Functional UDP-xylose Transport across the Endoplasmic Reticulum/Golgi Membrane in a Chinese Hamster Ovary Cell Mutant Defective in UDP-xylose Synthase*

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In mammals, xylose is found as the first sugar residue of the tetrasaccharide GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-O-Ser, initiating the formation of the glycosaminoglycans heparin/heparan sulfate and chondroitin/dermatan sulfate. It is also found in the trisaccharide Xylα1–3Xylα1–3Glcβ1-O-Ser on epidermal growth factor repeats of proteins, such as Notch. UDP-xylose synthase (UXS), which catalyzes the formation of the UDP-xylose substrate for the different xylosyltransferases through decarboxylation of UDP-glucuronic acid, resides in the endoplasmic reticulum and/or Golgi lumen. Since xylosylation takes place in these organelles, no obvious requirement exists for membrane transport of UDP-xylose. However, UDP-xylose transport across isolated Golgi membranes has been documented, and we recently succeeded with the cloning of a human UDP-xylose transporter (SLC25B4). Here we provide new evidence for a functional Golgi UDP-xylose transporter (SLC25B4). Here we provide new evidence for a functional Golgi UDP-xylose transporter (SLC25B4).

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2 The abbreviations used are: GAG, glycosaminoglycan; CHO, Chinese hamster ovary cell line; EGF, epidermal growth factor; FGF, fibroblast growth factor; HRP, horseradish peroxidase; UGDH, UDP-glucose dehydrogenase; UXS, UDP-xylose synthase; XylT, xylosyltransferase; AtXylT, A. thaliana B1,2-xylosyltransferase; ER, endoplasmic reticulum; MS, mass spectrometry; HPLC, high pressure liquid chromatography.
UDP-xylene metabolism in mammalian cells. A, UDP-Xyl is synthesized in two steps from UDP-Glc by the enzymes UGDH, forming UDP-GlcA, and UXs, also referred to as UDP-glucuronic acid decarboxylase. UGDH is inhibited by the product of the second enzyme, UDP-Xyl (42). B, in mammals, UDP-Xyl is synthesized within the lumen of the ER/Golgi, where it is substrate for different xylosyltransferases incorporating xylose in the glycosaminoglycan core (XylT1 and XylT2) or in O-glucose-linked glycans. The nucleotide sugar transporter SLC35D1 (52) has been shown to transport UDP-GlcA over the ER membrane and SLC35B4 (29) to transport UDP-Xyl over the Golgi membrane. The function of this latter transporter is unclear.

Here we describe the isolation of a third CHO cell line (pgsI-208) with the xyloside-correctable phenotype. The mutant is deficient in UDP-xylene synthase (UXS), also known as UDP-glucuronic acid decarboxylase. This enzyme catalyzes the synthesis of UDP-Xyl, the common donor substrate for the different xylosyltransferases, by decarboxylation of UDP-glucuronic acid. Importantly, UXs in the animal cell is localized in the lumen of the ER and/or Golgi (26–28), superseding at first sight the need for the Golgi UDP-xylene transporter, which has been recently cloned and characterized (29). Using this cell variant, experiments were designed that establish the functional significance of UDP-Xyl transport with respect to UDP-glucuronic acid production and xylosylation.

EXPERIMENTAL PROCEDURES

Cell Cultures and Plasmids—Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61; Manassas, VA). Mutant pgbsA-745 (XylT-deficient) and pgbsB-761 (GalTI-deficient) were characterized previously (18, 25), and the mutant line described here was named pgsl-208. Cells were grown under an atmosphere of 5% CO₂ in α-minimal essential medium containing ribonucleosides and deoxyribonucleosides (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Biochrom AG, Berlin, Germany).

