The Twisted Abdomen Phenotype of Drosophila POMT1 and POMT2 Mutants Coincides with Their Heterophilic Protein O-Mannosyltransferase Activity*

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Walker-Warburg syndrome, caused by mutations in protein O-mannosyltransferase-1 (POMT1), is an autosomal recessive disorder characterized by severe brain malformation, muscular dystrophy, and structural eye abnormalities. As humans have a second POMT, POMT2, we cloned each Drosophila ortholog of the human POMT genes and carried out RNA interference (RNAi) knockdown to investigate the function of these proteins in vivo. Drosophila POMT2 (dPOMT2) RNAi mutant flies showed a “twisted abdomen phenotype,” in which the abdomen is twisted 30–60°, similar to the dPOMT1 mutant. Moreover, dPOMT2 interacted genetically with dPOMT1, suggesting that the dPOMTs function in collaboration with each other in vivo. We expressed dPOMTs in S21 cells and measured POMT activity. dPOMT2 transferred a mannose to the dystroglycan protein only when it was coexpressed with dPOMT1. Likewise, dPOMT1 showed POMT activity only when coexpressed with dPOMT2, and neither dPOMT showed any activity by itself. Each dPOMT RNAi fly totally reduced POMT activity, despite the specific reduction in the level of each dPOMT mRNA. The expression pattern of dPOMT2 mRNA was found to be similar to that of dPOMT1 mRNA using whole mount in situ hybridization. These results demonstrate that the two dPOMTs function as a protein O-mannosyltransferase in association with each other, in vitro and in vivo, to generate and maintain normal muscle development.

O-Mannosylation is an important modification of proteins in various fundamental physiological processes. In the yeast Saccharomyces cerevisiae, O-linked oligomannose chains are required for the stability, correct localization, and/or function of proteins (1–6). Yeast O-mannosylation is initiated in the lumen of the endoplasmic reticulum by a family of protein O-mannosyltransferases, PMT1–PMT6,1 which catalyze the transfer of Man from dolichylphosphate Man to Ser or Thr residues of secretory proteins (7–9). The PMT family is classified phylogenetically into the PMT1, PMT2, and PMT4 subfamilies. The members of the PMT1 subfamily interact heterophilically with those of the PMT2 subfamily, whereas the single member of the PMT4 subfamily acts as a homophilic complex (7).

Protein O-mannosyltransferase homologs have been identified in many multicellular eukaryotes such as Drosophila melanogaster, mouse, and human (10–12). There are two human protein O-mannosyltransferase (POMT) homologs, hPOMT1 and hPOMT2, which belong to the POMT4 and POMT2 subfamilies, respectively (11). Mutations in the hPOMT1 gene give rise to the severe neuronal migration disorder, Walker-Warburg syndrome (12). Walker-Warburg syndrome is a recessive autosomal disorder characterized by congenital muscular dystrophy, severe brain malformation, and structural eye abnormalities. Muscle-eye-brain disease is also a recessive autosomal disorder characterized by congenital muscular dystrophy, brain malformation, and ocular abnormalities. Muscle-eye-brain disease is caused by mutations in the gene encoding UDP-N-acetylglucosamine:protein O-mannosyl-2,3-aminosaminyltransferase-1 (POMGnT1), contributing to the synthesis of the O-mannosylglycan, Siaα2–3Galβ1–4GlcnAcβ1–2Manα1–Ser/Thr (13–15). It is a laminin-binding ligand of α-dystroglycan (α-DG) (16, 17). These findings indicate that the O-mannosylation of proteins plays an important role in vivo in making and/or maintaining neuronal and muscular tissues. Most recently, hPOMT1 and hPOMT2 were shown to have POMT activity corresponding to the first step in O-mannosylglycan synthesis only when coexpressed with each other (18). Drosophila has two POMT orthologs, dPOMT1 and dPOMT2, which correspond to human hPOMT1 and hPOMT2, respectively (11). The dPOMT1 mutants are known to have reduced viability, whereas escaper flies show the so-called twisted abdomen phenotype that is caused by pronounced de-

1 The abbreviations used are: PMT and POMT, protein O-mannosyltransferase; h, human; d, Drosophila; POMGnT1, protein O-mannose β1,2,3-acetylglucosaminyltransferase; Sia, sialic acid; α-DG, α-dystroglycan; RNAi, RNA interference; EST, expressed sequence tag; MGAT, mannose β1,2,3-acetylglucosaminyltransferase; IR, inverted repeat; UAS, upstream activating sequence; RpL32, ribosomal protein L32; ORF, open reading frame; HA, hemagglutinin; GST, glutathione S-transferase; MES, 2-morpholinooethanesulfonic acid; PA, pyridylamine.

