Recognition of Acceptor Proteins by UDP-β-D-xylose Proteoglycan Core Protein β-D-Xylosyltransferase*

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The formation of chondroitin sulfate is initiated by xylosyltransferase (XT) transferring xylose from UDP-xylose to consensus serine residues of proteoglycan core proteins. Our alignment of 51 amino acid sequences of chondroitin sulfate attachment sites in 19 different proteins resulted in a consensus sequence for the recognition signal of XT. The complete recognition sequence is 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. This sequence was confirmed by determination of the Michaelis-Menten constants for in vitro xylosylation of different synthetic proteins and peptides using an enriched XT preparation from conditioned cell culture supernatant of human chondrocytes.

The highest acceptor activity was determined by the sequence Q-E-E-E-E-G-S-G-G-G-Q, which was found in the single chondroitin sulfate attachment site of bikunin, the inhibitory active component of the human inter-α-trypsin inhibitor.

We determined the Michaelis-Menten constant (K_m) of xylosylation of the synthetic bikunin analogue peptide Q-E-E-E-G-S-G-G-G-Q to be 22 μM, which was 9-fold decreased in comparison to deglycosylated core protein from bovine cartilage (188 μM), which was previously used as acceptor for the XT assay assay. The best XT acceptors were nonglycosylated recombinant wild-type bikunin (K_m = 0.9 μM) and the recombinant [Val_38,Val_39]_bikunin (K_m = 0.6 μM), a variant without any inhibitory activity against serine proteinases.

These results imply that the primary structure of the acceptor is not the only determinant for recognition by xylosyltransferase. Thus, protein conformation is also a main factor in determining xylosylation.

The functions of proteoglycans are highly diversified, ranging from mechanical functions, essential for maintaining the structural integrity of connective tissue, to effects on dynamic processes such as cell adhesion and motility and also cell differentiation and morphogenesis. Proteoglycans are the major components of the extracellular matrix, and their core proteins are substituted with glycosaminoglycans 10-fold their protein mass (1).

All glycosaminoglycans, except hyaluronic acid, are secreted as components of proteoglycans, covalently linked to a core protein. Chondroitin sulfate, dermatan sulfate, heparin, and heparan sulfate chains are bound to the protein by a serum-linked xylose-galactose-galactose bridge (2). The initial, apparently rate-limiting step in the biosynthesis of glycosaminoglycans is the transfer of xylose from UDP-xylose to serine residues of the core protein catalyzed by UDP-β-D-xylose:proteoglycan core protein β-D-xylosyltransferase (EC 2.4.2.26) (XT) (3, 4). Obviously, only selected serine residues were recognized by xylosyltransferase. Available information on the structure of proteoglycan core proteins has already shown that glycosylated serine residues are usually followed by a glycine residue (5).

Further investigations based on comparison of the amino acid sequences of three chondroitin sulfate attachment sites in different proteoglycans suggested a recognition sequence S-G-X-G (X = variable amino acid) with some N-terminal acidic amino acids (6).

Here we aligned the amino acid sequences of 51 known chondroitin sulfate attachment sites in 19 different proteins to identify the complete recognition signal for XT defined by the primary protein structure.

One of these proteins was bikunin (Fig. 1), the inhibitory component of inter-α-trypsin inhibitor (ITI) (7). Bikunin carries a single chondroitin sulfate chain, which binds to the heavy chains of ITI, so this glycosaminoglycan is essential for the structure of the inhibitor.

**EXPERIMENTAL PROCEDURES**

**Materials**

UDP-[14C]xylose (9.88 kBq/nmol) was purchased from DuPont (Bad Homburg, Germany), Immobilon-AV membrane from Millipore (Eschborn, Germany), and nitrocellulose discs (inner diameter, 25 mm) from Sartorius (Göttingen, Germany). The liquid scintillation counter LS 500TD and scintillation mixture were supplied by Beckman (Fullerton, CA).

