

# Identification of Glycosyltransferase 8 Family Members as Xylosyltransferases Acting on O-Glucosylated Notch Epidermal Growth Factor Repeats<sup>\*[5]</sup>

Received for publication, September 11, 2009, and in revised form, November 10, 2009  
Published, JBC Papers in Press, November 25, 2009, DOI 10.1074/jbc.C109.065409

Maya K. Sethi<sup>‡</sup>, Falk F. R. Buettner<sup>‡§</sup>, Vadim B. Krylov<sup>¶</sup>, Hideyuki Takeuchi<sup>||</sup>, Nikolay E. Nifantiev<sup>¶</sup>, Robert S. Haltiwanger<sup>||</sup>, Rita Gerardy-Schahn<sup>‡</sup>, and Hans Bakker<sup>‡\*1</sup>

From the <sup>‡</sup>Department of Cellular Chemistry, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany, the <sup>§</sup>Department of Infectious Diseases, Institute for Microbiology, University of Veterinary Medicine Hannover, 30173 Hannover, Germany, the <sup>¶</sup>Laboratory of Glycoconjugate Chemistry, N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospect 47, 119991 Moscow, Russian Federation, and the <sup>||</sup>Department of Biochemistry and Cell Biology, Institute for Cell and Developmental Biology, Stony Brook University, Stony Brook, New York 11794-5215

The epidermal growth factor repeats of the Notch receptor are extensively glycosylated with three different O-glycans. O-Fucosylation and elongation by the glycosyltransferase Fringe have been well studied and shown to be essential for proper Notch signaling. In contrast, biosynthesis of O-glucose and O-N-acetylglucosamine is less well understood. Recently, the isolation of the *Drosophila* mutant *rumi* has shown that absence of O-glucose impairs Notch function. O-Glucose is further extended by two contiguous  $\alpha$ 1,3-linked xylose residues. We have identified two enzymes of the human glycosyltransferase 8 family, now named GXYL1 and GXYL2 (glucoside xylosyltransferase), as UDP-D-xylose: $\beta$ -D-glucoside  $\alpha$ 1,3-D-xylosyltransferases adding the first xylose. The enzymes are specific for  $\beta$ -glucose-terminating acceptors and UDP-xylose as donor substrate. Generation of the  $\alpha$ 1,3-linkage was confirmed by nuclear magnetic resonance. Activity on a natural acceptor could be shown by *in vitro* xylosylation of a Notch fragment expressed in a UDP-xylose-deficient cell line and *in vivo* by co-expression of the enzymes and the Notch fragment in insect cells followed by mass spectrometric analysis of peptide fragments.

\* This work was supported, in whole or in part, by National Institutes of Health Grant GM061126 (to R. S. H.). This work was also supported by a grant from the Deutsche Forschungsgemeinschaft (DFG) (to R. G.-S.) for the Junior Research Group "Stem Cell Glycans" established under the roof of REBIRTH, a DFG cluster of Excellence at Hannover Medical School.

[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental "Experimental Procedures," Table S1, and Figs. S1 and S2.

<sup>1</sup> To whom correspondence should be addressed. Tel.: 49-511-5328245; E-mail: bakker.hans@mh-hannover.de.

More than half of the 29–36 epidermal growth factor (EGF)<sup>2</sup> repeats of the mammalian Notch receptors are modified with O-fucose (Fuc), O-glucose (Glc), or O-N-acetylglucosamine (GlcNAc) glycans. These O-glycans are linked to specific serine or threonine residues in distinct consensus sequences (1, 2), which are also found on EGF repeats of various other proteins that include blood coagulation factor VII and Notch ligands Delta and Serrate/Jagged (3).

Although further modifications of O-GlcNAc have not been identified yet, O-Fuc added by the protein-O-fucosyltransferase 1 (Pofut1) can be elongated to the tetrasaccharide Sia $\alpha$ 2,3/6Gal $\beta$ 1,4GlcNAc $\beta$ 1,3Fuc $\alpha$ 1-O-Ser/Thr. O-Fucosylation is clearly essential for Notch function, and *Pofut1* null mice exhibit an embryonic lethal phenotype (4–7). The enzyme has a chaperone function that, at least in *Drosophila*, seems more critical than its fucosyltransferase activity to functionalize Notch. Elongation of O-Fuc by the GlcNAc-transferase Fringe in *Drosophila* (8, 9) or its three mammalian homologs (10) modulates relative signaling intensities by different Notch ligands. In *Drosophila*, Fringe enhances signaling of Delta but reduces that of Serrate (11). Although more complicated in mammals, Fringe modification of Notch alters interactions with its ligands as well (3, 8, 12).

