N-Acetylgalactosaminyltransferase IX Acts on the GlcNAcβ1,2-Manα1-Ser/Thr Moiety, Forming a 2,6-Branchected Structure in Brain O-Mannosyl Glycer*

Received for publication, October 31, 2003, and in revised form, November 13, 2003
Published, JBC Papers in Press, November 14, 2003, DOI 10.1074/jbc.C300489200

Kei-ichiro Inamori†, Takeshi Endo*, Jianguo Gu‡, Ichiro Matsuoka, Yukishige Ito†, Shigero Fujii‡, Hiroko Iwasaki†, Hisashi Narimatsu†, Eiji Miyoshi‡, Koichi Honke‡‡, and Naoyuki Taniguchi†††

From the †Department of Biochemistry, Osaka University Medical School, Osaka, Japan and the ‡Laboratory of Chemistry, Kansai Medical University, Osaka, Japan and the ‡‡Laboratory of Glycobiology, National Institute of Advanced Industrial Science and Technology, Open Space Laboratory C-2, 1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan, and the †††Department of Molecular Genetics, Kochi Medical School, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan

Mammals contain O-linked mannosic residues with 2-mono- and 2,6-di-substitutions by GlcNAc in brain glycoproteins. It has been demonstrated that the transfer of GlcNAc to the 2-OH position of the mannosic residue is catalyzed by the enzyme, protein β1,2-N-acetylgalactosaminyltransferase (POMGnT1), but the enzymatic basis of the transfer to the 6-OH position is unknown. We recently reported on a brain-specific protein O-linked mannosic residue in the presence of POMGnT1. Collectively, these results strongly suggest that POMGnT1 is the first β1,2-N-acetylgalactosaminyltransferase that is responsible for the formation of the 2,6-branched structure in the brain O-mannosyl glycer.

Nearly all secreted and cell surface proteins in mammalian cells are glycosylated, and two major groups on glycoproteins are N- and O-glycans. In O-glycans, the mucin-type O-GlcNAc linkage and O-xylene linkage in proteoglycans are well known. In addition to these O-glycans, several other unique linkages such as O-linked fucose (1–3), O-linked glucose (2, 4), O-linked GlcNAc (5, 6), and O-linked mannosic (7) have also been reported. O-Mannosylation is one of the rare types of O-glycosylation in mammals, existing in a limited number of glycoproteins of brain, nerve, and skeletal muscle (7). One of the obvious functional roles of O-mannosylation has been shown to involve a sialyl O-mannosyl glycer, Siaβ2,3Galβ1,4GlcNAcβ1,2-Man, which is the laminin binding ligand of α-dystroglycan (8). Recently, protein O-mannose β1,2-N-acetylgalactosaminyltransferase (POMGnT1), which catalyzes the transfer of GlcNAc to O-linked mannosic glycans, was identified and the POMGnT1 gene was demonstrated to be responsible for muscle-eye-brain disease (MEB) (9, 10). MEB is congenital muscular dystrophy caused by a defect in the binding activity of dystroglycan to its ligands, including laminin, neurexin, and agrin due to the hypoglycosylation of α-dystroglycan (11).

Interestingly, O-mannosyl glycer is one of major O-glycans in the brain (its ratio to the O-linked GalNAc is about 1:3), which contains GlcNAcβ1,2-Man and GlcNAcβ1,2-GlcNAcβ1,6-Man structures (12). In addition, the brain contains the HNK-1 epitope (sulfoglucuronyl lactosamine) carried on O-mannosyl glycer that contains 2-mono- and 2,6-di-substituted mannose (13).

