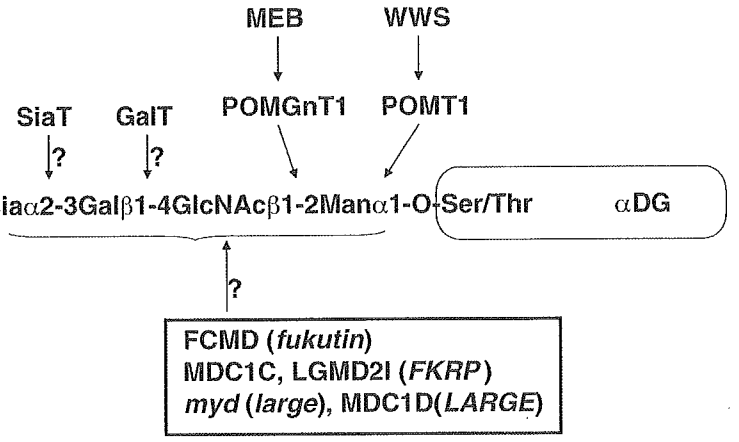


Fig. 4. Possible defects of *O*-mannosylglycosylation of α-dystroglycan in muscular dystrophy. Mutations in *POMGnT1*, *POMT1*, *fukutin*, *FKRP* and *LARGE* (*large*) cause defects in the glycosylation of α-dystroglycan resulting in muscular dystrophy. The substrates of these putative enzymes, with the exception of POMGnT1 and POMT1, are largely unknown. It is unclear whether other as yet uncharacterized forms of muscular dystrophy are caused by defects in galactosyltransferases (GalT) and sialyltransferases (SiaT).

ment in both vertebrates and invertebrates.

Fukuyama-type congenital muscular dystrophy (FCMD). Like MEB and WWS, FCMD is an autosomal recessive disorder that is characterized by congenital muscular dystrophy, lissencephaly, and eye anomalies and FCMD is a relatively common autosomal recessive disorder in the Japanese population.[55] It is the second most common form of childhood muscular dystrophy in Japan after Duchenne muscular dystrophy. Based on an average incidence of 3 per 100,000 population, one in ~90 persons could be a heterozygous carrier in Japan. Kobayashi *et al.*[56] identified a gene on chromosome 9q31 that is responsible for FCMD. The gene encodes a novel 461 amino acid protein of unknown function. The protein, named fukutin because of its association with FCMD, has an N-terminal hydrophobic region which would be a signal sequence or a transmembrane domain. A sequence analysis predicts that it could be an enzyme involved in glycosylation.[57] Consistent with this finding, highly glycosylated α-dystroglycan was selectively deficient in the skeletal muscle of FCMD patients.[58] Recently, Takeda *et al.*[59] generated chimeric mice using embryonic stem cells in which the *fukutin* gene was targeted for disruption. These mice developed severe muscular dystrophy, with a selective deficiency of α-dystroglycan and its laminin-binding activity. These mice also had central nervous and ocular abnormalities. Taken together, these results indicate that fukutin is necessary for the maintenance of muscle integrity, cortical histogenesis, and normal ocular development, and suggest a functional linkage between fukutin and α-dystroglycan.

Congenital muscular dystrophy type 1C (MDC1C) and limb-girdle muscular dystrophy 2I (LGMD2I). Defective glycosylation of α-dystroglycan has also been implicated in congenital muscular dystrophy type 1C (MDC1C), which is caused by a homologue of *fukutin* (fukutin-related protein, *FKRP*).[60] MDC1C is characterized by severe muscle weakness and degeneration, and cardiomyopathy. Mental retardation and cerebellar cysts have been observed in some cases. Allelic mutations in the *FKRP* gene also cause a milder and more common form of muscular dystrophy called limb-girdle muscular dystrophy 2I (LGMD2I: OMIM 607155), which is frequently associated with cardiomyopathy and shows variable onsets ranging from adolescence to adulthood.[61] Patients with the mutations in the *FKRP* gene invariably exhibit a reduced expression of α-dystroglycan, which is strongly correlated with disease severity. A western blot analysis showed an apparent loss of higher molecular weight forms of α-dystroglycan. Although the function of FKRP is unknown, it has been suggested that FKRP is involved in the glycosylation of α-dystroglycan as a glycosyltransferase or a kind of modulator. Because FKRP and fukutin are thought to be Golgi-resident proteins,[62] it is possible that defects of

these proteins cause abnormal processing of α-dystroglycan.

Congenital muscular dystrophy type 1D (MDC1D) and the myodystrophy (*myd*) mouse. The gene *large*, which is mutated in the myodystrophy (*myd*) mouse, encodes a putative glycosyltransferase.[63] However, its biochemical activity has not been confirmed. The causative mutation in *myd* was identified as a deletion of exons 5-7 of the *large* gene. This deletion results in a frameshift in the corresponding mRNA, leading to a premature termination codon. The *myd* mouse shows a progressive muscular dystrophy, ocular defects, and a central nervous system phenotype characterized by abnormal neuronal migration in the cerebral cortex, cerebellum, and hippocampus, and disruption of the basal lamina.[64),65] The *myd* mouse, like MEB and FCMD patients, showed hypoglycosylation of α-dystroglycan in muscle and brain. The human homologue of the *large* gene (*LARGE*) may be involved in novel forms of muscular dystrophy. A recent study[66] described a patient with congenital muscular dystrophy, profound mental retardation, white matter changes, and subtle structural abnormalities in the brain and a reduction of immunolabelling of α-dystroglycan. This type of muscular dystrophy was named as MDC1D. The patient was found to have a missense mutation and a 1bp insertion in the *LARGE* gene.

Perspectives. Unlike proteins and nucleic acids, which are linear molecules, sugar chains form branching, and positional and anomeric isomers, indicating the occurrence of a remarkable number of structures with a small number of units. Such complexity has made their structural analysis difficult and has obscured their functions for a long time. However, newly developed and sensitive methods to elucidate the structures and functions of the sugar chains have made it possible to precisely determine small amounts of sugar chains. Such studies have shown that glycans are highly abundant and exhibit diverse structures, with widely varying functions. Newly available genetic approaches accelerate discoveries of these functions. Furthermore, many examples of genetic alterations in glycan structures and expression have been found in humans, and have provided many clues to glycan functions. In the future, progress in understanding glycan functions will continue to rely on glycan structural analyses based on mutational analyses. Because the amount of material is often limited, it is essential to develop more sensitive methods for analyzing the structures of the sugar chains.

O-Mannosylation is an unusual type of protein modification and is present in a limited number of glycoproteins of brain, nerve, and skeletal muscle. *O*-Mannosyl glycans play critical roles in the following example. Hypoglycosylated α-dystroglycan, which is probably caused by a defect of *O*-mannosylation, has greatly reduced affinities for laminin, neurexin and agrin.[67] This suggests that defective glycosylation of α-dystroglycan due to the genetic defects of glycosyltransferases is the common trait of muscle cell degeneration and abnormal brain structure found in MEB, WWS, FCMD, MDC1C, MDC1D patients and the *myd* mouse (Fig. 2). Therefore, α-dystroglycan may be a potential target of future therapy for muscular dystrophy. However, the substrates of these enzymes (Table III), with the exception of POMGnT1 and POMT1, are largely unknown (Fig. 4). Identification and characterization of each enzyme will help to reveal the molecular pathomechanisms of congenital muscular dystrophies with brain malformation. Future studies may also reveal that presently uncharacterized forms of muscular dystrophy are caused by defects in galactosyltransferases and/or sialyltransferases. A major challenge will be to integrate the forthcoming structural, cell biological, and genetic information to understand how α-dystroglycan glycosylation contributes to muscular dystrophy.

Acknowledgements. This study was supported by Research Grants for Nervous and Mental Disorders (14B-4) and Research on Psychiatric and Neurological Diseases and Mental Health from the Ministry of Health, Labour and Welfare of Japan, and by a Grant-in-Aid for Scientific Research on Priority Area (14082209) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1) Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct. Glycobiology **3**, 97-130.
2) Bruckner, K., Perez, L., Clausen, H., and Cohen, S. (2000) Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. Nature **406**, 411-415.
3) Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S. *et al.* (2000) Fringe is a glycosyltransferase that modifies Notch. Nature **406**, 369-375.
4) Okajima, T., and Irvine, K. D. (2002) Regulation of Notch signaling by *O*-linked fucose. Cell **111**, 893-904.
5) Shi, S., and Stanley, P. (2003) Protein *O*-fucosyltransferase 1 is an essential component of Notch signaling pathways. Proc. Natl. Acad. Sci. USA **100**, 5234-5239.
6) Lowe, J. B., and Marth, J. D. (2003) A genetic approach to mammalian glycan function. Annu. Rev. Biochem. **72**, 643-691.

7) Endo, T. (1999) *O*-Mannosyl glycans in mammals. Biochim. Biophys. Acta **1473**, 237-246.
8) Freeze, H. H. (2002) Human disorders in *N*-glycosylation and animal models. Biochim. Biophys. Acta **1573**, 388-393.
9) Chantret, I., Dancourt, J., Dupre, T., Delenda, C., Bucher, S., Vuillaumier-Barrot, S., Ogier de Baulny, H., Peletan, C., Danos, O., Seta, N. *et al.* (2003) A deficiency in dolichyl-P-glucose: $Glc_1Man_9GlcNAc_2$-PP-dolichyl α3-glucosyltransferase defines a new subtype of congenital disorders of glycosylation. J. Biol. Chem. **278**, 9962-9971.
10) Thiel, C., Schwarz, M., Peng, J., Grzmil, M., Hasilik, M., Braulke, T., Kohlschutter, A., von Figura, K., Lehle, L., and Korner, C. (2003) A new type of congenital disorders of glycosylation (CDG-Ii) provides new insights into the early steps of dolichol-linked oligosaccharide biosynthesis. J. Biol. Chem. **278**, 22498-22505.
11) Wu, X., Rush, J. S., Karaoglu, D., Krasnewich, D., Lubinsky, M. S., Waechter, C. J., Gilmore, R., and Freeze, H. H. (2003) Deficiency of UDP-GlcNAc: dolichol phosphate *N*-acetylglucosamine-1 phosphate transferase (*DPAGT1*) causes a novel congenital disorder of glycosylation type Ij. Hum. Mutat. **22**, 144-150.
12) Tan, J., Dunn, J., Jaeken, J., and Schachter, H. (1996) Mutations in the *MGAT2* gene controlling complex *N*-glycan synthesis cause carbohydrate-deficient glycoprotein syndrome type II, an autosomal recessive disease with defective brain development. Am. J. Hum. Genet. **59**, 810-817.
13) Lubke, T., Marquardt, T., Etzioni, A., Hartmann, E., von Figura, K., and Korner, C. (2001) Complementation cloning identifies CDG-IIc, a new type of congenital disorders of glycosylation, as a GDP-fucose transporter deficiency. Nat. Genet. **28**, 73-76.
14) Luhn, K., Wild, M. K., Eckhardt, M., Gerardy-Schahn, R., and Vestweber, D. (2001) The gene defective in leukocyte adhesion deficiency II encodes a putative GDP-fucose transporter. Nat. Genet. **28**, 69-72.
15) Hansske, B., Thiel, C., Lubke, T., Hasilik, M., Honing, S., Peters, V., Heidemann, P. H., Hoffmann, G. F., Berger, E. G., von Figura, K. *et al.* (2002) Deficiency of UDP-galactose: *N*-acetylglucosamine β-1,4-galactosyltransferase I causes the congenital disorder of glycosylation type IId. J. Clin. Invest. **109**, 725-733.
16) Strahl-Bolsinger, S., Gentzsch, M., and Tanner, W. (1999) Protein *O*-mannosylation. Biochim. Biophys. Acta **1426**, 297-307.
17) Timpel, C., Strahl-Bolsinger, S., Ziegelbauer, K., and Ernst, J. F. (1998) Multiple functions of Pmt1p-mediated protein *O*-mannosylation in the fungal pathogen *Candida albicans*. J. Biol. Chem. **273**, 20837-20846.
18) Spiro, R. G., and Bhoyroo, V. D. (1980) Studies on the carbohydrate of collagens. Characterization of a glucuronic acid-mannose disaccharide unit from *Nereis* cuticle collagen. J. Biol. Chem. **255**, 5347-5354.
19) Krusius, T., Reinhold, V. N., Margolis, R. K., and Margolis, R. U. (1987) Structural studies on sialylated and sulphated *O*-glycosidic mannose-linked oligosaccharides in the chondroitin sulphate proteoglycan of brain. Biochem. J. **245**, 229-234.
20) Krusius, T., Finne, J., Margolis, R. K., and Margolis, R. U. (1986) Identification of an *O*-glycosidic mannose-linked sialylated tetrasaccharide and keratan sulfate oligosaccharides in the chondroitin sulfate proteoglycan of brain. J. Biol. Chem. **261**, 8237-8242.
21) Finne, J., Krusius, T., Margolis, R. K., and Margolis, R. U. (1979) Novel mannitol-containing oligosaccharides obtained by mild alkaline borohydride treatment of a chondroitin sulfate proteoglycan from brain. J. Biol. Chem. **254**, 10295-10300.
22) Chiba, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusunoki, S., Kanazawa, I., Kobata, A., and Endo, T. (1997) Structures of sialylated *O*-linked oligosaccharides of bovine peripheral nerve α-dystroglycan. The role of a novel *O*-mannosyl-type oligosaccharide in the binding of α-dystroglycan with laminin. J. Biol. Chem. **272**, 2156-2162.
23) Sasaki, T., Yamada, H., Matsumura, K., Shimizu, T., Kobata, A., and Endo, T. (1998) Detection of *O*-mannosyl glycans in rabbit skeletal muscle α-dystroglycan. Biochim. Biophys. Acta **1425**, 599-606.
24) Yuen, C. T., Chai, W., Loveless, R. W., Lawson, A. M., Margolis, R. U., and Feizi, T. (1997) Brain contains HNK-1 immunoreactive *O*-glycans of the sulfoglucuronyl lactosamine series that terminate in 2-linked or 2,6-linked hexose (mannose). J. Biol. Chem. **272**, 8924-8931.
25) Chai, W., Yuen, C. T., Kogelberg, H., Carruthers, R. A., Margolis, R. U., Feizi, T., and Lawson, A. M. (1999) High prevalence of 2-mono- and 2,6-di-substituted manol-terminating sequences among *O*-glycans released from brain glycopeptides by reductive alkaline hydrolysis. Eur. J. Biochem. **263**, 879-888.
26) Inamori, K. I., Endo, T., Gu, J., Matsuo, I., Ito, Y., Fujii, S., Iwasaki, H., Narimatsu, H., Miyoshi, E., Honke, K. *et al.* (2004) *N*-acetylglucosaminyltransferase IX acts on the GlcNAcβ1,2-Manα1-Ser/Thr moiety, forming a 2,6-branched structure in brain *O*-mannosyl glycan. J. Biol. Chem. **279**, 2337-2340.
27) Smalheiser, N. R., Haslam, S. M., Sutton-Smith, M., Morris, H. R., and Dell, A. (1998) Structural analysis of sequences *O*-linked to mannose reveals a novel Lewis X structure in cranin (dystroglycan) purified from sheep brain. J. Biol. Chem. **273**, 23698-23703.
28) Wing, D. R., Rademacher, T. W., Schmitz, B., Schachner, M., and Dwek, R. A. (1992) Comparative glycosylation in neural adhesion molecules. Biochem. Soc. Trans. **20**, 386-390.
29) Takahashi, S., Sasaki, T., Manya, H., Chiba, Y., Yoshida, A., Mizuno, M., Ishida, H., Ito, F., Inazu, T., Kotani, N. *et al.* (2001) A new β-1,2-*N*-acetylglucosaminyltransferase that may play a role in the biosynthesis of mammalian *O*-mannosyl glycans. Glycobiology **11**, 37-45.
30) Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M. *et al.* (2001) Muscular dystrophy and neuronal

