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Review

Human xylosyltransferases in health and disease

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Abstract. The xylosyltransferases I and II (XT-I, XT-II, EC 2.4.2.26) catalyze the transfer of xylose from UDP-xylose to selected serine residues in the proteoglycan core protein, which is the initial and rate-limiting step in glycosaminoglycan biosynthesis. Both xylosyltransferases are Golgi-resident enzymes and transfer xylose to similar core proteins acceptors. XT-I and XT-II are differentially expressed in cell types and tissues, although the reason for the existence of two xylosyltransferase isoforms in all higher organisms remains elusive. Serum xylosyltransferase activity was found to be a biochemical marker for the assessment of disease activity in systemic sclerosis and for the diagnosis of fibrotic remodeling processes. Furthermore, sequence variations in the XT-I and XT-II coding genes were identified as risk factors for diabetic nephropathy, osteoarthritis or pseudoxanthoma elasticum. These findings point to the important role of the xylosyltransferases as disease modifiers in pathologies which are characterized by an altered proteoglycan metabolism. The present review discusses recent advances in mammalian xylosyltransferases and the impact of xylosyltransferases in proteoglycan-associated diseases.

Keywords. Xylosyltransferase, proteoglycan, chondroitin sulphate, heparan sulphate, glycosaminoglycan, xylose.

Introduction

Proteoglycans are an important group of glycosylated macromolecules, which represent one of the largest and most complex molecular structures in animal cells. These glycoproteins are essentially involved in many biological processes, including extracellular matrix deposition, biomechanical lubrication, cell-cell interactions, tumor cell growth, viral infections or neurite outgrowth [1], and consist of a core protein to which varying numbers and types of glycosaminoglycan chains are covalently attached [2]. The biological roles of proteoglycans are closely related to the presence of the glycosaminoglycan chains, which contain binding sites for various growth factors and matrix proteins. The glycoaminoglycans chondroitin sulphate, dermatan sulphate, heparan sulphate and heparin are anionic linear polysaccharides consisting of alternating disaccharide units which are attached to the core protein by a uniform tetrasaccharide linker, GlcA-β1,3-Gal-β1,3-Gal-β1,4-Xyl-β-O-Ser (Fig. 1). Biosynthesis of the glycosaminoglycan chains occurs in the Golgi apparatus by the subsequent addition of monosaccharides, which may finally be subjected to N- or O-sulphation, acetylation, deacetylation or epimeriation [2]. The initial step in the posttranslational biosynthesis of the uniform tetrasaccharide linkage region is catalyzed by the UDP-D-xylose:proteoglycan core protein β-D-xylosyltransferase (EC 2.4.2.26). This enzyme is the only protein that transfers a xylose molecule to serine residues in proteins or

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peptides and represents, with the exception of some invertebrates that share a β1,2-xylosyltransferase involved in N-glycan metabolism, the only xylosyltransferase in metazoa. The first evidence that xylose is present in proteoglycans and that it is linked to serine residues via a β-glycosidic linkage was found in the middle of the last century [3–5]. The enzymatic transfer of radiolabeled xylose to endogenous acceptor proteins could then be detected in cartilage and other tissues [6, 7]. Hence, xylosyltransferase activity was one of the first glycosaminoglycan glycosyltransferase activities detected. However, another 35 years passed until the successful identification of the xylosyltransferase amino acid sequence, the discovery of the presence of two xylosyltransferase isoforms in higher organisms and the cloning of the corresponding genes.

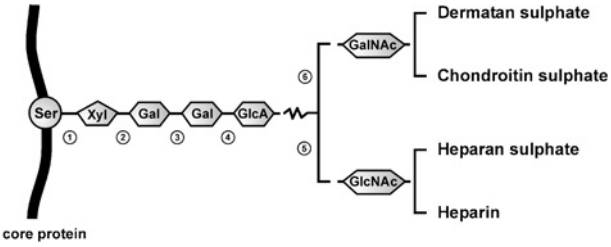

Figure 1. Biosynthesis of the uniform tetrasaccharide linker in chondroitin sulphate, dermatan sulphate, heparan sulphate and heparin glycosaminoglycans. The glycoaminoglycan chains in proteoglycans are synthesized in the Golgi apparatus by the transfer of monosaccharides, each of which is catalyzed by a specific glycosyltransferase. The biosynthesis of the common tetrasaccharide linker is initiated by the transfer of xylose from UDP-xylose to selected serine residues in the core protein and is catalyzed by the xylosyltransferases XT-I and XT-II. The 5th sugar residue is either a N-acetylglucosamine, which is found in chondroitin sulphate and dermatan sulphate chains, or a N-acetylgalactosamine, present in heparan sulphate or heparin chains. Thereafter, the glycosaminoglycan chains are elongated by alternating disaccharides, which are then partly modified by sulphation, epimerization or deacetylation. Ser, serine; Xyl, xylose; Gal, galactose; GlcA, glucuronic acid; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine. The numbers represent the glycosyltransferase catalyzing each step: 1, xylosyltransferase I and xylosyltransferase II (EC 2.4.2.26); 2, β1,4-galactosyltransferase 7 (EC 2.4.1.133); 3, β1,3-galactosyltransferase 6 (EC 2.4.1.134); 4, glucuronyltransferase I (EC 2.4.1.135); 5, N-acetylglucosaminyltransferase I (EC 2.4.1.223); 6, N-acetylgalacosaminyltransferase I (EC 2.4.1.223).

Purification and cloning of the xylosyltransferases

The purification of glycosyltransferases was hampered by difficulties in obtaining a sufficient amount of the source materials, as these enzymes are only present in minute amounts in cells and body fluids. Consequently, the amino acid sequence of the majority of the glycosyltransferases involved in glycosaminoglycan biosynthesis was elucidated in the last 10 years by genetic complementation studies, complementary DNA (cDNA) cloning and further molecular biological methods, rather than by purification of native enzymes [8–11]. While the existence of a xylose-transferring enzyme had been postulated since the 1960 s [4–7, 12], it took more than another 3 decades until the xylosyltransferase amino acid sequence was identified. Several attempts to purify the xylosyltransferase from diverse sources were published by different groups between 1972 and 2000 [13–16]. These included the isolation of xylosyltransferase from chicken cartilage, rat chondrosarcoma and rat ear cartilage, and postulated the xylosyltransferase to be a heterodimer with 25-kDa and 27-kDa subunits [14] or a monomer with a molecular mass of 78 kDa [16]. From today's point of view it is easy to see that those attempts were only partial purifications and enrichments of xylosyltransferase activity and that the visualized protein bands were only co-purified contaminating proteins. The successful isolation of human xylosyltransferase and the determination of peptide sequences were first reported by our group in 2000/2001 [17]. The enzyme was purified from 2000 liters of serum-free cell culture supernatant conditioned by *JAR* choriocarcinoma cells, which were grown in a hollow-fiber bioreactor. The isolation procedure included a combination of ammonium sulphate precipitation, heparin affinity chromatography, ion exchange chromatography and protamine affinity chromatography, and the XT-I was purified 4700-fold to apparent homogeneity with a 1% yield. The purified XT had a molecular mass of 120 kDa and was characterized by matrix-assisted laser desorption/ionization mass spectrometry-time of flight (MALDI-TOF) fingerprinting to be a hitherto unknown protein. The amino acid sequences of 11 peptides were then determined and used for the design of multiple degenerate oligonucleotide primers for cloning of the corresponding cDNA. In order to prove that the determined amino acid sequence is indeed identical to human XT-I, antibodies were raised against one of these peptide sequences. These antibodies were then used to enrich XT-I from an alternative source by immunoaffinity chromatography. Furthermore, XT-I was purified alternatively by aprotinin affinity chromatography, and the 120-kDa protein was detected by Western blot analysis in the XT-I-containing fraction [17].

