Biochemical and Biophysical Research Communications 391 (2010) 685–691

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Differences in gene expression of human xylosyltransferases and determination of acceptor specificities for various proteoglycans

Christina Roch, Joachim Kuhn, Knut Kleesiek, Christian Götting *

Institut für Laboratoriums- und Transfusionsmedizin, Herz- und Diabeteszentrum NRW, Universitätsklinik der Ruhr-Universität Bochum, 32545 Bad Oeynhausen, Germany

ARTICLE INFO

Article history:
Received 14 November 2009
Available online 26 November 2009

Keywords:
Glycosaminoglycan
Kinetic analysis
Proteoglycan
Xylosyltransferase

ABSTRACT

The xylosyltransferase (XT) isoforms XT-I and XT-II initiate the posttranslational glycosaminoglycan (GAG) synthesis. Here, we determined the relative expression of both isoforms in 33 human cell lines. The majority of tested cell lines showed dominant *XYLT2* gene expression, while only in 23132/87, JAR, NCI-H510A and THP-1 was the XT-I mRNA expression higher. Nearly equal expression levels were detected in six cell lines. Additionally, to shed light on putative differences in acceptor specificities the acceptor properties of potential acceptor sequences were determined. Peptides were expressed as glutathione-*S*-transferase fusion proteins containing putative or known GAG attachment sites of *in vivo* proteoglycans. Kinetic analysis showed that K_m and V_{max} values for XT-I mediated xylosylation were slightly higher than those for XT-II, and that XT-I showed a lesser stringency concerning the acceptor sequence. Mutagenesis of the bikunin peptide sequence in the G-S-G attachment site and flanking regions generated potential acceptor molecules. Here, mutations on the N-terminal side and the attachment site were found to be more susceptible to a loss of acceptor function than mutations in the C-terminus. Altogether the known consensus sequence a-a-a-a-G-S-G-a-a/G-a ('a' representing Asp or Glu) for XT-I mediated xylosylation could be approved and additionally extended to apply to XT-II as well.

Introduction

Proteoglycans (PG) represent a large group of polyanionic molecules which are expressed in a wide range of cell types. They consist of a core protein and varying numbers of covalently attached glycosaminoglycan chains [1]. Posttranslational proteoglycan biosynthesis is initiated by transferring an UDP-xylose residue to specific serine moieties of the glycosaminoglycan attachment sites of core proteins. This rate-limiting step is catalyzed by the isoenzymes XT-I and XT-II (EC 2.4.2.26) [2–4]. The consensus sequence a-a-a-a-G-S-G-a-a/G-a ('a' = Asp or Glu) for xylosylation by XT-I is consistent or similar to the short minimal motifs G-S-G and G-S-x-G ('x' representing any amino acid) [5–7]. Other glycosyltransferases complete the assembly of a uniform tetrasaccharide linker and in consecutive steps different alternating disaccharide units are attached to the linker building up the GAG chain [8].

To date there is little knowledge about functional differences and so the reason for the coexistence of both isoenzymes in higher organisms remains unclear. One difference monitored so far has been a diversity expression pattern of both isoenzymes in mouse tissues and diverse cell lines [2,3,9,10]. To examine these findings we accomplished a relative quantification of XT-I and XT-II mRNA expression in 33 human cell lines. Additionally, we determined the acceptor properties of potential acceptor sequences.

Materials and methods

Cell culture. Cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) or the German Resource Centre for Biological Material (Braunschweig, Germany). hTERT-BJ1 was supplied by Clontech (Mountain View, CA, USA). Cells were cultivated in media supplemented with FCS and required supplements according to the manufacturer's recommendations. Cells in the 2–5 passage were harvested after a period of 4 d when they reached sub-confluence. CHO pgsA-745 cells in which XT-I or XT-II expression was restored [3,11] were cultured as described previously [3]. Supernatants were collected and concentrated 10-fold by ultracentrifugation in Vivacell 100 concentrators (Vivascience, Hanover, Germany).

Construction of plasmids. Vector constructs were generated by annealing complementary oligonucleotides (Supplemental

Abbreviations: CHO, Chinese hamster ovary; CI, confidence interval; FCS, fetal calf serum; GAG, glycosaminoglycan; GST, glutathione-*S*-transferase; IPTG, isopropyl-β-D-thiogalactosid; ND, not detectable; PBS, phosphate buffered saline; PG, proteoglycan; SD, standard deviation; WT, wild-type; XT-I, xylosyltransferase I; XT-II, xylosyltransferase II

* Corresponding author. Address: Institut für Laboratoriums- und Transfusionsmedizin, Herz- und Diabeteszentrum NRW, Universitätsklinik der Ruhr-Universität Bochum, Georgstrasse 11, 32545 Bad Oeynhausen, Germany. Fax: +49 5731 972013.
E-mail address: cgoetting@hdz-nrw.de (C. Götting).

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doi:10.1016/j.bbrc.2009.11.121

Table 1). Produced cDNA inserts were cloned into the XhoI and EcoRI digested pGEX-6P-1 vector (Amersham Biosciences, CT, USA) and ligated by T4 DNA ligase. *Escherichia coli* BL21 DE3 cells (Novagen, Darmstadt, Germany) were transformed with the cloned plasmids. Plasmids were sequenced by using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

Site-directed mutagenesis. For the procedure of codon altering the QuickChange Site-directed mutagenesis Kit (Stratagene, La Jolla, Canada) was used. A vector coding for the bikunin proteoglycan attachment site was mutated by using oligonucleotides containing wobble-bases (Supplemental Table 2). Originated DNA of the mutants was sequenced.

Expression and purification of fusion proteins. Transformed *E. coli* BL21 DE3 cells were grown at 30 °C in LB medium supplemented with 75 μg/l ampicillin. Peptide expression was induced by 0.5 mM IPTG. Cells were harvested by centrifugation and lysed in 5 ml PBS by a threefold freezing/thawing cycle. Purification of the fusion proteins was performed by affinity chromatography using the GST-Bind Kit (Novagen) according to the manufacturer's instructions. Purified protein solutions were dialyzed with the 250-fold volume of PBS. Determination of protein concentrations was performed by the Bradford method using bovine serum albumin as standard.

