POMT1

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MWGFLKRPVV VTADINLSLV ALTGMGLLSR LWRLTYPRAV  40
VFDEVYYGQY ISFYMKQIFF LDDSGPPFGH MVLALGGYLG  80
GFDGNFLWNR IGAEYSSNVP VWSLRLLPAL AGALSVPMAY 120
QIVLELHFSH CAAMGAALLM LIKNALITOS RLMLLESVLT 160
FFNLLAVLSY LKFFNCQKHS PFSLSWWFWL TLTGVACSCA 200
VGIKYMGVFT YVLVLGVAAV HAWHLLGDQT LSNVGADVQC 240
CMRPACMGQM RMSQGVCVFC HLLARAVALL VIPVVLYLLF 280
FYVHLILVFR SGPHDQIMSS AFQASLEGGL ARITQGQPLE 320
VAFGSQVTLR NVFGKPVPCW LHSHQDTYPM IYENGRGSSH 360
QQQVTCYPFK DVNNWWIVKD PRRHQLVVSS PPRPVRHGDM 400
VQLVHGMTTR SLNTHDVAAP LSPHSQEVSC YIDYNISMPA 440
QNLWRLEIVN RGSDTDVWKT ILSEVRFVHV NTSAVLKLSG 480
AHLPDWGYRQ LEIVGEKLSR GYHGSTVWNV EEHRYGASQE 520
QRERERELHS PAQVDVSRNL SFMARFSELQ WRMLALRSDD 560
SEHKYSSSPL EWVTLDTNIA YWLHPRTSAQ IHLLGNIVIW 600
VSGSLALAIY ALLSLWYLLR RRRNVHDLPQ DAWLRWVLAG 640
ALCAGGWAVN YLPFFLMEKT LFLYHYLPAL TFQILLLPVV 680
LQHISDHLCR SQLQRNSFSA LVVAWYSSAC HVSNTLRPLT 720
YGDKSLSPHE LKALRWKDSW DILIRKH                747
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POMT2

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MPPATGGGLA ESELRPRRGR CGPQAARAAG RDVAAEAVAR  40
SPKRPAWGSR RFQAVGWWAL LALVTLLSFA TRFHRLDEPP  80
HICWDETHFG KMGSYYINRT FFFDVHPPLG KMLIGLAGYL 120
SGYDGTFLFQ KPGDKYEHHS YMGMRGFCAF LGSWLVPFAY 160
LTVLDLSKSL SAALLTAALL TFDTGCLTLS QYLLLDRILM 200
FFIMAAMLSM VKYNSCADRP FSAPWWFWLS LTGVSLAGAL 240
GVKFVGLFII LQVGLNTIAD LWYLFGDLSL SLVTVGKHLT 280
ARVLCLIVLP LALYTATFAV HFMVNSKSGP GDGFFSSAFQ 320
ARLSGNNLHN ASIPEHLAYG SVITVKNLRM AIGYLHSHRH 360
LYPEGIGARQ QQVTTYLHKD YNNLWIIKKH NTNSDPLDPS 400
FPVEFVRHGD IIRLEHKETS RNLHSHYHEA PMTRKHYQVT 440
GYGINGTGDS NDFWRIEVVN RKFGNRIKVL RSRIRFIHLV 480
TGCVLGSSGK VLPKWGWEQL EVTCTPYLKE TLNSIWNVED 520
HINPKLPNIS LDVLQPSFPE ILLESHMVMI RGNSGLKPKD 560
NEFTSKPWHW PINYQGLRFS GVNDTDFRVY LLGNPVVWWL 600
NLLSIALYLL SGSIIAVAMQ RGARLPAEVA GLSQVLLRGG 640
GQVLLGWTLH YFPFFLMGRV LYFHHYFPAM LFSSMLTGIL 680
WDTLLRLCAW GLASWPLARG IHVAGILSLL LGTAYSFYLF 720
HPLAYGMVGP LAQDPQSPMA GLRWLDSWDF              750
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—— SOSUI —— HMMTOP —— TMpred ▨TM consensus segment ▨The major splicing variant of POMT1 lacks amino acids 234-255.

Fig. 1 Analysis of secondary structures of human POMT1 and POMT2. The secondary structure was predicted using three algorithms specifically designed to detect transmembrane alpha helices: SOSUI, TMPred and HMMTOP. The consensus segments predicted by three different algorithms were defined as the transmembrane segments: Amino acids of 17–28, 106–122, 154–171, 194–213, 266–283, 597–614 and 638–656 of human POMT1; amino acids of 54–70, 147–164, 193–210, 241–250, 285–295, 601–619, 643–658, 670–684 and 705–720 of human POMT2.

Transmembrane topologies of POMT1 and POMT2 by analysis of N-glycosylation site occupancy

The results of Fig. 1 suggest that each of POMT1 and POMT2 have two possible membrane topologies: the N-termini of POMT1 and POMT2 are on the cytosolic or luminal sides of the ER membrane or they are on the same side (Fig. 2A and B). Because *N*-glycosylation occurs only on the ER luminal side, it is possible to determine the correct topology by examining the *N*-glycosylated status of each protein. Human POMT1 and POMT2 have four (Asn[16], Asn[435], Asn[471] and Asn[539]) and five (Asn[98], Asn[330], Asn[445], Asn[528] and Asn[583]) potential *N*-glycosylation sites, respectively. If the N-terminus of POMT1 is on the cytosolic side, Asn[16] would be predicted to be on the cytosolic side and Asn[435], Asn[471] and Asn[539] would be predicted to be on the luminal side. In this case, Asn[435], Asn[471] and Asn[539] could be *N*-glycosylated. On the other hand, if the N-terminus of POMT1 is on the luminal side, only Asn[16] could be *N*-glycosylated. In the case of POMT2, if the N-terminus is on the cytosolic side, all five Asns (Asn[98], Asn[330], Asn[445], Asn[528] and Asn[583]) could be *N*-glycosylated, while if it on the luminal side, none could be *N*-glycosylated.

To determine the occupancy of each potential *N*-glycosylation site, we constructed expression vectors in which the Asn residue of each site was substituted by Gln (Fig. 2C) and transfected them into HEK293 cells. Fig. 3A shows a western blot analysis of six cell

cultures, each doubly transfected with wild-type POMT2 and one of the POMT1 variants. Three of the mutant POMT1s (N435Q, N471Q and N539Q) (upper panel) were 2–3 kDa smaller than the wild type, a value corresponding to a single *N*-glycan chain. Only the N16Q mutant had the same molecular mass as the wild-type protein. The POMT2 sizes (lower panel) were unaffected, as expected. Similarly, Fig. 3B shows the sizes of POMT1 and POMT2 in cells doubly transfected with wild-type POMT1 and one of the POMT2 variants. All five of the POMT2 mutants were also 2–3 kDa smaller than the wildtype (lower panel), while POMT1 sizes (upper panel) were unaffected, as expected. When each mutant and wild counterpart was expressed in the presence of tunicamycin, an inhibitor of initiation of *N*-glycosylation, all expressed POMTs had the same molecular mass (data not shown). These results demonstrate that all but one of the Asn residues of the potential *N*-glycosylation sites of POMT1 and POMT2 (the exception being Asn[16] of POMT1) have an *N*-glycan. Because the glycosylated Asn residues must be on the luminal side, the N-termini of POMT1 and POMT2 must be on the cytosolic side of the ER membrane based on the proposed models shown in Fig. 2. It is noteworthy that Nilsson and von Heijine (23) reported that a potential *N*-glycosylation site needed to be a distance of 12–14 amino acid residues from the membrane to be modified actually. Thus, the proximity of Asn[16] of POMT1 to the membrane may hamper the glycosylation.
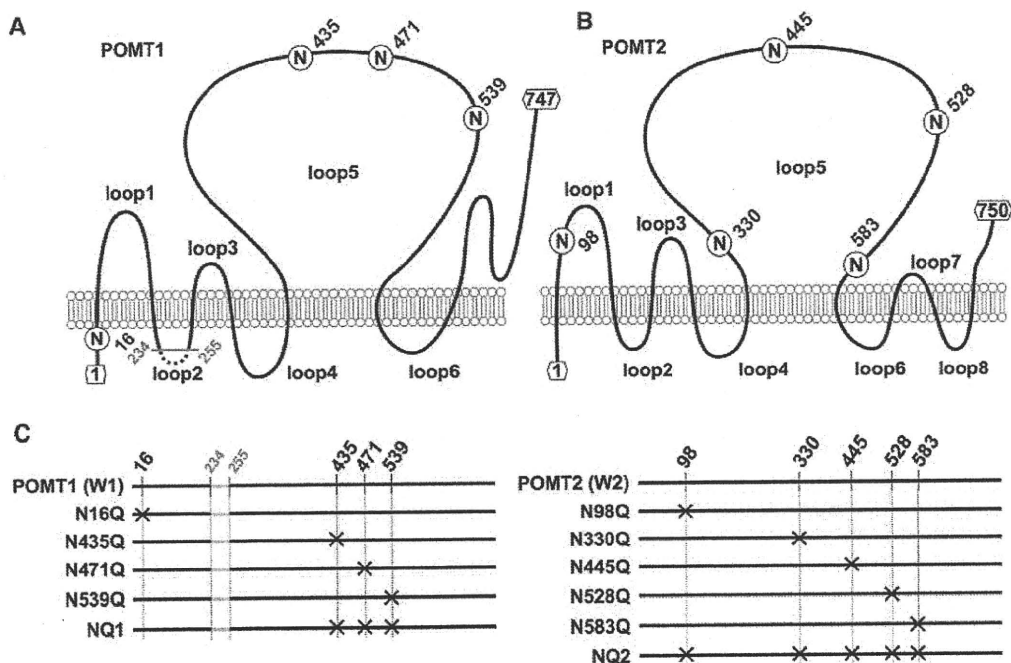
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Fig. 2 Possible transmembrane topologies of human POMT1 and POMT2 (A and B), and a summary of mutations of potential *N*-glycosylation sites (C). The letters N (in circles) show Asn residues of potential *N*-glycosylation sites and the numbers indicate amino acid positions. The crosses in (C) indicate the substitution of Gln for Asn. The major splicing variant of POMT1, which lacks amino acids 234–255, was used in this study (*15*). Although the model does not specify which side of the ER membrane is the cytosolic side or luminal side, our conclusion is that the N-termini of POMT1 and POMT2 are on the cytosolic side of the ER membrane. There are possible other models; for example, some transmembrane helices in (A) and (B) may be non-transmembrane manner and be embedded in the membrane. However, after taking account of seven-transmembrane helices models of yeast pmt1 (*17*), two possible membrane topologies are shown here.

Together, further work is necessary to determine membrane topologies of POMT1 and POMT2 in the future.

Effect of N-glycosylation on POMT activity

To evaluate the contribution of the individual *N*-glycans on POMT1 and POMT2 to POMT activity, the POMT1 or POMT2 mutants were co-expressed with wild-type POMT2 (W2) or wild-type POMT1 (W1), respectively. As shown in Fig. 3C and D (hatched bars), POMT activity was significantly greater in all transfectants than in the mock transfectant. However, these activities were not accurate because the expression levels of each mutant protein were not equal as shown in Fig. 3A and B. Therefore, each activity was normalized to the amount of expressed protein. The individual *N*-glycosylation site mutations had comparable POMT activity to wildtype, except the N98Q transfectant showed a lower enzymatic activity to ~50% of wildtype, and N16Q, N435Q and N471Q transfectants did to ~70% of wildtype (Fig. 3C and D, closed bars).

Next, we generated the NQ1 and NQ2 mutants that have no *N*-glycans by substitution of Gln for Asn residues of all *N*-glycosylation sites of POMT1 and POMT2, respectively (Fig. 2C). These mutants and wild-type POMTs were co-expressed in the following combinations: W1 + W2, NQ1 + W2, W1 + NQ2 and NQ1 + NQ2. In the western blot analysis, NQ1 and NQ2 migrated faster than wild-type POMT1 and

POMT2, respectively (Fig. 4A). As shown in Fig. 4B, the POMT activities of the microsomal fractions from three transfectants (NQ1 + W2, W1 + NQ2 and NQ1 + NQ2) were significantly lower than the activity of the W1 + W2 transfectant. These results clearly show that the *N*-glycans of both POMT1 and POMT2 are required for expression of POMT activity.

On the other hand, after the mock and W1 + W2 transfectants were cultured in media containing 1 μg/ml tunicamycin, W1 and W2 migrated to the same positions as NQ1 and NQ2 (Fig. 4A), respectively, on SDS-PAGE (Fig. 4C). As shown in Fig. 4D, the POMT activity of the microsomal fraction from [W1 + W2, TN(+)] was significantly lower than that from W1 + W2 without tunicamycin treatment [W1 + W2, TN(−)], indicating that the *N*-glycans of both POMT1 and POMT2 are required for POMT activity. Importantly, the POMT activities of the microsomal fractions from both tunicamycin-treated transfectants [Mock and W1 + W2, TN(+)] were strikingly decreased compared with the activity of the non-treated mock transfectant [Mock, TN(−)] (Fig. 4D). These results indicate that the inhibition of *N*-glycosylation by tunicamycin treatment inactivated not only the recombinant POMTs, but also the endogenous POMTs.

Role of N-glycosylation in solubility of POMTs

To understand why removing all the *N*-glycans on the POMT1 or POMT2 destroys POMT activity, we

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assayed the solubility of the POMTs in a buffer containing 0.5% *n*-octyl-β-D-thioglucoside, which is the most effective detergent for measurement of POMT activity (*15*). As shown in Fig. 4E, POMT1 and POMT2 were detected in the solubilized supernatant from the W1 + W2 transfectant but not from the other transfectants. Similarly, neither POMT1 nor POMT2 was detected in the solubilized supernatant from the tunicamycin-treated W1 + W2 transfectant (Fig. 4F). The fact that neither POMT1 nor POMT2 was detected in the solubilized supernatant from NQ1 + W2 and W1 + NQ2 transfectants (Fig. 4E) demonstrates that the *N*-glycans of both POMT1 and POMT2 are necessary for solubilization of the POMT1–POMT2 complex. These results suggest that the inactivation of POMT was caused by the decreased hydrophilicity of proteins as a result of removing the *N*-glycans.

