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The heparanome and regulation of cell function: structures, functions and challenges

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1. ABSTRACT

The cell-extracellular matrix interface is a crowded space whose structure is dependent on macromolecular assemblies that are dynamic in time, molecular composition and location. Signals travel from one cell to another (or to the same cell) by the regulated assembly / disassembly of molecular complexes. These signals can evoke relatively simple biological responses such cell proliferation and migration, but once integrated, they guide cell fate in complex biological phenomena such as embryonic development and organism homeostasis. Heparan sulfate proteoglycans are ubiquitous components of this space and important actors of these processes in all tissue-organized life forms. A key feature of heparan sulfate is its size, 40 nm to 160 nm, which enables it to integrate self-assembling macromolecular structures over substantial length scales. What is the structure of heparan sulfate? Why do we think heparan sulfate is so important? How do we try to explain its activity? What do we know about its interactions? These questions together with a final look to the future are the "menu" of this review.

2. INTRODUCTION

Heparin was identified in 1916 as a material extracted from dog liver able to inhibit clotting of plasma *ex vivo* (1, 2). For its anticoagulant activity, heparin entered the list of approved drugs of the American Food and Drug Administration (FDA) from 1939 and it is nowadays the largest, by weight, biotechnology product used in the clinic. More than thirty years after its discovery, a family of compounds related to heparin was identified. Because of their lower level of sulfation compared to heparin, they were named heparin monosulfuric acid (3). These compounds are now referred to as heparan sulfate (HS) and we know that it is more correct to consider heparin as a particular form of HS rather than *vice-versa*. In the last twenty years, HS took a curtain call in the theatre of the scientific community for its involvement in a large number of fields of major biomedical interest and fundamental biological importance. New therapeutic agents targeting HS function are today under investigation to tackle high impact pathologies such as cancer and atherosclerosis (4-6). Many scientists around the globe have contributed to the

production of an impressive amount of important, sometimes contradictory literature. For many of the main subjects related to HS detailed and complete reviews are available.

The aim of this review is to introduce the world of heparan sulfate proteoglycans through a brief summary of what we know about these fascinating molecules, highlighting the many questions that are still open and ones that are emerging, and to identify what may be important future research. In the first part the structural features that characterize HS and how its expression is finely regulated by the cell machinery are described. The second part outlines a series of *in vivo* evidences for the central role of HS in crucial biological processes and proceeds with the illustration of some of the mechanisms proposed to explain its activity. In the third part some thermodynamic and structural concepts underlying the interaction of HS with proteins are summarized. Our view of the future is put forward at the end.

3. COMPLEX STRUCTURES FINELY REGULATED

3.1. The heparan sulfate proteoglycans

Heparan sulfate (HS) is a linear polysaccharide containing a characteristic disaccharide repeating unit. It belongs to the family of glycosaminoglycans (GAGs) together with chondroitin sulfate (CS), dermatan sulfate (DS), hyaluronic acid (HA) and keratan sulfate (KS). First identified as a low sulfated form of heparin (3), HS attracted a growing number of scientists from different areas of biology for its involvement in the regulation of many events that take place at the interface between cell and extracellular matrix (ECM). Its expression is almost ubiquitous in mammalian cells and its complex structure differentiates HS from other GAGs (7).

HS polysaccharides represent one of the main components of the cell surface and ECM. They are synthesized as covalent complexes with core proteins containing sugar attachment consensus sequences, consisting in general of a Ser-Gly motif flanked by at least two acidic amino acid residues (8), forming heparan sulfate proteoglycans (HSPGs). Three major families of HSPGs have been characterized. They include the transmembrane proteins syndecans (four members in mammals), the glypicans, proteins attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (six members) and ECM proteins such as perlecan, agrin and collagen XVIII (for reviews see (9-14)). The nature of the core protein determines the localization of the HS chains at the cell surface (syndecans, glypicans) or in the ECM (perlecan, agrin, collagen XVIII). Other proteins such as CD44v3, betaglycan and testican are not synthesized by default as HSPGs but they can be modified with HS chains under particular circumstances (15).

The structural variability of these polysaccharides results from the arrangement of postpolymerization modifications, mainly sulfation, along the sugar backbone and on their polydispersity. The repeating unit consists of 1-4-linked pyranosyluronic acid and 2-amino-2-deoxyglucopyranose (glucosamine) residues. The uronic acid may be either beta D-glucuronic acid (GlcA) or its C-5 epimer alpha L-iduronic acid (IdoA). The beta D-glucosamine may be N-acetylated (GlcNAc), N-sulfated (GlcNS) or, rarely, unsubstituted (GlcN). Variable degrees of O-sulfation may occur at position 2 of the uronic acid and at positions 3 and 6 of the glucosamine (Figure 1A).

The complexity of HS derives from the characteristics of its biosynthesis (reviewed in (16)). Different sequences are assembled by a non template-driven process, in which a set of enzymes acts on the growing saccharide polymer introducing modifications to the sugar backbone. These modification reactions depend on the enzymes' specificity and cellular physiology and do not go to completion (Figure 2) (16). Three phases can be distinguished: chain initiation, polymerization and polymer modification. The first step is the assembly of a linker tetrasaccharide attached to specific serine residues of newly translated core proteins. The transfer of a UDP-xylose by xylosyltransferase, followed by the addition of two D-galactose residues by galactosyltransferases and a GlcA by glucuronosyltransferase I, forms the linker tetrasaccharide, GlcAbeta1-3Galbeta1-3Galbeta1-4Xyl-L-[ser]. This is common to heparin, HS, CS and DS. The following addition of an alpha4GlcNAc residue or of a beta4GalNAc residue to the linker region commits the process toward the synthesis of heparin or HS, in the first case, and CS or DS in the second (16). After hexosamine has been added, the polymerization of the heparin / HS chain occurs through the alternate transfer of UDP-GlcAbeta4 and UDP-GlcNAcalpha4 to the non-reducing end of the growing polysaccharide. The reaction seems to be catalyzed by hetero-oligomeric complexes of members of the EXT gene family (copolymerases, two isoforms in humans), which possess both transferase activities (17). The chain elongation proceeds to variable extents generating polysaccharides of different length (16).

Growing precursor polymers are subject to a maturation process in which the sugar backbone undergoes different modifications. These modifications are carried out by a series of enzymes, four classes of sulfotransferases and an epimerase, that act in an ordered and regulated way. The first modification is the replacement of the N-acetyl groups of GlcNAc with sulfate groups. Both reactions are catalyzed by dual functional N-deacetylase / N-sulfotransferases (NDSTs, four isoforms) and are normally tightly coupled. However, rarely they can occur in dissociative fashion, explaining the presence of small amounts of unsubstituted GlcN residues in heparin and in HS (16). The presence of N-sulfated groups is a prerequisite for all other modifications and thus this step plays a key role in determining the structure of polymer chain.

The C5 epimerization of some GlcA to IdoA is catalyzed by C5 epimerase (EPI, one isoform), whose substrates are GlcA attached to the reducing end of GlcNS. This enzyme does not react with uronic acids that are O-sulfated or adjacent to O-sulfated glucosamines (16), suggesting that this modification must occur immediately after the NDST reaction.

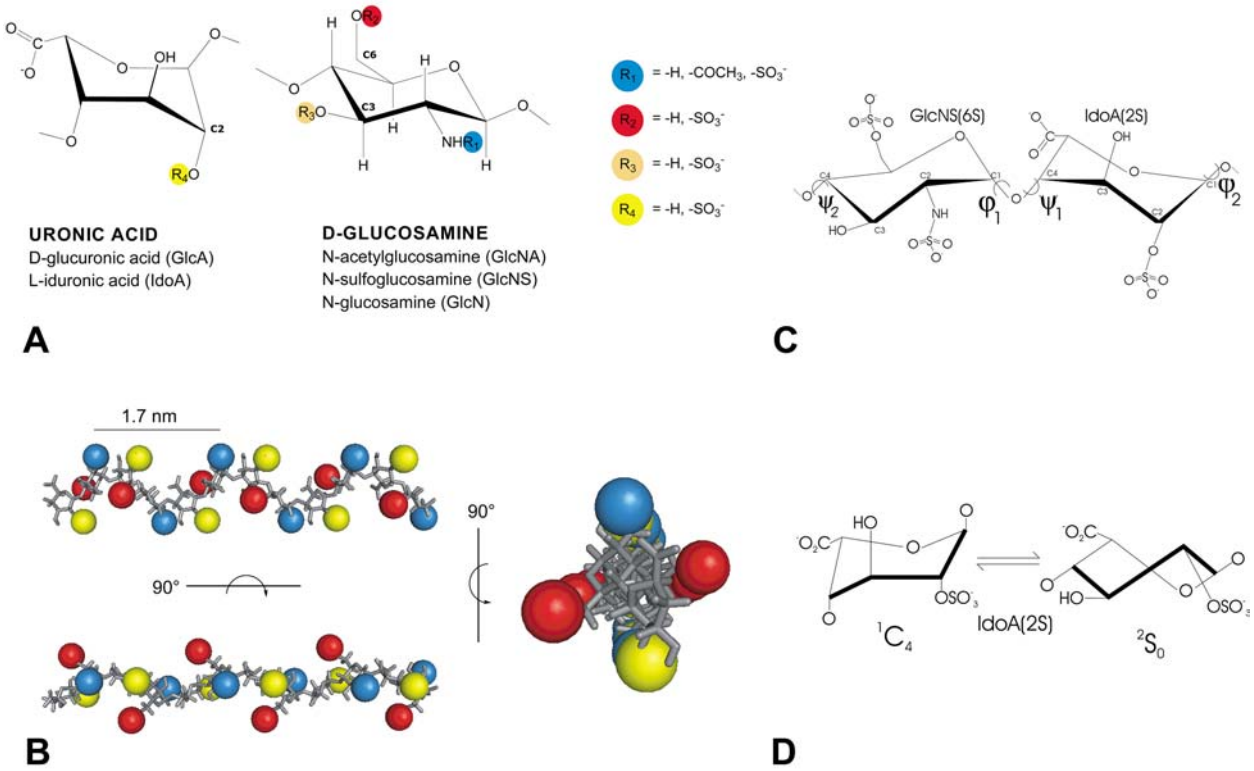

Figure 1. A. Heparin and HS monosaccharides. The repeating units consist of an iduronic acid, GlcA or its C5-epimer IdoA, 1-4-linked with a glucosamine residue. The glucosamine may be N-acetylated (R1= $COCH_3$), N-sulfated (R1= SO_3^-) or, rarely, unsubstituted (R1= H). Further complexity derives from variable degree of O-sulfation that may occur at position 2 of the uronic acid (R4) and at positions 3 (R3) and 6 (R2) of the glucosamine. B. Conformation of heparin-derived dodecasaccharide obtained by NMR (PDB: 1HPN). The sugar backbone is showed as sticks and the sulfate groups as spheres. The color code for sulfate groups is the same of figure 1A. Picture obtained from 1HPN file using PyMol (http://www.pymol.org). C. Glycosidic bond dihedral angles. The dihedral angles defining the rotation of the sugar chain around glycosidic bonds are defined as phi (H1-C1-O-C4') and psi (C1-O-C4'-H4'). D. The pyranose rings of heparin / HS can assume different conformations. GlcA and GlcN assume the rigid $^4C_1$ chair conformation, while IdoA tends to be more flexible, with its usual conformation being an equilibrium between the $^1C_4$ and $^2S_0$ skew-boat conformers.

