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Loss-of-function of an *N*-acetylglucosaminyltransferase, POMGnT1, in muscle–eye–brain disease

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Abstract

Muscle–eye–brain disease (MEB), an autosomal recessive disorder, is characterized by congenital muscular dystrophy, brain malformation, and ocular abnormalities. Previously, we found that MEB is caused by mutations in the gene encoding the protein O-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1), which is responsible for the formation of the GlcNAcβ1-2Man linkage of *O*-mannosyl glycan. Although 13 mutations have been identified in patients with MEB, only the protein with the most frequently observed splicing site mutation has been studied. This protein was found to have no activity. Here, we expressed the remaining mutant POMGnT1s and found that none of them had any activity. These results clearly demonstrate that MEB is inherited as a loss-of-function of POMGnT1.

Keywords: Muscle–eye–brain disease; Muscular dystrophy; Dystroglycan; Glycosylation; POMGnT1; Neuronal migration disorder

Muscular dystrophies are genetic diseases that cause progressive muscle weakness [1]. The best known is that described by Duchenne which results from mutations in the gene encoding a protein called dystrophin. Another subclass is congenital muscular dystrophies, where muscle weakness is apparent at birth or shortly afterwards. One of them is muscle–eye–brain disease (MEB: OMIM 253280). MEB is an autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormalities, and brain malformation (type II lissencephaly) [2]. Patients with MEB show congenital muscular dystrophy, severe congenital myopia, congenital glaucoma, pallor of the optic discs, retinal hypoplasia, mental retardation, hydrocephalus, abnormal electroencephalograms, and myoclonic jerks. All infants with MEB are floppy with generalized muscle weakness, including facial and neck muscles, from birth. Muscle biopsies show dystrophic changes and brain MRIs reveal pachygyria-type cortical neuronal migration disorder, flat brainstem, and cerebellar hypoplasia.

α-Dystroglycan is an extracellular peripheral membrane glycoprotein anchored to the cell membrane by binding to a transmembrane glycoprotein, β-dystroglycan. The α-dystroglycan–β-dystroglycan complex is widely expressed in a broad array of tissues and is thought to act as a transmembrane linker between the extracellular matrix and intracellular cytoskeleton. This is because α-dystroglycan binds to laminin and the intracellular domain of β-dystroglycan interacts with dystrophin in skeletal muscle [3]. α-Dystroglycan is heavily glycosylated and its sugars have a role in binding to laminin, neurexin, and agrin [3,4]. We previously found that the sugar moiety of α-dystroglycan includes *O*-mannosyl glycan, which is a rare type of glycan in mammals [5]. We also found that a sialyl *O*-mannosyl glycan, Siaα2-3Galβ1-4GlcNAcβ1-2Man, is a laminin-binding ligand of α-dystroglycan [6].

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Recently, we identified a glycosyltransferase, protein O-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1), which catalyzes the transfer of GlcNAc to *O*-mannose of glycoproteins, and showed that the *POMGnT1* gene is responsible for MEB [7]. We identified 13 independent disease-causing mutations in *POMGnT1* in MEB patients [7,8]. To confirm that the mutations observed in patients with MEB are responsible for the defects in the synthesis of *O*-mannosyl glycan, we expressed the protein with the most frequent mutation and found that it had lost enzymatic activity. It is important to determine whether the remaining mutations similarly cause loss of function of POMGnT1 because the 13 mutations are dispersed throughout the entire *POMGnT1* gene and some mutations seem to be located outside the catalytic domain. In the current study, we examined the enzymatic activity of mutant POMGnT1 proteins observed in patients with MEB.