migration disorder caused by mutations in a glycosyltransferase, POMGnT1. Dev. Cell **1**, 717-724.

31) Ioffe, E., and Stanley, P. (1994) Mice lacking *N*-acetylglucosaminyltransferase I activity die at mid-gestation, revealing an essential role for complex or hybrid *N*-linked carbohydrates. Proc. Natl. Acad. Sci. USA **91**, 728-732.

32) Metzler, M., Gertz, A., Sarkar, M., Schachter, H., Schrader, J. W., and Marth, J. D. (1994) Complex asparagine-linked oligosaccharides are required for morphogenic events during post-implantation development. EMBO J. **13**, 2056-2065.

33) Wang, Y., Tan, J., Sutton-Smith, M., Ditto, D., Panico, M., Campbell, R. M., Varki, N. M., Long, J. M., Jaeken, J., Levinson, S. R. *et al.* (2001) Modeling human congenital disorder of glycosylation type IIa in the mouse: conservation of asparagine-linked glycan-dependent functions in mammalian physiology and insights into disease pathogenesis. Glycobiology **11**, 1051-1070.

34) Holt, K. H., Crosbie, R. H., Venzke, D. P., and Campbell, K. P. (2000) Biosynthesis of dystroglycan: processing of a precursor propeptide. FEBS Lett. **468**, 79-83.

35) Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Sernett, S. W., and Campbell, K. P. (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature **355**, 696-702.

36) Winder, S. J. (2001) The complexities of dystroglycan. Trends Biochem. Sci. **26**, 118-124.

37) Huang, X., Poy, F., Zhang, R., Joachimiak, A., Sudol, M., and Eck, M. J. (2000) Structure of a WW domain containing fragment of dystrophin in complex with β-dystroglycan. Nat. Struct. Biol. **7**, 634-638.

38) Burton, E. A., and Davies, K. E. (2002) Muscular dystrophy—reason for optimism? Cell **108**, 5-8.

39) Williamson, R. A., Henry, M. D., Daniels, K. J., Hrstka, R. F., Lee, J. C., Sunada, Y., Ibraghimov-Beskrovnaya, O., and Campbell, K. P. (1997) Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in *Dag1*-null mice. Hum. Mol. Genet. **6**, 831-841.

40) Cote, P. D., Moukhles, H., Lindenbaum, M., and Carbonetto, S. (1999) Chimaeric mice deficient in dystroglycans develop muscular dystrophy and have disrupted myoneural synapses. Nat. Genet. **23**, 338-342.

41) Cohn, R. D., Henry, M. D., Michele, D. E., Barresi, R., Saito, F., Moore, S. A., Flanagan, J. D., Skwarchuk, M. W., Robbins, M. E., Mendell, J. R. *et al.* (2002) Disruption of *DAG1* in differentiated skeletal muscle reveals a role for dystroglycan in muscle regeneration. Cell **110**, 639-648.

42) Moore, S. A., Saito, F., Chen, J., Michele, D. E., Henry, M. D., Messing, A., Cohn, R. D., Ross-Barta, S. E., Westra, S., Williamson, R. A. *et al.* (2002) Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy. Nature **418**, 422-425.

43) Saito, F., Moore, S. A., Barresi, R., Henry, M. D., Messing, A., Ross-Barta, S. E., Cohn, R. D., Williamson, R. A., Sluka, K. A., Sherman, D. L. *et al.* (2003) Unique role of dystroglycan in peripheral nerve myelination, nodal structure, and sodium channel stabilization. Neuron **38**, 747-758.

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

45) Taniguchi, K., Kobayashi, K., Saito, K., Yamanouchi, H., Ohnuma, A., Hayashi, Y. K., Manya, H., Jin, D. K., Lee, M., Parano, E. *et al.* (2003) Worldwide distribution and broader clinical spectrum of muscle-eye-brain disease. Hum. Mol. Genet. **12**, 527-534.

46) Manya, H., Sakai, K., Kobayashi, K., Taniguchi, K., Kawakita, M., Toda, T., and Endo, T. (2003) Loss-of-function of an *N*-acetylglucosaminyltransferase, POMGnT1, in muscle-eye-brain disease. Biochem. Biophys. Res. Commun. **306**, 93-97.

47) Kano, H., Kobayashi, K., Herrmann, R., Tachikawa, M., Manya, H., Nishino, I., Nonaka, I., Straub, V., Talim, B., Voit, T. *et al.* (2002) Deficiency of α-dystroglycan in muscle-eye-brain disease. Biochem. Biophys. Res. Commun. **291**, 1283-1286.

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

49) Beltrán-Valero de Bernabé, D., Currier, S., Steinbrecher, A., Celli, J., van Beusekom, E., van der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W. B. *et al.* (2002) Mutations in the *O*-mannosyltransferase gene *POMT1* give rise to the severe neuronal migration disorder Walker-Warburg syndrome. Am. J. Hum. Genet. **71**, 1033-1043.

50) Willer, T., Amselgruber, W., Deutzmann, R., and Strahl, S. (2002) Characterization of POMT2, a novel member of the *PMT* protein O-mannosyltransferase family specifically localized to the acrosome of mammalian spermatids. Glycobiology **12**, 771-783.

51) Manya, H., Chiba, A., Yoshida, A., Wang, X., Chiba, Y., Jigami, Y., Margolis, R. U., and Endo, T. (2004) Demonstration of mammalian protein *O*-mannosyltransferase activity: Coexpression of POMT1 and POMT2 required for enzymatic activity. Proc. Natl. Acad. Sci. USA **101**, 500-505.

52) Jurado, L. A., Coloma, A., and Cruces, J. (1999) Identification of a human homolog of the *Drosophila rotated abdomen* gene (*POMT1*) encoding a putative protein *O*-mannosyl-transferase, and assignment to human chromosome 9q34.1. Genomics **58**, 171-180.

53) Cormand, B., Pihko, H., Bayes, M., Valanne, L., Santavuori, P., Talim, B., Gershoni-Baruch, R., Ahmad, A., van Bokhoven, H., Brunner, H. G. *et al.* (2001) Clinical and genetic distinction between Walker-Warburg syndrome and muscle-eye-brain disease. Neurology **56**, 1059-1069.

54) Martin-Blanco, E., and Garcia-Bellido, A. (1996) Mutations in the *rotated abdomen* locus affect muscle development and reveal an intrinsic asymmetry in *Drosophila*. Proc. Natl. Acad. Sci. USA **93**, 6048-6052.

55) Fukuyama, Y., Osawa, M., and Suzuki, H. (1981) Congenital progressive muscular dystrophy of the Fukuyama type—

clinical, genetic and pathological considerations. Brain Dev. **3**, 1-29.

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

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

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

59) Takeda, S., Kondo, M., Sasaki, J., Kurahashi, H., Kano, H., Arai, K., Misaki, K., Fukui, T., Kobayashi, K., Tachikawa, M. *et al.* (2003) Fukutin is required for maintenance of muscle integrity, cortical histiogenesis and normal eye development. Hum. Mol. Genet. **12**, 1449-1459.

60) Brockington, M., Blake, D. J., Prandini, P., Brown, S. C., Torelli, S., Benson, M. A., Ponting, C. P., Estournet, B., Romero, N. B., Mercuri, E. *et al.* (2001) Mutations in the fukutin-related protein gene (*FKRP*) cause a form of congenital muscular dystrophy with secondary laminin α2 deficiency and abnormal glycosylation of α-dystroglycan. Am. J. Hum. Genet. **69**, 1198-1209.

61) Brockington, M., Yuva, Y., Prandini, P., Brown, S. C., Torelli, S., Benson, M. A., Herrmann, R., Anderson, L. V., Bashir, R., Burgunder, J. M. *et al.* (2001) Mutations in the fukutin-related protein gene (*FKRP*) identify limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C. Hum. Mol. Genet. **10**, 2851-2859.

62) Esapa, C. T., Benson, M. A., Schroder, J. E., Martin-Rendon, E., Brockington, M., Brown, S. C., Muntoni, F., Kroger, S., and Blake, D. J. (2002) Functional requirements for fukutin-related protein in the Golgi apparatus. Hum. Mol. Genet. **11**, 3319-3331.

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

64) Michele, D. E., Barresi, R., Kanagawa, M., Saito, F., Cohn, R. D., Satz, J. S., Dollar, J., Nishino, I., Kelley, R. I., Somer, H. *et al.* (2002) Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. Nature **418**, 417-422.

65) Holzfeind, P. J., Grewal, P. K., Reitsamer, H. A., Kechvar, J., Lassmann, H., Hoeger, H., Hewitt, J. E., and Bittner, R. E. (2002) Skeletal, cardiac and tongue muscle pathology, defective retinal transmission, and neuronal migration defects in the Large(*myd*) mouse defines a natural model for glycosylation-deficient muscle-eye-brain disorders. Hum. Mol. Genet. **11**, 2673-2687.

66) Longman, C., Brockington, M., Torelli, S., Jimenez-Mallebrera, C., Kennedy, C., Khalil, N., Feng, L., Saran, R. K., Voit, T., Merlini, L. *et al.* (2003) Mutations in the human *LARGE* gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of α-dystroglycan. Hum. Mol. Genet. **12**, 2853-2861.

67) Michele, D. E., and Campbell, K. P. (2003) Dystrophin-glycoprotein complex: post-translational processing and dystroglycan function. J. Biol. Chem. **278**, 15457-15460.