Variable extents of O-sulfation expand the sequence complexity. The uronosyl-2-O-sulfotransferase (2OST, one isoform) catalyzes the O-sulfation in position 2 of both GlcA and IdoA, preferring the latter substrate. The glucosamine can be O-sulfated at position 6 and, rarely, at position 3. These reactions are catalyzed by 6-O-sulfotransferases (6OST, three isoforms) and 3-O-sulfotransferases (3OST, six isoforms), respectively (18).

The polymerization of the sugar chain begins in the lumen of the endoplasmic reticulum (ER) with the xylose incorporation into core proteins and proceeds in the Golgi apparatus. Despite the cloning of cDNAs encoding most of the biosynthetic enzymes involved and characterization of the enzymes, the architecture of the machinery and its regulation remain poorly understood (19). Nevertheless, the rapidity of the process and the ability of some of these enzymes to interact with each other suggest the existence of a biosynthetic complex ("gagosome") (16). How the whole process is coordinated and the regulatory mechanisms that determine the pattern of chain modification are not known (20).

Heparin shares with HS the same disaccharide units (Figure 1A). However, expression, localization, chain lengths and level of sulfation distinguish these two classes of molecules. Heparin presents a far more homogeneous and high level of sulfation (21) and its expression is a prerogative of connective-tissue mast cells and oligodendrocyte-type-2 astrocyte progenitors (22, 23). Heparin proteoglycan consists of a unique core protein (serglycin) with multiple heparin chains attached. In the mast cell the sugar chains are then cleaved at some of the GlcA residues by an endo-beta-glucuronidase, to give a mixture of smaller polydisperse heparin polysaccharides that are stored in the cytoplasmic secretory granules as non-covalent complexes with basic proteases (24). Nevertheless, due to its commercial availability and more homogeneous / simpler structure, heparin or its derivatives have been used in the last decades as a model for HS, especially in the study of sugar-protein interactions that often involve the sulfated region of the polysaccharide (25).

Their structural complexity makes heparin / HS extremely challenging molecules to characterize. A

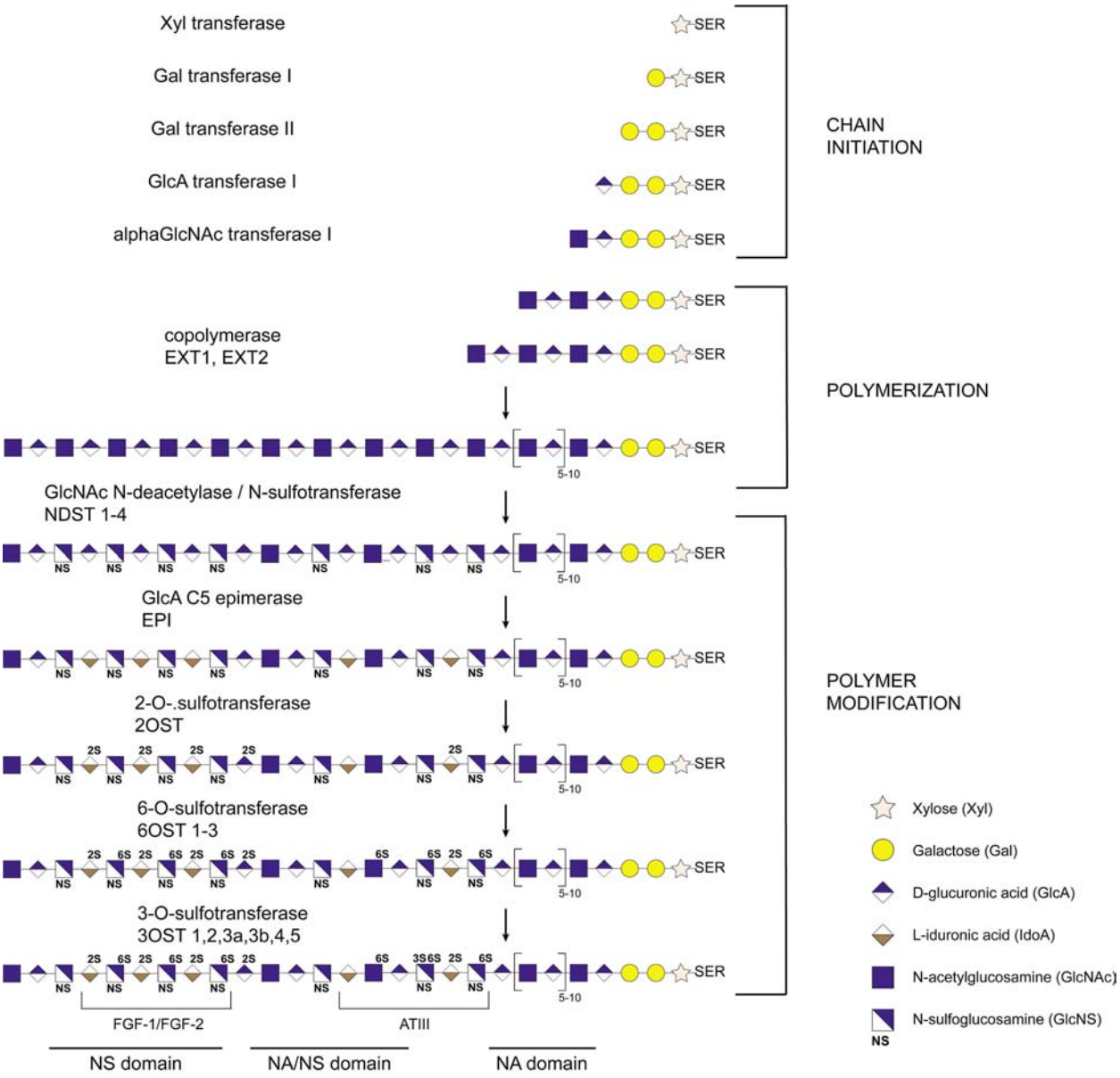

Figure 2. The polymerization and chain modification steps of HS are schematically represented. The size of the chains is arbitrary. Monosaccharides are represented according to the guidelines of the Nomenclature Committee of the Consortium for Functional Glycomics (http://glycomics.scripps.edu/CFGnomenclature.pdf).

complete structural analysis of these polysaccharides would require the identification of the chain composition, its sequence and the characterization of its solution conformation. The majority of data available derives from compositional analysis of HS preparations obtained by enzymatic / chemical degradation of the sugar chains. The existence of a domain organization in the HS chains was first demonstrated by pioneer works of Linker and Cifonelli (26, 27). They investigated the structural features of HS combining degradation with nitrous acid (27) and / or enzymatic digestion with heparinase I (26, 28) with separation techniques such as paper chromatography and gel filtration chromatography. The interpretation of the complex profiles obtained led the authors to propose a model for HS structure where more abundant unmodified regions are alternated with block of sulfated units along the chain (26-28). Furthermore, they observed that the region proximal to the linker tetrasaccharide is composed mainly of an N-acetylated sequence of 6 or 7 units resistant to nitrous acid treatment (27). The characterization of HS structure obtained from a single cell type allowed the definition of a more complete and accurate model (29-32). Turnbull and Gallagher investigated the structure of HS purified from human skin fibroblasts using an approach derived from the one of Linker and Cifonelli (30-32). In the proposed model regions with a high degree of modification and sulfation (NS domains) alternate with unmodified regions, rich in GlcA and GlcNAc, (NA domains) and

mixed regions containing both NA and NS units (NA/NS domains) lie at the boundaries of the NA and NS domains (32, 33) (Figure 2). The model includes the presence of a highly conserved N-acetylated region of eight disaccharide units contiguous with the protein-linkage region identified in human skin fibroblast HS, too (29) (Figure 2). Although informative, these data describe only the average composition of HS chains and are not sufficient for a comprehensive characterization of their structure. In the last decade two new sequencing strategies have been implemented. These methods allow the identification of the sequence of purified oligosaccharides (up to 10 sugars in length), combining partial chemical cleavage and enzymatic digestion with separation techniques such as gel electrophoresis, HPLC and MALDI-MS (34-36). However, sequencing of an entire chain (50-200 saccharides) is well beyond current technical capabilities.

Structural data have been obtained in the last decades by X-ray crystallography, NMR and molecular modeling performed on heparin or HS derivatives (37-41). NMR and crystallography studies suggested that heparin may assume a right handed helical structure with successive disaccharide units in the chain related by a rotation of 180□ and a translation of 0.82 to 0.87 nm (39) (Figure 1B).

The pyranose rings of heparin / HS can assume three main conformations. While the glucuronic acid and the glucosamine have always been observed in the rigid $^4C_1$ chair conformation, the iduronic acid tends to be more flexible and it can also assume the $^1C_4$ and $^2S_0$ skew-boat conformations (42) (Figure 1C). The IdoA conformation depends on its substitution pattern and on the location of the residue along the chain. When the residue is at the reducing end all three conformers $^4C_1$, $^1C_4$ and $^2S_0$ are permitted. The balance of this equilibrium depends both on the iduronate 2-O-sulfation and on the substitution of adjacent glucosamine residues (43). The flexibility introduced in the chain by IdoA is considered crucial for protein binding, as it facilitates the docking between anionic groups of the sugar and cationic patches on the protein surface (44).

