Analysis of the DXD Motifs in Human Xylosyltransferase I Required for Enzyme Activity*

Human xylosyltransferase I (XT-I) is the initial enzyme involved in the biosynthesis of the glycosaminoglycan linker region in proteoglycans. Here, we tested the importance of the DXD motifs at positions 314–316 and 745–747 for enzyme activity and the nucleotide binding capacity of human XT-I. Mutations of the $^{314}$DED$^{316}$ motif did not have any effect on enzyme activity, whereas alterations of the $^{745}$DWD$^{747}$ motif resulted in reduced XT-I activity. Loss of function was observed after exchange of the highly conserved aspartic acid at position 745 with glycine. However, mutation of Asp$^{745}$ to glutamic acid resulted in a 6-fold lower $V_{\text{max}}$, with $K_{m}$ values comparable with those of the wild-type enzyme. Despite the major effect of the DWD motif on XT-I activity, nucleotide binding was not abolished in the D745G and D747G mutants, as revealed by UDP-bead binding assays. $K_{m}$ values for inhibition by UDP were determined to be 1.9–24.6 $\mu$M for the XT-I mutants. The properties of binding of XT-I to heparin-beads, the $K_{i}$ constants for noncompetitive inhibition by heparin, and the activation by protamine were not altered by the generated mutations.

Glycosyltransferases represent a large group of enzymes involved in the biosynthesis of the highly variable sugar structures found in bacteria, plants, and animal cells. They transfer activated sugars, such as UDP-sugars, to specific acceptor molecules, which can be proteins, sugars, or lipids. In the last decade, a large number of glycosyltransferases involved in many biosynthetic pathways have been cloned (1). However, analysis of the structural properties and catalytic mechanisms is still challenging and complex, as they are type II transmembrane proteins localized in the endoplasmic reticulum and Golgi apparatus (2, 3).

One large group of glycosylated proteins found in animal cells is the proteoglycans. These abundant glycoproteins are polyanionic molecules present in the extracellular matrix and on the cell surface and serve a wide range of functions. Proteoglycans are increasingly implicated as important regulators in many biological processes, such as extracellular matrix deposition, cell membrane signal transfer, morphogenesis, cell migration, normal and tumor cell growth, and viral infection (4–6). Proteoglycans mediate diverse cellular processes through interaction with a variety of protein ligands. Electrostatic interactions with the glycosaminoglycan chains attached to the core protein are involved in most of these bindings (7).

Thus, the biological activity of proteoglycans is intimately related to glycosaminoglycan biosynthesis.

The sulfated glycosaminoglycans chondroitin sulfate, heparan sulfate, heparin, and dermatan sulfate are bound to the proteoglycan core protein by a xylose-galactose-galactose binding region (7). Xylosyltransferase I (XT-I; EC 2.4.2.26) is the chain-initiating enzyme involved in the biosynthesis of glycosaminoglycan-containing proteoglycans (8, 9). The enzyme catalyzes the transfer of $\alpha$-xylose from UDP-$\alpha$-xylose to specific serine residues of the core protein and is a regulatory factor in chondroitin sulfate biosynthesis (10). XT-I activity was found to be present in the cisternae of the rough endoplasmic reticulum of various species (11), and we have shown that the enzyme is secreted from the endoplasmic reticulum into the extracellular space together with chondroitin sulfate proteoglycans (12).

As XT-I is the initial enzyme in the biosynthesis of the glycosaminoglycan linkage region (10) and as it is secreted into the extracellular matrix to a great extent, XT-I activity was proposed to be a diagnostic marker for the determination of enhanced proteoglycan biosynthesis and tissue destruction (13). XT-I activities in synovial fluid were found to be significantly increased in chronic inflammatory joint diseases (14). Our recent studies have shown that serum XT-I activity is a confirmed biochemical marker for the determination of fibrotic activity in systemic sclerosis (12, 15) and that highly elevated XT-I activities are found in human follicular fluid as a consequence of the enhanced proteoglycan biosynthesis during folliculogenesis (16).

Recently, we purified human XT-I from 2000 liters of culture supernatant conditioned by JAR choriocarcinoma cells (17) and cloned the corresponding mammalian XT-I cDNAs (18). Furthermore, we identified another gene in the mammalian genomes coding for a novel protein, which was shown to be highly

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‡ To whom correspondence should be addressed. Tel.: 49-5731-972-033; Fax: 49-5731-972-013; E-mail: cgoetting@hdz-nrw.de.

1 The abbreviations used are: XT-I, xylosyltransferase I; XT-II, xylosyltransferase II; rXT-I, recombinant xylosyltransferase I; MES, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline; BisTris, 2-(bis-2-hydroxyethyl)aminomethyl-2-(hydroxymethyl)propane-1,3-diol; MOPS, 4-morpholinepropanesulfonic acid; contig, group of overlapping clones.
homologous to XT-I (18). However, the catalytic activity of this protein, which was termed xylosyltransferase II (XT-II), is not yet known.

To date, little structural information is available concerning the catalytic site of XT-I. Recently, a D\textsubscript{D} motif at positions 745–747 was found to be critical for enzyme activity (20–25). The D\textsubscript{D} motif in glycosyltransferases was proposed to be involved in the coordination of a divalent metal ion that is required for the binding of the nucleotide-sugar (21). However, mutations of the D\textsubscript{D} motif in the Fringe signaling molecule did not alter photolabeling by a UDP analog, but did eliminate the biological activity of Fringe (25). These findings, which were supported by other studies (26, 27), raised the possibility that not all D\textsubscript{D} motifs are functionally equivalent.

Amino acid sequence analysis of mammalian XT-I revealed two D\textsubscript{D} motifs located in the N- and C-terminal regions of this glycosyltransferase. In this study, we analyzed the importance of these motifs for the enzyme activity of human XT-I. We mutated residues within both D\textsubscript{D} motifs to determine their importance to XT-I activity and nucleotide binding. Our results show that the D\textsubscript{D} motif at positions 745–747 is required for obtaining full enzyme activity, but is not critical for the binding of UDP.

