Developmental Cell, Vol. 1, 717–724, November, 2001, Copyright ©2001 by Cell Press

Muscular Dystrophy and Neuronal Migration Disorder Caused by Mutations in a Glycosyltransferase, POMGnT1

Aruto Yoshida,[1,9] Kazuhiro Kobayashi,[2,9] Hiroshi Manya,[3] Kiyomi Taniguchi,[2] Hiroki Kano,[2] Mamoru Mizuno,[4] Toshiyuki Inazu,[4] Hideyo Mitsuhashi,[1] Seiichiro Takahashi,[3] Makoto Takeuchi,[1] Ralf Herrmann,[5] Volker Straub,[5] Beril Talim,[6] Thomas Voit,[5] Haluk Topaloglu,[7] Tatsushi Toda,[2,8,10] and Tamao Endo[3,8,10]

[1] Central Laboratories for Key Technology
Kirin Brewery Co., Ltd.
Kanazawa-ku, Yokohama 236-0004
Japan

[2] Division of Functional Genomics
Department of Post-Genomics and Diseases
Osaka University Graduate School of Medicine
Suita, Osaka 565-0871
Japan

[3] Department of Glycobiology
Tokyo Metropolitan Institute of Gerontology
Itabashi-ku, 173-0015
Japan

[4] Research Department
The Noguchi Institute
Itabashi-ku, Tokyo 173-0003
Japan

[5] Department of Pediatrics and Pediatric Neurology
University of Essen
D-45122 Essen
Germany

Departments of [6] Pediatric Pathology and [7] Pediatric Neurology
Hacettepe Children's Hospital
Ankara 06100
Turkey

Summary

Muscle-eye-brain disease (MEB) is an autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormalities, and lissencephaly. Mammalian *O*-mannosyl glycosylation is a rare type of protein modification that is observed in a limited number of glycoproteins of brain, nerve, and skeletal muscle. Here we isolated a human cDNA for protein *O*-mannose β-1,2-*N*-acetylglucosaminyltransferase (POMGnT1), which participates in *O*-mannosyl glycan synthesis. We also identified six independent mutations of the *POMGnT1* gene in six patients with MEB. Expression of most frequent mutation revealed a great loss of the enzymatic activity. These findings suggest that interference in *O*-mannosyl glycosylation is a new pathomechanism for muscular dystrophy as well as neuronal migration disorder.

[8] Correspondence: endo@tmig.or.jp (T.E.), toda@clgene.med.osaka-u.ac.jp (T.T.)

[9] These authors contributed equally to this work.

[10] These authors contributed equally to this work.

Introduction

Since the discovery of the Duchenne muscular dystrophy gene product dystrophin (Hoffman et al., 1987), many studies have focused on understanding the pathophysiology of muscular dystrophies and on developing therapeutic approaches. Dystroglycan is a component of the dystrophin-glycoprotein-complex (DGC) in skeletal muscle, and it is thought to have a crucial role in linking the extracellular basal lamina to the cytoskeletal proteins. Structural defects in the DGC can result in a loss of linkage between laminin-2 (merosin) in the extracellular matrix and actin in the subsarcolemmal cytoskeleton, and this can lead to various muscular dystrophies (Campbell, 1995). Neurons that constitute the cerebral cortex migrate hundreds of cell-body distances from their place of birth to reach their final position within the correct cortical layer, a process called cortical histogenesis. Several genes that are thought to function in the migration, and assembly of neurons during this process has been implicated in cortical dysgenesis disorders. These genes include *reelin*, *mdab1*, which is involved in the *scrambler* and *yotari* mouse mutations, *LIS1*, which is involved in Miller-Dieker syndrome, and *doublecortin*, which is involved in X-linked lissencephaly (Gleeson and Walsh, 2000). Muscle-eye-brain disease (MEB: MIM 253280) is an autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormalities, and brain malformation (type II lissencephaly) and is seen mainly in Finland (Santavuori et al., 1989). Patients with MEB manifest 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 (Figure 1). The gene responsible for MEB has been mapped to chromosome 1p32-34 (Cormand et al., 1999), but it has not been identified and the cause of this disease is not known.

In eukaryotes, proteins are frequently modified by *O*-glycosylation as well as by *N*-glycosylation. *O*-glycosylation occurs at Ser and Thr residues. In yeast and fungi, *O*-glycosylation mainly occurs in the form of *O*-mannosylation (Gemmill and Trimble, 1999). However, *O*-mannosylation is rare in mammals, occurring in a limited number of glycoproteins of brain, nerve, and skeletal muscle (Endo, 1999). The role of *O*-mannosylation is not clear. We demonstrated that sialyl *O*-mannosyl glycan is a laminin binding ligand of α-dystroglycan (Chiba et al., 1997). It is possible that defects in *O*-mannosylation may weaken the laminin binding and consequently are responsible for muscular dystrophy and neuronal migration disorders such MEB. Very recently, a deletion of a glycosyltransferase-like protein gene, *Large*, was found to be the basis of the myodystrophy (*myd*) mouse and to give rise to an α-dystroglycan