A cDNA clone of human UXs1 (Hugo Genome Nomenclature Committee ID: 17759) was amplified from prostate gland Marathon-ready cDNA (Clontech) using primers with EcoRI and XbaI-flanking start and replacing stop codon, respectively, and cloned in pcDNA3 (Invitrogen) already containing FLAG (N-terminal) and hemagglutinin (C-terminal) tags. XylT2 cDNA (Hugo Genome Nomenclature Committee ID: 15517) was amplified from a human embryonic kidney cDNA library (Invitrogen) using primers with BglII and XbaI flanking the start and stop codon, respectively, and cloned BamHIXbaI flanking the start codon and cloned in pcDNA3 containing an N-terminal FLAG tag. A cytoplasmic form of human UXs1 (cytUXS1) was amplified from the full-length clone by primers amplifying the region coding from amino acid 38 to the C terminus and cloned in pcDNA3-FLAG-hemagglutinin like the full-length construct. Arabidopsis thaliana UXs3 (30) (AT5G59290) was amplified by primers covering the complete open reading frame from a cDNA library (31) and cloned into pcDNA3-FLAG-hemagglutinin (AT5G55500) (32) was isolated from the same cDNA library using a sibling selection procedure with PCR primers based on a partial soybean XylT sequence (33).

Complementation Assay—Complementation tests were carried out by cell hybridization as described (34). Approximately 2 × 10⁵ cells of pgsl-208 were added to individual wells of a 24-well plate along with an equal number of pgbsA-745 or pgbsB-761 cells. After overnight incubation, the mixed cell monolayers were treated for 1 min with 50% (w/w) polyethylene glycol (M₉ = 3350) in F-12 medium. The cells were incubated for 1 day, replated in 100-mm diameter tissue culture dishes to obtain ~300 colonies/dish, and then overlaid with polyester cloth. The replica-plated colonies were incubated with 35SO₄ as described (21). Complementation was assessed by the appearance of colonies that regained the capacity to incorporate 35SO₄ into acid-precipitable material, as judged by autoradiography.

Complementation by cDNA was carried out by transient transfection using Metafectene (Biontex, Munich, Germany) of pgsl-208 with UXs1 or an empty vector control together with AtXylT. Two days after transfection, cells were fixed in 1.5% glutaraldehyde and stained with polyclonal rabbit anti-horse-radish peroxidase (HRP), recognizing xylosylated plant N-glycans (Rockland) followed by alkaline phosphatase-conjugated goat anti-rabbit antibody and developed with Fast-Red substrate (Sigma).
UDP-xylene Synthase-deficient CHO Cells

Flow Cytometry—Mutant cells were transfected with plasmids expressing UXs1, cytUXS1, or XyIT2 using Metafectene. Cells were selected for stable expression by G418 (Calbiochem), and selected colonies were checked for expression by immunofluorescence using anti-FLAG-M2 monoclonal antibody (Sigma). For flow cytometry, 5 × 10^5 cells were released with phosphate-buffered saline, 2 mM EDTA and incubated with RB4Ea12, a vesicular stomatitis virus glycoprotein-tagged single chain phage display antibody recognizing heparin sulfate (35, 36), followed by mouse anti-vesicular stomatitis virus glycoprotein tag monoclonal antibody (P5D4; Sigma) and fluorescein isothiocyanate-labeled anti-mouse. Cells (10^5) were analyzed by flow cytometry (FACScan; BD Biosciences).

Sequence Analysis of CHO UXSI—Chinese hamster UXSI cDNA was amplified using primers based on conserved parts of human, mouse, and rat sequences covering the sequence from amino acid 37 to 420 (C terminus); GTATGGTACCTTGGTTACATCGAGGCTGCTCAT and GACCTCTAGAGCTGTGCGGCTCGCCCTC and cloned BamHI/XbaI (underlined) in pcDNA3 (Invitrogen) for sequencing. Based on this sequence, CHO-specific primers were used to amplify UXSI1 mRNA from pgsI-208 in two parts, CAGGAAAATGGTGAACTAAAGTGTATGGTACCTT to cover amino acids 44–237 with CGACTCGTTTGCCCTCG to cover amino acids 196–406 from a total of 420 amino acids (numbered based on the human sequence). The fragments were cloned in TopoTA (Invitrogen) for sequencing. The genomic fragment around the identified mutation was amplified using AGGTATATGGATCTGGTTATTG to cover amino acids 196–406 and GTCTGCTCCTGGCCTCCACA with TGGGGATGTA-CTGTTATTTG to cover amino acids 44–237.

