The sweet spot: how GAGs help chemokines guide migrating cells

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ABSTRACT
Glycosaminoglycans are polysaccharides that occur both at the cell surface and within extracellular matrices. Through their ability to bind to a large array of proteins, almost 500 of which have been identified to date, including most chemokines, these molecules regulate key biologic processes at the cell–tissue interface. To do so, glycosaminoglycans can provide scaffolds to ensure that proteins mediating specific functions will be presented at the correct site and time and can also directly contribute to biologic activities or signaling processes. The binding of chemokines to glycosaminoglycans, which, at the biochemical level, has been mostly studied using heparin, has traditionally been thought of as a mechanism for maintaining haptotactic gradients within tissues along which cells can migrate directionally. Many aspects of chemokine-glycosaminoglycan interactions, however, also suggest that the formation of these complexes could serve additional purposes that go well beyond a simple immobilization process. In addition, progress in glyobiology has revealed that glycosaminoglycan structures, in term of length, sulfation, and epimerization pattern, are specific for cell, tissue, and developmental stage. Glycosaminoglycan regulation and glycosaminoglycan diversity, which cannot be replicated using heparin, thus suggests that these molecules may fine-tune the immune response by selectively recruiting specific chemokines to cell surfaces. In this context, the aim of the present text is to review the chemokine–glycosaminoglycan complexes described to date and provide a critical analysis of the tools, molecules, and strategies that can be used to structurally and functionally investigate the formation of these complexes.


Introduction
A cell’s ability to communicate with neighboring or distant cells is essential for most biologic processes. Soluble factors, which diffuse into the extracellular medium and bind to cell surface receptors to trigger specific biologic responses, have a key role in this process [1]. Most of these extracellular “messengers,” which include hundreds of chemokines, growth factors, cytokines, and morphogens, are endowed with pleiotropic and overlapping activities and are usually effective at very low concentrations. Their instructive potentials, however, are highly contextual and strictly regulated both spatially and temporally [2]. Such control recently appeared to be largely operated by a class of extraordinarily complex macromolecules known as PGs, which during the past 20 y have been found to be key regulators of most physiologic processes and their pathologic disorders [3]. PGs, as their names indicate, comprise a core protein to which glycan chains of the GaG family are covalently bound [4]. These molecules are widely expressed throughout the body and are the most abundant components of the pericellular zone where they are present, both in the extracellular matrix (through which soluble effectors diffuse to reach their target cells) and associated with the cell membrane (thus in close proximity to signaling receptors). As such, they are strategically positioned to interfere with any biologic processes occurring at the cell–tissue interface. PGs mainly exert their large functional repertoire through their GaG chains, HS in particular, which bind to most of the above-mentioned signaling proteins, thereby modifying their structure, stability, or local concentration or acting as a template for the assembly of supramolecular complexes [5–7].

A great deal of information regarding the regulatory action of HS has been obtained through the study of FGFs, a family of 23 proteins that induce cell proliferation and differentiation [8]. FGFs carry out their functions by binding to FGF tyrosine kinase receptors in a strictly HS-dependent manner, in which the polysaccharide bridges FGF and FGF tyrosine kinase receptor, thereby facilitating the formation of a ternary signaling complex. The observation that cell-surface HS can be replaced by soluble HS-like oligosaccharides when the cells are stripped of their own GaG chains in bioassays has

Abbreviations: 3D = 3-dimensional, CKR = chemokine receptor, CS = chondroitin sulfate, CSP = chemical shift perturbation, DPP-IV = dipeptidyl peptidase-4, DS = dermatan sulfate, FGF = fibroblast growth factor, GaG = glycosaminoglycan, GlcN = glucosamine, GlcA = glucuronic acid, HA = hyaluronic acid, HS = heparan sulfate, IdoA = iduronic acid, MNP =

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offered an easy way to investigate the HS characteristics required to induce FGF-mediated activity, and a tremendous amount of data has been acquired on that particular system [9, 10].

