Cloning and expression of a novel UDP-GlcNAc:α-D-mannoside β1,2-*N*-acetylglucosaminyltransferase homologous to UDP-GlcNAc:α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I

Wenli ZHANG*, Doron BETEL*† and Harry SCHACHTER*†[1]

*Program in Structural Biology and Biochemistry, The Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada, and †Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada

A TBLASTN search with human UDP-GlcNAc:α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT I; EC 2.4.1.101) as a probe identified human and mouse Unigenes encoding a protein similar to human GnT I (34 % identity over 340 amino acids). The recombinant protein converted Man(α1-6)[Man(α1-3)]Man(β1-)*O*-octyl to Man(α1-6)[GlcNAc(β1-2)Man(α1-3)]Man(β1-)*O*-octyl, the reaction catalysed by GnT I. The enzyme also added GlcNAc to Man(α1-6)[GlcNAc(β1-2)Man(α1-3)]Man(β1-)*O*-octyl (the substrate for β-1,2-*N*-acetylglucosaminyltransferase II), Man(α1-)*O*-benzyl [with K_m values of ≈ 0.3 and > 30 mM for UDP-GlcNAc and Man(α1-)*O*-benzyl respectively] and the glycopeptide CYA[Man(α1-)*O*-T]AV (K_m ~ 12 mM). The product formed with Man(α1-)*O*-benzyl was identified as GlcNAc(β1-2)Man(α1-)*O*-benzyl by proton NMR spectroscopy. The enzyme was named UDP-GlcNAc:α-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I.2 (GnT I.2). The human gene mapped to chromosome 1. Northern-blot analysis showed a 3.3 kb message with a wide tissue distribution. The cDNA has a 1980 bp open reading frame encoding a 660 amino acid protein with a type-2 domain structure typical of glycosyltransferases. Man(β1-)*O*-octyl, Man(β1-)*O*-*p*-nitrophenyl and GlcNAc(β1-2)Man(α1-6)[GlcNAc(β1-2)Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-)*O*-Asn were not acceptors, indicating that GnT I.2 is specific for α-linked terminal Man and does not have *N*-acetylglucosaminyltransferase III, IV, V, VII or VIII activities. CYA[Man(α1-)*O*-T]AV was between three and seven times more effective as an acceptor than the other substrates, suggesting that GnT I.2 may be responsible for the synthesis of the GlcNAc(β1-2)Man(α1-)*O*-Ser/Thr moiety on α-dystroglycan and other O-mannosylated proteins.

Key words: cytoskeleton, α-dystroglycan, extracellular matrix, laminin, O-mannosylated protein.

INTRODUCTION

N-glycosylation is one of the most common post-translational protein modifications. The synthesis of N-glycans begins with an oligosaccharyltransferase, which transfers $Glc_3Man_9GlcNAc_2$ *en bloc* from dolichol-pyrophosphate-$Glc_3Man_9GlcNAc_2$ to an Asn residue of the nascent polypeptide chain in the lumen of the endoplasmic reticulum [1,2]. Oligosaccharide processing in the endoplasmic reticulum and Golgi apparatus leads to the formation of $Man_5GlcNAc_2$-Asn-Xaa, which is the entry point for the conversion of oligomannose to hybrid and complex N-glycans due to the action of UDP-GlcNAc:α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I (GnT I; EC 2.4.1.101) [3,4]. GnT I is essential for the subsequent actions of several enzymes in the processing pathway [5,6]. Deletion of the GnT I gene in tissue-culture cells [7] and in the mouse [8,9] prevents complex N-glycan synthesis. Although somatic cell mutants lacking the GnT I gene show essentially normal growth, mouse embryos with a null mutation in this gene do not survive beyond 10.5 days post-fertilization, indicating the importance of complex N-glycans for metazoan development.

The GnT I gene has been cloned from several mammalian species, and also from frog, *Caenorhabditis elegans*, *Drosophila melanogaster* and tobacco (see [10] for references). Although several glycosyltransferase isoenzyme families have been reported, e.g. UDP-Gal:GlcNAc-R β1,4-galactosyltransferases [11], UDP-Gal:GlcNAc-R β1,3-galactosyltransferases [11] and GDP-Fuc:Galβ1-4GlcNAc-R (Fuc to GlcNAc) α1,3-fucosyltransferases [12] (where R can be an oligosaccharide or aglycone), to date only *C. elegans* has been reported to have more than a single gene encoding GnT I activity [13]. A TBLASTN screen of the EST database for genes sharing significant sequence similarity with GnT I showed the presence of human (Hs.183860) and mouse (Mm.2069) Unigenes encoding a protein (hypothetical protein FLJ20277) similar to human GnT I (34 and 45 % identities over 340 and 159 amino acids respectively).

We report here that the novel human protein not only has GnT I activity but can also incorporate GlcNAc into Man(α1-)*O*-R compounds (where R can be an oligosaccharide or aglycone), which are not acceptors for GnT I. The enzyme has therefore been named UDP-GlcNAc:α-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I.2 (GnT I.2; encoded by the *MGAT*1.2 gene). Preliminary reports of this work have been published elsewhere [14,15].

EXPERIMENTAL

Materials

The following materials were purchased from the indicated sources: Grace's insect medium, fetal calf serum, TC Yeastolate,

Abbreviations used: GnT I, UDP-GlcNAc:α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I; GnT I.2, UDP-GlcNAc:α-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I.2; Ni-NTA, Ni^{2+}-nitrilotriacetate; NBT, Nitro Blue Tetrazolium; BCIP, 5-bromo-4-chloroindol-3-yl phosphate; M2-octyl, Man(α1-3)Man(β1-)*O*-octyl; GnGn, GlcNAc(β1-2)Man(α1-6)[GlcNAc(β1-2)Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-)*O*-Asn; M3-octyl, Man(α1-6)[Man(α1-3)]Man(β1-)*O*-octyl; GnM3-octyl, Man(α1-6)[GlcNAc(β1-2)Man(α1-3)]Man(β1-)*O*-octyl.

[1] To whom correspondence should be addressed, at the Program in Structural Biology and Biochemistry (e-mail harry@sickkids.on.ca).

The nucleotide sequence data for the *MGAT1.2* gene have been deposited in the GenBank Nucleotide Sequence Database under the accession numbers AF250859 and AAF71270.

TC lactalbumin hydrolysate, gentamycin, amphotericin (Fungizone) and competent *Escherichia coli* DH5α (GIBCO Laboratories, Grand Island, NY, U.S.A.); Vent DNA polymerase (New England Biolabs); Sep-Pak C_{18} reverse-phase cartridges (Waters); restriction endonucleases and T4 DNA ligase (Pharmacia); BaculoGold baculovirus linearized DNA, pAcHLT-C baculovirus transfer vector, transfection buffers and cationic liposomes (PharMingen); *Spodoptera frugiperda* (Sf9) insect cells (A.T.C.C.); Triton X-100 and goat anti-mouse IgG (whole molecule) coupled to alkaline phosphatase (Sigma); and Ni^{2+}-nitrilotriacetate (Ni-NTA) resin (Qiagen).

Anti-Xpress antibody (Invitrogen) was used to detect recombinant protein secreted into the Sf9 cell medium by the Nitro Blue Tetrazolium (NBT)/5-bromo-4-chloroindol-3-yl phosphate (BCIP; Promega) method. UDP-[^{3}H]GlcNAc (New England Nuclear) was diluted with non-radioactive UDP-GlcNAc (Sigma) to a specific radioactivity of 2000–10000 d.p.m./nmol. Man(α1-6)[Man(α1-3)]Man(β1-)*O*-octyl (M3-octyl) was a gift from Dr Hans Paulsen (Institute of Organic Chemistry, University of Hamburg, Hamburg, Germany), or was purchased from Toronto Research Chemicals (Toronto, Canada). Man(α1-3)Man(β1-)*O*-octyl (M2-octyl) was purchased from Chemical Alta (Edmonton, Canada). Man(α1-)*O*-benzyl, Man(β1-)*O*-octyl and Man(β1-)*O*-*p*-nitrophenyl were purchased from Toronto Research Chemicals. Glycopeptides CYA[Man(α1-)*O*-T]AV and GlcNAc(β1-2)Man(α1-6)[GlcNAc(β1-2)Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-)*O*-Asn (GnGn) were respective gifts from Dr Sabine Strahl-Bolsinger (University of Regensburg, Regensburg, Germany) and Dr Erika Staudacher [Universität der Bodenkultur (BOKU), Vienna, Austria].

