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Cell Surface Heparan Sulfate Proteoglycan and the Neoplastic Phenotype

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Cell surface proteoglycans are strategically positioned to regulate interactions between cells and their surrounding environment. Such interactions play key roles in several biological processes, such as cell recognition, adhesion, migration, and growth. These biological functions are in turn necessary for the maintenance of differentiated phenotype and for normal and neoplastic development. There is ample evidence that a special type of proteoglycan bearing heparan sulfate side chains is localized at the cell surface in a variety of epithelial and mesenchymal cells. This molecule exhibits selective patterns of reactivity with various constituents of the extracellular matrix and plasma membrane, and can act as growth modulator or as a receptor. Certainly, during cell division, membrane constituents undergo profound rearrangement, and proteoglycans may be intimately involved in such processes. The present work will focus on recent advances in our understanding of these complex macromolecules and will attempt to elucidate the biosynthesis, the structural diversity, the modes of cell surface association, and the turnover of heparan sulfate proteoglycans in various cell systems. It will then review the multiple proposed roles of this molecule, with particular emphasis on the binding properties and the interactions with various intracellular and extracellular elements. Finally, it will focus on the alterations associated with the neoplastic phenotype and will discuss the possible consequences that heparan sulfate may have on the growth of normal and transformed cells.

Key words: heparan sulfate, transformation, cell surface proteoglycans, growth control, cancer glycosaminoglycans

There is a growing realization that proteoglycans are directly implicated in the biology of the cell membrane in most eukaryotic cells. Cell surface proteoglycans comprise a family of macromolecules endowed with diverse structural and functional properties. Their strategic location and their highly charged nature make them important biological participants in the cell-cell and cell-matrix interactions that take place during normal and neoplastic development. Although recent evidence suggests that proteoglycans bearing chondroitin sulfate chains may be associated with the cell surface [1–3], the vast majority of the reports indicate that the predominant proteoglycan associated with the cell surface is substituted by heparan sulfate side chains. Inasmuch as this area

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has been reviewed in considerable detail previously [4–8], this brief review will be selective rather than comprehensive. I will attempt to elucidate the structural diversity and clarify recent advances in understanding the biosynthesis and degradation of these macromolecules. I will then attempt to provide an overview of the various proposed functional roles and their alterations associated with the neoplastic phenotype.

STRUCTURAL DIVERSITY

Despite several recent advances in clarifying the structure and metabolism of heparan sulfate proteoglycans, a number of key questions still remain unanswered, particularly as they relate to the amino acid sequence of the protein core and the regulation of protein core biosynthesis, posttranslational modification and transport to the cell surface.

By analogy with the chondroitin sulfate proteoglycan [9], the first step in biosynthesis is the formation of the protein core on the ribosomes of the rough endoplasmic reticulum. The size of the message is not yet established with certainty, but it is likely that there are several protein cores. This is inferred from the high degree of variability in apparent M_r of the protein cores isolated from various tissues or cells. For example, the size of the protein core can vary from 30,000 to over ten times this value [8]. Furthermore, we do not know whether there is a domain that is specific for glycosaminoglycan binding or that may direct the proteoglycan across the various membrane-bound compartments to their final destination site. Interestingly, the sequence analysis of a noncartilaginous chondroitin sulfate proteoglycan cDNA [10] indicates that there is a Ser-Gly repeat that serves as the recognition and acceptor site for the chondroitin sulfate chains. This region is structurally related to the attachment region in the heparin proteoglycan [11] and a heparan sulfate proteoglycan from basement membrane [12]. It is conceivable, therefore, that proteoglycans bearing different glycosaminoglycan chains may undergo similar biosynthetic steps, at least at the level of their precursor protein core, and may share homologous amino acid sequences. This concept is supported by the recent demonstration [13] that three different protein cores contain a homologous 12-amino acid sequence that surrounds the Ser-Gly dipeptide region and is a good acceptor for xylosyltransferase, the enzyme that begins the assembly of glycosaminoglycan chains. From these studies it has been proposed that the recognition consensus sequence for the attachment of glycosaminoglycans to protein core consists of acidic amino acids closely followed by the tetrapeptide Ser-Gly-X-Gly [13]. Interestingly, at least one Ser-Gly-X-Gly tetrapeptide sequence is present in three known gene products that can be substituted with heparan sulfate chains, namely fibronectin [14], the transferrin receptor [15] and the product of the *Drosophila* per locus gene [16]; however, it is not known whether these sequences are always substituted with heparan sulfate chains. This preliminary but significant evidence thus indicated that Ser-Gly sequences may be crucial in the structure of the protein core and its posttranslational modifications. The protein core can be modified by the addition of N- and O-linked oligosaccharides and heparan sulfate chains [5–8]. The latter undergo a series of polymer modification reactions [17] which culminate in the rapid sulfation of the chains and the transfer of the completed proteoglycan to the cell surface [7].

An emerging generalization deduced from the studies summarized above is that there are several protein cores represented in this group, and the three following examples have been selected to reflect this structural diversity.

The heparan sulfate proteoglycan associated with the surface of hepatocytes is significantly smaller than that of skin fibroblasts or human colon carcinoma cells (Fig. 1). This molecule [18] appears to be quite simple with a short, intercalated protein core (M_r = 35,000) and about four heparan sulfate chains of M_r = 14,000; it is present on hepatocytes but restricted to the sinusoidal domain of the plasma membrane [19]. There is no available evidence, however, for the presence of N- and O-linked oligosaccharides. In contrast, skin fibroblasts express a significantly larger molecule (Fig. 1) with two disulfide-bonded protein cores of M_r = 90,000, each containing about five heparan sulfate chains of M_r = 20,000, and fewer N- and O-linked oligosaccharides [15]. The protein core in this case is structurally and biologically similar to the transferrin receptor, a membrane glycoprotein. In fact, they both consist of a disulfide-bonded dimer protein of identical size, they both bind transferrin in vitro, and both are immunoprecipitated by monoclonal antibodies against the transferrin receptor. This binding activity has been located to the protein core in close proximity to the hydrophobic domain [20]. Recent studies [21] have indicated that this biological activity can be found only in postconfluent skin fibroblasts. However, the inability of this proteoglycan to bind transferrin in situ makes the physiological significance of this phenomenon uncertain [7].