Radiochemical XT activity assay for determination of Michaelis–Menten constants and maximum reaction rates. Radiochemical determination of XT activity is based on a transfer of UDP-[^{14}C]-D-xylose (PerkinElmer, Waltham, MA, USA) to an acceptor protein and has been described previously [7]. Various concentrations of purified fusion protein solutions were incubated in triplicate with the concentrated supernatant of CHO pgsA-745-XT-I or CHO pgsA-745-XT-II cells under assay conditions with additional nine parts equimolar non-radioactive UDP-xylose (Sigma, Taufkirchen, Germany) for 4 h. K_m and V_{max} values were calculated from Hanes–Woolf plots.

Real-time quantitative RT-PCR analysis of XT expression. XT mRNA expression was quantified by a fluorogenic RT-PCR assay using the Realplex Mastercycler System (Eppendorf, Hamburg, Germany) and intron-spanning oligonucleotides (Supplemental Table 3). mRNA isolation and PCR reaction conditions are described elsewhere [3]. Transcriptional levels of target genes were normalized to constant mRNA levels of the housekeeping genes GAPDH, HPRT1 and β_2-microglobulin using a housekeeping-gene index and the geNorm algorithm. cDNA synthesized from total RNA of SW-1353 cells served as a calibrator to which gene expression values of other cells were compared to.

Results

Relative quantification of XT-I and XT-II mRNA expression

We analyzed the expression pattern of both xylosyltransferases in 33 human cell lines by using real-time RT-PCR to obtain further knowledge about XT expression profiles. The *XYLT1* gene was not expressed in A-431, BM-1604, CAPAN-2, HELA, HT-1080, K-562 and LNCAP (Fig. 1). Only marginal amounts of XT-I mRNA could be measured in the total mRNA of A-549, EA.hy 926, ECV-304, EFO-21, HEK-293, Hep G2, MHH-ES-1 and RPMI-2650. In comparison to these results a broad XT-II mRNA expression could be detected in every analyzed cell line. The highest xylosyltransferase expression levels were found in NCI-H510A for XT-I (4.74 ± 0.27, mean ± standard deviation (SD)) and in BT-474 for XT-II (6.29 ± 1.18). XT-I expression was higher than XT-II expression in 23132/87, JAR, NCI-H510A and THP-1. Nearly equal expression levels were determined in 1301, Hs 27, SAOS-2, SW-1353, WERI-RB-1 and Y-79. In every other analyzed cell line the XT-II expression was dominant.

Determination of acceptor properties of fusion proteins containing xylose attachment sites of in vivo acceptor proteins

To date only some kinetic data of limited numbers of sequences like bikunin, L-APP or bFGF are accessible [12–14]. To investigate acceptor specificities of XT isoforms we determined acceptor properties of a panel of fusion proteins composed of glutathione-*S*-transferase (GST) coupled with sequences containing known or putative GAG attachment sites of proteoglycans. We subdivided these proteoglycan sequences in dependency of their number of G-S-G-motifs into three groups (Table 1). All fusion proteins with one G-S-G-motif, as combined in group 1, bore good acceptor functionalities for both XT isoforms. Confinements of this observation were aggrecan and L-APP, where just a weak xylosylation with XT-II could be determined. K_m values were very similar for XT-I and XT-II, with slightly lower K_m values for XT-II mediated xylosylation. On the other hand, V_{max} values of the xylose transfer catalyzed by XT-I were moderately to considerably higher than those determined for XT-II.

SPACRCAN, agrin and glypican-1 containing two or three cumulated G-S-G-motifs are summarized in the second group. Glypican-1 featured the best acceptor properties with a K_m value of 17.1 μM (95% confidence interval (95% CI): 14.6–20.1 μM) for XT-I and a K_m value of 10.9 μM (95% CI: 8.5–14.1 μM) for XT-II. A XT-II catalyzed reaction revealed no product with SPACRCAN while agrin was xylosylated to a minor extent. K_m values for XT-I mediated reactions for these two fusion proteins were in a range between 100 and 1000 μM.

In the last group the G-S-G-motif is absent. The best acceptor function of fusion proteins containing a G-S-x-motif was detected for collagen α2(IX) with a K_m value of 3.4 μM (95% CI: 0.2–12.0 μM) for XT-I. A xylosylation mediated by XT-I revealed a weak or no product with brevican and epiphycan_2, respectively. None of these peptides was xylosylated by XT-II. Fusion proteins summarized with a x-S-G-motif bear different functions as acceptor molecules. K_m values for XT-I mediated glycosylation could be determined between 9.7 and 20.8 μM. In comparison to peptides with a G-S-G-motif the V_{max} values were considerably lower. Betaglycan_2 and versican-α were xylosylated in minimal measurable amounts with XT-I while epiphycan_1 was not xylosylated. For none of these peptides an acceptor function for XT-II mediated glycosylation could be demonstrated, except biglycan and phosphacan, where a weak xylosylation was detectable. Although we found some peptides which were xylosylated exclusively by XT-I, the maximum reaction rates were quite low, resulting in weak radioactive signals in the XT activity test.

Mutagenesis aminoterminal to the attachment site of the bikunin sequence

We generated a multiplicity of fusion proteins based on the bikunin sequence containing mutated amino acids in the G-S-G-G-motif and amino- or carboxy-terminal to this motif, respectively. Fusion protein of clone 1.1 with an E(−4)K mutation had similar K_m and V_{max} values for XT-I and XT-II as wild-type (WT) bikunin (Table 2). In the case of mutations of all acidic amino acids (peptides 1.2 and 1.3) to other than basic amino acids, the glycosylation for XT-II was abolished. However, these proteins were good substrates for XT-I xylosylation, with similar K_m values to WT-bikunin but considerably lower V_{max} values. Peptides possessing no acidic and one basic amino acid in the −4, −3 or −2 position (peptides 1.4–1.10) were not xylosylated by XT-II, but were still poor substrates for XT-I. An elevation of the number of basic amino acids caused a loss of acceptor function for both xylosyltransferases (peptides 1.11 and 1.12).