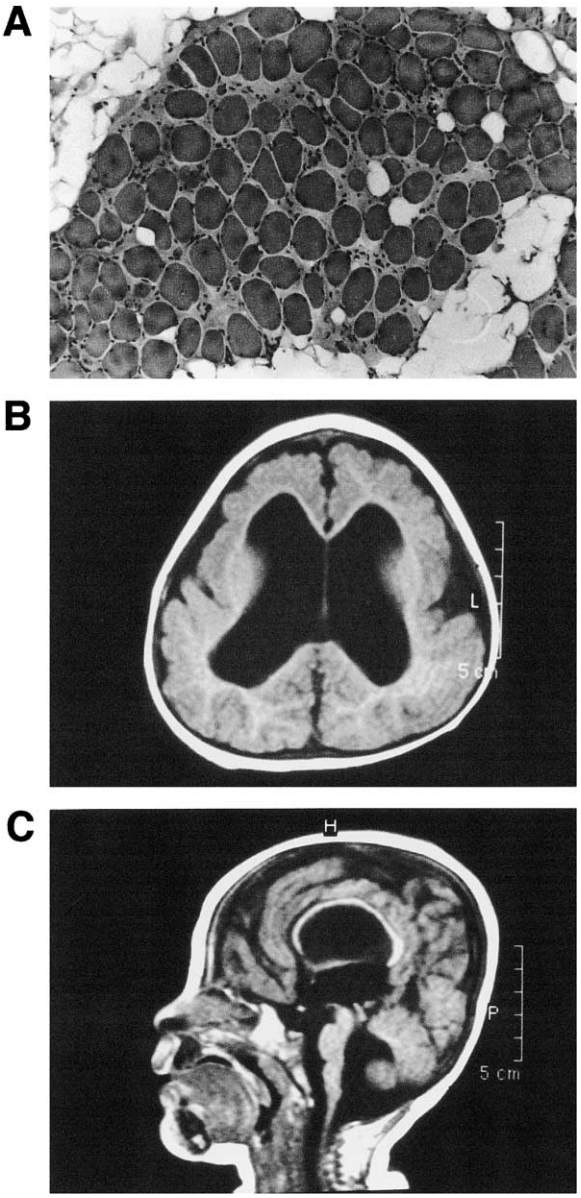

Figure 1. MEB Patient Aged 5 Years

(A) Muscle biopsy. Typical rounding fibers and variation in size with endomysial connective tissue proliferation. There is also prominent fatty infiltration. HE × 20.

(B) Cranial MRI, axial section. Ventricles are enlarged in the presence of cortical atrophy. There is diffuse thickening of the cerebral cortex from the frontal region to the parieto-occipital areas, denoting pachygyria.

(C) Cranial MRI, sagittal section. Cerebellar hypoplasia and brain stem strophy. Corpus callosum is thin.

that is markedly underglycosylated (Grewal et al., 2001). Others found a selective deficiency of highly glycosylated α-dystroglycan in Fukuyama-type congenital muscular dystrophy (FCMD) muscle (Hayashi et al., 2001). These findings suggest that interference in glycosylation is one cause of muscular dystrophies, although the mechanism by which this occurs is unclear.

A key difference between mammalian and yeast-type *O*-mannosyl glycans is that those in mammals have the GlcNAcβ1-2Man linkages (Endo, 1999). This linkage is assumed to be catalyzed by a glycosyltransferase, UDP-*N*-acetylglucosamine: protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase (POMGnT1). POMGnT1 catalyzes the transfer of *N*-acetylglucosamine from UDP-GlcNAc to *O*-mannosyl glycoproteins, according to the following reaction: UDP-GlcNAc + Man-R → GlcNAcβ1-2Man-R + UDP in which R is protein. POMGnT1 activity was recently found in brain homogenates of several mammals (Takahashi et al., 2001). Thus, characterization of this enzyme might help to elucidate the biosynthetic pathway of mammalian *O*-mannosyl glycans.

It should be noted that GlcNAcβ1-2Man linkages are also found in N-glycans, where they are catalyzed by two enzymes, UDP-*N*-acetylglucosamine: α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT-I) and UDP-*N*-acetylglucosamine: α-6-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase II (GnT-II) (Schachter, 1995). However, we found that recombinant GnT-I and -II, expressed by *Saccharomyces cerevisiae*, had no ability to catalyze the GlcNAcβ1-2Man linkage in *O*-mannosyl glycans (Takahashi et al., 2001).

In this study, we cloned and characterized POMGnT1. We also found that MEB is caused by mutations in the *POMGnT1* gene. These results provide a molecular basis for the defects in *O*-mannosyl glycan synthesis and suggest that interference in *O*-mannosyl glycosylation is a new pathomechanism for muscular dystrophy as well as neuronal migration disorder.

Results

Cloning of Human *POMGnT1*

A BLAST search of human cDNA sequences homologous to human *GnT-I* (Kumar et al., 1990) uncovered four human EST clones (accession numbers AA911248, F11377, W77826, and R20086), whose functions have not been identified. Further computer-aided retrieval of contiguous EST clones disclosed a cDNA sequence of approximately 1.6 kb. Based on this sequence, two cDNA fragments were amplified from human brain RNA by RT-PCR. An analysis of the sequences of these cDNA fragments showed a reading frame of 1.3 kb, whose predicted amino acid sequence is highly homologous to the C-terminal side of human GnT-I (Kumar et al., 1990). The remaining 5′-region of the cDNA was amplified from human brain cDNA by a modified 5′-RACE method. A 0.8 kb 5′-RACE fragment completed the *POMGnT1* open reading frame. The nucleotide sequence indicated that POMGnT1 is a 660 amino acid protein (Figure 2A) with a calculated molecular mass of 71.5 kDa. A hydrophobicity analysis and secondary structure prediction of the amino acid sequence suggested that human POMGnT1 is a type II membrane protein and the region from Phe38 to Ile58 is a putative transmembrane domain. This topology was similar to the topologies of other Golgi glycosyltransferases cloned to date.