A third prototype of cell surface heparan sulfate proteoglycans is that synthesized

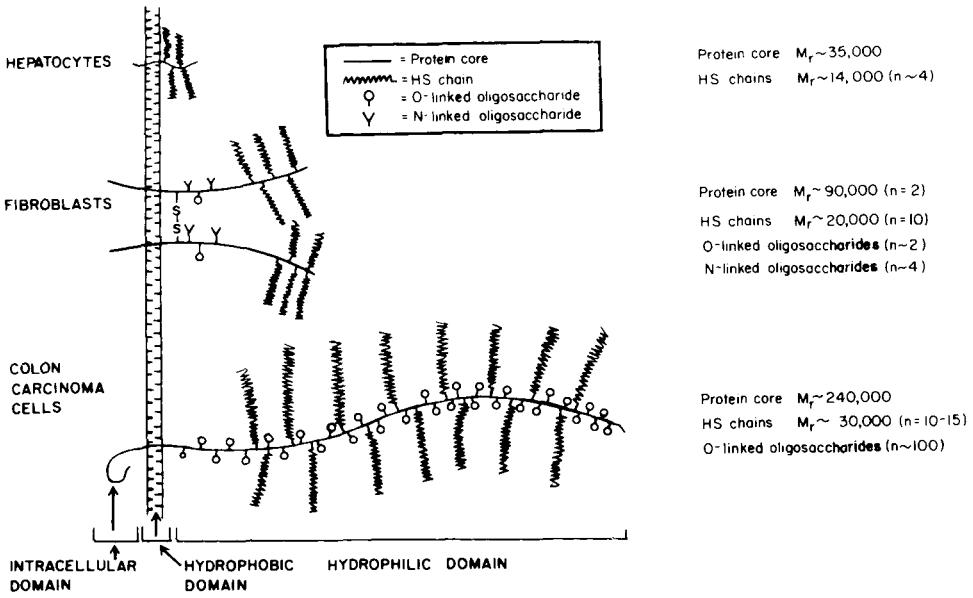

Fig. 1. Schematic representation of three different proteoglycans associated with the plasma membrane of hepatocytes, fibroblasts, and human colon carcinoma cells. Notice the variability in protein core size and degree of glycosylation. The fibroblast proteoglycan contains a region, located in the proximity of the hydrophobic domain, that has affinity for transferrin [15, 20]. In all the three examples illustrated here, and probably in most of the cell-surface proteoglycans, the hydrophilic domain can be cleaved by trypsin with the release of an intact proteoglycan (ectodomain) enriched in heparan sulfate chains. The existence of an intracellular trans-membrane domain has not been unequivocally established for any of these proteoglycans. For additional details see the text.

by human colon carcinoma cells [22,23]. This proteoglycan is even larger than the one from skin fibroblasts, with a protein core M_r about 240,000, substituted with 10–15 heparan sulfate chains with an M_r = 30,000, and about 100 O-linked oligosaccharides. Recent studies indicate that the protein core precursor may have an M_r of up to 400,000 (Iozzo RV, Hassell JR, unpublished results). This molecule, by analogy with other heparan sulfate proteoglycans of other systems [24,25], has three distinct domains: a) an extracellular or hydrophilic domain that contains most of the glycosaminoglycan side chains, b) a hydrophobic domain which anchors the protein core into the lipid bilayer; we have recently provided evidence [23] for the presence of a leucine-rich hydrophobic domain that can be cleaved from the intact proteoglycan by mild proteolytic treatment; and c) a hypothetical intracellular domain that presumably binds cytoskeletal or contractile elements (see below).

Recent evidence [26,27] indicates that some cell surface proteoglycans may be hybrid molecules containing both chondroitin and heparan sulfate side chains, thus adding another element of complexity to this family of macromolecules. A fundamental question that needs to be addressed in future studies is the manner by which various cells coordinate and regulate the glycosylation of the protein core. It is likely that differences in the primary structure of the protein core are directly involved in the regulation of these events.

LOCALIZATION AT THE CELL SURFACE

Since the original report of cell-associated heparan sulfate [28,29], several investigators have confirmed this observation in a variety of cell systems [15,18–27,30–37]. Most of the investigations have employed biochemical, enzymatic, or morphological approaches to assess the presence of such molecules at the plasma membrane. For instance, the heparan sulfate proteoglycan in human colon carcinoma cells can be visualized by using cationic dyes such as ruthenium red or cuprolinic blue [22,38,39]. When visualized by ruthenium red staining (Fig. 2A), these proteoglycans appear as spherical electron-dense granules, 20–100 nm in diameter, distributed along the plasma membrane. These structures can be completely removed by trypsin or heparitinase and can be metabolically labeled by radiosulfate as shown by electron microscopic autoradiography (Fig. 2B). These findings indicate that the cell surface granules do indeed represent heparan sulfate proteoglycans, the only sulfated molecules synthesized by these colon carcinoma cells [22,23]. When stained with cuprolinic blue using the critical electrolyte concentration approach [40], they appear as long filamentous structures (Fig. 2C), 40–220 nm in length. These filaments likely represent the protein core upon which the heparan sulfate side chains have partially collapsed after binding the cationic dye [40]. These filaments contain polyanionic sulfate ester groups, as indicated by their staining in the presence of 0.3 M $MgCl_2$ (Fig. 2C) and can be removed by pretreatment of the cells with trypsin or heparitinase. Finally, the cell surface of human colon carcinoma cells can be stained with an antibody raised against a heparan sulfate proteoglycan isolated from Engelbreth-Holm-Swarm (EHS) tumor [41]. Fluorescence reactivity can be observed in the form of punctate or linear deposits along the cellular contours (Fig. 2D). This pattern is visualized better at the immunoelectron microscopic level (Fig. 2D), where electron-dense deposits of antigen-antibody complexes decorate the microvillar surface of the carcinoma cells. Heparan sulfate proteoglycans from hepatocytes [19] and mouse mam-

