Human UDP-\(\beta\)-xylose:proteoglycan core protein \(\beta\)-\(\beta\)-xylosyltransferase (EC 2.4.2.26, XT) initiates the biosynthesis of glycosaminoglycan lateral chains in proteoglycans by transfer of xylose from UDP-xylose to specific serine residues of the core protein. In this study, we report the first isolation of the XT and present the first partial amino acid sequence of this enzyme. We purified XT 4,700-fold with 1\% yield from serum-free JAR choriocarcinoma cell culture supernatant. The isolation procedure included a combination of ammonium sulfate precipitation, heparin affinity chromatography, ion exchange chromatography, and protamine affinity chromatography. Among other proteins an unknown protein was detected by matrix-assisted laser desorption ionization mass spectrometry-time of flight analysis in the purified sample. The molecular mass of this protein was determined as 120 kDa by SDS-polyacrylamide gel electrophoresis. The isolated protein was enzymatically cleaved by trypsin and endoproteinase Lys-C. Eleven peptide fragments were sequenced by Edman degradation. Searches with the amino acid sequences in protein and EST data bases showed no homology to known sequences. XT was enriched by immunoaffinity chromatography with an immobilized antibody against a synthetic peptide deduced from the sequenced peptide fragments and was specifically eluted with the antigen. In addition, XT was purified alternatively with an aprostin affinity chromatography and was detected by Western blot analysis in the enzyme-containing fraction.

Proteoglycans are the major components of the extracellular matrices and are present in a variety of cell and basement membranes. Proteoglycans have different functions. They are essential for maintaining the structural integrity of connective tissue, are involved in cell adhesion and motility, in cell differentiation and morphogenesis, and may also be responsible, in part, for the nonthrombogenic properties of the vascular endothelium. Both heparan sulfate proteoglycans, present in the membrane of endothelial cells, and thrombomodulin, a chondroitin sulfate-containing proteoglycan, have been implicated in procoagulant and anticoagulant mechanisms (1, 2).

UDP-\(\beta\)-xylose:proteoglycan core protein \(\beta\)-\(\beta\)-xylosyltransferase (EC 2.4.2.26, XT)\(^1\) initiates the biosynthesis of glycosaminoglycan lateral chains in proteoglycans by the transfer of xylose from UDP-xylose to specific serine residues of the core protein (3, 4). Only selected serine residues of the core protein were recognized by XT. Several comparisons of amino acid sequences of known glycosaminoglycan attachment sites in different proteins resulted in a recognition sequence composed of the amino acids a-a-a-a-G-S-G-a-b-a, with a = E or D and b = G, E, or D (5). The highest sensitivity for XT activity in a radiochemical test was reached using a protein acceptor containing this recognition sequence. This was confirmed by the determination of Michaelis-Menten (\(K_m\)) constants for \(in\ vivo\) xylosylation of different proteins and synthetic peptides in comparison to silk, which was formerly used. The use of recombinant bikunin, which contains the recognition sequence as acceptor, enables an accurate and precise determination of XT activity even in serum. Bikunin is the inhibitory component of the human inter-\(\alpha\)-trypsin inhibitor and a natural core protein which is quantitatively modified by one chondroitin sulfate chain.

In contrast to almost all other glycosyltransferases the XT is secreted into the extracellular space (6). The addition of the glycosaminoglycans to the core protein in proteoglycans occurs \(in\ vivo\) in the endoplasmic reticulum or Golgi apparatus (7–9).

We found highly increased XT activities in synovial fluids of patients with chronic joint diseases (10). The elevation of enzyme activity in synovial fluids indicates the increased synthesis of proteoglycans in inflammatory processes. Increased XT activities were also determined in the serum of patients with systemic sclerosis, closely related to an elevated proteoglycan biosynthesis. These results demonstrate the validity of XT as a diagnostic marker for the determination of sclerotic activity in systemic sclerosis (11, 12).