EXPERIMENTAL PROCEDURES

Materials—High Five insect cells (Trichoplusia ni, BTI-Tn-5B1-4) were purchased from Invitrogen, and JAR choriocarcinoma cells were from American Type Culture Collection (Manassas, VA). Insect Xpress were purchased from Invitrogen, and JAR choriocarcinoma cells were from American Type Culture Collection (Manassas, VA). Insect Xpress were purchased from Invitrogen, and JAR choriocarcinoma cells were from American Type Culture Collection (Manassas, VA). Insect Xpress were purchased from Invitrogen, and JAR choriocarcinoma cells were from American Type Culture Collection (Manassas, VA). Insect Xpress were purchased from Invitrogen, and JAR choriocarcinoma cells were from American Type Culture Collection (Manassas, VA).

Table I

| Primer          | Sequence                                                | Mutation |
|-----------------|---------------------------------------------------------|----------|
| XTGly3Up        | 5'-CACAAAGACTGGCTAGTTGGGGCAGACTCCG-3'                   | D314G    |
| XTGly5Lo        | 5'-CGGAATAGCCTCGTCTCTACCGAGCCAGGAGGATGCCAAGGAGAG-3'     | D316G    |
| XTGly4Up        | 5'-TGAGTGGGAGGGGAGCCAGCCTGATTTGAGTCGAGCTCCAG-3'        | D316G    |
| XTGly4Lo        | 5'-CATGATCTCCACAGGACCGCCCTGCCAACCTGAGC-3'              | D316G    |
| XTGly1U         | 5'-CTCGAGCTGGCAGCTGTCCCAGCAGAGGAGGAGGAG-3'             | D745E    |
| XTGly1L         | 5'-CCCTCTCTGCTCAGCAGGAGGAGGAGGAGGAG-3'                 | D745E    |
| XTTrp1U         | 5'-CTGACAGCAATAGCCAAAAAGGAGG-3'                        | W746D    |
| XTTrp1L         | 5'-GATCATGCTCTCGAGCTGTGCAGAGGAGGAGGAGGAG-3'            | W746D    |
| XTTrp2U         | 5'-GATCATGCTCTCGAGCTGTGCAGAGGAGGAGGAGGAG-3'            | W746D    |
| XTTrp2L         | 5'-GATCATGCTCTCGAGCTGTGCAGAGGAGGAGGAGGAG-3'            | W746D    |
| XTTrp14U        | 5'-CTGAGCAGGAGGAGGAGGAGGAGGAGGAGGAGGAG-3'              | W746D    |
| XTTrp15L        | 5'-CGGAATAGCCTCGTCTCTACCGAGCCAGGAGGATGCCAAGGAGAG-3'    | W746D    |
| XTGly2Up        | 5'-GAGTGTGGGAGGGGAGCCAGCCTGATTTGAGTCGAGCTCCAG-3'       | D747T    |
| XTGly2Lo        | 5'-GAGTGTGGGAGGGGAGCCAGCCTGATTTGAGTCGAGCTCCAG-3'       | D747T    |
| XTIE1U          | 5'-CTCCCCATCCAGGCCCCAGC-3'                             | D747E    |
| XTIE1Lo         | 5'-CTCCCCATCCAGGCCCCAGC-3'                             | D747E    |

Novex (San Diego, CA). Pharmaceutical heparin from porcine intestinal mucosa was obtained from Ratiopharm (Ulm, Germany), and the QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Pfu DNA polymerase was obtained from PEQLab (Erlangen, Germany), and T4 polynucleotide and Hotstart T4 polymerase were from Cyagen (Hilden, Germany). The Quikprep spin plasmid kit and the PCR purification kit were also obtained from Qiagen (Hilden, Germany). The digital imaging system was from Raytest (Straubenhardt, Germany), and DNA sequencing was performed on an automated DNA sequencing system from PerkinElmer Life Sciences with the dye terminator cycle sequencing kit. The synthetic peptides and rabbit antiserum were purchased from Biosearch (Göttingen). Peroxidase-conjugated and alkaline phosphatase-conjugated AffiniPure F(ab’\textsubscript{2}) fragment goat anti-rabbit IgG (H + L) antibodies were purchased from Dianova (Hamburg, Germany). All other chemicals were analytical grade and obtained from Merck.

PCR Amplification and Cloning of Human XT-I cDNA—Synthetic oligonucleotide primers including 5'-HindIII and 3'-Xbal restriction sites (upper primer, 5'-cagccagcaatgctctactcctct-3'; and lower primer, 5'-gagttgatagcaatgctctactcctct-3', with restriction sites underlined) were designed based on the XT-I cDNA sequence (18). The primers were used to amplify the XT-I cDNA (2434 bp) from pCG2227 (18) under standard PCR conditions with Pfu DNA polymerase. The resulting PCR product was digested with HindIII and Xbal and inserted into the HindIII/Xbal sites of plasmid pMB-V5-A, resulting in pCG255-1. The constructed pCG255-1 plasmid encodes a soluble form of an XT-I/V5 epitope fusion protein and was transformed into chemically competent Escherichia coli TOP10 cells. Clones containing the correct vector were identified by single and double restriction digestion, followed by agarose gel analysis, and were confirmed by double-strand DNA sequencing. DNA sequencing was performed on an ABI 310 DNA sequencing system with the dye terminator cycle sequencing kit. Multiple clones were sequenced to compensate for misreading.

Site-directed Mutagenesis—Coads were altered using a method based on the QuikChange site-directed mutagenesis kit. The sequences of the sense and antisense mutated primers are indicated in Table I. The constructed vectors were listed in Table II. Mutated DNA was double-strand-sequenced to confirm the single codon change and to ensure that no additional changes were introduced. The various mutants were individually expressed in High Five insect cells as described below. At least two vectors encoding each XT-I variant and two independent transfection experiments were performed for the analysis of these mutants. In addition, all vectors encoding XT-I variants with altered enzyme activity were remodeled to the wild-type enzyme by site-directed mutagenesis as described above. These revertants were then expressed in High Five insect cells to ensure full restoration of enzyme activity.