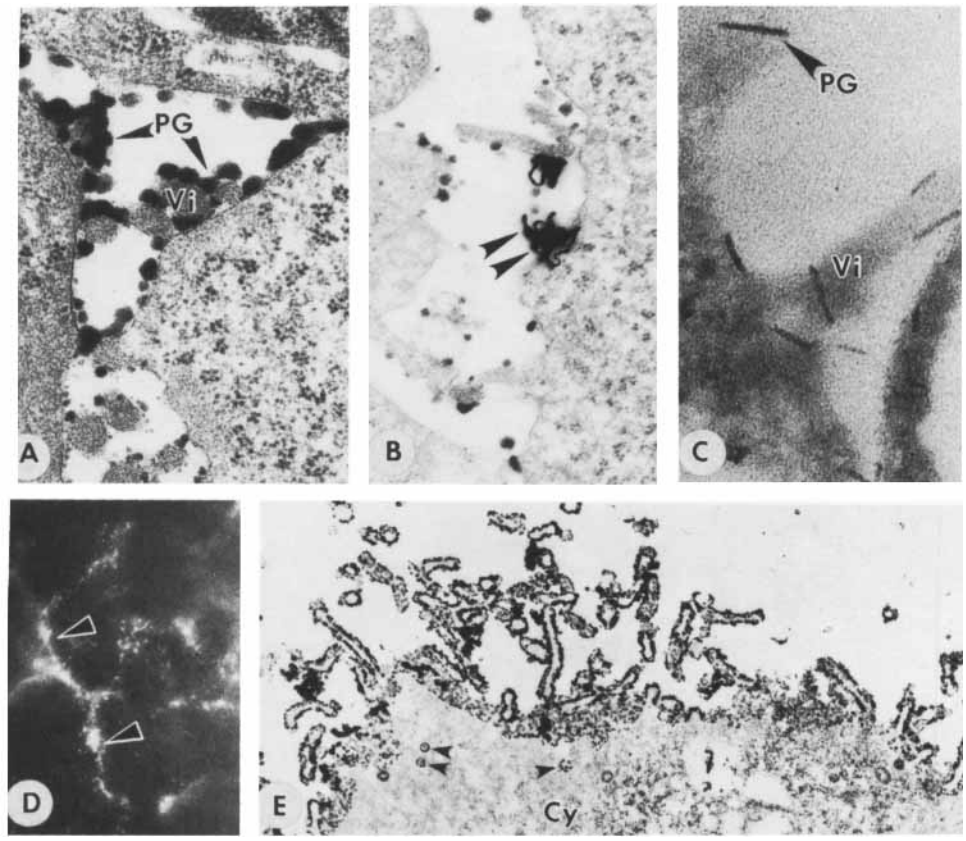

Fig. 2. Localization of heparan sulfate proteoglycan at the surface of human colon carcinoma cells. In **A,** the cells were fixed and processed for electron microscopy in the presence of ruthenium red, a cationic dye that binds to proteoglycans. Notice numerous spherical granules (PG) decorating the avillous as well as the villous (Vi) portions of the plasma membrane. These granules can be metabolically labeled by radiosulfate (**B,** double arrowheads) using electron microscope autoradiography. In **C,** the proteoglycans were visualized with cuprolinic blue in 0.3 M $MgCl_2$ and appear as electron-dense, filamentous structures (PC) whose length is proportional to the size of the protein core. Both the granules (A,B) and the filaments (C) can be removed by trypsin and heparitinase, but not by chondroitinase ABC (not shown). Using an antiheparan sulfate antiserum, punctate fluorescence deposits can be observed along the cellular contours (**D**). By immunoelectron microscopy (**E**), electron-dense antigen-antibody complexes decorate the surface of microvilli or label small subplasmalemmal vesicles (E, arrowheads) which carry proteoglycan to and from the plasma membrane; in contrast, the remaining cytoplasm (Cy) is unreactive. Control sections incubated with normal rabbit serum were totally unreactive (not shown). A, ×30,000; B, ×23,000; C, ×65,000; D, ×450; E, ×13,000.

mary epithelial cells [42] have been also localized on the cell surface using immunohistochemistry.

MODES OF CELL SURFACE ASSOCIATION

Cell surface heparan sulfate proteoglycans may exist in two forms, either bound to the plasma membrane by noncovalent interaction via "receptors" (Fig 3A) or as integral membrane components (Fig. 3B). The receptor-mediated attachment has been

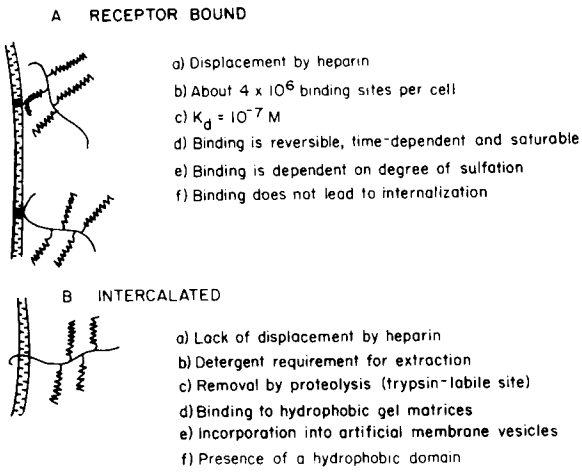

Fig. 3. Proposed mechanisms of cell surface association of heparan sulfate proteoglycans. Alternate mechanism not depicted here would involve (i) a proteoglycan-proteoglycan interaction mediated by copolymeric regions of the heparan sulfate side chains, and (ii) a binding to the lipid bilayer via a phosphatidylinositol moiety that is covalently linked to the protein core. See the text for additional details and discussion.

worked out by Höök and collaborators in hepatocytes [5,18,30,43,44] and has been demonstrated to occur in other cell systems [45–49]. The binding to the cell surface is most likely mediated through anionic sequences in the heparan sulfate side chains; however, the possibility that specific amino acid sequences in the protein core may mediate this binding cannot be ruled out. The major evidence for the receptor-mediated binding is provided by the ability of heparin, a much more anionic compound, to displace the cell surface proteoglycan and by the ability of various cells to bind radiolabeled heparin or heparan sulfate [5]. The hepatocytes contain about 4 million receptors per cell, and the binding of exogenous heparan sulfate is reversible, time-dependent, and saturable [43]. The dissociation constant is on the order of about 10^{-7} M and is dependent on the degree of sulfation of the heparan sulfate chains [50]. Finally, binding to the receptor is not a prelude to internalization and degradation of the ligand [43].

