CMLS, Cell. Mol. Life Sci. 61 (2004) 794–809
1420-682X/04/080794-16
DOI 10.1007/s00018-003-3278-2

CMLS Cellular and Molecular Life Sciences

Review

The never-ending story of peptide *O*-xylosyltransferase

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Received 11 July 2003; received after revision 4 September 2003; accepted 24 September 2003

Abstract. In a journey lasting 40 years from the first reports on its activity in the 1960s to its purification and the cloning of relevant complementary DNAs, peptide *O*-xylosyltransferase has finally arrived at the same point as many other enzymes. This enzyme, whose systematic name is UDP-α-D-xylose:proteoglycan core protein β-D-xylosyltransferase (EC 2.4.2.26), catalyses the first step in the biosynthesis of chondroitin, dermatan and heparan sulphates in the endoplasmic reticulum and/or the cis-Golgi cisternae. Analyses of their primary structure show that peptide *O*-xylosyltransferases are members of glycosyltransferase family 14 and so are homologous to β1,6-*N*-acetylglucosaminyltransferases involved in O-glycan and poly-*N*-acetyllactosamine branching. Furthermore, vertebrates appear to have two rather similar genes encoding xylosyltransferase I and xylosyltransferase II, but enzymatic activity was only detected for a recombinant form of the first isoform. On the other hand, *Caenorhabditis* and *Drosophila* have each only one peptide *O*-xylosyltransferase gene. In the worm *sqv-6* mutant, a mutation of the xylosyltransferase gene is associated with defective vulval morphogenesis, indicative of the importance of the glycosaminoglycan chains of proteoglycans in animal development. There remain, however, open questions, for instance, on the enzyme's intracellular localisation and structure-function relationships.

Key words. Xylosyltransferase; proteoglycan; glycosaminoglycan; UDP-xylose; chondroitin sulphate; heparan sulphate.

Introduction

Xylosyltransferases catalyse the transfer of xylose from UDP-xylose to other biomolecules; however, the common name, reflecting transfer of the same sugar, hides the fact that the various types of xylosyltransferases are related to each other neither at the primary structural level nor as to the linkages formed. Examples of these enzymes include α1,6-xylosyltransferases involved in xyloglucan formation in plants (EC 2.4.1.169)[1]; β1,2-xylosyltransferases which create immunogenic epitopes on N-glycans of all plants and some invertebrates (EC 2.4.2.38); an *O*-xylosyltransferase that modifies zeatin, a plant cytokinin (EC 2.4.1.204)[1]; and – last but not least – peptide-modifying xylosyltransferases, the subject of this review, required to transfer the first sugar of various types of glycosaminoglycan chains (EC 2.4.2.26; see figs 1 and 2). Specifically, chondroitin, dermatan and heparan sulphates (including heparin) share a common tetrasaccharide core (GlcAβ1,4Galβ1,3Galβ1,4Xyl-*O*-Ser) to which many disaccharide repeats in series are attached as part of the biosynthesis of many proteoglycans [1]. The linear repeating unit for chondroitin and dermatan sulphates is based on GalNAcβ1,4GlcAβ1,3, while heparan sulphates and heparin have GlcNAcα1,4GlcAβ1,4 repeats [2, 3]. Their monosaccharide units can be subject to epimerisation and sulphation events;

[1] In the case of EC 2.4.1.169 and 2.4.1.204, xylosyltransferases have been mistakenly allocated EC numbers of a hexosyltransferase; xylose is actually a pentose, and so all xylosyltransferases should have numbers of the form 2.4.2.x. A revision of these numbers is indeed amongst the latest (June 2003) proposed Enzyme Nomenclature changes (see: http://www.chem.qmw.ac.uk/iubmb/).

the sulphate and hexuronic acid moieties result in a high degree of negative charges on these molecules, enabling them to perform a wide variety of biologically significant functions in the extracellular spaces.

Investigations by Muir [4] were the first to suggest the involvement of serine in the linkage of chondroitin sulphate chains to protein and subsequent work in the mid1960s by Rodén's group proved that xylose β-linked to serine is the 'core' residue linking chondroitin sulphate [5] and heparin [6] to protein. Modification of proteins by chondroitin, dermatan and heparan sulphates can therefore be considered to be a type of O-glycosylation, seemingly specific to animals and absent from plants, protozoans and fungi. As with other O-glycans, these types of chain are synthesised by the sequential addition of monosaccharide units to protein (see fig. 1), rather than being initiated by the transfer en bloc of a precursor oligosaccharide as is the case with N-glycans and glycophosphatidylinositol membrane anchors [7]. In the subsequent pages, we will follow the progress of research on the first step in this pathway, starting with the first detection of the relevant xylosyltransferase activity and continuing with our current, sometimes confused, knowledge on its properties, intracellular localisation, genetic structure and wider biological significance.

Defining xylosyltransferase acceptor specificity

The first reports on the transfer of xylose to endogenous protein acceptors date from the mid 1960s, making peptide *O*-xylosyltransferase one of the first glycosyltransferases to be described. In particular, transfer of radioactive xylose into trichloroacetic acid precipitable material by hen oviduct, murine mastocytoma and embryonic chicken cartilage extracts, as sources containing both enzyme and acceptor, was reported by the Neufeld [8, 9] and Dorfman groups [10]. Subsequently Baker et al. tested embryonic chicken cartilage as a source of xylosyltransferase with a number of different acceptor substrates, ranging from Smith-degraded (i. e. deglycosylated) proteoglycan to short peptides such as Ser-Gly-Gly [11]. Another deglycosylation treatment of chondroitin sulphate proteoglycan from rat chondrosarcoma using hy-