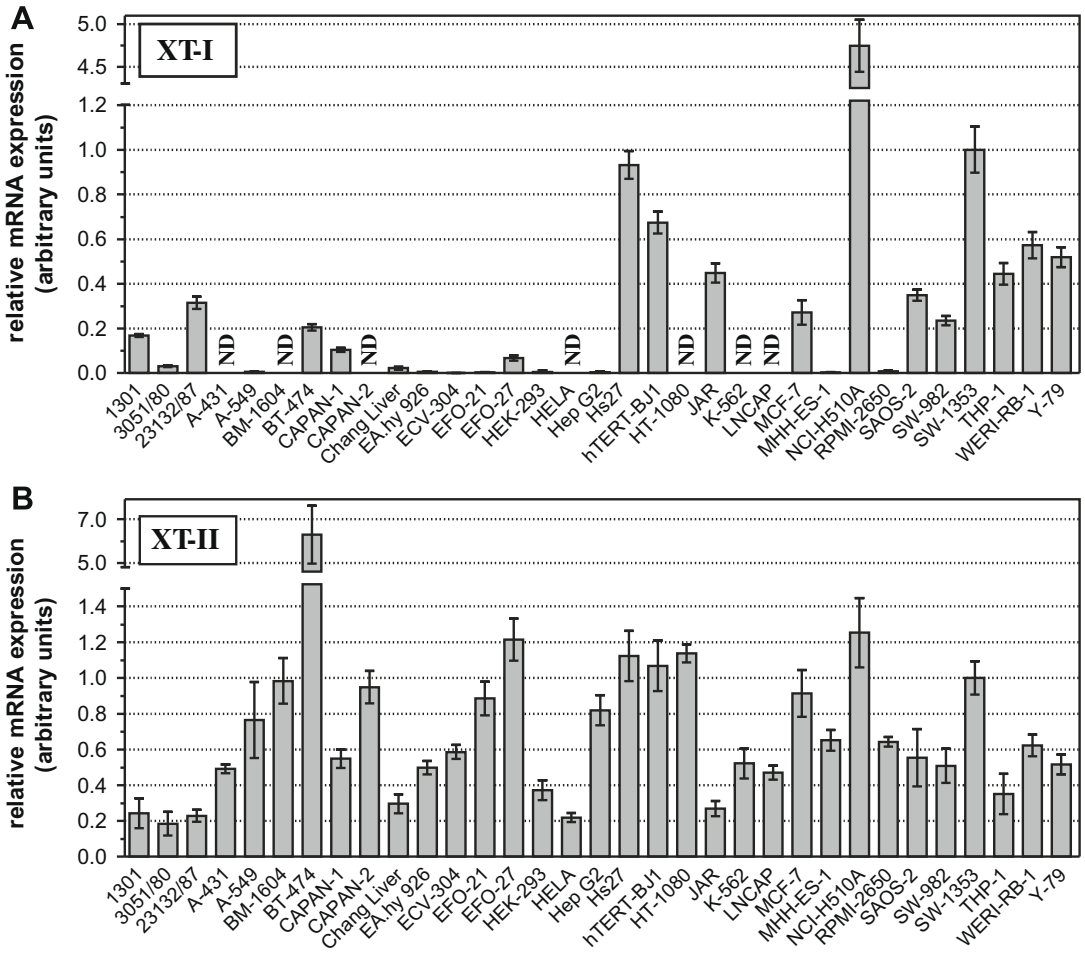

Fig. 1. XT-I and XT-II mRNA expression in human cell lines. Relative quantification of mRNA of human cells was accomplished by a fluorogenic real-time PCR assay. Transcriptional levels of the target peptides XT-I (A) and XT-II (B) were normalized by an index of the housekeeping genes GAPDH, HPRT and β_2-microglobulin. cDNA synthesised from total RNA of SW-1353 cells served as a calibrator to which gene expression of other cells were compared to. Relative mRNA expression levels are presented in arbitrary units. PCR reactions were run in triplicate and expression levels are shown as mean values with standard deviation. (ND, not detectable).

Mutagenesis of the G-S-G-G-motif of the bikunin sequence

Many PG attachment sites contain neighboring glycine residues as characterized by the consensus sequence. Here, we changed the glycine residues of the bikunin sequence. The G(−1)S mutant (peptide 2.1) was determined as having good acceptor functions for both isoforms, in contrast to the 2.2 peptide (G(−1)R mutation) which was not xylosylated by XT-II, but had a similar K_m value for a xylose transfer by XT-I (Table 3). Additionally, the maximum reaction rate for the G(−1)R mutant decreased dramatically. Expression products of the mutants 2.3 and 2.4 with single point mutations (G(+1)S exchange) had good acceptor properties for XT-I but not for XT-II. A complete loss or strong reduction of acceptor functionality was detected for peptides with a G(+1)R mutation (peptide 2.6) and a G(+1)C mutation (peptide 2.5). Peptides 2.7 and 2.8 with a GG(−1,+2)SR and a EG(−2,+2)del,R exchange, respectively, showed comparable K_m values for a xylosylation catalyzed by XT-I, but peptide 2.8 was a considerably better substrate in a XT-II catalyzed reaction. A double exchange of glycine residues in the positions +1 and +2 (peptides 2.9–2.15) caused a loss of acceptor functions for both isoforms except fusion proteins 2.9 and 2.10 containing a GG(+1,+2)SC and a GG(+1,+2)TS mutation. Both were good substrates for XT-I but poor substrates for XT-II. Peptide 2.19 with a GG(−1,+1)SS mutation within the peptides containing a double exchange of glycine residues in the positions −1 and +1 (peptides 2.16–2.19), was the sole exception being xylosylated by XT-I to a minor extent. None of these proteins had an acceptor function for XT-II. Peptides with a mutation in every glycine position (peptides 2.20–2.33) as well as a peptide with a S(0)T exchange (peptide 2.34) lost their acceptor function for both isoforms.