The glycosidic dihedral angles phi (H1-C1-O-C4') and psi (C1-O-C4'-H4') (Figure 1D), which define the overall chain conformation, have been studied by molecular modeling and NMR (39, 45). These early studies showed that rotation around the glycosidic bond is limited by steric interactions, in particular for the GlcNbeta1-4IdoA bond (39). Further studies revealed the relative flexibility of chemically modified heparin chains, in comparison to an unsubstituted polysaccharide (*E.Coli* polysaccharide K5) (46). NMR analysis of selectively modified heparin derivatives showed the influence of O- and N-sulfation on the glycosidic linkage geometry and iduronate ring conformation (40, 41), as exemplified by the effect of 2-O-sulfation on the iduronate conformer population ($^1C_4$:$^2S_0$ ratio) (40). The conformational changes induced by modifications do not affect only residues subjected to sulfation, but also more remote positions with a possible influence on the overall chain conformation and hence on the biological activity of the sugar (40).

Recently new spectroscopic approaches have opened the door to understanding the conformation of entire chain (47). In a recent work the solution conformation of heparin and eight systematically modified heparin derivatives has been investigated using a combination of synchrotron radiation circular dichroism (SRCD) and NMR spectroscopy. This study reports for the first time that the conformation and flexibility of heparin chain can be affected not only by the substitution pattern, but also by the nature of the cation associated with the sugar. Interestingly, the conversion to different cationic forms affects also the biological activity of heparin in an *in vitro* assay of fibroblast growth factor signaling activation (BaF3 proliferation assay) (47). The role of different cations have been implicated also for the binding of heparin / HS to annexin 2 (48), annexin V (49) and L-selectin (50) (Ca^{2+}), and endostatin (51) (Zn^{2+}). However, while for annexin 2 both the sugar and protein participate in the coordination of the cations, in the other cases the interaction with the cation seems to be restricted to the protein. In this last case the role of cations would be to induce a conformation of the protein more favorable for sugar binding (49, 51).

3.2. Regulation of chain expression

One of the main features of HS that emerged from early studies is the spatial and temporal regulation of its expression. Analysis of HS preparations obtained by enzymatic and chemical degradation revealed heterogeneity in the sugar composition between different samples. Reproducible variations have been observed between samples extracted from different species and tissues. Differences in chain length, total, N- and O-sulfation levels, IdoA / GlcA ratio as well as the distribution of the modification patterns along the chain characterize HS from different sources (21, 52-55). For example, rat liver heparan sulfate on syndecan-2 presents a terminal heparin-like structure with an unusually high content of N- and O-sulfate that result in a total sulfate content at least 50% higher than other HS species such as endothelial and skin fibroblast HS (53).

Characteristic changes of HS structure have been associated with certain pathological conditions. The structure of liver and spleen HS isolated from patients affected by AA amyloidosis and from patients control was compared. Interestingly, HS from AA amyloidosis patients showed structural similarity, independently from the organ of origin, and differences were observed when compared to control samples (56). This study characterized for the first time a specific modification of HS structure (variation of the O-sulfation profile, especially 6-O-sulfation) associated to a pathological degeneration of organ architecture (56). Using a similar approach, Lindahl and colleagues performed a structural comparison between HS extracted from cerebral cortex of patients affected by Alzheimer's disease (AD) and control subjects. In this case no significant variation was observed between AD patients and controls. Brain HS showed characteristic disaccharide

composition in comparison to other organs and homogeneity was observed between HS samples from different regions of the brain (57).

A gradual modulation of HS structure has been observed during ageing. Human aorta HS obtained from patients of different ages presented an age-dependent increase of GlcN 6-O-sulfation and, by consequence, of the trisulfated units IdoA(2S)beta1-4GlcNS(6S). This modification has been associated with an increased binding of plateled-derived growth factor A and B chains to HS extracted from elderly patients, suggesting a possible mechanism for the involvement of HS in the patophysiology of atherosclerosis (58).

Modification of HS chain composition has been correlated to the tumorigenicity of different cell lines (59). The association between HS structural changes and cell transformation has been further investigated using an *in vitro* model of the progression of human colon cancer from adenoma to carcinoma. The comparison of HS isolated from adenoma and carcinoma cell lines revealed differences in O-sulfation pattern (decrease of 2-O-sulfation associated with increase of 6-O-sulfation in carcinoma derived material), even if the overall structure of the sugar chains was conserved (60).

All the studies cited above take in consideration the total pool of HS chain extracted from whole organs or cell lines without any possible discrimination between polysaccharides deriving from different core proteins. A first work described a modification of the HS structure in two different murine neuroepithelial cell lines derived from embryonic days 9 and 11. At these early stages neuroepithelial cells secrete essentially one type of HSPG, a perlecan related proteoglycan (HSPG-PRM) (61). The HSPGs isolated from the medium of the two cell lines shared the same core protein, but they displayed different binding ability to FGF-1 and to FGF-2. A simple structural analysis of the polysaccharides revealed differences in the number of chains and chain length between the two samples. This report gave the first evidence that the nature of the sugar chains is sufficient to change the binding specificity of a proteoglycan (62). Two other works analyzed in more detail the structural features of HS chain obtained from a single type of HSPG (syndecan-1) expressed by different cell lines, that displayed variations in the binding affinity for HS interacting proteins such as collagen type 1 and FGF-2. These variations were associated to modification of the HS structure in term of content and pattern of sulfation (63, 64).

The use of antibodies able to recognize different HS epitopes provides a single and defined structural framework with which to probe HS structures. These antibodies have revealed specific epitopes distributions among different cell types in the context of the same organ (65-67). Using similar approaches, a spatial-temporal regulation of HS epitopes expression has been observed during development in hamster embryo, rat skeletal muscle and rat lung (65, 68, 69).

An alternative approach is to exploit the specificity of HS binding proteins to probe the spatial and temporal distribution of HS structure. Exogenous FGF-2 identifies changes in binding sites between basement membrane of rat mammary gland ducts and terminal end buds, the structures responsible for ductal growth and branching (70). Similarly, Rapraeger and colleagues used a panel of FGFs and FGF / FGFR to probe HS structures that bind the growth factors and are competent to assemble ligand-receptor complexes. Again, remarkable specificity was demonstrated in term of tissue domains possessing HS binding sites with such functions (71-73).

What are the mechanisms involved in the fine and dynamic regulation of HS expression at the cell and tissue level? Nowadays we do not have an answer to this question. However, many recent studies point out the existence of several possible levels of control. The regulation of HSPG biosynthesis is of course the most obvious. Due to its complexity many mechanisms could be involved. (i) The core protein expression can influence the number of HSPGs expressed by a cell, the localization of HS chains and their activity. (ii) The assembly of the biosynthetic machinery can affect the structure of the sugar. The existence of different isoforms (16) and different splice variants (74) of the biosynthetic enzymes possessing different tissue distribution and different specificity (18, 75) is the central core of HS regulation. How the machinery is assembled and what is its influence on the activity of individual modification enzymes are probably the keys to understand the regulation of the process (16, 19). (iii) The availability of substrates (e.g. rate of PAPS synthesis and import into the Golgi) can be a further level of control of the HS synthesis (16).

Furthermore, different post-synthesis mechanisms exist to edit the structure of HS. (i) The extracellular domain of both syndecan and glypican can be shed from the plasma membrane by proteolytic cleavage (syndecans and glypicans) or by cleavage of the GPI-anchor (glypicans) (9). The shedding can result in a drastic change in the concentration of HS chains at the cell surface and in the ECM, potentially affecting the activity and distribution of their ligands. Syndecan-1 shedding has been associated directly with mesenchymal induction of epithelium (76). Recently a new lipase (Notum) has been identified in *Drosophila* as negative regulator of Wingless (Wg) signaling pathway. The Wg inhibitor activity resides in its ability to release the extracellular domain of Dlp, a Drosophila glypican (77). (ii) The HS chains can be degraded by extracellular endo-beta-glucuronidase (heparanase) (78). In this case, the degradation of the sugar results in the liberation of associated proteins and in the release of active HS fragment of 5-7 kDa of size able to sustain the activation of growth factors signaling such as FGF-2 (79). Furthermore, acting on ECM proteoglycans the enzyme can take part in the remodeling of the extracellular environment promoting phenomena such as cell spreading and angiogenesis (79). Heparanase has become object of intense studies in the last year for its involvement in tumor growth and invasion (80). Several therapeutic approaches targeting the activity of this enzyme

are under investigation (6). (iii) Finally, the sulfation pattern of the HS chains can be modified by cell surface sulfatases (81). Sulf1 and Sulf2 are two 6-O-endosulfatases specific for HS chains anchored to the cell surface through interactions of a hydrophilic domain with cell components. Their activity influences the function of heparin / HS binding proteins such as Wnt, BMP and FGF-2 (82). These enzymes act as proper editors of the HS code; selectively removing 6-O sulfate they can affect conformation and binding properties of the sugar. The effects of these modifications depend on the nature of HS-protein interactions. The activity of the quail orthologue has been shown to enhance Wnt signaling (81). The proposed model is that the removal of 6-O sulfate drastically reduces the affinity of Wnt for HS, allowing the growth factor to interact with and activate its receptor Frizzled with higher efficiency (81). *Vice-versa*, in the case of the FGF system, where the 6-O-sulfation is required for the formation of an active signaling complex (83), the activity of Sulf shows an inhibitory effect (84).

4. REGULATION OF CELL FUNCTION

4.1. Human diseases and model organisms

When it was already demonstrated that the complex structures of HS are not generated randomly, instead the consequence of biosynthesis and post polymerization editing, the identification of *Drosophila melanogaster* defective phenotypes and human hereditary diseases associated to HSPG core proteins and biosynthetic enzymes provided the *in vivo* evidence of the central role of these molecules in the regulation of cell function (for reviews see (85-87)).

The EXT1 and EXT2 genes, encoding the enzymes responsible for the polymerization of the heparin / HS chains, are considered possible bone tumor suppressor genes, since heterozygous null mutations predispose to the development of benign cartilage capped tumors from the growth plates of bones, characteristic of the autosomal dominant disorder multiple exostoses (HME; MIM 133700) (88). Deletions and mutations in the gene encoding glypican-3 core protein (GPC3) are responsible for the X-linked Simpson-Golabi-Behmel overgrowth syndrome (SGBS; MIM 312870). This syndrome is characterized by a pre- and postnatal overgrowth and increased susceptibility to the formation of certain tumors (89, 90). Interestingly, Gpc3 null mice display a phenotype characterized by developmental overgrowth similar to the SGBS condition (90). Moreover, changes in glypican-3 expression have been associated to the progression of certain tumors (i.e. hepatocellular carcinoma) (91) and increased levels of glypican-1 have been found in cervical and pancreatic cancer (92). In these cases, a deregulation of HS expression affecting either its synthesis or its localization leads to an impairment of tissue homeostasis and aberrant cell growth, even though effects solely due to alteration of protein structure can not be excluded.