Oligonucleotides were synthesized on a Pharmacia DNA synthesizer and purified by the cartridge method (Hospital for Sick Children Pharmacia Biotechnology Center, Toronto, Canada). The baculovirus transfer vector pVT-Bac-His was constructed by D. Joziasse, G. Smit and R. van Elk (Faculty of Biology, Vrije Universiteit, Amsterdam, The Netherlands). A DNA fragment encoding six His residues followed by an enterokinase-sensitive site was ligated into the *Bam*HI and *Pst*I sites of pVT-Bac (donated kindly by Dr T. Vernet, Biotechnology Research Institute, Montreal, Quebec, Canada [16]). Recombinant proteins encoded by this vector contain at their N-terminus a cleavable signal sequence derived from the insect protein melittin and will be secreted from baculovirus-infected insect cells with an N-terminal $(His)_6$ epitope tag that can be removed by enterokinase cleavage [17]. IMAGE Consortium human cDNA clones 564176 (accession numbers AA121360 and AA121370) and 1573028 (accession number AA970316) were obtained from Genome Systems (St. Louis, MO, U.S.A.). Human clone HEP02567 (accession number AK000284; 2736 bp), encoding hypothetical protein FLJ20277 inserted into a modified form of the plasmid pME18S-FL, was a gift from Dr Sumio Sugano (NEDO Human cDNA Sequencing Project, Institute of Medical Science, University of Tokyo, Tokyo, Japan).

General molecular biology procedures

Unless otherwise stated, all molecular biology procedures were carried out using standard methods [18,19]. Plasmids were amplified in competent *E. coli* DH5α cells (Gibco BRL). Competent *E. coli* DH5α cells transformed with plasmid were inoculated into LB media containing 0.05 mg/ml ampicillin and grown overnight at 37 °C. Plasmid DNA was purified using the QIAprep Spin Plasmid Kit (Qiagen) following the manufacturer's protocol. Purity of the DNA was determined by the ratio of absorbances at 260 and 280 nm; a ratio of 1.8 was considered pure. DNA sequencing was carried out by the double-strand dideoxy method [20].

Cloning of human GnT I.2 cDNA

A TBLASTN screen of the EST database for genes sharing significant sequence similarity with GnT I showed the presence of human (Hs.183860) and mouse (Mm.2069) Unigenes encoding a protein (hypothetical protein FLJ20277) similar to human GnT I (34 and 45% identities over 340 and 159 amino acids respectively). PCR, Vent DNA polymerase, forward primers 5′-GCTAGCGAATTCGCACGAGAAATC-3′ and 5′-GCTAGCGAATTCCACGAGAAATC-3′ (with the *Eco*RI site underlined), reverse primer 5′-GATAGGTACCCCCTACCAGCATCTACAAAATCC-3′ (*Kpn*I site underlined) and IMAGE Consortium human cDNA clone 564176 as a template were used to obtain 1.4 kb DNA products RF1 and RF2 respectively. PCR conditions were as follows: denaturation at 94 °C for 3 min followed by 35 cycles at 94 °C for 40 s, 55 °C for 30 s and 72 °C for 100 s, with a final step at 72 °C for 10 min. PCR products were purified (QIAquick PCR Purification Kit, Qiagen) and subcloned downstream and in frame with the ATG start site of the baculovirus transfer vector pVT-Bac-His [17,21] using the *Eco*RI and *Kpn*I cloning sites to produce plasmids pVT-Bac-His-RF1 and pVT-Bac-His-RF2 respectively. Plasmid-insert sequences were confirmed by DNA sequence analysis (DNA Sequencing Facility of the Hospital for Sick Children). RF1 encodes the C-terminal end of GnT I.2 (amino acid residues 227–660) and RF2 is an 'out-of-frame' control in which the G immediately downstream of the *Eco*RI site had been removed.

The 2.6 kb IMAGE Consortium human cDNA clone 1573028 was also sequenced. This sequence, together with cDNA sequences deposited in the Hs.183860 Unigene database, was used to obtain a 2355 bp sequence encoding amino acids 84–660 of GnT I.2. This sequence has been deposited in the GenBank database under accession numbers AF250859 and AAF71270. Human clone HEP02567 (accession number AK000284) encodes 2736 bp of the GnT I.2 cDNA sequence, which includes 141 bp of 5′-untranslated terminus, the ATG start codon, the TGA stop codon and the polyadenylation sequence. Since the mRNA for GnT I.2 is about 3.3 kb (see the Results section), over 500 bp of 5′-untranslated sequence remains to be determined. A 2783 bp DNA fragment containing almost the full-length open reading frame of GnT I.2 (66 bp from the 5′-end of the open reading frame are not present but the full transmembrane domain is retained) was cut out of clone HEP02567 with *Kpn*I and subcloned into the *Kpn*I site of the baculovirus transfer vector pAcHLT-C downstream and in frame with the $(His)_6$ open reading frame. Both forward (FpAcHLT-FL) and reverse (RpAcHLT-FL) recombinant clones were isolated. The reverse clone was used as a negative control to detect endogenous Sf9 enzyme.

Expression of human GnT I.2 in the baculovirus/Sf9 system

The baculovirus transfer plasmids pVT-Bac-His-RF1 and pVT-Bac-His-RF2 (see above) were expressed in the baculovirus/Sf9 insect cell system as described previously [17,21]. Expression with the pVT-Bac-His transfer vector produces proteins, which are secreted from the Sf9 cells with an N-terminal $(His)_6$ tag. RF1 encodes a truncated human GnT I.2 (amino acid residues 227–660) lacking the cytoplasmic N-terminal domain, transmembrane hydrophobic domain and 165 residues of the stem region. RF2 has a stop codon near the 5′-end. The RF2 Sf9 supernatant was assayed routinely for enzyme activity as a control for endogenous Sf9 enzyme. As an expression control, truncated

rabbit GnT I in the pVT-Bac-His vector was expressed in the baculovirus/Sf9 system [17,21] and the Sf9 supernatants were assayed for GnT I activity whenever RF1 and RF2 expression runs were carried out.

The baculovirus transfer plasmids FpAcHLT-FL and RpAcHLT-FL encode recombinant proteins that are not secreted from the Sf9 cells. Enzyme assays require lysis of the Sf9 cells and purification of the enzyme from the lysate by adsorption to Ni-NTA beads. RpAcHLT-FL was used as a control for endogenous enzyme activity in the Sf9 cell lysates.

Sf9 cells were grown at 28 °C in Grace's insect medium supplemented with 10 % fetal calf serum, TC Yeastolate, TC lactalbumin hydrolysate, 50 mg·ml^{-1} gentamycin sulphate and 2.5 mg·ml^{-1} amphotericin (Fungizone) in Falconware T flasks. Recombinant baculovirus transfer plasmid (30 ng) was co-transfected with BaculoGold baculovirus linearized DNA (14 ng, PharMingen) into 5×10^4 Sf9 cells at 80 % confluency in a 96-well microtitre plate wells using cationic liposomes according to the PharMingen protocol [17,21,22]. Cells were incubated for 4–5 days at 28 °C to allow homologous recombination and release of virus into the culture medium. The recombinant baculovirus was amplified twice in 25 cm² flasks and the resulting virus was kept as a stock. Enzyme was prepared by adding 0.2 ml of virus stock to Sf9 cells in 175 cm² flasks at 80 % confluency at a multiplicity of infection of 2 pfu/cell. Cells and supernatant were harvested at 3–6 days post-infection. Supernatants were assayed for soluble secreted enzyme activity either directly or after adsorption on to Ni-NTA beads. Cells were lysed in the presence of proteinase inhibitors according to the protocol in the Qiagen Ni-NTA/QIAexpress kit and the recombinant protein was purified by adsorption on to Ni-NTA beads (see below). In some experiments, microsomes were prepared from the Sf9 cell lysates using the sucrose-gradient method as described by Takahashi et al. [23]. Four flasks of infected Sf9 cells (2×10^8 cells) were sonicated five times at 17 W for 1 min with 2 min intervals on ice. The nuclei and cell debris were removed by centrifugation at 1500 ***g*** for 30 min, the supernatant was centrifuged at 100000 ***g*** for 1 h, and the yellow translucent microsomal pellet was resuspended in 1.3 ml of buffer (140 mM Mes, pH 7.1/10 % glycerol/0.2 M GlcNAc/5 mM AMP/1 % Triton X-100/protease inhibitors) [23] and homogenized with a Micro Tissue Grinder (Fisher). The suspension was centrifuged at 100000 ***g*** for 1 h and the supernatant was used for enzyme assays as described in the Results section.

Analysis of recombinant GnT I.2 enzyme activity

Ni-NTA resin was prepared as described in the QIAexpress protocol. The beads were equilibrated in 50 mM sodium phosphate (pH 8.0), 0.3 M NaCl and 10 mM imidazole. Sf9 supernatant (0.7 ml) was added to 0.1 ml of Ni-NTA beads and the suspension was rocked gently overnight at 4 °C. The suspension was centrifuged at 900 ***g*** for 4 min and the supernatant was discarded. The beads were washed with 1 ml of wash buffer [0.1 M NaCl/20 mM imidazole/5 % (v/v) glycerol/10 mM Mes, pH 6.5]. After centrifugation, the beads were resuspended in 0.1 ml of wash buffer and stored at 4 °C. Attempts to elute active enzyme from the Ni-NTA resin were not successful and therefore the beads were used for enzyme assays in our initial experiments to avoid detection of endogenous Sf9 GnT I activity with the M3-octyl acceptor. Subsequent experiments with acceptors that were not acted on by GnT I were carried out with Sf9 supernatant as the enzyme source. The near full-length proteins expressed inside Sf9 cells (baculovirus transfer plasmids FpAcHLT-FL and RpAcHLT-FL) were released from whole cells by 1 % Triton X-100 in Qiagen's lysis buffer (50 mM sodium phosphate/0.3 M NaCl/10 mM imidazole, pH 8.2). Enzyme was adsorbed on to Ni-NTA beads as described in the Qiagen protocol. Enzyme was released from microsomes as described above and used directly for enzyme assays without adsorption on to Ni-NTA beads.