(Received Feb. 4, 2004; accepted March 12, 2004)

Glycoconjugate Journal 21, 3–7, 2004

Structure, function and pathology of *O*-mannosyl glycans

Tamao Endo

Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, Foundation for Research on Aging and Promotion of Human Welfare, 35-2 Sakaecho, Itabashi-ku, Tokyo 173-0015, Japan

Animal cells contain many glycoproteins, *i.e.*, proteins with covalently liked sugar chains. The major glycans of glycoproteins can be classified into two groups, *N*-glycans and *O*-glycans, according to their glycan-peptide linkage regions. Development of sensitive methods for the analyses of glycan structures have revealed a new type of glycosidic linkage to the peptide portion, the *O*-mannosyl linkage, in mammals, which used to be considered specific to yeast. *O*-Mannosylation is present in a limited number of glycoproteins of brain, nerve, and skeletal muscle. Recently *O*-mannosylation has been shown to be important in muscle and brain development. Glycobiology of *O*-mannosyl glycans is expected to produce remarkable advances in the understanding and treatment of congenital muscular dystrophies. In this article, I describe the structure, biosynthesis, and pathology of *O*-mannosyl glycans.
Published in 2004.

***Keywords:* O-mannosylation, congenital muscular dystrophy, dystroglycan, glycosyltransferase**

Introduction

The major sugar chains of glycoproteins can be classified into two groups according to their sugar-peptide linkages. Those that are linked to asparagine (Asn) residues of proteins are termed *N*-glycans, while those that are linked to serine (Ser) or threonine (Thr) residues are called *O*-glycans. In *N*-glycans, the reducing terminal *N*-acetylglucosamine (GlcNAc) is linked to the amide group of Asn via an aspartylglycosylamine linkage. In *O*-glycans, the reducing terminal *N*-acetylgalactosamine (GalNAc) is attached to the hydroxyl group of Ser and Thr residues. In addition to the abundant *O*-GalNAc forms, several unique types of protein *O*-glycosylation have been found, such as *O*-linked fucose, glucose, GlcNAc, and mannose, which have been shown to mediate diverse physiological functions. For example, *O*-fucosylation has recently been implicated in Notch signaling [1–4] and *O*-mannosylation has been shown to be important in muscle and brain development.

Structure and occurrence

O-Mannosylation is known as a yeast-type modification, and *O*-mannosylated proteins are abundant in the yeast cell wall [5]. All *O*-mannosyl glycan structures elucidated so far in yeast are neutral linear glycans consisting of 1 to 7 mannose residues. *O*-Mannosylation of proteins is essential for viability in yeast, and its absence may affect cell wall structure and rigidity. Additionally, a deficiency in protein *O*-mannosylation in the fungal pathogen, *Candida albicans*, leads to defects in multiple cellular functions including expression of virulence [6]. In addition to fungi and yeast, clam worm has an *O*-mannosyl glycan (a glucuronosylα1-6mannosyl disaccharide) in skin collagen [7].

Mammalian *O*-mannosylation is an unusual type of protein modification that was first suggested in chondroitin sulfate proteoglycans of brain [8] and is present in a limited number of glycoproteins of brain, nerve, and skeletal muscle [9]. One of the best known *O*-mannosyl-modified glycoproteins is α-dystroglycan [9], which is a central component of the dystrophin-glycoprotein complex isolated from skeletal muscle membranes [10]. We previously found that the glycans of α-dystroglycan include *O*-mannosyl oligosaccharides, and that a sialyl *O*-mannosyl glycan, Siaα2-3Galβ1-4GlcNAcβ1-2Man, of α-dystroglycan is a laminin-binding ligand of α-dystroglycan [11,12]. Further, α-dystroglycan from sheep brain has a Galβ1-4(Fucα1-3)GlcNAcβ1-2Man structure [13] and mouse J1/tenascin contains the *O*-mannosyl glycans [14]. Additionally, an *O*-mannosyl glycan containing the HNK-1 epitope (HSO_3-3GlcAβ1-3Galβ1-4GlcNAcβ1-2Man) was detected in total brain glycopeptides [15]. It is noteworthy that these oligosaccharides have not only 2-substituted mannose but also 2,6-substituted mannose [16]. Therefore, it is likely

To whom correspondence should be addressed: Tamao Endo, Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, Foundation for Research on Aging and Promotion of Human Welfare, 35-2 Sakaecho, Itabashi-ku, Tokyo 173-0015, Japan. Tel.: +81-3-3964-3241 (ext. 3080); Fax: +81-3-3579-4776; E-mail: endo@tmig.or.jp

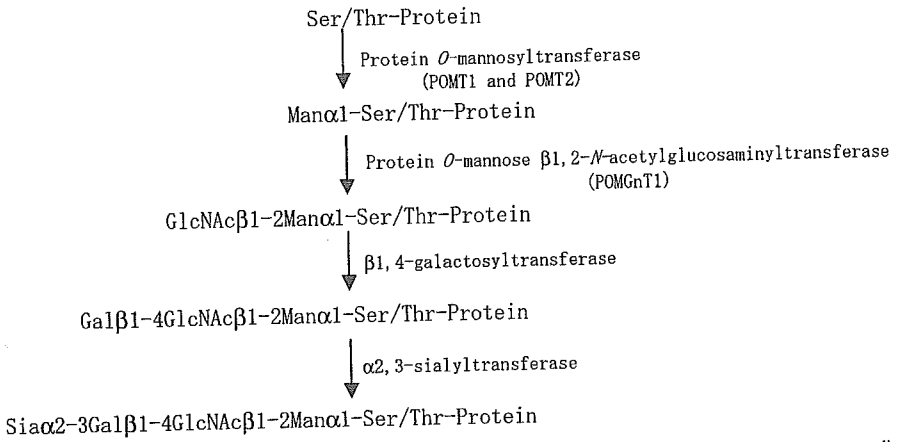


Figure 1. Biosynthetic pathway of mammalian *O*-mannosyl glycan. POMT1 and POMGnT1 are responsible for WWS and MEB, respectively. It is unclear whether others as yet characterized forms of muscular dystrophy are caused by defects in β1,4-galactosyltransferase and α2,3-sialyltransferase.

that a series of *O*-mannosyl glycans, with heterogeneity of mannose-branching and peripheral structures, is present in mammals. Further studies are needed to clarify the distribution of such *O*-mannosyl glycans in various tissues.

Biosynthesis

Identification and characterization of the enzymes involved in the biosynthesis of mammalian type *O*-mannosyl glycans will help to elucidate the function and regulation of these glycans (Figure 1).

Mammalian *O*-mannosyl glycans, unlike those of yeast, have the GlcNAcβ1-2Man linkage [9]. This linkage is assumed to be catalyzed by a glycosyltransferase, UDP-GlcNAc: protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase (POMGnT1). POMGnT1 catalyzes the transfer of GlcNAc from UDP-GlcNAc to *O*-mannosyl glycoproteins. Human *POMGnT1* gene has been cloned [17]. Based on its nucleotide sequence, human POMGnT1 is a 660-amino acid type II membrane protein. This topology was similar to the topologies of other Golgi glycosyltransferases [18]. *POMGnT1* was found to be expressed in all human tissues examined [17]. Careful examination of substrate specificity of POMGnT1 indicated that POMGnT1 did not have either UDP-GlcNAc: α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT-I) or UDP-GlcNAc: α-6-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase II (GnT-II) activity. On the other hand, GnT-I and GnT-II did not have any POMGnT1 activity [19]. Taken together, these results indicate that loss-of-function of POMGnT1 is not compensated by GnT-I and GnT-II.

As described above, mammalian *O*-mannosyl glycan has 2,6-substituted mannose [16]. A gene for encoding a 6-branching enzyme (GnT-IX, *i.e*, an enzyme that catalyzes the GlcNAcβ1-6Man linkage in *O*-mannosyl glycans) has recently been cloned [20]. Since GnT-IX is specifically expressed in the brain, identifying the functional roles of 2,6-branched *O*-mannosyl glycans in the brain is of great interest.

In *Saccharomyces cerevisiae*, the family of protein *O*-mannosyltransferases catalyzes the transfer of a mannosyl residue from dolichyl phosphate mannose (Dol-P-Man) to Ser/Thr residues of certain proteins [5]. There is no obvious consensus sequence for attachment of *O*-mannosyl glycans, though *in vitro* studies with peptide acceptors show that the presence of a proline residue in the vicinity enhanced *O*-mannosylation of nearby Ser or Thr residues [5]. However, attempts to detect protein *O*-mannosyltransferase activity and to characterize the enzyme(s) responsible for the biosynthesis of *O*-mannosyl glycans in vertebrates have not been successful. Two human homologues, *POMT1* and *POMT2*, were found, but their gene products did not show any protein *O*-mannosyltransferase activity [21,22]. POMT1 and POMT2 share almost identical hydropathy profiles that predict both to be integral membrane proteins with multiple transmembrane domains. Recently, we developed a new method to detect the enzymatic activity of protein *O*-mannosyltransferase in mammalian cells and tissues [23]. Initially, we attempted to detect mannose transferase activity based on an assay for protein *O*-mannosyltransferase activity in yeast using several synthetic peptides and Triton X-100 as a detergent. However, we did not detect any activity in several mammalian tissues and cells, possibly due to the specificity of the acceptor peptide sequence. If the enzyme recognizes a specific amino acid sequence, α-dystroglycan may be a suitable acceptor because it has *O*-mannosyl glycans [9]. Therefore, we prepared a GST fusion protein of α-dystroglycan for a candidate acceptor. However, using the α-dystroglycan-GST as an acceptor and the Dol-P-Man as a donor substrate did not observe any enzymatic activity. Next, we examined the effect of detergent because yeast protein *O*-mannosyltransferases are integral membrane proteins and thus hydrophobic proteins. Because Triton X-100 is a non-ionic detergent, we examined many ionic and ampholytic detergents, including alkyl-glycosides. Finally, we found that the best detergent was *n*-octyl-β-D-thioglucoside. With these changes, we succeeded in detecting mammalian protein *O*-mannosyltransferase activity [23].

Using this new method, we demonstrated that human POMT1 and POMT2 have protein *O*-mannosyltransferase activity, but only when they are co-expressed [23]. This suggests that POMT1 and POMT2 form a hetero-complex to express enzymatic activity similar to the complex in yeast [24]. *POMT1* and *POMT2* are expressed in all human tissues, but *POMT1* is highly expressed in fetal brain, testis and skeletal muscle, and *POMT2* is predominantly expressed in testis [21,22]. *O*-Mannosylation seems to be uncommon in mammals and only a few *O*-mannosylated proteins have been identified. It will be of interest to determine the regulatory mechanisms for protein *O*-mannosylation in each tissue.

No enzymes for galactosylation, sialylation, fucosylation, glucuronylation, or sulfation of *O*-mannosyl glycans have been identified.

Pathology

Muscular dystrophies are genetic diseases that cause progressive muscle weakness and wasting [25]. Recent data suggest that aberrant *O*-mannosylation of α-dystroglycan is the primary cause of some forms of congenital muscular dystrophy (Table 1).

Muscle-eye-brain disease [MEB: OMIM 253280, OMIM = Online Mendelian Inheritance in Man (http://www.ncbi.nih.gov/)], is an autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormalities and brain malformation (type II lissencephaly). After we screened the sequences of the *POMGnT1* gene for mutations in patients with MEB, we identified 13 independent disease-causing mutations in these patients [17,26]. We have not detected these 13 substitutions in any of 300 normal individuals, indicating that the *POMGnT1* gene is responsible for MEB. To confirm that the mutations observed in patients with MEB are responsible for the defects in the synthesis of *O*-mannosyl glycan, we expressed all of the mutant proteins and found that none of them had enzymatic activity [17,27]. These findings indicate that MEB is inherited as a loss-of-function of the *POMGnT1* gene. If POMGnT1 does not function, no peripheral structure can be formed on *O*-mannose residues. Because these structures are involved in adhesive processes, a defect of *O*-mannosyl glycan may severely affect cell migration and cell adhesion. Additionally, a selective deficiency of glycosylated α-dystroglycan in MEB patients was found [28]. This finding suggests that α-dystroglycan is a potential target of POMGnT1 and that hypoglycosylation of α-dystroglycan may be a pathomechanism of MEB. Thus, MEB muscle and brain phenotypes can be explained by abnormal *O*-mannosylation.

Walker-Warburg syndrome (WWS: OMIM 236670) is another form of congenital muscular dystrophy that is characterized by severe brain malformation and eye anomalies. Patients with WWS are severely affected from birth and usually die within their first year. Recently, 20% of WWS patients (6 of 30 unrelated WWS cases) have been found to have mutations in *POMT1* [29], but none of the 30 cases studied had mutations in another homologue, *POMT2*. This suggests that other as yet unidentified genes are responsible for this syndrome.