Materials and methods

Construction of POMGnT1 mutants. An expression vector encoding each mutant of *POMGnT1* was prepared by site-directed mutagenesis. Template cDNA for site-directed mutagenesis encoding full-length *POMGnT1* tagged with the His-tag and Xpress epitope was cloned into pcDNA 3.1 Zeo(+) (Invitrogen), as described previously [7]. Site-directed mutagenesis for missense and frameshift mutants (E223K, C269Y, P493R, H573fs, L611fs, and V626fs) was performed using a QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The primers used to make the different mutants were: E223K, 5′-GGAGGTCCTGTCTTCGGGAAGAAA CATTCTAAATC-3′ and 5′-GATTTAGAATGTTTCTTCCCGAAG ACAGGACCTCC-3′; C269Y, 5′-GCCGGCGCTTCTACAGCAAA GTTGAGGG-3′ and 5′-CCCTCAACTTTGCTGTAGAAGCGCCG GC-3′; P493R, 5′-CCGAGAGTGCATCATCCGTGACGTTTCCC G-3′ and 5′-CGGGAAACGTCACGGATGATGCACTC-3′; H573fs, 5′-CAGACACAGAGGGCCAACCTACGTGGCC-3′ and 5′-GGCC ACGTAGGTTGGCCCTCTGTGTCTG-3′; L611fs, 5′-GGCAACC ATCGGGGCCGTGGAGATTGTTTC-3′ and 5′-GAAACAATCTC CACGGCCCCGATGGTTGCC-3′; and V626fs, 5′-CTTCCTGGTG GTGGGGTCCCGGCTTCCC-3′ and 5′-GGGAAGCCGGGACCC CACCACCAGGAAG-3′. Site-directed mutagenesis for splicing site mutant (Δ536–550) was constructed by joining two PCR products. Two fragments were ligated at a *Sal*I site and introduced into the *Pst*I and *Xba*I sites of template plasmid. The primers used to make the mutant were: *Pst*I–*Sal*I, 5′-GCTTCTGCAGCAAAGTTGAGG-3′ and 5′-CTGTCGACATTCCTGAGCTGGACACC-3′; and *Sal*I–*Xba*I, 5′-GGGTCGACAGTGAGGCTGAGGTTCTGGACC-3′ and 5′-AACGGGCCCTCTAGACTCGAGG-3′. All mutant clones were sequenced to confirm the presence of the mutations.

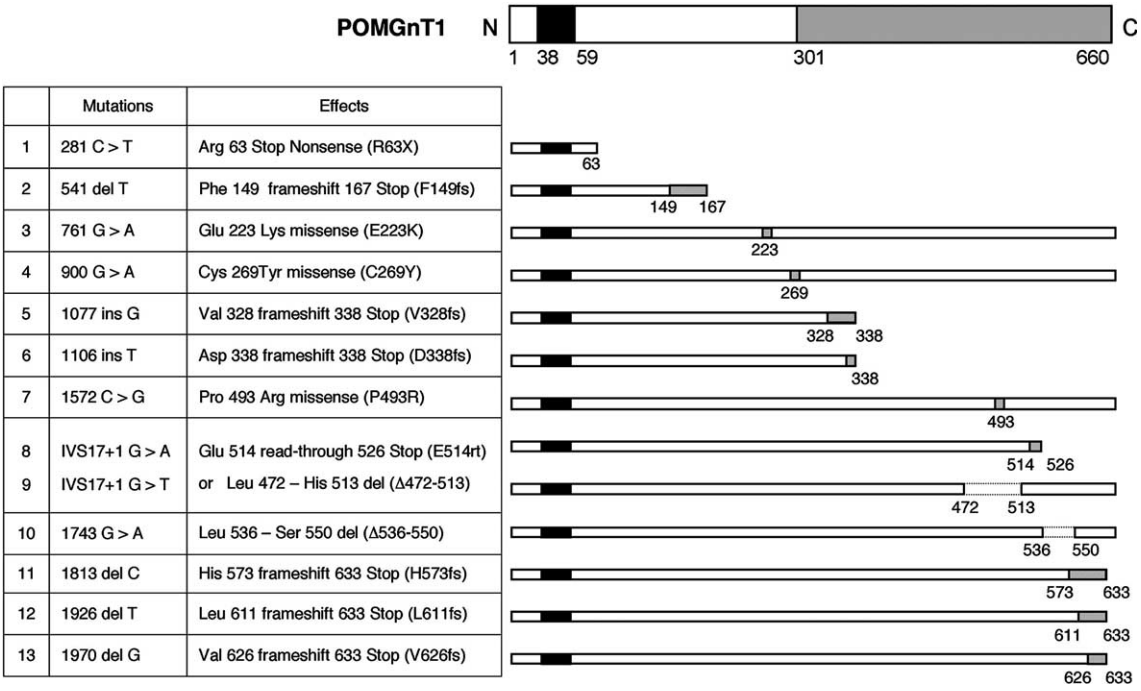
Cell culture and expression of POMGnT1 mutants. Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO_2. The expression plasmids of POMGnT1 mutants were transfected into HEK293T cells using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. The transfected cells were harvested and homogenized after being cultured for 2 days in complete medium.