Isolation of XT has been hampered by difficulties in obtaining a sufficient amount of the source materials. Therefore, we developed a method to produce a protein solution containing high XT activity but low protein concentration by serum-free cultivation of JAR choriocarcinoma cells using hollow fiber bioreactor technology. Using this material we isolated an unknown protein in trace amounts and sequenced 11 peptides from the enzymatically cleaved protein. The amino acid se-

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\(^{1}\) The abbreviations used are: XT, xylosyltransferase; HPLC, high performance liquid chromatography; MOPS, 3-(N-morpholino)propane-sulfonic acid; MALDI-TOF mass spectrometry, matrix-assisted laser desorption/ionization time of flight mass spectrometry; EST, expressed sequence tag; PAGE, polyacrylamide gel electrophoresis; bis-tris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxymethyl)propane-1,3-diol; MES, 4-morpholineethanesulfonic acid.

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Isolation of Human Xylosyltransferase

**Synthesis of the Protamine Affinity Matrix**

Protamine chloride was immobilized as ligand on POROS 20 AL. 30 mg of ligand was dissolved in 10 ml of 10 mM phosphate, 0.15 M NaCl, pH 7.4. After the protein had been dissolved, 5 ml of 100 mM phosphate, 1.50 M NaCl, pH 7.4, was added. NaCNBH$_3$ was dissolved in the ligand/buffer solution to a final concentration of 5 mg/ml and 1.0 g of POROS 20 AL was suspended in the same solution. The suspension was mixed gently on a shaker for 1 min at room temperature. An additional 2 ml of 100 mM phosphate, 1.50 M NaCl, pH 7.4, was added to the suspension and the mixture was shaken continuously. This step was repeated every 5 min until the mixture volume was 25 ml. After additional shaking for 2 h, the medium was filtered and a sintered glass funnel was used to filter 20 ml of 0.2 M Tri-HCl, 5 g/liter NaCNBH$_3$, pH 7.2, and mixed gently on a shaker for 30 min at room temperature. After the media had been washed in a sintered glass funnel using 100 ml of 10 mM phosphate, pH 7.4, 100 ml of 1.0 M NaCl, and another 100 ml of 10 mM phosphate, pH 7.4, the matrix was packed in a PEEK column (4.6 × 50 mm).

**Purification of Xylosyltransferase from JAR Cell Culture Supernatant**

Fractionated ammonium sulfate precipitation and chromatography steps were performed at room temperature, ultrafiltration and diafiltration were carried out at 4 °C. 18.5 liters of JAR cell culture supernatant was obtained from three hybrid hollow-fiber bioreactors. TECNOMOUSE, each containing 5 culture dishes, was concentrated to 800 ml with ultrafiltration using YM1 cellulose membranes. The reagent was centrifuged at 4,000 × g for 1 h. The supernatant was decanted, and the pellets discarded.

Step 1: Fractionated Ammonium Sulfate Precipitation—Solid ammonium sulfate was added to the supernatant to 28% saturation. After 1 h at room temperature the suspension was centrifuged at 4,000 × g for 2 h, the supernatant was decanted, and the precipitate was removed. Additional ammonium sulfate was added to the solution to the point of 40% saturation, and the suspension was allowed to stand for 1 h. To recover the precipitate the supernatant was decanted after the suspension was centrifuged at 4,000 × g for 2 h. Before chromatography on immobilized heparin the precipitate was dissolved in 460 ml of buffer A (20 mM sodium acetate, pH 6.0).

Step 2: Heparin Affinity Chromatography on POROS 20 HE2—The step 1 product was passed through a 0.2-μm filter. 4.0 ml of the filtrate was applied to a POROS 20 HE2 column (16 × 100 mm) equilibrated with buffer A at a flow rate of 40 ml/min. After washing the column with 100 ml of buffer A the XT activity was eluted with the same buffer containing 0.1 M NaCl. The NaCl concentration was increased stepwise to 20 ml of buffer A, 0.09 M NaCl; 20 ml of buffer A, 0.15 M NaCl; 30 ml of buffer A, 0.24 M NaCl; 24 ml of buffer A, 0.30 M NaCl; 24 ml of buffer A, 0.60 M NaCl; 24 ml of buffer A, 1.00 M NaCl; and 24 ml of buffer A, 1.89 M NaCl. Fractions of 38 ml each were collected and the XT activity was measured. The procedure was repeated 115 times by cyclic chromatography and the fractions containing XT activity (115 × 38 ml) were collected.