This paper is available on line at http://www.jbc.org

2337
We recently identified a novel β1,6-N-acetylglucosaminyltransferase, GnT-IX, as a homolog of N-acetylglucosaminyltransferase V (GnT-V), which is specifically expressed in the brain (14). GnT-IX was found to catalyze the transfer of GlcNAc to the 6-OH position of the mannose in the sequence of GlcNAcβ1,2-Manα, present in both the α1,3- and α1,6-linked mannose arms in the core structure of N-glycan. In the present study, we provide evidence to show that GnT-IX also catalyzes the transfer of GlcNAc in β1,6-linkage to O-mannosyl glycan, indicating that GnT-IX is responsible for the formation of the 2,6-branched structure of O-mannosyl glycans in the brain.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ser-linked mannos-containing saccharides, Manα1-O-Ser, GlcNAcβ1,2-Manα1-O-Ser, and Galβ1,4-GlcNAcβ1,2-Manα1-O-Ser were synthesized as reported previously (15). GlcNAcβ1,2-Manα1-O-Ser was fluorescence-labeled with N-[2-(2-pyridylaminooethyl)lysine]succinamidic acid 5-norbornene-2,3-dicarboxyimide ester (Wako, Osaka, Japan) and the labeled substrate (GnM-S-PAES) was purified by TSKgel Amide-80 column (21.5 × 300 mm; Tosoh) and TSKgel ODS-80TM column (7.8 × 300 mm; Tosoh) using a Shimadzu LC-VP HPLC system. The labeled substrate was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid for an amnic column and with 20 mM ammonium acetate (pH 4.0) from an ODS column and monitored with a fluorescence spectrophotometer (excitation, 320 nm; emission, 400 nm). The synthetic mannosyl peptide (Ac-Ala-Ala-Pro-Thr(Man)-Pro-Val-Ala-Ala-Pro-NH2) was kindly provided by Dr. Toshiyuki Inazu (Department of Applied Chemistry, School of Engineering, Tokai University). Reombinatable soluble GnT-IX was prepared as described previously (14).

**Activity Assay for GnT-IX**—The activity assay using the synthetic Ser-linked mannos-containing saccharides as acceptor substrates was performed in a mixture of 0.1 mM MOPS (pH 7.5), 10 mM EDTA, 21.9 μM UDP-[14C]GlcNAc (71.2 Ci/mmol), 0.5 mM substrate and recombinant soluble GnT-IX in a volume of 20 μl. After incubation for 12 h at 37 °C, the reaction mixture was boiled for 3 min, diluted with 80 μl of water, and then passed through an AG-X8 column (Bio-Rad, acetate form, 0.1 ml). The flow-through was evaporated and redissolved in 10 μl of water and then applied to a Silica gel 60 HPTLC plate (Merck). The plate was developed with methanol:butyl alcohol:acetic acid:water (2:2:1:1 by volume), and the incorporation of the radioactivity was visualized by a phosphorimager (BAS-2500, Fuji Film, Tokyo). The reaction using a fluorescence-labeled acceptor substrate GnM-S-PAES was analyzed by HPLC as described under “Experimental Procedures.” A, substrate; P, enzymatic product.

**MALDI-TOF MS Analysis**—MALDI-TOF MS was performed with a Perseptive Biosystems Voyager RP-DE instrument. The mass spectra were acquired in the reflectron mode under a 20 kV accelerating voltage with positive detection. 2,5-Dihydroxybenzoic acid (10 mg/ml) was used as the matrix.

**NMR Analysis**—For preparation of the NMR sample, a large scale reaction was carried out in a mixture of 0.1 mM MOPS (pH 7.5), 10 mM EDTA, 40 mM UDP-GlcNAc, 100 mM GlcNAc, 0.5 mM MnCl2, 40 mM UDP-GlcNAc, 200 mM GlcNAc, 0.5% Triton X-100, 40 mM thioglycollate, 40 μM phosphoramidase, with or without recombinant soluble GnT-IX and partially purified recombinant POMGnT1. The enzymatic product was separated by HPLC.