Expression and Characterization of Human POMGnT1 Protein

A transient expression of *POMGnT1* cDNA in HEK293T cells increased their microsomal POMGnT1 activity for

a mannosylpeptide approximately 100-fold (0.12 versus 12.8 pmol/h/μg protein). When we sonicated the microsomal fraction in a buffer containing 2% Triton X-100, about 60% of the enzyme still bound to the membrane tightly (data not shown). These results indicated that the cloned cDNA encodes a functional membrane-bound glycosyltransferase, POMGnT1. A soluble form of POMGnT1 was expressed in HEK293T cells to further characterize the enzymatic properties of POMGnT1. HX-sPOMGnT1 consisted of the C-terminal 595 amino acids (Ser66-Thr660 in Figure 2A). We also transfected HEK293T cells to produce a soluble enzyme, HX-sGnT-I, consisting of the C-terminal 407 amino acids (Ser39-Asn445 in Figure 2A) of human GnT-I (Kumar et al., 1990) to compare with HX-sPOMGnT1. The estimated molecular weights of HX-sPOMGnT1 and HX-sGnT-I were approximately 76 and 52 kDa.

HX-sPOMGnT1 exhibited POMGnT1 activity, and its activity increased in proportion to (1) the amount of enzyme, (2) the incubation time, (3) the amount of UDP-GlcNAc, and (4) the amount of acceptor mannosylpeptide. Also, the POMGnT1 activity depended on manganese ion (data not shown). The Km for mannosylpeptide was 1.85 mM, and the Km for UDP-GlcNAc was 0.73 mM when mannosylpeptide (0.4 mM) was used as an acceptor. The product synthesized by HX-sPOMGnT1 was identified as described previously (Takahashi et al., 2001). In brief, the product was completely digested by streptococcal β-*N*-acetylhexosaminidase, which cleaves the GlcNAcβ1-2Man linkage. The β-eliminated product was found to elute at the same position as the authentic GlcNAcβ1-2Man_{oH} by HPAEC-PAD (data not shown). Thus, we concluded that the GlcNAc residue is linked to the 2 position of the Man residue on the peptide. The substrate specificity of two soluble *N*-acetylglucosaminyltransferases is summarized in Figure 2B. Although HX-sPOMGnT1 was able to use a mannosylpeptide as a substrate, it did not transfer a GlcNAc to either mannose or *p*-nitrophenyl-α-D-mannopyranoside. These results suggested that POMGnT1 closely recognizes an acceptor structure consisting of mannose and peptide. Furthermore, HX-sPOMGnT1 did not show any detectable GnT-I activity when M5-PA or M3-PA was employed as an acceptor, even though it has a domain that is highly homologous with a part of human GnT-I. In contrast, HX-sGnT-I did not show any substantial POMGnT1 activity, although it exhibited obvious GnT-I activity for both M5-PA and M3-PA. Thus, we concluded that human POMGnT1 is a new *N*-acetylglucosaminyltransferase that is functionally different from GnT-I.

Northern Analysis of Human *POMGnT1* mRNA

Northern blot analysis of *POMGnT1* showed that a transcript of 2.7 kb was expressed in all 23 normal tissues tested (Figure 2C and Supplemental Data, Figure S1 [http://www.developmentalcell.com/cgi/content/full/1/5/717/DC1]), indicating that human *POMGnT1* is constitutively expressed in the body. An additional weaker band of 3.4 kb was also detected in spinal cord, lymph node, and trachea, suggesting that *POMGnT1* has two transcriptional initiation sites or alternative splicing in these tissues. The expression pattern of *POMGnT1* was apparently different from that of *GnT-I* (Kumar et al., 1990).

Phylogenic and Genomic Characters of *POMGnT1*

Whereas *GnT-I* is widely distributed in mammals, insects, nematodes, and plants (Kumar et al., 1990; Chen et al., 1999; Strasser et al., 1999), a BLAST search suggested that *POMGnT1* orthologs are restricted to mammals such as mouse, pig, and cattle. This raises the possibility that the GlcNAc-containing mammalian type *O*-mannosylation occurs in mammals and is biologically important for mammals only. Figure 2D shows a phylogenic tree of known GnT-I proteins, GnT-I homologs, and POMGnT1 proteins. Interestingly, human POMGnT1 was positioned between *Caenorhabditis elegans* and plant GnT-I species, and mammalian GnT-I made an independent cluster. The genomic organization of *POMGnT1* is significantly different from the single exon of the human *GnT-I* coding region (Yip et al., 1997). Based on a draft genomic sequence (AL360086) containing human *POMGnT1*, we performed a genomic PCR analysis and concluded that *POMGnT1* is divided into 22 exons and its coding region starts from exon 2 (Figure 2E). Taken together, these findings imply that a primordial gene was duplicated a long time ago.

The *POMGnT1* Gene Is Mutated in MEB

Two UniSTS entries (stSG22112 and stSG3089) suggested that the human *POMGnT1* gene exists at 1p33-34, whereas the *GnT-I* gene exists at 5q35. The 1p33-34 locus is supported by the finding that the clone containing human *POMGnT1* (AL360086) overlaps with another clone (AL122001) that contains a DNA segment of chromosome 1p31.3-33. We noticed that *MEB* has been also mapped to 1p32-34 (Cormand et al., 1999). After the initial mapping of the *MEB* gene to 1p32-p34, the *MEB* locus has been narrowed to a 1-cM interval including four microsatellite markers, D1S2134, D1S2677, D1S2824, and D1S2748, by founder-haplotype mapping (Dubowitz, 1999). We further found that the *POMGnT1* gene is located within this small candidate interval for *MEB*. Since defects of DGC cause muscular dystrophies (Campbell, 1995) and *O*-mannosyl type glycan is required for the laminin binding of α-dystroglycan in DGC (Chiba et al., 1997), it is possible that mutations in the *POMGnT1* gene are related to MEB.