The O-Glc glycan typically exists as the trisaccharide Xyl $\alpha$ 1,3Xyl $\alpha$ 1,3Glc $\beta$ 1-O-Ser in mammals (1, 13). On factor VII, this glycan is bound to serine 52, and mutation of this amino acid reduces the coagulant activity in a clotting assay (14). The gene encoding the protein O-glucosyltransferase (Poglut) has recently been identified via the *Drosophila* mutant *rumi* that showed a temperature-dependent *Notch* phenotype (15). Based on the role of O-Fuc modifications, it is anticipated that xylosylation of O-Glc is playing a similar role in Notch signaling as glycosylation by Fringe. Although enzymatic activities of the two  $\alpha$ 1,3-xylosyltransferases have been detected in mammalian cells (16–18), the genes encoding the xylosyltransferases have not yet been identified, hampering further functional studies of O-Glc modifications. Here we describe the identification of two genes, now named GXYL1 and GXYL2 (glucoside xylosyltransferase), encoding enzymes able to transfer xylose to the O-Glc residue bound to Notch EGF repeats both *in vitro* and *in vivo*.

## EXPERIMENTAL PROCEDURES

**Enzyme Expression**—Protein A fusions of human GLT8D1, GLT8D2, GXYL1, and GXYL2 (Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC)), for which constructs are described in the [supplemental Experimental Procedures](#), were expressed in Sf9 insect cells grown in Insect-Xpress medium (Lonza) by baculovirus

<sup>2</sup> The abbreviations used are: EGF, epidermal growth factor; GLT8, glycosyltransferase 8 family; GXYL, glucosyl xylosyltransferase; Poglut, protein O-glucosyltransferase; UGGT, UDP-glucose:glycoprotein glucosyltransferase; CHO, Chinese hamster ovary; MS, mass spectrometry; MS/MS, tandem MS; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight.

infection (Bac-to-Bac<sup>®</sup>; Invitrogen). After 72 h of incubation, secreted proteins were purified using 100  $\mu$ l of IgG-Sepharose-6 Fast Flow (GE Healthcare) per 100 ml of culture supernatant for 12 h at 4  $^{\circ}$ C, washed according to the manufacturer's guidelines, and stored at  $-20^{\circ}$  C in 50% glycerol, 2 mM MnCl<sub>2</sub>, 10 mM MOPS, pH 7.

**Xylosyltransferase Activity Assays**—Assays were performed in 50  $\mu$ l of reaction buffer (100 mM MOPS, pH 7.5, 10 mM MnCl<sub>2</sub>, 10 mM ATP). Acceptors were applied to a final concentration of 100  $\mu$ M, and UDP-[6-<sup>3</sup>H]Gal, UDP-[1-<sup>3</sup>H]Glc (GE Healthcare), or UDP-[U-<sup>14</sup>C]Xyl (PerkinElmer Life Sciences) was diluted with cold UDP sugars to obtain 5  $\mu$ M final concentration with a specific activity of 4 kBq/nmol for <sup>3</sup>H sugars and 0.75 kBq/nmol for [<sup>14</sup>C]Xyl. Reactions were started by the addition of 10  $\mu$ l of bead-coupled enzyme, incubated at 37  $^{\circ}$ C for 1 h, and terminated with 1 ml of ice-cold H<sub>2</sub>O. Radioactivity associated to the acceptors was determined after purification on C18 columns (Sep-Pak<sup>R</sup> Vac 3cc; Waters Corp.) as described (19) and counted by liquid scintillation (LS 6500; Beckman Coulter). Enzymatic activity was expressed per  $\mu$ g of protein as calculated from Coomassie Blue staining using protein A standard as reference (see Fig. 2E). Additionally, factor VII EGF1 (20) and Glc-R (in which R is a hydrophobic aglycon) as reference were tested at a final concentration of 2  $\mu$ M using 5  $\mu$ M UDP-[<sup>14</sup>C]Xyl (7.5 kBq/nmol). Incubation time was increased to 2 h, and washing was performed with 0.5 M acidic acid, pH 3.4, to disassociate bead-coupled enzyme-product complexes.

**Expression and Purification of Notch EGF1–5 and in Vitro Xylosylation**—His/Myc-tagged mouse Notch EGF1–5 was expressed in two 175-cm<sup>2</sup> flasks of pgsI-208 and control Chinese hamster ovary (CHO) cells as described previously (21). Media (80 ml) containing secreted proteins were loaded on nickel affinity columns (HisTrap HP, 1 ml; GE Healthcare), washed with 10 volumes of binding buffer (20 mM Tris-HCl, pH 8; 150 mM NaCl), and eluted with 20 mM Tris-HCl, pH 8; 150 mM NaCl; 500 mM imidazole using a 10-ml linear gradient. Protein fractions were pooled, desalted (HiPrep 26/10; GE Healthcare) with 10 mM MOPS, pH 7, and finally concentrated to 100  $\mu$ l using Amicon Ultra-4 centrifugal devices (Millipore). Purification was confirmed by SDS-PAGE followed by Coomassie Blue staining and immunoblotting with monoclonal antibody 9E10 (anti-Myc). Ten  $\mu$ l of purified Notch EGF1–5 was incubated under standard assay conditions in the presence of 100  $\mu$ M cold UDP-Xyl as donor and enzyme for 4 h, separated by SDS-PAGE, and analyzed by mass spectrometry.