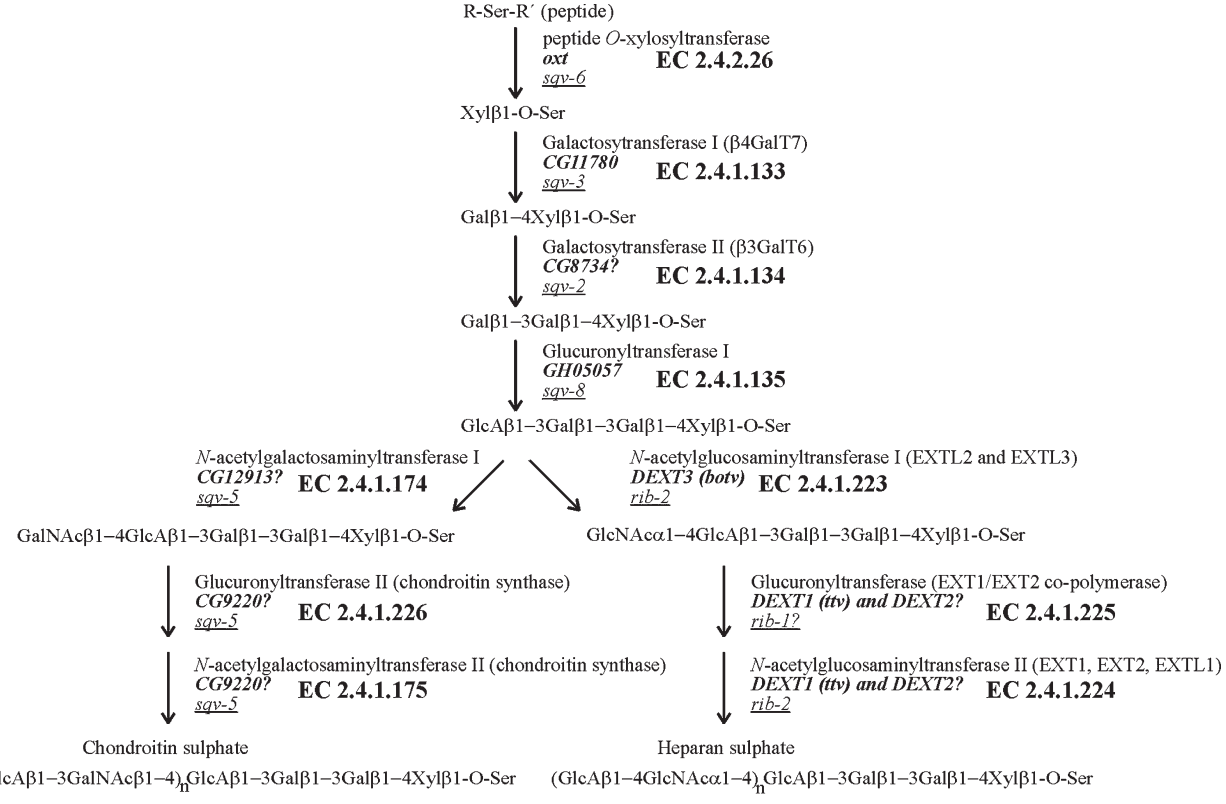

Figure 1. Biosynthesis of glycosaminoglycans. Mammals, flies and worms share many of the basic features of chondroitin and heparan sulphate biosynthesis, but probably that of worms is different in the phase of disaccharide repeat formation due to worm copolymerases also having initiating activity. Genes from flies (bold) and worms (underlined) that are proven to be, or are probably, involved in the relevant steps are indicated. Note: Fly genes are either named or are indicated by the relevant 'Celera Genome' reading frame number; the putative fly glucuronyltransferase I gene, though, was not automatically predicted as a reading frame, but is encoded by various ESTs such as the GH05057 indicated.

drofluoric acid also resulted in a suitable substrate [12], whereas silk fibroin from *Bombyx mori*, which contains Ser-Gly-Ala-Gly-Ala-Gly repeats, has approximately the same K_m value as Smith-degraded proteoglycan [13] (see also table 1). Another study showed transfer to Ser-Gly-Gly, Gly-Ser-Gly and Gln-Ser-Gly as well as Gly-Thr-Gly [14]. The latter result indicates that transfer by xylosyltransferase to threonine is, at least, theoretically possible. Indeed, site-directed mutagenesis of the Asp-Glu-Ala-Ser-Gly-Ile-Gly motif of decorin, which carries a single chondroitin/dermatan sulphate chain, showed that replacement of the serine by a threonine resulted in less than 10% of the mutant decorin molecules being modified; the majority of the threonine-containing form remained, however, unmodified [15]. Furthermore, the threonine-containing form of a bikunin-type peptide had a K_m value hundredfold higher than the corresponding serine-containing one [16].

The ability of xylosyltransferase to transfer to tripeptides containing the Ser-Gly motif reflects findings that this is a feature of many glycosaminoglycan attachment sites. Amongst the earliest determined sequences, bovine tra-

Table 1. K_m values for peptide substrates of peptide *O*-xylosyltransferase.

Enzyme source	Peptide substrate	K_m	Reference
Chicken embryonic cartilage	Smith-degraded chondroitin sulphate	0.064 mM 240 mg/L 143 mg/L	[11] [39] [13]
Rat chondrosarcoma	Smith-degraded chondroitin sulphate	0.18 mM	[14]
Human chondrocyte	Smith-degraded chondroitin sulphate	0.155 mM	[31]
Rat chondrosarcoma	HF-degraded chondroitin sulphate	1.02 mM (in terms of serine) 0.0029 mM 0.00011 mM	[14] [20] [28]
Chicken embryonic cartilage	silk fibroin $(SGAGAG)_n$	182 mg/L	[13]
Human chondrocyte	silk fibroin $(SGAGAG)_n$	0.545 mM	[31]
Human chondrocyte	bikunin protein	0.0009 mM	[31]
Chicken embryonic cartilage	SGG	20 mM 33 mM	[11] [13]
Rat chondrosarcoma	SGG GSG QSG GTG GSGSGSGSGS	55 mM 28 mM 8.5 mM 87 mM 0.04 mM (0.2 mM in terms of serine)	[14] [14] [14] [14] [14]
Rat chondrosarcoma	Decorin (PG40) peptide (CDEASGIGPDDRD) Rat L2 chondroitin sulphate precursor (PG19) peptide (SDDYSGSGSG) SAAYSGSGSG (replacement of acidic residues of L2 peptide)	0.037 mM 0.062 mM 0.26 mM 0.79 mM 1.39 mM	[20] [28] [20] [28] [20]
Rat ear cartilage	bikunin-type peptide (QEEEGSGGGQGG) QEEEGTGGGQGG (threonine-containing) QGGGGSGGGQGG (replacement of acidic residues)	0.008 mM 0.82 mM 8.6 mM	[16] [16] [16]
Human JAR choriocarcinoma	bikunin peptide (QEEEGSGGGQK) L-APP peptide (TENEGSGLTNIK) L-APLP2 peptide (SENEGSGMAQQK)	0.022 mM 0.020 mM 0.019 mM	[29] [29] [29]
Drosophila (*Pichia* recombinant)	syndecan peptide (DDDSIEGSGGR)	0.5 mM	[41]

cheal cartilage was found to contain modified Leu-Pro-Ser-Gly-Glu sequences [17], and the N-terminus of bovine skin proteodermatan sulphate has a xylosylated serine within the sequence Asp-Glu-Ala-Ser-Gly-Ile-Gly [18]; on the other hand, the single glycosaminoglycan attachment site of chicken type IX collagen is within the sequence Val-Glu-Gly-Ser-Ala-Asp [19]. This would indicate that a Ser-Gly motif is not an absolute acceptor site requirement; such a conclusion is supported by a site-directed mutagenesis study on the aforementioned decorin motif, in which substitution of either glycine residue by alanine had no significant effect on the degree of glycosaminoglycan modification [15].

