Cos-7 cells. (E) Double labeling of fukutin–FLAG or TMR–GFP (left panels, green) and POMGnT1–V5 (middle panels, red) in transfected Cos-7 cells.

arrows) but failed to detect endogenous fukutin (Fig. 1B, arrowheads), suggesting that endogenous fukutin levels are below the range detectable by antibodies. Deletion of the fukutin transmembrane region (TMR del) shifted the localization of fukutin from the Golgi to the cytoplasm (Fig. 1C), whereas the fukutin transmembrane region fused to GFP (TMR–GFP) remained in the Golgi (Fig. 1C). These observations indicate that the transmembrane region targets fukutin to the Golgi apparatus. Double staining of Cos-7 cells transfected with POMGnT1–V5 shows co-localization of POMGnT1–V5 with GM130 (Fig. 1D). We found that LARGE–V5 also localized to the Golgi apparatus (data not shown; also seen in Fig. 3B). Co-transfection and double staining of fukutin–FLAG and POMGnT1–V5 demonstrated co-localization of POMGnT1–V5 with fukutin–FLAG (Fig. 1E). In addition, TMR–GFP also co-localizes with POMGnT1–V5 (Fig. 1E). These data suggest that both proteins are Golgi residents and localize together.

Interaction of fukutin with POMGnT1 through the transmembrane region of fukutin

We performed immunoprecipitation experiments to further investigate potential associations between fukutin and POMGnT1 or LARGE. Since endogenous expression of these proteins is undetectable, we co-transfected fukutin–FLAG with either POMGnT1–V5 or LARGE–V5 into Cos-7 cells and immunoprecipitated fukutin–FLAG from the cell lysates (Fig. 2A and B). As shown in Fig. 2A, POMGnT1–V5 co-precipitated with fukutin–FLAG by anti-FLAG antibody (upper panel, lane 2). In addition, anti-V5 antibody immunoprecipitated both POMGnT1–V5 (upper panel, lane 4) and fukutin–FLAG (lower panel, lane 2) (Fig. 2A). These results indicate an interaction between fukutin and POMGnT1. In contrast, immunoprecipitation did not reveal significant interaction between fukutin–FLAG and LARGE–V5 (Fig. 2B). *In vitro* translated fukutin–FLAG and POMGnT1–V5 also co-precipitated from a reticulocyte cell-free system, suggesting that the interaction is direct (data not shown). We further verified the interaction using bacteria two-hybrid system (data not shown). Together, these data support a direct interaction between fukutin and POMGnT1.

Fig. 1B shows that the transmembrane region of fukutin possesses a Golgi-localization signal. To identify the POMGnT1 binding region in fukutin, we performed immunoprecipitation experiments using Cos-7 cells co-transfected with POMGnT1–V5 and a series of epitope-tagged fukutin truncations containing the transmembrane region [fukutin$_{6-27}$–GFP (TMR); fukutin$_{1-143}$–FLAG (F2); fukutin$_{1-235}$–FLAG (F3); fukutin$_{1-328}$–FLAG (F4); and fukutin$_{1-422}$–FLAG (F5)] (Fig. 2C). Using immunofluorescence, we confirmed the localization of all truncation proteins to the Golgi apparatus (data not shown). POMGnT1–V5 co-immunoprecipitated with the entire series of proteins (Fig. 2D). These experiments also showed that TMR interacts with POMGnT1–V5 (Fig. 2E), indicating that the fukutin transmembrane region is sufficient to bind to POMGnT1.

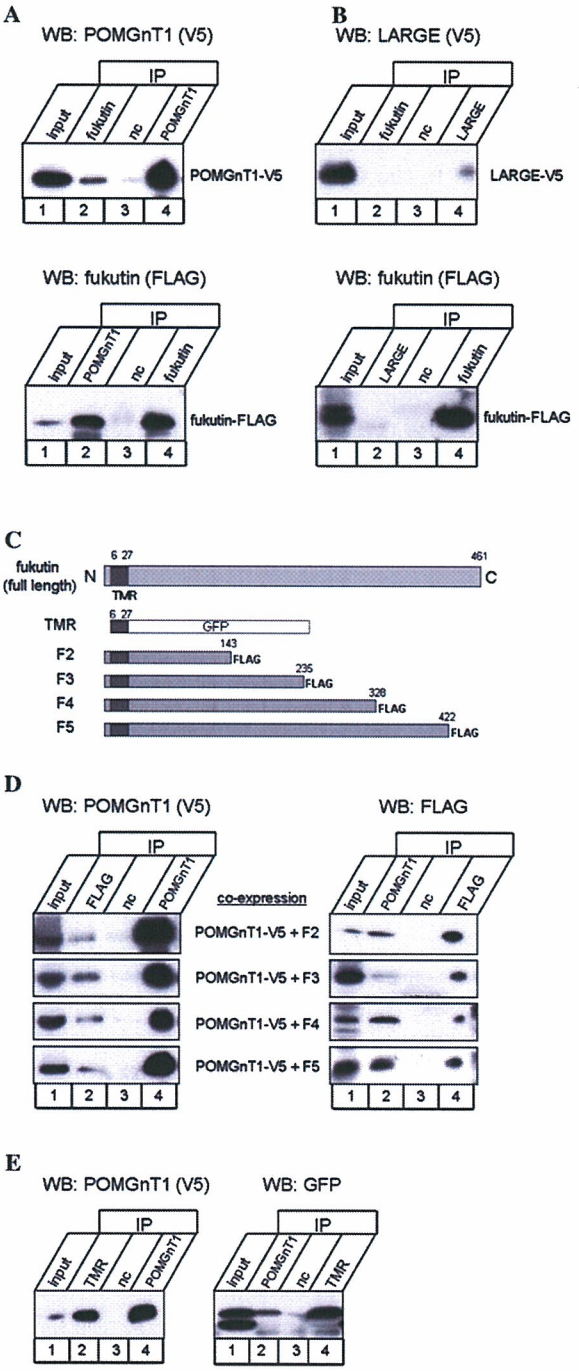

Fig. 2. Interaction of fukutin with POMGnT1 through the transmembrane region. (A) Immunoprecipitation from Cos-7 cells co-expressing fukutin–FLAG and POMGnT1–V5 using anti-FLAG (fukutin) or anti-V5 (POMGnT1) antibodies (IP) and Western blotting with anti-V5 or anti-FLAG antibodies (WB). Nc, non-immune mouse IgG as a negative control; Input, total lysate before immunoprecipitation. (B) Immunoprecipitation from Cos-7 cells co-expressing fukutin–FLAG and LARGE–V5 using anti-FLAG (fukutin) or anti-V5 (LARGE) antibodies (IP). (C) Schematic representation of full-length and truncated fukutin constructs (TMR, F2, F3, F4, and F5). TMR, transmembrane region. (D) Immunoprecipitation from Cos-7 cells co-expressing FLAG-tagged fukutin deletion mutants and POMGnT1–V5 using anti-FLAG (fukutin) or anti-V5 (POMGnT1) antibodies (IP) and Western blotting with anti-V5 or anti-FLAG antibodies (WB). (E) Immunoprecipitation from Cos-7 cells co-expressing fukutin TMR and POMGnT1–V5 using anti-GFP (TMR) or anti-V5 (POMGnT1) antibodies (IP) and Western blotting with anti-V5 or anti-GFP antibodies (WB).

A mutation in fukutin changes the localization of fukutin and POMGnT1

Although the prevalent mutation in FCMD patients is a 3-kb retrotransposal insertion, several missense mutations have been identified [4,16]. We examined the effects of known FCMD point mutations found in FCMD on the cellular localization of fukutin. One of these, Y371C fukutin–FLAG, localized to the ER instead of the Golgi (Fig. 3A). When co-expressed with Y371C, POMGnT1–V5 localization also shifted to the ER (Fig. 3B). The Golgi-specific localization of LARGE–V5 remained unchanged in cells co-expressing LARGE–V5 and Y371C (Fig. 3B). These data suggest that fukutin not only interacts with POMGnT1 but also influences its subcellular localization.

Fukutin-deficiency affects POMGnT1 enzymatic activity

We examined whether fukutin had enzymatic activity related to *O*-mannosylation of α-DG by measuring several sugar nucleotide transfer activities in microsome fractions prepared from cells overexpressing fukutin. These experiments detected no significant glycosyltransferase activity for fukutin (data not shown).

However, we have shown here that fukutin co-localizes and interacts with POMGnT1. Moreover, a similar reduction in the relative molecular weight of α-DG, about 60 kDa, occurs in both FCMD and MEB [11]. To investigate possible effects of fukutin deficiency on POMGnT activity, we measured the POMGnT activity levels (GlcNAc transfer to a mannosyl peptide) in brain microsome fractions prepared from wild type and fukutin knock-in mice that carry the retrotransposal insertion in *fukutin*. Western blotting analysis of wheat-germ agglutinin (WGA)-enriched fractions from wild type brain showed the α-DG core protein as a broad band with a molecular weight of 100–120 kDa, whereas fukutin knock-in mice had hypoglycosylated α-DG (~70 kDa) (Fig. 4A). POMGnT1 enzymatic activity in fukutin knock-in mice was reduced by approximately 30% compared to wild type (Fig. 4B). Real-time PCR analysis confirmed that POMGnT1 mRNA levels were not decreased in the presence of the retrotransposal insertion (data not shown). These data suggest that defects in fukutin may disrupt the *O*-mannosylation pathway of α-DG by affecting POMGnT1 activity.