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fcts in muscle development (10). The dPOMT1 gene was named after this phenotype as rotated abdomen (rt) (19). On the other hand, mutants of the dPOMT2 gene have not yet been isolated, and no biochemical report has documented the activities of both dPOMTs.

In this study, we report the production of mutant flies by RNA interference (RNAi) of two Drosophila POMT genes, dPOMT1 and dPOMT2. Both of the RNAi mutant flies showed the same rt phenotype as classical dPOMT1 mutants. Furthermore, genetic interaction analysis revealed a synergistic effect between these two mutations, suggesting that the two gene products function in the same genetic cascade. We also performed biochemical analyses to demonstrate that dPOMT1 functions as protein O-mannosyltransferase in association with each other. Reduction of in vivo POMT activity in each mutant fly also supports the heterophilic nature of these two enzymes. These data indicate that both dPOMT1 and dPOMT2 are required for functional POMT activity to contribute to normal muscle development in vivo.

EXPERIMENTAL PROCEDURES

Materials—The Drosophila expressed sequence tag (EST) clones RE38203 (dPOMT1), LP01681 (dPOMT2), LD43357 (dMGT1), and GH05379 (dMGT2) were obtained from Research Genetics (Huntsville, AL). Dolichylphosphate [3H]Man (125,000 dpm/pmol) and UDP-[3H]GlcNAc (400,000 dpm/nmol) were supplied by American Radiolabeled Chemicals, Inc. (St. Louis, MO) and PerkinElmer Life Sciences, respectively.

dPOMT1 and dPOMT2 RNAi Mutant Flies—The cDNA fragments corresponding to the N-terminal region (nucleotides 67–566 of the coding sequence) of dPOMT1 and the C-terminal region (nucleotides 792–1289) of dPOMT2 were amplified by PCR from EST clones RE38203 and LP01681, respectively, and inserted as an inverted repeat (IR) in a modified Bluescript vector, pSC1. IR-containing fragments were intro-duced into BL21(DE3) was cultured to an OD600 of 0.6 at 37 °C and maintained in 0.5 mM isopropyl-β-D-thiogalactopyranoside at 20 °C for 18 h. The cells were sonicated and centrifuged, and then the supernatant was applied to glutathione-Sepharose 4B beads (Amersham Biosciences). Eluted GST-fused dPOMT2 protein was injected into a New Zealand White rabbit. After four booster injections, the antiserum was used for Western blot analysis.

Preparation of Cellular Microsomal Membrane Fraction and Larval Extracts—The infected cells were homogenized in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, and 1 mM dithiothreitol with a protease inhibitor mixture (3 mM benzamidine-HCl, 1 mM leupeptin, 1 mM benzamidine-ethylacrylate, and 1 mM phenylmethylsulfonyl). After centrifugation at 900 × g for 10 min, the supernatant was subjected to ultracentrifugation at 100,000 × g for 1 h. The precipitate was used as the microsomal membrane fraction. Act5C-GAL4/Act5C-GAL4/dPOMT1-IR flies and Act5C-GAL4/Act5C-GAL4/dPOMT2-IR flies were raised at 25 and 28 °C, respectively. The larval larvae were homogenized in 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% n-octyl-β-D-thiogalactoside (Dojindo Laboratories, Kumamoto, Japan) with the protease inhibitor mixture (400 μl for every 20 larvae). The supernatant was obtained by ultracentrifugation at 100,000 × g for 1 h and used as larval extract. The protein concentration was determined by BCA assay.

Assay of POMT Activity—The POMT activity was based on the amount of [3H]Man transferred to GST-α-D as described previously (18). Briefly, the reaction mixture contained 20 mM Tris-HCl (pH 8.0), 100 mM dolichylphosphate [3H]Man (125,000 dpm/pmol), 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% n-octyl-β-D-thiogalactoside, 10 μg of each dPOMT1-IR and dPOMT2-IR proteins, and 0.1 μg enzyme source, nUSA. The reaction mixture was centrifuged at 10,000 g for 1 h, and the supernatant was applied to glutathione-Sepharose 4B beads (Amersham Biosciences). The radioactivity adsorbed by the beads was measured using a liquid scintillation counter.