**In Vivo Xylosyltransferase Activity**—Sf9 cells were co-infected with baculovirus encoding Notch EGF1–5 and GXYLT1, GXYLT2, or GLT8D1 for 72 h. Purification and further processing of secreted EGF1–5 was performed as described for EGF1–5 from CHO cells.

**Liquid Chromatography-MS/MS**—Protein bands were excised from gels and trypsinized, and peptides were recovered as described (22). Reverse-phase chromatography using acetonitrile as an eluent was performed on a Waters nanoACQUITY UPLC device equipped with an analytical column (Waters, BEH130 C18, 100  $\mu$ m  $\times$  100 mm, 1.7- $\mu$ m particle size) coupled online to an electrospray mass ionization Q-TOF Ultima (Waters). Spectra were recorded in positive ion mode, and pep-

tides were automatically subjected to fragmentation (MS/MS). Alternatively, MALDI-TOF MS was carried out on a VoyagerDE Pro (Applied Biosystems). Peptide solution was mixed with matrix ( $\alpha$ -cyano-4-hydroxy-cinnamic acid (Bruker Daltonics), 5 mg/ml in 50% acetonitrile with 0.1% trifluoroacetic acid) and then spotted on the target plate. Spectra were acquired in positive ion mode, averaging about 1000 laser shots.

## RESULTS

**Selection of Potential Xylosyltransferases**—UDP-glucose: glycoprotein glucosyltransferase (UGGT) catalyzes the transfer of glucose in  $\alpha$ 1,3-linkage to *N*-glycans of unfolded protein. Because the generated linkage is often conserved within glycosyltransferase gene families and considering that the only difference between xylose and glucose is the absence of the C6 primary alcohol group in xylose, we hypothesized that the xylosyltransferases involved in the generation of the Xyl $\alpha$ 1,3Xyl $\alpha$ 1,3Glc $\beta$ 1 trisaccharide occurring on Notch might be homologous to UGGT. The latter enzyme is much larger than typical glycosyltransferases, having a domain potentially involved in the recognition of unfolded proteins followed by a C-terminal catalytic domain. Using this catalytic domain in a position-specific iterated (PSI)-BLAST, a group of four human genes with unknown function was identified. The genes, which were at that time named *GLT8D1* to *GLT8D4* (glycosyltransferase 8 domain-containing), belong to glycosyltransferase gene family 8 of the Carbohydrate-Active EnZymes (CAZy) data base (23) and possess the glycosyltransferase DXD motif involved in metal binding. *GLT8D1* and *GLT8D2* as well as *GLT8D3* and *GLT8D4*, which are now renamed *GXYLT1* and *GXYLT2* (HGNC), showed  $\sim$ 50% identity with each other at the amino acid level, but between the *GLT8D1*–2 and *GXYLT1*–2 pairs and with UGGT, identity drops to about 20%. All four subfamily members show the typical type-II architecture of Golgi glycosyltransferases with the prediction of a small N-terminal cytoplasmic domain, a transmembrane domain followed by a stem region, and a C-terminal catalytic domain that aligns with UGGT (Fig. 1).

**GXYLT1 and GXYLT2 Are  $\alpha$ 1,3-Xylosyltransferases**—To assess the enzymatic activity of the four putative glycosyltransferases, constructs for expression of a secreted soluble form of the enzymes in Sf9 insect cells were generated. All enzymes were expressed with an N-terminal protein A tag and lacked the predicted cytoplasmic and transmembrane domains. The enzymes secreted in the medium at similar levels (as seen by Western blotting) were captured on IgG beads by the protein A tag and used in bound form for *in vitro* glycosyltransferase assays. Initial assays were carried out using the synthetic acceptors Glc-R and Xyl-Glc-R (24). These compounds mimic the natural acceptors of the two different xylosyltransferases involved in the synthesis of the Xyl $\alpha$ 1,3Xyl $\alpha$ 1,3Glc $\beta$ 1 trisaccharide on EGF domains and allow separation of reaction products from unincorporated donor by reverse-phase chromatography. Assays carried out with UDP-[<sup>14</sup>C]Xyl as donor substrate revealed the xylosyltransferase activity of *GXYLT1* and *GXYLT2* on Glc-R but not on Xyl-Glc-R. In contrast, *GLT8D1* and *GLT8D2* were inactive with both acceptors (Fig. 2A). Specificity for both