Discussion

Recent studies have revealed that α-DG glycosylation is required for maintenance of muscle integrity, organization of neuromuscular junction, and neural cell migration in the central nervous system [7,17]. Disruption of DG-matrix linkage due to abnormal glycosylation of α-DG is thought to be the primary cause for several forms of CMDs. The *O*-mannose-linked glycan Siaα2,3-Galβ1,4-GlcNAcβ1,

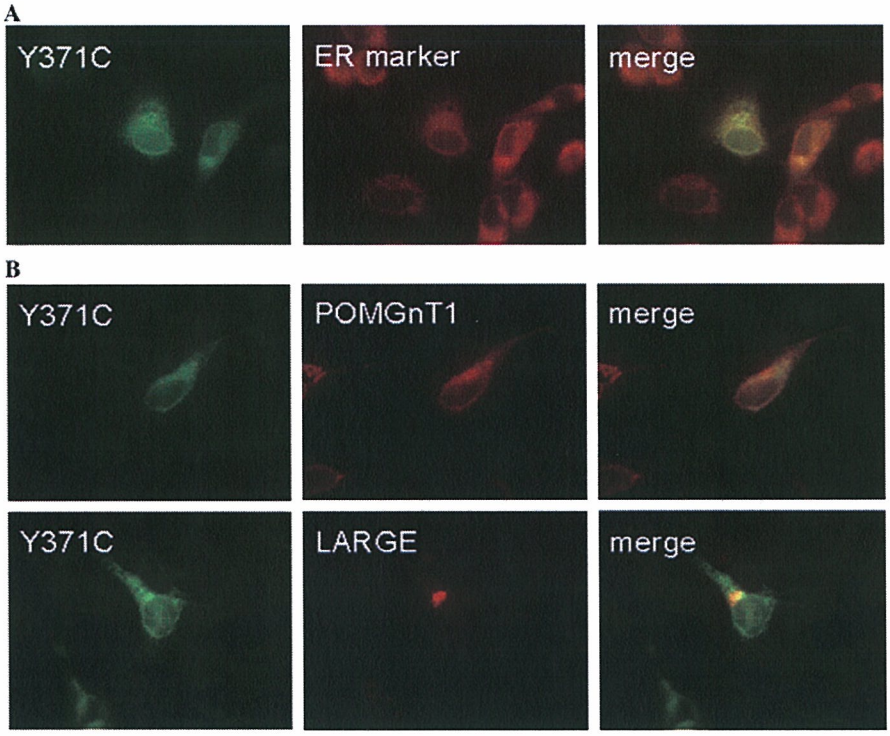

Fig. 3. Fukutin affects POMGnT1 localization. (A) Double labeling of mutant fukutin–FLAG (Y371C) (left panel, green) and the ER marker KDEL (middle panel, red) in transfected Cos-7 cells. Co-localization can be seen in the merged images (right panel). (B) Double labeling of Y371C fukutin–FLAG (left panels, green) and POMGnT1–V5 or LARGE–V5 (middle panels, red) in transfected Cos-7 cell cultures.

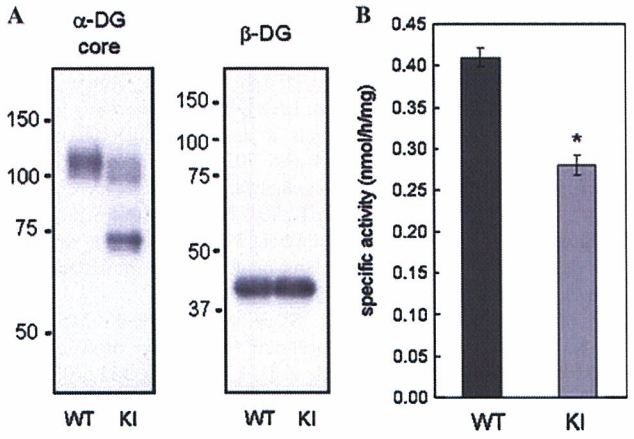

Fig. 4. Fukutin affects POMGnT1 enzymatic activity. (A) Western blotting analysis of WGA-enriched brain DG from wild type (WT) or fukutin knock-in (KI) mice with antibodies to α-DG core peptide (left) and β-DG (right). Staining of β-DG shows that equal amounts of DG were loaded on the gel. (B) POMGnT activities in brain microsome preparations from wild type (WT) and fukutin knock-in (KI) mice ($n = 3$, $p < 0.0001$).

2-Manα1-*O*-Ser/Thr has been identified in both brain and muscle tissue [18,19]. POMT1/POMT2 and POMGnT1 are glycosyltransferases known to be involved in the synthesis of this glycan [5,8]. It has been reported that similar decreases in the molecular weight of α-DG and loss of α-DG–laminin-binding activity occur in FCMD, MEB, WWS, and Large^{myd} mice [10,11]. Since mutations in POMTs and POMGnT1 result in abnormal glycosylation of α-DG in WWS and MEB, the molecular mass shift of α-DG can be attributed to abnormal *O*-mannosylation of α-DG [7]. Fukutin is predicted to belong to a family of enzymes involved in modifying cell surface molecules such as glycoproteins and glycolipids [7]. It is reasonable to hypothesize that fukutin possesses enzymatic activity that contributes to the synthesis of the *O*-mannosyl glycans in α-DG; however, our experiments detected no such activity. Alternatively, we suspected that fukutin might interact and function with other glycosyltransferases, participating indirectly in *O*-mannosylation of α-DG. Consistent with this hypothesis, we have demonstrated a direct interaction between fukutin and POMGnT1 that is mediated through the transmembrane region of fukutin.

This study demonstrates the Golgi-localization of POMGnT1 for the first time. Co-localization studies further support the fukutin–POMGnT1 interaction. Interestingly, our data indicate that a missense mutation in fukutin changes the subcellular localization of POMGnT1. The ~30% decrease in POMGnT1 enzymatic activity in the fukutin-defective (knock-in) mouse brain suggests that fukutin is associated with POMGnT1 activity *in vivo*. Barresi et al. found that overexpression of POMGnT1 cannot rescue the α-DG glycosylation defect in FCMD cells [10]. Thus, POMGnT1 might require fukutin to correct defects in α-DG glycosylation.

It has been reported that a unique molecular chaperone, Cosmc, is required for the activity of human core 1 β3-galactosyltransferase (C1β3Gal-T) that generates the core 1

O-glycan Galβ1-3GalNAcα1-Ser/Thr. Altered *O*-glycosylation caused by deficiency of C1β3Gal-T activity is associated with acquired human diseases such as IgA nephropathy and Tn syndrome, indicating roles for molecular chaperones in the pathogenesis of glycosylation-related human diseases [20]. Thus, proper *O*-mannosylation of α-DG may require chaperones as well as glycosyltransferases. Our results also suggest that fukutin may play a molecular chaperone-like role in *O*-glycosylation.

Only about 15 point mutations have been identified in FCMD patients to date. Recently, two non-Japanese individuals homozygous for truncating mutations in *fukutin* were reported to show more severe, WWS-like phenotypes [21,22]. It has been postulated that most individuals carrying two fukutin point mutations will be embryonic lethal [4]. Homozygous-null mouse embryos carrying targeted disruptions of fukutin die by E9.5 and show basement membrane fragility [23]. However, POMGnT1 mutations cause milder phenotypes even though the mutations deprive POMGnT1 of its enzymatic activity [5,6,24]. The fact suggests additional interaction of fukutin with other protein(s). Genetic studies have implicated POMTs, fukutin, and FKRP in WWS, but these data still only account for a minority of WWS cases [7]. It might be possible that unidentified enzymes and/or chaperones underlie α-DG glycosylation and the pathogenesis of α-dystroglycanopathies and that these proteins comprise a large multi-enzyme complex exerting their enzymatic functions together. Detailed analyses will be required for full understanding of the α-DG glycosylation pathway, which may lead to novel therapeutic strategies for α-dystroglycanopathies.

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References

[1] Y. Fukuyama, M. Osawa, H. Suzuki, Congenital progressive muscular dystrophy of the Fukuyama type—clinical, genetic and pathological considerations, Brain Dev. 3 (1981) 1–29.

[2] T. Toda, M. Segawa, Y. Nomura, I. Nonaka, K. Masuda, T. Ishihara, M. Sakai, I. Tomita, Y. Origuchi, K. Ohno, N. Misugi, Y. Sasaki, K. Takada, M. Kawai, K. Otani, T. Murakami, K. Saito, Y. Fukuyama, T. Shimizu, I. Kanazawa, Y. Nakamura, Localization of a gene for Fukuyama type congenital muscular dystrophy to chromosome 9q31-33, Nat. Genet. 5 (1993) 283–286.

[3] K. Kobayashi, Y. Nakahori, M. Miyake, K. Matsumura, E. Kondo-Iida, Y. Nomura, M. Segawa, M. Yoshioka, K. Saito, M. Osawa, K. Hamano, Y. Sakakihara, I. Nonaka, Y. Nakagome, I. Kanazawa, Y. Nakamura, K. Tokunaga, T. Toda, An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy, Nature 394 (1998) 388–392.

[4] E. Kondo-Iida, K. Kobayashi, M. Watanabe, J. Sasaki, T. Kumagai, H. Koide, K. Saito, M. Osawa, Y. Nakamura, T. Toda, Novel mutations and genotype-phenotype relationships in 107 families with Fukuyama-type congenital muscular dystrophy (FCMD), Hum. Mol. Genet. 8 (1999) 2303–2309.

[5] A. Yoshida, K. Kobayashi, H. Manya, K. Taniguchi, H. Kano, M. Mizuno, T. Inazu, H. Mitsuhashi, S. Takahashi, M. Takeuchi, R. Herrmann, V. Straub, B. Talim, T. Voit, H. Topaloglu, T. Toda, T. Endo, Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1, Dev. Cell 1 (2001) 717–724.

[6] K. Taniguchi, K. Kobayashi, K. Saito, H. Yamanouchi, A. Ohnuma, Y.K. Hayashi, H. Manya, D.K. Jin, M. Lee, E. Parano, R. Falsaperla, P. Pavone, R. Van Coster, B. Talim, A. Steinbrecher, V. Straub, I. Nishino, H. Topaloglu, T. Voit, T. Endo, T. Toda, Worldwide distribution and broader clinical spectrum of muscle-eye-brain disease, Hum. Mol. Genet. 12 (2003) 527–534.

[7] M. Kanagawa, T. Toda, The genetic and molecular basis of muscular dystrophy: roles of cell-matrix linkage in the pathogenesis, J. Hum. Genet. 51 (2006), doi:10.1007/s10038-006-0056-7.

[8] H. Manya, A. Chiba, A. Yoshida, X. Wang, Y. Chiba, Y. Jigami, R.U. Margolis, T. Endo, Demonstration of mammalian protein *O*-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity, Proc. Natl. Acad. Sci. USA 101 (2004) 500–505.

[9] K. Akasaka-Manya, H. Manya, A. Nakajima, M. Kawakita, T. Endo, Physical and functional association of human protein *O*-mannosyltransferases 1 and 2, J. Biol. Chem. 281 (2006) 19339–19345.

[10] R. Barresi, D.E. Michele, M. Kanagawa, H.A. Harper, S.A. Dovico, J.S. Satz, S.A. Moore, W. Zhang, H. Schachter, J.P. Dumanski, R.D. Cohn, I. Nishino, K.P. Campbell, LARGE can functionally bypass α-dystroglycan glycosylation defects in distinct congenital muscular dystrophies, Nat. Med. 10 (2004) 696–703.

[11] D.E. Michele, R. Barresi, M. Kanagawa, F. Saito, R.D. Cohn, J.S. Satz, J. Dollar, I. Nishino, R.I. Kelley, H. Somer, V. Straub, K.D. Mathews, S.A. Moore, K.P. Campbell, Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies, Nature 418 (2002) 417–422.

[12] H. Matsumoto, S. Noguchi, K. Sugie, M. Ogawa, K. Murayama, Y.K. Hayashi, I. Nishino, Subcellular localization of fukutin and fukutin-related protein in muscle cells, J. Biochem. 135 (2004) 709–712.

[13] K. Araki, M. Araki, K. Yamamura, Targeted integration of DNA using mutant lox sites in embryonic stem cells, Nucleic Acids Res. 25 (1997) 868–872.