Comparing the attachment sites on chicken type IX collagen and bovine skin proteodermatan sulphate highlights another potential requirement, in that both sequences have a glutamic acid residue at position –2 with respect to the serine. Indeed, a number of studies have indicated that acidic residues preceding Ser-Gly or Ser-Gly-Xaa-Gly are a common feature of glycosaminoglycan attachment sites [20–25]. Indicative of an accessory role of these acidic patches in substrate recognition is data showing that the respective replacement of the aspartate or glutamate residues within the peptides Ser-Asp-Asp-Tyr-Ser-Gly-Ser-Gly-Ser-Gly and Gln-Glu-Glu-Glu-Gly-Ser-Gly-Gly-Gly-Gln-Gly-Gly resulted in increased K_m values as compared with the 'parent' peptides [16, 20]. In one interesting case of control of glycosaminoglycan addition, removal of an exon in alternatively spliced isoforms of the messenger RNAs (mRNAs) of the Alzheimer β-A4-amyloid protein precursor (yielding L-APP, also known as appican) and an APP-like protein (yielding APLP2-751) result in generation of otherwise silent chondroitin attachment sites due to bringing a Ser-Gly motif in closer proximity to an upstream acidic patch [26, 27].

The various sequence alignment or site-directed mutagenesis studies have been used to design better peptide acceptors for in vitro assays. Partially purified rat chondrosarcoma xylosyltransferase has a K_m value of 37 or 62 µM for a peptide based on the sequence of human decorin (PG40) [20, 28], and human xylosyltransferase has K_m values of around 20 µM for peptides based on the sequences of human bikunin, L-APP and L-APLP2 [22, 29]. A peptide similar to the bikunin peptide also functions as an acceptor for *Caenorhabditis* (native and recombinant) and *Drosophila* (recombinant) xylosyltransferase [30, also J. Drexler, K. Paschinger and I. B. H. Wilson, unpublished data], showing a lack of species specificity of xylosyltransferases for their substrates. Recombinant forms of mammalian bikunins have also been used as substrates [31, 32]. In general, it seems that longer peptides, including deglycosylated proteoglycan core proteins or peptides containing multiple Ser-Gly motifs and/or acidic regions, are superior substrates compared with Ser-Gly-Gly. However, regardless of the amount of accumulated data on glycosaminoglycan attachment sites, there is no absolute certainty in predicting whether a particular site will actually be xylosylated; furthermore, electronic resources are limited in this respect, since the existing O-GLYCBASE database contains only 16 proven O-xylosylation sites [33].

As mentioned before, both heparan and chondroitin sulphate chains are attached to proteins through a xylose residue, and various studies have suggested that their attachment sites have somewhat different sequence contexts. For instance, a Trp residue and an acidic patch C-terminal to the target serine appears to direct heparan sulphate modification of a shortened form of betaglycan when this is expressed in Chinese hamster ovary cells; their replacement, however, generally results in a greater degree of chondroitin sulphate at this site, rather than a reduction in sulphate incorporation (used as a measure of overall glycosaminoglycan chain formation) [34]. Repetitive Ser-Gly sequences are another common feature of heparan sulphate attachment sites; increasing the distance between consecutive Ser-Gly motifs, however, reduces the percentage modification by heparan sulphate [35]. Other, more distant, regions of a core protein may also direct the specific type of glycosaminoglycan chain attached [36]. One may conclude that such effects are due not to the specificity or expression level of the xylo-

Table 2. K_m values for UDP-xylose of peptide *O*-xylosyltransferase.

Enzyme source	Peptide substrate	K_m UDP-Xyl	Reference
Rat chondrosarcoma	Smith-degraded chondroitin sulphate	10 µM	[40]
Chicken epiphyseal cartilage	Smith-degraded chondroitin sulphate	25 µM	[39]
Chicken embryonic cartilage	silk fibroin	12 µM	[13]
Rat chondrosarcoma	decorin peptide	180 µM	[28]
Rat ear cartilage	bikunin-type peptide	6.5 µM	[16]
Drosophila (*Pichia* recombinant)	syndecan peptide	100 µM	[41]

syltransferase, but of those of the later glycosyltransferases.

Origin of the donated xylose

Although the possibility that xylosylphosphoryldolichol is involved in glycosaminoglycan biosynthesis was once explored [37], the donor substrate for xylosyltransferase is indeed UDP-α-D-xylose. This nucleotide sugar is present in plants, some fungi and animals, but only in the latter case is the UDP-xylose generated used in vivo as a peptide *O*-xylosyltransferase donor. Whereas in animals proteoglycans are the main class of glycoconjugates containing xylose, plants, for instance, have xyloglucans and also generate UDP-L-Ara from UDP-D-Xyl [38]. Kinetic analyses of peptide *O*-xylosyltransferases from various sources have yielded K_m values for UDP-xylose ranging from 6.5 to 180 µM [13, 16, 28, 39–41] (table 2). Furthermore, it appears that in an ordered sequential reaction mechanism, UDP-xylose is the first substrate to bind xylosyltransferase, while the xylosylated peptide is the first product to be released [28]. Also, since the donor is UDP-α-D-xylose and the linkage formed is Xyl-β-Ser, xylosyltransferase can be defined as an inverting glycosyltransferase; however, a fuller examination of the enzyme's mechanism requires the still-awaited elucidation of its three-dimensional structure.

UDP-xylose is generated in vivo from UDP-glucose in two NAD^+-dependent steps (fig. 2). In the first, UDP-glucuronic acid is synthesised in the cytosol by the action of UDP-glucose 6-dehydrogenase (1.1.1.22); the gene encoding this enzyme is defective in the *Caenorhabditis elegans sqv-4*, *Danio rerio* (zebrafish) *jekyll* and *Drosophila melanogaster sugarless* (also known as *suppenkasper* or *kiwi*) mutants [42–46], which all display morphogenetic defects and/or lethality. The absence or near-absence of chondroitin or heparan sulphates in *sugarless* embryos and larvae was confirmed [45, 47], but no tests for the presence of the tetrasaccharide linker were performed. It is not clear whether the observed defects are specifically due to a lack of UDP-xylose or of UDP-glucuronic acid, since the latter is itself directly utilised by a number of enzymes in the later stages of chondroitin and heparan sulphate biosynthesis.

