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 chordoerin 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 mannos 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 (pomt1–7) 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 POMT1 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
are expressed. We also examined the distribution of protein O-mannosyltransferase activity in various rat tissues.

Results

\textit{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 \textit{t-Pomt2} and the latter is \textit{s-Pomt2} (somatic form), because \textit{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 \textit{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.

\textit{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 \textit{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 mannose 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).

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 Poms. 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 protein O-mannosyltransferases. The cDNA sequences of rat *Pomt1* (A) and *Pomt2* (B) 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 5′-terminal extension of the deduced testis-specific isoform is underscored and the testis-specific sequence is shaded.
H. Manya et al.

Fig. 1. continued
Fig. 2. Comparison of human, mouse, and rat POMTs. (A and B) ClustalW alignments of human, mouse and rat POMT1 and POMT2 amino acid sequences, respectively. Conserved amino acids are boxed. (C) ClustalW phylogenetic tree of human, mouse, rat, and Drosophila POMTs and Saccharomyces cerevisiae Pmt1, Pmt2, and Pmt4. The branch lengths indicate amino acid substitutions per site. h, human; m, mouse; r, rat.
therefore possible that mutations of other factors that regulate POMT activity may cause unidentified WWS, although further studies are needed to test this hypothesis.

Pomt2 was found to have two transcription-initiation sites, giving rise to the testis form (t-Pomt2) and the somatic form (s-Pomt2). Both t-Pomt2 and s-Pomt2 showed protein O-mannosyltransferase activity equally when coexpressed with rat Pomt1. In addition, human POMT1 and POMT2 could be exchanged for rat Pomt1 and Pomt2, without loss of activity. Human POMT1 and rat Pomt1 proteins share 96% similarity and 86% identity; human POMT2 and rat Pomt2 share 99% similarity and 90% identity. Such high similarity may explain the exchangeability of each component for enzymatic activity. The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) molecular weights of t-Pomt2 and s-Pomt2 expressed in HEK293T cells were 85 and 75 kDa, respectively. The difference was due to the presence of an additional 70 amino acids in the N-terminal region of t-Pomt2. However, this difference did
not affect protein O-mannosyltransferase activity. A testis-specific form of Pomt2 translation was also observed in mouse (Willer et al., 2002). In mice, the testis form of Pomt2 was localized to maturing spermatids and was distributed within the acrosome and the endoplasmic reticulum (ER). The authors of that study speculated that the ER-localized Pomt2 is involved in the synthesis of O-mannosyl glycans, and the acrosome-localized Pomt2 acts as a lectin that is involved in adhesive interactions of sperm and egg during fertilization (Willer et al., 2002). In Figure 3A (middle panel), a band corresponding to the 2.7 kb mRNA in testis is thought to be the s-Pomt2. This conclusion is consistent with data from the mouse, insofar as mRNAs of both s-Pomt2 and t-Pomt2 were detected in mouse testis (Willer et al., 2002).

In Figure 7B, the band was detected around the migration position of s-Pomt2 at 75 kDa in the cells transfected with t-Pomt2 (lanes 2 and 4). Because the t-Pomt2 cDNA has two ATG-start sites, it is likely that the 75-kDa band of t-Pomt2 is derived from the transcription starting at the second ATG-start site. The biological significance of the presence of different Pomt2s remains to be determined.

