Review

Heparan sulfate-protein interactions: therapeutic potential through structure-function insights

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Abstract. Heparin and the related glycosaminoglycan, heparan sulfate, bind a myriad of proteins. The structural diversity of heparin and heparan sulfates is enormous, but differences in the conformational flexibility of the mono-saccharide constituents add extra complexity and may influence protein binding. Silencing genes for heparin/ heparan sulfate biosynthetic enzymes profoundly affects mammalian development. Thus, altering the structure of heparan sulfate chains can alter protein binding and embryo development. Different heparan sulfate structures are located in particular tissue sites, and these structures are recognised by different sets of proteins. Regulation of certain heparan sulfate-protein interactions by pH or cations is described. Heparin/heparan sulfate structures are viewed as potential therapeutics for a variety of diseases. An understanding at the molecular and functional levels of the specificity and affinity of heparan sulfate-protein interactions is crucial for designing heparin-inspired drugs. How the development of synthesis techniques is facilitating structure-function analyses and drug development is discussed.

Introduction

Although heparin has been in clinical use for decades, the extent of the importance of heparin and the related glycosaminoglycans (GAGs), heparan sulfates, in biology and medicine has not been recognised until recently. Heparan sulfate and heparin-like structures appeared very early in metazoan evolution and have been preserved in modern organisms. Virtually all cells secrete, or have associated with their cell surface, a type of glycosaminoglycan. This means that all proteins outside of the cell, regardless of function, have evolved in the presence of sulfated polysaccharides. It is thus not surprising that there are large numbers of heparin/heparan sulfate binding proteins and that protein-GAG interactions have profound effects on vertebrate and invertebrate physiology. Heparin or heparan sulfate family members have been detected in a wide range of marine invertebrates [1]. Perhaps the best illustration that heparan sulfate-protein interactions were fundamental to metazoan development comes from the finding that a protein binding a sulfated polysaccharide mediates cell-cell adhesion in the simplest of all metazoans, a marine sponge [2]. Data from a partial characterisation of the sulfated polysaccharide revealed the presence of glucuronic acid, *N*-sulfated glucosamine and *O*-sulfates. As the polysaccharide was cleaved by nitrous acid, collectively these data point to it being a heparan sulfate family member [2]. Although

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many invertebrates have also been reported to have chondroitin sulfates, it has been proposed that the wide, virtually ubiquitous distribution of heparan sulfate-type structures indicates that this was the original GAG in the metazoan lineage [3].

It is now accepted that the heparin/heparan sulfate class of GAGs binds to a wide range of proteins of diverse function. Heparin was initially discovered because of its profound effect on coagulation, and it was in that capacity that in 1935 it was used in clinical trials and subsequently in the clinic [4]. Heparin was later found to bind to antithrombin III, causing a conformational change within that protein, which enhanced the neutralization of thrombin leading to anticoagulant effects [5]. However, only one-third of the chains in commercial heparins have this capacity, which indicated that heparin chains with high affinity for antithrombin III must contain particular oligosaccharide sequences. Subsequent structural analysis revealed that a unique pentasaccharide (fig. 1) was required for high-affinity binding and that the relatively rare modification of a 3-*O*-sulfate on the glucosamine, the third monosaccharide in the sequence, was essential [6]. The possibility that other proteins may bind heparin or heparan sulfate with similar exquisite specificity is a continuing source of interest and controversy.

In recent years, technological advances in the structural analyses of heparin and heparan-sulfate oligosaccharides [7, 8] and in the modeling of GAG-protein binding events [9, 10] have assisted our understanding of structural aspects of these interactions. However, studies to unravel the functional relevance of GAG-protein interactions must be performed alongside structural analyses before the biological implications can be ascertained. In some cases, the interaction with GAGs serves to regulate protein stability and activity. In others, the interaction of proteins with GAGs acts to sequester proteins or infectious agents to particular locations. Growth factors, particularly those of the fibroblast growth factor (FGF) family, are a well-studied example of the types of proteins that bind GAGs.

It was the finding in 1991 that heparan sulfate is required for the binding of basic FGF (or FGF-2) to its high-affinity receptor and for receptor activation [11, 12] that spearheaded heparan sulfate into a centre-stage position in cell biology. The idea that particular heparan sulfate structures of a certain length may be required to bind various FGF family members followed shortly thereafter [13]. In 1993 a paper was published entitled 'Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor' [14]. Indeed, different heparin/heparan sulfate structures were required to bind and activate different FGFs [15–17]. The possibility that heparin/heparan sulfate mediated dimerisation of FGF molecules triggered or facilitated receptor dimerisation, and hence activation, was proposed to explain the role of these oligsaccharides in receptor activation [13, 18–20]. A further key finding was that heparan sulfate also bound FGF receptors (FGFR). This was first demonstrated with FGFR-1 [21]. Crystal structures of FGF FGFR-heparin complexes confirmed that heparin makes contact with both the growth factor and the receptor [22, 23]. It is now known that the heparan sulfate structure and length required for activating FGFs is dictated by the particular FGF-FGFR pair [24]. The FGF-FGFR-heparin story is a complex one, and its pre-eminence in heparan sulfate biology has shaped, rightly or wrongly, much of current thinking in relation to heparin/heparan sulfate growth factor interactions. However, it is probable that not all growth factor interactions with these GAGs will be reminiscent of that of the FGF family. As we move into an era where heparin/heparan sulfate-like structures are being examined for their therapeutic potential, it is important not to have our thinking unduly biased by the FGF-FGFR-heparin story.

Nevertheless, as a result of the huge body of work on the FGF-FGFR-heparin/heparan sulfate interaction, GAGs and particularly heparin/heparan sulfate-like structures are now attracting considerable interest as a source of new therapeutics for the treatment of infectious diseases, inflammation and allergic diseases, and cancers. Crucial issues to be understood if the potential of GAGs as therapeutics is to be realised include how GAG-protein interactions are regulated in the tissues, whether particular GAG epitopes are localised within tissues, whether biological activity requires high-affinity GAG-protein binding, and how specific GAG-protein interactions are in vivo. Some of these issues will be examined in the course of this review.
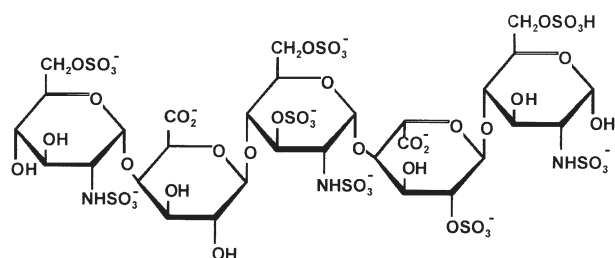
Structure of heparin and heparan sulfates

Saccharide composition and arrangement

Heparin and heparan sulfates are mixtures of linear chains that display extraordinary structural diversity, the different chains of these molecules having different patterns of sulfation. Yet, the underlying structural regularities of heparin-like-GAGs (HL-GAGs) allows the deduction of structural details from molecular weight data. Heparin and

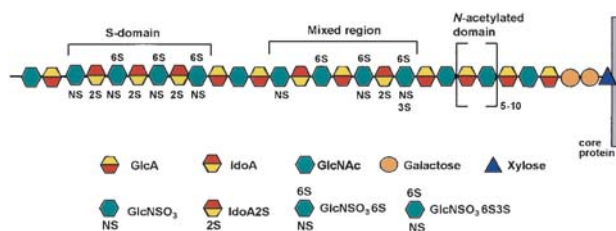Figure 1. The antithrombin III-binding pentasaccharide.