α-Mannosidase Digestion of Mannosyl-GST-α-D—To characterize the linkage of the mannosyl residue, the radioactive products absorbed to the glutathione-Sepharose 4B beads were incubated with jack bean α-mannosidase (0.8 units; Seikagaku Corp., Tokyo) in 50 μl of 0.1 M ammonium acetate buffer (pH 4.5) containing 1 mM ZnCl2 at 37 °C.
Jack bean α-mannosidase (0.8 units) was added freshly every 20 h and was incubated for up to 60 h. Inactivated jack bean α-mannosidase, prepared by heating the enzyme at 100 °C for 5 min, was used as a control. After incubation, the radioactivities of the supernatant and beads were measured using a liquid scintillation counter.

Whole Mount in Situ Hybridization—The BglII-EcoRI-digested fragment of dPOMT1 and the Ecl136II-NotI-digested fragment of dPOMT2 were subcloned into pBluescript SK (Stratagene) digested by BamHI-EcoRI and SmaI-NotI, respectively. The above templates were linearized and transcribed in vitro by T3 or T7 RNA polymerase with a digoxigenin RNA labeling mixture (Roche Applied Science). Each transcript was treated with an alkaline solution containing 80 mM NaHCO₃, 120 mM Na₂CO₃, and 10 mM dithiothreitol for reduction to 300 bases as a digested RNA probe. Fixed CantonS embryos were hybridized overnight at 55 °C with the digoxigenin-labeled probes in 50% formamide, 5/1 SSC (150 mM NaCl and 15 mM sodium citrate), 100 μg/ml heparin, 0.1% Tween 20, 20 μg/ml yeast RNA, 20 μg/ml heat-denatured salmon sperm DNA, and 10 μM dithiothreitol. After hybridization, the embryos were washed with 50% formamide, 5/1 SSC, and 0.1% Tween 20 for 20 min. The process of washing was continued by serial dilution from 50% formamide, 5/1 SSC, and 0.1% Tween 20 to phosphate-buffered saline containing 0.1% Tween 20. Detection was carried out by immunnoassay using an alkaline phosphatase-conjugated anti-digoxigenin antibody Fab' fragment (Roche Applied Science).

Construction and Purification of dMGAT1 and dMGAT2 Proteins—The ORFs of dMGAT1 and dMGAT2 were expressed in Sf21 insect cells by Gateway™ cloning technology as described above. The cDNA fragments of dMGAT1 and dMGAT2 were amplified from EST clones LD43357 and GH07804 using the primer set of dMGAT1 (forward primer, 5’-AAAAAGCAGGCTTCCATACGAGCCGGCATCAG-3’; and reverse primer, 5’-AGAAAGCTGGGTTACTCTGTCCTTAGCGTCGT-3’), and the primer set of dMGAT2 (forward primer, 5’-AAAAAGCAGGCTCCACCCTGCACAAGTATCTG-3’; and reverse primer, 5’-AGAAAGCTGGGTGCCTTACCTCGTGGCCAG-3’), respectively. The fragments amplified by two-step PCR were recombined with the pDONR201™ vector, and the inserts were transferred to yield pVL-FLAG-dMGAT1 and pVL-FLAG-dMGAT2, respectively. pVL-FLAG is an expression vector derived from pVL1393 containing the signal sequence of human immunoglobulin (MHFQVQIFSFLLISASVIMSRG) and the FLAG peptide (DYKDDDDK) at the N terminus. pVL-FLAG-dMGAT1 and pVL-FLAG-dMGAT2 were transfected into Sf21 cells using the same method as described above. The culture medium of each infected cell containing FLAG-dMGAT1 or FLAG-dMGAT2 recombinant protein was applied to anti-FLAG antibody M1 affinity gel FIG.1 .
The purified proteins were quantified by Western blotting using anti-FLAG monoclonal antibody as a standard of FLAG-BAP™ control protein (Sigma).

