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Analysis of perlecan structure and function.

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ANALYSIS OF PERLECAN STRUCTURE AND FUNCTION

by

PAIRATH TAPANADECHOPONE

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
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ABSTRACT OF DISSERTATION
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Title Analysis of Perlecan Structure and Function

During the development of a primary tumor and the establishment of metastatic foci, tumor cells interact with extracellular matrices. The relationship between tumor cells and extracellular matrix in the process of growth and invasion is intensively studied, but many questions remain. Perlecan, a major proteoglycan (PG) of all mammalian basement membranes and extracellular matrices, plays important roles in cell-matrix interactions. Many studies have shown that neoplastic transformation is associated with alterations in PG synthesis. These alterations are believed to promote tumorigenic growth, angiogenesis, and invasion. Therefore, the major goal of this research was to define the structural and functional roles of perlecan in this process.

Perlecan was first described as a heparan-sulfated molecule, but hybrid forms containing both heparan sulfate (HS) and chondroitin sulfate (CS)/dermatan sulfate (DS) side chains have also been demonstrated. Since the biological functions of different types of glycosaminoglycan (GAG) vary depending on their cellular origin and their structural modifications, we analyzed the expressions and biological functions of perlecan produced by normal and transformed murine epidermal cells. Expression of perlecan in tumorigenic cells was significantly increased. In addition, tumor-derived perlecan showed some structural differences. It contained both HS and CS/DS chains, whereas the wild type was expressed predominantly as a heparan-sulfated molecule. Moreover, the HS was composed

of different disaccharide subunits and had reduced sulfation. However, there was little change in binding affinities for heparin-binding growth factors. This study suggests that differential expression of perlecan plays important roles in the process of malignant transformation.

Perlecan has previously been shown to contain three GAGs attached on domain I. However, some evidence indicated that the C-terminal domains IV and V of this molecule can also be substituted with GAG chains, but the exact substitution sites were not identified. Recombinant domain IV and/or V of mouse perlecan was expressed in COS-7 cells and analyzed for GAG substitution. We found that both HS and CS chains could be detected on specific serine residues on domain V. The functional consequences of these substitutions remain unknown but may substantially contribute to the physicochemical and biological functions of perlecan.

DEDICATION

I dedicate this dissertation to everybody in my family for their love and support.

I also would like to dedicate this work to Thai Education.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
CS	chondroitin sulfate
CSPG	chondroitin sulfate proteoglycan
DGSDE	asp-gly-ser-asp-glu
DMEM	Dulbecco's modified Eagle's medium
DS	dermatan sulfate
DW	distilled water
ECM	extracellular matrix
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Swarm
EMEM	Eagle's minimal essential medium
ER	endoplasmic reticulum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FN	fibronectin
GAG	glycosaminoglycan
GalNAc	*N*-acetylgalactosamine
GC	gly-cys
GF	growth factor
GlcA	glucuronic acid

GlcNAc	*N*-acetylglucosamine
GPI	glycosylphosphatidylinositol
h	hour
HA	hyaluronic acid
HB-EGF	heparin binding epidermal growth factor
HGF/SF	hepatocyte growth factor/scatter factor
HIP	heparin/heparan sulfate interacting protein
HPLC	high-performance liquid chromatography
HS	heparan sulfate
HSD	highly sulfated domain
HSPG	heparan sulfate proteoglycan
IFN-γ	interferon gamma
IdoA	iduronic acid
Ig	Immunoglobulin
kDa	kilodalton
KGF	keratinocyte growth factor
KS	keratan sulfate
LDL	low-density lipoprotein
MALDI-MS	matrix-associated laser desorption ionization mass spectrophotometry
min	minute
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MW	molecular weight

N-CAM	neural cell adhesion molecule
NF-κB	nuclear factor-κB
PAGE	polyacrylamide gel electrophoresis
PBS	Dulbecco's phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PG	proteoglycan
PMSF	phenylmethylsulfonyl fluoride
PRM	perlecan-related molecule
RGD	arg-gly-asp
RPA	ribonuclease protection assay
RT-PCR	reverse transcriptase-polymerase chain reaction
SAX-HPLC	strong anion-exchange high-performance liquid chromatography
SDS	sodium dodecyl sulfate
SGD	ser-gly-asp
SGXG	ser-gly-x-gly
TBS	Tris-buffered saline
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha
TPA	12-*O*-tetradecanoylphorbol-13-acetate
UMD	unmodified domain

INTRODUCTION

Cell-Extracellular Matrix Interaction

During growth, differentiation, and migration, cells interact with the extracellular matrix (ECM). The harmony between cells and their environment is a key factor that maintains the normal architecture and function of tissues (Hay, 1991; Adams and Watt, 1993). The ECM is a complex and dynamic meshwork of collagens and elastin embedded in viscoelastic ground substance composed of glycoproteins and proteoglycans (PGs). The ECM exists as a three-dimensional supporting scaffolding that determines tissue architecture and isolates tissues into compartments. Originally, the ECM was only thought to contribute to the mechanical scaffolding and integrity of cells and organs. Although this is a very important function, we now know that the ECM is a dynamic environment that provides the cells with much more than physical support. One function of the ECM in mitogenesis and differentiation is to bind and sequester growth factors (GFs). GFs bound to the ECM often have altered potency and increased stability and are concentrated in the vicinity of the cells. One of the best described GF-matrix interactions involves the binding of fibroblast growth factors (FGFs) to heparan sulfate proteoglycans (HSPGs) (Basilico and Moscatelli, 1992). The ECM also acts as a selective macromolecular filter and plays a role in cell adhesion and migration (Mosher *et al.*, 1992; Scott, 1995), which are involved in both normal and pathological processes (Bruijn *et al.*, 1989; Adams and Watt, 1993). Basement membranes are specialized ECMs that form a thin layer of supporting structure on which epithelial and endothelial cells grow

and that surrounds muscle, fat, and nerve cells. In other words, it demarcates cells from connective tissues and serves a structural role in the support and organization of tissues. Recent evidence has demonstrated that basement membranes not only provide mechanical support and divide tissues into compartments but are known to play a significant role in fundamental biological processes; for example, they serve as selective permeability barriers, storage sites of GFs, and ligands for cell adhesion (Yurchenco and Schittny, 1990; Paulsson, 1992). All of these have an influence on behavior (differentiation, proliferation, spreading, migration) of cells by so-called cell-matrix interaction.

Basement Membrane

Structure and Composition

Basement membrane is a specialized ECM. It is usually composed of three layers (by conventional electron microscopy): an unstained layer of lamina lucida (or rara) next to the surface of associated cells; the main layer, or lamina densa; and lamina fibroreticularis or pars reticularis on the connective tissue side (Laurie and Leblond, 1985). Chan and Inoue (1994) proposed that the lamina lucida is an artifact formed during the process of conventional tissue preparation and suggested that in the living state, basement membrane is composed of a single layer of lamina densa materials. Immunostaining reveals that basement membranes are biochemically complex and heterogeneous structures containing at least five major classes of components: collagen type IV, laminins, entactin, fibronectin (FN), and PGs (Paulsson, 1992; Adachi *et al.*, 1997). Basement membranes contain both HS and chondroitin sulfate (CS) PGs (Timpl, 1994). Perlecan is a major PG in the basement membrane that is predominantly expressed

as a heparan-sulfated molecule (Noonan *et al.*, 1991). There is at least one CSPG which is widespread in basement membranes (McCarthy and Couchman, 1990). The cDNA sequence has recently been cloned and the proteoglycan is named bamacan (*ba*sement *m*embrane *a*ssociated *c*hondroitin sulfate proteoglyc*an*) (Couchman *et al.*, 1996; Wu and Couchman, 1997). Other basement membrane associated proteins are SPARC (secreted protein acidic and rich in cysteine)/BM40/osteopontin; collagen VII, which forms anchoring fibrils; agrin and type XVIII collagens (Paulsson, 1992; Halfter *et al.*, 1998). The compositions of basement membranes and ECMs result from the balance between the synthesis of their constituents and their degradation by proteolytic enzymes (Birkedal-Hansen, 1995). Different basement membranes differ quite markedly in their solubility and susceptibility to proteolytic degradation, which reflects differences in their molecular structure and functions (Dziadek and Mitrangas, 1989). Collagen IV provides a scaffold for the other structural molecules by forming a network via interaction between specialized N- and C-terminal domains (Paulsson, 1992; Adachi *et al.*, 1997). Laminin γ1 subunit is necessary for laminin assembly, which, in turn, is essential for the organization of other basement membrane components (Smyth *et al.*, 1999). Perlecan can self-assemble into dimers and stellate oligomers with peripherally oriented glycosaminoglycan (GAG) chains along the surfaces of the matrix (Yurchenco *et al.*, 1987; Laurie *et al.*, 1988). It also binds to laminin by GAG side chains and to entactin by a site in its protein core (Battaglia *et al.*, 1992). Recent data have shown that perlecan is essential for the integrity of the basement membranes (Costell *et al.*, 1999). In different basement membranes, the components arrange themselves in different ways and may account for differences in functional properties (Desjardins and Bendayan, 1989). All of these

constituent molecules are able to regulate cell behavior and maintain cell shape and polarity (Paulsson, 1992).

PGs

PGs are present in almost all ECMs. They consist of a protein core containing one or more covalently attached GAG side chains (Kjellen and Lindahl, 1991; Hardingham and Fosang, 1992). The PG superfamily now contains more than 30 full-time molecules that fulfill a variety of biological functions. PGs act as tissue organizers, influence cell growth and the maturation of specialized tissues, play a role as biological filters, modulate GF activities, regulate collagen fibrillogenesis and skin tensile strength, affect tumor cell growth and invasion, and influence corneal transparency and neurite growth (Iozzo, 1998).

PGs can be grouped into five categories based on their core protein and location. Large, extracellular PGs are the large, aggregating PGs associated with hyaluronic acid through an N-terminal hyaluronic acid-binding region, often stabilized by link proteins. Aggrecan (~220 kDa) in cartilage contains more than 100 CS chains with keratan sulfate (KS) chains, and versican (~265 kDa) in fibroblasts contains CS chains. The core protein of small interstitial PGs are approximately 35 kDa and contain one or two GAG chains. Decorin contains a dermatan sulfate (DS) or CS chain, biglycan which contains two DS or CS chains, and fibromodulin which contains KS chains. Perlecan (~400 kDa), which is a large PG, belongs to the third group of PGs, basement membrane PGs. Cell-surface PGs are expressed on the cell surface of most cells and represent a diverse set of structures. The transmembrane PGs, syndecans (~30 kDa), contain variable amounts of both CS and HS, giving rise to hybrid PGs. The glycosylphosphatidylinositol (GPI)-

anchored subset of cell surface PGs, termed the glypican family, contain HS only. Serglycin (~18 kDa) is an intracellular PG found in mast cell basophilic granules and contains either CS or HS chains.

Molecular Structure

PGs are proteins that are substituted with one or more GAG chains (Figure 1). GAGs are large carbohydrates of varying lengths that are composed of repeating disaccharide units consisting of hexosamine (*N*-acetylglucosamine: GlcNAc or *N*-acetylgalactosamine: GalNAc) and usually a uronic acid (glucuronic acid: GlcA or its C5 epimer, iduronic acid: IdoA). There are four different classes of GAGs. Hyaluronic acid (hyaluronan, hyaluronate, HA) consists of a copolymer of GlcNAc and GlcA. Chondroitin, the nonsulfated backbone of CS, has GalNAc and GlcA. The most common sites of sulfation are the 4 and 6 positions on the hexosamine, termed chondroitin-4 sulfate and chondroitin-6 sulfate, respectively. KS consists of a backbone structure with alternating GlcNAc and galactose. HS has the same backbone structure as that of heparin and consists of GlcNAc and GlcA or IdoA. However in comparison to heparin, HS chains typically contain a higher proportion of glucuronic acid that is less sulfated at *N*- and *O*-positions. Both heparin and HS chains undergo a series of modification reactions that include *N*-deacetylation, and *N*-sulfation of GlcNAc residues, *O*-sulfation at various positions, and epimerization of GlcA to IdoA. The extent of these reactions varies, giving rise to enormous structural heterogeneity. The high content of sulfate and the presence of hexuronic acid impart a large negative charge to GAGs and permit them to associate electrostatically with a large number of ligands. In addition to core protein interactions,

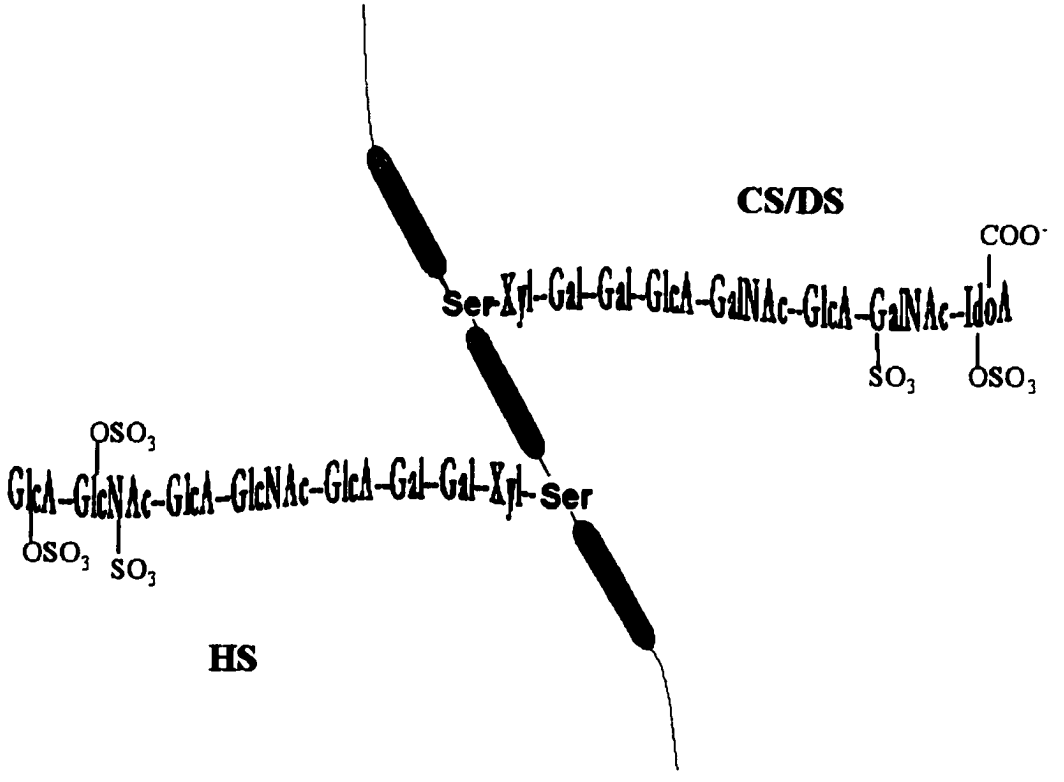

Figure 1. Structure of proteoglycans.

the specific GAG chains determine many biological activities of PGs (Jackson *et al.*, 1991; Hardingham and Fosang, 1992; Salmivirta *et al.*, 1996). With the exception of heparin, GAG chains are covalently attached to the core protein at their reducing end through an *O*-glycosidic linkage to a serine residue or *N*-linked to asparagine. As with CS/DS chains, the initial backbone of the HS chain is synthesized onto an appropriate core protein through a linkage sugar (glucuronosyl-β1,3-galactosyl-β1,3-galactosyl-β1,4-xylose) added in endoplasmic reticulum (ER) compartment. The typical HS substitution site is on serine residues in the core protein. HS can be modified by *N*-sulfation, epimerization of GlcA to IdoA, 2-*O*-sulfation of IdoA residues, and 6-*O*-sulfation of glucosamine residues (Yanagishita and Hascall, 1992). More rare modifications are 2- and 3-*O*-sulfation of glucosamine. In general, the HS chain comprises a series of spatially discrete highly sulfated domains (HSD or S-domain) alternating with large regions of the unmodified domain (UMD). The boundary between the HSD and the UMD is not sharp. It may contain short mixed sequences where *N*-acetylated disaccharides are in alternate sequence with *N*-sulfated ones (Gallagher, 1994). The length of the HSDs ranges from 2 to 8/9 disaccharides and they are separated on average by ~16-18 disaccharides of the UMDs. The alternative patterns for HSD and UMD can differ between HS chains derived from different cell types (Kato *et al.*, 1994; Sanderson *et al.*, 1994). The UMD provides more flexibility than the relatively rigid, highly anionic HSDs. Thus, together with chain length, sugar residue sequences, and sulfation patterns, the binding avidity of HS chains for proteins can vary with the spacing of the UMDs (Spillmann and Lindahl, 1994).

GAG-Protein Interactions

GAG chains, HS in particular, bind and regulate the functions of diverse proteins, including extracellular matrix components, peptide growth factors, cell adhesion molecules, lipolytic enzymes, protease inhibitors, and circulating lipoproteins. Their interactions are not simply by electrostatic forces between anionic GAG and basic proteins. Heparin binds to the intrinsic coagulation protease, thrombin, and to variety of serine protease inhibitors, including antithrombin III, and induces a conformational change to prevent coagulation. A specific pentasaccharide sequence containing a 3-*O*-sulfated glucosamine residue has been identified as a minimal sequence for the high-affinity binding to antithrombin III that leads to inhibition of factor Xa (Spillmann and Lindahl, 1994). Therefore, the interactions are specifically regulated, possibly by unique patterns of sulfation in the GAG chains and amino acid sequences and/or conformation of the proteins. Studies of GF-PG interactions, and of other functions of PGs, have mainly focused on the roles of GAG chains, although core proteins also appear to have significant functions.

Modulation of GFs

One of the most interesting functions of PGs is their ability to bind and modulate GF activity. A significant number of studies have focused on the effects of the binding of heparin or HS chains to members of the FGF family. The binding of heparin or HS chains of PGs protects the GF from chemical and proteolytic degradation and serves as a matrix-bound or cell-surface-bound reservoir of FGFs. Binding of HS to certain proteins has been suggested to induce a conformational change, which may lead to expressing novel reactive determinants, or confer stability of configuration. Specific saccharide sulfation

sequences within HS GAG chains seem to favor signaling by distinct members of the FGF family (Spillmann and Lindahl, 1994). HSPG found in intact ECM has been shown to induce dimerization of the bFGF receptor, which is required for receptor activation (Miao *et al.*, 1996; Moy *et al.*, 1997). Thus, sulfation of basement membrane PGs is the key determinant in the response of certain cells to selected GFs that may define pattern and differentiation (Sannes *et al.*, 1996). Both cell-surface and ECM HSPGs act in concert to regulate the bioavailability and growth promoting activity of bFGF. While the HS in subendothelial ECM functions primarily in sequestration of bFGF, HS on the cell surface plays a more active role in displacing the ECM-bound bFGF and subsequently presenting it to high-affinity signal transducing receptors (Miao *et al.*, 1996). Binding of bFGF to HS protects it from proteolytic activity (Tumova and Bame, 1996), and the concerted action of proteases that degrade the protein core and heparanases that remove the HS chains may, in turn, modulate the bioavailability of the GFs (Whitelock *et al.*, 1996). Because each GAG chain may have a unique arrangement of monosaccharide residues, individual PG molecules probably represent unique chemical entities. Thus, each PG carries enormous amounts of information in the form of different saccharide sequences. Some PGs even contain more than one type of GAG on the same core protein (e.g., syndecan-1, aggrecan, and perlecan) (Hardingham and Fosang, 1992).

Basement Membrane PGs

Basement membranes contain both HS and smaller amounts of CS/DSPGs (Noonan and Hassell, 1993). The relative amount of HSPGs and CS/DSPGs produced by different basement membranes can vary considerably. For example, the Engelbreth-Holm-Swarm (EHS) tumor produces 75% of the total GAG as HS and 19% as CS/DS,

whereas the L2 yolk sac carcinoma produces 21% as HS and 76% as CS/DS (Wewer *et al.*, 1985). Three HSPGs are characteristically present in vascular and epithelial basement membranes of mammalian organisms: perlecan, agrin, and collagen XVIII (Halfter *et al.*, 1998; Iozzo, 1998). Bamacan (Wu and Couchman, 1997) and leprecan (Wassenhove-McCarthy and McCarthy, 1999) are basement membrane CSPGs. Perlecan is the major PG of basement membranes and ECMs and can be expressed as a hybrid molecule, containing both HS and CS/DS GAGs (Couchman *et al.*, 1996). There are some other small PGs which remain to be characterized. The chimeric structure design of these PGs suggests that they may be involved in numerous biological processes (Iozzo, 1998).

Cell Biology of Perlecan

Perlecan and ECMs

Perlecan is the main PG of many basement membranes and ECMs. It is a large PG with a wide tissue distribution and multiple potential functions (Iozzo, 1998; Olsen, 1999). It was originally isolated as a large, low-density HSPG from the EHS tumor with a core protein of ~400 kDa (Hassell *et al.*, 1980; Paulsson *et al.*, 1987) and was named perlecan because of its appearance on rotary shadowing electron micrographs (Paulson *et al.*, 1987; Noonan *et al.*, 1991) as a variable number of globules separated by thin segments like beads on a string. Three side chains were seen attached to a globule at one end of the core protein (Paulsson *et al.*, 1987; Noonan *et al.*, 1991). Perlecan is one of the most important and interesting molecules of the ECM with numerous biological functions in cell-matrix interactions, in addition to being a structural component of ECMs and basement membranes (Iozzo, 1994). The history of perlecan began over 2 decades ago with three key observations: (a) a role of PG in branching morphogenesis, (b) the

demonstration of spatially ordered polyanionic sites in the basal lamina of breast ducts, and (c) the discovery of HSPG in glomerular basement membranes (Iozzo *et al.*, 1991). Recent research studies have extensively uncovered various aspects of perlecan structure, functions, and gene regulation. The major questions concerning perlecan are: how is perlecan expressed differently in different tissues, how are its diverse functions mediated, and what mechanism regulates all of these? Hassell and his collaborators have had a great impact on the understanding of perlecan. The molecule was first purified from a mouse tumor (EHS) that was known to synthesize excessive amounts of basement membrane constituents as a large, low-density HSPG (Hassell *et al.*, 1980; Kleinman *et al.*, 1982; Paulsson *et al.*, 1987). Molecular cloning and peptide sequence analysis have shown that perlecan is synthesized not only by the EHS tumor but also by a variety of cell types, including epithelial and mesenchymal cells, muscle, and peripheral nerve, and is concentrated at cell-matrix interfaces, especially in basement membranes (Heremans *et al.*, 1989; Yamane *et al.*, 1996). It has also been found in many organs, including the pituitary gland, skin, breast, thymus, prostate, colon, liver, pancreas, spleen, kidney, heart, lung, vascular basement membranes, and lymph node (Murdoch *et al.*, 1994); bone marrow and hematopoietic cells (Klein *et al.*, 1995; Schofield *et al.*, 1999); cartilage (SundarRaj *et al.*, 1995); synovium (Dodge *et al.*, 1995); placenta (Isemura *et al.*, 1987); and cornea (Hassell *et al.*, 1992). Since perlecan is found in all basement membranes and many ECMs, it not surprisingly has a wide range of regulatory controls, binding properties, and interactions. Perlecan is not only to be regulated by cytokines but to bind to them, as well as to GFs, with high affinity. Some of these interactions involve only GAG side chains and others involve the core protein. Perlecan has also been shown to bind most of the basement membrane components, as well as several ECM molecules;

these bindings are crucial for maintaining the integrity (Costell *et al.*, 1999) of tissue. In addition, the subsequent availability of immunological reagents recognizing specific domains of the core protein and the GAG chains (Couchman *et al.*, 1984, 1989, 1995; Murdoch *et al.*, 1994) has stimulated research in many cellular systems and undoubtedly set the stage for major discoveries.

Genomic Organization and Transcriptional Control

Perlecan is one of the most complex gene products because of its enormous size and extent of posttranslational modification (Iozzo, 1994). The human perlecan gene (HSPG2) is a single copy gene located on the telomeric region of the short-arm of human chromosome 1, band p36.1-35 (Figure 2) (Wintle *et al.*, 1990; Dodge *et al.*, 1991; Kallunki *et al.*, 1991). Mouse perlecan is located on mouse chromosome 4 (Chakravati *et al.*, 1991). Few human genetic diseases have been found to be located in or near this region of chromosome 1. However, several cancer markers and progressive musculoskeletal disorders were identified in this region (Cohen *et al.*, 1993). The human perlecan gene consists of 94 exons and spans at least 120 kb of genomic DNA. Similar to the protein, the exon arrangement was analyzed vis-à-vis the modular structure of perlecan, which has regions of homology to low-density lipoprotein receptor, laminin; neural cell adhesion molecule (N-CAM); and epidermal growth factor (EGF). Both exon and intron phases are remarkably conserved, suggesting that most of this PG has evolved from a common ancestor by gene duplication or exon shuffling. Exon I contains the 5' untranslated region and encodes for perlecan signal peptide. Exons II-VI encode domain I, which contains the sequence necessary for the three GAG attachment sites. The first ser-gly-asp (SGD) attachment sequence resides between exon II and III where the

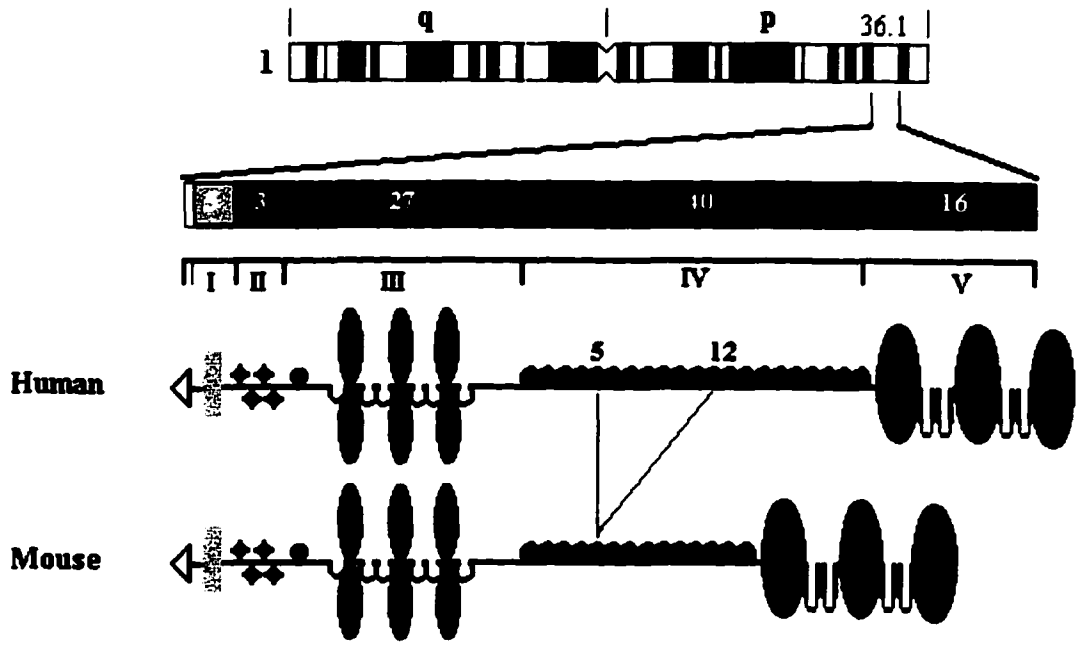

Figure 2. Genomic organization of perlecan.

junctional splicing occurs with the Asp residue. Exon III encodes the remaining two SGD sequences. It is possible that if the exon III is lost by alternative splicing, perlecan would lose all three GAG attachment regions and would be expressed as a glycoprotein, like inthe case of perlecan in colon carcinoma cells (Iozzo and Hassell, 1989) and in the nematode, *C. elegans* (Rogalski *et al.*, 1993). Domain II is encoded by five exons; three of them encode for the LDL receptor-like region, whereas the other two exons encode the immunoglobulin (Ig)-like module. Domain III is encoded by 27 separate exons of various sizes (50-231 bp). Domain IV, which contains the largest array of IgG-like repeats, is encoded by 40 separate exons and is similar in exon organization to N-CAM. The arrangement of the exons allows for alternative splicing between IgG domains such as in the case of mouse perlecan. Domain V is encoded by 16 exons, including the largest exon of 1.2 kb, harboring the C-terminal end of domain V and the 3' untranslated region (Cohen *et al.*, 1993). Primer extension and S1 nuclease protection assays revealed multiple transcription start sites distributed over 80 bp, suggesting that control of the perlecan gene is complex (Cohen *et al.*, 1993).

To date, only the promoter of human perlecan has been sequenced and tested for functional activity (Cohen *et al.*, 1993; Iozzo *et al.*, 1997). The putative promoter region is located within a typical gly-cys (GC) nucleotide-rich regions, lacks canonical TATA to CAAT boxes, and contains multiple cis-acting elements and palindromic inverted repeats, which may potentially form a secondary structure that could impact the regulation of gene expression (Cohen *et al.*, 1993). In this respect, it is similar to the promoter region of several oncoproteins, GFs, and housekeeping genes that are generally devoid of TATA or CAAT boxes and contain multiple transcription initiation sites. The proximal promoter region also contains 4 GC boxes and 15 consensus hexanucleotide-binding sites for the

zinc finger transcriptional factor Sp1. Another striking feature of the perlecan promoter is the presence of multiple AP2 motifs (Iozzo *et al.*, 1997). The AP2 transcription factor can be suppressed by SV40 T antigen (Mitchell and Tjian, 1989). SV40 T antigen inhibits transcription of perlecan in renal tubular epithelial cells (Piedagnel *et al.*, 1994), whereas perlecan expression is up regulated by phorbol ester in colon cancer (McBain *et al.*, 1990) and in erythroleukemic K562 cells (Grassel *et al.*, 1995). Other elements found in the perlecan promoter region include a PEA3 motif--EST-1, PU.1 box, and GATA-1 motifs that are involved in hematopoiesis (Orkin, 1995). A transforming growth factor-β (TGF-β)–responsive element is present which binds NF-1-like transcription factors and TGF-β induces both messenger ribonucleic acid (mRNA) and protein levels of perlecan (Iozzo *et al.*, 1997). In the distal region of the perlecan promoter, there is a binding site for nuclear factor-κB (NF-κB), which is involved in interleukin-induced transcription of several GF and cytokine genes (Mitchell and Tjian, 1989). Some other factors that have been shown to regulate perlecan gene transcription include glucose (Kasinath *et al.*, 1996), as well as cAMP and retinoic acid (Chakravarti *et al.*, 1993; Ko *et al.*, 1996). High glucose concentrations posttranscriptionally inhibit PG synthesis in glomerular and mesangial cells (Kanwar *et al.*, 1983; Kasinath *et al.*, 1996; Templeton and Fan, 1996). In contrast, long term exposure of human mesangial cells to high concentrations of glucose induces perlecan gene expression (Wahab *et al.*, 1996).

