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

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Abstract

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

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

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

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

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

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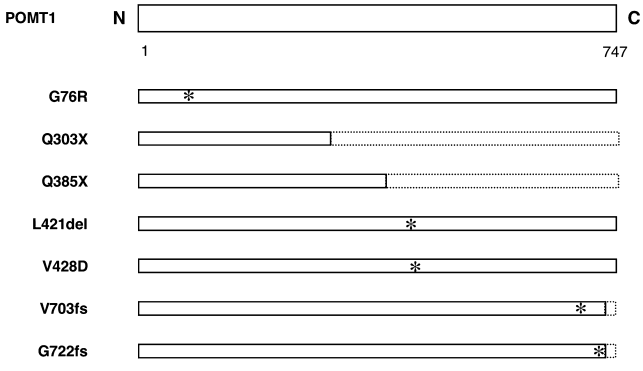
complex with POMT2 and is responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis [18]. Mutations in the *POMT1* gene are considered to be the cause of Walker–Warburg syndrome (WWS: OMIM 236670) [19], an autosomal recessive developmental disorder associated with congenital muscular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

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



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

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

Materials and methods

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

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

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

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

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

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

transferred to a glutathione-*S*-transferase fusion α-dystroglycan (GST-αDG) as described previously [18]. Briefly, the reaction mixture contained 20 mM Tris–HCl (pH 8.0), 100 nM [^{3}H]mannosylphosphoryldolichol (Dol-P-Man, 125,000 dpm/pmol) (American Radiolabeled Chemical), 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside, 10 μg GST-αDG, and enzyme source (80 μg of microsomal membrane fraction) in 20 μl total volume. After 1 h incubation at 22 °C, the reaction was stopped by adding 150 μl PBS containing 1% Triton X-100 (Nacalai Tesque), and the reaction mixture was centrifuged at 10,000*g* for 10 min. The supernatant was removed, mixed with 400 μl PBS containing 1% Triton X-100 and 10 μl of glutathione–Sepharose 4B beads (Amersham Biosciences), rotated at 4 °C for 1 h, and washed three times with 20 mM Tris–HCl (pH 7.4) containing 0.5% Triton X-100. The radioactivity adsorbed to the beads was measured using a liquid scintillation counter.

Results and discussion

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

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

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

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

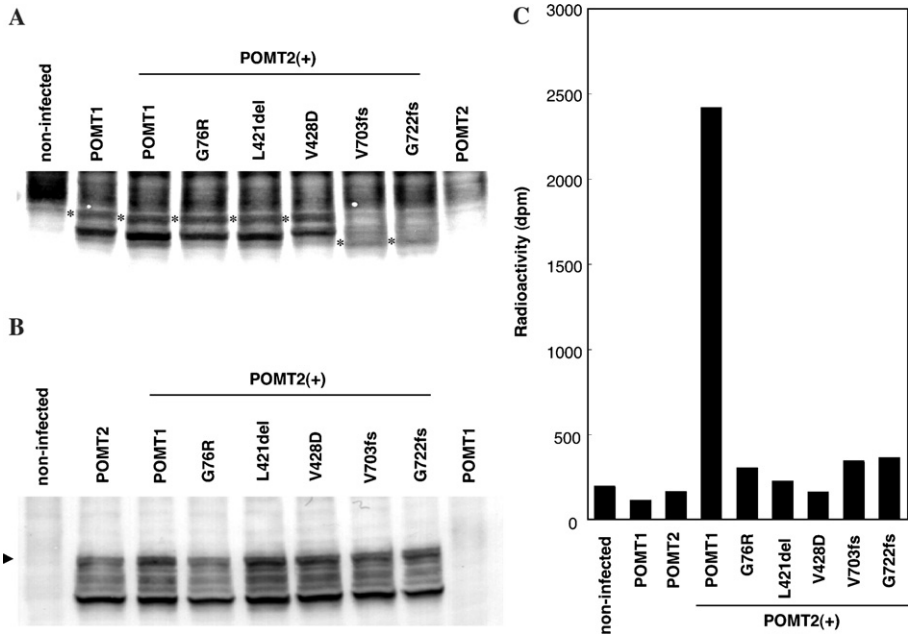


Fig. 2. Expression and activity of mutated POMT1 proteins. (A,B) Western blot analyses of recombinant POMT1 and POMT2 proteins detected by anti-POMT1 antibody (A) and anti-POMT2 antibody (B). The proteins were expressed in Sf9 cells by baculovirus, fractionated by SDS–PAGE (7.5% gel), transferred to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

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

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

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

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

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

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

O-mannosylation is an uncommon protein modification in mammals, but it is important in muscle and brain development. Since a few *O*-mannosylated proteins have been identified, further studies are needed to clarify the distribution of *O*-mannosyl glycans in various tissues. Future studies may also reveal that presently uncharacterized forms of muscular dystrophy are caused by defects in other glycosyltransferases. A major challenge will be to integrate the forthcoming structural, cell biological, and genetic information to understand how α-DG O-mannosylation contributes to muscular dystrophy and neuronal migration disorder.

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