**RESULTS AND DISCUSSION**

The reports of GlcNAc substitution at the 6-OH position of mannose in the O-mannosyl glycans (12, 13) prompted us to investigate whether GnT-IX was able to act, not only on the α1,3- and α1,6-linked mannose arms of the core of N-glycan, but on the GlcNAcβ1,2-Manα in the O-mannosyl glycan as well. Three synthetic Ser-linked mannos-containing saccharides, Manα1-O-Ser, GlcNAcβ1,2-Manα1-O-Ser, and Galβ1,4-GlcNAcβ1,2-Manα1-O-Ser, were separately incubated with recombinant soluble GnT-IX and UDP-[14C]GlcNAc. After incubation, unretracted UDP-[14C]GlcNAc was removed by anion-exchange column chromatography and the reaction product was separated on HPTLC. Interestingly, radioactivity was incorporated only when GlcNAcβ1,2-Manα1-O-Ser was used as an acceptor (Fig. 1A). When POMGnT1 and GnT-IX were simultaneously incubated with a synthetic mannosyl peptide, Ac-Ala-Ala-Pro-Thr(Man)-Pro-Val-Ala-Ala-Pro-NH2, two GlcNAc residues were incorporated into the substrate (m/z values for (M + Na+) of 1425.6 and (M + K+) of 1441.5), but no transfer was detected by GnT-IX in the absence of POMGnT1 (data not shown), as confirmed by HPLC and MS analysis (Fig. 2C) of the enzymatic product. It supports the view that GnT-IX acts on the GlcNAcβ1,2-Manα structure in O-mannosyl glycans of glycoproteins as a substrate, and the action is dependent on prior action of POMGnT1. This substrate specificity is similar to that of the homolog, GnT-V, which is not able to catalyze the transfer of GlcNAc to the galactosylated biantennary sugar chains, as reported previously (16).

To isolate the enzymatic product of GnT-IX, a fluorescence-labeled substrate with GlcNAcβ1,2-Manα1-O-Ser structure was synthesized and used in a large scale reaction. The fluorescence-labeled substrate GnM-S-PAES was incubated in the reaction mixture, and the enzymatic product was then separated from the substrate on an ODS column. The product peak (P) had a faster retention time than the substrate peak (S) as shown in Fig. 1B. The separated product peak and the substrate were subjected to MS analysis (Fig. 2A). The spectrum of the substrate showed an m/z value for (M + H)+ of 894.6, corresponding to that of the calculated value of GnM-S-PAES (891.7). On the other hand, the spectrum of the product peak showed an m/z value for (M + H)+ of 894.6, indicating that one GlcNAc had been transferred to the substrate.

NMR analyses were carried out to further confirm the structure of the enzymatic product. Proton NMR spectra of the substrate and the product are shown in Fig. 2B. In the spectrum of the substrate, anameric proton signals of Man and GlcNAc were observed at 4.838 and 4.546 ppm, respectively, and a methyl proton signal corresponding to the acetyl group of GlcNAc was detected at 2.057 ppm. The anameric proton signal of GlcNAc appeared as a doublet with a coupling constant of 8.2 Hz. In the spectrum of the product, a methyl proton signal of an
additional GlcNAc appeared at 2.038 ppm. The chemical shift value was similar to that of the methyl proton signal of GlcNAc, which was linked to the 6-OH position of Man 4 in the tetra-antennary sugar chain (14). A new anomic proton signal consistent with the GlcNAc was found at 4.594 ppm as a doublet with a coupling constant of 8.0 Hz. The coupling constant value indicates that the linkage of the GlcNAc is \( {1\rightarrow 2} \). The anomic proton signal of Man was observed at 4.797 ppm and showed a shift to higher field by 0.041 ppm relative to that of the substrate (spectra of \( S \) and \( P \) in Fig. 2B). A similar up-field shift of the Man anomic proton signal was observed when GlcNAc was attached to 6-OH position of Man (14). These results indicate that the additional GlcNAc of the product is linked to the 6-OH position of Man and that the anomic configuration is \( {1\rightarrow 2} \).