Figure 2. Cartoon of a heparan sulfate proteoglycan, showing the structure of the heparan sulfate chain. The symbols used are defined below the proteoglycan.

heparan sulfates consist of repeating disaccharide units comprising a hexuronic acid (HexA) and a D-glucosamine (GlcN) linked to each other and to other disaccharides by $1\rightarrow4$ linkages. The uronic acid may be either a β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA). Both occur as unmodified monosaccharides or as 2-O-sulfated residues (GlcA2S and IdoA2S). The glucosamine may be either N-sulfated or N-acetylated or, rarely, may exist as a free amine. N-sulfated-glucosamines (GlcNSO$_3$) may be O-sulfated at C3 (GlcNSO$_3$3S) (rarely), or at C6 (GlcNSO$_3$6S), or both C3 and C6 (GlcNSO$_3$3S6S), or carry no sulfates. Similarly, the N-acetylated glucosamines (GlcNAc) may be O-sulfated at C6 (GlcNAc6S) or unsulfated. The combination of these different structural units into disaccharides and the arrangement of the disaccharides along a chain creates an extraordinarily large potential for structural diversity. However, the theoretical diversity is not realised because of constraints manifest during chain biosynthesis.

Heparin and heparan sulfate chains are all synthesised attached to a core protein. Heparin is cleaved from its core protein, the mast cell protein serglycin, and is secreted from mast cells as a glycosaminoglycan chain. In contrast, heparan sulfates are attached to a protein core, to give a structure called a proteoglycan. Heparan sulfate proteoglycans are expressed by virtually all mammalian cells, and depending on the core protein, they may either be associated with the cell surface or deposited in the extracellular matrix. Heparin and heparan sulfate chains are synthesised as a non-sulfated precursor that is linked to a serine residue in the core protein by a tetrasaccharide linker: βGlcA-1,3 $\rightarrow$ βGal-1,3 $\rightarrow$ βGal-1,4 $\rightarrow$ βXyl-1 $\rightarrow$ Ser [25]. The polysaccharide chain is modified sequentially by a series of enzymic reactions, none of which goes to completion. Complex patterns of sulfation result. It is the regulated expression and activity of a number of glycotransferases, sulfotransferases and an epimerase that determines the fine structure of both heparin and heparan sulfate chains. Heparan sulfate structures are not dependent upon the core protein, as different structures are produced when the same core protein is expressed in different cell types [26]. The process of heparan sulfate biosynthesis that gives rise to this structural diversity has been reviewed in detail by Esko and Lindahl [27].

Heparin and heparan sulfates are structurally distinct. Heparan sulfate has a well-defined domain organisation that is not present in heparin [28, 29]. Commercial heparin is isolated from mast cell-rich tissues and comprises that HL-GAG fraction that has the highest anticoagulant activity. Heparan sulfates are extracted from tissues that contain few, if any mast cells, and make up the total HL-GAG pool isolated. However, the most highly sulfated fractions of heparan sulfate fit many of the criteria used to describe mast cell heparin. Generally the ratio of iduronic acid to glucuronic acid is low in heparan sulfates, and the numbers of N-sulfated-glucosamines and N-acetylated glucosamines are approximately equal. In contrast, heparin has a high ratio of iduronic acid to glucuronic acid, more N-sulfated glucosamines than N-acetylated glucosamines and is more highly sulfated than heparan sulfates [25]. Common features of heparin chains are stretches where the tri-sulfated disaccharide, IdoA2SGlcNSO$_3$6S-IdoA2SGlcNSO$_3$6S, is repeated [30]. Heparan sulfate chains have IdoA and GlcNSO$_3$ containing regions that are highly sulfated (S-domains) alternating with regions that are predominantly GlcA and relatively low in sulfates (fig. 2). There are also a relatively minor proportion of mixed sequences, which contain both GlcNSO$_3$ and GlcNAc. The S-domains may contain heparin-like trisulfated disaccharides as well as the disulfated disaccharide, Ido2SGlcNSO$_3$. C-6 sulfation of GlcNSO$_3$ also occurs variably in the mixed regions. The rare modifications of C-3 O-sulfation of GlcNSO$_3$ occurs in the mixed regions and the S-domains [31], and the deacetylation of GlcNAc to yield an unsubstituted amine occurs in the mixed regions and the N-acetylated-domains [32].

Three-dimensional structure affects protein binding

It is the three-dimensional structure of the heparin or heparan sulfate chain that is critical for protein binding. In solution, a heparin chain is a relatively stiff helix. Within the chain the GlcN and the GlcA residues are stable in the $^4C_1$ conformation, whereas the IdoA residues oscillate between two nearly equal energy conformations, the $^1C_4$ 'chair' and $^2S_0$ 'skew-boat' conformations (fig. 3). The energy barrier between these forms is not high, and so the oscillations between these two conformations are rapid. In
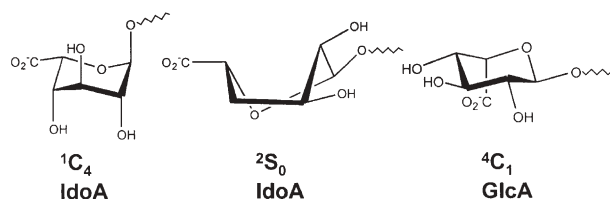

Figure 3. Ring conformations of uronic acids in heparin and heparan sulfate.

the main, internal IdoA residues favour the $^2S_0$ conformation, because in the $^1C_4$ form the bulky carboxyl group is equatorial and all other substituents are in axial positions [28]. However, the iduronate conformation is influenced by the substitution pattern of the glucosamine attached to its non-reducing end. For example, the $^1C_4$ chair form predominates when Ido2S has a GlcNAc attached at the 4-position [28]. The glycosidic linkages are quite stiff, and this means that the shape of the heparin chain does not change according to the conformation of the IdoA ring [10]. Moreover, the glycosidic linkage conformations remain similar regardless of the sulfation pattern [33].