General Structure of Perlecan

The complete amino sequences of mouse perlecan (a 396-kDa polypeptide) (Noonan *et al.*, 1991), human perlecan (a 467-kDa polypeptide) (Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992), and nematode (*C. elegans*) perlecan (a 270-

kDa polypeptide) (Rogalski *et al.*, 1993) have provided potential structure-function information. Analysis of mouse and human perlecan sequences shows that the proteins consist of five distinct domains (Figure 3) (Noonan *et al.*, 1991; Murdoch *et al.*, 1992) which correspond to previous electron micrographs (Paulsson *et al.*, 1987).

Domain I contains a start methionine followed by typical signal transfer sequence and a unique 172-amino-acid segment that is rich in acidic residues but lacks cysteine; the domain also contains three GAG attachment sites, SGD sequences. There is no internal repeat with the exception of the SGD sequences, and this segment appears to be unique to perlecan (Noonan *et al.*, 1991; Murdoch *et al.*, 1992). The SGD sites are predominantly substituted with HS chains and, in some circumstances, can be also substituted with the CS/DS also (Kokenyesi and Silbert, 1995; Costel *et al.*, 1997; Dolan *et al.*, 1997). Domain II contains four cysteine- and acidic amino acid-rich repeats, with a striking preservation of the binding site sequence of apolipoproteins, the pentapeptide asp-gly-ser-asp-glu (DGSDE). Immediately distal to the LDL receptor domain, intercalated between domain II and III, there is a single IgG repeat, similar to repetitive elements in domain IV (Bourdon *et al.*, 1987). Domain III consists of three cysteine-free globular regions which are flanked on either side by cysteine-rich extended domains and are similar to the short arm of laminin α1. In this domain of mouse (Noonan *et al.*, 1991) and nematode (Rogalski *et al.*, 1993) sequences but not in the human sequence, there is an arg-gly-apd (RGD) sequence that can be a binding site for β1 and β3 integrins Hayashi *et al.*, 1992; Battaglia *et al.*, 1993; Brown *et al.*, 1997). Domain IV, the largest domain in the human sequence, has Ig-like repeats that show the greatest similarity to N-CAM. There are 21 repeats in human perlecan (Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992) and 14 repeats in mouse and nematode perlecan (Noonan *et al.*,

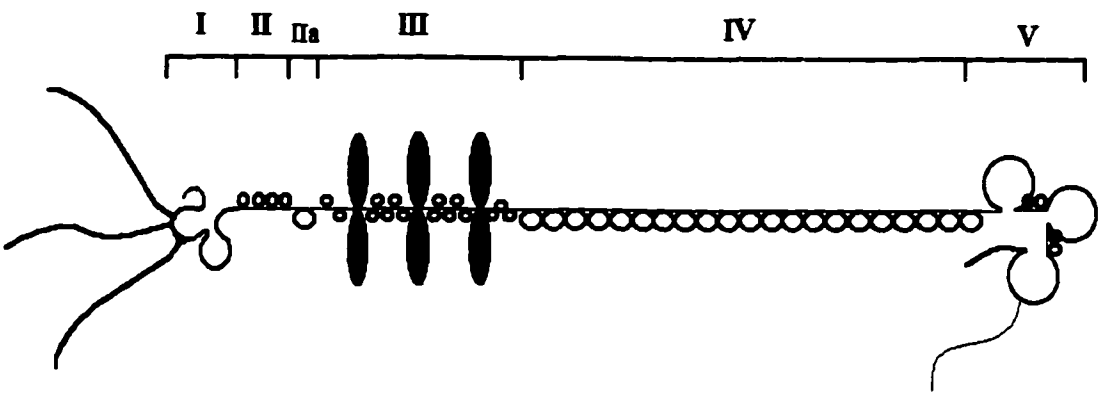

Figure 3 Structure of perlecan.

1991; Rogalski *et al.*, 1993). Domain V contains three repeats with similarity to agrin and laminin α chain G domains that are separated by EGF-like regions which are not found in laminin α chains (Noonan *et al.*, 1991; Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992). The complex molecular organization of perlecan suggests that this mosaic pattern has evolved through duplication of elements to form the functional domains (Cohen *et al.*, 1993). This composite, multidomain gene product appears to be evolutionarily related to molecules involved in functional cellular processes such as nutrient binding/delivery, mitogenesis, and the attachment/detachment of cells, which imply a versatile role for this protein (Iozzo *et al.*, 1997).

Perlecan Regulation and Expression

Perlecan, the mutidomain proteoglycan, is on eof the most complex gene products because of its large size and number of posttranslational modifications (Iozzo, 1994). Two primitive forms of perlecan have been identified in *Caenorabditis elegans* and in *Xenopus laevis* (Rogalski *et al.*, 1993). In *C. elegans*, a number of complex isoforms of this gene product are expressed through alternative splicing. To date, there are three major isoforms in the nematode: a short form lacking the Ig repeat and the C-terminal agrin-like regions, a medium form containing the Ig repeats but lacking the agrin-like region, and a newly identified long form that contains all five domains (Mullen *et al.*, 1999). Human perlecan is encoded by a 14.35-kb mRNA; however, in the mouse, an ~12-kb mRNA encodes for perlecan core protein. The sequence comparison among mouse, human, and nematode reveals the role of alternative splicing among species. Homology between the mouse and human sequences was found to range from 36% to 90%. The mouse perlecan sequence lacks 7 Ig-like motifs in domain IV, corresponding to

the mid-5th to the mid-12th Ig repeats present in the human sequence (Iozzo, 1994). Alternative splicing of the gene within species has been observed in mouse and nematode gene (Noonan and Hassell, 1993). A 45-kDa HSPG-PRM (perlecan-related molecule) was also isolated from embryonic mouse neuroepithelial cells (Nurcombe *et al.*, 1993). The cells have three mRNAs and a small amount of a 12.6-kb transcript, as well as a novel 6.5-kb transcript, and an abundance of 3.5-kb transcripts. All of these transcripts were generated from a single perlecan gene (Joseph *et al.*, 1996). The PRM molecule was also shown to be localized to several different tissues of developing embryos, suggesting that it plays an important role (Joseph *et al.*, 1996). Another study found differently sized transcripts of perlecan domain I by using reverse transcriptase-polymerase chain reaction (RT-PCR) to analyze the mRNA in rodent and human tissues. These differently sized transcripts were further characterized and found to be splice variants of perlecan and to have high affinity for bFGF (Maresh *et al.*, 1996).

Expression of perlecan can be regulated at transcriptional, translational, and/or posttranslational levels. The presence of perlecan during cell differentiation and tissue morphogenesis indicates that this molecule plays crucial roles in embryogenesis. During mouse development, perlecan can be detected early in preimplantation embryos before the formation of the basement membrane (Dziadek and Mitrangras, 1989). Besides being deposited inside the blastocysts, expression of perlecan at the trophectodermal surface of the blastocyst seems to play a role in attachment of the embryo to the uterine lining that may be HS chain dependent (Smith *et al.*, 1997). Perlecan mRNA is low in the morula stage but increases in day-4 blastocysts, attaining maximal expression in day-4.5 attachment-competent blastocysts. Perlecan core protein expression is also attenuated during delayed implantation and appears to respond to estrogen, probably by the

increased translation of preexisting perlecan mRNA (Smith *et al.*, 1997). A systematic study of perlecan expression in embryogenesis has shown that the expression generally correlates with tissue maturation (Handler *et al.*, 1997). Costell *et al.* (1999) have shown what happens when perlecan expression is completely abolished. In homozygous knockout embryos, there was no abnormality observed before embryonic day 10 (E10); however, most of the embryos died between E10 and E12. A few animals survived but died around birth with severe defects in brain and skeleton. All perlecan-null embryos that did not exhibit apparent heart defects continued their intrauterine development but died perinatally. Between E15 and the newborn stage, these animals developed a severe osteochondrodysplasia characterized by dwarfism, cleft palate, short limbs, and a short and abnormally bent vertebral column (Costell *et al.*, 1999). The lack of any defects before E10 is surprising since perlecan is first expressed in two-cell embryos and increases later on. Perhaps other PGs such as agrin and collagen XVIII can substitute for the loss of perlecan function in the early stages of development (Olsen, 1999).

Perlecan synthesis and deposition vary greatly between vascular and avascular tissues. In cardiovascular development, perlecan has been shown to be a potent growth inhibitor for vascular smooth muscle cells (Weiser *et al.*, 1996). Expression of perlecan was observed in early vasculogenesis (at E 10.5), including heart, pericardium, and major blood vessels (Handler *et al.*, 1997). In developing aorta, the tissue matures from a highly replicative to a quiescent differentiated state. As the tissue matures, perlecan expression increases as the replication of smooth muscle cells decreases. Perlecan may have roles in controlling smooth muscle replication by down regulating Oct-1, a transcriptional factor involved in vascular smooth muscle growth (Weiser *et al.*, 1996, 1997).

Perlecan expression is prominent in the endothelial basement membrane of all vascularized organs (*e.g.*, lung, liver, spleen, pancreas, and kidney). It is also expressed at critical sites of vasculogenesis, indicating a role in angiogenesis. Perlecan also plays important roles not only in early steps of blood vessel development but also in maturation and maintenance of a variety of differentiated mesenchymal tissues. In skeletal muscle differentiation, perlecan protein has been shown to localize at high concentration on the myoblast surface, while the mRNA decreases substantially during differentiation. Perhaps perlecan acts as an adhesion molecule for myoblasts and is not required once cells have fully differentiated into myotubes. FGF-2 is a strong inhibitor of skeletal muscle differentiation. The inhibitory actions of FGF-2 on myotube formation may be controlled by lowering perlecan expression to prevent cellular responses (Larrain *et al.*, 1997). Perlecan is essential for myoblast formation and muscle attachment in *C. elegans*. It is a product of muscle and is expressed in individual muscle cells. Disruption of perlecan can lead to paralysis (Moerman *et al.*, 1996). Recent data have also shown that perlecan mediates interaction of skeletal muscle cells to collagen IV through its core protein (Villar *et al.*, 1999).

In the mammalian respiratory system, perlecan has been found to be a major component of alveolar and airway basement membranes from birth through adulthood. It is prominent in pulmonary arteries but weakly expressed in pulmonary veins (Sannes and Wang, 1997).

In intestinal development, the overall distribution of perlecan at all stages of development is similar to that of the other basement membrane components, where it is prominent at subepithelial basement membrane and around muscle cells. However, the pattern of distribution undergoes a change around birth, where the expression of perlecan

in the basement membrane is discontinuous and irregular from the middle to the tip of the villi. Moreover, poorly or completely unsulfated HSs were found in early fetal intestines (Simon-Assmann *et al.*, 1995).

Perlecan is also expressed in matrices of hyaline cartilage, the articular surface, and the growth plate region of developing mouse embryos, particularly during endochondral ossification, but not in area of membranous bone formation. The accumulation is detected in cartilage primordia and is primarily of proliferating chondrocyte origin. Perlecan not only is a marker of chondrogenesis but also strongly potentiates chondrogenic differentiation (SundarRaj *et al.*, 1995; Handler *et al.*, 1997; French *et al.*, 1999). Perlecan in cartilage contains both CS and HS side chains (SundarRaj *et al.*, 1995).

In the central nervous system, perlecan is found only in the choroid plexus of the developing mouse brain (Handler *et al.*, 1997). In addition, homozygous mice with a null mutation in the perlecan gene exhibit defects in the basement membrane separating the brain from the adjacent mesenchyme, with invasion of brain tissue into the overlying ectoderm (Costell *et al.*, 1999). Perlecan expression increases during neuronal differentiation of murine embryonal carcinoma cells and contains mainly CS/DS GAGs with only minor levels of HS (Sekiguchi *et al.*, 1994). The increased expression of perlecan in differentiated cells coincides with an increase in the expression of β-amyloid precursor protein. It has been shown to bind with high affinity to Alzheimer amyloid precusor, suggesting a role for perlecan in Alzheimer's disease (Narindrasorasak *et al.*, 1995).

As a result of its complex molecular structure and widespread distribution, as well as its ability to interact with a variety of matrix components, perlecan seems to play a

crucial role not only in normal biological processes but also in pathophysiological conditions. The intrinsic structural diversity in perlecan contributes to the heterogeneity of the basement membranes and ECMs. Recent data reveal that perlecan may be involved in many diseases, including diseases with multiorgan involvement (*e.g.*, diabetes mellitus) (Makino *et al.*, 1993; Ljubimov *et al.*, 1996; Bollineni *et al.*, 1997), neurologic disease (*e.g.*, Alzheimer's disease) (Snow *et al.*, 1994; Narindrasorasak *et al.*, 1995), cardiovascular disease (*e.g.*, atherosclerosis) (Vijayagopal *et al.*, 1996), liver disease (Roskams *et al.*, 1996), and renal disease (Miner and Sanes, 1996). It also may be involved in rheumatologic disease (*e.g.*, arthritis) (Dodge *et al.*, 1995); hematological disease (Grassel *et al.*, 1995); and, inevitably, cancer (Cohen *et al.*, 1994).

Perlecan and GAG Side Chains

Perlecan was first isolated from the EHS tumor and was characterized as a basement membrane PG with the HS chains attached at the N-terminal region (Paulsson *et al.*, 1987; Noonan *et al.*, 1991). However, in addition to the SGD sequences in domain I, both mouse and human perlecans possess a large number of potential glycosylation sites with over 50 ser-gly dipeptides and ser-gly-x-gly (SG-X-G) tetrapeptides dispersed throughout their protein cores (Noonan *et al.*, 1991; Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992). The serine-glycine sequence is known to be a recognition consensus sequence for xylosyltransferase, which transfers xylose from UDP-xylose to the hydroxyl group of serine residues (Zhang *et al.*, 1995). Some of these sequences are flanked by acidic amino acids and thereby comprise a proposed consensus sequence for GAG attachment sites (Zhang and Esko, 1994; Zhang *et al.*, 1995).

GAGs play both structural and functional roles in biological processes. Interaction of GAGs with other ECM constituents contributes to specific architecture and permeability properties (Wight *et al.*, 1992; San Antonio *et al.*, 1993). GAGs may localize specific GFs and enzymes at their sites of action to facilitate their physiological functions and in some cases prevent their proteolytic degradation (Ruoslahti and Yamaguchi, 1991). GAGs have been shown to regulate protein secretion and gene expression in certain tissues (Busch *et al.*, 1992; Reichsman *et al.*, 1996). GAGs may play a critical role in the pathophysiology of basement membrane-related diseases, including epidermolysis bullosa (EB), diabetes mellitus (DM), atherosclerosis, and metastasis. These activities depend on interactions of the polysaccharides with proteins, mediated by saccharide sequences and expressed at various levels of specificity, selectivity, and molecular organization (Spillmann and Lindahl, 1994). The problem of specificity is highlighted in a series of studies involving members of the FGF family. These proteins all bind to heparin and HS GAG (Basilico and Moscatelli, 1992; Guimond *et al.*, 1993; Friedl *et al.*, 1997; Schulz *et al.*, 1997).

Perlecan was originally characterized as an HS molecule. Recent data from both *in vitro* and *in vivo* studies indicate that perlecan can also exist as a hybrid molecule with lesser amounts of CS/DS chains (Danielson *et al.*, 1992; Couchman *et al.*, 1996). Perlecan bearing only galactosaminoglycan in the absence of HS has also been detected (Hassell *et al.*, 1992; Couchman *et al.*, 1996; Groffen *et al.*, 1996), as well as core protein bearing no GAG, which as described in *C. elegans* (Rogalski *et al.*, 1993). Hassell and his colleagues (1997) have recently reported that all three serine residues in the SGD sequences in domain I can accept both HS and CS side chains but that the cluster of acidic amino acid residues N-terminal to this sequence is the primary determinant

responsible for priming for HS (Dolan *et al.*, 1997). The impact of alternate glycosylation of the perlecan core proteins has not yet been understood. There is a high possibility that substitution with galactosaminoglycan in place of or in addition to HS chains may impact the biological activity.

Molecular Interactions of Perlecan

Interaction with ECM molecules and their receptors. Perlecan has been shown to bind many other basement membrane and ECM components. Many interactions appear to be through the GAG chains. The binding of mouse perlecan to mouse laminin 1 involves the E3 fragment of the C-terminal globular domain of the laminin α1 chain, which contains a heparin-binding site; the binding appears to be HS dependent (Battaglia *et al.*, 1992). Beside laminin 1, perlecan can also bind to laminin 6 and 7 (Aumailley and Krieg, 1996), as well as *Drosophila* laminin (α5β1γ1) (Mayer *et al.*, 1997). Binding to *Drosophila* laminin is also HS dependent (Mayer *et al.*, 1997). The HS substitution on both domain I and domain V can facilitate interaction with the C-terminal globular domain of laminin α chain, and it is possible that these two molecules can form a linear copolymer in the basement membrane (Ettner *et al.*, 1998). The core protein of perlecan has also been shown to be able to bind to laminin, possibly through domain V (Brown *et al.*, 1997), and to collagen IV (Villar *et al.*, 1999). Recombinant domain IV of mouse perlecan has been shown to bind to FN, entactin-1, entactin-2, and laminin-1-entactin-1 complex with high affinity (Hopf *et al.*, 1999). Recombinant domain V of perlecan has also been shown to bind mainly to the G2 domain of entactin with some weaker affinity for the G3 domain (Reinhardt *et al.*, 1993; Brown *et al.*, 1997). It can also bind to fibulin-2 with a much lower affinity than either laminin or entactin (Brown *et al.*, 1997).

In addition to the HS chains, the core protein may interact with other components. Perlecan core protein has been reported to bind to fibronectin (Heremans *et al.*, 1990). The interaction of perlecan has been mapped to the second type III repeat located C-terminal to the gelatin-collagen binding domain and within the N-terminal region of 140 kDa "cell"-binding fragment of fibronectin (FN) (Heremans *et al.*, 1990). Thrombospondin 1, which is localized at the cell surface of endothelial cells, has been found to bind to perlecan in a specific and dose-dependent manner. The interaction is dependent on the HS chain on perlecan, which may organize thrombospondin 1 at the apical surface of vascular endothelial cells (Vischer *et al.*, 1997).

In *Xenopus* muscle cells, perlecan is precisely colocalized with α-dystroglycan, a transmembrane protein that interacts with ECM molecules, as well as the cytoskeleton, and is cluster with acetylcholine esterase receptors. The binding between these two molecules has been shown to be calcium and heparin sensitive (Peng *et al.*, 1998). The interaction probably occurs via the laminin G-like module at the C-terminus of perlecan (Hohenester *et al.*, 1999), which in turn binds to the collagenous domain of acetylcholine esterase through the HS on perlecan. The dystroglycan-perlecan complex thus serves as a cell-surface acceptor for acetylcholine esterase clustering at the neuromuscular junction (Peng *et al.*, 1999).

Another matrix molecule that has been shown to interact with perlecan is a heparin/HS interacting protein (HIP) on the cell surface. HIP functions as an adhesion molecule for several cell lines. The binding is apparently HS dependent (Rohde *et al.*, 1998) although any additional role of the core protein was not investigated. Perlecan has been shown to support endothelial cell attachment and to spread through both β1 and β3 integrins. Although the adhesion seemed to be modulated by GAG chains, the core

protein was clearly involved in mediating adhesion which is partially RGD independent (Hayashi *et al.*, 1992). Recombinant domain III containing an RGDS sequence supports adhesion of an epithelial-like mouse mammary tumor cell line, MMT 060562, and the adhesion was blocked by the RGD containing synthetic peptides (Chakravarti *et al.*, 1995). The interaction may be cell type specific, as well as species specific, since human perlecan does not contain the RGD sequence (Kallunki and Tryggvason, 1992). The C-terminal domain V of perlecan could also promote adhesion of several cell lines, and this could be blocked by antibodies against β1 integrin (Brown *et al.*, 1997). In *C. elegans*, the st549 mutant, a perlecan or UNC-52 knockout was identified by defects in the localization of β-integrin and vinculin in the dense body and M-line of developing body wall muscle cells (Hresko *et al.*, 1994; Rogalski *et al.*, 1995). These data indicate potential direct interactions between perlecan and integrins.

Interaction with GFs/cytokines. The strategic location of perlecan suggests that this gene product is involved in regulating growth of the cells through its ability to capture and store growth factors (Iozzo, 1994). The release of GF/HS complexes via controlled proteolytic processing is a physiologic mechanism that disengages biologically active molecules at the site of remodeling and tumor invasion (Iozzo, 1994; Whitelock *et al.*, 1996).

FGFs are a family of small monomeric proteins (~18 kDa) that regulate a diverse range of physiological processes such as cell proliferation and differentiation during development, homeostasis of adult tissue, as well as pathological processes involving angiogenesis, wound healing, and cancer (Basilico and Moscatelli, 1992). They play multiple roles during development and in adult tissues as paracrine and autocrine growth

and differentiation factors by signaling through transmembrane receptor tyrosine kinases. However, HS is also required for signaling (Basilico and Moscatelli, 1992). At least 18 members have been identified, FGF-1 through FGF-18 (Basilico and Moscatelli, 1992; Ohbayashi *et al.*, 1998; Xu *et al.*, 1999). Like many cytokines, FGFs have a high affinity for heparin and HS; however, unlike other molecules, they require heparin/HS binding in order to activate their receptors (Xu *et al.*, 1999). There are four FGF receptors (FGFRs) which are transmembrane receptor tyrosine kinases and, like other cytokine receptors, have promiscuous binding properties allowing for binding and activation by several FGFs with varying affinities. These properties allow different tissues or cells to have specific responses to various FGFs (Xu *et al.*, 1999). FGFs use a dual receptor system to activate signal transduction pathways (Nugent and Edelman, 1992; Gallagher, 1994) and HS is required for signaling (Basilico and Moscatelli, 1992). FGFs contain sites for binding FGFR and HS, and for nuclear targeting (Spivak-Kroizman *et al.*, 1994; Herr *et al.*, 1997). Strong electrostatic interactions between positively charged side chains in the growth factors to sulfate and carboxyl groups of the saccharides on the HS contribute significantly to the interaction (Thompson *et al.*, 1994). Different FGFs appears to have distinct HS requirements for receptor activation. These reflect different tissue requirements. FGF-1, -2, -4, -5, 7-, -8, and -9 have all been shown to require HS for signaling (Basilico and Moscatelli, 1992), with the HSPG, FGF, and the FGFR forming a complex that is stabilized by the presence of the HS chains. As indicated below, individual FGFs may have distinct binding sequences with HS, enabling regulation of FGF activities during development or disease by changes in HS content in perlecan, or the amount of perlecan.

FGF-2 (basic FGF) is the most extensively studied member of the FGF family. Its mitogenic properties largely depend on the presence of HS (Nugent and Edelman, 1992). Perlecan may have a unique role in bFGF regulation. It can act as a storage site, with regulated release of bFGF to cell surface HSPG or to the FGFR directly. (Aviezer *et al.*, 1994a). FGF-2 is present at low concentrations in tissue but at high levels in aqueous humor of the eye (Tripathi *et al.*, 1992). However, the capacity of perlecan in ECM to sequester and concentrate bFGF and to protect it from proteolytic degradation and increase it's stability and activity has a greater significance than generally appreciated (Sasisekharan *et al.*, 1997). Both basic and acidic FGFs bind strongly to heparin/HS on perlecan (Ishihara *et al.*, 1997), but the FGF binding sites in HS are quite specific. FGF-2 binds with high affinity to single HSD of 12-14 sugars in length (~6-7 disaccharides). A 14-mer HSD containing IdoA,2S-$GlcNSO_3$ repeats (ΔGlcA-$GlcNSO_3$-[IdoA,2S-$GlcNSO_3$]$_5$-IdoA-GlcNAc) binds to bFGF with similar affinity as the intact polysaccharides. This sequence does not contain *O*-sulfates at C-6 or C-3 of the glucosamine residues; the binding appeared to depend on the *N*-sulfate and 2-*O*-sulfate groups (Habuchi *et al.*, 1992; Turnbull *et al.*, 1992; Ishihara, 1994). A sequence of 5 sugars containing only one IdoA,2S (GlcA-$GlcNSO_3$-IdoA-$GlcNSO_3$-IdoA,2S) was shown as the minimal sequence for bFGF binding (Maccarana *et al.*, 1993). X-ray crystallography confirmed this view, although analysis of the site of the interaction in co-crystals of bFGF and a heparin hexasaccharide indicates that the most complementary minimal sequence is $GlcNSO_3$-IdoA,2S-$GlcNSO_3$-IdoA-$GlcNSO_3$ (Faham *et al.*, 1996). Most studies indicate that 10-14 sugar residues with a high content of IdoA,2S and $GlcNSO_3$ are necessary for bFGF activation (Habuchi *et al.*, 1992; Maccarana *et al.*, 1993; Tyrell *et al.*, 1993; Walker *et al.*, 1994). However, an unspecified number of 6-

sulfate groups in association with the IdoA,2S- $GlcNSO_3$ sequence was required for docking the HS/bFGF complex to the FGFR, although they were not required for recognition of bFGF (Guimond *et al.*, 1993; Ishihara, 1994; Pye *et al.*, 1998). A monomeric bFGF/oligosaccharide complex is the minimal functional unit required for the mitogenic stimulation (Pye and Gallagher, 1999). Beside HS, FGF-2 has been shown to bind DS, a large proportion of soluble GAG released into human wound fluid, with a Kd of 2.48 μM. This DS is functionally active and is a potent mediator of bFGF responsiveness during wound repair (Penc *et al.*, 1998).

The interaction and regulation of FGF-1 (acidic FGF) by HS have been less studied than those of FGF-2. FGF-1 shares similarities in requiring a relatively long sulfated sequence (10-14 mers) in a HSD sequence for efficient binding and activation (Guimond *et al.*, 1993; Ishihara, 1994; Ogura *et al.*, 1999). Some evidence indicates that aFGF interacts with a different pattern of sulfate groups from those interacting with bFGF. The specific interaction of FGF-1 to HS, compared to FGF-2, requires higher content of 6-*O*-sulfate groups in GlcNS residues on HS (Ishihara, 1994; Yardenah *et al.*, 1998). The structure of FGF-1-heparin co-crystals suggests a role of 6-*O*-sulfate groups in the interaction (DiGabriele *et al.*, 1998). The minimal FGF-1 binding sequence in HS comprises 5-7 sugars and contains a critical trisulfated IdoA(2-OSO_3)-$GlcNSO_3$(6-OSO_3) disaccharide unit (Kreuger *et al.*, 1999). 6-*O*-Sulfation of the GlcNAc unit seems to play an important role in regulation of various biological and pathological processes, including cell differentiation (Salmivirta *et al.*, 1996; Brickman *et al.*, 1998) and malignant transformation (Jayson *et al.*, 1998; Safaiyan *et al.*, 1998). Moreover, *N*-sulfated HS decasaccharide depleted of FGF-1 binding domains showed dose-dependent and saturatable binding to FGF-2. These indicate distinct, different FGF-1 and FGF-2 binding

sites in HS (Kreuger *et al.*, 1999). Furthermore, recent data from matrix-associated laser desorption ionization mass spectrophotometry (MALDI-MS), chemical cross linking, and crystal structure analysis have shown that FGF-1 and FGF-2 also dimerize differently. FGF-2 forms sequential oligomers, whereas FGF-1 forms only dimers.

Binding of FGF-4 (Kaposi FGF) to heparin/HS is similar to that of FGF-1 and FGF-2 in that the smallest oligosaccharide that binds to this GF and promotes mitogenic activity is a fully *N*-sulfated decasaccharide enriched in 2-*O*- and 6-*O*-sulfated disaccharide units (Guimond *et al.*, 1993; Ishihara, 1994). Heparin-binding secretory transforming gene (hst) encodes FGF-4. Tumors derived from in vivo subcutaneous injection of GH4 hst-transfected rat lactotrope cells strongly expressed FGF-4 and grew more aggressively than non-transfected cells (Shimon *et al.*, 1996). FGF-4 has been shown to have angiogenic activity through the autocrine induction of VEGF secretion by mouse mammary EF43 cells transfected with FGF-4 cDNA (Deroanne *et al.*, 1997).

Keratinocyte GF (FGF-7 or KGF) is synthesized exclusively by fibroblasts. It acts as a paracrine mediator of proliferation and differentiation of a wide variety of epithelial cells. It is cell or matrix associated and is an unusual FGF family member in that its activity is largely directed to epithelial cells. HSPG has been shown to be required for KGF signaling through interaction with both ligand and receptor (Jang *et al.*, 1997; Hsu *et al.*, 1999; LaRochelle *et al.*, 1999). Heparin fragments of octamer and decamer can promote formation of a 2:1 KGF/KGFR complex, while a hexamer cannot (Hsu *et al.*, 1999). FGF-7 has been shown to enhance cell proliferation and trigger the invasion of human prostate epithelial cells significantly through a paracrine and/or autocrine regulatory pathway (Ropiqeut *et al.*, 1999). In addition, FGF-7 binds strongly to HSPGs in subendothelial matrix of blood vessels and to the basement membrane of the papillary

renal cell carcinoma (Friedl *et al.*, 1997). Furthermore, perlecan promotes FGF-7 binding to its receptor in human colon carcinoma cells. Perlecan binds to FGF-7 in an HS-independent manner, thereby raising the possibility that the core protein is involved in modulation of FGF-7 activity (Sharma *et al.*, 1998).