[14] N.T. Man, G.E. Morris, Production of panels of monoclonal antibodies by the hybridoma method, in: G.E. Morris (Ed.), Methods in Molecular Biology, Epitope Mapping Protocols, vol. 66, Humana Press, Totowa, 1996, pp. 377–389.

[15] S. Takahashi, T. Sasaki, H. Manya, Y. Chiba, A. Yoshida, M. Mizuno, H. Ishida, F. Ito, T. Inazu, N. Kotani, S. Takasaki, M. Takeuchi, T. Endo, A new β-1,2-*N*-acetylglucosaminyltransferase that may play a role in the biosynthesis of mammalian *O*-mannosyl glycans, Glycobiology 11 (2001) 37–45.

[16] K. Kobayashi, J. Sasaki, E. Kondo-Iida, Y. Fukuda, M. Kinoshita, Y. Sunada, Y. Nakamura, T. Toda, Structural organization, complete genomic sequences and mutational analyses of the Fukuyama-type congenital muscular dystrophy gene, *fukutin*, FEBS Lett. 489 (2001) 192–196.

[17] M. Taniguchi, H. Kurahashi, S. Noguchi, T. Fukudome, T. Okinaga, T. Tsukahara, Y. Tajima, K. Ozono, I. Nishino, I. Nonaka, T. Toda,

Aberrant neuromuscular junctions and delayed terminal muscle fiber maturation in α-dystroglycanopathies, Hum. Mol. Genet. 15 (2006) 1279–1289.

[18] A. Chiba, K. Matsumura, H. Yamada, T. Inazu, T. Shimizu, S. Kusunoki, I. Kanazawa, A. Kobata, T. Endo, Structures of sialylated *O*-linked oligosaccharides of bovine peripheral nerve α-dystroglycan. The role of a novel *O*-mannosyl-type oligosaccharide in the binding of α-dystroglycan with laminin, J. Biol. Chem. 272 (1997) 2156–2162.

[19] T. Sasaki, H. Yamada, K. Matsumura, T. Shimizu, A. Kobata, T. Endo, Detection of *O*-mannosyl glycans in rabbit skeletal muscle α-dystroglycan, Biochim. Biophys. Acta 1425 (1998) 599–606.

[20] T. Ju, R.D. Cummings, Protein glycosylation: chaperone mutation in Tn syndrome, Nature 437 (2005) 1252.

[21] F. Silan, M. Yoshioka, K. Kobayashi, E. Simsek, M. Tunc, M. Alper, M. Cam, A. Guven, Y. Fukuda, M. Kinoshita, K. Kocabay, T. Toda, A new mutation of the *fukutin* gene in a non-Japanese patient, Ann. Neurol. 53 (2003) 392–396.

[22] D. Beltrán-Valero de Bernabé, H. van Bokhoven, E. van Beusekom, W. van den Akker, S. Kant, W.B. Dobyns, B. Cormand, S. Currier, B. Hamel, B. Talim, H. Topaloglu, H.G. Brunner, A homozygous nonsense mutation in the *fukutin* gene causes a Walker-Warburg syndrome phenotype, J. Med. Genet. 40 (2003) 845–848.

[23] H. Kurahashi, M. Taniguchi, C. Meno, Y. Taniguchi, S. Takeda, M. Horie, H. Otani, T. Toda, Basement membrane fragility underlies embryonic lethality in *fukutin*-null mice, Neurobiol. Dis. 19 (2005) 208–217.

[24] H. Manya, K. Sakai, K. Kobayashi, K. Taniguchi, M. Kawakita, T. Toda, T. Endo, Loss-of-function of an *N*-acetylglucosaminyltransferase, POMGnT1, in muscle-eye-brain disease, Biochem. Biophys. Res. Commun. 306 (2003) 93–97.

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Physical and Functional Association of Human Protein *O*-Mannosyltransferases 1 and 2*

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A defect of protein *O*-mannosylation causes congenital muscular dystrophy with brain malformation and structural eye abnormalities, so-called Walker-Warburg syndrome. Protein *O*-mannosylation is catalyzed by protein *O*-mannosyltransferase 1 (POMT1) and its homologue, POMT2. Coexpression of POMT1 and POMT2 is required to show *O*-mannosylation activity. Here we have shown that POMT1 forms a complex with POMT2 and the complex possesses protein *O*-mannosyltransferase activity. Results indicate that POMT1 and POMT2 associate physically and functionally *in vivo*. Recently, three mutations were reported in the *POMT1* gene of patients who showed milder phenotypes than typical Walker-Warburg syndrome. We coexpressed these mutant POMT1s with POMT2 and found that none of them had any activity. However, all POMT1 mutants, including previously identified POMT1 mutants, coprecipitated with POMT2. These results indicate that the mutant POMT1s could form heterocomplexes with POMT2 but that such complexes are insufficient for enzymatic activity.

Dystrophin-glycoprotein complex is composed of α-, β-dystroglycan (DG),[2] dystrophin, and some other molecules. Dystrophin-glycoprotein complex is thought to act as a transmembrane linker between the extracellular matrix and intracellular cytoskeleton (1). α-DG is a central component of the dystrophin-glycoprotein complex and is heavily glycosylated, and its sugars have a role in binding to extracellular matrixes such as laminin, neurexin, and agrin (2). Previously we reported 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 (3). We have also found that muscle-eye-brain disease (OMIM 253280), a congenital muscular dystrophy, was caused by mutations in the gene encoding POMGnT1 (protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1), which forms a GlcNAcβ1–2Man linkage of *O*-mannosyl glycans (4, 5).

Protein *O*-mannosyltransferase 1 (POMT1) and its homologue POMT2 are responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis (6). Mutations in the *POMT1* and *POMT2* genes are considered to be the cause of Walker-Warburg syndrome (WWS: OMIM 236670), an autosomal recessive developmental disorder associated with congenital muscular dystrophy, neuronal migration defects, and ocular abnormalities (7, 8). Previously, seven mutations in the *POMT1* gene (G76R, Q303X, Q385X, L421del, V428D, V703fs, and G722fs) were identified in patients with WWS (7, 9). We have demonstrated that these mutations in the *POMT1* gene lead to defects of POMT activity (10). This may cause a defect in α-DG glycosylation and result in failure of binding to laminin or other molecules in the extracellular matrix and interrupt normal muscular function and migration of neurons in developing brain. Recently, other mutations in the *POMT1* gene were found (11–13). Among them, three mutations (G65R, A200P, and M582C) display milder pathology than typical WWS. Patients with A200P mutation are characterized by mild mental retardation and microcephaly without brain malformation (14), and patients with G65R and M582C mutations are characterized by calf hypertrophy, microcephaly, and severe mental retardation, but no eye abnormalities (15). These findings suggested that these mutations would not completely abolish enzymatic activity, which prompted us to examine POMT activities of mutated POMT1 with milder phenotypes.

PMT, protein *O*-mannosyltransferase, is evolutionarily conserved from prokaryotes, such as *Mycobacterium tuberculosis*, to eukaryotes, such as yeast, *Drosophila*, mouse, and human (16–19). In yeast *Saccharomyces cerevisiae*, *O*-mannosylation is required for the stability, correct localization, and/or function of proteins. Yeast *O*-mannosylation is initiated in the lumen of the endoplasmic reticulum (ER) by a family of PMTs that catalyze the transfer of a mannosyl residue from dolichol phosphate mannose to Ser/Thr residues of proteins (16). *S. cerevisiae* has seven PMT homologues (Pmt1p-7p) that share almost identical hydropathy profiles. The hydropathy profiles predict that PMTs are integral membrane proteins with multiple transmembrane domains (16, 20–22). The PMT family is

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[2] The abbreviations used are: α-DG, α-dystroglycan; ER, endoplasmic reticulum; HEK, human embryonic kidney; WWS, Walker-Warburg syndrome; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonic acid; PMT, protein *O*-mannosyltransferase; POMT, protein *O*-mannosyltransferase.

classified phylogenetically into the PMT1, PMT2, and PMT4 subfamilies. Members of the PMT1 subfamily (Pmt1p and Pmt5p) interact heterophilically with those of the PMT2 subfamily (Pmt2p and Pmt3p), whereas the single member of the PMT4 subfamily (Pmt4p) acts as a homophilic complex (17, 23). Although Pmt1p-4p and Pmt6p have *O*-mannosyltransferase activity by themselves (23), complex formation is essential for maximal transferase activity of yeast PMT family members (17, 24).

In human, transferase activity may also require formation of a heterocomplex of POMT1 and POMT2, because cotransfection of *POMT1* and *POMT2* up-regulates POMT activity in human embryonic kidney (HEK) 293T cells whereas expression of only one of these proteins does not (6). However, no direct evidence for a physical interaction between POMT1 and POMT2 proteins has been obtained so far. Here, we have demonstrated that POMT1 and POMT2 form a functional complex *in vivo* using immunoprecipitating techniques. Furthermore, we showed that the mutations of POMT1 protein found in WWS patients do not prevent complex formation with POMT2 but they do abolish activity of the complex.

EXPERIMENTAL PROCEDURES

Vector Construction of POMT1 Mutants and POMT2—Human *POMT1* cDNA was used for site-directed mutagenesis and was cloned into pcDNA 3.1 (Invitrogen) as described previously (6). For each of the three mutations (G65R, A200P, M582C) examined in this study, the *POMT1* gene was modified with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions as described previously (10). The three mutants were generated with the following primer pairs: G65R, 5′-CTTCTTGGATGACAGTAGGCCGCCATTTGGCC-3′ and 5′-GGCCAAATGGCGGCCTACTGTCATCCAAGAAG-3′; A200P, 5′-GTCGCTTGTTCCTGTCCAGTGGGCATCAAG-3′ and 5′-CTTGATGCCCACTGGACAGGAACAAGCGAC-3′; M582C, 5′-CAATATTGCCTACTGCCTGCACCCCAGGAC-3′ and 5′-GTCCTGGGGTGCAGGCAGTAGGCAATATTG-3′. All mutant clones were sequenced to confirm the presence of the mutations. Other mutants (G76R, L421del, V428D) and human *POMT2* cDNA were obtained as described previously (10).

Detergents—CHAPS, CHAPSO, *N,N*-bis(3-D-gluconamidopropyl)cholamide (BIGCHAP), *N,N*-bis(3-D-gluconamidopropyl)deoxycholamide (deoxy-BIGCHAP), *n*-octyl-β-D-glucoside, *n*-heptyl-β-D-thioglucoside, *n*-octyl-β-D-thioglucoside, *n*-dodecyl-β-D-maltoside, *n*-octanoyl-*N*-methylglucamide (MEGA-8), *n*-nonanoyl-*N*-methylglucamide (MEGA-9), *n*-decanoyl-*N*-methylglucamide (MEGA-10), β-D-fructopyranosyl-α-D-glucopyranoside monodecanoate (SM-1000), β-D-fructopyranosyl-α-D-glucopyranoside monododecanoate (SM-1200), sodium cholate, sodium deoxycholate were purchased from Dojindo (Kumamoto, Japan), and digitonin and Triton X-100 were from Nacalai Tesque (Kyoto, Japan).