Step 3: Ion Exchange Chromatography on POROS 20 HQ—Collected fractions from step 2 were desalted using diafiltration with YM1 cellulose membranes and ultrafiltration cells. After concentration of the desalted protein solution to 0.05 liter using analogous techniques the XT-enriched solution was subjected to ion exchange chromatography. 4.0 ml of the XT solution was applied onto the POROS 20 HQ column (16 × 100 mm) previously equilibrated with buffer A at a flow rate of 40 ml/min. The column was washed with 80 ml of buffer A, and the adsorbed protein was eluted stepwise using the same buffer containing 0.07 M NaCl (88 ml), 0.18 M NaCl (120 ml), and 0.36 M NaCl (120 ml) followed by a linear gradient of 0.36–1.00 M NaCl (200 ml) and another step of buffer A, 2.0 M NaCl (120 ml). 50-m fractions were collected and assayed for activity and evaluated by SDS-PAGE. Chromatography was repeated 13 times, and the fractions exhibiting XT activity (13 × 50 ml) were collected for affinity chromatography.

Step 4: Affinity Chromatography on Protamine Chloride—XT-containing solution from step 3 was desalted as described above and concentrated to 5 ml by ultrafiltration with YM1 cellulose membranes. The ultrafiltration product was passed through a 0.2-μm filter. 100 μl of the filtrate was loaded onto a protamine chloride-POROS column (4.6 × 50 mm) equilibrated with buffer A. The flow rate was 10 ml/min. The column was washed with 10.0 ml of buffer A, and the adsorbed fraction was eluted with the same buffer containing NaCl by a stepwise increase of the NaCl concentration: 6.6 ml of buffer A, 0.04 M NaCl; 6.6 ml of buffer A, 0.06 M NaCl; 6.6 ml of buffer A, 0.23 M NaCl followed by a linear gradient of 0.23–1.20 M NaCl (4.2 ml) in buffer A. Fractions of 6.0...
ml were collected, assayed for XT activity, and evaluated by SDS-PAGE. Cyclic chromatography was repeated 50 times. The purified enzyme was collected, concentrated to 1.0 ml using ultrafiltration techniques and stored at –75 °C.  

Solvent 5: SDS-PAGE Buffer—The protein composition of various fractions was estimated by SDS-PAGE. Briefly, 12.1 μl of sample was added to 4.7 μl of sample buffer (1.0 mM Tris-HCl, 1.17 mM sucrose, 0.28 mM SDS, 2.08 mM EDTA, 0.88 mM Serva Blue G250, 0.70 mM phenol red, 0.10 mM dithiothreitol, pH 8.5) and heated for 10 min at 99 °C. After the sample had been loaded, SDS-polyacrylamide gel electrophoresis was carried out on a 4–12% bis-tris polyacrylamide gel with MOPS running buffer (1.0 mM Tris, 6.0 mM SDS, 20.5 mM EDTA, pH 7.1). Protein bands were detected by Coomassie Brilliant Blue or silver staining. The Coomassie bands were excised and characterized by MALDI mass spectrometry and amino acid sequence analysis.

MALDI Mass Spectrometry

Coomassie-stained proteins were excised from the gel, repeatedly washed with H2O and H2O/acetonitrile, and digested overnight with trypsin and endoproteinase Lys-C at 37 °C. The peptides generated in the supernatant were analyzed by MALDI mass spectrometry. Sample preparation was achieved following the thin film preparation techniques (13). Briefly, aliquots of 0.3 μl of a nitrocellulose containing saturated solution of a-cyano-4-hydroxycinnamic acid in acetone were deposited onto individual spots on the target. Subsequently, 0.8 μl of 10% formic acid and 0.4 μl of the digest sample was loaded on top of the thin film spots and allowed to dry slowly at ambient temperature. To remove salts from the digestion buffer the spots were washed with 10% formic acid and with H2O.