In WWS patients, as in MEB patients, the glycosylated α-dystroglycan was selectively deficient in skeletal muscle. WWS and MEB are clinically similar disorders, but WWS is a more severe syndrome than MEB. The difference of severity between the two diseases may be explained as follows. If POMGnT1, which is responsible for the formation of the GlcNAcβ1-2Man linkage of *O*-mannosyl glycans (Figure 1), is non-functional, only *O*-mannose residues may be present on α-dystroglycan in MEB. On the other hand, *POMT1* mutations cause complete loss of *O*-mannosyl glycans in WWS. Thus, it is possible that attachment of a single mannose residue on α-dystroglycan in MEB is responsible for the difference in clinical severity of WWS and MEB.

Interestingly, defective myogenesis in the *Drosophila rt* mutant was found to be due to a mutation in a homologue of *POMT1* [22,30]. Although the *rt* gene product is not known to be a component involved in the initial step of *O*-mannosyl glycan biosynthesis, *O*-mannosylation is an evolutionarily conserved protein modification [9], and may be essential for muscle development in both vertebrates and invertebrates.

Table 1. Possible muscular dystrophies caused by abnormal glycosylation of α-dystroglycan

Condition	*Gene*	*Protein function*
Muscle-eye-brain disease (MEB)	*POMGnT1*	GlcNActransferase
Walker-Warburg syndrome (WWS)	*POMT1*	*O*-Mannosyltransferase
Fukuyama-type congenital muscular dystrophy (FCMD)	*Fukutin*	Putative glycosyltransferase
MDC1C	*FKRP*	Putative glycosyltransferase
Limb-girdle muscular dystrophy 2I (LGMD2I)	(Fukutin-related protein)	
MDC1D	*LARGE*	Putative glycosyltransferase
myd mouse	*large*	

In addition to MEB and WWS, other muscular dystrophies have been suggested to be caused by abnormal glycosylation of α-dystroglycan, *e.g.*, Fukuyama-type congenital muscular dystrophy (FCMD, which is caused by the *fukutin* gene: OMIM 253800), congenital muscular dystrophy type 1C (MDC1C, which is caused by the *FKRP* gene, fukutin-related protein: OMIM 606612) and its allelic disease called limb-girdle muscular dystrophy 2I (LGMD2I: OMIM 607155), congenital muscular dystrophy type 1D (MDC1D, which is caused by the *LARGE* gene), and the myodystrophy (*myd*) mouse, which is caused by the *large* gene (Table 1). Highly glycosylated α-dystroglycan was found to be selectively deficient in the skeletal muscle of these patients and the mouse, as it was in MEB and WWS patients, and the gene products were thought be putative glycosyltransferases [31]. However, it is still unclear whether these gene products are involved in the *O*-mannosyl glycan biosynthesis. Future studies may also reveal that presently uncharacterized forms of muscular dystrophy are caused by defects in other glycosyltransferases. Identification of these defects may provide new clues to the glycopathomechanism of muscular dystrophy.

Perspectives

O-Mannosylation is an uncommon protein modification in mammals, but it is important in muscle and brain development. Further studies are needed to clarify the distribution of *O*-mannosyl glycans in various tissues and to examine their changes during development and pathological conditions. A major challenge will be to integrate the forthcoming structural, cell biological, and genetic information to understand how α-dystroglycan *O*-mannosylation contributes to muscular dystrophy and brain development.

Acknowledgments

This work was carried out in collaboration with many scientists, whose names appear in the list of literature citations. The author gratefully acknowledges the financial support of Research Grants for Nervous and Mental Disorders (14B-4) and Research on Psychiatric and Neurological Diseases and Mental Health from the Ministry of Health, Labour and Welfare of Japan, and a Grant-in-Aid for Scientific Research on Priority Area (14082209) from the Ministry of Education, Culture, Sports, Science and Technology of Japan throughout this project.

References

1 Bruckner K, Perez L, Clausen H, Cohen S, Glycosyltransferase activity of Fringe modulates Notch-Delta interactions, *Nature* **406**, 411–5 (2000).
2 Moloney DJ, Panin VM, Johnston SH, Chen J, Shao L, Wilson R, Wang Y, Stanley P, Irvine KD, Haltiwanger RS, Vogt TF, Fringe is a glycosyltransferase that modifies Notch, *Nature* **406**, 369–75 (2000).
3 Okajima T, Irvine KD, Regulation of notch signaling by O-linked fucose, *Cell* **111**, 893–904 (2002).
4 Shi S, Stanley P, Protein *O*-fucosyltransferase 1 is an essential component of Notch signaling pathways, *Proc Natl Acad Sci USA* **100**, 5234–9 (2003).
5 Strahl-Bolsinger S, Gentzsch M, Tanner W, Protein *O*-mannosylation, *Biochim Biophys Acta* **1426**, 297–307 (1999).
6 Timpel C, Strahl-Bolsinger S, Ziegelbauer K, Ernst JF, Multiple functions of Pmt1p-mediated protein *O*-mannosylation in the fungal pathogen *Candida albicans*, *J Biol Chem* **273**, 20837–46 (1998).
7 Spiro RG, Bhoyroo VD, Studies on the carbohydrate of collagens. Characterization of a glucuronic acid-mannose disaccharide unit from *Nereis* cuticle collagen, *J Biol Chem* **255**, 5347–54 (1980).
8 Finne J, Krusius T, Margolis RK, Margolis RU, Novel mannitol-containing oligosaccharides obtained by mild alkaline borohydride treatment of a chondroitin sulfate proteoglycan from brain, *J Biol Chem* **254**, 10295–300 (1979).
9 Endo T, *O*-Mannosyl glycans in mammals, *Biochim Biophys Acta* **1473**, 237–46 (1999).
10 Winder SJ, The complexities of dystroglycan, *Trends Biochem Sci* **26**, 118–24 (2001).
11 Chiba A, Matsumura K, Yamada H, Inazu T, Shimizu T, Kusunoki S, Kanazawa I, Kobata A, Endo T, Structures of sialylated *O*-linked oligosaccharides of bovine peripheral nerve α-dystroglycan. The role of a novel *O*-mannosyl-type oligosaccharide in the binding of α-dystroglycan with laminin, *J Biol Chem* **272**, 2156–62 (1997).
12 Sasaki T, Yamada H, Matsumura K, Shimizu T, Kobata A, Endo T, Detection of *O*-mannosyl glycans in rabbit skeletal muscle α-dystroglycan, *Biochim Biophys Acta* **1425**, 599–606 (1998).
13 Smalheiser NR, Haslam SM, Sutton-Smith M, Morris HR, Dell A, Structural analysis of sequences *O*-linked to mannose reveals a novel Lewis X structure in cranin (dystroglycan) purified from sheep brain, *J Biol Chem* **273**, 23698–703 (1998).
14 Wing DR, Rademacher TW, Schmitz B, Schachner M, Dwek RA, Comparative glycosylation in neural adhesion molecules, *Biochem Soc Trans* **20**, 386–90 (1992).
15 Yuen CT, Chai W, Loveless RW, Lawson AM, Margolis RU, Feizi T, Brain contains HNK-1 immunoreactive *O*-glycans of the sulfoglucuronyl lactosamine series that terminate in 2-linked or 2,6-linked hexose (mannose), *J Biol Chem* **272**, 8924–31 (1997).
16 Chai W, Yuen CT, Kogelberg H, Carruthers RA, Margolis RU, Feizi T, Lawson AM, High prevalence of 2-mono- and 2,6-di-substituted manol-terminating sequences among *O*-glycans released from brain glycopeptides by reductive alkaline hydrolysis, *Eur J Biochem* **263**, 879–88 (1999).
17 Yoshida A, Kobayashi K, Manya H, Taniguchi K, Kano H, Mizuno M, Inazu T, Mitsuhashi H, Takahashi S, Takeuchi M, Herrmann R, Straub V, Talim B, Voit T, Topaloglu H, Toda T, Endo T, Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1, *Dev Cell* **1**, 717–24 (2001).
18 Taniguchi N, Honke K, Fukuda M, *Handbook of Glycosyltransferases and Related Genes* (Springer-Verlag, Tokyo, 2002).
19 Takahashi S, Sasaki T, Manya H, Chiba Y, Yoshida A, Mizuno M, Ishida H, Ito F, Inazu T, Kotani N, Takasaki S, Takeuchi M, Endo T, A new β-1,2-*N*-acetylglucosaminyltransferase that may play a role in the biosynthesis of mammalian *O*-mannosyl glycans, *Glycobiology* **11**, 37–45 (2001).

20 Inamori KI, Endo T, Gu J, Matsuo I, Ito Y, Fujii S, Iwasaki H, Narimatsu H, Miyoshi E, Honke K, Taniguchi N, N-Acetylglucosaminyltransferase IX acts on the GlcNAcβ1,2-Manα1-Ser/Thr moiety, forming a 2,6-branched structure in brain *O*-mannosyl glycan, *J Biol Chem* **279**, 2337–40 (2004).

21 Jurado LA, Coloma A, Cruces J, Identification of a human homolog of the *Drosophila rotated abdomen* gene (*POMT1*) encoding a putative protein *O*-mannosyl-transferase, and assignment to human chromosome 9q34.1, *Genomics* **58**, 171–80 (1999).

22 Willer T, Amselgruber W, Deutzmann R, Strahl S, Characterization of POMT2, a novel member of the *PMT* protein *O*-mannosyltransferase family specifically localized to the acrosome of mammalian spermatids, *Glycobiology* **12**, 771–83 (2002).

23 Manya H, Chiba A, Yoshida A, Wang X, Chiba Y, Jigami Y, Margolis RU, Endo T, Demonstration of mammalian protein *O*-mannosyltransferase activity: Coexpression of POMT1 and POMT2 required for enzymatic activity, *Proc Natl Acad Sci USA* **101**, 500–5 (2004).

24 Girrbach V, Strahl S, Members of the evolutionarily conserved PMT family of protein *O*-mannosyltransferases form distinct protein complexes among themselves, *J Biol Chem* **278**, 12554–62 (2003).

25 Burton EA, Davies KE, Muscular dystrophy–reason for optimism? *Cell* **108**, 5–8 (2002).

26 Taniguchi K, Kobayashi K, Saito K, Yamanouchi H, Ohnuma A, Hayashi YK, Manya H, Jin DK, Lee M, Parano E, Falsaperla R, Pavone P, Van Coster R, Talim B, Steinbrecher A, Straub V, Nishino I, Topaloglu H, Voit T, Endo T, Toda T, Worldwide distribution and broader clinical spectrum of muscle-eye-brain disease, *Hum Mol Genet* **12**, 527–34 (2003).

27 Manya H, Sakai K, Kobayashi K, Taniguchi K, Kawakita M, Toda T, Endo T, Loss-of-function of an *N*-acetylglucosaminyltransferase, POMGnT1, in muscle-eye-brain disease, *Biochem Biophys Res Commun* **306**, 93–7 (2003).

28 Kano H, Kobayashi K, Herrmann R, Tachikawa M, Manya H, Nishino I, Nonaka I, Straub V, Talim B, Voit T, Topaloglu H, Endo T, Yoshikawa H, Toda T, Deficiency of α-dystroglycan in muscle-eye-brain disease, *Biochem Biophys Res Commun* **291**, 1283–6 (2002).

29 Beltrán-Valero de Bernabé D, Currier S, Steinbrecher A, Celli J, van Beusekom E, van der Zwaag B, Kayserili H, Merlini L, Chitayat D, Dobyns WB, Cormand B, Lehesjoki AE, Cruces J, Voit T, Walsh CA, van Bokhoven H, Brunner HG, Mutations in the *O*-mannosyltransferase gene *POMT1* give rise to the severe neuronal migration disorder Walker-Warburg syndrome, *Am J Hum Genet* **71**, 1033–43 (2002).

30 Martin-Blanco E, Garcia-Bellido A, Mutations in the *rotated abdomen* locus affect muscle development and reveal an intrinsic asymmetry in *Drosophila*, *Proc Natl Acad Sci USA* **93**, 6048–52 (1996).

31 Michele DE, Campbell KP, Dystrophin-glycoprotein complex: post-translational processing and dystroglycan function, *J Biol Chem* **278**, 15457–60 (2003).

Received 14 April 2004; accepted 7 May 2004

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 41, Issue of October 8, pp. 42638–42647, 2004
Printed in U.S.A.

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

Received for publication, May 3, 2004, and in revised form, July 12, 2004
Published, JBC Papers in Press, July 22, 2004, DOI 10.1074/jbc.M404900200

Tomomi Ichimiya‡§, Hiroshi Manya¶, Yoshiko Ohmae‡§, Hideki Yoshida‡§, Kuniaki Takahashi§‖, Ryu Ueda§‖, Tamao Endo¶, and Shoko Nishihara‡§**

From the ‡Laboratory of Cell Biology, Department of Bioinformatics, Faculty of Engineering, Soka University, 1-236 Tangi-cho, Hachioji Tokyo 192-8577, Japan, the §Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, 4-1-8 Hon-cho, Kawaguchi, Saitama 332-0012, Japan, the ¶Glycobiology Research Group, Tokyo Metropolitan Institute of Gerontology, Foundation for Research on Aging and Promotion of Human Welfare, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015, Japan, and the ‖Invertebrate Genetics Laboratory, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 441-8540, Japan

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 Sf21 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 PMT4 and PMT2 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*-mannose β1,2-*N*-acetylglucosaminyltransferase-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-

* This work was supported by the Core Research for Evolutional Science and Technology of Japan Science and Technology Agency. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank™/EBI Data Bank with accession numbers AB176550, AB176551, AB176552, and AB176553.