At the same time *Drosophila melanogaster* defective phenotypes bearing mutations in genes encoding enzymes involved in the biosynthetic pathway of GAGs were isolated. Mutations in both genes coding HS specific biosynthetic enzymes such as HS glycosyltransferases (*tout velu* (*ttv*)*, brother of tout velu* (*botv*)*, sister of tout velu* (*sotv*)) and NDST (*sulfateless* (*sfl*)) and in proteins involved in the synthesis and transport of biosynthetic precursors, as in the cases of *sugarless* (*sgl*), *fringe connection* (*frc*) and *slalom* (*sll*) (see (86, 87) and references therein) cause abnormal embryonic segment polarity indicative of the possible role of HSPGs in the control of development. The most intriguing observation from these studies is the similarity between these phenotypes and the ones bearing mutations in the Wnt / Wingless (Wg) and Hedgehog (Hh) signaling pathways. These pathways, together with the transforming growth factor-beta (TGFbeta) – Decapentaplegic (Dpp) and FGF signaling pathways, play a central role in development processes and all of them include heparin / HS binding proteins (see Table 1). Another gene, *pipe* (*pip*), involved in dorsal / ventral patterning has been associated to the mammalian gene encoding for 2OST, however its involvement in HS biosynthesis has been recently questioned (93). The functional role of the genes *hs3st* and *hs6st* has been investigated using RNAi-mediated knock down. These genes, encoding the fly homologues of 3OST and 6OST, show genetic interactions with *Notch* and *breathless* (FGFR) (87).

Genes encoding HSPG core proteins have also been associated with defects in development. Mutations of Dally and Dlp, two *Drosophila* glypicans, result in a weak segment polarity defect and defects in wing, antennae and genitalia morphogenesis (90, 94). Dally and Dlp are required for Hh diffusion and signaling during embryo development and they are essential for Wg gradient formation in the wing disc (95, 96). Their activities are also required for Dpp signaling and distribution during wing development. In contrast, it seems that the Dpp control of embryonic dorsoventral patterning is Dally / Dlp independent (97, 98).

The genes encoding the core proteins syndecan (*sdc*) and perlecan (*terribly reduced optic lobes* (*trol*)) are involved in central nervous system development. *sdc* mutants present defects in axonal guidance associated to alterations in Slit-Robo pathway, while Trol influences neural stem cell proliferation through modulation of FGF and Hh signaling (86).

Further data obtained from other model organisms have confirmed the role of HSPGs in the control of development and homeostatic processes (87, 99). Targeted deletion of genes involved in heparin / HS biosynthesis results in embryonic / perinatal lethality in mice. Homozygous deletion of Ext1 or Ext2 genes encoding HS polymerase enzymes causes failure of gastrulation (100, 101), while the heterozygous condition (Ext2) is associated to development of exostoses and multiple cartilage abnormalities (101). Mutations in two of the four Ndst genes result in very specific phenotypes. Ndst1 null mice present perinatal death due to respiratory failure. The phenotype is associated to forebrain defects, facial abnormalities and axon guidance defects (87). In the

Ndst2 mutants the abnormalities are restricted to mast cells, where the lack of correctly modified heparin affects the formation of secretory granules (102, 103). Alterations of enzymes involved downstream in the biosynthetic process are also responsible for severe phenotypes, indicating the importance of the fine structure of HS for its activity. HS-C5 glucuronyl epimerase and 2OST mutants (both enzymes have a single isoform in mouse) die perinatally and present renal agenesis. Inactivation of one of the five genes encoding for 3OST, Hs3st1, results in a mild phenotype with intrauterine growth retardation (87). Transgenic mice lacking the isoform 1 of 6OST (HS6ST1) have been recently created (104). Homozygous mutants present late embryonic lethality, impairment of placental function due to aberrant angiogenesis and lung deformation (104).

The loss of function of single core proteins produces milder phenotypes, probably due to a partial overlap of function between the different isoforms and the very modest selection pressures experienced by model laboratory animals. The deletion of syndecan 3 gene, for example, causes a subtle phenotype characterized by an alteration of feeding behavior (87). Further analysis revealed structural changes in the adult cerebral cortex of knockout mice related to an altered neural migration (105). More severe phenotypes are caused by the loss of the ECM core protein perlecan. Knockout mice display embryonic or perinatal lethality associated with aberrant brain development and cartilage abnormalities (106).

These data give an extraordinary indication of the central role of HSPGs in the control of development processes and organ physiology. However, the complexity of HS complicates the analysis of mutants' phenotypes and the understanding of underlying mechanisms. (i) Our poor understanding of the biosynthetic machinery is a major limitation. How the whole process is regulated, what is the role of different enzyme isoforms, how their expression is spatially / temporally regulated, what is the level of redundancy between their activities are still open questions that reduce our capabilities of interpretation. (ii) On the core protein side, the impossibility to discriminate between HS and core protein related effects has to be considered together with the limits deriving from the presence of multiple isoforms and from possible compensatory effects. Particularly informative from this point of view is the work of Rossi and colleagues who generate mice expressing a mutant perlecan lacking three of the four possible HS attachment sites (107). In this case mutants are viable, fertile and express perlecan with a reduced level of HS modification. The knockout mice present small eyes and they are affected by lens degeneration after birth (107). Interestingly, a similar phenotype has been obtained by deletion of collagen XVIII, a related ECM core protein (87). Further analysis revealed alterations of FGF-2 induced tumor growth and wound and tumor angiogenesis (108). The difference with the more severe phenotype deriving from the deletion of the whole perlecan gene indicates the presence of functions independent from HS chains. (iii) The limited viability of many of these mutants impairs the study of the impact of such mutations in adult physiology. This last issue has been overcome in some cases by the creation of conditional mutants (109-111). Two related, recently published papers reported the creation of mice bearing a targeted inactivation of the gene Ndst1. Using the Cre-*lox*P system the authors obtained viable and fertile mutants with Ndst1 deletion restricted to endothelial cells and leukocytes (110). The mutants developed normally and presented an altered structure of the endothelial HS. An impaired neutrophil infiltration was observed in different experimental models of inflammation. The authors showed how the undersulfation of endothelial HS affected neutrophil trafficking by increasing the rolling velocity of leukocytes and by weakening their adhesion to endothelial cells. These phenomena are correlated to alterations of HS binding abilities. In the first case, the increased rolling velocity depends on a decreased binding of L-selectin to the endothelial HS. In the second case the weaker adhesion is due to an improper presentation of chemokines to the leukocite unable to sustain the activation of firm adhesion (110). These are the first *in vivo* evidence of the role of HS in leukocyte / endothelium interaction and of its influence on chemokine activity. In a related paper, Fuster and colleagues used the same model to investigate the effect of Ndst1 inactivation on angiogenesis (111). The mutants presented no alterations of physiological angiogenesis during cutaneous wound repair. However, the lack of Ndst1 activity reduced the growth and angiogenesis of experimental tumors subcutaneously injected in the mice. The authors associated these changes to altered binding properties of undersulfated HS chains. In this case, endothelial cells isolated from mutants mice showed a reduced affinity for and altered signaling response to two growth factors, FGF-2 and $VEGF_{164}$, involved in tumor growth and control of angiogenesis (111).

4.2. Influence on protein function

The *in vivo* data cited above, when considered as a whole, give a quite strong taste of the central role of HSPGs in many fundamental biological processes. However, when they are evaluated in more detail, all the complexity of the phenomena described emerges dramatically. Its unique structural features allow HS to interact with a vast number of protein partners (Table 1). Its ability to influence their activity in a way that can be dynamically regulated (by changes of its structure and/or of localization) gives to HS a central role in the regulation of complex biological phenomena such as development. Many mechanisms have been proposed in the past years to describe at a molecular level the action of HS. In the next section some of the best-characterized systems will be summarized since they serve as our paradigms for the many less well understood systems.

4.2.1. Influence on protein transport and distribution: HS and morphogen gradients

Development in metazoans represents probably the most complex example of spatial-temporal coordination of biological processes. The distribution of morphogens is responsible for the transport of information during development and it determines cell fate and pattern formation. HSPGs play an important role in the distribution and activity of the main morphogens belonging to the Wnt, Hh, TGFbeta and FGF families. The interaction between