Heparin-binding epidermal growth factor (HB-EGF) is a heparin/HS-binding member of the EGF family. It is produced as a membrane-anchored form (pro-HB-EGF) and processed later to a soluble form (sHB-EGF). HB-EGF activates two EGF receptors, HER1 and HER4, and binds to HSPG (Raab and Klagsbrun, 1997). It has been implicated in a variety of both normal and pathological processes such as blastocyst implantation (Paria *et al.*, 1999), wound healing, tumor growth (Abramovitch *et al.*, 1999), smooth muscle hyperplasia, and atherosclerosis (Raab and Klagsbrun, 1997). In hyperplastic skin, expression of HB-EGF was strongly induced in all epidermal layers; however, expression is undetectable in normal skin (Rundhaug *et al.*, 1997). HB-EGF mRNA expression was increased 2-5 fold in human glioma cell lines; TGF-α, bFGF, or HB-EGF itself rapidly induced the mRNA expression (Mishima *et al.*, 1998). Furthermore, HB-EGF has been shown to be an effector of Jun-1-induced oncogenic transformation (Fu *et al.*, 1999).

Hepatocyte growth factor/scatter factor (HGF/SF) is an HS-binding GF that stimulates cell proliferation, motility, and morphogenesis of epithelial cells. It is produced by stromal fibroblasts (Rahmoune *et al.*, 1998) and activates cells by binding to its receptor, the c-met tyrosine kinase, through oligomerization of peptides in an HS-dependent manner (Sakata *et al.*, 1997). In addition, it also binds to DS (Lyon *et al.*, 1998; Deakin and Lyon, 1999) with a Kd of 19.7 nM, 10-100 fold weaker than that of HS. The smallest DS oligosaccharide sufficient for high-affinity binding is an

octasaccharide comprising unsulfated iduronate residues in combination with 4-*O*-sulfated *N*-acetylgalactosamine. The *N*-sulfation of hexosamines and 2-*O*-sulfation of iduronates are not absolutely required for binding (Lyon *et al.*, 1998). Interestingly, the activity of HGF/SF is not only dependent upon the presence of GAGs but requires an intact PG structure to activate the receptor (Deakin and Lyon, 1999). No specific evidence has shown the direct interaction of perlecan with HGF/SF. However, perlecan may play important roles in promoting HGF/SF activity since it can be expressed as a hybrid HS and CS/DS molecule.

Platelet-derived growth factor (PDGF) is a potent mitogen for several cell types and also acts as a chemoattractant during embryogenesis and wound healing (Heldin *et al.*, 1993). Perlecan has been shown to bind to both isoforms of PDGF (PDGF-AA and -BB) via its protein core. PDGF-AA binds to domain III, whereas PDGF-BB binds to domains I, III, IV, and V. These interactions of PDGFs with perlecan are unique since most interactions with GF binding reported to date involve the binding to the GAG chains of perlecan. Perlecan in the basement membrane may aid in storage of PDGF, which, when released, may play a pivotal role in cell-matrix interaction (Gohring *et al.*, 1998).

In addition to binding to a wide variety of ECM proteins, perlecan also binds to TGF-β (Iozzo *et al.*, 1997) and IFN-γ (Lortat-Jacob *et al.*, 1991), and is transcriptionally induced by these cytokines. Binding to IFN-γ is time and concentration dependent and is accomplished specifically via HS chains (Lortat-Jacob *et al.*, 1991; Sharma and Iozzo, 1998). TGF-β induced a 2- to 3-fold increase in perlecan mRNA and protein core level in human skin fibroblasts (Iozzo *et al.*, 1997). IFN-γ, a cytokine with known antiproliferative and antitumor activity, rapidly and effectively blocked perlecan gene expression with concurrent growth suppression of colon carcinomas (Sharma and Iozzo,

1998). This indicates that perlecan can be transcriptionally regulated by different mechanisms. Perlecan has also been shown to bind to transthyretin, one of the two specific proteins involved in thyroid hormone transportation in plasma. In this case, perlecan contained both CS (~20%) and HS (~80%) chains; the interaction with transthyretin appears to involve both the core protein and the GAG chains (Smeland *et al.*, 1997).

PGs and Neoplasia

During the development of primary tumors or the establishment of metastatic foci, there is a continuous remodeling of the ECM characterized by various degrees of biosynthesis and degradation. This results from a direct and specific interaction of tumor and host cells via three distinct but often interrelated mechanisms: (a) direct production of ECM and cytokines by tumor cells, (b) abnormal degradation of connective tissue components and, (c) neoplastic induction of ECM biosynthesis by host mesenchymal cells (Liotta *et al.*, 1983; Liotta, 1986). Tumor cells may condition their own environment by triggering changes in tumor stroma toward a permissive milieu that facilitates growth and invasion, or the stroma could represent an attempt by the host to wall off tumor cells, thereby exerting antagonistic biologic forces (Iozzo, 1995). Many studies have shown that neoplastic transformation dramatically alters PG synthesis in both the tumor and the surrounding tissues. These quantitative and qualitative alterations are believed to promote tumorigenic growth by (a) heightened tissue hydration, thereby favoring neoplastic cell movement, and invasion; (b) establishment of GF-enriched milieu conducive to tumor growth; and (c) enhanced neovascularization. These are all typically observed in invasive carcinomas (Esko *et al.*, 1988; Iozzo, 1988; Iozzo and Cohen, 1993; Hunzelmann *et al.*,

1995). Both transformed cells and normal cells can produce their own ECMs which may be different in quantity and quality. Different types of tumors may produce distinct types of GAGs, which could be responsible for differences in their proliferative and/or invasive properties. The qualitative alterations in PG structure that appear to be directly linked to transformation are the expression of different types of GAGs or a decrease in degree of sulfation (Iozzo, 1988). Previously, it has been demonstrated that some tumors produce higher amounts of CSPG (Iozzo *et al.*, 1981; Oguro *et al.*, 1991), whereas others produce HSPG predominantly (Bouziges *et al.*, 1990; Nakanishi *et al.*, 1992; Tsara *et al.*, 1995). In the stroma surrounding basal cell carcinomas, the deposition of biglycan and, to a lesser degree, of decorin, was increased when compared to normal human dermis (Hunzelmann *et al.*, 1995). The connective tissue stroma of human colon carcinoma has been shown to be rich in CS (Adany *et al.*, 1990). In B-16 melanoma cells, the secretion of a factor by tumor cells that stimulates synthesis of fibroblast collagenase was dependent on the binding of these cells to HSPG in the matrix. This binding was inhibited by heparin/HS and partially by DS (Biswas, 1988). In HT168 human colon carcinomas and 3LL-HH liver cells (Timar *et al.*, 1992, 1995), the cells with a high HS/CS ratio were more likely to have a highly metastatic potential. HS and CS PGs frequently have opposite biological functions in cell-matrix adhesion, as well as in the regulation of cell differentiation and proliferation. Differences in PG structure of the tumor cells could be, at least in part, responsible for differences in their proliferation and invasion properties.

Besides the type of GAGs that are expressed differently in different types of tumors, there can be some structural differences within the same type of GAG. HS species expressed by different cell and tissue type differ in their structural and functional properties. HS from highly metastatic tumors with more highly organized basement

membrane exhibited a higher degree of overall sulfation in the GAG chains (Nakanishi *et al.*, 1992). Disaccharide analysis of three tumor-derived HSs from Lewis lung carcinomas showed that the degree of sulfation of the HS correlated with the degree of morphological organization of tumor basement membranes. This was due to an increased amount of the repeating disaccharides having 6-*O*-sulfated glucosamine residues (Nakanishi *et al.*, 1992). In human colon carcinomas, the HS was compared from adenoma and carcinoma sources. The chain lengths of the HS from both sources were the same (~20 kDa), but the degree and pattern of sulfation were strikingly different. The iduronic 2-*O*-sulfate content of the carcinoma-derived HS was 33% decreased, and the overall level of *N*-sulfation was reduced by 20%. The level of 6-*O*-sulfation was increased by 24%. This 6-*O*-sulfation was mainly located in the mixed sequences of alternating *N*-sulfated and *N*-acetylated disaccharides (Jayson *et al.*, 1998).

Compositional analysis of disaccharides from the contiguous *N*-sulfated regions of HS from CaCo-2 colon carcinoma showed a greater proportion of 2-*O*-sulfated iduronic acid units and a smaller amount of 6-*O*-sulfated glucosamine units in differentiated than in undifferentiated cells. By contrast, the chain length, overall degree of sulfation, and size of distribution of the *N*-acetylated regions were similar, regardless of the differentiation status of the cells (Salmivirta *et al.*, 1998). Another study of HT 29 human colon carcinomas has shown that the differentiated cells incorporated twice as much sulfate as the undifferentiated cells. This difference was found to affect primarily 6-*O*-sulfation. The chain length was larger and more homogeneous in size than that produced by the undifferentiated HT29 cells (Molist *et al.*, 1997, 1998).

Different types of GAG and various degrees of sulfation may influence growth patterns (Nagasawa *et al.*, 1993; Delehedde *et al.*, 1996). For example, GAGs containing

iduronic acid accelerate the growth of Twa sarcoma cells during both fast and slow growth, whereas GAGs containing glucuronic acid inhibit the growth of these cells during fast growth but accelerate it during slow growth (Nagasawa *et al.*, 1993). The reason for this may come from the different binding affinities of GAGs for various cytokines and GFs. FGFs, for example, have been found to be involved in the process of tumorigenesis. FGF-1 and -2 are involved in epithelial malignancy (Arbeit *et al.*, 1996). FGF-3 (int-2), -4 (Kaposi's FGF or K-FGF), -5, -6, and -8 were identified as products of oncogenes (Basilico and Moscatelli, 1992; Aviezer *et al.*, 1994b); FGF-5 and -6 have also been found to be expressed in Karposi's sarcoma (Li *et al.*, 1993). FGF-7 (keratinocyte GF or KGF) was mitogenically active only on epithelial cells (Rubin *et al.*, 1995), and transfection of human FGF-9 cDNA into BALB/c mice induced cellular transformation (Ishihara *et al.*, 1997). The composition and sequence of HS isolated from human colon carcinoma differ in a defined and specific manner. Adenoma HS has been shown to bind to bFGF with 10-fold higher affinity than that of carcinoma HS (Jayson *et al.*, 1999).

Perlecan and Neoplasia

Perlecan mRNA has been shown to be markedly increased in F9 embryonal carcinoma cells (Chakravati *et al.*, 1993), and in metastatic melanomas compared to normal tissue. The change in metastatic melanoma was upregulated in vitro within 10 min of neurotrophin stimulation and correlated with a vast deposition of perlecan protein core in the pericellular matrix (Cohen *et al.*, 1994). Purified perlecan has been shown to enhance invasiveness of human melanoma cells (Marchetti *et al.*, 1993), and contact with basement membrane perlecan augments the growth of transformed cells but suppresses that of normal endothelial cells (Imamura *et al.*, 1991). This suggests that perlecan may

play a pivotal role in the initial onset of invasion. ACC3, adenoid cystic carcinoma cells of human salivary gland, have been shown to synthesize, secrete, and then degrade perlecan. These processes were strictly regulated by the growth of the cells (Kimura *et al.*, 1999). Cholangiocarcinomas, as well as hepatocellular carcinomas, showed an altered pattern of HSPGs compared to normal liver parenchyma. Perlecan expression was increased in the vessels of the stroma, suggesting a role in tumor angiogensis (Roskams *et al.*, 1998). Expression and assembly of perlecan in the basement membrane were variably lost in invasive squamous cell carcinomas and adenocarcinomas in the lung. However, occasionally, the stromal tissues of these carcinomas were strongly stained for perlecan. This could be a mechanism for the tumor cells to invade through the basement membrane (Nackaerts *et al.*, 1997). In human breast carcinomas (Nerlich *et al.*, 1997, 1998) and larynx carcinomas (Nerlich *et al.*, 1998), the mRNA for perlecan was increased but protein levels were not, possibly due to an increase in protease activity. Perlecan, in conjunction with bFGF was capable of inducing mitogenesis and angiogensis (Aviezer *et al.*, 1994a). High levels of perlecan deposition were present in newly vascularized stroma of breast, colon, and prostate (Iozzo, 1994), as well as liver (Roskams *et al.*, 1998). Blocking perlecan expression by stable transfection of human colon carcinoma cells with perlecan antisense cDNA leads to the suppression of tumor growth and neovascularization, as well as cellular invasion, mainly through lack of FGF-7 activity (Sharma *et al.*, 1998). Several proteases such as plasmin, heparanases, and the matrix metalloproteinases (MMPs), stromolysin (MMP-3), and collagenase-3 (MMP-13) have been shown to degrade perlecan and release significant levels of bFGF into the medium (Whitelock *et al.*, 1996).

The strategic location of perlecan at the interfaces of tumor invasion, coupled with its ability to store GFs in the basement membrane, suggest that it may be directly involved in the modulation of cell-surface events in the multistep process of invasion (Cohen *et al.*, 1994; Aviezer *et al.*, 1997). While its core protein serves as a ligand for integrin receptor binding (Hayashi *et al.*, 1992; Chakravarti *et al.*, 1995), its GAGs promote binding of bFGF to its receptorand are a potent inducer mitogenic and angiogenic activities (Aviezer *et al.*, 1994b). This evidence supports the notion that perlecan is directly involved in promoting the growth and invasion of tumor cells. However, there is recent evidence indicating that perlecan may inhibit the growth and invasiveness of fibrosarcoma cells in a bFGF-independent pathway. Mathiak and his colleagues (1997) have generated stable overexpression of an antisense perlecan cDNA in the HT-1080 human fibrosarcoma cell line, which leads to a reduced level of perlecan synthesis. The transfected cells grew faster, formed larger colonies in semisolid agar, and induced faster formation of subcutaneous tumors in nude mice than the wild type cells. This raises the possibility that perlecan may prevent the infiltration of mesenchymal neoplasm.

Taken together, these contradictory findings raise important questions about the expression and role of perlecan in modulating tumor growth and progression; for example, what is the precise role of perlecan in tumor cell growth, and by which mechanisms does perlecan promote or suppress tumor growth? Perlecan may play different roles in different types of tumors. The importance of an intact basement membrane, controlled deposition of perlecan in the pericellular matrix (which regulates the cellular stimulation by FGFs), and maintenance of normal HS chain composition (in particular, sulfation content) may be critical to the containment and growth control of a

variety of benign and malignant tumors. There is an apparent need for further research studies; therefore, to determining the expression and role of perlecan in tumor formation and progression is of considerable merit.

These studies focus on investigating the source of perlecan secretion, the expression of GAGs on perlecan, and the role of these GAGs on FGF binding. The recent advent of improved techniques, combined with specific immunological reagents, now makes detailed analysis of these questions feasible. New and unambiguous answers to these questions will serve as potential indicators for novel cancer treatment and prevention in the future.

STRUCTURAL CHANGES IN PERLECAN ON EPIDERMAL TRANSFORMATION DO NOT INFLUENCE HEPARIN-BINDING GROWTH FACTOR AFFINITY*

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Abstract

Perlecan, a PG of basement membrane and ECMs, has important roles in both normal biological and pathological processes. As a result of its ability to store and protect GFs, perlecan may have crucial roles in the process of tumor cell growth and invasion. Since the biological functions of different types of GAG vary depending on their cellular origin and their structural modifications, we analyzed the expression and biological functions of perlecan produced by an epidermal cell line (JB6) and its transformed counterpart (RT101). Expression of perlecan in tumorigenic cells was significantly increased at both mRNA and protein levels. JB6 perlecan was exclusively substituted with HS, whereas that of RT101 contained some additional CS. Detailed structural analysis of the HS chains extracted from perlecan of both cell types revealed that their overall sulfation and chain length were similar (~60 kDa) but that the HS chains of tumor-cell-derived perlecan were less sulfated and had larger unmodified regions. This resulted from reduced 2-*O*- and 6-*O*-sulfation and increased unsulfated disaccharides. HS of RT101- and JB6-derived perlecan bound FGF-1, -2, -4, and -7 and HB-EGF with similar affinity. Therefore, tumor-derived perlecan may support the angiogenic responses seen *in vivo* and may be a key player in tumorigenesis.

Introduction

During the development of primary tumors or the establishment of metastatic foci, there is a continuous remodeling of the ECM, which results from interactions between tumor and host cells (Iozzo, 1995). Many studies have shown that neoplastic transformation dramatically alters PG synthesis in both tumor and the surrounding tissues. These quantitative and qualitative alterations are believed to promote tumorigenic

growth and invasion (Iozzo, 1988; Iozzo and Cohen, 1993; Iozzo, 1995). The alterations in PG structure that appear to be directly linked to transformation can occur by expression of different types of GAG[1] chains or by an alteration in degree of sulfation (Timar *et al.*, 1992; Jayson *et al.*, 1998). Previously, it has been demonstrated that some tumors produce increased amounts of CSPG (Iozzo *et al.*, 1981; Adany *et al.*, 1990), whereas some produce HSPG predominantly (Timar *et al.*, 1992; Roskams *et al.*, 1998). Differences in PG structure in tumor cells could be responsible for differences in their proliferation and invasion properties. Different types of GAG and various degrees of sulfation may affect growth, partly because of the different binding affinities of GAGs to various cytokines and GFs (Nakanishi *et al.*, 1992).

Perlecan mRNA and protein deposition have been shown to be markedly increased in some tumor cells as compared to normal tissue (Cohen *et al.*, 1994; Nerlich *et al.*, 1998). This suggests that perlecan may play a pivotal role in invasion and/or tumor angiogenesis. High levels of perlecan deposition are present in newly vascularized stroma of some tumors, suggesting its role in tumor angiogenesis (Iozzo, 1995, 1998). In addition, blocking perlecan expression by stable transfection of human colon carcinoma cells with perlecan antisense cDNA resulted in suppression of tumor growth and neovascularization, and cellular invasion, mainly through the loss of FGF-7 activation (Sharma *et al.*, 1998). Perlecan core protein has been shown to serve as a ligand for integrin receptor binding (Chakravarti *et al.*, 1995), whereas the GAGs promote binding of bFGF (FGF-2) to its receptor, and induce mitogenic and angiogenic activities (Aviezer *et al.*, 1994; Whitelock *et al.*, 1996). This evidence supports the notion that perlecan is directly involved in promoting the growth and invasion of tumor cells. However, there is also a recent report indicating that perlecan may inhibit the growth and invasiveness of

fibrosarcoma cells through an FGF-2 independent pathway (Mathiak *et al.*, 1997). This raises the possibility that perlecan may prevent the infiltration of mesenchymal neoplasm.

Perlecan is predominantly expressed as an HSPG; however, in some circumstances, it can be expressed as a chondroitin/DSd or a hybrid molecule (Kokenyesi and Silbert, 1995; Couchman *et al.*, 1996; Dolan *et al.*, 1997). Furthermore, GAGs (HS in particular) can be composed of disaccharide subunits with sulfation patterns. These specific saccharide sequences contain information that determines the biological functions of PG (Lindahl *et al.*, 1998). For example, during neural development, the structure of HS chains on perlecan-related HSPG is altered, resulting in change of their affinities for aFGF (FGF-1) and FGF-2, GFs involved in developmental regulation (Nurcombe *et al.*, 1993; Joseph *et al.*, 1996; Brickman *et al.*, 1998). The structural and functional differences of perlecan in tumorigenic and nontumorigenic cells have not been examined conclusively. Therefore, we hypothesized that the GAGs of tumor-cell-derived perlecan are structurally distinct from those of the wild type. These differences may affect the functional roles of perlecan in tumor growth and contribute to the process of tumor formation and progression. To test this hypothesis, we used JB6 Cl 41.5a cells, a mouse epidermal cell model of tumor promotion. This cell line can be irreversibly transformed to an anchorage-independent and tumorigenic phenotype (RT101 cells) by treating of the cells with phorbol ester, 12-*O*-tetradecyanoylphorbol-13-acetate (TPA) (De Benedetti *et al.*, 1991; Singh *et al.*, 1995). Furthermore, when injected into immunocompromised rats, tumor growth and substantial angiogenic responses ensue.[2] We analyzed the expression of perlecan extracted from both cell types and found that the expression of perlecan core protein and fine structure of the GAG chains from these cell

types are different. However, there was no loss of high-affinity GF binding, indicating a potential role for perlecan to promote the tumor invasion process.

Experimental Procedures

Cell culture and metabolic labeling. JB6 Cl 41.5a cells, a mouse epidermal cell line, and its transformed counterpart (RT101) (De Benedetti *et al.*, 1991; Singh *et al.*, 1995) were grown in Eagle's minimal essential medium (EMEM) supplemented with 5% heat-inactivated fetal bovine serum and 2 mM L-glutamine. Cell were subcultured at 70-80% confluence in T-75 flasks and incubated at 37°C with 5% CO_2. Mycoplasma testing of cells by DNA fluorochrome staining was conducted monthly. Both cell lines were kindly provided by Dr. Nancy H. Colburn (National Cancer Institute, National Institute of Health) via Dr. Pi-Ling Chang and Dr. Charles Prince (University of Alabama at Birmingham). For labeling, cells were cultured in the presence of 50 μCi/ml of $^{35}SO_4$ (NEN™ Life Science, Boston, MA) and/or 30 μCi/ml D-[6-^{3}H] glucosamine-HCl (American Radiolabeled Chemicals, St. Louis, MO).

Ribonuclease protection assay. A fragment of 174 bp cDNA of mouse perlecan domain V was made by polymerase chain reaction (PCR) using a domain IV/V cDNA as a template. The product encompassed residues 10266-10439 of perlecan (Tapanadechopone *et al.*, 1999). The PCR product was subcloned into pBluescript sk(+) and then linearized by Sal I that cuts immediately downstream of the sequence. The linearized template DNA was subjected to phenol/chloroform extraction and ethanol precipitation. The DNA was then dissolved in RNase-free water.

The linearized DNA template was transcribed by T7 RNA polymerase by using MAXIscript™ in vitro transcription kits (Ambion, Austin, TX). The reaction was performed at 37°C for 30-60 min in the presence of 10 mM each of ATP, CTP, and GTP, as well as, 12.5 μM of [α-^{32}P]UTP and ribonuclease inhibitor. The DNA template was removed by incubating the reaction with 2 U of RNase-free DNase I at 37°C for 15 min. The RNA was precipitated by ammonium acetate/ethanol and resuspended in RNase-free water.

Total RNA was extracted from confluent JB6 and RT101 cells grown in T-75 flasks. Cells were lysed by addition of 3 ml of RNAzol™ B (Leedo Medical Laboratories, Houston, TX) and subjected to chloroform extraction. Cell debris was removed and high molecular weight (MW) nucleic acid was precipitated by isopropanol. The RNA pellets were dissolved in RNase-free water. For each sample, 5 μl of ^{32}P-labeled antisense probe (~10^4-10^5 cpm/μl) was mixed with an equal amount of total RNA from both JB6 and RT101 samples and incubated at 37°C overnight for hybridization. The mixtures were subsequently added to ribonuclease digestion buffer containing 5 U RNase A and 1,000 U RNase T1 and incubated at 37°C for 30 min. In each reaction 20 μl of 10% sodium dodecyl sulfate (SDS) and 10 μl Proteinase K (20mg/ml) were added and incubated for another 30 min. Hybridized RNAs were then extracted from the aqueous phase by isopropanol precipitation. The RNA pellets were dissolved in RNA loading buffer, incubated at 90°C for 3 min, and subjected to electrophoresis on 5% denaturing polyacrylamide gel. The gel was dried and subjected to autoradiography for quantitation.

RT-PCR analysis of perlecan mRNA expression. cDNAs were synthesized from 1 μg of each total RNA from both JB6 and RT101 cells using an Access RT-PCR system

(Promega, Madison, WI). Oligonucleotide primers for a C-terminal fragment of domain V perlecan (D FV; Tapanadechopone *et al.*, 1999) were used for amplification. The primers were 5'-GAGAATTCGACAGCACAGCCGAT-3' (forward) and 5'-GCGCGC GTCGACTACTCGACACTGTC-3' (reverse). The reactions were performed using 1 μM of each primer, 0.2 mM dNTP mix, 1 mM $MgSO_4$, 0.1 U AMV reverse transcriptase, 0.1 U Tfl DNA polymerase, and 1 μg of each total RNA in AMV/Tfl reaction buffer. For first strand cDNA synthesis, incubation was carried out at 48°C for 45 min, followed by 94°C for 2 min. For second strand cDNA synthesis and PCR amplification, 40 cycles of 94°C for 30 s, 60°C for 1 min, 68°C for 2 min were performed. A final extension of 68°C for 7 min was performed at the end of 40 cycles. β-Actin internal standard (Ambion, Austin, TX) was used as a control. The RT-PCR products were analyzed on 1% agarose gel. The perlecan primers were omitted for negative controls.

Extraction of PG from conditioned medium of JB6 and RT101 cells. Conditioned media from JB6 and RT101 cultures were centrifuged at 1,000 rpm for 15 min to remove cell debris and were run through 3- x 1-cm DEAE-Sephacel (Pharmacia Biotech) columns equilibrated with 4 M Urea, 0.2 M NaCl, 10 mM NEM, 20 mM EDTA, and 0.05 M Tris-HCl, pH 8.0, containing 0.2 μM phenylmethylsulfonyl fluoride (PMSF) and 1% Tween. The columns were washed with the above buffer but at pH 4.0 and containing 50 mM sodium acetate instead of Tris-HCl and were washed again with the same buffer but in the absence of urea. PGs were subsequently eluted with 4 M guanidine-HCl, pH 4.0, containing 50 mM sodium acetate and 0.1% Tween. Fractions of 1 ml were collected and UV absorbency at 280 nm was used to detect PGs. Corresponding fractions were pooled, and PGs were precipitated by adding 4 volumes of ice-cold ethanol and were incubated at

-20°C for at least 3 h. The precipitated pellets were dried and resuspended in appropriate buffers.

Immunoprecipitation of perlecan from radiolabeled cultured cells. Protein A-sepharose beads were added to radiolabeled (^{35}S and/or ^{3}H) conditioned media collected from both JB6 and RT101 cells and incubated for 1 h at RT to remove nonspecific binding components. After bead sedimentation, supernatants were transferred to new tubes and incubated with rabbit polyclonal antibody against perlecan core protein, EY#10 (gift from Dr. J.R. Hassell), that was first bound to further batches of protein A-sepharose beads. After incubation, extensive washing with 0.1% Tween in Tris-buffered saline (TBS) was performed until very low radioactivity background was detected in washes. Perlecan was eluted from the beads with 4 M Guanidine HCl and subjected to ethanol precipitation.

Gradient anion-exchange chromatography. Intact or chondroitinase ABC-treated [^{35}S]-radiolabeled perlecan samples from JB6 or RT101 cells were applied to a 3- x 1-cm column of DEAE-Sephacel as described above, except the labeled perlecan was eluted with a gradient of 0.2-1.5 M NaCl, 50 mM sodium acetate, and 10 mM NEM, pH 4.0. Fractions of 1 ml were collected and analyzed for conductivity and radiolabel content (Couchman *et al.*, 1985).

Gel electrophoresis and immunoblotting. Precipitated PG pellets from the previous step were dissolved in heparinase buffer (0.1 M sodium acetate and 0.1 mM calcium acetate, pH 7.0). Aliquots of 15 µl, with or without digestion by 0.5-1 mU heparinase III

(heparitinase I, EC 4.2.2.8) and/or 1-2 mU chondroitinase ABC (chondroitinase ABC lyase, EC 4.2.2.4, Seikagaku American Inc., Ijamsville, MD) at 37°C for 3-5 h, were resolved by 3-15% gradient SDS- polyacrylamide gel electrophoresis (PAGE), with subsequent transfer to nitrocellulose for immunoblotting as previously described (Tapanadechopone *et al.*, 1999). Primary rat monoclonal antibody H5L5 recognizing domain III of mouse perlecan and a rabbit polyclonal antibody EY#10 (Tapanadechopone *et al.*, 1999) recognizing all perlecan core protein domains were used. Alkaline phosphatase or horseradish peroxidase conjugates of goat anti-rat IgG or goat anti-rabbit IgG, diluted 1:3,000 in the same buffers as the primary antibodies, were used as secondary antibodies for 1 h at RT. Further extensive washing of membranes was followed by color development (BioRad) or ECL Western blotting analysis system (Amersham Lifescience) according to the manufacturers' instructions.

Slot blot analysis of perlecan. PGs were isolated from the conditioned media as described above. In this case, the monolayers were trypsinized and cell numbers were counted after media collection to normalize perlecan synthesis to cell number. Total PG concentrations (mg/ml) were calculated by measuring absorption at 280/260 nm (mg/ml of protein = 1.5 x A280-0.75 x A260). Aliquots of total PG produced by the same number of cells (or in other experiments, the same concentration of total PG regardless of the cell number) were subjected to enzymatic treatment with combined heparinase III and chondroitinase ABC to remove the GAGs. Two-fold serial dilutions of the samples were applied to nitrocellulose membrane (BioRad Laboratories) assembled in BioRad slot blot apparatus. Membranes were blocked in 5% dried milk in Dulbecco's phosphate-buffered saline (PBS) for 30-60 min. Hybridoma supernatant H5L5 recognizing mouse perlecan

domain III, diluted 1:8 in PBS containing 1% dried milk, 0.1% bovine serum albumin (BSA) and 0.1% Tween 20, was incubated to the membranes and processed as described above. The blots were developed by chemiluminescence detection and quantified by densitometer.

Perlecan GAG analysis. Purified perlecan immunoprecipitates were subjected to alkaline elimination as previously described (Couchman *et al.*, 1985). [^{35}S]-radiolabeled GAGs from both JB6 and RT101 perlecan were ethanol precipitated and then resuspended in heparinase or chondroitinase buffers (Tapanadechopone *et al.*, 1999). For size analysis, released GAGs were analyzed by high-performance liquid chromatography (HPLC) on TSK 4000 (TosoHass GmbH, Montgomeryville, PA) equilibrated with 4 M guanidine HCl, 50 mM Tris, and 0.5% Triton X-100, pH 6.0, at a flow rate of 0.5 ml/min. The column was calibrated with heparin, HS, and CS polysaccharides of known molecular weight. Aliquots of 0.5 ml were taken for scintillation counting.

To verify the type of GAGs, either separate or combined heparinase I and III, or chondroitinase ABC were added and incubated at 37°C to cleave HS and CS/DS GAGs, respectively. After 6 h of incubation, additional enzyme aliquots were added and incubated overnight to ensure completion. Both combined heparinase- and chondroitinase-treated radiolabeled products were resolved on Superdex® Peptide HR 10/30 HPLC column (Amersham Pharmacia Biotech) equilibrated with 0.5 M pyridine acetate, pH 5.0, at a flow rate of 0.5 ml/min. Aliquots of 0.25 ml were taken for radiolabel content measurement. Samples without treatment were run as controls. Fractions of intact HS after chondroitinase ABC treatment and disaccharide fractions

after combined heparinase treatment were pooled, lyophilized, and subjected to further analyses.

