Isolation and Characterisation of Mammalian Xylosyltransferase

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Abstract

Proteoglycans are macromolecules composed of glycosaminoglycan chains covalently bound to a core protein. They are a major structural component of all extracellular matrices as well as being important in many cell surface interactions with other matrix molecules, cytokines and growth factors. The synthesis of a glycosaminoglycan (GAG) chain on a proteoglycan begins with the transfer of a xylose molecule from UDP-xylose to the hydroxyl group of a selected serine residue in the core protein, catalysed by a xylosyltransferase. Investigations into this key stage of proteoglycan post-translational modification are central to understanding the mechanisms of synthesis of this important class of molecules. The aim of this thesis was to develop a purification method for xylosyltransferase to enable sequence information of the protein to be determined that would permit cloning of the enzyme.

An efficient assay system was developed to measure xylosyltransferase activity throughout the purification of the enzyme, using a synthetic peptide acceptor with a sequence derived from the chondroitin sulphate attachment region of human bikunin. A variety of different cellular/tissue sources of enzyme were tested as well as several size fractionation and affinity chromatography purification procedures. The most successful strategy was developed using a Swarm Rat Chondrosarcoma cell line. These cells when cultured in serum-free conditions with insulin secreted abundant enzyme into the medium. The medium was harvested and the enzyme was purified using a synthetic peptide affinity matrix combined with a lectin affinity system. The purification yielded a protein with an apparent mass of 100 kDa by SDS-PAGE. Upon trypsin digestion this protein yielded fragments from which a short peptide, QLYVALE_KR, was isolated. This was used to produce degenerate oligonucleotides for use in RT-PCR to produce cDNAs from Swarm Rat Chondrosarcoma mRNA. However this did not result in the detection of a specific mRNA, by RT-PCR or by Northern hybridisation.

During the course of this work it was discovered that xylosyltransferase was secreted from many cell types in an active form. This seemed unusual as xylosyltransferase has no apparent function outside of the endoplasmic reticulum / Golgi system. The mechanics surrounding the secretion of xylosyltransferase were investigated using a number of agents that disrupt the synthesis and secretory processes within the cell. It was found that xylosyltransferase appeared to be secreted by a process independent of the normal vesicular transport mechanisms common to other secretory proteins. It may therefore be released from the cell by some other novel mechanism.

Formal Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for any degree or qualification of the University of Manchester or any other university or institute of learning.

Autobiographical Statement

I gained a first class B.Sc. (Hons) in Molecular Biology with industrial experience from the University of Manchester in 1996. My year of industrial experience was spent in the Neurotoxicology Department at Zeneca Pharmaceuticals, Alderley Park, Cheshire.

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Abbreviations

bp / kb	base pairs / kilobases
BCA	bicinchoninic acid
BFA	brefeldin A
BSA	bovine serum albumin
CDP	cytidine 5'-diphosphate
CHAPS	3-[(Cholamidopropyl)dimethyl-ammonio]-1-propanesulphonate
ConA	concanavalin A
CPC	cetyl-pyridinium chloride
CS	chondroitin sulphate
DEPC	diethylpyrocarbonate
$DES4^{+}.2$	Syrian Hamster embryo chondrocyte cell line
DMEM	Dulbecco's modified eagle medium
DMMB	1,9-dimethylene blue
DMSO	dimethylsulphoxide
DS	dermatan sulphate
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
FCS	foetal calf serum
FITC	fluroscein isothiocyanate
GAG	glycosaminoglycan
Gal	galactose
GalNAc	N-acetylgalactosamine
GlcA	glucuronic acid
GlcNAc	N-acetylglucosamine
HBSS	Hank's balanced salt solution
HEPES	N-2-Hydroxyethylpiperazine-N-2'-ethanesulphonic acid
HS	heparan sulphate

IdoA	iduronic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilodaltons
K_m	Michaelis Meenten constant
KS	keratan sulphate
LRR	leucine-rich repeat
mAb	monoclonal antibody
MCS	multiple cloning site
MES	2-(N-Morpholino)ethanesulphonic acid
MOPS	3-(N-morpholino)propanesulphonic acid
NEM	N-ethylmaleimide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGSD	smith degraded proteoglycan
PMSF	phenylmethylsulphonylfluoride.
PVDF	polyvinyldifluoride
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLRPs	small leucine-rich proteoglycans
SRC	Swarm Rat Chondrosarcoma
Tris	tris(hydroxymethyl)amino-methane
TWEEN-20	polyoxyethylenesorbitan monolaurate
UDP	uridine di-phosphate
V_0	void volume
V_{max}	maximal velocity
V_t	total volume
Xyl	xylose
β-xyloside	p-nitrophenyl β-D-xylopyranoside

1. Introduction

1.1 The Extracellular Matrix

An extracellular matrix (ECM) surrounds all cells; it is a network of secreted extracellular molecules that can vary greatly in its form and constituents according to its function. It may be highly developed to have a structural role and to provide tissues with their specific mechanical and physiochemical properties. Cartilage is a tissue that comprises a highly specialised ECM. It is composed of a dense network of collagen fibres in which is embedded a high concentration of proteoglycans as well as hyaluronan, glycoproteins and other matrix proteins. In contrast, the ECM found around the plasma membrane of cells such as skeletal muscle cells forms a basement membrane which is a flat sheet made of laminin, collagen IV, entactin, fibronectin, and proteoglycans (Sanes, 1988).

1.1.1 Articular Cartilage

Articular cartilage is found at the weight bearing surfaces of joints. The function of the tissue is to allow nearly frictionless movement of the joint and bear loads without damaging wear for the duration of our lives.

Articular cartilage comprises a sparse population of cells called chondrocytes and is a highly hydrated tissue, consisting of up to 60-85% of its wet weight in water. The most abundant protein of cartilage is collagen type II, which accounts for ~20% of the tissue wet weight (Van der Rest and Garrone, 1991) followed by the proteoglycans, mainly aggrecan, at ~5% (Hardingham and Fosang, 1992), figure 1.1. There are a number of other components in the matrix, these include link protein, hyaluronan, biglycan, collagens type V, VI, IX, XI, cartilage oligomeric protein (COMP), other proteoglycans in small amounts (versican, biglycan, and perlecan), fibromodulin and thrombospondin. A cross section through articular cartilage shows variation in the matrix structural and compositional properties with distance from the cell (Poole *et al.*, 1982). The chondrocytes also vary in size and activity with their location within the tissue (Archer *et al.*, 1990).

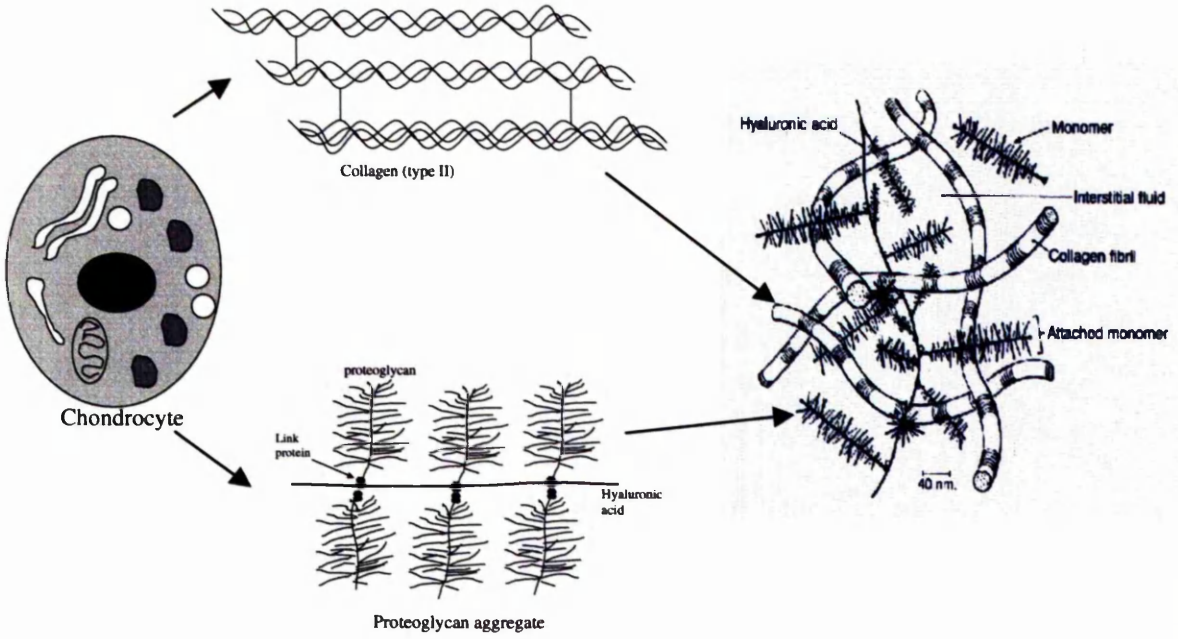


Figure 1.1. Chondrocytes synthesise collagen (structural framework) and hydrophilic proteoglycans, which interact with collagen fibrils to form the hydrated macromolecular matrix of articular cartilage.

1.1.2 Chondrocytes

Chondrocytes, whose prime function is to produce the matrix of cartilage, are mesenchymal cells that differentiate to become the sole architect of cartilage (Muir, 1995), figure 1. They make up only a very small percentage of the tissue volume in mature cartilage, however they alone produce and maintain the extracellular cartilage matrix in which they are sparsely distributed and isolated from their neighbours. As cartilage is avascular, the chondrocyte depends on diffusion from the synovial fluid to gain its nutrients and this may in turn limit the number of cells able to survive in a given volume (Stockwell, 1979). Chondrocytes encapsulate themselves in an elaborate network of fibrils known as a chondron (Wotton, 1991). Chondrons are specific to chondrocytes and can contain one or several chondrocytes. These units are filled with fluid that renders them resistant to compression, dampening the mechanical pressures and physico-chemical changes induced by dynamic loading.

1.1.3 Collagens

There are many members of the collagen family. All members share the basic structure comprising three polypeptide chains wound in a triple helical configuration. Each of the polypeptide chains forms a left-handed polyproline II-type helix. Then, three helices are coiled together to form a right-handed triple helix, designed to resist tension (Woo *et al.*, 1976). In order to form this helix, it is an absolute requirement that every third amino acid is a glycine residue to allow the close packing of the helix. Therefore, all collagens have the basic structure $(Gly\text{-}X\text{-}Y)_n$ where X and Y are commonly proline and hydroxyproline, respectively and n is the number of repeats (Rich and Crick, 1961). The number of repeats varies between collagen types; the fibrillar collagens can have up to 1000 consecutive amino acids consisting of this repeat in the three α-chains, whereas in the non-fibrillar collagens this repeat may be interspersed with non-collagenous sequences.

The major role of collagens in cartilage is to provide tensile strength and structural organisation within the matrix. Collagen type II is the most predominant form found in cartilage, but types V, VI, IX, and XI are also present. Type II is a homotrimer composed of three α-1(II) polypeptide chains, which polymerise extracellulary to form collagen fibrils. Fibrils of different diameters are found throughout the matrix, ranging from 12 nm in the pericellular region to up to 300 nm towards the outer regions of the matrix. Collagen type IX is composed of three distinct α chains and is a fibril associated collagen with interrupted helices (FACIT), it also bears a GAG chain. Collagen type IX is found in association with collagen type II and is thought to play a role in stabilising the collagen network in the matrix, and may assist in withstanding the compressive loads applied to articular cartilage (Olsen, 1989).

1.2 Proteoglycans

Proteoglycans are a large family of macromolecules ubiquitous in mammalian cells and tissues. They are composed of a protein core to which one or more glycosaminoglycan (GAG) chains are covalently attached. It is the presence of the GAG chain that

distinguishes proteoglycans from other glycoproteins and thus groups together a wide spectrum of family members, although the specific functions of the individual proteoglycans vary greatly. Many of the different proteoglycan core proteins have now been cloned, and whilst there is very little sequence homology between them as a whole family, certain motifs and domains identify family subgroups linked by similarities in structure. There are four main groups of GAGs; chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS) and heparan sulphate (HS)/heparin (Fosang and Hardingham, 1996). The type of GAG chains, their number, size, and sulphation pattern varies widely, giving these molecules a broad level of polydispersity and heterogeneity. Thus, both the wide spectrum of protein cores and the variation in GAG chain structure contribute to the diversity in structure and function of this class of molecules.

Proteoglycans can be found in a range of cellular and extracellular distributions and this depends on the type of proteoglycan and the cell type it is synthesised in. They may be found inserted in the membrane of secretory vesicles or in the cell surface plasma membrane as an intercalated, integral membrane component such as the neuronal CS-proteoglycan implicated in the binding of neuronal cells to glial cells (Stallcup and Beasley, 1987) and CD44 which is a hyaluronan binding receptor involved in lymphocyte homing (Stamenkovic *et al.*, 1991). They may also be found in storage granules such as the heparin proteoglycan in mast cells. Other groups may be packaged into secretory vesicles and then secreted into the ECM as a structural component, such as the major proteoglycan found in cartilaginous tissues, aggrecan, and perlecan the HS-proteoglycan found in basement membranes.

1.2.1 Classification and Structure of Proteoglycans

Proteoglycans comprise some of the most complex molecules found in mammalian tissues. They are a large family of molecules; over 40 independent full-length cDNAs of mammalian proteoglycans have been sequenced to date, each one belonging to a distinct gene. To add further diversity, some proteoglycans exist as structural variants or exist as part-time proteoglycans. Proteoglycans have been classified based on their overall structure and their biological properties of the protein cores (Iozzo and Murdoch, 1996). The secreted proteoglycans have for ease of classification been

divided into two main groups: the modular proteoglycans and the small leucine-rich proteoglycans (SLRPs).

1.2.1.1 Extracellular Matrix Modular Proteoglycans

1.2.1.1.1 The Hyalectins

The members of this family all share a similar protein structure. They all have multi-domain protein cores which consist of a highly glycosylated region and modules that share homology to other proteins known to be involved in cell growth, differentiation, adhesion and lipid metabolism. They have an amino-terminus that binds to hyaluronan, followed by a GAG-binding domain where most of the GAG chains attach, and a carboxyl terminus that contains modules found in the selectin family of proteins (fig 1.2). Through interactions with hyaluronan and lectins, these molecules may link the cell surface with other matrix molecules.

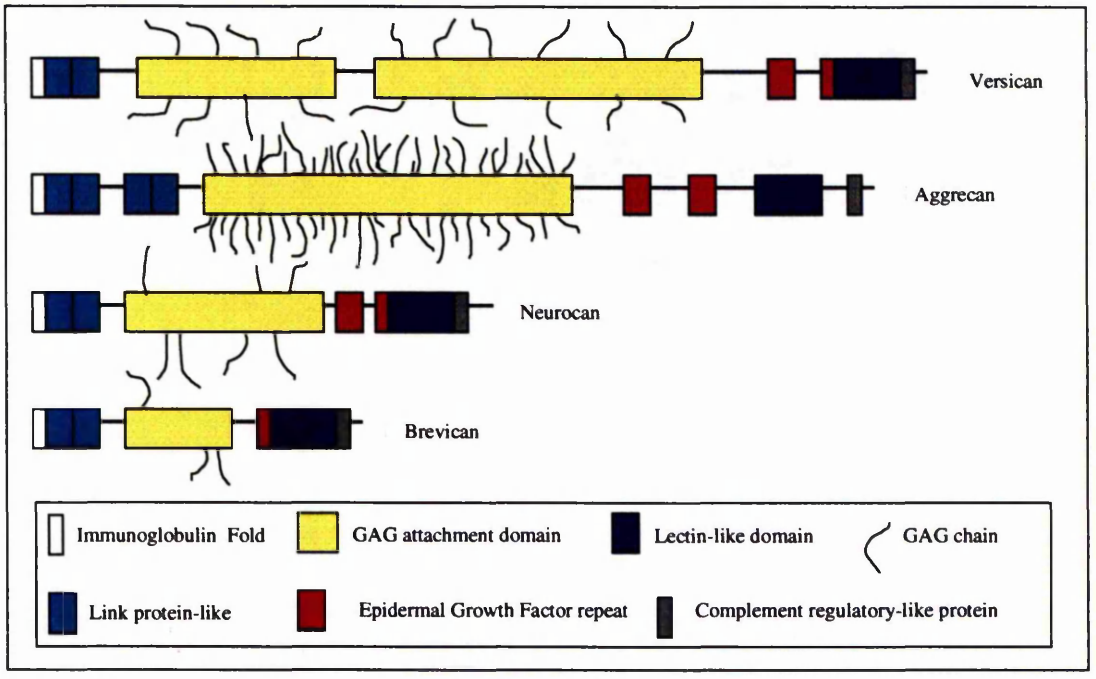


Figure 1.2. The hyalectins resident in the extracellular matrix. A key to the various protein modules and structural motifs is provided in the bottom panel. Adapted from Hardingham and Fosang, 1992.

Aggrecan

Aggrecan, the major proteoglycan of cartilage, has an overall mass of 1-4 x 10^6 Da. It comprises a protein core (220-250 kDa) encoded by a single gene that is expressed predominantly in cartilaginous tissue (Hardingham and Fosang, 1992). It has three globular domains and two extended domains (fig 1.2). Aggrecan is highly glycosylated with up to 100 chondroitin sulphate and 50 keratan sulphate chains attached (Hardingham and Fosang, 1992). The chondroitin sulphate chains are all found attached to the long extended domain between the globular domains, whereas the keratan sulphate chains are more widely distributed. Aggrecan also contains a variable number of O-linked oligosaccharides, which are sometimes extended during biosynthesis to form keratan sulphate chains. It also has some N-linked oligosaccharides.

Aggrecan forms large multi-molecule aggregates comprising numerous proteoglycan monomers noncovalently bound to hyaluronan. Hyaluronan is a long unbranched polysaccharide chain, which is capable of binding a large number of aggrecan monomers to form aggregates of up to several hundred million in molecular weight (Buckwalter and Rosenberg, 1982). A small glycoprotein, called the link protein, which is homologous to the amino terminal of aggrecan helps stabilise this large aggregate by forming a ternary complex with the first globular (G1) domain of aggrecan and hyaluronan (Hardingham, 1979). Aggrecan fills the interfibrillar matrix of collagen fibres drawing in water to the tissue, creating a large osmotic swelling pressure. The GAG chains on aggrecan carry a high number of negatively charged anionic groups, which attracts ions such as Na^+ into the tissue and thus the concentration of ions inside the tissue far exceeds that of the outside. Water is therefore drawn in, due to osmotic imbalance between the inside and outside of the matrix. Through swelling the tissue, proteoglycans give the tissue good compressive resilience, as well as preventing deformation of the tissue under great loads, as the size and aggregation of aggrecan greatly restricts its ability to move within the matrix. Further, the hyaluronan acts to trap and hold the aggrecan within the ECM.

Aggrecan has two globular domains at the amino-terminus, one that binds hyaluronan (G1), which is responsible for the formation of aggregates (Hardingham and Muir, 1972), and another that has a similar structure but whose function remains to be

elucidated (G2). The globular N-terminal domain that binds hyaluronan (G1) contains three motifs, and immunoglobulin fold (Ig-fold), and two link-protein motifs, also known as the proteoglycan tandem repeat (PTR). This module is related in structure to the mammalian type-C lectin family of carbohydrate binding motifs (Kohda *et al.*, 1996).

Towards the C-terminal of the protein from the globular domains G1 and G2, there is a KS-rich region and then a long extended CS attachment region. This GAG-binding domain is enriched with acidic amino acid residues, to which up to ~100 GAG chains can attach. The recognition sequence in aggrecan for the xylosyltransferase that initiates CS chain synthesis is a ser-gly dipeptide, although this is not constant throughout the family of proteoglycans, and not all ser-gly dipeptides are glycosylated.

The carboxyl terminal region of the core protein forms the G3 domain. It contains two EGF domains, which can be alternatively spliced (Roughley and Lee, 1994), a lectin-like domain, a complement regulatory-like domain, and a short tail region. In the nanomelic chick which has severe skeletal malformation, a stop codon was found in the translated region towards G3 which resulted in the expression of a truncated aggrecan that was not glycosylated nor secreted (Vertel *et al.*, 1993).

Versican

Versican, a proteoglycan widely expressed in vascular and avascular connective tissues (Zimmermann *et al.*, 1994) has a very similar structure to aggrecan. However, it lacks a hyaluronan-binding related domain (G2) and contains two GAG-binding regions, named α and β, which can be alternatively spliced (Naso *et al.*, 1994). Each GAG-binding region carries up to 30 GAG binding sites as well as sites for O- and N- linked oligosaccharides, but unlike aggrecan, versican has no KS chains attached. Alternative splicing of this region produces four different versican variants (Zimmermann and Zimmermann, 1994). V0, the largest variant of versican, contains both GAGα and GAGβ and has 17-23 GAG attachment sites (Naso *et al.*, 1994). The other three variants contain only GAGα (V1), GAGβ (V2), or neither (V3).

The carboxyl terminus of versican contains the same motifs as the G3 domain of aggrecan. A recombinant G3 region of avian versican has been shown to bind HS and heparin and simple carbohydrates (Ujita *et al.*, 1994), suggesting a role in ligand binding for this domain.

Brevican and Neurocan

Expression of brevican is highly specific in the brain and increases as the brain develops. It contains a hyaluronan-binding domain in its N-terminus and a lectin-like domain in its C-terminus. It has just one EGF-repeat in its globular C-terminal region which shows high sequence homology with the second EGF-repeat of versican and neurocan (Yamaguchi, 1996). It has the shortest GAG attachment region of the hyalectins but has a high content of acidic residues (Yamada *et al.*, 1994). Also of note is that two variants of brevican are found, one with and one without the GAG binding domain; the latter is created by proteolytic cleavage of this domain.

Neurocan is synthesised by neurons in early post-natal brain. Its functional role is not clearly understood, but results suggest that it is a potent inhibitor of neuronal and glial adhesion and neurite outgrowth (Margolis *et al.*, 1998). N-linked oligosaccharides attached to the neurocan core protein interact with neural cell adhesion molecules such as NCAM and axonin-1 and ECM molecules, suggesting neurocan plays a major role in modulating cell adhesion, neurite growth, and signal transduction across the plasma membrane during the development of the CNS.

Neurocan has a hyaluronan-binding domain at its N-terminus, and a lectin-like domain at its C-terminus as do the other hyalectin family members. It differs from the other hyalectins in that it carries just seven GAG attachment sites (Margolis and Margolis, 1994).

1.2.1.1.2 The Small Leucine-Rich Proteoglycans (SLRPs)

This group encompasses a growing number of proteoglycans that are structurally related: decorin (Krusius and Ruoslahti, 1986), biglycan (Fischer *et al.*, 1989), fibromodulin (Oldberg *et al.*, 1989), lumican (Blochberger *et al.*, 1992), epiphycan (Deere at al., 1996), keratocan (Funderburgh *et al.*, 1996), osteoglycan (Madisen at al., 1990), osteoadherin, PRELP (Bengtsson *et al.*, 1995) and opticin (Reardon *et al.*, 2000). In general, SLRPs are compact proteins whose distinctive feature is a structural motif, called the leucine-rich tandem repeat (LRR), which contributes towards the biological functions of this family of molecules (Iozzo, 1997). The length and number of LRRs varies between each member of the family and contributes to the variety of functions the individual proteoglycans have. As a family these proteoglycans contribute towards tissue organisation, interact with growth factors, bind to cell receptors, and also take part in a number of pathological processes such as wound healing and tissue repair.

The protein core of the SLRPs consists of three main regions: an amino terminus, to which the GAG chains or tyrosine sulphate is attached; a central domain containing the LRRs, which are flanked by cysteine-rich clusters; and a carboxyl end region (fig 1.3). The amino terminus, which bears the negatively charged GAGs or tyrosine sulphate, is thought to be involved in interactions with positively charged domains found in other ECM or cell-surface proteins.

Decorin, biglycan and epiphycan carry 1-2 DS or CS GAG chains each. The other members of the family bear tyrosine sulphate at their amino terminus. Adjacent to the N-terminus is a region that contains four highly conserved cysteine residues that are similarly spaced along 20 amino acids. The rest of the central domain comprises the tandem 24-residue repeats consisting of alternating hydrophobic and hydrophilic amino acids, with highly conserved leucine and asparagine residues (Patthy, 1987). At the carboxyl terminal are two highly conserved cysteine residues that are positioned 32 residues apart (except in keratocan), which create a loop structure by forming a disulphide bond (Neame *et al.*, 1989). In other LRR-containing proteins the flanking cysteine residue regions are thought to aid in cell-adhesion and binding to other molecules (Kobe and Deisenhofer, 1994).

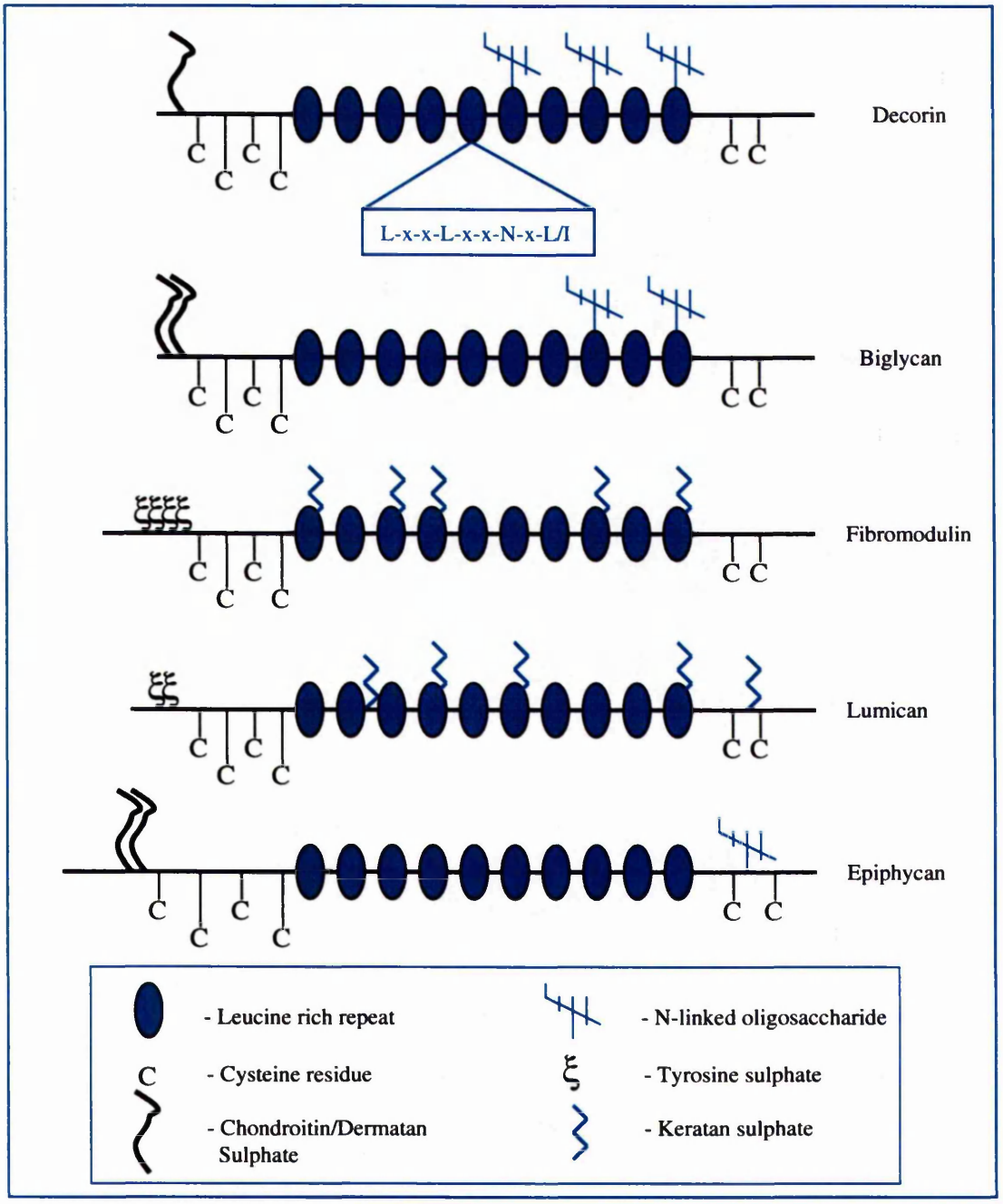


Figure 1.3. The family of small leucine-rich proteoglycans. The consensus sequence for the leucine rich repeat is shown in the rectangle. A key to the various structural components is given in the bottom panel. Adapted from Iozzo and Murdoch, 1996.

Decorin

Decorin is a small proteoglycan comprising a core protein substituted with just one GAG chain. In cartilage and skin the GAG chain attached is dermatan sulphate and in bone it is chondroitin sulphate. The composition of the GAG chain, in terms of iduronate content and pattern of sulphation also varies between tissues (Cheng *et al.*, 1994). The core protein of decorin consists of an N-terminal GAG attachment region, followed by a LRR flanked by cysteine clusters, and a C-terminal domain. There are also three consensus sequences in the core protein for N-linked oligosaccharide addition. Decorin, like a number of the SLRP family of proteoglycans, can exist as a part-time proteoglycan. Decorin lacking a CS chain has been detected in human skin (Fleischmajer *et al.*, 1991) and to make matters more complex, it has also been detected bearing two DS chains in chick sternal cartilage (Blaschke *et al.*, 1996). Over-expression of decorin in mammalian cells yields significant amounts of secreted core protein devoid of GAG chains (Hocking *et al.*, 1998), the proportion of which appears to be cell-type dependant and related to the endogenous levels of xylosyltransferase.

Decorin is localised in tissues rich in fibrillar collagens (Bianco *et al.*, 1990). The distribution of decorin supports the proposal that it has an important role in collagen fibril formation. *In vitro* studies have demonstrated a direct interaction between the core protein of decorin and fibrillar collagen, delaying collagen formation. This was a property of the protein, as the removal of the GAG chain had no effect on binding (Pringle and Dodd 1990). Decorin has also been shown to interact with other matrix components such as collagen type VI (Bidanset *et al.*, 1992) and fibronectin, inhibiting fibronectin-mediated cell adhesion (Schmidt *et al.*, 1991).

Biglycan

Biglycan is mainly expressed in the pericellular region in specialised cell types such as renal tubular epithelia, keratinocytes and skeletal myofibres (Bianco *et al.*, 1990). Biglycan has not been as widely studied as decorin but has been shown to interact with a variety of other proteins, such as fibronectin where it inhibits cell adhesion, thrombospondin, and heparin cofactor II. The GAG chain of biglycan is apparently critical for the enhancement of the inhibitory activity of heparin cofactor II to thrombin

(Whinna *et al.*, 1993). Interestingly, where biglycan is highly expressed decorin is expressed at low levels and *vice versa* (Bianco *et al.*, 1990). This suggests that decorin and biglycan differ in the interactions that determine their matrix distribution and that their expression is controlled independently.

The protein core structure of biglycan is very similar in structure to that of decorin (figure 1.3). It is a small proteoglycan comprising a core protein of approximately 40 kDa substituted with two GAG chains.

Fibromodulin and Lumican

The mRNA of fibromodulin and lumican has been found in a range of tissues such as articular cartilage, heart, lung, kidney, pancreas, placenta and skeletal muscle (Grover *et al.*, 1995). Functional studies indicate that both lumican and fibromodulin interact with collagen and inhibit fibrillogenesis. Lumican appears to affect the diameter of the fibrils, resulting in thinner fibrils (Rada *et al.*, 1992).

Fibromodulin contains leucine-rich repeat sequences similar to those of decorin and biglycan, but it contains no Ser-Gly GAG attachment sequences. However, within the leucine rich repeat region there are four N-linked oligosaccharide acceptor sequences, Asn-X-Ser/Thr, which can be substituted with N-linked keratan sulphate chains (Plaas *et al.*, 1990). The fifth potential keratan sulphate attachment site lies outside the leucine-rich region (Blochberger *et al.*, 1992). Close to the N-terminus of fibromodulin there is a tyrosine-rich sequence which can be sulphated. So even though fibromodulin carries no CS or DS GAG chains it can still be highly charged.

Lumican is expressed in the cornea, but it is also found in the walls of blood vessels. It is similar in sequence to fibromodulin compared to the other members of the SLRP family. It contains N-linked keratan sulphate chains, and has a tyrosine-rich sequence at its N-terminal but it is substituted with less tyrosine sulphate chains than fibromodulin so it is not as highly charged.

Keratocan

Keratocan was initially isolated from bovine cornea, but the mRNA of keratocan has also been detected in skeletal muscle, tendon skin, aorta lung and cartilage. It is a keratan sulphate proteoglycan consisting of a core protein, M_r ~38 kDa, substituted with up to three GAG chains (Corpuz *et al.*, 1996). Like fibromodulin and lumican, the core protein of keratocan has N-terminal tyrosine sulphation consensus sites and has cysteine residues flanking its leucine rich repeat domain. In addition, keratocan has five N-linked glycosylation sites.

Proline-Arginine-Rich and Leucine-rich repeat Protein (PRELP)

To date, PRELP mRNA has only been detected in articular chondrocytes, cultured fibroblasts and human lung (Grover *et al.*, 1995). PRELP has a core protein of ~42 kDa and shares 35% amino acid identity with lumican and fibromodulin (Bengtsson *et al.*, 1995). The unique feature of the PRELP core protein is an amino-terminal domain rich in arginine and proline residues. It also has four potential sites for N-linked glycosylation.

Chondroadherin

Chondroadherin, a 36 kDa protein, was found to interact along with PRELP to aid in chondrocyte adherence to tissue culture plastic (Sommarin *et al.*, 1998). Chondroadherin was shown to interact with chondrocytes via the integrin $\alpha 2\beta 1$ cell surface receptor (Camper *et al.*, 1997), the same integrin that promotes adhesion to collagen type II. The core protein of chondroadherin consists of ten leucine-rich repeats and has nine cysteine residues. The arrangement of the cysteine residues is unique to chondroadherin; four cysteines are at either end of the LRR domain.

Epiphycan and Osteoglycin

Epiphycan expression in mouse is restricted to epiphyseal cartilage and testis (Kurita *et al.*, 1996). The biological function of this dermatan sulphate proteoglycan is still unknown but its unique expression suggests that it may be involved in cartilage-tissue differentiation. Like many of the SLRP family, bovine epiphycan can interact with fibrillar collagens *in vitro* (Johnson *et al.*, 1997). Osteoglycin was first isolated from bovine bone (Bentz *et al.*, 1989), and was mistakenly classified as an osteoinductive factor due to contamination with bone morphogenic proteins (BMPs).

The core proteins of epiphycan and osteoglycan consist of six leucine-rich repeats flanked by cysteine clusters. Mouse epiphycan contains three potential GAG-attachment sites, two at the amino-terminal and another at the carboxy terminal. Three potential N-linked glycosylation sites have also been identified. Osteoglycin can exist as a keratan sulphate proteoglycan in bovine cornea (Funderburgh *et al.*, 1997), which adds to the theory that all the members of the SLRP family can exist as "part-time" proteoglycans.

1.2.2 Basement Membranes

Basement membrane is found wherever parenchymal cells come into direct apposition to connective tissue. Parenchymal cells include epithelial cells of the epidermis, of the genitourinary, respiratory and gastrointestinal tracts, exocrine glands, endothelial and mesothelial cells, cells in the central and peripheral nervous system, endocrine cells, muscle fibres and fat cells (Lindblom and Paulson, 1996). Basement membranes separate the different tissue compartments in the body by forming thin (20-300 nm) sheet-like structures encompassing them. They also regulate diffusion of solutes in and out of the tissue, which is best illustrated by filtration in the glomerulus of the kidney.

The molecules found in the most abundance in basement membranes are collagen type IV (Zhou *et al.*, 1993), laminin and nidogen (which form a complex), and proteoglycans. The three proteoglycans found in basement membrane are perlecan (Iozzo *et al.*, 1994), agrin (Ruegg, 1996) and bamacan (Wu and Couchman, 1997).

Perlecan

Perlecan has been localised to every basement membrane studied, and is considered to be the primary HS-proteoglycan of basement membranes, providing a layer of negative charge in a barrier function (Iozzo, 1994). It is one of the largest single-chain polypeptides of vertebrate animals, and because of its chimeric structural design plays a central role not only in growth and development but also in wound repair, cancer invasion and diabetes (Iozzo, 1994).

