Structural Insights into Biological Roles of Protein-Glycosaminoglycan Interactions

Review

Rahul Raman,[1] V. Sasisekharan,[2]
and Ram Sasisekharan[1,*]
[1]Biological Engineering Division
[2]Harvard-MIT Division of Health Sciences and
    Technology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

The extracellular environment is largely comprised of complex polysaccharides, which were historically considered inert materials that hydrated the cells and contributed to the structural scaffolds. Recent advances in development of sophisticated analytical techniques have brought about a dramatic transformation in understanding the numerous biological roles of these complex polysaccharides. Glycosaminoglycans (GAGs) are a class of these polysaccharides, which bind to a wide variety of proteins and signaling molecules in the cellular environment and modulate their activity, thus impinging on fundamental biological processes. Despite the importance of GAGs modulating biological functions, there are relatively few examples that demonstrate specificity of GAG-protein interactions, which in turn define the structure-function relationships of these polysaccharides. Focusing on heparin/heparan (HSGAGs) and chondroitin/dermatan sulfate (CSGAGs), this review provides structural insights into the oligosaccharide-protein interactions and discusses some key and challenging aspects of understanding GAG structure-function relationships.

Introduction

Complex polysaccharides are primary constituents of every eukaryotic cell surface and the extracellular environment. Many recent studies have shown that polysaccharides play important roles in numerous physiological and pathological processes such as growth and development [1–5], angiogenesis and cancer [6–9], and microbial pathogenesis [10–14]. Glycosaminoglycans (GAGs), a major class of extracellular complex polysaccharides, have been studied extensively [12, 15–17]. GAGs are linear acidic polysaccharides containing disaccharide repeat units of an uronic acid linked to a hexosamine, and there are four classes of GAGs based on the different chemical structures (Table 1). Each GAG backbone can be modified by sulfation at the uronic acid and hexosamine (Table 1), making them highly information dense. For example, HSGAGs could potentially contain up to 48 disaccharide building blocks based on the sulfation pattern, which can be con-

trasted with that of DNA (4 building blocks) and proteins (20 building blocks).

The high information density of GAGs in terms of their sequence diversity arises from their biosynthesis, which is a complex non-template-driven process involving several enzymes with tissue-specific isoforms [16, 18]. HSGAGs and CSGAGs are O-linked to a Ser-Gly/Ala-X-Gly consensus motif on a core protein (GAG and protein together known as proteoglycan). Hyaluronic acid is the simplest of the GAG structures containing an unsulfated backbone (Table 1) and it is not synthesized from the core protein. The biosynthesis of HSGAGs and CSGAGs begins with the attachment of a tetrasaccharide linker GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-O-(Ser) transferred to the core protein by four different enzymes. The actual chain building begins after this step where a multidomain glycosyltransferase GlcNAcT-II or GalNAcT-II successively transfers GlcA and N-acetyl-glucosamine (for HSGAG) or -galactosamine (for CSGAG), respectively, to the precursor chain leading to chain elongation.

After the elongating chain matures to a particular stage, it is acted upon by several enzymes, for example sulfotransferases (distinct for HSGAGs and CSGAGs) [16, 18] that respectively epimerize GlcA to IdoA and sulfate the backbone. For example, one of the first modification steps in HSGAGs is the action of the N-deacetylase N-sulfotransferase that results in –[G-H$_{NS}$]– units from –[G-H$_{NAc}$]–. This modification is followed by the action of the C5 epimerase enzyme, which converts some of the GlcA to IdoA. During chain maturation, the 2-O, 3-O, and 6-O sulfotransferases sulfate the monosaccharides of the HSGAG chain at different positions. The sequence diversity of a GAG chain is governed by the expression of these enzymes (and their tissue-specific isoforms) and their distinct substrate specificities. The action of the biosynthetic enzymes gives rise to regions of distinct sulfation patterns in different GAG chains. Heparin, which is primarily synthesized in mast cells, is comprised of long regions of a homogenous trisulfated disaccharide repeat –[I$_{2S}$-H$_{NS,6S}$]– interspersed with smaller variable regions containing GlcA and lower sulfation. Thus most of heparin is homogenous with fully sulfated disaccharide units. On the other hand, heparan sulfate is synthesized by the cell types and possesses a higher degree of tissue-specific variation in the regions of sulfation. Thus, by utilizing this complex biosynthetic machinery, the cell can maintain a diverse set of GAG chains on the cell surface and thus dynamically change its environment in response to biochemical signals.
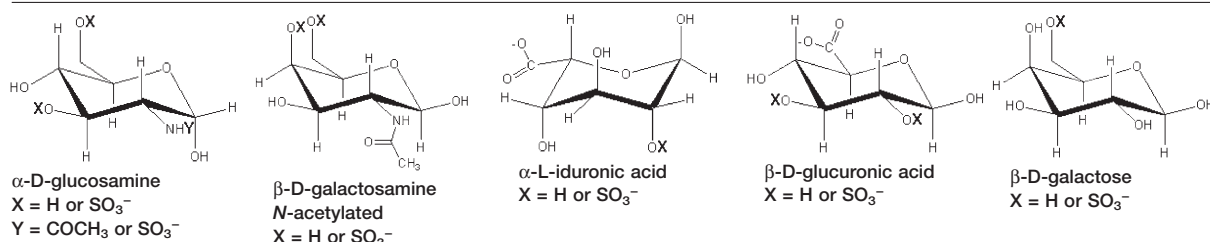
The chemical heterogeneity of HS/CSGAGs in terms of their sulfation pattern and backbone chemical structure facilitates binding to a variety of proteins such as growth factors, enzymes [12, 15, 17, 19], and morphogens [2, 4, 5, 17], and also proteins on microbial pathogens [10, 11, 13] in the extracellular environment (Figure 1). The emerging view is that there is specificity in these

*Correspondence: rams@mit.edu

Table 1. Different Classes of GAGs and Their Disaccharide Building Blocks

Category	Disaccharide	U	H	Modifications
Heparin/Heparan (HSGAG)	$U_{2X\ (\alpha/\beta1,4)}\ H_{NY,3X,6X\ (\alpha1,4)}$	IdoA/GlcA	Glucosamine	X, sulfated; Y, acetylated/sulfated
Chondroitin (CS)/ Dermatan (DS) (CSGAG)	$U_{2X,3X\ (\alpha/\beta1,3)}$ $H_{NAc,4X,6X\ (\beta1,4)}$	IdoA/GlcA	Galactosamine	X, sulfated; 3-O, sulfated only for GlcA
Keratan	$Gal_{6X\ (\beta1,4)}\ H_{NAc,6X\ (\beta1,3)}$	Gal	Glucosamine	X, sulfated
Hyaluronic Acid	$GlcA_{\ (\beta1,3)}\ H_{NAc\ (\beta1,4)}$	GlcA	Glucosamine	None

Monosaccharide Structures

α-D-glucosamine
X = H or SO_3^-
Y = $COCH_3$ or SO_3^-

β-D-galactosamine
N-acetylated
X = H or SO_3^-

α-L-iduronic acid
X = H or SO_3^-

β-D-glucuronic acid
X = H or SO_3^-

β-D-galactose
X = H or SO_3^-

IdoA or I, α-L-iduronic acid; GlcA or G, β-D-glucuronic acid; H, either α/β-D-glucosamine or β-D-galactosamine depending on the GAG class; Gal, β-D-Galactose. Acetylation [$COCH_3$] is indicated using Ac and sulfation [SO_3^-] using S.