MALDI mass spectra were recorded in the positive ion mode with delayed extraction on a Reflex II time-of-flight instrument equipped with a SCOUT multiprobe inlet and a 337-nm nitrogen laser. Ion acceleration voltage was set to 20.0 kV, the reflector voltage was set to 21.5 kV and the first extraction plate was set to 15.4 kV. Mass spectra were obtained by averaging 50–200 individual laser shots. Calibration of the spectra was performed internally by a two-point linear fit using the autolysis products of trypsin at m/z 842.50 and 2211.10.

Amino Acid Sequence Analysis of XT

The 120-kDa Coomassie-stained protein was excised from the gel, repeatedly washed with H2O and H2O/acetonitrile, and digested with trypsin and endoproteinase Lys-C overnight. For HPLC separation the excised gel fragment was extracted twice with 0.1% trifluoroacetic acid, 60% acetonitrile. The extracted enzymatic fragments were separated on a reversed-phase column (PE Biosystems), Acurate splitter (LC-Packings), UV absorbance at 214 nm and individual fractions were collected, assayed for XT activity, and evaluated by SDS-PAGE. Cyclic chromatography was repeated 50 times. The purified XT-containing fractions eluted from the heparin affinity matrix were desalted using diafiltration with YM1-cellulose membranes and passed through a 2-μm filter. 0.4 ml of filtrate was applied at 10 ml/min to the antigen column previously equilibrated with buffer B (50 mM Tris-HCl, pH 8.0). After the column was washed with 4.1 ml of buffer B and with 12.5 ml of buffer B, 0.15 mM NaCl, the adsorbed protein was eluted using 12.4 ml of 50 mM sodium citrate, 0.15 mM NaCl, pH 3.0, followed by 3.4 ml of 100 mM sodium citrate, 1.5 mM NaCl, pH 3.0. The eluate was collected as 10-ml fractions in tubes containing 2 ml of 0.5 M Tris-HCl, pH 8.0, to immediately neutralize the citric acid.

Preparation of Immunoaffinity Column

Purified antibody was concentrated to a protein concentration of 0.3 mg/ml using ultrafiltration with YM1 cellulose membranes. The antibody solution was adjusted to 10 mM phosphate, 0.15 mM NaCl, pH 7.4. After filtration of the solution through a 0.2-μm filter 100 μl of filtrate was applied at 0.2 ml/min to a POROS 20 PA column (2.1 × 30 mm). This step was repeated 17 times.

Adsorbed antibody was cross-linked using cross-linking solution (100 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, pH 8.5). After the column was washed with 5 ml of buffer C (10 mM phosphate, 0.15 mM NaCl, pH 7.4) 2 ml of cross-linking solution was applied at 0.5 ml/min onto the cartridge. The procedure was repeated 6 more times, using a total volume of 14 ml of cross-linking solution. To block unreacted functional groups on the cross-linking reagents 2 ml of 100 mM monoethanolamine, pH 9.0 (quenching solution), was loaded onto the cartridge at 0.5 ml/min. The column was washed with 0.2 ml of water, the cross-linker was removed using another 2-ml quenching solution. The immunoaffinity column was cycled between buffer C and 12 mM HCl, 0.15 mM NaCl 3 times using a total volume of 12 ml of solution.

Immunofinity Column Purification of XT

XT-containing fractions eluted from the heparin affinity matrix were desalted using diafiltration with YM1-cellulose membranes and passed through a 2-μm filter. 100 μl of this XT sample was applied to the immunoaffinity column equilibrated with buffer D (20 mM Tris-HCl, pH 8.0) at a flow rate of 1 ml/min. The column was washed with 1.4 ml of buffer D and with 8.5 ml of buffer D, 0.15 mM NaCl. The XT activity was eluted with 4.2 ml of 12 mM HCl followed by 1.2 ml of 12 mM HCl, 1.5 mM NaCl. Alternatively the elution was performed using 100 μl of antigen at 1.0 ml/min in buffer D. Fractions (1 ml) were collected into tubes containing 1 ml of 0.1 M Tris-HCl, pH 8.0. The XT activities of the fractions were determined.