It is the most complex molecule in this group (see figure 1.4), having five different modules: a GAG attachment region; a SEA (sperm protein, enterokinase, and agrin) module (Bork and Patthy, 1995); four LDL receptor like domains; a laminin-like module; 21 consecutive Ig fold repeats; and finally four EGF-like repeats interspersed with globular regions.

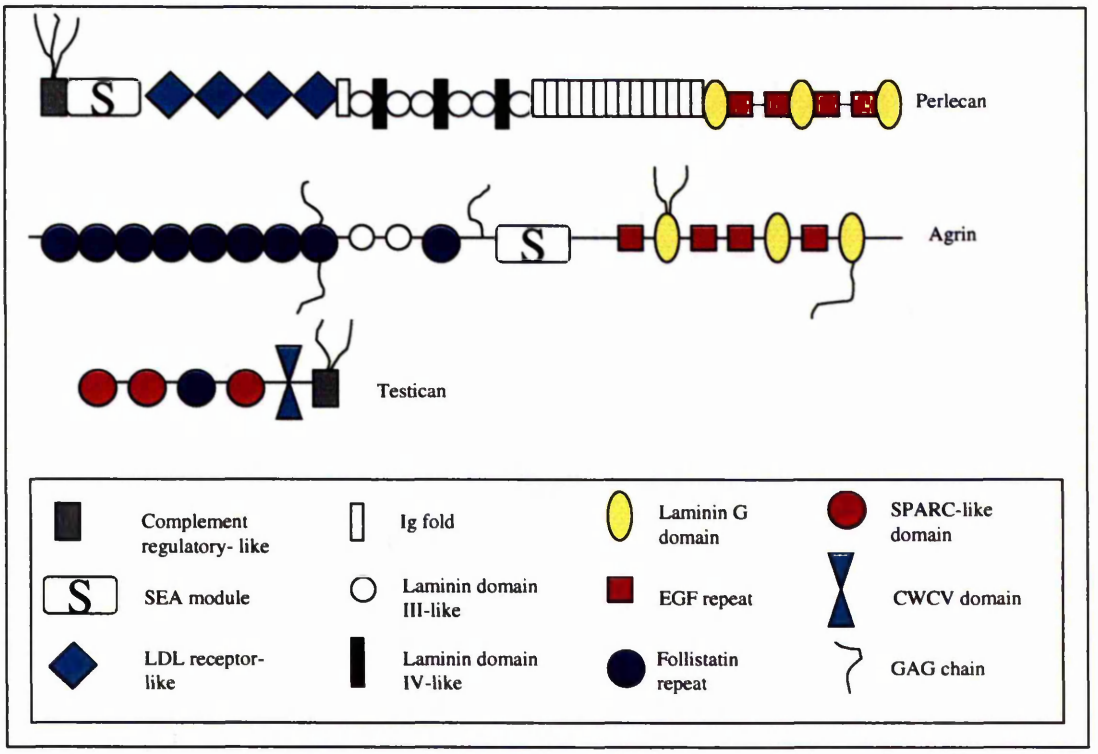


Figure 1.4. A schematic diagram of the basement membrane modular proteoglycans. A key to the various protein modules and structural motifs is provided in the bottom panel. Adapted from Iozzo and Murdoch, 1996.

Agrin and Testican

Agrin is an ECM proteoglycan that is involved in the aggregation of acetylcholine receptors during synaptogenesis at the neuromuscular junction (Ruegg, 1996). It has a core protein of 200 kDa, which contains four distinct domains. Agrin has both a SEA domain and the EGF-like repeats interspersed with globular repeats seen in perlecan (Tsen *et al.*, 1995). It has a highly glycosylated domain that contains two conserved GAG attachment sites and has several potential sites for the attachment of O-linked oligosaccharides. It also has nine follistatin-like domains that make up a significant portion of the molecule.

Testican, originally isolated from seminal fluid (Alliel *et al.*, 1993), also contains the follistatin-like repeats, but the GAG-attachment region is found at the carboxy terminus. The domains found within the testican molecule bear great resemblance to those found in proteins with adhesive and anti-adhesive properties, however its exact function is still not clear.

Bamacan

Bamacan expression has been found in the basement membranes of a variety tissues (McCarthy and Couchman, 1990). It is a large, chondroitin sulphate proteoglycan with a core protein of ~ 135 kDa. The core protein consists of five domains; a hydrophobic domain; a possible GAG attachment domain; a rod-like region that may be involved in cell attachment; a coiled-coil domain that may be involved in cell adhesion (Hunter *et al.*, 1989); and a hydrophilic domain that has two potential sites for GAG chain attachment.

1.2.3 The Cell Surface Proteoglycans

Not all proteoglycans are secreted out into the pericellular region or the ECM; some are resident in the plasma membrane of cells. Proteoglycans at the cell surface can either be directly intercalated in the lipid bilayer of the cell membrane as an integral membrane component, or by interacting with other cell surface molecules. The two major groups

of proteoglycans found to reside at the cell surface are the syndecans and the glypican families (Gallagher, 1996). In addition, betaglycan (Zhang and Esko, 1994), NG2 (Nishiyama *et al.*, 1991), CD44 (Jalkenen *et al.*, 1986, and Lesley *et al.*, 1992) and thrombospondin have also been classified as cell surface proteoglycans. All these molecules share a common form; they have a short cytoplasmic tail, a membrane-spanning domain and an extended extracellular region (fig 1.5).

The Syndecans

The syndecans are transmembrane proteins that interact with a wide range of molecules, such as extracellular matrix components, protease inhibitors, and growth factors (Couchman and Wood, 1996). It has been demonstrated that nearly all cell lines and tissues express at least one form of syndecan, and which types are expressed and to what level is individual to the tissue or cell type in question (Kim *et al.*, 1994). The syndecans are primarily HS bearing molecules, although they have been found to contain CS chains, or as a HS/CS hybrid molecule (Carey, 1997). They have been proposed to be receptors having a role in signal transduction, by interacting with the ECM through its CS/HS bearing extracellular domain, then relaying messages to the cell. The cytoplasmic region has conserved tyrosine residues in, which could act as phosphorylation sites for protein kinases, which are involved in many signal transduction pathways in the cell. There is also evidence that the syndecans play an important role as accessory signalling molecules modulating integrin-based adhesion (Woods and Couchman, 1998).

There are four members of the syndecan family, numbered 1-4 (Gallagher, 1996). Syndecan proteins range in size from ~45 kDa (syndecan-3) to ~20 kDa (syndecan-4) as deduced from cDNA cloning. Most of the core protein of the syndecans is extracellular, having just a small cytoplasmic tail region and just one membrane spanning domain. All the syndecans normally have three HS GAG chains attached, and they can sometimes also bear CS GAG chains (Carey, 1997).

The Glypicans

The glypican family of glycosylphosphatidylinositol-anchored HS proteoglycans comprises four members, glypican, cerebroglycan, OCL-5 and K-glypican, and the Drosophila protein, daily (Lander *et al.*, 1996). All the members of this family are expressed in the developing nervous system, with cerebroglycan being restricted to that tissue. The functions of the vertebrate members of this family are unknown, but in Drosophila the effects of mutations in the daily gene suggest a role for the glypicans in regulating cell cycle progression during the transition of neural cells from proliferation to neuronal differentiation.

These molecules share highly conserved protein structural features that distinguish them from the syndecan family. For instance, they are attached to the cell surface via a phophoinositol linkage instead of a transmembrane region (David *et al.*, 1990). However, the core protein of glypican contains three Ser-Gly repeats that are in a similar spatial arrangement to the HS GAG attachment sites in syndecan-1.

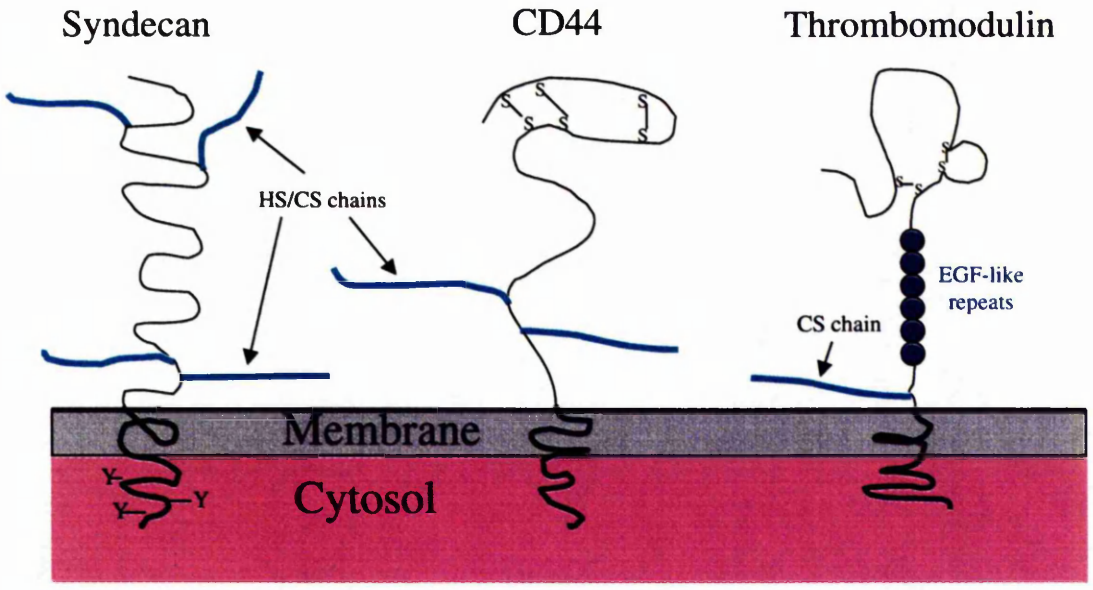


Figure 1.5. A schematic of some of the members of the cell surface proteoglycans. Adapted form Hardingham and Fosang, 1992.

Thrombomodulin

Thrombomodulin can occur in both proteoglycan and non-proteoglycan forms. It is found in the vascular endothelium and its function is as an anti-coagulant is mediated through the protein core (Esmon, 1989). Thrombomodulin inhibits thrombin-induced fibrinogen clotting, promotes inactivation of thrombin by antithrombin, and upon binding, activates the zymogen form of the anticoagulant protein C (Esmon, 1989).

Thrombomodulin is a 57 kDa integral membrane multi-domain glycoprotein. It encompasses an amino-terminal hydrophobic domain, an EGF-domain containing six EGF repeats, a short Ser/Thr-rich domain containing the GAG attachment site, a transmembrane domain and a carboxy-terminal cytosolic tail. There are four possible GAG attachment sites in domain 3 of thrombomodulin, but only one is substituted with a CS GAG chain.

CD44

CD44 proteoglycans are a class of membrane proteoglycans with several alternatively spliced forms that are expressed on the surfaces of many cell types. They can carry CS and HS chains, or a combination of both as well as O- and N- linked oligosaccharides, according to their location and function in a particular tissue. CD44 has been well characterised as a membrane receptor in lymphocytes where it is important in lymphocyte homing to lymphocyte tissue and recognition between lymphocytes and endothelial venules (Jalkanen *et al.*, 1986). In this function, only a minority of CD44 molecules bears GAG chains. It also is known to have roles in the control of cell adhesion and migration (Lesley *et al.*, 1992), and is a principal cell surface receptor for hyaluronan (Stamenkovic *et al.*, 1991).

The CD44 proteoglycans have been reported to contain keratan sulphate, chondroitin sulphate, heparan sulphate, or both chondroitin sulphate and heparan sulphate GAG chains in addition to N-linked oligosaccharides and O-linked lactosamine chains (Greenfield *et al.*, 1999).

NG2

NG2 is a transmembrane proteoglycan found on the surfaces of several different types of immature cells (Levine and Nishiyama, 1996). NG2 is associated with multipotential glial precursor cells, chondroblasts of the developing cartilage, brain capillary endothelial cells, aortic smooth muscle cells, skeletal myoblasts and human melanoma cells. The common feature amongst these cell types is their ability to divide throughout the lifetime of the organism. NG2 has functional properties analogous to those described for other proteoglycans such as the syndecans, betaglycan and neurocan. In vitro studies have shown that NG2 binds type VI collagen and inhibits neurite growth. NG2 is structurally unique chondroitin sulphate proteoglycan, with a core protein of ~ 300 kDa.

Betaglycan

Betaglycan is a membrane-anchored protein which acts as an accessory receptor presenting TGFβ to the signalling receptors, and enhancing cell responsiveness to this factor (Lopez-Casillas *et al.*, 1994). It binds TGFβ through its core protein and it also binds FGF-2 through its heparan sulphate side chain (Andreas *et al.*, 1992).

Betaglycan has six Ser-Gly repeats in its core protein for possible GAG attachment, as well as containing a single heparan sulphate chain. It is often present on the cell surface as a hybrid proteoglycan carrying both chondroitin sulphate and heparan sulphate chains (Zhang and Esko, 1994).

1.2.4 Proteoglycans of Intracellular Granules

Proteoglycans have been found in secretory granules of various leukocytes, including mast cells and basophils. The proteoglycans found here have core proteins with a long region of ser-gly repeats, to which are attached a high density of GAG chains. This renders the region highly protease resistant. This protein, serglycin, carries different GAG chains depending on the cell type it is expressed in (Ruoslahti, 1989). For

instance, in natural killer cells, the GAG chain is CS whereas in connective tissue mast cells, heparin is the major carbohydrate component (Stevens *et al.,* 1987). It has been demonstrated that serglycin bearing CS chains of a mouse T-cell line binds specifically to CD44 (Toyama-Sorimachi *et al.,* 1995), suggesting that serglycin may act as a ligand for CD44 in the lymphohematopoietic system.

1.2.5 The Expanding Family of Proteoglycans

The number of molecules classified as proteoglycans is constantly growing. Many molecules have been found to exist in glycanated and non-glycanated forms, and have thus been termed "part-time" proteoglycans. Collagens and proteoglycans were historically grouped as two distinct classes of molecules, however it is now clear that this is an over-generalisation. For instance, collagen type IX is found with a 70% incidence with a chondroitin sulphate chain attached on the α2(IX) chain (Huber et al., 1986). Proteoglycans have also been found to be involved in hunger responses; a membrane anchored proteoglycan termed "Satiotem" has been found to be involved in transducing activating signals and may also act as a source of second messenger for the regulatory mechanism of appetite (Upreti and Kidwai, 1996). New proteoglycans are also being found in the central nervous system, such as neurocan and phosphacan which are thought to play a primary role in the developing nervous system of vertebrates. The field of proteoglycans is constantly enlarging, with new avenues of research being opened all the time.

1.3 Glycosaminoglycan Chains

Sulphated GAGs are negatively charged, long chain unbranched polysaccharides composed of up to 100 repeating disaccharide units (Hardingham and Fosang, 1992). These highly charged, hydrophilic molecules have a major influence on tissue hydration and elasticity. They have a large hydrodynamic volume and they also have a propensity to interact with other molecules through ionic interactions: binding to ECM proteins, cell surface receptors, enzymes and cytokines.

Chondroitin sulphate (CS) and dermatan sulphate (DS) are both grouped together as the galactosaminoglycans and heparan sulphate (HS), heparin, keratan sulphate (KS) and hyaluronic acid (HA) are glucosaminoglycans. Sulphated GAG chains are diverse structures which display a considerable heterogeneity with respect to the length of polysaccharide chains, the disaccharide units they are built from, the number of chains, their distribution along the protein core, and the further chemical modifications they can undergo, figure 1.7. All these variables give proteoglycans even greater functional diversity.

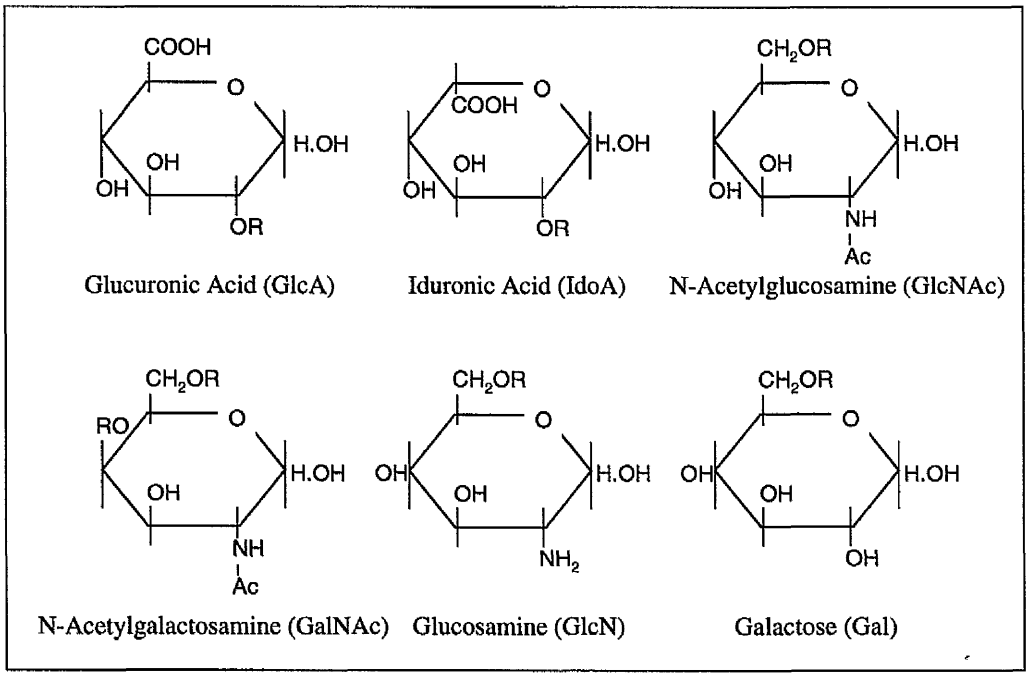


Figure 1.6. Amino sugar and hexuronic acid residues involved in glycosaminoglycan chain structure (where R = SO_4 or H). Adapted from Gallagher, 1989)

1.3.1 GAG Chain Units and Modification

GAG chains are formed by the sequential addition of monosaccharide units, figure 1.6, from the appropriate UDP-sugars to the core protein to form a growing sugar chain, through a series of polymerisation reactions. All the polymerisation products, except HA which exists as a GAG unattached to a protein core, are then further modified, possibly before completion of the whole chain (fig 1.7).

Hyaluronan

The structure of the polymerisation product is [(1→4)-β-D-glucuronosyl-(1→3)-β-D-N-acetylglucosaminyl]$_n$, where n is typically 1000-10,000. Hyaluronan is unsulphated and there are no further structural modifications to this molecule.

Chondroitin Sulphate

The structure of the polymerisation product, chondroitin, is [(1→4)-β-D-glucuronosyl-(1→3)-β-D-N-acetylgalactosaminyl]$_n$, where n is typically 20-80. This polymer is sulphated by the actions of sulphotransferases which catalyse the formation of O-sulphate groups at the C4 and C6 of the N-acetylgalactosamine residues (Roden and Schwartz, 1975). There is usually just one sulphate added per disaccharide unit, although there can be disulphated and even trisulphated disaccharides. Individual CS chains may contain both 4- and 6- sulphate groups or they may consist predominantly of one type. Minorities (<10%) of disaccharides are non-sulphated. CS is the least sulphated of the GAG chains.

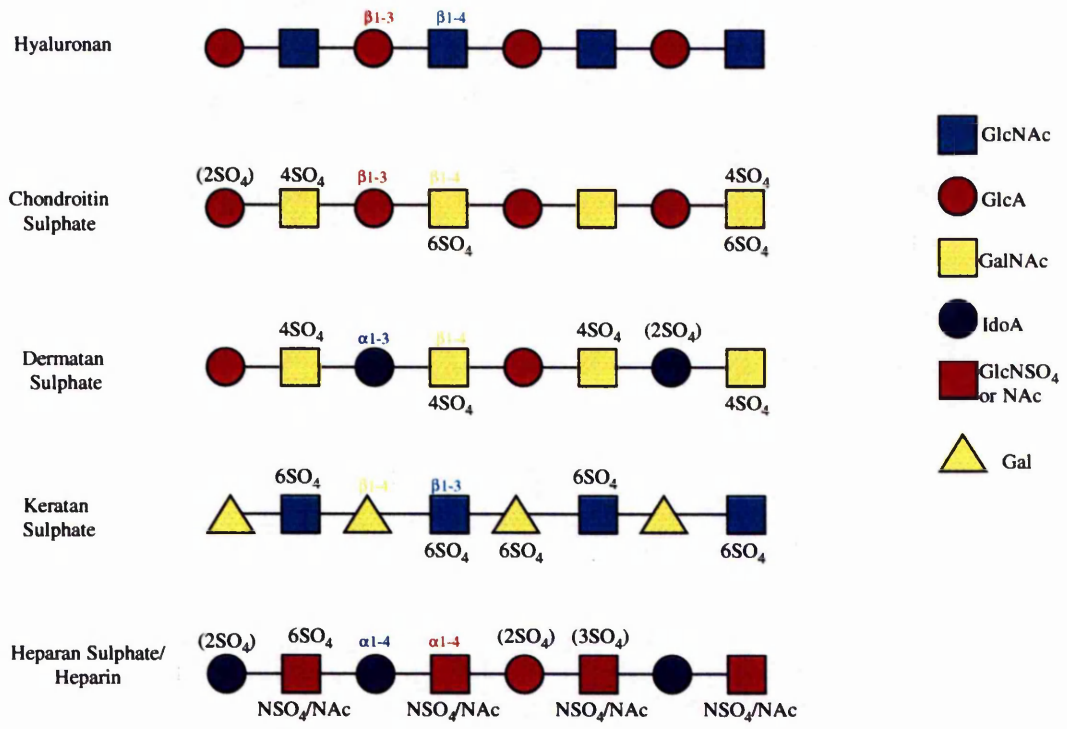


Figure 1.7. The repeating disaccharide structure of glycosaminoglycans. Also shown are the various modifications the chains can undergo to make them fully functional. GlcA – glucuronic acid, GlcNAc – N-acetylglucosamine, GalNAc – N-acteylgalactosamine, Gal - galactose, IdoA – iduronic acid. Reproduced from Hardingham and Fosang, 1992.

Dermatan Sulphate

The polymerisation product is identical to that of chondroitin. The chains can undergo O-sulphation as well as epimerisation of D-glucuronic acid units to L-iduronic acid. The L-iduronic acid content of the polymer can vary widely, from negligible amounts up to 90% of the total uronic acid present, leading to considerable structural diversity.

Heparan Sulphate and Heparin

The structure of the polymerisation product for both GAGs is $[(1\rightarrow4)$-β-D-glucuronosyl-$(1\rightarrow4)$-α-D-N-acetylglucosaminyl$]_n$, where n is typically 20-80. These molecules undergo modifications to epimerise D-glucuronic acid to L-iduronic acid as in DS as well as deacetylation of N-acetyl-D-glucosamine residues followed by sulphation of the resulting free amino groups. HS can contain low- and highly sulphated regions in the same chain and are D-glucuronic acid rich polysaccharides, whereas heparin is more highly sulphated than heparan sulphate and is also more L-iduronic acid rich. Heparin is the most highly sulphated of all the GAG chains.

Keratan Sulphate

KSs are generated by O-sulphate substitution of the structure, $[(1\rightarrow4)$-β-D-galactosyl-$(1\rightarrow3)$-β-D-N-acetylglucosaminyl$]_n$, where n is typically 10-60. Sulphation can occur at C6 of both or either of the Gal and GlcNAc units, giving rise to mono- and disulphated disaccharide units.

1.4 The Proteoglycan Synthesis Pathway

The biosynthetic assembly of all proteoglycans, except the KS chains typically found in cartilage and cornea, can be summarised in five sequential stages: (1) synthesis of the core protein; (2) xylosylation of specific serine residues of the core protein; (3) stepwise addition of two Gal residues followed by a GlcUA residue to the attached xylose residue to form the linkage region; (4) repeated addition of alternating hexosamine residues to form the long polymer GAG chains; and (5) modification of the chains by variable *N*-deacetylation/*N*-sulphation, and/or *O*-sulphation, and epimerisation of GlcUA to IdUA. In addition, during step (2) and/or (3), *N*- and/or *O*-linked

glycosylation can occur. The biosynthesis of proteoglycans is sequential and formed by the actions of many glycosyltransferases, sulphotransferases and transport molecules (Breton and Imberty, 1999). This is in contrast to the synthesis of nucleic acids and proteins, which are created by direct transfer of information from linear templates.

Core protein synthesis depends on the collaboration of several classes of RNA molecules and requires a series of preparatory steps. Initially, a molecule of messenger RNA (mRNA) must be copied from the DNA that encodes the core protein. Meanwhile, in the cytoplasm, each of the 20 amino acids from which the protein is to be built must be attached to its specific transfer RNA molecule, and the subunits of the ribosome on which the new protein is to be made must be pre-loaded with other protein factors. When all of these components are combined together to form a functional ribosome in the cytoplasm, protein synthesis can begin. The mRNA moves through the ribosome in a stepwise fashion to produce the corresponding chain of amino acids that makes up the protein core (Matthews and van Holde, 1990).

1.4.1 The Endoplasmic Reticulum in Proteoglycan and Glycoprotein Synthesis

From the ribosome, the newly synthesised protein core is directed into the endoplasmic reticulum (ER) through a special hydrophobic signal peptide it has attached to it. The extensive membrane system of the ER contains numerous biosynthetic enzymes, including those responsible for almost all of the cell's lipid biosynthesis and for the addition of *N*-linked oligosaccharides (Kimura *et al.*, 1984). *N*-linked oligosaccharides (N-acetyl-glucosamine, mannose, and glucose and containing a total of 14 sugar residues) are the most common ones found in the glycoproteins and are transferred to proteins in the ER. The complete side chain is always transferred to the NH_2 side chain of an asparagine residue of the protein, via a dolichol phosphate intermediate, almost as soon as the asparagine residue enters the ER lumen during translocation. Thus, *N*-linked glycosylation almost always occurs during protein synthesis, as most proteins are co-translationally imported into the ER (Roden, 1980). The reaction is catalysed by a membrane bound enzyme with the active site exposed on the luminal surface of the ER. The enzyme is thought to target asparagine residues within the signal sequences Asn-X-Ser or Asn-X-Thr (where X is any amino acid except proline). While still in the ER, the

N-linked oligosaccharide structures are modified to yield the diversity seen in mature glycoproteins (Hirschberg and Snider, 1987). This usually entails the removal of three glucose residues and one mannose residue. In addition, more complex *N*-linked polysaccharides are developed in the Golgi apparatus. The original structure can either be further trimmed down and then a variable number of galactose, sialic acid, and occasionally fucose residues are added on by the action of a number of glycosyl transferases (Kornfeld and Kornfeld, 1985).

Less frequently, oligosaccharides are formed by stepwise assembly of monosaccharides to the hydroxyl group on the side chain of a serine, threonine, or hydroxylysine residue (Schachter and Brockhausen, 1992). These *O*-linked oligosaccharides are formed in the Golgi apparatus.

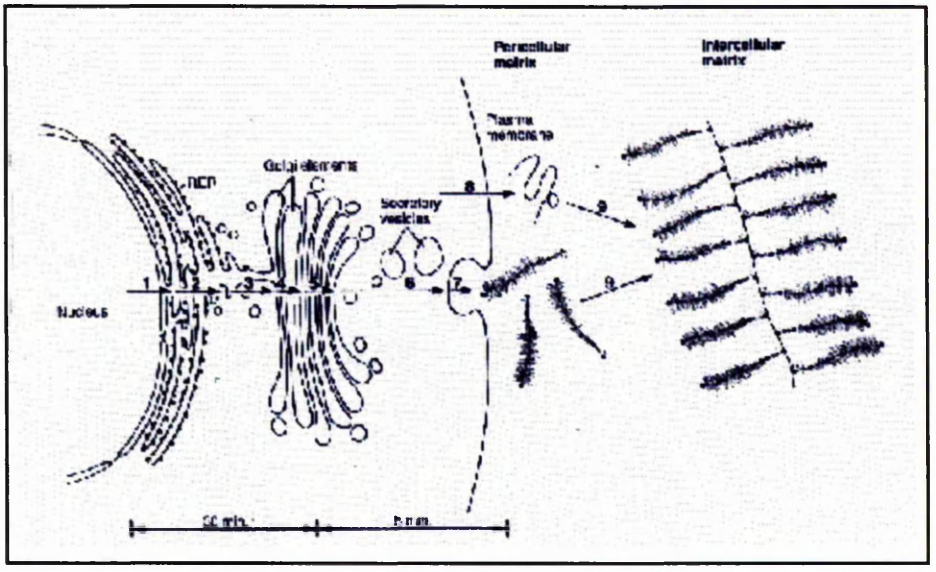


Figure 1.8. Schematic overview of synthesis and secretion of aggrecan, link protein and hyaluronan by a chondrocyte. The transcription of specific aggrecan and link protein genes to mRNA (1). The translation of the mRNA in the rough endoplasmic reticulum (RER) to form the protein core (2). The newly-formed protein is transported from RER (3) through the cis (4) and medial-trans (5) Golgi compartments for glycosylation and sulphation. The molecules are transported via secretory vesicles (6) to the plasma membrane where they are released (7). Hyaluronan is synthesised at the plasma membrane (8). Finally, aggrecan, link protein and hyaluronan form the aggregates within the extracellular matrix (9). Illustration adapted from an original by T. E. Hardingham.

From the ER the protein is then packed into transport vesicles that pinch off from specialised regions of the ER called the transitional elements and then fuse specifically with nearby cisternal elements of the Golgi apparatus. The membranes fuse, and the core protein then enters the lumen of a Golgi cisterna, for subsequent completion of the *N*-linked oligosaccharides, *O*-linked glycosylation and GAG formation (Varki, 1998).

Two different types of linkage, an *O*-linkage to serine or threonine, or an *N*-linkage to asparagine attaches keratan sulphate chains to the core protein. The *O*-linkage is typically found in cartilage aggrecan whereas the *N*-linkage is typically found in lumican in the cornea.

1.4.2 Glycosaminoglycan Attachment to the Core Protein: a Transitional ER and Golgi Event

Chondroitin sulphate, dermatan sulphate and heparan sulphate/heparin all have a common trisaccharide linkage region to the core protein. The first event in chain synthesis is the transfer of xylose from UDP-xylose to specific serine residues on the core protein. The transfer of xylose to serine residues has been located to the rough ER (Horwitz and Dorfman, 1968), as the activity of the enzyme which catalyses this reaction, xylosyltransferase, was found to be at a higher level here than in the smooth ER. In contrast, work in rat chondrosarcoma cells showed that xylosylation occurred within 5 minutes of Gal addition to *N*-linked oligosaccharides, which is a late Golgi event (Vertel *et al.,* 1993). This may imply a rapid transit through the Golgi cisternae.

The GAG linkage region is extended by the addition of two Gal residues from UDP-gal onto the xylosylated core protein. Meanwhile the membrane attached nascent proteoglycan has moved from the cis to the medial Golgi (Silbert and Sugumaran, 1995). A GlcUA is then transferred from UDP-GlcUA to the last Gal residue. This reaction is catalysed by a GlcUA transferase that appears to be distinct from the GlcUA transferase involved in the formation of the repeating disaccharide units (Helting and Roden, 1969). To this completed linkage region either a GalNAc or a GlcNAc is transferred, depending on whether there is to be a chondroitin/dermatan sulphate or

heparin/heparan sulphate chain, respectively, to be attached. The two transferases involved here also differ from those involved in polymerisation (Fritz *et al.*, 1984).

1.4.3 The GAG Attachment Signal

The tri-oligosaccharide linkage region of chondroitin sulphate, dermatan sulphate, heparin, and heparan sulphate is identical; therefore there must be some code or signal for which type of GAG is to be attached at a particular serine in the amino acid sequence of the protein.

The signal for the attachment of a GAG chain to the core protein of a proteoglycan appears to lie within the primary structure of the core protein. Xylosylation only occurs on serine residues, yet not all serine residues have a GAG attached. The other residues in close proximity to the glycosylated serines have been investigated however, no definitive consensus sequence has been reached. Bourdon and co-workers (1987) examined three different core proteins and revealed a sequence that had homology between them, from which they constructed synthetic peptides. The peptides they produced were shown to be effective xylosyltransferase acceptors and had the sequence, Ser-Gly-X-Gly (where X is any amino acid), preceded by a cluster of two to three acidic amino acids, which have before them, a group of hydrophobic amino acids. Substitutions for the amino acids in this sequence have shown almost complete loss of acceptor ability; for example, if the glycine following the serine was replaced with alanine, 99% of the acceptor activity was lost. Whilst this sequence appears to act as a good acceptor for xylosyltransferase, there are numerous exceptions where this sequence does not hold. Aggrecan appears to have a consensus sequence of Ser-Gly-X-X-Ser-Gly (Krueger *et al.*, 1990) whilst human versican has the sequence, acidicX-Gly-Ser-Gly-acidicX as it's acceptor site for xylosyltransferase. Collagen type IX also does not have a standard attachment sequence around the serines that are glycosylated (McCormick *et al.*, 1987) and is the only example with a Gly-Ser rather than a Ser-Gly sequence. To date, the presence of a glycine residue on the carboxy-terminal side of a serine residue is the most consistent feature of the known acceptor structures

As there seems to be no absolute consensus sequence for xylosyltransferase within the primary structure of the proteins that are xylosylated, the second and tertiary structure surrounding the GAG attachment site are likely to be important. Xylosyltransferase may require the Ser-Gly pair to be presented in a particular conformation to allow recognition and/or access for the enzyme active site to interact with the core protein. Furthermore, whilst there is no definitive sequence for where GAG chains are attached it is still not known what determines the addition of a particular CS, DS, or HS chain structure onto the linkage region. All these sugar chains are initiated with the same sugar: xylose. It could be that the glycosyltransferases present in the Golgi apparatus vary with the cell type synthesising the proteoglycan, or there may be additional signals in the core protein sequence and conformation that determine the nature of the GAG chain attached to the core protein. This too could explain why some proteoglycans, such as thrombomodulin, exist in both GAG attached and no GAG attached forms.

1.4.4 Glycosaminoglycan Polymerisation, Sulphation, Epimerisation and Secretion

The enzymes involved in polymerisation, namely the glycosyltransferases and polymer modifying enzymes, are organised in the medial to trans Golgi membranes (Noro *et al.*, 1983; Sugumaran and Silbert, 1991). CS, DS, and HS/heparin chains are elongated and modified as set out in section 1.6.1. Unlike protein synthesis, there is no obvious template-directed synthesis; rather the structure of a GAG chain is dependent on the membrane architecture and the organisation of the enzymes within it.

Once the proteoglycan synthesis is complete the molecules are moved on from the Golgi membranes to their final destination. Secretory vesicles are budded off from the Golgi membranes, which then travel through the cytoplasm to their final destination where they fuse with the plasma membrane to become cell-surface proteoglycans, or with internal organelles, such as intracellular granules, or they secrete their contents into the ECM.

1.5 Glycosyltransferases

Glycosyltransferases are the enzymes that synthesis oligosaccharides, polysaccharides and glycoconjugates. It is estimated that there are over 100 glycosyltransferases acting together in a co-ordinated manner to produce the great diversity seen in GAG chains alone (Paulson and Colley, 1989). These enzymes catalyse the transfer of sugar residues from an activated nucleotide sugar to an existing glycan chain or to a protein or lipid acceptor initiating a chain. Each glycosyltransferase, with few exceptions, can catalyse the synthesis of a single glycosidic linkage. In fact, most of the transferases involved in the biosynthesis of GAG chains and glycoproteins are themselves glycoproteins. The development of bioinformatics, together with new cloning strategies, has led to the identification of a wide range of transferase enzymes (for reviews see Field and Wainwright, 1995; Natsuka *et al.,* 1994). Sequence information for different transferases can easily be retrieved from a databank such as Genbank.