The Chang Liver cell line was obtained from ICN (Meckenheim, Germany), RNA Insta-Pure™ was from Eurogentec (Seraing, Belgium), the enzymes used for cDNA cloning were from Boehringer Mannheim (Mannheim, Germany), the vector pET 15b and the E. coli strain BL21(DE3) were from Novagen (Madison, WI), and Chelating Sepharose and Q-Sepharose were from Pharmacia (Uppsala, Sweden). Oligonucleotides were synthesized by Genosys (Cambridge, United Kingdom). Minimum essential medium, fetal calf serum, serum-free and protein-free hybridoma medium, Pronase E, collagenase XI, antibiotic/antimycotic solution, trypsin, chymotrypsin, granulocyte elastase, kalikrein, VLR-pNA, Suc-AAPF-pNA, and Suc-AAA-pNA were obtained from Sigma (Deisenhofen, Germany).

The peptides QEEGSGGQQKK, GVEGSADFLK, VCRSGSGLVGK, and PLVSSGEDEPK were synthesized by Quality Controlled Biochemicals (Hopkinton, MA). All other peptides were purchased from Bachem Biochemica (Heidelberg, Germany). Silk fibroin and hydrofluoric acid- and phenylmethylsulfonyl fluoride-degraded proteoglycan from bovine nasal septum cartilage were prepared as described previously (8). All other chemicals in pro analysis quality were purchased from Merck (Darmstadt, Germany).

*The abbreviations used are: XT, xylosyltransferase; ITI, inter-α-trypsin inhibitor; pNA, para-nitroanilide; PCR, polymerase chain reaction.

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Methods

XT Activity Assay—Varying amounts of the potential acceptors were incubated with partially purified and enriched XT solution from chondrocyte culture supernatant and UDP-[14C]xylose. The reaction mixture for the assay contained, in a total volume of 100 μL: 50 μL of XT solution, 25 mM 4-morpholineethanesulfonic acid (pH 6.5), 25 mM KCl, 5 mM KF, 5 mM MgCl₂, 5 mM MnCl₂, 1.0 μM UDP-[14C]xylose, and varying acceptor concentrations. After incubation for 1 h at 34 °C, the mixtures were placed on small discs of Immobilon-AV membrane, which immunobilizes even small peptides quantitatively by covalent links (9). After drying, the membrane discs were washed four times for 10 min with 0.1% Tween 20 in phosphate-buffered saline and measured by liquid scintillation counting. The enzyme activity was expressed in units (1 unit = 1 μmol of incorporated xylose-min⁻¹) (10).

Determination of the Acceptor Activities for Xylosylation of Different Acceptors—For a quantification of the acceptor activities of different proteins and peptides, the reaction rate of the maximal rate (Vₘₐₓ) and the Michaelis-Menten constants (Kₘ) was determined for their xylosylation. Kₘ is inversely proportional to the affinity of XT to the acceptor, and Vₘₐₓ reflects the turnover number of the enzyme; thus, Vₘₐₓ/Kₘ can be defined as acceptor activity. For calculation of Kₘ and Vₘₐₓ, the transfer rates were measured as a function of the acceptor/substrate concentrations.

The reaction mixture contained, in a total volume of 100 μL: 50 μL of XT solution, 25 mM 4-morpholineethanesulfonic acid (pH 6.5), 25 mM KCl, 5 mM KF, 5 mM MgCl₂, 5 mM MnCl₂, 1.0 μM UDP-[14C]xylose, and varying acceptor concentrations. After incubation for 1 h at 34 °C, the mixtures were placed on small discs of Immobilon-AV membrane. After drying, the membrane discs were washed four times for 10 min with 0.1% Tween 20 in phosphate-buffered saline and measured by liquid scintillation counting. The enzyme activity was expressed in units (1 unit = 1 μmol of incorporated xylose-min⁻¹) (10).