Mutagenesis carboxyterminal to the attachment site of the bikunin sequence

Mutagenesis in the C-terminus of the G-S-G-motif generated eight mutants. Each of them was xylosylated in a XT-I or XT-II catalyzed reaction (Table 4). K_m values differ slightly, but are in a similar range to the K_m value of WT-bikunin. Again, K_m and V_{max} values of a xylose transfer are slightly lower for XT-II mediated xylosylation compared to XT-I.

Discussion

Here, we determined XT mRNA expression of both xylosyltransferases in a large number of human cell lines. XT-II was broadly expressed in every tested cell line. In contrast, XT-I was not expressed in seven and only in marginal amounts in a multiplicity of cell lines. These results show that in cells with exclusive XT-II expression the GAG biosynthesis is initiated solely by XT-II

Table 1
Kinetic parameters for fusion proteins containing glycosaminoglycan attachment sites of proteoglycans.

	Protein	Peptide sequence	Number (position) of amino acids consistent with consensus sequence		K_m XT-I (95% CI) (μM)	K_m XT-II (95% CI) (μM)	V_{max} XT-I (95% CI) (pmol/h)	V_{max} XT-II (95% CI) (pmol/h)
			N-terminal	C-terminal				
Consensus sequence		aaaaGSGaba						
1	Syndecan-1	FSDNFSGSGAGAL	1 (−5)	1 (+3)	19.8 (16.1–24.6)	7.4 (5.6–9.6)	86.3 (75.6–100.4)	5.3 (4.7–6.0)
	Aggrecan	EFGDFTGSGDVSG	1 (−4)	1 (+2)	18.8 (14.6–24.9)	w. x.	17.1 (14.5–20.8)	<0.5
	Syndecan-4	DDFELSGSGDLDD	1 (−4)	2 (+2,+4)	3.5 (1.8–5.7)	14.1 (10.0–20.4)	50.9 (44.1–60.2)	12.5 (10.2–16.1)
	Neuroglycan C	FVTAEAGSGDAQA	1 (−3)	1 (+2)	23.6 (18.7–30.4)	3.2 (2.0–4.6)	57.8 (49.4–69.6)	2.5 (2.3–2.8)
	Thrombomodulin	FDGGDSGSGEPP	1 (−3)	1 (+2)	18.2 (15.1–22.2)	2.9 (2.5–3.4)	209.0 (185.2–239.8)	24.7 (23.8–25.7)
	Perlecan_3	PEFGDLGSGDFQ	1 (−3)	1 (+2)	18.7 (14.5–24.8)	9.4 (7.6–11.6)	132.2 (111.9–161.8)	23.0 (20.7–25.9)
	Testican-2	FFSGDFGSGVGWED	1 (−3)	1 (+3)	10.3 (8.0–13.3)	8.2 (5.0–13.0)	56.1 (49.7–64.4)	6.3 (5.2–8.0)
	Versican-β	FIPITEGSGEAEE	1 (−2)	2 (+2,+4)	14.6 (12.6–16.9)	12.7 (10.2–15.9)	100.2 (91.7–110.3)	54.4 (48.0–62.8)
	Serglycin	EFEDYSGSGFGSGS	2 (−5,−4)	1 (+3)	11.1 (9.4–13.1)	2.3 (1.9–2.8)	74.2 (68.1–81.6)	7.8 (7.5–8.1)
	Syndecan (D. m.)	DDDSIEGSGGR	2 (−5,−2)	–	17.8 (15.4–20.7)	12.2 (9.8–15.1)	105.2 (95.8–116.7)	12.0 (10.7–13.7)
	Perlecan_2	EFGDDLGSGDLG	2 (−4,−3)	1 (+2)	14.5 (10.4–20.8)	10.2 (8.5–12.2)	105.5 (86.0–136.6)	43.1 (39.0–48.1)
	bFGF-peptide	EFPEDGGSGAFPP	2 (−4,−3)	–	13.7 (11.6–16.2)	9.3 (6.9–12.4)	114.7 (103.8–128.2)	13.1 (11.3–15.6)
	L-APLP2	EFSENEGSGMAEQ	2 (−4,−2)	1 (+4)	13.4 (10.7–17.0)	10.4 (8.9–12.3)	145.5 (128.3–168.0)	59.9 (55.2–65.5)
	L-APP	EFTENEGSGLTNI	2 (−4,−2)	–	13.1 (9.9–17.8)	w. x.	14.6 (12.4–18.0)	<0.5
	Bamacan	FQDEGEGSGESER	3 (−5,−4,−2)	2 (+2,+4)	22.2 (18.6–27.0)	17.9 (14.3–22.6)	190.1 (168.5–218.1)	132.9 (115.8–156.0)
	Bikunin	EFQEEEGSGGGQLV	3 (−4,−3,−2)	1 (+3)	27.4 (20.3–38.9)	46.3 (29.8–88.3)	297.2 (239.6–391.1)	405.8 (281.9–724.1)
2	SPACRCAN	DGGLGSGSGQKVD *			i. x.	>10,000	<0.5	<0.5
	Agrin	FEGGSGGSGSGDG *			i. x.	w. x.	<0.5	<0.5
	Glypican-1	EFDDGSGSGSGDGC*			17.1 (14.6–20.1)	10.9 (8.5–14.1)	92.3 (83.6–102.8)	12.6 (11.0–14.6)
3	Collagen α2(IX)	FIQGLEGSADFLC	1 (−2)	1 (+2)	3.4 (0.2–12.0)	>10,000	2.0 (1.4–3.4)	<0.5
	Epiphycan_2	FPRLIDGSSPQEP	1 (−2)	1 (+4)	>10,000	>10,000	<0.5	<0.5
	Brevican	FESEETGSSRGAP	2 (−4,−3)	1 (+3)	w. x.	>10,000	<0.5	<0.5
	Perlecan_1	PEFADSISGDDLG	1 (−3)	2 (+2,+3)	13.3 (9.1–19.8)	>10,000	4.1 (3.3–5.3)	<0.5
	Biglycan	EEASGADTSGVLD	1 (−2)	1 (+4)	9.7 (7.2–13.2)	w. x.	24.4 (20.9–29.5)	<0.5
	Betaglycan_1	FPALGDSSGWPDG	1 (−2)	1 (+4)	20.8 (15.6–28.3)	>10,000	5.4 (4.5–6.7)	<0.5
	Phosphacan	EFGAEDSSGSSPAT	2 (−3,−2)	–	16.6 (9.8–30.3)	w. x.	3.7 (2.7–5.6)	<0.5
	CD44	FTDDDVSSGSSSE	3 (−5,−4, −3)	–	14.6 (10.9–19.9)	>10,000	11.8 (10.0–14.4)	<0.5
	Betaglycan_2	FGYEDLESGDNGF	2 (−4,−3)	1 (+2)	w. x.	>10,000	<0.5	<0.5
	Versican-α	FTPEEVFSGMKLS	2 (−4,−3)	–	w. x.	>10,000	<0.5	<0.5
	Epiphycan_1	FIATVMPSGNREL	–	1 (+4)	>10,000	>10,000	<0.5	<0.5