Glycosyltransferases are generally low abundance proteins, so it has been difficult to purify these proteins to get reliable sequence data for them, for use in molecular cloning. The technique of molecular cloning has enabled the discovery and subsequent characterisation of over 30 different mammalian glycosyltransferases (for examples, Nomura *et al.,* 1998; Sato *et al.,* 1998; Martin *et al.,* 1997). The cloning of a number of different Golgi-localised glycosyltransferases revealed that they shared the common topology of type II membrane proteins (fig 1.9), including an amino-terminal cytoplasmic domain (<25 residues), a transmembrane region, and a large carboxyl-terminal catalytic domain (>325 residues), which extrudes into the lumen of the Golgi apparatus (Paulson and Colley, 1989).

Many glycosyltransferases recognise identical donor or acceptor substrates, and are sub-classed accordingly. However, sequence analysis of cloned enzymes suggests there is little, if any, primary sequence similarity between the different members of the glycosyltransferase family, although those with similar catalytic properties do share some sequences (White *et al.,* 1995).

The glycosyltransferase families that have been studied most are those that add terminal sugars in glycoconjugates and that therefore are most likely to be involved in

recognition, interactive and signalling events. These include sialyltransferases (ST), fucosyltransferases (FucT), and the galactosyltransferases (GalT).

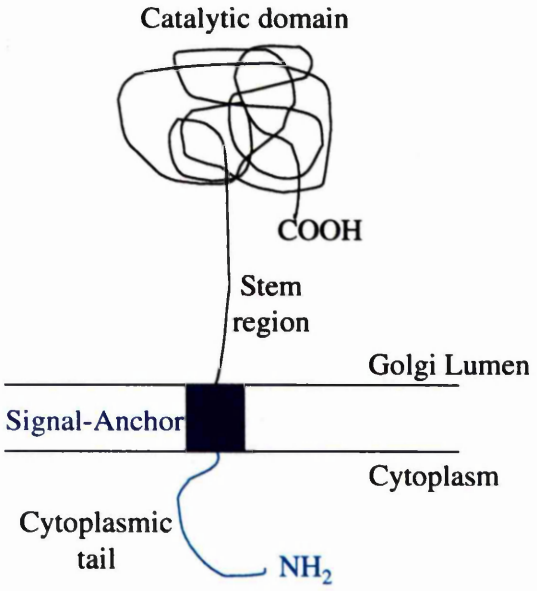


Figure 9. A schematic to show the common topology of cloned terminal glycosyltransferases. Deduced amino acid sequences of the terminal glycosyltransferases cloned to date predict that these enzymes have a characteristic topology in the Golgi apparatus consisting of a short amino-terminal cytoplasmic tail, a signal-anchor domain which spans the membrane, an extended stem region, and a large COOH-terminal catalytic domain orientated within the lumen of the Golgi cisternae.

The Sialyltransferases (STs)

All the eukaryotic STs cloned to date share several conserved peptide regions in their catalytic domain. These 'sialylmotifs' have been termed the large (50 amino acids), small (23, amino acids), or very small (6 amino acids) motifs (Datta and Paulson, 1997). Through site-directed mutagenesis, the functional significance of the different motifs was investigated (Datta *et al.*, 1998). By replacing the most conserved residues in the motifs with alanine it was found that the large motif was mainly involved in donor substrate binding, and the small motif was involved with both donor and acceptor binding. Both the large and small sialylmotifs contain highly conserved cysteine residues. If these residues are mutated the result is an inactive enzyme, suggesting that these two residues form a disulphide bridge essential for the formation of the active conformation of the STs.

The Fucosyltransferases (FucTs)

The FucTs have been further sub-divided on the basis of their function and sequence similarities into three groups - α2-, α3/4-, and α6–FucTs. Sequence analysis of their primary structure revealed that all α2- and α6–FucTs share a common peptide motif in their catalytic domains that is absent in the α3/4-FucTs, but this group does share other similar regions to the α2- and α6–FucTs (Breton *et al.*, 1998). To date, there is very little experimental data as to the functional role of these conserved regions (Sherwood, 1998).

The Galactosyltransferases (GalTs)

GalTs have been sub-divided into five different families on the basis of sequence similarities and conserved motifs (Breton *et al.*, 1998). However, despite their differences an acidic motif (DXD) was found to be present in almost all the different GalT families (Wiggins and Munro, 1998). This motif although common between the GalTs is not present in STs or FucTs.

1.6 Xylosyltransferase

The chain initiating reaction of the majority of the proteoglycans is catalysed by a xylosyltransferase, which transfers xylose from UDP-xylose to specific serines in the core protein, linking the GAG to the protein core by a glycosidic bond between xylose and the hydroxyl group of serine. This reaction initiates the formation of chondroitin-4-sulphate, chondroitin-6-sulphate, dermatan sulphate, heparin and heparan sulphate, thus would be expected to be an important regulatory step in proteoglycan synthesis due to its critical position in the synthesis pathway.

The majority of glycosyltransferases are bound to cell organelle membranes and are not readily available in a soluble form (Schwartz and Roden, 1974). Xylosyltransferase appears to be an exception, as substantial amounts of this enzyme have been found in soluble cell fractions, making it accessible to purify and study (Grebner *et al.*, 1966).

Previously, it was reportedly purified to apparent homogeneity from a number of sources, including embryonic chick cartilage (Stoolmiller *et al.,* 1972) and Swarm rat chondrosarcoma tissue (Stoolmiller *et al.,* 1975).

Xylosyltransferase activity was first measured using endogenous acceptors present in crude tissue extracts (Robinson *et al.,* 1966), but this method gave problems in interpretation and reproducibility due to the ill-defined nature of the acceptor. For purification of the enzyme various assays have been devised, to follow the enzyme through to apparent homogeneity (Schwartz and Dorfmann, 1975, Bourdon *et al.,* 1987, and Brinkman *et al.,* 1997).

In 1975, Schwartz and Dorfmann purified rat chondrosarcoma xylosyltransferase using differential centrifugation, Sephadex G-200 gel chromatography, and affinity chromatography on a matrix consisting of chemically degraded aggrecan core protein bound to Sepharose. The molecular weight of the enzyme was determined to be approximately 100 kDa and following treatment with dodecyl sulphate and mercaptoethanol, polyacrylamide-gel electrophoresis revealed two diffuse bands with approximate molecular weights of 23 kDa and 27 kDa. This result would suggest that the protein isolated was a tetramer, composed of two sets of subunits of different sizes. The enzyme was also analysed for carbohydrate content, as many glycosyltransferases are glycoproteins themselves, which was found to account for approximately 10% of the overall mass.

Comparisons between the xylosyltransferase purified from embryonic chick cartilage and the rat chondrosarcoma were performed (Schwartz and Dorfmann, 1975). It was found that while the size of the enzyme and the subunits were also similar in size (upon reduction) in addition to the specific activity of both enzymes, the carbohydrate content of the enzymes differed, and the antiserum prepared to each enzyme did not cross react between the two species. This would suggest that the enzymes from the two sources have structural differences. In addition to this early work it was demonstrated that bovine decorin carried only one GAG chain linked to the fourth amino acid in the sequence Asp-Glu-Ala-Ser-Gly (Chopra *et al.,* 1985). Whereas in chicken (Li *et al.,* 1992) and pigeon (Register *et al.,* 1993) decorin, the sequence is Asp-Glu-Ala-Gly-Ser, so that the GAG is attached to Ser^5 rather than Ser^4. From this result it has been

proposed that avian xylosyltransferase activity differs from mammalian xylosyltransferase activity (Li *et al.*, 1992)

It is still unclear whether there is one or more xylosyltransferase. The enzyme responsible for the initiation of chondroitin sulphate GAG chains may differ in structure and/or sequence from the enzyme involved in the initiation of synthesis of the other types of GAG chains. It is known that there are species differences in this enzyme, so it is also conceivable that there are differences in the enzymes expressed in different tissues. This may be related to the expression of different proteoglycans for the various roles they play in each tissue and this may require different xylosyltransferases to be expressed in different cell types.

It is also clear that there is no singular consensus sequence for the initiation of a GAG chain on a core protein. This could suggest that there are other determining factors around the Ser-Gly attachment sites that assist in the recognition of these sites by xylosyltransferase. As not all Ser-Gly repeats have a GAG chain attached it may be that the tertiary structure of the core protein is also an important factor. Biglycan and decorin are both expressed with a 16 residue and 14 residue signal peptide respectively (Fischer *et al.*, 1989). The signal peptide targets the nascent core protein to the rough endoplasmic reticulum (Silbert and Sugumaran, 1995) and, in general, is cleaved co-translationally and thus is absent in the mature protein. The amino acid sequence of the signal peptide contains a series of acidic acid residues that have been proposed to regulate the attachment of GAG chains (Ruoslahti, 1988). Therefore, the pro-peptide may function as a recognition/attachment site for xylosyltransferase (Sawhney *et al.*, 1991), assisting in the correct positioning of the enzyme so it can function efficiently. This view is further supported by transfection experiments using constructs containing various deletions of the decorin signal peptide region. These deletions led to the secretion of proteoglycans substituted with shorter GAG chains (Oldberg *et al*, 1996). The effect of these deletions may be to reduce the affinity between the protein substrate and xylosyltransferase, and maybe with the other glycosyltransferases, and/or inducing the proteoglycan to spend less time in the Golgi apparatus.

To date, there does not appear to be any common human or animal diseases associated with deficiencies in GAG biosynthesis, however Esko and co-workers (1985) produced

Chinese hamster ovary cell mutants which were defective in the biosynthesis of GAGs. They isolated mutants that appeared to have defective xylosyltransferase activity. However, whilst enzymatic evidence demonstrated deficient transfer of xylose, it was not determined whether the mutation was within the gene coding for the catalytic subunit of xylosyltransferase or a regulatory gene, as other mutants they isolated still had normal xylosyltransferase activity. Another possibility could be that there is more than one xylosyltransferase, but further work is needed to clarify this.

1.7 Aims of the Thesis

Xylosyltransferase is a key enzyme in the synthesis of proteoglycans, initiating GAG chain synthesis by xylosylation of selected serine residues on the protein core. It has been purified to apparent homogeneity before but there is no sequence data available or much information as to its structure and behaviour within the cell. By elucidating the sequence of xylosyltransferase it is hoped that more could be learned about the structure and function of the enzyme as well as having a better insight into the proteoglycan synthetic pathway and the significance of xylosyltransferase within it.

To produce a cDNA clone of xylosyltransferase the enzyme had to be purified. In order to purify the enzyme an efficient assay system was developed so the enzyme could be monitored throughout the purification procedures. Various sources of enzyme were tested for their level of xylosyltransferase activity, and then purified using gel-filtration and affinity chromatographic methods. Affinity purified proteins were used to gain peptide sequence data on xylosyltransferase.

The mechanisms surrounding the secretion of xylosyltransferase were investigated using a number of agents to further characterise the unusual cell biology of xylosyltransferase.

2. Materials

All chemicals were of the highest quality grade and of molecular biology grade when required.

Sigma Aldrich Chemical Company (Poole, Dorset)

Urea, guanidine-hydrochloride, 2-[N-morpholino]ethanesulfonic acid (MES) (sodium salt), uridine 5'diphosphoxylose (sodium salt), magnesium chloride, manganese chloride, ampicillin (sodium salt), streptomycin sulphate, papain, cetylpyridinium chloride, fluorescein isothiocyanate (FITC), polyoxylenesorbitan (Tween-20), potassium chloride, calcium chloride, CHAPS, Coomassie brilliant blue G, anti-mouse IgG (Fc specific), β-mercaptoethanol, dimethyl sulphoxide, 10 mM phosphate buffered saline (PBS) (sachets), collagenase, sodium metaperiodate, sodium perchlorate, N-ethyl-maleimide, Sephadex G-75, Sephacryl G-200, Sephacryl G-300, sodium azide, saturated phenol, chloroform, potassium fluoride, salmon testes DNA, insulin (porcine), bicinchonic acid solution, copper II sulphate solution, chondroitin sulphate C chains, bovine serum albumin protein standards (1 mg/ml), cytidine 5'-diphosphate, silver nitrate, sodium thiosulphate, ethanolamine, sodium carbonate, sodium chloride, Trypan blue, benzamidine hydrochloride, concanavalin A Sepharose conjugate, concanavalin-A peroxidase conjugate, Tris hydrochloride, Triton X-100, methyl -α-D-mannoside, Sigmacote™, 6-aminohexanoic acid, cyclohexamide, brefeldin A, monensin, Tri-reagent™, dimethyl sulphoxide (DMSO), 3-(N-morpholino)propanesulphonic acid (MOPS), X-Omat AR/BIOMAX™ MR films, p-nitrophenyl β-D-xylopyranoside, α-cyano-4-hydroxycinnamic acid.

BDH Laboratory supplies (Poole, Dorset)

SSC, sodium dodecyl sulphate, sodium dihydrogen orthophosphate, sodium acetate (anhydrous), 95-99% (v/v) ethanol, methanol (molecular biology grade), glacial acetic acid, acetonitrile, nitric acid, formic acid, sodium bicarbonate, concentrated hydrochloric acid, formaldehyde (36.5-38% (w/v) aqueous solution), formamide (molecular biology grade), glycerol, ethylenediamine tetra-acetic acid (EDTA) disodium salt, glycine, sodium hydroxide, potassium chloride.

Bio-Rad Laboratories (Hemel Hempstead, Hertfordshire)

Broad range protein standards, kaleidoscope protein standards, P2-biogel (fine), P4-biogel (fine), tris-glycine SDS-PAGE ready gels

GibcoBRL Life Technologies Ltd. (Scotland)

Dulbecco's PBS, dh5α *Escherichia coli* competent cells (subcloning efficiency), MEM media, Dulbecco's MOD Eagle Medium, Hank's balanced salt solution, 10X trypsin-EDTA, 1 Kb DNA ladder, 1 Kb RNA ladder, ultra-pure agarose, Foetal Calf Serum (FCS), 1 M Hepes buffer solution, 10X PBS.

Pharmacia Biotechnologies Ltd. (Milton Keynes)

PD-10 Sephadex G-25M columns, NHS-activated fast flow media, NHS-activated pre-packed Hi-Trap columns (5 ml)

Boehringer Mannheim UK Ltd. (Lewes, East Sussex)

Alkaline phosphatase and 10X alkaline phosphatase buffer, proteinase K, T4 DNA ligase and 10X ligase buffer and all restriction enzymes and 10X restriction buffers

Lithium thiocyanate and trifluoroacetic acid were obtained from FLUKA Chemicals (Gillingham, Dorset). Imidazole, [^{35}S]Sulphate (carrier free) and potassium borohydride were from ICN Biomedicals Ltd. (Thame, Oxfordshire). MES (free acid) was from Calbiochem (Beeston, Nottingham). Uridine diphosphate xylose [xylose-^{14}C (U)] and Renaissance Western Blot Chemiluminescence Reagent kit were from Du-pont/NEN (Stevenage, Hertfordshire). Cellu-Sep^{TM} T4 regenerated cellulose tubular dialysis membrane was acquired from Membrane Filtration Products Inc. (San Antonio, Texas, USA).

Exoklenow, IPTG and "Prime It" random primer labelling kit were obtained from Stratagene Ltd. (Cambridge). 5' 3' Perfect Prep kit was from CP Laboratories (Boulder, USA) and JETSORB gel extraction kit was obtained from GENOMED Inc. (North Carolina, USA). Pure nitrocellulose blotting membrane was from Gelman Sciences Ltd. (Northampton) and dimethyl methylene blue was purchased from SERVA (Heidelberg, Germany).

Tryptone, yeast extract and agar were bought from DIFCO Laboratories (Detroit, USA). Ecoscint A scintillation fluid was from National Diagnostics (Atlanta, USA). Dried skimmed milk (low fat) was purchased from SPAR (Harrow , Middlesex) and silk fabric from John Lewis (Cheadle, Cheshire). 8% (w/v) tris-glycine ready gels were obtained from NOVEX Electrophoresis GmbH. (Frankfurt M, Germany). Grade 3MM Chromatography paper was purchased from Whatman® International Ltd (Maidstone, Kent).

Oligonucleotide primers were from Oswel DNA Service (Lab 5005, Medical & Biological Sciences Building, University of Southampton) and GeneAmp® RNA PCR Core kits were from Perkin Elmer (Warrington, Cheshire).

Plasmid pKSC4 (containing an *Eco RI* insert coding for the C-terminal amino acids of the CS2 region and the G3 domain of aggrecan) was a kind donation from Dr. Jayesh Dudhia (Kennedy Institute of Rheumatology, Hammersmith, London). The Syrian Hamster embryo chondrocyte cell line, DES4+.2 were donated by Dr E. Kolettas and the Swarm rat chondrosarcoma cell line (RX) were donated by Dr. James Kimura, Henry Ford Hospital, Detroit, USA. Porcine trotters were from Newton Heath Abattoir (Manchester).

The peptide QEEEEGSGGGQKK derived from the human bikunin cDNA sequence, with two lysine residues added at the carboxyl terminal, was synthesised courtesy of Mr. Ian Moss (Charing Cross and Westminster Hospital Medical School, ABC Centre).

3. Methods

3.1 Analytical Methods

3.1.1 Protein Assay

Protein concentration was determined using the bicinchoninic acid protein (BCA) assay (Smith *et al.*, 1985). Briefly, samples (10 µl) were placed in a 96-well plate, in triplicate. Duplicate bovine serum albumin standards of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml were placed in separate wells. After mixing BCA (25 ml) with copper II sulphate solution (0.5 ml) an aliquot (200 µl) was added to the wells containing standards and sample(s). The plate was incubated at 37°C for 30 min then the absorbance was measured at 570 nm on a Titertek Multiscan PLUS® MKII plate reader. Sample concentrations were determined from a standard curve.

3.1.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method of Laemmli (1970). Samples were analysed on 4-20% (w/v) gradient or 7.5% or 8% (w/v) homogenous tris-glycine SDS-PAGE ready made mini-gels, under both non-reduced and reduced conditions. Samples were prepared for electrophoresis by adding an equal volume of 2x SDS-PAGE sample loading buffer (60 mM Tris-HCl (pH 6.8), 25% (v/v) glycerol, 2% (w/v) SDS, 0.1% (v/v) bromophenol blue), and were reduced with 200 mM β-mercaptoethanol. The samples and markers were boiled to denature the proteins prior to SDS-PAGE. Electrophoresis was performed in 25 mM Tris (pH 8.3), 192 mM glycine, and 0.1% (w/v) SDS at 125 V until the dye front migrated to within 1 cm of the bottom of the gel. The gels were then either silver stained (section 3.1.3) or stained with 0.02% (w/v) Coomassie Brilliant Blue in 40% (v/v) methanol / 10% (v/v) acetic acid for 1 h then de-stained with 7% (v/v) acetic acid/40% (v/v) methanol until the background was clear.

3.1.3 Silver Staining

After electrophoresis, the gel was briefly washed with deionised water, fixed in 50% (v/v) methanol / 5% (v/v) acetic acid (20 min), washed in 50% (v/v) methanol (10 min) and then washed in deionised water (10 min). The gel was placed in 0.02% (w/v) sodium thiosulphate (1 min), washed with deionised water (2 min) and submerged in chilled 0.1% (w/v) silver nitrate solution (20 min). The gel was washed with deionised water (1 min) then developed with 0.04% (v/v) formalin in 2% (w/v) sodium carbonate, on a plate shaker, until the desired intensity was achieved. The reaction was terminated by the addition of 5% (v/v) acetic acid. The gel was stored at 4°C in 1% (v/v) acetic acid.

3.1.4 Sulphated Glycosaminoglycan (S-GAG) Assay

The sulphated glycosaminoglycan (S-GAG) concentrations were determined using a colourimetric dye binding assay modified by Ratcliffe *et al.* (1988). The assay is based on a metachromatic shift in absorption maximum from 600 nm to 535 nm when a complex is formed between 1,9-dimethylmethylene blue (DMMB) and the S-GAG in the sample or standard.

Samples (50 µl) were placed in a 96 well plate, in triplicate. Standards were in the range of 0–40 µg/ml of S-GAG chains. DMMB (200 µl) was added to the samples and standards, mixed and the absorbance at 570nm measured immediately because a precipitate will form on standing. A standard curve was plotted and the concentration of S-GAG in each sample was calculated.

3.1.5 Cetyl-Pyridinium Chloride (CPC) Assay

Radiolabelled GAGs were isolated as described previously (Goodstone *et al.*, 1998). Briefly, cell or media samples (50 µl) containing [^{35}S]sulphate-labelled macromolecules, were adjusted to 1 ml with DMEM. Then 10% (w/v) CPC (100 µl) was added and samples were incubated at 37°C for 10 min to allow the CPC to bind to

the GAGs. The samples were then centrifuged for 12 min at 10,000 g_{av} at room temperature. The supernatants were discarded, and the pellets were resuspended in 1 ml of 0.5% (w/v) CPC / 20 mM Na_2SO_4, followed by centrifugation as before. After discarding the supernatants the pellets were resuspended in 1ml ethanol saturated with sodium acetate to help remove free radioactivity and centrifuged again. The pellets were then washed in ice-cold ethanol (1 ml) and centrifuged at 4°C for 12 min at 10,000 g_{av}. Finally, the pellets were resuspended in 100 mM sodium acetate, pH 7.0 (100 µl). Incorporated radioactivity was determined by liquid scintillation counting on a Wallac model 1409 liquid scintillation counter.

3.1.6 Separation of Macromolecular Material from Low Molecular Weight Material using PD-10 columns

Columns were equilibrated with 4 M guanidine-HCl (GuHCl), 10 mM CHAPS buffered with 25 mM sodium acetate, pH 6.0, containing a protease inhibitor cocktail. Samples were applied to the columns and incorporated radioactivity was eluted with 4 M GuHCl and fractions (250 µl) were collected. Incorporated radioactivity was determined by liquid scintillation counting on a Wallac model 1409 liquid scintillation counter.

3.1 Cell Culture and Preparation of Cell Supernatants

3.2.1 Cell Isolation from Swarm Rat Chondrosarcoma (SRC) Tissue and Preparation of the 105,000 g_{av} Tissue Supernatant

SRC tissue was maintained in laboratory rats as subcutaneous tumours by serial transplantation. Tumour tissue was maintained under the Home Office license of Dr. V. Duance (University of Cardiff, Wales). Animals were housed and tumour passage kindly performed by Mr. T. Simms (University of Bristol, Dept. of Clinical Veterinary Science).

Cell Isolation

SRC tissue was removed aseptically and sieved through a fine wire mesh, then washed in complete culture medium containing 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% (v/v) FCS (40 ml / 20 g tissue). The tissue mass was pelleted by centrifugation, 1000 rpm for 5 min, and resuspended in serum-free culture medium. This was repeated three times. The cell pellet was resuspended in 50ml serum-free culture medium containing 0.5% (w/v) trypsin and 1 mM disodium EDTA. The cells were incubated at 37°C for 1 h in a shaking water bath. The cells were pelleted and then resuspended in 50 ml serum-free culture medium containing 0.4% (w/v) collagenase, and incubated at 37°C for 1 h in a shaking water bath. The cells were collected by centrifugation, washed three times in the complete culture medium. Cells ($1x10^6$/ml) were resuspended in complete culture medium and incubated in 50 ml falcon tubes at 37°C in 5% CO_2, 95% air for 16 h.

Preparation of the 105,000 g_{av} Tissue Supernatant

The cells were washed once in standard MES buffer (see appendix, section 7.1) then centrifuged at 1200 rpm for 10 min. The cell pellet was resuspended in standard MES buffer (10 ml / 20 g tissue) containing a proteinase inhibitor cocktail (see appendix, section 7.1), sonicated for 5x 10 sec bursts followed by freeze thawing to thoroughly lyse the cells. The homogenate was centrifuged at 10,000 g_{av} at 4°C for 20 min, the supernatant removed and spun again at 105,000 g_{av} at 4°C for 60 min. The resultant supernatant was stored at –20°C, and referred to as the 105,000 g_{av} tissue supernatant.

3.2.2 Culture of RX SRC Cell Line and Preparation of the Medium Fraction

SRC cells were plated on tissue culture plastic at a density of $6x10^4$ cells / cm^2, in DMEM containing 20 mM HEPES, penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% (v/v) FCS. After an overnight incubation at 37°C, the media was removed and replaced reducing the FCS concentration reduced to 2% (v/v). After a further 24 h incubation period, the medium was replenished and a range of insulin concentrations added. After 72 h, the culture medium was removed and centrifuged at 10,000 g_{av} for 10 min at 4°C. To the medium supernatant the following were added to the final concentrations indicated: 50 mM MES, pH 6.5, 12 mM $MgCl_2$, 3 mM $MnCl_2$, 5 mM KF, 50 mM KCl, 0.1% (w/v) sodium azide, and a protease inhibitor cocktail. This chondrosarcoma medium fraction was stored at 4°C until it was used for the purification of xylosyltransferase.

3.2.3 Culture of Syrian Hamster Embryo Chondrocytes and Preparation of the Des4 Supernatant

Syrian hamster embryo chondrocytes immortalised with diethylstilbestrol (Koi and Barrett, 1986; McLachan *et al.*, 1982), were plated on tissue culture plastic T-75 flasks in Minimal Essential Medium containing 2 mM L-glutamine, non essential amino-acids, penicillin (100 units/ml), streptomycin (100 µg/ml) and 10% (v/v) FCS and incubated at 37°C. At confluence, monolayers were washed with Ca^{++} and Mg^{++}-free PBS and then trypsinised with 0.5% (w/v) trypsin in standard Hanks Balanced Salt Solution (HBSS). Cells were pelleted at 1300 g_{av} (10 min) and then resuspended in standard MES buffer containing a protease inhibitor cocktail (1 ml / 100 mg cells). Resuspended cells were sonicated and centrifuged as described in section 3.2.1, then stored at -20°C, and designated the Des4 supernatant.

3.3 Preparation of Xylosyltransferase Substrates

3.3.1 Smith Degradation of Proteoglycan (PGSD)

Smith degradation of aggrecan was carried out essentially as described by Baker *et al.* (1972). Porcine laryngeal aggrecan (1 g), prepared by Dr. Phillip Gribbon, was dissolved in 200 ml 0.05 M sodium metaperiodate and 0.25 M sodium perchlorate, pH 5.0, and stored in the dark at 25°C for 5 days. The mixture was dialysed against deionised water for 24 h, then potassium borohydride was added to a final concentration of 0.15% (w/v) and reduction was allowed to proceed for 24 h at 25°C. The solution was acidified to pH 1.0 with concentrated HCl and kept at 25°C for 72 h. After neutralisation and dialysis against deionised water for 5 h, the material was concentrated to 25 ml by freeze-drying. The sample was applied to a column of Sephadex G-75 (40 x 2 cm) and eluted with 0.2 M NaCl (5 ml/h). Fractions (2 ml) were collected and absorbance monitored at 280 nm. Fractions containing 280 nm absorbing material were pooled, dialysed against deionised water for 12 h then freeze-dried. CS chains were digested with chondroitinase ABC (0.5 units/ml) in 0.01 M Tris (pH 7.4) / 0.15 M NaCl for 24 h at 37°C. The sample was dialysed against deionised water for 12 h, freeze dried, then dialysed into standard MES buffer. The sample was analysed for protein content, using the BCA assay and sulphated GAG content, using the DMMB dye binding assay.

3.3.2 Preparation of Silk Fibroin

100% pure silk fabric (1 g), from John Lewis Cheadle, was dissolved in 10 ml of 60% (w/v) lithium thiocyanate at 60°C, according to the method described by Campbell *et al.*, 1994. The resultant solution was diluted with 80 ml deionised water, filtered through glass wool, and then exhaustively dialysed against deionised water until free of thiocyanate. The volume was adjusted to 100 ml to give a final concentration of 10 mg/ml. The solution was stable at 4°C for 2 weeks.

3.3.3 Recombinant Substrate

3.3.3.1 Plasmid Construction

Basic molecular biological techniques were as described in Sambrook *et al.* (1989) and Davis *et al.* (1994). Plasmid mini preps for verifying constructs, were made essentially using the alkaline lysis method of Birnboim and Doly (1979) with the perfect-prep miniprep plasmid kit. The vector used was pTrcHis (fig 4.5). Inserted cDNAs were derived from plasmid pKSC4 (Dudhia *et al.*, 1996) coding for part of CS attachment region and the globular G3 domain of the carboxyl terminal region of human aggrecan. Restriction fragments of the cDNA used in expression experiments are shown in fig 4.4. Colony hybridisation was as described in Sambrook *et al.* (1989), the probe used was the whole C4 EcoRI insert labelled with $\alpha[^{32}P]$dCTP using the 'prime-it' kit.

3.3.3.2 Construct Expression and Purification

Expression of the recombinant colony was performed according to the manufacturers instructions. Briefly, recombinant cultures were grown in SOB media at 37°C with vigorous shaking to an OD_{600}=0.6. IPTG was added to a final concentration of 1 mM and the culture grown at 37°C with vigorous shaking for a further 4 h. Protein purification was as detailed in the Xpress™ Purification Manual.

The expressed proteins were purified initially on a small scale following the procedure described for small-scale batch screening using denaturing conditions in the TALON™ Metal Affinity Resin User Manual (Clontech). Essentially, the cells were collected by centrifugation (14,000 rpm, 2 min) then lysed in denaturing lysis buffer containing 6 M guanidinium-HCl (pH 8.0), vortexed, then centrifuged as before. The supernatant was applied to TALON resin and agitated for 10 min at 25°C, then washed three times in denaturing lysis buffer containing 8 M Urea. Bound 6-His protein(s) were eluted by the addition of 100 mM EDTA (pH 8.0). For larger culture volumes (>500 ml) the batch/gravity flow column purification method from the same manual was followed.

This method was as described for the small-scale batch screening procedure, except gravity flow columns containing TALON resin were used, and bound 6-His proteins were eluted in 500 µl fractions by the addition of 100 mM EDTA (pH 8.0).

3.3.3.3 SDS-PAGE of TALON-Affinity Purified Proteins

Samples of total expressed protein and TALON-purified protein were subjected to SDS-PAGE. Samples were separated on 4-20% (w/v) tris-glycine ready-made gels, under non-reducing conditions, with pre-stained markers as standard. Bands were either visualised by staining with Coomassie Blue or transferred to nitrocellulose membrane for immunoblotting.

3.3.3.4 Immunoblot Detection of 6-His Recombinant Proteins

Resolved molecules were electrophoretically transferred from the gels to nitrocellulose filters (Towbin *et al.*, 1979) using a Mini Trans-Blot Cell. The gels were transferred in 15.6 mM Tris Base (pH 8.3) / 120 mM glycine at 100 V for 50 min. The resultant nitrocellulose filter was washed twice in PBS, pH 7.4, (5 min) and then blocked in PBS containing 3% (w/v) non-milk fat for 1 h at room temperature. The 6-His monoclonal antibody, was added to the filter at a 1:20,000 dilution in wash buffer for 1 h. The filter was placed in PBS containing 0.5% (w/v) non-milk fat for three washes (10 min). Bound primary antibody was detected by incubation for 1 h at room temperature with peroxidase conjugated anti-mouse (1:80,000). After three 10 min washes, a final rinse in PBS, the filters were incubated with chemiluminescence reagent (1 min), then exposed to X-omat AR-5 film for varying lengths of time. Films were developed using an automatic developer.

3.3.4 Purification of the Synthetic Peptide

The peptide (figure 4.9) was purified by reverse phase chromatography, using the SMART system (Pharmacia). The mass of the peptide, 1234 Da, was assessed by MALDI-TOF, and the concentration assessed using a refractometer; the sample was applied to a 5 ml Hi-trap column (Pharmacia) and the content of the void volume measured.

3.3.4.1 Preparation of FITC-Labelled Synthetic Peptide

The purified peptide (1 mg) was dissolved in 1 ml of a 4 mM carbonate- 0.6 mM bicarbonate buffer, pH 9.0, containing 50 µg of FITC. The mixture was rotated for 12 h at 4°C. FITC-conjugated peptide was separated from unconjugated-FITC by gel filtration on a P4 Biogel column (1 x 20 cm), equilibrated in PBS, at a flow rate of 10 ml/h. Fractions (500 µl) were collected and visualised on an UV-transilluminator, then fractions containing fluorescent material were pooled.

3.3.4.2 FITC-labelled Synthetic Peptide Xylosyltransferase Assay

Incubation mixtures contained the following components in a final volume of 500 µl in standard MES buffer: 33 nmol of UDP-[^{14}C]xylose (specific activity 30 µCi/µmol), 105,000 g_{av} tissue supernatant (250 µl of a 10 mg/ml solution) and FITC-conjugated synthetic peptide (200 µg). After incubation for 1 h at 37°C the mixture was applied to a P2 Biogel column (1 x 20 cm) equilibrated with standard MES buffer. The column was eluted with standard MES buffer, flow rate 10 ml/h, and 500 µl fractions collected. The fractions were visualised on an UV-transilluminator and incorporated radioactivity was determined by liquid scintillation counting on a Wallac model 1409 liquid scintillation counter.

3.4 Development of Xylosyltransferase Assays

3.4.1 Method 1: Xylosyltransferase Assay using Nitrocellulose Discs

The enzyme was assayed essentially according to the methods described by Roden *et al.* (1972). Briefly, incubation mixtures contained 1.5 nmol of UDP-[^{14}C]xylose (specific activity 30 μCi/μmol), varying volumes of the 105,000 g_{av} tissue supernatant, and varying amounts of exogenous substrate diluted in standard MES buffer to a final volume of 100 μl. After incubation for 1 h at 37°C, the mixture was placed on a nitrocellulose disc (2 cm^2) and air-dried. The discs were washed once in 10% (v/v) trifluoroacetic acid (TFA) and three times in 5% (v/v) TFA. Incorporated radioactivity was determined by liquid scintillation counting on a Wallac model 1409 liquid scintillation counter.

3.4.2 Method 2: Xylosyltransferase Assay using Gel Filtration

Incubation mixtures contained 3.3 nmol of UDP-[^{14}C]xylose (specific activity 30 μCi/μmol), varying amounts of enzyme protein and varying amounts of exogenous substrate diluted in standard MES buffer to a final volume of 100 μl. After incubation for 1 h at 37°C the mixture was applied to a P2 Biogel column (5 x 1 cm) pre-equilibrated with standard MES buffer. The column was eluted with standard MES buffer and fractions (200 μl) were collected. Incorporated radioactivity was determined by liquid scintillation counting on a Wallac model 1409 liquid scintillation counter.

3.5 Isolation of Xylosyltransferase

3.5.1 Size Exclusion Chromatography

A 1 ml sample of the 105,000 g_{av} tissue supernatant, FCS or chondrosarcoma medium fraction was applied to a column (0.9 x 60 m) of Sephadex G-200 or Sephacryl S-300 (fine), flow rate 0.25 ml/min, which previously had been equilibrated with standard MES buffer to pH 6.5. Fractions were collected (1 ml), tested for xylosyltransferase activity and the absorbance at 280 nm recorded for protein content.

3.5.2 Concentration Methods

3.5.2.1 Freeze Drying

Fractions containing xylosyltransferase activity from Sephacryl S-300 size fractionation were pooled and dialysed exhaustively at 4°C over 36 h against deionised water. The sample was freeze-dried, then reconstituted in standard MES at various fractions of the original volume prior to dialysis. The reconstituted and original samples were both tested for xylosyltransferase activity using the xylosyltransferase assay.

3.5.2.2 Centrifugal-Membrane Concentrators: AMICON Centriplus-30 and Microcon-3

Fractions containing xylosyltransferase activity from Sephacryl S-300 size fractionation were pooled and applied to either a Centriplus-30 or Microcon-3 membrane. The samples were centrifuged at 3000 rpm (Centriplus-30) or 12,000 rpm (Microcon-3) at 4°C until the volume had reduced to the required amount. The reconstituted and original samples were both tested for xylosyltransferase activity using the xylosyltransferase assay.

3.5.3 Affinity Purification

3.5.3.1 Preparation of Uridine Diphosphate Agarose

UDP-agarose (16 μmol UDP/ml gel slurry) was washed in 10 volumes of deionised water then resuspended 1:1 in sample loading buffer; 25 mM Mops (pH7.4), 25 mM $MnCl_2$ and 0.1 mM DTT.