To analyze the size of low and high sulfated regions of perlecan HS from the two cell lines, HS samples from the previous steps were subjected to separate heparinase I or III treatments and were applied to a Superdex® Peptide HR 10/30 HPLC column. Fractions of 0.5 ml were collected and counted for radioactivity in order to determine the profiles of heparitinase-I- and -III-resistant regions (low and high sulfated regions respectively).

Disaccharides derived from combined heparinase I and III treatments were analyzed by strong anion-exchange (SAX)-HPLC. Lyophilized [^{3}H]-HS disaccharides of both JB6 and RT101 perlecan were resuspended separately in 200 µl of 28 mM KH_2PO_4, pH 4.6. The samples were analyzed on a 0.4 x 25 cm Partisil SAX column (Whatman Inc., Clifton, NJ) using step gradients with increasing concentrations of 28 mM, 150 mM, and 400 mM KH_2PO_4 buffers (Bienkowski and Conrad, 1985; Fedarko and Conrad, 1986). The samples were run at 1 ml/min and fractions of 1 ml were collected for radiolabel measurement. The elution peaks were identified by comparison of their retention times with those of heparin disaccharide standards (Sigma, St. Louis, MO).

Affinity co-electrophoresis analysis of HS-GF interactions. The affinities of the interactions between GFs and HS from both JB6 and RT101 perlecan were determined by affinity co-electrophoresis (Lim *et al.*, 1991). [^{35}S]-radiolabeled perlecan GAG samples dissolved in loading buffer (5% sucrose, 0.05% xylene cyanal) were applied equally into small slots in a 1% (w/v) sea plaque agarose gel (FMC Bioproducts, Rockland, ME) and electrophoresed through precast 42-mm longitudinal lanes containing different

concentrations (2-folded serial dilutions ranging from 0-200 nM) of different growth factors; rh (recombinant human) FGF-1, -2 (Promega, Madison, WI), rh FGF-4 (R&D Systems, Inc., Minneapolis, MN), rh FGF-7 (KGF, Promega), and rh HB-EGF (R&D Systems, Inc.). The gels were prepared using 50 mM MOPSO, 125 mM sodium acetate, and 0.5% CHAPS, pH 7.0, as previously described (San Antonio *et al.*, 1991; Jayson *et al.*, 1999). The electrophoresis running buffer was identical to the buffer used to prepare gels, except CHAPS was excluded. Electrophoresis was performed at 50 V (constant voltage) for 2 h in a cooling system. Gels were dried under vacuum and subjected to autoradiography. The results of the mobility shift in this study were used to calculate the retardation coefficient (R), which is found by measuring the distance between migration of the HS in the presence of ligand (M) and the migration of the free HS (N) (R=M/N; 31). The binding affinities (Kd; slope $\approx -1/Kd$) were calculated from Scatchard plots of $R/[GF]_{free}$ against R (Lee and Lander, 1991; Lim *et al.*, 1991) using PSI-Plot version 5.5 (Poly Software International, Sandy, UT). The R values were determined as average from at least two repeated experiments.

Results

Increased expression of perlecan in tumorigenic cells. The expression of perlecan mRNA by transformed RT101 cells compared to that of wild-type JB6 cells was first examined by ribonuclease protection assay (RPA) (Figure 1A). The expected fragments (~173 bp, arrowheads) of perlecan transcript were detected in both JB6 (lanes 1) and RT101 (lanes 2) samples. Quantitation of the expression level by densitometric analysis indicated up to 5- to 7-fold increase in perlecan expression in tumorigenic cells. This was confirmed by RT-PCR analysis (Figure 1B). Increased message was detected in RT101

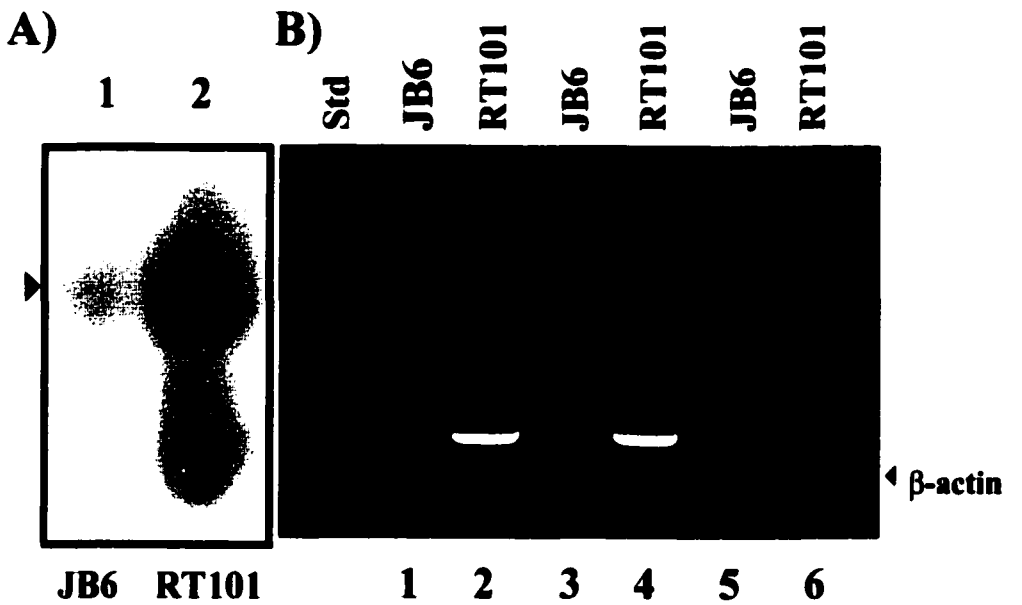

Figure 1. Increased mRNA expression of perlecan in tumorigenic cells. (A) Ribonuclease protection assay of JB6 and RT101 samples and (B) RT-PCR were analyzed for perlecan mRNA levels. (A) Perlecan mRNAs from total RNA preparation of JB6 (lane 1) and RT101 (lane 2) cells were separated on 5% denaturing polyacrylamide gel after hybridization with ^{32}P-labeled probe and ribonuclease treatment. (B) Amplification products of 550 bp were detected by RT-PCR analysis. JB6 samples, lanes 1, 3, and 5; RT101 samples, lanes 2, 4, and 6. Std, standard marker. β-Actin was used to normalize the amplified products (arrowhead). Perlecan primers were omitted in lanes 5 and 6.

samples (Figure 1B, lanes 2 and 4) compared to that of the wild-type JB6 samples (Figure 1B, lanes 1 and 3) when normalized to β-actin levels (Figure 1B, lanes 3-6). Also, a single transcript of ~14.5 kb was detected in both cell types by Northern blot analysis (data not shown). This indicates a lack of alternatively spliced variant forms in these cell types. In addition, slot blot analysis of perlecan core protein, normalized either to cell number (Figure 2A) or to total PG concentration (Figure 2B), showed 3- to 4-fold increase in the RT101 samples when detected with a rat monoclonal antibody specific to perlecan domain III.

Tumor-cell-derived perlecan contains CS/DS GAGs. Perlecan is predominantly expressed as an HSPG; however, in some circumstances it can be substituted with CS/DS GAGs. To identify the type of GAGs on perlecan produced by both cell types, perlecan was extracted from conditioned media of both wild-type JB6 and transformed RT101 cells. Gel electrophoresis and immunoblotting with polyclonal (Figure 3, A and B), and monoclonal (Figure 3, C and D) antibodies revealed a polypeptide of $M_r \approx 400$ kDa after heparinase III digestion (Figure 3, lanes H) in samples from JB6 (Figure 3, A and C) and RT101 cells (Figure 3, B and D). Interestingly, partial resolution of a core protein after chondroitinase ABC treatment (Figure 3, B and D; lanes C) was seen only in RT101 samples, suggesting the presence of some galactosaminoglycan (CS/DS). To confirm the presence of galactosaminoglycan on the core protein of the RT101-derived perlecan, intact radiolabeled GAGs were released from the core protein by alkaline borohydride treatment and resolved on a Superdex® Peptide HR10/30 HPLC column (Figure 4). JB6- and RT101-derived GAGs eluted in the Vo as a single population (Figure 4, A and D). Heparinase III treatments (Figure 4, B and E) degraded JB6 and most of the RT101

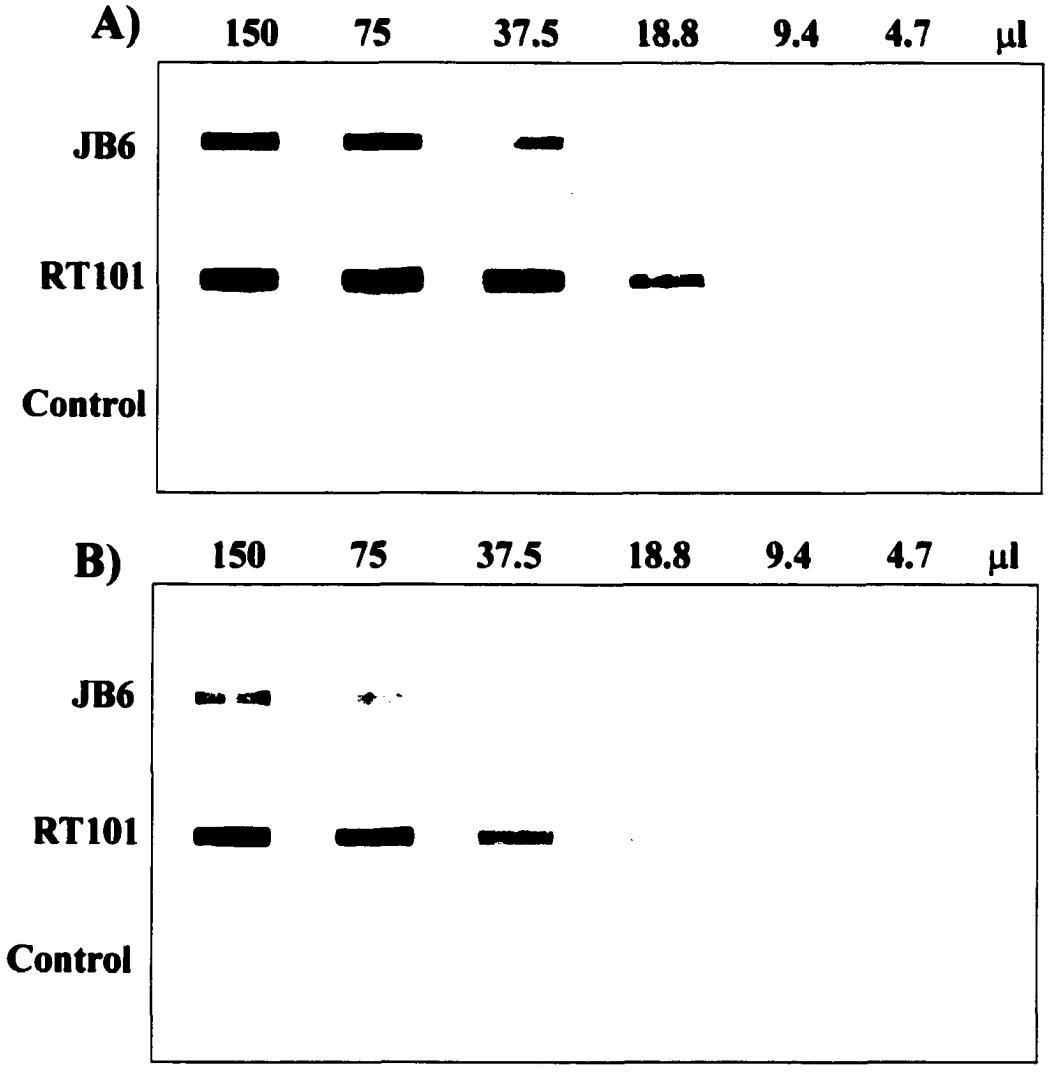

Figure 2. Increased perlecan protein levels in tumorigenic cells. Slot blot of perlecan from both JB6 and RT101 cells probed with H5L5, a rat monoclonal antibody against perlecan domain III. (A) Aliquots of serial dilutions of total PGs normalized by cell number. (B) Aliquots of serial dilutions normalized to the total amount of secreted PG. Serum-containing culture medium was used as a control.

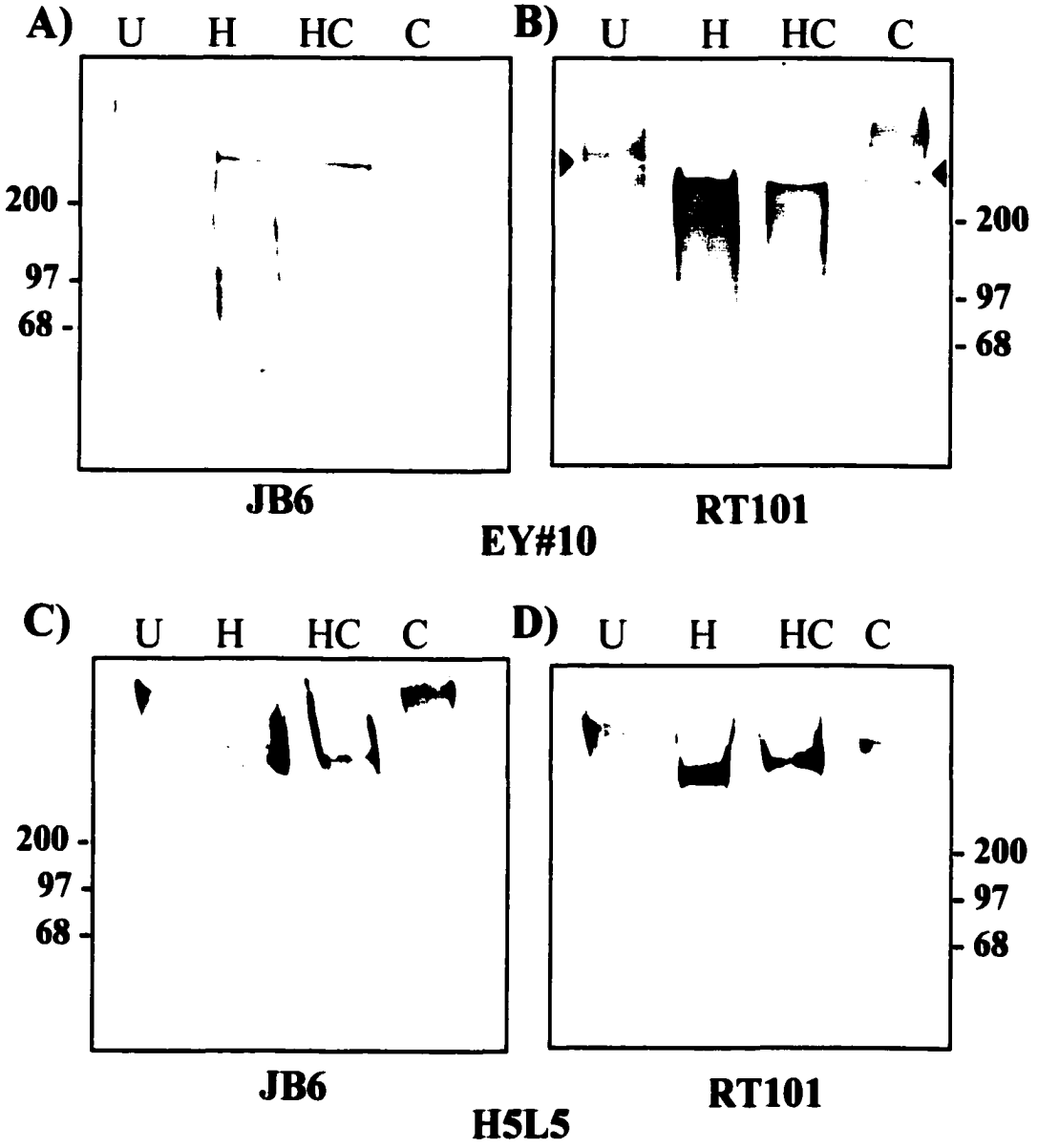

Figure 3. Tumor-cell-derived perlecan contains CS/DS GAGs. Immunoblotting of perlecan secreted by JB6 (A, C) and RT101 (B, D) cells with polyclonal antibody Ey#10 (A, B) against murine perlecan core protein and with H5L5 (C, D) monoclonal antibody against domain III of mouse perlecan. In each blot, the samples are untreated (U), heparinase III pretreated (H), heparinase III and chondroitinase ABC pretreated (HC), or chondroitinase ABC pretreated (C). Molecular weight standards in kilodaltons are shown. A discrete $\approx M_r$ 400-kDa polypeptide was visible after heparinase III pretreatment. In addition, partial resolution of core protein was seen after chondroitinase ABC pretreatment of RT101 but not JB6 perlecan. Arrowheads in panel B indicate junction of the stacking and resolving gels.

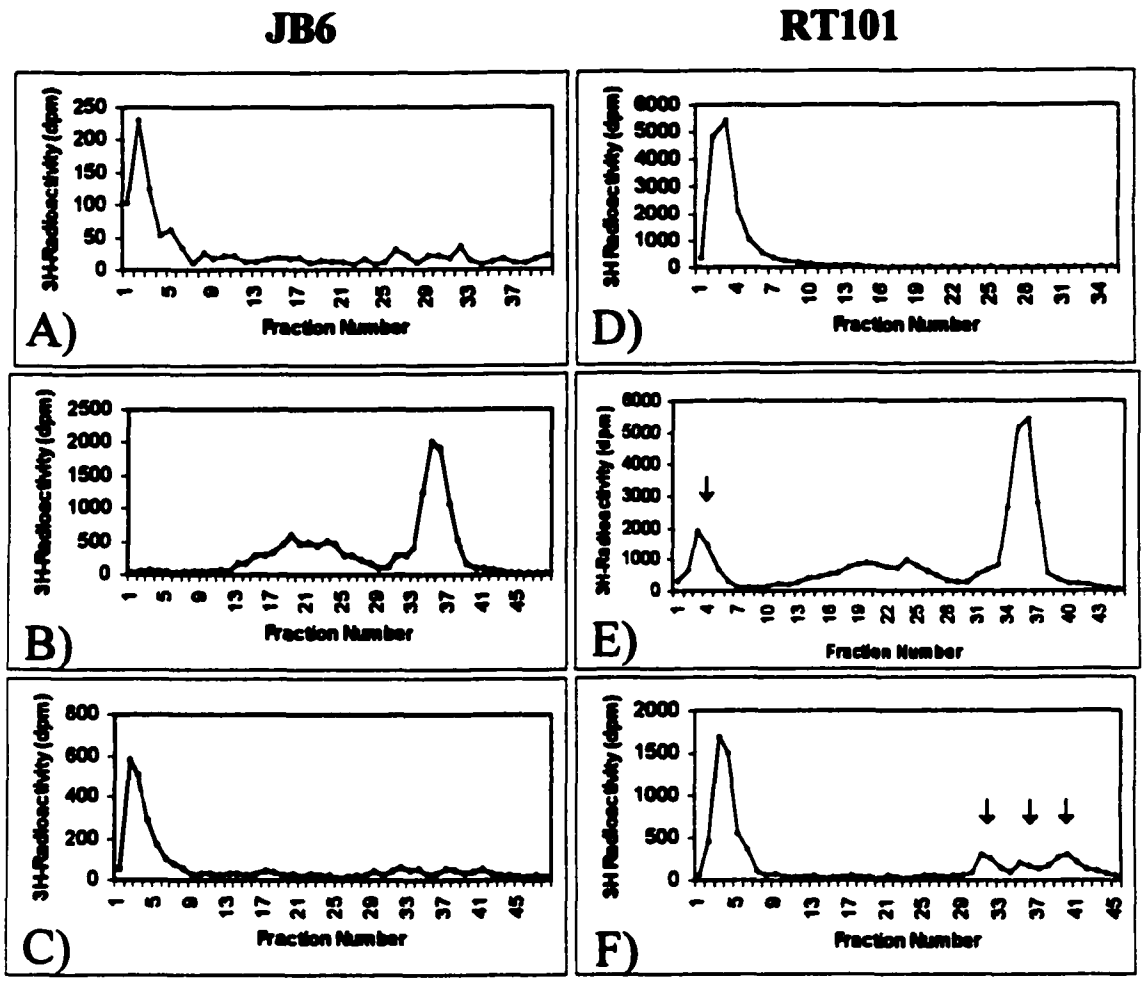

Figure 4. Perlecan GAG analysis. Superdex® Peptide HR 10/30 HPLC elution profiles of intact ^{3}H-labeled GAGs (A, D), heparinase III-pretreated GAGs (B, E), and chondroitinase ABC-pretreated GAGs (C, F). JB6 GAGs: A, B, and C; RT101 GAGs: D, E, and F. Fractions after Vo are shown.

sample to a mixture of short oligosaccharides and a prominent disaccharide fraction. However, the presence of intact GAG in the RT101 sample (Figure 4E, arrow) indicated the presence of CS/DS. To confirm this, samples were also treated with chondroitinase ABC. While the JB6 GAGs were not sensitive to chondroitinase ABC treatment, a subpopulation of RT101-derived GAGs (~25%) were degraded (Figure 4F, arrows), confirming the presence of CS/DS on the core protein of the RT101-derived perlecan but not on that of the non-transformed JB6 counterpart.

Structural analysis of GAG chains on perlecan of both cell types. For size analysis, GAGs were released from immunopurified perlecan by alkaline elimination and resolved by gel filtration HPLC on a TSK 4000 column (Figure 5). The column was calibrated with heparin, HS, and CS polysaccharides of known MW. Both samples eluted at a similar Kav (~60 kDa), indicating that there was no difference in size of the GAG chains on perlecan produced by both cell types. The relative sulfation level of perlecan GAG was determined by gradient anion-exchange chromatography (Figure 6). [^{35}S]-radiolabeled intact perlecan from both JB6 and RT101 cells eluted at the same position from the DEAE-Sephacel columns (Figure 6, A and B). To evaluate the influence of CS/DS chains on RT101-derived perlecan, some of the samples were also pretreated with chondroitinase ABC. This treatment did not change the elution profile of the JB6 perlecan (cf. Figure 6, A and C), consistent with the absence of CS/DS. However, the elution profile of the RT101 perlecan was altered after chondroitinase ABC, with samples eluting at a lower salt concentration (cf. Figure 6, B and D). The HS of the RT101 perlecan was, therefore, less sulfated than that of the JB6 cells; however, the additional

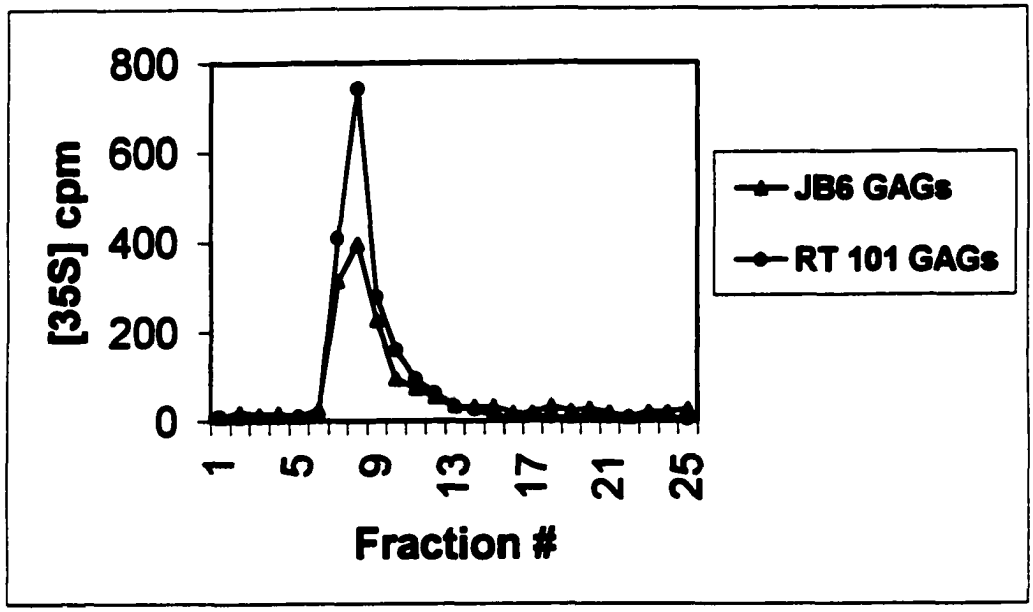

Figure 5. Comparison of the size of perlecan GAGs synthesized by JB6 and RT101 cells. $^{35}SO_4$-labeled GAGs were released from perlecan core protein by alkaline borohydride treatment and chromatographed on a TSK 4000 HPLC column. Aliquots of each fraction were analyzed by liquid scintillation counting and compared to polysaccharide standards of known mass. Fractions after Vo are shown.

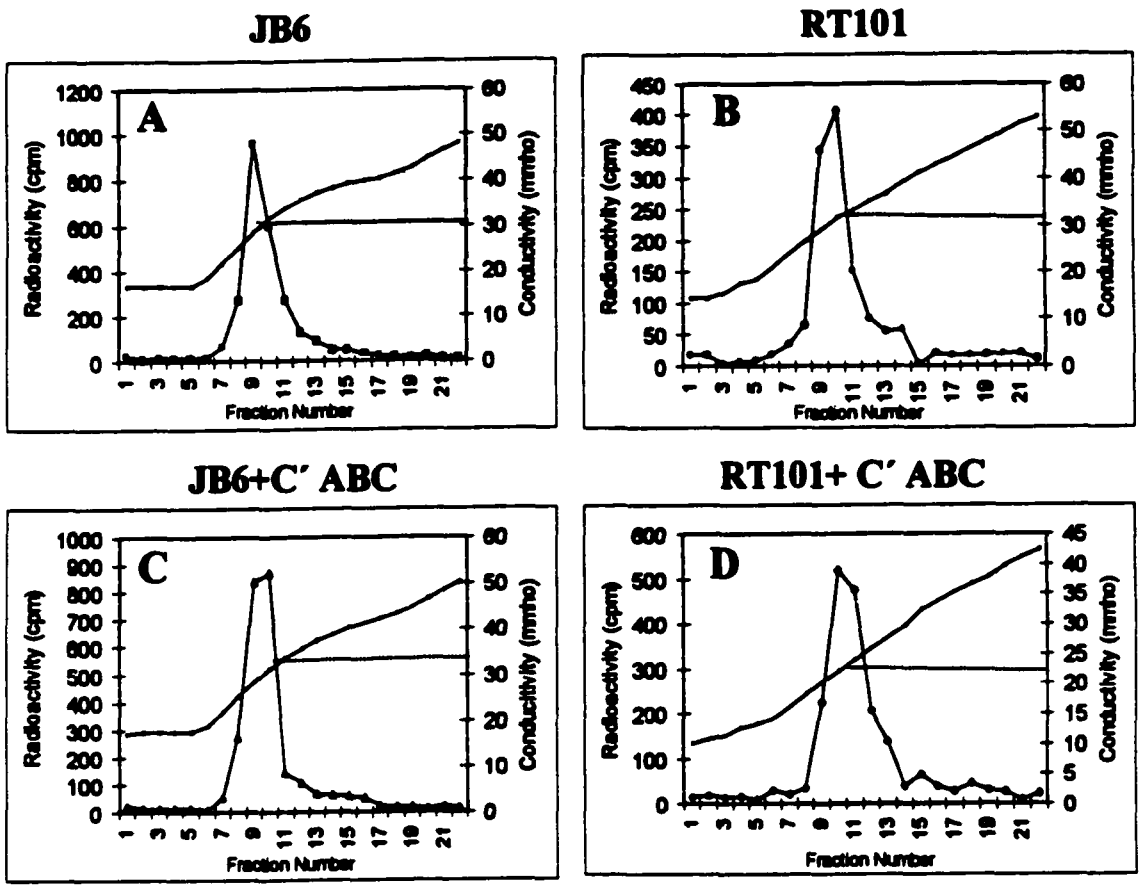

Figure 6. Comparison of overall sulfation levels of perlecan GAGs. Gradient anion-exchange chromatography of $^{35}SO_4$-labeled intact perlecan from both JB6 and RT101 cells. The samples, with or without chondroitinase ABC treatment, were applied to DEAE-Sephacel columns. The columns were washed and eluted with a linear gradient of 0.2-1.5 M salt. Fractions were collected and analyzed for conductivity and radiolabel. C'ABC: Chondroitinase ABC pretreated.

presence of CS/DS resulted in a similar overall sulfation. These data also confirmed that the RT101 perlecan contained CS/DS chains.

Total disaccharide compositions of perlecan HS. Changes in the sulfation level suggested that HS from wild-type and tumor-cell perlecan might have different disaccharides composition. To test this, ^{3}H-glucosamine-labeled HS from both JB6 and RT101 perlecan was depolymerized with combined heparinase I and III treatment. The disaccharides purified by gel filtration HPLC on Superdex® Peptide column were resolved by SAX-HPLC (Figure 7 and Table 1). Major peaks identified by comparison with heparin-derived disaccharide standards were quantified (Table 1). The major disaccharide of both HS species was ΔUA-GlcNAc, which comprised over half of the total disaccharide units. ΔUA-GlcNSO$_3$ was the second prominent constituent in both cases. The level of *N*-sulfation was similar for wild-type and tumorigenic cells (41.0 and 37.3%, respectively). Consistent with the anion-exchange chromatography results, progression to the tumorigenic phenotype was associated with a decrease in sulfation; the average number of sulfate groups per 100 disaccharides in the JB6 and RT101 perlecan HS was 75 and 62, respectively. In particular, perlecan HS from RT101 cells had lower levels of 2-*O*- and 6-*O*-sulfation and an increased amount of ΔUA-GlcNAc compared to the wild type. Based on the decrease of the trisulfated disaccharide ΔUA(2S)-GlcNS(6S) in perlecan from tumorigenic cells, the change in sulfation level mostly affected the highly sulfated S-domains, where this disaccharide can be primarily found.