The two conformations of IdoA orientate the 2-*O*-sulfate and carboxyl groups in different positions relative to the helix, and this can have profound effects on protein binding (fig. 4). The arrangement of the heparin helix is such that for sequences of repeating trisulfated disaccharides, the three sulfates are clustered on one side of the chain, with a similar cluster forming on the other side of the chain for the next trisulfated disaccharide in the sequence [10, 34]. The distance between sulfate clusters on one side of the chain is about 17Å [10]. However, when IdoA2S is in the $^1C_4$ chair configuration, the clusters of sulfates appear more dispersed than when IdoA2S is a skew-boat [34]. In solution these two conformations are in equilibrium; thus, it may be expected that a binding protein will perturb this equilibrium to select the conformation that is most energetically favourable for a stable interaction. Experimental data indicate that this is the case. The heparin pentasaccharide that binds antithrombin III has a single internal IdoA (fig. 1). A study using organic synthesis to lock IdoA derivatives in different conformations demonstrated a critical role for the skew-boat $^2S_0$ conformer in the activation of antithrombin by heparin [35]. In the case of FGF-2 binding to a heparin hexasaccharide, crystal structures of the complex revealed the IdoA at position 3 in the saccharide was in the $^1C_4$ conformation, whilst the IdoA at residue 5 was in the $^2S_0$ conformation [36]. The sulfate group in the IdoA locked in a particular conformation is not always directly involved in interacting with the basic amino acids of the binding protein. The 2-*O*-S on the IdoA of the antithrombin III binding pentasaccharide is not directly involved in binding; rather the skew-boat conformation of this IdoA facilitates electrostatic interactions between carboxyl groups in the saccharide and basic amino acids in the antithrombin III protein binding site [34]. In contrast, the Ido2S that is not directly involved in binding to FGF-2 adopts a $^1C_4$ conformation, whereas the one that is involved in binding adopts the skew-boat conformation [34]. These examples illustrate how similar monosaccharide sequences could display different conformations when bound to different proteins.
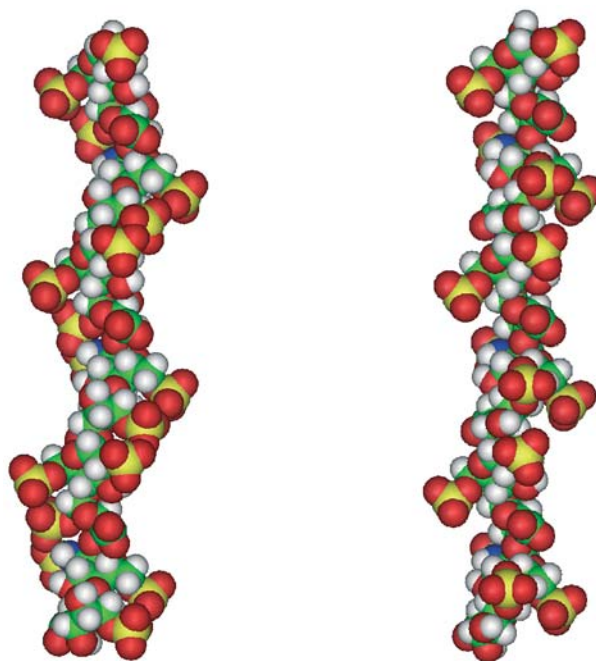

Figure 4. Space-filled model of the solution structure of heparin determined by nuclear magnetic resonance spectroscopy [132] and first published by Mulloy and Forster [10]. The iduronates are shown in their $^1C_4$ chair (left) and $^2S_0$ skew-boat (right) conformations. Sulfates are displayed in red and yellow. Reproduced with permission from [10].

There is very little information as to the three-dimensional structure of heparan sulfate. Although the highly sulfated S-domains of heparan sulfate presumably would adopt the helical structure of heparin, the likely conformation of the GlcA-GlcNAc domains is less certain. Clearly, these sequences lack the internal flexibility that is generated by IdoA, but they retain an ability to rotate about their glycosidic linkages. It appears that the degree of rotation that can occur at GlcA linkages, when GlcA is in the $^4C_1$ conformation, are greater than that observed with IdoA. This led Conrad [25] to argue that stretches of GlcA-containing disaccharides may bend more readily than IdoA-containing disaccharide sequences. Mulloy and Forster utilised data from model oligosaccharides, the K5 polysaccharide (GlcNAc-GlcA-$_n$), maltose and cellobiose. They argued that because more than one low-energy linkage conformation was detected for both linkage types, GlcNAc-GlcA and GlcA-GlcNAc, it is probable that the flexibility of regions rich in GlcA-GlcNAc repeating disaccharides would facilitate the appropriate positioning of two S-domains within a heparan sulfate chain onto an interacting protein [10]. Indeed, a heptadecasaccharide chain consisting of two S-domains linked by three GlcA-GlcNAc repeats has been proposed as a heparan sulfate binding domain for the chemokine macrophage inflammatory protein 1α (MIP-1α) [37]. This structure was modeled using coordinates of the solution structure of heparin to form the S-domains, with the non-sulfated mid-

dle region being modeled as an extended chain in which the glycosidic linkages adopt sterically allowed conformations. This type of model appears to be appropriate for heparan sulfate structures that bind other proteins, for example platelet factor 4, transforming growth factor β (TGF-β), interleukin-8 (IL-8) and regulated on secretion, normal T-cell expressed and secreted (RANTES) [38–41].

Heparan-sulfate chain configuration is affected by protein binding

The binding of some proteins to heparin and heparan sulfate is not only reliant on interactions between the sulfate groups on the saccharide and basic residues on the protein, but van der Waals contacts contribute substantially [22, 42]. The best example comes from FGF family members. Studies revealed that although the GAG chain maintained its overall helical structure when bound to the FGF, a kink in the helical axis appears upon binding. Moreover, this 'kink' is retained in the FGF-2-FGFR1-GAG complex and in the FGF-1-FGFR2-heparin complex [22, 42]. The extent of the kink is exaggerated by the $^1C_4$ conformation of the iduronic acid that is favoured upon FGF binding because it orientates the glycosidic bonds axially. Calculations of the interaction energies of a kinked oligosaccharide and an oligosaccharide with a standard helical structure indicated that the kinked oligosaccharide provides more favourable ionic and van der Waals contacts [42]. The biological implications of this seem to lie in the specificity of the GAG structure recognised. For FGF-1, a number of different structures were found to bind, but with graded affinities [43]. A detailed structural analysis of the oligosaccharides that bind FGF-1 indicated that although the number of sulfated groups is about equal, the difference lies in the abilities of these oligosaccharides to form a kink comprising an iduronic acid in the $^1C_4$ conformation flanked by two glucosamines. Optimal binding requires the sulfation pattern of the kink-spanning trisaccharide to be an N-sulfate on the non-reducing end glucosamine, 2-O-sulfate on iduronate and 6-O-sulfate on the reducing end glucosamine [42]. Analyses of other protein-oligosaccharide co-crystal structures also indicate a kink in the oligosaccharide chain, and this positions the sulfates optimally for ionic and van der Waals interactions. These proteins include antithrombin III, and the NK1 domain of hepatocyte growth factor [42].