HS/CS GAG-protein interactions that modulate the activities of the protein and thus affect its biological functions [20, 21]. While the list of the known proteins that HS/CS GAGs bind to is growing, the oligosaccharide sequence specificity of only a few of the HS/CS GAG-protein interactions is known at this point of time. Given the growing recognition of the important biological roles of HS/CS GAGs, it is important to understand how oligosaccharide sequence motifs in these translate into their biological roles by binding to and modulating the activity of proteins. This review summarizes what is currently known about sequence-specific HS/CS GAG-protein interactions by highlighting the well-characterized interactions and provides insights into how structure and conformation of the HS/CS GAG and the protein govern the specificity of their interactions.

Biological Roles of GAGs
The GAG chains of both the cell surface and secreted proteoglycans are present in an environment of various proteins such as growth factors, cytokines, morphogens, and enzymes (proteases, protease inhibitors) in the extracellular environment. GAGs play a critical role in assembling protein-protein complexes such as growth factor-receptor or enzyme-inhibitor on the cell surface and in the extracellular matrix that are directly involved in initiating cell signaling events or inhibiting biochemical pathways (Figure 1). Furthermore, extracellular GAGs can potentially sequester proteins and enzymes and present them to the appropriate site for activation (Figure 1). Thus for a given high-affinity GAG-protein interaction, the positioning of the protein binding oligosaccharide motifs along GAG chain determines if an active signaling complex is assembled at the cell surface or an inactive complex is sequestered in the matrix. It should be noted that high-affinity GAG-protein interactions are not the only biologically significant interactions. GAGs have been shown to play important roles in maintaining morphogen gradients across a cell or tissue, which have been implicated in developmental

processes [2, 5]. Maintaining a gradient in the concentration of growth factors or morphogens would involve graded affinities between different GAG sequences with the given protein. Thus, the nature of GAG-protein interactions coupled with their sequence diversity enables GAGs to "fine tune" or what can be viewed as "analog modulation" of the activity of proteins. As stated earlier, there have been numerous studies that have implicated the role of GAGs in important biological processes. Summarizing all these studies is beyond the scope of this review. Thus the recent investigations of the biological roles of GAGs are briefly discussed in the following.

The earliest known biological role of GAGs is that of heparin, which has been used as a clinical anticoagulant for several decades. Several studies have focused on biochemical characterization of the anticoagulant activity of heparin in terms of its specific interaction with antithrombin III (see below for details). The GAG biosynthetic enzymes have been the target of numerous genetic studies that have provided direct functional evidence on the involvement of GAGs in cell growth and developmental processes involving Wnt, Hedgehog, transforming growth factor (TGF), and fibroblast growth factor (FGF) signaling pathways [5]. Murine genetic studies have also directly implicated the role of mast cell heparins in acting as a reservoir for storing different proteases [22, 23]. More recently, RNA-mediated interference and deletion mutagenesis of a homolog of chondroitin synthase in *Caenorhabditis elegans* (involved in the biosynthesis of chondroitin sulfate) was shown to cause defects in early embryogenesis [3]. The phenotypic linkage between HSGAGs and growth factors was also identified by null mutation of the EXT1 gene in mice, which affected the morphogenesis and midline axon guidance during development of the brain [24].

In addition to looking at enzymes involved in GAG biosynthesis, extracellular GAG degrading enzymes have also been the focus of many studies. Through
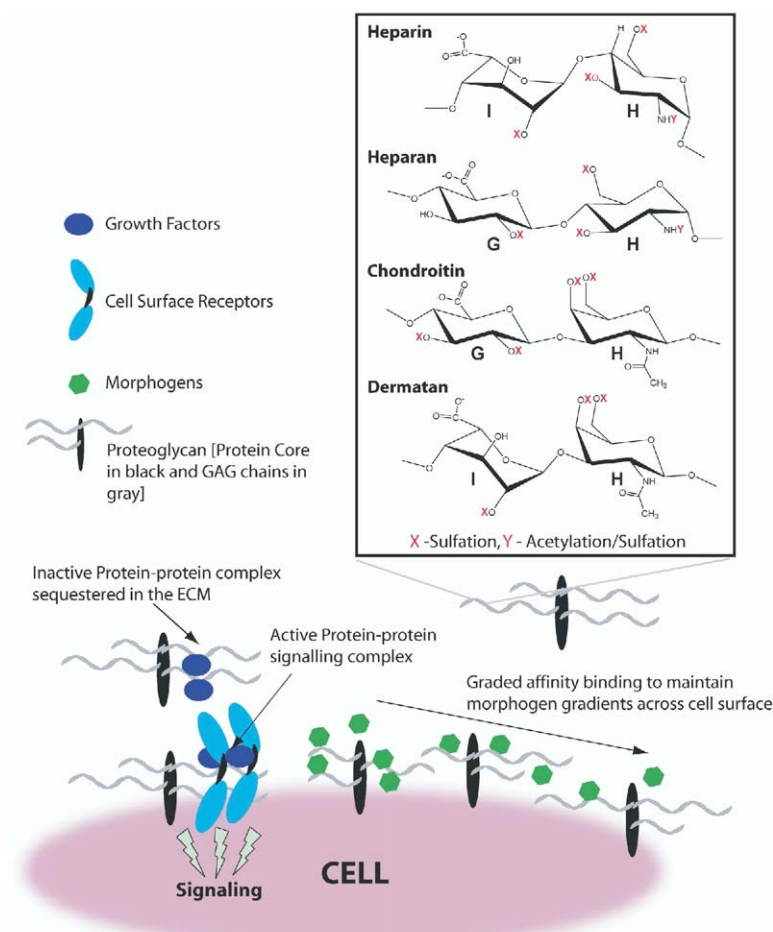
Figure 1. Structure and Biological Roles of GAGs