To test this hypothesis, we screened the whole coding region and the exon/intron flanking sequences of the *POMGnT1* gene for mutations in five Turkish patients and one French patient with MEB. The parents of each of these patients were either consanguineous or from the same village. We identified six independent disease-causing mutations in these patients (Figure 3A). Patients YA and KO carried a G to T substitution in intron 17 homozygously, which altered the conserved GT splicing donor sequence to TT (Figure 3B). Patient SA carried an A substitution in intron 17 homozygously, which altered the conserved GT splicing donor sequence to AT. RT-PCR analysis of skeletal muscle from patient SA revealed that this mutation caused read-through of intronic sequences, resulting in introduction of a premature termination codon. The mutation also caused skipping of the upstream exon 17, resulting in deletion of

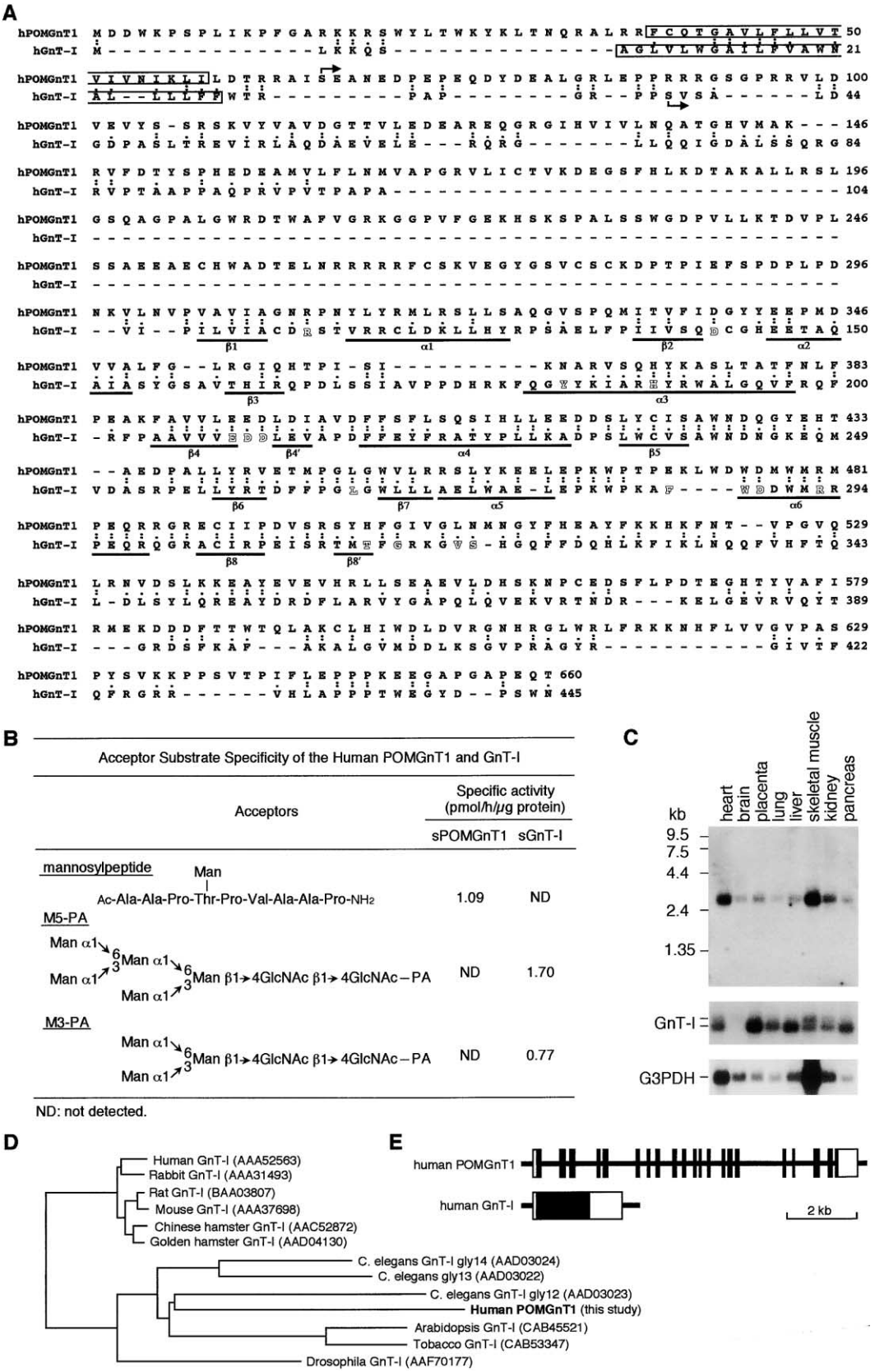

Figure 2. Characterization of Human POMGnT1

(A) Comparison of alignment of deduced amino acid sequences of human POMGnT1 and GnT-I (Kumar et al., 1990). Letters in outline indicate amino acids involved in the binding of GnT-I to UDP-GlcNAc and Mn^{2+} ion, and the secondary structures of GnT-I are represented by thick

A

Mutations of the *POMGnT1* Gene in MEB Patients

Patients	Mutation	Effect	Status
YA	IVS17 +1 G > T	Leu472 read-through 484Stop / Leu472-His513 del	homozygote
KO	IVS17 +1 G > T	Leu472 read-through 484Stop / Leu472-His513 del	homozygote
SA	IVS17 +1 G > A	Leu472 read-through 484Stop / Leu472-His513 del	homozygote
MK	1743 G > A	Ser550Asn	homozygote
CC	1813 del C	His573 frameshift 633Stop	homozygote
TLG	1572 C > G	Pro493Arg	compound heterozygote
	1970 del G	Val626 frameshift 633Stop	