** To whom correspondence should be addressed. Tel.: 81-426-91-8140; Fax: 81-426-91-8140; E-mail: shoko@t.soka.ac.jp.

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

fects 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* phenotypes 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 dPOMTs function 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 (dMGAT1), and GH07804 (dMGAT2) were obtained from Research Genetics (Huntsville, AL). Dolichylphosphate [^{3}H]Man (125,000 dpm/pmol) and UDP-[^{3}H]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 introduced into a transformation vector, pUAST. The cloning procedures will be described elsewhere.[2] Each of the *UAS-dPOMT1-IR* and *UAS-dPOMT2-IR* flies was mated with an *Act5C-GAL4* fly, and F_1 progeny were raised at 25 and 28 °C to observe phenotypes.

Quantitative Analysis of dPOMT1 and dPOMT2 Transcripts by Real-time PCR—Total RNA was extracted from *Act5C-GAL4/UAS-dPOMT1-IR* third instar larvae raised at 25 °C and from *Act5C-GAL4/UAS-dPOMT2-IR* and *Act5C-GAL4/w^{1118}* larvae raised at 28 °C. We could not collect *Act5C-GAL4/UAS-dPOMT1-IR* larvae at 28 °C because of low viability. First-strand cDNA was synthesized by RevaTra Dash (Toyobo, Osaka, Japan), and real-time PCR of the *dPOMT1* and *dPOMT2* transcripts was carried out for the region except for the sequences using the IR construction for the RNAi fly. The gene-specific primers were as follows: *dPOMT1*, 5′-ACACCTGTGGCAACTGCTCTAC-3′ (forward), 5′-ACTTATGGCATGCATCCATAGCT-3′ (reverse), and 5′-ACGCCGGTCTCACCGATCGC (probe); and *dPOMT2*, 5′-TTTCCGGCCTTGATCTTCAA-3′ (forward), 5′-TGGGCAGAACCCTCAAAATG-3′ (reverse), and 5′-TCCTTGCTGACGGGCGTTATGTACAACT-3′ (probe). To normalize the efficiency of cDNA preparation among individual samples, the measurement of *RpL32* mRNA in each cDNA was carried out using the following primers: *RpL32*, 5′-GCAAGCCCAAGGGTATCGA-3′ (forward), 5′-CGATGTTGGGCATCAGATACTG-3′ (reverse), and 5′-AACAGAGTGCGTCGCCGCTTCA-3′ (probe). The probes were labeled at the 5′-end with the reporter dye 3-carboxyfluorescein and at the 3′-end with the quencher dye carboxytetramethylrhodamine (Nippon EGT, Toyama, Japan). Amplifications involved 40 cycles of 94 °C for 30 s and 60 °C for 4 min, performed with an ABI PRISM 7700 sequence detection system (Applied Biosystems).

Vector Construction and Expression of dPOMT1 and dPOMT2 Proteins—The full-length open reading frames (ORFs) of *dPOMT1* and *dPOMT2* were expressed in insect cells according to the Invitrogen GATEWAY™ cloning technology instruction manual. The DNA fragments of *dPOMT1* and *dPOMT2* were amplified by two-step PCR. The first PCR used the plasmid DNA from EST clone RE38203 or LP01681 as a template for dPOMT1 or dPOMT2 amplification and the primer set of dPOMT1 (forward primer, 5′-AAAAAGCAGGCTTGTCTGCCACCTACACCA-3′; and reverse primer, 5′-AGAAAGCTGGGTAGTACAGGTGGTGGTTCTTG-3′) or the primer set of dPOMT2 (forward primer, 5′-AAAAAGCAGGCTTGGCAGCAAGTGTTGTTA-3′; and reverse primer, 5′-AGAAAGCTGGGTCTAGAACTCCCAGGTAGAAAG-3′), respectively. The second PCR used the first PCR product as a template, forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′, and reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′. The amplified fragments were recombined with the pDONR201™ vector (Invitrogen). Each insert was then transferred between the attR1 and attR2 sites of pVL1393g-HA or pVL1393g to yield pVL1393g-*dPOMT1-HA* or pVL1393g-*dPOMT2*, respectively. pVL1393g and pVL1393g-HA are expression vectors derived from pVL1393 (Invitrogen), and pVL1393g-HA contains a fragment encoding the three-HA peptide (YPYDVPDYA) at the C terminus. pVL1393-*dPOMT1-HA* and pVL1393-*dPOMT2* were cotransfected with BaculoGold viral DNA (Pharmingen) into Sf21 insect cells according to the manufacturer's instructions, and the cells were incubated for 5 days at 27 °C to produce recombinant viruses. Sf21 cells were infected with each recombinant virus at a multiplicity of infection of 2.5 and incubated for 96 h to express dPOMT1-HA and dPOMT2 proteins.

Preparation of Rabbit Anti-dPOMT2 Polyclonal Antibody—The N-terminal region (nucleotides 1–279 of the coding sequence) of dPOMT2 was amplified using a forward primer including the EcoRI site (underlined), 5′-GAATTCATGGCAGCAAGTGTTGTT-3′, and a reverse primer including the XhoI site, 5′-CTCGAGTTAGCCCATCTTGCCAAAGTG-3′. The amplified fragment was digested with EcoRI and XhoI and subcloned into pGEX-6P-1 (Amersham Biosciences), an N-terminal glutathione *S*-transferase (GST) fusion vector. A transformant of *Escherichia coli* BL21(DE3) was cultured to A_{600} = 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.

Western Blot Analysis—The Sf21 cells expressing dPOMT1-HA and dPOMT2 were suspended in an 8 M urea solution, and 15 μg of each protein was subjected to 2–15% SDS-PAGE. The separated proteins were transferred to membranes, which were probed with peroxidase-conjugated anti-HA monoclonal antibody (Santa Cruz Biotechnology) and rabbit anti-dPOMT2 polyclonal antibody and stained with HRP-1000 immunostain (Konica, Tokyo, Japan).

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 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 mM benzamidine-HCl, 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/UAS-dPOMT1-IR* flies and *Act5C-GAL4/UAS-dPOMT2-IR* and *Act5C-GAL4/w^{1118}* flies were raised at 25 and 28 °C, respectively. Third instar larvae were homogenized in 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% *n*-octyl-β-D-thioglucoside (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 [^{3}H]Man transferred to GST-α-DG as described previously (18). Briefly, the reaction mixture contained 20 mM Tris-HCl (pH 8.0), 100 nM dolichylphosphate [^{3}H]Man (125,000 dpm/pmol), 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside, 10 μg of GST-α-DG, and enzyme source (40 μg of total protein from the larval extract or 80 μg of microsomal membrane fraction from infected cells) in a total volume of 20 μl. After a 1-h incubation at 22 °C, the reaction was stopped by adding 200 μl of phosphate-buffered saline (130 mM NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO_4) containing 1% Triton X-100 (Nacalai Tesque, Kyoto, Japan), and the reaction mixture was centrifuged at 10,000 × *g* for 10 min. The supernatant was removed, mixed with 400 μl of phosphate-buffered saline containing 1% Triton X-100 and 10 μl of glutathione-Sepharose 4B beads, rotated at 4 °C for 1 h, and washed three times with 20 mM Tris-HCl (pH 7.4) containing 0.5% Triton X-100. The radioactivity adsorbed by the beads was measured using a liquid scintillation counter.

α-Mannosidase Digestion of Mannosyl-GST-α-DG—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 $ZnCl_2$ at 37 °C.

[2] R. Ueda and K. Saigo, manuscript in preparation.

A

```
hPOMT1 ------------------------------------------------------------------------------------------
dPOMT1 MSATYTNTITQRRKTAKVRQQQQHQWTGSDLSGESNERLHFRSRSTNSMQQHTAISNSPSPLCCNGARALTMLNCCVDVNCHLNAPLRGS  90
hPOMT1 ------------------------------------------------------------MWGFLKRPVVVTADINLSLVAL  22
dPOMT1 VNRHTTPTPTPTATPTPVATPKQASPSPTSDRSRSLSRSPSPSRSRSLSCQKQIDKNSAGAASAEERKTANASSQPFTVNLRIDLFSWTL 180
                                                                                . .:*..*.  *:*  :*

hPOMT1 TGWGLLSRLWRLTYPRAVVFDEVYYGQYISFYMKQIFFLDDSGPPFGHMVLALGGYLGGFDGNFLWNRIGAEYSSNVPVWSLRLLPALAG 112
dPOMT1 FLLAFGTRFYKLATPPHIVFDELHYGKYISMYMRNIFFFDQH-PPLGKQLIAGLVSLAGYDGNYTFTRIGEPYSPEMPIFWFRFLPAMCG 269
         :.: :*:::*: *  :****:::**:***:**::***:*:  **:*: ::*   *.*:***: :.***  **.::*:: :*:****:.*

hPOMT1 ALSVPHAYQIVLELHFSHCAAMGAALLMLIENALITQSRLMLLESVLIFFNLLAVLSYLKFFNCQKHSPFSLSWWFWLTLTGVACSCAVG 202
dPOMT1 SLLAPAVYNLLLEAKLSRWSSALGGLLVVLDNSLLTQSRFVLMESMLLLATTVGIACLLRFQRSRLG---SLEWFFTGTAAAVCLGAAGT 356
         :* .* .*:::** ::*: ::  ..**::::*:*:*****::*:**:*:: . :.: . *:* ..:     **.*:*  * :.*. ..*

hPOMT1 IKYMGVFTYVLVLGVAAVHAWHLLGDQTLSNVGADVQCCMRPACMGQMRMSQGVCVFCHLLARAVALLVIPVVLYLLFFYVHLILVFRSG 292
dPOMT1 VKYVGFLALGLAAFYLLCRHLWQLLYDAGLTDR----------QLWM--------HAISRLLIFVGIPLAVYLGVFYIHFKTLHRAG 424
         :**:*.::  *.: : . * *:** *  *::              *: *       * ::* : :: **:.:** .**:*:  :.*:*

hPOMT1 PHDQIMSSAFQASLEGGLARITQGQPLEVAFGSQVTLRNVFGKPVPCWLHSHQDTYPMIYENGRGSSHQQQVTCYPFKDVNNWWIVKDPR 382
dPOMT1 PHDSIMTIAFQASLDGGLASITKGQPLAVVHGSQITLRHTHGR--TCWLHSHAAVYPVRYPDKRGSSHQQQVTCYSFKDVNNWWLVKRPT 512
         ***.**: ******:**** **:**** *..***:***:..*:  .****** ..**: * : ************.********:** *

hPOMT1 RHQLVVSSPPRPVRHGDMVQLVHGMTTRSLNTHDVAAPLSPHSQEVSCYIDYNISMPAQNLWRLEIVNRGSDTDVWRTILSEVRFVHVNT 472
dPOMT1 KENLVVGDEPDIIRHGEIIQLVHGITSRALNSHDVAAAMTPQCQEVSCYIDYEIKMAGELLWRVEILNRDSEGDIWHAIKSEVRLVHVST 602
         :.:***.. *  :***:::******:*:*:**:*****.::*:.*********:*.*..: ***:**:**.*: *:*::* ****:***.*

hPOMT1 SAVLKLSGAHLPDWGYRQLEIVGEKLSRGYHGSTVWNVEEHRYGASQEQRERRRELHSPAQVDVSRN-LSFMARFSKLQWRMLALRSDDS 561
dPOMT1 EASLKFSGRQLPEWGFNQHEVVADRE-KAIHEDAIWNVEEHRYTQTEDHRERRRQMLTAEMIPTKRTRISFWAKLLELQSKMLFQTKSVP 691
         .* **:** :**:**:.* *:*.::  :. * .::******** ::::******:: :.  : ..*. :** *:: *** :**   .. .

hPOMT1 EHKYSSSPLEWVTLDTNIAYWLHPRTSAQIHLLGNIVIWVSGSLALAIYALLSLWYLLRRRRNVHDLPQDAWLRWVLAGALCAGGWAVNY 651
dPOMT1 NHMYSSMPHEWPLMDKGIAYWLDSQSSAQIYLLGNILLWYTATMGILVYAGLLAFYAMRRQRLCFDISEQEWQRFVLAGDTFFMGYVMHY 781
         :* *** * **  :*..*****..::****:*****::* :.::.: :** *  :* :**:*  .*:.:: * *:****     *:.::*

hPOMT1 LPFFLMEKTLFLYHYLPALTFQILLLPVVLQHISDHLCRSQLQR-----SIFSALVVAWYSSACHVSNTLRPLTYGDKSLSPHRLKALRW 736
dPOMT1 IPYFCVDRTLFLHNYLPAFVFRLLLLCFVVEHLDYLLRRFCTGRGVHLVRLYRLMLILWLVGVLSIFSKFIPFSYGARKMTLNEVRSLRW 871
         :*:* ::::*****:*****:.*::*** .*::*:.  * *    *      ::  ::: * ..  : ..: *::** :.:: :*:::***

hPOMT1 KDSWDILIRKH----- 747
dPOMT1 KDTWDFVLHKNHHLY 886
         **:**::::*:
```