Table 1. A partial heparin interactome

Growth factors, cytokine, chemokines and morphogens							
Heparin / HS interacting protein	UniProtKB ID[1]	Ref.	PDB ID[2]	Heparin / HS interacting protein	UniProtKB ID[1]	Ref.	PDB ID[2]
activin	P08476	(200)		FGF2	P09038	(172)	1BFB, 1BFC, 1FQ9
agouti-related protein	O00253	(201)		FGF3	P11487	(202)	
agouti-signaling protein	P42127	(201)		FGF4	P08620	(203)	
angiotensin-II	P01019	(204)		FGF5	P12034	(205)	
betacellulin	P35070	(206)		FGF6	P10767	(207)	
BMP2	P12643	(208)		FGF7	P21781	(209)	
BMP3	P12645	(210)		FGF8	P55075	(211)	
BMP4	P12644	(212)		FGF9	P31371	(213)	
BMP7	P18075	(214)		FGF10	O15520	(215)	
CCL1 (I-309)	P22362	(216)		FGF11	Q92914	(217)	
CCL2 (MCP1)	P13500	(218)		FGF14	Q92915	(217)	
CCL3 (MIP1-alpha)	P10147	(219)		FGF20	Q9NP95	(220)	
CCL4 (MIP1-beta)	P13236	(221)		FGF16-18, 22[3]	O43320, O60258, O76093, Q9HCT0		
CCL5 (RANTES)	P13501	(222)	1U4L, 1U4M	follistatin	P19883	(223)	
CCL7 (MCP3)	P80098	(224)		GDNF	P39905	(225)	
CCL8 (MCP2)	P80075	(226)		GMC-SF	P04141	(227)	
CCL11 (eotaxin)	P51671	(228)		HB-EGF	Q99075	(229)	
CCL13 (MCP4)	Q99616	(224)		HDGF	P51858	(230)	
CCL14[3] (NCC2)	Q16627			HGF	P14210	(231)	1GMN, 1GMO
CCL15 (HCC2)	Q16663	(232)		IGFBP2	P18065	(233)	
CCL16[3] (LEC)	O15467			IGFBP3	P17936	(234)	
CCL17 (TARC)	Q92583	(235)		IGFBP4	P22692	(236)	
CCL18 (PARC)	P55774			IGFBP5	P24593	(237)	
CCL19 (MIP3-beta)	Q99731	(238)		IGFBP6	P24592	(239)	
CCL20[3] (MIP3-alpha)	P78556			Ihh	Q14623	(112)	
CCL21 (SLC)	O00585	(235)		IL2	P60568	(240)	
CCL22 (MDC)	O00626	(241)		IL3	P08700	(242)	
CCL23 (MPIF1)	P55773	(243)		IL4	P05112	(244)	
CCL24 (MPIF2)	O00175	(245)		IL5	P05113	(246)	
CCL25 (TECK)	O15444	(238)		IL6	P05231	(247)	
CCL26[3] (MIP4-alpha)	Q9Y258			IL7	P13232	(248)	
CCL27 (CTACK)	Q9Y4X3	(226)		IL10	P22301	(249)	
CCL28 (MEC)	Q9NRJ3	(238)		IL12	P29460	(250)	
CCN1	O00622	(251)		INF-gamma	P01579	(252)	
CCN2	P29279	(253)		LTBP-1	P22064	(254)	
chordin	Q9H2X0	(255)		midkine	P21741	(256)	
CX3C (fractalkine)	P78423	(224)		neuregulin	Q02297	(257)	
CXCL1 (GRO-alpha)	P09341	(126)		noggin	Q13253	(258)	
CXCL2 (MIP2-alpha)	P19875	(259)		PDGF-A	P04085	(260)	
CXCL3[3] (MIP2-beta)	P19876			PDGF-B	P01127	(261)	
CXCL4 (PF4)	P02776	(126)		pleiotrophin (HB-GAM)	P21246	(139)	
CXCL5[3] (ENA78)	P42830			PlGF2	P49763	(262)	
CXCL6 (GPC2)	P80162	(263)		prolactin	P01236	(264)	
CXCL7 (NAP2)	P02775	(126)		R-spondin1-3	Q2MKA7, Q6UXX9, Q9BXY4	(265)	
CXCL8 (IL8)	P10145	(126)		sFRP1	Q8N474	(266)	
CXCL9[3] (MIG)	Q07325			Shh	Q15465	(112)	
CXCL10 (IP10)	P02778	(267)		SLIT	O75093	(268)	
CXCL11[3] (I-TAC)	O14625			SLPI	P03973	(269)	
CXCL12 (SDF1-alpha)	P48061	(270)	2NWG	TGF-beta1	P01137	(271)	
CXCL13 (BLC)	O43927	(238)		TGF-beta2	P61812	(271)	
CXCL14[3] (BRAK)	O95715			VEGF-A	P15692	(272)	
CXCL16 (SR-PSOX)	Q9H2A7	(238)		VEGF-B	P49765	(273)	
EMBP	P13727	(274)	2BRS	Wnt1	P04628	(113)	
endostatin	P39060	(51)		XCL1 (lymphotactin)	P47992	(275)	

FGF1	P05230	(276)	1AXM, 2AXM, 1E0O, 2ERM	XCL2[3] (SCM1-beta)	Q9UBD3		
Coagulation / complement cascade							
Heparin / HS interacting protein	UniProtKB ID[1]	Ref.	PDB ID[2]	Heparin / HS interacting protein	UniProtKB ID[1]	Ref.	PDB ID[2]
annexin-II	P07355	(48)	2HYU, 2HYV	factor-B	P00751	(277)	
annexin-V	P08758	(49)	1G5N	factor-D	P00746	(278)	
APC	P04070	(279)		factor-H	P08603	(277)	
C1inh	P05155	(280)		factor-I	P05156	(277)	
C1q	P02745, P02746, P02747	(277)		factor-P	P27918	(277)	
C2	P06681	(277)		factor-IXa	P00740	(281)	
C3	P01024	(277)		factor-Xa	P00742	(282)	2GD4
C4	P0C0L4	(277)		factor-Xia	P03951	(283)	
C4beta-BP	P04003	(284)		factor-XIIa	P00748	(285)	
C5	P01031	(277)		heparin cofactor II	P05546	(286)	1JMJ
C6	P13671	(277)		TFPI	P10646	(287)	
C7	P10643	(277)		thrombin	P00734	(288)	1XMN, 1TB6, 2B5T
C8	P07357, P07358, P07360	(277)		von Willebrand factor (vWF)	P04275	(188)	
C9	P02748	(277)					
Enzymes							
Heparin / HS interacting protein	UniProtKB ID[1]	Ref.	PDB ID[2]	Heparin / HS interacting protein	UniProtKB ID[1]	Ref.	PDB ID[2]
AChE	Q9Y215	(289)		HNE	P08246	(290)	
ADAMTS1	Q9UHI8	(291)		lipoprotein lipase (LPL)	P06858	(292)	
ADAMTS5	Q9UNA0	(293)		PAI	P05121	(294)	
ADAMTS9	Q9P2N4	(293)		PCI	P05154	(295)	
AT-III	P01008	(134)	1AZX, 1E03, 1NQ9, 1TB6, 2B5T, 1SR5, 2GD4	PCPE2	Q9UKZ9	(296)	
BACE1	P56817	(297)		PEDF	P36955	(298)	
CAP37	P20160	(299)	1A57, 1A7S	PN-1	P07093	(300)	
cathepsin-B	P07858	(301)		sPLA2	P14555	(302)	
cathepsin-G	P08311	(303)		TPA	P00750	(304)	
chymase	P23946	(305)		trypsin	P07477	(306)	
cyclophilin-B	P23284	(307)		tryptase-beta	Q15661, P20231	(308)	
diamine oxidase	P19801	(309)		urokinase	P00749	(310)	
EC-SOD	P08294	(311)		XOD	P47989	(312)	
hepatic lipase	P11150	(313)					
ECM components							
Heparin / HS interacting protein	UniProtKB ID[1]	Ref.	PDB ID[2]	Heparin / HS interacting protein	UniProtKB ID[1]	Ref.	PDB ID[2]
collagen-I	P02452, P08123	(314)		collagen-XXV	Q9BXS0	(315)	
collagen-II	P02458	(316)		fibrillin1-2	P35555, P35556	(317)	
collagen-III	P02461	(318)		fibrin	P02675	(319)	
collagen-IV	P53420	(320)		fibronectin	P02751	(321)	
collagen-IX	P20849	(322)		laminin	P25391, P24043	(323)	
collagen-V	P20908	(324)		PRELP	P51888	(325)	
collagen-XI	P12107, P13942	(326)		thrombospondin	P07996	(327)	1ZA4
collagen-XII	Q99715	(328)		vitronectin	P04004	(329)	
collagen-XIII	Q5TAT6	(330)		tenascin	P24821	(331)	
collagen-XIV	Q05707	(332)					
Adhesion molecules and receptors							
Heparin / HS interacting protein	UniProtKB ID[1]	Ref.	PDB ID[2]	Heparin / HS interacting protein	UniProtKB ID[1]	Ref.	PDB ID[2]
DCC	P43146	(333)		FGFR2	P21802	(167)	1E0O
HIP	P47914	(334)		FGFR3	P22607	(335)	
L-selectin	P14151	(50)		FGFR4	P22455	(336)	
Mac-1	P11215	(337)		neuropilin	O14786	(338)	
N-CAM	P13592	(339)		ROBO	Q9Y6N7	(268)	
P-selectin	P16109	(340)		VEGFR1	P17948	(341)	
AAMP	Q13685	(342)		VEGFR2	P35968	(343)	
FGFR1	P11362	(147)	1FQ9				
Others							
Heparin / HS interacting protein	UniProtKB ID[1]	Ref.	PDB ID[2]	Heparin / HS interacting protein	UniProtKB ID[1]	Ref.	PDB ID[2]
APLP1-2	P51693, Q06481	(344)		lactoferrin	P02788	(345)	
apoA5	Q6Q788	(346)		L-type Ca^{2+} channels	Q13698	(347)	
apoB	P04114	(348)		myosin ATPase	P24844	(349)	

apoE	P02649	(350)		OPG	O00300	(341)	
apoH	P02749	(205)		PDCD5	O14737	(351)	
APP	P05067	(352)		PrP	P04156	(353)	
cytochrome-C	P99999	(354)		SAP	P02743	(355)	
HMGB1	P09429	(356)		synuclein-alpha	P37840	(357)	
HRG	P04196	(358)		tau	P10636	(359)	

[1] The UniProtKB (http://beta.uniprot.org/) identifier (UniProtKB ID) of the human homologue of the heparin / HS interacting protein [2] The Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) identifier (PDB ID) when a structure of the interactor in complex with heparin / HS derivatives or synthetic analogues is available [3] Indicates a protein that lacks published evidence of interaction with heparin / HS, but presents high sequence homology with proved interactors

HS and morphogens has been characterized *in vitro* (112-115) and the results obtained in model organisms (see above) illustrate the impact of these interactions (or of the lack of) *in vivo*.

The ability of HSPGs to influence the molecular diffusion by creating preferential diffusive paths for their ligands was first proposed as possible mechanism to enhance growth factor-receptor interaction (116). Similar models have been proposed to explain the influence of HSPGs in the movement of Wg (*Drosophila* ortholog of vertebrate Wnt), Hh and Dpp (*Drosophila* ortholog of vertebrate BMP2 and BMP4) (95-97). In all these cases, the study of morphogen distribution in wing imaginal discs revealed the inability of Wg, Hh and Dpp to move through a line of clones with impaired HS synthesis (*sfl*, *ttv*) or bearing mutations in core protein genes (*dally*, *dlp*). The authors proposed a model of preferential cell-to-cell transport mediated by the glypicans *dally* and *dlp*. According to the model, morphogens travel from the producer cell to the target cell by sequentially binding different HS chains. The difference in concentration would guarantee the unidirectionality of the displacement toward the target cell (95-97). Other authors proposed alternative mechanisms of transport, such as direct transfer of glypicans between neighboring cells (117, 118) and HS-mediated transcytosis (118). Furthermore, it has been suggested that the influence of HSPGs on the shape of the morphogen gradient resides in their ability to decrease diffusivity through the ECM rather than increasing it (119). A recent study on the dynamics of Shh signaling during chicken embryo development shows that a decrease of the diffusivity of the morphogen can extend its range of action (120).