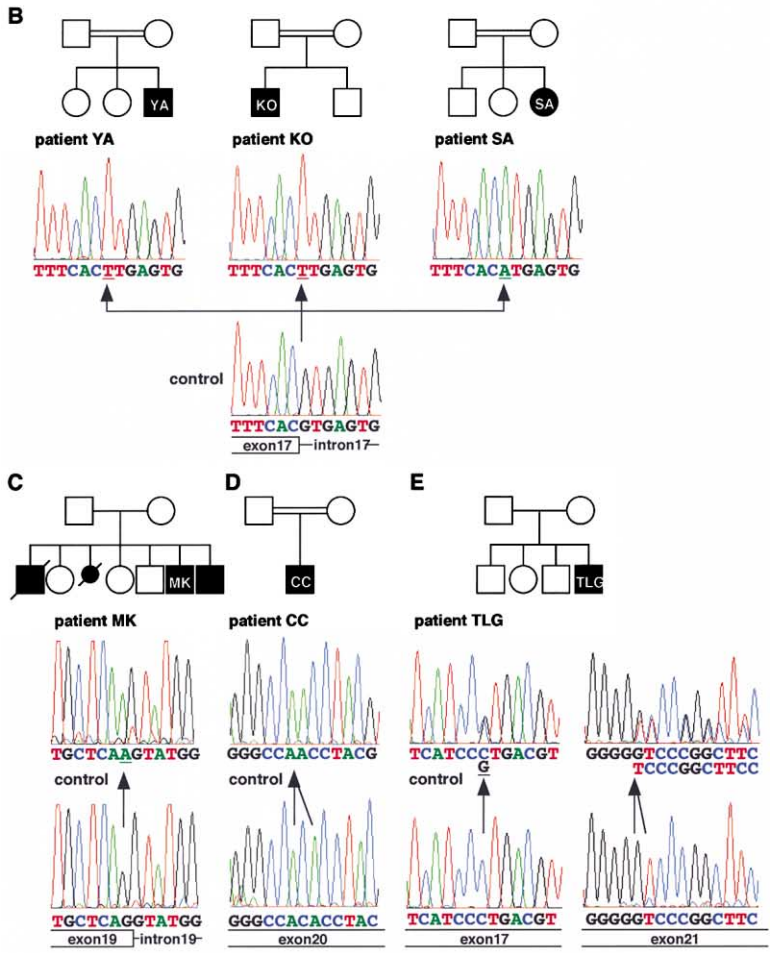

Figure 3. Point Mutations in Six Patients with MEB

(A) Summary of mutations of the *POMGnT1* gene in MEB patients.

(B) Patients YA, KO, and SA were homozygous for a G to T or A substitution allele in intron 17, which causes abnormal splicing.

(C) Patient MK was shown to carry a G1743A transition allele in exon 19 homozygously, which represents a missense mutation (Ser550Asn).

(D) Patient CC was homozygous for a 1 bp deletion at base1813 in exon 20, which causes a frameshift and a premature termination.

(E) Patient TLG is a compound-heterozygote who carries a C1572G transversion in exon 17 (Pro493Arg) and a 1 bp deletion at base 1970 in exon 21 (frameshift).

42 amino acids (data not shown). Patient MK carried a G1743A transition in exon 19 homozygously, which represents a Ser550Asn nonconservative amino acid change (Figure 3C). Patient CC had a 1 bp deletion at base 1813 in exon 20 homozygously, causing a frameshift and a premature termination at codon 633 (Figure 3D). Furthermore, we found that a French patient, TLG, is a compound heterozygote who carries a C1572G transversion in exon 17 (Pro493Arg) and a 1-bp deletion at base 1970 in exon 21 (frameshift and premature termination at codon 633) (Figure 3E). Each mutation cosegregated in each pedigree examined (families SA, MK,

lines under the sequence (Ünligil et al., 2000). The putative transmembrane domains are boxed. The soluble constructs (Ser66-Thr660 for POMGnT1, Ser39-Asn445 for GnT-I) are indicated by the arrows.

(B) Acceptor substrate specificity of the soluble form human POMGnT1 and GnT-I.

(C) Northern analysis of *POMGnT1* mRNA expression in human tissues. A ^{32}P-labeled *POMGnT1*-specific probe was hybridized to human multiple tissue Northern blot (Human MTN). The sizes of RNA marker bands are indicated on the left. The same blots were also probed with ^{32}P-labeled human cDNA fragments of *GnT-I* and *G3PDH* (middle and lower panels, respectively).

(D) Phylogenic tree of the GnT-I and POMGnT1 family. The phylogenic tree was constructed by the CLUSTAL W algorithm using amino acid sequences of human POMGnT1 and other GnT-I-related sequences retrieved from the Entrez protein database.

(E) Comparison of genomic organization in the coding region of human *POMGnT1* and *GnT-I*. The organization of human *GnT-I* was taken from a reference (Yip et al., 1997). Filled boxes and open boxes indicate exons in the coding region and the noncoding region, respectively.

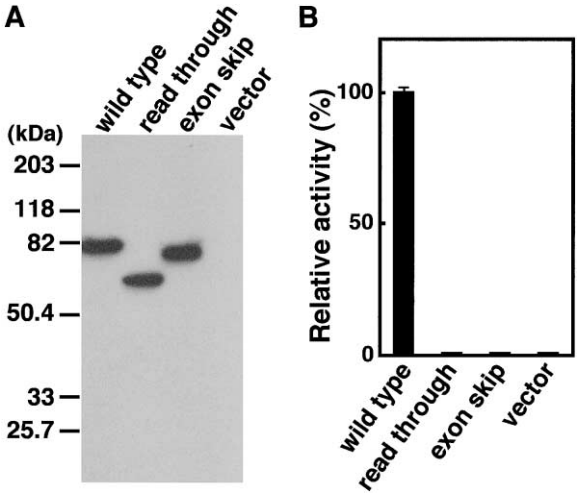

Figure 4. Function of the Mutant POMGnT1 Proteins

(A) Western blot analysis of Xpress epitope-tagged mutants expressed in HEK293T cells. Proteins in the membrane fractions were fractionated by SDS-PAGE and then detected by an anti-Xpress epitope antibody. Molecular weight standards are shown on the left. (B) The rate of GlcNAc transfer to a mannosyl peptide was measured using each of the membrane fractions from each transfectant. Analysis of enzyme activity is described in Experimental Procedures.