Endo-glycosidase treatment of POMTs

To elucidate the structures of the *N*-glycans on POMT1 and POMT2, W1 and W2 were treated with PNGase F and Endo H. As shown in Fig. 5, each glycosidase-treated W1 and W2 migrated to the same molecular mass as tunicamycin-treated POMT1 and POMT2, respectively, indicating that both PNGase F and Endo H digested all *N*-glycan chains on W1 and W2. Because Endo H preferentially cleaves high-Man type *N*-glycans (*24*, *25*), the *N*-glycans on both POMT1 and POMT2 were high-Man type. These results indicate that the POMT1 and POMT2 reside

in the ER, and it is consistent with our previous report (*15*).

Discussion

In this study, we investigated the roles of *N*-glycans attached to human POMT1 and POMT2. The helical consensus regions (Fig. 1) indicate that POMT1 and POMT2 have seven- and nine-transmembrane domains, respectively. Human POMT1 and POMT2 contain four and five potential *N*-glycosylation sites, respectively, based on their amino acid sequences. Analysis of the sizes and activities of POMT mutants lacking single potential *N*-glycosylation sites by site-directed mutagenesis (Fig. 3) demonstrate that three sites of POMT1 (Asn^{435}, Asn^{471} and Asn^{539}) and five sites of POMT2 (Asn^{98}, Asn^{330}, Asn^{445} Asn^{528} and Asn^{583}) are glycosylated. The finding that only Asn^{16} of POMT1 was not glycosylated indicates that the glycosylated Asns are located in the ER lumen and that Asn^{16} is located in the cytosol. Any single *N*-glycan chain of POMT1 or POMT2 was dispensable for POMT activity, but the elimination of all *N*-glycans of either POMT1 or POMT2 inhibited POMT activity. The cause of inactivation of POMT has been the decreased hydrophilicity following the loss of the *N*-glycans. Together, these findings suggest that the *N*-glycosylation of POMTs is required to maintain the conformation and activity of the POMT1–POMT2 complex.
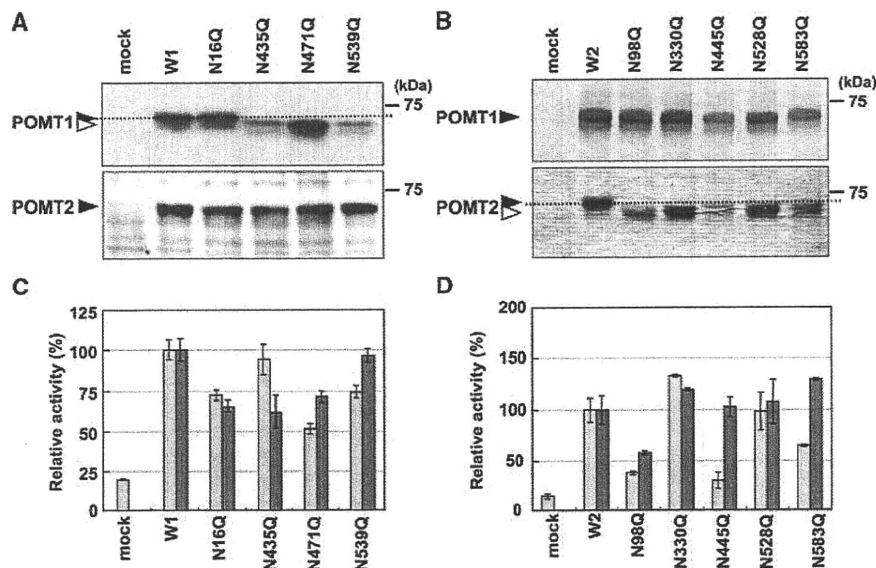

Fig. 3 Determination of the occupancy of the *N*-glycosylation sites on POMT1 and POMT2 and POMT activity of single *N*-glycosylation site mutants. (A) Western blot analyses of the microsomal fractions from the cells co-transfected with POMT1 mutants and W2. (B) W1 and POMT2 mutants. Closed triangles indicate the migration positions of fully glycosylated POMT1 and POMT2. Open triangles indicate the migration position of the mutant proteins, which lack single *N*-glycan chain. Molecular weight standards are shown on the right. The mock lanes were loaded with sample from cells transfected with vector only. The dotted lines of (A) and (B) show the migration position of fully glycosylated POMT1 and POMT2, respectively, for comparison with mutant proteins. The lower band of two bands detected by POMT1 western blots (by anti-myc antibody) was probably the degraded product. (C and D) POMT activities of the microsomal fractions obtained in (A) and (B). Hatched bars show total POMT activities in each microsomal fraction. The activity of mock lanes indicates endogenous POMT activity of HEK293 cells. Closed bars show the POMT activities corrected for expression levels of POMT1 and POMT2 proteins using NIH image. Average values ± SD of three independent experiments are shown.

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Loop 5 of both POMT1 and POMT2, which are the largest hydrophilic regions formed on the luminal side, have amino acid sequences that are similar to the catalytic domains of yeast pmt1 (*17, 19*) and thus are expected to be the catalytic domains of POMT1 and POMT2. Because the *N*-glycosylation sites are enriched on loop 5 (Fig. 2), it is possible that these *N*-glycans contribute to the hydrophilicity and affect the folding of the catalytic domain. The finding that removal of *N*-glycans from either POMT1 or POMT2 inhibited POMT activity (Fig. 4) suggests that loop 5 is

required for the correct folding of the catalytic center in the POMT1–POMT2 complex. However, the *N*-glycans do not appear to be necessary for formation of the POMT1–POMT2 complex. This is because, when glycosylated and non-glycosylated combinations of POMT mutants (NQ1 + W2 and W1 + NQ2) were expressed in the same cells, neither protein was solubilized (Fig. 4), indicating the formation of a stable complex. A somewhat similar result was obtained with yeast Pmt1, in which deletion of loop 5 eliminated enzymatic activity, but did not
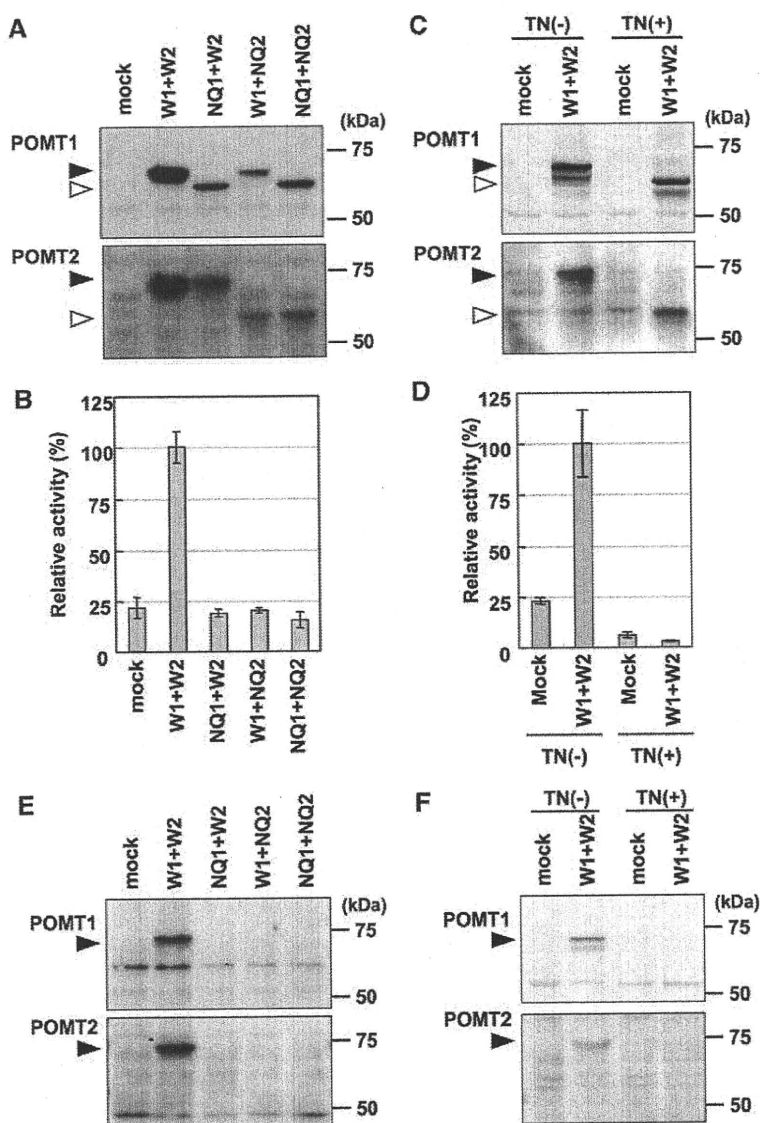

Fig. 4 Effect of *N*-glycan deficiency on POMT activity and on solubilization efficiency. (A and C) Western blot analyses of the microsomal fraction from cells expressing *N*-glycan-deficient mutants (A) and the W1 + W2 transfectants cultured with [TN(+)] or without tunicamycin [TN(−)] (C). (B and D) POMT activities of the microsomal fraction obtained in (A) and (C), respectively. Average values ± SD of three independent experiments are shown. (E and F) Western blot analyses of the solubilized supernatants from the cells expressing *N*-glycan-deficient mutants (E) and the W1 + W2 transfectants cultured with [TN(+)] or without tunicamycin [TN(−)] (F). Closed triangles indicate the migration positions of fully glycosylated POMT1 and POMT2. Open triangles indicate the migration positions of POMTs that have no *N*-glycan chains. Molecular weight standards are shown on the right. The mock lanes were loaded with sample from cells transfected with vector only, indicating endogenous POMT activity of HEK293 cells.
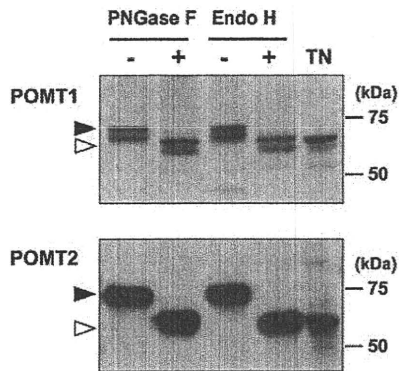
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Fig. 5 Endo-glycosidase digestions of POMT1 and POMT2. The microsomal fractions from the W1 + W2 transfectants were treated with PNGase F or Endo H. Lane TN, microsomal fraction from the W1 + W2 transfectant cultured with tunicamycin. Closed triangles indicate the migration positions of fully glycosylated POMT1 and POMT2. Open triangles indicate the migration positions of the deglycosylated forms of POMT1 and POMT2. Molecular weight standards are shown on the right.

prevent Pmt1–Pmt2 interactions (*19*). Together, these results suggest that the loop 5s of human POMT1 and POMT2 do not have an important role in complex formation. We previously showed that POMT1 mutants derived from WWS patients could form a complex with wild-type POMT2, although these complexes did not have any POMT activity (*14*). Taken together, these results indicate that complex formation of POMT1–POMT2 is necessary but not sufficient for enzymatic activity.

Another question is when the POMT1–POMT2 complex is formed. We previously showed that POMT1 and POMT2 could not associate when they were expressed individually in different cells and then mixed (*1*). This suggests that the POMT1–POMT2 complex is formed concomitantly with translation in the ER. Further studies are needed to clarify the mechanism and role of complex formation and enzymatic activity. However, the present results show that *N*-glycosylation is needed for the correct folding of proteins and for enzymatic activity.

In the PMTs of *S. cerevisiae* and *Mycobacterium tuberculosis*, loop 1 was found to have a role in both enzymatic activity and complex formation (*19, 26*). In POMT2, loop 1 may also contribute to activity because the activity of the N98Q transfectant (which lacks the *N*-glycan on Asn[98] on loop 1) was lower than the activities of the wildtype and other mutants (Fig. 3D).

Yeast PMTs and human POMTs are predicted to be integral membrane proteins and have similar topologies with multiple transmembrane domains. However, there are some differences. For example, Triton X-100 appeared to abolish human POMT activity (*15*) but did not inhibit yeast PMT activity (*27*). The difference in detergent sensitivities may be due to differences in the amino acid sequences of POMTs and PMTs and in the lipid compositions of the ER membranes of humans and yeast. In addition, it has recently been

reported that *O*-mannosylation of human POMTs requires a specific amino acid sequence while yeast *O*-mannosylation does not (*28*). This is consistent with the fact that *O*-mannosylation of proteins is common in yeast but rare in mammals. Differences in amino acid sequences and differences in the numbers, positions and structures of *N*-glycans between POMTs and PMTs may cause the difference of the substrate specificity of both. Further studies are necessary to elucidate the regulatory mechanism of protein *O*-mannosylation.

Conflict of interest
None declared.

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Protein *O*-mannosylation is necessary for normal embryonic development in zebrafish

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Two distinct cDNAs corresponding to two zebrafish protein *O*-mannosyltransferase genes, *zPOMT1* and *zPOMT2*, were cloned from early developmental embryos. Gene expression analysis revealed that *zPOMT1* and *zPOMT2* were expressed in similar patterns during early embryonic development and in all adult tissues. To study the regulation of *zPOMT1* and *zPOMT2* mRNA distribution during zebrafish embryogenesis, we injected *enhanced green fluorescent protein* (*EGFP*) mRNA fused to the 3′untranslated regions of each *zPOMT* gene. The distribution of EGFP resulting from the two constructs was similar. Injection of antisense morpholino oligonucleotides of *zPOMT1* and *zPOMT2* resulted in several severe phenotypes— including bended body, edematous pericaridium and abnormal eye pigmentation. Immunohistochemistry using anti-glycosylated α-dystroglycan antibody (IIH6) and morphological analysis revealed that the phenotypes of *zPOMT2* knockdown were more severe than those of *zPOMT1* knockdown, even though the IIH6 reactivity was lost in both *zPOMT1* and *zPOMT2* morphants. Finally, only when both *zPOMT1* and *zPOMT2* were expressed in human embryonic kidney 293T cells were high levels of protein *O*-mannosyltransferase activity detected, indicating that both zPOMT1 and zPOMT2 were required for full enzymatic activity. Moreover, either heterologous combination, *zPOMT1* and *human POMT2* (*hPOMT2*) or *hPOMT1* and *zPOMT2*, resulted in enzymatic activity in cultured cells. These results indicate that the protein *O*-mannosyltransferase machinery in zebrafish and humans is conserved and suggest that zebrafish may be useful for functional studies of protein *O*-mannosylation.