GAGs present as a part of proteoglycans on the cell surface and ECM, bind to numerous proteins and modulate their function. Shown in the box are the commonly occurring disaccharide repeat units of heparin, heparan, chondroitin, and dermatan sulfate. The monosaccharides are represented based on Table 1 and the hydrogen atoms are not shown for clarity. Regions of possible sulfation and acetylation modifications are indicated with a red X and Y.

cloning and characterization of the human heparanase enzyme (depolymerizes the cell surface HSGAGs), which is secreted by tumor cells during metastasis, these polysaccharides have been shown to be important barriers to tumor metastasis [25, 26]. Recently, a novel extracellular sulfatase enzyme was discovered and was shown to play a key role in embryogenesis through the Wnt signaling pathways [27, 28]. This was the first time that an extracellular enzyme *Qsulf1* (homologous with the lysosomal N-acetyl 6-O desulfatase) was shown to be involved in governing the function of HSGAGs in cell signaling.

Another approach to studying the biological roles of GAGs has been to utilize novel constructs of proteins for direct in situ monitoring of GAG-protein interactions. Using such a construct of FGF receptors with alkaline phosphatase and a ligand and carbohydrate engagement assay (LACE), the regulation of FGF signaling during mouse development by spatial and temporal expression of HSGAGs was investigated [29]. Enzymes that depolymerize GAGs have also been used in in vivo animal studies. By using heparinase I and heparinase III to depolymerize cell surface HSGAGs in a mouse model for cancer, it was shown that distinct HSGAG fragments either promoted or inhibited tumor growth and metastasis [7]. Chondroitinase ABC, which de-

polymerizes CSGAGs, has been shown to promote functional recovery after glial scar injury in adult rats [1].

The studies summarized above provide direct evidence of the biological roles of GAGs. However, it is important to understand these roles from the standpoint of structure-function relationships of GAG-protein interactions. Delineating the physiological context of GAG-protein interactions to truly define structure-function relationships in vivo is a challenging task. Nevertheless, there have been several approaches to both qualitatively and quantitatively investigate in vitro GAG-protein interactions, as summarized in a recent review [30]. The list of proteins that are known to interact with GAGs (Table 2) has been outlined in recent reviews [12, 15, 17, 31] and this list is constantly expanding. In the following section, the GAG sequence specificity of these interactions is discussed using prototypic examples of the best characterized GAG-protein systems.

Biochemical Characterization of GAG-Protein Interactions
Growth Factors
Fibroblast Growth Factor. The FGF family of proteins contains 23 different members and is primarily involved in cell signaling during growth and development. The most studied GAG-protein interactions are that of HSGAGs

Table 2. GAG-Protein Interactions

Protein-GAG	GAG Oligosaccharide	Biological Roles
FGF-1-HSGAG	$-[I_{2S}-H_{NS,6S}]_n-$ n>2 for binding > 5 for FGF-mediated cell signaling	FGF-oligomerization, assembling FGF-FGFR complexes leading to receptor oligomerization and cell signalling. Cell growth and development, angiogenesis.
FGF-2-HSGAG	$-[I_{2S}-H_{NS,6x}]_n-$ n>2 for binding > 5 for FGF-mediated cell signaling. Sulfation at 6-O position is not required for binding but may be required for cell signaling	
HGF/SF-DS	$I-H_{NAc,4S}-I-H_{NAc,4S}-I-H_{NAc,4S}-I-H_{NAc,4S}$	Hepatocyte regeneration, morphogenesis, cell motility, tumorigenesis and metastasis
Midkine, Pleotrophin-CS	$-[G-H_{NAc,4S,6S}]_n-$ or $-[G_{2S}-H_{NAc,6S}]_n-$	Neuronal adhesion, migration and neurite outgrowth
Other growth factors/ morphogens: FGFs (1–21), TGFβ, VEGF, PDGF, EGF Amphiregulin, Betacellulin, Neuregulin, IGF II Activin, Sonic Hedgehog, Sprouty peptides, Wnts (1-13), BMP-2, 4		Cell growth, morphogenesis and development
AT-III-heparin	$-[H_{NAc,6S}-G-H_{NS,3S,6S}-I_{2S}-H_{NS,6S}]-$	Anticoagulation and antithrombosis
Annexin V-heparin	$-[I_{2S}-H_{NS,6S}]_n-$	Antithrombosis
HCF II-DS	$I_{2S}-H_{NAc,4S}-I_{2S}-H_{NAc,4S}-I_{2S}-H_{NAc,4S}$	Anticoagulation and antithrombosis
Other coagulation factors: Factor Xa, Thrombin, Thrombomodulin		Anticoagulation and antithrombosis
HSV-1-heparin	$\Delta U-H_{NS}-I_{2S}-H_{NAc}-I_{2S}(orG_{2S})-H_{NS}-I_{2S}-H_{NH2,3S,6S}$	Microbial pathogenesis
FMDV-heparin	$-[I_{2S}-H_{NS,6S}]_n-$	Microbial pathogenesis
VCP-heparin	$-[I_{2S}-H_{NS,6S}]_n-$	Microbial pathogenesis
Cytokines/Adhesion: Interleukins, GM-CSF Interferonγ, TNF-α, Angiostatin, Endostatin L-selectin, N-CAM MAC-1, PECAM-1 CXC, Bac-5,7, PR-39		Inflammation/immune response

The GAG oligosaccharide sequences are shown for the prototypic examples of GAG-protein interactions.

with basic FGF (FGF-2) and acidic FGF (FGF-1). FGF-2 is implicated in development and differentiation of several tumors where it has been proposed to be a part of an autocrine loop or to induce tumor angiogenesis [6, 19]. FGF signaling involves the following steps—FGF binding to its cell surface tyrosine kinase receptors (FGFRs), oligomerization of FGF leading to receptor oligomerization, phosphorylation of other signaling molecules, and initiation of signaling cascade. HSGAGs play a critical role in FGF signaling by facilitating the formation of FGF-FGFR complexes (and/or stabilizing these complexes) and enhancing (and/or stabilizing) FGF oligomerization. Several crystal structures and cocrystal structures of different FGFs with HSGAG oligosaccharides and FGFRs have been solved [32–35]. These crystal structures indicate that FGF-1 and FGF-2 have different modes of oligomerization facilitated by binding to HSGAG oligosaccharides. A single HSGAG oligosaccharide bridges two FGF-1-FGFR complexes (2:2:1 stoichiometry) with no protein-protein contact between FGF-1 monomers. On the other hand, two HSGAG oligosaccharide chains bridge two FGF-2-FGFR complexes (2:2:2 stoichiometry) once again with no protein-protein contact between FGF-2 monomers. Also, using structural analysis of the FGF-2 crystals structures and protein engineering methods a novel dimeric FGF-2 was constructed and shown to have significantly enhanced HSGAG-mediated signaling [36]. It was also implicated that there was protein-protein contact in this engineered FGF-2 dimer that binds to HSGAG oligosaccharides, which was in contrast with the dimerization observed in the cocrystal structure [36]. These studies demonstrate the complexity of the actual molecular mechanism involved in HSGAG-mediated FGF oligomerization and signaling.

