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LARGE2 generates the same xylose- and glucuronic acid-containing glycan structures as LARGE

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LARGE (like-glycosyltransferase) and LARGE2 (glycosyltransferase-like 1B (GYLTL1B)) are homologous Golgi glycosyltransferases possessing two catalytic domains with homology to members of glycosyltransferase families GT8 and GT49. Mutations in human and mouse *Large* result in muscular dystrophy due to underglycosylation of dystroglycan. The systemic function of LARGE2 is unknown, but at a cellular level the enzyme can substitute for LARGE in glycosylating dystroglycan. Here, we show that LARGE2 catalyzes the same glycosylation reaction as LARGE. It is a bifunctional glycosyltransferase using uridine diphosphate (UDP)-xylose (Xyl) and UDP-glucuronic acid (GlcA) as donor sugars to produce a xyloglucuronan with alternating Xyl and GlcA residues.

Keywords: dystroglycan / glucuronyltransferase / glycosyltransferase / glycosyltransferase-like 1B / xylosyltransferase

Introduction

LARGE (like-glycosyltransferase) and LARGE2 (glycosyltransferase-like 1B, gene abbreviation: *Gyltl1b*) are glycosyltransferase like proteins that contain two putative catalytic domains (Grewal et al. 2005). The first domain is a member of the glycosyltransferase-8 (GT8) family, whereas the C-terminal domain shows similarity to human uridine diphosphate (UDP)-*N*-acetylglucosamine (GlcNAc):Galβ1,3-*N*-acetylglucosaminyltransferase 1, a GT49 family glycosyltransferase. LARGE is involved in glycosylation of α-dystroglycan, which is part of the dystrophin–glycoprotein complex. This complex connects the cytoplasm with extracellular matrix proteins in skeletal muscle cells, which is required to withstand the mechanical forces during muscle contraction and stretch (Michele and Campbell 2003). Mutations in human and mouse *Large* result in muscular dystrophy (Grewal et al. 2001; Longman et al. 2003; Moore and Hewitt 2009; Godfrey et al. 2011).

Recently, it has been shown that LARGE is an enzyme generating a polymer of alternating xylose (Xyl) and glucuronic acid (GlcA) residues (Inamori et al. 2012). Its first GT8 domain, which shows homology to other mammalian xylosyltransferases (Sethi et al. 2010, 2012), is responsible for xylosyltransferase activity and the second domain for glucuronyltransferase activity. How the polysaccharide is exactly attached to the protein is not known yet, but it is most likely mainly linked via *O*-mannose (Man) (Stalnaker et al. 2011). Interestingly, it has been proposed that overexpression of LARGE is capable of compensating for several congenital muscular dystrophies, including those arising from defects in *O*-mannosylation such as Walker–Warburg syndrome or Muscle–Eye–Brain disease (Barresi et al. 2004). More recently, compensation by LARGE was shown to occur only in cells from patients with less severe forms of muscular dystrophy consistent with residual mutant enzyme activity (Willer et al. 2012). LARGE is, however, capable of glycosylating α-dystroglycan or other proteins in distinct Chinese hamster ovary (CHO) cell lines with mutations in various types of glycosylation, including *O*-mannosylation, indicating that it can act on *N*-linked glycans as well (Patnaik and Stanley 2005; Aguilan et al. 2009). The same conclusion was drawn from experiments using *O*-mannosylation-deficient neural stem cells. These experiments furthermore showed that LARGE can additionally act on proteins other than α-dystroglycan (Zhang and Hu 2012). Nevertheless, LARGE most likely requires preexisting phosphorylated glycan structures on α-dystroglycan to act (Yoshida-Moriguchi et al. 2010) and *O*-mannosylated structures are considered the main targets in vivo (Hu et al. 2011; Willer et al. 2012).

Modification of α-dystroglycan by LARGE is required for binding to the extracellular matrix protein laminin (Kanagawa et al. 2004). Laminin or specific monoclonal antibodies (Ervasti and Campbell 1991) can be used to detect glycosylation of α-dystroglycan upon recombinant expression of LARGE (Patnaik and Stanley 2005). Using these tools, LARGE2 was even more efficient in glycosylating α-dystroglycan than LARGE (Fujimura et al. 2005). This indicated that LARGE or LARGE2 expression results in the appearance of the same or a similar glycan epitope on α-dystroglycan. A differential expression of LARGE and LARGE2 suggested that the enzymes might be of distinctive importance in different organs (Grewal et al. 2005).