A complementary example derives from the influence of the heparin-binding activity of VEGF-A on its control of vessel branching morphogenesis (121). Mouse embryos have been engineered to express only the VEGF-A isoform lacking the heparin-binding domains (HBDs) ($VEGF_{120}$). The mutants present normal vasculogenesis and angiogenesis able to support correct development of the embryo, but they display abnormal capillary branching (121). The complexity of the vascular branching is reduced and associated with an aberrant distribution of the endothelial cells within the growing vasculature (121). The lack of the heparin-binding activity causes a different localization of the secreted VEGF and a disruption of its concentration gradients in the ECM (121). The authors conclude that the heparin-binding VEGF-A isoforms are required to produce a gradient that guides endothelial cells during the formation of branching capillary networks (121).

4.2.2. Protein stabilization, oligomerization and presentation to receptors: chemokines

Chemokines are a family of small secreted proteins (8-12 kDa). More than 50 members have been classified in four families (C, CC, CXC and CX_3C) on the basis of the arrangement of a conserved cysteine rich motif located in the amino terminal region. Almost all the chemokines studied to date appear to interact with HS (122) (Table 1). Different models have been proposed to describe the influence of HS binding in chemokines activity. The proposed mechanisms include regulation of protein localization and transport, increase of their stability and induction / enhancement of oligomerization.

The formation of gradients of chemokines and their presentation on the luminal surface of endothelial cells are crucial events for the recruitment of circulating leukocytes in sites of inflammation (123). As in the case of morphogens, *in vivo* evidence supports the view of a fundamental role of HSPGs in the distribution and transport of their ligands (110, 124). Chemokines interact with GAGs expressed on the surface of endothelial cell *in vitro* and *in vivo* (125). Their ability to bind HS is a prerequisite for their chemotactic activity *in vivo* (124). Mutations in the GAG binding domain of MCP-1 (CCL2), MIP-1beta (CCL4) and RANTES (CCL5) impair their ability to recruit leukocytes in an *in vivo* model, whereas this activity is unaltered *in vitro* assays (124). Similar results have been obtained in mice lacking NDST-1 activity in endothelial cells and leukocytes (see above) (110). The authors report also that the presence of sulfated HS chains is required on endothelial cells, but not on leukocytes and they describe a mechanism of transcytosis of HS, which is required for the correct presentation of the chemokines on the endothelial lumen (124). HSPGs appear to be crucial in the transport of chemokines from the site of production to the endothelial cell surface and for the appropriate presentation to receptor expressed by the leukocytes. Additionally, given the selectivity of different chemokines for different GAGs structures, modification of the HS chain could result in the presentation of restricted groups of chemokines and, as a consequence, in the recruitment of different leukocyte subpopulations (126, 127).

Most of the chemokines tend to form dimers or tetramers in solution using different modes of oligomerization (122). It has been demonstrated that the binding to heparin / HS induces or enhances the formation of multimers *in vitro* (128, 129). Furthermore, multimeric complexes of MIP-1alpha (CCL3), MIP-1beta (CCL4) and RANTES (CCL5) associated with sulfated proteoglycans are secreted by activated cytotoxic lymphocytes (130).

Even though the oligomerization is not required for receptor activation (124), mutants of MCP-1 (CCL2), MIP-1beta (CCL4) and RANTES (CCL5) unable to form oligomers, but with receptor and GAG binding activity conserved, fail to recruit leukocytes in an *in vivo* model of chemotaxis (124). The oligomerization driven by HS chains would enhance the concentration of chemokines at the cell surface rendering the activation of the receptors more probable (124). In addition, the formation of GAG / chemokine complexes can influence the conformation of the protein and expose or mask different epitopes on the protein surface (122).

Finally, the interaction with heparin / HS increases the stability of chemokines (131, 132). Heparin binding drastically increases the plasma half-life of IFN-gamma and decreases its inactivation by preventing the proteolysis of its carboxy-terminal domain (131). Similarly the interaction with HS protects SDF-1 (CXCL12) from the inactivation mediated by the cell surface serine protease DPP IV / CD26, *in vitro* (132).

4.2.3. Enhancement of activity by conformational change

Antithrombin III (AT-III) is the prototypical heparin interacting protein. The elucidation of its mechanism of activation was the first example of heparin's influence on protein function to be described (133-136). The binding to heparin induces a structural rearrangement (described in detail in (137)) that results in the expulsion of a loop containing a protease-reactive site. The conformational change dramatically increases the inhibitor activity of ATIII against the coagulation factors thrombin and factor X of approximately 9000- and 17000-fold, respectively (137). The mechanism has been finely described and different crystal structures of ATIII complexed with different saccharides and in ternary complex with its substrates have been resolved (129, 137) (Table 1).

Conformational changes induced by heparin / HS binding have been observed also for FGF-2 by infrared spectroscopy (138), mucus proteinase inhibitor by fluorescence spectroscopy (138) and heparin-binding growth associated molecule (HB-GAM) by circular dichroism and NMR (139). However, in these cases the conformational changes have not been characterized in detail and no evident association with the protein function have been demonstrated.

Even in the absence of strong structural evidence, the association between heparin binding and conformational change can inspire speculative mechanisms in other systems. The FGF family of growth factors exemplifies one of these cases. Despite low sequence homology and different chemical-physical properties, the FGFs are characterized by a similar structure, described as "beta-trefoil", comprising a three-fold repeat of a four-stranded antiparallel beta-sheet (140). With exception of the study mentioned above, no structural changes induced by the heparin binding have been demonstrated. However, considerable published data argues persuasively for a conformational change in FGFs upon binding to the sugar. It is known from early studies that heparin stabilizes the conformation of FGF-1 and FGF-2, preventing physicochemical or enzymatic degradation (141). For FGF-1 it has been demonstrated that the protein exists in a partially folded state at physiological temperatures (142). This state, defined as "molten globule", is characterized by the alteration of tertiary structure without loss of secondary structure elements and it is considered an intermediate between the native and the unfolded form; binding to heparin induces the transition of the protein structure to the folded form (142). A recent NMR study compared the backbone relaxation data of FGF-1 in free-state or bound to a heparin-like hexasaccharide (143). The sugar binding induces a reduction of internal motions observed in the free state, especially in the HBD (143). Furthermore, the crystal structure of FGF-7 reveals the presence of just 10 well-defined beta-strands, folded according to the beta-trefoil motif, while strands 11 and 12 are poorly defined (144). Interestingly, the corresponding region (strand 11) has been described in a helix-like conformation in a NMR study of FGF-2 (145). This region comprises the primary HBD and the authors speculate that the interaction with the sugar could induce proper formation of the beta-strand, which is otherwise unstructured (146).

These data suggest that the induction of conformational changes may be a mechanism more common than expected. Our actual observations can be biased by the lack of techniques able to capture such structural rearrangements and the fact that protein crystallization may favor a minority, folded structure, in solution.

4.2.4. Assembly of active signaling complexes: the FGF system

The FGF family of growth factors is encoded by 22 genes in the human genome and most of them possess heparin-binding activity (115) (Table 1). Their signal transduction is mediated by cell-surface tyrosine kinase receptors (FGFR1-4) also able to interact with heparin / HS (147-149). The binding of the growth factor induces receptor dimerization and transphosphorylation of the cytoplasmic tyrosine kinase domains resulting in their activation and phosphorylation of downstream signaling mediators (149). Heparin / HS participate in the assembly of ternary signaling complexes (FGF-HS-FGFR) and modulate receptor activation (150-153). Though the growth factors are able to bind to the receptor in absence of HS (148, 154, 155), the interaction with the sugar stabilizes the signaling complex (148, 154) and results in sustained receptor signaling that is required for classic biological activities such as mitogenesis (151, 153).

Based on site-directed mutagenesis and biophysical measurements, the first proposed model for the assembly of the FGF-HS-FGFR is that the FGF-2 ligand is bivalent with respect to its receptor (148, 156). The authors proposed that the binding of the sugar to both FGF-2 and FGFR increased the affinity of ligand-receptor interaction through a primary, high affinity binding site on FGF (148). Furthermore, the presence of the heparin induces the

formation of a quaternary complex (2:1:1 FGFR, FGF, HS) stabilizing a further ligand-receptor interaction, mediated by a secondary, low affinity binding site on FGF, thus promoting the receptor dimerization required for signaling activation (148). The disruption of the secondary binding site by mutagenesis does not affect the primary interaction with the receptor and the interaction with the sugar, but leads to a significant reduction of the mitogenic activity of FGF-2 (156). This is known as the growth hormone model and is also supported by subsequent work with FGF-7 (157).

The almost contemporaneous publication of two different crystal structures of the ternary complex FGF-HS-FGFR fueled the debate about the mode of action of heparin / HS in the activation of FGF signaling (for a recent review see (158)), since they proposed two new different models for the assembly of the signaling complex. The first model (symmetric model) indicates a dimeric 2:2:2 complex of FGF-2, FGFR1 ectodomain and a heparin-derived hexasaccharide (PDB ID: 1FQ9) (159). The second model (asymmetric model) is also characterized by a dimeric assembly, but with a 2:2:1 stoichiometry ratio between FGF-1, FGFR2 and a heparin-derived decasaccharide (PDB ID: 1E0O) (160). In the first case, the sugar interacts with both ligand and receptor promoting the assembly of a 1:1:1 complex. The dimerization of two of these complexes is mediated by direct FGFR-FGFR interactions, secondary interactions between ligand in one ternary complex and receptor in the other and FGFR-FGFR interactions mediated by the sugar (also called the "two-end" model since the two sugar chains run in opposite directions with their non-reducing ends facing each other) (159). In the second case, an asymmetric heteropentamer is formed by the interaction of two 1:1 FGF-FGFR complexes associated through the interaction with heparin. Heparin interacts with both the ligands, but with just one of the two receptor's ectodomains. The role of the sugar is critical for the assembly of the heteropentamer due to the absence of significant protein-protein interfaces (160). The two models diverge drastically on the structural role of heparin and imply different mechanisms of assembly. In the symmetric model, the sugar is one of the components driving the dimerization of the two halves of the complex, which can occur also in the absence of the sugar through protein-protein interactions. The assembly of the active complex involves the formation of 1:1:1 complexes followed by dimerization, enhanced by the presence of the sugar. On the contrary, the asymmetric model requires the presence of the sugar for assembly of the complex and proposes an assembly mechanism based on the pre-formation of FGF-FGFR binary complexes followed by dimerization induced by heparin.