Cell Solubilization—HEK293T cells or transfected HEK293T cells were homogenized in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol with protease inhibitor mixture (3 μg/ml of pepstatin A, 1 μg/ml of leupeptin, 1 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride). 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. Microsomal fractions thus obtained were solubilized with buffer (20 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol, 10 mM EDTA) containing detergents at different concentrations at 4 °C. The solubilized microsomal membrane fractions thus obtained were subjected to POMT activity assay and Western blot analysis.

Expression of POMT1 with POMT2—Expression plasmids were transfected into HEK293T cells using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Cells were incubated for 3 days at 37 °C to produce POMT1 and POMT2 proteins.

Immunocytochemical Analysis—The expression vectors encoding Myc-tagged POMT1 and FLAG-tagged POMT2 were transfected into the HEK293T cells using Lipofectamine Plus reagent. Transfected HEK293T cells were grown on poly-D-lysine-coated culture slides (BD Biosciences), fixed with 4% formaldehyde for 30 min, and then permeabilized with cold methanol. After treatment with 1% bovine serum albumin in PBS at room temperature for 1 h, cells were incubated with anti-FLAG, anti-Myc (9E10), and anti-calreticulin (Santa Cruz Biotechnology, Santa Cruz, CA) for overnight at 4 °C. Next, cells were washed with PBS and incubated with Alexa Fluor 488-conjugated anti-mouse IgG or Alexa Fluor 546-conjugated anti-goat IgG (Molecular Probes, Eugene, OR) for 1 h at room temperature. After a final wash with PBS, cells were observed, using fluorescence microscopy. We also used the following fluorescent primary antibodies: fluorescein isothiocyanate-conjugated anti-FLAG (Sigma) and TRITC-conjugated anti-Myc (Santa Cruz Biotechnology).

Immunoprecipitation—Microsomal fractions were lysed with assay buffer (20 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside) in a final concentration of 2 mg/ml for 5 h at 4 °C. After solubilization, proteins were subjected to centrifugation at 10,000 × *g* for 30 min and precleaned with CL-6B-Sepharose (Sigma). Precleaned supernatants were mixed with anti-Myc (9E10)-agarose conjugate (Santa Cruz Biotechnology) and incubated overnight. After three washes with the assay buffer, the agarose beads were suspended in sample buffer. Samples were subjected to Western blot analysis. To assay POMT activity, the precipitated beads were suspended in 60 μl of assay buffer and used as the enzyme source.

Western Blot Analysis—The microsomal fractions (20 μg) or immunoprecipitated samples were separated by SDS-PAGE (7.5% gel), and proteins were transferred to a polyvinylidene difluoride 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 (6) or anti-Myc (A-14) antibody (Santa Cruz Biotechnology), and treated with anti-rabbit IgG conjugated with horseradish peroxidase or anti-mouse IgG conjugated with horseradish peroxidase (GE Healthcare). Proteins that bound to the antibody were visualized with an ECL kit (GE Healthcare). As reported previously (6), anti-POMT1 and anti-POMT2 polyclonal antibodies did not detect endogenous POMT1 and POMT2, respectively.

Each antibody is specific for the respective recombinant protein; that is, they do not cross-react with each other.

Assay for POMT Activity—POMT activity was based on the amount of mannose transferred from dolichol phosphate mannose to a glutathione *S*-transferase fusion α-DG (GST-αDG) as described previously (6) with a slight modification. Briefly, assays were carried out in a 20-μl reaction volume containing 20 mM Tris-HCl, pH 8.0, 100 nM ^{3}H-labeled dolichol phosphate mannose (Dol-P-[^{3}H]Man, 125,000 dpm/pmol; American Radiolabeled Chemicals, St. Louis, MO), 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside, 10 μg of GST-α-DG, and 80 μg of microsomal membrane fraction. Microsomal fractions were solubilized with buffer containing 20 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside for 1 h, and the reaction was initiated by adding Dol-P-[^{3}H]Man.

After a 1-h incubation at 25 °C, the reaction was stopped by adding 150 μl of PBS containing 1% Triton X-100, and the reaction mixture was centrifuged at 10,000 × *g* for 10 min. The supernatant was removed, mixed with 400 μl of PBS containing 1% Triton X-100 and 10 μl of glutathione-Sepharose 4B beads (GE Healthcare), 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 with a liquid scintillation counter.

RESULTS

Colocalization of POMT1 and POMT2 in the ER—To determine the subcellular localization of POMT1 and POMT2, HEK293T cells were transfected with expression constructs encoding each protein. Anti-POMT1 and anti-POMT2 antibodies did not stain untransfected HEK293T cells (data not shown), suggesting that HEK293T cells express little POMT1 and POMT2. Myc-tagged POMT1 colocalized precisely with anti-calreticulin (ER marker). Calreticulin staining localized around the nuclei and overlapped with POMT1-Myc staining (Fig. 1*A*). On the other hand, FLAG-tagged POMT2 also colocalized with calreticulin (Fig. 1*B*), in agreement with a previous finding that POMT2 localized to the ER membrane (25). Double staining of HEK293T cells by both POMT1 and POMT2 demonstrated their colocalization (Fig. 1*C*). Thus, we concluded that POMT1 and POMT2 reside in the ER.

Solubilization of POMT Activity—Colocalization of POMT1 and POMT2 in the ER and the requirement of coexpression of both components for protein *O*-mannosylation (6) suggest that POMT1-POMT2 complex formation is necessary for POMT activity. To obtain physical evidence for complex formation between POMT1 and POMT2, we solubilized membrane proteins with various detergents and attempted to detect a complex by immunoprecipitation. In yeast, members of the PMT1 family were found to form heteromeric complexes with members of the PMT2 subfamily *in vivo* by coimmunoprecipitation experiments after solubilization of membrane proteins with 0.35% sodium deoxycholate and 0.5% Triton X-100 (17). Human POMT activity could not be detected when Triton X-100 was used as a detergent, but it could be detected when *n*-octyl-β-D-thioglucoside was used as the detergent (6). Therefore, at first we examined the effect of various detergents on POMT activity of HEK293T cells, in addition to

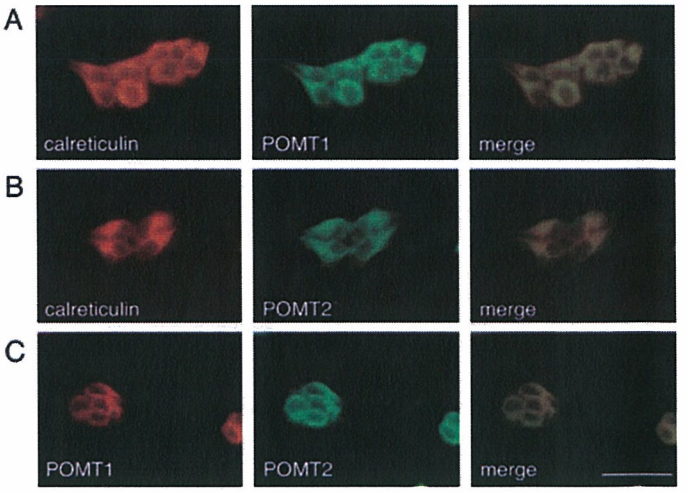

FIGURE 1. **Subcellular localization of POMT1 and POMT2.** *A*, transfected HEK293T cells were stained for calreticulin (*red*) and POMT1-myc (*green*). The merged images show precise co-localization of POMT1 with calreticulin. *B*, transfected HEK293T cells were stained for calreticulin (*red*) and POMT2-FLAG (*green*). The merged images show precise colocalization of POMT2 with calreticulin. *C*, transfected HEK293T cells were stained with fluorescent antibodies against POMT1-Myc (*red*) and POMT2-FLAG (*green*). The merged images show precise colocalization of POMT1 with POMT2. *Scale bar*, 50 μm.

Triton X-100 and *n*-octyl-β-D-thioglucoside. *n*-Octyl-β-D-thioglucoside at a concentration of 0.5% was found to be most effective under our assay conditions (Fig. 2). Then we tried to solubilize POMT activity under various incubation temperatures, incubation times, and detergent concentrations. We found that the optimal conditions for solubilizing POMT activity from the microsomal membrane were 0.5% *n*-octyl-β-D-thioglucoside at 4 °C for 5 h.

Coimmunoprecipitation of POMT1 and POMT2—To determine whether POMT1 and POMT2 form a heterocomplex, POMT1-Myc and POMT2 were cotransfected into HEK293T cells (Fig. 3, *A–C*). Microsomal membrane fractions of these cells were lysed with 0.5% *n*-octyl-β-D-thioglucoside at 4 °C for 5 h and immunoprecipitated with anti-Myc (9E10) antibody-conjugated agarose. A Western blot analysis of precipitates revealed that POMT1-Myc and POMT2 were coimmunoprecipitated (Fig. 3, *D* and *E*, *lanes 1*), indicating that POMT1 and POMT2 form a complex. We then confirmed that POMT2 does not bind to anti-Myc-agarose nonspecifically. Cells that were transfected with only POMT2 were solubilized by *n*-octyl-β-D-thioglucoside and subjected to immunoprecipitation with anti-Myc-agarose. In this case, POMT2 was not detected in the precipitates, indicating POMT2 did not bind to anti-Myc-agarose (Fig. 3, *D* and *E*, *lanes 3*). On the other hand, when POMT1-Myc and POMT2 were separately expressed in different cells and then solubilized, mixed, and immunoprecipitated with anti-Myc-agarose, no complex formation between POMT1-Myc and POMT2 was detected (Fig. 3, *D* and *E*, *lanes 5*). These results indicated that the POMT1 and POMT2 proteins could not associate with each other when they are expressed separately and suggest that assembly of POMT1 and POMT2 occurs in the ER membrane.