3.5.3.2 Lectin Affinity: Concanavalin-A Coupled to CNBr-activated Sepharose

Concanavalin-A (Con-A) Sepharose was equilibrated in 10 volumes of lectin buffer; 20 mM Tris-Cl (pH 8.0), 0.15 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 1% (v/v) Triton X-100. To the gel, an equal volume of either FCS or chondrosarcoma medium fraction and the same volume of lectin buffer was added, and rotated at 25°C for 16 h. The gel was washed extensively (30 bed volumes) with lectin buffer, then bound molecules were released from the gel with stepwise elution with five bed volumes of 10 mM, 20 mM, 50 mM, 100 mM, 250 mM, and 500 mM methyl-α-*D*-mannoside in lectin buffer. A portion of the eluted fractions were dialysed at 4°C against standard MES buffer and then tested for xylosyltransferase activity using the xylosyltransferase assay. The rest of the samples were dialysed at 4°C against deionised water, freeze dried and resolved on a 4-20% (w/v) tris-glycine SDS-PAGE ready gel, under non-reducing conditions, and visualised by silver staining.

3.5.3.2.1 Concanavalin-A-HRP Conjugate

Synthetic peptide affinity purified molecules resolved by SDS-PAGE on 8% (w/v) tris-glycine ready gels were electrophoretically transferred at 100 V (50 min), to PVDF filters pre-soaked in ethanol, using a Mini Trans-Blot Cell. The filters were washed briefly in deionised water, blocked in TBST at 25°C (30 min), then probed with 0.1 μg/ml of Con-A HRP conjugate in TBST containing 1 mM $CaCl_2$ at 25°C for 30min.

Following three 10 min washes in TBST, the filters were incubated with chemiluminescence reagent (1 min), then exposed to X-omat AR-5 film for varying lengths of time, and the film developed using an automatic developer.

3.5.3.3 Preparation of the Synthetic-Peptide Affinity Matrix

Synthetic peptide affinity columns were prepared using pre-packed Hi-Trap columns containing NHS-activated Sepharose, according to the manufacturer's instruction manual. Essentially, 6 column volumes of ice-cold 1 mM HCl was washed through the 5 ml column at a flow rate of 5 ml/min followed by circulating 10 ml of an 8 mg/ml solution of the synthetic peptide dissolved in coupling buffer (0.2 M $NaHCO_3$ (pH 8.3)/ 0.5 M NaCl) through the column. The gel was then washed and any un-substituted reactive groups deactivated through three alternative washes in high pH, 0.5 M ethanolamine (pH 8.3) / 0.5 M NaCl, and low pH, 0.1 M acetate (pH 4.0) / 0.5 M NaCl, buffers, allowing the gel to stand in the high pH buffer for 30 min after one set of washes. The gel was finally equilibrated in standard MES buffer (pH 6.5) and stored at 4°C until used.

Alternatively, fast-flow NHS-activated Sepharose media was used (not pre-packed). The method of conjugation was as described above.

3.6 Production of Peptide Sequences

3.6.1 In-Gel Digestion of Proteins for Internal Sequence Analysis

Synthetic peptide-affinity purified fractions resolved on Tris-glycine SDS-PAGE ready gels were silver-stained and stored at 4°C, as set out in section 3.1.2. Identical bands were excised from gels and placed in sterile Eppendorf tubes, and acetonitrile (100 µl) was added to dehydrate the gel slices. The gel slices were then completely dried out in a SpeedVac (Savant), then reduced in 50 µl 10 mM dithiothreitol / 100 mM NH_4HCO_3 (pH 8.0) at 56°C (1 h). Following cooling to room temperature, the dithiothreitol solution was replaced with 55 mM iodoacetamide (50µl) in 100 mM NH_4HCO_3, and the slices incubated in complete darkness for 45 min, with occasional vortexing. The gel slices were then washed in 100 mM NH_4HCO_3 (200 µl) (pH 8.0), followed by dehydration in 100 µl acetonitrile (5 min), then re-hydration in 100µl 100 mM NH_4HCO_3 (5 min), and then dehydrated again in acetonitrile, the liquid phase removed, and the gel slices dried out completely in a SpeedVac. The slices were prepared for digestion in 100 µl of 12.5 ng/µl trypsin in 50 mM NH_4HCO_3 at 4°C (45 min), the supernatant removed, and the slices left to digest at 37°C for 16 h in a minimum volume of 50 mM NH_4HCO_3. The tryptic peptides were extracted from the gel by the addition of 100 µl of 20 mM NH_4HCO_3 (20 min), with agitation, then the supernatant containing the peptides was removed. Three further 20 min washes in 100 µl of 5% (v/v) formic acid in 50% (v/v) acetonitrile, with agitation, were performed and the supernatants added to the initial extraction supernatant, to ensure all peptides had been removed from the gel pieces. The combined supernatants were concentrated to 20 µl using a SpeedVac, then diluted to 200 µl in deionised water, freeze dried, diluted again in deionised water, and then freeze dried again to remove any contaminating buffer salts.

3.6.2 Reverse-Phase HPLC Purification and Structural Characterisation

The peptides eluted from the polyacrylamide matrices were resuspended in 0.1% (v/v) TFA and were separated on a C2/C18 reverse phase column. The column was eluted with a linear gradient of acetonitrile, 0.1% (v/v) TFA at a flow rate of 0.3 ml/min using the SMART system. Absorbance was monitored at 218 nm. Fractions containing material were analysed using MALDI-TOF to produce a peptide-mass fingerprint of the protein. The N-terminal sequence analysis was performed on an Applied Biosystems 476A Protein Sequencer.

3.7 Production of cDNA Clones from the SRC Peptides

3.7.1 Immobilisation of SRC cell RNA and Hybridisation Analysis

Oligonucleotide probes were produced courtesy of Oswell. Oligonucleotide probes were constructed based on sequence data generated from in-gel digestion of synthetic peptide-affinity purified fractions resolved by SDS-PAGE.

3.7.2 SRC cell RNA Extraction and Northern Blot

Total RNA was extracted from SRC cells using TRI-REAGENT™ according to the manufacturer instructions (SIGMA). Briefly, SRC cells were grown to confluence, as detailed in section 3.2.2, and TRI-REAGENT (0.1 ml/cm^2) was added to the cells. The solution was left to stand at 25°C for 5 min then 0.2% (v/v) chloroform was added with vigorous shaking (15 sec), then the solution was kept at 25°C for 15 min followed by centrifugation at 12,000 g_{av} at 4°C for 15 min. The upper aqueous phase containing the RNA was removed and isopropanol added to half the volume of TRI-REAGENT initially used. The solution was mixed and allowed to stand at 25°C for 10 min. After centrifugation, the supernatant was discarded and the pellet washed with 1 ml of 75% (v/v) ethanol/ml of TRI-REAGENT by vortexing the sample then the pellet was collected by centrifugation at 12,000 g_{av} at 4°C for 5 min. The resultant pellet was air-dried then re-dissolved in deionised water and the absorbance at 260 and 280 nm measured using the Genequant (Pharmacia) to calculate the RNA concentration.

The extracted RNA (8 µg) was resolved by electrophoresis on 2.2 M formaldehyde/1% (w/v) agarose gel at 30 V for 16 h. The gel was washed extensively with deionised water, then placed in 10X SSC (1 h) prior to overnight transfer to a nitrocellulose filter using the method of Northern blotting, as described in Sambrook *et al.* (1989).

3.7.3 Hybridisation with Oligonucleotide Probe

The filter prepared in section 3.7.2 was hybridised in hybridisation buffer (6X SSC, 50 mM sodium phosphate buffer (pH 6.8), 5X Denhardts Solution, 0.1 mg/ml denatured sheared herring sperm DNA, and 1% (w/v) SDS at 37°C (1h) with gentle agitation. Meanwhile the oligonucleotide probe was prepared.

The oligonucleotide probe was end labelled with T4 Polynucleotide Kinase (T4 PNK). T4 PNK catalyses the transfer of the γ-phosphate from ATP to the 5'-terminus of polynucleotides or to mononucleotides bearing a 3' phosphate group (Richardson, 1965) and has been widely used to end-label short oligonucleotide probes (Agerwal, 1981). The reaction was set up as in the technical bulletin supplied by the manufacturer (Promega). The mix contained the oligonucleotide, T4 PNK buffer, [γ-^{32}P]ATP (at 3000 Ci/mmol), and T4 PNK, according to the manufacturers instructions. The reaction was incubated at 37°C (10 min) then stopped by the addition of EDTA to a final concentration of 10 mM. The reaction was then transferred to a G-25 spin column (3 x 0.5 cm) and centrifuged at 3000 rpm (3 min). The eluent was captured in a sterile eppindorf then transferred into a hybridisation bag containing 25 ml of hybridisation buffer. The filter was carefully placed in the bag, the bag sealed to prevent radioactive leakage, and incubated in a sealed container at 37°C with gentle agitation (16 h). The filter was removed from the bag, washed in 5X SSC / 0.1% (w/v) SDS at 25°C three times for 5 min each followed by a final wash in the same solution at 37°C. The filter was blotted to remove excess liquid, then exposed to X-omat AR-5 film for varying lengths of time, and the film developed using an automatic developer.

3.7.4 Polymerase Chain Reaction (PCR) Amplification of a Synthetic Peptide-Affinity Derived Oligonucleotide Primer

The degenerate oligonucleotide probe derived form synthetic peptide-affinity protein tryptic subunits, was subjected to reverse transcription (RT) according to the RT protocol supplied by the manufacturer (Perkin-Elmer). The cDNA template produced by RT was then amplified by PCR according to the manufacturers' instructions (Perkin

Elmer). The reaction conditions were varied to optimise PCR amplification. PCR products were resolved on 1% (w/v) agarose gels containing 0.1% (v/v) ethidium bromide in TAE buffer, then viewed on an UV-transilluminator.

3.8 Cellular Studies of Xylosyltransferase Activity

3.8.1 RX SRC Cell Culture

SRC cells were plated into 96-well plates at a density of 6×10^5 cells/well in medium containing 2% (v/v) FCS. The cells were left at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 16 h then the media was discarded and replaced with serum-free media containing 1 μg/ml insulin. The cells were incubated for a further 24 h prior to the initiation of each experiment. To initiate each experiment, media was removed and replaced with 50 μl of experimental or control media.

3.8.2 Preparation of Agents for Probing Intracellular Xylosyltransferase Activity

All experimental media were prepared in DMEM containing 20 mM HEPES, penicillin (100 units/ml), and streptomycin (100 μg/ml) containing 1 μg/ml insulin. Control media contained no further additions. Brefeldin A was dissolved in 100% (v/v) ethanol at 100 μg/ml and then diluted to a final concentration of 1 μg/ml in medium. Cycloheximide was dissolved in culture medium at 100 μg/ml just prior to use. Monensin was dissolved in 100% (v/v) ethanol to give a 0.5 M solution then diluted to a final concentration of 1 μM in culture medium.

3.8.3 Solubilisation of Cellular Components for Measurement of Xylosyltransferase Activity

The level of xylosyltransferase activity in the cell layer and media prior to and after the addition of the chemical agent was measured as follows: the media was removed, the cells washed in 50 μl of media (and added to the removed media), then 100 μl of media containing 10 mM CHAPS was applied to the cell layer, the cells were solubilised by vigorous pipetting, then left on ice for 60 min. CHAPS was also added to the media to

a final concentration of 10 mM and left on ice for 60 min. The cell layer and media solutions were then assayed for xylosyltransferase activity.

3.8.4 Metabolic Radiolabeling with [^{35}S]Sulphate

[^{35}S]Sulphate (2.5 μCi/well) was added to the cells either for the duration of the experiment or introduced for 30 min bridging the time point being measured. [^{35}S]-labelled macromolecules were extracted and isolated from the cell layer and media as laid out in section 3.8.5 and incorporated radiolabel was measured using either PD10 size-exclusion chromatography or the CPC assay.

3.8.5 Solubilisation and Isolation of [^{35}S] Labelled Macromolecules

At the end of the radiolabel periods, the medium and cell layer were separately solubilised using GuHCl. Briefly, medium fractions were diluted with an equal volume of 8 M GuHCl, 20 mM CHAPS buffered with 50 mM sodium acetate, pH 6.0, containing a protease inhibitor cocktail. The cell layer was solubilised with 4 M GuHCl. Unincorporated radioactivity was removed from incorporated radioactivity by either PD10 size-exclusion chromatography or the CPC assay.

4. Results

4.1 Development of an Assay to Measure Xylosyltransferase Activity

SRC tissue was chosen as the source of xylosyltransferase as it was a fast growing cartilaginous tumour producing a large amount of aggrecan and therefore rich in xylosyltransferase. In order to purify xylosyltransferase from SRC tissue it was necessary to produce an efficient reproducible assay that could measure xylosyltransferase activity throughout the purification procedures. The SRC tissue was removed from the rats, sieved through fine stainless steel (1 mm) mesh and enzyme-digested to isolate the cells (methods, section 3.2.1). The cells were lysed and centrifuged to produce a 105,000 g_{av} tissue supernatant that contained all soluble cell proteins. The 105,000 g_{av} tissue supernatant contained 10 mg/ml protein and it was used in the development of an assay for xylosyltransferase and further purification steps.

4.1.1 Substrates for Xylosyltransferase

The *in vivo* substrates for xylosyltransferase are proteoglycan core proteins bearing exposed serine residues within a consensus sequence that are presented in a conformation that allows xylosyltransferase access to attach a xylose residue. Xylosyltransferase activity was first measured using endogenous acceptors present in crude tissue extracts (Robinson *et al.*, 1966), but this method gave problems in interpretation and reproducibility due to the ill-defined nature of the acceptor, its unknown concentration and if one or more acceptors were present. To overcome this problem a number of exogenous substrates for xylosyltransferase, reported in the literature, have been used to give more reproducible and quantitative data.

4.1.2 Smith Degraded Proteoglycan (PGSD) as a Substrate for Xylosyltransferase

Smith degradation involves cleavage of the neutral sugars at the linkage serine and removes the CS chains but leaves the KS chains on the proteoglycan relatively intact. This makes available for xylosylation the reactive serines to which the CS chains were attached.

The PGSD was prepared by the published method (Baker *et al*, 1972) and tested as a substrate for xylosyltransferase. The source of enzyme in all subsequent experiments (unless otherwise stated) was the 105,000 g_{av} supernatant from rat chondrosarcoma cells, which had a protein concentration of 10 mg/ml. PGSD (200 µg, by weight) was incubated with the 105,000 g_{av} tissue supernatant (20 µl), and UDP-D-[^{14}C]xylose, with the relevant controls, as set out in section 3.4.1. There was no increase in incorporation of [^{14}C]xylose in the reaction as compared to the controls, figure 4.1. As no incorporation of [^{14}C]xylose was observed when using PGSD as the substrate its sulphated GAG content was measured using the DMMB dye-binding assay. The concentration of sulphated GAG chains in the stock preparation was found to be 5 mg/ml; over half the weight of the freeze dried sample, suggesting the CS GAG chains had not all been cleaved off.

The treatment by Smith degradation was repeated on the remainder of this sample and it was analysed by DMMB dye binding for sulphated GAG content. Less than 20% of the sample was now determined to be chondroitin sulphate / keratan sulphate material. The new preparation of PGSD (200 µg) was used as the substrate in a xylosyltransferase assay (section 3.4.1), but again there was no increase in incorporation of [^{14}C]xylose in the reaction as compared to the controls.

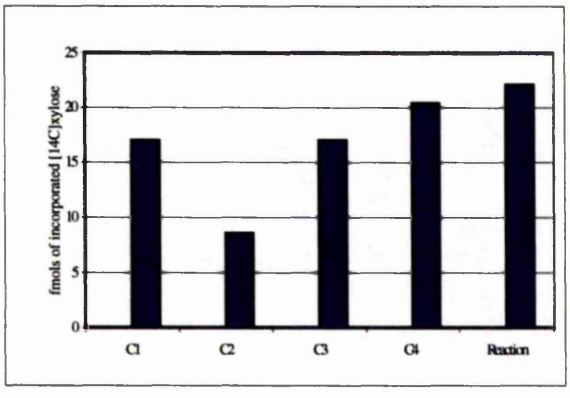


Figure 4.1. [^{14}C]xylose incorporation in the xylosyltransferase assay, using PGSD (200 µg) as the substrate and the 105,000 g_{av} as the source of enzyme activity (Reaction). C1 – no enzyme, C2 – no PGSD, C3 – no UDP-D-[^{14}C]xylose, C4 – no enzyme or PGSD. Reactions were performed in triplicate.

A second preparation of PGSD was given an extended period of acid cleavage (5 days) and was also digested with chondroitinase ABC. After the treatment the content of sulphated GAG was 40 µg/ml and the protein content of the sample was 200 µg/ml i.e. five fold more protein than sulphated GAG. When this PGSD preparation was used in a xylosyltransferase assay there was again no increase in incorporation of [^{14}C]xylose in the reaction compared to the controls. These results suggested that even if the sample contained available serine residues it also contained some inhibitor of enzyme action. Therefore an alternative substrate for xylosyltransferase was sought.

4.1.3 Silk Fibroin as a Substrate for Xylosyltransferase

Silk is not a natural substrate for xylosyltransferase as it is not thought to be glycanated in the silk worm. However, silk fibroin from the silk worm, *Bombyx mori*, has the repeating hexapeptide of Ser-Gly-Ala-Gly-Ala-Gly-, which contains the essential Gly-Ser-Gly for acceptor activity. It has previously been reported to be an acceptable substrate for xylosyltransferase and it also requires little experimental time to prepare it ready for assay (Campbell *et al.*, 1984).

Silk was prepared by dissolving commercial un-dyed silk in 60% (w/v) lithium-thiocyanate, then dialysing it into deionised water to give a final concentration of 1 mg/ml. Incubation of silk fibroin (200 µg) with the 105,000 g_{av} tissue supernatant and UDP-D-[^{14}C]xylose (section 3.4.1) resulted in the incorporation of [^{14}C]xylose, which was dependent on time, enzyme concentration, and the amount of silk in the reaction mixture. Silk fibroin (200 µg) was incubated with the 105,000 g_{av} tissue supernatant (50 µl) and UDP-D-[^{14}C]xylose, with the relevant controls (figure 4.2). Incorporation of [^{14}C]xylose was 20-fold greater in the reaction mixture than in all controls.

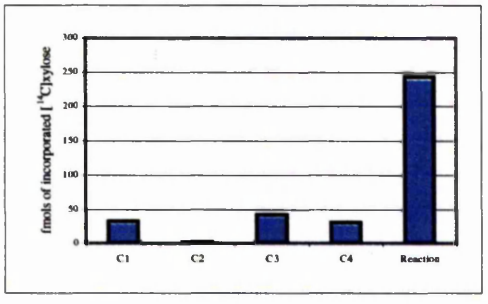


Figure 4.2. [^{14}C]xylose incorporation following a xylosyltransferase assay, using silk fibroin (200 µg) as the substrate and the 105,000 g_{av} as the source of enzyme activity. C1 – no enzyme, C2 – no silk fibroin, C3 – no UDP-D-[^{14}C]xylose, C4 – no enzyme or silk fibroin. Reactions were performed in triplicate.

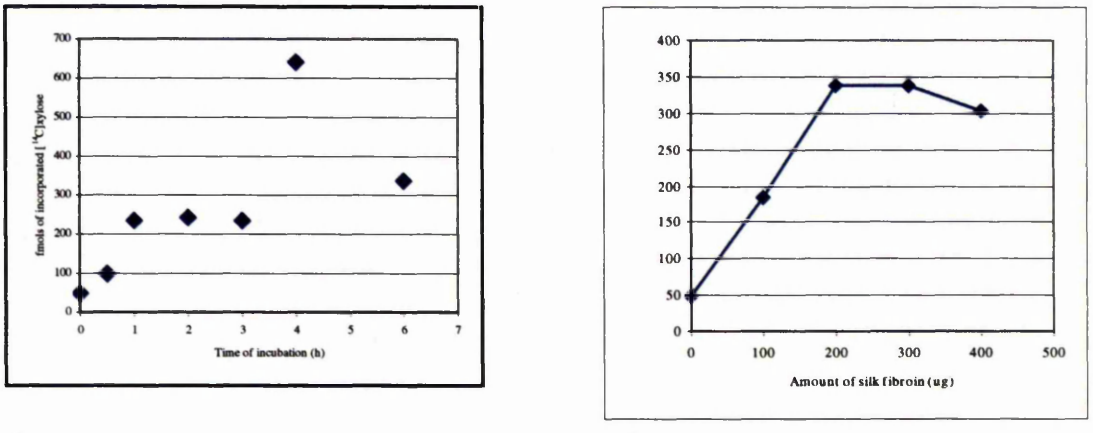
4.1.3.1 Investigations into the Reaction Conditions of the Xylosyltransferase Assay using Silk fibroin as Substrate

Time Course of Incorporation

Using silk fibroin as substrate the optimum time for incubation of the reaction mixture was investigated, figure 4.3 (panel A). Incorporation of [^{14}C]xylose on to silk fibroin with respect to time did not appear linear, rather it was inconsistent. The incorporation was similar between one hour to three hours and at six hours but with a spuriously high value at 4 hours of incubation. Low values at 3 h and 6 h are likely to be due to the substrate precipitating in some incubations and not others during longer incubation times. It was decided to incubate the reaction for just one-hour in future experiments to avoid this problem in the assay.

Concentration of Silk fibroin

The optimum amount of silk fibroin to use in an assay was investigated (figure 4.3 (panel B)). No increase in incorporation of [^{14}C]xylose was observed with increasing the silk content of the reaction mixture over 200 µg. This again may have resulted from the silk self-associating at higher concentrations.



A **B**

Figure 4.3. [^{14}C]xylose incorporation following a xylosyltransferase assay to measure the effect of varying the time of incubation at 37°C (panel **A**), using silk fibroin (200 µg) as the substrate, and the effect of increasing the amount of substrate (silk) in the assay (panel **B**). The 105,000 g_{av} (50 µl of a 10 mg/ml solution) was used as the source of enzyme activity.

PGSD and Silk

The second preparation of PGSD (200 μg) was again tested in a xylosyltransferase assay, with silk fibroin (200 μg) as a positive control. No incorporation of [^{14}C]xylose above background was seen when using PGSD as substrate. When PGSD and silk fibroin were added together to a reaction mixture, the PGSD preparation inhibited the incorporation of [^{14}C]xylose back down to control levels (results not shown)showing that it contained some inhibitor of xylosyltransferase action.

4.1.3.2 Xylosyltransferase Activity in the Chondrosarcoma Preparation

Standard xylosyltransferase assay conditions (section 3.4.1) were used to measure the level of xylosyltransferase activity in the crude Rat Chondrosarcoma homogenate, the 10,000 g_{av} and the 105,000 g_{av} supernatant fractions, respectively. Silk was used as the substrate and the protein content at each stage was measured. [^{14}C]xylose was incorporated in all the reaction mixtures. The protein concentration decreased from 0.9 mg/ml to 0.65 mg/ml when the crude homogenate was centrifuged at 10,000 g_{av}, but did not decrease any further after the 105,000 g_{av} centrifugation step. The incorporated [^{14}C]xylose/μg of protein was approximately two-fold higher in the 105,000 g_{av} supernatant fraction as compared to the crude homogenate (table 1).

	[^{14}C]xylose incorporation (pmols/ml)	Protein concentration (mg/ml)	[^{14}C]xylose incorporation (pmols/mg protein)
Crude homogenate	3.4	0.9	3.8
10,000 g_{av} supernatant fraction	2.2	0.65	3.4
105,000 g_{av} supernatant fraction	3.9	0.65	6.9

Table 1. Values extrapolated from xylosyltransferase assays using the crude homogenate, 10,000 g_{av} and 105,000 g_{av} supernatant fractions from the SRC tissue cells, (n=3, $p<0.05$).

Whilst silk fibroin had proved to be a substrate it was an unstable material to work with as it gradually gelled over a two-week period from its initial preparation. This made the experiments less reproducible, and so a better-defined acceptor for xylosyltransferase was sought.

4.1.4 The Recombinant Substrate

The CS-2 region of aggrecan is the major site of CS GAG chain attachment and has up to seventy possible xylosylation sites within it. By producing a cDNA containing this region and expressing the protein from it, it was hoped that it would provide a more reproducible recombinant substrate for xylosyltransferase.

A cDNA encoding the C-terminal region of aggrecan including a large segment of the CS-2 region was obtained from Dr. Jo Day. Various selected regions of this C4 cDNA from pKSC4, the human aggrecan cDNA containing a portion of the CS-2 region and the G3 domain (figure 4.4; panels A and B), were cloned into the bacterial expression vector, pTrcHis (figure 4.5) to produce expression constructs with a 6-histidine residue affinity tag. These were transformed into *E-coli*, dh5α cells and the recombinant protein expression induced by the addition of 1 mM IPTG. The recombinant protein was isolated using immobilised metal affinity chromatography selective for the 6-His tag. The purified recombinant protein was then used as a substrate in a standard xylosyltransferase assay (section 3.4.1).

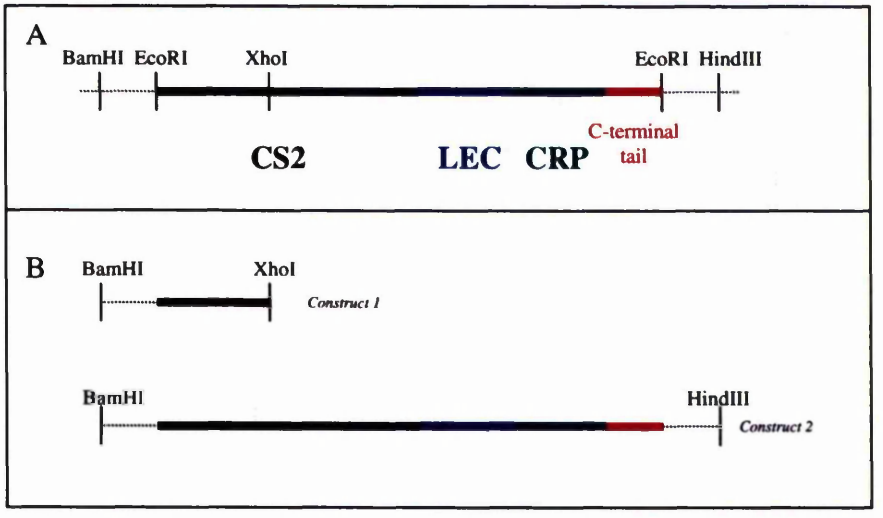


Figure 4.4. Panel A. The C4 cDNA insert (solid horizontal line) coding for part of the chondroitin sulphate attachment region (CS2), the lectin-like domain (LEC), the complement regulatory protein-like domain (CRP), and the C-terminal tail of human aggrecan. The dashed horizontal lines indicate DNA from the pBluescript polylinker region (not to scale).

Panel B. The bars indicate the restriction sites and portions of the cDNA used in the various recombinant constructs.

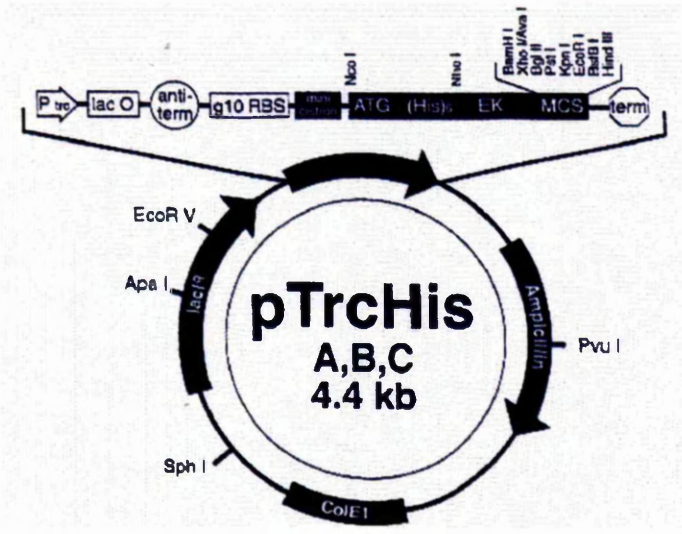


Figure 4.5. pTrcHis; a bacterial expression vector

Construct 1

pKSC4 was subjected to restriction digest using both *BamHI* and *XhoI* to produce a 750 base pair DNA fragment then ligated into pTrcHisA (figures 4.4 and 4.5). The integrity of the recombinant molecule was determined by restriction analysis. After induction with 1 mM IPTG samples of the culture were taken at time points of 0, 1, 2, 3, 4, and 5 h. The expected size of the recombinant protein containing a portion of the CS-2 region of aggrecan with a 6-His affinity tag was expected to be approximately 50 kDa. However, there was no marked increase of expression of any particular one of the resolved molecules on the Coomassie blue-stained SDS-PAGE gels (figure 4.6, panel A). The expressed products were purified using immobilised metal affinity chromatography, and the products resolved by SDS-PAGE and visualised by Coomassie blue staining. There was a very faint band at 50 kDa from the 2nd fraction (500µl) eluted from the column with 100 mM EDTA (gel not shown).

A range of IPTG concentrations and time periods were tried to optimise expression of the construct. The expressed proteins were resolved using SDS-PAGE, then immunoblotted using a 6-His monoclonal antibody to visualise any proteins that contained a 6-His tag (figure 4.6, panel B). Lanes 2 and 6, no IPTG, contained no immunoreactive material. There was immunoreactive material in all the lanes that had

been induced with IPTG. The size of the immunoreactive material was approximately 50 kDa. There was no marked increase of expression with increased amounts of IPTG or increasing the time of expression from 4 to 5 hours. The expression levels of this construct using this system was low and suggested that it would be insufficient to use in a xylosyltransferase assay.

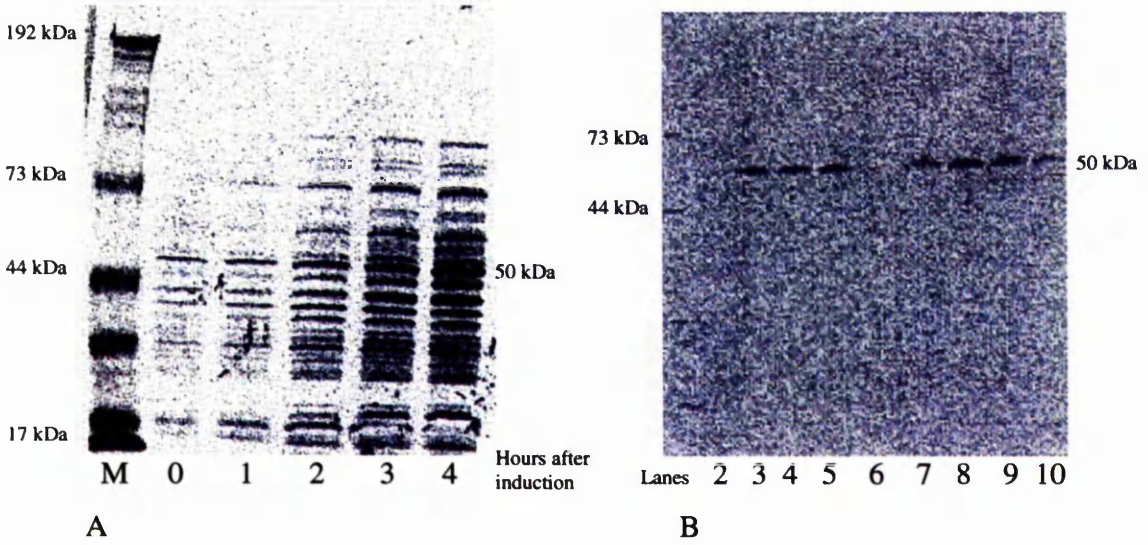


Figure 4.6. Panel A. SDS-PAGE analysis on a 4-20% (w/v) tris-glycine gel of total cell lysates following induction with 1 mM IPTG of *E.coli* containing plasmid construct 1. Samples were taken every hour for five hours following induction. Proteins were visualised by staining with Coomassie blue. M = marker.

Panel B. Immunoblot analysis of the expressed proteins from *E-coli* containing construct 1. The concentration of IPTG and increasing the time of expression was measured.

Lane 2 – uninduced, 4 h
Lane 3 – 1 mM IPTG, 4 h
Lane 4 – 5 mM IPTG, 4 h
Lane 5 – 10 mM IPTG, 4 h
Lane 6 – uninduced, 5 h
Lane 7 – 1 mM IPTG, 5 h
Lane 8 – 5 mM IPTG, 5 h
Lane 9 – 10 mM IPTG, 5 h
Lane 10 – 10 mM IPTG, 3 h

Construct 2

In this construct the whole of the C4 region, the CS-attachment region and G3 globular domains, were cloned into pTrcHis. pKSC4 was restricted with *BamHI* and *HindIII* to yield a fragment of 1953 base pairs which was cloned into pTrcHisA. The integrity of the construct was verified by restriction analysis. Positive clones were induced and purified as for construct 1. The purified product was resolved by SDS-PAGE on lanes 6

and 7 (figure 4.7). The expected size of the recombinant protein was approximately 100 kDa. A 100 kDa band

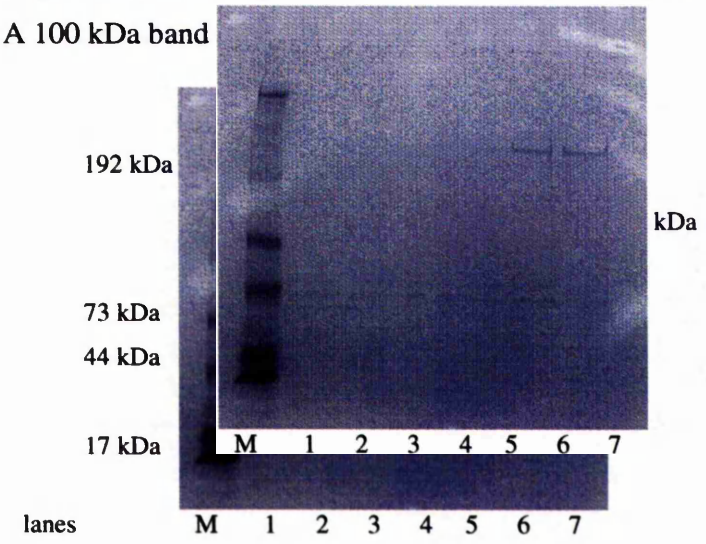


Figure 4.7. SDS-PAGE analysis on a 4-20% (w/v) Tris-glycine gel of the 'TALON' purified 6-His proteins from construct 3. Lanes 6-7; construct 3, Talon fraction 2. M = marker. Bands visualised by staining with Coomassie Blue.

4.1.4.1 The Recombinant Protein as a Substrate for Xylosyltransferase

The purified recombinant protein from construct 3 was dialysed against standard MES buffer. The recombinant protein (30 µg) was incubated with the 105,000 g_{av} tissue (50 µl) and UDP-D-[^{14}C]xylose, with the relevant controls, in a standard xylosyltransferase assay (section 3.4.1), using silk fibroin as a positive control (figure 4.8, column R1). No incorporation of [^{14}C]xylose was seen using the recombinant protein as substrate (figure 4.8, column R2) at the concentration tested.

Silk fibroin (200 µg) and recombinant protein (30 µg) were both added to the assay mixture. In this reaction there was no significant decrease in the incorporation of [^{14}C]xylose, compared to the reaction with silk alone as substrate (figure 4.8, column R3). This showed the recombinant protein was not inhibiting xylosyltransferase, but was also not acting as an acceptor for the enzyme. The main problem appeared to be that there was insufficient substrate and the enzyme was thus not transferring enough xylose to be easily detected. As the recombinant substrate was not being produced in large enough amounts sufficient to enable saturating concentration of substrate to be used with the enzyme it was decided to explore other more abundant substrates.