A much more general and ubiquitous form of cell surface heparan sulfate is the intercalated species in which a hydrophobic domain of the protein core is directly anchored within the lipid bilayer (Fig. 3B). Proteoglycans with these characteristics have been demonstrated in a variety of cell systems [18,22–27,30,32–34,47–51]. Although direct anchoring of the protein core into the lipid bilayer has not been unequivocally demonstrated, several supporting arguments have been used including a) heparin or other polyanionic compounds are unable to displace proteoglycans from the cell surface, b) detergent is required for efficient solubilization in monomeric form, c) mild proteolytic treatment with trypsin or chymotrypsin can cleave most of the ectodomain containing the glycosaminoglycan chains, d) the isolated proteoglycan has high affinity for hydrophobic matrices, e) the proteoglycan can be inserted into artificial membrane vesicles, and f) the protein core contains a hydrophobic domain that mediates cell attachment.

An example of the behavior of such an intercalated proteoglycan is observed in human colon carcinoma cells (Fig. 4). In this system, trypsin is capable of removing

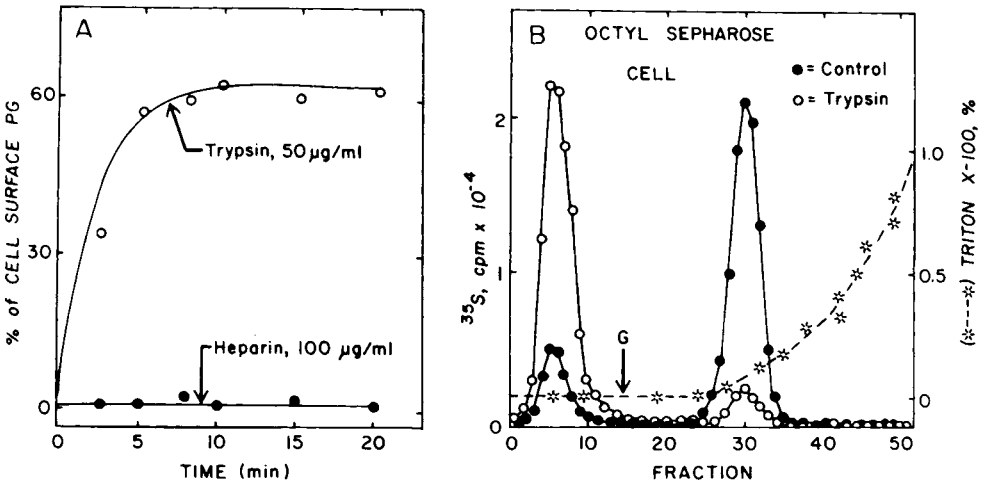

Fig. 4. Evidence for the intercalated nature of the heparan sulfate proteoglycan from human colon carcinoma cells. In **A**, confluent cultures were labeled for 30 h with radiosulfate, washed, and chased in isotope-free medium in the absence or presence of either trypsin (○) or heparin (●), at the designated concentrations. Notice that up to 60% of the cell surface proteoglycan (PG) is released by mild proteolysis in a time-dependent and saturable way, whereas heparin, a much more anionic compound, does not displace any material above background level. The purified cell surface proteoglycan binds to octyl Sepharose (**B,●**) and can be eluted with a Triton X-100 gradient (* - - *). The binding to the hydrophobic gel matrix can be prevented by pretreating the proteoglycan with trypsin (B,○). These results indicate that the cell surface proteoglycan is intercalated into the plasma membrane via a trypsin-labile domain in the protein core. (For additional information see text and [22,23].)

the majority of the cell surface proteoglycan in a time-dependent manner (Fig. 4A, open circles); heparin, however, does not remove any significant amount of it (Fig. 4A, closed circles), indicating that this proteoglycan is primarily membrane-intercalated. Furthermore, the majority of the cell surface proteoglycan from colon carcinoma cells binds to octyl Sepharose (Fig. 4B, closed circles), and this binding can be abolished by pretreatment of the cells with trypsin (Fig. 4B, open circles). We have shown [23] that this molecule contains a hydrophobic, leucine-rich, unsulfated domain with an M_r of about 5,000 [23]. This domain can be cleaved by trypsin from the intercalated proteoglycan, but it can be released neither from the proteoglycan recovered in the medium nor from the intracellular degradation species. We have proposed that this domain may indeed represent the intramembranous portion of the protein core. It is noteworthy, however, that the medium-released species has also shown some affinity for the hydrophobic matrix, suggesting that there may be other hydrophobic sequences interspersed between glycosylation sites. It is clear that direct evidence for the presence and location of hydrophobic domains in the protein core will have to await the unraveling of the amino acid sequence.

A novel cell surface association has been recently proposed to occur [52] for the heparan sulfate proteoglycan in a rat hepatocyte cell line [53]. Accordingly, the newly synthesized heparan sulfate proteoglycan is bound to the plasma membrane through a phosphatidylinositol moiety that is covalently linked to the protein core and has its fatty acyl chains buried in the lipid bilayer [52]. This linkage is subsequently cleaved by an

insulin-activated phospholipase C, and the released proteoglycan, which has the myo-inositol-phosphate moiety still attached to the protein core, is then internalized via a cell surface receptor that recognizes the myo-inositol-phosphate. It will be important to determine in future studies whether this type of membrane anchoring is operational in other cellular systems. This could be tested either indirectly by displacing the receptor-bound proteoglycan with exogenous inositol-phosphates or directly by releasing the proteoglycan with phospholipase C specific for myo-inositol-phosphate [52].