Aprotinin Affinity Chromatography

200 μl of desalted XT solution from the heparin purification step was applied at 10 ml/min to the aprotinin column previously equilibrated with buffer A. After washing the column with 8.6 ml of buffer A the adsorbed protein was eluted stepwise using the same buffer containing 0.3 mM NaCl (10.0 ml), 0.54 mM NaCl (10.0 ml), 1.00 mM NaCl (10.0 ml), and 1.50 mM NaCl (2.4 ml). Fractions of 5 ml were collected and assayed for XT activity.

Western Blot Analysis

For Western blot analysis, proteins were transferred to polyvinylidene difluoride membrane in a semi-dry instrument (Novex). After of the above antigen followed by booster injections at 3-week intervals, 4 times in total, into Charles rabbits.

Preparation of Solid-phase Antigen

The antigen CSRQKELLLKRKLLEQKEK was immobilized on POROS 20 EP. After 1.6 mg of antigen was dissolved in 12 ml of 10 mM phosphate, 0.15 mM NaCl, pH 7.4, 0.60 ml of 100 mM phosphate, 1.50 mM NaCl, pH 7.4, was added. 400 μg of POROS 20 EP was suspended in the solution and the suspension was mixed gently on a shaker at room temperature. At 10-min intervals, five times in total, an additional 0.24 ml of 100 mM phosphate, 1.50 mM NaCl, pH 7.4, was added to the suspension. After additional shaking for 5 days at room temperature the suspension was filtered on a sintered glass funnel. The matrix was suspended in 4 ml of 0.2 mM phosphate, 0.1 mM 2-mercaptoethanol, pH 7.4, and mixed on a shaker for 2 h at room temperature. The matrix was washed in a sintered glass funnel using 20 ml of 10 mM phosphate, 0.15 mM NaCl, pH 7.4, and 20 ml of 1.0 mM NaCl. After additional washing with 20 ml of 10 mM phosphate, pH 7.4, the matrix was packed in a PEEK column (4.6 × 50 mm).
transfer nonspecific antibody-binding sites were blocked with 2% bovine serum albumin in 0.1 M Tris-HCl, pH 7.2, for 1 h at room temperature. The membrane was incubated with antiserum in 50 mM phosphate, 0.15 M NaCl, 0.5 M/liter Tween 20, pH 7.4, at 1:1000 dilution for 1 h. Bound antibody was detected using a second anti-rabbit goat immunoglobulin coupled to horseradish peroxidase at a 1:1000 dilution. The blot was developed using 4-chloro-1-naphthol.

### Gel Filtration Chromatography

A sample of 100 μl from the heparin purification step was applied at 1.0 ml/min to a TSK G3000 SW column (30 cm × 7.5 mm, 10-μm particle size) which had previously been equilibrated with buffer A, 0.15 M NaCl. Proteins were eluted with the same buffer. Fractions of 200 μl were collected and tested for XT activity. Column calibration was performed using thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

### N-Glycosidase F Digestion

Aliquots (1 μg) of XT were digested with 3, 1 × 10⁻³ units of N-glycosidase F at 37 °C for 1 and 12 h according to the method recommended by the manufacturer. The samples were then subjected to SDS-PAGE, and protein bands were detected by silver staining.

### Measurement of Protein Concentration

Protein concentration was estimated by absorbance at 280 nm assuming ε²₈₀ = 10.0 or with the bicinchoninic acid protein assay using bovine serum albumin as a standard.

### RESULTS

#### Isolation of Xylosyltransferase from Cell Culture Supernatant

18.5 liters of enriched cell culture supernatant (equivalent to 2,000 liters normal cell culture supernatant) of serum-free cultivated JAR choriocarcinoma cells was required to isolate the XT in trace amounts. The so far unknown protein with a molecular mass of 120 kDa was enzymatically digested and a partial amino acid sequence was determined by Edman degradation and MALDI-TOF mass spectrometry. A summary of the purification is shown in Table I and explained in detail below.

#### Step 1: Fractionated Ammonium Sulfate Precipitation—XT of the ammonium sulfate precipitable fraction was dissolved in 0.46 liters of buffer A with solubilization of 79.5% of the original activity.