The heparin-derived oligosaccharides in the cocrystal structures contain predominant repeat unit of $-[I_{2S}-H_{NS,6S}]_n-$ where n = 2, 3, 4, 5, 6. The 2-O sulfate of the iduronate and the N-sulfate of the glucosamine are critical modifications that are required for both FGF-1 and FGF-2 signaling [37]. The 6-O sulfation has been shown to be critical for FGF-1 signaling but is not completely necessary for FGF-2 signaling. Typically, tetra- and hexasaccharides are sufficient to bind with high affinity to FGF-1 or FGF-2. However, octasaccharides or longer are required for bridging a dimeric FGF-2 along with the receptor to form a ternary signaling complex [34, 35].

Other GAG-Growth Factor Interactions. The biological functions modulated by the interactions of CS and DS with growth factors and cytokines have been summarized in recent reviews [12, 17]. Similar to HSGAGs, highly sulfated DS chains have been shown to bind FGF-2 and FGF-7 (with lower affinity compared to HSGAGs) and implicated in cell growth and wound repair. DS has also been shown to bind and activate Hepatocyte growth factor (HGF) signaling pathway through c-met receptor (protooncogene). Comparison of HSGAG and DS interactions with two truncated C-terminal variants (NK1 and NK2) of HGF revealed that the minimal length that binds with high affinity is a tetrasaccharide for HSGAGs and hexasaccharide for DS [38]. Oversulfated CS with specific sulfation patterns in the brain CS PGs have been implicated in neuronal adhesion, migration, and neuritogenesis via their

interactions with pleotrophin and midkine. Similar to the growth factor model, assembling of the pleotrophin complexes on cell surface by CS have been implicated in signaling, while CS on extracellular environment have been implicated in sequestering this cytokine.

Inhibitors of Coagulation Cascade

The coagulation cascade is controlled by a group of serine protease inhibitors including AT-III, heparin cofactor II, tissue factor pathway inhibitor, and other factors. AT-III is the most prominent because all of the coagulation proteases (except factor VIIa) are inhibited by AT-III, which forms equimolar covalent complexes with these enzymes.

Heparin-AT-III. Heparin forms a ternary complex with thrombin and AT-III, accelerating by 2000-fold the rate of thrombin inhibition by AT-III, thereby preventing coagulation [39, 40]. AT-III recognizes a specific pentasaccharide sequence motif [$H_{NAc,6S}$-G-$H_{NS,3S,6S}$-I_{2S}-$H_{NS,6S}$] within heparin. This pentasaccharide motif, which makes up for only 3% of all the chains in commercially prepared heparin, binds to AT-III with a $K_d \approx 0.6$ nM [39, 40]. The X-ray cocrystal structure of a synthetic mimic of this pentasaccharide motif with AT-III [39] indicates that the 3-O sulfate group on the glucosamine [$H_{NS,3S,6S}$] makes many ionic contacts with the protein. This 3-O sulfation is absolutely necessary for specific interaction between the pentasaccharide motif and AT-III. This cocrystal structure also implicated that pentasaccharide binding and subsequent conformational change activates AT-III for rapid inhibition of Factor Xa.

However, chemical synthesis of heparin mimetics have implicated that AT-III inhibition of thrombin requires a much longer heparin chain of at least 16–18 monosaccharides, in addition to requiring the presence of the pentasaccharide sequence [41, 42]. More recently, the cocrystal structure of ternary complex of AT-III-thrombin-heparin mimetic was solved [43]. This crystal structure demonstrated the bridging of AT-III and thrombin by a 16-mer synthetic mimic of a heparin containing a highly sulfated poly β-D-glucose tetrasaccharide at the nonreducing end followed by a stretch of neutral poly β-D-glucose heptasaccharide and the pentasaccharide motif at the reducing end. Thus, it is apparent that sequence and chain length is important for anticoagulant activity of heparin. In a recent study, the chain length was also shown to be an important determinant for heparin-mediated inhibition of thrombin by binding to heparin cofactor II. It was implicated that heparin binding to heparin cofactor II required a minimal chain length of 13 monosaccharides and the complete allosteric inhibition of thombin (2000-fold higher than without heparin) required a chain length of 26 monosaccharides [44].

Heparin-Annexin V. Exposure of phosphatidylserine (PS) on the outer membranes of activated cells is a physiological signal for the onset of processes such as coagulation and apoptosis. Extracellular annexin V (a Ca^{2+}-dependent membrane phospholipid binding protein) recognizes this signal, binds to PS components of the membrane, and self-assembles laterally into an organized array. This annexin array forms a shield that prevents excessive clot formation. A recent X-ray cocrystal structure of annexin V with a heparin-derived tetrasaccharide ΔU_{2S}-$H_{NS,6S}$-I_{2S}-$H_{NS,6S}$ suggested that Ca^{2+}-mediated annexin V-heparin interactions via these sequence motifs may facilitate the annexin self-assembly process and/or stabilize the oligomeric annexin layer [31].

DS-Heparin Cofactor II. A specific dermatan sulfate hexasaccharide [I_{2S}-$H_{NAc,4S}$]$_3$ has been shown to bind with high affinity to heparin cofactor II and inhibit thrombin [45]. This hexasaccharide has been reported to constitute only 2% of hexasaccharides in dermatan sulfate [45]—another example of a rare modification having a key functional role. It has been proposed that unlike heparin that inhibits thrombin by bridging it with heparin cofactor II, dermatan sulfate inhibits the thrombin-fibrin complex via heparin cofactor II [46].