Keywords: development/glycosylation/POMT1 and POMT2/ protein *O*-mannosyltransferase activity/zebrafish

Introduction

Posttranslational modification of proteins by glycosylation has critical biological functions at both the cellular and organismal levels (Haltiwanger and Lowe 2004; Ohtsubo and Marth 2006). In addition to the generally observed types of glycosylation such as *N*-glycosylation and mucin-type *O*-glycosylation, several unique glycans have recently been found to play important roles in a variety of biological processes. According to current knowledge, protein *O*-mannosylation in mammals is found on a relatively small number of proteins in the brain, nerves and skeletal muscle (Krusius et al. 1986; Chiba et al. 1997; Sasaki et al. 1998; Endo 1999). In contrast to yeast cells, where *O*-mannose is elongated by neutral, linear oligomannose chains (Strahl-Bolsinger and Tanner 1991), the mannose residue of mammalian *O*-mannosylglycans is extended with complex glycans terminating with sialic acid, sulfate or fucose (Endo 1999). The structure Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-Ser/Thr is required for binding between α-dystroglycan (α-DG) and laminin G domain (Chiba et al. 1997; Endo 1999; Montanaro and Carbonetto 2003).

Muscular dystrophies are genetic diseases characterized by progressive muscle degeneration and muscular weakening. They can be classified into a number of disease types, and some causative genes have been identified (Burton and Davies 2002). For example, dystrophin forms a dystrophin–glycoprotein complex (DGC), and Duchenne muscular dystrophy is caused by mutations in the gene encoding dystrophin. Mutations in other components of DGC are involved in other muscular dystrophies. Defects in glycosylation of α-DG, one of the DGC components, are responsible for certain congenital muscular dystrophies (Endo and Toda 2003; Michele and Campbell 2003). These kinds of muscular dystrophies, including diseases such as Walker–Warburg syndrome (WWS) and muscle–eye–brain disease, are called α-dystroglycanopathies. Protein *O*-mannosyltransferases (POMT1 and POMT2) catalyze the first step in *O*-mannosyl glycan synthesis (Manya et al. 2004), and defects in human POMT1 (hPOMT1) or hPOMT2 result in WWS, an autosomal recessive disorder associated with severe congenital muscular dystrophy, abnormal
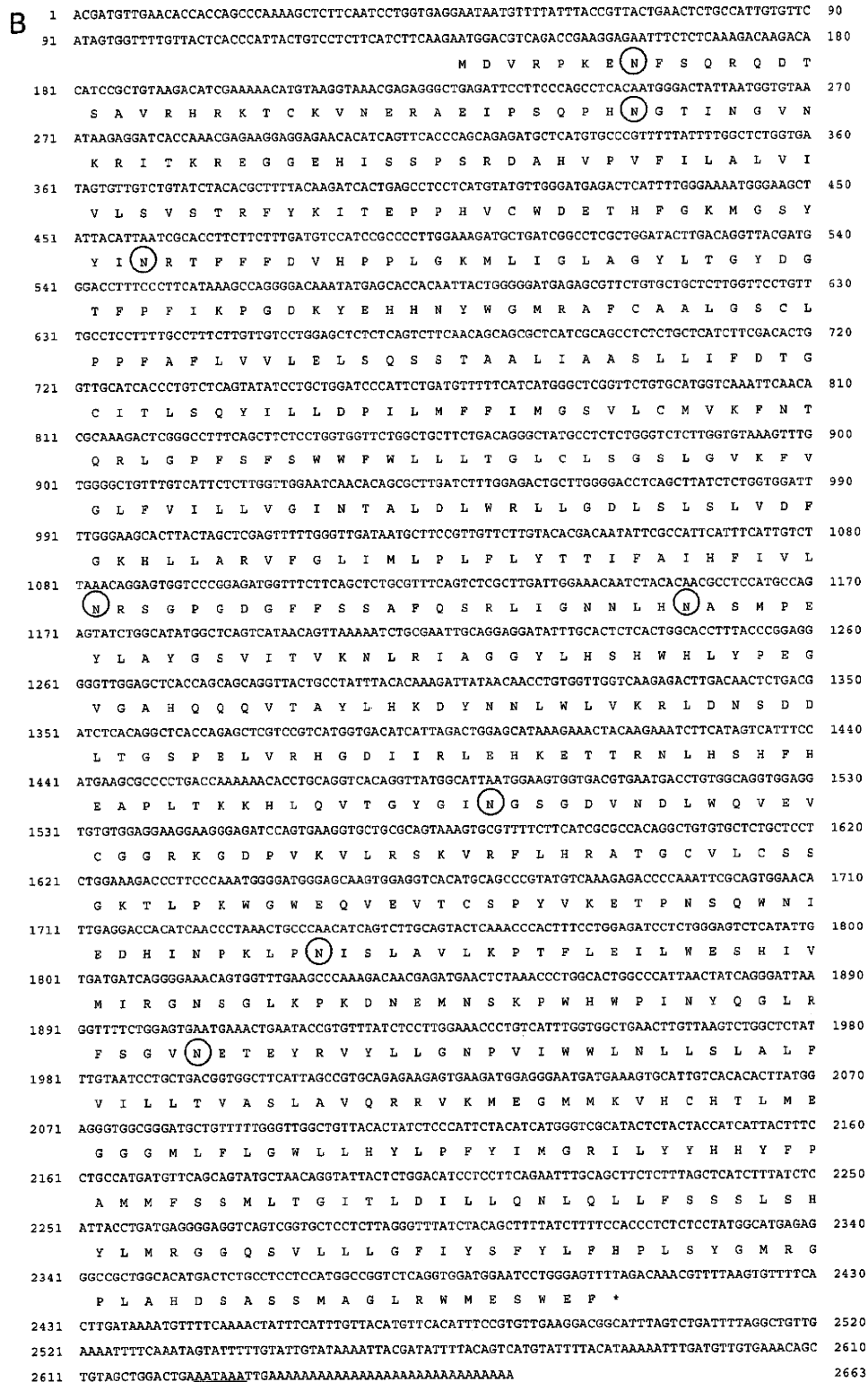
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A
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   1  GAACAGACCGTTGCTTTATTTACAGTGTTTAGTGGCTTTATTTAAAGCGTAAAGTTGTCGTATATGCCTAAGCAATGGCATTATTTATTT   90
  91  GTTTTTATTTTTCTTGACCAATTCCAGTTTTAGCGAATATTTTTATTCATAATTTACTGCTAAAAGACCGCGCATGTGGTCGTGAAAGCA  180
 181  GCACTGTTCACCTATGATTGCGAAATTCCAGAGGAAAAACTGAATCAAGTCAGGATTGAGCATGCAGTGTGTTAAACTGCCCGTCAGTGT  270
                                                                   M  Q  C  V  K  L  P  V  S  V
 271  GACAGTGGAGATAAATGTGCTGCTGCTGGCGGTTACAGCACTTGCCCTCTTTACTCGACTTTATGGCATTCACTTTCCCAAAGCTGTAGT  360
       T  V  E  I  N  V  L  L  L  A  V  T  A  L  A  L  F  T  R  L  Y  G  I  H  F  P  K  A  V  V
 361  GTTTGATGAGGTTTATTATGGACAGTTCCTGTCATTGTACATGAAGCAGGTTTTTTCATAGATGAAAGCGGTCCTCCTTTTGGACACAT   450
       F  D  E  V  Y  Y  G  Q  F  L  S  L  Y  M  K  Q  V  F  F  I  D  E  S  G  P  P  F  G  H  M
 451  GATACTTGCTTTAGGAGCGTATTTAGGAGGATTTGATGGCAACTTTGTTTGGAACAGAATTGGAGCAGAATACCCTGGTAATGTCCCTGT  540
       I  L  A  L  G  A  Y  L  G  G  F  D  G  N  F  V  W  N  R  I  G  A  E  Y  P  G  N  V  P  V
 541  TTGGAGCCTTCGACTGATACCGGCTCTAGCAGGCTCTTTTTGTGTGCCACTTGCATATCTTGTAGTAGTGGAGCTGGGATACTCCCACTT  630
       W  S  L  R  L  I  P  A  L  A  G  S  F  C  V  P  L  A  Y  L  V  V  V  E  L  G  Y  S  H  F
 631  CTCGGCACTGGGGGCCTGTGCACTTCCTCATGGAAAACTCCCTGATTGTGCAATCGCGCTTCATGCTGCTGGAGTCTGTTTTAATTTT   720
       S  A  L  G  A  C  A  L  L  L  M  E  N  S  L  I  V  Q  S  R  F  M  L  L  E  S  V  L  I  F
 721  CTTCTTGCTGTTGGCTGTGCTGTCTTACCTCCGCTTTCCCCAAGCACGCAACTCATTTTTCAAGTGGTTCTGGCTTGTGATCGTGGGGT  810
       F  L  L  L  A  V  L  S  Y  L  R  F  P  Q  A  R  N  S  F  F  K  W  F  W  L  V  I  C  G  V
 811  CAGCTGTGCATTCGGAATTGGGGTAAAGTACATGGGTATGTTCACATACTTTCTACTGCTGAGCCTGGCAGCTGTACACACCTGGCAGCT  900
       S  C  A  F  G  I  G  V  K  Y  M  G  M  F  T  Y  F  L  L  L  S  L  A  A  V  H  T  W  Q  L
 901  TATTGGAGATCGAACCTTGAGCCATGGCAAAGTAATGTTCCAGGTATTAGTTCGCTTCTTGGCACTCGTGGTGCTACCTGTCATCATTTA  990
       I  G  D  R  T  L  S  H  G  K  V  M  F  Q  V  L  V  R  F  L  A  L  V  V  L  P  V  I  I  Y
 991  CCTTGGGTTTTTCTACATTCACCTGACCTTGCTATATCGCAGTGGACCTTCTGACCAGATGATGAGCAGTGCCTTTCAAGCAAGTCTAGA  1080
       L  G  F  P  Y  I  H  L  T  L  L  Y  R  S  G  P  S  D  Q  M  M  S  S  A  F  Q  A  S  L  E
1081  GGGTGGTCTTGCCCGGATCACTCAGGGGCAGCCTTTAGATGTAGCGTTCGGCTCACAGGTCACTCTCCGCACAGTCTCCGGTAAACCTGT  1170
       G  G  L  A  R  I  T  Q  G  Q  P  L  D  V  A  F  G  S  Q  V  T  L  R  T  V  S  G  K  P  V
1171  GCCTTGTTGGCTTCACTCACACAAGGCCAACTATCCCATCAGGTATGAAAATGGCCGTGGAAGCTCCCACCAGCAGCAGGTGACCTGCTA  1260
       P  C  W  L  H  S  H  K  A  N  Y  P  I  R  Y  E  N  G  R  G  S  S  H  Q  Q  Q  V  T  C  Y
1261  TCCTTTCAAAGATGTCAACAACTGGTGGATTATCAAAGACCCTGGGCGGCAAAGTCTTGTGGTCAGCAGCCCCACCCAGACCTGTCAGACA  1350
       P  F  K  D  V  N  N  W  W  I  I  K  D  P  G  R  Q  S  L  V  V  S  S  P  P  R  P  V  R  H
1351  TGGGGATATTATTCAGTTGCTGCATGGAATGACAACTCGCTACCTGAACACACATGATGTTGCAGCCCCCATGAGTCCTCACTCACAGGA  1440
       G  D  I  I  Q  L  L  H  G  M  T  T  R  Y  L  N  T  H  D  V  A  A  P  M  S  P  H  S  Q  E
1441  AGTTTCGGGCTATATTGACTTTAACGTGTCTATGCCAGCCCAGAATCTCTGGAGAGTGGATATTGTGAACAGGGAGTCTGAGAAAGAGAT  1530
       V  S  G  Y  I  D  F (N) V  S  M  P  A  Q  N  L  W  R  V  D  I  V  N  R  E  S  E  K  E  I
1531  CTGGAAGACCATTTTATCAGAGGTGCGACTAGTCCACGTGAACACCTCAGCTGTTTTAAAGCTCAGTGGAGCCTCTCTGCCGGAGTGGGG  1620
       W  K  T  I  L  S  E  V  R  L  V  H  V (N) T  S  A  V  L  K  L  S  G  A  S  L  P  E  W  G
1621  TTTTAAACAGCTGGAGGTGGTGGGTGATAAGATTTATAAAGGCTACCAGCAGACCGGCATGTGGAACGTGGAGGAGCACCGCTATGGCAG  1710
       F  K  Q  L  E  V  V  G  D  K  I  Y  K  G  Y  Q  Q  T  G  M  W  N  V  E  E  H  R  Y  G  R
1711  AAGTCAGGAACCAAAGGAAAGGGAGTTGGAGCTGAAGTCTCCTACTCACAGCGATGTCAACAAAAATCTCACATTTATGGCCAAATTTCT  1800
       S  Q  E  P  K  E  R  E  L  E  L  K  S  P  T  H  S  D  V  N  K (N) L  T  F  M  A  K  F  L
1801  GGGAGCTGCAGTGGAAGATGCTGACAGTAAAGAACGAAGAGTCAGAGCACAAATATAGTTCATCACCTCTTGAATGGATCACAATGGACAC  1890
       E  L  Q  W  K  M  L  T  V  K  N  E  E  S  E  H  K  Y  S  S  S  P  L  E  W  I  T  M  D  T
1891  CAACATTGCATATTGGCTTCATCCTTCAAGTAATGCACAGATTCACTTTATAGGCAATATTGTTACTTGGACCACTGGAAACATTACACT  1980
       N  I  A  Y  W  L  H  P  S  S  N  A  Q  I  H  F  I  G  N  I  V  T  W  T  T  G (N) I  T  L
1981  GGTTGTGTACTGTCTTCTGTTTTTAACATACTTACTAAGACGGAGGAGGAAAGTTGAAGACATTCCACAAGACTCTTGGGAACAGTTGGC  2070
       V  V  Y  C  L  L  F  L  T  Y  L  L  R  R  R  R  K  V  E  D  I  P  Q  D  S  W  E  Q  L  A
2071  TTTGGCTGGTGTGGTTTGCTTTGGAGGCTGGGCAGTGAATTACCTGCCATTCTTCCTAATGGAGAAGACTCTCTTTCTATATCACTATCT  2160
       L  A  G  V  V  C  F  G  G  W  A  V  N  Y  L  P  F  F  L  M  E  K  T  L  F  L  Y  H  Y  L
2161  CCCAGCACTCACCTTCAAGATCTTGCAAATACCTATAGTCACAGAGCACCTGTACATCCACGTATTGAGATCCTCAGCTCAACAGAAAGC  2250
       P  A  L  T  F  K  I  L  Q  I  P  I  V  T  E  H  L  Y  I  H  V  L  R  S  S  A  Q  Q  K  A
2251  ATTTGGTGGTGTGATTTTAGCAGTTCTTTGTTCAGTATACATGTCCTACCACAGCTTGAGTCCTCTCACGTATGGCCAGCCAGCGTTAAC  2340
       F  G  G  V  I  L  A  V  L  C  S  V  Y  M  S  Y  H  S  L  S  P  L  T  Y  G  Q  P  A  L  T
2341  ATCAGACAAGCTCGCTGAGCTGCGCTGGAGGGAGAGTTGGGATATTCTCTTACGCAAACGCTAAAATATCCTTTATTTTTTTTAAGACAA  2430
       S  D  K  L  A  E  L  R  W  R  E  S  W  D  I  L  L  R  K  R  *
2431  GATAATTTCATGGTGTCAAAATTTTAAATCGAGATGACTGATAGGGCTGATGGATTTTTTGAGAAATGGATTCTGATTCGAAATGCATCA  2520
2521  CTTTTTCTGTCTTGAATGGATTCTGAGCTCAGTGTTTAACAGGAGATTATACCATATGCTTTACAAACTCTGCTTGCTTCCAATTCCTTAC  2610
2611  AAATACCACATGAATCTAAAGTAATCAGTAATGAAGTTCAAATTAGCCAATTACAAAAGTGTTTTTAGTGAGCTGTTTACATTAATCTTG  2700
2701  TCATAAAGGCCAACTTACATGAAGCACAAGCAATATATGGTTTGAAAACTAGTCAAGAGTGCAACATGCTGTTAAAAACTAAGTTTAGAA  2790
2791  TCACTTCAAGAGAGAATAGCAACGCATTTTGAATCAATTTTTCAATATTACACCATAGTTTTCATGGATTTCCAGAAAGCTGTGAAGCAC  2880
2881  TATTTCTAGGGTCTGTGCCCAAACATAATAATGTGAAAAAAAAACTCATGCTGCTGAATCTGAAAACTCTAAACCAAATGGACTCTGAAGC  2970
2971  ACAATTTCCGTGCTACATTTATCATGCTCGTATGTTTACATATTACTCTGTTTGTATAGCGATTTTATTGTATTGTTTTAGAGACTGTTA  3060
3061  AACTAATAAGATGCATTGCATTTCTTTAAATATGTTTTATTTGATTCTTGCACAACAGAAAAAAACGTTTAATAAACGTTTATATGACAT  3150
3151  TAAAAAAAAAAAAAAAAAA                                                                          3169
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B