Western blot analysis. The cells were homogenized in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 250 mM sucrose. After centrifugation at 900*g* for 10 min, the supernatant was subjected to ultracentrifugation at 100,000*g* for 1 h. The precipitate was used as the microsomal membrane fraction. Protein concentration was determined by BCA assay. The proteins in the microsomal fraction (20 μg) were separated by SDS–PAGE (10% gel) and transferred to a PVDF membrane. The membrane was blocked in PBS containing 5% skim milk and 0.5% Tween 20, incubated with anti-Xpress monoclonal antibody (Invitrogen), and treated with anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Amersham Biosciences). Proteins that bound to the antibody were visualized with an ECL Plus kit (Amersham Biosciences). To determine the amount of each mutant POMGnT1, we used Positope protein (Invitrogen) as a mass standard of Xpress-tagged protein and the intensities of bands in Western blotting. The band intensities were measured with a densitometer and NIH Image 1.61/ppc software.

Assay for POMGnT1 activity. POMGnT1 activity was based on the amount of [^{3}H]GlcNAc transferred to a mannosylpeptide [9]. Briefly, a reaction mixture containing 140 mM Mes buffer (pH 7.0), 200 μM UDP–[^{3}H]GlcNAc (~228,000 dpm/mol), 400 μM mannosylpeptide (Ac-Ala–Ala–Pro–Thr(Man)–Pro–Val–Ala–Ala–Pro-NH_2), 10 mM $MnCl_2$, 2% Triton X-100, 5 mM AMP, 200 mM GlcNAc, 10% glycerol, and enzyme solution was incubated at 37 °C for 1 h. After boiling for 3 min, the mixture was analyzed by reversed phase HPLC with a Wakopak 5C18-200 column (4.6 × 250 mm). The gradient solvents were aqueous 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.1% trifluoroacetic acid (solvent B). The mobile phase consisted of: (1) 100% A for 10 min, (2) a linear gradient to 75% A, 25% B over 25 min, (3) a linear gradient to 100% B over 5 min, and (4) 100% B for 5 min. The peptide separation was monitored at 214 nm and the radioactivity of each fraction (1 ml) was measured by a liquid scintillation counter.

Results and discussion

Previously, we identified 13 mutations in the *POMGnT1* gene in patients with MEB [7,8]. These mutations were all simple point mutations that caused nonsense, missense, frameshift and premature termination, and splicing-site mutation (read-through of intronic sequences and/or skipping of the upstream exon) (Fig. 1). They are dispersed through the entire *POMGnT1* gene. MEB patients have either homozygous or compound heterozygous mutations in the gene. Among these mutated enzymes, only two were examined and showed a loss of activity [7]. These are the splicing mutants IVS17+1 G > A and IVS17+1 G > T (mutations 8 and 9 in Fig. 1). Their products are characterized by a read-through at residue 514 (E514rt) and a deletion of amino acid residues 472–513 (Δ472–513), respectively. As predicted by computer analysis, the 660-amino acid POMGnT1 protein is divided into four domains: a cytoplasmic tail (Met1–Arg37), a transmembrane domain (Phe38–Ile58), a stem domain (Leu59–Leu300), and the catalytic domain (Asn301–Thr660) [7,10]. Nonsense or frameshift mutations near the 5′-terminus (281 C > T, 541 del T, 1077 ins G, and 1106 ins T in Fig. 1) shorten the POMGnT1 protein significantly. Because these products (R63X, F149fs, V328fs, and D338fs) probably result in a loss-of-function, we did not perform further studies. The remaining mutants were constructed and



