XT-II, the Second Isoform of Human Peptide-O-xylosyltransferase, Displays Enzymatic Activity*

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Peptide O-xylosyltransferase (EC 2.4.2.26) is the first enzyme required for the generation of chondroitin and heparan sulfate glycosaminoglycan chains of proteoglycans. Cloning of cDNAs has previously shown that, whereas invertebrates generally have a single xylosyltransferase gene, vertebrate genomes encode two similar proteins, xylosyltransferase I and II (XT-I and XT-II). To date, enzymatic activity has only been demonstrated for the human XT-I, Caenorhabditis SQV-6, and Drosophila OXT isoforms. In the present study, we demonstrate that a soluble form of human XT-II expressed in the xylosyltransferase-deficient pgSA-745 (S745) Chinese hamster ovary cell line is indeed capable of catalyzing the transfer of xylose to a variety of peptide substrates; its enzyme activity was also proven using a Pichia expressed form of XT-II. Its pH, temperature, and cation dependencies are similar to those of XT-I expressed in either mammalian cells or yeast. Our data suggest that XT-I and XT-II are, at least in vitro, functionally identical.

Morphogenesis is a highly complex process in animal development and requires the creation of growth factor gradients for its control. Central to the correct formation of such gradients and to the associated signal transduction pathways are the glycosaminoglycan chains, both chondroitin and heparan sulfates, of various proteoglycans (1–4). In the past few years, a number of mutations affecting glycosaminoglycan biosynthesis have shown the key role of these macromolecules. Particularly striking is the series of Caenorhabditis elegans squashed vulva (sqv) mutants (5, 6); each of these mutants is defective in a different gene involved in the formation of chondroitin sulfate. In mammals, the EXT1 and EXT2 genes involved in heparan sulfate biosynthesis are implicated in brain morphogenesis (7) and in the bone disease hereditary multiple exostoses (8, 9), whereas a form of heparan sulfate, heparin, acts as an anticoagulant and with symptoms in pseudoxanthoma elasticum (20–22). Both genes are, as judged by reverse transcription-PCR with mRNA from eight tissues, widely expressed (15), and green fluorescent protein-tagged forms of both enzymes were found to be targeted to the Golgi (23), a location consistent with some earlier cell fractionation studies (24). However, the enzymatic function of XT-II has remained elusive. Preliminary data in this laboratory suggested a rather minor activity in the culture supernatant of Pichia pastoris transformed with an XT-II construct; thus, as an alternative, we decided to attempt the expression in a mammalian cell line lacking xylosyltransferase activity (25). Indeed, for the first time, we report that human XT-II is an active enzyme with properties not significantly different from those of XT-I.

EXPERIMENTAL PROCEDURES

Vector Construction and Transfection—Partial reading frames of human XT-I and XT-II were isolated by PCR using the forward primers HsXTI/1/Fspl (5′-cccagaaaggccgcatctaagggtgcatctcaag-3′) or HsXTII/1/Fspl (5′-cccagaaaggccgccatctaagggtgcatctcaag-3′) and the reverse primers HsXTI/2/NotI (5′-tctttttgccgagccgcatctcaaggtgcatctcaag-3′) or HsXTII/2/NotI (5′-tctttttgccgagccgcatctcaaggtgcatctcaag-3′) with pGEM-T vectors previously prepared using fragments generated by PCR of human embryonic kidney (HEK-293) cell line cDNA (19) as templates and KOD polymerase (Novagen). Purified PCR fragments were then cut with Fspl and NotI and ligated into the pPA-TEV vector (26), which was the kind gift of Dr. James Rini, University of Toronto. This vector, derived from pIRESpuro3

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3 The abbreviations used are: XT-I, xylosyltransferase I; XT-II, xylosyltransferase II; AMPD, 2-amino-methyl-1,3-propanediol; MALDI-TOF, matrix-assisted laser-desorption ionization/time-of-flight; MS, mass spectrometry; HPLC, high pressure liquid chromatography; RP-HPLC, reverse phase HPLC; PNGase F, peptide N-glycosidase F.
(Clontech), is intended for the expression of secreted protein A fusion proteins under control of the cytomegalovirus promoter. The inserts of selected clones were sequenced in their entirety using the BigDye kit (Applera). Verified clones encoding XT-I and XT-II, as well as an empty vector control, were transiently transfected into the pgsA-745 (S745) Chinese hamster ovary cell line (kindly supplied by Dr. Jeffrey Esko, University of California, San Diego, CA; Ref. 25) using Lipofectamine 2000 reagent (Invitrogen). Independent transfections of XT-II were performed on separate days. Cells were cultivated for 24 h, and the culture supernatants were collected, stored at 4 °C and, as required, concentrated using Ultrafree microcentrifuge devices with an Mr 30,000 cut-off (Millipore). Protein content in the supernatants was determined using the MicroBCA protein assay kit (Pierce).