To determine whether GnT-V also catalyzes the same reaction, recombinant GnT-V, possessing equivalent enzymatic activity toward the GnGn-bi-PA substrate with GnT-IX, was incubated with GnM-S-PAES, but no activity was detected (data not shown). As a result, we conclude that GnT-IX, but not GnT-V, acts on the GlcNAc \( {1\rightarrow 2} \)-Man \( {1\rightarrow 6} \) structure in the \( O \)-mannosyl glycan, as a \( 1,6 \)-N-acetylg glucosaminyltransferase.

Fig. 3 shows a proposed biosynthetic pathway for the brain \( O \)-mannosyl glycan mediated by POMGnT1 and GnT-IX. It has been suggested that a putative \( O \)-mannosyltransferase POMT1 catalyzes the transfer of mannose in the first step in \( O \)-mannosyl glycan synthesis. POMGnT1 catalyzes the next step forming the GlcNAc\( {1\rightarrow 2} \)-Man structure. GnT-IX acts on the POMGnT1 product before the action of \( 4 \)-GalTβs. After the addition of Gal residues, ST3Gals forms the \( \alpha \)-dystroglycan (DG)-type \( O \)-mannosyl glycan or GlcAT and HNK-1ST form the HNK-1 epitope. Fuc-TIX is the enzyme most responsible for the synthesis of the Lewis X epitope in the brain (31, 32), but whether the Lewis X epitope-carrying \( O \)-mannosyl glycan contains a GlcNAc\( {1\rightarrow 6} \)-Man linkage is unknown. GlcAT, glucuronyltransferase; HNK-1ST, HNK-1 sulfotransferase.
GnT-IX Acts on the O-Mannosyl Glycan

O-glycan synthesis; however, GnT-IX has no similarity to these genes.

In conclusion, our results indicate that GnT-IX is a novel β1,6-N-acetylglucosaminyltransferase that acts not only on N-glycans but on O-mannosyl glycans as well. Although the biochemical function of the 2,6-branched structure remains unclear, the addition of GlcNAc to the 6-OH position of O-mannosyl glycan may contribute to an increase in terminal glycan structures which, in turn, may modify the affinities and avidities with respect to interacting counterparts.

Acknowledgment—We are grateful to Dr. Toshiyuki Inazu (Department of Applied Chemistry, School of Engineering, Tokai University) for providing a synthetic mannosyl peptide.