Heterologous Expression in High Five Insect Cells—Insect cell expression was done in High Five insect cells grown in 2 ml medium supplemented with 10 \textmu g/ml gentamycin. Transient expression experiments of the recombinant XT-I (XT-I) variants were performed in 3 ml High Five insect cell cultures (2 \times 10\textsuperscript{6} cells) seeded in a 60-mm tissue culture plate, transfected with each recombinant pMB-V5-His vector, and grown at 27 °C. At 48 h post-transfection, the culture medium
containing the recombinant protein was collected and centrifuged for 5 min at 1500 × g. The supernatant was enriched 50-fold by ultrafiltration using Microcon 3000 tubes, and the XT-I/V5 epitope fusion proteins were detected by Western blot analysis. A stable High Five/pCG255-1 insect cell clone expressing XT-I-(Δ1-48)-V5-His was generated as described in detail (28).

Synthesis of Recombinant Bikunin—Recombinant bikunin was expressed in E. coli strain BL21(DE3) as described previously (29). The purified protein was then used as an acceptor in the XT-I activity assay.

Determination of Total Protein Concentration—The method for determination of XT-I activity is based on the incorporation of D-[14C]xylose with recombinant bikunin as the acceptor. The reaction mixture for the assay, contained in a total volume of 100 μl, 50 μl of XT-I solution, 25 mM MES, pH 6.5, 25 mM KCl, 5 mM MgCl₂, 5 mM MnCl₂, 1.0 μM UDP-D-[14C]xylose, and 1.5 μM recombinant bikunin (13). After incubation for 1 h at 37 °C, the reaction mixtures were placed on nitrocellulose discs. After drying, the discs were washed for 10 min with 10% trichloroacetic acid and three times with 5% trichloroacetic acid solution. Incorporated radioactivity was quantified after the addition of 5 ml of scintillation mixture using a LS6500TD liquid scintillation counter. Enzyme activity is expressed in units (1 unit = 1 μmol of incorporated xylose/min). The linear range of the XT-I activity assay was determined as 0.02–5.5 milliunits/liter (13). To measure within the linear range, samples were diluted with phosphate-buffered saline (PBS) supplemented with 1% human serum albumin. The assay linearity and dilution procedure were validated using an enriched human XT-I solution prepared as described previously (30).

To investigate the influence of effectors on XT-I activity, commercial preparations were used: heparin and pharmaceutical heparin from porcine intestinal mucosa and protamine sulfate from salmon. To investigate the influence of UDP, the samples were incubated with the effecting reagents for 1 min, and XT-I activity was then assayed as described above.

For quantification of the Michaels-Menten constants for xylosylation of bikunin by different XT-I mutants, various concentrations of the acceptor protein were incubated with XT-I-containing solutions and UDP-[14C]xylose under assay conditions. The transfer rates were measured as a function of the acceptor concentrations, and Kₐ and Vₘₐₓ values were calculated on the basis of nonlinear regression analysis. For the determination of the interaction of heparin (mean molecular mass of 15,000 Da) and UDP with XT-I mutants, the apparent Kₐ and Vₘₐₓ values with and without the addition of inhibitor at different concentrations were used to calculate the Kₑ values.

Determination of Total Protein Concentration—The total protein concentration was determined using the bicinchoninic acid protein assay kit. Free amino acids in the samples were removed prior to protein determination by ultrafiltration with Microcon 3000 tubes according to the manufacturer's instructions.

Antibody Production—Anti-XT-I antibodies were produced as described previously (17). Briefly, the synthetic peptides CS-KF, CS-MG, CS-MN, and CS-V5 were synthesized, purified by high pressure liquid chromatography, coupled to keyhole limpet hemocyanin, and used for immunization. Rabbit anti-XT-I IgG polyclonal antibody was prepared by injection of the above antigen, followed by booster injections at 3-week intervals, four times in total, into rabbits.

SDS-PAGE—For SDS-PAGE, 12.1 μl of concentrated High Five cell culture supernatant were added to 4.7 μl of sample buffer (1.00 μl Tris-HCl, 1.17 μl sucrose, 0.28 μl SDS, 2.08 μl EDTA, 0.88 μl Serva Blue G-250, 0.70 μl phenol red, and 0.10 μl dithiothreitol, pH 8.5) and heated for 10 min at 99 °C. After the sample had been loaded, SDS-PAGE was carried out on a 4–12% Bis-Tris-polyacrylamide gel with MOPS running buffer (50 mM MOPS, 50 mM Tris, 3.47 mM SDS, and 0.03 mM EDTA, pH 7.7). Protein bands were detected by Coomassie Brilliant Blue.

Western Blot Analysis—Western blotting to polyvinylidene difluoride membrane was carried out using a semidry electroblotting apparatus. After transfer, nonspecific antibody-binding sites were blocked with 2% bovine serum albumin in 0.1 mM Tris-HCl, pH 7.2, for 1 h at room temperature. The membranes were incubated with horseradish peroxidase-coupled anti-V5 antibodies at 1:500 dilution, and bound antibodies were detected using 4-chloro-1-naphthol. Alternatively, anti-XT-I antibodies were used in 50 μl PBS, 0.15 mM NaCl, and 0.5 mM/liter Tween 20, pH 7.4, at 1:1000 dilution for 1 h for detection of human XT-I variants. Bound anti-XT-I IgG antibody was detected using a second horseradish peroxidase-coupled goat anti-rabbit IgG antibody at 1:1000 dilution. The blot was developed using 4-chloro-1-naphthol. If an increased sensitivity was needed to detect the XT-I/V5-His protein, alkaline phosphatase-coupled anti-V5 antibodies were used at 1:500 dilution, and the bound antibodies were detected using the Western Breeze kit and a digital imaging system according to the manufacturers' instructions.