Using degenerate primers based on the determined amino acid sequence of XT-I the cDNA sequences of human, rat and mouse XT-I were cloned, encoding a type II transmembrane protein with 959 amino acids [18, 19]. Soluble forms of human XT-I lacking the aminoterminal cytoplasmic tail and the transmembrane domain were successfully expressed in Chinese

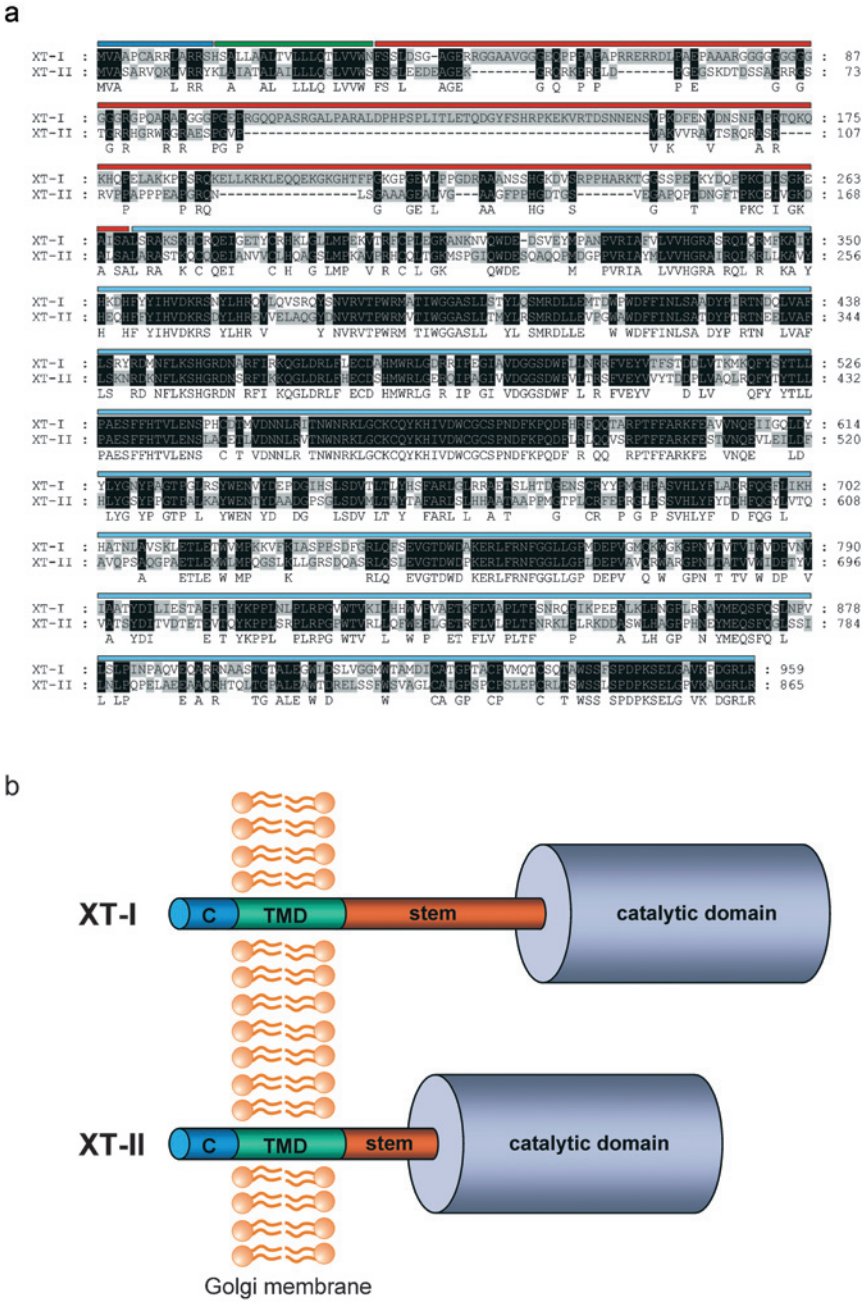

Figure 2. Sequence homology and topology of the human xylosyltransferases XT-I and XT-II. (**a**) Alignment of the amino acid sequences of human XT-I and XT-II. Introduced gaps are shown by dashes. Black boxes indicate identical amino acids in both proteins, and a consensus sequence is given below each aligned amino acid. The cytoplasmic region (dark blue), the transmembrane domain (green), the putative stem region (red) and the catalytic domain (light blue) are marked by colored boxes. (**b**) Proposed type II transmembrane topology of XT-I and XT-II. Both xylosyltransferases share a highly similar structure with a cytoplasmic region (C), a transmembrane domain (TMD), which anchors the enzyme in the Golgi membrane, and a variable stem region (stem). The catalytic domains of XT-I and XT-II share a high degree of sequence homology.

hamster ovary (CHO) cells [18], in *High five* insect cells [20–23] and in the yeast *Pichia pastoris* [24] in catalytically active form. The enzyme transferred xylose from UDP-D-xylose to serine residues in bikunin, silk and other core proteins, forming a β-linkage which was selectively labile to β-xylosidase treatment and alkaline β-elimination [18]. Full-length forms of human XT-I have also been successfully expressed in CHO [25], HEK293 and SaOS-2 cells [19]. During cloning the cDNA of human XT-I, another cDNA from human and rat sources was cloned which coded for a different protein with high homology to XT-I (Fig. 2) [18]. This putative protein was termed xylosyltransferase II (XT-II), although the enzymatic activity of this XT-I homolog remained elusive until the end of 2006. Based on the obtained sequence data, the xylosyltransferases of *Drosophila melanogaster* and *Caenorhabditis elegans* were identified [26, 27].

The xylosyltransferase genes *xylt-1* and *xylt-2*

The human XT-I and XT-II coding genes *xylt-1* and *xylt-2* were located on chromosomes 16p13.1 and 17q21.3–22, respectively [28]. The *xylt-1* gene has a total length of more than 300 kb and consists of 12 exons. The open reading frame is 2877 bp and encodes a 959-amino acid protein with a calculated molecular mass of 108 kDa and a putative type II transmembrane topology [20, 28, 29]. The purified native human XT-I has a molecular mass of approximately 120 kDa, indicating that the enzyme is glycosylated *in vivo* [17]. The exons 1 and 2, encoding the cytoplasmic tail, the transmembrane domain and parts of the stem region, are separated from the exons 3–12 by large introns of 112 and 98 kb, respectively. Interestingly, recombinant XT-I lacking the amino acids which are encoded by the first two exons displays full enzymatic activity. The human *xylt-2* gene comprises only 15 kb and consists of 11 exons with an open reading frame of 2598 bp [28, 29]. It codes for an 865-amino acid protein with a calculated molecular mass of 97 kDa. The exon-intron structure of the two xylosyltransferase genes is much conserved, with the splice sites of the last 9 exons being highly similar [29]. The corresponding murine genes are located on chromosomes 7 and 11, respectively, and share an exon-intron structure similar to the human xylosyltransferase genes. All higher organisms investigated so far have two xylosyltransferase genes encoding putative functional xylosyltransferases [18, 20, 22, 23, 25, 29], while only a single xylosyltransferase gene is present in the fly and the worm genomes [26, 27]. Yeast and bacteria possess no xylosyltransferase orthologs.

Xylosyltransferase II, an isoform of XT-I?

Although identified during a genetic screen for the XT-I cDNA sequences [28], the function of this putative enzyme was not clear at first. The amino acid sequences have a 52% overall sequence homology and share a type II transmembrane topology [18]. The homologous amino acids are not evenly distributed over the two protein sequences but display highly conserved domains and divergent parts of the xylosyltransferase proteins (Fig. 2). Regions with more than 80% identical amino acids are present in the human xylosyltransferases and are primarily located in the potential catalytic domain of the protein [18, 25]. Interestingly, the 20 amino acids at the carboxyterminal end of XT-II are also highly conserved [25], which is in concordance with the xylosyltransferase orthologs from *Drosophila melanogaster* and *Caenorhabditis elegans*, where this region was found to be necessary for enzyme stability and catalytic activity [27, 29].

Although the XT-II cDNA was cloned in 2000 [18], the catalytic activity and the physiological function of this protein remained a mystery. However, initial evidence came to light that XT-II is of biological importance and that the XT-II coding gene is not just a xylosyltransferase-like pseudogene [18], because sequence analysis of the *xylt-2* gene from healthy blood donors revealed only a very low rate of sequence variations, which was comparable to that identified in the *xylt-1* genes [30]. The detected sequence variations were predominantly silent alterations which did not result in any amino acid changes [30–32]. Furthermore, mutations in the *xylt-2* gene could be identified as genetic risk factors for diseases which are characterized by an altered proteoglycan metabolism [30–32]. Despite these facts, no enzyme activity could be proven for soluble recombinant XT-II [18, 24]. Just recently, three independent papers were published which describe the enzyme activitiy of XT-II and its role in proteoglycan biosynthesis [25, 33, 34].