The construction of the pPICZαC vector with the XT-II insert, transformation of P. pastoris strain GS115, and induction of expression with methanol has been described previously (19). Supernatants were concentrated 10-fold using Ultrafree microcentrifuge devices, diluted with 10 mM HEPES, pH 8, containing 0.01% (w/v) sodium azide and 1 mM phenylmethylsulfonyl fluoride, and again concentrated prior to storage at 4 °C.

Western Blotting—Aliquots of concentrated supernatants, equivalent to 12.5 μl of original culture medium, were subject to SDS-PAGE (10% gel) and Western blotting using rabbit anti-protein A antiserum (Sigma, 1:2000) and alkaline phosphatase-conjugated goat anti-rabbit (Vector Laboratories, 1:2000). SigmaFAST tablets were used to prepare the chromogenic substrate for detection. Aliquots of the supernatants were also digested overnight with PNGase F (1 unit, Roche Applied Science) at 37 °C in 10 mM HEPES, pH 8, prior to SDS-PAGE.

Assay of Xylosyltransferase Activity—Culture supernatants were assayed using the unmodified syndecan peptide (DDDS-IEGSGGR), the bikunin peptide (QEEEGSGGGQR), or a dansylated form of the perlecan peptide (DSISGDDLGSGLGSDLGDFQR) as substrates. Dansylation was performed as follows: 3 mg of peptide were dissolved in 7 ml of Li2CO3 (40 mM, pH 9.5); thereafter, 40 mg of dansyl chloride in 16 ml of acetone were added to a solution of the peptide in 15 ml of Li2CO3 (40 mM, pH 9.5). The solution was incubated at 37 °C for 16 h and then diluted with 30 ml of 40 mM Li2CO3 (pH 9.5) and 30 ml of 200 mM Li2CO3 (pH 9.5). The solution was then centrifuged at 5000 g for 10 min.

FIGURE 1. Expression of recombinant human xylosyltransferases in pgsA-745 cells. A, Western blotting of aliquots of culture supernatants of pgsA-745 cells transfected with empty vector (Control), human XT-I, or human XT-II were concentrated and, where indicated, subject to PNGase F digestion (+). Bands were detected using an anti-protein A antiserum as described. On the control blot, bands of M, 26,000–34,000 were observed (region not shown), consistent with the expression of protein A alone in the control samples. Protein standards are indicated in kDa. B–D, the culture supernatants were assayed overnight using a MALDI-TOF MS-based method. The syndecan peptide substrate (m/z 1108) was incubated in the presence of UDP-Xyl with culture supernatants of pgsA-745 cells transiently transfected with plasmids with human XT-I (B), human XT-II (C), or no insert (D). The increase in m/z of 132 is indicative of the transfer of xylose.

FIGURE 2. Assay of recombinant human xylosyltransferases by RP-HPLC. The syndecan (Syn) peptide substrate was incubated for 90 min with culture supernatants of pgsA-745 cells transiently transfected with plasmids with either human XT-I (A and B) or human XT-II (C and D) in the absence (A and C) or presence (B and D) of UDP-Xyl. The peaks eluting at 10 and 11 min were verified by MALDI-TOF MS to contain the xylosylated product and the substrate, respectively.
added, and the mixture was incubated in a water bath for 60 min at 37 °C. After the addition of 14 μl of ethylamine (70% (w/v)), the sample was subject to rotary evaporation and purified by gel filtration (Sephadex G25 fine, 1 × 50 cm in 25% methanol) and RP-HPLC. Detection of the dansylated product was performed using fluorescence at 315/550 nm. The syndecan (1 mM final concentration), bikunin (1 mM) or dansylated perlecan (30 μM) peptides were then incubated with supernatants of xylosyltransferase-expressing cells in the presence or absence of 1 mM UDP-Xyl, and the products were analyzed by MALDI-TOF MS (Perspective Biosystems Voyager-DE STR work station) as described previously (17, 19) or by RP-HPLC at a flow rate of 1.5 ml/min (Hypersil ODS). In the case of syndecan, the RP-HPLC gradient was of 11.4–13.3% acetonitrile from 5 to 13 min with UV detection at 214 nm (19), whereas for dansylated perlecan, a gradient of 34.2–41.8% acetonitrile was applied from 6 to 22 min with fluorescence detection at 315/550 nm.