Microbial Pathogens

A growing number of pathogens, including bacteria, viruses, and parasites, have been shown to utilize cell surface GAGs as receptors to target their host cells. It was shown that a heparan sulfate octasaccharide ΔU-H_{NS}-I_{2S}-H_{NAc}-I_{2S}[or G_{2S}]-H_{NS}-I_{2S}-$H_{NH2,3S,6S}$ containing a rare 3-O sulfated free amine bound to the gD glycoproteins of herpes simplex virus [13]. Another well-characterized HSGAG-virus interaction is that of a HSGAG oligosaccharide involved in binding to the virus shell protein of the foot and mouth disease virus (FMDV). In fact, the cocrystal structure of this oligosaccharide with the FMDV shell protein has been solved [11]. More recently, the cocrystal structure of the vaccinia virus complement control protein (VCP) with a heparin-derived decasaccharide [I_{2S}-$H_{NS,6S}$]$_5$ provided valuable insights into the regulation of the complement pathway by pathogens interacting with cell surface polysaccharides on host cells [10]. The adhesion of erythrocytes infected by the malarial parasite to placenta requires a low sulfated CS (G-$H_{NAc,4S}$ repeat unit) with a minimum length of a dodecasaccharide [17].

Although hyaluronic acid (HA) is the simplest of GAG families in terms of chain modification, it has been shown to bind to many proteins at the cell-ECM interface as well as in the pericellular environment [47]. HA binding proteins are known as hyaladherins, and the best-studied HA-protein interaction is that with CD44, which plays a key role in cancer [48, 49].

The above examples summarize some important aspects of the biological role of GAG-protein interactions in terms of specific GAG sequences. The oligosaccharide motif that is recognized by a protein is often found in low abundance. This strengthens the idea of specificity in GAG-protein interactions given the diversity of GAG sequences. Most of these interactions involve formation of multimeric complexes (self-oligomers, protein-receptor, enzyme-inhibitor, etc.) that are facilitated by GAGs. Thus in addition to just looking at the abundance of the oligosaccharide motif that binds to a specific protein, it is important to determine the position of this motif in the context of other protein binding motifs in a given GAG chain length. Given that different classes of GAGs can interact with the same protein, albeit with different affinities, there can be compensating effects. For example, the increased abundance of FGF binding DS GAGs can achieve the same effect of FGF binding HSGAGs in lower abundance. On the other hand, it is also possible that the difference in affinities between different classes of GAGs for a given protein plays an
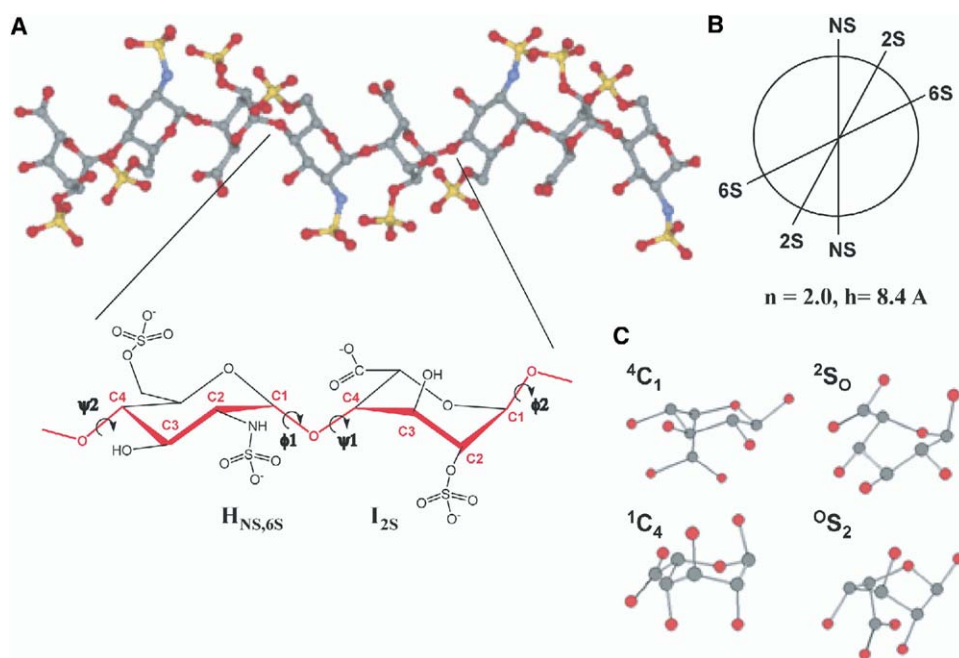
Figure 2. Three-Dimensional Structure of GAGs

Shown in (A) is a ball and stick representation of a heparin oligosaccharide with –[I_{2S}-$H_{NS,6S}$]– repeat unit (carbon, oxygen, nitrogen, and sulfur atoms colored gray, red, blue, and yellow, respectively). Also shown is the chair conformation of the expanded disaccharide unit where the backbone atoms (colored red) are labeled along with the glycosidic torsion angles (ϕ1, ψ1) and (ϕ2, ψ2). Shown in (B) is a helical wheel projection (i.e., viewing along the line of helical axis perpendicular to the plane of the paper) of the sulfate groups of the structure shown in (A). Note that the heparin structure adopts a uniform 2-fold helical symmetry with n = 2.0 and h = 8.4 Å. (C) Commonly observed low-energy ring conformations of iduronic acid (atoms colored the same way as in [A]).

important role in maintaining gradient in protein concentration.

Structural Specificity of Protein-GAG Interactions

The specificity of GAG-protein interactions is governed by the ionic interactions of the sulfate and carboxylate groups of GAGs with the basic amino acids on the protein as well as the optimal structural fit of a GAG chain into the binding site of the protein. The topology and distribution of the basic amino acids of the GAG binding site on the protein influences its specificity in molecular recognition of GAG sequences. The binding affinity of the interaction depends on the ability of the oligosaccharide sequence to provide optimal charge (orientation of sulfate groups) and surface (van der Waals contact) complimentarity with the protein, which is governed by the three-dimensional structure and conformation of GAGs.

GAG Conformation

X-ray fiber diffraction analyses of the different GAGs [50] and NMR studies [51] have indicated that the GAG backbone (Figure 2) adopts uniform helical structures where helical parameters *n* (the number of disaccharide units per turn of the helix) and *h* (the axial rise per disaccharide) depend on the nature of the cationic counter ions. For example, a heparin oligosaccharide containing the predominant –[I_{2S}-$H_{NS,6S}$]– repeat unit would adopt a 2-fold helical structure with n = 2 and h = 8.4 Å (Figure 2). The main parameters that deter-

mine the topology of the GAG chain are the glycosidic torsion angles (ϕ1, ψ1) and (ϕ2, ψ2) and the ring conformation of the monosaccharides. The hexosamine and GlcA pyranose puckers in GAGs adopt a $^4C_1$ chair conformation. On the other hand, the IdoA pyranose ring has the ability to adopt multiple low-energy conformations such as $^1C_4$, $^4C_1$, ^2S_O, and $^OS_2$ [52] (Figure 2).