Altering heparan sulfate structures has profound biological outcomes

Morphological consequences of silencing biosynthetic enzymes

Recent genetic experiments have thrust heparan sulfate-protein interactions into a centre-stage position in the field of developmental biology. The genes *EXT1* and *EXT2* encode glycosyltransferases that are required for heparan sulfate chain elongation in mammals. The EXT proteins transfer GlcA or GlcNAc residues to the nonreducing end of the polysaccharide. Heparan sulfate synthesis does not take place in EXT1$^{-/-}$ ES cells [44]. Similarly, when EXT2 expression is diminished in mammalian cells by gene-silencing techniques, heparan sulfate synthesis is blocked [45]. Mice rendered EXT1 deficient by gene targeting failed to gastrulate and lacked organised mesoderm and extra-embryonic tissues. Disruption of the EXT1 gene selectively in the murine nervous system caused death in the first day of life [46]. Moreover, mutations in either EXT1 or EXT2 cause hereditary multiple exostoses, an autosomal dominant bone disorder [44, 45]. These studies have indicated that heparan sulfate chain biosynthesis is critical for normal embryonic development.

The effects of alterations in heparan sulfate structure on development have been investigated by making knockout mice that lack expression of the enzymes required for modifying the heparan sulfate chain. The first step in the modification of the heparin/heparan sulfate chain is the removal of acetyl groups from GlcNAc residues to give free amino groups, which are then sulfated. The enzymes catalyzing these reactions are one of four isoforms of N-acetylglucosamine N-deacetylase/N-sulfotransferase (NDST). Knockout mice have been generated for two of these enzymes. Although NDST-2 is widely distributed during development and in the adult, mice lacking this enzyme had a phenotype that was restricted to connective-tissue mast cells. Heparan sulfates from the liver of NDST-2$^{-/-}$ mice show no real differences in the N-sulfation pattern from control mice, but heparin was absent indicating the essential role of this enzyme for heparin biosynthesis [47]. In contrast, a lack of NDST-1 is lethal. The heparan sulfates produced by NDST-1$^{-/-}$ mice have reduced N-sulfation and O-sulfation, and the epimerization of GlcA to IdoA occurs at reduced levels [48]. Around a third of embryos die during the prenatal period, whilst new-born pups have abnormal lungs that produce insufficient surfactants, and as a consequence of lung failure, the pups die shortly after birth [48, 49]. Skeletal defects and other defects which contribute to embryonic death are reviewed in Grobe et al. [48].

Loss of glucuronyl C5-epimerase activity is also lethal for neonates. Targeted disruption of the murine glucuronyl C5-epimerase gene (*Hsepi*) caused biosynthesis of heparan sulfate chains devoid of IdoA and with an abnormal sulfation pattern [50]. The phenotype of the *Hsepi*$^{-/-}$ mice was loss of kidneys, poorly inflated and immature lungs, bilateral iris coloboma, abundant skeletal abnormalities but normal brain, heart, liver, gastrointestinal tract, pancreas and skin [50]. *Hs2st* is the single gene that encodes heparan sulfate 2-O-sulfotransferase, and mice lacking this enzyme die in the neonatal period.

The phenotype of *Hs2st*⁻/⁻ mice has some similarities with that of *Hsepi*⁻/⁻ mice. Given that neither of these knockout mice have heparan sulfate with 2-*O*-sulfated IdoA, it is probably not surprising that they both fail to develop kidneys and display several skeletal abnormalities resembling those of *Hsepi*⁻/⁻ mice [50, 51].

Complications in the interpretation of data from biosynthetic enzyme silencing experiments may arise when one member of a multi-enzyme family is silenced. There are six members of the 3-*O*-sulfotransferase (3-OST) family, and these enzymes transfer sulfate groups to the 3-OH of glucosamine. Interestingly, the different enzyme isoforms preferentially recognise different saccharide structures around the glucosamine that is to be sulfated [52]. As these isoforms are expressed at different levels in different tissues this finding provides an explanation for the formation of tissue specific saccharide structures. The 3-OST-1 isoform is primarily responsible for 3-*O*-sulfation of the glucosamine within the antithrombin III-binding pentasaccharide. Thus, it may be expected that silencing this gene (*Hs3st1*) would give rise to mice with a procoagulant phenotype. This was not the case, even though heparan sulfate isolated from various tissues of *Hs3st1*⁻/⁻ mice had markedly reduced anti-Xa activity compared to heparan sulfates isolated from normal mice [53]. Unexpectedly, *Hs3st1*⁻/⁻ mice exhibited intrauterine growth retardation and genetic background-specific lethality. Possibly, in the absence of 3-OST-1 other members of this family perform the 3-*O*-sulfation of the glucosamine to a level that is sufficient to protect against thrombosis but not against the other abnormalities. The finding that the 3-OST-5 isoform is capable of generating antithrombin III-binding heparan sulfate in a cell line supports this view [54].

Functional consequences of silencing biosynthetic enzymes

Although the knockout studies indicate that the correct biosynthesis of heparan sulfate chains is critical for development, they also reveal how little is understood about the role of particular heparan sulfate structures in these processes. Experiments designed to address what structural changes in the heparan sulfate chains mean for protein binding and signaling are required. These studies are beginning. An analysis of heparan sulfate structures produced in *Hs2st*⁻/⁻ embryos revealed that the domain structure is conserved but the *N*-sulfates are clustered into longer S-domains. Despite the lack of 2-*O*-sulfate, the charge density is maintained by a dramatic increase of 6-*O*-sulfate in the GlcNS repeat regions [55]. The ability of the mutant heparan sulfate to bind fibronectin and hepatocyte growth factor (HGF) is maintained, but binding to FGF-1 and -2 was weaker. It would be interesting to perform X-ray crystallography on FGF-2 binding to

oligosaccharides derived from *Hs2st* mutant mice to determine how structures lacking 2-*O*-sulfation, but with increased 6-*O*-sulfation, make contacts with amino acids in the GAG binding site. Unexpectedly, growth factor signaling was very similar for FGF-1, -2 and HGF [55]. Clearly the strength of FGF-1 and -2 binding to heparan sulfate without 2-*O*-sulfate is sufficient for signaling in embryonic fibroblasts. This finding indicates that in vitro assessments of the strength of protein-heparin/heparan sulfate interactions do not necessarily predict the functional outcomes of that interaction. It also illustrates that novel heparan sulfate structures produced as a consequence of silencing heparan sulfate biosynthetic enzymes may have unexpected binding activities, further complicating interpretation of the molecular basis of the phenotypes of mice in which these genes have been silenced.