Peptide sequences of proteoglycans contain one (group 1), more than one (group 2) or none G-S-G-motif (group 3). Amino acids consistent with the consensus sequence are indicated by white letters in black shaded boxes. Serine residues of the GAG attachment sites are additionally printed in bold letters. Amino acids illustrated in grey shaded boxes are coded by nucleotide sequences of the plasmid and do not occur in the native *in vivo* proteoglycan. Fusion protein sequences end with the last shown carboxyterminal amino acids. Asterisks indicate PG sequences where the GAG attachment site cannot be assigned to a definite serine residue. For some of the peptides no K_m value could be determined because the amounts of xylosylated peptides were too low. Here we discriminated between peptides with K_m values in a range between 100 and 1000 μM (i. x.) and peptides where only a weak xylosylation was measurable (w. x.). For peptides with a K_m value higher 10,000 μM no xylosylation product could be detected. (a, Asp or Glu; b, Asp, Glu or Gly; D. m., *Drosophila melanogaster*; CI, confidence interval; i. x., intermediate xylosylation; w. x., weak xylosylation).

Table 2
Kinetic parameters for bikunin fusion proteins with mutations aminoterminal to the glycosaminoglycan attachment site.

Protein	Peptide sequence	Number (position) of basic amino acids	K_m XT-I (95% CI) (μM)	K_m XT-II (95% CI) (μM)	V_{max} XT-I (95% CI) (pmol/h)	V_{max} XT-II (95% CI) (pmol/h)
WT	QEEEGSGGGQLV	–	27.4 (20.3–38.9)	46.2 (29.8–88.3)	297.2 (239.6–391.1)	405.8 (281.9–724.1)
1.1	Q**K**EEGSGGGQLV	1 (−4)	20.0 (15.9–25.9)	35.6 (26.6–50.7)	134.2 (114.8–161.6)	179.2 (142.7–240.8)
1.2	QLTTGSGGGQLV	–	31.8 (25.1–41.6)	>10,000	12.6 (10.5–15.6)	<0.5
1.3	QPQTGSGGGQLV	–	65.6 (45.2–109.6)	>10,000	28.8 (21.0–45.6)	<0.5
1.4	Q**K**LQGSGGGQLV	1 (−4)	w. x.	>10,000	<0.5	<0.5
1.5	QT**K**QGSGGGQLV	1 (−3)	w. x.	>10,000	<0.5	<0.5
1.6	QP**R**IGSGGGQLV	1 (−3)	w. x.	>10,000	<0.5	<0.5
1.7	QT**R**QGSGGGQLV	1 (−3)	w. x.	>10,000	<0.5	<0.5
1.8	QL**R**PGSGGGQLV	1 (−3)	w. x.	>10,000	<0.5	<0.5
1.9	QIQ**R**GSGGGQLV	1 (−2)	w. x.	>10,000	<0.5	<0.5
1.10	QTI**R**GSGGGQLV	1 (−2)	w. x.	>10,000	<0.5	<0.5
1.11	Q**K**I**R**GSGGGQLV	2 (−4,−2)	>10,000	>10,000	<0.5	<0.5
1.12	Q**RRK**GSGGGQLV	3 (−4,−3,−2)	>10,000	>10,000	<0.5	<0.5

K_m values were measured using the radiochemical XT activity assay. Mutated amino acids are indicated by white letters in black shaded boxes and basic amino acids are additionally illustrated by bold letters. Valine residues are the last amino acids of the fusion proteins. (WT, wild-type; CI, confidence interval; w. x., weak xylosylation).

Table 3
Kinetic parameters for bikunin fusion proteins with mutations in the G-S-G-G-motif.