GnT I.2 activity was assayed using various acceptor substrates and concentrations (listed in the Results section), 0.8–1.2 mM UDP-[^{3}H]GlcNAc (2000–10000 d.p.m./nmol) as a donor substrate, 10–25 mM $MnCl_2$, 50–150 mM Mes buffer, pH 6.0 or 6.5, and various amounts of enzyme in a total volume of 0.02 or 0.05 ml [21]. Incubations were carried out at 37 °C for times ranging from 0.5 to 18 h depending on the enzyme preparation and acceptor substrate. SepPak C_{18} cartridges were used to determine the amount of radioactive product as described previously [21,24]. Incubations with GnGn, which lacks a hydrophobic group, were assayed by passage of the incubation mixture through a column of AG1-X8 (chloride form, 100–200 mesh) as described previously [25]. Kinetic parameters were determined for the substrate Man(α1-)*O*-benzyl by a series of reciprocal velocity/substrate plots at five concentrations of both acceptor and donor substrates [1, 5, 10, 15 and 20 mM Man(α1-)*O*-benzyl and 0.02, 0.04, 0.06, 0.08 and 0.1 mM UDP-GlcNAc] [21,26]. Controls were carried out routinely in the absence of acceptor and the value obtained was subtracted. Control enzyme assays were also carried out on the supernatants from Sf9 cells infected with the truncated out-of-frame RF2 baculovirus or on cell lysates from Sf9 cells infected with the near full-length 'reverse sequence' baculovirus. Since Sf9 cell lysates and supernatants contain endogenous GnT I and GnT II enzyme activities, it was essential to correct all GnT I.2 assays using substrates acted on by GnT I and GnT II with the endogenous insect cell enzyme levels. There was no detectable endogenous Sf9 enzyme activity with Man(α1-)*O*-benzyl or CYA[Man(α1-)*O*-T]AV, indicating the absence of GnT I.2 in insect cells and supernatants. Metal requirements and pH optima were determined as described previously [13,25]. All assays were carried out at least in duplicate and results are reported as means of these determinations.

Product identification by proton NMR spectroscopy

Twenty 0.05 ml assay mixtures containing 0.02 ml of RF1 supernatant as the enzyme source and 0.25 M Man(α1-)*O*-benzyl as the acceptor were incubated overnight at 37 °C. A further 0.02 ml of RF1 supernatant was then added and incubation was continued for a further 2.5 days at 37 °C. Enzyme product was purified by SepPak C_{18} cartridges and the pooled products were concentrated to a final volume of 0.1 ml by vacuum evaporation at 37–45 °C. Product was further purified by preparative TLC on silica gel plates (150A K5; Whatman) using chloroform/methanol/water (10:8:1) as a solvent. The product band was cut from the plate after charring a sample marker in a separate lane. Gel particles were washed with four aliquots of 0.3 ml of water and the eluate was dried by vacuum evaporation at 37–45 °C. The dry product was resuspended in 1 ml of 99 % ^2H_2O, exchanged repeatedly in 1 ml of ^2H_2O (99.9 atom % ^2H) and finally dissolved in 0.8 ml of ^2H_2O (99.96 atom % ^2H). Proton NMR spectra were recorded at 500 MHz at 25 °C using a Varian Unity Plus 500 spectrometer (University of Toronto NMR Centre). One- and two-dimensional TOCSY (^{1}H-^{1}H TOCSY) spectra were recorded. Chemical shifts (δ) were expressed in p.p.m. relative to an internal acetone standard (δ 2.225).

Western-blot analysis

Sf9 supernatant (0.01 ml) was analysed by SDS/PAGE (12.5 % gel; Protogel; National Diagnostic) [27] and transferred by

electrophoresis to an Immobilon PVDF membrane (0.45 μm; Millipore) in the presence of 0.1 % SDS. The membranes were probed with 1:5000 diluted mouse monoclonal antibody raised against the enterokinase cleavage site (Anti-Xpress antibody kit; Invitrogen). This enterokinase site is encoded by pVT-Bac-His and is expressed near the N-terminus of recombinant GnT I.2. Proteins were visualized by treatment with a 1:2500 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG followed by staining with BCIP and NBT (Promega) according to the manufacturer's instructions.

Northern-blot analysis

A 951 bp DNA fragment was excised from PCR product RF1 (see above) and used as the detecting probe for Northern blots. The probe was labelled by the random-primer method using [α^{32}P]dATP (3000 Ci/mmol; NEN DuPont) and the T$_7$Quick-primer Kit (Pharmacia). The labelled probe was purified by the G50 NICK column method (Pharmacia) to remove unincorporated [α-^{32}P]dATP. A Human Multiple Tissue cDNA Panel (Clontech, catalogue number 7780-1) was hybridized with the ^{32}P-labelled probe following the user manual accompanying ExpressHyb™ hybridization solution (Clontech, catalogue number 8015-1). The blot was exposed to X-ray film (BioMax RM; Kodak) at −70 °C for 25 h. The GnT I.2 probe was removed from the blot as described in the ExpressHyb Hybridization Solution User Manual and the Human Multiple Tissue cDNA Panel was hybridized once again with β-actin cDNA. The blot was exposed to X-ray film as above.

RESULTS

Cloning of human GnT I.2 cDNA

A TBLASTN search of the human and mouse EST databases with the amino acid sequence of human GnT I as a probe showed the presence of human (Hs.183860) and mouse (Mm.2069) Unigenes encoding a protein similar to human GnT I. PCR with IMAGE Consortium human cDNA clone 564176 as a template was used to obtain the 1.4 kb DNA product RF1, which encodes amino acid residues 227–660 of GnT I.2 (Figure 1). We also sequenced the 2.6 kb IMAGE Consortium human cDNA clone

```
1   MDDWKPSPLI KPFGARKKRS WYLTWKYKLT NQRALRRFCQ TGAVLFLLVT
51  VLVNLKLILD TRRAISEANE DPEPEQDYDE ALGRLEPPRR RGSGPRRVLD
101 VEVYSSRSKV YVAVDGTTVL RDFARFQGRG THVTVLNQAT GHVMAKRVFD
151 CYSPEDDEAM VLFLNMVAPG RVLICTVKDE GSFHLKDTAK ALLRSLGSQA
201 GPALGWRDTW AEVCRKGGPV HGLKHSKSPA LSSWGDPVLL KIDVPLSSAE
251 EAECEWADTE LNRRRRRFCS KVEGYGSVCS CKDPTPIEFS PDPLPDNKVL
301 NVPVAVTAGN RPNYLYRMLR SLLSAQGVSP QMTCVFTDGY YEEPMDVVAL
351 PGLRGIQHTP ISIKNA

GnT I.2  367 RVSQHYKASLTATFNLYPEAKPAVVLEEDLDLAVDPTSFLSQSIHLLEEDDSLYCISAWN
GnT I    184 KIAREYRWALGQVFRQF-RFPAAVVVEDDLEVAPDFFEYFRATYPLLKADPSLWCVSAWN
                **   *   *  *     *** * **  * ***        **  * ** * ****
GnT I.2  427 DQGYEHT--AEDPALLYRVETMPGLGWVLRRSLYKEELEPKWPTEEKLWDWDMWMRMPEQ
GnT I    243 DNGKEQMVDASRPELLYRTDFFPGLGWLLLAELWAE-LEPKWPKAF----WDDWMRRPEQ
             * * *    *  * ****    ***** *   *  * ******       ** *** ***
GnT I.2  485 RRGRECIIPDVSRSYHFGIVGLNMNGYFHEAYFKKEKFN
GnT I    298 RQGRACIRPEISRTMTFGRKGVS-HGQFFDQHLKFIKLN
             * ** ** *  **   **  *    * *     *  * *

524                          TVPGVQL RNVDSLKKEA YEVEVHRLLS
551 RAEVLDHSKN PCFDSFLPDT RGHTYVAFTR MEKDDDFTTW TQLAKCLHW
601 DLDVRGNERG LWRLFRKKNH FLVVGVPASP YSVKKPSVT PIFLEPPPKE
651 EGAPGAPEQT
```

Figure 1 Amino acid sequence of human GnT I.2

The non-cleavable hydrophobic signal-anchor domain is underlined. Alignment of amino acid sequences of human GnT I.2 and GnT I is shown. The proteins show 45% identity over 159 residues (indicated by asterisks). Human GnT I.2 residues in bold italics are identical to residues in rabbit GnT I in contact with UDP-GlcNAc-Mn^{2+}, as indicated by the crystal structure [28].