GLT8D1	MSF-RKVNIIILVLAVALFLLVHNNFSLSS--LLRNEVTDGIVGVPQPI-----DFV	51
GLT8D2	MALLRRTNQVILFLLVILVLCVILKVKVHGTV--PKNDADD-ES-----	41
GXYLT1	M--RRLVLRVW-VLCVACGFCSTLYAESQLVAS--LEEGTGGGGKQAAVASWL-AGGGRGAVRGA-----GVAGPAAHPG	70
GXYLT2	MKLRSSRAALIILALAAALLLALISLRAGRAEPPALPARPASAPQRPAPVPAWPGGALPGASPGVRRRRPFRFRFRAGRRGAARLEKLARRPG	95
GLT8D1	PNALRHAVDGRCEELPVVIAASEERLGAIA-AINSTQHNRSNVIYIVTLNNTAD---HRLGWLNSDSKGIKIRKRVNFDPKLLEKGVKEDP	141
GLT8D2	-----ETPEELCEELPVVIAAAGRMGAMA-AINSTYNTDANILYVVGLRNTLT---RIRKWIIEHSKREINFEVFNPMVLKGIKIRPDS	126
GXYLT1	VSDRYSLKIQVPRKMLHVAVACGRLEREMVT-VLKSAIIFSIKPLQCHHFAEDDQHHSEKGRDINWSEF---DQSNNTLYPIIT---F--PSENAA	156
GXYLT2	EPRSFQAVLPELWHLAVAVACGRLERELVAVLKVAVLFSHRKTIQCHHFTEDSLKPEKDKQKLPDPS-YTKKEHFEVPIIT---F--SVGNPQ	183
UGGT	QKTEEVKQDKDDIINIFSAKSHLYRFRFLRIMLIVLKNLKTVPVKEWF-LKNYLSPTKEFFIPLYMAN---EYNEPHELEWQYK---W--PRMLHQ	1327
GLT8D1	DQGESMKPLTFARFVPIIVP-SAKAIIMDDVDVIYQGLLQYNTAF---KHCHAAAFSEDCSASTKVVVRSANQYVIG-YDVKKERIRKRL	232
GLT8D2	SRPELQPLNIVFVPEPIIHL-QHEKVIHLDVDVIVQGLIQIQLYDTLH---ALCHAAAFSDQDLPSAQDINLVGLQNTYMG-YDVKRKAIKDL	217
GXYLT1	EMKRLRKPASQRLRPELILK-EVDSLAVDPIELFLRPVDDIWSLLKFNSTQIRAMA-SHEEERIGWYVRAHPH---F--PSENAA	234
GXYLT2	EMKRLRKPASQRLRPELILK-EVDSLAVDPIELFLRPVDDIWKLLRNFNSTQIRAMA-SHEEERIGWYVRAHPH---F--PSENAA	261
UGGT	QTEKRIWYKYLLELDVLPFVLDKRLVDAQVLRDLKLRDFNFI---DCAPYGTPECDSSRREMDGYRQKSG--VWASHA	1408
GLT8D1	SMKASTCSNENGVVAVNTEWKRQNIINOLEKWMKINVBEGDLS--R--TLAGSITL---TPFLIIVHLYQOQHSST--I-DPMNVRHLGSSAKRY	316
GLT8D2	GISPTCSNENGVVAVNTEWKRQNIINOLEKWMKINVBENGLDLS--S--SLGGGVA---TSPMLIVHGHKYST--I-NPMLHRLGWNPDARY	301
GXYLT1	-----GKTGVNSGVMLMNTFRIRKRYFKNEMTIV-RVQWGDHIMPLLK---KYKLNITWGDQDILLNIFHFNESLFFVFCOMNRYRPHDICYGNSC	321
GXYLT2	-----GSAGVNSGVMLMNTFRIRKRYFKNEMTIV-RVQWGDHIMPLLYQ---KYKNAITWGDQDILLNIFHFNESLFFVFCOMNRYRPHDICYGNSC	348
UGGT	-----GKRYHISALYVVDIKKFKIA-----AGDRRGYQGLSQDPNLSNLDDQDIPNMIHQV---IKSHQDQRLWCETWCDASRKK	1485
GLT8D1	SPQFVKAALKLEHNGHLKPGWRGTSY-----DVEKWIYDPTGKFN-----LIRRF-----EISNIK	371
GLT8D2	SEHFLQEAALKLEHNGRHKPKDFPSVH-----NDLMEWVFPDPAQIF-----KLNHS	349
GXYLT1	QBAEEGGIFILEHNRGVYHDKQAFRAVYDARINCSFEQDNIRSLKPLLELELQKTVHVCYKIKYKIFIKQLAKSVDRDRA-----RSPKEK	409
GXYLT2	RBAEHGVSVLEHNRGVYHDKQAFRAVYDARINCSFEQDNIRSLKPLLELELQKTVHVCYKIKYKIFIKQLAKSVDRDRA-----RSPKEK	443
UGGT	RAKTI-----D--GNNPM---TKSEKLEA---AVIVPEWC---YDQEKIKQIRFOKEKEH---SALYKEKTRPSS---REGP-----QKRREL	1555

FIGURE 1. **Sequence alignment.** The complete amino acid sequence of four human GLT8 family members is aligned with the N-terminal catalytic domain of UGGT starting at amino acid 1243. Sequence conservation is shown when three out of five amino acids are identical. Putative transmembrane domains are shown shaded, and the conserved DXD motif is underlined.