Human XT-II is involved in the biosynthesis of both chondroitin sulphate and heparan sulphate proteoglycans, as demonstrated by complementation of the proteoglycan biosynthesis defect of a CHO cell line [25, 34]. The CHO pgsA-745 cell line is not capable of synthesizing chondroitin sulphate or heparan sulphate proteoglycans, and has a more than 15-fold reduced xylosyltransferase activity [35]. Previous studies have already shown restoration of proteoglycan biosynthesis capacity in these cells by transfection with *Caenorhabditis elegans* xylosyltransferase cDNA [27]. Expression of human XT-I or XT-II in pgsA-745 cells completely restored the proteoglycan biosynthesis capacity as judged by cell-surface binding of fibroblast growth factor 2, glycosylation of recombinant biglycan or glycosaminoglycan staining [25, 34]. Analysis of the cell proteoglycans showed identical chondroitin sulphate and heparan sulphate levels in XT-I- and XT-II-transfected cells, and even a detailed look at the glycosaminoglycan disaccharide content could not elucidate any significant differences between XT-I- and XT-II-initiated glycosaminoglycan biosynthesis [25] [M. Ambrosius, C. Pönighaus and C. Götting, unpublished data]. Consequently, no differences between XT-I- or XT-II-transfected cells were observed, indicating that both enzymes are capable of complementing the glycosaminoglycan biosynthesis defect in pgsA-745 cells [25].

Enzyme characteristics and the xylosylation consensus sequence

Xylosyltransferases are the only glycosyltransferases involved in chondroitin sulphate and heparan sulphate metabolism, which transfer a sugar to an acceptor protein or peptide. Different acceptor proteins have been successfully used as xylosyltransferase acceptors, including endogenous acceptor proteins from crude cartilage extracts [6, 7, 12], deglycosylated proteoglycan core proteins [36, 37] and artificial acceptor proteins, which are not proteoglycans *in vivo*, like silk fibroin from *Bombyx mori* [38] or fibroblast growth factor 2 [39]. Furthermore, multiple peptides derived from the glycosaminoglycan-attachment sites of different proteoglycans served as an acceptor for xylosyltransferase-mediated xylosylation [16, 24, 26, 29, 36, 38, 40–45]. The best XT-I acceptor protein known so far is human bikunin with a Michaelis-Menten constant of 0.9 μM [20–23, 40, 41]. Bikunin is the inhibitory component of the inter-α-trypsin inhibitor and is modified *in vivo* with a chondroitin sulphate chain, which is essential for the inhibitor structure [46]. The chondroitin sulphate attachment site of bikunin has the amino acid sequence Gln-Glu-Glu-Glu-Gly-Ser-Gly-Gly-Gly-Gln, which is similar to the XT-I acceptor consensus sequence [40, 41]. This xylosylation consensus sequence a-a-a-a-Gly-Ser-Gly-a-Gly/a-a (with 'a' representing an acidic amino acid) was determined by comparing the glycosaminoglycan attachment sites of 50 different proteoglycans [40] and is similar to the minimal motifs Gly-Ser-Gly and Ser-Gly-x-Gly (with 'x' representing any residue) for core protein xylosylation [44, 47–49].

Human XT-II has just recently been shown to be a true xylosyltransferase that catalyzes the transfer of xylose residues from UDP-D-xylose to consensus serine residues in the core protein [25, 33]. Characterization of the enzyme properties revealed that XT-I and XT-II are highly similar enzymes with a comparable temperature optimum [16, 26] [J. Kuhn and C. Götting, unpublished data] and a broad pH optimum ranging from 6.5 to 8.0 [33]. The majority of glycosyltransferases have been shown to require the presence of divalent cations for enzymatic activity [50]. Cation-mediated bridging of the pyrophosphate from the UDP sugar to a common aspartic acid-any residue-aspartic acid sequence motif has been shown for many glycosyltransferases [51–57]. XT-I and XT-II share a similar cation dependency, as the highest activity of both enzymes was observed after addition of Mg^{2+}, Mn^{2+} or Ca^{2+}, whereas enzyme activity was abolished by Zn^{2+}, Cu^{2+} or Ni^{2+} [16, 26, 33, 58] [J. Carrera Casanova and C. Götting, unpublished data; J. Kuhn and C. Götting, unpublished data]. XT-II catalyzes the transfer of xylose to similar peptide acceptors as XT-I but with different efficiencies [25, 33]. The best acceptor known so far is a modified bikunin-related peptide, biotin-NH-Gln-Glu-Glu-Glu-Gly-Ser-Gly-Gly-Gly-Gln-Lys-Lys(5-fluorescein)-$CONH_2$, with an apparent Michaelis-Menten constant of 5.2 μM [25]. Until now, an XT-II-mediated xylosylation was shown for different peptide and protein substrates, including bikunin peptides [25, 33], syndecan peptide [33], perlecan peptide [33], fibroblast growth factor 2 peptide [25], bikunin [25], biglycan [34] and silk fibroin [25]. XT-II catalyzes the transfer of xylose to the hydroxyamino acid serine of the acceptor peptide through a β-linkage [25]. However, it has not yet been clarified whether the XT-II acceptor consensus sequence is identical to that of XT-I.

Tissue-specific expression of XT-I and XT-II

Complementation studies and characterization of the enzyme properties of XT-II were able to shed light on the xylosyltransferase activity of this enzyme and its involvement in initiating glycosaminoglycan biosynthesis, but a crucial question has remained unanswered. As XT-I and XT-II are highly similar enzymes which are both capable of initiating the proteoglycan biosynthesis, why do all higher organisms have two xylosyltransferase genes? This is somewhat surprising as, for the other glycosyltransferases which are involved in the biosynthesis of the common GlcAβ1,3-Galβ1,3-Galβ1,4-Xyl-O-Ser tetrasaccharide linker, only single isoforms exist [8–11].

Besides their similarity regarding enzymatic function, the xylosyltransferase genes have been shown to be differentially expressed in mammalian tissues and cell lines [18, 25, 34]. In most tissues, both xylosyltransferase genes are expressed [18, 25], and in certain cell lines XT-II is the major xylosyltransferase [34]. In liver tissues, in the HeLa cervical carcinoma cell line and in K562 erythroleukemia cells XT-II has been found to be exclusively expressed [25, 34].

The promoters of the xylosyltransferase genes have not yet been characterized, and therefore cell-type-specific transcriptional response elements are not yet identified. However, it has been shown that both xylosyltransferase genes are differentially regulated during transforming growth factor (TGF) β_3-induced chondrogenic differentiation of mesenchymal stem cells [59]. XT-I is the predominant xylosyltransferase in the early phase of chondrogenic stem cell differentiation and XT-II is upregulated 7 days after induction. A similar counterregulation of both xylosyltransferase genes was observed during the differ-

entiation of mesenchymal stem cells into osteoblasts [B. Müller and C. Götting, unpublished data]. Extracellular matrix biosynthesis requires the coordinated synthesis of collagens, proteoglycans and other matrix molecules, and the expression of many extracellular matrix components is induced by cytokines from the TGF-β-family [60, 61]. The *xylt-1* gene is also induced by TGF-β_1 in human cardiac fibroblasts in a dose-dependent manner and is a key player in fibrotic remodeling of the extracellular matrix in dilated cardiomyopathy [62]. TGF-β_1-inducible elements have not been identified for the *xylt-2* gene, and a strong response of the latter gene to TGF-β_1 was not observed in human dermal fibroblasts [S. Busch and C. Götting, unpublished data].