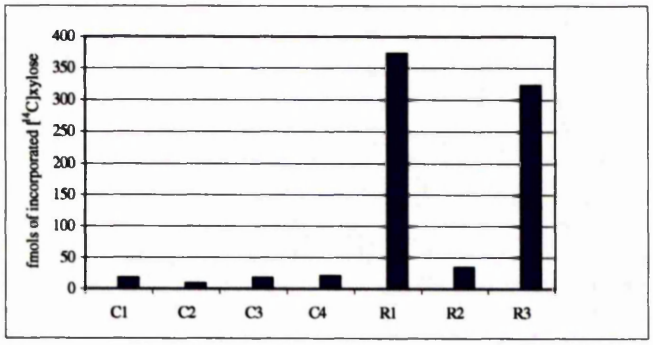


Figure 4.8. [^{14}C]xylose incorporation following a xylosyltransferase assay using the recombinant substrate (30 µg) as the substrate with silk fibroin (200 µg) as a positive control. The 105,000 g_{av} (50 µl) was used as the source of enzyme activity. C1 – no 105,000 g_{av}, C2 – no silk fibroin, C3 – no UDP-D-[^{14}C]xylose, C4 – no 105,000 g_{av} or silk fibroin, R1 – silk (only) reaction, R2- recombinant substrate reaction, R3 - silk and recombinant substrate together reaction (reactions performed in triplicate).

4.1.5 The Synthetic Peptide

Small peptides containing suitable amino acid sequences for transfer of xylose have also been used as substrates for xylosyltransferase. These have varied from tri-peptides containing the essential Gly-Ser-Gly sequence to peptides containing a number of possible active serines within them (Roden *et al.*, 1985). One of the most active acceptor peptides reported is derived from the sequence surrounding the single CS attachment site of bikunin, the inhibitory component of human inter-alpha-trypsin inhibitor, QEEEGSGGGQKK, (Weilke *et al.*, 1997). The sequence of the attachment site contains all the elements required for recognition by xylosyltransferase i.e., Ser-Gly-X-Gly, which is also preceded by a group of acidic amino acids (figure 4.9).

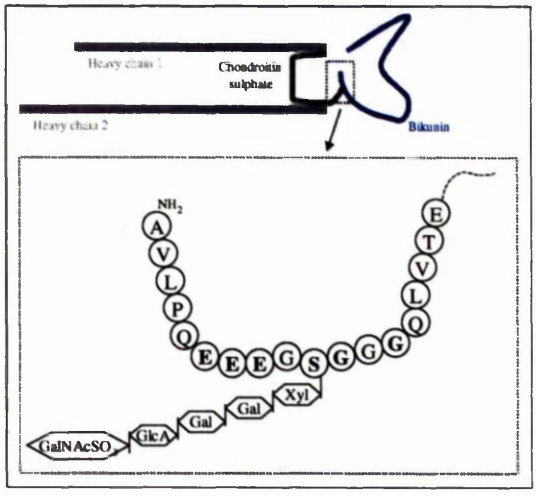


Figure 4.9. The structure of human inter-α-trypsin inhibitor and the chondroitin sulphate attachment site of bikunin, from Weilke *et al.* (1997).

Bold amino acids are thought to be essential or important for recognition by xylosyltransferase in chondroitin sulphate biosynthesis. Gal – galactose, GlcA – glucuronic acid, $GalNAcSO_3$ – N-acetylgalactosamine sulphate.

4.1.5.1 The Synthetic Peptide as a Substrate in the Xylosyltransferase Assay

In order to detect the peptide, some of it was labelled with FITC under mild conditions. This would label either the free N-terminal amino group or one of the C-terminal lysine residues. In either case, the FITC would not be close to the serine that forms the acceptor for xylose transfer. Gel chromatography on a Biogel P2 column showed fluorescence eluting in the position of unmodified peptide and earlier than free FITC. The FITC-conjugated peptide (250 μg) was incubated with the 105,000 g_{av} tissue supernatant (250 μl) and 8 nmols of UDP-D-[^{14}C]xylose for one hour at 37°C, with the relevant controls. The reaction products were separated by gel filtration on a P2 Biogel column (1 x 20 cm). The radiolabel trace showed a small peak around fractions 9-11 and a large peak around fractions 14-18. Fractions that contained fluorescent material eluted with the first small peak of radioactivity, suggesting that xylose was being transferred to the FITC-conjugated synthetic peptide. Controls with no enzyme, no peptide or no incubation period showed only the large non-incorporated peak on their respective radiolabel traces (figure 4.10). Using non-fluorescent synthetic peptide in the assay showed a radiolabel trace identical to that of the FITC-conjugated synthetic peptide, suggesting that FITC was not playing any role in the mechanics of the reaction. So by using gel filtration it was possible to separate incorporated [^{14}C]xylose attached to the FITC-peptide from unincorporated UDP-D-[^{14}C]xylose.

It was then attempted to use PVDF membranes to immobilise the peptide-xylose product, as this could lead to a simple rapid assay method. The FITC-conjugated synthetic peptide (10 μg) and unlabelled synthetic peptide (10 μg) were added to a standard xylosyltransferase assay (method 3.4.1) for one hour at 37°C. The reaction mixture was placed on PVDF membrane discs and allowed to dry, then precipitated with TFA, as for the reactions with the silk substrate and washed. The results (not shown) indicated that no [^{14}C]xylose labelled material was immobilised on the PVDF membrane in any of the control or experimental reactions. The xylose labelled peptide or FITC-peptide was not retained on the PVDF membrane.

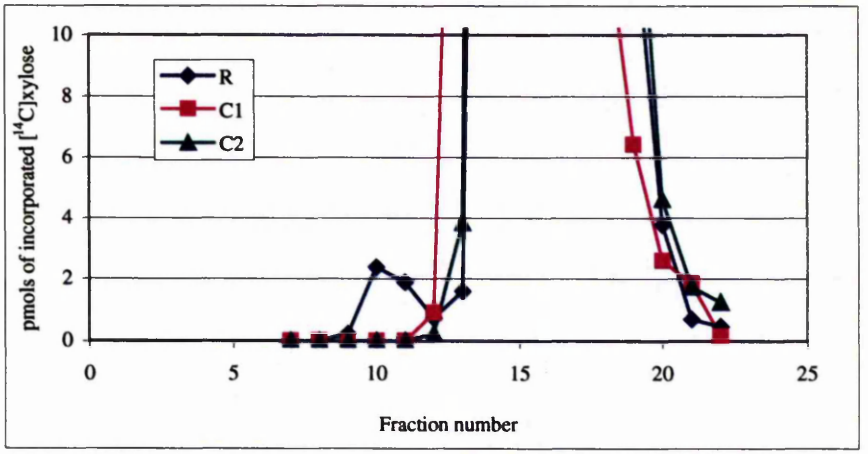


Figure 4.10. [^{14}C] counts from the respective fractions (500µl) from a P2 Biogel column (1 x 20 cm), flow rate 125 µl/min.

R – UDP-D-[^{14}C]xylose incubated with the FITC-conjugated or non-conjugated synthetic peptide and the 105,000 g_{av} tissue supernatant

C1 - UDP-D-[^{14}C]xylose incubated with the FITC-conjugated synthetic peptide

C2 - UDP-D-[^{14}C]xylose incubated with the 105,000 g_{av} tissue supernatant

4.1.5.2 Development of a Reproducible Xylosyltransferase Assay using the Synthetic Peptide as the Substrate

As it was possible to separate incorporated [^{14}C]xylose from unincorporated UDP-D-[^{14}C]xylose using gel filtration with the synthetic peptide as substrate, as described above, this method was investigated further to establish a more reproducible and quantitative method for measuring xylosyltransferase activity.

Various different separation media and columns of different dimensions were tested for their ability to separate the incorporated [^{14}C]xylose from unincorporated UDP-D-[^{14}C]xylose. The best results were obtained using columns made from cell-culture stripettes, cut to hold 5 ml of pre-swollen P2-biogel in MES buffer, held in place by a small bolus of glass wool. The FITC-conjugated peptide (10 µg) was incubated with the 105,000 g_{av} tissue supernatant (50 µl) and 3.3 nmols of UDP-D-[^{14}C]xylose at 37°C for 1 h, as set out in section 3.4.2, then applied to a 5 ml P2 Biogel column. Aliquots (100 µl) of standard MES buffer were applied to the column and collected. Each

fraction was counted for their [^{14}C] content (figure 4.11). Fractions 17 to 24 contained the incorporated peak. This method separated incorporated [^{14}C]xylose from unincorporated UDP-D-[^{14}C]xylose, in an efficient and reproducible manner.

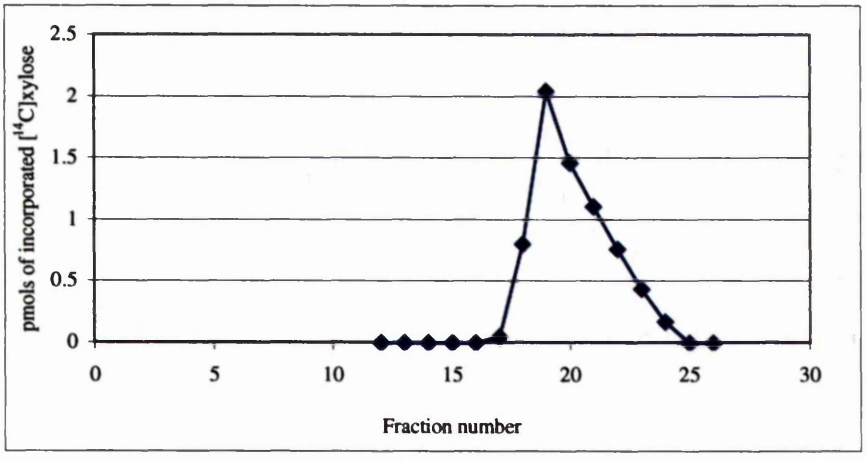


Figure 4.11. [^{14}C] counts from the respective fractions (100 µl) from a P2 Biogel column (1 x 5 cm), flow rate 100 µl/min, from a reaction containing UDP-D-[^{14}C]xylose incubated with the FITC-conjugated or non-conjugated synthetic peptide and the 105,000 g_{av} tissue supernatant

4.2 Optimisation of Xylosyltransferase Assay Reaction Conditions: using the synthetic peptide as substrate and the 105,000 g_{av} supernatant as the source of xylosyltransferase activity

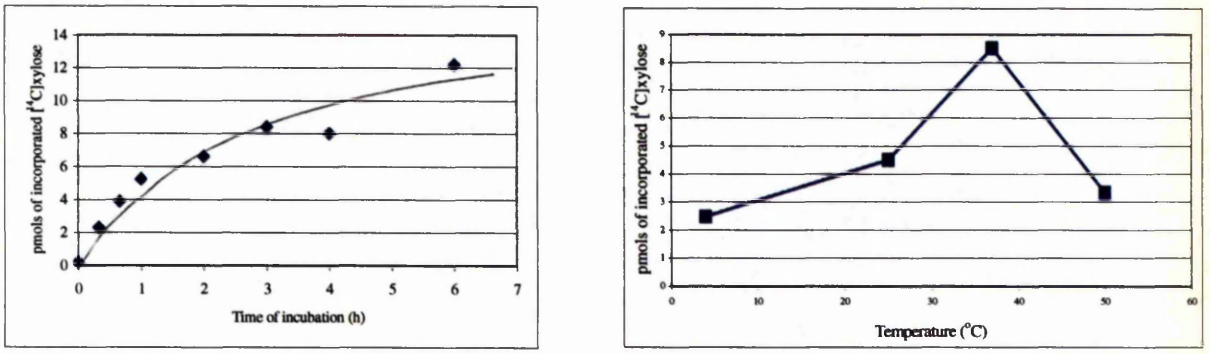
Using the synthetic peptide (2 µg) as the substrate for xylosyltransferase, the 105,000 g_{av} tissue supernatant (50 µl) as the source of xylosyltransferase and UDP-D-[^{14}C]xylose (1.65 nmols) to follow the reaction, the different factors contributing to the substitution of xylose onto the synthetic peptide were investigated, with the relevant controls. All reactants were applied to a 5 ml P2 Biogel column to separate the incorporated [^{14}C]xylose from the unincorporated UDP-D-[^{14}C]xylose. The column was eluted with standard MES buffer and collected in 100 µl fractions. Fractions 17-24 were pooled and their [^{14}C] content measured for incorporated xylose.

4.2.1 Duration of Incubation at 37°C

The reactions, with the relevant controls, were set up as above and incubated for a range of times, from 0 – 6 h. There was a steady increase in incorporation that was approaching a maximum at 6 h, figure 4.12 (panel A).

4.2.2 Temperature of Incubation

The reactions were set up as above but were incubated for 1 h at different temperatures. The incorporation increased with temperature and was maximal at 37°C, figure 4.12 (panel B). Enzyme activity was greatly reduced at 50°C.

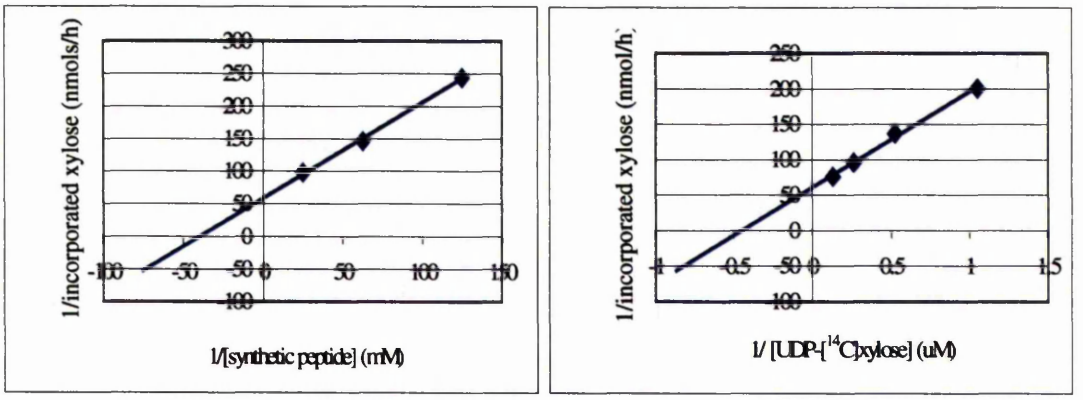


A B

Figure 4.12. The effect of time (panel A) and temperature (panel B) of incubation on xylosyltransferase activity.

4.2.3 Substrate Concentrations

The non-conjugated synthetic peptide and UDP-D-[^{14}C]xylose were added to a typical xylosyltransferase assay, section 3.4.2, at a range of concentrations to determine the optimum amount of substrate to add to an assay. The synthetic peptide was not fully saturating the assay at 25μM, figure 4.13, therefore in future experiments, 10 μg of synthetic peptide was added to each xylosyltransferase activity reaction. From the double reciprocal plot, the K_m for UDP-xylose was found to be 2.2 μM and a V_{max} of 0.017 nmol/h. The intercept for the synthetic peptide went straight through zero, so it was not possible to give values for the K_m or V_{max} for xylosyltransferase as a function of synthetic peptide concentration. However, if only the 3 higher substrate concentrations were used it gave a K_m of 2.2 μM.



A B

Figure 4.13. Determination of the K_m for the xylosyltransferase reaction as a function of synthetic peptide concentration (panel A) and UDP-D-xylose concentration (panel B), using a standard xylosyltransferase assay with the 105,000 g_{av} tissue supernatant as the source of xylosyltransferase activity. The concentration of substrate is plotted against the radioactivity incorporated. The K_m was calculated from the double reciprocal plots (as shown) of these data.

4.2.4 Enzyme Stability

Enzyme activity with time was investigated in order to determine how well the enzyme activity survived storage. This was important to know for further purification experiments. The 105,000 g_{av} tissue supernatant was stored at both room temperature (25°C) and at 4°C for varying lengths of time, then tested in a xylosyltransferase assay as described above. Storing the enzyme at room temperature showed a rapid decrease in xylosyltransferase activity, figure 4.14, and after 4 days there was no xylosyltransferase activity remaining. However, storing the enzyme at 4°C showed no significant reduction in xylosyltransferase activity even after 21 days, figure 4.14.

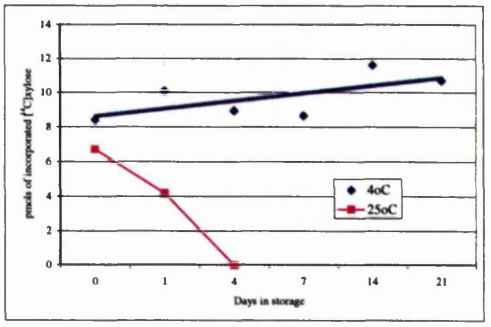


Figure 4.14. The effect of storage temperature on the xylosyltransferase activity of the 105,000 g_{av} tissue supernatant.

4.2.5 Addition of Detergent and Increased Salt Concentrations

In future purification steps detergents and increased levels of salts in buffer systems might be used, so their effects on xylosyltransferase activity were measured. Various concentrations of a detergent, CHAPS, and potassium chloride were added to a typical xylosyltransferase assay. The addition of 0.5% (w/v) CHAPS showed a reduction in xylosyltransferase activity by approximately one third, whilst increasing the concentration of KCl from 0.05 M up to 0.25 M in the MES buffer reduced the activity of xylosyltransferase by half (results not shown). Enzyme activity was thus still easily detected in the presence of 0.5% (w/v) CHAPS and in 0.25 M KCl.

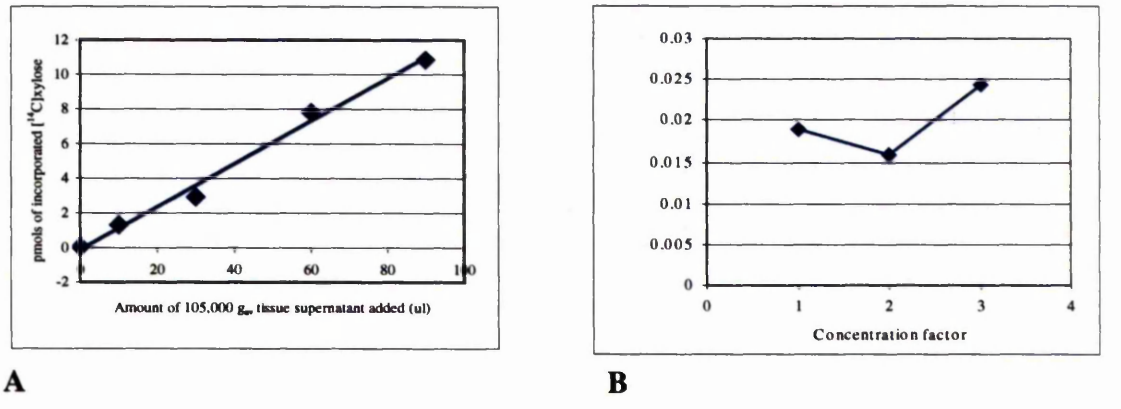
4.2.6 The Effect of Cytidine 5' Diphosphate

Glycogenin is an enzyme, which is responsible for glycogen synthesis, that mainly utilises glucose as its substrate, but when glucose is in short supply it can also utilise xylose as a sugar substrate. It might therefore be responsible for incorporation of [^{14}C]xylose into a high molecular weight product in a xylosyltransferase assay. Cytidine 5' Diphosphate (CDP) is a potent inhibitor of glycogenin but it does not effect xylosyltransferase activity. CDP was added to a typical xylosyltransferase assay to determine whether or not the enzyme activity that was measured was indeed xylosyltransferase activity and not glycogenin. The addition of CDP up to 100 µM had no effect on xylose incorporation (results not shown). Therefore glycogenin is not contributing in any way to the transfer of xylose in the assay.

4.2.7 Increasing the Concentration of the 105,000 g_{av} Tissue Supernatant

In a typical assay, 50 µl of the 105,000 g_{av} tissue supernatant (10 mg/ml) was used as the source of xylosyltransferase activity. The amount of the 105,000 g_{av} tissue supernatant added to the assay was varied from 10 – 90 µl, figure 4.15 (panel A), as well as increasing the other assay components to contain 10 µg of the synthetic peptide and 3.8 nmol of UDP-D-[^{14}C]xylose in a final volume of 100 µl. However, as the

amount of incorporated xylose was still increasing linearly it was hoped that by concentrating the stock solution with centrifugal membrane concentrators, more xylosyltransferase activity could be measured. Unfortunately, concentrating the 105,000 g_{av} tissue supernatant by this method did not increase the concentration of xylosyltransferase activity by the same factor, figure 4.15 (panel B). Decreasing the volume to one half or one third resulted in no significant increase in the concentrations of xylosyltransferase activity. There was therefore a significant loss of xylosyltransferase activity on concentration.



A **B**

Figure 4.15. The effect of increasing the amount of 105,000 g_{av} tissue supernatant (10 mg/ml stock) (panel **A**), and increasing the concentration of the 105,000 g_{av} tissue supernatant by centrifugal concentrators (Centriplus-30) (panel **B**) on xylosyltransferase activity.

4.3 Purification of Xylosyltransferase from the 105,000 g_{av} Tissue Supernatant

4.3.1 Size Fractionation

The first step in the purification strategy was to separate the molecules in the 105,000 g_{av} tissue supernatant by size and to monitor enzyme activity and protein concentration. This step was also useful in determining an approximate size for the enzyme.

Sephadex G-200 Chromatography

Initially the 105,000 g_{av} tissue supernatant was fractionated using Sephadex G-200 in standard MES buffer, column size 1x 20 cm. The V_0 and V_t of the G-200 column were determined using aggregated aggrecan (mw >30 x 10^6) and sodium azide (mw 65) respectively. The 105,000 g_{av} tissue supernatant (200 µl of a 10 mg/ml stock) was applied to the column (flow rate 0.2 ml/min), the sample eluted with standard MES buffer and fractions (500 µl) collected. The fractions were tested for protein content, using the BCA assay, and xylosyltransferase activity (section 3.4.2), figure 4.16. BSA (0.1 mg/ml), in a separate column run, was used as a size marker and eluted in fractions 17-18.

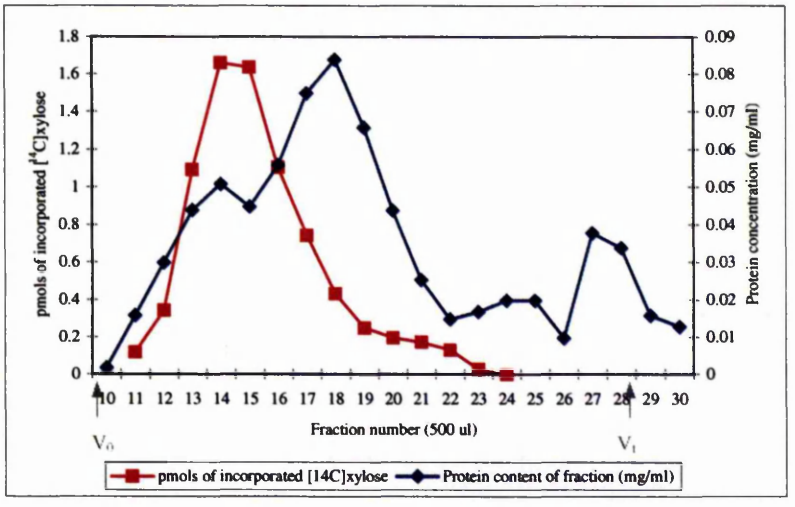


Figure 4.16. Purification of SRC xylosyltransferase by gel filtration. A 200 µl sample of the 105,000 g_{av} tissue supernatant (specific activity, 10 pmols [^{14}C]xylose transferred/mg of protein) was applied to a column of Sephadex G-200 (fine) which had previously been equilibrated with standard MES buffer, pH 6.5. Fractions of 500 µl were collected and the absorbance at 280 nm was recorded (blue line). Aliquots (90 µl) of every fraction were tested for xylosyltransferase activity, expressed as pmols of [^{14}C]xylose transferred per hour under standard assay conditions (red line).

The BSA marker eluted from the column between fractions 18 and 19, whilst the peak of xylosyltransferase activity eluted between fractions 14 and 15. This would suggest that xylosyltransferase has a larger molecular weight than BSA; approximately 100 kDa. The total amount of xylosyltransferase activity that was eluted from the Sephadex G-200 column was twice that applied to the column. This suggested that gel filtration removed an inhibitor of xylosyltransferase activty. However, using Sephadex G-200 there was still significant overlap between the enxyme activity and the other proteins in the original sample so further gel-fractionation media were investigated.

Sephacryl S-300 Chromatography

The 105,000 g_{av} tissue supernatant was fractionated using Sephacryl S-300 media in standard MES buffer, column size 0.9x 60 cm. The V_0 and V_t of the Sephacryl S-300 column were determined as before using aggregated aggrecan (mw >30 million) and sodium azide (mw 65) respectively. The 105,000 g_{av} tissue supernatant (1 ml of a 10 mg/ml protein stock) was applied to the column (flow rate=0.3 ml/min), the sample eluted with standard MES buffer and fractions (1 ml) collected. The fractions were tested for protein content, using the BCA assay, and xylosyltransferase activity (section 3.4.2), figure 4.17.

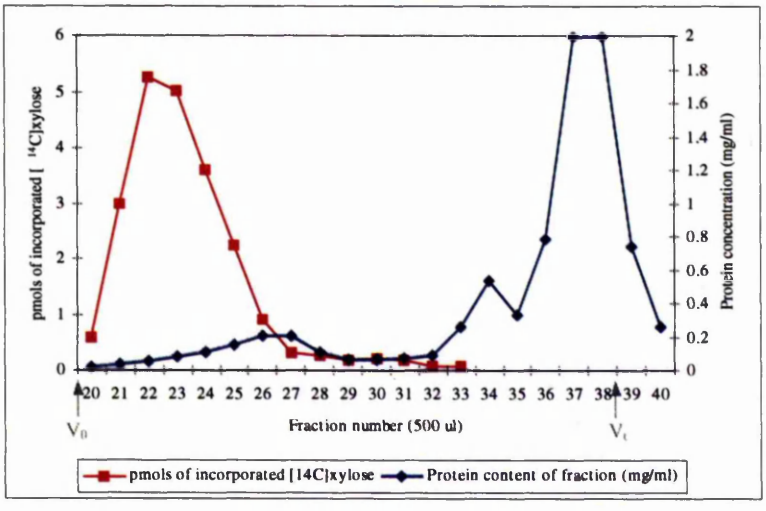


Figure 4.17. Purification of SRC xylosyltransferase by gel filtration. A 1 ml sample of the 105,000 g_{av} tissue supernatant (specific activity, 10 pmols [^{14}C]xylose transferred/mg of protein) was applied to a column of Sephacryl S-300 (fine) which had previously been equilibrated with standard MES buffer, pH 6.5. Fractions of 1 ml were collected and the absorbance at 280 nm was recorded (blue line). Aliquots of (90 µl) of every fraction were tested for xylosyltransferase activity, expressed as pmols of [^{14}C]xylose transferred per hour under standard assay conditions (red line).

The total amount of xylosyltransferase activity that was eluted from the Sephacryl S-300 column was also twice that applied to the column, confirming that gel filtration may remove an inhibitor of xylosyltransferase activty. Sephacryl S-300 appeared to separate over 95% of the other proteins from the enzyme activity. Therefore it was the chosen method for the initial step in purifying xylosyltransferase.

4.3.2 Protein Concentration Methods

Whilst size fractionation removed a high proportion of contaminants, it also left the sample dilute, which is undesirable for further purification stages. Fractions 21-25, from the Sephacryl S-300 column, contained the peak of xylosyltransferase activity. These fractions were pooled together and concentrated.

The pooled fractions were concentrated using centriplus-30^{TM} centrifugal membrane concentrator or by freeze drying, followed by reconstitution in standard MES buffer. Fractions were tested for xylosyltransferase activity before and after the concentration procedure using a standard xylosyltransferase assay, section 3.4.2. Both methods proved unsuccessful at concentrating xylosyltransferase activity. Using the centrifugal concentrators only one fifth of the original starting activity was present after concentration (activity/volume) and after freeze drying only one tenth was recovered (results not shown).

It was also attempted to block the centriplus-30^{TM} membrane by pre-treatment with casein, to test whether xylosyltransferase was sticking to the membrane, hence the loss of activity. However this did not effect the results.

It was necessary to concentrate the pooled fractions to increase the concentration of xylosyltransferase activity and this was not achieved by any of the methods tested with the 105,000 g_{av} tissue supernatant. Another problem with this source of enzyme was its limited amount due to the time taken to grow up and transplant the chondrosarcoma tissue. So, other more readily available sources of xylosyltransferase were sought.

4.4 Alternative Sources of Xylosyltransferase

It had been reported in the literature that xylosyltransferase activity had been found in human serum (Weilke *et al.*, 1997). Therefore it could also be present in bovine serum, so FCS was tested for its level of xylosyltransferase activity. The FCS used was of cell culture quality. For xylosyltransferase activity to be present in serum, it would suggest that xylosyltransferase is being secreted out of the cell in an active form. With this rationale the spent medium from the chondrocyte-like Syrian hamster cells, Des4^{+}.2, was also tested for xylosyltransferase activity.

Standard xylosyltransferase assays were set up containing either FCS or the Des4^{+}.2 media as the source of xylosyltransferase. The Des4^{+}.2 cells were plated at a density of $6x10^{4}$ cells/cm^{2}, after 24 h and 72 h the medium was removed and centrifuged at 10,000 g_{av} for 10 min at 4 °C. As the solutions did not contain the essential enzyme co-factors (Mg^{++} and Mn^{++}), half the volume (45 µl) was from the source, and half (45 µl) from standard MES buffer, which contains the ions required. The synthetic peptide and UDP-D-[^{14}C]xylose were added and a typical xylosyltransferase assay carried out, section 3.4.2.

The results (figure 4.18) showed strong xylosyltransferase activity in FCS with incorporation about 200% of that found in the 105,000 g_{av} tissue supernatant. The medium from Des4^{+}.2 cells also contained xylosyltransferase activity, which increased with time in culture. This suggested that various cells might release xylosyltransferase activity. It was decided to use FCS as the new source of xylosyltransferase activity as it contained a high level of xylosyltransferase activity and was readily available in large quantities.

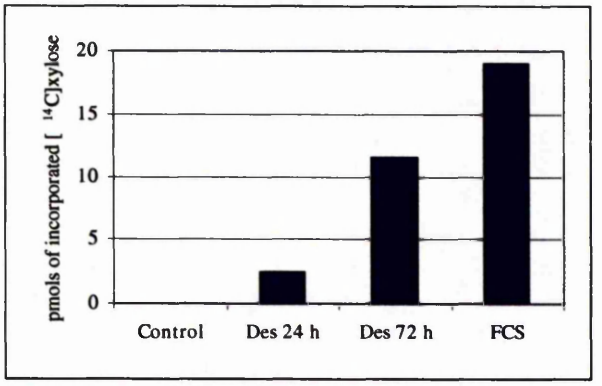


Figure 4.18. [^{14}C]xylose incorporation following a typical xylosyltransferase assay using foetal calf serum or the spent culture medium of the Syrian Hamster Embryo Fibroblasts, Des4^{+}.2 after 24 and 72 h as the source of xylosyltransferase activity. The control reaction was culture medium alone as a source of xylosyltransferase activity. All reactions were in duplicate.

4.4.1 The Effect of Cytidine 5' Diphosphate on Xylosyltransferase Activity in FCS

As for the 105,000 g_{av} SRC tissue supernatant, the transfer of xylose from UDP-xylose on to the synthetic peptide could be from both the activity of xylosyltransferase and/or glycogenin as previously described in section 4.2.5. Again, the addition of 200 µM CDP had no effect on the transfer of [^{14}C]xylose compared to the control reaction with no CDP added (results not shown). Therefore, glycogenin is not contributing in any way to the transfer of xylose in the assay when using FCS as the source of xylosyltransferase activity.

4.4.2 Size Exclusion Chromatography using Sephacryl S-300 on FCS

FCS was fractionated using Sephacryl S-300 media in standard MES buffer, column size 0.9x 60 cm. The V_0 and V_t of the Sephacryl S-300 column were determined as before using aggregated aggrecan (mw >30 million) and sodium azide (mw 65) respectively. The FCS (1 ml of a 20 mg/ml protein stock) was applied to the column (flow rate=0.3 ml/min), the sample eluted with standard MES buffer and fractions (1 ml) collected. The fractions were tested for protein content, using the BCA assay, and xylosyltransferase activity (section 3.4.2), figure 4.19.

The total amount of xylosyltransferase activity that was eluted from the Sephacryl S-300 column was twice that applied to the column, confirming that gel filtration may remove an inhibitor of xylosyltransferase activity, as was the case for the 105,000 g_{av} SRC tissue supernatant. However, unlike the 280 nm trace seen for the 105,000 g_{av} SRC tissue supernatant, the 280 nm trace from the FCS co-eluted with the peak of xylosyltransferase activity. This suggested that when using FCS as the source of xylosyltransferase, size exclusion chromatography using Sephacryl S-300 media was a less effective step in enzyme purification.

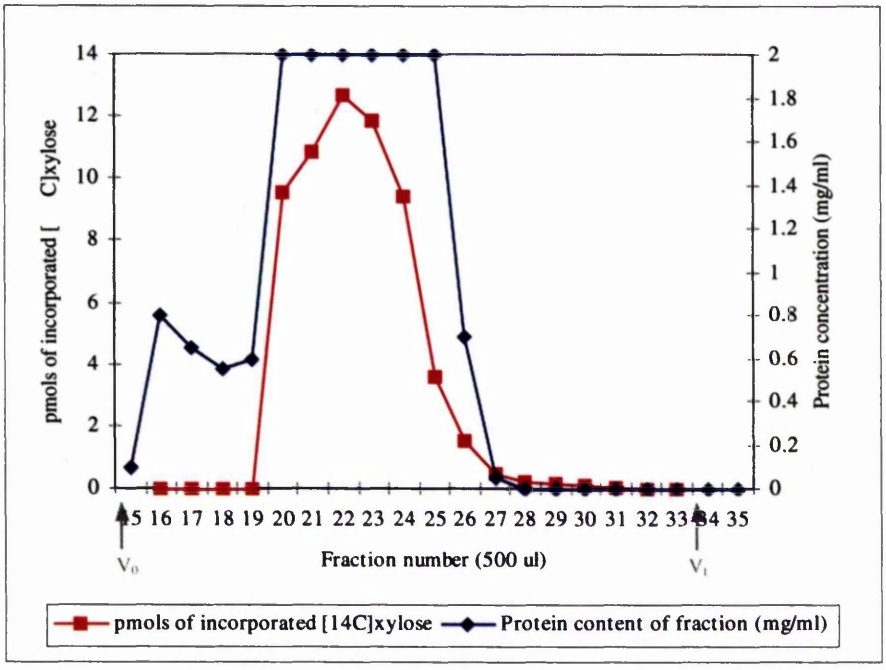


Figure 4.19. Purification of FCS xylosyltransferase by gel filtration. A 1 ml sample of FCS (specific activity, 10 pmols [^{14}C]xylose transferred/mg of protein) was applied to a column of Sephacryl S-300 (fine) which had previously been equilibrated with standard MES buffer, pH 6.5. Fractions of 1 ml were collected and the absorbance at 280 nm was recorded (blue line). Aliquots of (90 µl) of every fraction were tested for xylosyltransferase activity, expressed as pmols of [^{14}C]xylose transferred per hour under standard assay conditions (red line).

4.4.3 UDP-Agarose Affinity Gel Purification

Xylosyltransferase catalyses the following reaction:

UDP-xylose + acceptor → acceptor-xylose + UDP

Xylosyltransferase recognises both UDP-xylose and the protein acceptor sequence to which it attaches the xylose and then releases UDP. Therefore it is possible that a matrix support, such as agarose to which UDP is conjugated may have an affinity for xylosyltransferase and be an effective step in the purification of the enzyme.

The UDP-agarose slurry was washed in 10 volumes of deionised water then resuspended 1:1 in sample loading buffer (25 mM Imidazole (pH 7.4), 20 mM $MnCl_2$, 0.2 M NaCl, and 0.1% (w/v) Triton X-100). The gel was mixed with an equal volume of FCS (pre-dialysed against standard MES buffer) containing 0.1% Triton X-100 for 16 h at 25°C to allow the enzyme to bind to the gel support. The supernatant was discarded, the gel washed three times in three column volumes of sample loading buffer, then two column volumes of elution buffer (25 mM Imidazole (pH 7.5), 2 M NaCl and 0.2% Triton X-100) added and rotated at room temperature for 2 h. Samples (50 µl) of the supernatant were removed at various time intervals and tested for their level of xylosyltransferase activity, section 3.4.2.