Why protein O-mannosyltransferase activity requires coexpression of Pomt1 and Pomt2 is unclear. One possibility is that Pomt1 is a catalytic molecule and Pomt2 is a regulatory molecule or vice versa. Another possibility is that assembly of Pomt1 and Pomt2 forms a catalytic domain. That is the reason why expression of Pomt1 or Pomt2 alone does not show any enzymatic activity. Recently, it has been reported that complex formation between a glycosyltransferase and
its homologue changed its enzymatic character. Human chondroitin synthase exhibits glucuronyltransferase II and N-acetylgalactosaminy transferase II activities but cannot polymerize the chondroitin chain in vitro (Kitagawa et al., 2001). A recent study indicated that chondroitin-polymerizing activity requires the coexpression of chondroitin-polymerizing factor with chondroitin synthase (Kitagawa et al., 2003). Although the amino acid sequence of chondroitin-polymerizing factor displayed 23% identity to that of chondroitin synthase, chondroitin-polymerizing factor did not show any enzymatic activity. Heparan sulfate polymerization is another case. Heparan sulfate polymerization in vitro requires both EXT1 and EXT2 that have N-acetylgalactosaminytransferase II and glucuronyltransferase II activities. A heterocomplex formation of EXT1 and EXT2 is required for chain elongation of heparan sulfate and to be present in the appropriate intracellular locations (McCormick et al., 2003; Kim et al., 2005). Further studies are needed to understand the regulation of protein O-mannosylation by two Pomt homologues.

Mutations in the human POMT1 and POMT2 genes give rise to WWS, a congenital muscular dystrophy with severe neuronal migration disorder (Beltran-Valero de Bernabe et al., 1996; van Reeuwijk et al., 2005). Elucidating the regulation of O-mannosylation in brain will help to understand the molecular pathology of WWS. To address the pathogenesis of WWS, it is also important to determine what proteins in addition to α-DG may be modified by O-mannosylation. The mannosyl-O-Ser/Thr linkage was first identified in chondroitin sulfate proteoglycans, neurocan, phosphacan, and phosphacan-keratan sulfate of brain (Finn et al., 1979; Krusius et al., 1986, 1987; Margolis et al., 1996). The content of mannosyl-O-Ser/Thr linkage in these proteoglycans is regulated developmentally. On the basis of the yield of mannitol in hydrolysates of oligosaccharides after alkaline borohydride treatment of neurocan, the proportion of mannosyl-O-Ser/Thr linkage was calculated to increase from an insignificant level in one week postnatal rat brain to 15% of the total mannose in adult brain, whereas in the cases of phosphacan and phosphacan-keratan sulfate, the corresponding values were 26–31% at 7 days postnatal and 28–52% in adult brain (Rauch et al., 1991). On the other hand, N-acetylgalactosamine (GalNAc)-linked oligosaccharides disappeared from phosphacan during the course of postnatal brain development, and these were replaced in adult brain by a significant proportion of oligosaccharides and keratan sulfate chains containing mannosyl-O-Ser/Thr linkages (Rauch et al., 1991). If O-mannosylation and O-GalNAcylation occur on the same Ser/Thr residues of these proteoglycans, a developmental change of O-mannosylation may affect O-GalNAcylation, because O-mannosylation occurs in the ER and O-GalNAcylation takes place at a later processing step in the Golgi apparatus (Rottger et al., 1998). Elucidating the regulation of O-mannosylation should therefore help in understanding the developmental roles of O-glycosylation in brain.

Materials and Methods

Cloning of rat Pomt1

Two degenerate oligonucleotide primers were designed on the basis of the amino acid sequences of Saccharomyces cerevisiae PMT1 corresponding to amino acids 486–496 and 652–662: 5′-cctcctcctgtagggtt(c/t)i(a/g)icaiga(a/g)gt-3′ (sense) and 5′-cgaactcagcggia(a/g)(a/g)t(a/g)(g/l)(a/g)(a/g)(a/g)(a/g)aa-3′ (antisense) (XhoI sites are underscored). mRNAs was isolated from adult Sprague-Dawley rat brain using the FastTrack kit (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s directions, and RT–PCR was performed using a GeneAmp RNA PCR kit (Perkin Elmer, Wellesley, MA). Amplified products were ligated into pBluescript SK(-) (Stratagene, La Jolla, CA) after XhoI digestion. One clone was obtained for each side, and a sequence from portions of the clone were used in two Pomt homologues.