**Assay for POMGnT Activity**—The level of POMGnT activity was based on the amount of [3H]GlcNAc transferred to a mannosylpeptide (Ac-Ala-Ala-Pro-(Thr/Man)-Pro-Val-Ala-Ala-Pro-NH2) as described pre-
The abdomen phenotype, twisted clockwise 30°

The H9252 RNAi fly (UAS-dPOMT1-IR RNAi mutant flies. dPOMT2 promoter, a target of GAL4. In the F1 progeny of these flies, the has a transgene containing an IR of the target gene ligated to the UAS sylpeptide, 10 mM MnCl2, 2% Triton X-100, 5 mM AMP, 200 mM GlcNAc, /H9262 X-100, and recombinant enzyme in a total volume of 20 /H9262 GlcNAc, 5 mM AMP, 0.2% bovine serum albumin, 20 m M MnCl2,1m M /H9262 previously (20). Briefly, the reaction mixture contained 140 m M MES (pH 6.0) 100 mM GlcNAc, 5 mM AMP, 50 µM of microsomal membrane fraction in a total volume of 50 µl. After a 2-h incubation at 37 °C the peptide was eluted at a flow rate of 1 ml/min using a linear gradient of 1–25% solvent B. The peptide separation was monitored continuously at 214 nm, and the radioactivity of each fraction was measured using a liquid scintillation counter.

Assay for β1,2-N-acetylglucosaminyltransferase I and II Activities—
The β1,2-N-acetylglucosaminyltransferase I activity was measured as follows. The reaction mixture contained 100 mM MES (pH 6.0), 100 mM GlcNac, 5 mM AMP, 0.2% bovine serum albumin, 20 mM MnCl2, 1 mM UDP-GlcNAc, 10 µM pyridylaminated oligosaccharide, 0.5% Triton X-100, and recombinant enzyme in a total volume of 20 µl. The pyridylaminated acceptor oligosaccharide Manα1–6(Manα1–3)Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc-PA (PA100.2) was obtained from Seikagaku (Otsu, Japan). After a 2-h incubation at 37 °C, the product was separated on a Cosmosil 5C18-AR column (4.6 × 250 mm; Wako Pure Chemical Industries, Osaka). Solvent A was 0.1% trifluoroacetic acid in distilled water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The peptide was eluted at a flow rate of 1 ml/min using a linear gradient of 1–25% solvent B. The peptide separation was monitored continuously at 214 nm, and the radioactivity of each fraction was measured using a liquid scintillation counter.

RESULTS

Comparison of the PMT Family of Protein O-Mannosyltransferases—We performed a BLAST search of all Drosophila data bases using hPOMT1 and hPOMT2 as queries and obtained two Drosophila PMT genes, dPOMT1 and dPOMT2, whose Drosophila EST clones are RE38203 and LP01681, respectively. RE38203 contains a 2658-bp ORF encoding a dPOMT1 transcript of 886 amino acids (GenBankTM/EBI accession number AB176550), and LP01681 contains a 2295-bp ORF encoding a dPOMT2 transcript of 765 amino acids (GenBankTM/EBI accession number AB176551) (11). A ClustalW alignment of dPOMT1 and dPOMT2 showed 42 and 52% homology to hPOMT1 and hPOMT2, respectively (Fig. 1, A and C). Hydrophobicity analyses of the amino acid sequences revealed that dPOMT1 and dPOMT2 are type III transmembrane proteins.
with nine and seven transmembrane domains, respectively (Fig. 1, B and D).

The PMT family of protein O-mannosyltransferases was vastly conserved from yeast to human in eukaryotes and is classified into three subfamilies, PMT1, PMT2, and PMT4. A phylogenetic tree of the representative PMT families, six S. cerevisiae Pmt proteins (Pmt1–Pmt6), two human POMTs (hPOMT1 and hPOMT2), and two Drosophila POMTs (dPOMT1 and dPOMT2), indicates that dPOMT1 and hPOMT1 are in the PMT4 subfamily and that dPOMT2 and hPOMT2 are in the PMT2 subfamily (Fig. 1E). It is characteristic that only a few members of the PMT families are found in invertebrates and vertebrates.

**dPOMT1 and dPOMT2 RNAi Mutant Flies**—To obtain information about the function of dPOMT1 and dPOMT2 in vivo, we tried to make RNAi mutant flies using the GAL4-UAS-IR system. The Act5C-GAL4 fly has a transgene containing the yeast transcription factor GAL4, the expression of which is under the control of the cytoplasmic actin promoter. We used Act5C-GAL4 as a GAL4 driver fly to induce dPOMT1 and dPOMT2 gene silencing in all cells and at all developmental stages of the fly. The Act5C-GAL4/UAS-dPOMT1-IR fly showed a viability of 19% at 25 °C, but 0% at 28 °C. RNAi knockdown is more effective at 28 °C because of the temperature dependence of the GAL4-UAS expression system. Meanwhile, the Act5C-GAL4/UAS-dPOMT2-IR fly revealed a viability of 63% even at 28 °C. F1 escapers of Act5C-GAL4/UAS-dPOMT1-IR showed a clockwise twisted abdomen phenotype, after which the gene was named rotated abdomen (rτ). Interestingly, all escaper flies of Act5C-GAL4/UAS-dPOMT2-IR also exhibited the same phenotype, suggesting that these two PMT genes function with strong interaction in muscle development in the fly (Fig. 2).