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  1  ACGATGTTGAACACCACCAGCCCAAAAGCTCTTCAATCCTGGTGAGGAATAATGTTTTATTTACCGTTACTGAACTCTGCCATTGTGTTC   90
 91  ATAGTGGTTTTGTTACTCACCCATTACTGTCCTCTTCATCTTCAAGAATGGACGTCAGACCGAAGGAGAATTTCTCTCAAAGACAAGACA  180
                                          M  D  V  R  P  K  E (N) F  S  Q  R  Q  D  T
181  CATCCGCTGTAAGACATCGAAAAACATGTAAGGTAAACGAGAGGGCTGAGATTCCTTCCCAGCCTCACAATGGGACTATTAATGGTGTAA  270
       S  A  V  R  H  R  K  T  C  K  V  N  E  R  A  E  I  P  S  Q  P  H (N) G  T  I  N  G  V  N
271  ATAAGAGGATCACCCAAACGAGAAGGAGGAGAACACATCAGTTCACCCAGCAGAGATGCTCATGTGCCCGTTTTTATTTTGGCTCTGGTGA  360
       K  R  I  T  K  R  E  G  G  E  H  I  S  S  P  S  R  D  A  H  V  P  V  F  I  L  A  L  V  I
361  TAGTGTTGTCTGTATCTACACGCTTTTACAAGATCACTGAGCCTCCTCATGTATGTTGGGATGAGACTCATTTGGGAAAATGGGAAGCT  450
       V  L  S  V  S  T  R  F  Y  K  I  T  E  P  P  H  V  C  W  D  E  T  H  F  G  K  M  G  S  Y
451  ATTACATTAATCGCACCTTCTTCTTTGATGTCCATCCGCCCCTTGGAAAGATGCTGATCGGCCTCGCTGGATACTTGACAGGTTACGATG  540
       Y  I (N) R  T  F  F  F  D  V  H  P  P  L  G  K  M  L  I  G  L  A  G  Y  L  T  G  Y  D  G
541  GGACCTTTCCCTTCATAAAGCCCAGGGGACAAATATGAGCACCACAATTACTGGGGGATGAGAGCGTTCTGTGCTGCTCTTGGTTCCTGTT  630
       T  F  P  F  I  K  P  G  D  K  Y  E  H  H  N  Y  W  G  M  R  A  F  C  A  A  L  G  S  C  L
631  TGCCTCCTTTTGCCTTTCTTGTTGTCCTGGAGCTCTCTCAGTCTTCAACAGCAGCGCTCATCGCAGCCTCTCTGCTCATCTTCGACACTG  720
       P  P  P  A  F  L  V  V  L  E  L  S  Q  S  S  T  A  A  L  I  A  A  S  L  L  I  F  D  T  G
721  GTTGCATCACCCTGTCTCAGTATATCCTGCTGGATCCCATTCTGATGTTTTTCATCATGGGCTCGGTTCTGTGCATGGTCAAATTCAACA  810
       C  I  T  L  S  Q  Y  I  L  L  D  P  I  L  M  F  F  I  M  G  S  V  L  C  M  V  K  F  N  T
811  CGCAAAGACTCGGGCCTTTCAGCTTCTCCTGGTGGTTCTGGCTGCTTCTGACAGGGCTATGCCTCTCTGGGTCTCTTGGTGTAAAGTTTG  900
       Q  R  L  G  P  F  S  F  S  W  W  F  W  L  L  L  T  G  L  C  L  S  G  S  L  G  V  K  F  V
901  TGGGGCTGTTTGTCATTCTCTTGGTTGGAATCAACACAGCGCTTGATCTTTGGAGACTGCTTGGGGACCTCAGCTTATCTCTGGTGGATT  990
       G  L  F  V  I  L  L  V  G  I  N  T  A  L  D  L  W  R  L  L  G  D  L  S  L  S  L  V  D  F
991  TTGGGAAGCACTTACTAGCTCGAGTTTTTGGGTTGATAATGCTTCCGTTGTTCTTGTACACGACAATATTCGCCATTCATTTCATTGTCT 1080
       G  K  H  L  L  A  R  V  F  G  L  I  M  L  P  L  F  L  Y  T  T  I  F  A  I  H  F  I  V  L
1081 TAAACAGGAGTGGTCCCGGAGATGGTTCTTCAGCTCTGCGTTTCAGTCTCGCTTGATTGGAAACAATCTACACAACGCCTCCATGCCAG 1170
     (N) R  S  G  P  G  D  G  F  F  S  S  A  F  Q  S  R  L  I  G  N  N  L  H (N) A  S  M  P  E
1171 AGTATCTGGCATATGGCTCAGTCATAACAGTTAAAAATCTGCGAATTGCAGGAGGATATTTGCACTCTCACTGGCACCTTTACCCGGAGG 1260
       Y  L  A  Y  G  S  V  I  T  V  K  N  L  R  I  A  G  G  Y  L  H  S  H  W  H  L  Y  P  E  G
1261 GGGTTGGAGCTCACCAGCAGCAGGTTACTGCCTATTTACACAAAGATTATAACAACCTGTGGTTGGTCAAGAGACTTGACAACTCTGACG 1350
       V  G  A  H  Q  Q  Q  V  T  A  Y  L  H  K  D  Y  N  N  L  W  L  V  K  R  L  D  N  S  D  D
1351 ATCTCACAGGCTCACCAGAGCTCGTCCGTCATGGTGACATCATTAGACTGGAGCATAAAGAAACTACAAGAAATCTTCATAGTCATTTCC 1440
       L  T  G  S  P  E  L  V  R  H  G  D  I  I  R  L  E  H  K  E  T  T  R  N  L  H  S  H  F  H
1441 ATGAAGCGCCCCTGACCAAAAAACACCTGCAGGTCACAGGTTATGGCATTAATGGAAGTGGTGACGTGAATGACCTGTGGCAGGTGGAGG 1530
       E  A  P  L  T  K  K  H  L  Q  V  T  G  Y  G  I (N) G  S  G  D  V  N  D  L  W  Q  V  E  V
1531 TGTGTGGAGGAAGGAAGGGAGATCCAGTGAAGGTGCTGCGCAGTAAAGTGCGTTTTCTTCATCGCGCCACAGGCTGTGTGCTCTGCTCCT 1620
       C  G  G  R  K  G  D  P  V  K  V  L  R  S  K  V  R  F  L  H  R  A  T  G  C  V  L  C  S  S
1621 CTGGAAAGACCCTTCCCAAATGGGGATGGGAGCAAGTGGAGGTCACATGCAGCCCGTATGTCAAAGAGACCCCAAATTCGCAGTGGAACA 1710
       G  K  T  L  P  K  W  G  W  E  Q  V  E  V  T  C  S  P  Y  V  K  E  T  P  N  S  Q  W  N  I
1711 TTGAGGACCACATCAACCCTAAACTGCCCAACATCAGTCTTGCAGTACTCAAACCCACTTTCCTGGAGATCCTCTGGGAGTCTCATATTG 1800
       E  D  H  I  N  P  K  L  P (N) I  S  L  A  V  L  K  P  T  F  L  E  I  L  W  E  S  H  I  V
1801 TGATGATCAGGGGAAACAGTGGTTTGAAGCCCAAAGACAACGAGATGAACTCTAAACCCTGGCACTGGCCCATTAACTATCAGGGATTAA 1890
       M  I  R  G  N  S  G  L  K  P  K  D  N  E  M  N  S  K  P  W  H  W  P  I  N  Y  Q  G  L  R
1891 GGTTTTCTGGAGTGAATGAAACTGAATACCGTGTTTATCTCCTTGGAAACCCTGTCATTTGGTGGCTGAACTTGTTAAGTCTGGCTCTAT 1980
       F  S  G  V (N) E  T  E  Y  R  V  Y  L  L  G  N  P  V  I  W  W  L  N  L  L  S  L  A  L  F
1981 TTGTAATCCTGCTGACGGTGGCTTCATTAGCCGTGCAGAGAAGAGTGAAGATGGAGGGAATGATGAAAGTGCATTGTCACACACTTATGG 2070
       V  I  L  L  T  V  A  S  L  A  V  Q  R  R  V  K  M  E  G  M  M  K  V  H  C  H  T  L  M  E
2071 AGGGTGGCGGGATGCTGTTTTTGGGTTGGCTGTTACACTATCTCCCATTCTACATCATGGGTCGCATACTCTACTACCATCATTACTTTC 2160
       G  G  G  M  L  F  L  G  W  L  L  H  Y  L  P  F  Y  I  M  G  R  I  L  Y  Y  H  H  Y  F  P
2161 CTGCCATGATGTTCAGCAGTATGCTAACAGGTATTACTCGGACATCCTCCTTCAGAATTTGCAGCTTCTCTTTAGCTCATCTTTATCTC 2250
       A  M  M  F  S  S  M  L  T  G  I  T  L  D  I  L  L  Q  N  L  Q  L  L  F  S  S  S  L  S  H
2251 ATTACCTGATGAGGGGAGGTCAGTCGGTGCTCCTCTTAGGGTTTATCTACAGCTTTTATCTTTTCCACCCTCTCTCCTATGGCATGAGAG 2340
       Y  L  M  R  G  G  Q  S  V  L  L  L  G  F  I  Y  S  F  Y  L  F  H  P  L  S  Y  G  M  R  G
2341 GGCCGCTGGCACATGACTCTGCCTCCTCCATGGCCGGTCTCAGGTGGATGGAATCCTGGGAGTTTTAGACAAACGTTTTAAGTGTTTTCA 2430
       P  L  A  H  D  S  A  S  S  M  A  G  L  R  W  M  E  S  W  E  F  *
2431 CTTGATAAAATGTTTTCAAAACTATTTCATTTGTTACATGTTCACATTTCCGTGTTGAAGGACGGCATTTAGTCTGATTTTAGGCTGTTG 2520
2521 AAAATTTTCAAATAGTATTTTTGTATTGTATAAAATTACGATATTTTACAGTCATGTATTTTACATAAAAATTTGATGTTGTGAAACAGC 2610
2611 TGTAGCTGGACTGAAATAAATTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                                    2663
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Fig. 1. Nucleotide sequences and deduced amino acid sequences of zPOMT1 and zPOMT2. The cDNA sequences of zebrafish POMT1 (A) and POMT2 (B) genes are presented on the upper line. Deduced amino acid sequences are indicated by the single-letter amino acid codes. Potential N-glycosylation sites are indicated by circles. Consensus polyadenylation sites are underlined.

neuronal migration and eye anomalies (Beltran-Valero de Bernabe et al. 2002; van Reeuwijk et al. 2005). POMT orthologs have been identified in many animals, including *Drosophila* (Martín-Blanco and García-Bellido 1996; Willer et al. 2002), mouse (Willer et al. 2002; Willer et al. 2004), rat (Manya et al. 2006) and humans (Jurado et al. 1999; Willer et al. 2002). *Drosophila*, rat and mouse have orthologs of both of human *POMT* genes, and their products have protein *O*-mannosyltransferase activity when only they are co-expressed (Ichimiya et al. 2004; Manya et al. 2006; Lommel et al. 2008). In contrast, *Drosophila* does not have orthologs to human or murine protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase1 (POMGnT1) (Ichimiya et al. 2004). POMGnT1 catalyzes the transfer of GlcNAc from UDP-GlcNAc to the protein *O*-mannosyl residue (Yoshida et al. 2001; Liu et al. 2006; Miyagoe-Suzuki et al. 2009). Therefore, it seems that the structures of *O*-mannosylglycans of invertebrates are quite different from those of vertebrates. Moreover, it has been reported that hPOMT1 and hPOMT2 must form a heterocom-

plex for protein *O*-mannosyltransferase activity (Akasaka-Manya et al. 2006).