Intact heparin and heparan sulfate chains are also modified by extracellular endosulfatases, and these enzymes appear to play a role in regulating embryo patterning. Two endosulfatases, designated HSulf-1 and HSulf-2, have been cloned in mice and humans, as well as a quail homologue, QSulf1 [56, 57]. These enzymes remove sulfate from the 6-position of glucosamine in the disaccharide IdoA2S-GlcNS6S and to a lesser extent in GlcA-GlcNS6S disaccharides, recognising only a small subset of these disaccharides, but saccharide sequences containing IdoA are not recognised [56, 58]. Data from another study suggest that Qsulf1 recognises the 6-*O*-sulfates when GlcNS6S is flanked by IdoA2S. That is, the activity of Qsulf1 is confined to the S-domains [59]. An explanation for the increase in 6-*O*-sulfation in heparan sulfates from *Hs2st* mutant mice could be a marked reduction in the activity of HSulf-1 or HSulf-2, as these enzymes primarily recognise the IdoA2S-containing trisulfated disaccharide which is common in heparin and S-domains of heparan sulfate.

Given the role of FGF family members in development, and that heparan sulfate is an obligate cofactor for FGF/receptor signaling, it is logical that discussion of the molecular basis for the phenotypes observed when genes encoding heparan sulfate biosynthetic enzymes are silenced should focus on FGF family members. Merry and Wilson [60] have examined phenotypes of mice carrying targeted mutations in growth factors and receptors, principally of the FGF family and FGF receptors, with a view to determining whether a mutation in these molecules recapitulates the *Hs2st* mutant phenotype. Although the *Hs2st* mutant phenotype overlaps with the phenotype of mice depleted of some of these growth factors and receptors, no single mutation gives rise to a phenotype that resembles the *Hs2st* mutant. This is not unexpected given the large number of vertebrate proteins that bind to heparan sulfate, but which are not FGF family members or their receptors. Some of these, like NCAM [61], are

involved in cell adhesion and cell migration during embryogenesis, whilst others are extracellular matrix proteins, e.g. fibronectin, some laminins, thrombospondin and BM-40 [62–65]. Furthermore, other heparan sulfate-binding proteins may indirectly influence embryogenesis via the proteins they bind, e.g. the heparan sulfate-binding protein follistatin binds and neutralises activin, a molecule that plays a critical role in differentiation and early embryo development [66]. Unraveling which vertebrate molecular pathways are affected by mutations of heparan sulfate biosynthetic enzymes will be complex and challenging.

Tissue specific heparan sulfate structures are functionally relevant

The heparan sulfate enzyme knockout experiments are unable to demonstrate whether subtle differences in heparan sulfate structure are found in particular tissue sites and whether they have biological relevance. These are crucial, yet difficult questions to address because of the problems of isolating the very small quantities of heparan sulfates synthesised by different cell types, and of the difficulty of performing structural and biological analyses with very little material that has underlying heterogeneity. The use of epitope specific antibodies should assist in examining these questions.

Heparan sulfates are generally poor immunogens, but the recent use of phage display technology has allowed the generation of a panel of apparently epitope specific anti-heparan sulfate and anti-heparin antibodies [67–69]. Screening of these antibodies against panels of modified heparan sulfate and heparin molecules [67–69] as well as against heparan sulfate oligosaccharides of known sequence [68] indicated that the antibodies had different binding patterns. Assessment of antibody reactivity to heparan sulfate oligosaccharides of known sequence also provided data on the type of structure likely to be contained in the preferred epitope [68]. However, two of the antibodies selected against lung heparan sulfate were identical to antibodies selected against bovine kidney and human skeletal muscle, indicating that these tissues share heparan sulfate epitopes [70]. Antibody staining has revealed defined topological distributions of various heparan sulfate epitopes in the rat kidney and spleen, human lung, and human, rat and mouse skeletal muscle [67, 68, 70, 71]. In the spleen some of the antibodies co-localised with interleukin-2 which was bound to heparan sulfate [71]. Similarly, in the lung some of the antibodies blocked FGF-2 and VEGF (vascular endothelial growth factor) binding [70]. Collectively, these data indicate that heparan sulfate biosynthesis is controlled and differently regulated by the cell types within tissues, probably creating specific extracellular microenvironments.

If particular heparan sulfate epitopes are expressed in tissue sites, then are these different epitopes selectively recognised by heparan sulfate binding proteins? Two studies by Allen and colleagues suggest that they are [72, 73]. They have generated a series of probes for heparan sulfates based on the fact that both FGFs and their receptors bind heparan sulfates to form a signaling complex. The probes used were FGF-2, FGF-4 and their receptors: soluble FGF receptor 1-IIIc (FR1c) and FGF receptor 2-IIIc (FR2c), and in the second study, FGF-1 and FGF-8b with FR2c and soluble FGF receptor 3-IIIc (FR3c). Collectively, the data from these studies indicated that there are changes in the heparan sulfate structures expressed during development, and these changes are reflected in the different binding patterns of the various FGFs and FGF/receptor complexes. As is expected from in vitro studies of various FGFs binding to different heparan sulfate structures, the binding patterns of the growth factors differed. FGF-2 was found to bind heparan sulfate in a ubiquitous fashion in the developing mouse embryo, whereas FGF-4 was more selective, failing to bind heparan sulfate in the heart and large blood vessels, nor to aortic endothelial cells in culture [72].