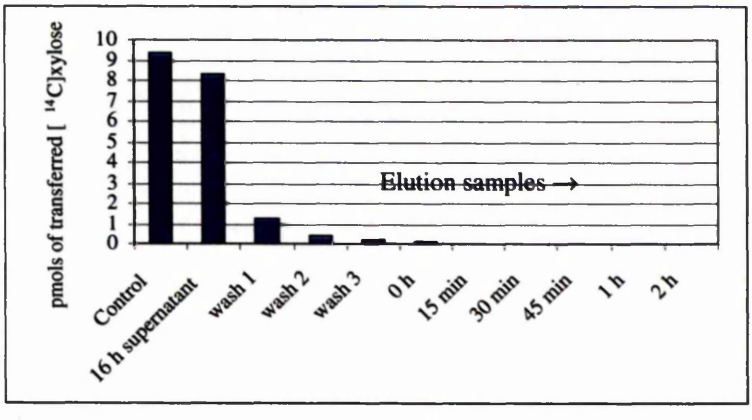


Figure 4.20. Elution of FCS xylosyltransferase activity from UDP-agarose affinity gel by the removal of manganese ions. The control was the original FCS used, not rotated with the gel, and diluted 1:1 with standard MES buffer then used in a standard xylosyltransferase activity. All samples (50 µl) were monitored for xylosyltransferase activity.

It was clear that xylosyltransferase did not have an affinity for the UDP-gel, under the test conditions, as the activity was either in the supernatant after the 16 h incubation or was lost in the subsequent washes, figure 4.20. There was no xylosyltransferase activity detected in the eluted fractions.

4.4.4 Synthetic Peptide Affinity Chromatography

As the synthetic peptide, QEEEGSGGGQKK, had proved to be a good substrate for xylosyltransferase, an affinity matrix using the synthetic peptide as the ligand was developed. The peptide, being only 1234 Da in size, is very small so it was covalently attached to a matrix that contained 12 carbon spacer arms in the hope that it would project out from the matrix sufficiently for xylosyltransferase to bind to it.

The synthetic peptide (80 mg) was conjugated to a 5 ml pre-packed Hi-trap NHS-activated column (10 μmol NHS-groups/ml gel) according to the manufacturer instructions. It was equilibrated in standard MES buffer containing 0.1% (w/v) sodium azide. To the column, FCS (5 ml) was applied and left for 1 h at 37°C to allow the enzyme to bind. The column was washed with 4 column volumes of standard MES buffer, then xylosyltransferase activity was eluted (5 ml fractions) by increasing the concentration of potassium chloride from 0.05 M to 0.25 M. All washes and elution fractions were monitored for protein content and xylosyltransferase activity.

Some of the applied FCS xylosyltransferase was adsorbed to the synthetic peptide affinity column, figure 4.21. The bound activity could be eluted by standard MES buffer containing 0.25 M KCl and was approximately 20% of the total activity. The specific activity of the eluted xylosyltransferase was difficult to calculate as the protein concentration of the eluent fell below the level detectable with the spectrophotometer. This suggested that the specific activity of the eluted fraction was very much higher than the starting material and that affinity chromatography using the synthetic peptide as a ligand was a useful step in the purification of xylosyltransferase.

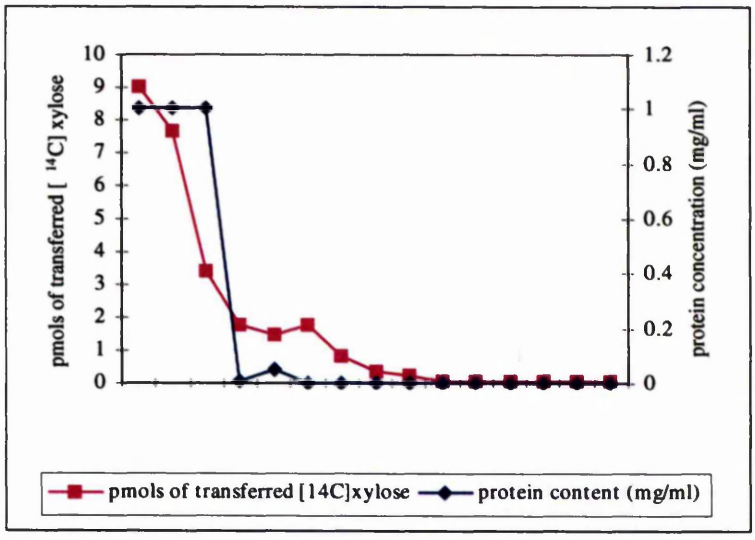


Figure 4.21. Affinity chromatography of FCS xylosyltransferase using the synthetic peptide as the affinity ligand. The synthetic peptide Hi-trap affinity column was equilibrated in standard MES buffer , pH 6.5, containing 0.05 M KCl. FCS, pre-dialysed in standard MES buffer, was applied to the column (5 ml; total activity 100 pmols of transferred [^{14}C]xylose/mg protein), which resulted in binding of approximately 0.5 nmols (50%), while nearly all the protein passed through the column. After washing with standard MES buffer, the enzyme was eluted with MES buffer containing 0.25M KCl with a recovery of approximately 30% of the bound fraction (400 pmols of transferred [^{14}C]xylose). Enzyme activity was expressed as pmols of transferred [^{14}C]xylose (pink line) and the protein content of each fraction as mg/ml (blue line). All fractions were 5 ml.

The rest of the eluted sample not used in the xylosyltransferase assay was dialysed exhaustively against deionised water, freeze dried, then reconstituted in SDS-PAGE sample loading buffer, and resolved on 4-20% (w/v) tris-glycine SDS-PAGE ready gels, under non-reducing conditions, and silver stained (gel not shown).

It was clear from the protein gels that there was still many contaminating proteins in the sample and the proportion of total xylosyltransferase activity bound to the column was not high, so the conditions of the affinity purification were modified to optimise the method. The different strategies included; increasing the number of washes; increasing the incubation time (4 – 16 h); varying the incubation temperature to 4°C, 25°C, or 37°C; changing the elution buffer to contain EDTA and/or no magnesium and manganese ions; re-applying the recovered active fraction to the re-equilibrated column. Overall the best recovery of activity was observed by incubating the column overnight

at 25°C, increasing the number of washes (12 column volumes), and eluting the enzyme in 0.25 M KCl. This method resulted in 40% of the total activity being bound, eluted and recovered. However, using SDS-PAGE, under non-reducing conditions, to visualise the proteins in the eluent samples, figure 4.22, it was clear that there were still many contaminating proteins, so an additional method of purification was sought.

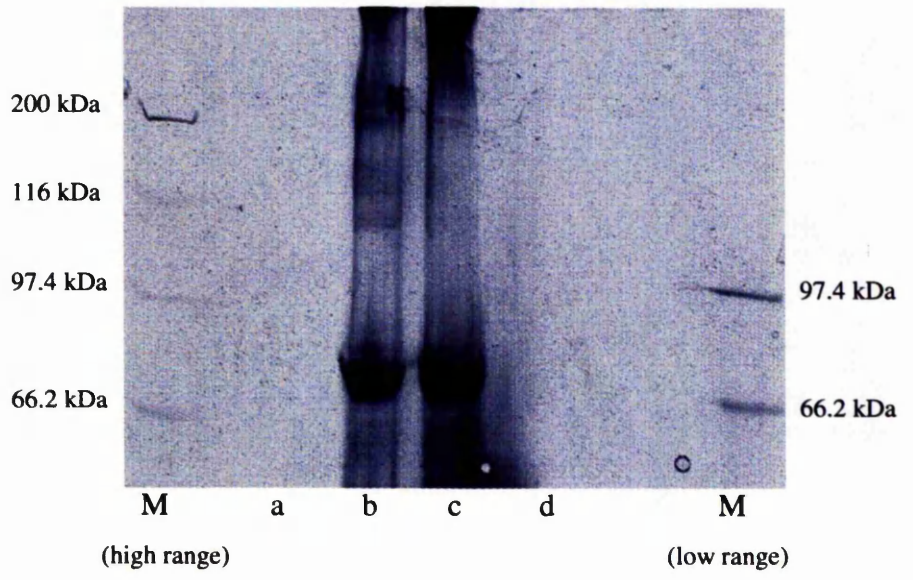


Figure 4.22. SDS-PAGE of synthetic peptide affinity fractions. All samples were from 5 ml column volumes, dialysed into deionised water, freeze dried then reconstituted in SDS-PAGE sample loading buffer. The samples are (a) wash 12, (b) first 5 ml eluted, (c) second 5 ml eluted, (d) third 5 ml eluted, M = markers. The gel was a 4 - 15% (w/v) tris-glycine ready gel and bands were visualised by silver staining.

4.4.5 Lectin Affinity Chromatography

Lectins are proteins that reversibly bind carbohydrates. As many glycosyltransferases are themselves glycosylated, lectins can be used as affinity ligands for these enzymes. Although they are not highly selective, lectin columns can play a role in a purification strategy. Concanavalin A (Con A) binds glucose- or mannose- containing proteins and usually binds to soluble glycoproteins. It has also been reported that many proteins found in the endoplasmic reticulum (ER) of the cell have affinity for Con A (Breuer and Bause, 1995). As xylosyltransferase is readily soluble and believed to be located in the ER / Golgi system, Con A lectin was tested for its ability to purify the enzyme.

Con A Sepharose (1 ml) was washed in 10 column volumes of Con A buffer (20 mM tris-HCL (pH 8.0), 0.15 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1% (v/v) Triton X-100). FCS (500 µl, 0.5 column volumes), was diluted 1:1 with Con A buffer then added to the gel. The mixture was left for 16 h with rotation at 25°C and then washed three times with five column volumes of Con A buffer. Bound proteins were eluted from the gel with five column volumes of 10 mM methyl-α-mannoside, followed by stepwise elution in five column volumes of 20 mM, 50 mM, 100 mM, 250 mM, and 500 mM methyl-α-*D*-mannoside. An aliquot of each fraction (200 µl) was removed, dialysed against standard MES buffer then tested for xylosyltransferase activity, figure 4.23.

FCS xylosyltransferase was adsorbed to the Con A affinity matrix, figure 4.23. The bound activity was eluted by the addition of 10 – 20 mM methyl-α-mannoside and accounted for approximately 40% of the applied activity. The specific activity of the eluted xylosyltransferase could not be calculated as the Con A buffer absorbed strongly at 280 nm. However, it was clear that xylosyltransferase was binding to the Con A gel, and this could be a useful step in the purification strategy.

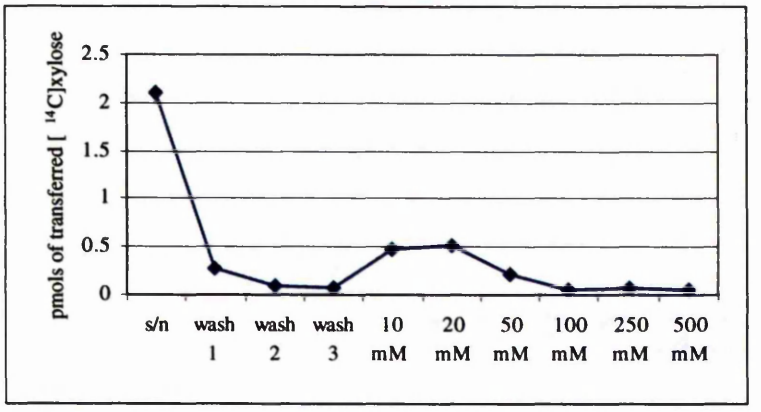


Figure 4.23. Affinity chromatography of FCS xylosyltransferase using the Con A as the affinity ligand. The Con A Sepharose was equilibrated in Con A buffer, pH 8.0. FCS, pre-dialysed in standard MES buffer, diluted 1:1 with Con A buffer was applied to the gel (0.5 ml; total activity 100 pmols of transferred [^{14}C]xylose/mg protein) and rotated for 16 h at 25°C. The gel was washed three times with five column volumes of Con A buffer. Bound molecules were released using step elutions with five bed volumes of 10 mM, 20 mM, 50 mM, 100 mM, 250 mM, and 500 mM methyl-α-*D*-mannoside. Approximately 40% of the bound fraction was recovered containing enzyme (65 pmols of transferred [^{14}C]xylose). Enzyme activity was expressed as pmols of transferred [^{14}C]xylose (blue line).

The eluent fractions were dialysed exhaustively against deionised water, freeze dried, reconstituted in sample loading buffer and the molecules resolved by SDS-PAGE, under non-reducing conditions, on 8% (w/v) tris-glycine ready gels. Unfortunately as lectins bind to glycoproteins, the gels (not shown) contained lanes of smears rather than discreet bands, so a comparison of Con A affinity selected proteins versus the synthetic peptide affinity selected proteins, resolved on the same percentage gel did not reveal any coincident bands.

Due to the many contaminating proteins seen in the eluent of both the synthetic peptide and Con A affinity gels it was proving difficult to reduce the number of proteins seen on a protein gel sufficiently to enable protein sequencing to identify xylosyltransferase. Whilst FCS was a good source of xylosyltransferase activity it was also very difficult to remove the very high proportion of the other proteins that co-eluted with the xylosyltransferase. The observation that cells appear to release xylosyltransferase into culture medium suggested an alternative strategy for obtaining enzyme in a source with a lower content of other proteins.

4.5 Swarm Rat Chondrosarcoma Cells (RX) as a Source of Xylosyltransferase

From previous work it was discovered that xylosyltransferase was secreted out of Syrian hamster embryo chondrocytes, cell line Des4^{+}.2, into the cell media in an active form. Therefore it was possible that xylosyltransferase would also be found secreted out into the media of other cell types. SRC cells (RX), were chosen as they would be expected to have a high level of proteoglycan synthesis and hence a high level of xylosyltransferase activity.

Chondrosarcoma cells were plated at a density of 6 x 10^4 cells / cm^2 in DMEM containing 20 mM HEPES, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% (v/v) FCS. After 48 h and 96 h the media was removed, spun at 10,000 g_{av} and dialysed against standard MES buffer ready for use in purification. Initially the media was tested for its level of xylosyltransferase activity. Chondrosarcoma media (98 μl) was incubated with the synthetic peptide and UDP-D-[^{14}C]xylose in a standard

xylosyltransferase assay (section 3.4.2). The SRC (RX) media had a level of xylosyltransferase activity comparable to FCS after 48 h in culture, 10 pmols of [^{14}C]xylose transferred per reaction. However, a further 48 h in culture showed a four-fold increase in xylosyltransferase activity, approximately 40 pmols of [^{14}C]xylose transferred per reaction. This culture medium provided a much higher concentration of xylosyltransferase activity than was obtained from any other source tested. This source was then used for further purification.

4.5.1 Size Exclusion Chromatography using Sephacryl S-300 on SRC (RX) Media (96 h in culture)

SRC (RX) 96 h media was fractionated using Sephacryl S-300 media in standard MES buffer, column size 0.9 x 60 cm. The V_0 and V_t of the Sephacryl S-300 column were determined as before using aggregated aggrecan (mw >30 x 10^6) and sodium azide (mw 65) respectively. The SRC 96 h media (1 ml of a 5 mg/ml protein stock) was applied to the column (flow rate 0.3 ml/min), the sample eluted with standard MES buffer and fractions (1 ml) collected. The fractions were tested for protein content, using the BCA assay, and xylosyltransferase activity (section 3.4.2), figure 4.24.

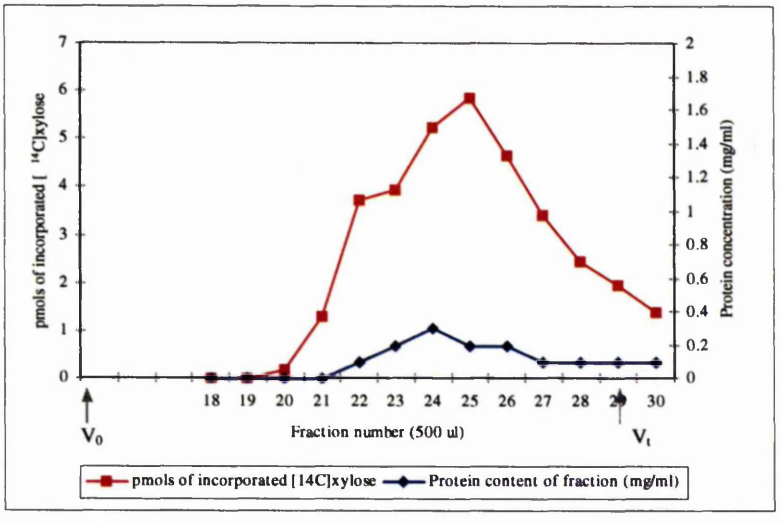


Figure 4.24. Purification of SRC (RX) 96 h media xylosyltransferase by gel filtration. A 1 ml sample of SRC 96 h media (specific activity, 100 pmols [^{14}C]xylose transferred/mg of protein) was applied to a column of Sephacryl S-300 (fine) which had previously been equilibrated with standard MES buffer, pH 6.5. Fractions of 1 ml were collected and the absorbance at 280 nm was recorded (blue line). Aliquots (90 µl) of every fraction were tested for xylosyltransferase activity, expressed as pmols of [^{14}C]xylose transferred per hour under standard assay conditions (red line).

The total amount of xylosyltransferase activity that was eluted from the Sephacryl S-300 column was half that applied to the column, which was a poor yield compared with the 105,000 g_{av} SRC tissue supernatant and FCS which showed a two fold increase of activity following size exclusion chromatography, figure 4.19. The 280 nm trace co-eluted with the peak of xylosyltransferase activity, as for FCS. However, there was four times less protein in the original sample, and only 30% of the total protein added to the column eluted with the xylosyltransferase activity peak. Overall, this size exclusion of the SRC (RX) 96 h media on Sephacryl S-300 gave a purification factor of 1.15. These results suggested that when using SRC 96 h media as the source of xylosyltransferase, size exclusion chromatography using Sephacryl S-300 media was not a very effective step in enzyme purification.

4.5.2 Synthetic Peptide Affinity Chromatography using SRC (RX) 48 h Media as the Source of Xylosyltransferase

The synthetic peptide affinity column was prepared as described previously in section 4.4.4. To the column, SRC (RX) 48 h media (5ml) was applied and left for 16 h at 25°C to allow the enzyme to bind. The column was washed in 12 column volumes of standard MES buffer, then xylosyltransferase activity was eluted (2.5 ml fractions) by increasing the concentration of potassium chloride in the buffer from 0.05 M to 0.25 M, containing 5 mM EDTA with no manganese or magnesium ions. All washes and elution fractions were monitored for protein content and xylosyltransferase activity, figure 4.25. SRC (RX) media xylosyltransferase was adsorbed to the synthetic peptide affinity column, figure 4.25. The bound activity (approximately 30% of the total activity) could be eluted by standard MES buffer containing 0.25 M KCl, 5 mM EDTA and with no magnesium or manganese ions.

The rest of the eluent sample not used in the xylosyltransferase assay was dialysed exhaustively against deionised water, freeze dried, then reconstituted in SDS-PAGE sample loading buffer, and resolved on 4-20% (w/v) tris-glycine SDS-PAGE ready gels, under non-reducing conditions. The resolved molecules were visualised by silver staining (gel not shown). The gel showed a number of bands with two predominant bands at 70 kDa and 90 kDa. There were still many contaminating proteins in the

sample, and the 70 kDa band was running concurrently with BSA. Previous attempts to purify xylosyltransferase from bands on a gel (results not discussed) had shown BSA contamination in bands of 100 kDa and 200 kDa, and it appeared that this was still likely to be the case. An alternative strategy for SRC (RX) cell growth was therefore investigated in order to produce a SRC (RX) media preparation free of BSA from which xylosyltransferase could be purified. It has been reported that SRC cells are sensitive to insulin and that this can replace their need for serum in culture medium (Foley *et al.*, 1982).

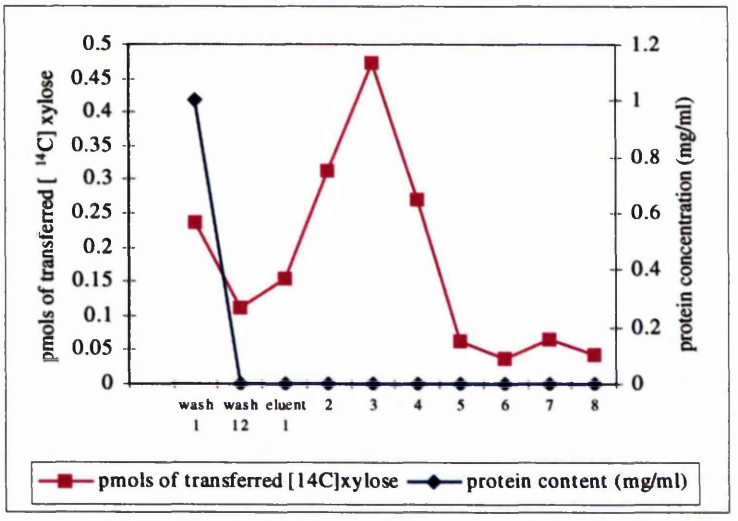


Figure 4.25. Affinity chromatography of SRC (RX) 48 h media xylosyltransferase was using the synthetic peptide as the affinity ligand. The synthetic peptide Hi-trap affinity column was equilibrated in standard MES buffer , pH 6.5, containing 0.05 M KCl. SRC 48 h media, pre-dialysed in standard MES buffer, was applied to the column (5 ml; total activity 200 pmols of transferred [^{14}C]xylose/mg protein). After washing with standard MES buffer, the enzyme was eluted with MES buffer containing 0.25 M KCl, 5 mM EDTA and no magnesium or manganese ions, with a recovery of approximately 30% of the bound fraction (800 pmols of transferred [^{14}C]xylose). Enzyme activity was expressed as pmols of transferred [^{14}C]xylose (pink line) and the protein content of each fraction as mg/ml (blue line). The wash fractions were 5 ml, and the elution fractions 2.5 ml.

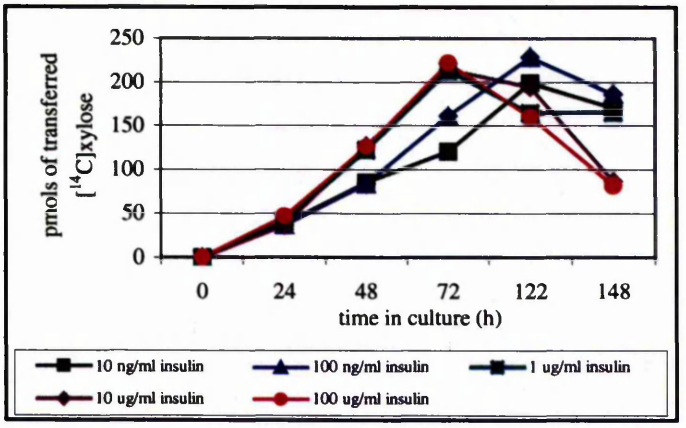
4.5.3 SRC (RX) Xylosyltransferase Production using Insulin as a Serum-free Media Supplement

SRC (RX) cells were plated at 56,000 cells/cm^2 in 9 cm^2 wells, in 2.5 ml of SRC media containing 1% (v/v) FCS. After 24 h the media was removed, the cells washed once in serum free media then a range of concentrations of FCS and insulin in media were added to the cells. The media was removed from the cells and tested for xylosyltransferase activity at 0 h, 24 h, 48 h, 72 h, 122 h, and 148 h, using a standard xylosyltransferase assay, figure 4.25. The media (98 µl) was added straight to an assay without dialysis into standard MES buffer.

The SRC (RX) cells with no supplement added to the media still secreted xylosyltransferase out into the media, figure 4.26 A, but after 2 days in culture the cells became non-adherent and lost their chondrocytic appearance. The highest level of xylosyltransferase activity was secreted from the culture that had medium added to it containing 2% (v/v) FCS and 10 µg/ml insulin (figure 4.26, panel A).

However, there was no significant difference between the maximum level of xylosyltransferase activity between this culture and those cultures that had insulin alone added at a concentration of 1 µg/ml and above (figure 4.26, panel B). Xylosyltransferase activity peaked in the cultures after 72 h in the test media and then slowly declined, at which stage the media was also becoming acidic. As there was less than 10% difference between the addition of 1 µg/ml and 10 µg/ml insulin on the secretion of xylosyltransferase, 1 µg/ml insulin with no FCS was added as a supplement to SRC (RX) cells for 72 h and the resultant media used in the characterisation and purification of xylosyltransferase.

A



B

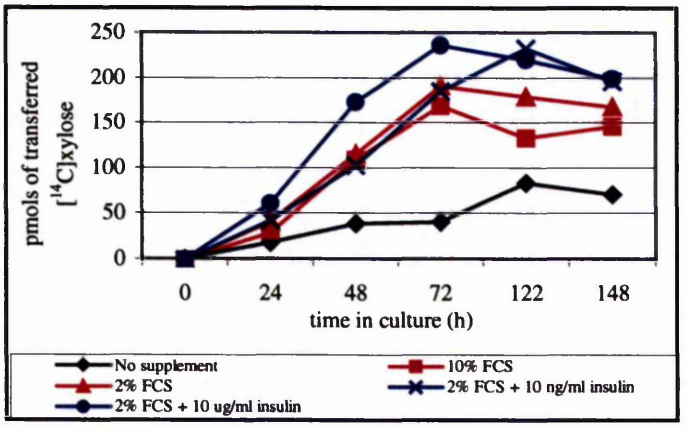


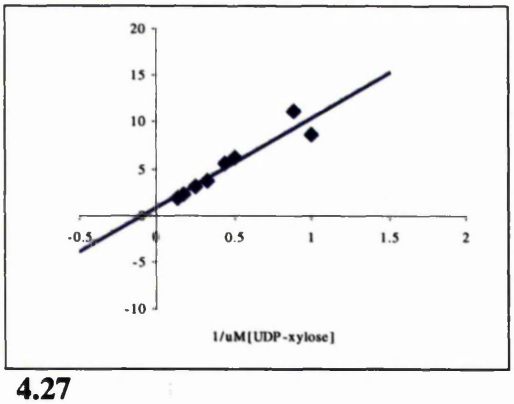
Figure 4.26. The effect of different supplements on the secretion of xylosyltransferase activity from SRC cells in culture. SRC (RX)cells were plated at 55,555 cells/cm^2 in 9 cm^2 wells, in 2.5 ml of SRC media containing 1% (v/v) FCS. After 24 h the media was removed, the cells washed once in serum free media then a range of concentrations of FCS and insulin in media were added to the cultures, and the media tested for xylosyltransferase activity at various time points. Graph **A** shows the level of xylosyltransferase activity secreted from SRC (RX) cells supplemented with FCS and FCS with insulin. Graph **B** shows the level of xylosyltransferase activity secreted from SRC (RX) cells supplemented with insulin alone (at various concentrations).

4.5.4 The Effect of Cytidine 5' Diphosphate on Xylosyltransferase Activity in SRC (RX) 72 h media (supplemented with 1 μg/ml insulin)

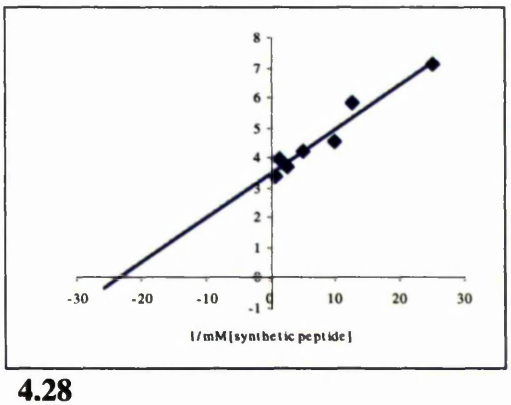
As for the other sources of xylosyltransferase, the transfer of xylose from UDP-xylose on to the synthetic peptide could be from both the activities of xylosyltransferase and/or glycogenin, section 4.2.5. The addition of 200 μM CDP had no effect on the transfer of [^{14}C]xylose compared to the control reaction with no CDP added (results not shown). Therefore, glycogenin is not contributing in any way to the transfer of xylose in the assay when using SRC (RX) 72 h media (supplemented with 1 μg/ml insulin) as the source of xylosyltransferase.

4.5.5 SRC (RX) Xylosyltransferase Reaction Kinetics

Reactions were set up varying the concentrations of UDP-D-[^{14}C]xylose and synthetic peptide to determine the Velocity, K_m, of the xylosyltransferase reaction as a function of UDP-D-xylose concentration and synthetic peptide concentration, figure 4.27 and 4.28 respectively. The SRC (RX) 72 h media (supplemented with 1 μg/ml insulin) was used as the source of xylosyltransferase. From the double reciprocal plot, the K_m for UDP-xylose was found to be 4 μM and a V_{max} of 0.5 nmol/h. The K_m for the synthetic peptide was 45 μM and a V_{max} of 0.29 nmol/h.



4.27



4.28

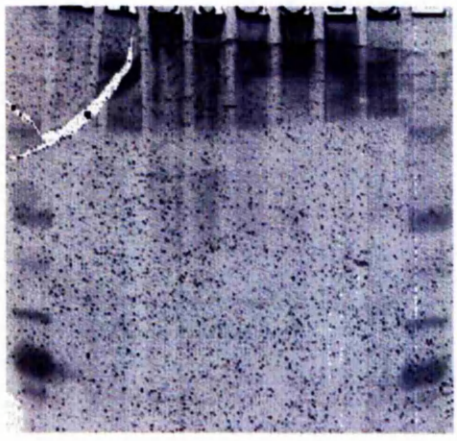
Figure 4.27 and 4.28. Determination of the K_m for the xylosyltransferase reaction as a function of UDP-D-xylose concentration (A) and synthetic peptide concentration (B), using a standard xylosyltransferase assay with the SRC (RX) 72 h media (supplemented with insulin) as the source of xylosyltransferase activity. The concentration of substrate is plotted against the radioactivity incorporated. The K_m was calculated from the double reciprocal plots (as shown) of these data.

4.5.6 Con A Sepharose Chromatography using the SRC (RX) 72 H media Supplemented with Insulin as the source of Xylosyltransferase

SRC (RX) 72 h media (supplemented with insulin) was applied to a Con A Sepharose affinity column to see if xylosyltransferase from this source also had an affinity for Con A and therefore could be used in the purification strategy. Con A Sepharose (5ml) was washed in 10 column volumes of Con A buffer (20 mM Tris-HCL (pH 8.0), 0.15 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1% (v/v) Triton X-100). SRC (5 ml, 1 column volume), was diluted 1:1 with Con A buffer then added to the gel. The mixture was left for 16 h with rotation at 25°C and then washed three times with five column volumes of Con A buffer. Bound proteins were eluted from the gel with 10 column volumes of 20 mM methyl-α- mannoside. An aliquot of each fraction (200 µl) was removed, dialysed against standard MES buffer then tested for xylosyltransferase activity.

SRC (RX) 72 h media (supplemented with insulin) xylosyltransferase was adsorbed to the Con A affinity matrix (results not shown). The bound activity was eluted by the addition 20 mM methyl-α-mannoside, approximately 16% of the xylosyltransferase activity applied to the column bound and eluted in an active form. The specific activity of the eluted xylosyltransferase could not be calculated as the Con A buffer absorbed at 280 nm. However, it was clear that xylosyltransferase from this source was binding to the Con A gel, and could be a useful step in the purification strategy.

The eluent fractions were dialysed exhaustively against deionised water, freeze dried, reconstituted in sample loading buffer and the molecules resolved by SDS-PAGE, under non-reducing conditions, on a 4-20% (w/v) tris-glycine ready gel. Unfortunately no distinct bands were resolved. There was heavy staining of proteins with large molecular weights (> 200 kDa), but the bands were diffuse and merged into each other, figure 4.29.



M W E1 E2 E3 E4 E5 E6 M

Figure 4.29. SDS-PAGE of Con A affinity fractions using SRC (RX) 72 h media (supplemented with insulin) as the source of xylosyltransferase. All samples are from 5 ml column volumes, dialysed into deionised water, freeze dried then reconstituted in SDS-PAGE sample loading buffer. The samples are W–last wash, E1–6 are the first six elution fractions respectively. The gel was a 4-20% (w/v) tris-glycine ready gel and bands were visualised by silver staining. Markers (M) are 200, 116, 97.4, 66.2, 45 kDa in size (descending order).

4.5.7 Synthetic Peptide Affinity Chromatography using SRC (RX) 72 h Media Supplemented with Insulin as the Source of Xylosyltransferase

The synthetic peptide affinity column was prepared as described previously in section 4.4.4. To the column, SRC (RX) 72 h media (supplemented with insulin) (5ml) was applied and left for 16 h at 25°C to allow the enzyme to bind. The column was washed in 12 column volumes of standard MES buffer, then xylosyltransferase activity was eluted (2.5 ml fractions) by increasing the concentration of potassium chloride in the buffer from 0.05 M to 0.25 M, containing 5 mM EDTA with no manganese or magnesium ions. The 2nd, 3rd, and 4th eluted fractions were pooled. All washes and elution fractions were monitored for protein content and xylosyltransferase activity, figure 4.28. SRC (72 h media, supplemented with insulin) xylosyltransferase was adsorbed to the synthetic peptide affinity column, figure 4.30.

The bound activity could be eluted by standard MES buffer containing 0.25 M KCl, 5mM EDTA and with no magnesium or manganese ions. The amount of xylosyltransferase activity eluted was comparable to that eluted when SRC media containing 10% (v/v) FCS was used. The rest of the eluent sample not used in the xylosyltransferase assay was dialysed exhaustively against deionised water, freeze dried, then reconstituted in SDS-PAGE sample loading buffer, and resolved on 8%

(w/v) tris-glycine SDS-PAGE ready gels, under non-reducing conditions. The resolved molecules were visualised by silver staining, figure 4.31.

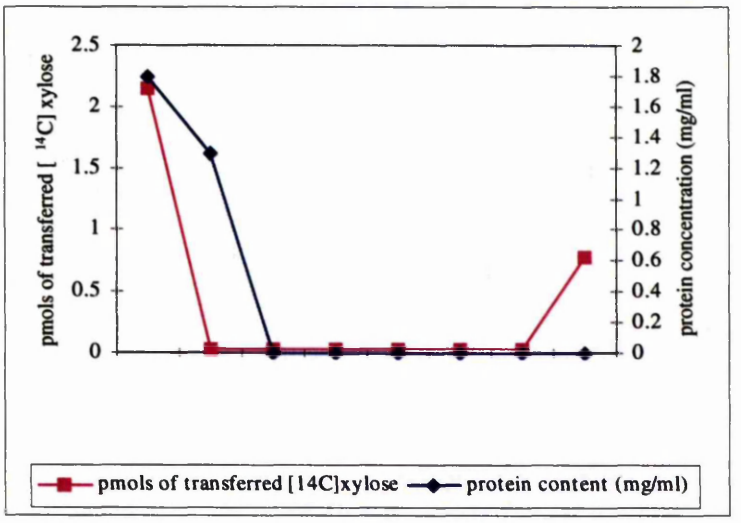


Figure 4.30. Affinity chromatography of SRC (RX) 72 h media xylosyltransferase was using the synthetic peptide as the affinity ligand. The synthetic peptide Hi-trap affinity column was equilibrated in standard MES buffer, pH 6.5, containing 0.05 M KCl. SRC 72 h media (supplemented with insulin) was applied to the column (5 ml; total activity 200 pmols of transferred [^{14}C]xylose/mg protein). After washing with standard MES buffer, the enzyme was eluted with MES buffer containing 0.25 M KCl, 5 mM EDTA and no magnesium or manganese ions, approximately 800 pmols of [^{14}C]xylose was transferred. Enzyme activity was expressed as pmols of transferred [^{14}C]xylose (pink line) and the protein content of each fraction as mg/ml (blue line). The wash fractions were 5 ml, and the elution fractions 2.5 ml. The 2nd, 3rd, and 4th fractions eluted were pooled and assayed for xylosyltransferase activity.