The zebrafish (*Danio rerio*) provides a readily accessible model for human muscle diseases such as muscular dystrophies (Bassett and Currie 2003). Muscle specification and differentiation follow a well-characterized time course and allow detailed analysis with single-cell resolution (Devoto et al. 1996). Zebrafish orthologs of proteins in the human DGC have been implicated in muscle development, and some zebrafish DGC orthologs have uses as models to study human muscular dystrophy and congenital myopathy (Parsons et al. 2002; Bassett and Currie 2003; Guyon et al. 2003). More recently, it has been reported that the zebrafish has orthologs of *POMT1*, *POMT2*, *POMGnT1* and other putative glycosyltransferases expected to contribute to the synthesis of mammalian-type *O*-mannosylglycan (Steffen et al. 2007; Moore et al. 2008). Taken together, the structures of *O*-mannosylglycans are thought to be similar in diverse vertebrates. Therefore, zebrafish may be a useful model for analyses of the biosynthetic

A

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hPOMT1    1:--------------------MWGFLKRPVVVTADINLSLVALTGMGLLSRLWRLTYPRAVVFDEVYYGQYISFYMKQIFFLDDSGPPF  68
mPOMT1    1:MGSHSTGLEETLGVLPSWLFCKMLRFLKRPLVVDINLNLVALTGLGLLTRLWQLSYPRAVVFDEVYYGQYISFYMKRIFFLDDSGPPF  90
rPOMT1    1:MGNRSMGREDTLGVLPSLLFCKMLRFLKRPLVVTIDINLNLVALTVLGLLTRLWQLSYPRAVVFDEVYYGQYISFYMKRVFFLDDSGPPF  90
zPOMT1    1:--------------------MQ-CVKLPVSVTVEINVLLLAVTALALFTRLYGIHFPKAVVFDEVYYGQFLSLYMKQVFFIDESGPPF  67

hPOMT1   69:GHMVLALGGYLGGFDGNFLWNRIGAEYSSNVPVWSLRLLPALAGALSVPMAYQIVLELHFSHCAAMGAALLMLIENALITQSRLMLLESV  158
mPOMT1   91:GHMLLALGGWLGGFDGNFLWNRIGAEYSSNVPIWSLRLLPALAGALSVPMAYQIVLELHFSHGAAIGAALLMLIENALITQSRLMLLESI  180
rPOMT1   91:GHMLILALGGWLGGFDGNFLWNRIGAEYSSNVPVWSLRLLPALAGALSVPMAYQIVLELHFSHCTAMGAALLMLIENALITQSRLMLLESI  180
zPOMT1   68:GHMILALGAYLGGFDGNFVWNRIGAEYPGNVPVWSLRLIPALAGSFCVPLAYLVVVELGYSHFSALGACALLLMENSLIVQSRFMLLESV  157

hPOMT1  159:LIFFNLLAVLSYLKFFNCQKHSPFSLSWWFWLTLTGVACSCAVGIKYMGVFTYVLVLGVAAVHAWHLLGDQTLSNVCVFCHLLARAVALL  248
mPOMT1  181:LIFFNLLAVLSYLKFFNSQTHSPFSVHWWLWLLLTGVSCSCAVGIKYMGIFTYLLVLGIAAVHAWNLIGDQTLSNMRVLSHLLARIVALL  270
rPOMT1  181:LIFFNLLAVLSYLKFFNSQTHSPFSVHWWLWLMLTGVSCSCAVGIKYMGIFTYLLVLSIAAVHAWHLIGDQTLSNICVLSHLLARAVALL  270
zPOMT1  158:LIFFLLLAVLSYLRFHKARN----SFFKWFWLVICGVSCAFGIGVKYMGMFTYFLLLSLAAVHTWQLIGDRTLSHGKVMFQVLVRFLALV  243

hPOMT1  249:VIPVVLYLLFFYVHLILVFRSGPHDQIMSSAFQASLEGGLARITQGQPLEVAFGSQVTLRNVFGKPVPCWLHSHQDTYPMIYENGRGSSH  338
mPOMT1  271:VVPVPFLYLLFFYVHLMLLYRSGPHDQIMSSAFQASLEGGLARITQGQPLEVAFGSQVTLKSVSGKPLPCWLHSHKNTYPMIYENGRGSSH  360
rPOMT1  271:VVPVPFLYLLFFYVHLMLLYRSGPHDQIMSSAFQASLEGGLARITQGQPLEVAFGSQVTLKSVSGKPLPCWLHSHKNTYPMIYENGRGSSH  360
zPOMT1  244:VLPVIIYLGFFYIHLTLLYRSGPSDQMMSSAFQASLEGGLARITQGQPLDVAFGSQVTLRTVSGKPVPCWLHSHKANYPIRYENGRGSSH  333

hPOMT1  339:QQQVTCYPFKDVNNWWIVKDPRRHQLVVSSPPRPVRHGDMVQLVHGMTTRSLNTHDVAAPLSPHSQEVSCYIDYNISMPAQNLWRLEIVN  428
mPOMT1  361:QQQVTCYPFKDINNWWIVKDPGRHQLVVNNPPRPVRHGDIVQLVHGMTTRLLNTHDVAAPLSPHSQEVSCYIDYNISMPAQNLWKLDIVN  450
rPOMT1  361:QQQVTCYPFKDINNWWIVKDPGRHQLVVNNPPRPVRHGDIVQLVHGMTTRLLNTHDVAAPLSPHSQEVSCYIDYNISMPAQNLWKLDIVN  450
zPOMT1  334:QQQVTCYPFKDVNNWWIIKDPGRQSLVVSSPPRPVRHGDIIQLQLHGMTTRYLNTHDVAAPMSPHSQEVSGYIDFNVSMPAQNLWRVDIVN  423

hPOMT1  429:RGSDTDVWKTILSEVRFVHVNTSAVLKLSGAHLPDWGYRQLEIVGEKLSRGYHGSTVWNVEEHRYGASQEQRERERELHSPAQVDVSRNL  518
mPOMT1  451:RESNRDTWKTILSEVRFVHVNTSAILKLSGAHLPDWGFRQLEVVGEKLSPGYHESMVWNVEEHRYGKSHEQKERELELHSPTQLDISRNL  540
rPOMT1  451:RESNQDTWKTILSEVRFVHVNTSALLKLSGAHLPDWGFRQLEVVGEKLSLGPHESMVWNVEEHRYGRGHEQKERELELHSPTQHDISRNL  540
zPOMT1  424:RESEKEIWKTILSEVRLVHVNTSAVLKLSGASLPEWGFKQLEVVGDKIYKGYQQTGMWNVEEHRYGRSQEPKERELELKSPTHSDVNKNL  513

hPOMT1  519:SFMARFSELQWRMLALRSDDSEHKYSSSPLEWVTLDTNIAYWLHPRTSAQIHLLGNIVIWVSGSLALAIYALLSLWYLLRRRRNVHDLPQ  608
mPOMT1  541:SFMARFSELQWKMLTLKNEDLEHQYSSTPLEWLTLDTNIAYWLHPRTSAQIHLLGNIVIWTSASLATVVYTLLFFWYLLRRRRSICDLPE  630
rPOMT1  541:SFMARFSELQWKMLTLKNEDLEHQYSSTPLEWLTLDTNIAYWLHPRTSAQIHLLGNIVIWTSASLATVAYTLLFFWYLLRRRRNICDLPE  630
zPOMT1  514:TFMAKFLELQWKMLTVKNEESEHKYSSSPLEWIIMDTNIAYWLHPSSNAQIHFIGNIVTWTTGNITLVVYCLLFLTYLLRRRRKVEDIPQ  603

hPOMT1  609:DAWLRWVLAGALCAGGWAVNYLPFFLMEKTLFLYHYLPALTFQILLLPVVLQHISDHLCRSQLQRSIFSALVVAWYSSACHVSNQLRPLT  698
mPOMT1  631:DAWSRWVLAGALCTGGWALNYLPFFLMERVLFLYHYLPALTFQILLLPIVLQHASDHLCRSQLQRNVFSALVVAWYSSACHVSNMLRPLT  720
rPOMT1  631:DAWSHWVLAGALCIGGWALNYLPFFLMERMLFLYHYLPALTFQILLLPIVMQHASDHLCRSQLQRNVFSALVVAWYSSACHVSNMLRPLT  720
zPOMT1  604:DSWEQLALAGVVCFGGWAVNYLPFFLMEKTLFLYHYLPALTFKILQIPIVTEHLYIHVLRSSAQQKAFGGVILAVLCSVYMSYHSLSPLT  693

hPOMT1  699:YGDKSLSPHELKALRWKDSWDILIRKH                                                                 725
mPOMT1  721:YGDTSLSPGELRALRWKDSWDILIRK                                                                  746
rPOMT1  721:YGDTSLSPGELRALRWKDSWDILIRKY                                                                 747
zPOMT1  694:YGQPALTSDKLAELRWRESWDILLRKR                                                                 720
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B

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hPOMT2   1:------------------------------------------------------------MPPATGGGLAESELRPRRGR  20
mPOMT2   1:MLYASGRLLAAREATTLSAPPRARGPALRGKRRELQIPWHLETPSYDPLTGQRTRPGVPPARRVILRKGRMPPAIGGGLAGSELRPRRGR  90
rPOMT2   1:MFYASGRLLAAREATTLYAPPRARGPALRGKRRELQIPWHLETPSYDSLTGQRTRPGVPPARRVILRKGRMPPAIGGGLAGSELRPRRGR  90
zPOMT2   1:------------------------------------------MDVRPKENFSQRQD--TSAVR-HRKTCKVNERA--  30

hPOMT2  21:CGPQAARAAGRDVAAEAVARSPKRPAWGSRRFEAVGWWALLALVTLLSFATRFHRLDEPPHICWDETHFGKMGSYYINRTFFFDVHPPLG  110
mPOMT2  91:CVPQAARAVSRDVVPQAAARKLKRPAWSSRRFQAAGWWATLAVVTLLSFATRFHRLDQPAHICWDETHFGKMGSYYINRTFFFDVHPPLG  180
rPOMT2  91:SVQQAARAVSRDVVPEAATRKLKRPAWSSRRFQAAGWWATLAVVTLLSFATRFHRLDQPAHICWDETHFGKMGSYYINRTFFFDVHPPLG  180
zPOMT2  31:EIPSQPHNGTINGVNKRITKREGGEHISSPSRDAHVPVFILALVIVLSVSTRFVKITEPPHVCWDETHFGKMGSYYINRTFFFDVHPPLG  120

hPOMT2 111:KMLIGLAGYLSGYDGTFLFQKPGDKYEHHSYMGMRGFCAFLGSWLVPFAYLTVLDLSKSLSAALLTAALLTFDTGCLTLSQYILLDPILM  200
mPOMT2 181:KMLIGLAGYLSGYDGTFLFQKPGDRYEHHSYMGMRGFCAFLGSWLIPFAYLTVLDLSKSFPAALLTAALLTFDTGCLTLSQYILLDPILM  270
rPOMT2 181:KMLIGLAGYLSGYDGTFLFQKPGDRYEHHSYMGMRGFCAFLGSWLIPFAYLTVLDLSKSFPAALLTAALLTCDTGCLTLSQYILLDPILM  270
zPOMT2 121:KMLIGLAGYLTGYDGTFPFIKPGDKYEHHNYWGMRAFCAALGSCLPPFAFLVVLELSQSSTAALIAASLLIFDTGCTTLSQYILLDPILM  210

hPOMT2 201:FFIMAAMLSMVKYNSCADRPFSAPWWFWLSLTGVSLAGALGVKFVGLFIILQVGLNTIADLWYLFGDLSLSLVTVGKHLTARVLCLIVLP  290
mPOMT2 271:FFIMAAMLSMVKYNSCANRPFSAPWWFWLSLTGISLAGALGVKFVGLFIIVQVGLNTISDLWHLFGDLSLSLVTVGKHLTARILCLIVLP  360
rPOMT2 271:FFIMAAMLSMVKYNSFANRPFSAPWWFWLSLTGISLAGALGVKFVGLFIIVQVGLNTISDLWHLFGDLSLSLVTVGKHLTARILCLIVLP  360
zPOMT2 211:FFIMGSVLCMVKFNTQRLGPFSFSWWFWLLLTGLCLSGSLGVKFVGLFVILLVGINTALDLWRLGDLSLSLVDFGKHLLARVFGLIMLP  300

hPOMT2 291:LALYTATFAVHFMVLSKSGPGDGFFSSAFQARLSGNNLHNASIPEHLAYGSVITVKNLRMAIGYLHSHRHLYPEGIGARQQQVTTYLHKD  380
mPOMT2 361:LVLYVTIFAVHVMVLNKSGPGDGFFSSAFQARLSGNSLHNASIPEHLAYGSVITVKNLRMAIGYLHSHRHLYPEGIGARQQQVTTYLHKD  450
rPOMT2 361:LVLYVTIFAVHVMVLNKSGPGDGFFSSAFQARLSGNSLHNASIPEHLAYGSVITVKNLRMAIGYLHSHRHLYPEGIGARQQQVTTYLHKD  450
zPOMT2 301:LFLYTTIFAIHFIVLNRSGPGDGFFSSAFQSRLIGNNLHNASMPEYLAYGSVITVKNLRIAGGYLHSHWHLYPEGVGAHQQQVTAYLHKD  390