Intracellular localization of the human xylosyltransferases

Intracellular localization of the xylosyltransferases and initiation of the glycosaminoglycan chain biosynthesis have been controversially discussed in the last 20 years. Some groups have favored localization of xylose transfer in the endoplasmic reticulum [63–67], while others have presented data showing localization of xylosyltransferases in the Golgi compartments [68–70]. Furthermore, analysis of the pH optimum of the xylosyltransferases could not provide the deciding indication for their subcellular localization. Different studies have determined the optimal enzyme activity to be achieved at pH levels of between 6 and 8 [7, 12, 13, 16, 26, 58], which would be consistent with a localization in either the endoplasmic reticulum or the Golgi apparatus [71]. Subcellular localization in different Golgi compartments could be determined for other enzymes involved in glycosaminoglycan biosynthesis using co-localization studies with known Golgi markers and high-resolution fluorescence microscopy [72–74]. Enzymes which are involved in the elongation of glycosaminoglycan chains are localized in the medial Golgi apparatus, whereas the sulphotransferases, which are involved in the final modification of the glycosaminoglycan chains, are situated in the *trans*-Golgi network [75–78]. β1,4-Galactosyltransferase 7 (β4GalT7) is the enzyme which transfers the first galactose residue to the xylosylated core protein and was recently located in the *cis*-Golgi compartments [74, 79, 80]. The mechanism of intracellular targeting of glycosyltransferases is not completely understood, but two models for Golgi retention, the *bilayer thickness* and *kin recognition hypotheses*, are currently being discussed [81, 82]. The former postulates that Golgi retention is achieved by the length of the transmembrane domain, while the latter proposes oligomerization of the enzyme as a mechanism. However, none of these models could completely explain the complex retention mechanisms of glycosyltransferases, which include the interaction of transmembrane domain, stem region or even the catalytic domain regions [75, 83–85]. The intracellular localization of human XT-I was recently identified by co-localization experiments with cell compartment-specific markers and the use of recombinant green fluorescent protein (GFP)-fusion proteins in HEK293 and SaOS2 cells [19]. Different variants of recombinant GFP-tagged XT-I could be located in the early Golgi compartments, and the amino terminal 214 amino acids of human XT-I could be determined necessary for Golgi targeting [19]. Furthermore, native XT-I could also be located in the early Golgi apparatus using XT-I antibodies, confirming that the results obtained with recombinant tagged protein are not artificial localizations due to high expression levels [19]. In addition, human XT-II was also found to be a Golgi-resident enzyme present in the early compartments [19]. The amino acid sequences of the XT-I and XT-II stem regions are rather divergent [22], with less than 15% identical amino acids (Fig. 2). The stem region in glycosyltransferases is supposed to be responsible for keeping the catalytic domain distant to the membrane of the subcellular compartiments in order to enable efficient glycosylation of the substrates. Furthermore, the stem region is also involved in subcellular targeting of the enzyme, while the exact molecular mechanisms are not yet known [19]. Interestingly, XT-II is 94 amino acids shorter than XT-I, and the alignment analyses show that these differences are primarily due to different lengths in the stem regions of both proteins (Fig. 2) [19]. Whether or not the differently sized stem regions influence the catalytic activities and the acceptor properties of XT-I and XT-II is not known. However, we could show that those parts of the stem region that are responsible for a proper localization of the xylosyltransferases in the Golgi apparatus are different in XT-I and XT-II [19]. Using GFP-tagged xylosyltransferase variants, the aminoterminal 45 amino acids of XT-II could be identified as required for the Golgi targeting, while more than 200 amino acids are needed for correct compartment localization of human XT-I [19]. The underlying molecular mechanisms and the sterical influence of the stem region on the XT-II protein structure are as yet unknown and will only be accessible after resolving the crystal structure of XT-II. Furthermore, motifs for *cis*-Golgi targeting have not yet been identified in the amino acid sequence of human XT-I or XT-II, which is in

concordance with other results showing that no conserved sequence motifs for glycosyltransferase targeting are known [86].

Secretion of XT-I and XT-II into extracellular space

Although xylosylation of the core protein occurs in the Golgi apparatus, more than 90% of the xylosyltransferase activity in cultured cells is found in the culture supernatant, where it is accumulated in a time-dependent manner [28, 87]. Only a small fraction of total xylosyltransferase activity is located membrane-bound in the cells within the Golgi apparatus [28]. The release of xylosyltransferase activity could either be an active secretion process after cleavage of the membrane-bound Golgi enzyme or a release of cellular content after cell damage and disruption of membrane integrity. Colchicine inhibits microtubule-mediated vesicular transport and has been shown to inhibit the release of glycosaminoglycans into the extracellular matrix [88] without influencing protein synthesis [89, 90]. Colchicine treatment of human chondrocytes blocked xylosyltransferase secretion, indicating that the enzyme is present in the extracellular space due to an active secretion process. Some glycosyltransferases are shed from the Golgi surface by protease cleavage of the stem region [91]. Although some proteases, like cathepsin-D, Alzheimer's β-scretase BACE1 or γ-secretase [92–95], have been identified as being involved in glycosyltransferases shedding, the majority of proteases involved in this process, as well as the molecular mechanisms controlling glycosyltransferase cleavage, remain unknown. Interestingly, both xylosyltransferases are predominantly secreted into the extracellular space despite the low degree of sequence homology in the stem regions and the aminoterminal part of the proteins [18, 25]. More than 90% of the total enzyme activity was found to be located in the culture supernatant in wild-type CHO cells and in CHO cell clones transfected with XT-I or XT-II coding expression vectors [25]. Until now, the amino acids in the xylosyltransferase stem regions where the proteolytic cleavage occurs have not been identified. Xylosyltransferase secretion seems to be independent of cell type and organ system, as xylosyltransferase activity has been identified in culture supernatants of all cell lines investigated [42, 59, 87] and in many human body fluids, including blood, synovial fluid, semen, follicular fluid and *liquor cerebrospinalis* [28, 42, 87, 96–99]. Until now, no UDP-xylose has been found in the extracellular space, indicating that no xylosyltransferase-mediated xylose transfer to protein substrates occurs due to the lack of donor substrates. Consequently, the question of the role of secreted xylosyltransferases in the extracellular space arises, and this problem has not yet been satisfactorily solved. It has been shown that the xylosyltransferases are secreted into the extracellular space together with large chondroitin sulphate proteoglycans, probably being attached to the modified core protein [87, 97]. Therefore, the hypothesis was formulated that the shed xylosyltransferases might be involved in proteoglycan trafficking throughout the Golgi compartments or in controlling glycosaminoglycan biosynthesis. The latter is supported by different studies demonstrating that both XT-I and XT-II catalyze the rate-limiting step in galactosaminoglycan biosynthesis [62, 100, 101]. Furthermore, shedding of glycosyltransferases has been shown to be a mechanism for downregulating their cellular activity [102]. With the role of the xylosyltransferases being the committing step enzymes of chondroitin sulphate, dermatan sulphate, heparan sulphate and heparin biosynthesis, the release of the xylosyltransferases would be an elegant mechanism for controlling the synthesis of the lateral glycosaminoglycan chains. Although the biological role of xylosyltransferase secretion has not been solved, this feature offers new diagnostic opportunities to measure the proteoglycan biosynthesis rate. The simultaneous secretion of xylosyltransferase and large proteoglycans makes the quantification of xylosyltransferase activity in body fluids a valuable tool for non-invasive determination of the actual proteoglycan synthesis rate, which is of importance in pathological tissue remodeling processes [28, 32, 87, 96, 97, 103, 104].

Enzyme structure

To date little information is known regarding the protein structure of the human xylosyltransferases. In the last few years, X-ray crystal structures of a couple of glycosyltransferases in the presence and absence of their donor substrates have been reported [105], and although glycosyltransferases have been classified into more than 80 different families based on sequence similarity [106], only two major fold types have been observed in crystal structures [107]. The GT-A fold is a single-domain α/β/α structure and was found in all metal ion-dependent glycosyltransferases. On the other hand, the GT-B fold represents a two-domain α/β/α structure and is characteristic for metal-ion-independent glycosyltransferases [108]. Both xylosyltransferases share the type II transmembrane topology which is typical for Golgi-resident glycosyltransferases, with a cytoplasmic tail, a single membrane-spanning domain, and a luminal part consisting of the stem region and the catalytic domain. A crystal

structure of either xylosyltransferase has not been resolved and, due to the low degree of sequence similarity to other glycosyltransferases, *in silico* approaches using database-deposited structural information have not yet been successful. Just recently, the crystal structure of the leukocyte-type core 2 β1,6-N-acetylglucosaminyltransferase was published [108], which is a key enzyme in the biosynthesis of branched O-glycans and which also belongs to the glycosyltransferase family 14 in the CAZy database (http://www.cazy.org) [106]. As the xylosyltransferases form a subgroup within the family 14 glycosyltransferases [29, 106], this novel structure might facilitate gaining information on the xylosyltransferase enzyme model. Among the conserved residues in XT-I are 14 of the 15 cysteine residues which are completely conserved among all mammalian XT-I [23]. Conserved cysteine residues occur in many glycosyltransferases [109, 110], and intra- and intermolecular disulphide bonds have been demonstrated [111]. These disulphide bonds may facilitate or stabilize protein folding, or even be important for glycosyltransferase activity [112, 113]. Only the cysteine residues Cys^{276}, Cys^{471}, Cys^{561}, Cys^{572} and Cys^{574} are critical for the active conformation of XT-I, as alterations of these residues lead to a complete loss of function [23]. Substitution of the other conserved cysteine residues does either not affect enzyme activity or results in a less than 50% reduced catalytic activity. Soluble recombinant human XT-I has been shown to form seven disulphate bonds and to have no free thiol groups [23]. Analysis of the electrophoretic mobility of XT-I under non-reducing and reducing conditions indicated that soluble XT-I does not form homodimers [23]. Furthermore, 260 amino acids at the aminoterminal end of human XT-I can be deleted without any loss of enzyme activity [22]. However, deletion of 272 amino acids results in an inactive protein, indicating a crucial role for the 12-amino acid motif Gly^{261}-Lys-Glu-Ala-Iso-Ser-Ala-Leu-Ser-Arg-Ala-Lys^{272} for proper folding, which is supported by the observation that a selected deletion of this motif leads to a complete loss of activity [22].