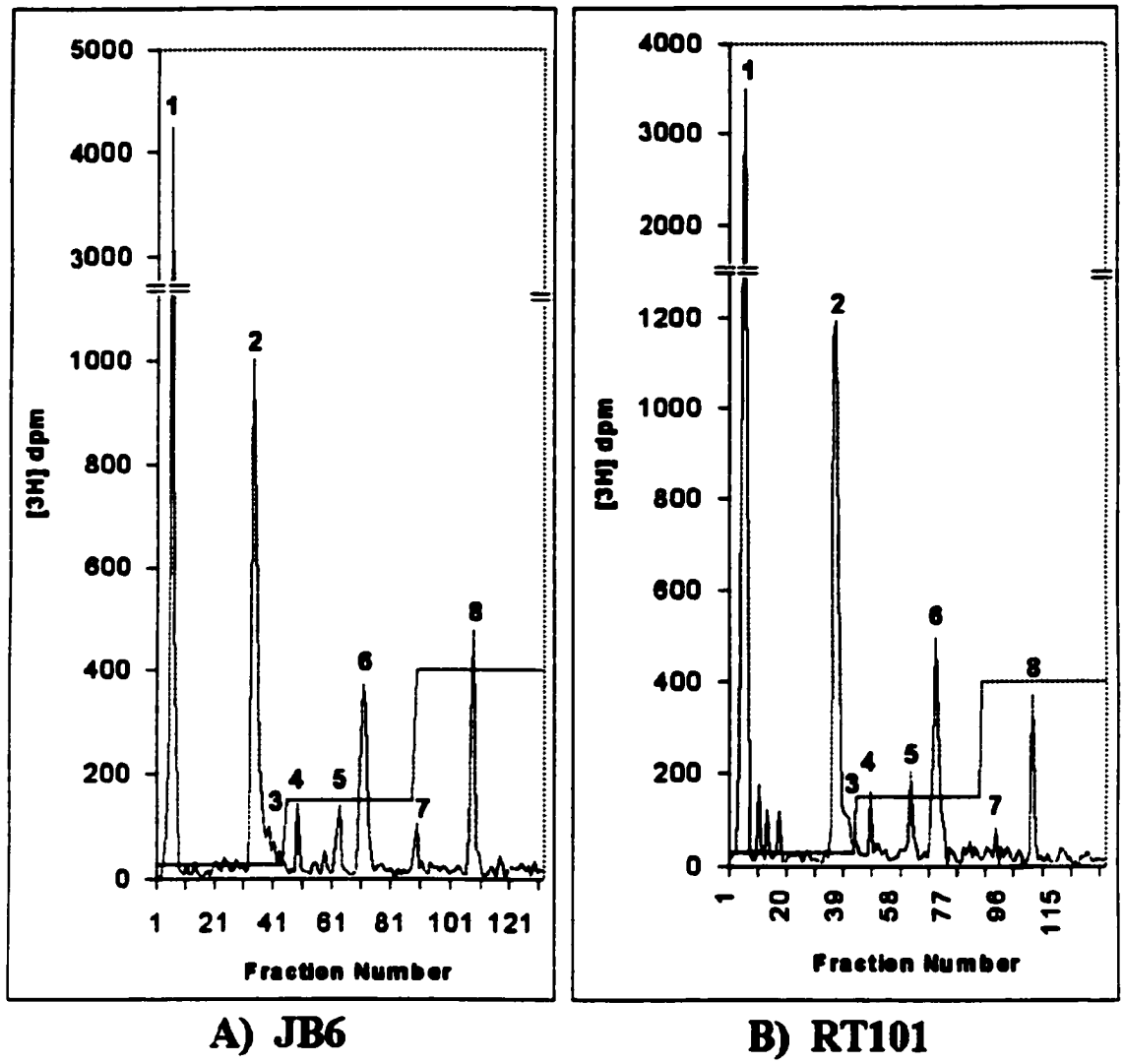

Figure 7. Strong anion-exchange SAX-HPLC analyses of ^{3}H-labeled HS disaccharides derived from JB6 (A) and RT101 (B) cells. Panels A and B show the separation of disaccharides resulting from depolymerization of HS using combined heparinases I and III. The elution of heparin disaccharide standards was as follows: Peaks 1: ΔUA-GlcNAc; 2: ΔUA-GlcNS; 3: ΔUA-GlcNAc(6S); 4: ΔUA(2S)-GlcNAc; 5: ΔUA-GlcNS(6S); 6: ΔUA(2S)-GlcNS; 7: ΔUA(2S)-GlcNAc(6S); and 8: ΔUA(2S)-GlcNS(6S).

Table 1. Comparative disaccharide compositions of HS from JB6 and RT101 perlecan. HS samples were degraded by combined heparinase I and III digestion. The resulting disaccharides were analyzed by SAX-HPLC. The results represent the mean values obtained from two determinations.

Disaccharide Subunits	% in JB6	% in RT101
1). ΔUA-GlcNAc	51.72	57.72
2). ΔUA-GlcNS	21.73	21.30
3). ΔUA-GlcNAc(6S)	4.34	3.35
4). ΔUA(2S)-GlcNAc	1.87	1.07
5). ΔUA-GlcNS(6S)	2.43	3.00
6). ΔUA(2S)-GlcNAc	10.45	9.60
7). ΔUA(2S)-GlcNAc(6S)	1.09	0.60
8). ΔUA(2S)-GlcNS(6S)	6.37	3.36
% N-S	40.98	37.26
% 2-*O*-S	19.78	14.63
% 6-*O*-S	14.23	10.31
Number of Sulfate/ 100 Disaccharides	75.00	62.00

Analysis of heparinase-I- and- III-resistant regions on perlecan HS. In general, HS chains comprise spatially discrete, highly sulfated, iduronate-rich domains (S-domain) interspersed with larger regions of UMDs. The boundary between S-domain andUMDs contains short mixed (M) sequences where *N*-acetylated disaccharides alternate with *N*-sulfated ones (Bernfield *et al.*, 1999). Both the composition and spacing of the domains are important for HS ligand-binding abilities and can be different for HS derived from different sources. To identify these regions in HS of JB6- and RT101-derived perlecan, HS samples were digested with either heparinase I (cleaves predominantly the sequence

IdoUA(2S)-GlcNS±(6S) in highly sulfated regions) or heparinase III (cleaves predominantly the sequence GlcUA-GlcNA±(6S) in unmodified regions) and the products were resolved on a Superdex® Peptide 10/30 HPLC column. Heparinase I treatment generated two classes of products, which were similar in size and composition for the JB6 and RT101 perlecan HS (Table 2). Over 80% of the products were longer resistant fragments originating from the U-domains, which were at least 14-20 sugar residues in length, and the rest was a mixture of shorter oligosaccharides from the susceptible regions containing 2-*O*-sulfated hexuronic acid, which primarily occur in S-domains (Table 2). There was no clear decrease in the short oligosaccharides from tumorigenic RT101 cell perlecan HS, although the decrease in 2-*O*-sulfation in perlecan HS from these cells could be expected to alter the susceptibility to heparinase I. However, it is possible that the moderate amounts of radiolabeled immunoprecipitated perlecan HS that were available for the analysis were not sufficient to allow sensitive detection of small changes in the small product amount.

Heparinase III degrades predominantly U-domains and therefore can be used to determine the size of S-domains, which remain intact after the treatment. These resistant S-domains were on average hexadecasaccharides and for JB6 and RT101 perlecan represented 57 and 43% of the total chain length, respectively (Table 2). SAX analysis detected lower sulfation levels and more *N*-acetylated glucosamine in perlecan HS from RT101 cells, which should lead to higher susceptibility to heparinase III. Consist with this, the amount of heparinase-III-generated disaccharides is higher for the RT101 sample (Table 2), indicating that the tumorigenic RT101 cell-derived perlecan contains HS with longer U-domains. These profiles were consistent with the SAX-HPLC profiles (Figure

7 and Table 1). Combining these results, it appears that the HS of RT101 consists of smaller S-domains but slightly larger U-domains compared to those of the JB6 wild type.

Table 2. Susceptibility of perlecan HS to heparinases I and III. Perlecan HS was degraded by either heparinase I or III and subjected to Superdex® Peptide HR 10/30 HPLC. The results represent the mean values from three sets of data for heparinase I treatment and two sets of data for heparinase III treatment.

	Average JB6 (%)	Average RT101 (%)
Heparinase I:		
14-20 sugars (fr 1-16)	80.3 ± 6.0	84.9 ± 0.8
4-12 sugars (fr 17-30)	2.2 ± 3.4	9.0 ± 1.8
Disaccharides (fr 31-40)	7.5 ± 2.7	6.1 ± 1.1
Average size of the HI-resistant domain (U-domain)	~20 sugar residues (elutes at fraction 7)	~20 sugar residues
Heparinase III:		
14-20 sugars (fr 1-16)	57.3 ± 5.3	42.8 ± 8.3
4-12 sugars (fr 17-30)	19.8 ± 3.0	15.3 ± 2.0
Disaccharides (fr 31-40)	22.8 ± 2.3	41.8 ± 6.3
Average size of the HIII-resistant domain (S-domain)	Hexadecasaccharide (elutes at fraction 10)	hexadecasaccharide

Binding of perlecan-derived HS to GFs. The data suggest that structural differences in HS are associated with the process of tumorigenic transformation in these murine epidermal cells. Potentially, these changes could affect the HS ability to bind various ligands. Therefore, we used affinity co-electrophoresis to assess the interaction between perlecan-derived HS from both JB6 and RT101 cells and several GFs, which are involved in regulation of epithelial cell growth. Four members of the FGF family (FGF-1,

-2, -4, and -7) and HB-EGF were examined. Affinity co-electrophoresis measures relative retardation of radiolabeled HS in the presence of various ligand concentrations, which represents ligand-bound fraction of HS and therefore can be used to calculate affinity. Dissociation constants for the complex of HS and various GFs were determined by Scatchard analysis (Lee and Lander, 1991; Lim *et al.*, 1991). The Scatchard plots and corresponding dissociation constants are shown in Figure 8. The R values are average from at least two independent experiments. In the range of tested concentrations (12-200 nM), perlecan HS from both cell types bound with high affinity to all GFs. However, HS from RT101 perlecan bound with slightly higher affinity to FGF-1 (Kd ≈ 40 versus 66.7 nM), FGF-2 (Kd ≈ 12.5 versus 29.2 nM), FGF-7 (Kd ≈ 29.8 versus 48.8 nM), and HB-EGF (Kd ≈ 26.4 versus 35.8 nM) but with a marginally lower affinity to FGF-4 (Kd ≈ 40.2 versus 28.8 nM) compared to the JB6-derived perlecan HS.

Discussion

Perlecan is a widespread basement membrane and ECM PG. It is among the most complex of ECM molecules, having a multidomain core protein of approximately 400 kDa, substituted with GAG chains, which may be HS and sometimes CS/DS (Iozzo, 1998). The substitution of the core protein is most commonly seen on the N-terminal domain I but has also been recorded on the C-terminal domain V (Brown *et al.*, 1997; Tapanadechopone *et al.*, 1999). Virtually all basement membranes possess perlecan; interestingly, though, where the gene has been deleted, as recently reported (Mullen *et al.*, 1999), the major impact appears to be on skeletal tissues. This is perhaps because perlecan is a component of cartilage (Costell *et al.*, 1999). In addition, perlecan has been suggested to be an important component in tumor biology. Perlecan may form a scaffold

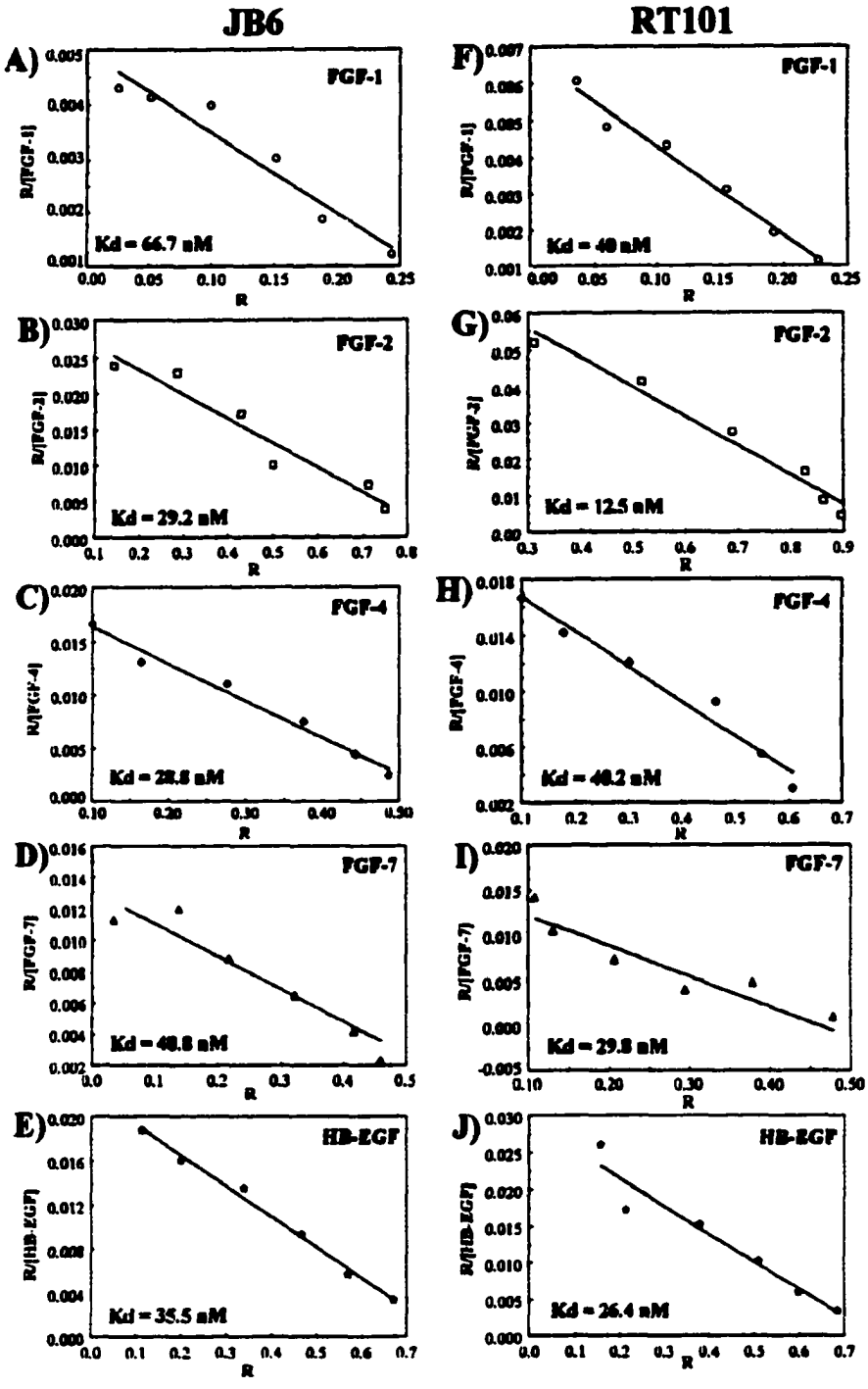

Figure 8. Scatchard analyses of perlecan HS binding to GFs. Kd values were calculated from the slopes (slope = -1/Kd) of the fitted straight lines using PSI-Plot program. The y-axis represents the values of R/[GF] (in nM) and the x-axis is the fractional retardation R. The R values are the average from at least two repeated experiments (see Experimental Procedures). FGF-1: A and F; FGF-2: B and G; FGF-4: C and H; FGF-7: D and I; and HB-EGF: E and J. JB6-derived perlecan HS is shown in panels A-E, RT101-derived perlecan HS in panels F-J.

for cellular interactions, since the core protein can interact with integrins (Iozzo, 1998). An additional important feature of perlecan is that its HS chains may bind many GFs, including some which are potent inducers of angiogenesis (Aviezer *et al.*, 1994). Therefore, perlecan may be an important component in tumor angiogenesis, and it has been suggested that tumor-released proteases such as stromelysin (MMP-3) and collagenase 3 (MMP-13) may cleave perlecan core protein to release GAG chains with attached GFs (Whitelock *et al.*, 1996), which may then promote angiogenic responses. Therefore, it is important to understand the biology of perlecan in the context of tumor progression.

The model we have used is convenient, since it allows a direct comparison of a wild-type epidermal cell line with its transformed counterpart. The JB6 epidermal cell line, when exposed to phorbol esters, can be irreversibly transformed (De Benedetti *et al.*, 1991; Singh *et al.*, 1995). We have compared the structure of perlecan as a first stage in ascertaining whether this molecule may be important in epidermal tumorigenesis. The major differences seen between JB6 and RT101 perlecan were as follows: First, both the mRNA and protein levels of perlecan core protein appear to be increased in the transformed cell line, in agreement with previous reports which have shown that other transformed cells can be prolific producers of this matrix PG (Cohen *et al.*, 1994; Nerlich *et al.*, 1998). Second, while GAG chain size is not different between the two cell types, the presence of a small amount of galactosaminoglycan was detected in the transformed but not in the wild-type epidermal cells. The presence of CS and/or DS on perlecan has been noted several times, and this is one of several PGs that can be synthesized as a hybrid PG, where the core protein may possess both galactosaminoglycan and glucosaminoglycan (Couchman *et al.*, 1996; Iozzo, 1998). The impact of alternate

glycanation on the biology of perlecan is not understood. For instance, it is not known whether the interaction of GFs or other HS-binding moieties is influenced by the presence of galactosaminoglycan. In some cases, DS does possess GF-binding capabilities similar to but usually with lower affinity than those seen with the equivalent interaction with HS (Lyon *et al.*, 1998; Penc *et al.*, 1998). It has been shown previously that hybrid molecules may be synthesized with substitution of both chain types present on domain I of the core protein (Kokenyesi and Silbert, 1995; Couchman *et al.*, 1996; Dolan *et al.*, 1997). In the present study, however, it appears that the presence of hybrid perlecan molecules is a correlate of the transformed state. This may represent a case where the increased synthesis of perlecan leads to some of the glycanation being of the "default" pathway (i.e., galactosaminoglycan). Similar cases have been reported previously but not directly in the context of transformation (Groffen *et al.*, 1996).

In keeping with the increased synthetic rate of perlecan, we also determined that the HS chains of RT101 perlecan were of lower overall sulfation than the normal counterpart. However, this difference is subtle and is even masked where the CS/DSs are present. Under these circumstances the perlecan from wild-type and transformed mouse epidermal cells eluted equivalently in gradient anion-exchange chromatography. It was only when the galactosaminoglycans were removed by chondroitinase ABC treatment that the lower sulfation of the HS chains became apparent. Consistent with these data, we ascertained that there were small differences in the fine structure of the HS synthesized by JB6, compared with RT101 cells. The RT101-derived perlecan HS had a decrease in 2-*O*- and 6-*O*-sulfation, with a concomitant increase in unsulfated disaccharides. A schematic illustration of the overall structure of the HS chains from these two cell types is shown in Figure 9.

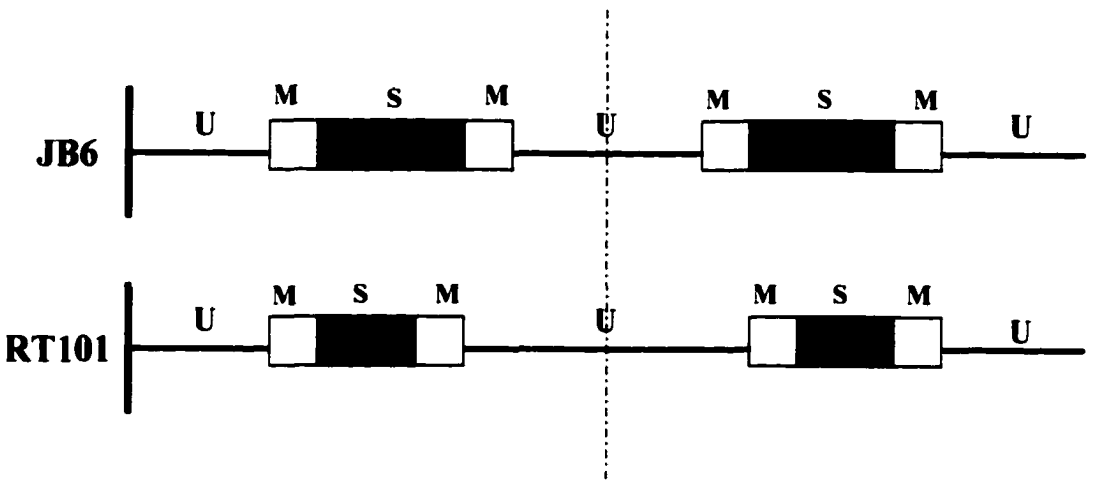

Figure 9. Schematic model of JB6 and RT101 perlecan HS domain structure. S-domains (filled rectangles) are spaced along the chain from the core protein (left) to the non-reducing ends (right) separated by U-domains (straight lines). The boundaries between S- and U-domains are mixed sequences (M, open rectangles).

Our results have some similarity to a recent comparison of colon adenoma and carcinoma HS fine structure (Jayson *et al.*, 1998). The latter had HS with one-third less 2-*O*-sulfate and one-fifth less *N*-sulfate. In our study *N*-sulfation was virtually unchanged, but there was around 25% less 2-*O*-sulfation. Our studies also revealed a similar decrease in 6-*O*-sulfation, a modification that was seen to increase in the previous study of transformation (Jayson *et al.*, 1998). The reasons are unclear and may be related to tissue-specific expression of sulfotransferases. In addition, our study is distinct from previous work, concentrating on one PG, perlecan, rather than total HS. The impact of this subtle change in HS fine structure on transformation is not entirely clear, especially as further analysis showed that the affinity of the HS chains was essentially unchanged with respect to GF interactions. Four members of the FGF family were chosen for this study, all of which have been implicated in epithelial tumorigenesis (Basilico and Moscatelli, 1992; Arbeit *et al.*, 1996). In the case of the FGF-7, these interactions may not be restricted to the HS chains but may also extend to an involvement of the perlecan core protein (Sharma *et al.*, 1998). However, in no case was a sharp difference in affinity seen in the interactions of these four members of the FGF family or HB-EGF with perlecan from normal and transformed cells. The affinities are high, in the nanomolar range, and therefore indicate that perlecan of both cell types has the ability to bind these potent growth, differentiation, and angiogenesis-promoting factors. This is the first time that perlecan-derived HS has been shown to interact with FGF-4 and HB-EGF. Future in vivo work will determine just how important the presence of perlecan in the tumor matrix is and whether there are distinctions between tumor-derived and host-derived perlecan.

However, given exciting data which show that antisense targeting of perlecan can block both tumor growth and angiogenesis in vivo (Sharma *et al.*, 1998), it may well

be that detailed understanding of the role of this complex PG will be highly relevant for future understanding of tumor invasiveness.

Acknowledgments

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Footnotes

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[1] The abbreviations used are: GAG, glycosaminoglycan; CS, chondroitin sulfate; PG, proteoglycan; HS, heparan sulfate; FGF, fibroblast growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RPA, ribonuclease protection assay; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; SAX, strong anion-exchange chromatography. GF, growth factor; HB-EGF, heparin-binding epidermal growth factor, HGF/SF, hepatocyte growth factor/scatter factor; MMP, matrix metalloproteinase.

[2] P. Tapanadechopone, X. Jiang, and J. Couchman, unpublished data.

LOCALIZATION OF GLYCOSAMINOGLYCAN SUBSTITUTION SITES ON DOMAIN V OF MOUSE PERLECAN

by

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Abstract

Perlecan, the predominant basement membrane PG, has previously been shown to contain GAGs attached at serine residues, numbers 65, 71, and 76, in domain I. However, the C-terminal domains IV and V of this molecule may also be substituted with GAG chains, but the exact substitution sites were not identified. The amino acid sequence of mouse perlecan reveals many ser-gly sequences in these domains that are possible sites for GAG substitution. We expressed recombinant domain IV and/or V of mouse perlecan in COS-7 cells and analyzed GAG substitution. Both HS and CS chains could be detected on recombinant domain V. One site, ser-gly-glu (serine residue 3593), toward the C-terminal region of domain V is a substitution site for HS. When this sequence was absent, CS/DS substitution was deleted, and the likely site for this galactosaminoglycan substitution was ser-gly-ala-gly (serine residue 3250) on domain V.

Introduction

One of the major components of all mammalian basement membranes is the large, low-density PG, perlecan, which has a protein core of M_r 369-450 K (Hassell *et al.*, 1985; Paulsson *et al.*, 1987; Kallunki and Tryggvason, 1992; Noonan *et al.*, 1991; Murdoch *et al.*, 1992; Murdoch and Iozzo, 1993; Iozzo, 1998). It was named perlecan because of the appearance of the molecule by rotary shadowing electron microscopy, appearing as a series of globules separated by rods, or "beads on a string" (Paulsson *et al.*, 1987; Yurchenco *et al.*, 1987). The protein core consists of a single polypeptide (Noonan *et al.*, 1991; Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992) and complete sequence analysis and data base analysis of the deduced sequence from both

mouse and human perlecan show the protein to consist of five distinct domains, most of which consist of multiple repeated motifs (Noonan *et al.*, 1991; Kallunki and Tryggvason, 1992; Cohen *et al.*, 1993). The N-terminal half of domain I contains a unique small segment of 84 amino acids with three serine sites for GAG attachment, consisting of SGD sequences (Kokenyesi and Silbert, 1995; Dolan *et al.*, 1997; Costell *et al.*, 1997; Graham *et al.*, 1999). The C-terminal half of domain I consists of a SEA module which has been shown to enhance HS synthesis (Bork and Patthy, 1995; Dolan *et al.*, 1997). Domain II contains four cysteine- and acidic amino acid- rich repeats that are very similar to those found in the low-density lipoprotein (LDL) receptor and proteins such as GP330. Domain III shares homology to the short arm of laminin α chains and contains four cysteine-rich regions intercalated among three globular domains. Domain IV is a highly repetitive region with 14 (Noonan *et al.*, 1991) or 21 (Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992) Ig-like repeats that show greatest similarity to N-CAM. In this region lies the potential for alternate mRNA splicing (Noonan and Hassell, 1993). One of these repeats also separates domain II and III. Domain V is similar to the carboxy-terminal globular G-domain of agrin and laminin α chains; it contains three globular regions and four EGF-like repeats (see Murdoch and Iozzo, 1993; Iozzo, 1998; Noonan and Hassell, 1993 for reviews).

Perlecan is tightly bound to other matrix macromolecules and both the protein core and the polyanionic GAG chains may be involved in these interactions (Visher *et al.*, 1997; Giry-lozinguez *et al.*, 1998; Hopf *et al.*, 1999; Talts *et al.*, 1999). Perlecan can also self assemble noncovalently into linear dimers, and to a lesser extent, oligomers. It is possible that GAG chains may modulate this self assembly (Yurchenco *et al.*, 1987). Several functions including roles in cell proliferation, embryogenesis, morphogenesis,

and selective permeability filtration in basement membranes have been suggested for perlecan (Murdoch and Iozzo, 1993; Handler *et al.*, 1997; French *et al.*, 1999; Peng *et al.*, 1999). This PG also serves as an attachment substrate for cells probably through integrin and HIP, a process that may be modulated by HS (Battaglia *et al.*, 1993; Chakravarti *et al.*, 1995; Rohde *et al.*, 1998; Slater and Murphy, 1999; Whitelock *et al.*, 1999).

Perlecan was originally described as an HSPG (Hassell *et al.*, 1985). However, while the three consecutive SGD sequences in domain I are often attachment sites for HS side chains, they can also be substituted with CS (Kokenyesi and Silbert, 1995; Costell *et al.*, 1997; Dolan *et al.*, 1997). Hybrid forms containing both HS and CS/DS side chains have also been described (Danielson *et al.*, 1992; Couchman *et al.*, 1996). In addition to the consensus sequences in domain I, perlecan also possesses a large number of other potential GAG attachment sites. These include ser-gly dipeptides and ser-gly-x-gly (SGXG) consensus sequences dispersed throughout the core protein (Noonan *et al.*, 1991; Murdoch *et al.*, 1992), some of which are flanked by acidic amino acids and therefore comply with the proposed consensus sequence for GAG attachment sites (Bourdon *et al.*, 1987; Zhang and Esko, 1994; Zhang *et al.*, 1995). In the human perlecan sequence, there are 53 ser-gly dipeptides including 3 SGXG sequences, one in domain IV and two in domain V (Murdoch *et al.*, 1992). In the predicted sequence of mouse domain IV, there are four DSGE sequences, five less than in the human sequence, and in domain V there are one SGXG and two additional tetrapeptides, EGSG and GSGE (Noonan *et al.*, 1991). These may potentially be substitution sites for GAG chains. Domains II and III, however, do not accept GAG chains (Dolan *et al.*, 1997).

GAG substitution may substantially alter the physicochemical and biological functions of perlecan, and recently the C-terminal domain of this molecule was shown to be potentially substituted with GAG chains (Brown *et al.*, 1997; Ettner *et al.*, 1998) but the specific substitution sites are not known. In this study, recombinant proteins from domain IV and/or V of mouse perlecan were expressed in COS-7 cells and then analyzed. We show that GAG substitution sites are present on domain V which can be substituted with HS and CS/DS chains. The serine (amino acid residue 3593) in the ser-gly-glu sequence at the C-terminus of murine domain V was identified as a glycanation site and was usually substituted with an HS side chain. When this site is unavailable for substitution, CS/DS substitution was observed, and the serine (amino acid residue 3250) in the ser-gly-ala-gly sequence could be the site for this CS/DS substitution.

Materials and Methods

Preparation of murine perlecan cDNA constructs. Recombinant domain I/II/III was constructed as described previously (Dolan *et al.*, 1997). A cDNA construct encoding domain IV/V was similarly prepared from cDNA BPG 7, clone 12 and clone 2D (Noonan *et al.*, 1991) in pBluescript II SK$^+$ (Strategene), using restriction enzyme sites in the multicloning region of the vector and the inserts. *Hind*III linkers (New England Biolabs) were used to add *Hind*III sites in the same reading frame to provide for interchangeability of some constructs and facilitate construction. Short cDNA segments used to mutagenize specific amino acid in murine perlecan sequence were prepared by synthesizing upper and lower strands with the required amino acid sequence, and containing the desired overhang at the ends for in-frame ligation into unique restriction sites in the insert. *Not*I and *Xba*I sites at the 5' and 3' ends,

respectively, of constructs were used for ligation into the multicloning site of the pRc/CMV expression vector. All constructs were sequenced to confirm appropriate construction.