Binding to UDP-Beads—A 20-μl aliquot of fresh UDP-beads was washed with PBS to remove stabilizing reagents and contaminants and was then resuspended in 50 μl of PBS. The UDP-beads were mixed with 50 μl of 10–50-fold enriched culture supernatant from High Five insect cells expressing the XT-I variants containing either 5 mM MgCl₂ or 5 mM EDTA in the presence or absence of 25 μM UDP. The reaction mixtures were incubated at 25 °C for 1 h with rotation, then washed with PBS. The beads were then centrifuged for 1 min at 1000 × g and boiled in sample buffer for 10 min. The samples were loaded onto an SDS-polyacrylamide gel and electrophoresed as described above. The bound XT-I/V5-His fusion proteins were then detected after Western blotting using anti-V5 antibodies.

Analysis of XT-I Amino Acid Sequences—The hydrophobic plot of human XT-I was performed with the Winpep software (31) using the algorithm of Kyte and Doolittle (32) with a word size of 11. Homology analysis of the DXD motif of XT-I was carried out with the ClustalX and GeneDoc software packages using the following sequences: human XT-I (GenBank™/EBI accession number AJ277441) (18), chimpanzee (Pan troglodytes) XT-I (Ensembl Gene ENSPTRG00000007819), Mus musculus XT-I (GenBank™/EBI accession number AJ297015), Rattus norvegicus XT-I (GenBank™/EBI accession number AJ295748) (18), Fugu rubripes XT-I (Ensembl Gene SFRFRUG000000122544), zebrabfish (Danio rerio) XT-I (Ensembl Gene contig ctg26551), human XT-II (GenBank™/EBI accession number NM_022167) (18), chimpanzee (P. troglodytes) XT-II (Ensembl Gene ENSTPRG00000007819), Mus musculus XT-II (GenBank™/EBI accession number AJ297157), R. norvegicus XT-II (GenBank™/EBI accession number AJ295749) (18), F. rubripes XT-II (GenBank™/EBI accession number NM_139448), and Anopheles gambiae xylosyltransferase (Ensembl Gene ANAGNGG000000004589). If an amino acid sequence was not available, the corresponding cDNA or genomic DNA sequence was used to derive the amino acids.

Statistical Analysis—Statistical analysis was performed using the t test and the Kolmogorov-Smirnoff test. p values of 0.05 or less were considered significant.

RESULTS

General Structural Features of XT-I—In common with other glycosyltransferases that localize to the Golgi complex, XT-I
is a type II transmembrane protein with a short N-terminal cytoplasmic tail (1–14 amino acids) (Fig. 1), followed by a transmembrane anchor (amino acids 15–35) and a stem region. The large segment (>900 amino acids), which is located in the Golgi lumen, contains the catalytic domain. Analysis of human XT-I identified two DXD motifs located at positions 314–316 and 745–747. We have shown in previous studies that in addition to the XT-I gene, higher organisms share another gene coding for XT-II, a protein highly homologous to XT-I (18). However, *D. melanogaster* (33), *A. gambiae* (34) and *Caenorhabditis elegans* (34) have only one xylosyltransferase ortholog. Alignment of the amino acid sequences of XT-I and XT-II from human, chimpanzee, mouse, rat, pufferfish, zebrafish, fruit fly, and malaria mosquito showed that both DXD motifs are conserved among these species (Fig. 1). However, the DXD motif at positions 314–316 is less conserved than the 745DXD747 motif and is obviously not present in the insect XT-I orthologs. The consensus sequence of the C-terminal motif is EV(G/S)T(D/E)DXDKE(L/I).

**Expression of Human XT-I and XT-I Mutants in High Five Insect Cells**—A soluble form of human XT-I lacking the cytoplasmic tail and transmembrane domain was expressed in High Five insect cells. Histidine- and V5-tagged rXT-I lacking amino acids 1–148 was secreted into the cell culture supernatant and was highly active. The XT-I activity of the culture medium conditioned by High Five/pCG255 cells was deter-

**Fig. 1.** Hydropathy analysis of human xylosyltransferases and sequence analysis of both DXD motifs. A, the hydropathy plot of human XT-I shows the common topology of a type II transmembrane glycosyltransferase. The transmembrane domain (TM) is located at positions 15–35, and the localization of the N-terminal DED motif (DXD 1) and the C-terminal DWD motif (DXD 2) is marked. B, XT-II shows also a type II transmembrane protein topology, with the transmembrane domain located at positions 14–38. The two DWD motifs at positions 219–221 and 651–653 are marked by arrows. C and D, ClustalX was used to align the XT-I and XT-II sequences from human, chimpanzee, mouse, rat, zebrafish, and pufferfish and the xylosyltransferase (*XylT*) sequences from fruit fly (*Drosophila* (*Dros.*)) and malaria mosquito (*Anopheles* (*Anoph.*)). The DXD motif located at positions 314–316 is less conserved among these species (C) than the C-terminal DXD motif found at positions 746–747 (D). The positions of both DXD motifs are indicated by brackets, and conserved amino acid residues are boxed (gray and black).
The comparison with rXT-I. However, the graph indicates the percent XT-I activity relative to wild-type rXT-I-V5-His.

DWD (positions 745–747) mutated in the N-terminal DED (positions 314–316) and C-terminal DWD (positions 745–747) motifs. The recombinant mutants were expressed in insect cells and detected by Western blotting using anti-V5 antibodies (inset), and XT-I activity was measured. The bar graph indicates the percent XT-I activity relative to wild-type rXT-I-(Δ1–148)-V5-His. Error bars represent S.D.

Detected as 220 milliunits/liter, which is ~200-fold higher in comparison with the XT-I activity secreted by JAR choriocarcinoma cells (17) and with the XT-I activity in human blood (12). XT-I mutants were also expressed in parallel in High Five insect cells and could also be detected by Western blotting using anti-V5 antibodies (Fig. 2) and monospecific polyclonal antibodies raised against peptides homologous to human XT-I (data not shown).