Cloning of rat Pomt1 and Pomt2 genes give rise to WWS, a congenital muscular dystrophy with severe neuronal migration disorder (Beltran-Valero de Bernabe et al., 2002; van Reeuwijk et al., 2005). Elucidating the regulation of O-mannosylation in brain will help to understand the molecular pathology of WWS. To address the pathogenesis of WWS, it is also important to determine what proteins in addition to α-DG may be modified by O-mannosylation. The mannosyl-O-Ser/Thr linkage was first identified in chondroitin sulfate proteoglycans, neurocan, phosphacan, and phosphacan-keratan sulfate of brain (Finn et al., 1979; Krusius et al., 1986, 1987; Margolis et al., 1996). The content of mannosyl-O-Ser/Thr linkage in these proteoglycans is regulated developmentally. On the basis of the yield of mannitol in hydrolysates of oligosaccharides after alkaline borohydride treatment of neurocan, the proportion of mannosyl-O-Ser/Thr linkage was calculated to increase from an insignificant level in one week postnatal rat brain to 15% of the total mannose in adult brain, whereas in the cases of phosphacan and phosphacan-keratan sulfate, the corresponding values were 26–31% at 7 days postnatal and 28–52% in adult brain (Rauch et al., 1991). On the other hand, N-acetylgalactosamine (GalNAc)-linked oligosaccharides disappeared from phosphacan during the course of postnatal brain development, and these were replaced in adult brain by a significant proportion of oligosaccharides and keratan sulfate chains containing mannosyl-O-Ser/Thr linkages (Rauch et al., 1991). If O-mannosylation and O-GalNAcylation occur on the same Ser/Thr residues of these proteoglycans, a developmental change of O-mannosylation may affect O-GalNAcylation, because O-mannosylation occurs in the ER and O-GalNAcylation takes place at a later processing step in the Golgi apparatus (Rottger et al., 1998). Elucidating the regulation of O-mannosylation should therefore help in understanding the developmental roles of O-glycosylation in brain.

To obtain further 5′-cdNA sequence, 5′-rapid amplification of cDNA ends (RACE) was carried out with a GeneAmp RNA PCR kit, terminal deoxynucleotidyl transferase (Promega) and Taq DNA polymerase (Perkin Elmer). The
specific antisense primers were 5'-catcgaatctccttgacacc-3' comprising the nucleotides 377–356 for the reverse transcription and 5'-ggtgtcagccttgacaggaag-3' comprising the nucleotides 326–304 for the nested PCR. The sense primers with linker were 5'-tgaatgagcggccgcgtattttttttttttttt-3' and 5'-tgaagaaggcccggcagc-3' (EcoRI sites are underscored). The amplified products were ligated into the pGEM-7Z vector and sequenced.

Cloning of rat Pomt2

Using a cDNA sequence of human POMT2 as probe, we identified a sequence (accession number XM_345708.1) that appeared likely to encode a part of rat Pomt2 by BLAST search in the GenBank database. On the basis of this sequence, we obtained further 5'- and 3'-cDNA sequences of somatic form Pomt2 (s-Pomt2) by RT–PCR from rat testis poly(A)+ RNA (BD Biosciences, Franklin Lakes, NJ) using a SMART RACE cDNA Amplification Kit (BD Biosciences), according to the manufacturer's instructions. The specific primers for 5'-RACE and 3'-RACE were 5'-ageccacattgagaatc-3' and 5'-agatcttgactgacca-3', respectively. To obtain more of the 5'-cDNA sequence of the testis form Pomt2 (t-Pomt2), RT–PCR was carried out with SuperScript III RNase H- Reverse Transcriptase (Invitrogen) and KOD-Plus-DNA polymerase (Toyobo Co., Osaka, Japan) using 5'-agatcttgactgacca-3' for the sense primer, based on the cDNA sequence of mouse Pomt2 (NM_153415), and 5'-aatctccggtgtaaggac-3' for the antisense primer, based on rat s-Pomt2 mentioned above. The amplified cDNAs were cloned into pCR4Blunt-TOPO (Invitrogen) and sequenced.