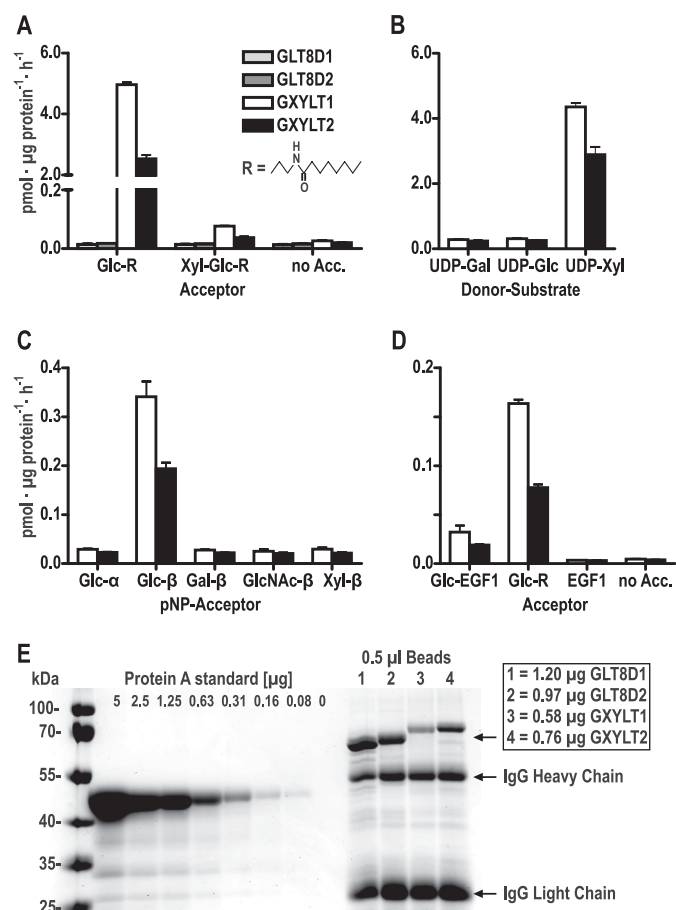


FIGURE 2. **In vitro enzymatic activity.** A, activity of GLT8D1, GLT8D2, GXYLT1, and GXYLT2 with acceptors mimicking the two naturally occurring xylosyltransferase substrates on EGF domains using UDP-Xyl as donor substrate. *no Acc.*, no acceptor. B, donor substrate specificity using Glc-R as substrate. C, acceptor substrate specificity of GXYLT1 and GXYLT2 using para-nitrophenol (pNP)-linked monosaccharides as acceptor substrates. D, glucosylated EGF1 of factor VII when compared with unglucosylated EGF1 and Glc-R as acceptor, now measured at 2  $\mu$ M acceptor concentration (when compared with 100  $\mu$ M in A-C) to adapt to the low availability of factor VII EGF1. E, quantification of enzyme bound to 0.5  $\mu$ l of IgG beads using a protein A standard and Coomassie Blue staining, of which 10  $\mu$ l was used per assay in A-D.

the donor substrate UDP-Xyl and the  $\beta$ -linked glucose acceptor was demonstrated by a series of assays using different donor substrates and a panel of acceptor sugars linked to para-nitrophenol (Fig. 2, B and C). In addition, the linkage formed by GXYLT1 was investigated. The product obtained from a reaction with 1 mg of Glc-R was purified on a C18 cartridge and analyzed by high performance liquid chromatography and  $^1$ H-NMR. This showed that the product was identical to the synthetic Xyl-Glc-R (24), which confirmed the generation of an  $\alpha$ 1,3-linkage (supplemental Fig. S1).

#### The $\alpha$ 1,3-Xylosyltransferases Act on EGF Repeats—The activity of

GXYLT1 and GXYLT2 on two different EGF repeat-containing substrates was subsequently tested. Using a radioactive assay similar to the one described above, we first demonstrated that recombinant factor VII EGF repeat 1, produced in *Escherichia coli* and *in vitro* glucosylated using Poglut (15, 20), was functioning as an acceptor, but not the unglucosylated EGF repeat (Fig. 2D). Similarly, activity of both enzymes on a protein containing the first five EGF repeats (EGF1–5) of mouse Notch1 (25) was established. For this purpose, the Notch acceptor structure was expressed in CHO pgs1-208 cells, which are deficient in UDP-xylose synthase. Although in wild type CHO cells the dominant glycan structure found on EGF1–5 is Xyl-Xyl-Glc-O, expression of EGF1–5 in pgs1-208 resulted in the exclusive substitution by Glc-O (21). After purification, the glucosylated EGF1–5 was incubated with the IgG bead-linked enzymes in the presence of UDP-Xyl, and the reaction products were analyzed by mass spectrometry after in-gel trypsinization (Fig. 3). EGF1–5 contains two O-Glc consensus sequences on EGF repeats 2 and 4. Fig. 3 shows the analysis of the glucosylated peptide derived from repeat 4, but repeat 2 is identically modified by the xylosyltransferases. The dominant peak at  $m/z$  1210 demonstrated the addition of a pentose residue to the O-glucosylated peptide by GXYLT1 and GXYLT2 (Fig. 3, B and C). In contrast, only the initial acceptor visible at  $m/z$  1166 is found in the negative control (Fig. 3A) and is not changed by incubation with GLT8D1 or GLT8D2 (data not shown).