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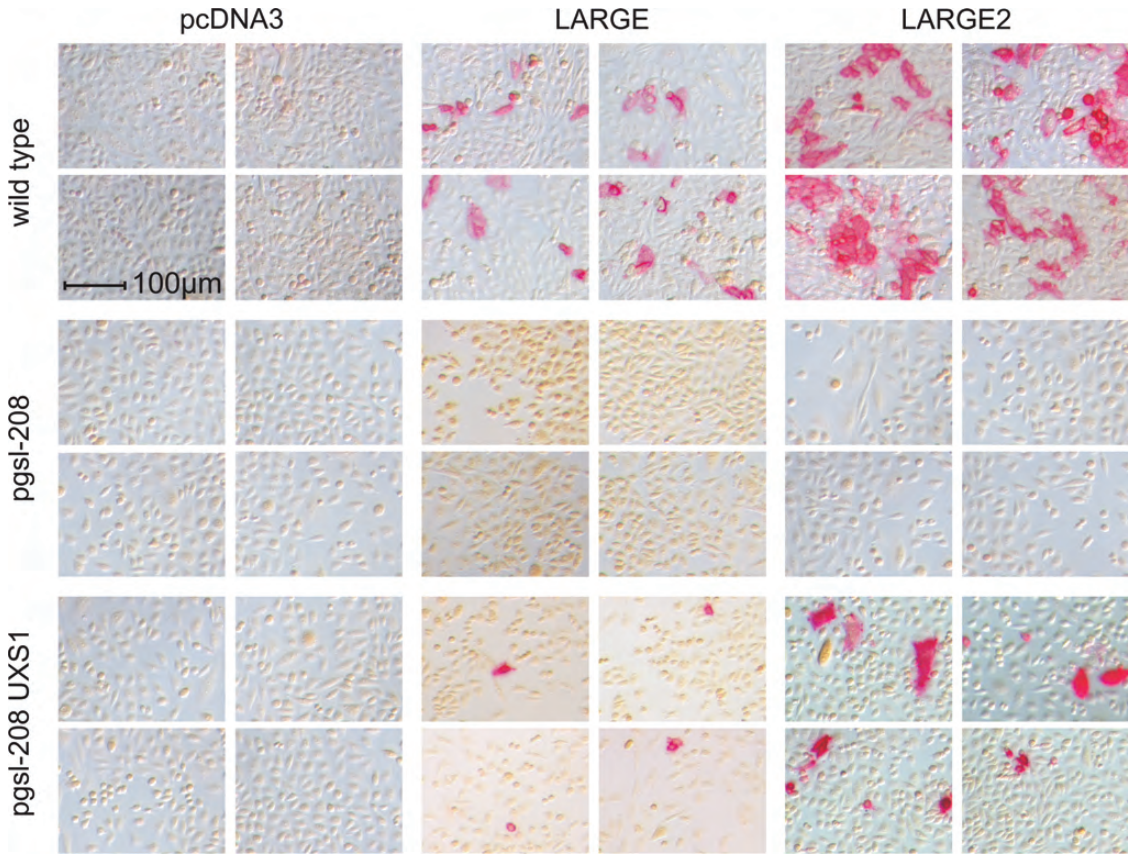


Fig. 1. LARGE and LARGE2 do not act in pgsI-208 cells, a CHO line deficient in UXS. LARGE, LARGE2 or an empty vector control (pcDNA3) was transiently expressed in wild-type (upper row) and mutant CHO cells (middle row) and the appearance of the product of LARGE was detected on the cell surface by monoclonal antibody IIH6. Only by co-expression of UXS1 (lower row), pgsI-208 cells became positive for IIH6 after expression of LARGE or LARGE2. Note that pgsI-208 cells have lower transfection efficiency than CHO cells.

Here, we analyze the enzymatic activity of LARGE2 and show that it has enzymatic activity identical to that of LARGE.

Results

LARGE and LARGE2 are not active in CHO cells lacking UDP-Xyl

LARGE has been shown to be active in several CHO cell mutants deficient in the biosynthesis of different nucleotide sugars (Patnaik and Stanley 2005; Aguilan et al. 2009). On the basis of these studies, CMP-sialic acid, GDP-fucose (Fuc), UDP-galactose (Gal) and UDP-*N*-acetylgalactosamine (GalNAc) could be excluded as substrates. Recently, a new CHO cell line (pgsI-208), with a deficiency in UDP-Xyl synthase 1 (UXS1), has been described (Bakker et al. 2009). This cell line is devoid of UDP-Xyl and it has been shown that both LARGE (Inamori et al. 2012) and LARGE2 (H. Bakker, unpublished observation, presented at the 2010 Meeting of the Society for Glycobiology, November 7–10, St. Pete Beach, FL.) are not capable of forming their product in these cells, providing the first indication that LARGE and LARGE2 require UDP-Xyl. To directly compare both enzymes, mouse LARGE and LARGE2 were transfected in CHO cells. In wild-type cells, the expression of LARGE and LARGE2 resulted in cell-surface appearance of the glycan epitope detected by monoclonal antibody IIH6 (Figure 1). As observed before, the presence of acceptor structures for LARGE in CHO cells does not depend on co-expression of dystroglycan (Patnaik and Stanley 2005). In contrast, pgsI-208 cells transfected with LARGE or LARGE2 were not stained by IIH6 unless co-transfected with UXS1. This indicated that UDP-Xyl is required by LARGE and LARGE2, but did not reveal whether it is a direct substrate of the enzymes or is required to generate the acceptor structure. In these experiments, expression of LARGE2 resulted in a more intense staining compared with LARGE expression (Figure 1), in agreement with a previous report, showing stronger staining with IIH6 on western blot (Fujimura et al. 2005). Thus, further experiments were carried out with LARGE2.