Interactions of human xylosyltransferases and heparin

The polysulphated glycosaminoglycan heparin is a non-competitive inhibitor of XT-I [39] and the enzyme binds to heparin with high affinity, which could be successfully utilized for efficient purification of native [17, 42, 97, 114] and recombinant XT-I [21]. Heparin binding sites are composed of either short stretches of positively charged amino acids, a combination of amino acids which are brought into close proximity due to protein conformation, or follow the Cardin-Weintraub rules, which identified a b-Arg/Lys-Arg/Lys-b-Arg/Lys-b or b-Arg/Lys-Arg/Lys-Arg/Lys-b-b-Arg/Lys-b (with 'b' representing nonpolar or hydrophobic amino acids) consensus sequence for heparin binding [115–117]. Heparin binding of XT-I is independent of protein conformation, as binding is not abolished by protein denaturation [22]. Furthermore, a sequence motif similar to the Cardin-Weintraub consensus sequences was located in human XT-I. However, substitution of the basic amino acids within this motif or deletion of the whole motif did not result in significantly reduced heparin binding [22], suggesting that this motif is not the primary binding site for heparin and that other positions in the amino acid sequence of XT-I, which do not follow the Cardin–Weintraub rules, mediate the interaction with heparin. Many short clusters of basic amino acids are scattered throughout the sequence of XT-I, leading to the hypothesis that strong binding of the enzyme to heparin depends on a multiplicity of basic clusters in the sequence and not just on one binding site [22]. Recently, it was shown that XT-II is also strongly inhibited by heparin and binds to immobilized heparin [J. Carrera Casanova and C. Götting, unpublished data]. The biological role of this endproduct inhibition is not yet clear, as the high binding affinity and catalytic inhibition seem to be specific for heparin. Chondroitin sulphate and heparan sulphate have also been shown to inhibit xylosyltransferase activity at higher concentrations [23, 97]. Therefore, it cannot be excluded that the interaction between the xylosyltransferases and heparin occurs simply due to the polyanionic character of this oversulphated glycosaminoglycan. Furthermore, it is doubtful whether this interaction is of relevance *in vivo*, as heparin is not present in the Golgi apparatus where xylosylation of the core protein occurs. As XT-I and XT-II are released from the Golgi compartments into the extracellular space, xylosyltransferases and heparin would be able to interact in the extracellular matrix, but the lack of donor substrates would preclude the inhibition of any catalytic function.

Determination of xylosyltransferase activity in biochemistry and clinical diagnostics

The quantification of xylosyltransferase activity in blood or body fluids has been successfully used to monitor proteoglycan biosynthesis during physiological and pathological tissue remodeling processes. Consequently, the development of methods suitable for the sensitive and precise determination of xylosyltransferase activity was demanded for use in diagnos-

tic applications. However, the xylosyltransferases are only present in minute amounts within cells and body fluids, resulting in major technical difficulties when setting up a robust and convenient assay. The methods for quantifying xylosyltransferase activity are based either on the incorporation of radiolabeled xylose into a suitable acceptor protein or the quantification of xylosylated peptides by mass spectrometry.

First assays of xylose transfer contained the xylosyltransferase enzyme solution as well as endogenous unknown xylose acceptors [6, 7, 12]. However, such crude systems contained two unknown dependent variables, enzyme concentration and acceptor concentration, which did not allow for reliable quantification of enzyme levels. After identification of the xylose acceptor proteins and production of exogenous acceptors, the development of specific radiochemical XT activity assays became possible. A first assay used Smith-degraded proteoglycan from bovine nasal cartilage as an exogenous acceptor [13, 36]. Many small molecular weight compounds had been tested as potential acceptors, including free serine, serine derivatives, and serine-containing peptides, but the best xylose acceptor known at that time was obtained by Smith degradation of the entire proteoglycan molecule. However, this assay was very disagreeable and time-consuming. For instance the preparation of Smith-degraded proteoglycan involves sequential periodate oxidation, borohydride reduction and mild acidic hydrolysis of the isolated proteoglycans over a period of 6 days. A modified procedure for preparation of Smith-degraded proteoglycan was published in 1979 [118]. This new method involved the separation of periodate-oxidized core proteins from chondroitin sulphate by size exclusion chromatography and thereafter reduction of the former in $H_3BO_3/NaBH_3$ at pH 8.5. The resulting product exhibited high acceptor activity for xylosyltransferase from embryonic-chick cartilage with an apparent K_M of 160 μg/ml or 45 μM (calculated per serine residue). Although the characterization of the [^{14}C]xylose-labeled Smith-degraded proteoglycans was shown with different methods like electrophoretic mobility, paper chromatography and thin-layer chromatography [36], no definitive identification of the enzyme product was reached. In 1980 a further method for removing polysaccharides from chondroitin sulphate proteoglycans was described [37]. Here, core proteins with high xylosyltransferase acceptor activity were prepared by hyaluronidase digestion from rat chondrosarcoma chondroitin sulphate proteoglycans, followed by treatment with 70% polyhydrogen fluoride. Since the preparation of macromolecular core protein substrates by Smith degradation or polyhydrogen fluoride treatment of cartilage proteoglycans entailed a substantial experimental effort and resulted in acceptor proteins with a high degree of variability, efforts where made to find commercially available proteins containing Ser-Gly sequences, which are suitable as xylosyltransferase acceptors. Silk fibroin from the silkworm *Bombyx mori* was found to be an excellent xylose acceptor [38]. Pieces of silk could be used as xyloyltransferase acceptors directly in the reaction mixtures, but a substantially greater incorporation of [^{14}C]xylose into silk fibroin was measured with preparations which had been dissolved in 60% lithium thiocyanate and subsequently dialyzed exhaustively. It was shown that the incorporated [^{14}C]xylose measured by liquid scintillation spectrometry was dependent on time, enzyme concentration and the amount of silk in the reaction mixture. A comparison between silk fibroin and Smith-degraded proteoglycan showed that silk was clearly the better substrate and had a V_{max} value two to three times higher than that of proteoglycan derivative [38]. However, a comparison based on the molar concentrations of reactive serine residues could not be made because precise information regarding the relative proportions of xylosylated and unxylosylated serine residues was not available for both substances. Unfortunately, the xylosyltransferase acceptors used until then were not suitable for a reliable determination of xylosyltransferase activity in blood and body fluids. The development of an assay which was sensitive enough to quantify xylosyltransferase activity in serum with high precision was possible using non-glycosylated recombinant bikunin, shown to be an excellent xylosyltransferase acceptor [40, 41]. The acceptor activity of recombinant non-glycosylated bikunin was more than 250-fold higher than that for deglycosylated cartilage proteoglycan and therefore allowed for the measurement of xylosyltransferase in human serum [41]. In addition, the recombinant protein is a single, well-defined compound in contrast to deglycosylated cartilage proteoglycan and silk fibroin, which have a high number of potential glycosylation sites per molecule. Synthetic peptides derived from the sequence of the chondroitin sulphate attachment site in bikunin ^{6}Glu-Glu-Glu-Gly-Ser-Gly-Gly-Gly^{13} were good xylosyltransferase acceptors and were used in radiochemical xylosyltransferase activity assays (Fig. 3) [16, 41]. Furthermore, the commercially available fibroblast growth factor 2 (1–24) peptide is well suited as a xylosyltransferase acceptor and was used in a radiochemical assay to measure xylosyltransferase activity in cell culture supernatant and human body fluids [39]. The radiochemical xylosyltransferase activity assays developed so far allowed for an accurate and sensitive determination of xylosyltransferase activity. But these xylosyltransferase assays have significant disadvantages,