Next, we examined whether the coprecipitated POMT1-POMT2 complex has POMT activity. Distinct activity was detected in the precipitates from POMT1-Myc-POMT2

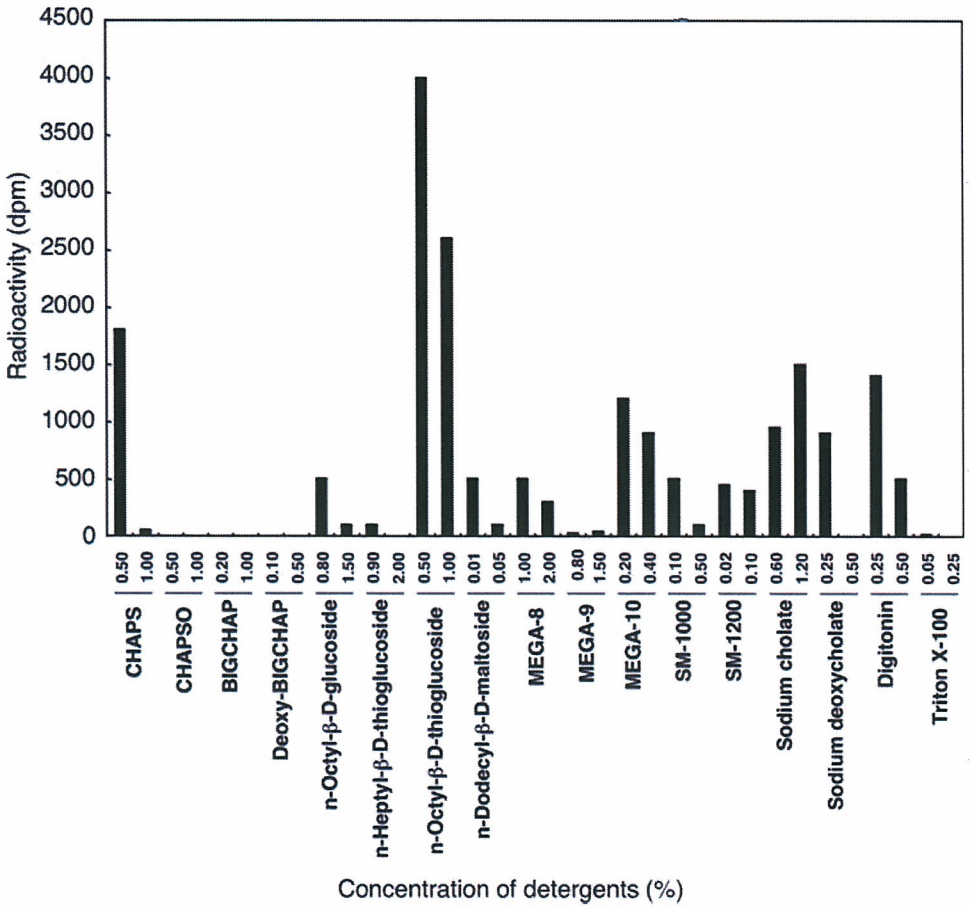

FIGURE 2. **Effect of detergents on POMT activity.** *CHAPS*, 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonic acid; *CHAPSO*, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonic acid; *BIGCHAP*, *N,N*-bis(3-D-gluconamidopropyl) cholamide; *Deoxy-BIGCHAP*, *N,N*-bis(3-D-gluconamidopropyl)deoxycholamide; *MEGA-8*, *n*-octanoyl-*N*-methylglucamide; *MEGA-9*, *n*-nonanoyl-*N*-methylglucamide; *MEGA-10*, *n*-decanoyl-*N*-methylglucamide; *SM-1000*, β-D-fructopyranosyl-α-D-glucopyranosidemonodecanoate; *SM-1200*, β-D-fructopyranosyl-α-D-glucopyranoside monododecanoate.

cotransfected cells (Fig. 3*F*, *lane 1*). The precipitates from cell membranes expressing only POMT1-Myc had slight POMT activity (Fig. 3*F*, *lane 2*). This weak activity may be due to complex formation between transfected POMT1-Myc and endogenous POMT2. The POMT activity in a mixture of individually expressed POMT1-Myc and POMT2 was similar to the background level (Fig. 3*F*, *lane 5*). Based on these results, we concluded that POMT1 and POMT2 associate physically *in vivo* and that this state becomes functional.

Effect of Mutations on POMT Activity—Recently, three mutations (G65R, A200P, and W582C) in the *POMT1* gene were found (Table 1) (11–13). Because these patients have milder phenotypes than typical WWS patients, we expected the mutated POMT1s to have some POMT activity. To test this hypothesis, these mutations were introduced into *POMT1-myc* cDNA and cotransfected into HEK293T cells with *POMT2* (Fig. 4, *A–C*). However, none of the POMT1 mutants, like the other mutants (10), showed any POMT activity (Fig. 4*D*).

Immunoprecipitation of Mutant POMT1 and POMT2— Eight mutations in the *POMT1* gene of patients with WWS (G76R, L421del, V428D, G65R, A200P, W582C, V703fs, and G722fs) were found to abolish POMT activity in both the present and previous studies (10). We examined six of these mutant POMT1s (all but the latter two) to determine whether they prevented complex formation with POMT2 (Table 1). The mutated *POMT1-mycs* were cotransfected into HEK293T cells with *POMT2*. POMT2 was found to precipitate with each of the POMT1 mutants (Fig. 5, *A* and *B*), indicating that these mutations did not affect complex formation with POMT2.

DISCUSSION

Protein *O*-mannosylation is an essential post-translational modification (19). In yeast and fungi, protein *O*-mannosylation is indispensable for cell wall integrity and normal cellular morphogenesis (16). In *Drosophila*, a defect of *O*-mannosylation causes a rotation of the abdomen due to abnormal muscle development (26, 27). In mouse, targeted disruption of the *Pomt1* gene is embryonically lethal (28). In human, impairment of α-DG *O*-mannosylation leads to congenital muscular dystrophy and neuronal migration disorders, WWS (7, 10). Protein *O*-mannosylation requires at least two components, POMT1 and POMT2 (6). The present results have shown that POMT1 and POMT2 form a heterocomplex *in vivo*. The immunoprecipitated complex of POMT1 and POMT2 possessed POMT activity. In addition, we demonstrated that mutant POMT1s that have been found in WWS patients have the ability to form a complex with POMT2, although they lost POMT activities.

In yeast, the members of the PMT1 subfamily interact heterophilically with those of the PMT2 subfamily, whereas the single member of the PMT4 subfamily acts as a homophilic complex (17, 24). On the other hand, in human the single member of the PMT2 subfamily (POMT2) interacts with a member of the PMT4 subfamily (POMT1). These results suggest that the combination of interacting molecules has changed during evolution. It is noteworthy that only a single PMT member has been found in *M. tuberculosis* and that it has POMT activity (18). It is unclear whether it forms a homophilic complex *in vivo*.

Yeast PMTs and human POMTs are predicted to be integral membrane proteins with multiple transmembrane domains (25, 29). However, human and yeast POMT proteins showed differences in detergent sensitivity. Triton X-100 appeared to abolish human POMT activity (6) but did not inhibit yeast PMT activity (30). This difference may be due to the lipid compositions in human and yeast, which would affect the efficiency of

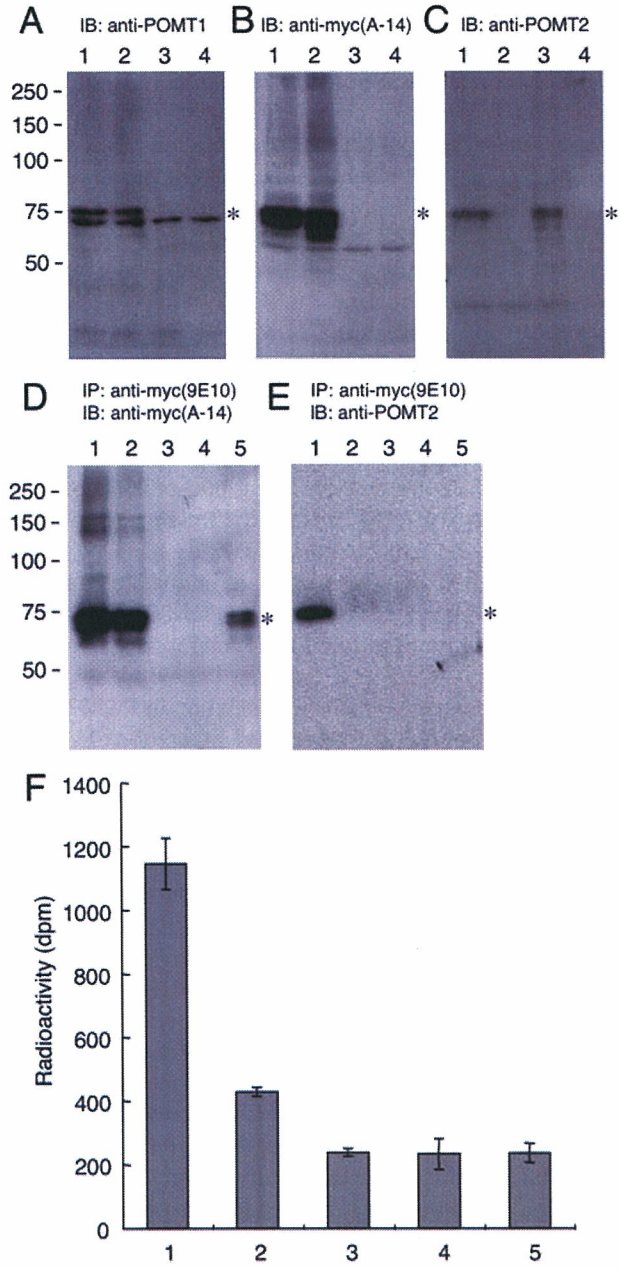

FIGURE 3. **Physical and functional association of POMT1 and POMT2.** *A–C*, expression of POMT-Myc and POMT2 in HEK293T microsomal membrane fractions was determined by anti-POMT1 antibody (*A*), anti-Myc antibody (A-14) (*B*), and anti-POMT2 antibody (*C*). *D* and *E*, POMT1-POMT2 complex formation *in vivo*. POMT1-Myc and POMT2 were transfected into HEK293T cells and immunoprecipitated by anti-Myc (9E10) antibody-conjugated agarose. The resulting precipitates were analyzed by immunoblotting with anti-Myc antibody (A-14) (*D*) and anti-POMT2 antibody (*E*). *F*, POMT activity of immunoprecipitates. *Lane 1*, POMT1-Myc and POMT2 were transfected into cells; *lane 2*, cells were transfected with POMT1-Myc alone; *lane 3*, cells were transfected with POMT2 alone; *lane 4*, mock transfectant; *lane 5*, a mixture of individually expressed POMT1-Myc and POMT2. *Asterisks* indicate positions of corresponding molecules. Molecular weight standards are shown on the *left*. POMT activity was based on the amount of mannose transferred to a GST-αDG. Average values of three independent experiments are shown.

protein solubilization by detergents and the stability of proteins after the removal of lipids by detergents.