In the second step, formation of UDP-xylose itself is catalysed by UDP-glucuronic acid decarboxylase (UDP-D-glucuronate carboxy-lyase; EC 4.1.1.35). Nicotinamide adenine dinucleotide (NAD^+) is required to generate the putative transition state intermediate, which is then decarboxylated before being reduced by enzyme-bound NADH; the overall NAD^+ balance remains, thereby, unaffected [48]. Decarboxylase complementary DNAs (cDNAs) have been cloned from *Cryptococcus (Filobasidiella) neoformans* (a pathogenic fungus), *Pisum sativa* (pea), *Arabidopsis thaliana*, *Rattus norvegicus* and *Caenorhabditis elegans* (encoding the SQV-1 protein) [49–53]. Compatible with the trypsin insensitivity of UDP-xylose synthesis within intact chicken chondrocyte organelles [54], the decarboxylase in animals is apparently a lumenal enzyme with a type II transmembrane topology [50,53], whereas in plants cytoplasmic and membrane-bound forms are known [52]. This would suggest that in animals at least, UDP-glucuronic acid and not UDP-xylose is transported across the endoplasmic

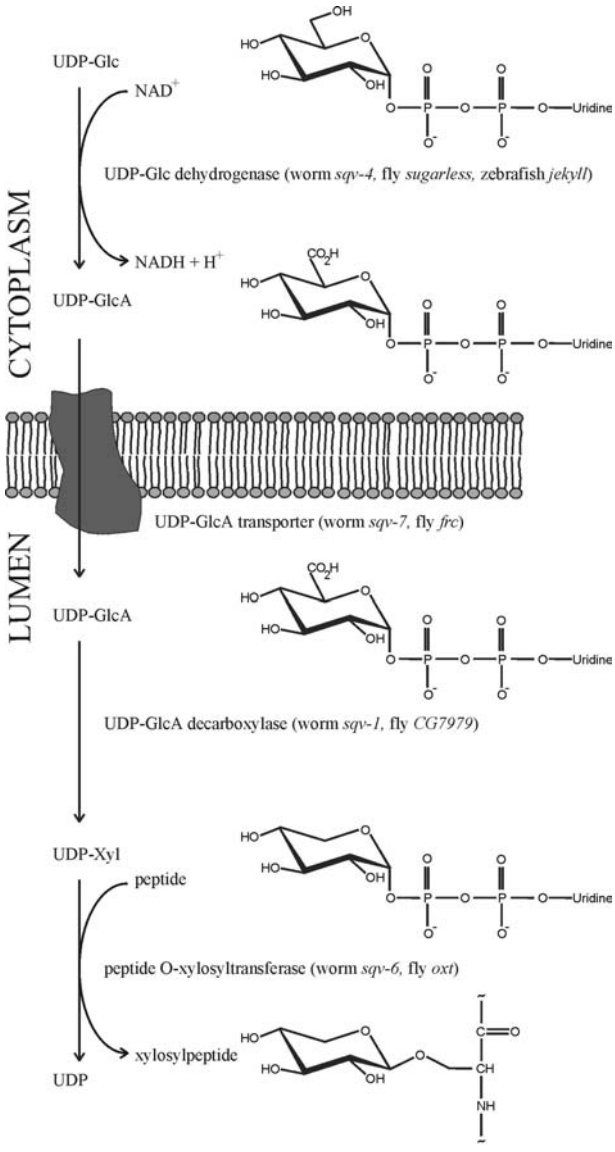

Figure 2. Biosynthesis of UDP-xylose and the peptide *O*-xylosyltransferase reaction. As described in the text, UDP-xylose is generated in two steps from UDP-glucose; the intermediate UDP-glucuronic acid is transported across the endoplasmic reticulum membrane and converted into UDP-xylose prior to its utilisation by xylosyltransferase. The names of relevant worm, fly or zebrafish genes are given in italics in brackets beside the enzyme/transporter names.

reticulum/Golgi membrane. The requisite transporter in *Caenorhabditis elegans* is the SQV-7 protein [55].

Cofactor and pH requirements

Many glycosyltransferases are sensitive to the presence of divalent cations; indeed, these cations may, at least in some cases, form a bridge between the pyrophosphate moiety of the nucleotide sugar and the enzyme [56]. Although the rat ear cartilage and recombinant *Drosophila* xylosyltransferases have some activity in the presence of EDTA, the enzyme is activated in the presence of Mn(II), Mg(II) and Ca(II) ions, but inhibited completely by Zn(II) ions [16, 41]. An aortic wall xylosyltransferase was also found to be activated by Mn(II) [57], while that in chicken embryo was activated by Mn(II) and Mg(II) ions [10]. The relevance of these cation requirements in vitro to the physiological state is unclear, but in yeast cells lacking the Pmr1p Ca(II)/Mn(II)-ATPase, it is Mn(II) and not Ca(II) that can partially restore normal glycosylation [58]. Other components often included in xylosyltransferase assays are potassium chloride and fluoride, but their presence is not necessary, and their inclusion in extraction buffers 30 years ago was probably an attempt to stabilise the enzyme and inhibit phosphatases.

The pH optima for xylosyltransferases lie between 6.0 and 8.0, depending on the enzyme source and the buffer used [8, 10, 16, 39, 41, 57], with only the two first studies suggesting an optimum of or below pH 6.5. How do these data compare with the intracellular pH values? A recent study suggested a pH value of 7.4 ± 0.2 in the endoplasmic reticulum and 6.2 ± 0.4 within the Golgi of live endocrine cells [59]. Thus, a pH optimum of 7–8, for instance, would be consistent with an endoplasmic reticulum location.

Purification of xylosyltransferase

The first attempts to purify the enzyme were made in the mid-1970s using chicken cartilage or rat chondrosarcoma extracts [39, 40, 60], the initial procedure of ammonium sulphate precipitation and gel filtration being supplemented by affinity chromatography on Smith-degraded proteoglycan. Gel filtration analyses of the enzyme from both sources suggested the native molecular mass was around 100,000, while SDS-polyacrylamide gel electrophoresis (PAGE) indicated the presence of bands of 23,000 and 27,000; this result led to the hypothesis that xylosyltransferase was composed of two sets of nonidentical subunits. However, for another 25 years there was no significant progress on this front.

In the year 2000 two groups reported the purification of xylosyltransferases from either rat ear cartilage [16] or cultured human choriocarcinoma cells [61]. In the former case, ammonium sulphate fractionation, heparin-affinity chromatography, gel filtration and chromatography on the bikunin dodecapeptide and again on heparin resulted in an apparently homogeneous protein with a molecular mass of 78,000 in 27% yield; in the latter case, ammonium sulphate fractionation, heparin-affinity, ion-exchange and protamine-affinity chromatography enriched a number of proteins, one of which was an N-glycosylated protein with a molecular mass of 120,000 (as judged by SDS-PAGE), a figure which is in agreement with the molecular masses deduced by gel filtration of the xylosyltransferases purified in the 1970s. Peptides from the putative human enzyme were sequenced and used not only to design polymerase chain reaction (PCR) primers, but also in order to synthesise a peptide against which an antibody was raised; this antibody then found application in affinity-enriching the enzyme and so proving that the M_r 120,000 species was indeed a xylosyltransferase.

Overall, the results of the recent studies suggest that the molecular masses deduced in the 1970s by SDS-PAGE were incorrect and that the observed bands of M_r 25,000 and 27,000 were due to a dominant copurifying set of polypeptides; this, therefore, perhaps complicates interpretation of data obtained with antibodies raised against 'old' preparations of the enzyme. Furthermore, since the gel filtration data suggest a native molecular weight of 100,000, one may conclude that xylosyltransferase exists not in an $(\alpha\beta)_2$ form, but as a monomer.