#### Step 2: Heparin Affinity Chromatography on POROS 20 HE—4 ml of XT-enriched solution from step 1 was loaded onto the POROS 20 HE column. XT activity was completely retained on the column. More than 70% of total protein passed through the column. Contaminating protein was eluted at a low NaCl concentration. 44% of the XT activity bound to the heparin matrix emerged at 0.5 M NaCl (Fig. 1A).

#### Step 3: Ion Exchange Chromatography on POROS 20 HQ—4 ml of the desalted XT-containing fraction from step 2 was loaded onto the POROS 20 HQ column equilibrated in buffer A. More than 98% of the XT activity bound to the resin. The column was then eluted stepwise with NaCl in buffer A (Fig. 1B). XT-containing fractions were collected.

#### Step 4: Affinity Chromatography on Protamine Chloride—The product of step 3 was desalted and concentrated using dia-and ultrafiltration. 100 μl of the protein solution was applied to the POROS protamine chloride column previously equilibrated with buffer A. Approximately 95% of the transferase activity bound to the column, whereas 75% of the contaminating protein did not. Additional proteins were eluted with buffer A containing low NaCl concentrations. Enzyme activity was eluted at ~0.15 M NaCl (Fig. 1C). The enzyme activity was stable for at least 6 months at ~75 °C.

#### Step 5: SDS-PAGE—XT-containing fractions from steps 1–4 were subjected to SDS-PAGE on a 4–12% gradient polyacrylamide gel (Fig. 2, panel A). Coomassie-stained protein bands were excised and characterized by MALDI-TOF mass spectrometry after tryptic digestion. The molecular mass of an unknown protein was determined as 120 kDa (Fig. 2, panel B).

### Amino Acid Sequence Analysis of XT

The 120-kDa protein from the excised band was digested with trypsin and endoproteinase Lys-C. The proteolytic fragments were separated by reversed-phase HPLC, and selected peptides were subjected to automatic amino acid sequence analysis. Table II shows the obtained amino acid sequences.

### Immunochemical Detection of XT

Polyclonal antibodies against the synthetic peptide CS-RQKELLRKLEQQEK deduced from the peptides 2 and 10 of the enzymatically cleaved 120-kDa protein were covalently bound on POROS 20 PA. About 50% of the XT activity of an applied sample was bound (Fig. 3, panel A) when a partially purified XT sample obtained by heparin affinity chromatography (puriﬁcation step 2) was loaded onto the column. 58% of the adsorbed XT activity was eluted with 150 mM NaCl, and the rest was eluted with 12 mM HCl. Furthermore, the adsorbed XT activity was also eluted from the solid phase when 100 μl (1 mg/ml) of the synthetic peptide was added to the mobile phase (Fig. 3, panel C). When immobilized preimmune serum was used as affinity matrix (negative control) no XT activity was adsorbed to or eluted from the matrix (Fig. 3, panel B).

### Determination of the Molecular Weight of XT

100 μl of heparin affinity purified XT was separated under nonreducing and nondenaturing conditions using a TSK G3000 SW column. Two XT activity maxima were detected at 500 and 120 kDa (Fig. 5, panel A). The molecular mass of the 120-kDa protein was reduced about 3% after N-glycosidase F digestion as shown by SDS-PAGE (Fig. 5, panel B).

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**TABLE I**

**Purification of XT**

| Step                                      | Volume | Total activity | Total protein | Specific activity | Purification | Recovery |
|-------------------------------------------|--------|----------------|---------------|------------------|--------------|----------|
| JAR high density cell culture supernatant | 18,500 | 525.8 mg       | 89,355.0 mg   | 0.006            | 1            | 100      |
| Ammonium sulfate precipitation            | 460    | 426.0 mg       | 8,937.8 mg    | 0.048            | 8            | 79       |
| Heparin affinity chromatography           | 50     | 108.3 mg       | 473.0 mg      | 0.229            | 40           | 35       |
| Ion exchange chromatography               | 5      | 91.0 mg        | 43.1 mg       | 2.090            | 348          | 17       |
| Protamine affinity chromatography         | 1      | 6.83 mg        | 0.24 mg       | 28.458           | 4,743        | 1        |