RESULTS

A CHO Cell Mutant Deficient in Xylose Incorporation—A CHO cell clone, designated pgsI-208 according to the nomenclature of previously identified proteoglycan synthesis mutants (21), was selected from a mutagenized population using FGF-2-Saporin, a recombinant chimera consisting of FGF-2 fused to the plant cytotoxin saporin-6 (Selective Genetics, Inc., La Jolla, CA), as described for the isolation of a glucuronoyltransferase 1 mutant (21). Since FGF-2 binds to heparan sulfate, this procedure selects for mutants deficient in heparan sulfate biosynthesis. The addition of naphthol-β-o-xyloside restored the incorporation of ^35SO_4 in GAGs in pgsI-208 (Table 1) as in the previously isolated mutant pgsA-745, exhibiting a defect in the GAG initiating XylT (4–6, 18). This result indicated that all of the downstream enzymes were present, and mutation in an upstream step involved in xylosylation or production of precursors was anticipated.

The cell line was then fused pairwise to mutants pgsA-745 and pgsB-761, the two previously isolated mutants in which xylosides restore GAG synthesis (18, 25), to test for genetic complementation (34). Fusion of pgsI-208 cells with pgsA-745 and pgsB-761, the two previously isolated mutants in which xylosides were expressed by transient transfection. This approach provides a convenient way to measure UDP-Xyl indirectly, since the epitope formed by this enzyme on N-glycans is absent in mammalian cells and can be readily detected on the cell surface using rabbit anti-HRP, which is reactive to plant type N-glycans containing β-linked xylose (40). In wild type CHO cells, transient expression of AtXYIT resulted in anti-HRP-positive cells, whereas transfection of mutant pgsI-208 cells did not (Fig. 2A). The simultaneous absence of GAG biosynthesis and xylosyla-

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<th>Strain</th>
<th>^6^H</th>
<th>Glucosamine</th>
<th>^35SO_4</th>
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<th>With Xylβ-O-Np</th>
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<tr>
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<td>142</td>
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<tr>
<td>pgsB-761</td>
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<td>3</td>
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<td>pgsI-208</td>
<td>38</td>
<td>14</td>
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UDP-xylose Synthase-deficient CHO Cells

**Figure 2. Complementation of pgsI-208 by UXS.** A, CHO or pgsI-208 cells transfected with AThyT. The product of AThyT, xylosylated N-glycans, was detected on the cell surface using anti-AThyT, reactive to xylosylated N-glycans of this protein (40). B and C, pgsI-208 and pgsA-745 cells were stained with antibody RB4 Ea12 (35, 36) against heparan sulfate and analyzed by flow cytometry. B, CHO cells (blue) compared with pgsI-208 (black) and pgsI-208 stably transfected with human UXS1 (red). C, CHO cells (blue) compared with xylotransferase-deficient mutant pgsA-745 (black) and pgsA-745 stably transfected with human XylT2 (red).

O-Glucose-linked Glycans on EGF Repeats of Notch—Since pgsI-208 was selected for resistance to FGF-2-saporin, the absence of heparan sulfate was anticipated. To examine this possibility, pgsI-208 cells were analyzed by flow cytometry using antibody RB4 Ea12 against heparan sulfate (35, 36). Wild-type CHO cells exhibited strong staining by this antibody, whereas pgsI-208 did not and behaved like pgsA-745 cells, defective in XylT2 (Fig. 2). Expression of human UXS1 and XylT2 in pgsI-208 and pgsA-745, respectively, restored antibody binding to a level comparable with wild-type CHO cells (Fig. 2, B and C).