The transcript levels of third instar larvae of each inducible dPOMT1 and dPOMT2 RNAi fly were determined by real-time PCR. The dPOMT1 transcript level of the Act5C-GAL4/UAS-dPOMT1-IR RNAi fly raised at 25 °C was 32% of that of the Act5C-GAL4/+ control fly (Fig. 3A). The dPOMT2 transcript level of the Act5C-GAL4/UAS-dPOMT2-IR fly raised at 28 °C was 21% (Fig. 3B). However, in each of these two mutant flies, the expression of non-targeted genes was not influenced at all (Fig. 3, A and B). These results, indicating that expression of each gene was specifically suppressed by RNAi, led to the conclusion that the same twisted abdomen phenotype of these mutants was caused by the reduction in each transcript. It was revealed that the transcript level of dPOMT2 was more reduced by RNAi, whereas the mutant fly showed less lethality compared with that of dPOMT1. This discrepancy might come from their independent and unknown functions in development.

**Genetic Interaction between dPOMT1 and dPOMT2**—The same phenotype of dPOMT1 and dPOMT2 RNAi flies suggests an intimate genetic interaction in muscle development. If so, the double mutant of dPOMT1 and dPOMT2 should have a synergistic effect on the phenotype. To test this possibility, first, we combined one copy of the dPOMT1 mutation with the dPOMT2 RNAi mutant allele. To do this, the Act5C-GAL4/SM1;UAS-dPOMT2-IR/TM6B fly was crossed with the +/+; rτ/TM3 fly. Progenies of this cross were reared at 18 °C. RNAi knockdown is so weak at this temperature that the Act5C-GAL4/+;UAS-dPOMT2-IR/TM3 fly showed no aberrant phenotype (Fig. 4C). Also, one copy of the dPOMT1 mutation (rτ) gave no twisted abdomen phenotype because of the recessive character of this allele (Fig. 4B). However, the resulting Act5C-GAL4+/rτ/UAS-dPOMT2-IR fly showed a clear twisted abdomen phenotype (Fig. 4A), indicating that one copy of dPOMT1 enhances the dPOMT2 phenotype. Second, we conducted a cross to make a dPOMT1 and dPOMT2 double mutant by RNAi. Each of the dPOMT1 and dPOMT2 RNAi flies survived to adulthood and showed the twisted abdomen phenotype at 25 °C; however, the double mutant fly did not emerge, showing complete lethality (data not shown). These genetic interactions between dPOMT1 and dPOMT2 indicate an intimate interaction between dPOMT1 and dPOMT2 in muscle development.

**POMT Activity of dPOMT1 and dPOMT2**—We prepared recombinant dPOMT1 and dPOMT2 to identify POMT activity. pVL1393-dPOMT1-1 HA and/or pVL1393-dPOMT2 was cotransfected with BaculoGold viral DNA into Sf21 insect cells, and microsomal membrane fractions were collected from each infected cell. The specific expression of recombinant proteins was confirmed by Western analysis using anti-HA monoclonal antibody and anti-dPOMT2 antibody (Fig. 5, A and B). POMT activity toward GST-α-DG was measured using each microsomal membrane fraction as described under “Experimental Procedures.” Whereas there was no enzyme activity when either dPOMT1 or dPOMT2 was expressed independently, POMT-specific activity appeared when dPOMT1 and dPOMT2 were coexpressed (Fig. 5C). We then characterized the linkage of the mannose residue to α-DG by α-mannosidase digestion (Fig. 5D). The radioactive reaction product of dPOMT1 and dPOMT2 absorbed to glutathione-Sepharose 4B beads released the radioactivity to the supernatant, indicating that a mannose residue is linked to Ser/Thr in GST-α-DG by α-O-glycosidic linkage. Recently, POMT activity was demonstrated in human POMTs, and coexpression of hPOMT1 and hPOMT2 was shown to be indispensable for enzyme activity (18). The above result also demonstrated that coexpression of dPOMT1 and dPOMT2 is an essential factor for revealing their activities.
Furthermore, we measured POMT activity toward GST-α-DG in third instar larval extracts of dPOMT1 and dPOMT2 RNAi flies to test whether enzyme activity is affected by the reduction of each transcript in the mutants. Both Act5C-GAL4/UAS-dPOMT1-IR flies raised at 25°C and Act5C-GAL4/UAS-dPOMT2-IR flies raised at 28°C showed a decrease in POMT activity to 40 and 12%, respectively, compared with the Act5C-GAL4/H9252 control fly in proportion to the reduction in each transcript level (Fig. 5E). These results also support that dPOMT1 and dPOMT2 works as protein O-mannosyltransferases interacting with each other in vitro and in vivo.