Screening for potential sugar donors of LARGE2

The experiments described above did not reveal whether UDP-Xyl was used as a direct substrate by LARGE2 or was required for its acceptor structure. It also did not disclose the second sugar used by LARGE2, which has two different putative glycosyltransferase domains and was potentially using two different donor sugars. Therefore, an in vitro assay was

set up to determine the putative donor substrates of LARGE2. For this assay, a construct, in which the putative N-terminal membrane anchor of LARGE2 was replaced by protein A, was generated to allow the production and secretion of the glycosyltransferase from Sf9 insect cells. The protein A tagged enzyme was then coupled to IgG sepharose beads and used directly for enzymatic assays.

A suitable way to determine the donor specificity of a glycosyltransferase without prior knowledge about the acceptor specificity is to investigate the enzyme hydrolyzing activity toward nucleotide sugars. The reaction rate can, moreover, be increased by small molecules like ethanol, which possibly function as acceptor molecules. The reaction product (free monosaccharide, or monosaccharide linked to ethanol) can be separated from the intact nucleotide sugars by anion exchange. This, however, limits the application to noncharged sugars. The hydrolysis of different radiolabeled nucleotide sugars was determined in the presence of 1 mM ethanol, since this had been shown to increase Xyl transfer of the Notch xylosyltransferase GXYLT1, used as control in our assay. This assay ascertained that UDP-Xyl is a donor substrate for LARGE2 (Figure 2). Still, the putative second sugar donor used by LARGE2 had not been identified.

After the identification of Xyl as one sugar transferred by LARGE2, an assay was established in which radiolabeled UDP-Xyl was combined with various (cold) nucleotide sugars to test whether one of these could increase transfer of Xyl. The reaction product was applied to a nitrocellulose filter, retaining proteins without binding the nucleotide sugars. Initially, a fragment of α-dystroglycan, produced in pgsI-208 CHO cells, was used as acceptor substrate. Experiments revealed, however, that adding an α-dystroglycan fragment did not improve activity of LARGE2 in this assay (not shown). All assays presented in Figure 3 were, therefore, carried out without adding any external acceptor. This suggested that acceptor molecules for LARGE2 were present in the enzyme preparation, possibly on LARGE2 itself or contaminating proteins from the insect culture, or that the enzyme was able to generate unconjugated polymers. This assay showed that only addition of UDP-GlcA increased retention of [^{14}C]Xyl on the filter, strongly suggesting that GlcA is the second sugar transferred by LARGE2 (Figure 3A). In addition, we show that the formation of a product retained on the filter was probably enzyme catalyzed (Figure 3B) since no product was formed in the presence of ethylenediaminetetraacetic acid (EDTA) and a great reduction in activity was seen if manganese was omitted from the reaction mixture. In addition, supplementing the reaction with UDP, a known inhibitory product of glycosyltransferase reactions (Koeller and Wong 2000), inhibited the activity as well.

Direct evidence for transfer of Xyl and GlcA

The previous experiments showed that the two sugars used by LARGE2 are Xyl and GlcA. If LARGE2 uses these sugars alternatingly, one monosaccharide potentially serves as acceptor for the other. To demonstrate this, LARGE2 was incubated with radiolabeled UDP-Xyl or UDP-GlcA in the presence of p-nitrophenyl (pNP) linked acceptor sugars. LARGE2 was able to transfer Xyl to GlcAβ-pNP but not to Xylα-pNP (Figure 4A). Vice versa, GlcA was only transferred to α-linked Xyl but not to GlcA, glucose or GlcNAc (Figure 4B). In addition to the assays using radiolabeled donor sugars, nonradioactive assays were carried out and products were analyzed by high-performance liquid chromatography (HPLC, Figure 4C–E). Assignment of the peaks to Xyl-GlcA-pNP and GlcA-Xyl-pNP was confirmed by mass spectrometry (MS) (Figure 5).