hPOMT2 381:YNNLWIIKKHNTNSDPLDPSFPVEFVRHGDIIRLEHKETSRNLHSHYHEAPMTRKHYQVTGYGINGTGDSNDFWRIEVVNRKFGNRIKVL  470
mPOMT2 451:YNNLWIIKKYNANTDPLDPSFPVEFVRHGDIIRLEHKETTRNLHSHYHEAPLTRKHYQVTGYGINGTGDSNDFWRIEVVNRKFGNRIKVL  540
rPOMT2 451:YNNLWIIKKYNTNTDPLDPSFPVEFVRHGDIIRLEHKETTRNLHSHYHEAPLTRKHYQVTGYGINGTGDSNDFWRIEVVNRKFGNRIKVL  540
zPOMT2 391:YNNLWLVKRLD-NSDDLTGSP--ELVRHGDIIRLEHKETTRNLHSHFHEAPLTKKHLQVTGYGINGSGDVNDLWQVEVCGGRKGDPVKVL  477

hPOMT2 471:RSRIRFIHLVTGCVLGSSGKVLPKWGWEQLEVTCTPYLKETLNSIWNVEDHINPKLPNISLDVLQPSFPEILLESHMVMIRGNSGLKPKD  560
mPOMT2 541:RSRIRLFIHLVTGCVLGSSGKILPKWGWEQLEVTCTPYLKETTNSIWNIEEHINPKLPNISLDVLQPSFPEILLESHMVMIRGNNGLKPKD  630
rPOMT2 541:RSRIRFIHLVTGCVLGSSGKILPKWGWEQLEVTCNPYLKETSNSIWNIEEHINPKLPNISLDVLQPSFPEILLESHMVMIRGNNGLKPKD  630
zPOMT2 478:RSKVRFLHRATGCVLCSSGKTLPKWGWEQVEVTCSPYVKETPNSQWNIEDHINPKLPNISLAVLKPTFLEILWESHIVMIRGNSGLKPKD  567

hPOMT2 561:NEFTSKPWHWPINYQGLRFSGVNDTDFRVYLLGNPVVWWLNLLSIALYLLSGSIIAVAMQRGARLPAEVAGLSQVLLRGGGQVLLGWTLH  650
mPOMT2 631:NEFTSKPWHWPINYQGLRFSGANDTDFRVYLLGNPVVWWLNLVSIVLYLLSGSTIAVAMQRGIQLPAELQGLTKVLLRGGGQLLLGWMLH  720
rPOMT2 631:NEFTSKPWHWPINYQGLRFSGANDTDFRVYLLGNPVVWWLNLVSLVLYLLTGSTIAIAMQRGIQLPAELQGLTKLLLRGGGQLLLGWTLH  720
zPOMT2 568:NEMNSKPWHWPINYQGLRFSGVNETEYRVYLLGNPVIWWLNLLSLALFVILLTVASLAVQRRVKMEGMMKVHCHTLMEGGGMLFLGWLLH  657

hPOMT2 651:YFPFFLMGRVLYFHHYFPAMLFSSMLTGILWDTLLRLCAWGLASWPLARGIHVAGILSLLLGTAYSFYLFHPLAYGMVGPLAQDPQSPMA  740
mPOMT2 721:YFPFFLMGRILYFHHYFPAMLFSSMLTGILWDTLLRLCAWGLAPSPLGRRIHAVGILSLLLTTAYSFYLFHPLAYGMVGPLAQEPESPMA  810
rPOMT2 721:YFPFFLMGRVLYFHHYFPAMLFSSMLTGLLWDTLLRFCAWALAPSPLGRRIHMVGILSLLLATAYSFYLFHPLAYGMVGPLAQEPESPMA  810
zPOMT2 658:YLPFYIMGRILYYHHYFPAMMFSSMLTGITLDILLQNLQL-LFSSSLSHYLMRGGQSVLLLGFIYSFYLFHPLSYGMRGPLAHDSASSMA  746

hPOMT2 741:GLRWLDSWDF  750
mPOMT2 811:GLRWLESWDF  820
rPOMT2 811:GLRWLESWDF  820
zPOMT2 747:GLRWMESWEF  756
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pathway of *O*-mannosylglycans in vertebrates, the mechanisms of muscular dystrophies, and myogenesis.

In this study, we isolated and cloned full-length cDNAs encoding two zebrafish *POMT* genes, *zPOMT1* and *zPOMT2*, and examined whether they have protein *O*-mannosyltransferase activity. We also investigated the expression patterns of both genes during embryogenesis and in adult tissues. Furthermore, we analyzed the distribution and localization of a protein expressed from constructs containing the 3′untranslated region (3′UTR) of *zPOMT1* or *zPOMT2*. Finally, knockdown analysis using antisense morpholino oligonucleotides (MOs) was performed to assess the function of protein *O*-mannosylation during zebrafish development.

Results

cDNA cloning and sequencing of zPOMT1 and zPOMT2

The full-length cDNAs encoding *zPOMT1* and *zPOMT2* genes were cloned by reverse transcriptase-polymerase chain reaction (RT-PCR) using zebrafish embryos. The complete cDNAs and deduced amino acid sequences of *zPOMT1* and *zPOMT2* are shown in Figure 1 (GenBank accession nos. AB281275 and AB281276, respectively). *zPOMT1* consisted of an open reading frame (ORF) of 2160 bases encoding a conceptual translation product of 720 amino acids with a predicted molecular mass of 82,036 Da (Figure 1A). *zPOMT2* consisted of an ORF of 2268 bases encoding conceptual translation product of 756 residues with a predicted molecular mass of 85,710 Da
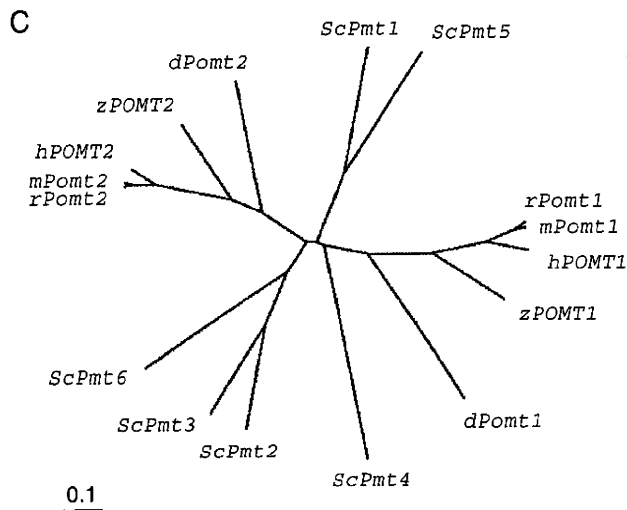
1093

C

Fig. 2. Comparisons of human, mouse, rat and zebrafish POMTs. ClustalW alignments of human (h), mouse (m), rat (r) and zebrafish (z) POMT1 (A) and POMT2 (B) amino acid sequences are indicated by single-letter amino acid codes, respectively. Conserved amino acids are highlighted. (C) ClustalW phylogenic tree of human, mouse, rat, zebrafish and *Drosophila* (d) POMTs and *S. cerevisiae* (Sc) Pmts. The amino acid sequence of hPOMT1 is a major type that was used for assay of protein *O*-mannosyltransferase activity in this study. The amino acid sequences of mPOMT2 and rPOMT2 belonged to the testis form. The branch lengths indicate amino acid substitutions per site.

(Figure 1B). A consensus polyadenylation site (AATAAA) was located downstream of the translation termination codon in both *zPOMT1* and *zPOMT2*. As shown in Figure 2, the deduced amino acid sequences in both zPOMT1 and zPOMT2 were similar to those of mammals such as human, mouse and rat (Jurado et al. 1999; Willer et al. 2002; Willer et al. 2004; Manya et al. 2006). zPOMT1 had 66% identity to hPOMT1 (Figure 2A), whereas zPOMT2 showed 70% identity to hPOMT2 (Figure 2B). A phylogenetic analysis of 16 proteins—six *Saccharomyces cerevisiae* Pmts (ScPmt1-6) (Willer et al. 2002), two human (hPOMT1 and hPOMT2), two mouse (mPOMT1 and mPOMT2), two rat (rPOMT1 and rPOMT2), two *Drosophila* (dPOMT1 and dPOMT2) and two zebrafish (zPOMT1 and zPOMT2)—indicates that zPOMT1 is in the pmt4 subfamily and zPOMT2 is in the pmt2 subfamily (Figure 2C).

Gene expression of zPOMT1 and zPOMT2

Quantitative PCR was performed with early developmental stages (Figure 3A) and all adult tissues (Figure 3B). There

were significant differences in the levels of *zPOMT1* and *zPOMT2* expression during embryogenesis. At 0 h post fertilization (hpf), both genes, *zPOMT1* and *zPOMT2*, were highly expressed. While *zPOMT1* expression decreased after 6 hpf, *zPOMT2* expression increased at 6 hpf and then decreased at 12 hpf. Furthermore, *zPOMT2* expression increased again around 24 hpf. There were no significant differences in *zPOMT1* and *zPOMT2* expression levels in males and females in any adult tissue except for the liver. Interestingly, *zPOMT1* and *zPOMT2* were highly expressed in both testis and ovary (Figure 3B). By means of whole-mount in situ hybridization, mRNAs of *zPOMT1* and *zPOMT2* were detected during early developmental stages. Both *zPOMT1* (Figure 3C) and *zPOMT2* (Figure 3D) transcripts were ubiquitously expressed throughout the gastrulation, tailbud and somite stages. At 24 hpf, both *zPOMT1* and *zPOMT2* mRNAs were detected predominantly in eyes and somites.

Localization of 3′UTRs in zPOMT1 and zPOMT2

The 3′UTR of an mRNA can affect the expression and/or localization of the mRNA during development within particular cells, including primary germ cells (Hashimoto et al. 2009). Therefore, to investigate the function of 3′ UTRs of *zPOMT1* and *zPOMT2*, we fused the 3′UTR of each *zPOMT* to the *enhanced green fluorescent protein (EGFP)* gene (Figure 3E). Capped mRNAs of *EGFP*-3′UTR of *zPOMT1* and *zPOMT2* were synthesized and injected into embryos at the one- to two-cell stage. With both constructs, EGFP was distributed throughout the whole body at 6 hpf, and EGFP was highly expressed in the eye, hindbrain and somite from 18 to 24 hpf (Figure 3F).

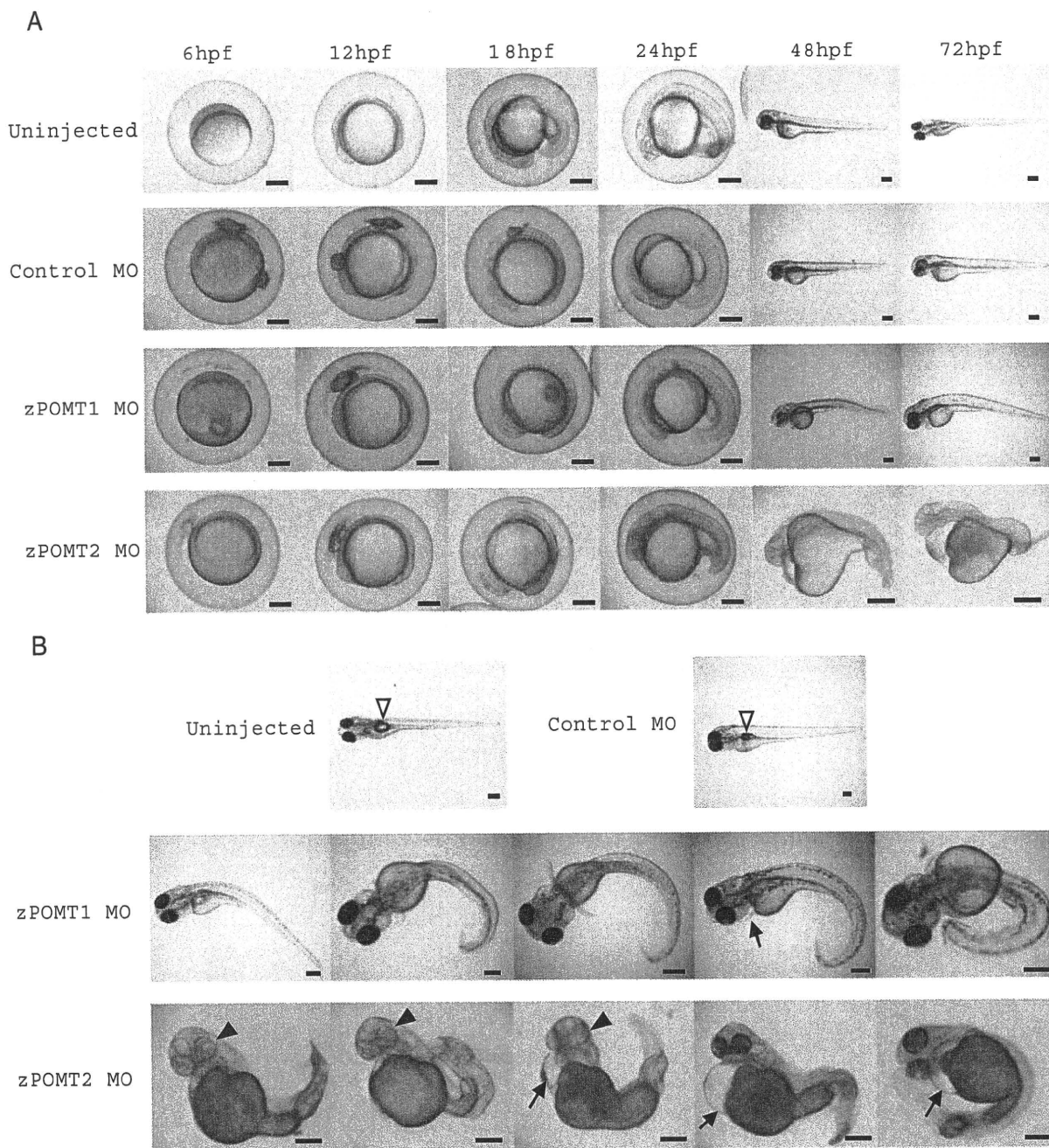### Knockdown analysis of zPOMT1 and zPOMT2