B

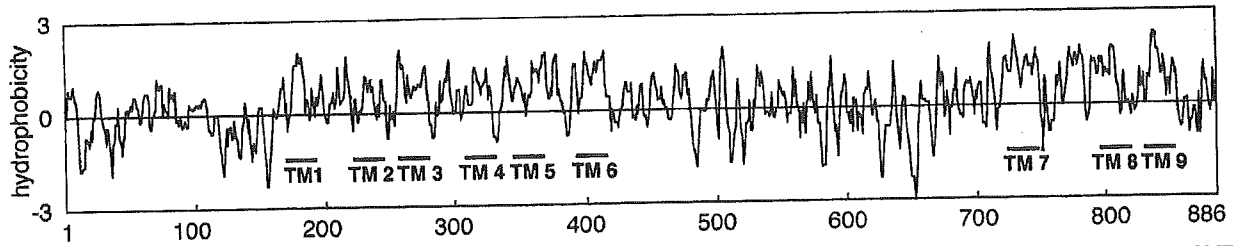


FIG. 1. **Comparison of *Drosophila*, human, and *S. cerevisiae* POMTs.** *A* and *C*, ClustalW alignment of the *Drosophila* and human POMT1 and POMT2 amino acid sequences, respectively. The *asterisks* indicate identical amino acids. The *colons* indicate conserved amino acids defined by a score of >0.5. The *periods* indicate conserved amino acids defined by a score of <0.5. The *underlined* amino acids are putative transmembrane regions obtained using the SOSui program developed by Mitsui Knowledge Industry Co., Ltd. *B* and *D*, hydrophobicity plots of dPOMT1 and dPOMT2, respectively. The *bars* indicate putative transmembrane (*TM*) regions. *E*, ClustalX phylogenetic tree of dPOMT1, dPOMT2, hPOMT1, hPOMT2, and *S. cerevisiae* Pmt1–Pmt6. The branch lengths indicate amino acid substitutions per site.

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.

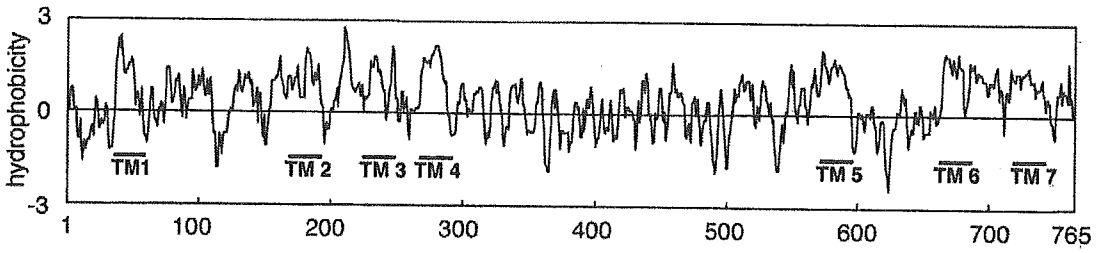
Whole Mount in Situ Hybridization—The BglII-EcoRI-digested fragment of *dPOMT1* and the Ecl136II-NotI-digested fragment of *dPOMT2* were subcloned into pBluescript SK⁻ (Stratagene) digested by BamHI-EcoRI and SmaI-NotI, respectively. The above templates were linearized and transcribed *in vitro* by T3 or T7 RNA polymerase with a digoxigenin RNA labeling mixture (Roche Applied Science). Each transcript was treated with an alkaline solution containing 80 mM $NaHCO_3$, 120 mM Na_2CO_3, 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× SSC (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 mM dithiothreitol. After hybridization, the embryos were washed with 50% formamide, 5× SSC, and 0.1% Tween 20 for 20 min. The process of washing was continued by serial dilution from 50% formamide, 5× SSC, and 0.1% Tween 20 to phosphate-buffered saline containing 0.1% Tween 20. Detection was carried out by immunoassay 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 κ (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

C

```
hPOMT2 MPPATGGGLAESELRPRRGRCGPQAARAAGRDVAAEAVARSPKRPAWGSRRFEAVGWWALLALVTLLSFATRFHRLDEPPHICWDETHFG  90
dPOMT2 ----MAASVVKTPKCPRRG--------------SVKDVAQNAPRTAPTSSKEANWNWWLLLATVFLVTFATRFYKVTEPDHICWDETHFG  72
hPOMT2 KMGSYYINRTFFFDVHPPLGKMLIGLAGYLSGYDGTFLFQKPGDKYENHSYMGMRGFCAFLGSWLVPFAYLTVLDLSKSLSAALLTAALL 180
dPOMT2 KMGSWYINRTFFFDVHPPLGKMLIGLSGYLTGYNGTFPFEKPGDKYNETRYQGMRYFCTTLGALIMPMGPDTVYDLTRSHEAALLAAAYL 162
hPOMT2 TFDTGCLTLSQYILLDPILMFFIMAAMLSMVKYN--SCADRPFSAPWWFWLSLTGVSLAGALGVKFVGLFIILQVGLNTIADLWYLFGDL 268
dPOMT2 IFDVGLLTLNQYILLDPILLFFMMASVWGMVKVSKSTASGGSYGLRWWLWLFLTGTMLSCTISVKFVGLFVVLLVGLHTATELWLILGDL 252
hPOMT2 SLSLVTVGKHLTARVLCLIVLPLALYTATFAVHFMVLSKSGPGDGFFSSAFQARLSGNNLHNASIPEHLAYGSVITVKNLRMAIGYLHSH 358
dPOMT2 GQPILETVKQLACRAITLIVWPVLLYILFFYIHLSVLNRSGNGDGPYSSAFQSRLIGNSLYNASMPRDVAYGSLVTIKNRKTGGGYLHSH 342
hPOMT2 RHLYPEGIGARQQQVTTYLHKDYNNLWIIKKHNTNSDPLDPSFPVEPVRHGDIIRLEHKETSRNLHSHYHEAPMTRKHYQVTGYGINGTG 448
dPOMT2 HHLYPKGSGARQQQVTTYTHKDENNKWLIRPHNK---PGPPKGKVQILRHGDLVRLTHMATRRNLHSHNEPAPMTKKHLQVTGYGELGLG 429
hPOMT2 DSNDFWRIEVVNRKFGNRIKVLRSRIRFIHLVTGCVLGSSGKVLPKWGWEQLEVTCTPYLKETLNSIWNVEDHINPKLPNISLDVLQPSF 538
dPOMT2 DANDVWRVLIVGGKVNETVHTVTSRLKFIHLLQNCALTSSGKQLPKWGFEQQEVSCNPNVRD-KNSQWNVEDNEHKLMPSVSFSVYAPGF 518
hPOMT2 PEILLESHMVMIRGNSGLKPKDNEFTSKPWHWPINYQGLRFSGVNDTDFRVYLLGNPVVWWLNLLSIALYLLSGSIIAVAMQRGARLPAE 628
dPOMT2 FARFLESHAVMLQGNAGLKPKEGEVTSRPWQWPINYRGQPFSGS---SYRIYLLGNPLIWWSNLVFLALFVTVFLCNAVVQQRRAGFARS 605
hPOMT2 VAG-----------------------------------------------------LSQVLLRGGGQVLLGWTLHYFPPFLMGRVLYFHHYFPAML 671
dPOMT2 AAQNQAQVPDSETVAQDEESEHSTTDICSCCTPAKEIVPKAVPSGSPEAPNPAQSLRAAAWLFLGWMLHYLPFWANGRVLYFHHYFPALI 695
hPOMT2 FSSMLTGILWDTLLRLCAWGLASWPLARGIHVAGILSLLLGTAYSFYLFHPLAYGMVGPLAQDPQSPMAGLRWLDSWDF 750
dPOMT2 FNSLLTGVMYNYILR----------VLPKWIHHVILGLVLSILVYSFAAFSPLAYGMSGPLANEPNSTMYNLKWLSTWEF 765
```

D



E

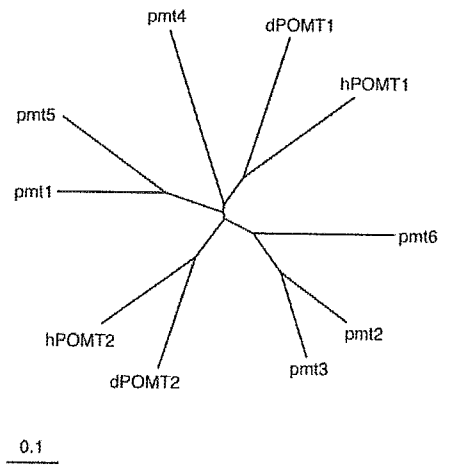


FIG. 1—*continued*

(Sigma). 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 [^{3}H]GlcNAc transferred to a mannosylpeptide (Ac-Ala-Ala-Pro-(Thr/Man)-Pro-Val-Ala-Ala-Pro-NH_2) as described pre-

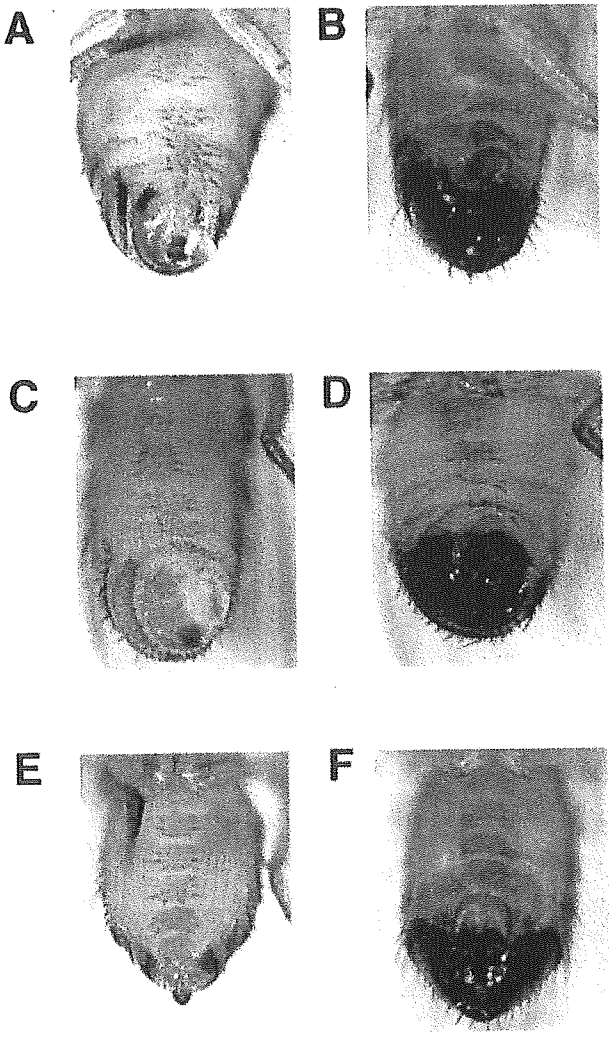


FIG. 2. **Twisted abdomen phenotypes of the *dPOMT1* and *dPOMT2* RNAi mutant flies.** The phenotypes of the *Act5C-GAL4/UAS-dPOMT1-IR* RNAi fly (*A* and *B*), *Act5C-GAL4/UAS-dPOMT2-IR* RNAi fly (*C* and *D*), and CantonS fly (*E* and *F*). The *UAS-dPOMT-IR* fly has a transgene containing an IR of the target gene ligated to the UAS promoter, a target of GAL4. In the F_1 progeny of these flies, the double-stranded RNA of the target gene, *dPOMT*, is expressed ubiquitously in all cells under *Act5C* promoter control to induce gene silencing. *A*, *C*, and *E* are female flies. *B*, *D*, and *F* are male flies. *Act5C-GAL4/UAS-dPOMT1-IR* and *Act5C-GAL4/UAS-dPOMT2-IR* flies were raised at 25 and 28 °C, respectively. Each RNAi line had the twisted abdomen phenotype, twisted clockwise 30–60°, as viewed from behind.