The complex pattern with which each FGF/receptor pair bound heparan sulfates in the embryos suggested that the different FGF/receptor combinations recognised distinct heparan sulfate structures that are spatially and temporally regulated [72, 73]. Analysis of whether 2-*O*-sulfation or 6-*O*-sulfation is a requirement of the heparan sulfate that is involved in the particular FGF/receptor/heparan sulfate signaling complex indicated differences in sulfation requirements between FGF/receptor complexes [73]. Importantly, the data suggest that the heparan sulfate binding site displayed by an FGF/receptor pair differs from that displayed when the same FGF combines with a different receptor. These data are discussed in the light of the finding that FGF-1 can signal using heparan sulfate from *Hs2st*−/− mice. It is suggested that for FGF-1, the heparan sulfate structure that binds FGF-1 requires 2-*O*-sulfation, similarly 2-*O*-sulfation is required for it to form a complex with heparan sulfate and FR2c, but this is not the case if it forms a complex with heparan sulfate and FR2b [73]. Although the sulfation patterns involved have not been determined, the situation could be similar for FGF-4 and its receptors. Considering the pattern with which these probes bind embryo sections, it appears that the heparan sulfate structures required to bind FGF-4/FR1c complexes differ from those that bind FGF-4 alone. On the other hand, FR2c seems to be less selective, recognising all FGF-4-heparan sulfate complexes [72]. Thus, the heparan sulfate structure that binds the various FGFs in isolation will not necessarily be the same structure that is required for signaling.

Another example of subtly different heparan sulfate structures displaying markedly variant capabilities for

FGF signaling comes from a study on murine embryos. The structure of heparan sulfates isolated from murine embryonic day 10 (E10) and embryonic day 12 (E12) neuroepithelial cells differed. There were differences in the levels of 2-*O*-sulfation, the patterns of 6-*O*-sulfation, total chain length and the number of sulfated domains per chain [74]. E10 heparan sulfate was strongly active in supporting FGF-8 signaling via FR3c, whereas the E12 heparan sulfate had no activity. In contrast, both heparan sulfate preparations supported FGF-2 signaling via FR1c [75]. These data are in concordance with the developmental stages at which FGF-8 and FGF-2 function in the embryo. Interestingly, clear differences were also evident in the levels of 2-*O*-sulfotransferase expressed and the isoforms of 6-*O*-sulfotransferases expressed in E10 and E12 neuroepithelial cells. Moreover, there were differences in the isoforms of NDSTs expressed [75], consistent with the finding of altered patterns of *N*-sulfation (reflected in the differences in sulfated domains) between E10 and E12 heparan sulfate. This study provides evidence in support of isozyme expression patterns giving rise to certain heparan sulfate structures that are functionally specific and is an important adjunct to the enzyme knockout experiments.

An example of heparan sulfates in a particular tissue location preferentially binding a protein is that of the chemokine MCP-1 binding. Although heparan sulfates are also found in the extracellular matrix secreted by these cells, MCP-1 focused on the apical surface even when added to the basal side of the endothelial cell layer [76]. Presumably the secreted heparan sulfate is structurally different from the cell surface-bound heparan sulfate, and this is reflected in the pattern of MCP-1 binding. Clearly, studies of the binding patterns of other chemokines and cytokines that bind heparan sulfate are warranted. Although little structural data on the types of heparan sulfate structures recognised by these proteins are available, it is frequently hypothesised that particular heparan sulfates act to localise chemokines and cytokines to specific tissue sites and by so doing contribute to regulating their function. This appears to be true for FGF family members, but whether it is true for a range of cytokines and chemokines remains to be determined.

Regulation of heparan sulfate-protein interactions in the tissues

The data indicate that certain heparan sulfate structures bind particular proteins both in vivo and in vitro but do the in vitro data reflect what is happening biologically? The ionic nature of buffers used for in vitro binding assays, for example, may not always reflect the extracellular milieu in which proteins bind GAGs in vivo. There are numerous proteins, which bind heparin/heparan sulfate

with higher affinity if cations are present, particularly zinc or copper ions. These proteins include beta-amyloid precursor protein, histidine-proline-rich glycoprotein (HPRG), interleukin-5, high molecular weight kininogen, prion protein, heparin cofactor II and endostatin, to name a few [77–83]. Occasionally, differences in the binding patterns of a particular protein to heparin can be explained by variations in the losses of cations associated with the protein, depending on the purification method used. For example, variable binding to GAGs of different endostatin preparations appeared to be due to differing losses of cations (probably zinc) according to the purification protocol employed, and the existence, or otherwise, of zinc-dependent dimers [83]. Although oligomerization may be stabilised by cations, and this facilitates interactions with heparin or heparan sulfate [81], cations may also induce a conformational change in a protein which assists an interaction with heparin. For example, Zn^{++} induces a conformational change in heparin cofactor II that enhances its interaction with heparin [82].

In the above examples the cations are bound by the protein, but heparin/heparan sulfate chains also bind strongly to divalent metal ions [25]. The binding of cations to GAG chains is not always a simple electrostatic interaction between the negatively charged groups on the carbohydrate and the positively charged cation because Zn^{++} binds selectively to heparin rather than to other GAGs [84]. NMR evidence indicates that iduronic acid is the main binding site in heparin for heavy metal cations. Moreover, the spectral data suggest that Zn^{++} binding alters the ring conformation of iduronic acid such that the $^1C_4$ conformation is stabilised over the $^2S_0$ conformation [85–87]. If metal ion binding similarly controls the ring conformation of iduronate in heparin and heparan sulfate under physiological conditions, this may be expected to influence the specificity and affinity of protein interactions. The concentration of Zn^{++} in body fluids is generally quite low, but platelets contain zinc at 30–60-fold higher concentrations than plasma, and abundant zinc binding proteins such as decorin or biglycan could serve as storage pools for these ions [88, 89]. It is feasible that local concentrations of Zn^{++} may be much higher than that of plasma, particularly around sites of platelet activation. Thus, microenvironmental concentrations of cations are likely to influence/regulate the in vivo affinity and specificity of numerous heparan sulfate-protein interactions.

In vitro binding assays are almost invariably performed at neutral pH; however, in vivo the local pH is not always neutral. For example, local interstitial acidification is commonly associated with inflammatory lesions, which is attributed to primarily the local increase in lactic acid production caused by the anaerobic glycolysis of infiltrating neutrophils [90]. Measurements of the pH of fluids drained from sites of inflammation document

extracellular pH values as low as 6.1 [91]. Moreover, it has been known for many years that tumour microenvironments are usually more acidic than normal, with pH values as low as 5.5 [92, 93]. Hypoxia or ischemia is commonly associated with local acidosis. Alteration of pH can have profound effects on the ability of some proteins to bind heparin or heparan sulfate. This is particularly so when the GAG binding site involves histidines. Histidine-proline-rich glycoprotein is a prime example, for at neutral pH binding to heparin is minimal, but increases to a maximum at pH 6.5. However, zinc ions supplant the requirement for low pH. At intermediate pH, both protonation of histidine and the binding of zinc promote the interaction with heparin [94]. Prion protein also binds GAGs in a pH- and metal ion-dependent fashion; at pH values above the histidine pKa, prion protein-GAG complexes are stabilised by Cu^{++} or Zn^{++} [81].