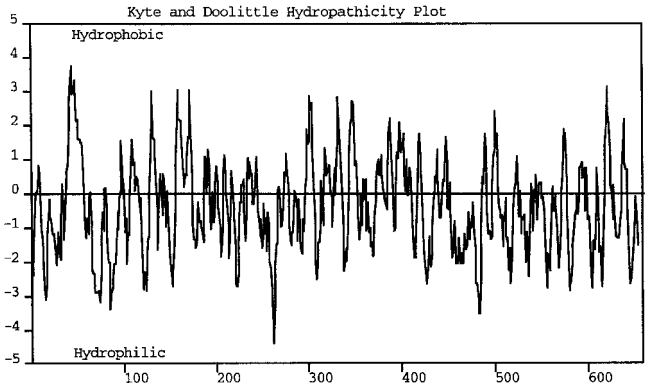

Figure 2 Kyte and Doolittle hydropathicity plot [46] for human GnT I.2

The 24 amino acid-residue hydrophobic transmembrane segment can be seen at residues 38–61. The *y*-axis shows units of hydrophobicity and the *x*-axis shows amino acid residue numbers.

Table 1 Functions of GnT I residues that align with the human GnT I.2 sequence

Functions are based on the crystal structure of rabbit GnT I [28].

GnT I.2 residue	GnT I residue	Function
Asp-338	Asp-144	Uracil N-3 contact
His-371	His-190	Uracil O-2 contact
Glu-394	Asp-212	Ribose O-2′, O-3′ contact
Arg-311	Arg-117	α-Phosphate O-2α contact
Glu-393	Glu-211	GlcNAc O-3, O-4 contact
Trp-475	Trp-290	GlcNAc O-4 contact
Asp-395	Asp-213	Mn^{2+} contact
Cys-421–Cys-490	Cys-239–Cys-305	Essential Cys–Cys bridge

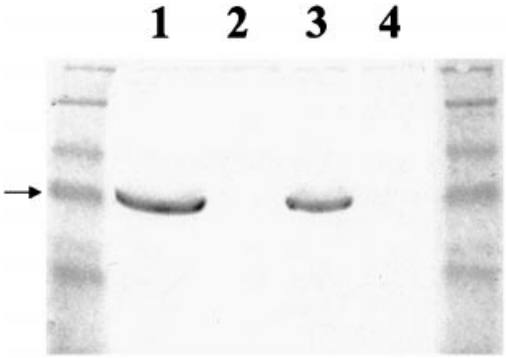

Figure 3 Western-blot analysis of Sf9 supernatants containing the RF1 and RF2 products

The membranes were probed with mouse monoclonal antibody raised against the enterokinase cleavage site (Anti-Xpress antibody kit; Invitrogen). Protein was visualized with alkaline phosphatase-conjugated goat anti-mouse IgG. The origin is the band at the top. The bands in the first and last columns indicate protein standards of (from top to bottom) 175, 83, 62, 47.5 and 32.5 kDa (prestained protein markers from New England Biolabs). RF1 shows a $\approx$ 56 kDa protein whereas only small peptide material (not seen in the figure) is present in the RF2 sample. Lanes 1 and 3, RF1; lanes 2 and 4, RF2. Protein was obtained at 3 (lanes 1 and 2) and 4 (lanes 3 and 4) days after infection with baculovirus. The arrow shows the 62 kDa marker.

Table 2 Enzyme assays with M3-octyl and GnGn glycopeptide as acceptors

Assays were carried out with Sf9 supernatants from cells infected with baculovirus encoding RF1 (GnT I.2), RF2 (out-of-frame control) and rabbit GnT I. Assays were carried out under the conditions described in Experimental section with acceptor concentrations of 0.5 mM, 0.8 mM UDP-[^{3}H]GlcNAc (10000 d.p.m./nmol), at 37 °C for 15 h using 0.01 ml of Ni-NTA bead suspension. Results are means from the number of determinations shown in parentheses.

	Activity (nmol/15 h per 0.01 ml of Ni-NTA bead suspension)			
Acceptor substrate	RF1	RF2	RF1–RF2	GnT I
M3-octyl	0.31 (5)	0.034 (2)	0.28	1.2 (1)
GnGn glycopeptide	0.18 (4)	0.19 (1)	0	

1573028. This sequence and sequences deposited in the Hs.183860 Unigene database were used to obtain a 2355 bp sequence encoding amino acids 84–660 of GnT I.2 (Figure 1). Human clone HEP02567 (accession number AK000284) encodes 2736 bp of the GnT I.2 cDNA sequence, which includes 141 bp of 5′-untranslated region, the ATG start codon, the TGA stop codon, the 3′-untranslated region and the polyadenylation sequence. We have made three corrections to the sequence of AK000284 (T2048 → C, G2315 → A and CCCC at 2512 to CCC) and the sequence of the resulting full-length GnT I.2 protein is shown in Figure 1. GnT I.2 has a 1980 bp open reading frame encoding a 660 amino acid protein and shows the membrane protein type-2 domain structure typical of glycosyltransferases. That is, a 37-residue N-terminal cytoplasmic domain, a 24-residue non-cleavable hydrophobic signal-anchor domain, a relatively long stem region (≈ 239 amino acids) and a catalytic domain (≈ 360 amino acids; Figures 1 and 2). Since the mRNA for GnT I.2 is about 3.3 kb (see below), over 500 bp of 5′-untranslated sequence remains to be determined. Recently, further cDNA sequences have appeared for both the human (accession numbers AK022727, NM_017739 and BC001471) and mouse (accession numbers AK002638, AK019640 and AK015478) genes. The database entry for Unigene Hs.183860 indicates that the human *MGAT1.2* gene maps to chromosome 1, whereas GnT I maps to 5q35. The mouse and human GnT I.2 amino acid sequences are 98.5 % identical over 620 residues. The identities between human GnT I.2 and the three *C. elegans* GnT I sequences are 31 % over 269 residues (*gly-12*), 37 % over 156 residues (*gly-13*) and 32 % over 352 residues (*gly-14*); it is therefore not possible to determine by sequence comparisons alone whether any of these three genes is an orthologue for GnT I.2.

Several amino acids in the human GnT I.2 sequence are aligned with residues in the GnT I sequence identified as contact regions for UDP-GlcNAc-Mn^{2+} in the X-ray crystal structure of rabbit GnT I [28] (Table 1). It should be noted that the so-called DxD motif found in many glycosyltransferases is EDD in GnT I and EED (residues 393–395) in both human and mouse GnT I.2.

Properties of recombinant human GnT I.2 expressed in the baculovirus/Sf9 system

Truncated human GnT I.2 (construct pVT-Bac-His-RF1, amino acid residues 227–660) was expressed in the baculovirus/Sf9 system as a secreted protein containing a $(His)_6$ tag. Western-blot