Cloning of xylosyltransferase cDNAs

The final breakthrough in 'taming' xylosyltransferase was cloning of the relevant mammalian cDNAs; exploiting the peptide sequence information they gained (see previous section), Kleesiek's group cloned one partial (XT-I) and one full-length (XT-II) cDNA each from human, mouse and rat sources [62]. Although the initially isolated XT-I cDNAs lacked a 5′ end and contained no region encoding a transmembrane domain, they obviously encoded the entire catalytic region since the human XT-I was successfully expressed in Chinese hamster ovary cells in a soluble form as judged by assays in which transfer of radioactive xylose to recombinant human bikunin or to artificial peptides was measured. More recently the 5′ end of the human XT-I has been isolated (GenBank accession No. AJ539163) and does indeed encode a potential transmembrane domain and a stem region with a polyglycine sequence – the full-length reading frame predicting a protein of 959 amino acids (M_r 107,568). On the other hand, even though complete and predicting a protein of 865 amino acids (M_r 96,711) with regions of more than 80% homology to XT-I (overall 51% identity), the

XT-II cDNAs did not direct expression of enzymatically active protein. Whether XT-II has a different peptide sequence specificity or requires substrates that already carry a xylose residue at another site, analogous to the specificity of some polypeptide *N*-acetylgalactosaminyltransferases involved in mucin-type glycosylation [63], is unknown.

Considering the interest in the role of proteoglycans in the development of genetic model organisms [64, 65], I thought it appropriate to search for a homologue of mammalian xylosyltransferases in *Drosophila melanogaster* and *Caenorhabditis elegans* genomes. BLAST searching revealed potential xylosyltransferases in the genomes of both organisms. In the case of the insect, the identified homologue (*oxt*; CG17771) was annotated in Flybase as being a core 2 β1,6-*N*-acetylglucosaminyltransferase, but having 876 amino acids (M_r 99,097) rather than the 400 or so normal for core 2- and I-synthesising enzymes. The percentage identity is, if one excludes the first 77 residues (i.e. the probable cytosolic, transmembrane and stem regions), 36 and 37%, respectively, with the corresponding parts of human xylosyltransferases I and II. Expression of a soluble form of this enzyme in *Pichia pastoris* verified that the protein had peptide *O*-xylosyltransferase activity as indicated by use of a novel mass spectrometric assay allowing simultaneous product identification and quantitation [41].

In the case of the nematode, cloning of the xylosyltransferase cDNA was complicated by the absence of a putative transmembrane domain in the predicted protein derived from analysis of reading frame Y50D4C.4 and the lack of any relevant expressed sequence tags (ESTs) (strangely, there are ESTs matching repetitive sequences within the introns of this gene). Closer examination of the relevant cosmid sequence, in conjunction with comparisons of sequences of other xylosyltransferase cDNAs (from insect or mammals) or putative xylosyltransferase ESTs (from the ascidians *Halocynthia roretzi* and *Ciona intestinalis*) and a bit of guesswork on my part, allowed the identification of a possible 5′ end. Two overlapping reverse-transcription PCR (RT-PCR) products were cloned and allowed reconstruction of the cDNA sequence predicted to encode a protein of 806 amino acids (GenBank accession No. AJ496235). A soluble form of the enzyme has also been successfully expressed in *Pichia pastoris* in an enzymatically active form [J. Drexler and I. B. H. Wilson, unpublished data]. The recently published data of Hwang et al. agrees with my identification of the *Caenorhabditis* xylosyltransferase sequence; they show that the nematode xylosyltransferase gene has a premature stop codon in the *sqv-6* mutant (see also later) and demonstrates that the wild-type nematode cDNA can complement the defect in xylosyltransferase-deficient Chinese hamster ovary pgsA-745 cells [66].

Sequence similarities of xylosyltransferases

The mammalian and invertebrate peptide *O*-xylosyltransferases identified so far have no obvious homology to the N-glycan-modifying β1,2-xylosyltransferases [67] or α1,6-xylosyltransferases [68] of plants, but rather constitute a distinct subgroup in the CAZy glycosyltransferase family 14 [69][2] phylogenetic tree (see fig. 3). Götting et

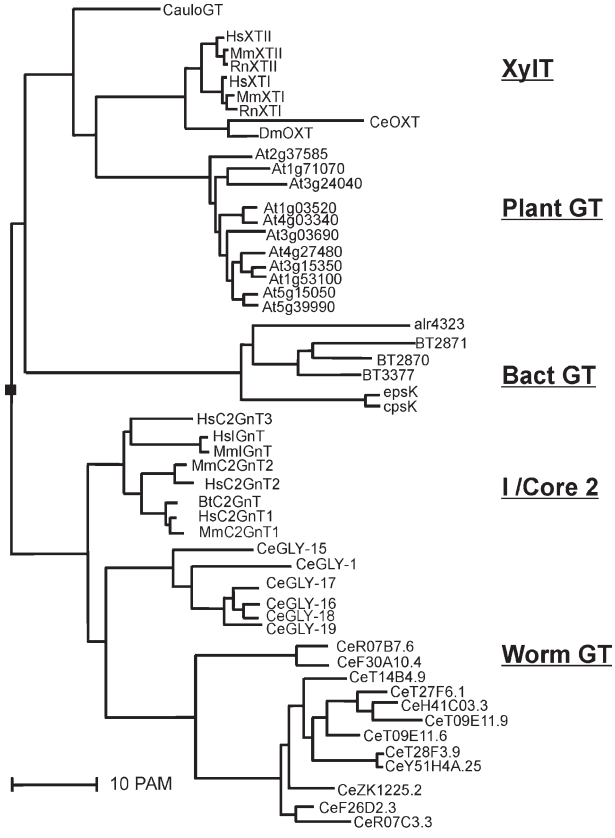

Figure 3. Possible phylogeny of CAZy family 14 glycosyltransferases. A selection of family 14 glycosyltransferases and putative glycosyltransferases were aligned using the MultAlin program (http://prodes.toulouse.inra.fr/multalin/multalin.html), specifically: *Caulobacter* reading frame CC2860, *Caenorhabditis*, *Drosophila*, human, mouse and rat peptide *O*-xylosyltransferases (excluding the stem and extended C-terminal domains), *Arabidopsis* putative glycosyltransferases (designated with the relevant reading frame number), *Bacteroides thetaiotaomicron* putative glycosyltransferases BT2870, BT 2871 and BT3377, *Nostoc* sp. PCC 7120 alr4323, *Streptococcus thermophilus* CpsK and EpsK proteins, bovine core 2 β1,6-*N*-acetylglucosaminyltransferase, human and murine core 2 β1,6-*N*-acetylglucosaminyltransferases 1, 2 and 3 (C2GnT 1, 2 and 3), human and murine I β1,6-*N*-acetylglucosaminyltransferases (IGnT; A transcripts), *Caenorhabditis* GLY-1 glucosyltransferase and related proteins (GLY-15, -16, -17, -18, -19) and other *Caenorhabditis* family 14 putative glycosyltransferases (designated with the relevant Wormbase reading frame number).