TURNOVER OF PLASMA-MEMBRANE PROTEOGLYCANS

The fate of the cell surface proteoglycan has been studied in several systems, primarily employing pulse-chase experiments with radiosulfate as precursor [22,34,54–58]. The emerging evidence is that the membrane-bound proteoglycan is processed through two mechanisms: a) by limited cleavage of the protein core and release into the medium, and b) by internalization and intracellular degradation. We have recently used a combination of quantitative biochemistry and electron microscopic autoradiography to study the turnover of the heparan sulfate proteoglycan in human colon carcinoma cells [59]. This was possible primarily because these cells incorporate radiosulfate exclusively into heparan sulfate, thus allowing the possibility to correlate the two sets of information. According to the model generated from these studies (Fig. 5), about 55% of the membrane proteoglycan is released into the medium ($t_{1/2}$ = 2.5 hr) where it resides as an intact macromolecule and is neither endocytosed nor degraded further. The remaining 45% is endocytosed and converted to a smaller intermediate species ($t_{1/2}$ = 6 hr), initially involving proteolytic cleavage of the protein core and partial endoglycosidic cleavage of the heparan sulfate chains. This first degradative step generates an intermediate glycosaminoglycan-peptide species with chains of M_r = 10,000, approximately one-third their original size. Subsequently, these fragments are degraded to yet smaller species (M_r = 5,000) and then are finally depolymerized to free sulfate and oligosaccharides ($t_{1/2}$ = 20 hr). Although the intracellular degradation of this proteoglycan is markedly inhibited by chloroquine, the conversion into the first glycosaminoglycan-peptide intermediate (Fig. 5) is not significantly influenced by increases in pH [59], suggesting that this step may occur in weakly acidic compartments, probably endosomes. When pulse-chase experiments are performed in the presence of cytochalasin B, a drug that disrupts the microfilamentous network and also inhibits endocytosis [60], the newly synthesized proteoglycans are quantitatively released into the medium (Fig. 6) without being significantly internalized and degraded as described above. These results raise the possibility that the structural integrity of microfilaments is required for internalization and degradation of cell surface proteoglycans, in contrast to the process of exocytosis/secretion that apparently continues undisturbed (Fig. 6). The possibility, however, that cytochalasin B might have affected other mechanisms of intracellular catabolism cannot be totally ruled out.

The basic degradative pathways elucidated in our study are compatible with those described in ovarian granulosa cells [55,57,58] and fit patterns also observed in fibroblasts [34]. Furthermore, they are consistent with the presence of endoglycosidases [61,62] capable of generating heparan sulfate fragments nearly identical in size with the one we reported [59]. There are, however, several differences among cell systems, particularly as they relate to the kinetics of secretion and the presence of slow and fast pathways of intracellular catabolism. These quantitative and possibly qualitative differences in turn-

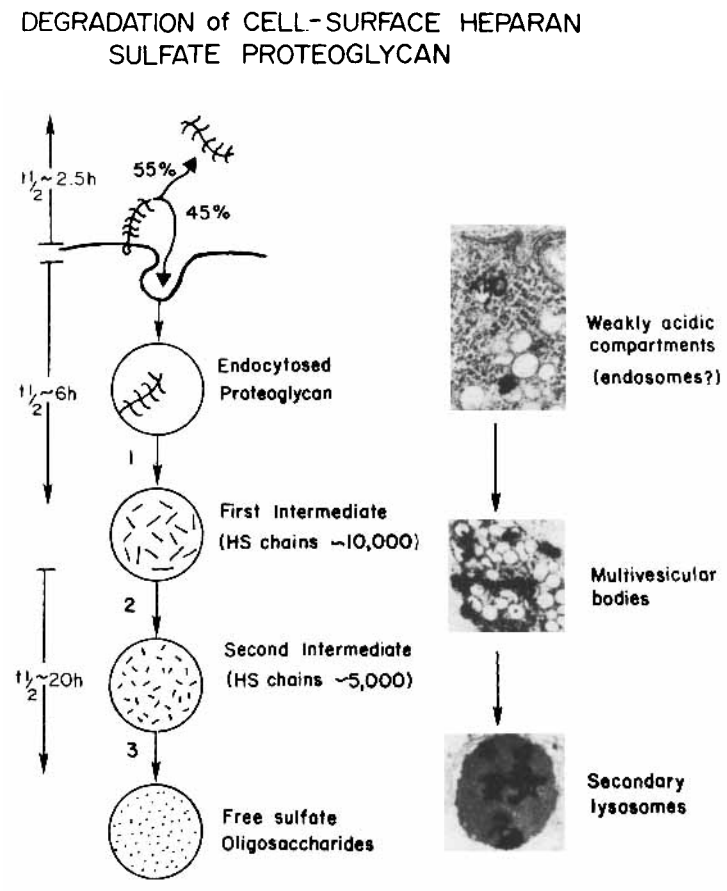

Fig. 5. Schematic representation (**left**) and electron microscope autoradiographs of various steps involved in the degradation of heparan sulfate proteoglycan from human colon carcinoma cells. The electron microscope autoradiographs were prepared from cells incubated with radiosulfate for 40 hr and chased for 8 hr in isotope-free medium [59]. As detailed in the text, steps 2 and 3 are markedly inhibited by chloroquine, a drug that quickly raises the pH of intracellular organelles, but step 1 is only slowed down by chloroquine. This suggests that the initial depolymerization of the endocytosed proteoglycan, ie, the generation of the first intermediate with heparan sulfate (HS) chains of M_r = 10,000, may occur in a weakly acidic compartment, probably within an endosome. The time constants depicted on the left were generated from kinetic studies using a combination of quantitative biochemistry and autoradiography [59].

over at various steps suggest the points at which metabolic differences among cells could occur, and provide the beginning of comparative data for understanding the functional role of these metabolic pathways in the normal and neoplastic state.