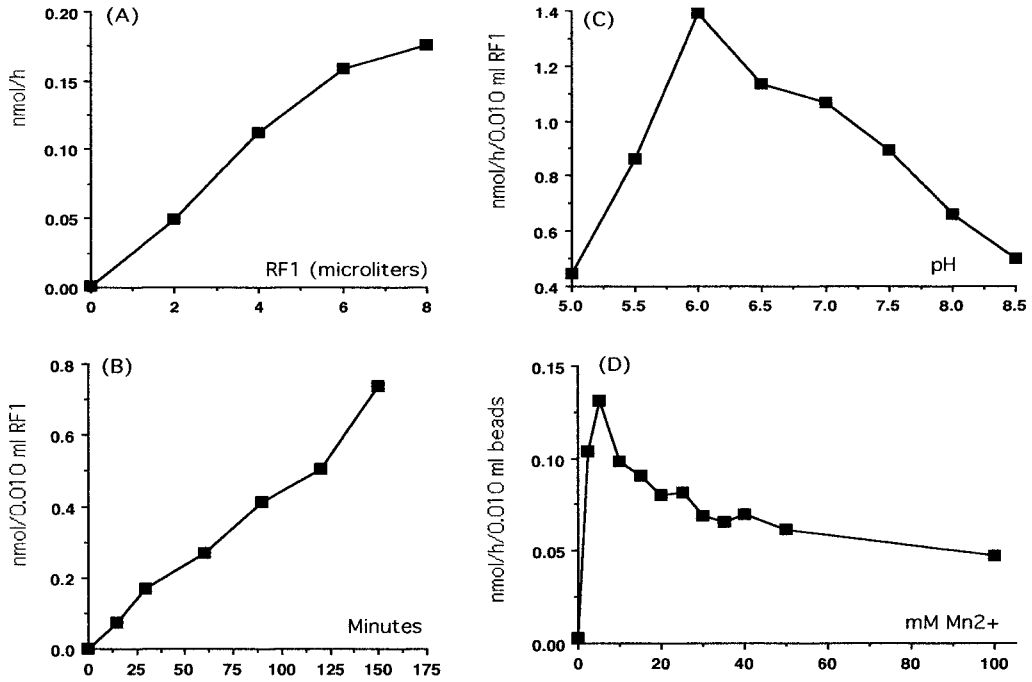

Figure 4 Kinetic studies on human GnT I.2

(**A**) The effect of enzyme concentration on GnT I.2 activity. Assays were carried out with 5 mM Man(α1-)*O*-benzyl as acceptor, 1 mM UDP-[^{3}H]GlcNAc (6000 d.p.m./nmol), 20 mM $MnCl_2$, 120 mM Mes (pH 6.0) and volumes of RF1 supernatant as indicated, at 37 °C for 2 h. (**B**) The effect of time of incubation on GnT I.2 activity. Assays were carried out as in (**A**) with 0.008 ml of RF1 supernatant. (**C**) The effect of pH on GnT I.2 activity. Assays were carried out with 15 mM Man(α1-)*O*-benzyl as acceptor, 1 mM UDP-[^{3}H]GlcNAc (2000 d.p.m./nmol), 20 mM $MnCl_2$, 120 mM buffer (Mes, pH 5.0–7.0; Hepes, pH 7.5–8.5) and 0.02 ml of RF1 supernatant, at 37 °C for 1 h. (**D**) The effect of $MnCl_2$ concentration on GnT I.2 activity. Assays were carried out with 0.5 mM M3-octyl as acceptor, 1 mM UDP-[^{3}H]GlcNAc (9000 d.p.m./nmol), $MnCl_2$ concentrations as indicated, 100 mM Mes (pH 6.5) and 0.01 ml of enzyme (near full-length recombinant GnT I.2 from Sf9 cell lysates, adsorbed on to Ni-NTA beads), at 37 °C for 6 h. Assays carried out with enzyme adsorbed on to Ni-NTA beads do not show endogenous insect cell activity.

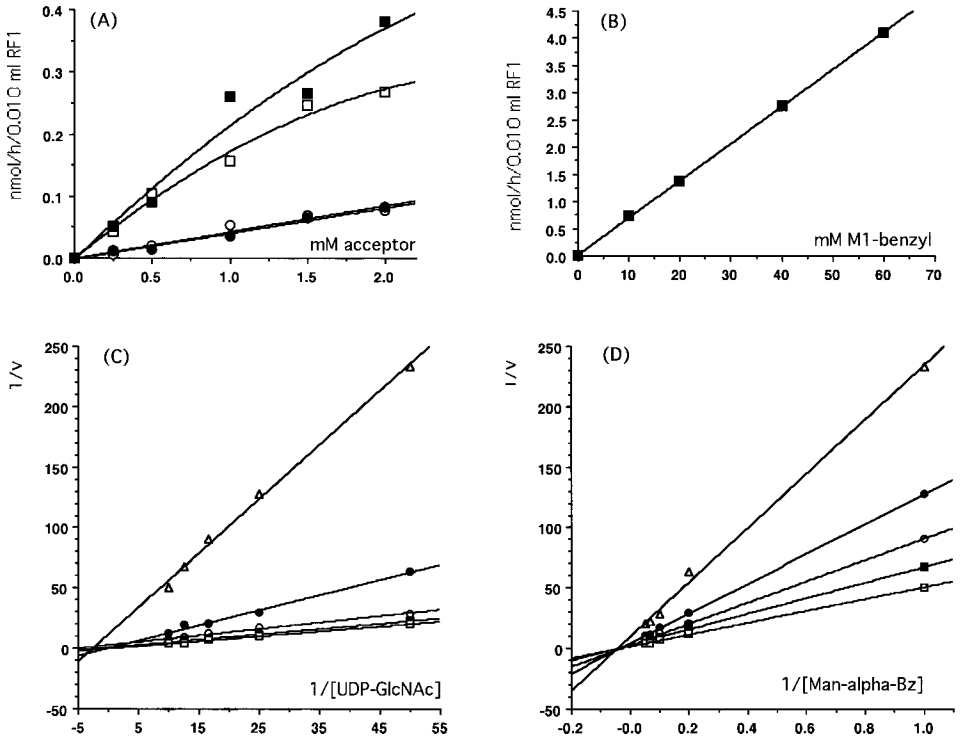

Figure 5 Kinetic studies on human GnT I.2

(**A**) Velocity/substrate plots for RF1 enzyme activity with acceptors Man(α1-)*O*-benzyl (○, ●) and CYA[Man(α1-)*O*-T]AV (□, ■) at UDP-GlcNAc concentrations of 2 (□, ○) and 4 (■, ●) mM. Acceptor concentrations were as indicated. Assays were run with UDP-[^{3}H]GlcNAc (8000 d.p.m./nmol), at 25 mM $MnCl_2$, 3% Triton X-100, 150 mM Mes (pH 6.0) and 0.008 ml of GnT I.2 (RF1 supernatant), at 37 °C for 3 h. The K_m value for CYA[Man(α1-)*O*-T]AV was calculated to be $\approx$ 12 mM from these data. (**B**) Velocity/substrate plot for RF1 enzyme activity with acceptor Man(α1-)*O*-benzyl (M1-benzyl) at high concentrations (as indicated). Assays were run at 2 mM UDP-[^{3}H]GlcNAc (7500 d.p.m./nmol), 7.5 mM $MnCl_2$, 120 mM Mes (pH 6.0) and 0.02 ml of RF1 supernatant, at 37 °C for 1 h. It can be concluded that the K_m for Man(α1-)*O*-benzyl is well over 30 mM. (**C**, **D**) Kinetic parameters were determined by a series of reciprocal velocity/substrate plots at five concentrations of both acceptor and donor substrates using 0.02 ml of RF1 supernatant in 120 mM Mes (pH 6.0) at 37 °C for 1 h. Reciprocal plots are shown for UDP-GlcNAc [**C**; with Man(α1-)*O*-benzyl concentrations (mM) of 1, Δ; 5, ●; 10, ○; 15, ■; 20, □] and Man(α1-)*O*-benzyl [**D**; with UDP-GlcNAc concentrations (mM) of 0.02, Δ; 0.04, ●; 0.06, ○; 0.08, ■; 0.1, □]. Velocity (v) is expressed as nmol/h per 0.02 ml of RF1 supernatant. The data indicate K_m values of about 0.3 and $>$ 15 mM for UDP-GlcNAc and Man(α1-)*O*-benzyl respectively.

Table 3 The effect of bivalent cations on GnT I.2 activity

Assays were carried out with 15 mM Man(α1-)*O*-benzyl as an acceptor, 1 mM UDP-[^{3}H]GlcNAc (2000 d.p.m./nmol), 5 mM cation, 120 mM Mes buffer (pH 6.0) and 0.02 ml of RF1 supernatant, at 37 °C for 1 h. Results are means from duplicate assays.

Bivalent cation	GnT I.2 activity (nmol/h per 0.01 ml of RF1)
Manganese	0.96
Cobalt	0.39
Iron	0.26
Cadmium	0.18
Magnesium	0.063
Nickel	0.047
Calcium	0.037
Barium	0.03
Zinc	0.023
Copper	0.01
No cation + EDTA	0.021
No cation	0.023

analysis using mouse monoclonal antibody raised against the enterokinase cleavage site [17] showed a protein band at about 56 kDa (Figure 3); the predicted molecular mass was 55 kDa. Assays with GnT I.2 (RF1), the RF2 out-of-frame control and M3-octyl as a substrate (Table 2) showed that there is minimal endogenous insect cell enzyme activity when Ni-adsorbed enzyme is used. However, there is significant transfer of GlcNAc to M3-octyl when RF2-containing Sf9 supernatant is assayed directly (results not shown). The product formed by RF1 was identified as Man(α1-6)[GlcNAc(β1-2)Man(α1-3)]Man(β1-)*O*-octyl (GnM3-octyl) by co-migration with standard oligosaccharide [29] on TLC in the following three solvent systems: (i) acetonitrile/water, 5:1; (ii) dichloromethane/methanol/water, 6:2:1; and (iii) chloroform/methanol/water, 6:2:1 (results not shown).

Both GnT I.2 (RF1) and GnT I can transfer GlcNAc to 10 mM M3-octyl; however, GnT I.2 can also use 10 mM Man(α1-)*O*-benzyl as an acceptor under conditions in which GnT I is totally inactive with this acceptor (results not shown). The advantage of using Man(α1-)*O*-benzyl is that RF2-containing Sf9 supernatant has no detectable enzyme activity with this substrate, showing that Sf9 cells have no endogenous GnT I.2 activity. GnT I.2 was therefore characterized with Man(α1-)*O*-benzyl as an acceptor.