Immunological Inhibition of XT-I Activity—To analyze the importance of the 745DWD747 motif for the enzyme activity of XT-I, monospecific polyclonal antibodies directed against the DWD motif were added to XT-I isolated from the supernatant of JAR choriocarcinoma cells and rXT-I-(Δ1–148)-V5-His. The relative enzyme activity was reduced by 42% compared with the negative controls, and no inhibition was observed when preimmune serum was added. No significant differences were observed between native human XT-I and rXT-I-(Δ1–148)-V5-His.

Effect of Alterations of the Two DXD Motifs on XT-I Activity—To determine the importance of these two DXD motifs for the activity of human XT-I, we mutated the conserved aspartate residues at positions 314 and 316 in the N-terminal DED motif and at positions 745 and 747 in the C-terminal DWD motif. All XT-I mutants were detected in the Western blot using anti-V5 (Fig. 2) and anti-XT-I (data not shown) antibodies and were expressed at comparable levels. Analysis of the XT-I activity of each individual mutant revealed that alterations of the N-terminal DXD motif did not affect enzyme activity, whereas mutations of the DWD motif resulted in reduced XT-I activity. The D745G mutation resulted in a loss of enzyme activity of at least 60%, the kinetic analysis revealed that the Ki values determined for the wild-type enzyme (Table III).

In Vitro Effects of UDP and Protamine Sulfate on the Enzyme Activity of XT-I Mutants—UDP is a competitive inhibitor of many glycosyltransferases (35, 36), including human XT-I. Inhibition of enzyme activity by UDP at different concentrations was investigated for each of the generated XT-I mutants. The addition of 0.5 mM UDP inhibited wild-type XT-I activity by 95%, whereas the addition of 2.5 mM UDP resulted in a loss of activity of >99% (Table IV). The kinetic study revealed that inhibition by UDP was of the competitive type, and the apparent substrate inhibition constant (Ki) was determined to be 10.8 μM for rXT-I (Table III). Most of the alterations of the N- or C-terminal DXD motif did not have any influence on the UDP-mediated inhibition of XT-I activity, as similar Ki values were determined for these XT-I variants. However, inhibition of enzyme activity by 0.5 mM UDP was significantly reduced in all mutants with a replaced tryptophan residue at position 746 (Table IV). This is supported by the substrate inhibition constant, which was determined to be elevated in comparison with the wild-type enzyme (Table III). Furthermore, mutant D745E showed an increased sensitivity to UDP, with a Ki of 1.9 μM. The inhibitory effect of UDP on mutant D745G could not be reliably determined because of the low enzyme activity of this mutant. We have shown in previous studies that the addition of protamine sulfate results in increased XT-I enzyme activity (16). Protamine sulfate was then added to the enzyme preparations of the XT-I mutants to investigate the potential effect of the altered DXD motifs on protamine activation. The addition of 100 μg/ml protamine sulfate resulted in a 15–35% increase in enzyme activity, with no significant differences between each of the XT-I variants (Table IV).

Effect of Mutations on Binding to UDP-Beads—To determine the role of the DWD motif at positions 745–747 in nucleotide binding in human XT-I, we analyzed the binding of soluble rXT-I and mutants D745G and D747E to UDP-agarose beads (Fig. 3). For each experiment, crude enzyme preparations were incubated with UDP-beads, and after washing, the bound enzyme was detected by Western blotting using anti-V5 antibodies. Only a single band with the expected molecular mass was observed in all experiments. The wild-type enzyme bound to the UDP-beads with high affinity in the presence of Mn2+, but binding was inhibited by 25 mM UDP. No binding to the UDP-beads was observed after the addition of 5 mM EDTA, indicating the impor-

Interestingly, the Km values were not significantly altered in these mutants, but the maximum transfer rate was reduced by a factor of 4–6 (Table III).

Effect of Alterations of the Two DXD Motifs on XT-I Activity—To determine the importance of the 745DWD747 motif for the enzyme activity of XT-I, monospecific polyclonal antibodies directed against the DWD motif were added to XT-I isolated from the supernatant of JAR choriocarcinoma cells and rXT-I-(Δ1–148)-V5-His. The relative enzyme activity was reduced by 42% compared with the negative controls, and no inhibition was observed when preimmune serum was added. No significant differences were observed between native human XT-I and rXT-I-(Δ1–148)-V5-His.

Analysis of the XT-I activity of each individual mutant revealed that alterations of the N-terminal DXD motif did not affect enzyme activity, whereas mutations of the DWD motif resulted in reduced XT-I activity. The D745G mutation resulted in a loss of enzyme activity of >95%, indicating the necessity of this residue for enzyme activity. However, changing this aspartate residue to glutamate, an amino acid with acidic properties also, did not affect the catalytic activity of human XT-I. The Km and Vmax values determined for xylosylation of recombinant XT-I activity of at least 60%. The kinetic analysis revealed that the Vmax was not changed in mutants D747G and D747E in comparison with rXT-I. However, the Km of both mutants was increased by ~6-fold, indicating reduced substrate binding affinity. To elucidate the importance of the central tryptophan residue in the DWD motif, we changed this tryptophan to glycine (W746G), aspartate (W746D), and asparagine (W746N). All of these mutants showed a striking reduction in XT-I activity of ~75% compared with the wild-type enzyme.