Chemokines are another large family of important signaling proteins, comprising some 50 members, which mostly control the migratory patterns and the positioning of immune and non-immune cells through interaction with 7-transmembrane–spanning receptors coupled to G-proteins, 20 of which have been identified to date [11, 12]. Similar to FGFs, most chemokines bind to HS [13], but, in contrast to FGFs, GAGs do not generally appear to assist chemokines in receptor recognition, and chemokines can signal either free or HS bound cells, at least in vitro. Chemokine functions have thus mostly been investigated in the absence of GAGs, although mounting evidence is showing that, in vivo, this interaction is critical for chemokine activities [14–18]. This interaction has been proposed to control the diffusion potential of chemokines through the extracellular matrix and to determine local concentrations, potentially through modulation of monomer–dimer equilibriums, thus providing positional information within tissues in such forms as chemotactic gradients along which cells can migrate directionally [19].

Many aspects of chemokine–GAG interactions also suggest that the formation of these complexes could serve additional purposes that go well beyond a simple immobilization process. However, because of the complexity of the experimental setup required for in vivo investigations and the difficulty in characterizing GAGs, the structure–function relationships of this system have remained difficult to establish. In addition, many studies have been based on heparin, used as a surrogate for HS. This particular GAG, although chemically related to HS, is nevertheless highly distinct from HS in its structural organization [20, 21], diversity, and tissue and cellular distribution [22], and it does not permit the specificity and the physiology of the interaction to be addressed.

In this context, the aim of this review is to describe the different mechanisms by which GAGs could interfere with chemokine activity, highlight salient features of HS structure and its distribution and regulation in chemokine biology, and provide a critical analysis of the tools and strategies that can be used to investigate the formation of chemokine–GAG complexes.

CHEMOKINES HAVE 2 LIGANDS: CKRs AND GAGs

The chemokine–CKR interaction: a common model of signaling complexes

Chemokines comprise a family of approximately 50 structurally related chemotactic cytokines of 8–12 kDa in molecular mass. These molecules are key mediators of immune cell migration and also control the activation, differentiation, and survival of leukocytes. As such, they regulate many critical processes, including immune cell development, differentiation, and homeostasis, routine immune surveillance, the recruitment of cells into injured tissues, and inflammation [23]. Functionally, this group of cytokines can be categorized as homeostatic chemokines, which are those constitutively expressed and involved in homeostatic migration and cellular homing or as inflammatory chemokines, which are those up-regulated and active in the recruitment of leukocytes toward inflamed areas, with some of these chemokines falling into both categories [24]. Chemokines also promote the oriented migration of non-immune cells and are thus active in many other physiopathologic conditions, including development, tissue remodeling and repair, metastasis, and angiogenesis. Accordingly, CKRs, which belong to the subgroup of rhodopsin-like 7-transmembrane receptors, are differentially expressed by all leukocytes, as well as by many nonhematopoietic cells. CKRs can be either G-protein–coupled receptors, which signal cells through activating G_{i}-type G proteins, causing integrin activation and the polarization of the cytoskeleton, or ACKRs, which appear to be involved in scavenging their ligands in a G-protein–independent manner and could mediate β-arrestin–based responses [23, 25].

From a structural point of view, chemokines share a remarkably conserved tertiary structure, consisting of a more or less disordered N-terminal domain of 6–10 residues, followed by a long loop, a 3_{10} helix, and a triple-stranded antiparallel β-sheet overlaid by a C-terminal α-helix. According to the structure of a conserved cysteine-containing motif in the amino-terminal region of the molecule, they are classified as CC, CXC, CX3C, or C chemokines.

With 50 chemokines and 20 receptors, this system signals cells through various combinations, including homodimerization and heterodimerization of the ligand–receptor pairs [26], presumably considerably enhancing the network of interactions and their biologic outputs. Despite this large diversity, at the biochemical level, the interactions between chemokines and CKRs seem to be conserved in all chemokines, irrespective of the subgroup to which they belong or the type of cell they activate. Early studies on CXCL8 [27] and CXCL12 [28] demonstrated that chemokine binding to, and activation of, their corresponding receptors occur independently and rely on 2 distinct domains. According to that model, which has been generalized to most of the chemokine–CKR pairs studied to date, the N-terminal tail of the receptor (approximately 30–40 more or less unfolded residues) first interacts with amino acids from the core domain of the chemokine, facilitating a rapid anchoring on the extracellular side of the CKR. Subsequently, the N-terminal peptide of the chemokine binds to a cavity buried within the transmembrane helices of the receptor and triggers the conformational changes required to induce G-protein signaling, as shown with numerous mutagenesis studies and confirmed with molecular modeling [29] and structural analyses conducted using N-terminal fragments [30–32] or full-length [33, 34] receptors. One should note at this stage that the N-terminal domain of many G-protein–coupled receptors, including CKRs, are subjected to posttranslational modifications resulting in tyrosine sulfation [35]. Similar to GAGs, this region of CKRs thus displays clusters of sulfate groups (see below).