To investigate the presence of xylose in O-glucose-linked glycans, a secreted construct containing the first five EGF-like repeats of mouse Notch1 (37), was expressed in CHO, pgsA-745, and pgsI-208 cells. The C-terminal His tag on this protein allowed its purification from the medium. Tryptic peptides were analyzed for the presence of O-glucose glycans using liquid chromatography-MS/MS (38). We have previously identified a tryptic peptide from EGF 4 bearing an O-glucose trisaccharide when analyzing samples derived from Lec1-CHO cells (38). In contrast, analysis of the sample from pgsI-208 revealed the presence of the peptide modified with a single hexose residue (m/z = 1255.1; Fig. 3A). In contrast, analysis of the sample from pgsI-208 revealed the presence of the peptide modified with a single hexose elutes slightly later (53.7 min; Fig. 3B) than that with the trisaccharide (52.9 min; Fig. 3A), consistent with the predicted effects of two fewer sugars on retention time. Searches of the data showed that the trisaccharide form was present in the sample from pgsA-745 but not pgsI-208 (Fig. 3C), whereas the monosaccharide form was found in pgsI-208 but not pgsA-745 (Fig. 3D). These data suggested that the defect in UXS prevents the addition of xyloses to O-glucose on EGF repeats.

The UDP-GlcA Content of pgsI-208 Is Dramatically Increased—The enzyme UDP-glucose dehydrogenase (UGDH) (Fig. 1) is known to be inhibited by UDP-Xyl (42). In Cryptococcus neoformans, the absence of cytosolic UXS results in an drastic increase of the UDP-GlcA concentration due to lack of feedback inhibition of the dehydrogenase by UDP-Xyl (43). To investigate the consequences of reduced UDP-Xyl production in the mammalian cell line where UXS is localized in the ER/Golgi lumen, the composition of the pool of relevant nucleotide sugars was determined in wild-type, XylT2 (pgsA-745), and UXS (pgsI-208) mutants using formic acid extraction and HPLC analyses (39). pgsA-745 and wild-type cells showed the same content of UDP-Glc, UDP-GlcA, and UDP-Xyl (Fig. 4, A and B), indicating that the absence of GAG synthesis had no influence on the ratio of these nucleotide sugars. Conversely, UDP-Xyl was completely absent in pgsI-208, whereas the concentration of UDP-GlcA was dramatically increased to a level.
FIGURE 3. **Glycan analysis of Notch EGF1–5 by mass spectrometry.** pgsA-745 and pgsI-208 cells were transiently transfected with a construct encoding EGF repeats 1–5 of mouse Notch1. Tryptic peptides of the purified secreted product were analyzed by liquid chromatography-MS/MS for the presence of peptides modified with O-glucose. A, identification of the O-glucose trisaccharide form of a peptide from the pgsA-745 sample. The top panel shows an MS spectrum of material eluting at 52.9 min. The ions labeled \([M+3H]^3+\) and \([M+4H]^4+\) match the predicted mass for triply and quadruply charged forms of the O-glucose trisaccharide form of SCQQADPCASNPCCANGGQCLPFESSYICR, a tryptic peptide from EGF 4 previously demonstrated to be modified with O-glucose trisaccharide.† Collision-induced dissociation fragmentation of the triply charged form of this peptide resulted in the MS/MS spectrum shown in the bottom panel. The major product ions at \(m/z\) 1210.9, 1167.1, and 1112.9 correspond to sequential losses of a pentose, a second pentose, and a hexose. Numerous sequence fragment ions (y-ions are shown) are observed that confirm the identity of the peptide. Ions selected for fragmentation in the MS spectrum are identified by red diamonds. The position of the parent ion fragmented in the MS/MS spectrum is identified with a blue diamond. B, identification of the O-glucose monosaccharide form of the peptide from the pgsI-208 sample. The top panel shows an MS spectrum of material eluting at 53.7 min, slightly later than the trisaccharide form (consistent with the loss of two hydrophilic xylose residues). The ions labeled \([M+3H]^3+\) and \([M+4H]^4+\) match the predicted mass for triply and quadruply charged forms of the glycopeptide. Collision-induced dissociation fragmentation of the triply charged form resulted in the MS/MS spectrum shown in the bottom panel. The major product ion, \(m/z\) 1112.8, matches the mass for the unglycosylated peptide. C, the trisaccharide form is present in samples from pgsA-745 but not pgsI-208. The data from both samples was searched for the ion corresponding to the triply charged form of the trisaccharide form, \(m/z\) 1255.1 (see A). D, the monosaccharide form is present in samples from pgsI-208 but not pgsA-745. The data from both samples was searched for the ion corresponding to the triply charged form of the monosaccharide form, \(m/z\) 1166.7 (see B).
about 200 times higher than the level in wild-type cells (Fig. 4). This finding strongly suggested that the luminally produced UDP-Xyl is controlling the cytoplasmically produced UDP-GlcA pool.