[2] The full listing of proposed members of glycosyltransferase family 14 is given at the CAZy database website (http://afmb.cnrs-mrs.fr/CAZY/index.html). The classification of glycosyltransferase families is most recently reviewed in [69].

al. [62] stated that the xylosyltransferase sequences had no homology to proteins known at that time; however, reflecting their common membership of glycosyltransferase family 14, peptide *O*-xylosyltransferases do indeed have sequence similarity to the previously characterised core 2 and I β1,6-*N*-acetylglucosaminyltransferases involved, respectively, in branching of mucin-type O-glycans and polylactosaminoglycans, as well as a number of eukaryotic and prokaryotic glycosyltransferases of unknown function. Peptide *O*-xylosyltransferases, though, differ from other family 14 enzymes from animals first of all by size, being around 800 (rather than 400) amino acids long, and also in the position of the cysteine residues within the homologous region of around 300 amino acids (see also fig. 4); in this region the degree of identity is ~30%. One would expect that the region common to all family 14 members is involved in the binding of the UDP-sugar donors used by these enzymes and in the catalytic mechanism.

It is a matter of speculation as to the role of regions of the xylosyltransferases that are not homologous to the other family 14 members. The N-terminal regions of the peptide *O*-xylosyltransferases are quite variable in length, with many gaps needed to align them (fig. 4). However, they all share a Pro-Xaa-Cys-Asp/Glu motif, which according to preliminary data is necessary for catalytic activity of the *Drosophila* xylosyltransferase [D. Schuster and I. B. H. Wilson, unpublished data]. Fewer gaps are necessary to align the last 300 amino acids or so of the insect and mammalian peptide *O*-xylosyltransferases, whereas the nematode sequence is more divergent except for a block of amino acids close to the C-terminus. The whole C-terminal domains of these proteins have no obvious homology to any other protein, and it can be speculated that they have a role in either peptide substrate recognition or in phosphorylation of xylose. Presence of 2-*O*-phosphate on the xylose is a variable feature of the tetrasaccharide core of mammalian, shark and insect glycosaminoglycans, but apparently not of *Caenorhabditis* chondroitin sulphate [70–73]. Whether the lack of phosphorylation of, at least, the chondroitin sulphate of *Caenorhabditis* is related to the highly divergent nature of the C-terminal domain sequence is unknown. Relevant or not to any function of the C-terminal domain, an Asp/Glu-Xaa-Asp motif, which is found in many glycosyltransferases, is located in this region in insect and mammalian sequences. Mutation of this motif in human XT-I results in a lack of activity [74], although whether this is really due to its being a 'true' DXD motif involved in binding the nucleotide sugar is doubtful, since it is not in the region homologous to other family 14 members which also require UDP sugars and is absent from the *Caenorhabditis elegans* and predicted *C. briggsae* sequences. It is clear, though, that the C-terminal domain is necessary for either stability of the protein or acceptor recognition since deletion of any amino acids from the terminus appears to result in abolition of activity of human XT-I [74] or *Drosophila* xylosyltransferase [I. B. H. Wilson, unpublished data]. Furthermore, the form of the *Caenorhabditis* xylosyltransferase protein encoded by the inactive *sqv-6* mutant is predicted to lack the C-terminal 42 amino acids [66].

Further homology analyses yield data compatible with *O*-xylosylation of peptides being an animal-specific post-translational modification; probable proteoglycan xylosyltransferases are present, as judged by analysis of the genomic and EST databases, in fish (*Tetraodon nigroviridis, Oncorhynchus mykiss* and *Oryzias latipes*), amphibians (*Xenopus laevis* and *Silurana tropicalis*), birds (*Gallus gallus*), mammals (*Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Bos taurus* and *Sus scrofa*), insects (*Drosophila melanogaster*, *Anopheles gambiae* and *Apis mellifera*), ascidians (*Halocynthia roretzi* and *Ciona intestinalis*) and nematodes (*Caenorhabditis elegans*, *C. briggsae* and *Heterodera glycines*).

Structure of xylosyltransferase genes

Comparison of the complete *Drosophila* xylosyltransferase cDNA sequence with the genome sequence (AE003474) indicated the presence of three exons following the usual :GU...AG: rule. In contrast, the human xylosyltransferase II gene (chromosome 17; AC004707 or AC015909) has 11 exons, whereas the human xylosyltransferase I gene (chromosome 16; AC109495, AC02 1115 and AC109946) has 12; furthermore, the last nine splice sites within these human genes are conserved (see fig. 5). In addition, as judged from analysis of sequences from the relevant genomes, the same genomic structure appears to be also valid for the rat xylosyltransferase I and II genes (AC103474 and AC103440), the murine xylosyltransferase I and II genes (AC122836 and AL6457 64) and an estimated two pufferfish (*Tetraodon nigroviridis*) xylosyltransferase genes. It is possible that the existence of two xylosyltransferase genes is a characteristic of vertebrates since *Drosophila* and *Caenorhabitis* only have one such gene; an exception to this is the presence of two genes tandemly arranged in the genome of the mosquito *Anopheles gambiae* (GenBank accession No. AAAB01008960.1). However, more genome sequences are required to determine how and when duplication (or loss) of xylosyltransferase genes occurred.

Regardless of the discrepancy in the numbers of exons, there is some conservation of the genomic structure between fly and humans, since the second fly *oxt* exon/intron junction (nucleotides 852–853; see also fig. 5) corresponds to splice sites within both human xylosyltransferase genes. However, none of the other mammalian junctions are present in the fly gene, while the first fly


Dm_XT
Ce_XT
H_XT1
H_XT2
C2GT1
C2GT2
C2GT3
I_GnT

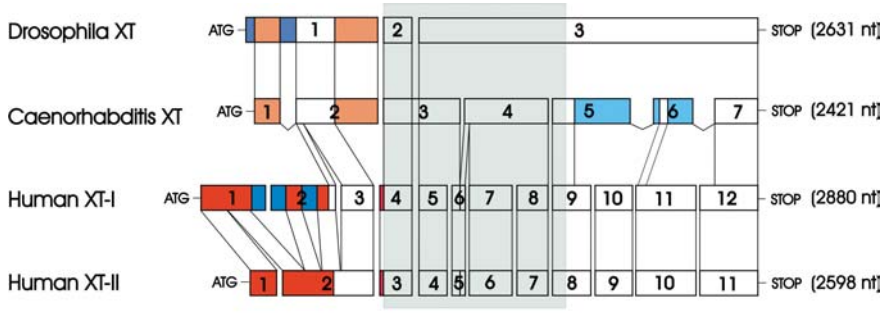

Figure 5. Schematic presentation of the coding regions and exonic structures of human, fly and worm peptide *O*-xylosyltransferase genes. Sections present in more than one gene are linked with lines to indicate their relative positions within the respective genes. Transparent sections denote regions common to all four genes. Orange-coloured sections denote regions homologous only between the fly and worm sequences, while red coloured sections are common only to both human genes. Blue-coloured sections denote regions specific only for that particular gene. Shaded section indicates larger region of genes similar to other family 14 glycosyltransferases. Introns are not shown.

exon/intron boundary is in a region lacking homology to the mammalian sequences. As deduced from analysis of probable reading frames, the two mosquito genes have an exonic arrangement identical to that of the fly, except that there are at least two exons corresponding to the exon 1 of the fly gene.