**Further Characterization of XT-I and XT-II**—The enzymatic parameters of both human xylosyltransferases expressed in pgsA-745 cells were examined using the aforementioned RP-HPLC method with the syndecan peptide as substrate. For the pH dependence, HEPES and AMPD buffers of various pH values (6.5–9.0) were used. Various cations were tested at a final concentration of 10 mM; the effects of temperature during the reaction and of substrate concentration were examined using incubations containing 40 mM HEPES, pH 8, and 10 mM MnCl₂. All assays were performed in duplicate and within the linear range of product formation with respect to time. Relative activities (for pH, temperature, and cation dependence) were determined based on HPLC peak areas (for the product peak and the sum of product and substrate peaks); the percentage conversion of product was then converted to an activity relative to that under the condition giving the highest rate of conversion to product. Specific activities were also calculated based upon the amount of substrate, as determined by the relative HPLC peak areas for product and substrate, converted within the linear range, in the presence of Mn(II) at 37 °C, and are expressed in terms of milliunits/mg, where 1 milliunits/mg corresponds to 1 nmol substrate turned over per minute per mg of protein in the culture supernatant.

**RESULTS AND DISCUSSION**

**Expression of Xylosyltransferases**—In previous studies, we have found that forms of *Caenorhabditis SQV-6, Drosophila OXT*, and human XT-I could be expressed using *P. pastoris* in an active form (17, 19). These forms were truncated so as to delete the cytosolic, transmembrane and stem regions but retained the Pro-Xaa-Cys-(Asp/Glu) motif noted as being the most N-terminal conserved region in invertebrate and verte-

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*FIGURE 3. Characterization of recombinant human xylosyltransferases. RP-HPLC-based assays using the syndecan peptide substrate were performed for 90 min with culture supernatants of pgsA-745 cells transfected with XT-I and XT-II at different temperatures (A), with AMPD buffers with different pH values (B), or with different divalent cations (C). Data from duplicate experiments were normalized so that 100% corresponds to the condition resulting in the highest activity. The dependence of reaction rate for XT-I and XT-II with respect to either syndecan peptide acceptor concentration (D) or UDP-Xyl donor concentration (E) was measured using RP-HPLC-based assays; the assays were performed in duplicate, and the averaged data analyzed using Hanes’ plots are shown. For all panels, the individual data points are indicated by the error bars.
brate peptide O-xlyosyltransferases (11); the two human xylosyltransferase sequences display 61% identity (75% similarity) in the region beginning with this motif. However, although the expression in 

Pichia was easy to perform, the HPLC- and MS-based assays using the culture supernatants were sometimes complicated by the peptones in the medium and, furthermore, a barely detectable activity was found in preliminary trials with human XT-II. Considering that there was no published record of XT-II displaying activity, we decided that another system should be used to determine whether this protein is indeed enzymatically active. Previously, human XT-I has been expressed in an active soluble form in wild-type Chinese hamster ovary cells (15) and insect cells (27), as well as in a full-length form in HEK-293 cells (23). On the other hand, others have used the Caenorhabditis enzyme to rescue the defect in the pgsA-745 xylosyltransferase-deficient Chinese hamster ovary cell line (18). Specifically, an unknown genetic defect in this cell line results in a xylosyltransferase activity in crude cellular extracts at least 15-fold lower than in the parental line and, as a result, an incorporation of sulfate into glycosaminoglycan that is 2% of normal levels (25). Therefore, as an alternative to 
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Upon transient expression, Western blotting of culture media indicated that for both XT-I and XT-II, bands of the same insert sequences as the previously used yeast vectors and generated mammalian expression plasmids containing the Pichia that is 2% of normal levels (25). Therefore, as an alternative to

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enzymes displayed significant activity at pH 6.5, which contrasts with the apparent lack of activity of the *Pichia*-expressed XT-I using AMPD at pH 6.5 (19). There is no difference, however, between the actual optima of XT-I expressed in the different systems. These values, however, are still somewhat higher than that which may be expected for a Golgi enzyme, considering that the pH inside this organelle has been estimated in one study as being pH 6.2 ± 0.4 (28). Others have found pH optima for natural forms of mammalian xylosyltransferases of between pH 6.5 and 7.5 (29–31), although it is not known whether these native forms were mixtures of both XT-I and XT-II or not.