Yeast Pmt1p has been proposed to consist of seven transmembrane helices (31). The Pmt1p N terminus and loops 2, 4, and 6 are located in the cytoplasm, and the C terminus and

TABLE 1
Summary of mutations in the *POMT1* gene of WWS patients

Mutations	Effects
G193A	$Gly^{65} \rightarrow$ Arg missense (G65R)
G598C	$Ala^{200} \rightarrow$ Pro missense (A200P)
G1746C	$Trp^{582} \rightarrow$ Cys missense (W582C)
C226A	$Gly^{76} \rightarrow$ Arg missense (G76R)
1260 to 1262 del CCT	Leu^{421} deletion (L421del)
T1283A	$Val^{428} \rightarrow$ Asp missense (V428D)

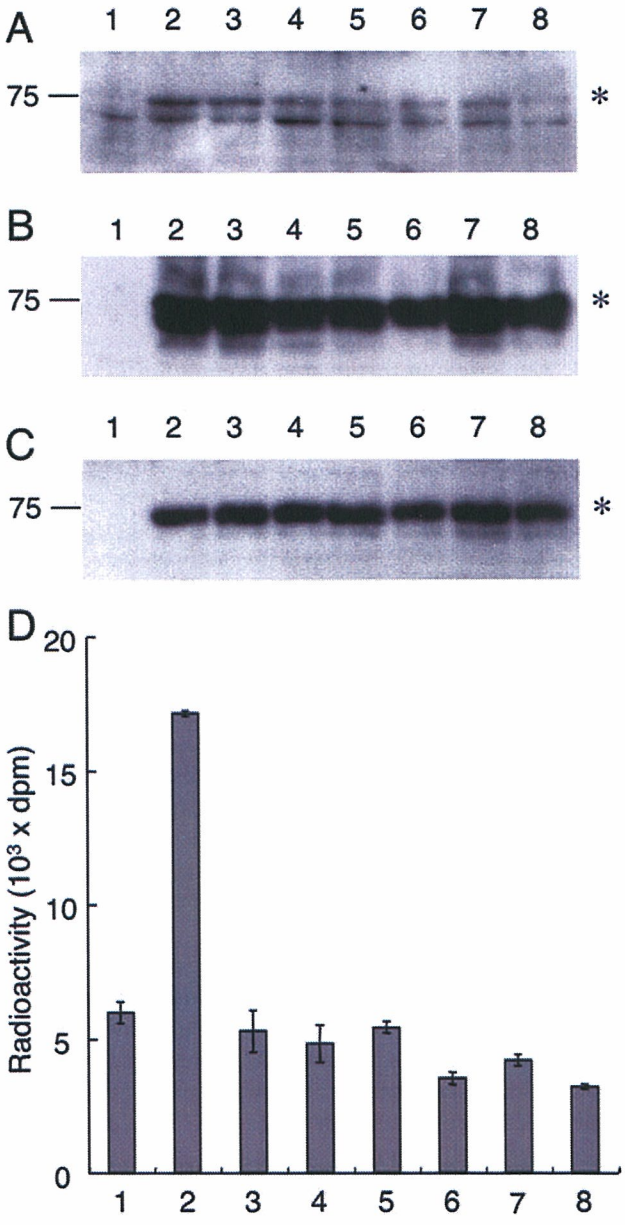

FIGURE 4. **Enzymatic activity mutated POMT1-Myc with POMT2.** *A–C*, Western blot analyses of POMT1-Myc and POMT2 proteins detected by anti-POMT1 antibody (*A*), anti-Myc antibody (A-14) (*B*), and anti-POMT2 antibody (*C*). *D*, POMT activities of the POMT1-Myc mutants coexpressed with POMT2. *Lane 1*, mock; *lane 2*, POMT1-Myc and POMT2; *lane 3*, G65R and POMT2; *lane 4*, A200P and POMT2; *lane 5*, W582C and POMT2; *lane 6*, G76R and POMT2; *lane 7*, L421del and POMT2; *lane 8*, V428D and POMT2. *Asterisks* indicate the migration positions of each POMT1-Myc protein (*A*, *B*) and POMT2 protein (*C*). Molecular weight standards are shown on the *left*. POMT activity was based on the amount of mannose transferred to a GST-αDG. Average values of three independent experiments are shown.

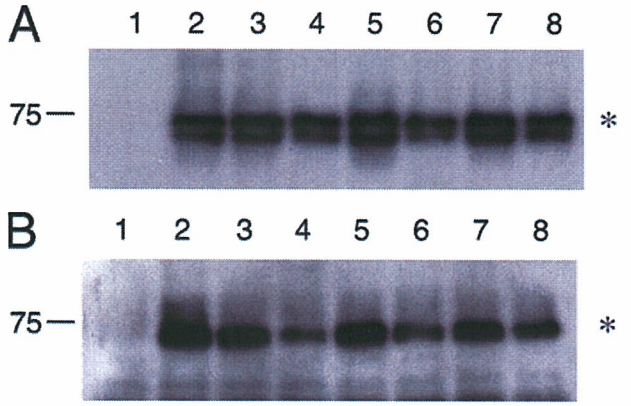

FIGURE 5. **Complex formation of mutated POMT1-Myc with POMT2.** *A* and *B*, immunoprecipitates of mutated POMT1-Myc and POMT2 proteins analyzed with anti-Myc antibody (A-14) (*A*) and anti-POMT2 antibody (*B*). *Lanes 1*, mock; *lanes 2*, POMT1-Myc and POMT2; *lanes 3*, G65R and POMT2; *lanes 4*, A200P and POMT2; *lanes 5*, W582C and POMT2; *lanes 6*, G76R and POMT2; *lanes 7*, L421del and POMT2; *lanes 8*, V428D and POMT2. *Asterisks* indicate the migration positions of each POMT1-Myc protein (*A*) and POMT2 protein (*B*). Molecular weight standards are shown on the *left*.

loops 1, 3, and 5 are located in the ER lumen. A large hydrophilic region (loop 5) and loop 1 are important for enzymatic activity (17, 31, 32). Based on this model, seven of ten *POMT1* mutations identified in WWS patients appear to be located in loops 1 and 5 (7, 9, 11–13), and three of four *POMT2* mutations identified in WWS patients are located in loop 5 (8). Taken together, these results indicate that loops 1 and 5 are important for catalysis, as they are in yeast PMTs. Furthermore, deletion of loop 5 in yeast Pmt1 eliminates enzymatic activity, but not Pmt1-Pmt2 interactions (32). Similarly, mutant POMT1 has the ability to form a complex with POMT2, although the complex does not have POMT activity. Further studies are needed to clarify the role of each domain in POMT activity and complex formation. Additionally, it has been shown that an Arg residue in the transmembrane domain is necessary for complex formation in yeast PMTs and a Glu residue in loop 1 is necessary for enzymatic activity (32). These amino acid residues are conserved in human POMT1 (33), and so it will be interesting to see whether they also are necessary for complex formation and activity. It is also of interest to screen uncharacterized WWS patients for the Arg and Glu mutations.

There is growing evidence that the glycans of glycoproteins play several roles in cellular differentiation and developmental events as well as in disease processes (34). Glycosylation is basically controlled by the combined action of many glycosyltransferases. The level and the strict substrate specificity of glycosyltransferases cooperate to synthesize specific sugar sequences and sugar linkages found in glycoproteins. Glycosyltransferase activities are regulated by other factors or by complex formation. For example, human core 1 β3-galactosyltransferase activity requires the expression of Cosmc (35). Cosmc is a molecular chaperone that specifically assists the folding/stability of core 1 β3-galactosyltransferase and is required for a glycosyltransferase expression. Mutations of *COSMC* were recently found in patients with Tn syndrome who could not produce core 1 structure (Galβ1–3GalNAc) (36). Another glycosyltransferase with complex regulation is human chondroitin synthase, which cannot polymerize chondroitin sulfate *in vitro*; rather. its activity requires the coexpression of chondroitin-polymerizing factor (37). As a third example, the bifunctional glycosyltransferases EXT1 and EXT2, which polymerize heparan sulfate, need to form a hetero-oligomeric complex to exert their optimal catalytic activities and to exist in the appropriate intracellular locations (38, 39). In the present study, we observed that protein *O*-mannosylation can be initiated by direct complex formation of POMT1 and POMT2, but not by either enzyme by itself. POMT1 or POMT2 are thus different from EXT1 and EXT2 because the latter enzymes are active by themselves. One possibility is that formation of the POMT1-POMT2 complex creates a new catalytic domain. Further studies are needed to elucidate the mechanism of complex formation between POMT1 and POMT2 and the regulation of POMT activity. Our results, together with previous studies of glycosyltransferases, indicate that glycosylation is regulated in a complicated fashion.

Our findings that POMT1 and POMT2 associated physically and functionally *in vivo* and that POMT1 and POMT2 could not associate when they are expressed individually and then mixed suggest that the assembly of POMT1 and POMT2 requires specific conditions in the ER membrane. However, a heterocomplex POMT2 and mutated POMT1s suggest that single amino acid substitutions and deletion in POMT1 found in WWS patients do not affect assembly of POMT1 and POMT2. These mutations would abolish dolichol phosphate mannose or acceptor (α-DG) binding. In the POMT assay, cells transfected with mutated POMT1 and wild-type POMT2 (Fig. 4*D*, *lanes 3–8*) had decreased enzymatic activity compared with the mock transfectant (Fig. 4*D*, *lane 1*). The decrease of endogenous enzymatic activity may be caused by the disturbance of endogenous POMT1-POMT2 formation.

WWS patients carrying three mutations (G65R, A200P, and M582C) showed milder phenotypes than typical WWS (14, 15), which led us to expect that these mutations would not completely abolish activity. Our finding that these mutant proteins did not have any enzymatic activity is thus puzzling. One possibility is that the levels of LARGE expression may be greater in these patients than in previous reported severe WWS patients. Barresi *et al.* (40) report that overproduction of LARGE caused hyperglycosylation of α-DG and improvement of function, *e.g.* laminin binding, in WWS fibroblasts. However, the change of the LARGE expression level in WWS patients may be rare because these patients had hypoglycosylated α-DG (14, 15). Measurement of POMT activities of tissues from these patients would help to explain their mild symptoms. If each patient showed some POMT activities, other factor(s) that regulate POMT activity should be considered. In fact, some WWS patients have no mutations in *POMT1* or *POMT2* (7, 11). It is possible that mutations of such factors may cause unidentified WWS. Further studies are needed to test this hypothesis.