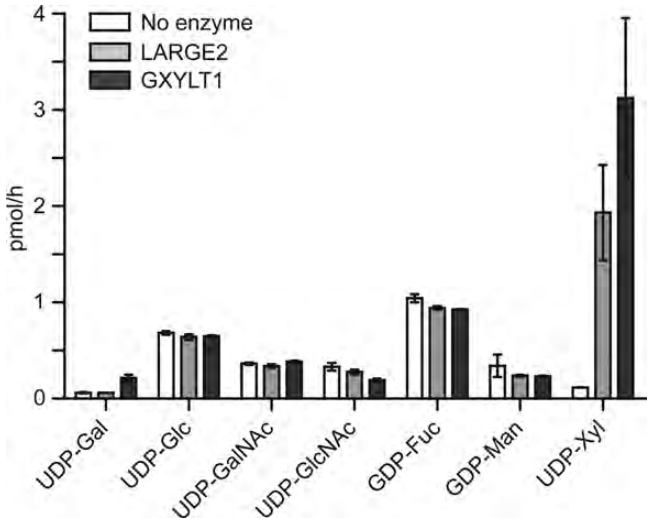


Fig. 2. Nucleotide sugar hydrolysis assays revealed that UDP-Xyl is a substrate of LARGE2. Assays to determine the donor substrates (see materials and methods) were carried out with different radiolabeled nucleotide sugars. The analytical method only allowed using noncharged sugars as donor. As control, a known xylosyltransferase (GXYLT1) was used, which showed clear specificity for UDP-Xyl.

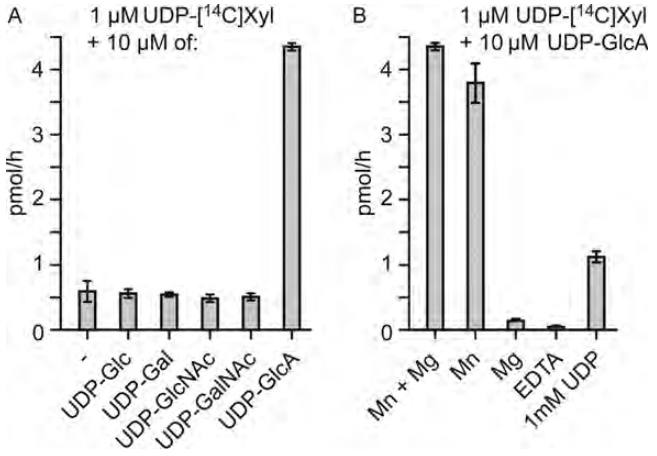


Fig. 3. "Filter assays" show that UDP-GlcA is the second nucleotide-sugar used by LARGE2. (**A**) Protein A beads bound LARGE2 was incubated with radiolabeled UDP-[^{14}C]Xyl and a variety of other nonlabeled nucleotide sugars, without addition of an external acceptor (see text for details). After incubation, the radioactivity that was retained on a nitrocellulose filter was determined. Monosaccharides and small oligosaccharides will pass the nitrocellulose filter, but proteins and possibly larger sugar polymers will be retained. (**B**) The enzymatic reaction depends on the presence of manganese and is inhibited by EDTA and UDP.

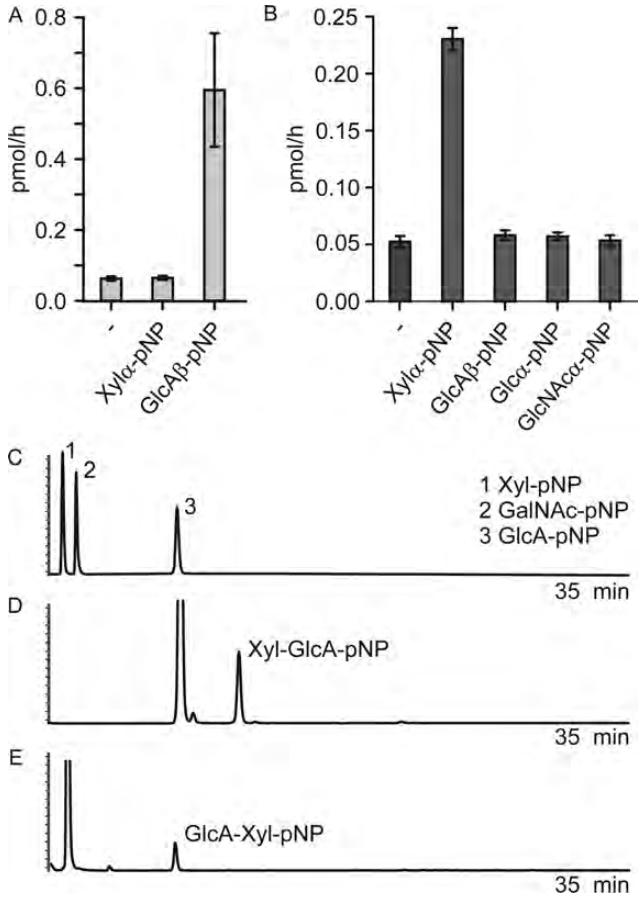
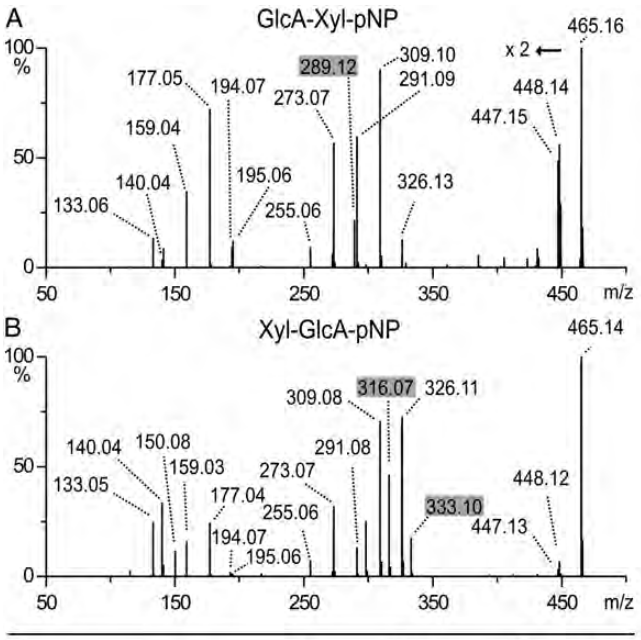