Structural Implications of GAG-Protein Interactions

Several cocrystal structures of HSGAG oligosaccharides with different proteins have been solved. These structures have highlighted the ionic interactions between specific sulfate groups and carboxylate groups of HSGAGs with the basic amino acids in the GAG binding site on the protein. However, ionic contacts are not sufficient to explain the optimal structural fit of a GAG oligosaccharide to the binding site of the protein that influences the affinity of the interaction. From the standpoint of GAG conformation, it can be envisioned that protein binding would induce local distortions in an otherwise uniform helical structure of GAGs, which are manifested as changes in the glycosidic torsion angles. These conformational changes would enable an optimal structural fit in terms of both ionic and van der Waals contact between the oligosaccharide motif and the protein.

This structural aspect of GAG-protein interaction was investigated in a recent study, where a systematic analysis of the helical parameters of HSGAGs in all the available protein-HSGAG cocrystal structures revealed local conformational changes causing a kink in the oli-
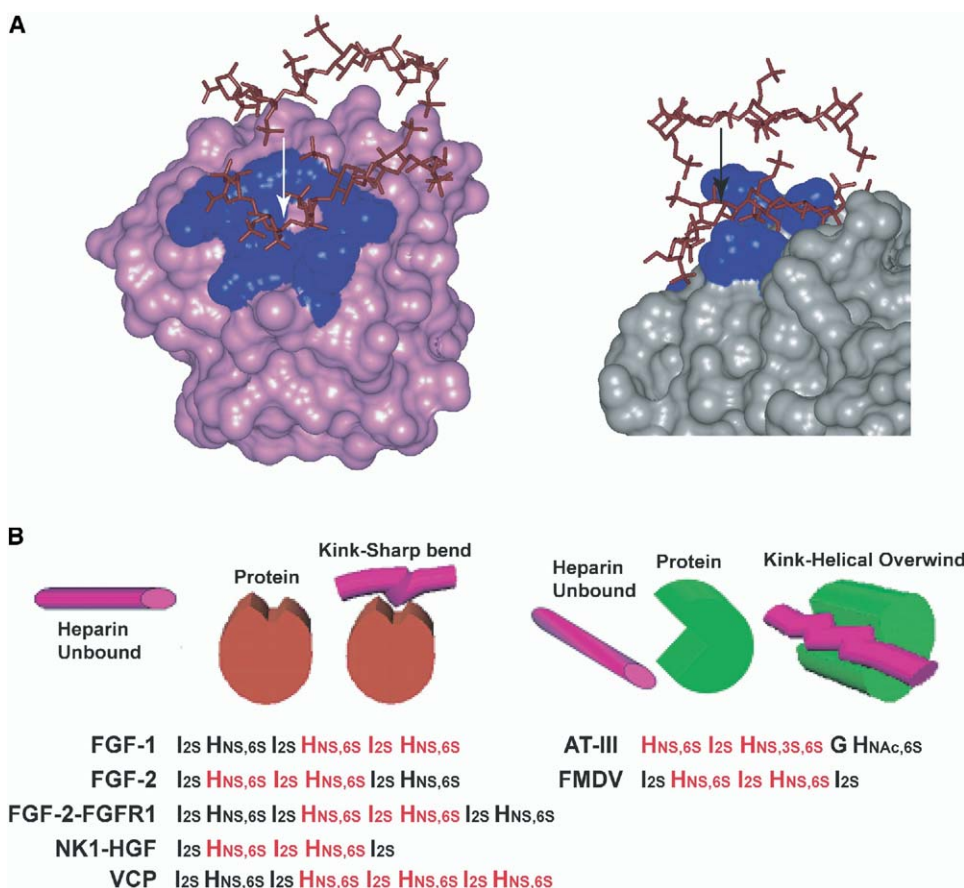
Figure 3. Kink in GAG Helical Structure

Shown in (A) is the grasp rendering of FGF-2 (pink) and AT-III (gray) cocrystal structures with HSGAG oligosaccharides (brown) where the HSGAG binding site on the protein is in blue. Only that part of AT-III close to the pentasaccharide binding site is shown for clarity. Note the kink in the helical axis (indicated by an arrow) from the unbound to bound structure. (B) Schematic of the two kinds of kink, sharp bend (left) and helical overwind (right), observed in the different protein-HSGAG cocrystal structures. The oligosaccharide sequences in the cocrystal structures are indicated under the corresponding schematic where the region of the kink is colored in red.

gosaccharide helical structure that directly interacted with the protein [53]. These deviations were captured by measuring localized helical parameters n_L and h_L obtained by repeating each successive disaccharide unit with the experimentally observed glycosidic torsion angles. Thus any local deviation from the helical structure would be reflected as significant changes in these parameter values when compared to those of a uniform helical structure. The observation of the oligosaccharide kink in the different protein-GAG cocrystal structures is summarized below.

FGF-HSGAG Complexes. In all the cocrystal structures of HSGAG oligosaccharides with FGF-1 and FGF-2 and their receptors [32–35], the kink in the FGF binding oligosaccharide spanned a trisaccharide [$H_{NS,6S}$-I_{2S}-$H_{NS,6S}$] where an iduronate is flanked by two glucosamines (Figure 3) and it contained the critical NS and 6-O sulfate groups on the hexosamine and 2-O sulfate groups on IdoA implicated in high-affinity interaction with FGFs. The sharp curvature of the kink in the oligosaccharide was reflected in the deviation of the h_L value from around 8.4 Å (unbound region) to around 7.6 Å

(kink region), whereas the n_L value was around 2.0 throughout the structure. The formation of the kink was associated with changes in two specific glycosidic torsion angles where change in one caused the helical axis to bend below and change in the other caused the helical axis to bend above the reference axis of the unbound oligosaccharide structure, thus forming a kink. Further, in both FGF-1 and FGF-2 bound oligosaccharides, the ring conformation of the IdoA in the trisaccharide spanning kink was in $^1C_4$. Thus, the axial positioning of the glycosidic bonds due to the $^1C_4$ IdoA conformation enhanced the degree of the kink. Binding of HSGAGs to FGFs does not induce any conformational changes on the protein. In fact, the topology of the HSGAG binding loop regions on FGF-1, -2, -4, -7, and -9 (whose crystal structures have been solved) almost coincide with each other [53]. On the other hand, the distribution of the basic amino acids in the binding loops is different for each of the FGFs [53, 54]. Thus the narrow binding pocket formed by the HSGAG binding loops on each FGF imposes structural constraints on the HSGAG oligosaccharide that induce a kink for

optimal surface contact with the protein. The distribution of the basic residues within the HSGAG binding loops imposes unique constraints on the orientation and spatial arrangement of the sulfate groups. Together, these structural constraints determine the specificity of oligosaccharide sequence to bind to different FGFs.