Characterization of the Inhibitory Activity of Recombinant Wild-type Bikunin and Recombinant [Val³⁶,Val³⁸]⁻¹,Gly⁹²,Ile⁹⁴−¹,bikunin—We tested the inhibitory activity of bikunin and the mutated bikunin variant against different serine proteinases. Different amounts of the inhibitors were added to the proteinases in a total volume of 150 μL. After a 30-min incubation at 37 °C, 50 μL of chromogenic substrate solutions with different concentrations were added. The optical density at 405 nm was measured periodically. The increase during the approximate linear phase was determined as a function of the substrate concentrations and served for calculation of the Michaelis-Menten constants. The Kₘ with and without inhibitor were used for calculation of the dissociation constants of the enzyme-inhibitor complex (Kᵢ).

Trypsin (19)—The end concentrations in the reaction mix were: 25 μg trypsin, 50 μg Tris/HCl (pH 7.8), 20 mM CaCl₂, 0.02% polyethylene glycol 8000, 0–1.2 μM bikunin or [Val³⁶,Val³⁸]⁻¹,Gly⁹²,Ile⁹⁴−¹,bikunin.

The nomenclature of the bikunin variant is in accordance with Refs. 46 and 47.
TABLE I

Partial amino acid sequences of chondroitin sulfate attachment sites in different proteins

The arrow over sequence indicates the glycosylated serine residue. Amino acids with a supposed importance for recognition by xylosyltransferase are boldface. CSF, colony-stimulating factor; PG, proteoglycan; MHC, major histocompatibility complex; a, acidic amino acid (E or D).

| Protein | Chondroitin sulfate attachment site |
|---------|----------------------------------|
| Aggrecan (chondrocytes) (21, 22) | ... |
| Collagen IX (chondrocytes) (23) | ... |
| CS-PG II (chondrocytes) (24) | ... |
| Versican (fibroblasts) (25) | ... |
| Betaglycan (fibroblasts) (26) | ... |
| Biglycan (fibroblasts) (27) | ... |
| Appican (fibroblasts) (28) | ... |
| Fibrinectin receptor (fibroblasts) (29) | ... |
| Vitronectin (fibroblasts) (30) | ... |
| PG 19 (fibroblasts) (31) | ... |
| Decorin (fibroblasts) (32) | ... |
| Syndeean (epithelial cells) (33, 34) | ... |
| CD 44 (epithelial cells) (35) | ... |
| Tenascin (brain) (36) | ... |
| Neurocan (brain) (37) | ... |
| CSF-1 (thrombocytes) (38) | ... |
| Platelet glycoprotein IIIa (39) | ... |
| Human MHC class II (40) | ... |
| Apolipoprotein J (hepatocytes) (41) | ... |
| Bikunin (hepatocytes) (7) | ... |

| Relative occurrence of D/E (%) | 21 10 15 |
| Relative occurrence of G (%) | 6 8 4 12 |
| Position | -8 -7 -6 -5 -4 -3 -2 -1 0 |
| Consensus sequence | a a a a a a G G a a/G a a a |

respectively, and 10, 50, 100, or 250 μM V-L-R-pNA.

Chymotrypsin (19)—The end concentrations in the reaction mix were: 25 nM chymotrypsin, 50 mM Tris/HCl, pH 7.8, 20 mM CaCl₂, 0.05% Triton X-100, 0.1–2 μM bikunin or [Val³⁶⁵,Val³⁶⁶⁺¹⁺²⁺³⁺⁴⁺⁵⁺⁶⁺⁷][Gly²⁹,He³⁸⁴⁺²]bikunin, respectively, and 10, 50, 100, or 250 μM Suc-A-A-P-F-pNA.