matrix-metalloproteinase, NDST = N-desacetylase/N-sulfotransferase, NMR = nuclear magnetic resonance, OST = O-sulfotransferase, PG = proteoglycan, SPR = surface plasmon resonance
GAG-binding sites of chemokines: a variety of complexes and binding modes

If the general organization of chemokine–CKR complexes is similar, chemokines can associate with GAGs using a large variety of different configurations and binding regions, giving rise to different modes of association. In proteins, the characterization of HS binding sites remains a complex issue. HSs have the highest charge density of any known biologic macromolecules. Given their high content of negative charges (sulfate and carboxyl groups), it is not surprising that protein–HS interactions are significantly (but not exclusively) driven by long-range electrostatic interactions. Consistently, HS-binding proteins feature cationic surfaces, and early studies based on HS-binding sequence comparisons led to the proposal of a number of HS-binding sequences characterized by clusters enriched in Lys, Arg, and occasionally, His [36]. However, this does not exclude the contribution of other forces, such as van der Waals’ interactions and hydrogen bonds [37]. Protein surface analysis and energy calculations applied to various chemokine structures led to the proposal of 4 different binding modes: one for each of the CC and CX3C types of chemokines and 2 different modes within the CXC family, enabling the classification of chemokines according to the HS-binding cluster they display [13]. Here, we provide such an analysis for all human chemokine sequences, based on existing experimental data. For that purpose, chemokine sequences were aligned according to their secondary structures, either extracted from available protein data base files or from 3D models calculated by a Modeler server (https://modbase.compbio.ucsf.edu/modweb/) and grouped by the nature of their cyst motif. Amino acids involved in binding to GAGs, as determined by NMR spectroscopy (underlined residues) or mutagenesis (yellow highlighted residues), were then mapped into the sequence alignment (Fig. 1). These residues, which usually occur in clusters, were boxed in different colors.

Figure 1. Alignment of chemokine sequences, based on the 3D structure fit, highlighting HS binding amino acids. Chemokines have been grouped according to their cysteine motif and amino acid sequences have been aligned based on secondary structure alignment. Within each class, chemokine sequences are ordered according to the phylogenetic tree issued by ClustalΩ (http://www.clustal.org). The secondary structures of the first member of each class (CCL1, CXCL1, CX3CL1, and XCL1) are schematically depicted either as a cylinder for helix or as a thick arrow for beta-strand. Available HS-binding data for human chemokines are mapped into the sequences (except for CXCL1 and CXCL2, for which data were obtained with the Mus musculus homologs) and shown underlined for the residues that experience chemical shift perturbation upon GAG binding and are highlighted in yellow for those in which mutations reduced binding affinity. The different binding clusters, corresponding to distinct binding modes, have been highlighted in green for cluster 1, pink for cluster 2, blue for cluster 3 and orange for cluster 5.
according to their position within the sequence. In addition, all Arg and Lys residues are shown in red to highlight the position of the various basic residues.

Cluster 1 comprises basic residues present in both the C-terminal and β10 helices and in the 40s loop (in green; Fig. 1) and is the main HS-binding determinant for CXCL1 [58], CXCL2 [39], CXCL4 [40], CXCL8 [41], CXCL10 [42], and CXCL11 [43]. Most of the other CXC chemokines, except for CXCL12 and CXCL16 (see below), feature basic residues within this cluster type and are thus likely to interact with GAGs similarly. The GAG-binding site of XCL1 [44] is also typical of the cluster 1 group.

Cluster 2 defines another binding mode that, so far, has been experimentally observed only for the 3 isoforms of CXCL12 (α, β, and γ). It involves the β-sheet (in red; Fig. 1), with the β1-strand as the main contributor [45–47]. CXCL16, which also features basic residues clustered within the β-sheet, but not in the C-terminal helix, is likely to belong to this group.