On the other hand, the organisation of the *Caenorhabditis elegans* xylosyltransferase gene (seven exons; see fig. 5) is different again, but is closer to that of the human genes than to that of the fly. It can be surmised that three splice sites of the worm mRNA correspond to those at the ends of exons 2, 4 and 7 of the human xylosyltransferase II gene. However, two pairs of exons (3/4 and 6/7) that are separated by relatively small introns (ca. 300 and 100 nt, respectively) in the human gene correspond to single exons in the worm gene, whereas the splice site conserved between fly and humans is absent from the nematode sequence. One possible explanation is that the positions of at least many of the introns in xylosyltransferase genes predate the divergence of *Drosophila*, *Caenorhabditis* and vertebrate lineages, with different introns being lost during evolution of insects and nematodes, this process of intron loss being most apparent in *Drosophila*. A similar conclusion was made recently for γ-glutamyl carboxylase genes: a snail has the same number of introns in the analysed region as humans, whereas the fly has only one [75]. However, a definitive phylogeny for the organisation of xylosyltransferase genes cannot be constructed, since relevant fish cDNAs, as well as other invertebrate genes and cDNAs, are not yet cloned.

Localisation of the xylosyl transfer reaction

Even though the first xylosyltransferase cDNA was isolated 3 years ago, there has, to date, been no study in which a xylosyltransferase has been localised by the now usual modern technique of determining the location of a glycosyltransferase using a tagged form of part of the protein or with antibodies raised against the recombinant protein. Using other methods, no single location has been defined for xylosyltransferase: there are some data suggesting a cis-Golgi localisation, others an endoplasmic reticulum one [76]. A similar degree of uncertainty is also apparent for mucin-type O-glycosylation (i.e. addition of GalNAc to serine or threonine residues), in that there is a range of data suggesting either an endoplasmic reticulum and/or early Golgi localisation, perhaps dependent on cell type and stage of differentiation or transformation [77, 78]. This may be partly a result of differential expression of perhaps up to 15 different polypeptide *N*-acetylgalactosaminyltransferases; however, such an explanation may not hold to the same extent for O-xylosylation since there are only two xylosyltransferase genes present in mammals.

Probably the first study on the subcellular localisation of the xylosylation reaction was that of Horwitz and Dorf-

Figure 4. Sequence comparison of peptide *O*-xylosyltransferases and core 2/I β1,6-*N*-acetylglucosaminyltransferases. The alignment was constructed using sequences of the fly (Dm_XT), worm (Ce_XT) and two human xylosyltransferases (H_XT1 and H_XT2), as well as the three human core 2 (C2GT1, 2 and 3) and one human I-branching (I_GnT) β1,6-*N*-acetylglucosaminyltransferases. Residues identical in a majority of sequences in one subfamily and present in at least one sequence of the other subfamily of the CAZy family 14 glycosyltransferases are in purple. Residues identical in at least one invertebrate and one human xylosyltransferase, but not present in core 2/I sequences, are in green, whereas those identical in the majority of core 2/I β1,6-*N*-acetylglucosaminyltransferases, but not a hallmark of xylosyltransferases, are in grey. Potential N-glycosylation sites are in yellow and cysteine residues are in green; the red Gln residue in the *Caenorhabditis* sequence is at the position altered to a stop (amber) codon in the *sqv-6* mutant. Amino acid residues at intron junctions are double underlined; transmembrane domains and potential DXD motifs are single underlined.

man, published in 1968 [79]. The conclusion was that in cartilage tissue the xylosylation reaction takes place particularly in the rough endoplasmic reticulum. Indeed, one conference abstract suggested that nascent proteins (i.e. still attached to the ribosome) could become xylosylated [80], whereas the use in electron microscopy of ferritin-coupled antibodies raised against a xylosyltransferase preparation indicated an endoplasmic reticulum location in embryonic cartilage cells [81]. Although one may have doubts about the quality of the antigen used to raise this antibody, since it recognised copurified polypeptides of M_r 27,000 and 25,000 (as compared with the theoretical molecular mass based on the now-cloned human xylosyltransferase I cDNA sequence of 107,568), there is other evidence to suggest an endoplasmic reticulum location, at least in chicken embryonic chondrocytes [82]. Subcellular fractionation of radiolabelled chondroitin sulphate proteoglycan precursors and electron microscopic examination of the location of UDP-[^{3}H]xylose suggested that xylosylation already occurred in the rough endoplasmic reticulum, but continued also in the cis-Golgi [54, 83]. Potentially consistent with an endoplasmic reticulum residency of xylosyltransferase are data obtained upon inhibition of ATP-dependent intracellular transport in human skin fibroblasts by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation; phosphorylation of xylose residues on dermatan sulphate still occurred in the presence of this reagent [84], suggesting that the xylosyltransferase, too, is in a pre-Golgi compartment.

However, there are also data suggestive of a Golgi localisation of xylosyltransferase. One study suggests a higher specific activity of xylosyltransferase in rat liver Golgi membranes than in endoplasmic reticulum membranes [85], whereas the kinetics of rat chondrosarcoma proteoglycan biosynthesis were considered to be more consistent with a Golgi localisation for this enzyme [86]. A recent paper indicates that only a minor portion of decorin molecules carrying the endoplasmic reticulum KDEL retention signal became O-xylosylated, presumably during 'escape' from, and retrieval back to, the endoplasmic reticulum: the conclusion being that xylosyltransferase is present in the intermediate compartment [87].

Evidence consistent with either an endoplasmic reticulum or Golgi localisation comes from the use of brefeldin A, a compound which reversibly disrupts Golgi function and architecture, results in the redistribution of Golgi enzymes to the endoplasmic reticulum and prevents transport to the still-extant trans-Golgi network. In cell lines treated with this reagent, proteoglycans are still modified by xylose and galactose [88], but further elongation and sulphation of chondroitin sulphate precursors is prevented [88, 89], whereas heparan sulphate biosynthesis still proceeds relatively normally [90]. This suggests that further modifications of chondroitin sulphate take place in the trans-Golgi network, but is consistent with enzymes involved in heparan sulphate elaboration being localised in the Golgi [91, 92], potentially as a multi-enzyme complex called by some a gagosome [93]. However, the 'mixing' of endoplasmic reticulum and Golgi membranes upon the use of brefeldin A precludes any specific conclusion about localisation of xylosyltransferase – it only excludes that xylosyltransferase is located in the trans-Golgi network.