Protein	Peptide sequence	Position of mutated amino acid	K_m XT-I (95% CI) (μM)	K_m XT-II (95% CI) (μM)	V_{max} XT-I (95% CI) (pmol/h)	V_{max} XT-II (95% CI) (pmol/h)
WT	QEEEGSGGGQLV		27.4 (20.3–38.9)	46.3 (29.8–88.3)	297.2 (239.6–391.1)	405.8 (281.9–724.1)
2.1	QEEE**S**SGGGQLV	−1	10.3 (8.3–12.9)	8.5 (6.6–11.1)	119.3 (106.7–135.3)	28.5 (25.3–32.7)
2.2	QEEE**R**SGGGQLV	−1	9.9 (7.5–13.1)	>10,000	3.7 (3.2–4.3)	<0.5
2.3	QEE-GS**S**GGQLV	−2,+1	12.3 (9.7–15.7)	w. x.	10.5 (9.3–12.2)	<0.5
2.4	QEEEGS**S**GGQLV	+1	15.3 (11.8–19.9)	w. x.	25.2 (21.7–30.0)	<0.5
2.5	QEEEGS**C**GGQLV	+1	w. x.	>10,000	<0.5	<0.5
2.6	QEEEGS**R**GGQLV	+1	>10,000	>10,000	<0.5	<0.5
2.7	QEEE**S**SG**R**GQLV	−1,+2	24.3 (18.9–32.1)	w. x.	30.1 (25.4–36.9)	<0.5
2.8	QEE-GSG**R**GQLV	−2,+2	20.5 (16.9–25.4)	6.4 (5.5–7.4)	79.1 (69.2–92.3)	27.4 (25.9–29.1)
2.9	QEEEGS**C**GQLV	+1,+2	13.3 (10.4–17.1)	w. x.	12.8 (11.2–15.0)	<0.5
2.10	QEEEGS**T**SLV	+1,+2	28.8 (23.8–35.5)	w. x.	27.0 (23.5–31.7)	<0.5
2.11	QEEEGS**I**MLV	+1,+2	>10,000	>10,000	<0.5	<0.5
2.12	QEEEGS**R**CGQLV	+1,+2	>10,000	>10,000	<0.5	<0.5
2.13	QEEEGS**R**ALV	+1,+2	>10,000	>10,000	<0.5	<0.5
2.14	QEEEGS**R**LLV	+1,+2	>10,000	>10,000	<0.5	<0.5
2.15	QEE-GS**RR**GQLV	−2,+1,+2	>10,000	>10,000	<0.5	<0.5
2.16	QEEE**C**S**C**GGQLV	−1,+1	>10,000	>10,000	<0.5	<0.5
2.17	QEEE**R**S**S**GGQLV	−1,+1	>10,000	>10,000	<0.5	<0.5
2.18	QEE-S**S**RGGQLV	−2,−1,+1	>10,000	>10,000	<0.5	<0.5
2.19	QEEE**S**S**S**GGQLV	−1,+1	w. x.	>10,000	<0.5	<0.5
2.20	QEEE**C**S**RS**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.21	QEEE**C**S**FT**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.22	QEEE**C**S**NC**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.23	QEEE**F**S**LL**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.24	QEEE**N**S**TP**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.25	QEEE**R**S**CR**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.26	QEEE**R**S**RC**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.27	QEEE**R**S**RS**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.28	QEEE**R**S**SR**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.29	QEEE**S**S**CR**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.30	QEEE**S**S**RS**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.31	QEEE**T**S**PC**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.32	QEEE**T**S**IC**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.33	QEEE**Y**S**CC**GQLV	−1,+1,+2	>10,000	>10,000	<0.5	<0.5
2.34	QEEEG**T**GGGQLV	0	>10,000	>10,000	<0.5	<0.5

Mutated amino acids are indicated by white letters in black shaded boxes. Valine residues indicate the end of the fusion proteins. (WT, wild-type; −, deletion of amino acid; CI, confidence interval; w. x., weak xylosylation).

and it might be speculated that dominant XT-II expression could indicate for a preferred XT-II catalysis. Equipollent co-expression in some cases might correlate to the lesser stringency of XT-I concerning the acceptor protein sequences. For instance, the fibroblast cell lines Hs 27 and hTERT-BJ1, as well as cardiac fibroblasts [15], show broad expression levels of both isoforms. Betaglycan was shown as being expressed in fibroblasts [16] and its GAG chains were defined as being attached to Ser^{535} and Ser^{546} [17]. Both serine residues were solely xylosylated by XT-I in our acceptor study. Recently, it was shown that the proteoglycan form of betaglycan as a TGF-β superfamily co-receptor was able to inhibit TGF-β mediated signaling. If the GAG chain was absent, the inhibitory effect was abolished [18]. Some core proteins occurring as part-time proteoglycans might be correlated to their sequences and the expressed XT in the according tissue. For example, if there is a functional necessity requiring betaglycan with attached GAG chains, there must be a XT-I expression for initiating GAG biosynthesis. Simultaneous expression of XT-II might be ascribed to

Table 4
Kinetic parameters for bikunin fusion proteins with mutations carboxyterminal to the glycosaminoglycan attachment site.

Protein	Peptide sequence	K_m XT-I (95% CI) (μM)	K_m XT-II (95% CI) (M)	V_{max} XT-I (95% CI) (pmol/h)	V_{max} XT-II (95% CI) (pmol/h)
WT	QEEEGSGGGQLV	27.4 (20.3–38.9)	46.3 (29.8–88.3)	297.2 (239.6–391.1)	405.8 (281.9–724.1)
3.1	QEEEGSGGGQLV	15.1 (12.5–18.4)	22.7 (17.0–31.4)	81.4 (72.5–92.8)	123.0 (101.3–156.8)
3.2	QEEEGSGGRSVLV	25.1 (20.9–30.8)	15.0 (13.3–17.1)	134.6 (118.1–156.4)	44.9 (41.7–48.6)
3.3	QEEEGSGHNMLV	12.5 (10.6–14.8)	7.7 (6.7–8.9)	120.0 (109.2–133.0)	68.9 (64.7–73.7)
3.4	QEEEGSGSLALV	19.9 (15.4–26.1)	9.3 (7.9–11.0)	224.3 (191.6–207.8)	89.7 (83.2–97.4)
3.5	QEEEGSGCYKLV	30.1 (24.4–38.2)	21.5 (18.1–25.8)	154.4 (132.5–185.0)	94.8 (84.8–107.5)
3.6	QEEEGSGCRVLV	54.3 (40.2–79.1)	50.8 (40.7–65.9)	191.8 (149.8–266.4)	194.4 (161.8–241.3)
3.7	QEEEGSGRHLLV	57.2 (41.1–88.4)	24.2 (20.5–29.1)	140.9 (106.6–207.8)	17.0 (15.0–19.5)
3.8	QEEEGSGRRALV	58.6 (41.3–94.2)	13.7 (10.8–17.6)	82.7 (61.5–126.4)	15.8 (13.8–18.6)

Amino acids are indicated by white letters in black shaded boxes. Fusion proteins end with the C-terminal valine residue. (WT, wild-type; CI, confidence interval).

defined acceptors being exclusively xylosylated by XT-II. But this seems to be rather improbable because of the high number of tested core protein sequences with contrary results. Other reasons might be different transcriptional regulation of xylosyltransferases ([19], Müller unpublished results) or small differences in efficiencies and reaction rates of catalysis.