Xylose Incorporation in Glycans Is Restored by Cytoplasmic Expression of UXS—Previous in vitro experiments have shown that UDP-Xyl can be transported across the Golgi membrane (27, 44), and a Golgi-localized UDP-Xyl transporter has been identified (29). The in vivo significance of these findings is not known. To investigate if the transporter can function in vivo, two UXS expressing plasmids, cytoplasmic Arabidopsis UXS3 (30) and a human UXS expressed without the putative N-terminal transmembrane domain (cytUXS1), were transiently expressed together with AtXylT in pgsI-208 cells. Cytoplasmic localization of the UXS proteins was confirmed by immunofluorescence (not shown). Cytoplasmic UXS rendered cells positive for rabbit anti-HRP, which recognizes xylosylated N-glycans (Fig. 5A). More quantitative data were provided by stable expression of cytUXS in pgsI-208 and analyses of the cells with the anti-heparan sulfate antibody by flow cytometry (Fig. 5B). CHO cells (blue) compared with pgs-208 (black) and pgs-208 stably transfected with human UX3 expressed in the cytoplasm (red). C and D, nucleotide sugar composition analysis (as in Fig. 4) of pgs-208 expressing human cytUXS1 (C) or UX3 (D). Identity of the UDP-Xyl peak has been confirmed by mass spectrometry.

DISCUSSION

In this report, we describe a new CHO mutant, named here pgsI-208, defective in UXS, the enzyme generating UDP-Xyl. Lack of UDP-Xyl results in absence of both GAG biosynthesis and the addition of xylose to O-glucose glycans of EGF repeats present in Notch. Since CHO cells make both heparan sulfate and chondroitin sulfate, both types of GAGs are anticipated to be lacking in this cell line. Notch is the only protein carrying the Xyl1–3Xyl1–3GlcB-O-Ser trisaccharide tested in the system (3), but presumably other proteins bearing this sequence would be affected as well. The mutant also has the interesting phenotype of deregulated production of UDP-GlcA and led to the discovery that when provided with a cytosolic source of UDP-Xyl, this nucleotide sugar can be imported into Golgi and/or ER.