### Table III

|          | Km (μM) | Vmax (pmol/h·μM) | Ki (μM) | UDP | Heparin |
|----------|---------|-----------------|--------|-----|---------|
| Wild-type| 0.9 ± 0.3| 764 ± 69        | 10.8 ± 2.6 | 0.12 ± 0.04 |
| D314G    | 1.1 ± 0.3| 744 ± 59        | 7.2 ± 1.9 | 0.15 ± 0.07 |
| D316G    | 0.9 ± 0.2| 780 ± 74        | 11.3 ± 2.8 | 0.14 ± 0.05 |
| D745G    | NA      | NA              | NA      | NA   | NA      |
| D745E    | 1.1 ± 0.4| 717 ± 53        | 1.9 ± 1.1 | 0.07 ± 0.06 |
| W746D    | 1.2 ± 0.3| 132 ± 23        | 19.6 ± 6.1 | 0.13 ± 0.03 |
| W746N    | 1.3 ± 0.2| 165 ± 34        | 17.9 ± 5.6 | 0.11 ± 0.03 |
| W746G    | 1.2 ± 0.3| 82 ± 19         | 24.6 ± 9.3 | 0.12 ± 0.04 |
| D747G    | 6.9 ± 0.7| 688 ± 94        | 17.2 ± 7.4 | 0.15 ± 0.03 |
| D747E    | 4.4 ± 0.6| 709 ± 34        | 14.7 ± 5.3 | 0.11 ± 0.08 |

Means ± S.D. are shown. The kinetic constants for mutant D745G could not be reliably determined because of the low enzyme activity of this mutant. NA, data not available.

### Table IV

|          | Km (μM) | Vmax (pmol/h·μM) | Ki (μM) | UDP | Heparin |
|----------|---------|-----------------|--------|-----|---------|
| Wild-type| 0.9 ± 0.3| 764 ± 69        | 10.8 ± 2.6 | 0.12 ± 0.04 |
| D314G    | 1.1 ± 0.3| 744 ± 59        | 7.2 ± 1.9 | 0.15 ± 0.07 |
| D316G    | 0.9 ± 0.2| 780 ± 74        | 11.3 ± 2.8 | 0.14 ± 0.05 |
| D745G    | NA      | NA              | NA      | NA   | NA      |
| D745E    | 1.1 ± 0.4| 717 ± 53        | 1.9 ± 1.1 | 0.07 ± 0.06 |
| W746D    | 1.2 ± 0.3| 132 ± 23        | 19.6 ± 6.1 | 0.13 ± 0.03 |
| W746N    | 1.3 ± 0.2| 165 ± 34        | 17.9 ± 5.6 | 0.11 ± 0.03 |
| W746G    | 1.2 ± 0.3| 82 ± 19         | 24.6 ± 9.3 | 0.12 ± 0.04 |
| D747G    | 6.9 ± 0.7| 688 ± 94        | 17.2 ± 7.4 | 0.15 ± 0.03 |
| D747E    | 4.4 ± 0.6| 709 ± 34        | 14.7 ± 5.3 | 0.11 ± 0.08 |

Means ± S.D. are shown. The kinetic constants for mutant D745G could not be reliably determined because of the low enzyme activity of this mutant. NA, data not available.

### References

1. J. Kuhn, C. Gotting, and K. Kleesiek, personal communication.
**TABLE IV**  
Effect of UDP, heparin, and protamine on the XT-I activity of DXD mutants

| UDP (control 0 μm = 100%) | Heparin (control 0 IU/ml = 100%) | Protamine (control 0 μg/ml = 100%), 100 μg/ml |
|--------------------------|---------------------------------|------------------------------------------|
|                          | 0.5 μm | 2.5 μm | 0.9 IU/ml | 9 IU/ml | 90 IU/ml | %                                      |
| Wild-type                | 1.6 ± 0.4 | 0.4 ± 0.1 | 5.5 ± 1.2 | 6.6 ± 0.3 | 3.8 ± 0.3 | 135.2 ± 15.8 |
| D314G                    | 2.1 ± 0.6 | 0.4 ± 0.1 | 5.8 ± 0.6 | 4.8 ± 0.8 | 3.1 ± 0.4 | 118.6 ± 19.7 |
| D316G                    | 2.3 ± 1.0 | 0.4 ± 0.2 | 6.1 ± 1.8 | 5.8 ± 0.5 | 4.2 ± 1.2 | 127.3 ± 11.4 |
| D745G                    | NA      | NA      | 1.6 ± 0.1 | 0.9 ± 0.1 | 0.5 ± 0.2 | 132.9 ± 6.9 |
| D747E                    | 2.2 ± 0.6 | 0.6 ± 0.2 | 1.9 ± 0.9 | 0.9 ± 0.2 | 0.9 ± 0.4 | 133.1 ± 12.3 |
| W746D                    | 4.9 ± 0.6 | 0.5 ± 0.1 | 0.6 ± 0.1 | 0.5 ± 0.2 | 0.5 ± 0.3 | 125.2 ± 8.8 |
| W746N                    | 4.1 ± 0.7 | 0.6 ± 0.4 | 2.4 ± 0.6 | 0.5 ± 0.2 | 2.2 ± 0.8 | 113.3 ± 17.3 |
| W746G                    | 6.2 ± 1.1 | 0.4 ± 0.3 | 1.0 ± 0.3 | 0.9 ± 0.1 | 0.5 ± 0.2 | 113.7 ± 11.9 |
| D747G                    | 2.3 ± 0.6 | 0.3 ± 0.1 | 3.7 ± 0.4 | 3.8 ± 0.8 | 2.7 ± 0.4 | 126.2 ± 19.1 |
| D747E                    | 3.5 ± 0.3 | 0.4 ± 0.2 | 6.4 ± 1.3 | 4.7 ± 0.9 | 2.5 ± 0.5 | 126.0 ± 15.6 |

* p < 0.05.

**Fig. 3.** Binding of human rXT-I and XT-I mutants D745G and D747G to UDP-beads. UDP-beads were incubated with the samples, centrifuged, washed, boiled in sample buffer, and electrophoresed as described under "Experimental Procedures." A, detection of rXT-I bound to UDP-beads by immunoblotting using horseradish peroxidase-coupled anti-V5 antibodies. Lanes M, multicolor prestained molecular mass standard; lanes 1–4, incubation in the presence of 5 mM MnCl₂, 25 mM UDP, 5 mM EDTA, or a combination thereof; lane 5, untreated control sample. A specific single band was detected in all experiments. B, binding of rXT-I, D745G, and D747G to UDP-beads and detection of bound enzyme by alkaline phosphatase-coupled anti-V5 antibodies. Lanes 1–4, incubation in the presence of 5 mM MnCl₂, 25 mM UDP, 5 mM EDTA, or a combination thereof; lanes C, untreated control sample.