Cluster 3, which primarily includes residues of the 40s loop and the N-terminal loop (in blue, Fig. 1), is characteristic of most CC chemokines. The 40s loop is the main determinant of binding for CCL3 [48], CCL4 [49], CCL5 [50], CCL14 [51], and CCL18 [52], whereas the N-terminal loop predominates for CCL2 [53] and CCL7 [54]. Although all basic amino acids of CCL3 and CCL4 fall into cluster 3, a number of such residues spread out of this cluster for CCL2, CCL5, CCL7, CCL14, and CCL18, which might also participate in binding. This was shown at least for CCL2 and CCL7, for which mutation-based analysis demonstrated the involvement of residues within the C-terminal helix, although to a smaller extent when compared with the cluster 3 residues [53–55].

A cluster 4 was previously proposed [13], based on the crystal structure of CX3CL1 [56], which comprises a flat area composed of the 30s and 40s loops. As this prediction has not yet been validated by experimental data, this cluster is not represented here.

In addition to these 4 binding modes, we define here a new cluster (cluster 5) based on the study of the β- and γ-isomers of CXCL12 [46]. These 2 isoforms share with CXCL12α the first 68 amino acids (thus comprising a cluster 1-type HS binding site) but are characterized by distinctive 5- (for CXCL12β) or 30- (for CXCL12γ) residue extension, downstream of the C-terminal helix. Remarkably, these C-terminal peptides contain as much as 3 of 5 and 18 (of 30) basic residues, clustered into 1 or 4 putative “BBXB” HS-binding domains. Both NMR titration analysis and mutagenesis coupled to SPR studies showed that if cluster 1 remains the main HS-binding site for all CXCL12 isoform, these C-terminal extensions, defining cluster 5, very strongly enhanced the binding affinity and, in the case of CXCL12γ, also enabled the chemokine to interact with dermatan sulfate [46]. Based on sequence analysis (Fig. 1), cluster 5 can also be found in several other chemokines, such as CXCL9, CXCL13, and CXCL14 and CCL1, CCL7, CCL19, CCL20, CCL21, CCL23, CCL24, and CCL28. Although no direct mutagenesis studies have confirmed the involvement of this cluster in GAG binding for the above-mentioned chemokines, the CXCL9 C-terminal peptide (residues 74–103) strongly binds to HS [57], whereas processing of the C-terminal extension of CCL21 was shown to release a soluble form of the tissue-immobilized chemokine [58], experimentally demonstrating the HS-binding activity of cluster 5 for these 2 chemokines.

The relationships between GAG and CKR binding: how GAGs can interfere with chemokine-induced signaling

Current models usually suggest that HS functionally presents its chemokine ligands to CKR-expressing cells. For this to occur, however, the binding sites for the receptor and for the GAG need to be spatially distinct and uncompetitive. The analysis described above nevertheless indicates that GAG-binding clusters occupy a variety of possible positions on the chemokine surface, which may or may not overlap with the receptor-binding site (Fig. 2). As chemokines recognize different receptors, this process may control receptor selectivity. In CCL5, for example, the 3 basic residues in the 40s loop, forming a typical cluster 3 GAG-binding motif, appear to contribute to receptor-binding affinity and specificity in a receptor-dependent manner; mutation of these residues resulted in a significant decrease of CCRI binding, whereas the affinity for CCR5 remained unaffected [50], although it is not known whether the addition of GAG to wild-type CCL5 would have the same effect. In any case, as our comprehension of chemokine-induced receptor activation improves, the data in Figs. 1 and 2 should provide a structural basis for understanding how GAGs may modulate receptor recognition and chemokine function.