Fig. 4. Xylosyl- and glucuronyltransferase activities of LARGE2 using synthetic acceptors. (**A**) LARGE2 activity was assayed using UDP-[^{14}C]Xyl, incubated without acceptor, with Xylα-pNP or GlcAβ-pNP. (**B**) LARGE2 activity was assayed using UDP-[^{14}C]GlcA, incubated without acceptor or with Xylα-pNP, GlcAβ-pNP, Glcα-pNP, or GlcNAcα-pNP. (**C**) HPLC profile of standards of different pNP linked sugars. (**D**) HPLC profile of the product of LARGE2 with UDP-Xyl and GlcAβ-pNP. (**E**) HPLC profile of the product of LARGE2 with UDP-GlcA and Xylα-pNP. Reaction products of D and E were analyzed by tandem-MS (Figure 5).



C	m/z	Calc.	Observed	
			A	B
[GlcA-Xyl-pNP + NH_4]$^+$ *		465.14	465.16	465.14
[GlcA-Xyl-pNP + H]$^+$ *		448.11	448.14	448.12
[GlcA-Xyl-pNP minus H_2O + NH_4]$^+$ *		447.13	447.15	447.13
[GlcA-pNP + NH_4]$^+$		333.09	n.p.	333.10
[GlcA-Xyl minus H_2O + NH_4]$^+$ *		326.11	326.13	326.11
[GlcA-pNP + H]$^+$		316.07	n.p.	316.07
[GlcA-Xyl minus H_2O + H]$^+$ *		309.08	309.10	309.08
[GlcA-Xyl minus 2x H_2O + H]$^+$ *		291.07	291.09	291.08
[Xyl-pNP + NH_4]$^+$		289.10	289.12	n.p.
[GlcA-Xyl minus 3x H_2O + H]$^+$ *		273.06	273.07	273.07
[GlcA-Xyl minus 4x H_2O + H]$^+$ *		255.05	255.06	255.06
[GlcA + H]$^+$		195.05	195.06	195.06
[GlcA minus H_2O + NH_4]$^+$		194.07	194.07	194.07
[GlcA minus H_2O + H]$^+$		177.04	177.05	177.04
[GlcA minus 2x H_2O + H]$^+$		159.03	159.04	159.03
[Xyl minus H_2O + NH_4]$^+$		150.08	n.p. **	150.08
[pNP + H]$^+$		140.03	140.04	140.04
[Xyl minus H_2O + H]$^+$		133.05	133.06	133.05

*: For these structures, the mass does not reveal the monosaccharide order GlcA-Xyl or Xyl-GlcA.
n.p.: Not present
**: Absence of the NH_4-adduct of xylose is not indicative of the structure, the H-adduct (133.05) is observed.

Fig. 5. Fragmentation of reaction products of LARGE2 by tandem-MS. The reaction products of Figure 4D and E, Xyl-GlcA-pNP (**A**) and GlcA-Xyl-pNP (**B**), respectively, have identical masses of 465.14 as ammonium adducts. Tandem-MS confirmed their structures. The appearance of fragmentation ions with masses corresponding to Xyl-pNP (289.12 Da) and GlcA-pNP (333.10 and 316.07 Da), which are marked with a gray background, and of both Xyl and GlcA confirmed the structure of the original disaccharides. (**C**) Assignment of *m/z* values observed after fragmentation of reaction products shown in panel A and B.