Expression Patterns of dPOMT1 and dPOMT2 mRNAs—We investigated the expression patterns of dPOMT1 and dPOMT2 mRNAs in vivo using whole mount in situ hybridization. In stage 10 embryos, each of the dPOMT1 and dPOMT2 antisense probes stained almost all cells weakly but steadily, whereas the germ band and invaginating gut showed a more intense signal (Fig. 6, A and B). The dPOMT2 sense probe gave no signal (Fig. 6C). The similarity in their staining patterns indicates that dPOMT1 and dPOMT2 are coexpressed in vivo. Next, the developmental expression profiles of dPOMT1 and dPOMT2 mRNAs were obtained by quantitative analysis using real-time PCR. Whereas dPOMT1 mRNA was highly expressed in 0–2 h, suggesting a strong maternal expression, dPOMT2 mRNA was highly expressed in the zygotic stage after 4 h (Fig. 6D). In the early developmental stage, dPOMT1 may perform other functions alone.

Examination of the POMGnT Activity of dMGAT1 and dMGAT2—The mammalian extended O-mannosylglycan on α-DG, Siaβ2–3Galβ1–4GlcNAcβ1–2Manα1-Ser/Thr, is known as a laminin-binding ligand (16, 17). hPOMGnT1 is the enzyme responsible for the first step of its elongation (13–15). To determine whether the extended O-mannosylglycans are present in Drosophila or not, we ran a BLAST search of all Drosophila
were divided by that of the RpL32 script for normalization.

dPOMT1
tative analysis of dPOMT1 mRNAs at various developmental stages by real-time PCR. Black bars, dPOMT1; gray bars, dPOMT2. The actual amounts of dPOMT1 and dPOMT2 transcripts were divided by that of the RpL32 tran-
script for normalization.

FIG. 6. Expression of dPOMT1 and dPOMT2 mRNAs in vivo. A–C, whole mount in situ hybridization at stage 10 with digoxigenin-labeled RNA probes: antisense dPOMT1, antisense dPOMT2, and sense dPOMT2, respectively. The staining carried out using the antisense dPOMT1 and antisense dPOMT2 probes gave a very similar pattern in that the germ band and invaginating gut (asterisks) were remarkably stained. D, quantify-
tative analysis of dPOMT1 and dPOMT2 mRNAs at various developmental stages by real-time PCR. Black bars, dPOMT1; gray bars, dPOMT2. The actual amounts of dPOMT1 and dPOMT2 transcripts were divided by that of the RpL32 tran-
script for normalization.

data bases using hPOMGnT1 as a query. Nevertheless, we could not obtain Drosophila homologs of the hPOMGnT1 gene. Accordingly, we tested two Drosophila manose β1,2-N-acetylglucosaminyltransferases, dMGAT1 (21) and dMGAT2 (22), as candidates for POMGnT. The Drosophila EST clones LD43357 and GH07804 encoded the complete ORFs of dMGAT1 and dMGAT2, respectively (Fig. 7A). FLAG-tagged recombinant dMGAT1 and dMGAT2 were expressed in Sf21 insect cells and purified to determine whether or not they have POMGnT activity. There was no POMGnT activity in the FLAG-dMGAT1 or FLAG-dMGAT2 recombinant protein (Fig. 7, B and C), whereas they showed β1,2-N-acetylglucosaminyltransferase I or II activity for a proper substrate (Table I). These results imply that the Siaα2–3Galβ1–4GlcNAcβ1–2Mano1-Ser/Thr found in humans is absent in Drosophila because there is no POMGnT (Fig. 7D).