Antisense MOs against *zPOMT1* and *zPOMT2* were injected into the zebrafish embryos at the one- to two-cell stage, and the developmental progress of the morphants was compared with that of uninjected and control MO embryos (Figure 4). There were no significant differences between control MO and morphant embryos until 12 hpf. At 18 hpf, both *zPOMT1* MO and *zPOMT2* MO embryos were developmentally delayed in comparison with control MO (Figure 4A) and uninjected (data not shown) embryos. At 48 and 72 hpf, *zPOMT1* morphants showed slightly curved tails and curvature of the somite boundaries. In contrast, *zPOMT2* morphants at the same stage showed more severe phenotypes—including twisted tails, aberrant pericardium and abnormal eye pigmentation—than did the *zPOMT1* morphants. Quantitative analyses of embryos from all treatment groups were performed at 96 hpf. Each

Fig. 3. Gene expressions and whole-mount in situ hybridization for *zPOMT1* and *zPOMT2*. Quantitative PCR analyses of *zPOMT1* and *zPOMT2* mRNAs during early developmental stages (A) and in adult tissues (B). PCR products of *zPOMT1* and *zPOMT2* were detected throughout early developmental stages and in all tissues predominantly in gonads. *zβ-actin2* and *zCox1* were used as internal controls for quantitative PCR in early developmental stages and adult tissues, respectively. B, brain; E, eye; H, heart ; M, muscle ; T, testis; O, ovary; LM, liver (male); LF, liver (female); IM, intestine (male); IF, intestine (female); KM, kidney (male); KF, kidney (female); SM, spleen (male); SF, spleen (female). All reactions were performed in triplicate, and average values ± SD are shown. *zPOMT1* (C) and *zPOMT2* (D) mRNAs were detected. Both genes were expressed ubiquitously throughout early developmental stage, and high levels of expression were detected at 24 hpf. Arrowheads and boxes shown in the right columns at 24 hpf indicate eyes and the central parts of the somites, respectively. Boxes in 24 hpf were enlarged and shown in the far right panels. Scale bars = 100 μm. Illustration of capped mRNA structure (E) and localization of *EGFP*-3′UTR of control (upper panel), *zPOMT1* (middle panel) and *zPOMT2* (lower panel) (F). Arrows and arrowheads indicate the proteins corresponding to EGFP-*zPOMTs* 3′UTR. At 24 hpf, EGFP-3′UTR of *zPOMT1* was located predominately to the eye (arrowhead), whereas EGFP-3′UTR of *zPOMT2* was expressed highly in eye (arrowhead), hindbrain (arrow), and somite (bracket). Scale bars = 100 μm.

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A

zPomt1

zPomt2

B

zPomt1

zPomt2

C

6hpf 12hpf 18hpf 24hpf 24hpf

D

6hpf 12hpf 18hpf 24hpf 24hpf

E

5'——[EGFP | 3'UTR] AAAAAAA——3'

F

6hpf 12hpf 18hpf 24hpf

Control

3'UTR-zPOMT1

3'UTR-zPOMT2

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embryo with or without MO treatment was categorized as having normal, moderate or severe phenotypes according to morphological characteristics (Table I). The frequency of moderate and severe phenotypes increased with injection of increasing amounts of *zPOMT1* and *zPOMT2* MOs. The *zPOMT2* MO embryos had more severe deformities than did the *zPOMT1* MO embryos (Figure 4B, Table I). For example, aberrant eye pigmentation was observed only in *zPOMT2* MO embryos. Finally, swim bladders were not completely formed in *zPOMT1* and *zPOMT2* morphant embryos in comparison with uninjected or control MO embryos (Figure 4B).

To investigate the glycosylation status of α-DG in *zPOMT1* and *zPOMT2* morphant embryos, we immunostained embryos at 48 hpf with the anti-glycosylated α-DG antibody IIH6 (Figure 4C). Strong signals were detected with IIH6 in untreated embryos. Moreover, IIH6 reactivity in control MO embryos was also detected in the horizontal and vertical myosepta. However, the reactivity was almost completely lost in *zPOMT1* and *zPOMT2* morphants.

C

Heamatoxylin and eosin   IIH6

Untreated

Control MO
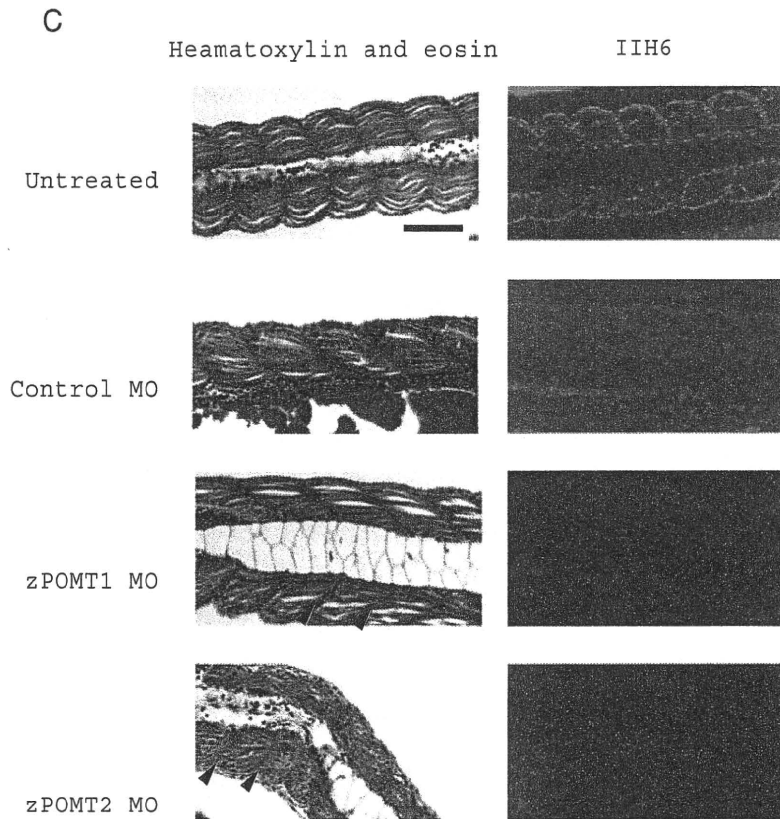
zPOMT1 MO

zPOMT2 MO

Fig. 4. Knockdown analysis of *zPOMT1* and *zPOMT2*. (**A**) Sequential changes during early developmental stages (6–72 hpf) of uninjected embryos (top panel), embryos injected with control MO (middle upper panel), embryos injected with *zPOMT1* MO (middle lower panel) and embryos injected with *zPOMT2* MO (bottom panel). Zebrafish embryos were injected with each MO at the one- to two-cell stage and were observed at 6, 12, 18, 24, 48 and 72 hpf. Scale bars = 200 μm. (**B**) 96 hpf morphants. Top panel: uninjected embryo (left side) and injected control MO (right side), embryos injected *zPOMT1* MO (middle panel), and embryos injected *zPOMT2* MO (bottom panel). Zebrafish embryos were injected with each MO at the one- to two-cell stages and were observed at 96 hpf. White arrowheads indicate swim bladder. Both morphant embryos revealed curved tail, and some had abnormal pericardium (arrows). Some of the *zPOMT2* morphants showed aberrant eye pigmentation (arrowheads). Scale bars = 200 μm. (**C**) Whole-mount immunohistochemistry with anti-glycosylated α-DG antibody IIH6 in 48-hpf embryos. Left panels, hematoxylin and eosin staining; right panels, IIH6 staining. IIH6 immunoreactivity was detected in uninjected and control MO but decreased in *zPOMT1* and *zPOMT2* morphants. Arrowheads represent vertical myosepta. Scale bars = 50 μm.

Protein O-mannosyltransferase activity of zPOMT1 and zPOMT2

To analyze the protein *O*-mannosyltransferase activity of zPOMT1 and zPOMT2, the cDNAs were cloned into an ex-

Table I. Quantification of *zPOMT* morphant phenotypes at 96 hpf

	Concentration (mM)	Normal	Moderate	Severe
Uninjected	-	97 (100.0%)	0 (0%)	0 (0%)
Control MO	1.0	65 (95.6%)	3 (4.4%)	0 (0%)
zPOMT1 MO	1.0	65 (79.3%)	14 (17.1%)	3 (3.6%)
	0.5	72 (93.5%)	4 (5.2%)	1 (1.3%)
	0.25	72 (98.6%)	0 (0%)	1 (1.4%)
zPOMT2 MO	1.0	33 (35.5%)	31 (33.3%)	29 (31.2%)
	0.5	55 (61.1%)	26 (28.9%)	9 (10.0%)
	0.25	79 (86.8%)	5 (5.5%)	7 (7.7%)

The phenotypic data shown were obtained from three independent experiments. The number of embryos observed for each phenotypic class is shown and also presented as a percentage of the total number of embryos studied for each morpholino (MO). Moderate (curved tails and curvature of the somite boundaries); Severe (twisted tail and aberrant pericardium).
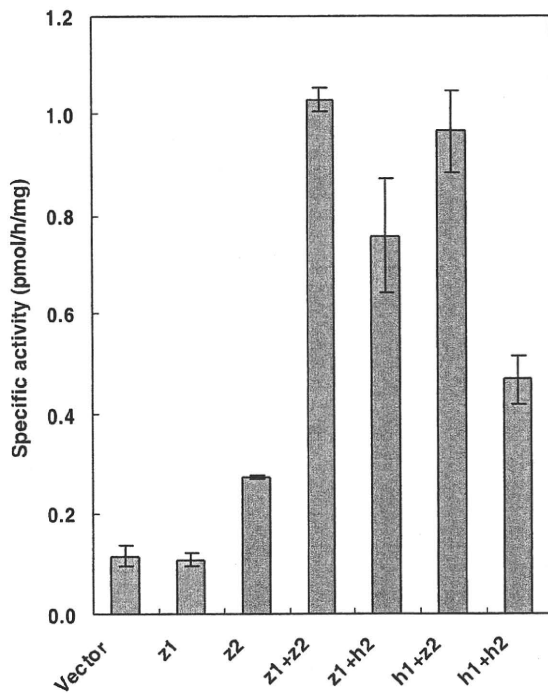
pression vector. The resulting expression constructs were transfected into human embryonic kidney 293T (HEK293T) cells, and microsomal membranes were used for enzyme assay. High levels of protein *O*-mannosyltransferase activity was observed only when the *zPOMT1* and *zPOMT2* genes were co-expressed in HEK293T cells (Figure 5). Jack bean α-mannosidase digestion showed that the mannosyl residue was linked to GST-α-DG by α-linkage (data not shown), as reported previously (Manya et al. 2004). Although a single transfection of *zPOMT1* in HEK293T cells did not show any enzymatic activity, transfection of *zPOMT2* alone did result in low levels of activity. Cells co-transfected with *zPOMT1* and *hPOMT2* and cells co-transfected with *hPOMT1* and *zPOMT2* showed robust levels of *O*-mannosyltransferase activity. In contrast, cells co-transfected with *zPOMT1* and *hPOMT1* and cells co-transfected with *zPOMT2* and *hPOMT2* did not show any enzymatic activity (data not shown). These results indicated that POMT1 and POMT2 from zebrafish and humans are interchangeable and that POMT1 and POMT2 have different functional roles in POMT enzymatic activity.

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Fig. 5. Protein *O*-mannosyltransferase activities of *zPOMTs*. Protein *O*-mannosyltransferase activity was based on the rate of mannose transfer from mannosylphosphoryldolichol to a GST-α-DG. Vector, cells transfected with vector only; z1, cells transfected with *zPOMT1*; z2, cells transfected with *zPOMT2*; z1+z2, cells co-transfected with *zPOMT1* and *zPOMT2*; z1+h2, cells co-transfected with *zPOMT1* and *hPOMT2*; h1+z2, cells co-transfected with hPOMT1 and *zPOMT2*; h1+h2, cells co-transfected with *hPOMT1* and *hPOMT2*. Average values ± SD of three independent experiments are shown.

Discussion

Zebrafish have been useful for the study of human muscular dystrophies and congenital myopathies (Parsons et al. 2002; Bassett and Currie 2003; Bassett et al. 2003; Guyon et al. 2003) because zebrafish have orthologs of genes implicated in human muscular dystrophies, including *POMT1*, *POMT2*, *POMGnT1*, *dystrophin*, *fukutin* and fukutin-related protein (*FKRP*) (Steffen et al. 2007; Moore et al. 2008). Recently, it has been reported that knockdown analysis of *FKRP*, one of the causative genes in α-dystroglycanopathies, resulted in morphants that showed a pathological spectrum similar to those of human muscular dystrophies associated with mutations in *FKRP* (Thornhill et al. 2008). However, the function of FKRP is not clear yet (Esapa et al. 2002; Esapa et al. 2005; Matsumoto et al. 2004; Dolatshad et al. 2005; Torelli et al. 2005; Beedle et al. 2007). In contrast, POMTs are known to be protein *O*-mannosyltransferases. *FKRP* MO and *zPOMTs* morphant embryos showed a reduction in the glycosylated α-DG staining, indicating that FKRP may affect the biosynthetic pathway of *O*-mannosylglycans (Thornhill et al. 2008).

In mammals, two protein *O*-mannosyltransferase (POMTs) family members, POMT1 and POMT2, are known to exist. hPOMT1 and hPOMT2 catalyze protein *O*-mannosyl transfer

to α-DG, which serves as a protein substrate, and mutations in the *hPOMT1* and *hPOMT2* genes result in WWS, a severe muscular dystrophy that also results in structural alterations in eyes and brain malformations, such as cobblestone lissencephaly. In this study, we have identified, cloned and expressed the full-length cDNAs of the *zPOMT1* and *zPOMT2* genes, and our results suggest that high levels of protein *O*-mannosyltransferase activity depends on expression of both genes. Co-expression of both *zPOMT1* and *zPOMT2* genes showed high protein *O*-mannosyltransferase activity similar to results from analyses of hPOMTs. Although transfection of *zPOMT1* alone did not show any enzymatic activity, transfection of *zPOMT2* alone showed slight activity. These results suggest that zPOMT2 itself has enzymatic activity, or zPOMT2 may form a complex with endogenous hPOMT1 resulting in low levels of enzymatic activity. Two heterologous protein combinations, zPOMT1 and hPOMT2 or zPOMT2 and hPOMT1, resulted in robust levels of enzymatic activity (Figure 5). This result suggests that a single mechanism of *O*-mannosylation is common to humans and zebrafish. On the other hand, cells co-transfected with *zPOMT1* and *hPOMT1* and cells co-transfected with *zPOMT2* and *hPOMT2* did not show any enzymatic activity, indicating clearly that POMT1 and POMT2 have different functional roles in POMT enzymatic activity. It is not clear why protein *O*-mannosyltransferase activity requires co-expression of POMT1 and POMT2 (Ichimiya et al. 2004; Manya et al. 2006; Manya et al. 2004; this study). 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. To further understand the mechanism of protein *O*-mannosylation, it is necessary to perform a structural study of a complex formed by POMT1 and POMT2.