Cathepsin G (19)—The end concentrations in the reaction mix were:
**Table II**

| Acceptor | $K_M$ (µM) | $V_{max}/K_M$ |
|----------|------------|---------------|
| Proteins |            |               |
| [Val$^{36}$, Val$^{38}$]$^{11}$, [Gly$^{92}$, Ile$^{94}$]$^{11}$ bikunin | 0.6 | 5133 |
| Wild-type bikunin | 0.9 | 3467 |
| TFMS-degraded proteoglycan | 155 | 15 |
| HF-degraded proteoglycan | 188 | 14 |
| Silk (OSGAGA)$_{n}$ | 545 | 5.5 |
| Peptides |            |               |
| GQEESGSGGK (bikunin) | 22 | 129 |
| NFDEIRDGGFPN | 128 | 21 |
| PLVSEGGEDFPK (neurocan) | 392 | 7.2 |
| WAGGDAEG | 418 | 6.1 |
| VCRSGGLVKE (apolipoprotein J) | 943 | 2.5 |
| GVESADFLX (collagen IX) | 2,668 | 1.1 |
| KTKGGDFVY | 3,625 | 0.7 |
| KKDGGFY | 8,273 | 0.3 |
| SGG | >50,000 |
| LNFTGW | >50,000 |

25 mM cathepsin G, 50 mM Tris/HCl, pH 7.8, 0–1.2 µM bikunin or [Val$^{36}$,Val$^{38}$]$^{11}$, [Gly$^{92}$, Ile$^{94}$]$^{11}$ bikunin, respectively, and 10, 50, 100, or 250 µM Suc-A-A-P-F-pNA.

**Table III**

| Proteinase | $K_i$ (µM) | Wild-type bikunin | ITI (29) |
|------------|------------|-------------------|---------|
| Trypsin (bovine) | >10$^{-6}$ | 2.3 · 10$^{-9}$ | 2–10 · 10$^{-10}$ |
| Chymotrypsin (bovine) | >10$^{-4}$ | 6.2 · 10$^{-9}$ | 1–10 · 10$^{-9}$ |
| Cathepsin G (human) | 4.2 · 10$^{-6}$ | 4.5 · 10$^{-7}$ | 2–6000 · 10$^{-9}$ |
| Elastase (human leucocytes) | >10$^{-4}$ | 2.8 · 10$^{-6}$ | 6–15 · 10$^{-8}$ |

**RESULTS**

Sequence Homology of the Glycosylation Sites of Different Proteoglycans—Alignment of the amino acid sequences of 51 known chondroitin sulfate attachment sites in unrelated proteoglycans (Table I) showed a significant homology and revealed a consensus sequence of 10 amino acids: a-a-a-a-G-N-a-b-a, where a = E or D and b = G, E, or D.

Preparation of an XT Solution from Cell Culture Supernatant—The serum-free cell culture medium conditioned by human chondrocytes had an XT activity of about 0.8 millilitre/liter and a total protein concentration of 0.09 g/liter. Fractions from ion exchange chromatography eluted by a NaCl gradient from 0.40 to 0.45 mM NaCl showed high XT activities. These fractions were collected and desalted. The XT activity of the preparation was about 4.5 millilitres/liter; the total protein concentration was 0.03 g/liter. So the specific XT activity referring to the total protein content was increased 17-fold from 8.9 millilitres/g to 152 millilitres/g.

Acceptor Activities for the Xylosylation of Different Acceptors—$K_m$ and $V_{max}$ were determined for the xylosylation of different proteins and peptides (Table II). The relation $V_{max}/K_m$ was defined as acceptor activity. As specific consensus sequence components appeared in proximity to the serine residue, the lower was $K_m$ and the higher was the acceptor activity. Testing the tripeptide SGG and the peptide LNFTGW, which contain a serine but do not match the consensus sequence, no xylosylation was detectable.

**Inhibitory Activity of Bikunin and the Mutated Variant**—In the reactive site of the two Kunitz-type domains of bikunin the amino acid sequence Met$^{36}$, Gly$^{37}$-Met$^{38}$ in domain $\delta_1$ was changed to Val$^{36}$, Gly$^{37}$, Val$^{38}$ and in domain $\delta_2$ Arg$^{92}$-Ala$^{93}$, Phe$^{94}$ was changed to Gly$^{92}$, Ala$^{93}$-Ile$^{94}$ by site-directed mutagenesis. The calculated $K_i$ values for wild-type bikunin and the variant [Val$^{36}$, Val$^{38}$]$^{11}$, [Gly$^{92}$, Ile$^{94}$]$^{11}$ bikunin with trypsin, chymotrypsin, cathepsin G, and elastase showed that the mutated bikunin variant lacked the inhibitory activity (Table III).