**Discussion**

XT-I belongs to the large group of glycosyltransferases involved in the biosynthesis of a large variety of complex oligosaccharides. The clarification of the structure and function of these enzymes is of great importance because of their increasing implications in many pathological processes and their potential as pharmacological target molecules. XT-I is the initial and committing step enzyme in the biosynthesis of the glycosaminoglycan chains in chondroitin sulfate and heparan sulfate proteoglycans. Proteoglycans are biologically important molecules that are proving to be involved in many central physiological and pathological processes. For example, proteoglycans are involved in cell entry strategies of pathogens (37–41), tumor growth (42, 43), and formation of the glial scar after spinal cord injury (44, 45). In all of these processes, inhibition of glycosaminoglycan biosynthesis or degradation of proteoglycans can be a potential as pharmacological target molecules.

**Table IV** shows the relative XT-I activity of the DXD mutants in the presence of different concentrations of UDP, heparin, and protamine. The inhibition constants of heparin and protamine were determined for each mutant. The inhibition of the wild-type enzyme by heparin was 19% at 0.9 IU/ml, while the inhibition of the D747G mutant was only 1.8% at the same concentration. The inhibition of the wild-type enzyme by protamine was 20% at 90 IU/ml, while the inhibition of the D747G mutant was only 2.2% at the same concentration.

The data suggest that the DXD motifs affect the inhibitory effects of heparin. rXT-I was strongly inhibited by coagulatory active heparin, and the kinetic study revealed that this inhibition was predominantly of the noncompetitive type, with an apparent Kᵢ of 0.12 μM (Table III). As shown in Table IV, heparin inhibited the enzyme activity of all mutants at concentrations ranging from 0.9 to 90 IU/ml. No significant differences between any of the mutants and wild-type rXT-I were observed. The inhibition constants were measured to be 0.07–0.15 μM. However, the effect of heparin on mutant D745G could not be determined because of the low enzyme activity of this mutant. The binding of rXT-I and the DXD mutants to heparin was investigated using heparin-agarose beads. Heparin-beads were incubated with the crude enzyme preparations, and the remaining enzyme activity in the supernatant was determined. Proper controls, as described under "Experimental Procedures," were performed to ensure that no heparin released from the beads during the incubation period led to false negative results. The efficient binding of rXT-I to the heparin-coated beads was shown by Western blot analysis using anti-V5 antibodies (Fig. 4). The maximum binding for the wild-type enzyme was 74% of the input enzyme. All rXT-I mutants efficiently bound to the heparin-beads, and no significant difference between the binding properties of each mutant was observed (Fig. 4).

**Fig. 3.** Binding of human rXT-I and XT-I mutants D745G and D747G to UDP-beads. UDP-beads were incubated with the samples, centrifuged, washed, boiled in sample buffer, and electrophoresed as described under "Experimental Procedures." A, detection of rXT-I bound to UDP-beads by immunoblotting using horseradish peroxidase-coupled anti-V5 antibodies. Lanes M, multicolor prestained molecular mass standard; lanes 1–4, incubation in the presence of 5 mM MnCl₂, 25 mM UDP, 5 mM EDTA, or a combination thereof; lane 5, untreated control sample. A specific single band was detected in all experiments. B, binding of rXT-I, D745G, and D747G to UDP-beads and detection of bound enzyme by alkaline phosphatase-coupled anti-V5 antibodies. Lanes 1–4, incubation in the presence of 5 mM MnCl₂, 25 mM UDP, 5 mM EDTA, or a combination thereof; lanes C, untreated control sample.
DXD Motif Analysis of Human Xylosyltransferase I

The xylosyltransferase family comprises only two members, XT-I and XT-II, which are both type II transmembrane proteins (Fig. 1) (18) containing one or two DXD motifs and which have been recently cloned by us (18). The two DXD motifs of XT-I are localized in the central and C-terminal regions of the enzyme. Hydrophobic cluster analysis confirmed the hydrophobic character of the amino acids surrounding both DXD motifs (18) (data not shown). Alignment of the XT-I amino acid sequences from a variety of species revealed that the 745DWD747 motif is evolutionarily more conserved than the 314DED316 motif, which is not present in the insect orthologs (Fig. 1). The C-terminal DXD motif is highly conserved, with only small variations in the xylosyltransferase proteins from Drosophila (DFD) and Anopheles (EYD). These findings indicate the importance of the latter motif for the proper function of XT-I.

Alterations of the two DXD motifs of human XT-I and the subsequent analysis of the rXT-I mutants support this conclusion, as alterations of the 314DED316 motif did not affect enzyme activity (Fig. 2). On the other hand, mutations of the 745DWD747 motif resulted in reduced XT-I activity. Mutation of the highly conserved aspartic acid at position 745 to glycine had the greatest effect on activity, as it was reduced by 95% compared with the wild-type enzyme. However, changing this aspartic acid to glutamic acid, an amino acid with similar physical properties, restored full enzyme activity. Glutamic acid is also found in the Anopheles xylosyltransferase ortholog (EYD) and in the C-terminal DXD motif from rat XT-II (18). This leads us to suggest that an amino acid with acidic properties is a prerequisite for the proper function of this xylosyltransferase motif. However, future extensive studies with additional mutants are necessary to elucidate the importance of Asp745 for enzyme function. The 745DWD747 motif of the xylosyltransferases is a rather unique DXD motif within the glycosyltransferases families, as none of the other families investigated so far has an aromatic amino acid at the central position of the consensual motif (19, 20). Therefore, we investigated the importance of the tryptophan at position 746 for XT-I activity.