Additionally, we analyzed XT acceptor specificities for fusion proteins. XT activity is determined by measuring the amount of transferred xylose residues to acceptor molecules [7,20]. At present it is impossible to differentiate between the isoforms because acceptors taken so far have acceptor functions for both enzymes. Thus measured values always reflect the total activity which is a combination of XT-I and XT-II mediated xylosylation.

Most expressed fusion proteins with a single G-S-G-motif had similar K_m values for XT-I and XT-II mediated xylosylation. But in general, affinity to the acceptor proteins seemed to be slightly higher for XT-II as indicated by lower K_m values. However, maximum reaction rates reached higher values for XT-I. This points towards a faster XT-I mediated reaction. Position and number of acidic amino acids seem to have only little influences on acceptor function because all K_m values were in a narrow range. This might be due to the naturally occurring combination of the amino acids. Nevertheless, an influence of acidic amino acids cannot be denied. A mutagenesis of the proteoglycan modification site of syndecan-1 in *Caenorhabditis elegans* showed that aminoterminal acidic amino acids in defined positions were required [21]. Difference between L-APP and L-APLP2 could also be attributed to an influence of acidic amino acids. Here, the main difference between the sequences is a glutamic acid residue in the +4 position present in L-APLP2 and absent in L-APP. This additional acidic residue might be the reason for a better acceptor function of the L-APLP2 peptide in a XT-II catalyzed reaction. The same effect might explain differences in xylosylation reactions between aggrecan and syndecan-4.

If putative GAG attachment sites were cumulated, it seems that the density of the negative charge and the positions of the acidic amino acids had a higher impact on the acceptor function. Here, a higher negative charge density and a closer localization to the xylosylation site appear to increase the function as an acceptor molecule. It cannot be excluded that this effect might be influenced by the length of the cumulated G-S-G-motif.

Peptides with divergences in the G-S-G-motif were mainly xylosylated by XT-I, but only in exceptional cases by XT-II. This demonstrates the lesser stringency of XT-I concerning the acceptor sequence. Here, characters of the amino acids in the +1 and −1 position, as well as position and density of the acidic amino acids, seem to exert a distinct influence on acceptor functionality, as seen by the sequence-dependent magnitude of xylosylation.

With this study we could verify putative GAG attachment sites to be xylosylated *in vitro* and thus suggest those sites likely to be modification sites *in vivo*. Furthermore, for every tested proteoglycan at least one sequence was xylosylated by one or both xylosyltransferases. This evidence could only not be provided for epiphycan. It might be possible that, other than the tested serine residues, like Ser^{320} might act as modification sites [22]. A second reason could be a cluster of seven glutamic acids being located between the two tested sequences of human epiphycan, which might contribute to acceptor recognition of these sequences by xylosyltransferases *in vivo*.

Mutations on the N-terminal side and the attachment site of bikunin were found to be more susceptible to a loss of acceptor function than mutations in the C-terminus. Results of the N-terminal mutagenesis indicate that with increasing numbers of basic amino acids the functionality of the fusion protein as an acceptor for a xylose residue decreases. Additionally, it has been ascertained that XT-I is less stringent concerning the acceptor protein than its isoform XT-II and furthermore the acidic amino acids are not essential for XT-I, but increase the amount of xylosylated product enormously. Similar findings for acceptor specificities of the dodecapeptide Q-E-E-E-G-S-G-G-G-Q-G-G and the same peptide with reductions in the number of glutamic acid residues were published [23].

Alterations in the G-S-G-G-motif caused a loss of acceptor function in the majority of cases. Some of the generated peptides with acceptor functions are consistent with the minimal consensus sequence motifs G-S-G or S-G-x-G. But some exceptions, like a G-S-S-G-G-, a G-S-S-C-G- and a G-S-T-S-L-motif show no consistence with one of these consensus sequences, but are xylosylated nevertheless. These results point towards an extension of the XT-I consensus sequences including these motifs. No xylosylation product could be detected for the S(0)T mutated bikunin fusion protein similar to the results of Pfeil and Wenzel [23] and Wang et al. [21]. Serine and threonine differ in an additional methyl group which might act as a sterical barrier and might impede a xylosylation reaction.

Acceptor functions of each peptide with mutations in the C-terminal side of the G-S-G-motif were not affected by amino acid exchanges as only small alterations of kinetic parameter values were determined. This is in agreement with the scanning mutagenesis of syndecan-1 where a low impact of C-terminal acidic amino acids for xylosylation was detected and no essentiality of aspartic acid residues could be verified [21].

In summary, a broad XT-II expression in contrast to an intermittent XT-I expression in the tested cell lines could be demonstrated confirming the XT expression patterns described by our group and others so far. Furthermore acceptor specificities of human xylosyltransferases were investigated. The consensus sequence a-a-a-a-G-S-G-a-a/G-a for XT-I postulated by Brinkmann et al. [7] could be approved and additionally extended as being the consensus sequence for XT-II. It could be ascertained that XT-I has a lesser stringency with regard to the acceptor sequences, and XT-II shows slightly higher affinities to acceptor peptides. Notably strong differences between the catalyzed reactions and the used substrates of the XT isoforms were not detected, showing that nearly the complete pool of proteoglycans is xylosylated by both isoforms.

However, in order to distinguish between the isoforms it would be of great interest to have acceptor molecules which are exclusively xylosylated by XT-I or XT-II. Thus the search for an applicable and specific acceptor molecule for both xylosyltransferases is still ongoing.

Acknowledgments

We thank Tim Take for his excellent technical assistance and Sarah L. Kirkby for her linguistic advice. This work was supported by the Forschungsförderung an der Medizinischen Fakultät der Ruhr-Universität Bochum (FoRUM), Grant F609-2007.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.121.