Designating the start methionine in the signal peptide as amino acid 1, the domain I/II/III construct (D I/II/III) encoded amino acids 1-1680 (Figure 1). The domain IV/V (D IV/V) construct encoded amino acids 1-35 (perlecan signal peptide plus 16 amino acids of domain I) and residues 1682-3707. The D IV/V construct was also modified by deleting amino acids 3584-3614 which removed the SGE sequence at amino acids 3593-3595 in domain V to produce the D IV/V (SGE-) construct. A further D IV/V truncated construct was prepared by deleting 158 amino acids from the C-terminus of domain V which removed the SGE sequence at amino acids 3593-3595 to produce the D IV/V (T) construct. Constructs consisting of domain V only were made by PCR using the D IV/V and D IV/V (SGE-) cDNAs as templates. Oligonucleotide primers for domain V (D V) of perlecan were 5'-GTGATCTCGAGCTTACCCAGACA-3' (forward) and 5'-TTAATAAA AGCTTACTCGACACTGTC-3' (reverse) and for a C-terminal fragment of domain V (D fV) of perlecan were 5'-GAGAATTCGACAGCAC AGCCGAT-3' (forward) and 5'-GCGCGCGTCGACTACTCGACACTGTC-3' (reverse). These were synthesized by the Oligonucleotide Core Facility of the Comprehensive Cancer Center, the University of Alabama at Birmingham. The forward primer of domain V corresponded to the nucleotide sequences 9525-9548 and contained an added restriction endonuclease site for *Xho*I at the 5' end. The forward primer of the C-terminal fragment of domain V (D fV) corresponded to the nucleotides 11220-11249 and contained an added restriction endonuclease site for *Eco*RI at the 5' end. The reverse primers for both constructs corresponded to the nucleotides 11746-11759 and contained

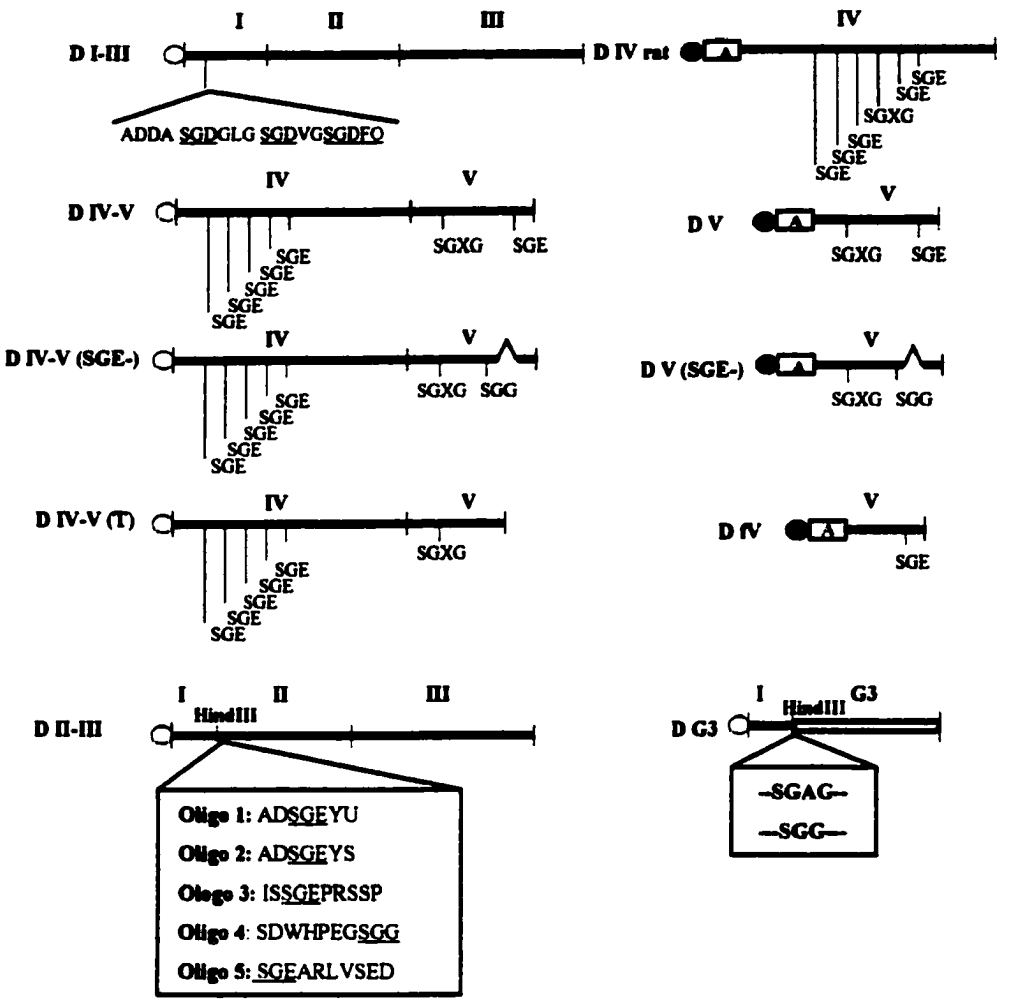

Figure 1. Diagram of perlecan constructs. D I/II/III, D II/III, D IV/V, D IV/V (SGE-), and D IV/V (T) constructs contained the perlecan signal peptide (open circle) to target the recombinant product for secretion. D IV/V (SGE-) has the SGE region (residues 3593-3614) deleted from domain V; D IV/V (T) terminates at residues 3550; D V constructs were ligated in frame with the IgG binding portion of protein A (A) and an upstream signal sequence of transin (solid circle). D fV includes the C-terminal of domain V comprising the residues 3550 to the stop codon of this molecule. D IV (rat) construct encoded Ig repeats 6.5-12.5 of rat perlecan. D II/III (Oligos 1-5) contained possible GAG attachment sequences from domains IV or V, inserted between the C-terminal portion of domains I and II/III. D G3 constructs encoded amino acids 1-35 of perlecan and amino acids 1780-2111 of aggrecan G3 domain. Inserted between these two regions were fragments of possible CS/DS substitution sites from domain V of perlecan.

added restriction endonuclease sites for *Hind*III or *Sal*I at the 5' end. The recombinant polypeptides corresponded to amino acids 2983 to the stop codon of this molecule for the whole of domain V, and amino acids 3550 to the stop codon of this molecule for the fragment of domain V (D fV). The same primers for the whole domain V were used to create D V (SGE-) and the final recombinant protein had amino acid 3584-3614 deleted from domain V. The primer pair oligonucleotides for each domain were used in PCRs under standard reaction conditions. The cDNA for D V and D V (SGE-) were treated with *Xho*I and *Hind*III. The cDNA for D fV was treated with *Eco*RI and *Sal*I then ligated into the same restriction sites in the pRK5/protein A vector (gift from Dr. J. D. Esko, University of California at San Diego).

Recombinant domain IV of rat perlecan. A cDNA expression library prepared from L2 rat yolk sac carcinoma cells (Stratagene) was screened with a polyclonal antibody against basement membrane CSPGs as previously described (Couchman *et al.*, 1996; Wu and Couchman, 1997). Of 11 clones detected from preliminary screening and subsequent rounds of clonal selection, two different polypeptides were identified by DNA sequencing. Two overlapping clones (5a and 11a) of 2.2 Kb yielded portions of domain IV of rat perlecan core protein, judged by very high homology to previously reported murine and human sequences (Noonan and Hassell, 1993). These clones were prepared and sequenced as described previously (Wu and Couchman, 1997). cDNA from clone 5a which contained six IgG repeats of rat perlecan, corresponding to human IgG repeats at amino acid sequence 2096-2702 (Murdoch *et al.*, 1992), was treated with *Eco*RI and *Sal*I, subcloned into the pRK5/protein A vector designated clone P5a.

Recombinant domain II/III constructs containing potential GAG attachment sequences from domain IV or V. A previously prepared construct of domain II/III (Dolan *et al.*, 1997) was also used to test DNA sequences from domain IV and domain V of mouse perlecan that encoded potential GAG attachment sites. These utilized a *Hind*III site between proline residue 35 and the start of domain II of mouse perlecan sequence (Doege *et al.*, 1997; Dolan *et al.*, 1997). The constructs were assembled in the pRc/CMV vector and consisted of DNA encoding perlecan signal peptide followed by 16 amino acids from the beginning of domain I (this region of domain I does not receive GAG side chains) followed by domains II and III. The possible GAG attachment sequences to be tested were named "Oligos 1-5". Oligos 1, 2, 3 were from domain IV of murine perlecan (Oligo 1: ADSGEYV corresponded to amino acid residues 2014-2020; Oligo 2: ADSGEYS corresponded to residues 2208-2214; and Oligo 3: ISSGEPRSSP corresponded to residues 2457-2466). Oligos 4, 5 were from domain V of mouse perlecan (Oligo 4: SDWHPEGSGG corresponded to amino acid residues 3503-3512; and Oligo 5: SGEARLVSED corresponded to residues 3593-3602). Domain II/III with no insert in the *Hind*III site served as a negative control.

Recombinant aggrecan G3 domain constructs containing potential CS/DS substitution sites from domain V. A previously prepared aggrecan G3 domain construct (Doege *et al.*, 1997) was also used to test sequences for potential GAG substitution sites. Briefly, the construct contained amino acid residues 1-35 of murine perlecan and amino acid residues 1780-2111 of aggrecan domain G3. Like the perlecan constructs, these were coupled by a *Hind*III site in the same reading frame was added to allow the subsequent constructions of chimeras (Figure 1). *Not*I and *Xba*I sites at the 5'/3' ends,

respectively, were used for ligation into the multicloning site of the pRc/CMV expression vector (Invitrogen). Two sequences containing possible CS/DS substitution sites were made by PCR using the D IV/V perlecan cDNA as a template. Oligonucleotide primers for D G3/SGAG construct were 5'-CACCTGGGAAGCTTCG GGGTGAGGTGT-3' (forward), corresponding to murine nucleotide sequence 10266-10292, and 5'-CCCAAAGCTTAGTCAGATCCCATTGGG-3' (reverse), corresponding to nucleotide sequence 10413-10439. For D G3/SGG construct, the paired primers were 5'-CGCTGAAGCTTAGGCGCTGGATATGGG-3' (forward), corresponding to nucleotide sequence 11049-11075, and 5'-CCGAAGCTTGAATTAAATGGTCTCGGG-3' (reverse), corresponding to nucleotide sequence 11208-11234. These contained an added *Hind*III cutting site in the appropriate reading frame. The cDNAs of these two constructs were treated with *Hind*III and then ligated into the same restriction site in G3 domain constructs. The orientation of the sequences were verified by DNA sequencing. Aggrecan G3 domain construct without an insert in the *Hind*III site was used as a control.

Cell culture and transient transfection. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and glutamine. The cells were plated into T25 flasks and allowed to reach 70% confluency. After rinsing the cell layer with serum free medium, 3 ml of serum free medium containing 4.5 μg of recombinant DNA/12 μl of lipofect-AMINE (Gibco BRL) complex was added to each flask, incubated for 5 h after which 3 ml of DMEM containing 20% fetal bovine serum was added. The medium was replaced with 6 ml of serum free DMEM after 24 h.

Incubation was continued for another 24 h, when the medium was collected for PG purification.

Constructs of domain IV/V, domain IV/V (SGE-), and Oligo 1-5 were also transfected into Chinese hamster ovary (CHO) cells grown in Ham's F-12 medium containing 10% fetal bovine serum and glutamine with the same procedures as above.

GAG analysis of recombinant domain IV/V. COS-7 cell cultures transfected with D I/II/III, D IV/V, and D IV/V(T) constructs, in the presence of $^{35}SO_4$ were analyzed as previously (Dolan *et al.*, 1997). Briefly, the radiolabeled recombinant protein was captured by protein G-Sepharose beads (Pharmacia Biotech Inc.) to which was bound rabbit antibodies to perlecan. The material bound to the beads was eluted with 4 M guanidine HCl and any remaining unincorporated $^{35}SO_4$ was removed by chromatography on PD-10 columns. The macromolecular material was then fractionated on Superose 6. Aliquots of each fraction were taken for measurement of radiolabel content by liquid scintillation counting. Fractions from Superose 6 chromatography were pooled and GAG side chains were released by treatment with 1 M sodium borohydride in 0.05 M NaOH. The proportion of HS and CS was determined by digestion with nitrous acid or chondroitinase ABC, followed by precipitation of macromolecular material with 95% ethanol containing 1% potassium acetate and measuring digested material in the supernatant by liquid scintillation counting. The proportion was also determined by chromatography of undigested and chondroitinase-digested material on Superose 6, and calculating the proportion of radiolabel eluting in the low MW pool.

D V (SGE-) in the pRK5/protein A vector, as well as empty vector were also transfected into COS-7 cells in the presence of 50 μCi/ml of D-[6-^{3}H]glucosamine HCl

(American Radiolabeled Chemicals). The radiolabeled recombinant products were captured by immunoprecipitation with rabbit IgG-agarose as described above. Unincorporated materials were removed by extensive washing. The recombinant products were eluted from the agarose beads with 4 M guanidine HCl and subjected to ethanol precipitation in the presence of a small amount of cold CS as a carrier. The precipitated pellets were dissolved in chondroitinase buffer (50 mM Tris, 30 mM sodium acetate, 20 mM EDTA, 10 mM NEM, 0.2 mM PMSF, and 0.02% sodium azide, pH 8.0; Couchman *et al.*, 1996). Some samples were then incubated with 2-2.5 mU of chondroitinase ABC (Seikagaku) at 37°C overnight. Samples were analyzed by HPLC chromatography on Superdex Peptide HR 10/30 column (Amersham Pharmacia Biotech) equilibrated with 0.5 M pyridine acetate, pH 5.0. Aliquots of each fraction were taken for measurement of radioactivity by liquid scintillation counting.

PG preparation. The serum-free media from the cells transfected with recombinant D I/II/III, D IV/V, D IV/V (SGE-), D G3/SGAG, and D G3/SGG were removed from the flasks, any debris removed by low speed centrifugation, and dialyzed against distilled water (DW) for 36-40 h. The dialyzed media were lyophilized and reconstituted in 60 μl of heparinase buffer (0.1 M sodium acetate and 0.1 mM calcium acetate, pH 7.0; Couchman *et al.*, 1996) for each 1 ml of original medium. Recombinant D V, D V (SGE-), and D fV expressed as protein A fusion proteins were purified from medium collected from transfected COS-7 cells by immunoprecipitation with rabbit IgG-agarose beads (Sigma); 1 μl of gel slurry was added to 1 ml of each sample. The mixtures were incubated at RT for 2 h. The beads then were washed 3 times with 1X PBS containing 0.02% sodium azide and once with the heparinase buffer (0.1 M

sodium acetate, 0.1 mM calcium acetate, pH 7.0; Couchman *et al.*, 1996). Each sample was solubilized in 60 µl of heparinase buffer.

Western blotting. Aliquots of 15 µl, with or without digestion by heparinase III (heparitinase, EC 4.2.2.8) and/or chondroitinase ABC (chondroitinase ABC lyase, EC 4.2.2.4, Seikagaku American Inc., Ijamsville, MD) at 37°C for 3-5 h as before (Couchman *et al.*, 1996) were resolved by 3-15% gradient SDS-PAGE, with subsequent transfer to nitrocellulose for immunoblotting as previously described (Couchman *et al.*, 1996). Primary rat monoclonal antibodies used were, G9L1 and H5L5 recognizing domain III of mouse perlecan, C11L1 and A7L6 recognizing domain IV of mouse perlecan or A10L4 recognizing domain V of mouse perlecan (Couchman *et al.*, 1995). Also used were murine monoclonal antibody, F69-3G10 (Seikagaku) which reacts with an HS neo-epitope, generated by digesting HS with heparinase (David *et al.*, 1992; Bai *et al.*, 1994) and 2B6 (Seikagaku) which reacts with a chondroitin 4-sulfate equivalent epitope which remained after chondroitinase ABC digestion (Couchman *et al.*, 1984; Sorrell *et al.*, 1988). Rabbit polyclonal antibodies (diluted 1:1000) R63 and Ey#9 (Couchman *et al.*, 1996) which recognized perlecan core protein, and rabbit antisera against aggrecan core protein (Doege *et al.*, 1997) were also used. Alkaline phosphatase or horseradish peroxidase conjugates of goat anti-mouse IgG or goat anti-rabbit IgG, diluted 1:3000 in the same buffers as the primary antibodies, were used as secondary antibodies for 1 h at RT. Further extensive washing of membranes, were followed by color development (BioRad) or ECL Western blotting analysis system (Amersham Lifescience) according to the manufacturers' instructions.

In some cases, antibodies were stripped from the membranes by submerging the membranes in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.6 mM Tris-HCl, pH 6.7) at 50°C for 30 min with occasional agitation. The membranes were washed for 2 x 10 min in TBS or PBS containing 0.1% Tween 20 (TBS-T or PBS-T) at RT using large volumes of wash buffer, and were then incubated with the ECL detection reagents and exposed to film to ensure that antibodies had been removed. The membranes were washed again to remove the reagents and were subsequently blocked with 5% nonfat dried milk in Tris buffered or phosphate-buffered saline (Zhang and Esko, 1994; Couchman *et al.*, 1996) for 30 min. Immunodetection with new antibodies was then performed as above.

Results

N- and C-terminal halves of mouse perlecan can both be glycanated. The products released by COS-7 cells transfected with the perlecan D I/II/III construct were analyzed by Western blotting with domain specific antibodies. Monoclonal antibody G9L1 recognizes domain III of mouse perlecan (Wu and Couchman, 1997) and reacted with a polypeptide of $M_r \approx 200$ K after heparinase III digestion (Figure 2A, lane H), and weakly after chondroitinase ABC treatment (lane C). A strongly reacting polypeptide was detected after both heparinase III and chondroitinase ABC treatments (lane HC), indicating that some galactosaminoglycan was present on this N-terminal half of perlecan. Material not enzyme treated was not detected, since it did not enter the resolving gel and/or transferred poorly to the membrane. These results confirm earlier data showing that D I/II/III can be HS and/or CS substituted (Costell *et al.*, 1997; Dolan *et al.*, 1997).

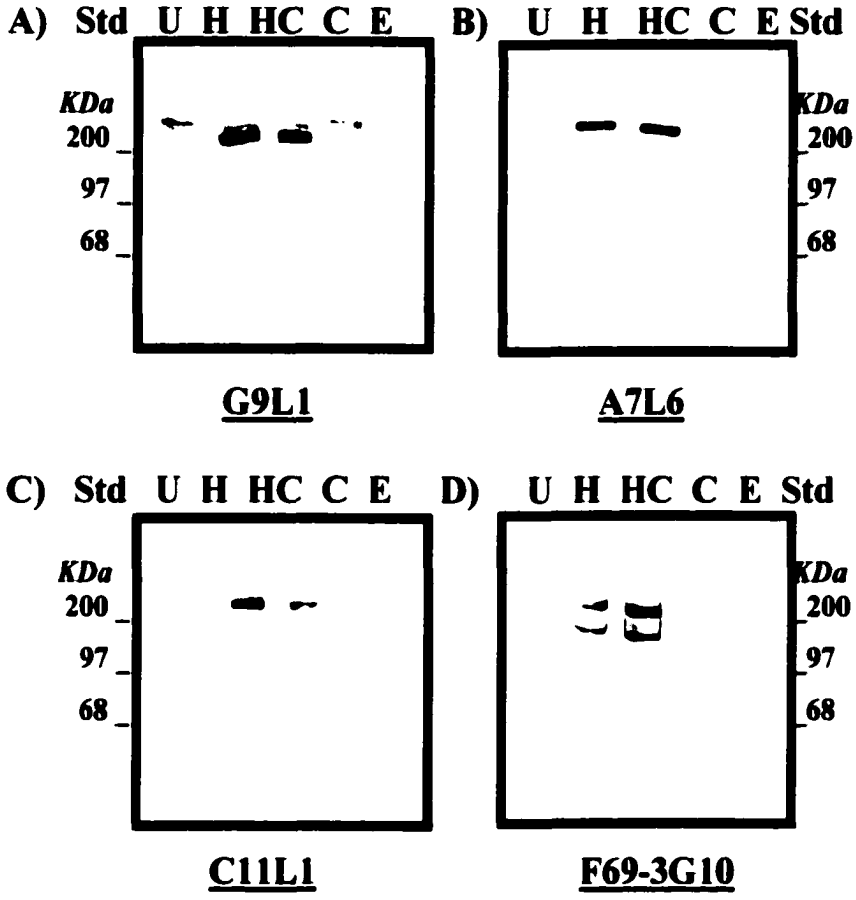

Figure 2. The N- and C-terminal halves of perlecan can both be heparan sulfated and chondroitin/dermatan sulfated. Immunoblotting of recombinant D I/II/III from transfected COS-7 cells with monoclonal antibody G9L1 (A), D IV/V from transfected COS-7 cells with A7L6 (B), C11L1 (C) against perlecan domain IV, and F69–3G10 (D) reacting with HS stubs that remain after heparitinase treatment. In each blot, the PGs are untreated (U), heparitinase-pretreated (H), heparitinase- and chondroitinase-pretreated (HC), or chondroitinase-pretreated (C). Lanes E contain heparitinase and chondroitinase only. Samples in D were reduced and denatured and the migration of molecular mass standards in kilodaltons are shown.

Monoclonal antibodies A7L6 and C11L1 recognize domain IV of mouse perlecan (Wu and Couchman, 1997), and were able to detect D IV/V transfection products after heparinase III treatment (Figure 2, B and C). Undigested material, and similarly sized material after chondroitinase ABC treatment were sometimes detected in these unreduced samples. The results suggested that domain IV/V of mouse perlecan could also be substituted with HS, but products bearing CS/DS chains only were not seen. PG bearing both types of GAG could not be readily resolved in these assays. A monoclonal antibody recognizing the "stubs" remaining after heparinase III digestion (David *et al.*, 1992; Couchman *et al.*, 1995) was used (Figure 2D) to confirm the presence of HS chains. This detected the heparinase III-treated D IV/V product.

Further immunoblots of D IV/V transfection products resolved under reducing conditions were carried out, with monoclonal antibody A10L4 against domain V of mouse perlecan (Figure 3A), or combined antibodies recognizing HS and chondroitin-4 sulfate "stubs" (Couchman *et al.*, 1984; Isamura *et al.*, 1987; Sorrell *et al.*, 1988; David *et al.*, 1992; Bai *et al.*, 1994, Figure 3B). As with A10L4, specific antibody against domain V of mouse perlecan clearly detected D IV/V products after heparinase III, or both enzyme treatments. Further support for this was seen with the antibodies that recognize "stubs," where a discrete polypeptide was detected after both heparinase III and chondroitinase ABC treatments.

GAG analysis of perlecan transfection products. Four transfections of COS-7 cells were carried out in the presence of $^{35}SO_4$ for GAG analysis. The D I/II/III construct yielded chains which resolved as a single population by gel filtration chromatography (Figure 4) and were predominantly HS (Table 1). In contrast, the D IV/V construct

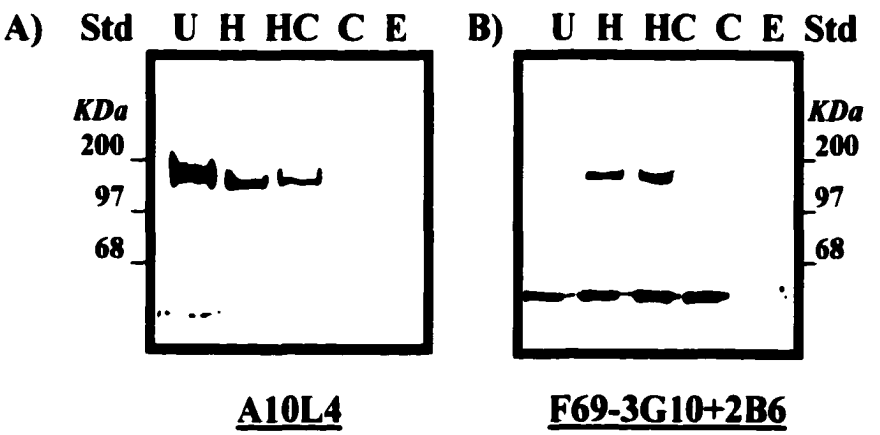

Figure 3. Perlecan domain IV/V is substituted with HS. Immunoblotting of recombinant product D IV/V from transfected COS-7 cells with polyclonal antibodies A10L4 (A) against domain V of mouse perlecan, and monoclonal antibodies F69-3G10 and 2B6 (B) against HS and CS stubs remaining after heparitinase and chondroitinase ABC treatment. All samples were resolved under reduced and heat-denatured condition. In each blot, the PGs were untreated (U), heparitinase-pretreated (H), heparitinase- and chondroitinase-pretreated (HC), or chondroitinase-pretreated (C). Lanes E contain heparitinase and chondroitinase only and molecular mass standard in kilodaltons are shown.

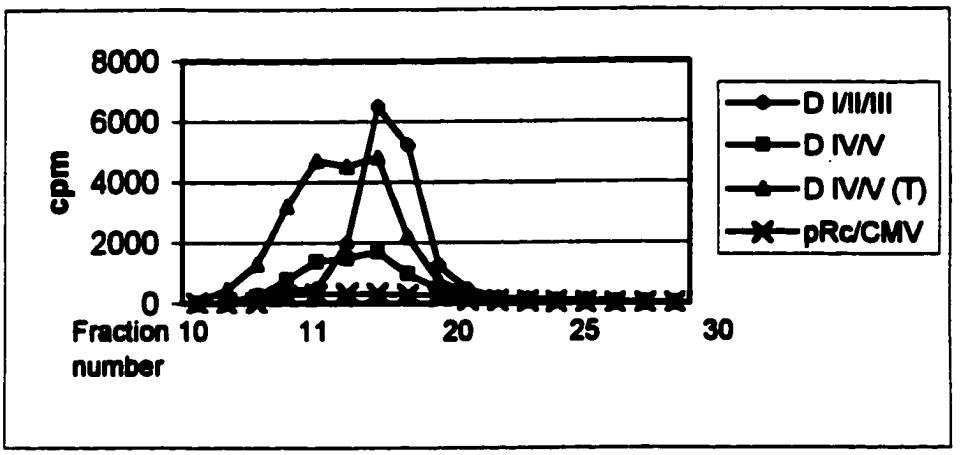

Figure 4. Superose 6 chromatography of $^{35}SO_4$-labeled GAG prepared from recombinant perlecan constructs from transfected COS-7 cells. The recombinant products were isolated from the media using antisera to perlecan coupled to protein G. The bound material was eluted and applied to a column of Superose 6. Aliquots of each fraction were taken for measurement of liquid scintillation counting. TRUNK: Domain IV/V truncated at residue 3593. pRc/CMV: empty vector.

Table 1. GAG composition. Characterization of the GAG type on the $^{35}SO_4$-adiolabeled products of the D I/II/III, D IV/V, D IV/V (T), and D V (SGE-) constructs (CS, chondroitin sulfate; HS, heparan sulfate)

Construct	CS	HS
D I/II/III	19-27%	78-81%
D IV/V	66-79%	21-34%
D IV/V (T)	96-98%	2-4%
D V (SGE-)	100%	-

yielded about one-third HS, and two-thirds galactosaminoglycan, resolving as a broad peak by gel filtration, with some longer chains than those of the D I/II/III constructs. A control, empty vector construct pool (pRc/CMV) yielded very little material (Figure 4), representing the very small pool of endogenous PG. One further construct used consisted of D IV/V truncated at residue 3550, therefore missing the most C-terminal SGE sequence. This was still glycanated, but almost exclusively with CS/DS chains which yielded a heterogeneous population by gel filtration chromatography. The data suggested that the SGE site in domain V (serine residue 3593) may be HS substi- tuted, while other sites were predominantly CS/DS-bearing.

The C-terminal SGE sequence of perlecan domain V can bear HS chains. Four approaches were used to define serine residue 3593 as a potential glycanation site. First, the whole of domain V (D V) and the C-terminal one-third of domain V (D fV), containing the SGE sequence, were subcloned into a protein A-containing vector

and expressed as fusion proteins in COS-7 cells. Precipitated recombinant proteins were analyzed by immunoblotting with a polyclonal anti-perlecan antibody (Figure 5A) and the HS "stub" antibody (Figure 5B). Both D V and D fV were detected, and both appeared to be preferentially HS substituted. In particular, HS stubs were apparent on D fV, containing serine 3593 (Figure 5B).

The second experiment was to delete the SGE from the D IV/V construct (D IV/V SGE-). Products from COS-7 cell transfections were analyzed as before, immunoblotting with polyclonal antibody Ey#9 or a mixture of monoclonal antibodies recognizing HS and CS "stubs" remaining after appropriate enzyme treatments. In both cases, polypeptides were clearly detected after chondroitinase ABC treatment (Figure 6, A and B, arrowheads), but not after heparinase III treatment, indicating a preponderance of galactosaminoglycan substitution in domain IV and V of perlecan when the C-terminal SGE in domain V was deleted.

Third, whole domain V (D V) and domain V with the SGE deleted (D V SGE-) were subcloned into pRK5 vector and expressed as protein A-containing fusion proteins. Immunoblot analysis showed strikingly that with the SGE present, HS substitution predominated, while in its absence, the fusion proteins were exclusively CS/DS substituted (Figure 7). These results confirmed serine 3593 as a predominantly HS substitution site, and its absence appears to increase the extent of CS/DS substitution, both in D IV/V, and in D V constructs.

Fourth, a series of constructs were made in which small segments from domain IV and V were spliced between the signal peptide and domain II/III of murine perlecan. The purpose of expressing of these constructs in both COS-7 and CHO cells, was to determine, in a high efficiency expression system, whether specific ser-gly dipeptides

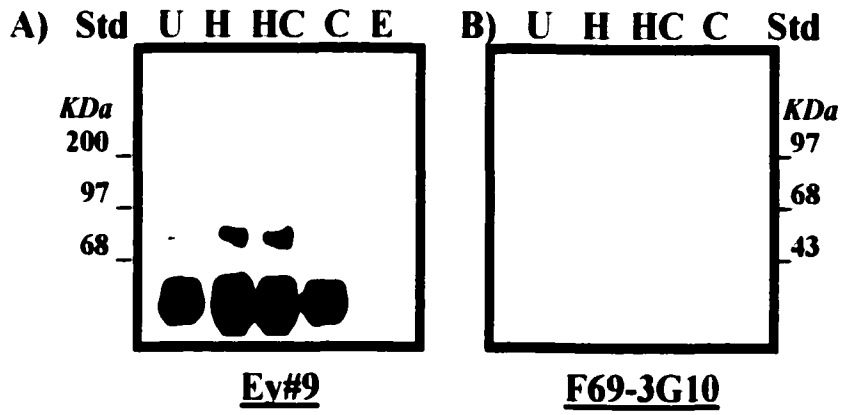

Figure 5. Domain V alone can be substituted with HS. Immunoblotting of recombinant D V with polyclonal antibody Ey#9 (A) against perlecan core protein, and D fV (B) with monoclonal F69-3G10 anti-HS stub antibody (B). In each blot, the PGs are untreated (U), heparitinase-pretreated (H), heparitinase- and chondroitinase-pretreated (HC), or chondroitinase-pretreated (C). Lanes E contain heparitinase and chondroitinase only and lanes Std are standard marker.