AT-III-Pentasaccharide Complex. In contrast with FGF, it has been implicated that binding of the pentasaccharide motif to AT-III induces conformational change that causes widening of the heparin binding grove of AT-III [39]. These conformational changes around the heparin binding grove also lead to allosteric conformational changes leading to the expulsion of the reactive site loop of AT-III (which is distant from the heparin binding site), which enhances the binding of this loop to Factor Xa [39]. The recently solved AT-III-thrombin-synthetic heparin mimetic cocrystal structure demonstrated that bridging of thrombin and AT-III by heparin is relatively insensitive to the expulsion of the reactive site loop, consistent with the earlier studies [43].

The kink in the AT-III bound pentasaccharide motif [$H_{NAc,6S}$-G-$H_{NS,3S,6S}$-I_{2S}-$H_{NS,3S,6S}$] spanned a trisaccharide [$H_{NS,3S,6S}$-I_{2S}-$H_{NS,3S,6S}$] that contains the critical 3-O sulfate group (of nonreducing end glucosamine) required for AT-III binding (Figure 3). Interestingly, the kink in the pentasaccharide is reflected by a transition in n_L value from 2 (unbound region) to 3 (bound region). This transition in the n_L value causes helical *overwind* compared to the *sharp bend* in the FGF-bound oligosaccharide. Thus in the case of AT-III-pentasaccharide complex, the optimal ionic and *van der Waals* contact with the protein requires the overwinding of the helix of the pentasaccharide, resulting in a kink. Chemical synthesis of heparin mimics containing IdoA conformation locked to either $^1C_4$ or ^2S_O form and NMR studies have shown that ^2S_O conformation of the IdoA is important for high- affinity binding to AT-III. Also NMR analysis of the free pentasaccharide in solution indicates that conformational equilibrium of the IdoA is shifted toward $^1C_4$. Thus the transition of the IdoA conformation to ^2S_O in the bound pentasaccharide enhances the helical overwind to provide optimal interactions with AT-III.

Other Protein-HSGAG Structural Complexes. In the other protein-HSGAG cocrystal structures, for example FMDV [11], annexin V [55], NK1 domain of HGF [56], and the most recently solved vaccinia virus complement protein (VCP) [10], the heparin-derived oligosaccharides predominantly contained the trisulfated disaccharide repeat unit –[I_{2S}-$H_{NS,6S}$]–. The oligosaccharide kink in the FMDV cocrystal structure was similar to that of the AT-III-bound pentasaccharide (Figure 3), in that there was a helical overwind, the n_L value going from 2 (unbound) to 3 (protein bound). This region spans three monosaccharides [$H_{NS,6S}$-I_{2S}-$H_{NS,6S}$] with the iduronate in the $^2S_0$ conformation. On the other hand, the oligosaccharide kink in the NK1 domain cocrystal structure was similar to that of the FGF-1 and FGF-2 bound oligosaccharides (Figure 3). Since annexin V was cocrystallized with only tetrasaccharides it was difficult to make a meaningful interpretation of the n_L and h_L values for quantifying the deviations from the overall uniform helical structure.

In the case of the VCP cocrystal structure, the kink spans a hexasaccharide showing two distinct transitions in h_L values (n_L was around 2), one from 8.0 to 7.3 and the other from 7.3 to 6.3. There were two structural VCP-oligosaccharide complexes in the asymmetric unit related by an approximate 2-fold symmetry. The region on the oligosaccharide that showed a transition in h_L from 8.0 to 7.3 interacted with the other VCP molecule in the asymmetric unit and the sharper bend (h_L transition from 7.3 to 6.3) interacted with the VCP in the structural complex. All the IdoAs in the region spanning the kink was observed to adopt the ^2S_O conformation. The cocrystal structure studies also indicated that there is a conformational change manifested as a hinge movement in the protein that is stabilized by heparin binding. The hinge movement exposes the binding site of VCP for proteins in the complement pathway and enhances the slowing down of the complement pathway activation.

Chondroitinase B-Dermatan Sulfate Complex. More recently, the oligosaccharide kink was also implicated in an enzyme-substrate complex, mainly chondroitinase B, and its substrate dermatan sulfate. Chondroitinase B cleaves DS oligosaccharides at the [-$H_{NAc,4S}$–I-$H_{NAc,4S}$–I-$H_{NAc,4S}$-] linkages (cleavage sites marked with "–") via β elimination to give ΔU-$H_{NAc,4S}$ containing reaction products where ΔU is a uronic acid with $\Delta 4,5$ unsaturated linkage. The cocrystal structure of chondroitinase B with three disaccharide reaction products of DS depolymerization was solved recently [57] and two of the products were in the catalytic site of cleavage. In addition, the cocrystal structure also contained a Ca^{2+} ion that was coordinated by the oxygen atoms on the enzyme as well as the carboxylate of the ΔU of the reaction product in the catalytic site. Based on the interactions between the active site amino acids and the Ca^{2+} coordination, the conformation of the intact substrate in the active site was modeled based on the two disaccharide reaction products. Analysis of the final substrate conformation indicated that there was almost a perpendicular local bending of the DS helical structure to position the substrate in an optimal fashion for catalysis. In fact, the cocrystal structures of chondroitinase B with GlcA containing CS oligosaccharides [57] indicated that the substrate did not occupy the catalytic site completely. This was due to the inability of the rigid chair conformation of GlcA to accommodate the bending of the oligosaccharide. Thus the topology and distribution of basic amino acids of the chondroitinase B active site and the Ca^{2+} ion coordination imposes unique structural constraints in terms of sulfation pattern and conformation of the substrate that was satisfied only by an IdoA containing DS substrate.

NMR studies have implicated that the overall conformation of HA in solution is flexible and this flexibility of HA is implicated to assemble different hyaladhereins where each protein captures distinct conformations of the different oligosaccharide binding motifs in HA [47, 58]. However, there are no cocrystal structures of HA binding proteins complexed with HA oligosaccharides. Thus availability of these crystal structures would provide better structural insights into the specificity of HA-protein interactions.