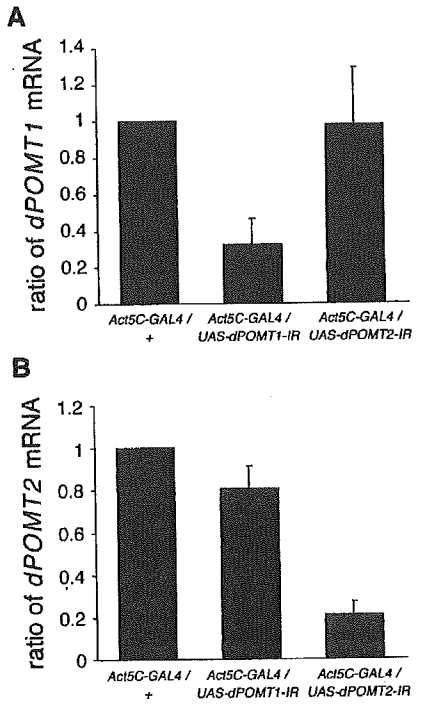


FIG. 3. **Quantitative analysis of *dPOMT1* and *dPOMT2* mRNAs in each *dPOMT1* and *dPOMT2* RNAi mutant fly by real-time PCR.** *A* and *B*, *dPOMT1* and *dPOMT2* transcript levels of *Act5C-GAL4/UAS-dPOMT1-IR* and *Act5C-GAL4/UAS-dPOMT2-IR* RNAi flies of third instar larvae raised at 25 and 28 °C, respectively, were determined by real-time PCR. *Act5C-GAL4/+* (w^{1118} crossed with the *Act5C-GAL4* fly) corresponds to the wild type. The actual amounts of *dPOMT1* and *dPOMT2* transcripts were divided by that of the *RpL32* transcript for normalization.

viously (20). Briefly, the reaction mixture contained 140 mM MES (pH 7.0), 400 μM UDP-[^{3}H]GlcNAc (400,000 dpm/nmol), 400 μM mannosylpeptide, 10 mM $MnCl_2$, 2% Triton X-100, 5 mM AMP, 200 mM GlcNAc, 10% glycerol, and 50 μg of microsomal membrane fraction in a total volume of 50 μl. After a 2-h incubation at 37 °C, the peptide was separated on a Wakopak 5C18-200 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.

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 $MnCl_2$, 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 (PA017) was obtained from Takara Shuzo (Otsu, Japan). After a 2-h incubation at 37 °C, the product was separated on a Cosmosil 5C18-AR column (4.6 × 250 mm; Nacalai Tesque). The solvent was 0.15% 1-butanol in 100 mM ammonium acetate (pH 6.0). The product was eluted at a rate of 1.2 ml/min at 45 °C by isocratic elution and was detected by fluorescence of the PA (excitation at 320 nm and emission at 400 nm).

The β1,2-*N*-acetylglucosaminyltransferase II activity was measured as follows. The reaction mixture contained 50 mM MES (pH 6.0) 100 mM GlcNAc, 100 mM NaCl, 5 mM AMP, 0.2% bovine serum albumin, 20 mM $MnCl_2$, 1 mM UDP-GlcNAc, 10 μM pyridylaminated oligosaccharide, 1% Triton X-100, and recombinant enzyme in a total volume of 10 μl. The acceptor oligosaccharide Manα1–6(GlcNAcβ1–2Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc-PA (PA100.2) was obtained from Seikagaku Corp. After a 2-h incubation at 37 °C, the product was separated as described above, except that the temperature was 50 °C.

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 POMT* genes, *dPOMT1* and *dPOMT2*, whose *Drosophila* EST clones are RE38203 and LP01681, respectively. RE38203 contains a 2658-bp ORF encoding a dPOMT1 protein of 886 amino acids (GenBank™/EBI accession number AB176550), and LP01681 contains a 2295-bp ORF encoding a dPOMT2 protein of 765 amino acids (GenBank™/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. 1*E*). 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. F_1 escapers of *Act5C-GAL4/UAS-dPOMT1-IR* showed a clockwise twisted abdomen phenotype, after which the gene was named *rotated abdomen* (*rt*). Interestingly, all escaper flies of *Act5C-GAL4/UAS-dPOMT2-IR* also exhibited the same phenotype, suggesting that these two *POMT* 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. 3*A*). The *dPOMT2* transcript level of the *Act5C-GAL4/UAS-dPOMT2-IR* fly raised at 28 °C was 21% (Fig. 3*B*). 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 *+/+;rt^P/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. 4*C*). Also, one copy of the *dPOMT1* mutation (*rt^P*) gave no twisted abdomen phenotype because of the recessive character of this allele (Fig. 4*B*). However, the resulting *Act5C-GAL4/+;rt^P/UAS-dPOMT2-IR* fly showed a clear twisted abdomen phenotype (Fig. 4*A*), 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.

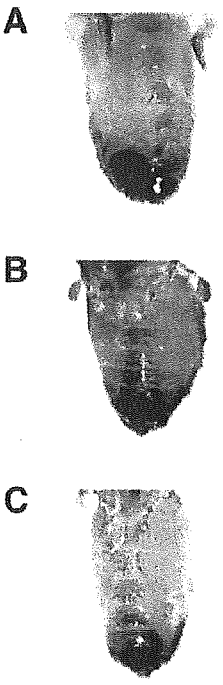


FIG. 4. **Genetic interaction between *dPOMT1* and *dPOMT2*.** The *Act5C-GAL4/SM1;UAS-dPOMT2-IR/TM6B* fly was crossed with the *+/+;rt^P/TM3* fly. Progenies of this cross were grown at 18 °C. *A*, *Act5C-GAL4/+;rt^P/UAS-dPOMT2-IR* male fly; *B*, *Act5C-GAL4/+;rt^P/TM6B* male fly; *C*, *Act5C-GAL4/+;UAS-dPOMT2-IR/TM3* male fly. The *Act5C-GAL4/+;rt^P/UAS-dPOMT2-IR* fly (*A*) showed a twisted abdomen phenotype, whereas the *Act5C-GAL4/+;rt^P/TM6B* fly (*B*) and the *Act5C-GAL4/+;UAS-dPOMT2-IR/TM3* fly (*C*) showed no aberrant phenotypes.

POMT Activity of dPOMT1 and dPOMT2—We prepared recombinant dPOMT1 and dPOMT2 to identify POMT activity. pVL1393-*dPOMT1-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. 5*C*). We then characterized the linkage of the mannosyl residue to α-DG by α-mannosidase digestion (Fig. 5*D*). The radioactive reaction product of dPOMT1 and dPOMT2 absorbed to glutathione-Sepharose 4B beads released the radioactivity to the supernatant, indicating that a mannosyl 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.

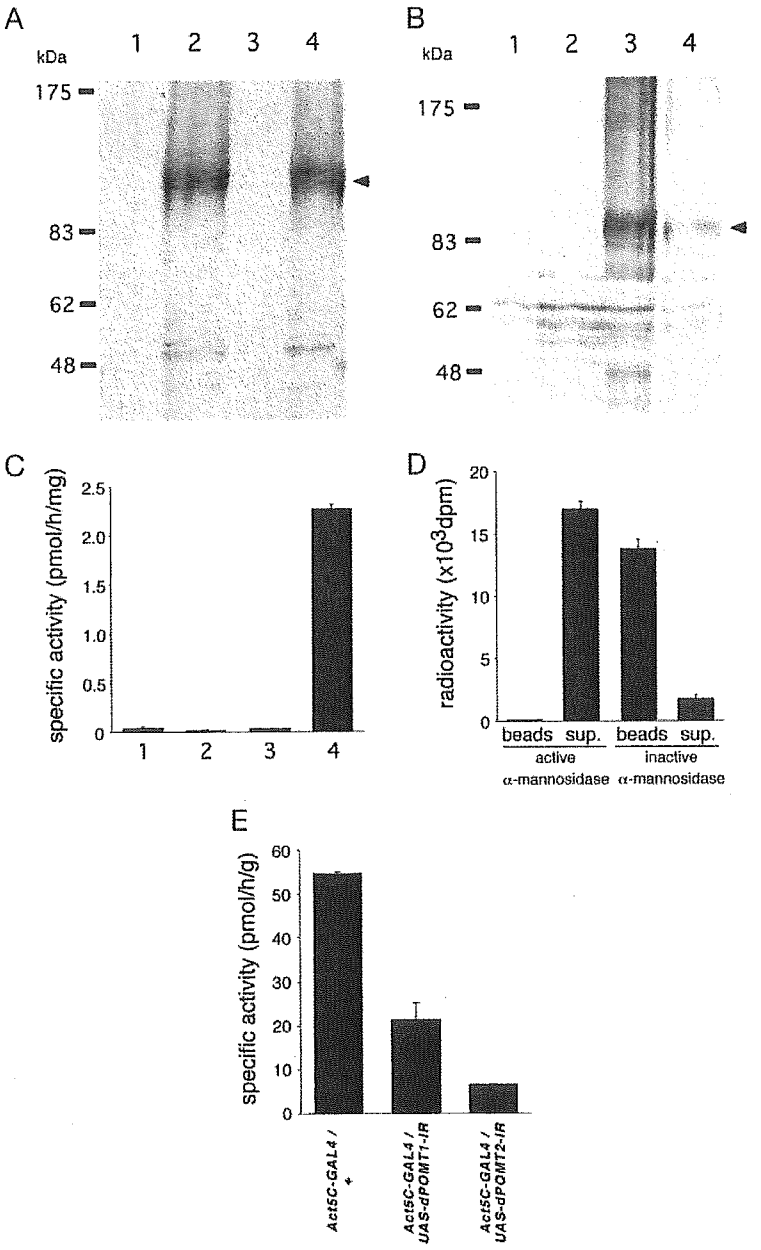


FIG. 5. **POMT activity of recombinant dPOMT1 and dPOMT2 and RNAi mutant flies.** *A* and *B*, Western blot analysis of HA-tagged dPOMT1 using anti-HA monoclonal antibody and of dPOMT2 using anti-dPOMT2 antibody, respectively. The prepared microsomal membrane fractions of infected cells were applied at 15 μg to a 2–15% gradient gel. *Lanes 1*, transfectants with vector alone; *lanes 2*, transfectants with *dPOMT1-HA*; *lanes 3*, transfectants with *dPOMT2*; *lanes 4*, cotransfectants with *dPOMT1-HA* and *dPOMT2*. *C*, POMT activity for GST-α-DG of recombinant dPOMT1-HA and dPOMT2. *Bar 1*, transfectants with vector alone; *bar 2*, transfectants with *dPOMT1-HA*; *bar 3*, transfectants with *dPOMT2*; *bar 4*, cotransfectants with *dPOMT1-HA* and *dPOMT2*. *D*, α-mannosidase digestion of mannosyl-GST-α-DG, the reaction product obtained by recombinant dPOMT1-HA and dPOMT2. Glutathione-Sepharose 4B beads bearing [^{3}H]mannosyl-GST-α-DG were incubated with active and inactive jack bean α-mannosidase. The radioactivity was released to the supernatant (*sup.*) by α-mannosidase. *E*, POMT activity for GST-α-DG of *dPOMT1* and *dPOMT2* RNAi flies. Third instar larvae of RNAi flies (*Act5C-GAL4/UAS-dPOMT1-IR* and *Act5C-GAL4/UAS-dPOMT2-IR*) were prepared at 25 and 28 °C, respectively, for the assay of POMT activity.

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/+* control fly in proportion to the reduction in each transcript level (Fig. 5*E*). These results also support that dPOMTs 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. 6*C*). 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. 6*D*). 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, Siaα2–3Galβ1–4GlcNAcβ1–2Manα1-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*

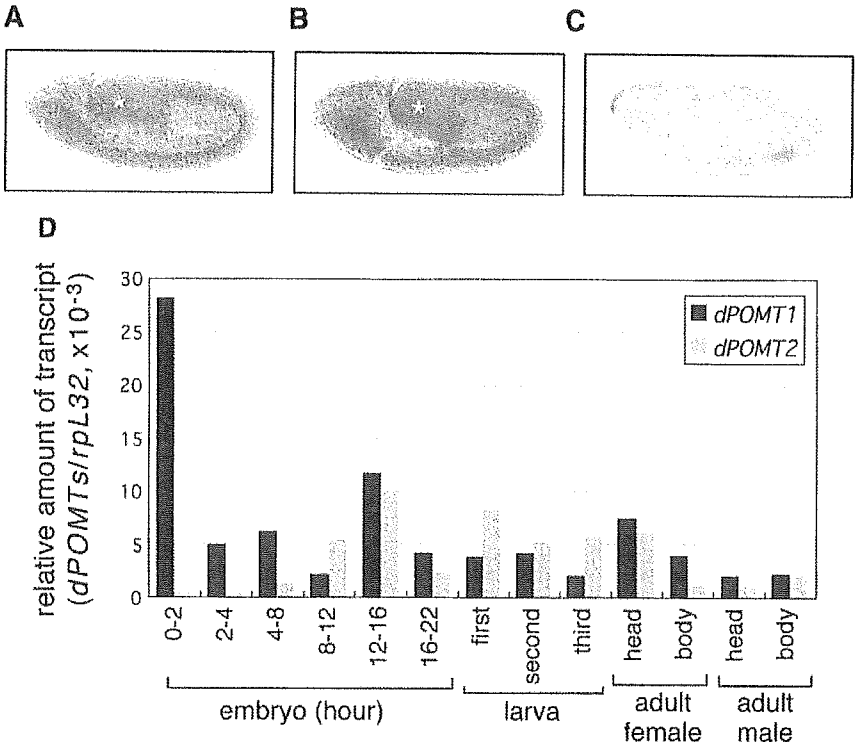


FIG. 6. **Expression of *dPOMT1* and *dPOMT2* mRNAs *in vivo*.** *A–C*, whole mount *in situ* hybridization at stage 10 with digoxigenin-labeled RNA probes: antisense *dPOMT1*, antisense *dPOMT2*, and sense *dPOMT2*, respectively. The staining carried out using the antisense *dPOMT1* and antisense *dPOMT2* probes gave a very similar pattern in that the germ band and invaginating gut (*asterisks*) were remarkably stained. *D*, quantitative 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* transcript 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* mannose 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. 7*A*). 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 Siaα2–3Galβ1–4GlcNAcβ1–2Manα1-Ser/Thr found in humans is absent in *Drosophila* because there is no POMGnT (Fig. 7*D*).