References

[1] L. Kjellen, U. Lindahl, Proteoglycans: structures and interactions, Annu. Rev. Biochem. 60 (1991) 443–475.
[2] K. Cuellar, H. Chuong, S.M. Hubbell, M.E. Hinsdale, Biosynthesis of chondroitin and heparan sulfate in Chinese hamster ovary cells depends on xylosyltransferase II, J. Biol. Chem. 282 (2007) 5195–5200.
[3] C. Pönighaus, M. Ambrosius, J.C. Casanova, C. Prante, J. Kuhn, J.D. Esko, K. Kleesiek, C. Götting, Human xylosyltransferase II is involved in the biosynthesis of the uniform tetrasaccharide linkage region in chondroitin sulfate and heparan sulfate proteoglycans, J. Biol. Chem. 282 (2007) 5201–5206.
[4] J. Voglmeir, R. Voglauer, I.B. Wilson, XT-II, the second isoform of human peptide-*O*-xylosyltransferase, displays enzymatic activity, J. Biol. Chem. 282 (2007) 5984–5990.
[5] A.E. Kearns, S.C. Campbell, J. Westley, N.B. Schwartz, Initiation of chondroitin sulfate biosynthesis: a kinetic analysis of UDP-D-xylose: core protein beta-D-xylosyltransferase, Biochemistry (Mosc) 30 (1991) 7477–7483.
[6] M.A. Bourdon, T. Krusius, S. Campbell, N.B. Schwartz, E. Ruoslahti, Identification and synthesis of a recognition signal for the attachment of glycosaminoglycans to proteins, Proc. Natl. Acad. Sci. USA 84 (1987) 3194–3198.
[7] T. Brinkmann, C. Weilke, K. Kleesiek, Recognition of acceptor proteins by UDP-D-xylose proteoglycan core protein beta-D-xylosyltransferase, J. Biol. Chem. 272 (1997) 11171–11175.
[8] C. Götting, J. Kuhn, K. Kleesiek, Human xylosyltransferases in health and disease, Cell. Mol. Life Sci. 64 (2007) 1498–1517.
[9] C. Götting, J. Kuhn, R. Zahn, T. Brinkmann, K. Kleesiek, Molecular cloning and expression of human UDP-D-xylose:proteoglycan core protein beta-D-xylosyltransferase and its first isoform XT-II, J. Mol. Biol. 304 (2000) 517–528.
[10] E. Condac, R. Silasi-Mansat, S. Kosanke, T. Schoeb, R. Towner, F. Lupu, R.D. Cummings, M.E. Hinsdale, Polycystic disease caused by deficiency in xylosyltransferase 2, an initiating enzyme of glycosaminoglycan biosynthesis, Proc. Natl. Acad. Sci. USA 104 (2007) 9416–9421.
[11] J.D. Esko, T.E. Stewart, W.H. Taylor, Animal cell mutants defective in glycosaminoglycan biosynthesis, Proc. Natl. Acad. Sci. USA 82 (1985) 3197–3201.
[12] E. Condac, G.L. Dale, D. Bender-Neal, B. Ferencz, R. Towner, M.E. Hinsdale, Xylosyltransferase II is a significant contributor of circulating xylosyltransferase levels and platelets constitute an important source of xylosyltransferase in serum, Glycobiology 19 (2009) 829–833.
[13] J.C. Casanova, J. Kuhn, K. Kleesiek, C. Götting, Heterologous expression and biochemical characterization of soluble human xylosyltransferase II, Biochem. Biophys. Res. Commun. 365 (2008) 678–684.
[14] S. Schön, G. Huep, C. Prante, S. Müller, R. Christ, F.W. Hagena, J. Kuhn, K. Kleesiek, C. Götting, Mutational and functional analyses of xylosyltransferases and their implication in osteoarthritis, Osteoarthritis Cartilage 14 (2006) 442–448.
[15] C. Prante, H. Milting, A. Kassner, M. Farr, M. Ambrosius, S. Schön, D.G. Seidler, A.E. Banayosy, R. Korfer, J. Kuhn, K. Kleesiek, C. Götting, Transforming growth factor beta1-regulated xylosyltransferase I activity in human cardiac fibroblasts and its impact for myocardial remodeling, J. Biol. Chem. 282 (2007) 26441–26449.
[16] C. Bellucci, C. Lilli, T. Baroni, L. Parnetti, S. Sorbi, C. Emiliani, E. Lumare, P. Calabresi, S. Balloni, M. Bodo, Differences in extracellular matrix production and basic fibroblast growth factor response in skin fibroblasts from sporadic and familial Alzheimer's disease, Mol. Med. 13 (2007) 542–550.
[17] F. Lopez-Casillas, H.M. Payne, J.L. Andres, J. Massague, Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites, J. Cell Biol. 124 (1994) 557–568.
[18] O. Eickelberg, M. Centrella, M. Reiss, M. Kashgarian, R.G. Wells, Betaglycan inhibits TGF-beta signaling by preventing type I-type II receptor complex formation. Glycosaminoglycan modifications alter betaglycan function, J. Biol. Chem. 277 (2002) 823–829.
[19] B. Müller, C. Prante, K. Kleesiek, C. Götting, Identification and characterization of the human xylosyltransferase I gene promoter region, J. Biol. Chem. 284 (2009) 30775–30782.
[20] J. Kuhn, C. Prante, S. Schön, C. Götting, K. Kleesiek, Measurement of fibrosis marker xylosyltransferase I activity by HPLC electrospray ionization tandem mass spectrometry, Clin. Chem. 52 (2006) 2243–2249.
[21] H. Wang, K. Julenius, J. Hryhorenko, F.K. Hagen, Systematic analysis of proteoglycan modification sites in *Caenorhabditis elegans* by scanning mutagenesis, J. Biol. Chem. 282 (2007) 14586–14597.
[22] M. Deere, J. Johnson, S. Garza, W.R. Harrison, S.J. Yoon, F.F. Elder, R. Kucherlapati, M. Hook, J.T. Hecht, Characterization of human DSPG3, a small dermatan sulfate proteoglycan, Genomics 38 (1996) 399–404.
[23] U. Pfeil, K.W. Wenzel, Purification and some properties of UDP-xylosyltransferase of rat ear cartilage, Glycobiology 10 (2000) 803–807.