Measurement of Protein Concentration

Protein concentration was estimated by absorbance at 280 nm assuming ε²₈₀ = 10.0 or with the bicinchoninic acid protein assay using bovine serum albumin as a standard.
We have purified UDP-D-xylose:proteoglycan core protein β-D-xylosyltransferase to apparent homogeneity from JAR choriocarcinoma cell culture. The isolated protein was a single-stranded polypeptide with a molecular mass of 120 kDa. The protein was enzymatically cleaved and 11 peptide fragments were sequenced by Edman degradation. Our results do not agree with previous reports on the isolation of the enzyme from embryonic chicken cartilage (15) and rat chondrosarcoma (16). No amino acid sequence data of the protein were reported.
however, a complex heteromeric tetramer structure of a 120-kDa protein was postulated.

Like other glycosyltransferases, XT is present only in very small amounts in animal tissues but unlike other glycosyltransferases, more than 90% of XT is enriched in the medium of cultured cells (11). The highest secretion of XT activity was measured in JAR choriocarcinoma cell culture as shown in our previous study (6), in which sternal cartilage chondrocytes and 21 different human cell lines were examined. To produce a highly enriched XT solution for the isolation of XT, we adapted JAR choriocarcinoma cells to hollow fiber culture conditions using a novel bioreactor (TECNO MOUSE) and Ultradroma-PF medium without serum addition as nutrient.

For purification of XT a combination of classic separation methods and new affinity matrices was employed. Previous studies have shown that heparin reduced XT activity indicating a strong interaction of heparin with the enzyme (10). Therefore, we used a heparin matrix as an affinity ligand for the XT. When applied to immobilized heparin, XT was completely adsorbed at the matrix and the XT activity was eluted only with a high salt concentration after most contaminating proteins were removed from the matrix.

Protamine chloride is well known as cationic activator for several sulfotransferases (17–20), so we investigated the effect of protamine chloride on the XT. An increased XT activity was measured when protamine chloride was added to the XT assay solution indicating an interaction of these arginine-rich proteins with XT. Therefore, we synthesized a protamine chloride affinity matrix using an aldehyde-activated perfusion medium.

**FIG. 3.** Immunoaffinity chromatography of xylosyltransferase. Panel A, 100 μl of the desalted XT-containing fractions eluted from heparin affinity matrix was applied to a column of immobilized polyclonal antibodies. After washing with buffer D, the column was eluted as indicated by the arrow with buffer D, 0.15 M NaCl (1) followed by 12 mM HCl (2). Fractions of 1 ml were collected into tubes containing 1 ml of 0.1 M Tris-HCl, pH 8.0. Protein elution was monitored at 280 nm (black line) and the XT activities (II) of each fraction were assayed.

**Panel B,** negative control of the immunoaffinity chromatography with immobilized preimmune serum. For conditions see panel A. Panel C, immunoaffinity chromatography with immobilized polyclonal antibodies. The column was eluted with buffer D containing 100 μl (1 mg/ml) of the peptide antigen indicated by the arrow. Fractions of 1 ml were collected into tubes containing 1 ml of 0.1 M Tris-HCl, pH 8.0. Protein elution was monitored at 280 nm (black line) and the XT activities (II) of each fraction were assayed.

**FIG. 4.** Aprotinin affinity chromatography of partially purified XT and immunoblot analysis of the separated fractions. Panel A, 200 μl of desalted XT fraction from the heparin purification step was applied to an aprotinin column previously equilibrated with buffer A. After washing with buffer A, the adsorbed proteins were eluted stepwise with NaCl (gray line). Protein elution was monitored at $A_{280}$ (black line) and fractions of 2 ml were assayed for XT activity (II). Panel B, aliquots of fractions 3, 7, 12, and 16 were analyzed by Western blot. The 120-kDa protein was detected in the XT-containing fractions 7 and 12. Immunological detections were performed using a polyclonal rabbit antiserum raised against the synthetic peptide CSRQKELLK-RKLEQQEK deduced from peptides 2 and 10 of the enzymatically cleaved unknown protein. Prestained molecular size standard were myosin (191 kDa), bovine serum albumin (64 kDa), and glutamic dehydrogenase (51 kDa).