REFERENCES

1. Michele, D. E., and Campbell, K. P. (2003) *J. Biol. Chem.* **278,** 15457–15460
2. Michele, D. E., Barresi, R., Kanagawa, M., Saito, F., Cohn, R. D., Satz, J. S., Dollar, J., Nishino, I., Kelley, R. I., Somer, H., Straub, V., Mathews, K. D., Moore, S. A., and Campbell, K. P. (2002) *Nature* **418,** 417–422
3. Chiba, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusunoki, S., Kanazawa, I., Kobata, A., and Endo, T. (1997) *J. Biol. Chem.* **272,** 2156–2162

4. Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., Talim, B., Voit, T., Topaloglu, H., Toda, T., and Endo, T. (2001) *Dev. Cell* **1,** 717–724
5. Manya, H., Sakai, K., Kobayashi, K., Taniguchi, K., Kawakita, M., Toda, T., and Endo, T. (2003) *Biochem. Biophys. Res. Commun.* **306,** 93–97
6. Manya, H., Chiba, A., Yoshida, A., Wang, X., Chiba, Y., Jigami, Y., Margolis, R. U., and Endo, T. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 500–505
7. Beltran-Valero de Bernabe, D., Currier, S., Steinbrecher, A., Celli, J., van Beusekom, E., van der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W. B., Cormand, B., Lehesjoki, A. E., Cruces, J., Voit, T., Walsh, C. A., van Bokhoven, H., and Brunner, H. G. (2002) *Am. J. Hum. Genet.* **71,** 1033–1043
8. van Reeuwijk, J., Janssen, M., van den Elzen, C., Beltran-Valero de Bernabe, D., Sabatelli, P., Merlini, L., Boon, M., Scheffer, H., Brockington, M., Muntoni, F., Huynen, M. A., Verrips, A., Walsh, C. A., Barth, P. G., Brunner, H. G., and van Bokhoven, H. (2005) *J. Med. Genet.* **42,** 907–912
9. Kim, D. S., Hayashi, Y. K., Matsumoto, H., Ogawa, M., Noguchi, S., Murakami, N., Sakuta, R., Mochizuki, M., Michele, D. E., Campbell, K. P., Nonaka, I., and Nishino, I. (2004) *Neurology* **62,** 1009–1011
10. Akasaka-Manya, K., Manya, H., and Endo, T. (2004) *Biochem. Biophys. Res. Commun.* **325,** 75–79
11. Currier, S. C., Lee, C. K., Chang, B. S., Bodell, A. L., Pai, G. S., Job, L., Lagae, L. G., Al-Gazali, L. I., Eyaid, W. M., Enns, G., Dobyns, W. B., and Walsh, C. A. (2005) *Am. J. Med. Genet. A.* **133,** 53–57
12. Balci, B., Uyanik, G., Dincer, P., Gross, C., Willer, T., Talim, B., Haliloglu, G., Kale, G., Hehr, U., Winkler, J., and Topaloglu, H. (2005) *Neuromuscul. Disord.* **15,** 271–275
13. van Reeuwijk, J., Maugenre, S., van den Elzen, C., Verrips, A., Bertini, E., Muntoni, F., Merlini, L., Scheffer, H., Brunner, H. G., Guicheney, P., and van Bokhoven, H. (2006) *Hum. Mutat.* **27,** 453–459
14. Dincer, P., Balci, B., Yuva, Y., Talim, B., Brockington, M., Dincel, D., Torelli, S., Brown, S., Kale, G., Haliloglu, G., Gerceker, F. O., Atalay, R. C., Yakicier, C., Longman, C., Muntoni, F., and Topaloglu, H. (2003) *Neuromuscul. Disord.* **13,** 771–778
15. Villanova, M., Mercuri, E., Bertini, E., Sabatelli, P., Morandi, L., Mora, M., Sewry, C., Brockington, M., Brown, S. C., Ferreiro, A., Maraldi, N. M., Toda, T., Guicheney, P., Merlini, L., and Muntoni, F. (2000) *Neuromuscul. Disord.* **10,** 541–547
16. Strahl-Bolsinger, S., Gentzsch, M., and Tanner, W. (1999) *Biochim. Biophys. Acta* **1426,** 297–307
17. Girrbach, V., and Strahl, S. (2003) *J. Biol. Chem.* **278,** 12554–12562
18. VanderVen, B. C., Harder, J. D., Crick, D. C., and Belisle, J. T. (2005) *Science* **309,** 941–943
19. Endo, T. (2004) *Glycoconj. J.* **21,** 3–7
20. Strahl-Bolsinger, S., Immervoll, T., Deutzmann, R., and Tanner, W. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8164–8168
21. Immervoll, T., Gentzsch, M., and Tanner, W. (1995) *Yeast* **11,** 1345–1351
22. Lussier, M., Gentzsch, M., Sdicu, A. M., Bussey, H., and Tanner, W. (1995) *J. Biol. Chem.* **270,** 2770–2775
23. Gentzsch, M., Strahl-Bolsinger, S., and Tanner, W. (1995) *Glycobiology* **5,** 77–82
24. Gentzsch, M., Immervoll, T., and Tanner, W. (1995) *FEBS Lett.* **377,** 128–130
25. Willer, T., Amselgruber, W., Deutzmann, R., and Strahl, S. (2002) *Glycobiology* **12,** 771–783
26. Martin-Blanco, E., and Garcia-Bellido, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6048–6052
27. Ichimiya, T., Manya, H., Ohmae, Y., Yoshida, H., Takahashi, K., Ueda, R., Endo, T., and Nishihara, S. (2004) *J. Biol. Chem.* **279,** 42638–42647
28. Willer, T., Prados, B., Falcon-Perez, J. M., Renner-Muller, I., Przemeck, G. K., Lommel, M., Coloma, A., Valero, M. C., de Angelis, M. H., Tanner, W., Wolf, E., Strahl, S., and Cruces, J. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 14126–14131
29. Jurado, L. A., Coloma, A., and Cruces, J. (1999) *Genomics* **58,** 171–180
30. Strahl-Bolsinger, S., and Tanner, W. (1991) *Eur. J. Biochem.* **196,** 185–190
31. Strahl-Bolsinger, S., and Scheinost, A. (1999) *J. Biol. Chem.* **274,** 9068–9075
32. Girrbach, V., Zeller, T., Priesmeier, M., and Strahl-Bolsinger, S. (2000) *J. Biol. Chem.* **275,** 19288–19296
33. Willer, T., Valero, M. C., Tanner, W., Cruces, J., and Strahl, S. (2003) *Curr. Opin. Struct. Biol.* **13,** 621–630
34. Haltiwanger, R. S., and Lowe, J. B. (2004) *Annu. Rev. Biochem.* **73,** 491–537
35. Ju, T., and Cummings, R. D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 16613–16618
36. Ju, T., and Cummings, R. D. (2005) *Nature* **437,** 1252
37. Kitagawa, H., Izumikawa, T., Uyama, T., and Sugahara, K. (2003) *J. Biol. Chem.* **278,** 23666–23671
38. McCormick, C., Duncan, G., Goutsos, K. T., and Tufaro, F. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 668–673
39. Kim, B. T., Kitagawa, H., Tanaka, J., Tamura, J., and Sugahara, K. (2003) *J. Biol. Chem.* **278,** 41618–41623
40. Barresi, R., Michele, D. E., Kanagawa, M., Harper, H. A., Dovico, S. A., Satz, J. S., Moore, S. A., Zhang, W., Schachter, H., Dumanski, J. P., Cohn, R. D., Nishino, I., and Campbell, K. P. (2004) *Nat. Med.* **10,** 696–703

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Molecular cloning and characterization of rat Pomt1 and Pomt2

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Mammalian *O*-mannosylation, although an uncommon type of protein modification, is essential for normal brain and muscle development. Defective *O*-mannosylation causes congenital muscular dystrophy with abnormal neuronal migration [Walker–Warburg syndrome (WWS)]. Here, we have identified and cloned rat *Pomt1* and *Pomt2*, which are homologues of human *POMT1* and *POMT2*, with identities of 86 and 90%, respectively, at the amino acid level. Coexpression of both genes was found to be necessary for enzymatic activity, as is the case with human *POMT1* and *POMT2*. Northern blot and reverse transcriptase polymerase chain reaction (RT–PCR) analyses revealed that rat *Pomt1* and *Pomt2* are expressed in all tissues but most strongly in testis. *In situ* hybridization histochemistry of rat brain revealed that *Pomt1* and *Pomt2* mRNA are coexpressed in neurons (dentate gyrus and CA1-CA3 region of the hippocampus and cerebellar Purkinje cells). Two transcription-initiation sites were observed in rat *Pomt2*, resulting in two forms: a testis form and a somatic form. The two forms had equal protein *O*-mannosyltransferase activity when coexpressed with rat Pomt1. Coexpression studies also showed that the human and rat protein *O*-mannosyltransferases are interchangeable, providing further evidence for the closeness of their structures.

Key words: glycosylation/Pomt1 and Pomt2/protein *O*-mannosyltransferase activity/rat

Introduction

Mammalian *O*-mannosylation is an uncommon type of protein modification that was first identified in chondroitin sulfate proteoglycans of brain and is present in a limited number of glycoproteins of brain, nerve, and skeletal muscle (Finne *et al.*, 1979; Krusius *et al.*, 1986, 1987; Endo, 1999). α-Dystroglycan (α-DG) is an *O*-mannosyl-modified glycoprotein that is a central component of the dystrophin–glycoprotein complex isolated from skeletal muscle membranes (Michele and Campbell, 2003). 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 (Chiba *et al.*, 1997). Subsequently, a series of *O*-mannosyl glycans with different mannose branching and peripheral structures were found in mammals (Yuen *et al.*, 1997; Sasaki *et al.*, 1998; Smalheiser *et al.*, 1998; Endo, 1999).

Initiation of protein *O*-mannosylation has been partially characterized. In yeast, a family of protein *O*-mannosyltransferases (*pmt1~7*) catalyzes the transfer of a mannosyl residue from dolichyl phosphate mannose (Dol-P-Man) to Ser/Thr residues of certain proteins (Strahl-Bolsinger *et al.*, 1999). In humans, two homologues, *POMT1* and *POMT2*, are present (Jurado *et al.*, 1999; Willer *et al.*, 2002, 2004). Human POMT1 and POMT2 share almost identical hydropathy profiles that predict both to be integral membrane proteins with multiple transmembrane domains. Recently, we demonstrated that human POMT1 and POMT2 have protein *O*-mannosyltransferase activity, but only when they are coexpressed, and later we found that human POMT1 and POMT2 form a heterocomplex to express enzymatic activity (Manya *et al.*, 2004). This has also been found to be the case in *Drosophila*. Two orthologs of human *POMT* genes, *dPOMT1* and *dPOMT2*, are present, and both are required for protein *O*-mannosylation (Ichimiya *et al.*, 2004).

Protein *O*-mannosylation is important for normal brain and muscle development, because a defect of *O*-mannosylation causes congenital muscular dystrophy with abnormal neuronal migration (Endo, 2004), the so-called Walker–Warburg syndrome (WWS: OMIM 236670) (Dobyns *et al.*, 1989). Patients with WWS are severely affected from birth and usually die within their first year. Recently, WWS patients have been found to have mutations in both *POMT1* and *POMT2* (Beltran-Valero de Bernabe *et al.*, 2002; van Reeuwijk *et al.*, 2005). In WWS patients, a highly glycosylated α-DG was selectively deficient in skeletal muscle (Beltran-Valero de Bernabe *et al.*, 2002; Jimenez-Mallebrera *et al.*, 2003; van Reeuwijk *et al.*, 2005). This finding suggests that α-DG is a potential target of POMT1 and POMT2 and that hypoglycosylation of α-DG may be a pathomechanism of WWS. In fact, *POMT1* mutations found in WWS patients led to a defect of protein *O*-mannosyltransferase activity even when the defective *POMT1* was coexpressed with wild-type *POMT2* (Akasaka-Manya *et al.*, 2004). In *Drosophila*, functional dPOMT1 and dPOMT2 are required for normal muscle development (Ichimiya *et al.*, 2004).