MULTIFUNCTIONAL ROLES OF CELL SURFACE PROTEOGLYCANS

Heparan sulfate proteoglycans are true "political" molecules. As such they occupy strategic positions, interact with a variety of parties of diverse composition, and eventually influence the behavior of cells. This interaction ranges from cooperative electrostatic

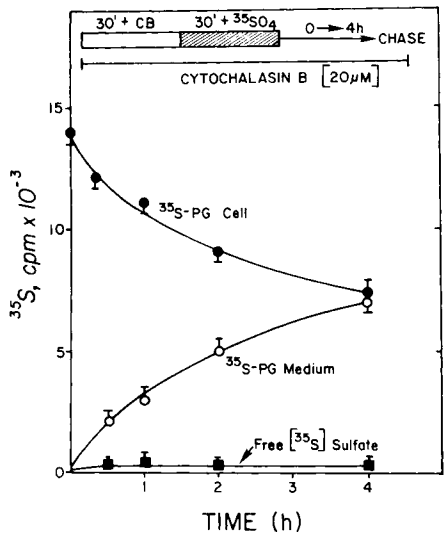

Fig. 6. Pulse-chase experiment in the presence of cytochalasin B. Colon carcinoma cells were preincubated for 30 min with 20 μM cytochalasin B (CB), a drug that disrupts the microfilaments and inhibits endocytosis [60]. The cells were subsequently pulsed for 30 min with 100 μCi/ml radiosulfate, washed, and chased in isotope-free medium for up to 4 h in the presence of same drug concentration. Notice that the proteoglycan (^{35}S-PG) released into the medium (○) accounts for the entire product lost from the cell layer (●), and that, for up to 4 h-chase, there is no significant generation of free [^{35}S]sulfate (■). These results indicate that the cell-surface proteoglycan is quantitatively released into the medium without being appreciably internalized and degraded, and implicate microfilaments in the translocation of the cell-surface proteoglycan to an intracellular degradative compartment.

binding of low specificity to precise lock-and-key interactions which depend upon the fine structure of the polysaccharide chain or the protein core. The list of proposed interactions and consequent functional implications is increasing at high speed. However, universally accepted roles for this family of macromolecules are still elusive. Therefore, the concepts reported in this section should be taken cautiously until any one of the proposed roles is confirmed by future investigations.

Transmembrane Interactions: A Link to the Cytoskeleton

The intercalated heparan sulfate proteoglycan has been proposed to bind cytoskeletal elements via an intracytoplasmic domain of the protein core, thus providing a linkage between the intracellular and extracellular environment. Work by Bernfield and collaborators [63] has shown that the putative membrane-bound proteoglycan from mammary epithelial cells interacts with polymerized actin in vitro. Using immunological approaches [64], these authors have demonstrated that this molecule is present in vivo solely on epithelial cell surfaces, where it localizes at the interface between epithelial and connective tissue. Interestingly, when the cells are maintained in confluent cultures, the proteoglycan is lost from the cell surface and is visualized at the basolateral regions, where it codistributes with actin microfilaments [65]. Further, when the cell surface proteoglycans are cross-linked with specific antibodies, they become detergent-insoluble, immobile complexes which are likely to be bound directly or indirectly to cytoskeletal

elements [65]. It has been proposed that the cell surface proteoglycan may represent a receptor for matrix molecules that is involved in maintaining cell shape and anchoring the cells to insoluble substrata [66]. The fact that the intercalated proteoglycan can be efficiently cleaved by trypsin in mammary epithelium [67] as well as in other cell systems [18,22,34,36,61] attests to a mechanism by which cells can loosen their attachment to the matrix.

Additional evidence for the role of intercalated proteoglycans in transmembrane interaction with the cytoskeleton is provided by the immunocytochemical codistribution of this molecule with microfilaments in cultured fibroblasts [68]. This proteoglycan becomes enriched in focal adhesion sites [68], which are left over on the culture dish following detachment of the cells with chelating agents [54]. Furthermore, when fibroblasts are extracted with detergent alone, a proportion of the hydrophobic proteoglycan remains bound to the cytoskeleton-matrix complex [69]. Copurification of heparan sulfate proteoglycan with cytoskeletal elements, such as spectrin, vimentin, and actin, has been demonstrated in Schwann cells [70]. More recently [71], it has been shown that purified liver cytoskeleton fractions are associated with a hydrophobic proteoglycan and that laminin is also tightly, but noncovalently, bound to this fraction.

In summary, the evidence discussed above indicates that at least in certain systems the cell surface heparan sulfate proteoglycan is closely associated with cytoskeletal elements, thus providing structural stability and support to the cell.

Self-Association: A Specialized Recognition Signal

Heparan sulfate chains express binding affinity for molecules of similar structure under physiological ionic conditions [72–75] and may provide a recognition signal for the cells to self-associate. This property is dependent on cooperative interactions between copolymeric regions of heparan sulfate chains, and the strength of binding is dependent on the interactions of a number of such zones. Self-association appears to be favored by alternating sequences of iduronic acid-rich and glucuronic acid-rich regions along the polymer as well as by an intermediate degree of sulfation and by a larger size. Interestingly, both the cell surface and the medium-released proteoglycans from fibroblasts have affinity for agarose-bound heparan sulfate chains [76], and this binding can be abolished by native or N-desulfated and N-acetylated heparin, but not by periodate-oxidized heparin. It has been suggested that these alternating sequences present in heparin, which are maintained by N-desulfation and N-acetylation but not by periodate oxidation, can compete with the putative contact zones responsible for heparan sulfate self-association [76]. These results raise also the possibility that the release of receptor-bound heparan sulfate proteoglycan from the cell surface by heparin may be due to interference with a proteoglycan/proteoglycan association mediated by the glycosaminoglycan chains [76].

In summary, given the elevated microheterogeneity in heparan sulfate composition among tissues, it is plausible that important contacts between cells or between cells and matrix may be mediated by specific heparan sulfate self-interactions. At present, however, the occurrence of such phenomenona in vivo and their physiologic relevance are unclear.

Interactions With Matrix Constituents: A Receptor for Extracellular Matrix

The interaction of interstitial proteoglycans with collagen is thought to be an important step in the formation and structural integrity of connective tissues [5,7,77], and recent evidence indicates that the protein core is directly involved in regulating the

formation of collagen fibers [78]. A role for the cell surface heparan sulfate glycosaminoglycans and proteoglycans in binding collagen has also been proposed. For instance, heparan sulfate chains isolated from mouse 3T3, SV3T3, and myeloma cells bind to native type I collagen, and this binding can be prevented by heparin and to a lesser extent by dermatan sulfate [79,80]. Bernfield and collaborators [81–83] have elegantly demonstrated that the isolated cell surface proteoglycan from mammary epithelial cells binds specifically to native type I collagen under physiological salt conditions. This binding shows high affinity, with $K_d = 10^{-9}$ M, and requires the intact proteoglycan. However, binding appears to be mediated through the glycosaminoglycan chains since it can be prevented by homologous molecules like heparin or dextran sulfate [81]. Interestingly, both isolated proteoglycans and living cells bind avidly to native type I, III, and V collagens, but not to denatured type I or native types II and IV [82]. This proteoglycan does not bind to laminin under physiological salt conditions [83]. It is intriguing that this heparan sulfate proteoglycan does not exhibit affinity for two components of the basal lamina (ie, laminin and type IV collagen) which are both produced by the same cells, but has affinity for interstitial collagens. On this basis, it has been proposed that the proteoglycan may represent a receptor for interstitial products typically secreted by stromal cells [83]. This mechanism of epithelial-stromal interaction may also favor the orderly deposition of basal lamina.