From the examples discussed above, it is becoming increasingly evident that the propensity of GAG oligo-

saccharides to form distinct *kinks* in their otherwise uniform helical conformation provides a *structural signature* or *molecular recognition motif* for sequence specific protein binding. This kink in the GAG oligosaccharide is reminiscent of DNA kink upon binding to proteins (such as transcription factors, restriction endonucleases, etc.), a hallmark for regulating the specificity of DNA-protein interactions. Protein binding induces distinct conformational changes to the sugar phosphate backbone of DNA leading to the kink formation. Although more restrictive compared to the multiple ring conformations sampled by IdoA in GAGs, the ribose sugar pucker can also adopt different ring conformations such as 2′ endo and 3′ endo.

Based on the examples discussed above, one would imagine that the conformationally flexible IdoA is necessary for formation of the kink. However, it should be noted that the kink in helical axis can be induced by changes in glycosidic torsion angles of GlcA containing GAG backbone in the case of heparan and chondroitin sulfate. Nevertheless, the IdoA in HSGAGs and dermatan sulfate would provide additional conformational flexibility to the GAG backbone and thus influence the degree of the kink. It is important to note that the motif that spans the kink is a high-energy structure since it is a sharp bend or overwind of the otherwise low-energy helical structure. Thus, from an energetics standpoint, unbound GAG oligosaccharides would not favor the formation of kink. The internal steric energy of the kink spanning motif is compensated by the favorable van der Waals and ionic contact energy with the binding site of the protein [53]. On the protein side, the topology of the binding site and distribution of basic residues in the binding site provide structural constraints on the GAG oligosaccharide.

The structural signature based on the kink provides a framework for understanding the specificity of GAG-protein interactions in terms of GAG sequence and how that impinges on the function of the protein. For instance, the kink in the FGF bound HSGAG oligosaccharides provided a framework to interpret the graded affinities of a library of heparan sulfate-derived oligosaccharides to FGF-1 and FGF-2 published in a recent study [59]. The propensity of the oligosaccharides to form the trisaccharide spanning kink [$H_{NS,6S}$-I_{2S}-$H_{NS,6S}$] correlated well with their low-, moderate-, and high-affinity binding to FGF-1 (Figure 4). Thus by determining the frequency of trisaccharide sequence motifs that can potentially form the kink, it is possible to qualitatively assign the affinity of any oligosaccharide to FGF-1 and FGF-2. The kink in oligosaccharide chains has also been observed in other polysaccharides. The cocrystal structure study of pectate lyase with its pectate substrate indicates a local deviation in the helical symmetry of the substrate from 3_1 to 2_1 at the catalytic site of the enzyme [60].

Intriguing and Challenging Aspects of GAG Structure-Function Relationships

The examples discussed above show that understanding the relationship between GAG sequence, its specific interaction with proteins, and how that modulates protein function is both a fascinating as well as challenging area of research.
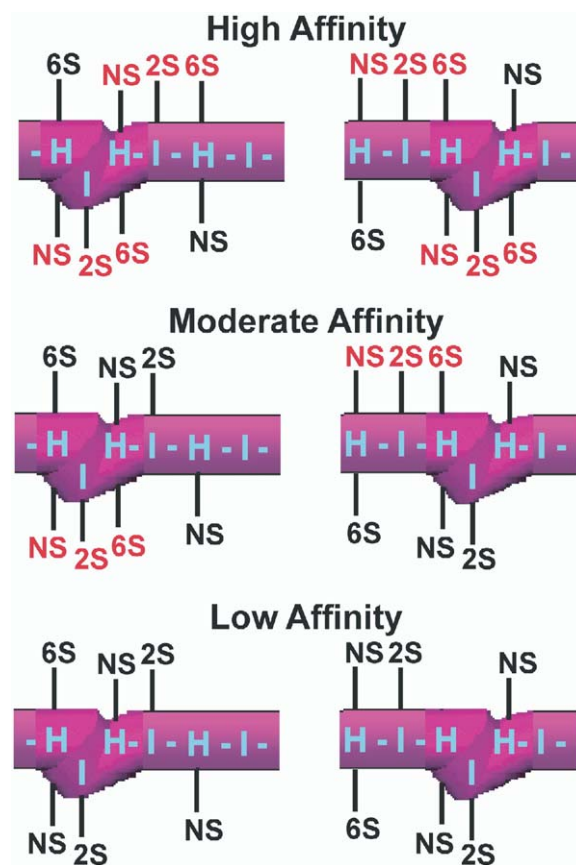

Figure 4. Affinity to FGF Reflected on Propensity of Kink Formation

Shown from top to bottom are schematic representations of heparin oligosaccharide sequences with high to moderate to low affinity for FGF-1 [43] where the kink is represented as a *bulge*. For each oligosaccharide, there are two possible orientations (shown on left and right) of the sulfate groups relative to the helical axis (up or down) that account for the 2-fold helical symmetry of heparin. The critical sulfate groups interacting with FGF are shown in red.

Unlike DNA, RNA, or proteins, the biosynthesis of complex polysaccharides like GAGs is a nontemplate-driven process involving several enzymes and their tissue-specific isoforms. The complexity of the biosynthetic machinery complicates the understanding of structure-function relationships. Due to the nontemplate-driven biosynthesis, it is difficult to amplify specific GAG oligosaccharides such as those containing AT-III binding pentasaccharide, which are known to play important biological roles. Further, the redundancy of the biosynthetic machinery based on different enzyme isoforms makes it challenging to take a functional genomics approach by knocking in or knocking out specific biosynthetic enzymes in order to understand the effect of a specific sulfation pattern in vivo.

Given that GAGs modulate the biological roles of proteins by facilitating the formation of protein-protein complexes, it is obvious that the relative locations of different molecular recognition motifs in the context of overall chain length of the GAGs are important determinants of their structure-function relationships. This further adds to the challenges to just isolating and identi-

fying oligosaccharide motifs with high binding affinity to a single protein. As discussed in the examples above, it is evident that even in terms of affinity it is not always the case where only high-affinity GAG-protein interactions produce biologically significant responses. This is particularly applicable to growth and developmental processes where a gradient of protein concentration is necessary for the required biological outcome. Taken together, it is clear that by contrasting the role of GAG-protein interactions with other biomolecular interactions such as DNA-protein and protein-protein interactions, the former provides an "analog" means to modulate protein function while latter provides a "digital" means to completely switch on or switch off the activity of the protein. Thus, understanding the structure-function relationships of GAGs could provide a framework for developing novel approaches for therapeutic intervention in diseases such as cancer, cardiovascular, and those related to microbial pathogenesis.

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