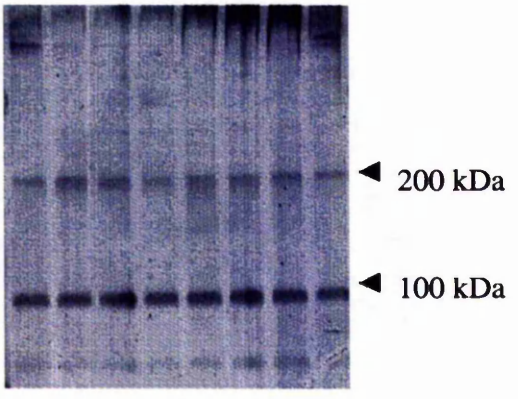


Figure 4.31. SDS-PAGE of synthetic peptide affinity fractions using SRC (RX) 72 h media (supplemented with insulin) as the source of xylosyltransferase. All lanes contain the pooled eluent fractions and are from 5 ml column volumes, dialysed into deionised water, freeze dried then reconstituted in SDS-PAGE sample loading buffer. The gel was an 8% (w/v) tris-glycine ready gel and bands were visualised by silver staining.

4.5.8 Visualisation of Synthetic Peptide Affinity Proteins that also have an Affinity for Concanavalin A

Synthetic peptide affinity proteins were obtained as above in section 4.5.7. The eluted fractions containing xylosyltransferase activity were resolved on an 8% (w/v) tris-glycine ready gel in duplicate, under non-reducing conditions. One gel was silver stained to visualise the synthetic peptide affinity proteins, figure 4.32 A, whilst the other gel was blotted on to PVDF membrane and probed with a Con A-peroxidase conjugate, figure 4.32 B. Silver staining the synthetic peptide affinity proteins showed there were two distinct bands at 200 kDa and 100 kDa. These bands were also con A positive so either band could potentially be xylosyltransferase. However, data from size exclusion chromatography using Sephacryl G-300 suggested that xylosyltransferase had an approximate size of 100 kDa so it was this band that was used to attain peptide sequences for xylosyltransferase.

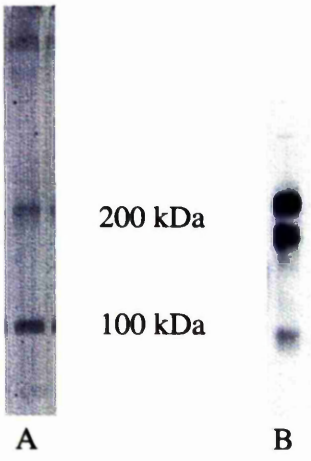


Figure 4.32. SDS-PAGE of synthetic peptide affinity fractions using SRC (RX) 72 h media (supplemented with insulin) as the source of xylosyltransferase. Bound fractions containing xylosyltransferase activity were dialysed into deionised water, freeze dried then reconstituted in SDS-PAGE sample loading buffer. The gel was an 8% (w/v) tris-glycine ready gel and bands were visualised by silver staining (**A**), or blotted to PVDF and probed with a Con A peroxidase conjugate (**B**).

4.6 Amino Acid Sequence Derived from the Synthetic Peptide Affinity 100 kDa Protein

To the synthetic peptide affinity column, SRC (RX) 72 h media (supplemented with insulin) was injected and left for 16 h at 25°C to allow the enzyme to bind. The column was washed in 12 column volumes of standard MES buffer, then xylosyltransferase activity was eluted (2.5 ml fractions) by increasing the concentration of potassium chloride in the buffer from 0.05 M to 0.25 M, containing 5 mM EDTA and no manganese or magnesium ions. The 2nd, 3rd, and 4th eluted fractions were pooled. A total of 60 ml of media was applied to the column, from which 0.5 pmols of [^{14}C]xylose was bound and eluted in an active form from the synthetic peptide column. The eluted fractions containing xylosyltransferase activity were pooled, dialysed against deionised water, freeze dried, reconstituted in sample loading buffer. and resolved on 8% (w/v) tris-glycine SDS-PAGE ready gels. The resolved molecules were visualised by silver staining, figure 4.31.

The 100 kDa Band was excised from all the lanes of two identical gels. Equivalent pieces of gel were cut out as a control sample. The gel pieces were subjected to a number of dehydration and re-hydration steps to remove interfering contaminants, followed by an overnight tryptic digestion. The tryptic peptides were eluted from the polyacrylamide matrices and analysed by MALDI-TOF, figure 4.33, then separated on a C2/C18 reverse phase column. Fractions containing material (absorbance was monitored at 218 nm) were again analysed by MALDI-TOF to produce a peptide–mass fingerprint of the protein. Fractions 17 contained a single peptide peak that was not observed in the control sample; peptide mass of 1174.87 Da, figure 4.34. This fraction was sequenced.

Fraction 19 contained insufficient amount of peptide from which to gain reliable peptide sequence from. Fraction 17 yielded a double sequence: QLYVALE_KR and FTNTQKW, the first sequence being the more predominant. The peptide mass of the first sequence was related back to the mass fingerprint gained from mass spectrometry, which suggested that the missing amino acid was alanine. Proteomic databases were searched with the peptide mass fingerprint and the amino acid sequence. Using Mascot and Profound (Zhang and Chait, 1995-1999) with a peptide mass tolerance of +/- 0.5 Da

with one missed cleavage there were no significant matches in the databases. The amino acid sequence showed no matches, suggesting that it was a novel sequence.

From the first sequence, QLYVALE, degenerate oligonucleotide probes were constructed for both the sense and anti-sense strand of DNA to attempt to gain the cDNA sequence of the protein containing this peptide sequence.

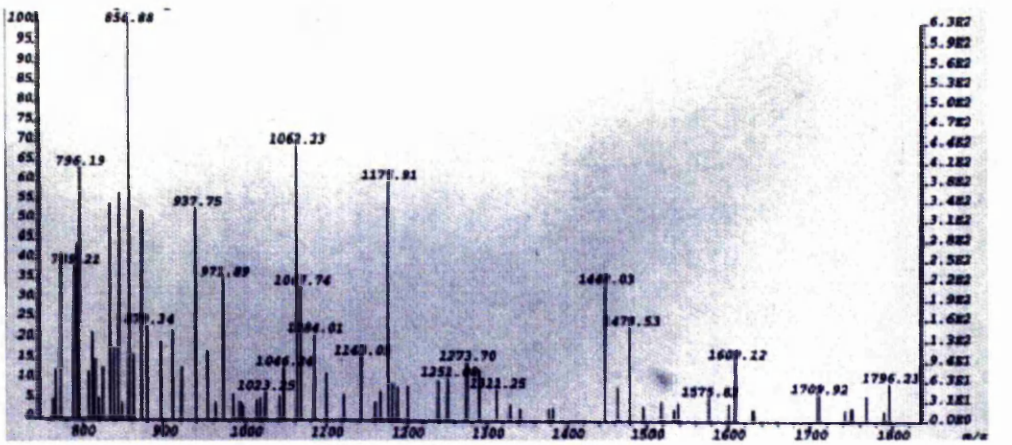


Figure 4.33 MALDI-TOF analysis of the peptide spectra resulting from a trypsin digest of the 100 kDa synthetic peptide affinity band. Mass, in Dalton, can be seen along the x-axis.

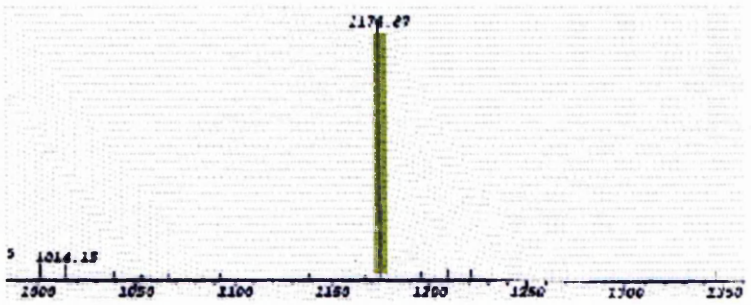


Figure 4.34 MALDI-TOF analysis of fraction 17 eluted from a C2/C18 reverse phase column using the SMART system (Pharmacia Biotech). The peptide in fraction 17 originated from the tryptic digest of the 100 kDa synthetic peptide affinity band. Mass, in Dalton, can be seen along the x-axis.

4.7 Production of a cDNA Clone using the Degenerate Oligonucleotides Deduced from the Amino Acid Sequence from the 100 kDa Synthetic Peptide Affinity Protein

As it was possible that the 100 kDa band that had affinity for the synthetic peptide was xylosyltransferase, the amino acid sequence identified from that band should enable the identification of further sequence information on the protein. Degenerate oligonucleotides were synthesised to correspond to both the template and reverse complement of the DNA sequence of the amino acid sequence to be used in Northern Blot Hybridisation and PCR amplification.

4.7.1 Northern Blot Analysis of RNA Extracted from SRC (RX) Cells

RNA was extracted from SRC (RX) cells using the Tri-REAGENT^{TM} protocol. The RNA concentration was determined, and approximately 10 μg of RNA was resolved on a 1% agarose/2.2 M formaldehyde gel in denaturing buffer. The RNA was visualised on a transilluminator, which showed two bands representing ribosomal RNA. The gel was transferred to nitrocellulose over a 16 h period in preparation for hybridising the oligonucleotide probe prepared in section 4.6.

4.7.2 Hybridisation of the Degenerate Oligonucleotide Probe to the SRC (RX) Northern Blot

The reverse complement degenerate oligonucleotide probe (YTCIARIGCIACRTAIARYTG, where Y= C + T or , R = A + G or , and I = inosine) deduced from the amino acid sequence purified from the 100 kDa protein band, was labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labelled probe was then added to the nitrocellulose filter prepared in section 4.7.1, which had been submerged in hybridisation buffer for 1 h at 37°C prior to the addition of the probe. The probe was left to hybridise to the filter for 16 h at 37°C, then washed in 5X SSC/0.1% (w/v) SDS three times at room temperature followed by a more stringent wash for 5

minutes at 37°C. The filter was then exposed to film for 1 week at –80°C. The developed film showed no bands where hybridisation had taken place (filter not shown). The filter was removed from the film cassette and washed in the same buffer as before for 10 minutes and the temperature of the wash was increased to 42°C. The filter was exposed to film for 1 week at –80°C, but again there were no visible bands. Under the experimental conditions no hybridisation had taken place, suggesting that the probe was not binding to any sequences present in the RNA of SRC (RX) cells. This may be because no corresponding mRNA sequence was present or the degenerate sequence had only low affinity for the specific mRNA.

4.7.3 PCR Amplification of SRC (RX) cDNA using the Degenerate Oligonucleotide Probe

RNA (1 µg) extracted from SRC (RX) cells using the Tri-REAGENT™ protocol was reverse transcribed according to the instruction manual (Perkin-Elmer). The SRC cDNA was then amplified using the template degenerate oligonucleotide, deduced from the amino acid sequence purified from the 100 kDa synthetic peptide affinity band, as the primer and Oligo dT to extend the DNA strand. As the primer had a high degree of degeneracy it was not possible to calculate an accurate annealing temperature so initially a temperature of 45°C was chosen. After 35 cycles the reaction was terminated and the product(s) resolved on a 1% (w/v) agarose gel. Unfortunately no bands were visible. The PCR amplification step was repeated with increased annealing temperatures of 50 and 55°C, however there were still no bands visible on a 1% (w/v) agarose gel (gels not shown). A range of magnesium concentrations (1 mM to 5 mM) were used in the amplification step but no bands were visible at any of the concentrations tested.

A different approach was then taken where various concentrations of Oligo dT were included in the initial reverse transcription reaction. The products of these reactions were then amplified using only the template degenerate oligonucleotide primer and no Oligo dT in 35 cycles of PCR, annealing temperature 45°C. The products of these reactions were then subjected to another round of 35 cycles, but this time further Oligo

dT was added to the reaction to extend the cDNA strands. The products were resolved on a 1% (w/v) agarose gel, but again there were no visible bands. No further clues to sequence information from the purified peptide were thus achieved through PCR methods.

4.8 Cellular Studies of Xylosyltransferase

During the isolation of xylosyltransferase it was found that xylosyltransferase was present in serum and was secreted by cells into the culture media. Xylosyltransferase is believed to be located in the late ER or early Golgi system where it is anchored to the membrane with its active site located within the cisternae, where it adds xylose residues onto certain serine residues in the core protein of proteoglycans. Why it should be found in the serum and media in an active form was therefore unclear, as no proteoglycan synthesis occurs outside the cell. It was an interesting discovery, and so the mechanisms surrounding the secretion of xylosyltransferase were investigated by blocking the secretory pathway and protein synthesis in the SRC (RX) cell culture system that was set up to purify xylosyltransferase. All experiments were performed in duplicate with a positive control. The positive control chosen was following the sulphation of proteoglycans as they are secretory proteins and if the Golgi system is disrupted they are no longer sulphated as the enzyme involved in sulphation are located within the Golgi system.

4.8.1 The Effect of Monensin on Xylosyltransferase Activity in the Cell and Media Layer

Monensin is a Na^+/K^+ ionophore that causes the break up the Golgi system so that proteins normally translocated and secreted from the cell remain trapped within it and accumulate. If xylosyltransferase was being secreted out of the cell in this manner then an accumulation of activity would be seen in the cell layer, together with a reduction in the level of xylosyltransferase secreted out into the media.

SRC (RX) cells were plated out at 60,000 cells/well (96 well plate) in medium containing 2% (v/v) FCS, after 24 h the media was removed and replaced with media containing 1 μg/ml insulin (no FCS). The cells were incubated in this media for 24 h after which the cells were washed once in the same media and the experiment started. Duplicate plates were set up, one to follow xylosyltransferase activity and a positive control to measure proteoglycan sulphation levels.

At t=0, media containing 1 μM monensin or media alone was added to the cell layer in the relevant cultures. For the measurement of xylosyltransferase activity at t=0, the media was removed immediately, the cells washed once in media, then the cell layer was solubilised in 10 mM CHAPS. Both the cell layer and media were than tested for xylosyltransferase activity. Xylosyltransferase activity was measured at 0, 1, 2, 3, 6, 8, and 24 h, figure 4.35. For the positive control cells where the level of sulphation was followed a 2.5 μCi spike of [^{35}S] was added at the beginning of each time period and removed at the end, i.e., 0-1 h, 1-2 h etc. At the end of each labelling period the media was removed from the cells and solubilised in 8M Gu-HCl extraction buffer, the cells washed once in PBS, then the cells were solubilised in 4M Gu-HCl extraction buffer. Incorporated [^{35}S] was separated from unincorporated [^{35}S] by gel filtration using PD10 columns, figure 4.36.

It was clear from the positive controls, figure 4.36 that in monensin treated cultures proteoglycans were not being sulphated, demonstrating that the Golgi system had been disrupted with monensin. In cultures not treated with monensin, xylosyltransferase activity increased in both the media and cell layers from 0 to 19 h in culture, figure 4.35. After 19 h the level of xylosyltransferase activity declined, possibly due to depletion of nutrients and some enzyme inactivation. In monensin treated cultures the level of xylosyltransferase showed little change in both the cell and media layer up to 8 h, with some increase in xylosyltransferase activity in the media at 19 h and 25 h. It was expected that by disrupting the Golgi, xylosyltransferase would not be secreted and a build up of activity would be seen in the cell layer, however there was little evidence of an intracellular accumulation of enzyme, but the amount secreted was greatly reduced. The results showed that over the time scale tested monensin had a significant effect on xylosyltransferase release from the cell. Monensin was therefore blocking the secretion of xylosyltransferase, but it was unclear why there was no intracellular increase in enzyme.

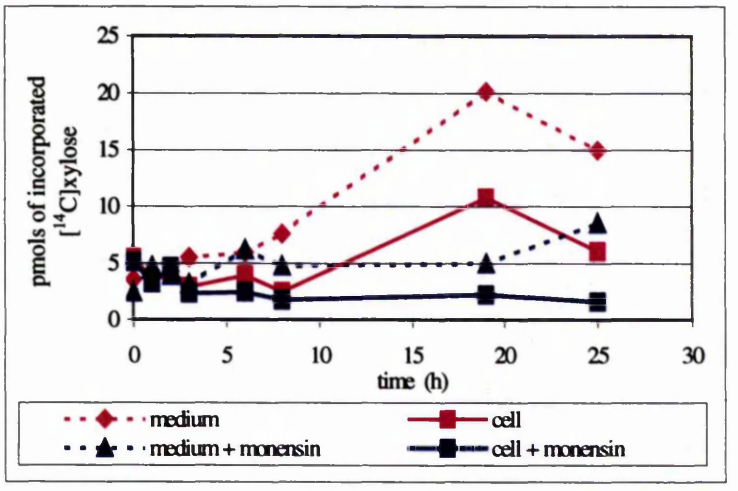


Figure 4.35. SRC (RX) xylosyltransferase activity in the cell layer and media following treatment with monensin (1 μM). Xylosyltransferase activity was measured using a typical xylosyltransferase assay and expressed as pmols of incorporated [^{14}C]xylose per reaction. All reactions were performed in duplicate.

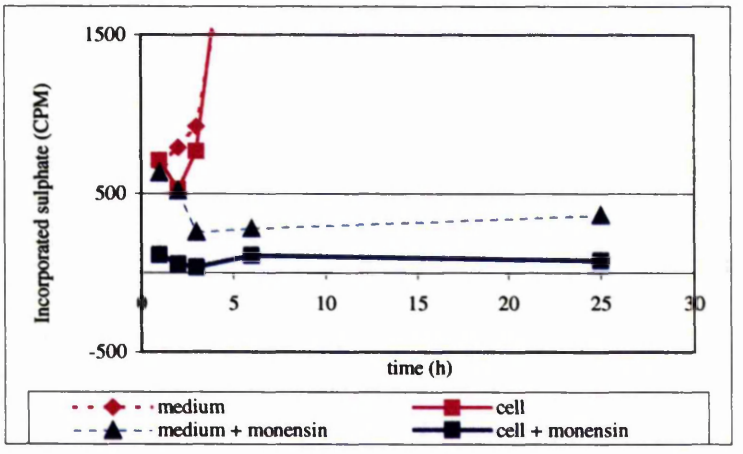


Figure 4.36. [^{35}S] incorporation into proteoglycan in SRC (RX) cells following treatment with monensin (1 μM). To follow sulphation, a 2.5 μCi spike of [^{35}S] was added at the beginning of each time period and removed at the end, i.e., 0-1 h, 1-2 h, 2-3 h, 6-8 h, 8-25 h. Incorporated sulphate was separated from free radiolabel by gel filtration on PD10 columns. All reactions were performed in duplicate.

4.8.2 The Effect of Cycloheximide on Xylosyltransferase Activity in the Cell and Media Layer

Cycloheximide rapidly inhibits protein synthesis in cells, therefore suspending any further synthesis of proteins soon after the cycloheximide has been applied. Measurement of xylosyltransferase activity after cycloheximide addition therefore enables the half-life of the enzyme within the cell to be determined.

The experiment was set up as described in section 4.8.1. At t=0, either media containing cycloheximide (1 mM) or media alone was added to the cell layer in the relevant cultures. For the measurement of xylosyltransferase activity at t=0, the media was removed immediately, the cells washed once in media, then the cell layer solubilised in 10 mM CHAPS. Both the cell layer and media were than tested for xylosyltransferase activity. Xylosyltransferase activity was measured at 0, 1, 3, 6, 8, and 24 h, figure 4.37. For the positive control cells, where the level of sulphation was followed, a 2.5 μCi spike of [^{35}S] was added 15 minutes before the start of each time period and removed 15 minutes after. At the end of each labelling period the media and the cell layer were solubilised together in 8M Gu-HCl extraction buffer. Incorporated [^{35}S] was separated from unincorporated [^{35}S] by CPC precipitation, figure 4.38. In addition β-xylosides were added to identical cultures as the positive controls to see the effect of cycloheximide on the other enzymes involved further down the pathway of GAG synthesis.

The positive control sulphate data in figure 4.38 showed that cycloheximide was halting protein synthesis. After 6 h in culture the level of sulphation was decreasing compared to the control that had not been treated with cycloheximide, suggesting that cycloheximide was stopping proteoglycan core protein synthesis. The addition of xyloside to cultures increased the level of sulphation seen, which was to be expected as they provide another substrate to have sugar chains attached, which can then be sulphated. The addition of cycloheximide to xyloside containing cultures showed a decline in sulphation levels after 6 hours to the same level as cycloheximide alone treated cultures. This suggested that the enzymes involved further down the pathway in GAG synthesis to xylosyltransferase were effected by cycloheximide after 6 h in

culture. Xylosyltransferase activity in the cell layer treated with cycloheximide was seen to decline after 1 h in culture and significantly after 6 h compared to the control. After 6 h in culture in the presence of cycloheximide there was only a low level xylosyltransferase activity remaining in the cell, and from the log plot it was determined that the enzyme has a half-life of approximately 2.5 h in the cell (figure 4.37a). The depletion in xylosyltransferase activity observed in the cell layer was accompanied by a reduced increase in xylosyltransferase activity in the media. After 24 h in culture the media from the cycloheximide treated cells contained less than half the xylosyltransferase activity found in the control media.

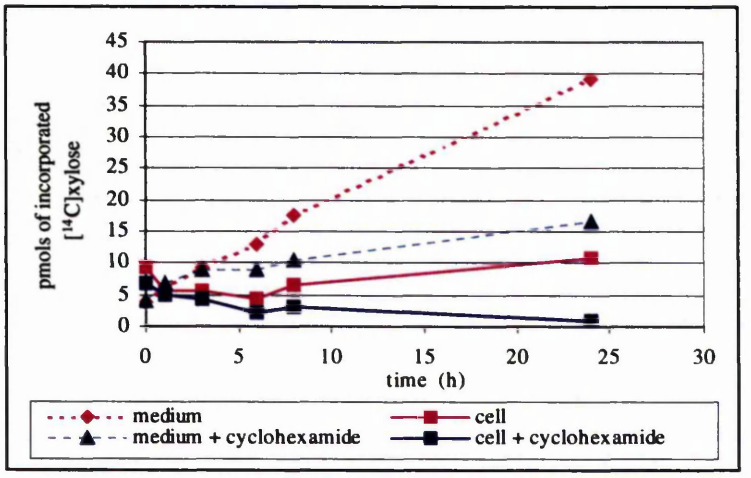


Figure 4.37. SRC (RX) xylosyltransferase activity in the cell layer and media following treatment with cycloheximide (1 mM). Xylosyltransferase activity was measured using a typical xylosyltransferase assay and expressed as pmols of incorporated [^{14}C]xylose per reaction. All reactions were performed in duplicate.

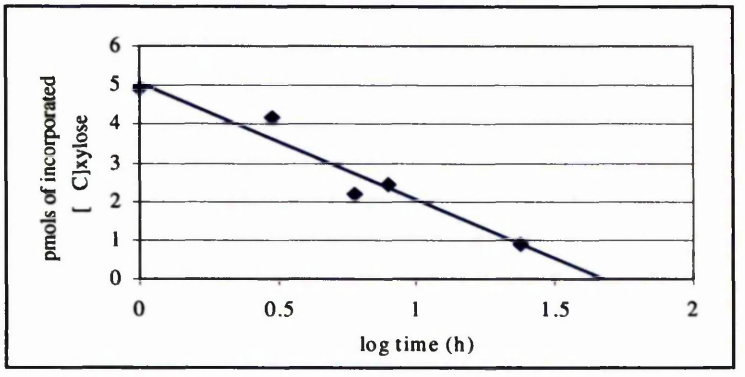


Figure 4.37a. Log plot of SRC (RX) xylosyltransferase in the cell layer following treatment with cyclohexamide.

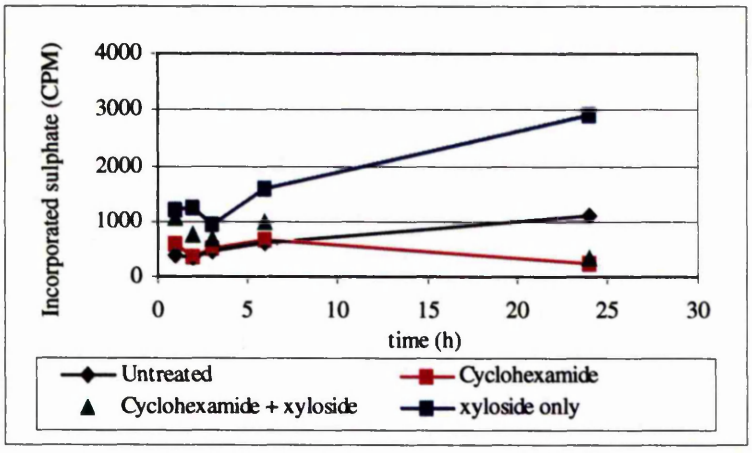


Figure 4.38. [^{35}S] incorporation into proteoglycan in SRC (RX) cells following treatment with cycloheximide (1 mM). To follow sulphation, a 2.5 μCi spike of [^{35}S] was added 15 minutes prior to the relevant time point and removed 15 minutes after. Incorporated sulphate was separated from free radiolabel by CPC precipitation. All reactions were performed in duplicate.

4.8.3 Time Course of Proteoglycan Core Protein Secretion

In the previous experiment to measure the effect of cycloheximide on xylosyltransferase activity it was possible to determine the approximate half-life of xylosyltransferase in the cell. A comparison was made between the time xylosyltransferase resides in the cell before it is secreted with a known secretory protein; proteoglycan core protein. SRC (RX) cells were in culture as for the previous experiment and at various time points were spiked with [^{35}S] (2.5 μCi) 15 minutes prior to the time point and removed 15 minutes after. At the end of each labelling period the media was removed from the cells and solubilised in 8M Gu-HCl extraction buffer. Incorporated [^{35}S] was separated from unincorporated [^{35}S] by gel filtration using PD10 columns to observe the level of sulphate secreted during the time course, figure 4.39. The half life of proteoglycan core protein in SRC (RX) cells was approximately 90 minutes, which is approximately half the time that xylosyltransferase was observed to reside in the cell, section 4.8.2.

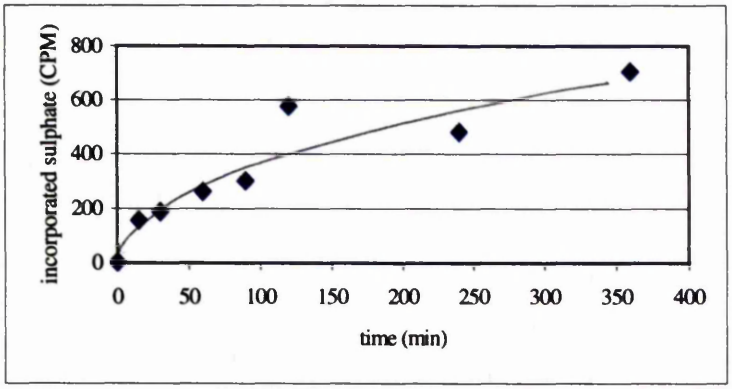


Figure 4.39. Time course of secreted core protein from SRC (RX) cell in culture. The level of incorporated sulphate secreted out into the media was monitored and expressed as CPM of incorporated sulphate. All reactions were performed in duplicate.

4.8.4 The Effect of Brefeldin A on the Secretion of Xylosyltransferase from SRC (RX) Cells in Culture

The fungal metabolite, brefeldin A (BFA), inhibits nucleotide activation and therefore, membrane binding of ADP-ribosylation factors. This prevents recruitment of coat proteins and formation of transport vesicles. BFA has been reported to interfere with vesicular transport causing disassembly of the Golgi complex with redistribution of Golgi components to the endoplasmic reticulum, and isolation of the trans-Golgi cisternae from the trans-Golgi network. It has also been suggested to disrupt protein synthesis. As monensin had failed to cause an accumulation of xylosyltransferase activity it may be that xylosyltransferase is secreted out of the cell by a different pathway to the normal protein secretory pathway. As BFA disrupts anterograde secretion form the Golgi but still allows retrograde (back to the endoplasmic reticulum) traffic of proteins it was investigated what effect BFA would have on xylosyltransferase in order to help elucidate the pathway of xylosyltransferase secretion.

The experiment was set up in an identical fashion to the cycloheximide experiment described in section 4.8.2, except that BFA (1 μg/ml) was added in place of cycloheximide. Xylosyltransferase activity and sulphate incorporation was measured in both the cell layer and media at 0, 0.5, 1, 2, 3, 6, and 8 h. It was clear from the sulphate

incorporation data that BFA had an immediate effect on sulphation levels, giving almost complete inhibition of sulphation compared to the control levels, figure 4.40. This result confirmed that BFA was disrupting the Golgi complex. However, BFA had no significant effect on the level of xylosyltransferase activity in the cell during 8 h in culture or in its secretion from the cell to the media, compared to control levels, figure 4.41. There was no detectable accumulation of xylosyltransferase activity in the cell in the presence of BFA.

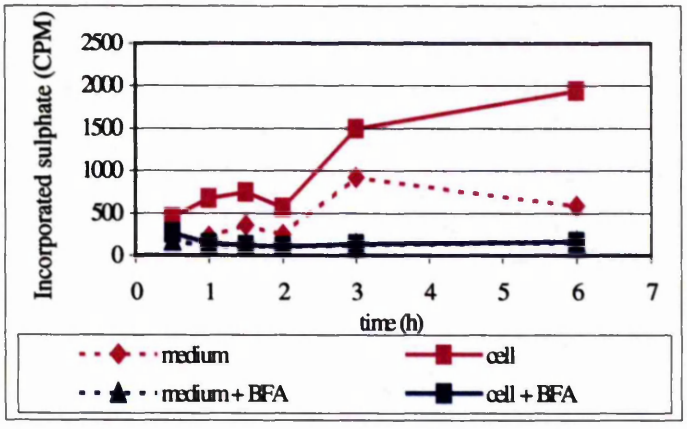


Figure 4.40. [^{35}S] incorporation into proteoglycan in SRC (RX) cells following treatment with brefeldin A (1 µg/ml). To follow sulphation, a 2.5 µCi spike of [^{35}S] was added 15 minutes prior to the relevant time point and removed 15 minutes after. Incorporated sulphate was separated from free radiolabel by gel filtration using PD10 columns. All reactions were performed in duplicate.

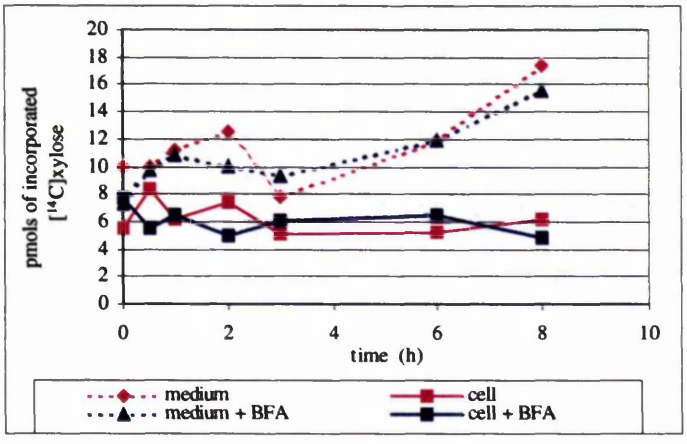


Figure 4.41. SRC (RX) xylosyltransferase activity in the cell layer and media following treatment with brefeldin A (1 µg/ml). Xylosyltransferase activity was measured using a typical xylosyltransferase assay and expressed as pmols of incorporated [^{14}C]xylose per reaction. All reactions were performed in duplicate.

5. Discussion

5.1 The Purification of Xylosyltransferase

Many glycosyltransferases involved in the biosynthesis of glycoproteins and proteoglycans have now been cloned and characterised (Field and Wainwright, 1995; Breton and Imberty, 1999). These enzymes transfer the sugar moiety from an activated nucleotide-sugar to an acceptor, which may be a growing oligosaccharide, a lipid or a protein. Despite the fact that many glycosyltransferases recognise the same donor and acceptor substrates, few regions of sequence homology has been found between the different classes of eukaryotic glycosyltransferases. However, the cloning of the first glycosyltransferases residing in the Golgi system revealed that they all shared a common topology of a type II membrane protein, and all had a molecular weight of approximately 50-60 kDa. Bioinformatics and new cloning strategies have since allowed the identification of a wide range of new enzymes, that contain a series of conserved motifs that has allowed them to be classified into a number of families, including the sialyltransferases, the fucosyltransferases and the galactosyltransferases.

Xylosyltransferase is the enzyme that catalyses the first reaction of transferring a xylose residue onto specific serines in the core protein of proteoglycans, however as yet, it has not been cloned and has not been assigned to any of the cloned glycosyltransferase families. It was reported that xylosyltransferase was purified to apparent homogeneity from a high-speed supernatant fraction after homogenisation of Swarm rat chondrosarcoma tissue (Schwartz and Dorfmann, 1975) and embryonic chick cartilage (Stoolmiller *et al.,* 1972) however there has been no significant follow up to these reports. Unlike many other glycosyltransferases, xylosyltransferase can apparently be obtained in a soluble form without deliberately rupturing cells (Stoolmiller *et al.,* 1975). As a purification procedure for this enzyme was reported, it seemed reasonable that it should be possible to purify the enzyme from rat chondrosarcoma tissue, and using micro-sequencing technology, which was unavailable in the 1970's, to obtain peptide and cDNA sequence in order to further characterise the enzyme.

The previous purification procedures used a variety of exogenous substrates (Baker *et al.,* 1972; Campbell *et al.,* 1983; Weilke *et al.,* 1997), and a range of these were used to attempt to purify xylosyltransferase from the high-speed supernatant fraction of rat chondrosarcoma tissue.

Historically, Smith degraded proteoglycan was used as a substrate for xylosyltransferase (Baker *et al*, 1972). Porcine laryngeal aggrecan was chemically degraded by this procedure, to cleave off any sulphated GAG chains from the core protein. Preliminary results using this preparation in an assay to test for xylosyltransferase activity proved unsuccessful, showing no incorporation of xylose when using the PGSD as the substrate, figure 4.1. The sample was analysed further and found to still have a high sulphated GAG content in comparison to its total weight. However, after extending the acid cleavage stage, the acceptor activity was destroyed. A third preparation from aggrecan included a digestion step using chondroitinase ABC to digest any sulphated GAG chains left in the preparation. The disaccharides produced from the chondroitinase ABS digestion were dialysed away. The chondroitinase digestion was included in order to reduce any possible inhibition of the transferase reaction by intact CS chains. The sample contained around 50% less (total weight) GAG constituents, but it still did not display any acceptor activity. Other chemical degradation methods involve hydrogen fluoride treatment of cartilage proteoglycans (Coudron *et al.*, 1980), and deglycosylation of aggrecan using trifluoromethanesulphonic acid (TFMS: Edge, 1981). TFMS treatment completely deglycosylates proteins, suggesting that it may be more reproducible than the protracted chemical procedure involved in Smith degradation. TFMS treated aggrecan was tested for its use as an acceptor for xylosyltransferase (results not shown) but it proved to be no better an acceptor than Smith degraded proteoglycan.

As the results from the Smith degraded proteoglycan preparations were proving unsuccessful, other possible exogenous substrates were sought. Campbell and co-workers (1983) had used silk fibroin as a substrate for xylosyltransferase. They had found silk to be a better acceptor than PGSD, as well as being more economical in both time of preparation and cost. The silk fibroin preparation was found to be a good acceptor for xylosyltransferase (figure 4.2). Incorporation of xylose was approximately 20-fold higher in the reaction experiments than in the relevant controls. Silk fibroin was acting as an acceptor for xylose transfer, and this confirmed that the high-speed supernatant fraction from Rat Chondrosarcoma homogenate did possess xylosyltransferase activity. Silk fibroin was then used to attempt to optimise reaction conditions (figure 4.3) and as a positive control alongside other acceptors, although its acceptor activity was variable, as there was up to 30% difference in the amount of

incorporated [^{14}C]xylose between different silk preparations. Following more extensive experiments with silk fibroin it became apparent that it was rather unstable in solution, making it intractable to work with, and giving a less reliable assay.

The crude Rat Chondrosarcoma tissue homogenate and 10,000 g_{av} and 105,000 g_{av} supernatant fractions were all found to contain xylosyltransferase activity when silk was used as substrate. The specific activity of the enzyme increased at each stage of purification, i.e. as the protein concentration decreased the level of xylose transferred increased (table 1). The increase of the specific activity from the crude homogenate to the 105,000 g_{av} was however only approximately two-fold, whereas previous publications using PGSD as substrate had reported the increase to be almost seven-fold (Schwartz and Dorfman, 1975).