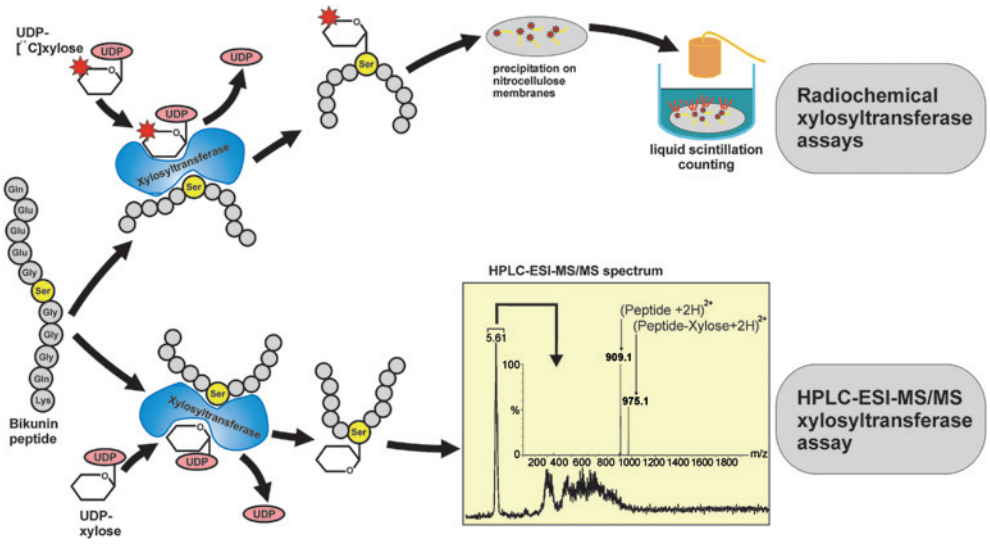

Figure 3. Determination of serum xylosyltransferase activity by incorporation of [^{14}C]xylose and by HPLC-electrospray ionization tandem mass spectrometry. Schematic outline of the principles of xylosyltransferase assays which are suitable for determining of xylosyltransferase activities in blood and other body fluids and which are applicable in clinical laboratories. The upper part illustrates the xylosyltransferase-catalyzed incorporation of [^{14}C]xylose into a bikunin-derived synthetic peptide. Unbound UDP-[^{14}C]xylose is removed from the assay mixture by trichloroacetic acid precipitation of proteins and peptides on nitrocellulose membranes. The amount of radiolabeled xylosylated peptide is then quantified by liquid scintillation counting. The lower part shows the novel HPLC-ESI-MS/MS assay for determining of xylosyltransferase activity. A modified bikunin-homologous peptide (m/z 909.1) is subjected to xylosyltransferase-mediated xylosylation, and the amount of xylosylated peptide (m/z 975.1) is quantified by HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS). This automated assay allows a precise and rapid determination of xylosyltransferase activity for a broad range of clinical applications with a total analysis time per sample including sample preparation of less than 90 min compared to 6 h using the radiochemical activity assay.

including time-consuming drying and washing steps and the use of expensive radiolabeled UDP-[^{14}C]xylose. In addition, automation of these assays is not possible. In 2002 a MALDI-TOF-coupled xylosyltransferase assay was described within the context of characterizing the *Drosophila melanogaster* xylosyltransferase [26]. The assay was used to demonstrate the transfer of xylose from UDP-xylose to the peptide Asp-Asp-Asp-Ser-Ile-Glu-Gly-Ser-Gly-Gly-Arg and to generate the kinetic data of fly xylosyltransferase, but no further characterization of the assay was performed. A novel, rapid, sensitive and specific HPLC (high-performance liquid chromatography) -electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) method to assay xylosyltransferase activity in serum and other body fluids using the synthetic peptide biotin-NH-Gln-Glu-Glu-Glu-Gly-Ser-Gly-Gly-Gly-Gln-Lys-Lys(5-fluorescein)-$CONH_2$ as a xylosyltransferase acceptor was recently described (Fig. 3) [119]. The sample preparation of this liquid chromatography-tandem mass spectrometric (LC-MS/MS) xylosyltransferase activity assay includes simple incubation, heat denaturation and centrifugation steps, and was very easy and relatively fast in comparison with the radiochemical assays. Furthermore, this method required only small amounts of biological materials (for example 5–50 µl serum). Measurement with the LC-MS/MS system was automated and, with a cycle time of approximately 3 min, very fast. Validation of the LC-MS/MS xylosyltransferase activity assay was performed according to the requirements for bioanalytical method validations and diagnostic accuracy [120–122]. The measurement of xylosylated peptide was linear over a wide range of concentration, and excellent sensitivity allowed for the precise quantification of serum xylosyltransferase, even in patients with severely reduced xylosyltransferase activities [119]. Due to the central role of xylosyltransferases in proteoglycan biology, the measurement of its enzyme activity in body fluids is of great interest. Recent developments in mass spectrometry have finally made the introduction of LC-MS/MS into clinical laboratories possible [123, 124]. The later xylosyltransferase activity assay uses this technology, facilitating the quantification of human xylosyltransferases in diagnostic applications.

Xylosyltransferases, biochemical markers of fibrosis and tissue remodeling

Proteoglycans play an important role in numerous physiological and pathological processes. Many of these processes, especially those which are accompanied by an accumulation of extracellular matrix components and connective tissue remodeling, are

characterized by increased proteoglycan biosynthesis and deposition. The diagnosis of pathological conditions and the monitoring of disease activity is of high clinical relevance, and many biochemical markers are utilized for the assessment of disease states and organ disorders. Especially proteins and enzymes present in the peripheral blood are attractive target structures for laboratory diagnostics, as they are accessible by non-invasive or minimally invasive standard procedures like venous blood drawing. However, an increased proteoglycan synthesis in the organ systems cannot be determined by analysis of marker proteins or metabolites in the peripheral blood, as proteoglycans are, on the one hand, localized in the extracellular matrix, and, on the other hand, represent a protein family with a high degree of structural diversity. Consequently, the search began for other marker proteins which are present in the peripheral blood and which can be quantified with high sensitivity and analytical precision. The properties of xylosyltransferases made them attractive targets as biochemical markers for the quantification of the actual proteoglycan biosynthesis rate: a) they catalyze the initial and rate-limiting step in the biosynthesis of glycosaminoglycans [25, 100, 101]; b) they are shed from the Golgi membrane and are secreted together with the proteoglycan in the extracellular space [19, 25, 42, 87]; c) assays have been developed that allow the sensitive and precise determination of xylosyltransferase in body fluids [41] and that are also suitable for high-throughput analyses in routine diagnostics [119]. While the biological role of xylosyltransferase secretion is not understood, the quantification of xylosyltransferase activity in serum and other body fluids could be successfully validated as a marker of the proteoglycan biosynthesis rate (Table 1). Furthermore, it is independent of patients' renal function, which makes it broadly applicable in many patient groups, including those with renal impairment [104]. Recently, XT-II was shown to have xylosyltransferase activity [25, 33, 34] and also to be secreted into the extracellular space [25]. As no acceptor peptides or proteins have yet been identified that are specific for either xylosyltransferase isoform, the xylosyltransferase activity in body fluids has to be considered to be a mixture of XT-I and XT-II activity.

Fibrosis is a systemic process of tissue remodeling where the functional cell types are replaced by connective tissue. The biosynthesis of proteoglycans and collagens and the excessive accumulation of extracellular matrix components are characteristic for fibrotic and sclerotic processes [125–127]. An increased proteoglycan synthesis in the affected organs could be shown in many tissue alterations, including systemic sclerosis (SSc), where increased chondroitin sulphate and dermatan sulphate contents were found in skin biopsies and where dermal fibroblasts from sclerotic lesions have an elevated proteoglycan biosynthesis [128–132]. Furthermore, elevated proteoglycan biosynthesis was also found in other fibrotic processes, like liver fibrosis [133–135] or dilated cardiomyopathy, which is characterized by structural remodeling processes of the ventricular wall [136–138]. These findings allude to the important role of proteoglycans in fibrotic tissue alterations, making them potential target molecules for exogenous modeling of connective tissue. In systemic and progressive diseases the assessment of disease activity is important for evaluation of the patient's prognosis and therapeutic options. Serum xylosyltransferase activity was found to be a suitable marker for the assessment of disease activity in SSc [28, 87]. Patients suffering from diffuse SSc, which is characterized by rapid disease progression and a more fatal outcome, have higher xylosyltransferase activity than patients with limited SSc. Furthermore, xylosyltransferase levels changed less than 10% within 1 year in patients with a constant clinical disease activity score [87]. High xylosyltransferase activities were also found in other fibrotic states. In lung tissues from rats with bleomycin-induced pulmonary fibrosis [139] and in human heart biopsies from patients with dilated cardiomyopathy, high xylosyltransferase expression was observed [62]. In the latter, samples from the left ventricular wall had higher XT-I expression than specimens from right ventricles, presumably due to the increased mechanical stress in the left ventricle. Furthermore, the implantation of ventricular assist devices in patients with dilated cardiomyopathy resulted in a subsequently decreased XT-I expression [C. Prante, H. Milting and C. Götting, unpublished data]. In a cell culture cardiomyopathy model, human cardiac fibroblasts subjected to cyclic mechanical stress also showed increased XT-I expression and glycosaminoglycan content, providing the mechanistic link between the xylosyltransferase, proteoglycans and heart fibrosis. Here, XT-I expression was regulated by the TGF-β_1 signaling pathway [62].