The rate of product formation was proportional to the volume of RF1 (Figure 4A) and time of incubation up to 150 min (Figure 4B). The optimum pH (Figure 4C) and Mn^{2+} concentration (Figure 4D) were 6.0 and 5 mM respectively. Since the enzyme velocity increases linearly with Man(α1-)*O*-benzyl concentration to 60 mM (the highest concentration tested), the K_m value for this acceptor must be well over 30 mM (Figures 5A and 5B). Kinetic analysis of GnT I.2 with Man(α1-)*O*-benzyl as substrate gave linear plots of $1/v$ against 1/[substrate] (Figures 5C and

Table 4 Acceptor specificity of GnT I.2

Assays with RF1 and RF2 were run with 1 mM acceptor, 1 mM UDP-[^{3}H]GlcNAc (5600 d.p.m./nmol), 6 mM $MnCl_2$, 1% Triton X-100, 50 mM Mes buffer (pH 6.0) and 0.008 ml of enzyme (RF1 or RF2 supernatants), at 37 °C for 2 h. All RF1 assays were corrected for endogenous activity (RF2). Assays with Ni-NTA beads were run with 66 mM acceptor, 0.3 mM UDP-[^{3}H]GlcNAc (10000 d.p.m./nmol), 16 mM $MnCl_2$, 8 mM AMP, 80 mM GlcNAc, 0.2 M Mes (pH 6.5) and 0.01 ml of Ni-NTA beads adsorbed with lysates of Sf9 cells infected with a baculovirus preparation expressing near full-length GnT I.2 (encoded by plasmid FpAcHLT-FL), at 37 °C for 6 h. Assays carried out with enzyme adsorbed on to Ni-NTA beads do not show endogenous insect cell activity. Means ± S.D. are shown, with the number of assays given in parentheses.

	GnT I.2 activity (nmol/h per 0.01 ml of enzyme)	
Acceptor	RF1	Ni-NTA beads
M3-octyl	0.082 ± 0.04 (4)	
GnM3-octyl	0.1 ± 0.02 (4)	
M2-octyl	0.01 ± 0.01 (2)	
Man(α1-)*O*-benzyl	0.025 ± 0.01 (4)	0.42 ± 0.02 (2)
Man(β1-)*O*-*p*-nitrophenyl		< 0.01 (2)
Man(β1-)*O*-octyl		< 0.01 (2)
CYA[Man(α1-)*O*-T]AV	0.37 ± 0.12 (2)	

Table 5 Proton NMR data at 25 °C for GnT I.2 substrate Man(α1-)*O*-benzyl (M1-benzyl) and product GlcNAc(β1-2)Man(α1-)*O*-benzyl (GnM1-benzyl)

Chemical shifts (δ) in p.p.m. with coupling constants (*J*, Hz) in parentheses. Peak numbers refer to the spectra shown in Figure 6.

Proton	M1-benzyl	GnM1-benzyl	Peak number (Figure 6)
Mannose			
H-1	4.974	4.94	1
H-2	3.942	4.047	3
GlcNAc			
H-1		4.484 (8.0 Hz)	4
N-acetyl		2.012	Not shown
Benzyl			
CH-2	4.585 (11 Hz)	4.582 (12 Hz)	2

5D). K_m values of ≈ 0.3 and > 15 mM were obtained for UDP-GlcNAc and Man(α1-)*O*-benzyl respectively. GnT I.2 showed a strong preference for Mn^{2+} and was inactive without addition of a bivalent cation (Table 3).

The enzyme was also active with M2-octyl and GnM3-octyl (the substrate for UDP-GlcNAc:α-6-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase II [22]) (Table 4). The product formed with M3-octyl may therefore be a mixture of GnM3-octyl and GlcNAc(β1-2)Man(α1-6)[Man(α1-3)]Man (β1-)*O*-octyl, but we have not examined this possibility. The enzyme shows a 3–7-fold increase over Man(α1-)*O*-benzyl activity with the glycopeptide CYA[Man(α1-)*O*-T]AV (Figure 5A and Table 4). The K_m value for CYA[Man(α1-)*O*-T]AV was calculated to be ≈ 12 mM (Figure 5A). Whereas Man(α1-)*O*-benzyl acted as an acceptor for GnT I.2, Man(β1-)*O*-octyl and Man (β1-)*O*-*p*-nitrophenyl were totally inactive (Table 4), indicating a strict preference for the α-anomer. We also showed that GnGn glycopeptide is not an acceptor (Table 2), indicating that the enzyme has no detectable GnT III, IV, V, VII or VIII activities; as a positive control for GnGn, we showed that the same preparation of GnGn serves as an excellent substrate for a plant β1,2-xylosyltransferase under study in our laboratory (S. Pagny, unpublished work). The following oligosaccharides and glycosides were tested as acceptors using radioactive UDP-GlcNAc, UDP-Gal and UDP-GalNAc as donors and were found to be ineffective: GlcNAc(α1-)*O*-benzyl, GlcNAc(β1-)*O*-benzyl, GalNAc(α1-)*O*-*p*-nitrophenyl, GalNAc(β1-)*O*-*p*-nitrophenyl, GalNAc(α1-)*O*-benzyl, Gal(α1-)*O*-*o*-nitrophenyl, Gal(β1-)*O*-*o*-nitrophenyl, Gal(β1-4)GlcNAc(α1-)*O*-*p*-nitrophenyl, Gal(β1-4) Glc(β1-)*O*-benzyl, Gal(β1-4)Glc, Gal(β1-4)GlcNAc, L-Fuc(α1-) *O*-*p*-nitrophenyl and L-Fuc(β1-)*O*-*p*-nitrophenyl.

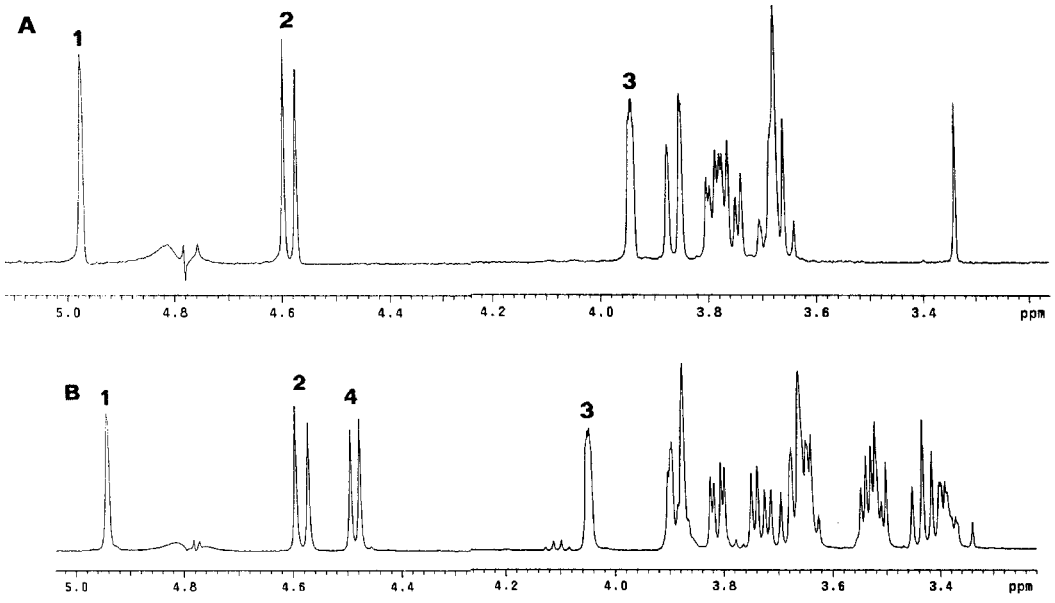

Figure 6 Proton NMR spectra taken at 25 °C of GnT I.2 substrate Man(α1-)*O*-benzyl (A) and product GlcNAc(β1-2)Man(α1-)*O*-benzyl (B)

See Table 5 for assignment of peaks.

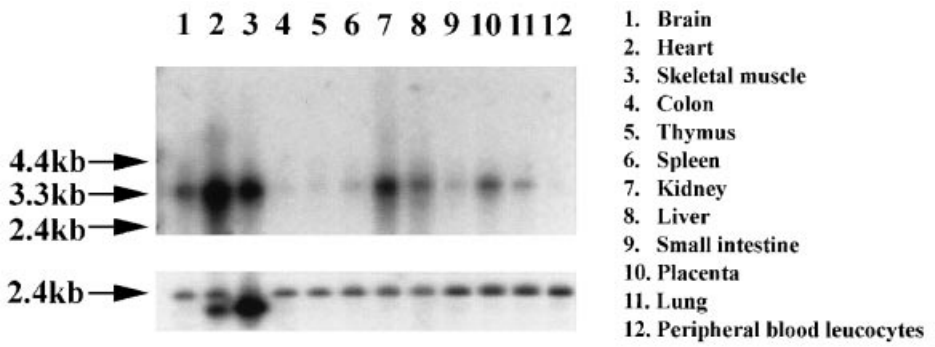

Figure 7 Northern-blot analysis

Top panel: a Human Multiple Tissue cDNA Panel (Clontech) was hybridized with ^{32}P-labelled probe (a 951 bp DNA fragment from RF1). GnT I.2 mRNA is seen at 3.3 kb. Standards at 4.4 and 2.4 kb are indicated. Bottom panel: the GnT I.2 probe was removed from the blot and the cDNA panel was hybridized once again with β-actin cDNA. The tissues analysed are listed on the figure.