CC, and TLG), and we have not detected these six substitutions in any of 246 normal chromosomes (data not shown), indicating that these mutations are pathogenic and that the *POMGnT1* gene is responsible for MEB.

Loss of Function of the Products from Mutant *POMGnT1* cDNA

To confirm that the mutations observed in patients with MEB are responsible for the defects in the synthesis of *O*-mannosyl glycan, we examined the function of the expressed mutant cDNAs. Since both read-through of intronic sequences and skipping of the upstream exon 17 were found in a patient with MEB who carries the most frequent mutation, we examined two forms of *POMGnT1* mRNA that either contained the intron 17 sequence (intron 17 read-through form) or lack the exon 17 sequence (exon 17 skipping form). The enzyme activity of either POMGnT1 mutant was $<1\%$ of that of those expressing the wild-type protein (Figure 4B), despite the expression levels of these products being almost equivalent (Figure 4A). The estimated molecular weights of wild-type, read-through, and exon-skipping were approximately 82, 65, and 78 kDa, respectively.

Discussion

In the present study, we have shown that mutations in the *POMGnT1* gene, which catalyzes the second glycosyl transfer step in the biosynthesis of mammalian *O*-mannosyl glycans, are the primary genetic defect in MEB and that they are inherited in a loss-of-function manner based on identification of the gene mutations and characterization of the gene products. The results reported here clearly indicate the importance of the intact synthesis of *O*-mannosyl glycans in muscular dystrophy as well as neuronal migration disorder.

Structural defects in DGC, leading to a loss of linkage between laminin-2 (merosin) in the extracellular matrix and actin in the subsarcolemmal cytoskeleton, cause various muscular dystrophies (Campbell, 1995). In this study, however, MEB is found to be caused by a defect in a glycosylation enzyme. MEB clinically resembles FCMD (Fukuyama et al., 1981) and Walker-Warburg syndrome (Dobyns et al., 1989), sharing the unusual combination of muscle, eye, and brain abnormalities. The *FCMD* gene has been positionally cloned (Kobayashi et al., 1998). Although the function of its gene product, fukutin, is not clear, computer analysis predicts that fukutin is an enzyme that modifies cell-surface glycoproteins or glycolipids (Aravind and Koonin, 1999), having similarity with the results in the current study. Since *O*-mannosyl glycan is required for the binding between α-dystroglycan and laminin (Chiba et al., 1997), the subsequent aberrant *O*-mannosylation may weaken binding and allow disruption of the sarcolemmal linkage of skeletal muscle or abnormal migration of neuronal cells. This is consistent with the observation that immunostaining of the laminin α2 chain was weaker in MEB muscle (Auranen et al., 2000) and that the dystrophic phenotypes were induced by an antibody blocking the muscle α-dystroglycan and laminin interaction (Brown et al., 1999).

The deduced amino acid sequences of POMGnT1 and GnT-I are compared in Figure 2A. Although the overall identity between them was only 23.2%, a particularly conserved region was observed at amino acids 367–505. The hydrophobicity profile and the predicted secondary structure indicated that the conserved regions have a high structural similarity. The detailed protein structure of rabbit GnT-I recently reported by Ünligil et al. (2000) showed that this conserved region corresponds to structures from α3 to β8′ in domain 1. A disulfide bridge connects two β strands (β5 and β8) in this region of GnT-I, and the corresponding two cysteine residues (Cys421 and Cys490) were also detected in POMGnT1. Thus, the frameworks of the conserved regions in POMGnT1 and GnT-I appeared to be very similar. A D/E-X-D motif, which is flanked by apolar residues, has been detected in GnT-I as well as several other glycosyltransferases (Chen et al., 1999; Ünligil et al., 2000). Three amino acids in the motif and another 13 amino acids of GnT-I are critical for the binding to UDP-GlcNAc and Mn^{2+} ion (Ünligil et al., 2000). Ten of 16 residues were detected at the corresponding positions (Arg311, Asp338, His371, Glu393, Asp395, Leu449, Trp475, Asp476, Arg480, and Gly502) of human POMGnT1. Interestingly, the putative catalytic base (Asp289) of GnT-I was conserved at Asp476 in POMGnT1. This high conservation of the catalytic domain suggested that the catalytic pocket and the reaction mechanism of POMGnT1 are at least partly similar to those of GnT-I.

On the other hand, POMGnT1 and GnT-I showed several structural differences. The cytoplasmic tail of this enzyme was predicted to be the N-terminal 37 amino acids, which is long relative to the cytoplasmic tails of other known glycosyltransferases including GnT-I. More than 110 amino acids of the additional residues were found in the putative stem domain of POMGnT1. In addition, the amino acid sequence from β8′ to the C terminus of GnT-I was less conserved in

POMGnT1 (Figure 2A). Since the loop following β8′ is involved in the acceptor binding of GnT-I (Ünligil et al., 2000), differences in the region from β8′ to the C termini may affect the precise specificity of these enzymes for acceptor oligosaccharides. It is interesting that all mutations found in this study occurred from exon 17 to the C terminus of POMGnT1, although the biological significance of this remains to be established. As shown in Figure 2C and the Supplemental Figure S1 [http://www.developmentalcell.com/cgi/content/full/1/5/717/DC1], *POMGnT1* expression appears to be mainly constitutive in many tissues. This suggests that *O*-mannosyl glycosylation is strictly regulated by the substrate specificity of POMGnT1 and/or the expression of an unidentified protein *O*-mannosyltransferase, which transfers a mannose to Ser/Thr in proteins. Consequently, a defect in *O*-mannosyl glycosylation may lead to the unusual combination of muscle, eye, and brain abnormalities.