Surprisingly, this tryptophan residue is also important for XT-I activity, as alterations of this aromatic residue to neutral, basic, or acidic amino acids resulted in a 60% reduction of enzyme activity compared with the wild-type enzyme. The kinetic analysis revealed that in contrast to the other mutants investigated in this study, Trp746 alterations resulted in a reduced Vmax value, with a Km similar to that of the wild-type enzyme. These results show that the tryptophan is important for enzyme activity, possibly being necessary for the structural orientation of the adjacent acidic residues. The 40% remaining enzyme activity after tryptophan replacement indicates that this aromatic amino acid is not part of the active-site mechanism, but appears to be necessary for the efficiency of the catalytic mechanism of core protein xylosylation. This importance of this residue is supported by the phylogenetic XT-I alignment demonstrating that this central tryptophan residue is highly conserved among humans, monkeys, rodents, and fish, giving an indication of its importance for XT-I. Furthermore, the significance of the 745DWD747 motif for XT-I activity is also supported by the successful inhibition of enzyme activity by polyclonal antibodies directed against the human DWD motif.

UDP is a strong competitive inhibitor of many glycosyltransferases, including XT-I. At a low concentration of 0.5 mM, XT-I activity was reduced by >95% in comparison with controls. The kinetics of inhibition of rXT-I-(Δ1–148)-V5-His by UDP was comparable with that of native human XT-I isolated from the culture supernatant of JAR choriocarcinoma cells. Inhibition by UDP was not significantly altered in the mutants with the invariant aspartic residue replaced with another amino acid (Table IV). The kinetic study revealed that UDP inhibition was of the competitive type, and comparable Kd values were obtained for the wild-type enzyme, the mutants with alterations of the N-terminal DXD motif, and those with modifications at position 747 (Table III). Interestingly, an increased sensitivity to UDP was noted for D745E, the mutant with Kd and Vmax values comparable with those of the wild-type enzyme. These results lead us to suggest that an acidic amino acid at position 745 is sufficient for binding of UDP-xylene and to preserve enzyme activity, although the glutamic acid at position 745 induces changes in the three-dimensional structure of the substrate-binding pocket that lead to elevated sensitivity to UDP. Consequently, a resolved crystal structure of XT-I is required to identify the intramolecular rearrangements in D745E and the mechanisms underlying this increased competitive effect of UDP. Mutants W746D, W746N, and W746G exhibited a slightly reduced response to the nucleotide UDP, although the significance of these results has to be clarified in further studies. The binding of rXT-I-(Δ1–148)-V5-His and XT-I mutants D745G and D747G to UDP-beads was divalent ion-dependent and was inhibited by EDTA and the soluble competitor UDP. No difference in the binding properties of the wild-type and mutant enzymes was observed, taking into consideration that the binding assay used allows only semiquantitative or qualitative analysis of UDP binding. The glycosyltransferase DXD motif was proposed to be involved in the coordination of a divalent metal ion that is required for the binding of the nucleotide-sugar (21). However, mutations of the DXD motif in some glycosyltransferases, such as the Fringe signaling molecule, did not alter the photolabeling by a UDP analog or the binding of UDP, but affected the enzyme activity (25–27). These findings raise the possibility that not all DXD motifs are functionally equivalent. The results of our in vitro studies using XT-I mutants show that the 745DWD747 motif of human

3 J. Kuhn, personal communication.
XT-I is also important for enzyme activity, but alterations in the amino acid sequence of this motif do not abolish the divalent cation-dependent binding of UDP. Although it is still possible that manganese coordination of UDP may contribute to the binding of UDP-xylene in XT-I, this contribution is not critical for binding of the nucleotide.

Promotase has been shown to serve as an activator of enzyme activity in glycosyltransferases and sulfotransferases (50–52). We have shown in previous studies that these arginine-rich proteins interact with XT-I secreted by JAR choriocarcinoma cells and that promotase chloride affinity chromatography can be employed to purify this enzyme (17). Therefore, we investigated the effect of promotase sulfate on the enzyme activity of the XT-I mutants. The addition of promotase sulfate at a concentration of 100 μg/ml resulted in an increase in XT-I activity of ~25%, with no significant differences between each of the mutants. This indicates that neither DxD motif is directly involved in the response of XT-I to promotase, although the molecular mechanisms for this property are not understood.

We have also shown previously that heparin is a potent inhibitor of XT-I and that the enzyme strongly binds to heparin during affinity chromatography (17, 18, 28). Here, we have demonstrated that coagulopathy active heparin strongly inhibited the residual activity of all XT-I mutants, even at low concentrations. The kinetic analysis has shown that heparin is a predominantly noncompetitive inhibitor of XT-I, with apparent inhibition constants ranging from 0.07 to 0.15 μM. No significant differences between any of the individual mutants were observed. The binding experiments performed revealed that all mutants were efficiently bound to immobilized heparin, with no significantly different binding properties. These results indicate that the DxD motifs are not directly involved in heparin binding and that the alterations do not induce misfolding of the heparin-binding site. This interpretation is in concordance with our results from a previous study, in which we showed that heparin is a predominantly noncompetitive inhibitor of native human XT-I (53). Further experiments using different XT-I mutants and probably also the crystal structure are necessary to localize the exact heparin-binding site of human XT-I.

Human XT-I seems to differ from the other mammalian glycosyltransferases, as it is a very large glycosyltransferase that is secreted mainly into the extracellular matrix (12), whereas the other glycosyltransferases are retained mostly in the Golgi apparatus. These unique properties suggest that human XT-I might possess additional functions, including a potential regulatory role in glycosaminoglycan biosynthesis. However, the biochemical mechanisms of the underlying secretion process and the biological role of XT-I in the extracellular matrix are not yet understood and have to be elucidated in the future. In this study, we have shown that the C-terminal DWD motif is required for enzyme activity, but is not exclusively necessary for UDP binding, promotase activation, or heparin inhibition. However, only a high resolution crystal structure of human XT-I will shed light on the detailed catalytic mechanism and the importance of the xylosyltransferase DDX motifs. This will also help in the design of specific XT-I inhibitors that might be, when locally administered, useful for the direct inhibition of proteoglycan biosynthesis, e.g. after spinal cord injury. This prevention of glial scar formation might be a successful approach to promote axon regeneration without the use of immunogenic bacterial chondroitinases.

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