The cation dependence was also broadly comparable for XT-I expressed in both the *Pichia* and the mammalian systems (Fig. 3C); the highest activities were again observed with Ca(II), Co(II), Mg(II), and Mn(II). For XT-II, the pattern was similar except that the activity in the presence of Co(II) was lower than that in the presence of EDTA and that XT-II, in contrast to the XT-I expressed in the mammalian system, is almost completely inhibited by Ni(II). Both enzymes were still active in the absence of any added cation and in the presence of EDTA. Recently, this lack of a necessity for a divalent cation has been structurally explained for an enzyme, core 2 β1,6-N-acetylglucosaminyltransferase, from the same glycosyltransferase family (CAZY family 14); indeed, all members of this enzyme family lack the degenerate DXD sequence motif involved in metal ion binding and catalysis in other so-called GT-A glycosyltransferases (32). Although mammalian xylosyltransferase sequences actually contain an (Asp/Glu)-Trp-Asp sequence necessary for the activity of XT-I (33), it does not correspond to a canonical DXD motif. It is located in the unique C-terminal region, which displays no homology to other glycosyltransferases, and we assume that it does not play a direct role in the catalytic mechanism or in metal ion binding.

Finally, the substrate concentration dependence of XT-I and XT-II was examined (Fig. 3, D and E). The apparent $K_m$ values for UDP-xylose were determined to be in the region of 250 μM; these values are somewhat higher than the value of 100 μM found with the *Drosophila* enzyme using the same method (17) but similar to the 300 μM estimated for the *Pichia*-expressed form of XT-I (data not shown). In comparison, values of between 10 and 180 μM were found with various native mammalian enzymes (31, 34–36), whereas ~1 μM was found by others for a form of XT-I expressed in insect cells and assayed using a protein acceptor (33). For syndecan, the $K_m$ values were in the region of 1 mM, which are comparable with those found for the same substrate with *Caenorhabditis* and *Drosophila* xylosyltransferases expressed in *Pichia* (17, 19). Considering that the various parameters, including $K_m$ values, for XT-I and XT-II are approximately the same, there is no obvious enzymological reason for the previous lack of success in detecting the activity of the latter; we can only assume that some aspect of the process was not optimal in the earlier attempts.

**FIGURE 5.** Assay of recombinant human xylosyltransferases with a peptide containing multiple acceptor sites. Xylosyltransferase activity was measured by RP-HPLC (A–F) or MALDI-TOF MS (G) after incubation of the dansylated perlecan (Perl) acceptor substrate with concentrated culture supernatants of pgsA-745 cells transfected with human XT-I (A and B) or human XT-II (C, D, and G), untransfected cells (E), or cells transfected with the pPA-TEV vector lacking an insert (F) in the presence (A, C, E, and F) or absence (B and D) of UDP-xylose for 12 h. To minimize the appearance of a degraded form of the substrate (eluting ~1.5 min earlier), these assays were performed in the presence of EDTA. The xylosylation status of the annotated individual HPLC peaks was verified after collection by MALDI-TOF MS.
vector construction, exact insert sequence, promoter, medium or cell line employed plays a role.

**Use of Alternative Substrates by XT-II**—Previously, we have assayed recombinant xylosyltransferases from vertebrate (XT-I) and invertebrate (OXT and SQV-6) sources with a variety of peptide substrates: *i.e.* those based on portions of *Drosophila* syndecan, human bikunin, or human perlecans (17, 19). As shown above, an initial hypothesis that XT-II may require some particular unique substrate (19) was disproved in the course of the present study since the syndecan peptide was a substrate when this enzyme was expressed in pgsA-745 cells. However, it was still of interest to test XT-II with the bikunin and perlecans substrates. Indeed, as for XT-I, transfer of xylose by XT-II was observed with the bikunin peptide as judged by the appearance of a peak with m/z 1322 (Fig. 4A). Furthermore, encouraged by the results with XT-II in the mammalian expression system, we reattempted the expression of XT-II in *Pichia* and, this time, a significant transfer of xylose to the bikunin peptide was also observed (Fig. 4B). In controls (empty vector or no donor), no species with m/z 1322 was present (Fig. 4, C and D). This result is an independent indication that XT-II is indeed truly active since yeasts are not known to possess an endogenous peptide O-xylosyltransferase.