REFERENCES

1. Nishimura, H., Takao, T., Hase, S., Shimomishy, Y., and Iwanaga, S. (1992) J. Biol. Chem. 267, 17520–17525
2. Harris, R. J., and Spellman, M. W. (1993) Glycobioscience 3, 219–224
3. Shao, L., and Haltiwanger, R. S. (2003) Cell. Mol. Life Sci. 60, 241–250
4. Hase, S., Kawabata, S., Nishimura, H., Takeya, H., Suyeshi, T., Miyata, T., Iwanaga, S., Takao, T., Shimomishy, Y., and Ikenaka, T. (1988) J. Biochem. (Tokyo) 104, 867–868
5. Holt, G. D., and Hart, G. W. (1986) J. Biol. Chem. 261, 8049–8057
6. Wells, L., Whalen, S. A., and Hart, G. W. (2003) Biochem. Biophys. Res. Commun. 302, 435–441
7. Endo, T. (1999) Biochim. Biophys. Acta 1445, 237–246
8. Chiha, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusanuki, S., Kanazawa, I., Kobata, A., and Endo, T. (1997) J. Biol. Chem. 272, 2156–2162
9. Santavuori, P., Somer, H., Sainio, K., Rapola, J., Kraus, S., Nikitin, T., Ketomaa, L., and Leisti, J. (1989) Brain Dev. 11, 147–153
10. Yoshida, A., Kobayashi, K., Many, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsushashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., Talim, B., Voit, T., Topalghi, H., Toda, T., and Endo, T. (2001) Dev. Cell 1, 717–724
11. Michele, D. E., Barres, R., Kanagawa, M., Saito, F., Cohn, R. D., Sattz, J. S., Dollar, J., Nishino, I., Kelley, R. I., Somer, H., Straub, V., Mathews, K. D., Moore, S. A., and Campbell, K. P. (2002) Nature 418, 417–422
12. Chai, W., Yue, C. T., Kogelberg, H., Carruthers, R. A., Margolis, R. U., Feizi, T., and Lawson, A. M. (1999) Eur. J. Biochem. 263, 879–888
13. Yue, C. T., Chai, W., Loveless, R. W., Lawson, A. M., Margolis, R. U., and Feizi, T. (1997) J. Biol. Chem. 272, 8924–8931
14. Inamori, K., Endo, T., Ide, Y., Fuji, S., Gu, J., Honke, K., and Taniguchi, N. (1997) J. Biol. Chem. 272, 43192–43199
15. Seifert, J., Ogawa, T., Kurono, S., and Ito, Y. (2000) Glycoconjug J. 17, 407–423
16. Gu, J., Nishikawa, A., Tsuuka, N., Ohno, M., Yamaguchi, N., Kanagawa, K., and Taniguchi, N. (1993) J. Biochem. (Tokyo) 113, 614–619
17. 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., Lehejski, A. E., Cruces, J., Voit, T., Walah, C. A., van Bokhoven, H., and Brunner, H. G. (2002) Am. J. Hum. Genet. 71, 1023–1041
18. Dobyns, W. B., Pagot, R. A., Arndt, D., Curey, C. J., Greenberg, F. G., Grix, A., Holmes, L. B., Laxova, R.,Michels, V. R., Robinow, M., and Zimmerman, R. L. (1989) Am. J. Med. Genet. 32, 185–210
19. Fukuyama, Y., Osawa, M., and Suzuki, H. (1981) Brain Dev. 3, 1–29
20. Amado, M., Almeida, R., Schwientek, T., and Clausen, H. (1999) Biochim. Biophys. Acta 6, 35–53
21. Terayama, K., Oka, S., Seki, T., Miki, Y., Nakamura, A., Kozutsumi, Y., Takio, K., and Kawasaki, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6093–6098
22. Shimoda, Y., Tajima, Y., Nagaee, T., Harri, K., Osumi, N., and Sanai, Y. (1999) J. Biol. Chem. 274, 17115–17122
23. Seki, T., Oka, S., Terayama, K., Imiya, K., and Kawasaki, T. (1999) Biochem. Biophys. Res. Commun. 255, 182–187
24. Bakker, H., Friedmann, J., Oka, S., Kawasaki, T., Nifant’ev, N., Schachner, M., and Mantei, N. (1997) J. Biol. Chem. 272, 29942–29946
25. Ong, E., Yeh, J. C., Ding, Y., Hindsaul, O., and Fukuda, M. (1998) J. Biol. Chem. 273, 5190–5195
26. Smalheiser, N. R., Haslam, S. M., Sutton-Smith, M., Morris, H. R., and Dell, A. (1998) J. Biol. Chem. 273, 23698–23703
27. Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshikawa, K., Sato, K., Osawa, M., Hamano, K., Sakakihara, Y., Nenaka, I., Nakagome, Y., Kanazawa, I., Nakamura, Y., Tokunaga, K., and Toda, T. (1998) Nature 394, 388–392
28. Brinklinton, M., Blake, D. J., Pandini, P., Brown, S. C., Torelli, S., Benson, M. A., Ponting, C. P., Estornet, B., Romero, N. B., Mercuri, E., Voit, T., Sewry, C. A., Guihenney, P., and Muntoni, F. (2001) Am. J. Hum. Genet. 69, 1198–1209
29. Peyrard, M., Seroussi, E., Sandberg-Nordqvist, A. C., Xie, Y. G., Han, F. Y., Fransson, I., Collins, J., Dunham, I., Kost-Alimova, M., Imreh, S., and Dumanski, J. P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 598–603
30. Kudo, T., Ikehara, Y., Togayachi, A., Kaneko, M., Hiraga, T., Sasaki, K., and Mori, H. (2003) J. Biochem. 134, 445–455