*In vivo* activity was shown using a similar approach in insect cells. Sf9 cells express Poglut (20) and, based on the observed structures, the first xylosyltransferase. Upon transfection with an EGF1–5 construct, a mixture of peptides modified with Glc-O and Xyl-Glc-O in an approximate 2:1 ratio is produced (not shown but identical to Fig. 4A, where GLT8D1 is co-expressed). Co-expression of EGF1–5 and GXYLT1 or GXYLT2 resulted, however, in a considerable increase of the xylosylated peptide, now analyzed by more quantitative MALDI mass spectrometry (Fig. 4).



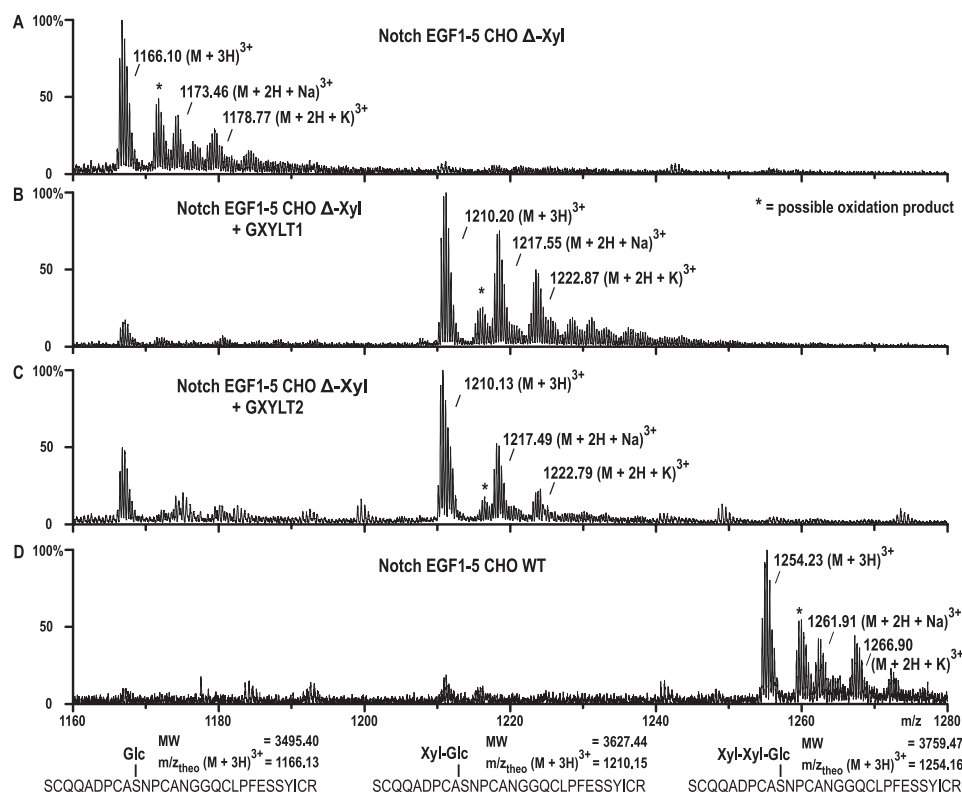


FIGURE 3. Xylosyltransferase activity using Notch EGF1-5 as acceptor. EGF1-5 produced in UDP-Xyl synthase-negative cells (A) was incubated with GXYLT1 or GXYLT2 (B and C), and tryptic peptides were analyzed by liquid chromatography-electrospray ionization-TOF mass spectrometry. Shown is the O-glucosylated peptide of EGF4. GXYLT1 and GXYLT2 were able to extend the O-glucosylated peptide by one xylose but not to produce the full trisaccharide that is produced in wild type CHO cells (D). To confirm that the  $m/z$  values shown represent the differentially glycosylated peptides of EGF4, sequencing was done by tandem mass spectrometry and shown in supplemental Fig. S2. MW, molecular weight.