To further demonstrate that LARGE2 uses the reaction product of one reaction for its second activity, nonlabeled Xyl-GlcA-pNP and GlcA-Xyl-pNP reaction products were generated and used as acceptors in second reactions using radiolabeled donor sugars. As shown in Figure 6, products of the first transfer reaction could serve as an acceptor for a second transfer indicating that LARGE2 is capable of elongating Xyl-GlcA and GlcA-Xyl disaccharides and generates an oligomer or polymer of alternating Xyl and GlcA.

Discussion

We have shown here that mouse LARGE2 uses the two nucleotide sugars UDP-Xyl and UDP-GlcA as donor sugars and likely generates a polymer alternating these sugars. The function of LARGE2 is thus identical to that of LARGE, which was shown to be a bifunctional glycosyltransferase using UDP-Xyl and UDP-GlcA, while this study was in progress (Inamori et al. 2012). Inamori et al. 2012 derived the function of LARGE from analyzing the monosaccharide composition of natural dystroglycan. They observed a high concentration of Xyl and GlcA that could not be explained by enzymatic activities of other glycosyltransferases acting on dystroglycan. Alternatively, we predicted LARGE and LARGE2 function

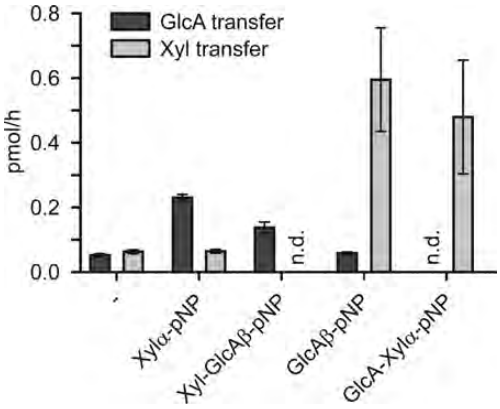


Fig. 6. Extension of reaction products by a third sugar. Products of the first (nonlabeled) reaction were used as acceptor molecules in a second reaction using radiolabeled UDP-[^{14}C]GlcA or UDP-[^{14}C]Xyl substrates. Xyl-GlcA-pNP functions as an acceptor for further transfer of GlcA, whereas GlcA-Xyl-pNP functions as an acceptor for transfer of Xyl. n.d.: not determined.

from their homology to xylosyltransferases that we previously identified (Sethi et al. 2010, 2012) and tested this hypothesis by expressing the enzyme in a CHO cells defective in UDP-Xyl biosynthesis. A systematic search then led us to the definition of the second activity.

Interestingly, Nakagawa et al. (2012) have recently found that the HNK-1 sulfotransferase is capable of inhibiting generation of the product of LARGE. The known structure that is generated by this sulfotransferase is the HNK-1 epitope (SO_4–GlcA–Gal), but in vitro, this sulfotransferase has been shown to act on GlcA alone, without the underlying galactose (Bakker et al. 1997). Therefore, it may be capable of modifying terminal GlcA present on LARGE and LARGE2 products and thereby inhibit further extension of the polymer. Consequently, glycosylation of dystroglycan might not only be regulated by expression of LARGE, but also by competing glycosyltransferases.

Owing to alternating linkage of Xyl and GlcA in α and β conformation, the overall structure produced by LARGE and LARGE2 is similar to those of heparin and heparan sulfate, which are glycosaminoglycans of alternating α-linked GlcNAc and β-linked GlcA units. Interestingly, laminin binds both dystroglycan and heparin/heparan sulfate via the same domain (Andac et al. 1999). Moreover, heparin has been shown to interfere with the interaction between dystroglycan and laminin (Pall et al. 1996). Heparin and the xyloglucuronan produced by LARGE and LARGE2 likely occupy the same or an overlapping binding pocket of laminin.

UDP-Xyl is unique among nucleotide sugars because it is generated from UDP-GlcA in the lumen of the endoplasmic reticulum (ER) by UXS1. LARGE, on the other hand, is localized in the Golgi lumen. We have previously shown that at least one UDP-Xyl transporter, encoded by SLC35B4, is present in mammals (Ashikov et al. 2005) and that xylosylation of Notch and glycosaminoglycan biosynthesis can be restored in cell lacking UXS1 by expression of UXS in the cytoplasm, proving that UDP-Xyl can be transported from the cytoplasm in the ER/Golgi lumen. Although transport of UDP-Xyl occurs, it is not known how UDP-Xyl is transported from the ER to the Golgi. A transporter of UDP-Xyl might be required to provide substrates for LARGE and LARGE2.