DISCUSSION

The dPOMT2 RNAi mutant flies, in addition to the dPOMT1 mutant, showed the twisted abdomen phenotype, as previously reported for the dPOMT1 classical mutant, rt (10). RNAi knockdown was performed with a heritable and inducible RNAi system using the GAL4-UAS-IR system (23, 24). The mRNA levels of dPOMT1 and dPOMT2 in each of the RNAi flies were reduced to 32 and 21% of those in wild-type flies, respectively, and no effects of RNAi on the mRNA levels of the other members of the dPOMT family were observed (Fig. 3). So the clock-

wise twisted abdomen phenotype in each RNAi fly (Fig. 2) was caused by the specific reduction of each transcript. This is the first report of the phenotype caused by a deficiency of dPOMT2. dPOMT2 was cytogenetically mapped to 1C4 on the chromo-
some. This region is included in the area 1C3–D4, where twisted (tw) mutations have been mapped genetically (25). The tw mutants show abdomens twisted −30° clockwise as viewed from the posterior and reduced viability. Considering the simi-
larity in phenotype of the dPOMT2 RNAi fly and tw mutants, dPOMT2 might be the tw gene, although genomic aberration of tw mutants has not been investigated.

The genetic interaction between dPOMT1 and dPOMT2 (Fig. 4) and POMT activity assay for recombinant enzymes (Fig. 5C) and RNAi flies (Fig. 5E) clearly demonstrated that both dPOMTs function as protein O-mannosyltransferases interacting with each other in vitro and in vivo. dPOMT1 and dPOMT2 are classified into the PMT4 and PMT2 subfamilies, respectively. In yeast, members of the PMT1 subfamily interact in pairs with members of the PMT2 subfamily, whereas the only member of the PMT4 subfamily forms a homodimer (7). In invertebrates and vertebrates, that is in Drosophila (this work) and humans (18), a single member of the PMT2 subfamily interacts with that of the PMT4 subfamily. The combi-
nation of interacting molecules might have changed during evolution.

Experiments with dPOMT1 and dPOMT2 RNAi flies revealed that both dPOMTs are indispensable for muscle develop-
ment. dPOMT1 and dPOMT2 are the Drosophila orthologs of hPOMT1 and hPOMT2, respectively. hPOMT1 localizes to 9q34 (26), and 20% of Walker-Warburg syndrome patients have mutations in hPOMT1 (12). But no mutations in hPOMT2, the human ortholog of dPOMT2, have yet been reported in Walker-Warburg syndrome patients, although dPOMT2 is needed for normal muscle development. hPOMT2 has been mapped to chromosome 14 at q24.3. We could not find any diseases with defects in muscular development in this region in a data base search. A deficiency of hPOMT2 may be found in Walker-Warburg syndrome patients upon further investigation.

Muscle-eye-brain disease is one of the congenital muscular dystrophies, although it does not show symptoms as sever as Walker-Warburg syndrome. A deficiency of POMGnT1, which transfers GlcNAc to Mano1-Ser/Thr with a β1,2-linkage, has been reported to induce muscle-eye-brain disease (14, 15). As shown in Fig. 7A, Drosophila does not have any orthologs of
hPOMGnT, suggesting that it does not possess any extended O-mannosylglycans. Moreover, the other mannose/H9252 1,2-N-acetylglucosaminyltransferases, dMGAT1 and dMGAT2, did not show any 1,2-N-acetylglucosaminyltransferase activity toward O-mannosylpeptides. Considering the above results, a single mannosyl modification might be enough in Drosophila, although extended O-mannosylglycans are needed in humans.

In mammals, O-mannosylglycans show a rare type of glycosylation that was first identified in chondroitin sulfate proteoglycans of the brain (27). They are present on a limited number of glycoproteins of brain, nerve, and skeletal muscle (16). The most well known O-mannosyl-modified glycoprotein is α-DG (16), which is a central component of the dystrophin-glycoprotein complex isolated from skeletal muscle membranes (28). In Drosophila, dystroglycan has been demonstrated to be required non-cell autonomously for the organization of the planar polarity of basal actin in follicle cells and required cell autonomously for cellular polarity in epithelial cells and oocytes in analyses using classical and RNAi mutants (29). But no dystroglycan mutant phenotype has been reported yet, which suggests a relation between O-mannosylation and dystroglycan. Further
investigation will be necessary to clarify to which core proteins, including dystroglycan, dPOMTs transfer Man and the role of O-mannosylation in the core protein.

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