Other proteins that bind GAGs in a pH-dependent fashion include the non-fibrillar form of beta-amyloid peptide, selenoprotein P, granulocyte macrophage colony stimulating factor (GM-CSF) and VEGF [95–98]. These proteins are dissimilar in overall structure, but their binding sites for GAGs involve one or more histidines. A VEGF isoform that lacks the native heparin binding domain (VEGF$_{121}$) was found only to bind heparin and heparan sulfate at low pH, whereas binding of the isoform, VEGF$_{165}$, to heparin increased at acidic pH. Thus, low pH appears to expose a new heparin binding site within the regions shared by the two isoforms [98]. The binding of both these VEGF isoforms to fibronectin also increased at low pH, an effect that was further enhanced by heparin. Under hypoxic conditions, like those found in and around tumours or wounds, the generation of an acidic extracellular environment may lead to storage of VEGF in a stable complex of fibronectin and heparan sulfate proteoglycans. As at neutral pH active VEGF is readily released, the pH-sensitive matrix storage and release of VEGF could set up a VEGF gradient which directs and stimulates the growth of new blood vessels into hypoxic or ischemic regions in tissues [99].

GM-CSF is a cytokine involved in regulating haemopoiesis in the bone marrow and at extramedullar sites. In the late 1980s it was suggested that an interaction of GM-CSF with stromal heparan sulfates contributed to its biological activity [100, 101], but there were no follow-up reports directly demonstrating heparan sulfate structures bound to GM-CSF. Other data indicated that even in the presence of cytokines, not all stromal cells could sustain myelopoiesis. The nature of the glycoconjugates in the stromal cell layer was found to be a determinant [102]. If GM-CSF undergoes a pH-induced conformational change that allows heparin to bind, as has been suggested [97], this could explain the lack of in vitro binding data. The interaction of haemopoietic cells with supporting stroma leads to an accumulation of sialylated glycoconjugates and proteoglycans at the interface of the two cell types [97, 103]. This may produce a local acidic microenvironment that supports the binding of GM-CSF to membrane heparan sulfates. Within the bone marrow, precursor cells of particular lineages are known to favour particular sites giving foci of developing cells of one lineage [104]. Thus, local pH and the presence of particular GAG structures may act together to regulate GM-CSF localisation, thereby producing the microenvironmental niche necessary for sustained myelopoiesis. Indeed, highly *O*-sulfated stromal cell heparan sulfate is an important component of the bone marrow 'niche' that acts with cytokines and chemokines to regulate cell proliferation and differentiation [105].

GAG chain presentation and activity

Many in vitro assays do not take into account the fact that the presentation of GAG chains may alter their activity. In vivo more than one heparan sulfate chain is frequently attached to a core protein, and proteoglycans may be expressed on cell surfaces which permit molecular clustering. Soluble and cell membrane forms of syndecan-1 and glypican-1 differed markedly in their ability to stimulate FGF-2-induced FGFR1 phosphorylation [106]. Membrane associated forms were active, whereas corresponding soluble forms were inactive. Interestingly, cells expressing a mutant glypican-1 that carried only one heparan sulfate chain also strongly stimulated FGF-2 induced FGFR1 signaling, suggesting that multivalency of heparan sulfate chains on the same core protein is not a requirement for the activity of membrane-associated proteoglycans in this system [106]. In contrast, when the syndecan-1 functions of collagen binding, cell-cell adhesion and cell invasion of collagen gels were assayed, multivalency was important. The function of syndecan-1 was modulated according to the number of heparan sulfate chains it carried, and the position of these chains on the core protein, even though the overall levels of cell surface heparan sulfate expression did not vary appreciably [107].

Towards the use of heparin or heparan structures as therapeutics

Heparin and heparan sulfates bind a multitude of different proteins that have a variety of biological functions. The requirement for these GAGs in mammalian development has been demonstrated. It is also clear that tissue-specific, different heparan sulfate structures bind different sets of proteins. Frequently the local tissue environment modulates the affinity of the GAG-protein interaction, and this may have biological outcomes in

terms of establishing gradients of cytokines or chemo-kines. If the goal is to define a GAG structure that has therapeutic applications, it is appropriate to determine optimal GAG structures that bind the protein in question with high affinity when GAG chains are in solution, as an oligosaccharide that binds tightly in vivo is the requirement. Thus, the pH of the target tissue and the possibility that cations will contribute to the binding affinity deserve consideration.

There is enormous potential for the development of heparin-like structures as drugs for a range of diseases in addition to the current antithrombotic target. The most obvious of these are cancer, inflammatory diseases and virus infections [108]. There have been a number of approaches to the development of heparin-based therapeutics. The production of mixtures of heparin-like structures that interact with and alter the function of numerous proteins is one approach. A drug currently in clinical trials in cancer patients that was designed through this approach is the phosphosulfomannan, PI-88 [109]. PI-88 is structurally heterogeneous [110], and as well as inhibiting heparanase it also binds FGF-1, FGF-2 and VEGF [111].

Another approach is the synthesis of particular structures based on a heparin/heparan sulfate template that are designed to bind specifically to the target protein. Although the diversity of heparin/heparan sulfates and the nature of their monosaccharide components make synthesis a major challenge for chemists, the first synthetic molecules were produced 20 years ago. Pioneers in the field were Choay, Petitou and colleagues who synthesised tetrasaccharides and pentasaccharides to understand the structural basis for heparin's antithrombin III-binding, and anticoagulant activity [112–114]. This early work has led to the registration of the first fully synthetic heparin structure for clinical use. Fondaparinux (Arixtra, Sanofi-Synthelabo) has been approved for use in thromboprophylaxis following orthopedic surgery. It is the antithrombin III-binding pentasaccharide sequence (fig. 1), but with a methyl group stabilizing the anomeric end [115]. Fondaparinux does not bind to platelet factor 4 (PF4) and does not cross-react with antibodies generated as a result of heparin-induced thrombocytopenia. Nor does fondaparinux stimulate endotoxin-induced interleukin-8 production by monocytes [116]. The data also suggest that fondaparinux will be well tolerated by patients who have a tendency to develop delayed-type hypersensitivity reactions to subcutaneously injected heparin, probably because fondaparinux does not bind dermal proteins [117, 118]. Clearly, careful selection of a heparin structure for activity against a target protein, in this case antithrombin III, does eliminate many of the undesirable effects of heparin therapy.