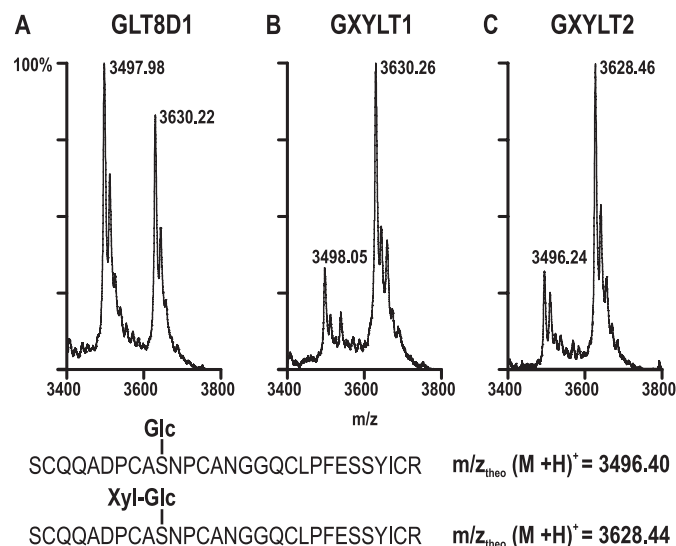


FIGURE 4. *In vivo* xylosyltransferase activity. Shown are the MALDI-mass spectra of insect cell-produced EGF1-5 that was co-infected with GLT8D1 as a negative control (A), GXYLT1 (B), or GXYLT2 (C). The glucosylated peptide with the calculated nominal mass of 3498 Da is most dominant in A but is reduced in favor of the Xyl-Glc-peptide (3630 Da) after incubation with GXYLT1 or GXYLT2. As for Fig. 3, peptide sequencing was performed and is presented in supplemental Fig. S2.

## DISCUSSION

Considering that similar glycosyltransferase reactions are often catalyzed by homologous glycosyltransferases, we

hypothesized that the xylosyltransferases acting on EGF domains and UGGT might show a significant degree of homology. UGGT uses UDP-glucose as donor substrate, a nucleotide sugar very similar to UDP-xylose, and transfers the substrate in an  $\alpha$ 1,3-linkage. Moreover, it possesses a protein binding domain as expected for the EGF-specific xylosyltransferases. In the CAZy data base, the glycosyltransferase family 24 (GLT24) contains exclusively UGGTs from different species (23). In contrast, the candidate xylosyltransferases selected by their homology to UGGT belong to the huge GLT8 family. Their putative activity was difficult to predict from this affiliation because the GLT8 family contains enzymes of various origin transferring a broad variety of sugars. Other mammalian members of the GLT8 family are LARGE, a putative glycosyltransferase acting on  $\alpha$ -dystroglycan (26), and glycogenin, a protein catalyzing its own oligo-glucosylation in  $\alpha$ 1,4-linkage to prime glycogen biosynthesis (27). Despite their limited homology to UGGT, we could, however, demon-

strate that GXYLT1 and GXYLT2 are xylosyltransferases catalyzing a similar reaction as UGGT.

The activity of GXYLT1 and GXYLT2 as  $\alpha$ 1,3-xylosyltransferases was unambiguously demonstrated using both synthetic and natural acceptor substrates. Assays with synthetic acceptors first provided evidence for the xylose donor specificity and  $\beta$ -glucose acceptor specificity. Subsequently, activity on EGF repeats was explicitly established by taking advantage of the availability of the UDP-xylose synthase-deficient CHO cell line pgsI-208 (21). This cell line is devoid of UDP-Xyl and allowed the production of EGF repeats exclusively modified with unsubstituted O-Glc and thus representing the natural substrate for the identified xylosyltransferases. The elongation of this substrate by GXYLT1 and GXYLT2 was then displayed by mass spectrometry. Finally, the enzymes were shown to act *in vivo* in Sf9 insect cells.

In evolution, GXYLT1 and GXYLT2 seem to have split only in the vertebrates. Orthologs of both xylosyltransferases are found in zebrafish, but the cephalochordate *Branchiostoma floridae* and other sequenced eumetazoans until the sea anemone *Nematostella vectensis* (28) only present a single gene. *Caenorhabditis elegans* seems to have lost the gene, and no ortholog is found in the unicellular choanoflagellate *Monosiga brevicollis*, which is the closest living relative of metazoans. Emerging of the xylosyltransferase therefore appears to match that of Notch signaling (29).

Amino acid sequence differences between GXYL1 and GXYL2 are most apparent in the putative stem regions where GXYL2 exhibits an arginine-rich and a proline-rich insertion. Although GXYL1 appears more active than GXYL2 in the assays performed in this study, no differences in specificity could be observed. As only three different EGF domains have been utilized, it cannot be excluded that there is preference for specific EGF domains. However, such differences have not been revealed for the three different mammalian Fringe GlcNAc transferases acting on *O*-fucose (10). The enzymatic activity of the two xylosyltransferases could thus as well be redundant and the enzymes only differentially expressed.

Extension of *O*-Fuc glycans by the enzyme Fringe has been shown to play a key role in modulation of Notch signaling, but the role of xylosylation of *O*-Glc could until now only be speculated about. Identification of two human genes encoding xylosyltransferases of the EGF *O*-glucosylation pathway will now enable functional studies that resolve the role of *O*-Glc xylosylation.