For purification of XT a combination of classic separation methods and new affinity matrices was employed. Previous studies have shown that heparin reduced XT activity indicating a strong interaction of heparin with the enzyme (10). Therefore, we used a heparin matrix as an affinity ligand for the XT. When applied to immobilized heparin, XT was completely adsorbed at the matrix and the XT activity was eluted only with a high salt concentration after most contaminating proteins were removed from the matrix.

Protamine chloride is well known as cationic activator for several sulfotransferases (17–20), so we investigated the effect of protamine chloride on the XT. An increased XT activity was measured when protamine chloride was added to the XT assay solution indicating an interaction of these arginine-rich proteins with XT. Therefore, we synthesized a protamine chloride affinity matrix using an aldehyde-activated perfusion medium.

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J. Kuhn, C. Göttling, M. Schnölzer, T. Kempf, T. Brinkmann, and K. Klesiecki, unpublished observations.
weight on SDS-PAGE and gel filtration. However, nonreducing and nondenaturing gel filtration chromatography with heparin affinity-purified XT from JAR cell culture supernatant shows an additional peak of XT activity at a molecular mass of ~500 kDa. Our previous results demonstrated that the enzyme is secreted simultaneously with chondroitin sulfate proteoglycans into the extracellular space, suggesting that XT is associated with proteoglycans (11).

Treatment of XT with N-glycosidase F resulted in a decrease of the molecular mass from 120 to 116 kDa, suggesting that the XT is a glycoprotein. Most glycosyltransferases purified so far are glycoproteins, which contain up to 15% carbohydrate by weight.

In eukaryotes, most glycosyltransferases are located in the endoplasmic reticulum and Golgi membranes and require the addition of detergents for solubilization. However, glycosyltransferases were also found in body fluids as soluble enzymes, and were purified to homogeneity (22, 23). Glycosyltransferases have virtually no sequence homology to each other, although these Golgi enzymes share a common domain structure. They are all type II membrane proteins, composed of a short NH₂-terminal cytoplasmic domain, a transmembrane domain, a stem region of variable length, and a large COOH-terminal globular catalytic domain (24, 25). Several investigations suggest that soluble glycosyltransferases were formed from membrane-bound enzymes by proteolytic cleavage between the catalytic domain and the transmembrane domain (26). Most reactions catalyzed by glycosyltransferases take place in the lumen of the Golgi apparatus, but xylosylation is performed in a pre-Golgi compartment in chicken chondrocytes (4, 9).

A comparison of the molecular mass of XT with other glycosyltransferases involved in biosynthesis of proteoglycans shows that the XT is larger than the other enzymes. Another difference is that nearly all proteoglycan glycosyltransferases are tightly bound to the membrane of the endoplasmic reticulum, whereas XT is secreted into the extracellular space (11).

The size of the XT and its secretion into the extracellular space may suggest that the enzyme is involved in additional molecular processes. Phosphorylation of C-2 of xylose has been discovered in both chondroitin sulfate (27) and heparan sulfate proteoglycans (28). Xylose is generally not phosphorylated in proteoglycans (29, 30), because the addition of the first glucuronic acid residue is followed by a rapid dephosphorylation of the xylose (31). It is possible that the XT is involved in the phosphorylation or dephosphorylation process. The function of the xylose phosphorylation is not clear, but it might provide a signal for secretory transport of proteoglycans (32).

Like other glycosyltransferases, XT requires a divalent metal ion for enzyme activity. Manganese is most effective for XT activity followed by magnesium (33). Many glycosyltransferases contain a DxD motif (34–36), suggesting that this motif is involved in binding the metal-ion cofactor and the donator substrate (37). A DxD sequence was also found in peptide 8 obtained from the enzymatically cleaved XT.

In conclusion, we have determined the first partial amino acid sequence of human XT, and produced antibodies raised against this enzyme that initiates the glycosaminoglycan synthesis in proteoglycans. On the basis of this sequence, it should be possible to clone the cDNA encoding the XT using degenerated primers and polymerase chain reaction cloning strategy. Furthermore, the antibodies against the XT can be used to develop an immunological test system for the rapid and sensitive measurement of this protein as a new diagnostic tool in clinical praxis.

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