Evidence supporting the asymmetric model derives from a biochemical characterization of the complexes used for the crystallization. Combining size-exclusion chromatography, analytical ultracentrifugation and mass spectrometry data, Harmer and collegues show that both the complexes can be obtained during sample preparation, independently of the method used, but the 2:2:1 complex of the asymmetric model predominates over the 2:2:2 symmetric model (161). The authors conclude that both the complexes can be relevant in the formation of signaling complexes *in vivo* (161). However, other biochemical and biophysical data are inconsistent with the asymmetric model. (i) The dimerization solely dependent on interactions established by the sugar fails to explain the transient receptor activation induced by FGF-2 in chlorate-treated fibroblasts, deficient in sulfated HS, and the fact that in the same system a tetrasaccharide is sufficient to stimulate cell proliferation (162). (ii) Site-directed mutagenesis experiments reveal that disrupting the secondary receptor binding site (adjacent to the primary one, not to be confused with the low affinity second binding site of the growth hormone model, see above) in FGF results in an attenuation of the signaling due to decreased dimerization (163). This binding site is required for the secondary FGF-FGFR contacts described in the symmetric model, but not in the asymmetric one (164).

A more intriguing hypothesis is that HS not only acts as co-receptor, but its structural features determine the specificity of the FGF system, promoting the assembly of different signaling complexes (73). Once again more than one model has been proposed. Based on differences in the interaction with the sugar displayed by both different FGFs and different FGFRs, in terms of specific structural requirements (165, 166) or kinetic parameters (167), it has been proposed that the assembly of a specific ternary complex is determined by the existence on the HS chain of a domain containing optimal binding sites for the specific growth factor and receptor involved (73). Other authors contrast this model and propose that the specificity for the optimal HS structure required depend on the unique binding site determined by the association of receptors and ligands in the signaling complex (164).

Furthermore, the binding to HSPGs can influence the distribution of the growth factors at the cell surface and in the ECM (70, 73). The localization of FGFs in the ECM mediated by the binding to matrix proteoglycan or as a result of the shedding of cell surface HSPGs can act as a storage mechanism. In this state the binding to the sugar has an inhibitory effect in part because the growth factors are unable to reach their receptors. The release of the growth factor, mediated, for example, by enzymatic degradation of HS chains associated with inflammation (168) or following mechanical injury (169) induces its immediate activation and the delivery of a strong mitogenic signal (168, 169). HSPGs are involved in the formation of FGF gradients during development (109). The targeted disruption of the EXT1 (HS polymerase) gene in the mouse embryonic brain leads to patterning defects and aberrant axon guidance (109). The defects are associated to altered distribution of morphogens, in particular FGF-8 (109).

5. HEPARIN – PROTEIN INTERACTION

5.1. Thermodynamics of interaction

The interaction between heparin / HS and proteins is driven by electrostatic bonding (24). At physiological pH, heparin / HS are polyanions with a fraction of their negative charges neutralized by bound

counterions. The binding of counterions reduces the repulsive forces between the charged groups on the chain, but it is entropically unfavorable, because it reduces the disorder of the solvated ions (170).

Protein binding at this level represents an ion-exchange process where positively charged residues of the protein replace counter ions. The entropically favorable release of counter ions is called the polyelectrolyte effect and is one of the main components of the free energy DELTAG of the interaction of heparin / HS and proteins (171).

The contribution of the non-ionic component has been evaluated for the interaction between FGF-2 and heparin by isotermal calorimetric titration (ITC). This study showed that electrostatic interactions are responsible for only about 30% of the binding free energy. Thus, the non-ionic component, due to hydrogen bonding and hydrophobic interactions, contribute the majority of the free energy of this binding reaction (172). The analysis of crystal structures of FGF-1 and FGF-2 complexed to heparin-derived oligosaccharides confirmed this hypothesis, showing the major contribution of van der Waals contacts to the protein-sugar interaction (159, 160). Similar studies applied to thrombin (173) and mucus proteinase inhibitor (174) showed more substantial contributions of the electrostatic component: for thrombin, almost all of the binding free energy is due to the polyelectrolyte effect (173).

A unique model for the interactions of proteins and heparin / HS is probably an over-simplification: the surfaces of different proteins can establish different interactions with the sugar chain, resulting in binding reactions with variable thermodynamic and kinetic parameters. In addition, entropic contributions can derive from structural rearrangements that can occur following the initial electrostatic interaction (25). Conformational changes have been observed both at protein (see above) and sugar level for some interactions. The influence of AT-III binding on the conformation of heparin-derived oligosaccharides has been demonstrated by NMR (175, 176). Variation of glycosidic dihedral angles and IdoA ring conformation has been observed comparing the three-dimensional structures of the sugars in free and bound state (175, 176). Thus, such structural rearrangements can influence the thermodynamic parameters of binding reaction, promoting the fitting of sugar and protein surfaces (176).

5.2. Protein binding sequences

Interactions between heparin / HS and proteins generally depend on sulfate groups. For these reason, NS or NA/NS domains are considered the protein binding motifs of HS chains. The specific requirements, in term of composition and minimal length of the chain, have been investigated for a number of interactors. As usual, it is difficult to delineate a single model for the assembly of protein binding sites; parameters influencing different levels of the sugar structure must be evaluated for each case.

The AT-III binding pentasaccharide was the first protein binding site characterized in the sugar (136). This is the paradigmatic example of binding site containing a rare modification of the sugar backbone. The sequence GlcNAc6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S contains several unusual features. The most relevant is the rare sulfation at position 3 of the central glucosamine, which is absolutely essential for high affinity binding. The pentasaccharide is sufficient for AT-III binding and for its inhibitory activity towards factor Xa (177), while a minimum 16 saccharide sequence is required for the inhibition of thrombin (178). 3-O-sulfation has also been suggested to be important for the interactions of FGF-7 (17). Another unusual feature of HS chains is the presence of N-unsubstituted glucosamine. These rare free amino groups are enriched in sequences able to bind L- and P-selectin (179). Herpes simplex glycoprotein gD and cyclophilin B require both these uncommon modifications; their binding motif combines an unusual N-unsubstituted, 3-O sulfated glucosamine residue, though whether this reflects a requirement for a free amine or the unusual specificity of 3OST-3A for a free amine is not fully understood (180, 181).

However, the majority of the binding sites are assembled using more common modifications, arrayed in specific patterns. For the FGF family of growth factors, for example, the binding sites localize in sequences containing the disaccharide IdoA2S-GlcNS with variable degrees of 6-O-sulfation. These sequences are common in the highly sulfated heparin, but less frequent in HS chains, where they localize at the center of NS domains. Members of the FGF family show differences in the optimal sugar substitution pattern recognized. For example, FGF-2 requires sulfation at position 2 of the uronic acid, but not at position 6 of the glucosamine, a substitution that is required for high affinity binding of FGF-1 (165, 166). Also, in this last case longer saccharides of 10-14 sugars in length, as well as the presence of 6-O-sulfation are required to promote mitogenic signal (83). However, in some cases the stimulation of cell proliferation has been obtained with shorter saccharides (162) even in absence of sulfate groups (182).

The high affinity binding of some interactors require longer sequences (20). Platelet factor 4 (PF-4) and interleukin 8 (IL-8) are oligomeric chemokines that under physiological condition exist as tetramer and dimer, respectively. The oligomers form 1:1 complexes with long sugar sequences of 12-20 saccharides. It has been proposed that sugar chains wrap around these complexes, interacting with spatially separated binding sites exposed on their surface (17). Similar structural arrangements have been proposed for other proteins such as VEGF (183) INF-gamma (184) and chemokines (122). The assembly of these long binding sites requires short NS domains separated by spacer region containing N-acetylated residues (SAS domains) (20). Similar structures, where the spacing between the sulfated regions seems to be more relevant than the specific sequence, could be implicated also in binding reactions involving more than one partner, as in the case of signaling complexes (20). Importantly, this spacing

may be inappropriate until there is an external stimulus. For example, it has been shown that cells (185) and tissues (168) can express HS chains that bind FGF ligands but are not able to support the assembly of a signaling complex capable of stimulating cell proliferation. Scission of these HS chains then liberates sulfated oligosaccharides that are very potent co-receptors for FGF-mediated cell proliferation (168, 185).

Finally, some binding reactions require the presence of divalent cations. Annexin A2 and A5, serum amyloid protein (SAP) and L- and P-selectins interact with heparin / HS in a calcium-dependent manner. The structure of annexin A2 and annexin A5 in complex with heparin derived oligosaccharide have been analyzed by X-ray crystallography (48, 49). In the annexin A2 / heparin oligosaccharide complex two carboxyl groups of the sugar participate in the coordination of one calcium ion together with two oxygens of the protein main chain (48). In the case of annexin A5 the contacts between the sugar and calcium are indirect: the presence of cations seems to be required to induce the protein conformation required for the binding (49).

There is still an open discussion about the level of structural requirements at the sugar level. Are protein binding sites determined by the assembly of specific sequences of saccharides or by a more generic distribution of modification patterns along the chain? Probably these two aspects are not mutually exclusive. The conformation of the polysaccharide is determined by the local sequence, but it is also influenced by its localization along the chain (25). In addition, different modalities of interaction for different proteins cannot be excluded, with variable degrees of specificity and, as consequence, structural requirements (20, 25).

A recent work questioned the existence of a selectivity of FGFs for different HS structures (186). Comparing the affinity of HS derived oligosaccharides for different FGFs, the authors show that a very low level of selectivity exists and that different growth factors bind with similar affinity to identical HS epitopes (186). They conclude that FGFs share the same binding sites on HS and that the affinity is mainly determined by the charge density of the sugar (186). However, it has to be noted that the affinities have been evaluated on the basis of the salt concentration required to elute the oligosaccharides from immobilized growth factors, thus taking in account only the electrostatic component of the interaction that for FGFs has been shown to be of minor relevance (see above) (172).