UXS was originally cloned from the pathogenic fungus C. neoformans (45) and later from other eukaryotes (28, 30, 46).
Mutants in the enzyme have been isolated from *C. neoformans* (47), where it is one of the capsule structure mutant strains (Cas2), and in *Caenorhabditis elegans* (26) as one of the squashed vulva (sqv-1) mutants affecting chondroitin and heparan sulfate biosynthesis. In CHO cells, like in *C. neoformans* (43), inactivation of UXS resulted in an increase of cellular UDP-GlcA. *In vitro* experiments have shown that UGDH, which generates UDP-GlcA from UDP-Glc, is inhibited by UDP-Xyl (42). Inhibition evidently occurs *in vivo* as well, since the absence of UDP-xylose resulted in a dramatic increase in UDP-GlcA. The accumulation of UDP-GlcA was most likely due to deregulated synthesis as opposed to a lack of consumption of UDP-GlcA. This conclusion is based on the observation that in pg5A-745, which, like pgsl-208, cannot incorporate UDP-GlcA into glycosaminoglycans, the ratio of UDP-Glc, UDP-GlcA, and UDP-Xyl was unchanged compared with wild-type CHO cells. In yeast, a system that does not utilize UDP-GlcA and UDP-Xyl, expression of UDPG can convert more than half of the UDP-Glc pool into UDP-GlcA (39). After additional expression of UXS, UDP-GlcA was no longer detectable due to substoichiometric accumulation of UDP-Xyl.

In contrast to fungi, where the enzyme is cytoplasmic (43), UXS of mammals (28) and *C. elegans* (26) and two of the three plant enzymes (30) are localized in the ER/Golgi lumen. Theoretically, physical separation of UDP-Xyl production from UDP-GlcA production provides a mechanism for cells to avoid inhibition of UGDH by UDP-Xyl. However, our current data do not support this hypothesis, because they clearly show that UDP-GlcA production is regulated by UDP-Xyl in mammalian cells. This conclusion was supported by the fact that cytoplasmically expressed UXS inhibited UDP-GlcA production, but less efficiently than the luminally expressed enzyme. The lower inhibition of cytUXS might be explained by differences in the rate of production of UDP-xylase or by other mechanisms. More data are needed for a conclusive interpretation of this interesting finding.

Although, we cannot exclude the possibility that a small portion of UDP-Xyl is produced in the cytoplasm, it seems more likely that UDP-Xyl generated in the Golgi/ER is transported to the cytoplasm to inhibit UGDH. This assumption is supported by observations of Bossuyt and Blanckaert (48) that show that luminal UDP-Xyl stimulates uptake of UDP-GlcA into ER vesicles, possibly by providing an antiport substrate, suggesting the existence of a UDP-GlcA/UDP-Xyl antipporter in the ER membrane. Transport of UDP-Xyl in the other direction, into the ER/Golgi lumen, has also been shown by transport of UDP-Xyl into isolated microsomes (44) or by incorporation of xylose from UDP-[14C]Xyl in permeabilized cells (27). Such a function could be provided by SLC35B4, a recently identified UDP-Xyl transporter of the Golgi (29). According to our results, UDP-Xyl is indeed transported into the Golgi/ER network, since cytosolic UXS allowed the formation of GAGs and xylosylation of N-glycans in the mutant. Thus, UDP-Xyl appears to be transported in both directions across the ER/Golgi membrane.

The exact localization of all proteins in this pathway is not completely resolved. The proteoglycan xylosyltransferases have been shown to be Golgi-localized (49), but evidence exists for co-translational xylosylation (50). Although evidence has been presented for UXS in the Golgi (28), our tagged constructs appear only in the ER.4 The ER and Golgi are also not uniform structures, and production and consumption of UDP-Xyl can take place at different subcellular locations. Whether most of the luminally produced UDP-Xyl is directly used in xylosylation reactions or is transported out of the ER or Golgi and back into the Golgi lumen remains unsolved. Methods to decrease the expression of different potential transporters in this process could provide insight into this problem.

Although a salvage pathway for the regeneration of xylose is anticipated in plants (51), no indication for its occurrence exists in mammalian cells. Feeding of CHO cells with xylose did not restore the mutant phenotype of pgsl-208. Furthermore, auxotrophic mutants dependent on exogenous xylose have not been identified in screening mutagenized cells. No ortholog of the plant enzyme (51) that is able to biosynthesize UDP-Xyl from xylose-1-phosphate is found in mammalian genomes. The pathway via UDP-GlcA, catalyzed by UXS, therefore, appears to be the only way to generate UDP-Xyl in CHO cells and probably all mammals.

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