Materials and methods

Materials

Insect-XPRESS protein-free insect cell medium (Lonza), alpha-MEM (Invitrogen) IgG Sepharose 6 Fast Flow (GE Healthcare), C18 3 mL 200 mg vacuum cartridges (Waters or Macherey-Nagel), AG1-X8 resin (Bio-Rad), multi screen plates (Millipore), nitrocellulose filter (MF™ membrane filters Millipore) and UDP-Xyl (Complex Carbohydrate Research Center, Athens GA).

Radiolabeled nucleotide sugars: UDP-[^{3}H]Glc, UDP-[^{3}H]GalNAc, and GDP-[^{3}H]Fuc were from American Radiolabeled Chemicals. UDP-[^{3}H]Gal, UDP-[^{3}H]GlcNAc, UDP-[^{14}C]Xyl, GDP-[^{3}H]Fuc, and UDP-[^{14}C]GlcA were from PerkinElmer.

All other chemicals were purchased from Sigma-Aldrich.

Cloning and expression of mouse LARGE, LARGE2 and α-dystroglycan

To generate a mammalian expression vector with the complete open-reading frame of LARGE2 (glycosyltransferase-like 1B, Gyltl1b), it was amplified from mouse brain cDNA by PCR with primers introducing BglII and XhoI restriction sites flanking the start and stop codons. In the same way the LARGE sequence was PCR-amplified from Image clone IMAGp998A1610981Q acc. Nr. BG975581. Both PCR products were inserted in pcDNA3 (Invitrogen) utilizing BamHI and XhoI sites. Mouse α-dystroglycan was amplified in two steps. The first PCR product harbors the native secretion signal (first 28 amino acid) fused to a short sequence encoding an HA epitope. The second PCR product contains the sequence from position 32 to amino acid 633. Both PCR products were inserted in pcDNA3 vector containing a human Fc fragment. The obtained construct leads to expression of a secreted α-dystroglycan fragment between amino acid 32 and 633 with an N-terminal HA tag and C-terminal human Fc fragment.

To generate a baculoviral vector for expression of soluble secreted LARGE2, the cDNA was amplified lacking the sequence of the transmembrane domain starting at amino acid 29. This was cloned in pFastBac (Invitrogen) already containing an N-terminal Protein A tag (Sethi et al. 2010, 2012). Expression and purification were carried out as described previously (Sethi et al. 2010). Briefly, the viral stock harboring the LARGE2 construct was prepared according to the Bac-to-Bac manual supplied by Invitrogen. Sf9 Insect cells were infected with the third viral passage and 72 h after infection the medium was clarified by centrifugation for 10 min at 4°C followed by filtration through a 0.22 µm Millex-GP filter. The medium was incubated overnight at 4°C with 1 µL/mL of IgG-Sepharose-6 Fast Flow beads. The beads were washed according to the manufacturer's protocol, and stored at −20°C in 50% glycerol, 2 mM $MnCl_2$ and 10 mM MOPS, pH 7. Prior to assays, the beads were washed twice with the same buffer without glycerol.

Cell surface staining

CHO (wild-type) and 208pgsI cells (Bakker et al. 2009) were seeded in 6-well plates in alpha-MEM supplied with 10% FCS and were transfected at 40% confluence by Metafectene (Biontex Laboratories) with vectors harboring the complete open-reading frame of LARGE or LARGE2. Forty eight hours after transfection, the cells were fixed and surface-stained with IIH6-C4 antibody (Millipore). HRP-conjugated goat anti-mouse (SouthernBiotech) was used as secondary antibody and signal application by tyramide signal amplification (Speel et al. 2006) was applied as described (Bakker et al. 2009) and visualized by Fast-Red staining (Sigma).

Nucleotide-sugar hydrolysis assays

AG1-X8 resin was suspended in 100 mM sodium acetate pH 5.8 (1.5 mL/g) and kept at 4°C. Radiolabeled nucleotide sugars were diluted with cold nucleotide sugars to obtain a final concentration of 1 µM with a specific activity of 3.7 Bq/µL for the ^{14}C isotopes and 37 Bq/µL for ^{3}H isotopes. Hydrolysis was performed in 50 µL reactions containing 100 mM MOPS pH 7.0, 10 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM ethanol, 1 µM radiolabeled nucleotide-sugar and 10 µL IgG beads for 2 h on a thermoshaker. Multi screen plates with low binding capacity filters were overlaid with 200 µL per well of AG1-X8 resin suspension and spun down at 100× *g* for 1 min. The resin was washed with 100 µL of 100 mM MOPS pH 7 and the plate was centrifuged at 100 × *g* for 1 min. To retain the unreacted nucleotide sugars on AG1-X8, the reaction mixtures were gently overlaid on the plate, centrifuged at 100 × *g* for 1 min and washed once with 100 mM MOPS. The flow-through was collected and the radioactivity was measured by liquid scintillation counting (Beckman Coulter LS 6500). The results were expressed in pmol of released sugar per hour incubation time (average of three independent preparations, measured as duplicates, with standard deviation). The calculated activity was compared with the hydrolysis detected in reactions without enzymes.