Recent investigations have revealed that glycosylation is related to several biological and pathological phenomena such as Notch signaling, cancer metastasis, and immunity (Dennis et al., 1999; Moloney et al., 2000; Lowe, 2001). Our findings suggest a new pathomechanism, in which defects of *O*-mannosyl glycosylation cause muscular dystrophies and further neuronal migration disorders. A putative glycosyltransferase gene, *Large*, was recently found to be mutated in the *myd* mouse (Grewal et al., 2001). Others found a selective deficiency of highly glycosylated α-dystroglycan in FCMD muscle (Hayashi et al., 2001). These findings also indicate that altered glycosylation is one cause of muscular dystrophies. In either case, however, the type of change in glycosylation that has occurred remains to be elucidated. It is noteworthy that α-dystroglycans in different species and different tissues all have *O*-mannosyl glycans. Therefore, *O*-mannosyl glycan may be important in the basic function of α-dystroglycan, which acts as a linker between the cytoskeleton and the extracellular matrix. Taken together, it is possible that α-dystroglycan is a potential target relevant for disease. Further studies will help us to better understand (1) the biological function and the regulation of this protein modification and (2) the pathophysiology of these complex disorders consisting of the simultaneous occurrence of central nervous, ocular, and muscular manifestations as well as muscular dystrophy in general.

Experimental Procedures

Isolation of Human *POMGnT1* cDNA Fragments by RT-PCR and 5′-RACE

Human cDNA sequences encoding a protein homologous to human GnT-I (Kumar et al., 1990) were surveyed in the GenBank database by BLAST. Based on EST sequences, we amplified two cDNA fragments by the Access RT-PCR system (Promega) using total RNA from human brain (Clontech). 5′-RACE was carried out by nested PCR using a Human Brain Marathon-Ready cDNA (Clontech). The amplified cDNAs were cloned into pCR-TOPO 2.1 (Invitrogen) and sequenced on an ABI Model 377 DNA sequencer (Perkin-Elmer).

Expression of Full-Length and Soluble-form Human POMGnT1

The cDNA for full-length *POMGnT1* was constructed by joining two RT-PCRs. Two fragments were ligated at a PstI site and introduced into the NheI and XhoI sites of pcDNA 3.1 Zeo(+) (Invitrogen). The expression plasmid POMGnT1/pcDNA 3.1 Zeo(+) was transfected into HEK293T cells, and the microsomal fraction of the cells was obtained.

A soluble form of POMGnT1 tagged with the His-tag and Xpress epitope (HX-sPOMGnT1) was secreted from HEK293T cells by using the Ig kappa chain secretion signal. The DNA encoding the secretion signal, His-tag, and Xpress epitope was prepared from pSecTag2 A and pcDNA3.1/His C (Invitrogen) by overlap extension PCR. The DNA was linked to the cDNA encoding Ser66-Thr660 of POMGnT1 and introduced into pcDNA3.1 Zeo(+). The resulting expression plasmid was transfected into HEK293T cells as described above. The culture supernatant was collected after 2 days culture. Similarly, HX-sGnT-I containing Ser39-Asn445 of human GnT-I (Kumar et al., 1990) was prepared.

The GnT-I activity and the POMGnT1 activity were measured as described by Takahashi et al. (2001). Pyridylaminated oligosaccharide acceptors, M5-PA and M3-PA, were obtained from Takara Shuzo (Ohtsu, Japan). The amount of protein was estimated with a BCA protein assay reagent kit (Pierce).

Western and Northern Blot Analyses

The microsomal fraction of each transfectant was separated on 10% SDS-PAGE and then transferred to a piece of polyvinylidene difluoride membrane. The anti-Xpress epitope (Invitrogen) was used as a primary antibody. The reactive protein was visualized using ECL western blotting detection reagents (Amersham Pharmacia Biotech).

Probe DNA fragments for human *POMGnT1*, *GnT-I*, and glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) were labeled with [α-^{32}P]dCTP using a Megaprime DNA labeling system (Amersham Pharmacia Biotech). Northern blots of human tissues (Human MTN, MTN II, MTN III; Clontech) were hybridized with a ^{32}P-labeled DNA probe in Rapid hybridization buffer (Amersham Pharmacia Biotech) at 65°C.

MEB Patients and Mutation Analysis

Five Turkish and one French linkage-proven MEB families permitted analysis of DNA samples. Primers to amplify each exon and surrounding intronic sequences were designed from genomic sequence of the *POMGnT1* gene. PCR products were excised from gels and directly sequenced.

Construction of Mutant POMGnT1s

An expression vector encoding each mutant of POMGnT1 was prepared by site-directed mutagenesis. In brief, we synthesized mutagenic primers, amplified cDNA with mutations from the full-length cDNA clone, and cloned into pcDNA 3.1 Zeo(+) (Invitrogen). All mutant clones were sequenced to confirm the presence of the mutations.

Acknowledgments

We thank Y. Nakabayshi and H. Soga for assistance, and Drs. N. Kotani and S. Takasaki for HPAEC-PAD analysis. This study was supported by the New Energy and Industrial Technology Development Organization (NEDO) as a part of the Research and Development Projects of the Industrial Science and Technology Frontier Program in Japan, Mizutani Foundation for Glycoscience, Health Science Research Grant, "Research on Brain" Science from the Ministry of Health, Labor, and Welfare, a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and grants from the Association Française contre les Myopathies and the Deutsche Forschungsgemeinschaft.

Received August 14, 2001; revised September 20, 2001.

References

Aravind, L., and Koonin, E.V. (1999). The fukutin protein family—predicted enzymes modifying cell-surface molecules. Curr. Biol. *9*, R836–R837.