A key factor regulating chemokine–CKR interactions, which has recently emerged and remains poorly investigated in the context of GAG-binding, is the presence of sulfated tyrosine residues on the receptor’s N terminus. Tyrosine sulfation, a common posttranslational modification of membrane-associated proteins, is catalyzed in the Golgi apparatus by the tyrosylprotein sulfotransferases, 2 isoforms of which exist in mammals. These enzymes preferentially add sulfate to tyrosine residues found within or near clusters of acidic amino acid residues (Asp and Glu), a motif that is present in most CKR N termini. The presence of such modifications has been shown experimentally...
in CCR2 [59, 60], CCR5 [61], CCR8 [62], CXCR3 [63], CXCR4 [64], CX3CR1 [65], and ACKR1 [66]. Mutagenesis of these residues [59, 62, 65, 66] or abrogation of their sulfation [30, 60] invariably causes a reduction in chemokine-binding affinity and a significantly attenuated response to the chemokine. On the other hand, sulfopeptides have been developed, in particular, to mimic GAGs. In several studies, such sulfopeptides have been shown to compete with GAGs to bind to various proteins, including VEGF [67] and the HIV-1 envelope glycoprotein gp120 [68]. Such HS mimetic sulfopeptides were shown to block gp120 binding to CXCR4, thus suggesting that CKR and GAGs may also interfere in binding to their chemokine ligands.

A recent study reported that the sulfation of 2 adjacent tyrosines (positions 16 and 17) in CXCR3 significantly enhanced the binding and subsequent activity of CCL11 [30]. Functionally, this has been demonstrated by growing CXCR3-expressing cells in the presence of sodium chlorate, a compound that competitively inhibits the formation of 3'-phosphoadenosine 5'-phosphosulfate, the sulfate donor in cellular-sulfation reactions, including that of GAGs [69]. It thus remains possible that, in bioassays, reduced CCL11 activity was also partly due to a reduced ability of the GAG chain to bind and concentrate the chemokine at the cell surface. Nevertheless, the structural basis of this interaction, as shown with NMR spectroscopy, and the CXCR3 N-terminal sulfopeptide, clearly support the importance of the 2 sulfotyrosines for CCL11 binding, with a 100-fold reduction in affinity for the nonsulfated peptide. The structure of the CCL11–CXCR3 peptide complex additionally revealed the involvement of the chemokine Arg-16 and Lys-47 in establishing salt bridges with the sulfotyrosines. Although the residues involved in GAG binding of CCL11 have not yet been determined experimentally, this protein belongs to the cluster 3–type chemokine, in which residue 16 (6 residues downstream of the CC motif in the N-loop) and 47 (3 upstream of the last C residue in the loop connecting the second and third β-strands) are systematically involved (Fig. 1), suggesting that CCR3 and GAGs may compete with each other to bind CCL11.

Inhibition of CCR2 sulfation has also been reported to reduce the strength of its response to CCL2. Interestingly, it was found that although sulfated peptides derived from the N-terminal region of CCR2 bound to both the monomeric and dimeric forms of CCL2, they shifted the equilibrium to favor the monomeric form, which is more active than the dimeric one [60]. Thus, whereas GAGs usually promote chemokine dimerization, it is tempting to propose that the sulfated CKR N-terminal domain helps chemokines to dissociate from GAGs, releasing their monomeric and active forms. This speculation is experimentally supported by a study employing nanoelectrospray ionization mass spectrometry to measure the affinity of another ligand of CCR2—CCL7—with the CCR2 N-terminal peptides containing 0, 1, or 2 sulfotyrosines. It was first found that the disulfated CCR2 has a 16-fold greater binding affinity than did the unmodified peptide [70]. Interestingly, the same methodology did not detect the formation of any trimolecular complexes following the incubation of equal concentrations of CCL7, the CCR2 sulfopeptide, and a heparin-derived pentasaccharide, which does form a complex with CCL7 in the absence of CCR2. Competition experiments indicated that the oligosaccharide was displaced by the disulfated CCR2 peptide, indicating that there is a partial overlap between the GAG-binding and the CCR2-binding domains [70]. Similarly, NMR spectroscopy also showed that among the 4 potential sulfotyrosines of the N-terminal tail of CCR5, the sulfation of tyrosine 10 and 14 strongly enhanced binding to CCL5, exclusively in its monomeric form. Chemical-shift mapping of the interaction indicated that the binding surface consisted of basic residues overlapping with the CCL5 dimer interface, providing a rationale for the exclusive binding of the chemokine’s monomeric form [71]. Altogether, these studies suggest that the CKR sulfopeptides help release chemokines from their HS-bound form, enabling them to productively recognize and activate their receptor.