5.3. Heparin-binding domains: looking for models

The first attempt to delineate the determinants of HBDs was made by Cardin and Weintraub and was reported in 1989 (187). They analyzed twelve known heparin-binding regions, in vitronectin, apoE, apoB-100 and PF-4. On the basis of the distribution of basic and hydropathic residues they proposed two consensus sequences: -XBBXBX- and -XBBBXXBX-, where B represent a basic residue and X the probability of a hydropathic residue. Molecular modeling of these sequences showed their tendency to assume conformations facilitating the interaction between basic residue of the protein and sulfated-groups of the sugar. The -XBBXBX- consensus tends to assume a beta-strand conformation with basic residues aligned on one face of the sheet. In a similar way, the -XBBBXXBX- forms an alpha helix with basic residues aligned on one side and hydropathic amino acids pointing back into the protein core (170, 187). A third consensus -XBBBXXBBBXXBBX-, where B represent a cationic residue, was proposed on the basis of the HBD of the von Willebrand factor (188).

Margalit and co-workers compared the three-dimensional structures of defined heparin-binding sequences (189). They suggested the relevance of the basic residues arrangement for the heparin binding, independently from the secondary structure assumed. They observed that a distance of around 20 Angstrom between basic residues is important for the interaction. In their model basic amino acids are exposed on opposite sides of secondary structures, suggesting a possible wrapping of the sugar chain around the peptide backbone (189).

The general requirements for heparin and HS binding have been investigated through affinity studies. The importance of specific amino acids for the interaction has been assessed using a library of random generated peptides. As expected, the basic residues arginine and lysine were essential for the binding to the highly sulfated heparin. In contrast, their role was less determinant for the interaction with HS, where neutral amino acids like glycine and serine were surprisingly more important (190).

The role of arginine and lysine in heparin binding was compared using homopeptides and peptides derived from the FGF-1 HBD. Arginine containing peptides bound heparin more tightly than the lysine analogous species. Interestingly, a FGF-1 derived peptide having only arginines as basic residues showed an increased affinity for heparin, while the analogous peptide containing only lysines bound less tightly than the native peptide (191).

Fromm and co-workers tested the role of spacing in linear heparin-binding sequence using peptides where two clusters of basic residues (arginines) were separated by a variable number of neutral amino acids (glycines) (192). The relative affinities of two series identified by the sequences -RRGmRR- and -RRRGmR- were compared. The greatest affinity for heparin was achieved when the spacer was absent (-RRRR-) and with the sequence -RRGGGRR-. In contrast, HS bound more tightly to peptide containing more spaced basic residues: -RRRGGGR- and -RRRGGGGR- (192).

The overall HBDs topology was evaluated analyzing the structural characteristics of those in FGF-1 and in FGF-2. Three turns are present in both binding sites determining a triangular shape where basic residues are concentrated in a small area. In order to evaluate the relevance of this topology, three different peptides derived from the FGF-1 HBD were generated and their affinities compared. These experiments confirmed the importance of

the peptide topology. A peptide containing an unusual D-proline residue in place of L-proline bound less tightly than the native peptide, while locking the heparin-binding peptide in a cyclic conformation produced an increase of the affinity (193). Interestingly, super-secondary structure characterized by reverse turns has been predicted in other heparin-binding proteins. Comparing X-ray and NMR structural data for FGF-1, FGF-2 and TGF-beta, Hileman and co-worker proposed a heparin-binding motif described as -TXXBXXTBXXXTBB-, where T defines a turn, B a basic amino acid (arginine, lysine or glutamine) and X a hydropathic residue (170).

Taken together, these studies delineate some important aspects of the interactions between proteins and heparin / HS. Basic residues are important for their ability to interact with the sulfate and carboxyl groups present on sugar chains. Their role is determinant for interaction with heparin and, presumably, with the highly sulfated regions within HS (NS domains) (192). Arginine residues show a higher affinity in comparison to lysines, probably due to the ability of the guanidino group to establish stronger hydrogen bonding to an oxygen of a sulfate group. Combining arginines and lysines in HBDs could be a mechanism to modulate the affinity of interaction (191). For the interactions with the more heterogeneous HS, neutral amino acids are also required. They can generate sequence able to accommodate a wider range of structure, increasing the flexibility of the peptide backbone and acting as spacers between basic residues (192). The role of non-ionic interactions could be more relevant for HS binding. Glutamine and asparagine residues seem to play an important role for the interaction of FGF-2, presumably for their ability to form hydrogen bonds with the hydroxyl groups of the sugar (172).

The topology of HBDs plays a critical role to orientate appropriately the residues involved in the binding reactions and it determines specificity, affinity and kinetic of the binding to the sugar. Despite a vast number of interactors (hundreds) being described (Table 1), the definition of models able to delineate general structure characteristics of HBDs is still not possible (194). Once again, the main responsibility can be ascribed to the structural features of HS. The unique characteristics of these molecules (polydispersity, different arrangement of modification patterns, flexibility) allow them to accommodate a variety of protein structures. Recent studies aimed to study the interactions between proteins and polyanions such as heparin, actin, tubulin and DNA give an idea of the extent of this phenomenon (195, 196). Using simple pull-down experiments performed on COS-7 cell extracts and 2D gel electrophoresis, the authors show the ability of heparin to interact with an impressive number of proteins (944 out of a total of 1,751), twice the number of actin and tubulin interactors and almost ten time higher when compared to DNA (195). Furthermore, the structural heterogeneity of HS chains and their ability to assume multiple conformations make structural characterization of protein-sugar complexes a challenge (129, 197). The structure of protein-sugar complexes has been resolved at atomic resolution only for few interactors (less than twenty), using short heparin derived oligosaccharide (mainly tetra- hexasaccharides) or synthetic mimetics (129). Molecular modeling has been proven to be a useful tool for the investigation of protein-heparin interaction when the structure at atomic resolution of the complex is not available (194). However, also in this case, the complexity of the interactions represents a major limit. In order to render the modeling achievable, in fact, restrictions have to be imposed especially to the conformational mobility of the sugar (194, 198).

An interesting approach has been used to elucidate the interaction between heparin and chemokines (122). The authors analyzed the accessible surfaces of twelve chemokines whose three-dimensional structures have been resolved. First of all, they identified exposed positively charged areas, more likely to interact with the negatively charged sugars, and then they predicted areas with high affinity for sulfate groups (122). The selected surfaces have then been probed for the docking of heparin fragments. In order to test different conformations of the sugar, different conformers generated by variation of the glycosidic linkage geometries have been applied to the docking procedure (122). On the basis of lowest energy models, the authors proposed four different modes of chemokine-heparin interaction related to structural characteristics and oligomerization of the protein (122). One interesting observation is that the oligomers selected for different models possess important variations of their conformation and they contact different clusters of basic amino acids on the protein surface, giving two important cues for a possible mechanism of GAG-chemokine specificity (122). This study shows that when sufficient structural data are available it is possible to generate models of interaction, at least for a group of related proteins, that fit with available data and that can be extremely useful for the investigation of molecular processes and for the design of new therapeutics.

6. CHALLENGES FOR THE FUTURE: STRUCTURES, MODELS AND SYSTEM BIOLOGY

Since the mechanism of activation of ATIII by heparin was established almost thirty years ago, an impressive volume of literature on heparin / HS has been produced. The efforts of biochemists, glycobiologists, structural and cell biologists has completely changed our perception of these molecules. Their structural complexity has emerged dramatically and still represents one of the major challenges of the field. Not only complex *per se*, the structure of HS is also subjected to a dynamic tuning, which involves regulation of its biosynthesis as well as post-synthetic mechanisms. How the expression of HS on the cell surface and the ECM / basement membrane is coordinated in space and time is still not known. The heterogeneity and flexibility of these molecules allow them to interact with a large number of partners (Table 1). Indeed, heparin-binding activity appears nowadays to be an extremely widespread, though not completely universal property of proteins that exert their activity at the cell-matrix interface. This fact feeds a debate about the specificity of these interactions that is still far from closure.

In the last ten years model organisms have provided the evidence that HS really participates in the control of biological processes in an *in vivo* context. Morphogenesis, angiogenesis, inflammation and neural development are just some examples of complex biological phenomena whose control is influenced by HS. Also in this case, the complexity of the observations gave rise to important questions such as the context dependency of HS function (98). These open questions represent the main challenge for the future.

A more detailed characterization of the solution conformation of heparin / HS chains and how it is influenced by substitution patterns, flanking sequences, counterions and bound proteins is required. Improvement in synthetic techniques can provide homogenous samples of defined structure ready to be applied to NMR and other spectroscopy techniques (47). The optimization of purification and sequencing techniques is fundamental to obtain defined pictures of the full repertoire of HS sequences expressed by particular cells or tissues, the heparanome (7), and possibly to follow dynamic changes of its expression. The combination of established and new techniques and an integration of the data set generated are the keys for the understanding of protein-heparin interactions. X-ray crystallography has provided almost all the structural information nowadays available and will take advantage in the future from the availability of more homogenous sugar samples. NMR and new spectroscopy techniques (199) will provide a more sophisticated level of structural information, being able to capture rearrangements of protein and sugar structure that eventually occur during the interaction. Microarray technologies could provide an additional tool to investigate the determinants of the interaction at sugar (glycoarrays) and protein level (peptide-arrays). Techniques such as isotermal titration calorimetry (ITC) and optical biosensors are already available and they can provide reliable affinity and kinetic data, taking in account all the energetic components of the interactions. Finally, a systematic investigation of heparin binding domains and protein binding motifs, together with a rationalization of data already available and their integration with new data set generated (the definition of the heparin interactome), would drastically speed up the process of modeling the interactions between heparin and proteins, with an obvious impact on the design of new therapeutics targeting these interactions. The establishment of new techniques able to track dynamically the behavior of molecules in living cells is a strong requirement for every field of biology. The opportunity to observe how heparin-protein molecular complexes are assembled in the real, crowded context of the cell surface environment and how they evolve in time in terms of composition and location would allow us to understand how HS carries out its function in the coordination of molecular networks.

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Abbreviations: ECM: extracellular matrix; GAG: glycosaminoglycan; GlcA: glucuronic acid; GlcN: glucosamine; GlcNAc: N-acetylated glucosamine; GlcNS: N-sulfated glucosamine; HBD: heparin / HS binding domain; HS: heparan sulfate; HSPG: heparan sulfate proteoglycan; IdoA: iduronic acid

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