**DISCUSSION**

We determined $K_m$ and $V_{max}$ for the xylosylation of different potential acceptors reflecting the affinity of XT to the acceptor and the turnover number, respectively (Table II). The acceptor activities ($V_{max}/K_m$) of the peptides confirmed the consensus sequence a-a-a-a-G-N-a-b-a revealed by comparison of chondroitin sulfate attachment sites in different proteins.

The acceptor tests showed that the minimal requirement for xylosylation was a polypeptide including serine and a C-terminally following glycine. For collagen IX alone, a chondroitin sulfate attachment site was described with alanine instead of glycine and an extent of glycosylation of about 70% (23). However, the sequence homologous peptide GVESADFLK showed only a weak acceptor activity in our test.

The acceptor activity of peptides including serine-glycine was higher, when more acidic amino acids were in the vicinity of serine, except for the positions directly adjacent to serine. In our acceptor test, acidic amino acids in the positions $-5$ to $-2$ and $+2$ to $+4$ as well as glycine residues in $-1$ and $+3$ improved the xylosylation rate by XT. The acidic amino acids were more effective, the closer their positions were to the serine.

In contrast to UDP-N-acetylgalactosamine:polypeptide N-acetylgalactosaminyltransferase, which transfers N-acetylgalactosamine to small peptides like NGT (42), XT recognizes only longer polypeptides. In our XT acceptor test, no xylosylation of the peptide SGG was observed and the acceptor activity of bikunin was 27-fold higher than that of the sequence homologous peptide with 11 amino acids.

In some core proteins, potential Ser-Gly xylosylation sites often escape xylosylation (43). We compared different proteoglycans and found that those with chondroitin sulfate chains of substantial importance for the function of the protein, e.g. human bikunin, match the consensus sequence better than proteoglycans that are naturally not quantitatively glycosylated, e.g. collagen and aggrecan.

Bikunin, the inhibitory component of the ITI, is quantitatively glycosylated by a chondroitin sulfate chain in position 10. This chain binds to the heavy chain of ITI and is essential for the structure of the inhibitor. The amino acid sequence of the chondroitin sulfate attachment site (QGEEESGGGQG$^{35}$) corresponds to the determined consensus sequence.

An additional example for quantitatively glycosylated chondroitin-4-sulfate proteoglycan is the human C1q inhibitor (C1qI) (44). The amino acid sequence of this 30-kDa circulating complement inhibitor is still unknown, but it should be expected that the amino acid sequence of the chondroitin sulfate attachment site also matches the consensus sequence.

Among the tested acceptors, recombinant [Val$^{36}$, Val$^{38}$]$^{11}$, [Gly$^{92}$, Ile$^{94}$]$^{11}$ bikunin proved to be the most effective. The mutations in the reactive sites were far from the xylosylation site and should have no substantial effect on secondary protein structure. In wild-type bikunin, the N-terminal Kunitz-type domain $\delta_1$ with the reactive site Met$^{36}$-Gly$^{37}$-Met$^{38}$ (position...
P$_1$-P$_1'$-P$_2$) (45) is a competitive inhibitor for elastase and cathepsin G; domain 82 with Arg$_{92}$Ala$_{93}$Phe$_{94}$ inhibits proteinases like trypsin and chymotrypsin. The determined dissociation constants ($K_d$) for the proteinase/inhibitor complexes showed that the mutated bikunin variant had no inhibitory activity. Thus, in the XT assay, it should not interact with proteinases contained in the partially purified enzyme.

The acceptor activity of [Val$_{36}$Val$_{38}$]$_{1}$, [Gly$_{92}$Ile$_{94}$]$_{2}$ bikunin was 24-fold higher than of the synthetic peptide QEEEGLSGGQK. These results imply that the amino acid sequence of the recognition site is not the only regulatory factor that determines the priority for glycosylation of this site. Protein conformation is another important factor.

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