Vector construction of rat Pomt1 and Pomt2

The Pomt1 fragment in the pGEM-7Z vector was digested and then introduced into the EcoRI sites of the pcDNA3.1 vector (Invitrogen) to express Pomt1. To make expression plasmid vectors of s- and t-Pomt2, the cDNAs containing putative open reading frame (ORF) of s- and t-Pomt2 were amplified by PCR using cloned cDNAs in pCR4Blunt-TOPO as template. Primers of s-Pomt2 were 5'-tgaatgagcggccgctggcc-3' and 5'-caactgagtaaagccgctattc-3' (HindIII and XhoI sites are underscored). Primers of t-Pomt2 were 5'-ctaagcttcctgatgtaagctg-3' and 5'-gctgtggtgctcg-3' (HindIII site is underscored). The PCR product of s-Pomt2 was cloned into the HindIII and XhoI sites of pcDNA3.1 vector (pcDNA3.1-s-Pomt2), and the PCR product of t-Pomt2 was introduced into the HindIII and Kpnl sites of pcDNA3.1-s-Pomt2. Human POMT1 and POMT2 cDNAs were cloned into pcDNA3.1 as described previously (Manya et al., 2004).

Nucleotide sequence and protein sequence analyses

Protein sequences were aligned and placed in a phylogenetic tree with ClustalW (http://www.ddbj.nig.ac.jp/searches-e.html). Similarities and identities were analyzed using the GENETYX-Mac program (GENETYX Corp., Tokyo, Japan), based on Lipman-Pearson’s method, and the gap was not counted.

Northern blot analysis

Northern blots of rat tissues (Rat MTN blot) were purchased from BD Biosciences. Probe DNA fragments for rat Pomt1 and Pomt2 were prepared by PCR, and β-actin was supplied with the Rat MTN blot. Primers for Pomt1 were 5'-cactgctgtgctgg-3' and 5'-gctgtggtgctcg-3'. Primers for Pomt2 were 5'-cggattcacaagctccaccc-3' and 5'-cggattcacaagctccaccc-3'. Each probe was labeled with [α-32P]dCTP using a Random Primer DNA labeling kit (Takara Bio Inc., Shiga, Japan). Blots were hybridized with a 32P-labeled DNA probe in ExpressHyb Hybridization solution (BD Biosciences) at 68°C for 1 h, followed by washing according to the manufacturer’s instruction.

RT–PCR analysis

First-strand cDNAs were synthesized from poly(A)+ RNAs of rat tissues (Rat MTC Panel I; BD Biosciences) using SuperScript III RNase H- Reverse Transcriptase. PCR was carried out with KOD-Plus-DNA polymerase using the following primers: 5'-acagctctctgcgttctt-3' and 5'-ctggcaagcagc-3' for the testis-specific sequence of Pomt2 (841 bp); 5'-gagacattgtacagctcgtt-3' and 5'-aatctccggtgtaaggac-3' and 5'-gctgtggtgctcg-3' for Pomt1 (618 bp); and 5'-taatctccggtgtaaggac-3' and 5'-gctgtggtgctcg-3' for Pomt2 (399 bp). Primers for rat glyceraldehyde 3-phosphate dehydrogenase (Rat G3PDH Control Amplimer Set) were purchased from BD Biosciences. The cycling parameters for PCR were 94°C for 15 s, 60°C for 30 s, and 68°C for 1 min, and cycle numbers were 40 cycles for testis-specific sequence of Pomt2 and 35 cycles for Pomt1, and G3PDH.