Microsome extracts from Sf9 cells infected with baculovirus expressing near full-length GnT I.2 (FpAcHLT-FL) showed GnT I.2 activity of 0.62 nmol/h per 0.02 ml at 37 °C with 20 mM Man(α1-)*O*-benzyl as an acceptor, 1 mM UDP-[^{3}H]GlcNAc (2500 d.p.m./nmol), 20 mM $MnCl_2$ and 140 mM Mes (pH 6.0). Similar incubations with the reverse control (RpAcHLT-FL) showed no activity. The data show that at least some of the GnT I.2 is membrane bound.

Identification of the product of GnT I.2 action

Comparison of the proton NMR spectra of purified GnT 1.2 product and Man(α1-)*O*-benzyl substrate (Figure 6 and Table 5) shows the presence of a β-linked GlcNAc in the product (H-1 chemical shift of 4.484 p.p.m. and an 8 Hz coupling constant). The product Man H-2 shows a 0.105 p.p.m. downfield shift characteristic of a 1,2 linkage [29–31]. The Man H-2 was identified by the presence of a strong cross-peak between Man H-1 and H-2 on the TOCSY spectra of both compounds (results not shown). The product formed with Man(α1-)*O*-benzyl was therefore identified as GlcNAc(β1-2)Man(α1-)*O*-benzyl.

Northern-blot analysis

Northern-blot analysis of human GnT I.2 (Figure 7) showed a 3.3 kb message with a wide tissue distribution similar to that of GnT I [32–34]. The EST database shows that GnT I.2 mRNA has been obtained from liver, spleen, adrenal, brain, breast, colon, oesophagus, small intestine, heart, skeletal muscle, kidney, lung, lymph node, ovary, pancreas, pancreatic islet, parathyroid, placenta, prostate, spleen and testis.

DISCUSSION

Here we have reported the cloning and expression of a human gene encoding a novel enzyme, GnT I.2, with homology to GnT I. An orthologous gene is present in the mouse. Recombinant human GnT I.2 does indeed exhibit GnT I enzyme activity but the K_m value for the acceptor substrate M3-octyl is appreciably higher with GnT I.2 (> 30 mM) than with GnT I (≈ 1 mM). GnT I.2 showed similarly weak transferase activities with another GnT I substrate, M2-octyl, and with compounds that are not substrates for GnT I, GnM3-octyl and Man(α1-)*O*-benzyl. The product formed with Man(α1-)*O*-benzyl was identified as GlcNAc(β1-2)Man(α1-)*O*-benzyl by proton NMR spectroscopy. There was no activity with Man(β1-)*O*-R (where R is *p*-nitrophenyl or octyl). Furthermore, studies on the embryonic lethal GnT I-null mouse [8,9,35] indicate that GnT I.2 cannot replace GnT I during embryogenesis. The conclusion from these data is that although GnT I.2 acts on terminal α-linked Man residues, the enzyme is clearly different from GnT I.

A clue as to the possible biological significance of GnT I.2 comes from recent studies on dystroglycan (dystrophin-associated glycoprotein), a cytoskeleton-linked extracellular matrix receptor expressed in many cell types and composed of α- and β-subunits encoded by a single mRNA [36]. Dystroglycan is synthesized as a precursor propeptide that is post-translationally cleaved and differentially glycosylated to yield α- and β-dystroglycans. β-Dystroglycan is a transmembrane protein which binds dystrophin and the intracellular cytoskeleton. In the basal lamina of muscle cells and Schwann cells, α-dystroglycan binds both β-dystroglycan and extracellular laminin and acts as a link between them [37–40]. The major sialylated O-glycosidically linked oligosaccharide of bovine peripheral nerve α-dystroglycan is a novel O-mannosyl-type oligosaccharide, sialyl(α2-3)Gal(β1-4)GlcNAc (β1-2)Man(α1-)*O*-Ser/Thr [41,42]. This oligosaccharide constitutes at least 66% of the sialylated O-linked sugar chains. Furthermore, a laminin-binding inhibition study suggested that the sialyl(α2-3)Gal(β1-4)GlcNAc moiety of this sugar chain is involved in the interaction of α-dystroglycan with laminin. The same oligosaccharide was found in rabbit skeletal-muscle α-dystroglycan [43]. O-mannosyl glycans are present in other mammalian glycoproteins and several structures other than sialyl(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-)*O*-Ser/Thr have been described: Gal(β1-4)R, Gal(β1-4)[Fuc(α1-3)]R and 3-*O*-sulpho-GlcA(β1-3)Gal(β1-4)R, where R is GlcNAc(β1-2 or β1-3)Man(α1-)*O*-Ser/Thr (see [42] for references).

A novel UDP-GlcNAc:O-linked Man β-1,2-*N*-acetylglucosaminyltransferase was recently found in the microsome fraction of newborn rat brains [23]. The enzyme was shown to incorporate GlcNAc in β-1-2 linkage into the Man residue of *N*-acetyl-AAP(Man-T)PVAAP-NH_2; it is not clear from the paper whether the Man was α- or β-linked to Thr. The authors found that the peptide ATP(Man-T)PVTA, corresponding to residues 316–323 of α-dystroglycan, was not suitable for assays because the non-glycosylated version ATPTPVTA was also a substrate for their crude enzyme fraction, presumably due to the action of O-GlcNAc-transferase on the free Thr residues. We found that the activity of human GnT I.2 using CYA[Man(α1-)*O*-T]AV ($K_m \approx$ 12 mM) as a substrate was between three and seven times more rapid than with our other substrates, implying that GnT I.2 may

be the same enzyme as described by Takahashi et al. [23]. It should be pointed out that CYA[Man(α1-)*O*-T]AV does not have a free Thr and is therefore not a substrate for O-GlcNAc-transferase.

Dystroglycan is a central component of the dystrophin–glycoprotein complex, a protein assembly that plays a critical role in a variety of muscular dystrophies [44]. Mouse embryos with a homozygous null mutation in the dystroglycan gene exhibit gross developmental abnormalities beginning at about 6.5 days of gestation [44]. Analysis of the mutant phenotype indicates that an early defect is a disruption of Reichert's membrane, an extra-embryonic basement membrane. Specifically, the localization of two critical structural elements of Reichert's membrane (laminin and collagen IV) are disrupted. Dystroglycan is also required for formation of a basement membrane in embryoid bodies, probably by binding soluble laminin and organizing it on the cell surface [45]. These results suggest that disruption of basement-membrane organization might be a common feature of muscular dystrophies linked to the dystrophin–glycoprotein complex. If the sialylated O-mannosyl oligosaccharide is required for the binding of α-dystroglycan to laminin [38,41,42], mice with a null mutation in the GnT I.2 gene should develop myodystrophy. Study of GnT I.2-null mutant mice should be able to determine whether GnT I.2 is the enzyme required for the synthesis of the GlcNAc(β1-2)Man(α1-)*O*-Ser/Thr moiety on α-dystroglycan and other O-mannosylated glycoproteins.

Note added in proof (received 16 November 2001)

The following paper has recently been published [47]. This paper shows that six patients with muscle–eye–brain disease (MEB, MIM 253280), a disease characterized by congenital muscular dystrophy, have point mutations in the same gene as we report in our present study.

We thank Dr Henrik Clausen, School of Dentistry, Copenhagen, Denmark, for his help during the early parts of this investigation and Dr Sabine Strahl-Bolsinger, University of Regensburg, Regensburg, Germany, Dr Erika Staudacher, Universität der Bodenkultur (BOKU), Vienna, Austria, and Dr Sumio Sugano, NEDO Human cDNA Sequencing Project, Institute of Medical Science, University of Tokyo, Tokyo, Japan, for their kind gifts of materials. The NMR spectra were run by David Froom. This research was supported by the Medical Research Council of Canada.