Auranen, M., Rapola, J., Pihko, H., Haltia, M., Leivo, I., Soinila, S., Virtanen, I., Kalimo, H., Anderson, L.V.B., Santavuori, P., and Somer,

H. (2000). Muscle membrane-skeleton protein changes and histopathological characterization of muscle-eye-brain disease. Neuromuscul. Disord. *10*, 16–23.

Brown, S.C., Fassati, A., Popplewell, L., Page, A.M., Henry, M.D., Campbell, K.P., and Dickson, G. (1999). Dystrophic phenotype induced in vitro by antibody blockade of muscle α-dystroglycan-laminin interaction. J. Cell Sci. *112*, 209–216.

Campbell, K.P. (1995). Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage. Cell *80*, 675–679.

Chen, S., Zhou, S., Sarkar, M., Spence, A.M., and Schachter, H. (1999). Expression of three *Caenorhabditis elegans N*-acetylglucosaminyltransferase I genes during development. J. Biol. Chem. *274*, 288–297.

Chiba, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusunoki, S., Kanazawa, I., Kobata, A., and Endo, T. (1997). Structures of sialylated *O*-linked oligosaccharides of bovine peripheral nerve α-dystroglycan. J. Biol. Chem. *272*, 2156–2162.

Cormand, B., Avela, K., Pihko, H., Santavuori, P., Talim, B., Topaloglu, H., de la Chapelle, A., and Lehesjoki, A.-E. (1999). Assignment of the muscle-eye-brain disease gene to 1p32-p34 by linkage analysis and homozygosity mapping. Am. J. Hum. Genet. *64*, 126–135.

Dennis, J.W., Granovsky, M., and Warren, C.E. (1999). Glycoprotein glycosylation and cancer progression. Biochim. Biophys. Acta *1473*, 21–34.

Dobyns, W.B., Pagon, R.A., Armstrong, D., Curry, C.J., Greenberg, F., Grix, A., Holmes, L.B., Laxova, R., Micheis, V.V., and Robinow, M. (1989). Diagnostic criteria for Walker-Warburg syndrome. Am. J. Med. Genet. *32*, 195–210.

Dubowitz, V. (1999). 68th ENMC international workshop (5th international workshop): on congenital muscular dystrophy. Neuromuscul. Disord *9*, 446–454.

Endo, T. (1999). *O*-Mannosyl glycans in mammals. Biochim. Biophys. Acta *1473*, 237–246.

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

Gemmill, T.R., and Trimble, R.B. (1999). Overview of *N*- and *O*-linked oligosaccharide structures found in various yeast species. Biochim. Biophys. Acta *1426*, 227–237.

Gleeson, J.G., and Walsh, C.A. (2000). Neuronal migration disorders: from genetic diseases to developmental mechanisms. Trends Neurosci. *23*, 352–359.

Grewal, P.K., Holzfeind, P.J., Bittner, R.E., and Hewitt, J.E. (2001). Mutant glycosyltranferase and altered glycosylation of α-dystroglycan in the myodystrophy mouse. Nat. Genet. *28*, 151–154.

Hayashi, Y.K., Ogawa, M., Tagawa, K., Noguchi, S., Ishihara, T., Nonaka, I., and Arahata, K. (2001). Selective deficiency of α-dystroglycan in Fukuyama-type congenital muscular dystrophy. Neurology *57*, 115–121.

Hoffman, E.P., Brown, R.H., and Kunkel, L.M. (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell *51*, 919–928.

Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M., et al. (1998). An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. Nature *394*, 388–392.

Kumar, R., Yang, J., Larsen, R.D., and Stanley, P. (1990). Cloning and expression of *N*-acetylglucosaminyltransferase I, the medial Golgi transferase that initiates complex N-linked carbohydrate formation. Proc. Natl. Acad. Sci. USA *87*, 9948–9952.

Lowe, J.B. (2001). Glycosylation, immunity, and autoimmunity. Cell *104*, 809–812.

Moloney, D.J., Panin, V.M., Johnston, S.H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K.D., Haltiwanger, R.S., and Vogt, T.F. (2000). Fringe is a glycosyltransferase that modifies Notch. Nature *406*, 369–375.

Santavuori, P., Somer, H., Sainio, K., Rapola, J., Kruus, S., Nikitin, T., Ketonen, L., and Leisti, J. (1989). Muscle-eye-brain disease (MEB). Brain Dev. *11*, 147–153.

Schachter, H. (1995). Glycosyltransferases involved in the synthesis of N-glycan antennae. In Glycoproteins, J. Montreuil., J.F.G. Vliegenthart., and H. Schachter, eds. (Amsterdam: Elsevier), pp. 153–199.

Strasser, R., Mucha, J., Schwihla, H., Altmann, F., Glossl, J., and Steinkellner, H. (1999). Molecular cloning and characterization of cDNA coding for β1,2N-acetylglucosaminyltransferase I (GlcNAcTI) from *Nicotiana tabacum*. Glycobiology *9*, 779–785.

Takahashi, S., Sasaki, T., Manya, H., Chiba, Y., Yoshida, A., Mizuno, M., Ishida, H., Ito, F., Inazu, T., Kotani, N., 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.

Ünligil, U.M., Zhou, S., Yuwarai, S., Sarkar, M., Schachter, H., and Rini, J.M. (2000). X-ray crystal structure of rabbit *N*-acetylglucosaminyltransferase I. EMBO J. *19*, 5269–5280.

Yip, B., Chen, S.H., Mulder, H., Höppener, J.W., and Schachter, H. (1997). Organization of the human β-1,2-*N*-acetylglucosaminyltransferase I gene (*MGAT1*), which controls complex and hybrid N-glycan synthesis. Biochem. J. *321*, 465–474.

Accession Numbers

The DDBJ/GenBank/EBI accession number for the sequence reported in this paper is AB057356.