In situ hybridization histochemistry

An EcoRI-XbaI restriction fragment of Pomt1 comprising 331 nucleotides (nucleotides 2424–2754) and an EcoRI-PstI restriction fragment of Pomt2 comprising 463 nucleotides (nucleotides 1902–2364) were subcloned into the pGEM-3Z vector (Promega), and the vectors were linearized with EcoRI for antisense probes of Pomt1 and Pomt2, XbaI for sense probe of Pomt1, or PstI for sense probe of Pomt2. For PstI digestion, the linearized template end was converted to a blunt end with T4 DNA polymerase (Promega). Digoxigenin-labeled RNA probes were synthesized with the Riboprobe System (Promega) and digoxigenin-11-dUTP (Roche Diagnostics, Tokyo, Japan). To detect the expression of Pomt1 and Pomt2 in brain, frozen sections of rat brain tissues were fixed with 3% paraformaldehyde/10 mM phosphate-buffered saline (PBS) and then analyzed with an In situ Hybridization kit (NIPPON GENE, Tokyo, Japan) according to the manufacturer’s directions except that the sections were washed at 48°C after hybridization.

Expression of POMTs and cell extract preparation

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, and 100 units/mL penicillin–50 μg/mL streptomycin at 37°C with 5% CO2. The expression plasmids of pcDNA3.1-rat Pomt1, pcDNA3.1-rat s-Pomt2, pcDNA3.1-rat t-Pomt2, pcDNA3.1-human POMT1-myc,
and pcDNA3.1-human POMT2 were transfected into HEK293T cells using LipofectAMIN PLUS reagent (Invitrogen) according to the manufacturer’s instructions. The transfected cells were cultured for 3 days in complete medium, harvested and homogenized. The cells were homogenized in 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, with a protease inhibitor cocktail (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 ultra centrifugation at 100,000 × g for 1 h. The precipitates were used as the microsomal membrane fraction. Protein concentration was determined by BCA assay (PIERCE, Rockford, IL).

Preparation of rat tissues
Heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis were obtained from 4-month-old Wistar rats. Tissue samples were homogenized with nine volumes (weight/volume) of 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 ultra centrifugation at 100,000 × g for 1 h. The precipitates were used as the microsomal membrane fraction. Protein concentration was determined by BCA assay. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology. All efforts were made to minimize the number of animals used and their suffering.

Western blot analysis
Rabbit antibodies specific to POMT1 and POMT2 were described previously (Manya et al., 2004). The microsomal fractions (20 μg) were separated by SDS–PAGE (10% gel), and proteins were transferred to a PVDF membrane. The membrane was blocked in PBS containing 5% skim milk and 0.5% Tween 20, incubated with each antibody, and treated with anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Biosciences Corp., Piscataway, NJ). Proteins bound to antibody were visualized with an ECL kit (Amersham Biosciences). As reported previously (Manya et al., 2004), anti-POMT1 and anti-POMT2 polyclonal antibodies did not detect endogenous POMT1 and POMT2, respectively. Each antibody is specific for the respective recombinant protein.

Assay for protein O-mannosyltransferase activity
Protein O-mannosyltransferase activity was based on the amount of [3H]-mannose transferred from Dol-P-Man to a glutathione-S-transferase fusion α-DG (GST-αDG) as described previously (Manya et al., 2004). Briefly, the reaction mixture contained 20 μM Tris–HCl (pH 8.0), 100 mM of [3H]-mannosylphosphoryl dolichol (Dol-P-Man, 125,000 dpm/μmol) (American Radiolabeled Chemicals Inc., St. Louis, MO), 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% n-octyl-β-D-thiogalactoside, 10 μg GST-αDG, and enzyme source (80 μg of microsomal membrane fraction) in 20 μL total volume. After 1 h incubation at 25°C, the reaction was stopped by adding 150 μL PBS containing 1% Triton X-100 (Nacalai Tesque, Kyoto, Japan), 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 (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 with a liquid scintillation counter.

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Conflict of interest statement
None declared.

Abbreviations
α-DG, α-dystroglycan; Dol-P-Man, dolichyl phosphate mannose; ER, endoplasmic reticulum; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; GalNAc, N-acetylgalactosamine; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcriptase polymerase chain reaction; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; WWS, Walker–Warburg syndrome.

References