In this study, we isolated rat orthologs of *POMT1* and *POMT2* cDNA clones and determined in which tissues they

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The nucleotide sequences reported in this article have been submitted to the DDBJ/GenBank/EBI Data Bank with accession numbers AF192388 for rat Pomt1 and AB246667 for rat Pomt2.

are expressed. We also examined the distribution of protein *O*-mannosyltransferase activity in various rat tissues.

Results

cDNA cloning of rat Pomt1 and Pomt2

A rat *Pomt1* cDNA was obtained by reverse transcriptase polymerase chain reaction (RT–PCR) using a rat brain cDNA library. The nucleotide sequence of the cDNA is predicted to encode a protein of 748 amino acids (Figure 1A). In addition, two rat *Pomt2* cDNAs were obtained by RT–PCR using a rat testis cDNA library. These two cDNAs originated from alternative initiation sites. The longer and shorter forms are predicted to contain proteins of 810 and 740 amino acids, respectively (Figure 1B). The former is named *t-Pomt2* and the latter is *s-Pomt2* (somatic form), because *t-Pomt2* expression is highly specific to the testis as described below.

ClustalW alignments show that human, mouse, and rat POMT1s (Figure 2A) are closely related and that human, mouse, and rat POMT2s (Figure 2B) are closely related. Rat Pomt1 and Pomt2 showed 86 and 90% identities to human POMT1 and POMT2, and 96 and 97% identities to mouse Pomt1 and Pomt2, respectively.

Expression of rat Pomt1 and Pomt2 genes

To examine the expression patterns and the size of rat *Pomt1* and *Pomt2* mRNAs, northern blot analyses were performed (Figure 3A). The mRNA band of around 3.3 kb represents the basic transcript of *Pomt1*. *Pomt1* mRNA was expressed in all tissues and predominantly expressed in testis. The basic transcript of *Pomt2* was around 2.7 kb, but due to alternative polyadenylation, 3.7 and 4.7 kb mRNAs were also detected (closed triangles in Figure 3A, middle panel). In testis, the transcript sizes were slightly larger due to differential transcription initiation (open triangles in Figure 3A, middle panel). Like *Pomt1*, *Pomt2* was expressed in all tissues but predominantly in testis.

The more sensitive RT–PCR analyses of rat *Pomt1*, *Pomt2*, and *t-Pomt2* were performed (Figure 3B). PCR products of *Pomt1* and *Pomt2* were detected in all tissues (top and second panels in Figure 3B). However, *t-Pomt2* mRNA was predominantly expressed in testis and slightly detected in brain, lung, and liver (third panel of Figure 3B). Differential transcription initiation of *Pomt2* gene was observed in mouse (Willer *et al.*, 2002), and the longer transcript is restricted to testis.

As shown by *in situ* hybridization, the messages of *Pomt1* and *Pomt2* were coexpressed in rat brain hippocampus and cerebellar cortex (Figure 4). Both mRNAs were mainly expressed in the cells of gray matter and strongly expressed in neurons of the dentate gyrus and CA1-CA3 region in the hippocampus formation and in Purkinje cells in the cerebellar cortex.

Protein O-mannosyltransferase activities in rat tissues

High protein *O*-mannosyltransferase activities were observed in brain, kidney, and testis (Figure 5). The activity in spleen was low, in agreement with the low levels of expression of *Pomt1* and *Pomt2* in spleen (Figure 3A).

Protein O-mannosyltransferase activity of the cloned cDNA products

To analyze the protein *O*-mannosyltransferase activity of rat Pomt1 and Pomt2, the expression vector of the cloned cDNAs was transfected into HEK293T cells, and the microsomal membranes were used for enzymatic assay as described under *Materials and Methods*. Expressed proteins were shown by staining with anti-POMT1 antibody (Figure 6A) and anti-POMT2 antibody (Figure 6B). Protein *O*-mannosyltransferase activity was observed when rat Pomt1 and s-Pomt2 were coexpressed (Figure 6C, lane 6), but not when they were expressed independently (Figure 6C, lanes 2 and 3). α-Mannosidase digestion showed that the mannosyl residue was linked to α-DG by α-linkage (data not shown), as reported previously (Manya *et al.*, 2004).

Cells cotransfected with rat *Pomt1* and human *POMT2* (Figure 6C, lane 8) and cells cotransfected with human *POMT1* and rat *s-Pomt2* (Figure 6C, lane 9) showed comparative protein *O*-mannosyltransferase activities with cells cotransfected rat *Pomt1* and rat *s-Pomt2* (Figure 6C, lane 6) and cells cotransfected human *POMT1* and human *POMT2* (Figure 6C, lane 7). As expected, cells cotransfected rat *Pomt1* and human *POMT1* and cells cotransfected rat *s-Pomt2* and human *POMT2* did not show enzymatic activity (data not shown).

Protein O-mannosyltransferase activities of rat s-Pomt2 and t-Pomt2

Rat *s-Pomt2* and *t-Pomt2* were expressed with or without rat *Pomt1* in HEK293T cells as shown by staining with anti-POMT1 antibody (Figure 7A) and anti-POMT2 antibody (Figure 7B). Cells cotransfected with rat *Pomt1* and *s-Pomt2* (Figure 7, lane 3) and cells cotransfected with rat *Pomt1* and *t-Pomt2* (Figure 7, lane 4) had comparable protein *O*-mannosyltransferase activities, but cells expressing only t-Pomt2 or s-Pomt2 had little activity (Figure 7C, lanes 1 and 2). These results demonstrate that coexpression of t-Pomt2 or s-Pomt2 with rat Pomt1 showed similar enzymatic activities.

Discussion

In this study, we identified and cloned rat *Pomt1* and *Pomt2*. We also proved that protein *O*-mannosyltransferase activity is encoded in both genes, because coexpression of both genes was necessary for the enzymatic activity. Northern blot and RT–PCR analyses revealed that rat *Pomt1* and *Pomt2* are expressed strongly in the testis and weakly in all other tissues examined. However, enzyme activity is almost the same in brain, kidney, and testis. This may be due to the fact that mRNA levels do not always correlate well with actual protein expression. We do not know the expression levels of Pomt proteins in each tissue because we do not have antibodies that recognize the endogenous Pomts. Another possibility is variations in the presence or absence of activators, cofactors, or inhibitors of protein *O*-mannosyltransferase activity in the different tissues. In fact, some WWS patients have no mutations in *POMT1* or *POMT2* (Beltran-Valero de Bernabe *et al.*, 2002; van Reeuwijk *et al.*, 2005). It is

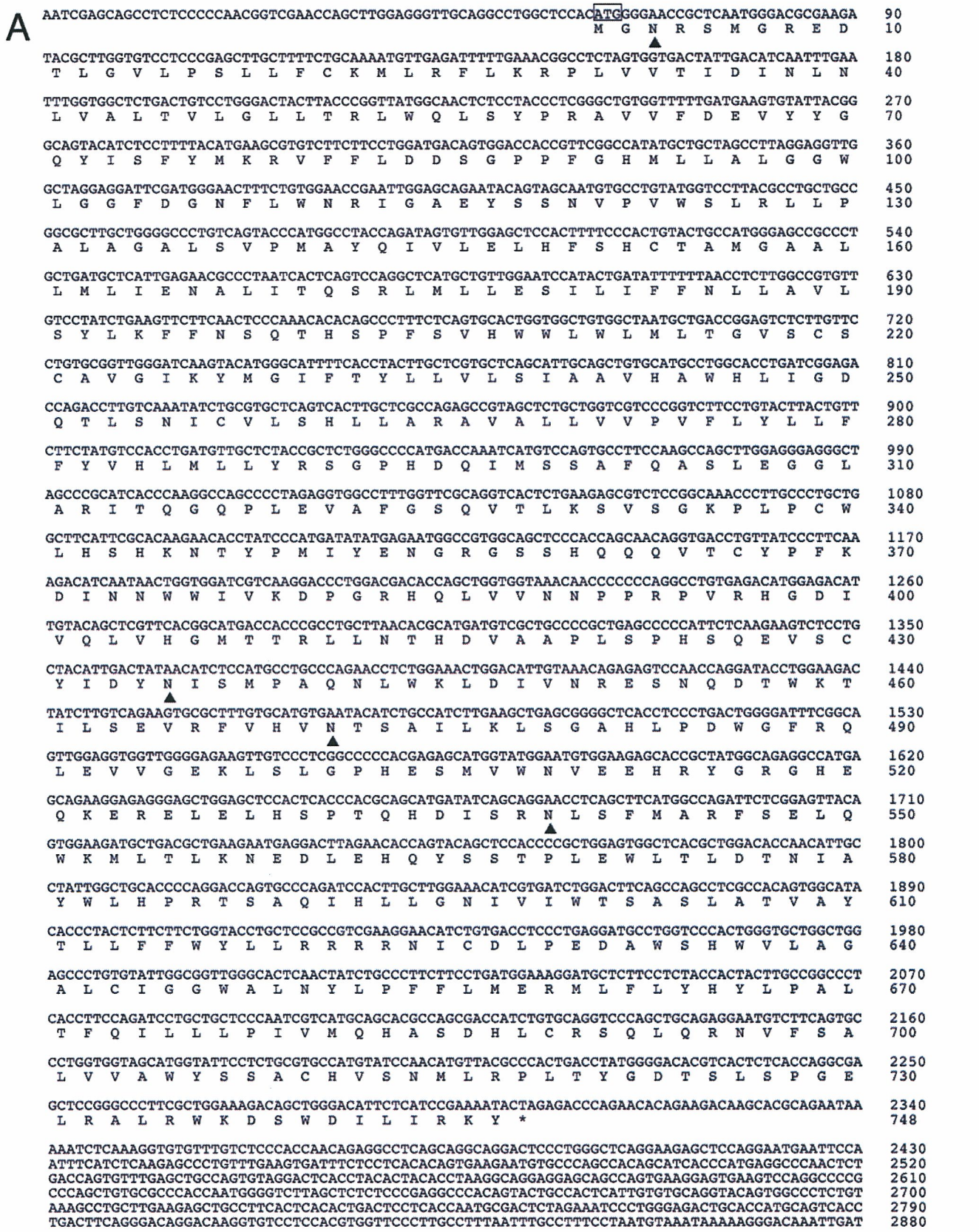

Fig. 1. Nucleotide and deduced amino acid sequences of rat *Pomt1* (**A**) and *Pomt2* (**B**). The cDNA sequences of rat *Pomt1* and *Pomt2* are listed in the top line. Deduced amino acid sequences are indicated by the single-letter amino acid codes. Potential *N*-glycosylation sites are indicated by filled triangles. ATG-start codons are boxed. In *Pomt2* (**B**), the *N*-terminal extension of the deduced testis-specific isoform is underscored and the testis-specific 5′-sequence is shaded.