Pseudoxanthoma elasticum (PXE) is a hereditary degenerative connective tissue disease which is characterized by progressive calcification and fragmentation of elastic fibers in the connective tissues and the occurrence of angioid streaks in the retina. Mutations in the *ABCC6* gene encoding an ABC transporter are causative for this disease, although the pathobiochemical mechanisms still remain elusive [140–142]. Prominent changes in the extracellular matrix occur in PXE patients, including massive accumulation of proteoglycans [143, 144] and altered proteoglycan metabolism [145, 146]. This stimulated proteoglycan biosyn-

Table 1. Xylosyltransferase levels in physiological and pathological states.

Condition	Organism	Body fluid	Alteration	Clinical relevance / remark[a]	Publication
Rheumatoid arthritis	human	synovial fluid	↑	higher XT activity in synovial fluid from rheumatoid arthritis patients compared to synovia from patients with arthritis urica and osteoarthrosis	[98]
Osteoarthritis	human	blood serum	↑	high XT activity in woman with osteoarthritis	[32]
Osteoarthritis with NSAIDS treatment	human	hip cartilage	↓	salicylates and indomethacin treatment results in reduced XT activity	[168]
Lathyrism	chicken	embryonic cartilage	↓	reduced XT activity in embryonic cartilage after β-aminopropionitrile treatment	[169]
Manganese deficiency	chicken	epiphysial cartilage	↑	increased XT activity in chicken cartilage after manganese-deficient diet	[170]
Fetal skeletal growth	sheep	costal cartilage	→	XT activity correlates with growth rate of fetal vertebral column	[149]
Juvenile bone formation	human	blood serum	↑	high XT activity correlates with osteogenesis marker bone alkaline phosphatase	[C. Götting, unpublished data]
Cartilage aging	rat	costal cartilage	↓	low XT activity in cartilage from old rats	[150]
Systemic sclerosis	human	blood serum	↑	suitable marker for the assessment of disease activity in SSc	[28, 87]
Dilated cardiomyopathy	human	heart biopsy	↑	elevated XT activity and XT-I mRNA expression in end-stage heart fibrosis	[62]
Pulmonary fibrosis	rat	lung tissue	↑	high XT activity after bleomycin-induced lung fibrosis	[139]
Menstrual cycle	human	blood serum	→	XT activity correlates with β-estradiol concentration during menstrual cycle	[41]
In vitro fertilization with superovulation	human	follicular fluid	↑	highest activity ever found in body fluids	[97]
Male infertility	human	seminal plasma	↓	low XT activity present in semen of men with oligo-, astheno-, teratozoospermia	[96]
Renal insufficiency	human	blood serum	→	serum XT activity is independent from renal function. This is an important feature for its broad use as fibrosis marker	[104]
Disturbed blood-brain barrier	human	liquor cerebro-spinalis	↑	high XT activity in patients with impaired blood-brain barrier	[42]
Pseudoxanthoma elasticum	human	blood serum	↑	high serum XT activity in PXE patients. Hypertensive PXE patients had higher activities compared to normotensive patients	[103]

[a] XT, xylosyltransferases; XT-I, xylosyltransferase I; PXE, pseudoxanthoma elasticum; SSc, systemic sclerosis; NSAIDS, non-steroidal anti-inflammatory drugs.

thesis is reflected by increased serum xylosyltransferase activities in PXE patients [103]. Interestingly, hypertensive patients had higher xylosyltransferase activities than age- and sex-adjusted normotensive patients, which correlates with an elevated proteoglycan biosynthesis rate in vascular tissues during hypertension [147, 148].

Proteoglycans play an important role in cartilage and bone formation, where they build up the extracellular matrix or serve as a scaffold for bone mineralization. This increased proteoglycan biosynthesis during skeletal growth and bone formation also results in increased xylosyltransferase activity, which was shown to correlate with the growth rate of the vertebral column in sheep [149] and with bone formation markers in humans [C. Götting, unpublished data]. Furthermore, the osteogenic differentiation of mesenchymal stem cells also leads to a stage-dependent increase in xylosyltransferase expression [B. Müller and C. Götting, manuscript submitted], which is also observed during chondrogenic differentiation of stem cells [59]. On the other hand, reduced xylosyltransferase levels are found during cartilage aging [150]. Degenerative joint diseases are characterized by a progressive erosion of the cartilage, including degradation and release of proteoglycans. Elevated xylosyltransferase levels were found in rheumatoid arthritis and osteoarthritis, reflecting the progressive damage of the articular cartilage and the cellular proteoglycan re-synthesis mechanism [32, 98].

In osteoarthritis, higher serum xylosyltransferase levels were found in patients with a longer disease course [32].

Human xylosyltransferases are subjected to hormonal regulation, with serum xylosyltransferase activities changing in women during the menstrual cycle and correlating with β-estradiol levels [41]. In follicular fluids from women undergoing *in vitro* fertilization with hormone-induced superovulation, the xylosyltransferase activities determined were the highest ever found in body fluids [97]. This correlates with massively increased proteoglycan biosynthesis during folliculogenesis [151, 152]. Proteoglycans also affect sperm motility and capacity [153, 154], and reduced xylosyltransferase activities were determined in seminal plasma from infertile males suffering from oligo-, astheno- and teratozoospermia [96].

Chondroitin sulphate proteoglycans are important components of the extracellular matrix in the nervous system and the brain. On the other hand, these molecules are now known to play a fatal role after spinal cord injury, as they form the glia scar, which prevents axon outgrowth and nerve regeneration [155]. Recent progress for achieving axon outgrowth after spinal cord injury was made by degradation of glia scar proteoglycans through chondroitinase ABC treatment [156]. A different approach selected the xylosyltransferases as target molecules to inhibit the formation of the glia scar. XT-I antisense oligonucleotides were used to inhibit glycosaminoglycan biosynthesis, which resulted in reduced chondroitin sulphate proteoglycan content and an elevated axon regeneration [157, 158]. These studies point to the crucial role of proteoglycans in biology and to the xylosyltransferases as potent effector molecules for the modulation of proteoglycan synthesis.

Sequence variations in the xylosyltransferase genes

Glycosylation defects are causative for a larger number of hereditary diseases and are often associated with a severe phenotype with multisystemic defects, including psychomotoric and neurological retardation. Defects in O-glycosylation also affect the proteoglycan superfamily, where pathologies with defects in glycosaminoglycan biosynthesis, sulfatation or core-protein synthesis are known (for review see [159] and [160]). To date, no naturally occurring xylosyltransferase defect is known, pointing to the essential role of the proteoglycans and the xylosyltransferases for life. Xylosyltransferase defects would presumably result in severe phenotypes, comparable to those of the progeroid variant of Ehlers-Danlos syndrome, which is caused by defects in the β1,4-galactosyltransferase 7 (β4GalT7) [161, 162]. The progeroid variant of Ehlers-Danlos syndrome is a very rare disorder, where mutations in β4GalT7, which catalyzes the transfer of galactose to the xylosylated core protein, result in reduced enzyme activity. As two xylosyltransferase genes are present in the human genome, it is a matter of speculation whether one xylosyltransferase can rescue a defect in the other xylosyltransferase gene in order to ensure a vital phenotype.

Besides those pathologies where enzymes involved in proteoglycan metabolism were identified to be causative, other diseases with malfunctions in organs and tissues where proteoglycans ensure structural and functional integrity have been investigated. Here, mutations in the genes of enzymes involved in proteoglycan assembly can serve as disease modifiers. Recently, the XT-I and XT-II coding genes were scanned for sequence variations in order to identify mutations in these genes that may act as risk factors in proteoglycan-associated pathologies [30–32, 163, 164]. In total, 9.4 kb of the coding sequence of the *xylt-1* and *xylt-2* genes in more than 250 patients and healthy blood donors were analyzed by denaturating HPLC and DNA sequencing. Furthermore, selected sequence variations were determined in more than 1500 humans. The xylosyltransferase coding genes were found to be not polymorphic, as only 47 sequence variations were identified. Seven missense variations were detected in the *xylt-1* gene (Table 2) and eight missense variations in the *xylt-2* gene (Table 3), which were all present at frequencies lower than 5% and, with the exception of p.A115S in the *xylt-1* gene, occurred only in heterozygous states. These results and the absence of nonsense mutations, frame-shift alterations and splice-site mutations point to the important role of the xylosyltransferases for human life. However, some sequence variations in xylosyltransferase genes that serve as major risk factors for proteoglycan-associated diseases could be identified (Table 2, Table 3) [31, 32, 163].