## REFERENCES

- Moloney, D. J., Shair, L. H., Lu, F. M., Xia, J., Locke, R., Matta, K. L., and Haltiwanger, R. S. (2000) *J. Biol. Chem.* **275**, 9604–9611
- Matsuura, A., Ito, M., Sakaidani, Y., Kondo, T., Murakami, K., Furukawa, K., Nadano, D., Matsuda, T., and Okajima, T. (2008) *J. Biol. Chem.* **283**, 35486–35495
- Luther, K. B., and Haltiwanger, R. S. (2009) *Int. J. Biochem. Cell Biol.* **41**, 1011–1024
- Okamura, Y., and Saga, Y. (2008) *Mech. Dev.* **125**, 663–673
- Shi, S., and Stanley, P. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100**, 5234–5239
- Stahl, M., Uemura, K., Ge, C., Shi, S., Tashima, Y., and Stanley, P. (2008) *J. Biol. Chem.* **283**, 13638–13651
- Okajima, T., Xu, A., Lei, L., and Irvine, K. D. (2005) *Science* **307**, 1599–1603
- 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., and Vogt, T. F. (2000) *Nature* **406**, 369–375
- Brückner, K., Perez, L., Clausen, H., and Cohen, S. (2000) *Nature* **406**, 411–415
- Rampal, R., Li, A. S., Moloney, D. J., Georgiou, S. A., Luther, K. B., Nitalazar, A., and Haltiwanger, R. S. (2005) *J. Biol. Chem.* **280**, 42454–42463
- Xu, A., Haines, N., Dlugosz, M., Rana, N. A., Takeuchi, H., Haltiwanger, R. S., and Irvine, K. D. (2007) *J. Biol. Chem.* **282**, 35153–35162
- Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F., and Weinmaster, G. (2000) *Nat. Cell Biol.* **2**, 515–520
- Hase, S., Nishimura, H., Kawabata, S., Iwanaga, S., and Ikenaka, T. (1990) *J. Biol. Chem.* **265**, 1858–1861
- Bjoern, S., Foster, D. C., Thim, L., Wiberg, F. C., Christensen, M., Komiyama, Y., Pedersen, A. H., and Kisiel, W. (1991) *J. Biol. Chem.* **266**, 11051–11057
- Acar, M., Jafar-Nejad, H., Takeuchi, H., Rajan, A., Ibrani, D., Rana, N. A., Pan, H., Haltiwanger, R. S., and Bellen, H. J. (2008) *Cell* **132**, 247–258
- Ishimizu, T., Sano, K., Uchida, T., Teshima, H., Omichi, K., Hojo, H., Nakahara, Y., and Hase, S. (2007) *J. Biochem.* **141**, 593–600
- Omichi, K., Aoki, K., Minamida, S., and Hase, S. (1997) *Eur. J. Biochem.* **245**, 143–146
- Minamida, S., Aoki, K., Natsuka, S., Omichi, K., Fukase, K., Kusumoto, S., and Hase, S. (1996) *J. Biochem.* **120**, 1002–1006
- Palcic, M. M., Heerze, L. D., Pierce, M., and Hindsgaul, O. (1988) *Glycoconj. J.* **5**, 49–63
- Shao, L., Luo, Y., Moloney, D. J., and Haltiwanger, R. (2002) *Glycobiology* **12**, 763–770
- Bakker, H., Oka, T., Ashikov, A., Yadav, A., Berger, M., Rana, N. A., Bai, X., Jigami, Y., Haltiwanger, R. S., Esko, J. D., and Gerardy-Schahn, R. (2009) *J. Biol. Chem.* **284**, 2576–2583
- Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.* **68**, 850–858
- Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009) *Nucleic Acids Res.* **37**, D233–D238
- Krylov, V., Ustyuzhanina, N., Grachev, A., Bakker, H., and Nifantiev, N. (2007) *Synthesis* **2007**, 3147–3154
- Shao, L., Moloney, D. J., and Haltiwanger, R. (2003) *J. Biol. Chem.* **278**, 7775–7782
- Kanagawa, M., Saito, F., Kunz, S., Yoshida-Moriguchi, T., Barresi, R., Kobayashi, Y. M., Muschler, J., Dumanski, J. P., Michele, D. E., Oldstone, M. B., and Campbell, K. P. (2004) *Cell* **117**, 953–964
- Pitcher, J., Smythe, C., and Cohen, P. (1988) *Eur. J. Biochem.* **176**, 391–395
- Putnam, N. H., Srivastava, M., Hellsten, U., Dirks, B., Chapman, J., Salamov, A., Terry, A., Shapiro, H., Lindquist, E., Kapitonov, V. V., Jurka, J., Genikhovich, G., Grigoriev, I. V., Lucas, S. M., Steele, R. E., Finnerty, J. R., Technau, U., Martindale, M. Q., and Rokhsar, D. S. (2007) *Science* **317**, 86–94
- King, N., Westbrook, M. J., Young, S. L., Kuo, A., Abedin, M., Chapman, J., Fairclough, S., Hellsten, U., Isogai, Y., Letunic, I., Marr, M., Pincus, D., Putnam, N., Rokas, A., Wright, K. J., Zuzow, R., Dirks, W., Good, M., Goodstein, D., Lemons, D., Li, W., Lyons, J. B., Morris, A., Nichols, S., Richter, D. J., Salamov, A., Sequencing, J. G., Bork, P., Lim, W. A., Manning, G., Miller, W. T., McGinnis, W., Shapiro, H., Tjian, R., Grigoriev, I. V., and Rokhsar, D. (2008) *Nature* **451**, 783–788