	Mutations	Effects
1	281 C > T	Arg 63 Stop Nonsense (R63X)
2	541 del T	Phe 149 frameshift 167 Stop (F149fs)
3	761 G > A	Glu 223 Lys missense (E223K)
4	900 G > A	Cys 269Tyr missense (C269Y)
5	1077 ins G	Val 328 frameshift 338 Stop (V328fs)
6	1106 ins T	Asp 338 frameshift 338 Stop (D338fs)
7	1572 C > G	Pro 493 Arg missense (P493R)
8	IVS17+1 G > A	Glu 514 read-through 526 Stop (E514rt)
9	IVS17+1 G > T	or Leu 472 – His 513 del (Δ472-513)
10	1743 G > A	Leu 536 – Ser 550 del (Δ536-550)
11	1813 del C	His 573 frameshift 633 Stop (H573fs)
12	1926 del T	Leu 611 frameshift 633 Stop (L611fs)
13	1970 del G	Val 626 frameshift 633 Stop (V626fs)

Fig. 1. Schematic representation of POMGnT1 and the predicted products corresponding to each mutation and a summary of mutations of MEB patients. Each protein is represented by a box with its N-terminus to the left. Each box from the left indicates a cytoplasmic tail (white box), a transmembrane domain (black box), a stem domain (white box), and the catalytic domain (gray box). The hatched boxes and dotted-line boxes represent mutated amino acids and deletion regions, respectively. The numbers below the boxes indicate the amino acid residue numbers of POMGnT1 and each mutant. rt, read-through; Δ, deletion; and fs, frameshift.

expressed in HEK293T cells and then we examined the enzyme activity of each product.

The 1743 G > A mutation (G-to-A base substitution at position 1743; mutation 10 in Fig. 1) caused skipping of exon 19, resulting in the deletion of 15 amino acids. The product (Δ536–550) did not show any enzymatic activity (Fig. 2). Three mutants with deletions near the 3′-terminus of the *POMGnT1* coding region (1813 del C, 1926 del T, and 1970 del G in Fig. 1) result in a frameshift and premature termination codon 633. These products (H573fs, L611fs, and V626fs) did not show any enzymatic activity (Fig. 2). They were thought to retain some ability to transfer a GlcNAc residue because they have the sequence of the normal POMGnT1 protein

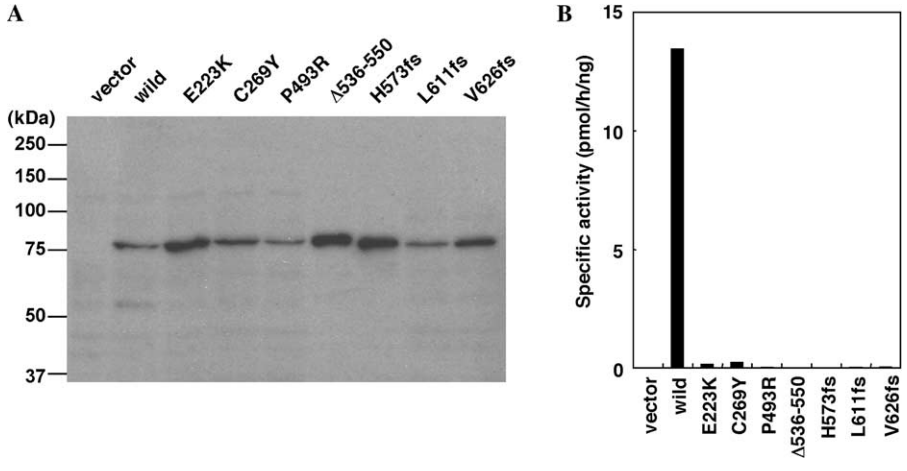


Fig. 2. Expression and activity of POMGnT1 mutant proteins. (A) Western blot analysis of Xpress-tagged POMGnT1 mutants expressed in HEK293T cells. The proteins (20 μg each) were subjected to SDS–PAGE (10% gel) and the separated proteins were transferred to a PVDF membrane. The PVDF membrane was stained with anti-Xpress monoclonal antibody. Molecular weight standards are shown on the left. (B) POMGnT1 activity was based on the rate of GlcNAc transfer to a mannosylpeptide using each of the membrane fractions from each transfectant. The product was separated by HPLC.