Recent data have shown that glycosaminoglycan galactosyltransferase I (β4GalT7; SQV-3) is a Golgi-resident protein, at least in *Drosophila* Schneider and Chinese hamster ovary cells, as judged from the colocalisation of a Golgi-specific lipid marker or overlapping localisation of α-mannosidase II and chimaeras of the *Drosophila* or murine proteins with green fluorescent protein [92, 94]. Subfractionation of chick embryo cartilage cells supports a cis-Golgi localisation for this galactosyltransferase I [95]. How may this localisation compare with that of xylosyltransferase? An overlapping localisation or an interaction of xylosyltransferase with galactosyltransferase I is possible since a similar antibody preparation to that used in the electron microscopy study described above could immunoprecipitate both xylosyltransferase and galactosyltransferase activities [96]; galactosyltransferase I bound to semipurified and immobilised xylosyltransferase [97] and a subcellular fractionation experiment suggested that both enzymes are present in rough and smooth membranes [98]. It has been proposed that the membrane-bound galactosyltransferase I may help 'dock' a soluble xylosyltransferase/core-protein complex to the Golgi membranes, so allowing further processing and translocation of the core protein to occur [99] (presumably such a soluble form resulting from proteolytic loss of the transmembrane domain). However, since data on xylosyltransferase localisation was performed at different times and in different ways, it is still difficult to come up with a unifying conclusion as to where it is located or with which proteins it may interact. The cloning of peptide *O*-xylosyltransferase cDNAs opens up the way to revisit this topic using more modern and specific tools. Until such data is acquired, the safest conclusion is that xylosylation occurs in a compartment preceding that in which conversion of oligomannosidic to complex N-glycans takes place [100].

Biological role and clinical correlations

Proteoglycans are important components of the extracellular environment and have roles as, e.g., structural components, coreceptors of growth factors, anticoagulants and 'clustering agents' for neurotransmitter hydrolases [101–105]. Certain lethal mutations and other disorders in vertebrates such as chondrodysplasias and exostoses

are associated with defects in genes encoding proteoglycan core proteins and enzymes involved in synthesising the side chains [106, 107]. Thus it would seem obvious that xylosyltransferase is, as the first enzyme in the biosynthesis of many proteoglycan side chains, probably crucial for animal life.

Cultured cells can, though, survive without xylosyltransferase, as shown by the existence of the mutant Chinese hamster ovary cell line S745 (pgsA-745) [108], which has different adhesion properties but the same proliferation time as wild-type K1 cells [109]; this shows that xylosyltransferase is not required for survival of single eukaryotic cells (considering that yeasts lack this enzyme anyway). However, proteoglycans often mediate interactions between cells; thus, one would expect an obvious phenotype to be mediated by xylosyltransferase mutants in whole multicellular organisms. In the absence of a *Drosophila* mutant or 'knockout' mammal, the only evidence to date for the importance of this enzyme comes from studies of the nematode *Caenorhabditis elegans*. A number of so-called squashed vulva (*sqv*) mutants were isolated which corresponded to eight genes [110]; three of these genes were noted to encode proteins that could be components of a glycosylation pathway [111]. Indeed, all eight genes are now known to have functions as glycosyltransferases, as nucleotide sugar biosynthesising enzymes or as nucleotide sugar transporters necessary for chondroitin sulphate biosynthesis [42, 53, 66, 112–114] (see also [115] for a recent review). It is therefore not surprising that one of the *sqv* mutants affects the worm's xylosyltransferase gene [66]; this particular mutation (*sqv-6*), like the others, results in defective vulval morphogenesis (hence the name) and an arrest of cell division in embryos of homozygous mothers. This phenotype is a clear indication of the importance of glycosaminoglycans in general and of xylosyltransferase in particular.

From a clinical perspective, there are as yet no known disorders involving a mutation of peptide *O*-xylosyltransferases: perhaps the presence of two isoforms in humans or an absolute necessity during development means that diseases due to homozygous mutations will not be found. However, secreted xylosyltransferase levels are increased in the sera of patients with systemic sclerosis, a chronic inflammatory disease of the connective tissue [116, 117], and in the seminal plasma of infertile men [118]. It also varies in the sera of men in an age-related manner, while in the sera of women the xylosyltransferase levels change during the menstrual cycle [31]; follicular fluid, however, contains the highest per volume concentration of any human body fluid [119]. That xylosyltransferase is secreted is not surprising; other glycosyltransferases are present in secretions (e.g. Lewis-type α1,4-fucosyltransferase, β1,4-galactosyltransferase I or α2,6-sialyltransferase [120–122]). But even if released glycosyltransferases are catalytically active in vitro, the lack of donor substrates would presumably preclude catalytic function in vivo – their release being due to either proteolytic cleavage of the stem region [123] followed by secretion by the default pathway or due to tissue damage. Suggestive of the former, the secretion of xylosyltransferase by human chondrocytes is inhibited by colchicine [116]; this agent prevents secretion since it interferes with microtubule-mediated vesicular transport.

Some changes in the xylosyltransferase activities of rat tissues in response to certain treatments have also been reported. The activity of this enzyme is decreased in the costal cartilage of diabetic, thyroidectomised and hypophysectomised rats [124], while bleomycin-induced lung injury resulted in an increase in the enzyme's activity in lung [125]. Both these results correlate with a respective decrease or increase in chondroitin sulphate content. Overall the data on alteration of either tissue or serum xylosyltransferase suggest that the expression of this enzyme is subject to hormonal and transcriptional/posttranscriptional control.

Conclusion

A large amount of data on xylosyltransferase have been accumulated in the last 40 years; however, there remain a number of questions. How is its activity controlled? Is it necessary for mammalian existence? What is the role of its large C-terminal domain? What is its three-dimensional structure? Where is it localised within the cell? Is this localisation cell-type dependent? Is the mammalian XT-II active? Is there any real significance in xylosyltransferase being secreted? The cloning of xylosyltransferase cDNAs may well constitute the main advance in research on this enzyme in recent years: certainly, one can say it has been 'reined in', but its story will still preoccupy biochemists for some time to come.

Acknowledgements. I thank Katharina Paschinger for critical reading of the manuscript, Dubravko Rendić for preparing figure 5 and also David Schuster and Jasmin Drexler, project students in the laboratory, for generating some of the relevant unpublished data. My work on *Caenorhabditis elegans* and *Drosophila melanogaster* xylosyltransferases has been funded in part by grants from the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (P13810 and P15475).

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