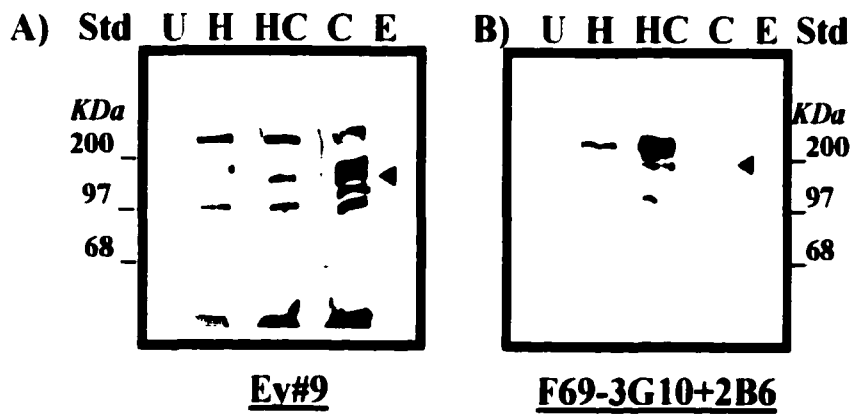

Figure 6. Recombinant perlecan D IV/V (SGE-) contains CS/DS. Sample were immunoblotted with polyclonal antibody Ey#9 or monoclonal F69-3G10 and 2B6 antibodies. Sample were untreated (U), heparitinase-pretreated (H), heparitinase- and chondroitinase-pretreated (HC), or chondroitinase-pretreated (C). Enzymes alone are shown in E. Arrowheads denote the expected recombinant products. Molecular mass standards in kilodaltons are shown.

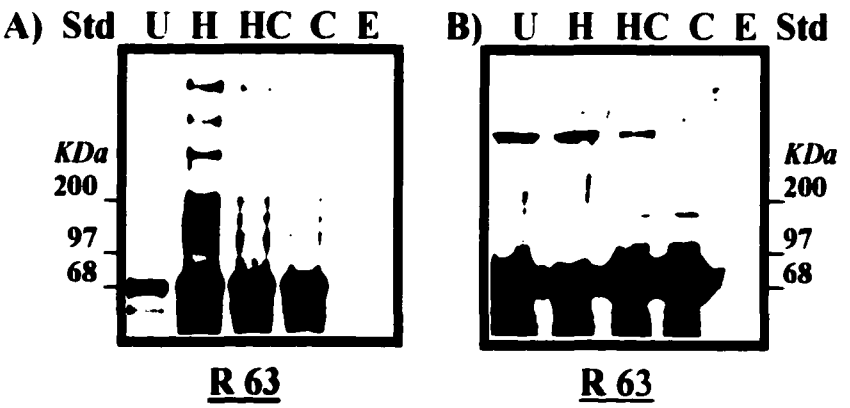

Figure 7. Recombinant D V (A) is HS substituted while D V (SGE-) (B) is CS/DS. COS-7 cell recombinant proteins were blotted with polyclonal antibody R63 against perlecan core protein. In each blot, the PGs are untreated (U), heparitinase-pretreated (H), heparitinase- and chondroitinase-pretreated (HC), or chondroitinase-pretreated (C). Higher order oligomers have formed of the recombinant protein are observed. Lanes E contains heparitinase and chondroitinase only and molecular mass standards in kilodaltons are shown.

could be glycanated. Six constructs were made, from which recombinant proteins could be immunologically detected in only four (oligos 2, 3, 5, and the D II/III constructs). HS substitution in the construct containing the C- terminal serine 3593 (oligo 5) could be readily detected in both cell types (Figure 8, B, E, and F). The data provided further substantiation that this serine residue is favorable for HS chain addition. This substitution can, therefore, take place when the region of perlecan is out of its structural context, as well as within; furthermore, it can also occur in different cell types. GAG substitution, could not, however, be detected in any of the other three constructs where protein expression occurred.

We also noted that complete domain IV/V constructs could not be detected in CHO cells, potentially resulting from extensive proteolytic processing of this region. Full- length endogenous perlecan could not be detected for similar reasons.

Analysis of perlecan domain IV for glycanation. Murine and human perlecan domain IV contain numerous potential sites for glycanation (Noonan *et al.*, 1991; Kallunki and Tryggvason, 1992; Cohen *et al.*, 1993; Iozzo, 1998;). However, a detailed analysis of all the potential substitution sites is hampered by the large size of this domain, and the extreme difficulty in expressing this domain recombinantly on its own, but in its entirety. To circumvent these problems, a segment, corresponding to Ig repeats (6.5-12.5) of human perlecan was cloned from a L2 rat yolk sac λZAP cDNA library. Two clones were obtained from screening with the R63 polyclonal antibody to give the predicted amino acid sequence shown in Figure 9. This sequence of domain IV corresponds to sequence not contained in the original 12 kb cDNA cloning of murine perlecan, and later described as a region of potential alternate splicing (Couchman *et al.*,

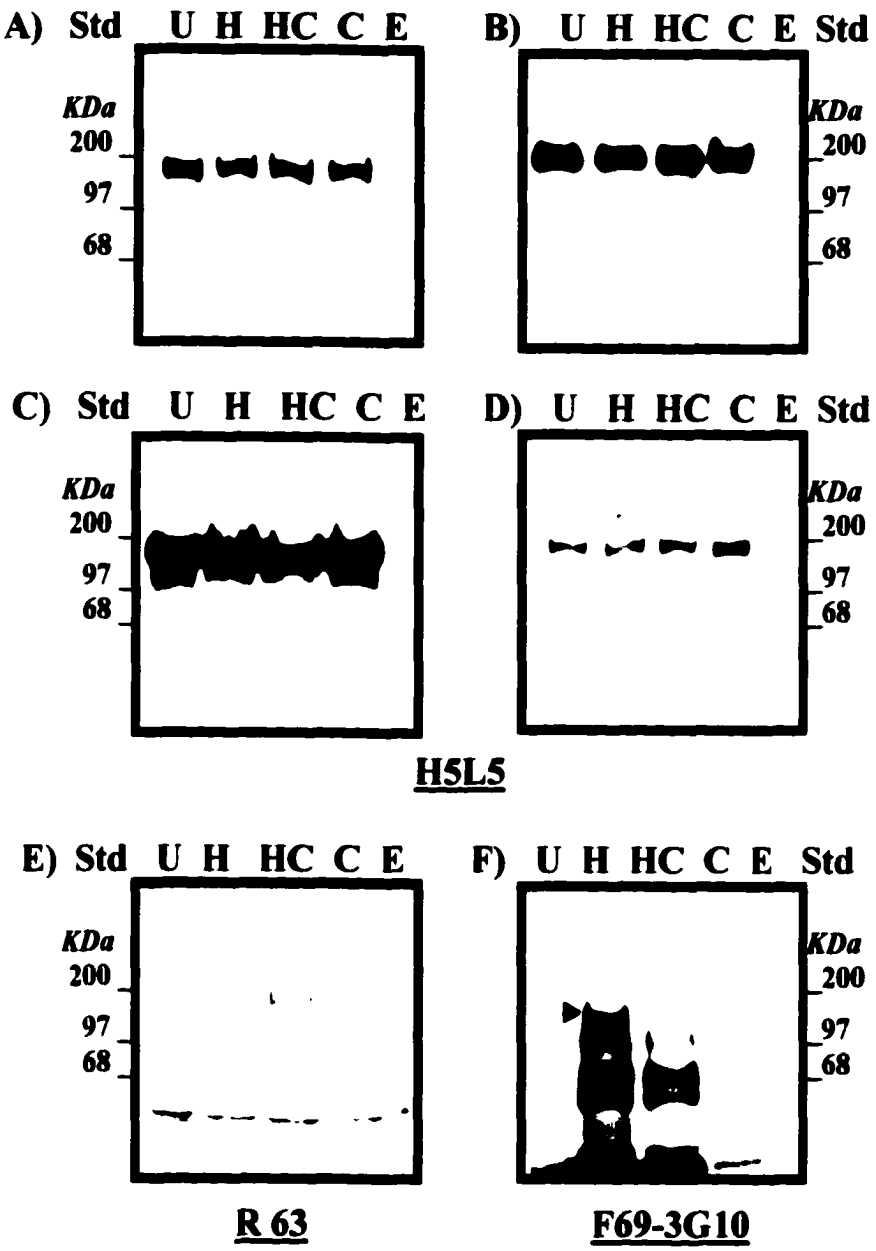

Figure 8. The SGE (residues 3593-3595) of domain V can be glycanated when translocated to perlecan domain II/III. Immunoblotting of D II/III containing Oligo 2 (A), 3 (B), 5 (C), and D II/III alone (D) from transfected cells with monoclonal antibody H5L5 against domain III of mouse perlecan core protein. Recombinant product Oligo 5 from transfected CHO cells immunoblotted with polyclonal antibody R63 (E) against perlecan core protein, then stripped and reprobed with monoclonal antibody F69-3G10 (F, arrowhead) against HS stubs. In each blot, the PGs are untreated (U), heparitinase-pretreated (H), heparitinase- and chondroitinase-pretreated (HC), or chondroitinase-pretreated (C). Lanes E contain heparitinase and chondroitinase only and molecular mass standards in kilodaltons are shown.

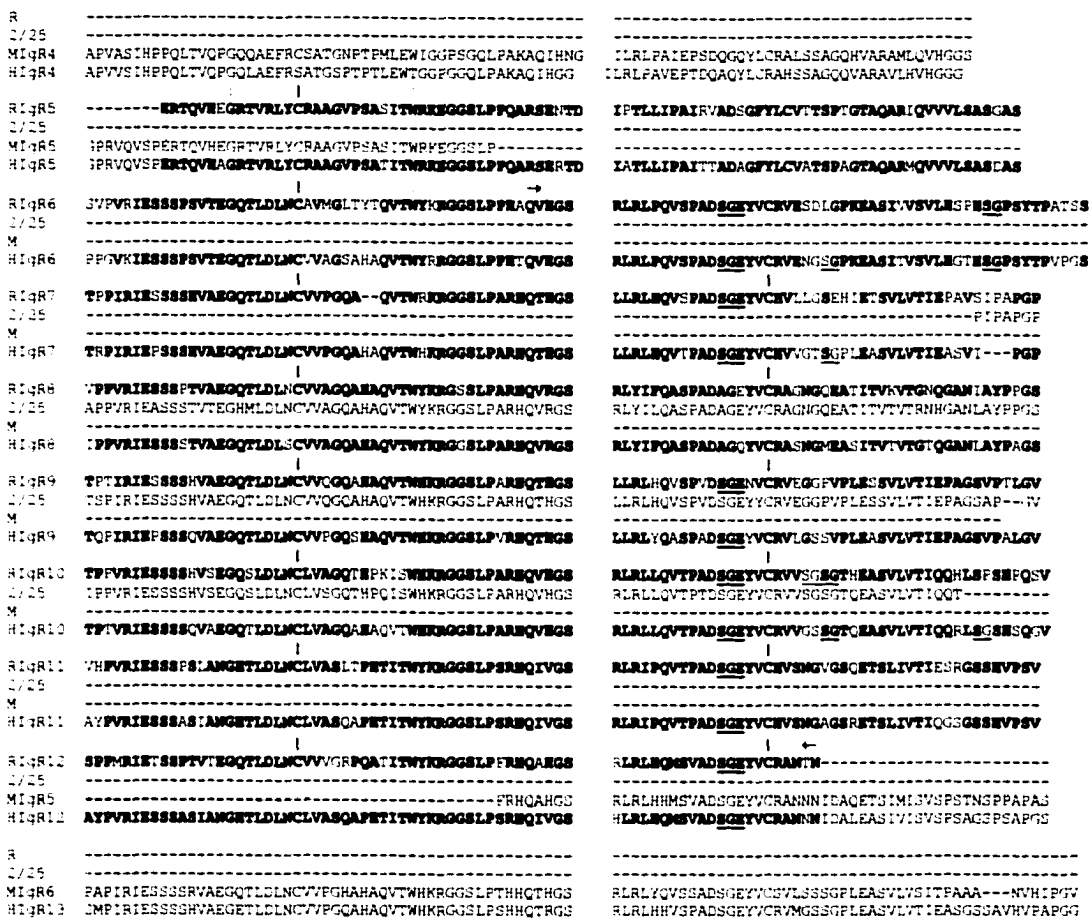

Figure 9. Partial sequence of rat domain IV, and alignment with human and murine sequences. Alignment of the Ig-like repeats (repeats 4-13) of rat (RIgR), murine clone 2 and 25 (2/25) and repeats 4-6 (MIgR), and human (HIgR) perlecan domain IV sequences. → indicates starting amino acid residue (2090) for construct P5a, ← indicates ending amino acid residue (2702) of the construct. Bold letters represent identical rat and human sequences. SG, SGE, and SGXG residues of the rat, and human sequences are underlined.

1996). Repeat 6.5-12.5 of the rat cDNA was subcloned into the pRK5 vector and expressed as protein A-fusion proteins in COS-7 cells. The immunoprecipitated fusion proteins were probed with R63 antibody after the same enzyme treatments as before. Despite the presence of one ser-gly dipeptide in each repeat, and one ser-gly-x-gly sequence, known to be favorable for glycanation (Bourdon *et al.*, 1987), no GAG chain substitution could be detected on the recombinant product present in the media (data not shown). This data suggests either that all glycanation is in domain V of perlecan, or that this C-terminal region of perlecan domain regulates glycanation of the upstream domain IV.

The SGAG sequence of domain V can be a substitution site for CS/DS. To confirm the CS/DS substitution on D V (SGE-), recombinant products were radiolabeled with D-[6-^{3}H] glucosamine HCl. pRK5/protein A vector with no insert was used as a control. The D V (SGE-) construct yielded PG product, which resolved as a single population by Superdex Peptide HR 10/30 column (Figure 10). After treatment with chondroitinase ABC, all of the radiolabel eluted in low MW fractions (Figure 10 and Table 1). These data confirm the presence of CS/DS GAGs on D V in the absence of the SGE sequence (residues 3593-3595).

Two possible CS/DS substitution sites, SGAG (residues 3250-3253) and SGG (residues 3510-3512) from domain V were tested by cloning approximately 154 and 188 bp, respectively, of cDNAs from PCR into aggrecan G3 domain construct. This construct has been shown to promote GAG substitution sites to the CS region of aggrecan or domain I of perlecan (Doege *et al.*, 1997). The recombinant constructs were transfected into COS-7 cells and the secreted recombinant products were examined by

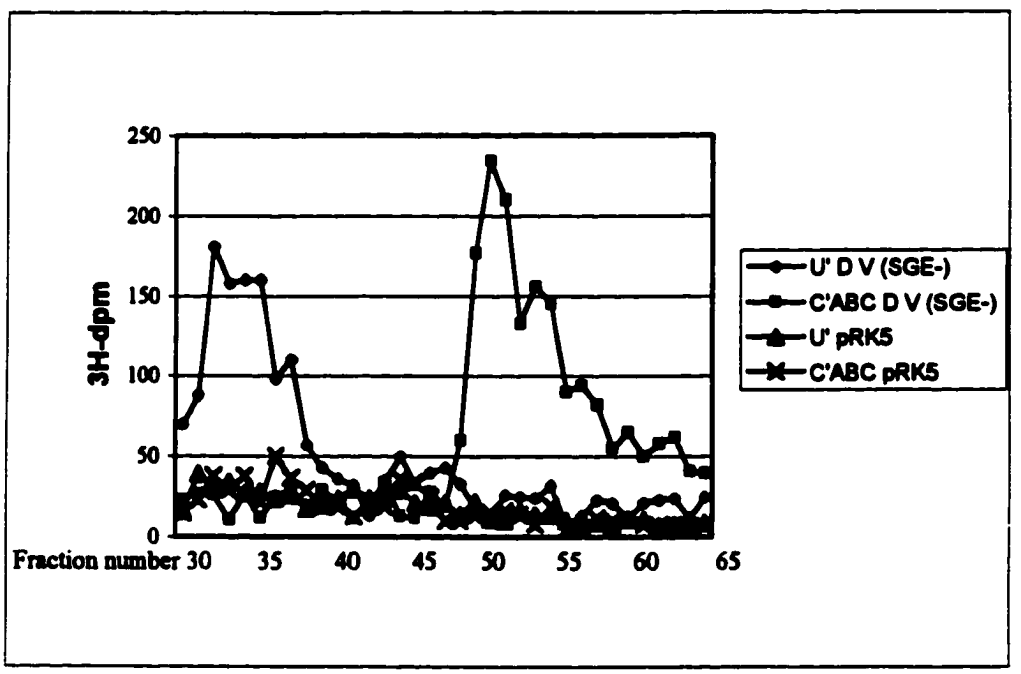

Figure 10. Perlecan domain V (SGE-) can be galactosaminoglycan substituted. Superdex Peptide HR 10/30 HPLC shows the elution positions of intact and chondroitinase-digested materials of recombinant pRK5/protein A and D V (SGE-) products. U', untreated materials; C'ABC, chondroitinase-treated materials.

immunoblotting with rabbit antisera against aggrecan (Figure 11). The products of D G3 (Figure 11A) and D G3/SGG constructs (Figure 11B) were detected, with no effect of enzymatic treatment, as polypeptides of approximately 29 kDa and 35 kDa. The D G3/SGAG recombinant products were resolved on the gels as smears (Figure 11C, lanes U and H), which appeared as much sharper products after chondroitinase ABC treatment (Figure 11C, lanes HC and C). Heparinase III had no effect, indicating that while residues 3510-3512 could not be glycanated, the SGAG representing residues 3250-3253 of domain V could be CS/DS substituted.

Discussion

These studies show perlecan can be substituted with both HS and CS/DS in the C-terminal half of the core protein. Expression of domain IV and V combined or domain V only in COS-7 cells yielded glycanated products. These were analyzed by immunoblotting with GAG- and core protein-specific antibodies, as well as by metabolic labeling and gel filtration chromatography. Taken together, the results indicate that a SGE residue (serine 3593) located on the last globular domain toward the C-terminal end of domain V (LAM G3 module; Iozzo, 1998), is preferentially HS substituted. This substitution was seen in two different cell lines and occurred both when this region of perlecan was present in its normal context, and also when a SGEARLVSED amino acid region including serine 3593 was translocated to a different site adjacent in domain II of perlecan. Therefore, this region alone contains sufficient information for glycanation. However, when this region was unavailable for substitution (in C-terminal deleted constructs of domain V alone or domain IV/V), CS/DS substitution was observed at site(s) within domain IV and/or V. Two fragments of domain V perlecan core protein

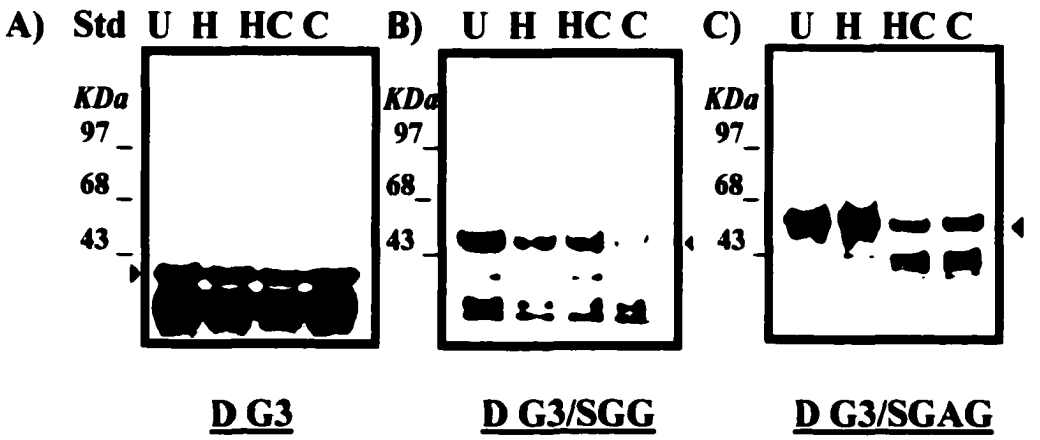

Figure 11. SGAG sequence on domain V can be CS/DS substituted. Immunoblotting of D G3 (A), D G3/SGG (B), and D G3/SGAG (C) from transfected COS-7 cells with rabbit antisera against aggrecan core protein. Sample were untreated (U), heparitinase-pretreated (H), heparitinase- and chondroitinase-pretreated (HC), or chondroitinase-pretreated (C). Arrowheads indicate the expected recombinant products. Molecular mass standards in kilodaltons are shown.

containing SGAG (serine 3250) and SGG (serine 3510) sequences were tested individually to identify these site(s). We found that the SGAG sequence on the second globular domain on domain V (LAM G2 module; Iozzo, 1998) can be substituted with CS/DS GAGs. It is interesting that such substitution was apparently upregulated by removal of a more C-terminal core protein fragment. This region was not immediately adjacent (in terms of primary structure) to the chondroitin/dermatan sulfated region. In contrast, when a portion of rat perlecan domain IV containing several SG dipeptides and one SGXG site was recombinantly expressed in the COS-7 cells, no glycanation resulted. In other core proteins, SGXG motifs are suitable for glycanation (Bourdon *et al.*, 1987), but this is apparently not the case for the Ig repeats of perlecan domain IV. This could be explained by the predicted secondary structure of domain IV that the SGE containing regions exist in between the Ig loops, but perhaps not exposed to the surface of the native protein. This may prevent the glycanation.

It is also appreciated that while acidic and hydrophobic residues within about 10-12 residues of SG dipeptides can favor glycanation (Zhang and Esko, 1994; Dolan *et al.*, 1997), recent work has shown that there may be long distance effects on both the extent and specificity of such substitution (Doege *et al.*, 1997; Dolan *et al.*, 1997). The nature of these long distance effects is not understood, but may operate in the C-terminal region of perlecan. Clearly the possession of SG dipeptides or SGXG motifs in potential favorable contexts, while necessary, are not sufficient for glycanation. It is perhaps noteworthy that each DSGEY in the rat domain IV sequence examined had an arginine 9, and usually 11, residues upstream. Similarly the single SGXG motif had an arginine four residues upstream. Therefore, while acidic residues in these locations may be favorable for glycanation, basic residues may prevent substitution. Moreover, the SGXG

motif in domain IV, while common to murine and rat sequences, is not conserved in the human homologue (Figure 9). Others have also shown a lack of glycanation of domain IV (Hopf *et al.*, 1999).

Previous studies have shown that GAG side chains can be attached to domain I of perlecan but not to domain II or III (Dolan *et al.*, 1997). A previous report also suggested HS and/or CS substitution of the C-terminal portion of perlecan although the sites were not identified (Brown *et al.*, 1997; Ettner *et al.*, 1998). It therefore appears that both ends of the core protein (domain I and V) can bear GAGs, with as yet unknown effects on function. Agrin has a C-terminal domain homologous in structure to that of perlecan domain V, and while it contains SG dipeptides, none appear to be very favorable for substitution based on flanking amino acid motifs (Rupp *et al.*, 1991; Tsim *et al.*, 1992; Patthy and Nikolics, 1994).

Perlecan is widespread basement membrane and ECM component. It is believed to interact with other matrix components such as entactin/nidogen, laminins, type IV collagen, thrombospondin, fibronectin, fibulin, and α-dystroglycan (Visher *et al.*, 1997; Giry-lozinguez *et al.*, 1998; Hopf *et al.*, 1999; Talts *et al.*, 1999). It may also interact with integrin receptors, though these have not been specifically identified (Battaglia *et al.*, 1993; Chakravarti *et al.*, 1995). Murine perlecan, but not human, contains an RGD motif in domain III which can promote cell adhesion (Kallunki and Tryggvason, 1992; Noonan *et al.*, 1991; Murdoch *et al.*, 1992; Chakravarti *et al.*, 1995). In some cases, integrin-perlecan interactions may be HS dependent, though the reasons for this are unclear (Battaglia *et al.*, 1993; Whitelock *et al.*, 1999). Additionally, perlecan may sequester heparin-binding GFs (e.g., FGF-2) through its HS chains, and basement membranes in vivo have been demonstrated to be a site of these potent molecules

(Aviezer *et al.*, 1994; Guillonneau *et al.*, 1996; Whitelock *et al.*, 1996). In the light of our data, it could be interesting to know what the impact of alternate sites of glycanation is on the biology of perlecan. While the functional attributes of domain V glycanation reported here are still far from clear, it has been reported that domain V derived perlecan is heparin-binding. A possibility is, therefore, that self association of perlecan (Yurchenco *et al.*, 1987) is affected by glycanation in this region. Additionally, perlecan domain V binds to α-dystroglycan (Peng *et al.*, 1999; Talts *et al.*, 1999). The dystroglycan-perlecan complex then serves as a receptor for acetylcholinesterase enzyme enabling the enzyme to be localized at the synapse (Peng *et al.*, 1999). This interaction and interactions with other ECM molecules may be similarly influenced.

In addition to perlecan, three other basement membrane PGs have been characterized and sequenced. As far as is known, agrin and collagen XVIII are only substituted with HS chains (Tsen *et al.*, 1995; Halfter *et al.*, 1998), while bamacan (*ba*sement *m*embrane-*a*ssociated *c*hondroitin sulfate proteoglyc*an*) is only galactosaminoglycan substituted, both in vivo and in vitro (Couchman *et al.*, 1996; Wu and Couchman, 1997). Perlecan is, therefore, different in its variability. It is, moreover, ubiquitous in mammalian basement membranes, in contrast to the other two PGs, and found in other connective tissues (Murdoch and Iozzo, 1993; Iozzo, 1998). Apparently perlecan core protein has properties independent of GAG chains, but the large hydrodynamic volume of these chains suggests that where present, they can profoundly influence the properties of the macromolecule. It is therefore important in the future to ascertain the patterns of perlecan glycanation in tissues with respect to development, function and disease.

Acknowledgments

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Footnotes

Abbreviations used: GAG, glycosaminoglycan; HS, heparan sulfate; CS, chondroitin sulfate; DS, dermatan sulfate; LDL, low density lipoprotein; N-CAM, neural cell adhesion molecule; EGF, epidermal growth factor; ESH, Engelbreth-Holm-Swarm; DMEM, Dulbecco's modified Eagle's medium; DW, distilled water; PBS, Dulbecco's phosphate-buffered saline; TBS, Tris-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FGF, fibroblast growth factor.

DETECTION OF PROTEOGLYCAN CORE PROTEINS WITH GLYCOSAMINOGLYCAN LYASES AND ANTIBODIES

by

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Introduction

PGs are quite abundant components of many ECMs, while most cell surfaces also bear these macromolecules. Frequently, the profiles are complex. For example, several members of the syndecan and glypican families of cell surface HSPGs may be present on a single cell type (David *et al.*, 1992; Bernfield *et al.*, 1999). Some ECMs (e.g., from brain) may also contain a variety of PGs, including several members of the hyalectans or aggregating PGs such as neurocan, brevican, and versican (Iozzo, 1998). Frequently, it is useful to monitor the nature and variety of PGs in a pool from tissues or cell cultures in a simple manner before moving on to further purification steps, use of core protein-specific antibodies, or pursuit of a potentially novel core protein.

While PGs require some specialized techniques for analysis, advantage can be taken of their glycanation to identify core proteins, even when their precise characteristics remain unresolved. Specific enzymes are readily available, first from bacterial sources but more recently of recombinant origin, which selectively degrade GAGs. Chondroitinase ABC will degrade virtually all CSs and DSs, while leaving HS and KS chains intact. Conversely, heparitinase enzymes will degrade nearly all forms of HS, while unable to degrade CS, DS, or KS (Figure 1). Further, the consequences of GAG removal can be monitored by SDS-PAGE. The heterogeneous nature of PGs largely ensues from the variable number, length, and charge of GAGs in a pool of a single core protein (e.g., aggrecan from cartilage or perlecan from a basement membrane preparation). PGs are frequently seen as broad smears or sometimes, when large, may not even penetrate a 3% resolving gel (Figure 2A). Once GAG lyases have removed most of the chains, the core proteins become much more readily resolved by SDS-PAGE as discrete polypeptides (see Figure 2).

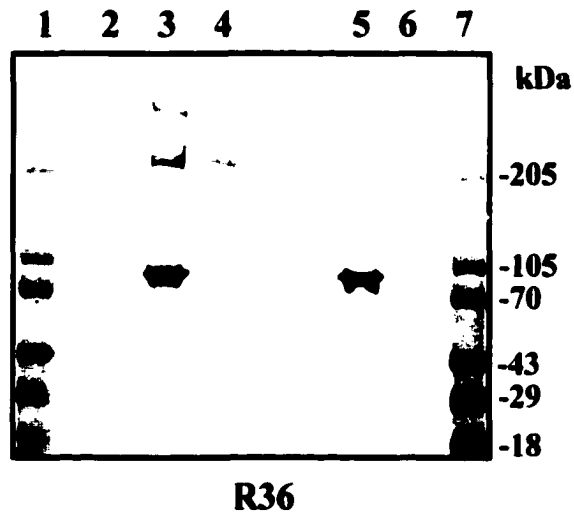

Figure 1. Detection of CS/DS PG core proteins from EHS tumor with R36, a polyclonal antibody recognizing CS/DS stubs remaining after chondroitinase ABC treatment. Lanes 1 and 7 are standards whose MW in kilodaltons is indicated. Lane 2, untreated sample; lane 3, sample treated with chondroitinase ABC and heparinase II and III; lane 4, sample treated with chondroitinase ABC only; lane 5 contains heparinase II and III only, while lane 6 contains chondroitinase ABC alone. The common polypeptide seen in lanes 3 and 5 is present in the heparinase II preparation. The data show that a CS/DS PG with a core protein of $M_r \approx$ 200,000 (in lanes 3 and 4) is accompanied by a second, large core protein that is revealed only after additional heparinase treatment. This, therefore, represents a hybrid form of perlecan bearing both HS and CS/DS chains.