Other matrix constituents that may exhibit binding affinity for heparin and related molecules are fibronectin and laminin. Both glycoproteins have affinity for the polysaccharide and contain regions that bind heparin [for detailed review, see 84]. It has been postulated that the attachment and spreading of cells to their substrata is mediated by cell surface heparan sulfate proteoglycans [85,86] which also abound at the focal footpad adhesion site [87] and codistribute with fibronectin [88]. Further evidence for a direct role of cell surface proteoglycan in adhesion is provided by the finding that heparin, but not chondroitin sulfate, prevents the binding of 3T3 cells to substrate linked to platelet factor 4 [89]. Heparitinase treatment of these cells prior to adhesion to fibronectin inhibits their spreading but does not block their attachment [89]. Similarly, it has been shown that heparan sulfate is not required for maintaining cell attachment after it has taken place [90]. These binding studies [89] have also demonstrated a reorganization of the microfilamentous network and are thus in agreement with the transmembrane concept outlined above. Additional support for this functional role derives from the codistribution of the proteoglycan with actin and fibronectin [68]. In spite of these reports, it appears that the membrane-intercalated proteoglycan is not in itself the primary cell surface receptor mediating cell adhesion. In fact, a fibronectin domain that assures attachment of normal kidney cells in vitro does not contain the heparan sulfate binding site [91] and a purified heparin-binding domain of fibronectin lacks cell-spreading activity [92]; similarly, an isolated cell-binding domain of laminin appears to lack affinity for heparan sulfate [93]. Interestingly, cells adhere to substrata coated with a cell-binding domain of fibronectin but, unlike those adhering to intact fibronectin, they do not form focal attachment sites and associated stress fibers [94]. When larger fragments are used, encompassing both cell- and heparin-binding domains, the cells adhere and form contact zones as they do with the entire fibronectin. These results indicate that an additional external stimulus is necessary for the complete and possibly physiologic attachment of cells, and this second stimulus may involve the cell surface proteoglycan [94].

In conclusion, the data summarized so far indicate that the cell surface proteoglycan can mediate some properties involved in the recognition, attachment, and spreading of

cells and can thus be considered a general receptor for extracellular matrix proteins. The conflicting reports about specificity and activity may reflect experimental variations or, more importantly, an intrinsic multivalency of this class of macromolecules.

REGULATION OF CELL GROWTH

Since its original discovery [28,29], it has been postulated that the cell surface heparan sulfate may be intimately involved in the regulation of cell proliferation in eukaryotic cells. Heparan sulfate is released during premitosis [95] and a model for this molecule as growth modulator has been proposed [96]. Accordingly, the cell-cycle-dependent exposure of heparan sulfate to the cell surface would negatively affect the transport of nutrients and ions across the plasma membrane, with a consequent deleterious effect on cell growth [96]. The loss of the cell surface heparan sulfate could be a necessary event for the cells to enter mitosis [96]. A correlation between heparan sulfate metabolism and cell division derives from the observation that the amount of nuclear heparan sulfate is markedly increased when hepatocytes reach confluency and become contact-inhibited [97]. A further correlation between nuclear heparan sulfate and growth has been provided by the findings that the amounts of nuclear heparan sulfate in hepatoma cells is only 3–10% of that found in normal hepatocytes, and that culture conditions that reduce the amount of nuclear heparan sulfate also induce a loss of contact inhibition [52]. It is clear from these studies that nuclear heparan sulfate may play a role in cell division; however, it is premature to conclude that there is a direct cause-and-effect relationship between cell division and levels of nuclear heparan sulfate or that this effect is shared by other cell systems. That other control mechanisms may be involved in this process is offered by the fact that the levels of nuclear heparan sulfate can be regulated by insulin availability [52].

Glutaraldehyde-fixed confluent cultures of fibroblasts, which are enriched in cell surface heparan sulfate proteoglycans, inhibit the growth of freshly seeded cells, and this effect can be abolished by pretreatment of the former with heparitinase or nitrous acid [98]. These findings thus suggest that this class of molecules may exert a local control on cell proliferation.

In addition to this local growth-inhibitory capacity, the released heparan sulfate has been demonstrated to affect the growth of neoplastic and normal cells, thus providing a functional role for the released molecule. For instance, glycosaminoglycans isolated from liver can restore density-dependent inhibition of growth in Yoshida hepatoma cells [99]. Similarly, liver plasma membranes or heparan sulfate proteoglycan prepared therefrom inhibit the growth of AH-130 hepatoma cells in vitro [100]. Heparin is capable of preventing smooth muscle cell proliferation in vivo following experimental arterial injury [101], and this effect can be obtained with anticoagulant as well as non-anticoagulant species of heparin in vivo [102] and in vitro [103]. Castellot et al [104] have shown that confluent cultures of endothelial cells release a heparinlike molecule or a highly sulfated heparan sulfate that inhibits the growth of smooth muscle cells in vitro, thus providing a plausible explanation for the results obtained in vivo [101]. These active molecules are apparently released from the cell surface by a platelet endoglycosidase present in the serum [105,106]. Finally, heparan sulfate proteoglycans isolated from postconfluent smooth muscle cells contain about eight times more growth-inhibitory capacity toward themselves than those isolated from subconfluent cultures [107]. These studies suggest that some cells may use the cell surface proteoglycan as regulator of

their own growth and that the antiproliferative activity resides in specific, yet undetermined, polysaccharide sequences.