Other synthetic heparin antithrombin III pentasaccharide analogues, e.g. Idraparinux (SANORG 34006), are cur-rently in clinical development [115, 119]. A second generation of heparin mimetics designed to treat various thrombotic disorders are in the pipeline. These structures consist of two functional domains, an antithrombin III binding domain and a thrombin binding domain separated by a spacer [120]. The goal was to obtain a mimetic with a favourable antithrombotic/bleeding ratio but which does not bind to PF4 or lead to heparin-induced thrombocytopenia. The preclinical data on a synthetic hexadecasaccharide indicate it is more active than heparin in in vivo models of thrombosis, yet it did not activate platelets or compete with heparin for binding to PF4 [121, 122].

A few laboratories are investigating utilising a modular approach for the synthesis of heparin oligosaccharides. The ready synthesis of heparin-like structures in quantities suitable for biological and biochemical assays, and eventually drugs, is the aim. The modular approach is feasible because different combinations of 20 disaccharides arranged in a linear sequence can determine the structure of native heparan sulfate chains. The laboratories of Seeberger and Boons have independently published their strategies for synthesis of six monosaccharide building blocks which contain different chemical protecting groups on key positions that are involved in chain linkage or modification by carboxyl or sulfate groups [123–125]. The linkage of these monosaccharides, first into disaccharides and then into larger well-defined oligosaccharides that display the variety of structures found in native heparan sulfate, is being explored.

Rosenberg and colleagues developed a method utilising the polysaccharide isolated from *Escherichia coli* K5 as a starting material for the synthesis of classical and non-classical heparan sulfate-like structures [126]. The production of *N*-sulfated glucosamines was performed chemically, but all subsequent modifications were performed by a set of recombinant heparan sulfate biosynthetic enzymes. The authors suggest this 'chemosynthetic' approach will allow the generation of libraries of homogeneous oligosaccharides, the definition of critical functional groups for target proteins, and eventually the design of heparan sulfate-like drugs [126]. Using this method they have prepared non-classical heparan sulfate-like structures that lack IdoA2S groups but are 3-*O*- and 6-*O*-sulfated. These structures possess anticoagulant activity indicating that the 2-*O*-sulfate of IdoA2S is a minor contributor to antithrombin III binding [126]. As IdoA2S groups seem critical for PF4 binding to GAGs and for heparanase cleavage, these non-classical anticoagulants should be more biologically active than their native counterparts. Idraparinux is an *O*-methylated, *O*-sulfated pentasaccharide, which although modeled on the antithrombin III pentasaccharide (fig. 1) similarly lacks a IdoA2S, but binds antithrombin III with an affin-

ity 10 times that of the natural structure [119]. These data highlight the importance of critical groups within a GAG structure for specific protein binding rather than the native heparin/heparan sulfate sequence itself being critical.

Concluding remarks

The study of heparin/heparan sulfate-protein interactions is now poised to make major advances in the next few years spearheaded by new technologies being developed for the structural analysis of heparin and heparan sulfates and the synthesis of heparin-like structures. These new technologies will greatly assist the development of microarrays of structurally defined GAG fragments. Such tools will assist in answering crucial questions as to the specificity of many of the GAG-protein interactions and whether motifs that possess a similar level of specificity as displayed by the antithrombin III-binding pentasaccharide exist for other heparin/heparan sulfate-binding proteins. However, binding specificity and affinity do not fully address questions of function. The example of the heparan sulfate produced by *Hs2st*$^{-/-}$ mice being able to initiate FGF-1 and FGF-2 signaling despite its reduced binding affinity [55] demonstrates that in biology, increased affinity does not always directly correlate with increased activity. Often once a certain threshold of binding stability is achieved, further increases in affinity are functionally immaterial. Moreover, an appropriate multivalent presentation of heparan sulfate structures each with suboptimal binding affinity may produce the same stability of binding, and hence biological activity, as a more specific but monomeric heparan sulfate structure.

It is important to consider the biological role of a particular heparin/heparan sulfate-protein interaction when discussing specificity and affinity issues. The recent discovery that heparan sulfate proteoglycans on macrophages, endothelia and genital epithelial cells capture cell-free human immunodeficiency virus (HIV) and for the latter two cells types facilitate the transfer of virus to CD4$^+$ T lymphocytes is an example where exquisite specificity appears not to be required for biological activity [127–129]. In contrast, herpes simplex virus type 1 (HSV-1) envelope glycoprotein D (gD) recognises an octasaccharide that includes the rare 3-*O*-sulfated glucosamine generated by the 3-*O*-sulfotransferase isoforms 3 and 5 [130, 131]. The gD-octasaccharide interaction is a critical event for HSV-1 entry into permissive cells, and as the virus infects only mucosal epithelium and very rarely neuronal cells, a quite specific interaction may be expected. Clearly, some GAG-protein interactions have evolved to be relatively non-specific, whereas others are quite specific.

The biosynthetic-enzyme silencing experiments have taught us a number of things. First, that heparan sulfate chains are critical for normal development. Second, that the structure of those chains is critical. Third, how little is understood about the tissue-specific regulation of the various isoforms of these enzymes, and hence what structures are expressed where, and finally how little is known of the biological consequences of normal heparan sulfate-protein interactions, the loss of which causes the phenotypes that are observed. As has been discussed, it is clear that particular heparan sulfate structures are expressed in different tissue types and at different times during development, and these different structures are selectively recognised by heparan sulfate-binding proteins. The tissue environment may also contribute to the regulation of GAG-protein interactions by changes in pH or cation composition. Thus, in vivo the specificity question takes on a different complexion because the microenvironmental milieu could prohibit binding, or many proteins may never encounter particular heparan sulfate structures. The fact that a protein may bind a structure in vitro that it would never encounter in vivo would normally have no biological relevance, but it could be important in a pharmaceutical context.

The design of an effective GAG-based therapeutic should be appropriate for the heparan sulfate-protein interaction that is targeted. A novel, very specific GAG structure may be inappropriate for inhibiting the localisation of HIV, for example. Frequently the side effects of a relatively non-specific GAG-based drug can be minimised by the choice of the drug delivery route. For example, an anti-HIV intravaginal application could obviate the lack of specificity. Among the multitude of heparan sulfate-protein interactions that exist in biology, there will be a spectrum of affinities and specificities. Nevertheless, because the diversity of heparan sulfate structures and the complexity of their biosynthetic pathways has been maintained throughout animal evolution, it is likely that many of these heparan sulfate-protein interactions will require a pronounced level of specificity. The newly evolving technologies in GAG synthesis and structural analysis will assist in resolving the relationship between structure and activity. An understanding of structure-activity relationships could well lead to the design of well-tolerated drugs based around heparan sulfate structures that target a range of diseases outside of the thrombosis-anticoagluation axis. The next decade is likely to be an exciting time for heparan sulfate-inspired therapeutics.

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