Glycosyltransferase assays

The specific activity of radiolabeled UDP-[^{14}C]Xyl and that of UDP-[^{14}C]GlcA were as for hydrolysis assays. Transferase reactions were performed in 50 µL of 100 mM MOPS pH 7.0, 10 mM $MnCl_2$, 5 mM $MgCl_2$, 10 mM ATP and 1 mM pNP-linked sugars. Reactions were started by the addition of 10 µL of bead-coupled enzyme followed by incubation on a thermoshaker at 37°C for 2 h. The hydrophobic acceptor was retained on 3 cc C18 cartridges, unincorporated radioactivity removed by washing with water and pNP-sugars eluted two times with 2 mL of methanol. After drying, 3 mL scintillation cocktail (Lumasafe plus, PerkinElmer) was added and counted. Transfer was calculated as the average of three independent experiments in pmol transferred sugars per hour.

Transfer of a second sugar on a product of the first glycosyltransferase reaction

To generate an acceptor for a second transfer, the transferase assay was performed with 0.1 mM pNP-Xyl or pNP-GlcA and 0.1 mM cold UDP-GlcA and UDP-Xyl, respectively. The reaction conditions were the same as described above. The products were purified by Sep-Pak C18 cartridges and dried in a vacuum concentrator. The pellets were dissolved in water and used as an acceptor in a subsequent glycosyltransferase assay using radiolabeled donor sugars (see above).

Glycosyltransferase assays using nitrocellulose filters

Essentially, the reaction was carried out as described above. To the 1 µM UDP-[^{14}C]Xyl with a specific activity of 3.7 Bq/µL, 10 µM cold other nucleotide sugars were added. After two hours of incubation at 37°C, the transfer was stopped by adding 200 µL 100 mM Glycine buffer pH 2.9. The acidic conditions allow the elution of IgG-bound protein. To separate the beads from the liquid phase, a centrifugation step at 500 × *g* was applied. Two additional washing steps with 200 µL glycine buffer were performed and the supernatants were combined. The solution was neutralized by 50 µL 1 M MOPS pH 7.0. The proteins were retained on a prewetted (10 mM MOPS pH 7.0) nitrocellulose filter by vacuum-assisted filtration. After three washes with 1 mL 10 mM MOPS, the filters were dried at room temperature and 3 mL of Filtersafe plus (Zinsser analytic) was added and counted by liquid scintillation.

HPLC assay of glycosyltransferase reaction products

The glycosyltransferase reaction was carried out as described above with slight modifications. The concentration of acceptor pNP-sugar was reduced to 0.1 mM and the nucleotide-sugar concentration increased to 0.1 mM. The reaction was carried out overnight at 37°C. Separation of products was performed on a HILIC Glykosep™ N-plus column (ProZyme) 4.6 mm/150 mm on a Shimadzu Prominence UFLC system. Absorptions were measured by a SPD-20AV UV/Vis detector at 254 and 262 nm for nucleotide sugars and 300 nm for the pNP-substrates. Buffer A was 100% acetonitrile and buffer B was 250 mM ammonium formate pH 4.4. Normal phase separation was obtained using a gradient starting at 80% buffer A and reaching 35% in 48 min. Interesting peaks were collected and dried in a rotational vacuum concentrator. The dried material was dissolved in 10 µL of 50% acetonitrile in 15 mM ammonium acetate (v/v). MS was performed in positive ion mode applying direct sample infusion into an ESI-Q-TOF (Q-TOF Ultima, Waters, Milford, MA) using nanoflow sample tips (Nanoflow Probe Tip, long; Waters). The mass matching to that of the ammonium adducts of GlcA-Xyl-pNP and Xyl-GlcA-pNP was selected for collision-induced fragmentation (tandem mass spectrometry) and several hundred spectra were recorded. Spectra were analyzed using the MassLynx V4.1 Software (Waters).

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Conflict of interest

None declared.

Abbreviations

CHO, Chinese hamster ovary; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; GT, glycosyltransferase (family); GT8, glycosyltransferase-8; GYLTL1B, glycosyltransferase-like 1B; HPLC, high performance liquid chromatography; Man, mannose; MS, mass spectrometry; pNP, p-nitrophenyl; UDP, uridine diphosphate; UXS, UDP-xylose synthase; Xyl, xylose.

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