Remarkably, overlapping expression patterns of *zPOMT1* and *zPOMT2* were observed by whole-mount in situ hybridization (Figure 3C). Such overlapping pattern suggests that the two proteins may collaborate functionally in vivo. This expression data are consistent with data suggesting that simultaneous expression of zPOMT1 and zPOMT2 is required for POMT enzymatic activity. Interestingly, similar co-expression of *POMTs* was observed in various tissues and during different developmental stages of embryogenesis in mouse and *Drosophila* (Ichimiya et al. 2004; Lyalin et al. 2006; Lommel et al. 2008). These results suggest that both protein *O*-mannosylation machinery and biological importance of protein *O*-mannosylation may have been conserved during metazoan evolution, although further analyses are necessary to understand the molecular mechanisms of protein *O*-mannosylation and its evolution.

The expression levels of the two *POMTs* genes differed at various developmental stages and in specific tissues of mouse, *Drosophila* and zebrafish. For example, while mouse *POMT2* was highly expressed in testis (Willer et al. 2002), *zPOMT1* and *zPOMT2* were highly expressed in ovary and testis (Figure 3B). Since *POMT1* knockout in mice results in embryonic lethality, protein *O*-mannosylation is necessary for normal development (Willer et al. 2004). In the case of *Drosophila POMTs*, the expression level of *dPOMT1* was higher than that of *dPOMT2* from 0 to 2 h in the embryo (Ichimiya et al. 2004), whereas the expression level of *zPOMT2* was higher than that of *zPOMT1* at from 0 to 6 hpf. It was assumed that both *zPOMT1* and *zPOMT2* mRNAs at these stages were derived

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from maternal expression. Furthermore, the expression level of *zPOMT1* decreased from 0 to 6 hpf, whereas *zPOMT2* expression was high from 0 to 6 hpf (Figure 3A). These results suggest that the expression of *zPOMT1* and *zPOMT2* may be regulated differently.

In humans, defects of protein *O*-mannosylation lead to WWS (Manya et al. 2003; Akasaka-Manya et al. 2004). To understand the importance of protein *O*-mannosylation in zebrafish development, we carried out the knockdown analysis of antisense MOs against *zPOMT1* and *zPOMT2*. As a result, *zPOMT1* and *zPOMT2* morphant embryos showed curved tail, and some had edematous pericardium (Figure 4B). Since both *zPOMT1* and *zPOMT2* morphants showed these phenotypic aberrations, they could not swim straight or feed at all, and they eventually died. At 96 hpf, the phenotypes of *zPOMT2* morphant embryos showed a higher incidence of more severe phenotypes than the *zPOMT1* morphant embryos did (Table I), yet immunoreactivity of IIH6 in *zPOMT1* and *zPOMT2* morphants was similar (Figure 4C). We predicted that the phenotypes of zebrafish embryos injected with MOs against *zPOMT1* and *zPOMT2* would be the same or similar because the expression patterns of the two genes were similar before 24 hpf (Figure 3). Therefore, the difference of phenotypes observed between *zPOMT1* and *zPOMT2* morphants in early development of the zebrafish might be explained by the variance of knockdown efficiency or by another function of zPOMT2 in addition to enzymatic activity. It may be consistent with severe phenotypes observed in *zPOMT2* embryos that only *zPOMT2* morphant embryos showed aberrant pigmentation in eyes. Fukutin-deficient chimeric mice revealed abnormalities in eyes, indicating that corneal opacification with vascular infiltration and eye abnormality was quite remarkable according to their aberrant pigmentation (Takeda et al. 2003). Further studies are necessary to reveal the role of *O*-mannosylglycans in the pathogenesis of eye abnormalities.

Here, we demonstrated that zebrafish POMTs possess protein *O*-mannosyltransferase activity when co-expressed in HEK293T cells. This result suggested that the protein *O*-mannosylation machinery is conserved in mammals and zebrafish. Therefore, to elucidate whether the function and mechanism of protein *O*-mannosylation related to *POMTs* are evolutionarily conserved in the vertebrates, the zebrafish should be a useful model. It was also suggested that the zebrafish may be a useful model for understanding the functions of glycans in the whole body. In the knockdown analyses of *zPOMT1* and *zPOMT2* by MOs, no glycosylated α-DG was detected in 48 hpf embryos (Figure 4C). Therefore, it appears that the enzymatic activity was completely lost. Furthermore, zebrafish α-dystroglycanopathy models may be useful to search for chemicals that treat α-dystroglycanopathies; the simple addition of candidate molecules to water could be developed as assays for theraputic effectiveness.

Materials and methods
Zebrafish and embryos
Zebrafish adults were maintained at 28°C under light condition of 14 h light period and 10 h dark period. Embryos were collected from pair-wise mating of adults and kept in filter-sterilized fresh water at 28°C.

Cloning and sequencing of the full-length cDNAs
Total RNAs were purified from 24 and 48 hpf embryos by using QIAzol (Qiagen, Hilden, Germany), and cDNA fragments were generated by RT-PCR using oligo dT primer and Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA). Degenerated oligonucleotide primers were designed by mRNA sequence of zebrafish POMT1 (zPOMT1) (accession no. NM_001048067.2). zPOMT2 gene (accession no. AB281276) was cloned in a zebrafish embryonic cDNA library that was synthesized with a SMART cDNA Library Construction Kit (Clontech, Mountain View, CA). Both zPOMT1 and zPOMT2 genes were amplified with the forward primers, 5′-atgcagtgtgt-taaactgcccgtcagtgt-3′ and 5′-atggatgtcagaccgaaggagaatttc-3′, and the reverse primers, 5′-ttagcgtttgcgtaagagaatatcccaactctc-3′ and 5′-ctaaaactcccaggattccatccacc-3′, respectively. The amplified cDNA fragments were cloned into pT7Blue vector (Novagen, Madison, WI), and the sequences were confirmed by CEQ™ 2000 DNA Analysis System (Beckman Coulter, Inc., Fullerton, CA) with a DTCS Quick Start kit (Beckman Coulter, Inc.). The nucleotide sequence was subjected to the basic local alignment with a BLAST search provided by the National Center for Biotechnology Information. The sequences were obtained from GenBank and aligned using CLUSTAL W (Thompson et al. 1994). A phylogenetic tree was generated using the neighbor-joining method. TREE-VIEW software generated visual representations of clusters (Page 1996).

Quantitative PCR analyses
Total RNA was extracted from embryos at 0, 6, 12, 18, 24, 48, 72 and 96 hpf and the tissue samples (brain, heart, liver, kidney, spleen, intestine, muscle, testis and ovary) of either male or female adult zebrafish. One microgram of total RNA was used for cDNA synthesis. First-strand cDNA was synthesized as described in the section of *cDNA cloning and sequencing of zPOMT1 and zPOMT2*. Quantitative PCR was carried out with SYBR Green Realtime PCR Master Mix (TOYOBO Co. LTD., Osaka, Japan). Two microliters of cDNA (0.1 μg/μL) was used for a template. The primers used to detect the messages of *zPOMT1* and *zPOMT2* were 5′-tgttggctgtgctgtcttacc-3′ (forward) and 5′-catggctcaaggttc-gatctc-3′ (reverse), 5′-cctcatgtatgttgggatgagac-3′ (forward) and 5′-gaaccaagagcagcacagaac-3′ (reverse), respectively. The primers for *zβ-actin2* and *zCox1*—5′-agttcagccatggatgatgaaa-3′ (forward) and 5′-accatgacaccctgatgtct-3′ (reverse), 5′-ttggccacccagaagtctac-3′ (forward) and 5′-gctcgggtgtctacatc-cat-3′ (reverse), respectively—were used as internal controls. Annealing temperatures were 63°C for *zPOMT1* and *zPOMT2*, 52°C for *zβ-actin2* and 54°C for *zCox1*. Melting curves were calibrated by LineGene (NIPPON Genetics Co. LTD., Tokyo, Japan).

Whole-mount in situ hybridization
Antisense probe synthesis was performed using a Digoxigenin (DIG) RNA Labeling Kit (Roche Diagnostics, Basel, Switzerland). Zebrafish embryos were collected after spawning and maintained at 28°C. Embryos at 0, 6, 12, 18, 24, 48, 72 and 96 hpf were fixed with 4% paraformaldehyde (PFA)-phosphate-buffered saline (PBS), dehydrated and kept in methanol

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at −80°C. The embryos were rehydrated in PBS, and hybridization of a DIG-labeled RNA probe was carried out in a hybridization buffer containing 50% formamide, 25% 20×SSC, 1% Tween20, 9 mM citric acid and 1 mg/ml heparin at 65°C for 16 h. Embryos hybridized with a DIG-labeled RNA were incubated with anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics) (1:2000) at 4°C for 12 h, stained with BM purple (Roche Diagnostics) at room temperature for 2 h and fixed with 4% PFA-PBS. The embryos were observed using ECLIPSE E600 (Nikon Corp., Tokyo, Japan).

Localization of 3′UTRs in zPOMT1 and zPOMT2 genes

The *EGFP* gene was cloned into pSP72 vector (Promega, Madison, WI). The3′UTRs of *zPOMT1* and *zPOMT2* were fused separately downstream of *EGFP* gene. The 3′UTR of zebrafish *NUDT2* was used as a control. Capped mRNAs of *EGFP-zPOMTs-3′UTR* were synthesized using mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (SP6, Ambion Inc., Austin, TX). Capped mRNAs at a concentration of 0.05 µg/µL were injected into fertilized eggs from one to two cells. The embryos were observed until 24 hpf using ECLIPSE E600 and a mercury lamp (Nikon).

Knockdown analysis of zPOMT1 and zPOMT2

Antisense MOs targeted to interfere with *zPOMT1* and *zPOMT2* translation were purchased from Gene Tools LLC (Philomath, OR). The antisense sequences of *zPOMT1* and *zPOMT2* genes were designed using the 50 sequence around the putative start of translation of *zPOMT1* and *zPOMT2* mRNA (accession nos. AB281275 and AB281276). The morpholino sequences were *zPOMT1-MO*: 5′-gacgggcagtttaacacactgcatg-3′ and *zPOMT2-MO*: 5′-gtccattcttgaagatgaagaggac-3′. The sequence of control MO was 5′-gtacgtcacacaatttgacgggcag-3′. MOs at a concentration of 0.25, 0.5 or 1.0 mM were injected into embryos at the one- to two-cell stage.

Immunohistochemistry

For immunohistochemistry, embryos were fixed overnight in 4% paraformaldehyde solution, embedded in paraffin and sectioned at 10 µm and mounted on slides. Sections were left to dry for 2 h. After being dewaxed and rehydrated, some sections were stained with hematoxylin and eosin, while others were subjected to immunohistostaining as described in (Mulero et al. 2007). Anti-glycosylated α-DG IIH6 (Upstate, Millipore, Billerica, MA) was used at a dilution of 1:100 with PBS. Slides were washed 3× 10 min with PBS containing 0.1% Tween (PBSTw) and incubated with secondary antibody for 1 h. The secondary antibody used was Alexa Fluor 488 Goat Anti-Mouse IgM (Molecular Probes Invitrogen Life Technologoies Corp., Tokyo, Japan) at a dilution of 1:500 with PBS. Embryos were fixed in 4% PFA-PBS and transferred into 100% methanol. The embryos were rehydrated in PBS containing 0.1% Tween-20 (PBT) and incubated in PBT containing anti-glycosylated α-DG antibody (IIH6) overnight at 4°C followed by several washes with PBT and incubation with secondary antibody (goat anti-mouse IgM AlexaFluor-488,). The embryos were observed using ECLIPSE E600 and a mercury lamp (Nikon).

Assay for protein O-mannosyltransferase activity

Expression plasmids of zPOMT1 and zPOMT2 were constructed using pcDNA3.1 Hygro (+) vector (Life Technologoies Corp., Tokyo, Japan) and confirmed by the sequencing. The expression plasmids were transfected into HEK293T cells, and the cells were cultured for 3 days in complete medium, harvested, and homogenized. Protein O-mannosyltransferase activity was based on the amount of [^{3}H]-mannose transferred from [^{3}H]-mannosylphosphoryldolichol to a glutathione-S-transferase fusion α-DG (GST-α-DG) as described previously (Manya et al. 2004). Approximately 80 µg of microsomal membrane fractions were collected from HEK293T cells coexpressing combinations of *POMT1* and/or *POMT2* genes from either human or zebrafish, suspended in a 20-µL reaction buffer containing 10 µg of GST-α-DG. The reaction mixture was incubated at 22°C for 1 h, and GST-α-DG was purified using glutathione-Sepharose 4B beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The radioactivity adsorbed to the beads was measured by using liquid scintillation counter.

Abbreviations

α-DG, α-dystroglycan; DGC, dystrophin–glycoprotein complex; DIG, Digoxigenin; *EGFP, enhanced green fluorescent protein*; FKRP, fukutin-related protein; HEK293T cells, human embryonic kidney 293T cells; MO, morpholino oligonucleotide; ORF, open reading frame; PBS phosphate-buffered saline; PBT PBS containing 0.1% Tween-20; PFA paraformaldehyde; POMGnT1, protein O-mannose β1,2-N-acetylglucosaminyltransferase1; POMT, PMT, protein O-mannosyltransferase; RT-PCR, reverse transcriptase-polymease chain reaction; SD, standard deviation; WWS, Walker–Warburg syndrome.

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