REFERENCES

1 Hemming, F. W. (1995) The coenzymic role of phosphodolichols. In Glycoproteins, vol. 29a (Montreuil, J., Vliegenthart, J. F. G. and Schachter, H., eds.), pp. 127–143, Elsevier, Amsterdam
2 Silberstein, S. and Gilmore, R. (1996) Biochemistry, molecular biology, and genetics of the oligosaccharyltransferase. FASEB J. **10**, 849–858
3 Verbert, A. (1995) From $Glc_3Man_9GlcNAc_2$-protein to $Man_5GlcNAc_2$-protein: transfer 'en bloc' and processing. In Glycoproteins, vol. 29a (Montreuil, J., Vliegenthart, J. F. G. and Schachter, H., eds.), pp. 145–152, Elsevier, Amsterdam
4 Schachter, H. (1995) Glycosyltransferases involved in the synthesis of N-glycan antennae. In Glycoproteins, vol. 29a (Montreuil, J., Vliegenthart, J. F. G. and Schachter, H., eds.), pp. 153–199, Elsevier, Amsterdam
5 Schachter, H. (1986) Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides. Biochem. Cell Biol. **64**, 163–181
6 Schachter, H. (1991) The "yellow brick road" to branched complex N-glycans. Glycobiology **1**, 453–461
7 Stanley, P. (1992) Glycosylation engineering. Glycobiology **2**, 99–107
8 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. U.S.A. **91**, 728–732
9 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
10 Sarkar, M. and Schachter, H. (2001) Cloning and expression of *Drosophila melanogaster* UDP-GlcNAc:alpha-3-D-mannoside beta1,2-N-acetylglucosaminyltransferase I. Biol. Chem. **382**, 209–217
11 Amado, M., Almeida, R., Schwientek, T. and Clausen, H. (1999) Identification and characterization of large galactosyltransferase gene families: galactosyltransferases for all functions. Biochim. Biophys. Acta **1473**, 35–53
12 Oriol, R., Mollicone, R., Cailleau, A., Balanzino, L. and Breton, C. (1999) Divergent evolution of fucosyltransferase genes from vertebrates, invertebrates, and bacteria. Glycobiology **9**, 323–334
13 Chen, S. H., Zhou, S. H., Sarkar, M., Spence, A. M. and Schachter, H. (1999) Expression of three *Caenorhabditis elegans* N-acetylglucosaminyltransferase I genes during development. J. Biol. Chem. **274**, 288–297
14 Betel, D., Zhang, W. and Schachter, H. (2000) Human UDP-GlcNAc:alpha-3-D-mannoside beta-1,2-*N*-acetylglucosaminyltransferase I.2 (GnT I.2), a homologue of GnT I, is enzymatically active. Glycobiology **10**, 1103–1104
15 Betel, D., Zhang, W. and Schachter, H. (2000) Human UDP-GlcNAc:alpha-3-D-mannoside beta-1,2-*N*-acetylglucosaminyltransferase I.2 (GnT I.2), a homologue of GnT I, is enzymatically active. Glycoconj. J. **17**, 41
16 Tessier, D. C., Thomas, D. Y., Khouri, H. E., Laliberté, F. and Vernet, T. (1991) Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide. Gene **98**, 177–183
17 Sarkar, M., Pagny, S., Unligil, U., Joziasse, D., Mucha, J., Glossl, J. and Schachter, H. (1998) Removal of 106 amino acids from the N-terminus of UDP-GlcNAc:alpha-3-D-mannoside beta-1,2-N-acetylglucosaminyltransferase I does not inactivate the enzyme. Glycoconj. J **15**, 193–197
18 Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1993) Current Protocols in Molecular Biology, John Wiley and Sons, New York
19 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor
20 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
21 Sarkar, M. (1994) Expression of recombinant rabbit UDP-GlcNAc:alpha 3-D-mannoside beta-1,2-N-acetylglucosaminyltransferase I catalytic domain in Sf9 insect cells. Glycoconj. J. **11**, 204–209
22 Tan, J., D'Agostaro, G. A. F., Bendiak, B., Reck, F., Sarkar, M., Squire, J. A., Leong, P. and Schachter, H. (1995) The human UDP-N-acetylglucosamine: alpha-6-D-mannoside-beta-1,2-N-acetylglucosaminyltransferase II gene (MGAT2) – cloning of genomic DNA, localization to chromosome 14q21, expression in insect cells and purification of the recombinant protein. Eur. J. Biochem. **231**, 317–328
23 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 beta-1,2-N-acetylglucosaminyltransferase that may play a role in the biosynthesis of mammalian O-mannosyl glycans. Glycobiology **11**, 37–45
24 Palcic, M. M., Heerze, L. D., Pierce, M. and Hindsgaul, O. (1988) The use of hydrophobic synthetic glycosides as acceptors in glycosyltransferase assays. Glycoconj. J. **5**, 49–63
25 Nishikawa, Y., Pegg, W., Paulsen, H. and Schachter, H. (1988) Control of glycoprotein synthesis. XV. Purification and characterization of rabbit liver UDP-N-acetylglucosamine:α-3-D-mannoside β-1,2-N-acetylglucosaminyltransferase I. J. Biol. Chem. **263**, 8270–8281
26 Dixon, M. and Webb, E. C. (1964) Enzymes, Longman, London
27 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) **227**, 680–685
28 Ünligil, U. M., Zhou, S., Yuwaraj, S., Sarkar, M., Schachter, H. and Rini, J. M. (2000) X-ray crystal structure of rabbit N-acetylglucosaminyltransferase I: catalytic mechanism and a new protein superfamily. EMBO J. **19**, 5269–5280
29 Reck, F., Springer, M., Paulsen, H., Brockhausen, I., Sarkar, M. and Schachter, H. (1994) Synthesis of tetrasaccharide analogues of the N-glycan substrate of beta-(1 → 2)-N-acetylglucosaminyltransferase II using trisaccharide precursors and recombinant beta-(1 → 2)-N-acetylguucosaminyltransferase I. Carbohydr. Res. **259**, 93–101
30 Mulder, H., Dideberg, F., Schachter, H., Spronk, B. A., De Jong-Brink, M., Kamerling, J. P. and Vliegenthart, J. F. G. (1995) In the biosynthesis of *N*-glycans in connective tissue of the snail *Lymnaea stagnalis*, incorporation of GlcNAc by beta-2-GlcNAc-transferase I is an essential prerequisite for the action of beta-2-GlcNAc-transferase II and beta-2-Xyl-transferase. Eur. J. Biochem. **232**, 272–283
31 Möller, G., Reck, F., Paulsen, H., Kaur, K. J., Sarkar, M., Schachter, H. and Brockhausen, I. (1992) Control of glycoprotein synthesis: substrate specificity of rat liver UDP-GlcNAc:Manalpha3R beta2-N-acetylglucosaminyltransferase I using synthetic substrate analogues. Glycoconj. J. **9**, 180–190

32 Kumar, R., Yang, J., Larsen, R. D. and Stanley, P. (1990) Cloning and expression of N-acetylglucosaminyltransferase I, the medial Golgi transferase that initiates complex N-linked carbohydrate formation. Proc. Natl. Acad. Sci. U.S.A. **87**, 9948–9952
33 Sarkar, M., Hull, E., Nishikawa, Y., Simpson, R. J., Moritz, R. L., Dunn, R. and Schachter, H. (1991) Molecular cloning and expression of cDNA encoding the enzyme that controls conversion of high-mannose to hybrid and complex N-glycans: UDP-N-acetylglucosamine:alpha-3-D-mannoside beta-1,2-N-acetylglucosaminyltransferase I. Proc. Natl. Acad. Sci. U.S.A. **88**, 234–238
34 Yip, B., Chen, S. H., Mulder, H., Hoppener, J. W. M. and Schachter, H. (1997) Organization of the human beta-1,2-N-acetylglucosaminyltransferase I gene (MGAT1), which controls complex and hybrid N-glycan synthesis. Biochem. J. **321**, 465–474
35 Ioffe, E., Liu, Y. and Stanley, P. (1997) Complex N-glycans in Mgat1 null preimplantation embryos arise from maternal Mgat1 RNA. Glycobiology **7**, 913–919
36 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
37 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 (London) **355**, 696–702
38 Yamada, H., Chiba, A., Endo, T., Kobata, A., Anderson, L. V., Hori, H., Fukuta-Ohi, H., Kanazawa, I., Campbell, K. P., Shimizu, T. and Matsumura, K. (1996) Characterization of dystroglycan-laminin interaction in peripheral nerve. J. Neurochem. **66**, 1518–1524
39 Matsumura, K., Chiba, A., Yamada, H., Fukuta-Ohi, H., Fujita, S., Endo, T., Kobata, A., Anderson, L. V., Kanazawa, I., Campbell, K. P. and Shimizu, T. (1997) A role of dystroglycan in schwannoma cell adhesion to laminin. J. Biol. Chem. **272**, 13904–13910

Received 25 June 2001/6 September 2001; accepted 30 October 2001

40 Montanaro, F., Lindenbaum, M. and Carbonetto, S. (1999) alpha-Dystroglycan is a laminin receptor involved in extracellular matrix assembly on myotubes and muscle cell viability. J. Cell. Biol. **145**, 1325–1340
41 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 alpha-dystroglycan. The role of a novel O-mannosyl-type oligosaccharide in the binding of alpha-dystroglycan with laminin. J. Biol. Chem. **272**, 2156–2162
42 Endo, T. (1999) O-mannosyl glycans in mammals. Biochim. Biophys. Acta **1473**, 237–246
43 Sasaki, T., Yamada, H., Matsumura, K., Shimizu, T., Kobata, A. and Endo, T. (1998) Detection of O-mannosyl glycans in rabbit skeletal muscle alpha- dystroglycan. Biochim. Biophys. Acta **1425**, 599–606
44 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
45 Henry, M. D. and Campbell, K. P. (1998) A role for dystroglycan in basement membrane assembly. Cell **95**, 859–870
46 Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**, 105–132
47 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