The studies summarized above implicate this class of macromolecules in the regulation of cell division. This function can be accomplished either proximally, by direct contact with cell surface receptors, or distally by proteolytic or endoglycosidic release of biologically active components of the molecule. It is conceivable that some of the functions could occur at a location distant from the site of production, thus mimicking some of the known effects of heparin in vivo.

HEPARAN SULFATE AND TRANSFORMATION

There is compelling evidence to implicate changes in the structural and functional organization of the cell surface in the altered behavior of malignant cells. Surface changes are normally viewed as contributing factors to the altered responsiveness of tumor cells to cell-cell contact interactions or to various hormonal and immunologic signals [108]. Given the strategic location, the structural heterogeneity and the functional multivalency of heparan sulfate described in the preceding sections, it is quite clear that specific qualitative or quantitative changes associated with transformation could lead to important changes in the behavior of the malignant cells.

Perhaps the most consistent alteration in cell surface heparan sulfate structure is a reduced degree of sulfation in a variety of cell systems. Over a decade ago, Underhill and Keller [109] reported that simian virus 40 (SV40)-transformed 3T3 cells synthesize undersulfated heparan sulfate chains. Since then, similar findings have been reported both in vitro [47,50,110] and in vivo [33,111,112]. The primary difference in sulfation between normal and transformed cells does not appear to be due to changes in the average hydrodynamic size, but rather to a decrease in the extent of O-sulfation in carbon position 2 of iduronic acid or carbon position 6 of glucosamine moiety [113]. Further support for a specific linkage between undersulfation and transformation is provided by the report that cells selected for high tumorigenicity from two independent clones of established cell lines, as well as SV40-transformed subclones, synthesize heparan sulfates with a reduced degree of sulfation [47]. Moreover, in vivo studies of human hepatomas have demonstrated that the heparan sulfate contains a lower sulfate/uronic acid ratio than that present in normal livers [112]. These alterations were detected in neither chondroitin sulfate nor dermatan sulfate fractions isolated from the same liver tumors [112]. Similarly, both the heparan sulfate isolated from ascites hepatoma cells [111] and that synthesized by a hepatoma cell line [50] exhibit a lower degree of sulfation than the heparan sulfate isolated from either normal liver or hepatocytes. In the latter case, however, the undersulfation appears to involve a reduction in N-sulfated groups, and it has been proposed that this alteration may be secondary to an inefficient N-deacetylase [50]. The N-deacetylation of glucosamine moieties has been identified as a regulatory step [114], and the extent of initial N-deacetylation appears to control the subsequent polymer modification reactions [17,115]. It is likely that the enzymes involved in the biosynthesis of heparan sulfate are arranged in a multi-enzyme complex in the Golgi region and the topology and structural integrity of this complex may regulate the production of heparan sulfate. If this were the case, then any abnormal heparan sulfate associated with the transformed phenotype may derive from selective disruption of this multi-enzyme complex.

Regardless of the mechanisms involved, it is clear that a qualititative change in

the fine structure of heparan sulfate would generate a multiplicity of effects. For instance, it has been reported that transformed cells are incapable of assembling a pericellular matrix, although they continue to synthesize all the necessary matrix constituents [116]. Also, there is a concomitant loss of fibronectin and laminin in transformed cells and this change is due to neither impairment of the cellular ability to bind fibronectin nor to alterations in the overall functional properties of the fibronectin molecule [117]. It is plausible that the lack of matrix assembly may be a consequence of the undersulfation of the cell surface heparan sulfate [5]. It is noteworthy that the heparan sulfate from nonadhering myeloma cells [80] or that from hepatoma cells [50], both of which lack a detectable pericellular matrix, are undersulfated and show low or no affinity for fibronectin.

Transformation-dependent structural changes in heparan sulfate have been also associated with abnormal self-affinity [118,119]. In quiescent cells heparan sulfate is retained at the cell surface, but during growth a significant proportion of it is lost, and the heparan sulfate remaining has a reduced degree of self-association [118]. In SV40-transformed cells the heparan sulfate chains have no affinity for agarose gels substituted with corresponding heparan sulfate species [119]. It is obvious that anomalous sulfation together with other not yet established changes in polymer structure may be directly involved in these functional alterations, and may lead to altered cell-cell and cell-matrix interactions discussed above. Finally, a reduced degree of sulfation of the heparan sulfate would reduce the overall charge of the cell surface and would thus contribute positively to cell growth, by derepressing the cells and favoring entrance into the mitotic phase. At present, however, there are no substantial data to support this idea.

CONCLUDING REMARKS

In this brief synopsis I have endeavored to highlight the diversity of forms and structures of heparan sulfate proteoglycans and to provide current evidence that they are important structural and functional molecules of most cell surfaces. It has become increasingly apparent that they play a multitude of key physiological roles. They can function as receptors for circulating molecules such as transferrin or for more static molecules such as collagen and other extracellular matrix proteins. As such they can constitute a link between the outer and inner cellular environment, a specialized form of communication mediated by the protein core and involving cytoskeletal elements. They can self-interact through copolymeric sequences in the glycosaminoglycan moiety; this ability has obvious implications in cell-cell and cell-matrix recognition. Their binding to specific matrix proteins helps in establishing epithelial cell polarity and normal morphology, and contributes to the maintenance of a differentiated phenotype.

In quiescent cells in vitro or in differentiated organ structures in vivo, these proteoglycans probably express one or the other of the above-mentioned functions. During neoplastic growth and invasion, however, these molecules undergo profound changes which parallel, in a sense, the marked remodeling of plasma membrane constituents that occurs during mitosis. At this time, structural changes in the heparan sulfate proteoglycan may have a multiplicity of effects. For instance, undersulfation of heparan sulfate chains may prevent the formation of a physiologic matrix around tumor cells and the assembly of a normal basement membrane, and thus may favor tumor growth and infiltration of host tissues. Biologically active portions of the molecule, released by proteases or endoglycosidases, may reach distant cells and influence their growth capacities. The

abnormal heparan sulfate associated with the transformed phenotype may also help in understanding the decreased cohesiveness and the lack of contact inhibition, two established properties of malignant cells. It is tempting to speculate that the presence of an altered microenvironment around neoplastic cells may significantly contribute to the establishment of the transformed phenotype.

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