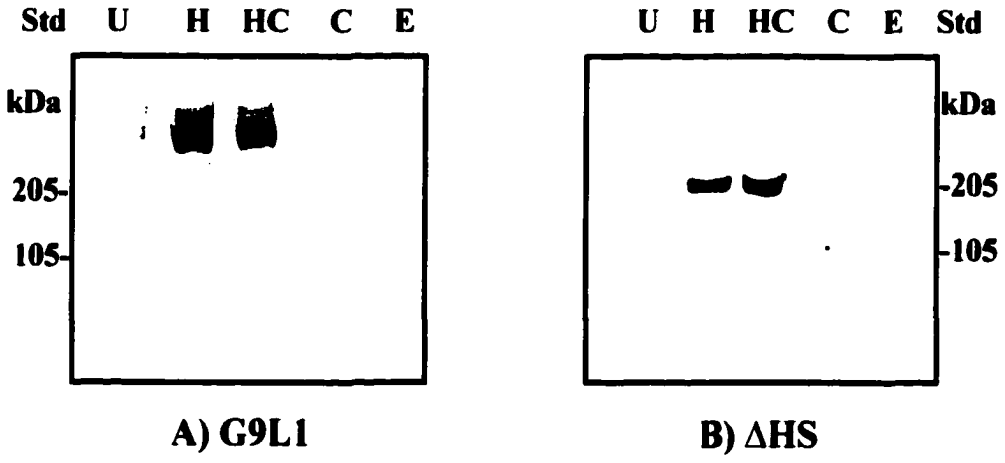

Figure 2. Detection of intact murine perlecan (A) and recombinant domain IV-V of mouse perlecan transfected into COS-7 cells (B) with a rat monoclonal antibody specific to perlecan core protein (A) and monoclonal Δ HS antibody recognizing HS stub after heparinase III (heparitinase) treatments (B). A shows that intact perlecan core protein is not clearly visible until the HS chains have been removed, while B shows HS substitution on the recombinant perlecan. In each blot, the PGs are untreated (U), heparitinase pretreated (H), heparitinase and chondroitinase pretreated (HC), or chondroitinase pretreated (C). Lanes E contain heparitinase and chondroitinase enzymes only.

Chondroitinases and heparitinases are eliminases, so that the remaining core proteins have serine (usually) residues bearing not only the stem oligosaccharide (xylose-galactose-galactose-uronic acid) but also a disaccharide or larger oligosaccharide with a terminal unsaturated uronic acid residue. This, it turns out, is quite antigenic; monoclonal (Couchman *et al.*, 1984; Tapanadechopone *et al.*, 1999) and polyclonal antibodies have been raised (Couchman *et al.*, 1996) which recognize the carbohydrate "stubs" remaining after chondroitinase or heparitinase treatments. They are also very specific. An antibody recognizing an HS "stub" with a terminal unsaturated uronic acid residue will not recognize the equivalent "stub" generated by a chondroitinase enzyme, and vice versa. Therefore, the combined use of enzymes and antibodies can be used (e.g., in Western blotting) to estimate the sizes of core proteins and the type of GAG present. This can be particularly useful where a particular core protein (e.g., perlecan), can be substituted with HS and/or CS chains (Figure 1). It can provide evidence of hybrid PGs that bear more than one GAG type. Further, since the antibodies do not recognize core protein epitopes, a mixed population of HS and/or CS and DS PG can be quickly analyzed for their number, size, and glycanation profiles. Such evidence can be supported by more traditional metabolic labeling methods and combined with chemical or enzymatic degradation techniques, followed by gel filtration analysis.

Materials

1. Samples for analysis dissolved in suitable buffers.
2. Buffers:
    a. Heparitinase buffer: 0.1 M sodium acetate, 0.1 mM calcium acetate, pH 7.0.

b. Chondroitinase buffer: 50 mM Tris, 30 mM sodium acetate, 20 mM EDTA, 10 mM NEM, 0.2 mM PMSF, and 0.02 sodium azide, pH 8.0 (see note 1).

3. Protease inhibitor for all heparitinase treatments (see note 2):

   10X trypsin inhibitor type III-O (ovomucoid 100 μg/ml, Sigma).

4. GAG lyases (Seikagaku or Sigma):

   a. HS: heparinase III (EC 4.2.2.8). This enzyme is also known as heparitinase and heparitinase I.

   b. CS and DS: chondroitin ABC lyase (EC 4.2.2.4).

   c. CS: chondroitin AC II lyase (EC 4.2.2.5).

   d. DS: chondroitin B lyase (no EC number).

5. GAG carriers (optional): CS type A (chondroitin 4-sulfate) or type C (chondroitin 6-sulfate), HS (Sigma).

6. 2X SDS-PAGE s ample buffer (Sigma) with or without reducing agent (e.g., 40 mM dithioerythreitol).

7. Prestained protein MW standards (Sigma or BioRad).

8. SDS-PAGE gels. If a wide size range of core proteins is suspected, 3-15% gradient gels can be useful.

9. Electroblotting and transfer buffers and apparatus.

10. Transfer membrane: 0.45-μm nitrocellulose (BioRad Trans-Blot® Transfer Medium or Schleicher & Schuell #BA85), PVDF (Millipore Immobilon P), or positively charged nylon (BioRad Zetabind) membranes.

11. Blocking buffer: 5% nonfat dried milk in 0.1% Tween 20 is optional in PBS (TPBS).

12. Diluting buffer: 1% nonfat dried milk, 0.1% BSA, and 0.1% (v/v) Tween 20 in PBS (for monoclonal antibodies) or TBS (for polyclonal antibodies).

13. Primary antibodies recognizing carbohydrate "stubs" (Seikagaku):

    a. Monoclonal Δ HS (for HS GAG).

    b. Monoclonal anti-PG Δ di-0S, -4S, -6S (for CS/DS GAGs, see note 3).

    These antibodies are also available as biotin conjugates.

    c. Equivalent antibodies recognizing protein of interest.

14. Secondary antibodies: horseradish peroxidase- or alkaline phosphatase- anti-Ig conjugate. Alternately, streptavidin-horseradish peroxidase conjugate should be used where the primary antibodies are biotin conjugates.

15. Chromogenic and chemiluminescence visualization system: e.g., ECL™ Western blotting detection reagents (Amersham Pharmacia Biotech) for peroxidase conjugates or alkaline phosphatase-conjugate substrate kit (BioRad).

Methods

1. Divide the PG sample to be analyzed into equal aliquots. The number depends on the enzyme treatments to be performed. For example, if a PG pool is suspected to contain CS and HS PGs, four aliquots should be used. One sample is left untreated, while others receive chondroitinase ABC, heparitinase, or both enzyme treatments. Ideally, each sample should contain 1-10 μg PG. The choice of buffer depends on the enzymes to be used (see note 1). Further controls contain buffer with enzyme only (no PG).

2. Treat samples with appropriate enzymes at 37°C. The amount and duration of enzyme treatment depend on the PG concentration. For 1-10 μg PG, a 2- to 3-h incubation with 1-2 mU chondroitinase ABC or 0.5-1 mU heparitinase in the presence of protease inhibitor (1X ovomucoid, see note 2) is suggested. Where concentrations of PG are

higher, adding a second aliquot of enzyme after 2 h for a further incubation can be beneficial. Where PG concentrations are very low (below 100-200 ng per sample), approximately 0.5 μg of appropriate free GAG can be added as carrier to aid efficiency and recovery (but see note 4).

3. If enzyme activity needs to be verified, set up samples of free GAG (approximately 0.5 mg/ml) in buffer, to which the enzymes are added, and incubate simultaneously. Enzyme activity is monitored spectrophotometrically at 232 nm.
4. Enzyme treatments are terminated by adding SDS-PAGE sample buffer (with or without reducing agent, see Materials) and heating to 100°C if required. Samples can be frozen at –20°C or immediately resolved by SDS-PAGE.
5. Samples are applied to SDS-PAGE gels for conventional electrophoresis and transfer to nitrocellulose or other medium (note 5). If a range of core protein masses is suspected or not known, acrylamide gradients are preferable (e.g., 3-15%).
6. Membranes are blocked conventionally (for example, in 5% dried milk powder in phosphate-buffered saline for at least 1 h). They are then probed with monoclonal or polyclonal antibodies recognizing carbohydrate "stubs" created by GAG lyases. These are available as purified IgG and sometimes in biotinylated form and should be used at 10-25 μg/ml. Incubation can be at 4°C overnight at room temperature or 37°C for shorter periods of at least 1 h. Constant gentle agitation is advised.
7. Thorough washing is followed by secondary antibody (e.g., affinity-purified goat anti-mouse IgG conjugated to horseradish peroxidase) in the same buffer for 1 h at room temperature. Antibody concentrations should be in accordance with manufacturer's instructions. Extensive washes are then followed by visualization as preferred, such as chemiluminescence.

Notes

1. A suitable buffer for chondroitinase ABC or AC II is listed in Materials, as is one suitable for heparitinase (also known as heparinase III). However, where samples are to be treated with both enzymes, we have found the heparitinase buffer to be suitable. It should be noted that chondroitinase B is inhibited by phosphate. Heparinase activity is increased by the presence of calcium ions, but it is reported that the activity of heparinase III is not much decreased by its absence.

2. Polysaccharide lyases are primarily of microbial origin. Protease contamination can be present in the enzyme preparation (especially all heparinase enzymes). This can cause misleading results. Thus, protease inhibitor should be added in case of heparitinase treatments. Chondroitinase ABC is available in protease-free form.

3. Separate, specific antibodies are available which, while all recognizing the terminal unsaturated uronic acid residue as described, have specificity for the presence and position of sulfate on the adjacent galactosamine residue. The three antibodies can be used combined. At the current time they are only available separately. Most commonly, the prevalence of sulfation is 4S > 6S > 0S.

4. We have found that the use of chondroitinase ABC and heparinase III together leads to a less efficient identification of CSPG core proteins than the use of the former enzyme alone. The reasons are not clear, but it may be that products of HS lyases are slightly inhibitory to chondroitinase enzymes. It is known that heparin will inhibit chondroitinases and should therefore not be used as a carrier.

5. Intact PGs transfer poorly to nitrocellulose or similar membrane. The more GAG present on a core protein, the more difficult it becomes. This is a result of high mass, as well as charge. Therefore, while decorin with one chain can be quite efficiently transferred, aggrecan with >100 chains may not. Transfer to cationic membranes can enhance PG capture.

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CONCLUSIONS

Many discoveries during the past decade have improved our understanding of the molecular process of malignant transformation and tumor progression. This is a large and complex issue, which has been studied for years in order to develop basic molecular insight to find new treatments for clinical problems. It is my intention that this project and some other relevant projects will be part of providing some of the missing jigsaw pieces, and will add to that already available about the basic knowledge of the molecular biology of cancer. The experiments have been performed in a manner intended to withstand further testing and to facilitate further experimentation beyond the scope of this project.

The focus of the first part of the studies has been to uncover parts of the mechanisms in the process of neoplastic transformation. Perlecan is one of the most complex gene products because of its multidomain structure and extent of post-translational modification (Iozzo, 1994). Perlecan from different sources can be expressed with distinct fine structure. The complex molecular organization and spatial/temporal expression patterns of this molecule suggest that it is involved in multifunctional cellular processes, including binding/delivery of mitogens and regulation of cell adhesion and migration (Iozzo, 1998). These could also involve the process of tumor formation and progression. The structure and function of perlecan in tumorigenic and nontumorigenic cells have not been examined conclusively. Therefore, the central hypothesis of this study is that perlecan in tumorigenic cells is structurally different from

that of nontumorigenic cells. These differences may affect some of its functional roles in tumor growth and contribute to the process of tumor formation and progression.

In this study, we used a mouse epidermal cell culture system as our model. Our findings indicate that expression of both perlecan in both mRNA and protein messages is increased in RT101 tumorigenic cells compared to that of the JB6 wild-type cells. In addition to an increased expression of perlecan in tumor cells, we have demonstrated that tumor-cell-derived perlecan is expressed differently as a hybrid molecule containing both HS and CS/DS chains on its core protein. Since the presence of hybrid perlecan molecules is a correlate of the transformed state, therefore, there is a very high possibility that substitution with galactosaminoglycan in place of or in addition to HS chains may impact the biological activity of perlecan produced by tumor cells. Although the effect of CS/DS substitution is still not clear, the highly negative charge on CS/DS GAGs may promote detachment of tumor cells in the process of invasion and metastasis. Timar *et al.* (1995) have shown that the migratory phenotype of tumorigenic cells is often associated with increased expression of CS. In addition, perlecan oligomerization (Yurchenco *et al.*, 1987) and interactions with other ECM components such as laminin (Battaglia *et al.*, 1992; Aumailley and Kreig, 1996; Brown *et al.*, 1997), entactin (Reinhardt *et al.*, 1993; Brown *et al.*, 1997), fibronectin (Heremans *et al.*, 1990), thrombospondin 1 (Vischer *et al.*, 1997), fibulin (Brown *et al.*, 1997), α-dystroglycan (Peng *et al.*, 1999; Talts *et al.*, 1999), or the cell surface molecule HIP (Rohde *et al.*, 1998) appear to be HS dependent. Galactosaminoglycan substitution of tumor-derived perlecan may alter these interactions and affect the integrity of basement membrane and/or ECM, enhancing a permissive milieu for growth and invasiveness of tumors. Some galactosaminoglycans have also been shown to have specific ligand-binding abilities; for example, DS GAGs are able to

bind bFGF (Penc *et al.*, 1998) or HGF/SF (Lyon *et al.*, 1998), usually with lower affinity than HS GAGs. All of these factors could be used by tumor cells to promote their growth and differentiation.

The progression to the tumorigenic phenotype was also accompanied by some structural changes of the HS chains. The perlecan HS from the tumor cells contains fewer sulfate groups by a reduction in 6-*O*- and 2-*O*-sulfation, primarily in S-domains, and an increase of N-acetylated disaccharides in the spaced sequences. Interestingly, the addition of highly negatively charged CS/DS to the core protein of the RT101 perlecan maintains the net charge at the same level as in the wild-type JB6 cells. Although the reduction in sulfation is consistent with previous findings in tumorigenic cells, a detailed comparison shows some differences, depending on the system studied. For example, transformed fibroblasts also displayed reduced 6-*O*-sulfation of HS when compared with the parental cell line (Winterbourne and Mora, 1981). However, unlike our study, this change occurred primarily in mixed sequences. Transformed mammary epithelial cells also exhibited reduced 6-*O*-sulfation, but other sulfation levels remained unchanged (Safaiyan *et al.*, 1998). The malignant transformation of human adenoma to carcinoma, which was associated with a decrease in 2-*O*-sulfation in S-domains, was, in contrast to our findings, accompanied with an increase in 6-*O*-sulfation in the mixed sequences (Jayson *et al.*, 1998). This indicated that, although the sulfation pattern of HS in S-domains and mixed sequences is regulated during tumorigenic processes, specific changes may occur, depending on cell type or tumor stage.

The fine structure of HS is an important determinant of affinity for various growth factors in both normal and pathological processes such as cell proliferation and differentiation, angiogenesis, wound healing, and cancer. We have tested whether the

structural differences we observed for HS affect their affinity for binding several growth factors that regulate epithelial cell growth (FGF-1, -2, -4, and -7 and HB-EGF). Our results show that these GFs bind perlecan HS chains from both sources with very high affinity, as previously determined for other heparin-like GAGs (Lee and Lander, 1991; Basilico and Moscatelli, 1992; LaRochelle *et al.*, 1999). However, HS from RT101 perlecan displayed only a minor increase in affinity for FGF-1, -2, and -7, and for HB-EGF. The difference in HS fine structure does not seem to affect binding affinity. However, there might be other important determining factors. We have not tested the binding to any ECM proteins; however, since the major structural difference between JB6 and RT101 perlecan is the change in the amount of HexUA(2S)-GlcNS(6S), it is possible that the affinity for laminin, which requires repetitive sequence of this disaccharide, would be lowered in perlecan from tumorigenic RT101 cells. In addition, CS/DS GAGs and the protein core, which were not included in our binding study, may also have specific ligand-binding abilities, which would have an impact on the interactive potential of the tumor perlecan.

Our findings suggest that the expression of perlecan in the process of tumorigenesis not only may confer a growth advantage but may be essential for the multifactorial and highly complex process that leads to growth and differentiation of tumors. By altered expression of perlecan, tumorigenic cells may contribute to creating an environment favoring tumor growth and invasion. We have shown that during this process, perlecan expression can be regulated at both transcriptional and post-translational levels. Therefore, the role of this complex molecule in the disease processes including tumorigenesis needs to be examined from multiple aspects. However, there are many questions that remaining to be resolved.

Why is perlecan level up-regulated in the process of malignant transformation? Does it promote growth and invasiveness of the tumors? To address the importance of perlecan in the process of transformation, antisense perlecan cDNA could be transfected into the RT101 tumorigenic cells. The growth rate of the transfected cells can be compared to those of the nontransfected or vector-transfected cells. In vitro tumorigenicity by growth in soft agar (Sharma *et al.*, 1998) or in vivo study by injection of control RT101 and the antisense transfected cells into nude mice could be performed to determine the rate of tumor growth. Invasion assay of the RT101 cells on the collagen gel or Boyden chamber containing perlecan extracted from both cell types may reveal the effects of perlecan on the migration of tumor cells.

Which factors stimulate increased expression of perlecan in tumor cells? The perlecan gene has been shown to be transcriptionally activated by TGF-β via a nuclear factor 1-binding element (Iozzo *et al.*, 1997). TGF-β has also been shown to promote the synthesis of perlecan when the cell density is high but not when the cell density is low (Kaji *et al.*, 2000). Furthermore, CS/DS PG synthesis is stimulated by TGF-β (Chen *et al.*, 1991; Maniscalco and Campbell, 1994). This may be responsible for changes in glycanation that can readily be verified in tissue culture. The rate of DNA synthesis, measured by ^{3}H-thymidine incorporation in the presence or absence of TGF-β, can also be determined. Perlecan synthesis in response to TGF-β can be measured by metabolic labeling and subsequent immunoprecipitation.

Why do tumor cells express perlecan as a hybrid molecule? Which mechanism regulates this process? The enzymes responsible for GAG synthesis are largely located in

the Golgi apparatus. A tetrasaccharide "linkage region" (GlcA-Gal-Gal-Xyl-) attached to specific serine residue on the core protein provides the starting point for GAG biosynthesis. Both glucosaminoglycans (heparin/HS) and galactosaminoglycans (CS/DS) use the same linkage region for their chain elongation. Once formed, this linkage region will serve as an acceptor for the first GlcNAc or GalNAc unit in a reaction that will commit the process toward generation of either a glucosamino- or a galactosaminoglycan chain. The factors that determine whether a GlcNAc or a GalNAc unit is added remain unclear. However, peptide sequence motifs close to the substitution sites have been implicated as signals for addition of a GlcNAc unit (Zhang and Esko, 1994), which may initiate HS formation by α-GlcNAc transferase (Fritz *et al.*, 1994). The absence of such a signal would lead to a "default" substitution of a GalNAc unit by β-GalNAc transferase, resulting in CS/DS formation (Rohrmann *et al.*, 1985). These two enzymes have recently been cloned (Hagen and Nehrke, 1998; Strasser *et al.*, 1999). In the process of malignant transformation, the α-GlcNAc transferase may be down-regulated. To test this hypothesis, detection of the mRNAs of these enzymes in tumor cells compared to those of the wild-type cells can be carried out. The enzyme activities of both α-GlcNAc- and β-GalNAc transferases can be determined by extraction of these enzymes from both cell types, and the activities of the enzymes can be compared by enzyme kinetic assay (Fritz *et al.*, 1994).

What are the functions of CS/DS GAGs on perlecan of the tumor? The presence of the CS/DS chains on perlecan has been described several times (Danielson *et al.*, 1992; Couchman *et al.*, 1996). However, the functions of these CS/DS substitutions remain unknown. The CS/DS GAGs contain a higher negative charge than HS GAGs. The large

hydrodynamic volume of these chains could profoundly influence the properties of the matrix macromolecules. These could affect GF binding or matrix assembly or could promote a permissive environment for growth and migration of the tumor cells. CS/DS chains have been shown to bind some growth factors (Lyon *et al.*, 1998; Penc *et al.*, 1998). Also, decorin (a DSPG) binds to collagen I and II, playing an important role in the organization of the collagen fibrils. The specific blends of PG and collagen determine the elasticity and hydrodynamic properties of the tissue (Coster, 1991). The galactosamino-glycan content of decorin, biglycan, and thrombomodulin has been shown to perform local anticoagulant and antithrombotic properties (Linhardt *et al.*, 1991; Andrew *et al.*, 1992). The presence of the CS/DS GAGs on perlecan may have a similar influence. We have not tested the effects of this CS/DS substitution in these studies and this question definitely needs to be addressed. The interactions between intact perlecan or the CS/DS GAGs from RT101 cells and other ECM molecules such as collagen IV, laminin, entactin, fibronectin, and thrombospondin, as well as various GFs, can be determined by various techniques such as affinity co-electrophoresis, solid-phase binding, or surface plasmon resonance assays. Comparison of the binding affinity of perlecan from both sources will show us the effect of CS/DS on the molecular interactions. Also, immunohistochemical staining of both JB6 and RT101 cells with antibodies against various ECM components will tell us if there are any differences in matrix composition and assembly. Cell attachment, spreading, and migration assays on perlecan substrates extracted from both cell types could also be tested to verify the effects of the CS/DS substitution on cell migration, which could affect invasiveness of the tumor cells.

The differences in the fine structure of the HS derived from both cell types did not have much influence on the binding affinity of the GFs tested. There was no loss of

the binding affinity. However, there are some other GFs that have been shown to bind HS chains and promote angiogenesis such as HGF/SF (Bhargava *et al.*, 1992; Grant *et al.*, 1993) and VEGF (Plate *et al.*, 1992). PDGF, a potent mitogen, has been shown to bind perlecan (Gohring *et al.*, 1998). Also, TGF-β (Iozzo *et al.*, 1997), and IFN-γ (Lortat-Jacob *et al.*, 1991) can induce perlecan transcription. The HS from the RT101 cells may bind to these potential molecules with a significantly higher affinity compared to those of the JB6 wild-type perlecan. All of these molecules should be tested and also should be included in the CS/DS binding assays.

What happens in vivo? The experiments in these studies were all in vitro studies. Future in vivo work will determine how important the presence of perlecan in tumor matrix is and whether there are distinctions between tumor-derived and host-derived perlecan. To test these questions, tumor formation can be induced in nude mice. Perlecan can be extracted from the solid tumor and can be analyzed by using the same techniques that we have used in these studies.

In our preliminary study, we injected the RT101 cells into athymic rats. Immunohistochemistry of tissue sections was performed and we found that perlecan could be secreted by both tumor and host cells. In addition to perlecan from the tumor cells, perlecan of the host cells was incorporated into the basement membranes of the newly formed blood vessels in the tumor. This indicates that perlecan may have crucial roles in the process of angiogenesis, which is essential for the process of tumorigenesis (Hanahan and Folkman, 1996). The importance of perlecan in this process can be verified by inducing tumor formation in a transgenic animal model with over or under perlecan expression to see whether transgenic animals carrying high copy numbers of perlecan are

predisposed to higher tumor growth rates than those of the ones carrying lower copy numbers of perlecan. Tissue sections of the tumors can also be used to determine potential differences in the angiogenic patterns in these animals. The results of these further studies will show us how important perlecan is in this process. Detection of angiogenic factors by immunohistochemistry or in situ hybridization such as FGF-1 and -2, VEGF, and HGF/SF may correlate with the distribution of perlecan in tumor tissue.

Recently, Sharma *et al.* (1998) published exciting data which show that antisense targeting of perlecan can block both tumor growth and angiogenesis in vivo. The results of these studies support the notion that perlecan is directly involved in promoting the growth and differentiation of epithelial-cell tumors. However, another study has indicated that perlecan can also inhibit the growth and invasion of fibrosarcoma (Mathiak *et al.*, 1997). These contradictory findings raise important questions about the expression and roles of perlecan in modulating tumor growth and progression in different tissues. Furthermore, it has been shown that perlecan synthesized by vascular endothelial cells is a potent inhibitor of smooth-muscle-cell proliferation. It is up-regulated and required to mediate the antiproliferative effect of apolipoprotein E (Paka *et al.*, 1999). That means perlecan may have dual roles, depending on the histologic origins of the cells. Perlecan may express differently and play different roles in different types of tumor. Further studies need to be carried out to establish the precise roles of perlecan in sarcomas and to determine whether it may play different roles in adenomas and carcinomas. A role of perlecan in modulating important signaling pathways that regulate tumorigenesis is also a strong possibility and needs to be considered in future studies.

In the second part of my studies, glycanation of perlecan was examined. Perlecan has previously been shown to contain three GAGs attached on domain I; however, the C-

terminal domains IV and V of this molecule can also be substituted with GAG chains (Brown *et al.*, 1997; Ettner *et al.*, 1998). The exact substitution sites were not identified. The amino acid sequence of mouse perlecan reveals many ser-gly residues in these domains, where the serine residues are possible sites for GAG substitution. We expressed recombinant domain IV and/or V of mouse perlecan in COS-7 cells and analyzed GAG substitution. We found that both HS and CS chains could be detected on domain V. It therefore appears that both ends of the core protein (domains I and V) can bear GAG. The SGE motif located on the last globular domain toward the C-terminal end of domain V is preferentially HS substituted. However, when this region was unavailable, CS/DS substitution was observed on the second globular region of the same domain. The functional consequences of these substitutions remain unknown but may substantially contribute to physicochemical and biological function of perlecan.

Perlecan is a widespread basement membrane and ECM component. It is believed to interact with other matrix components. It may also interact with integrin receptors, although these have not been specifically identified (Battaglia *et al.*, 1993; Chakravarti *et al.*, 1995). Murine perlecan, but not human, contains an RGD motif in domain III which can promote cell adhesion (Iozzo, 1998). In some cases, integrin-perlecan interactions may be HS dependent; however, the reasons for this are unclear (Battaglia *et al.*, 1993). Additionally, perlecan may sequester heparin-binding GFs (e.g., FGF-2) through its HS chains, and basement membranes in vivo have been demonstrated to be a site of these potent molecules (Aviezer *et al.*, 1994b; Whitelock *et al.*, 1996). It could be interesting to find out more about the impact of alternate glycanation sites on the biology of perlecan. While the functional attributes of domain V glycanation reported here are still far from clear, it has been reported that domain V from perlecan is heparin binding. A possibility

is, therefore, that self-association of perlecan (Yurchenco *et al.*, 1987) is affected by glycanation in this region. Additionally, perlecan domain V binds to α-dystroglycan (Peng *et al.*, 1999; Talts *et al.*, 1999). The dystroglycan-perlecan complex then serves as a receptor for acetylcholinesterase enzyme, enabling the enzyme to be localized at the synapse (Peng *et al.*, 1999). Furthermore, the globular modules (laminin-G-like modules) of other ECM molecules (laminin and agrin, including perlecan) have been shown to mediate the binding to α-dystroglycan, and HS seems to influence this binding (Hohenester *et al.*, 1999). The interactions with other ECM molecules may be similarly influenced. HS substitution on domain V may play this important role. These interactions may be crucial to basement membrane integrity. It is therefore important in the future to ascertain the patterns of perlecan glycanation and its function in tissues with respect to development, function, and disease.

To determine the roles of this GAG substitution, first, the ability of this HS substitution to bind various GFs can be examined. To test this, the immunopurified recombinant domain V with/without the presence of this HS substituion by treatment of the recombinant products with GAG lyases can be used for the analysis. Comparison of the binding affinities of these two products by affinity co-electrophoresis will show the roles of this HS substitution in GF binding. Second, HS substitution may play roles in the molecular interactions with other ECM molecules. These can be tested in the same manner as those of the GFs. Previous study has shown that perlecan can self-assemble via its C-terminal ends (Yurchenco *et al.*, 1987). The HS substitution may play a crucial role in the process of self-assembly, contributing to basement membrane assembly. To test this hypothesis, rotary shadowing and electron microscopy of the recombinant domain V

in the presence or absence of GAG substitution can be performed by using these techniques as previously described (Brown *et al.*, 1997; Ettner *et al.*, 1998).

The expression of this GAG substitution in vivo also needs to be verified. This can be done by extraction of perlecan from normal tissues or an induced tumor. After treating of the immunopurified perlecan with enzymes that cleave the perlecan core protein (Whitelock *et al.*, 1996), the C-terminal domain of this molecule can be captured by antibody specific to domain V of mouse perlecan. The presence of the GAG substitution can be verified by immunoblotting as we have described in these studies.

The progression in understanding the roles of perlecan may provide some information that could be beneficial, and may potentially improve cancer treatments. Sharma and colleagues (1998) have shown in in vivo experiments that tumor growth and angiogenesis in colon carcinoma were reduced in perlecan antisense-expressing cells. Also, suppression of perlecan synthesis in melanoma cells by using antisense targeting reduced cell proliferation, in vitro invasion, and metastasis potential in an animal model (Adatia *et al.*, 1997) TNF-α and IFN-γ have been shown to be potent down-regulators of perlecan gene expression, and both are anti-angiogenic (Iozzo *et al.*, 1997; Sharma *et al.*, 1998). These indicate that reduction of perlecan level could lead to a delay in tumor growth, suggesting a new target for the development of effective means for treatment and prevention of cancer. However, more studies covering various aspects of perlecan need to be carried out. In addition, malignant transformation is a multicellular processes; more research studies to covering various aspects of these processes need to be performed to gain more insights. Each new insight into the pathophysiology of malignant transformation will provide an improved understanding of the molecular basis of cancer progression. Arrival at this point has been due to discoveries arising from a basic

research-clinical interface, a familiar paradigm that will undoubtedly lead to translation into novel therapies. Collaboration among academic scientists, the pharmaceutical and biotechnology industry, and clinicians will rapidly improve the search for the cure of cancer.

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