Silk fibroin was used as a positive control in the xylosyltransferase assay with the second PGSD preparation. Reactions containing both PGSD and silk fibroin showed no acceptor activity, suggesting that some component in the PGSD preparation was inhibiting the transfer of xylose onto silk. The degradation to remove the GAG chains from the core protein may therefore have been successful, but the acceptor activity may have been masked by an inhibitory factor.

The failure of the PGSD to act as an acceptor led to the idea of producing a recombinant protein containing GAG attachment sites. A similar protocol has been described using recombinant bikunin as acceptor (Weilke *et al.*, 1997). A cDNA available in the lab, pKSC4, contained a region coding for part of the CS attachment region from the human aggrecan gene (figure 4.4, panel A). The rationale for this work was to create a construct that contained multiple GAG attachment sites that could act as a good substrate for xylosyltransferase, ligated to a polyhistidine tag to enable simple purification of the substrate. The recombinant protein could then be used in an affinity column to specifically bind the enzyme, in the absence of UDP-xylose, to purify the enzyme for Rat Chondrosarcoma tissue.

The first construct comprised the majority of the CS-2 region of pKSC4, containing 20 potential CS attachment sites, ligated to a polyhistidine tag. This was transfected in a bacterial expression vector into *E. coli* cells, but the expression levels of the construct

were very low and it was only by immunoblotting with a monoclonal antibody specific for the 6-His tag that the expression could be visualised (figure 4.6, panel A). The concentration of IPTG and time of expression was varied in order to try and increase the expression, but higher levels of expression were not achieved.

The second construct contained both the CS-2 region and the G3 region of aggrecan. The G3 region of aggrecan contains a folded globular domain which it was thought might help increase expression levels. The expression of bacterial fusion proteins depends on a number of factors, including the nature of the expressed protein, and the fusion partner. This construct was similarly transfected into *E-coli* cells. It was expressed, but the level of protein produced was again very low (figure 4.7). An alternative vector (pET16b) was also tried, but this also produced only a low yield of product.

The purified protein from the second construct, even when tested at the maximum concentration achievable, showed no incorporation of [^{14}C] (figure 4.8, R2). It was therefore difficult to consider that the expression systems tested could provide sufficient substrate for routine assay of xylosyltransferase. Unlike the PGSD preparation, the recombinant substrate did not inhibit the incorporation into silk (figure 4.8, R3). Since this was a prokaryotic expression system, the GAG attachment sites on the cDNA should not have undergone post-translational modifications and should have been available for xylosylation as described for the recombinant bikunin molecule (Weilke *et al.*, 1997). It was most likely that the concentration of acceptor protein included in the assay was too low for any incorporation to be measured. However the likely increase in concentration necessary for it to be an efficient acceptor made in difficult to envisage being able to increase the scale of the culture volumes to produce sufficient amounts of protein for a large number of assays.

Other substrates were then sought that would be available in larger amounts. Weilke and co-workers (1997) had shown that whilst recombinant bikunin could act as a good acceptor for xylosyltransferase, so could a small peptide containing a sequence found in bikunin. The peptide sequence surrounding the CS attachment site of bikunin (figure 4.9) was synthesised to produce a synthetic peptide, with a molecular weight of 1234 Da. Experiments using an FITC-conjugated version of the peptide indicated that it was

not immobilised on nitrocellulose, Hybond™ or PVDF membranes, which would have provided a method to isolate the peptide-xylose product. So a method using gel-filtration was employed to demonstrate xylose incorporation onto the synthetic peptide. Using gel filtration it was possible to separate incorporated [^{14}C]xylose-peptide from unincorporated UDP-D-[^{14}C]xylose. The incorporated [^{14}C]xylose peak ran concurrently with a peak of UV-excitable fluorescence, suggesting that xylose was being transferred to the FITC-conjugated synthetic peptide. The synthetic peptide was therefore shown to act as a good acceptor for xylosyltransferase (figure 4.10). Non-FITC labelled peptide showed a radiolabel trace identical to that of the FITC-conjugated synthetic peptide, suggesting that FITC was not playing any role in enhancing or inhibiting the enzyme action, and that peptide alone could be used as the substrate for xylosyltransferase.

Once a reproducible assay system had been developed using gel-filtration (results, section 4.1.4.2) it was possible to determine the optimum conditions for a xylosyltransferase assay. The temperature and duration of incubation and the substrate concentrations were tested so as to maximise the amount of xylose incorporation seen using the synthetic peptide as acceptor (figures 4.12 and 4.13). In addition, the stability of the enzyme, the effect of salt concentration, the addition of detergent and the effects of concentrating the Rat Chondrosarcoma high-speed supernatant fraction were measured, as these would be important factors in the further purification of the enzyme. Xylosyltransferase could be stored at 4°C for over 24 days without any loss in activity occurring, however the enzyme was unstable at room temperature, so any steps that had to be done at this temperature had to be kept to a minimum (figure 4.14). Both CHAPS and increasing the concentration of salt (KCl) reduced the level of xylose transferred in an assay, but the effect was consistent, so a simple calculation could account for the actual level of xylosyltransferase activity in a sample. Unfortunately, attempts to concentrate the 105,000 g_{av} tissue supernatant using centrifugal concentrators were unsuccessful. This was a set back to plans for purification as it would have been useful in further purification strategies to increase the concentration of xylosyltransferase at several stages during the process. This was a continued problem throughout the purification of the enzyme as it was very difficult to concentrate the enzyme without a major loss of activity.

Another issue of importance was the possibility that the enzyme activity being measured was not xylosyltransferase but glycogenin, as glycogenin can use xylose as an acceptor if glucose is in short supply (Manzella *et al.*, 1995). CDP inhibits glycogenin but not xylosyltransferase. The addition of CDP had no effect on the apparent level of incorporation of xylose, suggesting that the xylose was incorporated on to the synthetic peptide catalysed by xylosyltransferase and it was not being incorporated into a high molecular weight glycogen acceptor catalysed by glycogenin. This test was applied to all the enzyme sources used to confirm that any transfer of xylose was from the action of xylosyltransferase.

Having characterised the basic properties of xylosyltransferase and optimised the assay conditions it was then attempted to purify the enzyme from the high-speed tissue supernatant. In previous purification strategies (Schwartz and Roden, 1974) the initial step was using gel filtration to separate the melee of proteins in the sample according to their size. Previously, Sephadex G-200 was the matrix of choice, but this did not provide clear separation of xylosyltransferase active fractions from the majority of other proteins in the sample, so other media were investigated (figure 4.16). However, this method did produce an approximate size of 100 kDa for xylosyltransferase, as compared to the standard, BSA (67 kDa). Sephacryl S-300 was also tested and this was found to separate the xylosyltransferase active fractions from over 95% of the other proteins in the sample. Gel filtration also increased the level of xylosyltransferase activity recovered form the column (figure 4.17). Therefore Sephacryl S-300 size fractionation was a useful initial step in purifying xylosyltransferase, as not only did it separate xylosyltransferase from the majority of proteins but it also appeared to separate it from an inhibitor of xylosyltransferase activity.

Whilst size fractionation using Sephacryl S-300 proved to be a useful purification step, it also greatly increased the volume of the sample, making the activity more dilute. For future purification steps, such as affinity-chromatography, it was necessary to have a high level of enzyme activity in as small a volume as possible. To overcome the dilution, it was attempted to concentrate the sample using centriplus-30^{TM} centrifugal membrane concentrators, and by freeze drying the sample then reconstituting it in standard MES buffer. Unfortunately both of these methods were unsuccessful in concentrating xylosyltransferase activity and showed a loss of activity of 80 – 90% of

the original, respectively. Due to the inability to concentrate the size-fractionated sample together with the limited amount of chondrosarcoma tissue available, there was a need for a more readily available source of xylosyltransferase.

Weilke and co-workers (1997) reported that they could detect xylosyltransferase activity in human serum. Using this information it seemed likely that xylosyltransferase may also be found in bovine serum, so FCS was tested for xylosyltransferase activity. In addition, it was reasoned that as xylosyltransferase is located intracellularly in the endoplasmic reticulum, where it catalyses the addition of xylose onto proteoglycans, it must be secreted from cells in an active form, so the media from cells in culture may also contain xylosyltransferase activity. The spent media from chondrocyte-like Syrian hamster cells, Des4^{+}.2, was therefore also tested for xylosyltransferase activity. FCS and cell culture medium contained a high level of xylosyltransferase activity (figure 4.18), however FCS had a greater enzyme activity per unit volume and as it was readily available in large quantities it was evaluated further as a new enzyme source.

Purification was attempted using size-fractionation on Sephacryl S-300. Unlike the high-speed supernatant tissue source, Sephacryl S-300 did not remove the majority of the proteins from the peak of xylosyltransferase activity, so for FCS this was not an effective purification step (figure 4.19).

Although previous attempts to purify xylosyltransferase had used an initial gel-filtration step, this offered no advantage with FCS as the enzyme source, so it was decided to develop affinity-matrices that were selective for xylosyltransferase.

The first affinity matrix tested was a UDP agarose gel, which had been used by Barker and co-workers to purify a galactosyltransferase in 1972. Xylosyltransferase recognises both an acceptor sequence within the core protein and UDP-xylose, brings them together at the active site and catalyses the transfer of xylose. Therefore, it was reasonable that xylosyltransferase would have an affinity for an agarose derivative of UDP. Whilst it was recognised that a number of other transferases would also have an affinity for this substrate, it would greatly reduce the number of proteins in the FCS sample, if indeed any other transferases are secreted into bovine serum. However,

xylosyltransferase did not appear to have an affinity for UDP-agarose (figure 4.20), under the conditions tested, so other affinity matrices were tested.

As the synthetic peptide had proved to be a good substrate for xylosyltransferase, an affinity matrix using the synthetic peptide as the ligand was developed. The synthetic peptide was very small, only 1234 Da, and it was feared that when it was attached to a matrix the acceptor sequence would be too close to the matrix to allow xylosyltransferase access. Therefore the peptide was attached to a matrix with a 12 carbon atom spacer molecules, so the peptide would be projected out from the supporting matrix. Xylosyltransferase activity from FCS was adsorbed to this matrix. The yield of active xylosyltransferase eluted from the affinity matrix, by increasing the concentration of salt (KCl) from 0.05 M to 0.25 M, was approximately 20% of the total activity applied to the column. The protein concentration of the eluted fractions was below the level easily detected with the spectrophotometer; therefore the specific activity of the eluted fraction was very much higher than the starting material (figure 4.21). The conditions of binding the xylosyltransferase to the column and eluting the enzyme from the column were varied to see if a greater yield could be achieved. The best recovery of active xylosyltransferase was obtained after an overnight binding step at 25°C, followed by eluting the bound proteins in standard MES buffer containing 0.25 M KCl / 0.05 M EDTA with no magnesium or manganese ions. This method resulted in 40% of the bound activity being eluted and recovered.

Having developed an affinity matrix that bound and eluted xylosyltransferase it was then necessary to see how selective it was for this enzyme. The eluted affinity-fractions contained a very low protein concentration, which suggested that this column was effective in removing a high proportion of contaminating proteins, but it still remained to be seen how many proteins were present in the high affinity fraction. The eluted fractions containing xylosyltransferase activity were pooled and dialysed against deionised water to remove the salts contained in the elution buffer, then freeze dried to reduce the volume of the sample. Consequently the fractions were resolved by SDS-PAGE (figure 4.22). It was clear from the gel that there were still many proteins that were co-eluting with xylosyltransferase, or were binding to the enzyme and being eluted with it. Therefore a further affinity matrix was sought that could help reduce the number of contaminating proteins.

To choose a different affinity matrix that could be selective for xylosyltransferase, the basic properties of the enzyme were taken into consideration. It was known that xylosyltransferase is soluble (Schwartz and Roden, 1974) and xylosylation is an ER to Golgi event (Vertel *et al.*, 1993), suggesting that xylosyltransferase is an ER protein. Both these factors suggested that concanavalin A (con A) might be an effective affinity ligand for xylosyltransferase. Con A is a lectin, which binds carbohydrates reversibly, and is selective for glucose- and mannose- containing proteins and usually to those that are soluble. Many glycosyltransferases are themselves glycosylated and previous attempts to purify xylosyltransferase found that it had a carbohydrate component (Schwartz and Roden, 1975) and it was also readily soluble. In addition, it has also been reported that many proteins found in the ER have an affinity for Con A (Breuer and Bause, 1995). All these factors suggested that Con A was a good candidate ligand for an affinity matrix for xylosyltransferase. The Con A-Sepharose proved to be an effective affinity matrix for xylosyltransferase as approximately 40% of the total activity was recovered in the bound fraction (figure 4.23). The eluted fractions containing xylosyltransferase activity were pooled and dialysed against deionised water to remove the salts, then freeze dried to reduce the volume of the sample. Consequently the fractions were resolved by SDS-PAGE to reveal how many proteins were recovered in the fraction. As lectins bind glycoproteins the gels contained lanes of smears rather than discreet bands. However it was apparent that the use of this method alone or in conjunction with the synthetic peptide affinity matrix may not be sufficient to isolate xylosyltransferase free of all other proteins.

Due to the number of contaminating proteins in both the synthetic peptide and Con A affinity matrix it was not feasible to cut out all the bands on the gels to determine their sequence and by a process of elimination find the sequence for xylosyltransferase. However it was apparent that the two affinity column systems both showed good potential for the purification of xylosyltransferase that might be realised if a more enriched source of enzyme was available.

Previously, xylosyltransferase activity had been found in the spent media of a Syrian hamster embryo chondrocyte-like cell line. This result showed that xylosyltransferase was secreted from these cells in culture. As chondrocytes would clearly be a good

source of enzyme the spent media from cultured Swarm Rat Chondrosarcoma (SRC (RX)) cells (RX) was also tested as a source of xylosyltransferase. They were expected to have a high level of proteoglycan synthesis and hence a high level of xylosyltransferase activity. Xylosyltransferase was detected in the media from the SRC (RX) cells and the activity increased to a maximum after 96 h in culture. The concentration of xylosyltransferase activity present in the 96 h medium from these cells was far greater than that in any other source tested so far. The initial purification step was to size-fractionate the SRC (RX) 96 h media using Sephacryl S-300. As found with FCS, there was poor separation between the peak of xylosyltransferase activity and the majority of the proteins eluted form the column, so a size-fractionation step was not very effective in the purification of xylosyltransferase from SRC (RX) media (figure 4.24). The SRC (RX) 96 h media was then tested with the synthetic peptide affinity matrix (figure 4.25), using the same conditions as for FCS. The eluted fractions containing xylosyltransferase activity were pooled and the proteins analysed by SDS-PAGE. Once again there were a number of bands that were visualised by silver stain on the gel, with two predominant bands at 70 kDa and 90 kDa. The 70 kDa band ran concurrently with the BSA band in the marker lane, suggesting that there was BSA contamination in the sample. Indeed, previous attempts at producing tryptic peptides from dissected bands originating from synthetic peptide selected affinity bands had shown sequence data for BSA. BSA contamination was proving to be a big problem in purifying xylosyltransferase. However a new strategy was developed to produce SRC (RX) media free of BSA.

Serum contains a number of factors that assist the cell in adhering to tissue culture plastic as well as maintaining them in culture. It had previously been reported that chondrosarcoma cells could be maintained in culture with medium supplemented with insulin and in the absence of serum (Stevens *et al.*, 1981). Stevens found that insulin could stimulate and maintain proteoglycan synthesis at the physiological concentration of 1 ng/ml. If SRC (RX) cells could be maintained in culture without serum in the medium then this would solve the problem of BSA contamination. It was found that the SRC (RX) cells required medium containing 1% (v/v) serum to adhere to the substrata, but after 24 h this medium could be replaced with serum-free medium containing insulin. A range of concentrations of insulin and serum were tested to see the effect on xylosyltransferase activity over 5 days in culture. Whilst the maximum

level of xylosyltransferase activity was found in the cultures supplemented with 2 % (v/v) FCS and 10 μg/ml insulin after 72 h in culture, there was no significant difference in the level of activity in cultures that were supplemented with insulin alone at a concentration of 1 μg/ml (figure 4.26). Therefore SRC (RX) cells could be supplemented with serum-free media containing 1 μg/ml insulin and they still secreted comparable amounts of xylosyltransferase activity into the medium as occurred in the presence of serum.

The kinetics of the transfer of xylose using SRC (RX) xylosyltransferase was also investigated. Previous studies had measured the kinetics of xylosyltransferase as a function of the various acceptors they had tested. Bourdon and co-workers (1987) had found the K_m of cartilage proteoglycan to be 2.9 μM, whilst Kearns (1991) had used hydrogen fluoride treated proteoglycan core protein as their substrate, which had a K_m of 0.11 μM and a V_{max} of 13 μmol/h; both used rat chondrosarcoma tissue as the enzyme source. In comparison to these more traditional substrates, Brinkmann (1997) used the synthetic peptide as a substrate for xylosyltransferase from serum and found the K_m to be 22 μM. This suggested that the deglycosylated core proteins were possibly better acceptors as they had a lower K_m. The SRC (RX) xylosyltransferase with the synthetic peptide as the acceptor gave a K_m of 45 μM, which was a rather higher K_m than with the human serum enzyme. The V_{max} was lower than that reported with the other substrates and other sources also, at 0.29 nmol/h, suggesting that xylosyltransferase secreted from SRC (RX) cells has a lower affinity for the synthetic peptide substrate and has a slower turnover. Another reason for the different kinetics is that the enzymes are from different species and it is also possible that there are more than one xylosyltransferase, which could also account for the differences seen in the kinetic values.

The SRC (RX) insulin supplemented media was applied directly to the Con A affinity matrix, where approximately 16% of the xylosyltransferase activity was bound and eluted in an active form. SDS-PAGE analysis of the eluted fractions did not resolve any distinct bands (figure 4.29). The bands were diffuse and of high molecular weight (>200 kDa). The synthetic peptide affinity matrix bound SRC (RX) xylosyltransferase; the amount of active xylosyltransferase eluted was comparable to that eluted when SRC (RX) media containing 10 % (v/v) FCS was used (figure 4.30). However, SDS-PAGE

analysis of the eluted fractions containing xylosyltransferase activity suggested a breakthrough, as there were just two distinct bands of 100 kDa and 200 kDa (figure 4.31). Previous work using Sephadex G-200 had suggested an approximate size of 100 kDa for xylosyltransferase, and this was similar to previous published data (Schwartz and Roden, 1975). Furthermore when the SDS-PAGE synthetic peptide affinity gel was blotted to PVDF and probed with a Con A peroxidase conjugate (figure 4.32) the 100 kDa band was also con A positive making it a strong candidate for being xylosyltransferase.

The 100 kDa band was excised from the gel and digested with trypsin to produce tryptic peptides. The peptides contained in the sample were analysed by MALDI-TOF, which produced a peptide-mass fingerprint of the protein (figure 4.33). Proteomic tools such as Mascot and EMBL, which have the tryptic masses of all the peptides possible for all the sequenced proteins on their databases, were used to match the peptides produced from the 100 kDa band with all the known protein peptide sequences. Using a peptide mass accuracy of 0.5 Da, with a protein mass range of 70-120 kDa, with 2 missed cleavage sites there were no statistically significant matches found. This suggested that the peptides belonged to a novel protein that had no sequence data available on the major proteomic www sites. The peptides were then separated by reverse phase chromatography, to obtain fractions containing single peptide peaks. Fraction 17 contained a strong signal for a peptide of mass 1174.87 Da, with an amino-acid sequence of QLYVALE_KR. This sequence was again compared with all known tryptic peptides available form the EMBL and BLAST databases and no statistically significant matches were found, suggesting that the peptide was novel and could belong to the protein sequence of xylosyltransferase.

The peptide sequence was used to create degenerate oligonucleotides in order to produce a full-length cDNA sequence of the protein containing this peptide sequence. Initially the reverse complement degenerate oligonucleotide probe was hybridised to an SRC (RX) Northern Blot to confirm that the sequence was present in the mRNA of the SRC (RX) cells. Unfortunately the probe did not hybridise to the filter. This may have been because there was no corresponding mRNA sequence in the RNA preparation or the degenerate probe had only a low affinity for the specific mRNA under the conditions tested. It was then attempted to amplify the sense degenerate oligonucleotide

using the polymerase chain reaction (PCR). SRC (RX) RNA was reverse transcribed to produce SRC (RX) cDNA which was then amplified using the template degenerate oligonucleotide. A variety of different annealing temperatures, magnesium concentrations and reactions conditions were varied to try to optimise the amplification stage. However, none of the reactions produced any amplified sequence, so no further clues to sequence information from the purified peptide was achieved through PCR methods.

Since both the hybridisation to the Northern blot and amplification of the sequence by PCR were unsuccessful using the degenerate oligonucleotide there was no further sequence data gained on the 100 kDa band that may have been xylosyltransferase and time precluded further larger scale preparations in order to obtain additional sequence information.

5.2 Intracellular Studies on Xylosyltransferase

Xylosyltransferase is the chain-initiating enzyme of the biosynthesis of chondroitin sulphate. Previous investigations have suggested that xylosylation of serine residues in the core protein begins in the endoplasmic reticulum and continues into the Golgi system (Kearns *et al.*, 1993), suggesting that xylosyltransferase, the enzyme which catalyses this reaction, must also reside within these cellular compartments. In addition, Hoffman and co-workers in 1984 apparently purified xylosyltransferase to homogeneity to produce antibodies to UDP-D-xylose; core protein xylosyltransferase in order to locate xylosyltransferase in fragments of embryonic cartilage cells. However this purification has not been followed up or confirmed. These studies also indicated that the enzyme was located within the cisternae of the rough endoplasmic reticulum. With this background, it seemed unusual that xylosyltransferase activity was found in serum (section 4.4). Why xylosyltransferase was being secreted out of the cell in an active form was unclear. There are no known reactions for this enzyme to catalyse out of the cell, so it would be expected to be degraded or retained in the cell, as it is in-efficient to synthesis an enzyme that is readily lost from the cell. Weilke and co-workers (1997) also found xylosyltransferase activity in the serum of blood donors, and found an increase in xylosyltransferase activity in the serum at the onset of pregnancy and in the synovial fluids of patients with chronic joint diseases (Kleesiek *et al.*, 1987). These results indicate that under certain conditions there is an increase in the level of xylosyltransferase in the circulation, which may be caused by an increased secretion by cells, but why the enzyme is secreted and by what mechanism is unclear.

There are a number of agents available which have an effect on the secretion and synthesis pathways of the cells, allowing the investigator to discover how different molecules are synthesised and secreted out of the cell. Monensin is a chemical, which interferes with the intracellular translocation of many secretory proteins (Tartakoff and Vassalli, 1977) by breaking up the Golgi system so proteins normally transported out of the cell remain trapped within. Therefore proteins that are normally secreted through this pathway remain in the cell where synthesis continues and thus a build up is seen. Monensin has been used to accumulate secretory proteins before; Burditt *et al* in 1985 treated chondrocytes with monensin, which led to the intracellular accumulation of proteoglycan core protein, which was detected in the immunoprecipitates of cell

extracts. If xylosyltransferase was secreted from the cell along this pathway, it should accumulate in the cell following treatment with monensin, and this should be accompanied by a reduction in the level of xylosyltransferase secreted into the cell media. SRC (RX) cells in culture that were treated with monensin showed a reduced level of sulphate incorporation indicating that the Golgi had been disrupted so the sulphotransferases that normally reside in the Golgi cisternae could not function (figure 4.36). However, the level of xylosyltransferase in monensin treated cells remained constant throughout the experiment for up to 19 hours, and there was no significant fall in secretion (figure 4.35). By disrupting the Golgi it was though that an accumulation of xylosyltransferase activity would be seen in the cell alongside a concurrent loss of secreted activity into the media, however this was not the case. These results indicated that xylosyltransferase secretion was only weakly affected by monensin, and that it was not being secreted out through the Golgi pathway. It could be that xylosyltransferase levels are controlled by a feedback loop, such that when levels reach a maximum it is either destroyed in the cell or its synthesis is slowed down or halted.

As it appeared that xylosyltransferase was not being secreted through the Golgi system with other secretory proteins, the half-life of xylosyltransferase was measured and compared to a secretory protein, such as aggrecan. Cycloheximide inhibits protein synthesis in the cell; therefore blocking any further synthesis of proteins. The half-life of the proteoglycan core protein in the cell was determined to be 90 minutes (figure 4.39), which was in agreement with previous studies measuring this factor (Mitchell and Hardingham, 1980; Kimura *et al.,* 1980). In comparison, the half-life of xylosyltransferase in the cell was approximately 3 hours (figure 4.37). The route through which xylosyltransferase is lost from the cell takes over twice as long as the secretory protein aggrecan. The cycloheximide treated cells continued to secrete xylosyltransferase throughout the course of the experiment (24 h), leaving no activity detectable in the cell layer. This indicated that SRC (RX) cells do not retain a certain level of xylosyltransferase in the cell, it is just secreted or lost from the cell with a turnover time of about 6 h.

Many glycosyltransferases that have been characterised share a common topology (figure 1.7). Within their structure is a region called the clip region which is located within the cytoplasmic portion of the structure, just prior to the membrane spanning

domain (Field and Wainwright, 1995). This region is thought to be liable to cleavage, releasing the cytoplasmic portion of the enzyme into the cytoplasm, where it could then be secreted out from the cell. Whilst this could be an explanation for the secretion of xylosyltransferase, it is not very probable. The active site of xylosyltransferase would be expected to lie within the cisternae of the ER, where it adds xylose to the serine residues on the core protein. Many of the glycosyltransferases located within the Golgi have their active sites within the cisternae also. If just the cytoplasmic portion of xylosyltransferase were clipped off then the secreted enzyme would not contain the active site and therefore no enzyme activity would be seen in the serum/media.

It was then investigated whether xylosyltransferase was simply lost from the ER and managed to avoid the cells' degradation enzymes, or was secreted out via the cells vesicular transport system using brefeldin A (BFA). BFA prevents the recruitment of coat proteins and the formation of transport vesicles, and causes the re-assembly of the Golgi system into the ER. Calabro and Hascall (1994) used BFA to show that hyaluronan synthesis occurred at the plasma membrane and was independent of vesicular transport, whilst chondroitin sulphate synthesis was rapidly inhibited (to 1% within 15 min). SRC (RX) cells treated with BFA showed the same response as seen in Calabro's experiments (figure 4.40) in relation to chondroitin sulphate synthesis. However, xylosyltransferase secretion into the media was unaffected by the addition of BFA, indicating that xylosyltransferase is secreted independently of vesicular transport by some as yet undefined mechanism. It may be that xylosyltransferase is made in abundance in some cells and tissues, but not so in others. It would be interesting to know if there is a mechanism for secreting xylosyltransferase out of certain cell types, into the serum, followed by its uptake and function in other cell types. Mannose-6-P has been shown to be involved in other systems where this occurs (Hauri *et al.,* 2000).

Xylosyltransferase is an intriguing enzyme, which has an unusual cell biology. It is an intracellular enzyme with an intracellular function, yet it is found in an active form in serum. The data from the monensin and brefeldin A studies suggests that it is not a secretory protein, but it is lost from the cell via some undefined pathway through the cell. The fact that xylosyltransferase was secreted from the cell was also useful in isolating the enzyme. Whilst no cDNA sequence was gained from the purification protocol developed, it was clear that the combination of a synthetic peptide and con A

affinity matrix could produce a sample enriched for xylosyltransferase. Given more time, increasing the volume of media applied to the columns, and re-applying the purified samples back over the affinity columns a greater level of purification could be achieved. However, re-applying the unbound material and bound active material was tried on several occasions, but no more complete binding of xylosyltransferase was demonstrated. This could have been due to loss of enzyme activity after 24-36 h at room temperature, rather than the failure of binding xylosyltransferase. However, with the existing purification methods, increasing the scale to produce a larger amount of purified enzyme to start with, would give a better chance of gaining a number of peptide sequences from which degenerate oligonucleotide primers could be made in order to produce a full-length cDNA clone of xylosyltransferase.

Furthermore, whilst the chromatographic assay developed to measure xylosyltransferase was robust and gave extremely reproducible results, it was not very effective in terms of time taken to set up and complete each gel filtration analysis. Ideally, if this procedure was to be repeated it would be worth investigating the possibility of coupling the synthetic peptide (1234 kDa) to a larger molecule, such as ovalbumin. Ovalbumin can bind 2/3 molecules per mole, and would bind the synthetic peptide at its N-terminus, thus not affecting the reactive serine, positioned in the middle of the peptide. Upon coupling the peptide to a larger molecule, it may be possible to use a filter paper assay, as originally intended, but due to the unavailability of a paper able to retain the peptide conjugated to xylose, was not used. This would increase the speed and number of assays, thus making the whole purification procedure more streamlined.

Whilst the peptide data gained from the tryptic fragments of the synthetic peptide affinity purified SRC (RX) proteins, the degenerate oligonucleotides used in the Northern Blot and PCR methods gave no further sequence data. Cloning, using an oligo dT sequence combined with a degenerate primer has some limitation and the chances of it being successful are low. An alternative method to try would include the use of a random primer, which could attach to a number of regions within the cDNA, enhancing the opportunities of detecting some further sequences.

The traditional purification strategy chosen to attain xylosyltransferase sequence data was not the only option considered; the yeast two-hybrid method of retrieving

molecules with known interactions was also investigated. Through the course of research, it was clear that the synthetic peptide had a good affinity for xylosyltransferase and it was hoped that this could be taken advantage of by using it as the "bait" protein in the yeast two-hybrid system. Unfortunately, due to lack of time it was not possible to have this system working before the end of this study, but it should be considered by any future investigators with an interest in isolating and cloning xylosyltransferase.

6. References

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7. Appendix

7. Buffers, Media and Solutions

7.1 General Buffers

Standard MES buffer

50 mM MES (pH 6.5)

12 mM $MgCl_2$

3 mM $MnCl_2$

5 mM KF

50 mM KCl

0.1 % (w/v) sodium azide

MES buffer plus proteinase inhibitors

60 ml standard MES buffer

125 mg N-ethylmaleimide

100 µl cocktail I

100 µl cocktail II

Proteinase inhibitor cocktail I

2.5 mg leupeptin

5 mg antipain

25 mg benzamidine

2.5 mg aprotinin

Dissolve in 2.5 ml deionised water and store at –20 °C.

Proteinase inhibitor cocktail II

2.5 mg chymostatin

2.5 mg pepstatin

Dissolve in 2.5 ml DMSO and store at –20°C.

Protease inhibitor cocktail

0.1 M 6-aminohexanoic acid

100 mM Na_2-EDTA

5 mM benzamidine HCl

0.5 mM phenylmethylsulphonylfluoride

Dimethyl Methylene Blue Dye (DMMB)

16 mg dimethyl methylene blue, dissolved in 5 ml ethanol

1 g formate

2 ml formic acid

Dissolve in 1 litre of deionised water and adjust pH to 3.5. Store at 4°C in the dark.

7.2 Protein Analysis

2X SDS-PAGE sample loading buffer

60 mM Tris-HCl (pH 6.8)

25% (v/v) glycerol

2% (w/v) SDS

0.1% (v/v) bromophenol blue

SDS-PAGE running buffer

25 mM Tris Base (pH 8.3)

192 mM glycine

0.1 % (w/v) SDS

Fixer / Coomassie destain

30% (v/v) methanol

10% (v/v) glacial acetic acid

Pre-drying solution

30% (v/v) methanol

1% (v/v) glycerol

SDS-PAGE transfer buffer

3.03 g Tris base

14.4 g glycine

20% (v/v) methanol

Make up to 1 litre with deionised water

7.3 Western Blot / Immunoblot Buffers for Histidine Tagged Proteins

5X phosphate-buffered saline

82.3 g Na_2HPO_4

20.4 g $Na_2H_2PO_4$

40 g NaCl

Dissolve in 1.9 litres of deionised water and adjust pH to 7.4 with 0.1 M NaOH. Add deionised water to a final volume of 2 litres.

Wash buffer

200 ml 5X PBS

1 ml Tween 20

1 g non-fat dry milk

Dissolve in 1 litre of deionised water.

Blocking buffer

20 ml 5X PBS

0.1 ml Tween-20

5 g non-fat dry milk

Dissolve in 100 ml of deionised water.

7.4 DNA Analysis

2X SSC

175.3 g sodium chloride

88.2 g sodium citrate, di-hydride

Dissolve in 900 ml of deionised water, pH to 7.0 with concentrated hydrochloric acid then correct volume to 1 litre with deionised water.

50X TAE

242 g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (di-sodium salt), pH 8.0

Dissolve Tris in deionised water, add acetic acid and EDTA then make up to 1 litre with deionised water.

DNA sample loading buffer

2 ml glycerol

500 µl saturated bromophenol blue (in deionised water)

400 µl 10X TAE

Make up to 4 ml with deionised water

7.5 Colony Hybridisation Buffers

Solution I

20 g NaOH, dissolve in 500 ml deionised water.

Solution II

43.8 g NaCl

30.3 g Tris-HCl, pH 7.4

Dissolve in 500 ml deionised water.

Solution III

43.8 g NaCl, dissolve in 250 ml of deionised water and add 250 ml of 2X SSC.

100X Denhardt's solution

10 g polyvinylpyrrolidine

10 g bovine serum albumin

10 g ficoll

Make up to 500 ml with deionised water and sterilise through a 0.2 μm filter. Store at –20°C.

Pre-hybridisation solution

10 ml 20X SSC

28.4 ml deionised water

0.8 ml 100X Denhardt's solution

0.4 ml 10% (w/v) SDS

Add the above in order, then add 0.4 ml of pre-boiled 10 mg/ml salmon sperm DNA. Use immediately.

7.6 RNA Determination and Northern Blotting

DEPC-treated deionised water

0.1% (v/v) diethylpyrocarbonate in deionised water

Mix; allow to sit at room temperature for at least 6 hours, and autoclave.

MOPS buffer (10X)

41.9 g MOPS

6.8 g disodium EDTA

Make up to 1 litre with deionised water and autoclave.

Denaturing buffer (for RNA gel samples)

200 μl formamide

20 μl 10X MOPS buffer

70 μl formaldehyde

Northern Blot / Pre-hybridisation Solution

50% (v/v) formamide

4X SSC

1X Denhardt's solution

0.1% (w/v) SDS

0.3 mg/ml salmon testes DNA

7.7 Bacterial Cell Culture

1000X Ampicillin Stock

Make up solution of 100 mg/ml in deionised water and sterilise through a 0.2 μm filter. Store at –20°C.

LB media

10 g tryptone

5 g yeast extract

10 g NaCl

Make up to 1 litre with deionised water then autoclave.

For LB plates, add 15 g agar to the media, make up to 1 litre with deionised water and then autoclave. Allow the solution to cool to 60°C and then pour plates.

SOB Media

20 g tryptone

5 g yeast extract

0.5 g NaCl

Dissolve in 950 ml of deionised water. Add 10 ml of a 250 mM solution of KCl and adjust the pH to 7.0 with 5 M NaOH. Sterilise by autoclaving then add 5 ml of sterile 2 mM $MgCL_2$.

SOC media

As for SOB, but after autoclaving allow the solution to cool to around 60°C then add 20 ml of a 1 M solution of glucose.

E. Coli Glycerol Stocks

To 0.85 ml of overnight culture add 0.15 ml of glycerol (sterilised by autoclaving), followed by vigorous vortexing. Transfer the solution to a freezing vial and freeze in an ethanol / dry ice bath. Store at –80°C.

7.8 Mammalian Cell Culture

Complete chondrocyte medium (for Syrian hamster embryo chondrocytes)

500 ml minimum essential medium

5 ml 100X L-glutamine (200 mM)

5 ml 100X penicillin-streptomycin (10,000 iu/ml – 10,000 μg/ml)

50 ml foetal bovine serum (FCS)

5 ml non-essential amino-acids

Complete culture medium (for SRC tissue)

500 ml Dulbecco's modified essential medium

5 ml 100X L-glutamine (200 mM)

5 ml 100X penicillin-streptomycin (10,000 iu/ml – 10,000 μg/ml)

50 ml foetal bovine serum (FCS)

SRC (RX) cell culture medium

500 ml Dulbecco's modified essential medium

5 ml 100X HEPES (200 mM)

5 ml 100X penicillin-streptomycin (10,000 iu/ml – 10,000 μg/ml)

To this culture medium various concentrations of FCS and insulin were added.