until positions 573, 611, and 626, respectively. However, this was not the case. The results suggest that the C-terminal portion of the POMGnT1 protein is necessary for enzymatic activity. The remaining three mutants with base substitutions (761 G > A, 900 G > A, and 1572 C > G in Fig. 1) result in amino acid substitutions, Glu223Lys, Cys269Tyr, and Pro493Arg, respectively. These products did not show any enzymatic activity (Fig. 2). Because position 493 is located in the probable catalytic domain of the protein [7], the amino acid substitution P493R may have an effect on enzymatic activity. On the other hand, positions 223 and 269 are located in the stem domain. Although the function of the stem region is not clear, it may contain a targeting signal for the Golgi apparatus [11]. Because we used a crude microsomal membrane fraction as an enzyme source in this study, we cannot exclude the possibility that, in addition to a loss of enzymatic activity, the enzyme was not translocated to the Golgi apparatus correctly. In any case, we conclude that an amino acid substitution of 223 or 269 leads to a loss-of-function. In summary, none of the mutated POMGnT1 in MEB patients have any enzyme activity. This suggests that MEB patients have a defect of *O*-mannosyl glycosylation. However, measurement of the POMGnT1 activity or a structural analysis of the sugars of α-dystroglycan in each patient tissue will be necessary to reach this conclusion, although it is very hard to perform it at this stage due to the limited amount of the sample and the ethical problem.

In a previous study of MEB patients with mutations in *POMGnT1* [8], we observed that patients with mutations near the 5′-terminus of the coding region had relatively severe brain symptoms, especially hydrocephalus, while patients with mutations near the 3′-terminus had milder phenotypes. The fact that all mutant POMGnT1s lost all activity suggests that additional factors play a role in determining disease severity in the brain.

Like MEB, Fukuyama-type congenital muscular dystrophy (FCMD) and Walker–Warburg syndrome (WWS) are autosomal recessive disorders that are characterized by congenital muscular dystrophy, lissencephaly, and eye anomalies [12,13]. FCMD is caused by mutations in the gene encoding fukutin, a protein of unknown function [14]. A sequence analysis predicts it to be an enzyme that modifies cell-surface glycoproteins or glycolipids. Recently, 20% of WWS patients have been found to have mutations in *POMT1*, a putative *O*-mannosyltransferase that catalyzes the transfer of Man to a Ser or Thr residue [15]. However, it is unclear whether the POMT1 protein actually catalyzes the reaction. Interestingly, in each of these diseases, a highly glycosylated α-dystroglycan was selectively deficient in skeletal muscle [3,15–18]. Additionally, defective glycosylation of α-dystroglycan has been implicated in congenital muscular dystrophy type 1C (MDC1C). The defective glycosylation is caused by mutations in a gene encoding a putative glycosyltransferase (FKRP, fukutin-related protein) [19]. The gene *large*, which is mutated in the myodystrophy (*myd*) mouse, encodes a putative glycosyltransferase [20]. Moreover, hypoglycosylated α-dystroglycan in the muscle membrane of MEB, FCMD, and the *myd* mouse has greatly reduced affinities for laminin, neurexin, and agrin [4]. In other words, interference in O-mannosylation of α-dystroglycan may lead to a combination of muscle, eye, and brain abnormalities and is a new pathomechanism for muscular dystrophy as well as neuronal migration disorder. Some forms of muscular dystrophy may be due to defects of glycosyltransferases, but the substrates of these enzymes, with the exception of POMGnT1, are largely unknown. Identification and characterization of each enzyme will help to reveal the molecular pathomechanisms of congenital muscular dystrophies with brain malformation.

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