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 clockwise 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 chromosome. This region is included in the area 1C3~D4, where *twisted* (*tw*) mutants have been mapped genetically (25). The *tw* mutants show abdomens twisted ~30° clockwise as viewed from the posterior and reduced viability. Considering the similarity 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. 5*C*) and RNAi flies (Fig. 5*E*) 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 combination of interacting molecules might have changed during evolution.

Experiments with *dPOMT1* and *dPOMT2* RNAi flies revealed that both dPOMTs are indispensable for muscle development. *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 Manα1-Ser/Thr with a β1,2-linkage, has been reported to induce muscle-eye-brain disease (14, 15). As shown in Fig. 7*A*, *Drosophila* does not have any orthologs of

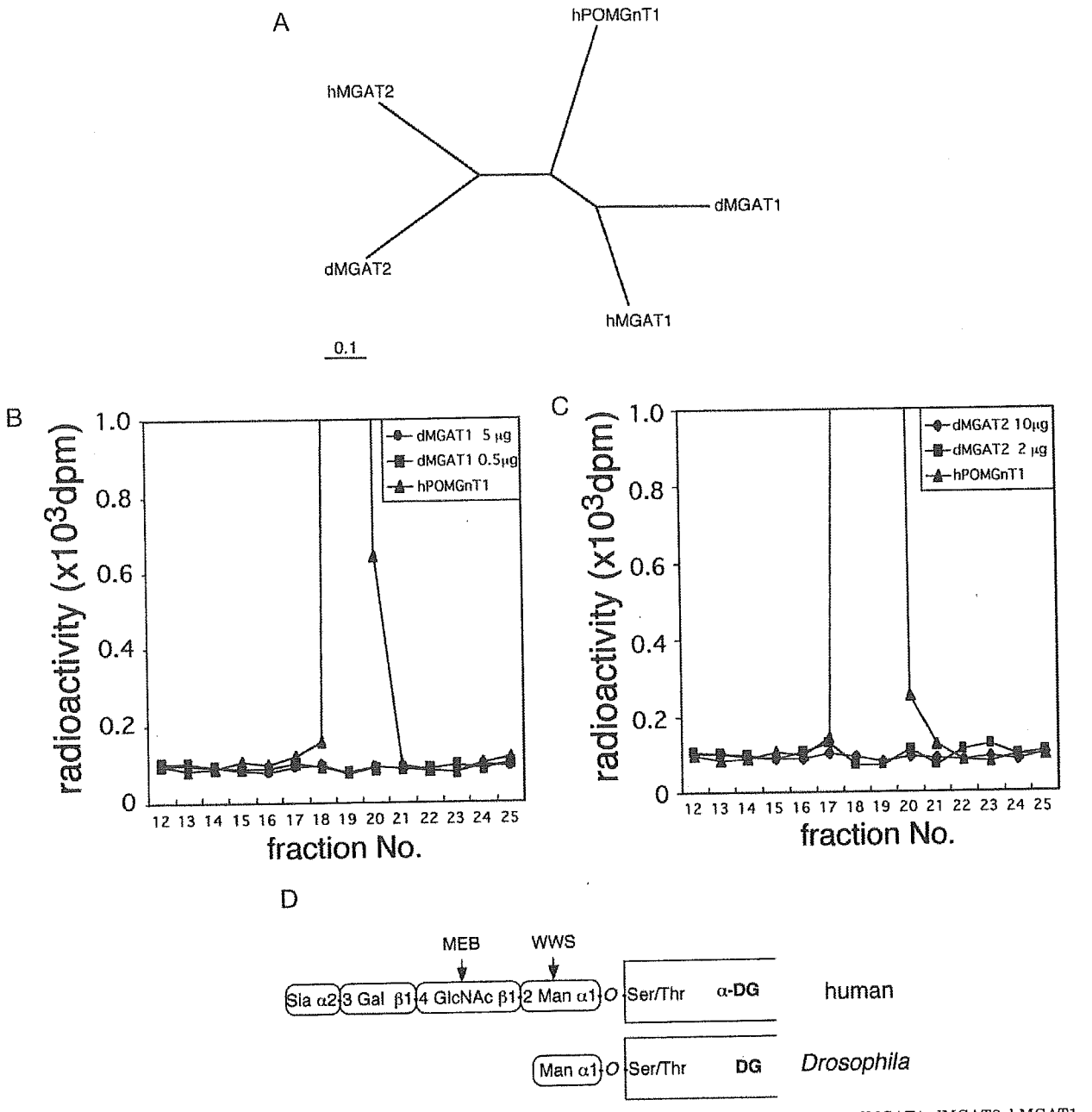


FIG. 7. **Examination of the POMGnT activities of dMGAT1 and dMGAT2.** *A*, ClustalX phylogenetic tree of dMGAT1, dMGAT2, hMGAT1, hMGAT2, and hPOMGnT1. The branch lengths indicate amino acid substitutions per site. *B* and *C*, POMGnT activity of recombinant FLAG-tagged dMGAT1 and FLAG-tagged dMGAT2, respectively. Each recombinant protein was prepared and assayed for POMGnT activity based on the amount of [^{3}H]GlcNAc transferred to a mannosylpeptide (Ac-Ala-Ala-Pro-(Thr/Man)-Pro-Val-Ala-Ala-Pro-NH_2). *D*, hypothetical model of the human and *Drosophila* *O*-linked mannosylglycan structure on dystroglycan (*DG*). *MEB*, muscle-eye-brain disease; *WWS*, Walker-Warburg syndrome.

hPOMGnT, suggesting that it does not possess any extended *O*-mannosylglycans. Moreover, the other mannose β1,2-*N*-acetylglucosaminyltransferases, dMGAT1 and dMGAT2, did not show any β1,2-*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

TABLE I
β1,2-N-acetylglucosaminyltransferase I and II activities of dMGAT1 and dMGAT2

Substrate	Specific activity	
	dMGAT1	dMGAT2
	pmol/h/μg	
Manα1–6(Manα1–3)Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc-PA	55.0	ND[a]
Manα1–6(GlcNAcβ1–2Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc-PA	ND	11.7

[a] Not determined.

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.

Acknowledgments—We thank H. Takemae, M. Suzuki, R. Okubo, K. Ohtsu, W. Awano, R. Tatsumi, R. Shimamura, A. Doi, and M. Taniguchi for technical assistance. We are grateful to Dr. S. Goto (Mitsubishi Kagaku Institute of Life Science) for helpful advice and discussion.

REFERENCES

1. Ecker, M., Mrsa, V., Hagen, I., Deutzmann, R., Strahl, S., and Tanner, W. (2003) *EMBO Rep.* **4,** 628–632
2. Lommel, M., Bagnat, M., and Strahl, S. (2004) *Mol. Cell. Biol.* **24,** 46–57
3. Bourdineaud, J. P., van der Vaart, J. M., Donzeau, M., de Sampaio, G., Verrips, C. T., and Lauquin, G. J. (1998) *Mol. Microbiol.* **27,** 85–98
4. Harty, C., Strahl, S., and Romisch, K. (2001) *Mol. Biol. Cell* **12,** 1093–1101
5. Sanders, S. L., Gentzsch, M., Tanner, W., and Herskowitz, I. (1999) *J. Cell Biol.* **145,** 1177–1188
6. Zakrzewska, A., Migdalski, A., Saloheimo, M., Penttila, M. E., Palamarczyk, G., and Kruszewska, J. S. (2003) *Curr. Genet.* **43,** 11–16
7. Girrbach, V., and Strahl, S. (2003) *J. Biol. Chem.* **278,** 12554–12562
8. Girrbach, V., Zeller, T., Priesmeier, M., and Strahl-Bolsinger, S. (2000) *J. Biol. Chem.* **275,** 19288–19296
9. Lussier, M., Gentzsch, M., Sdicu, A. M., Bussey, H., and Tanner, W. (1995) *J. Biol. Chem.* **270,** 2770–2775
10. Martin-Blanco, E., and Garcia-Bellido, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6048–6052
11. Willer, T., Amselgruber, W., Deutzmann, R., and Strahl, S. (2002) *Glycobiology* **12,** 771–783
12. Beltran-Valero de Bernabe, D., Currier, S., Steinbrecher, A., Celli, J., van Beusekom, E., van der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W. B., Cormand, B., Lehesjoki, A. E., Cruces, J., Voit, T., Walsh, C. A., van Bokhoven, H., and Brunner, H. G. (2002) *Am. J. Hum. Genet.* **71,** 1033–1043
13. Endo, T., and Toda, T. (2003) *Biol. Pharm. Bull.* **26,** 1641–1647
14. Manya, H., Sakai, K., Kobayashi, K., Taniguchi, K., Kawakita, M., Toda, T., and Endo, T. (2003) *Biochem. Biophys. Res. Commun.* **306,** 93–97
15. Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., Talim, B., Voit, T., Topaloglu, H., Toda, T., and Endo, T. (2001) *Dev. Cell* **1,** 717–724
16. Endo, T. (1999) *Biochim. Biophys. Acta* **1473,** 237–246
17. Chiba, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusunoki, S., Kanazawa, I., Kobata, A., and Endo, T. (1997) *J. Biol. Chem.* **272,** 2156–2162
18. Manya, H., Chiba, A., Yoshida, A., Wang, X., Chiba, Y., Jigami, Y., Margolis, R. U., and Endo, T. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 500–505
19. Bridges, C. D., and Morgan, T. (1923) *The Third Chromosome Group of Mutant Characters of Drosophila melanogaster*, pp. 1–251, Carnegie Institute of Washington, Washington, D. C.
20. Takahashi, S., Sasaki, T., Manya, H., Chiba, Y., Yoshida, A., Mizuno, M., Ishida, H., Ito, F., Inazu, T., Kotani, N., Takasaki, S., Takeuchi, M., and Endo, T. (2001) *Glycobiology* **11,** 37–45
21. Sarkar, M., and Schachter, H. (2001) *Biol. Chem.* **382,** 209–217
22. Tsitilou, S. G., and Grammenoudi, S. (2003) *Biochem. Biophys. Res. Commun.* **312,** 1372–1376
23. Kamiyama, S., Suda, T., Ueda, R., Suzuki, M., Okubo, R., Kikuchi, N., Chiba, Y., Goto, S., Toyoda, H., Saigo, K., Watanabe, M., Narimatsu, H., Jigami, Y., and Nishihara, S. (2003) *J. Biol. Chem.* **278,** 25958–25963
24. Takemae, H., Ueda, R., Okubo, R., Nakato, H., Izumi, S., Saigo, K., and Nishihara, S. (2003) *J. Biol. Chem.* **278,** 15571–15578
25. Demerec, M., Kaufmann, B. P., Fano, U., Sutton, E., and Sansome, E. R. (1942) *The Gene* Vol. 41, pp. 190–199, Carnegie Institute of Washington, Washington, D. C.
26. Jurado, L. A., Coloma, A., and Cruces, J. (1999) *Genomics* **58,** 171–180
27. Finne, J., Krusius, T., Margolis, R. K., and Margolis, R. U. (1979) *J. Biol. Chem.* **254,** 10295–10300
28. Winder, S. J. (2001) *Trends Biochem. Sci.* **26,** 118–124
29. Deng, W. M., Schneider, M., Frock, R., Castillejo-Lopez, C., Gaman, E. A., Baumgartner, S., and Ruohola-Baker, H. (2003) *Development (Camb.)* **130,** 173–184