In the case of the perlecans substrate, we decided to develop an HPLC-based assay. In this case, the peptide was modified by dansylation to yield a fluorescently tagged form, whose detection was not expected to be subject to interference by other substances in the culture medium. Initial tests with recombinant *Drosophila* OXT showed that the dansylated perlecans peptide was a suitable substrate for sensitive detection of xylosyltransferase activity (data not shown). Thus, we also tested this substrate with supernatants of cells transfected with the XT-I and XT-II constructs. In both cases, a number of products were observed by RP-HPLC (Fig. 5, A and C), which were not present in incubations with the control supernatants (Fig. 5, E and F) or in the absence of the donor substrate (Fig. 5, B and D). The various fractions were collected and analyzed by MALDI-TOF MS either separately (see annotations on Fig. 5, A and C) or as a pool (as shown for the incubation with XT-II; Fig. 5G).

In contrast to the transfer of only two xylose residues to the non-dansylated form of the perlecans peptide in incubations of either the mammalian-expressed XT-I and XT-II (data not shown) or the *Pichia*-expressed XT-I and OXT (19), up to three xylose residues were transferred by both XT-I and XT-II to the dansylated perlecans peptide; this corresponds to the number of Ser-Gly motifs in this peptide. We assume that the modification of the N terminus by the dansyl group facilitates transfer to the first Ser-Gly motif (this serine residue being the fourth residue of the peptide); in the case of the non-dansylated form xylosylated with *Pichia*-expressed enzymes, this residue does not, as shown by tandem MS, serve as a xylosylation acceptor site (19). These data may be compatible with the effects of peptide length on the degree of xylosylation previously noted by others (29, 37) and show that relatively small changes in a peptide substrate can have significant effects on the results obtained.

**Conclusions**—In this study, we have shown that both isoforms of human xylosyltransferase, XT-I and XT-II, are indeed active enzymes when expressed in the Chinese hamster ovary mutant pgsA-745 cell line, thereby showing activity of the latter for the first time. The demonstration of XT-II activity also in recombinant *Pichia* confirms this result and suggests that the activity of the supernatants of the transformed pgsA-745 cells is indeed due to XT-II activity and not due to some indirect rescue of the endogenous defective xylosyltransferase gene(s). Thus, now knowing that both xylosyltransferase isoforms are active and assuming their relatively ubiquitous expression (15), it may be that many or most mammalian cells have two enzymes capable of initiating chondroitin and heparan sulfate biosynthesis, although the situation in, for example, wild-type Chinese hamster ovary cells has been unknown. On the other hand, there is only one isoform of the next enzyme in the glycosaminoglycan biosynthetic pathway (38), β4Gal-T VII, and it is interesting to note that some cases of Ehlers-Danlos syndrome, a connective tissue disorder, are associated with mutations in the B4GALT7 gene, which result in reduced enzymatic activity and defective proteoglycan glycosylation (39). This raises the issue as to whether mutations in one xylosyltransferase gene will result in strong phenotypes or whether only ablation of both will result in lethality in mammals. This is in contrast to the situation in invertebrates, which generally only have a single xylosyltransferase gene, but may be akin to the situation with another Golgi enzyme pair, mannosidase II and mannosidase II*. In the case of the latter, although the single knock-outs do show either immunological or reproduction defects, both mannosidase genes must be ablated for either in utero or postnatal lethality to occur in the mouse (40). Perhaps the possession of two xylosyltransferases with similar activities is an evolutionary accident or is indeed a reflection of an essential role for proteoglycans in many developmental and physiological processes in mammals.

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**Note Added in Proof**—Two papers independently confirm the activity of mammalian XT-II and also show that the corresponding gene in the pgsA-745 cell line has a premature stop codon, while wild-type Chinese hamster ovary cells express only extremely low levels of the XT-I transcript (41, 42).

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