Proteoglycans are important components of the glomerular basement membrane and are responsible for the size- and charge-dependent permeability of the basement membrane [1, 165]. Qualitative and quantitative alterations in the glomerular proteoglycans lead to increased permeability and impaired renal function [165]. These alterations are found for example in patients with diabetic nephropathy, which affects approximately 1/3 of all patients with type 1 diabetes. Diabetic nephropathy is the major cause for terminal renal insufficiency, which is associated with high mortality [166]. The pathogenesis of diabetic nephropathy is multi-factorial, and multiple genetic risk factors are discussed [167]. Scanning of xylosyl-

Table 2. Naturally occurring sequence variations in the human *xylt-1* gene.

Polymorphism[a]	Amino acid	Region	Allelic frequency[b]	Clinical relevance	Publication
IVS1–5C>G		5'-UTR	C		[30–32]
c.343G>T	p.A115S	exon 1	A	type 1 diabetes [164], diabetic nephropathy [163], high XT serum levels [31]	[30–32, 163, 164]
IVS2+6T>C		intron 2	A		[32]
IVS2+29A>G		intron 2	A		[32]
IVS3+10C>T		intron 3	A	type 1 diabetes [164]	[32, 164]
IVS3+30G>C		intron 3	A	type 1 diabetes [164]	[32, 164]
c.1077C>T		exon 4	B		[30, 31, 164]
IVS4+47A>G		intron 4	D		[30, 32, 164]
c.1154C>T	p.P385L	exon 5	A		[30, 32]
c.1216C>T	p.R406W	exon 5	A		[31]
c.1284C>G		exon 5	C		[30–32, 164]
IVS6–34G>A		intron 5	A		[164]
IVS8–3G>A		intron 7	A		[30, 164]
c.1655T>G	p.I552S	exon 8	A		[32]
c.1756C>T	p.R586C	exon 8	A		[164]
c.1875G>A		exon 9	A		[30]
c.1883G>A	p.R628H	exon 9	A		[30]
c.1918G>A	p.G640S	exon 9	A		[164]
c.1989T>C		exon 9	C	low blood pressure in type 1 diabetes [30, 163]	[30–32, 163, 164]
c.1994C>T	p.T655M	exon 9	A		[31, 164]
IVS10–35C>A		intron 9	D		[164]
IVS10+100A>G		intron 10	n.d.		[32]
c.2515G>A		exon 11	A		[164]
c.2631C>T		exon 12	C		[30–32, 164]
c.2675G>A	p.R892Q	exon 12	A		[30]
c.2766G>A		exon 12	A		[30]

[a] Numbering of the *xylt-1* variations is based on human cDNA sequence (GenBank accession no. NM_022166) with the A of the ATG translation initiation start site as nucleotide +1.
[b] Classified allelic frequency of the mutant allele in the investigated populations: A, <5%; B, 5–10%; C, 10–50%; D, >50%; n.d., not determined

transferase genes in type 1 diabetic patients showed that the *xylt-1* variation p.A115S confers a 2.5-fold increased risk for diabetic nephropathy [30, 163]. This is the first evidence that mutations in the enzymes involved in proteoglycan assembly can serve as risk factors for diabetic nephropathy. p.A115S is located in the stem region of XT-I and probably results in altered shedding or Golgi retention. Therefore, it was proposed that this mutation can affect the proteoglycan re-synthesis capacity of the glomerular basement membrane and lead to susceptibility for renal impairment. Other mutations in the xylosyltransferase-coding genes are also suspected to affect the proteoglycan re-synthesis rate and cartilage repair, for example the sequence variation c.1569C>T in the *xylt-2* gene, which was associated with an early disease onset of osteoarthritis [32].

Patients with PXE suffer from prominent extracellular matrix alterations and altered proteoglycan metabolism, which is also reflected in increased serum xylosyltransferase activity [103]. Four different mutations in the *xylt-2* gene could be linked to a more severe disease course and early disease onset [31]. Carriers of the p.D56N mutation had a more than 13-fold increased risk of multiple organ involvement and a severe disease course, showing that this XT-II mutant is a prominent disease modifier for the PXE phenotype. Furthermore, patients harboring the p.A115S mutation had higher serum xylosyltransferase levels, indicating an influence of this sequence

Table 3. Sequence variation in the human *xylt-2* gene.

Polymorphism[a]	Amino acid	Region	Allelic frequency[b]	Clinical relevance	Publication
IVS1–86delG		5'-UTR	C		[30–32]
IVS1–84G>A		5'-UTR	A		[30–32]
IVS1–72G>C			n.d.		[31]
IVS1–35G>C			n.d.		[31]
c.166G>A	p.D56N	exon 2	A	severe disease course in PXE [31]	[30–32]
c.177A>G		exon 2	D	high blood pressure in type 1 diabetic patients [30]	[30–32]
c.342T>C		exon 2	D	high blood pressure in type 1 diabetic patients [30]	[30–32]
c.344C>T	p.P115L	exon 2	A		[31]
c.359G>A	p.R120H	exon 2	A	type 1 diabetes [30]	[30]
c.453C>T		exon 2	A		[30]
c.556G>A	p.A186T	exon 2	A		[30]
c.693G>A		exon 3	A		[30]
c.914C>G	p.T305R	exon 4	D	high blood pressure in type 1 diabetes [30]	[30–32]
IVS6–9T>C		intron 5	A		[30–32, 163]
IVS6–14_IVS6–13insG		intron 5	A		[30–32, 163]
c.1216C>T	p.R406C	exon 6	A		[32]
c.1253C>T	p.P418L	exon 6	A		[30–32]
IVS7+81A>C		intron 7	A		[30, 32]
c.1569C>T		exon 8	C	severe disease course in PXE [31]; osteoarthritis [32]; early disease onset in osteoarthritis [32]	[30–32]
c.2379G>A		exon 11	A		[30]
c.2391C>T		exon 11	A		[30]
c.2402C>G	p.T801R	exon 11	C	high blood pressure in type 1 diabetes [30]; early disease onset and severe disease course in PXE [31]	[30–32, 163]

[a] Numbering of the *xylt-2* variations was based on human cDNA sequence (GenBank accession no. NM_022167) with the A of the ATG translation initiation start site as nucleotide +1.
[b] Classified allelic frequency of the mutant allele in the investigated populations: A, <5%; B, 5–10%; C, 10–50%; D, >50%; n.d., not determined; PXE, Pseudoxanthoma elasticum.

variation on XT-I shedding or impaired Golgi retention [31].

Perspectives

Much progress has been made in recent years regarding the role of xylosyltransferases in biological processes and their involvement in disease states. XT-II, the function of which remained elusive for a long time, could now be proven to be a xylosyltransferase involved in the proteoglycan metabolism. Consequently, mammals do possess two active proteoglycan xylosyltransferases, but the differences between the xylosyltransferases and their role in cellular processes remain to be clarified in future studies. The observed differences in the tissue-expression patterns of XT-I and XT-II are probably not the only dissimilarity, as both enzymes are expressed in most tissues. It is likely that some of the hitherto untested core protein sequences are acceptor proteins specific for either XT-I- or XT-II-mediated xylosylation. The presence of two differentially regulated xylosyltransferase genes encoding proteoglycan xylosyltransferases with similar but not identical acceptor specificity is then proposed to be a potential tool in higher organisms to regulate posttranslational glycosaminoglycan biosynthesis and to adjust the proteoglycan pool in the cell differentially.

The simultaneous secretion of xylosyltransferase and proteoglycans into the extracellular space has provided an opportunity to validate the determination of xylosyltransferase activity as a biochemical marker of an altered proteoglycan biosynthesis rate. Increased proteoglycan biosynthesis occurs during fibrotic tissue remodeling, and serum xylosyltransferase activity

could be used as a first biochemical marker for the diagnosis of systemic fibrotic activity in humans. These findings provide new opportunities for using xylosyltransferase activity in laboratory diagnostics for the assessment of altered proteoglycan metabolism in disease states. Proteoglycans are crucially involved in many biological processes, and it is not surprising that sequence variations in the proteoglycan pathway genes can influence the proteoglycan biosynthesis rate and proteoglycan regeneration capacity. Mutations in the XT-I and XT-II genes were identified as disease modifiers and significant risk factors in multigenic diseases, like osteoarthritis and diabetic complications. These findings allude to hitherto unidentified sequence variations in the proteoglycan glycosyltransferase genes in proteoglycan-associated pathologies, which offer new diagnostic tools and a potential for understanding the role of proteoglycan biosynthesis in health and disease.

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