The importance of sulfotyrosines has also been investigated within the CXC chemokine subfamily, using CXCR4 and its ligand CXCL12. The N-terminal domain of CXCR4 displays up to 3 sulfotyrosines, at positions 7, 12, and 21, which contribute to CXCL12 binding [64]. From a structural point of view, the N-terminal peptide of CXCR4, in its nonsulfated form, sulfated on Tyr-21, and sulfated on all its tyrosine residues, has been studied in complexes with a covalently cross-linked CXCL12 homodimer. The chemokine–peptide complexes were similar, regardless of the sulfation status of the CXCR4 peptide, which wraps around both subunits of the CXCL12 dimer, placing tyrosines 12 and 21 in distinct sites on one subunit, whereas the tyrosine 7 occupies a cleft at the dimer interface that would not exist in the wild-type CXCL12 monomer [32]. Further study based on shorter peptides, each containing only 1 sulfotyrosine, indicated that, of the 3 sulfated residues, only Tyr-21 interacts at a specific position, making salt bridges with Arg-47 and promoting CXCL12 dimerization. Free sulfotyrosine, however, also promotes CXCL12 dimerization [72], so that the exact role of the CXCR4 N-terminal domain in this process remains not completely clear. Based on these data, it is proposed that HS can present chemokines in a bound form (the 2 binding sites do not overlap), can modify the presentation of chemokines to their receptors (e.g., by inducing structural modifications, such as oligomerization) and can release chemokines to enable functional CKR recognition, a displacement process that may be spontaneous or competitively induced by CKR sulfopeptides.

Methodological aspects of studying chemokine–GAG interactions

Numerous biophysical techniques have been developed to analyze the interactions between GAG and chemokines, including affinity chromatography on heparin columns [73], capillary electrophoresis [74], coupling radioactively labeled GAG-derived oligosaccharides to protein-binding filter assays [47], isothermal fluorescence titration [75], isothermal titration calorimetry [73], SPR [47, 76] various mass spectrometry techniques [77, 78], and NMR spectroscopy [79]. The characterization may be either at the molecular scale (e.g., heparin affinity chromatography, isothermal titration calorimetry, or SPR), or at the residue to atomic scale (e.g., NMR spectroscopy or X-ray diffraction crystallography). Each of these methods has its advantages and disadvantages, and they are best used in combination. In the context of chemokine–GAG interactions, SPR is certainly one of the most appropriate; it is a very sensitive
GAGS HAVE HUNDREDS OF LIGANDS: WHAT IS RECOGNIZED BY CHEMOKINES?

Having examined the chemokine residues involved in binding to HS, we now describe the GAG family to highlight the structural determinant responsible for chemokine recognition. These polysaccharides are highly complex molecules and are complex to investigate. Although their structure appears to be tightly regulated (see below), unlike other biopolymers (DNA, RNA, polypeptides), their biosynthesis is based on a nontemplate-driven process. Therefore, they cannot be amplified, produced, or modified recombinantly, and there is no simple method to isolate them in large amount and homogeneity. Consequently, detailed correlations between GAG structure and GAG function in the field of chemokines are only slowly emerging.

The GAG family—variations on a common scheme

GAGs comprise heparin, HS, CS, DS, and HA. These polysaccharides, except for HA, occur covalently bound to various core proteins. Based on differences in the deuterium exchange rate, the GAG-binding interface can thus be identified by mass spectrometry peptide mapping. CXCL8 binding to a chondroitin sulfate–derived hexasaccharide has been examined recently using this approach, and despite the observation of some deuteration levels that could also be linked to allosteric effects, the results are in good agreement with previous models of this complex [85]. This method consumes only few micrograms of label-free protein and GAG and is suitable at low concentrations.

NMR is another spectroscopic method that has been extensively used to study protein–ligand complexes, especially dynamic and versatile ones, such as those involving GAGs and chemokines [79, 86]. This method needs the protein NMR signals to be assigned, which, for small and well-packed proteins, such as chemokines, is generally straightforward. The standard, and so far, the easiest way, to identify residues involved in chemokine–GAG binding is to measure the perturbations in the protein $^{13}$N/$^1$H chemical shift that results from the interaction. The CSPs are triggered by electronic environment changes around the monitored nuclei. Generally, the bigger the perturbation, the more closely the monitored residue is involved in the molecular event (for instance, the binding). Careful interpretation of the results is nevertheless needed to properly decipher the involved molecular events. Indeed, amides could either be localized at the binding interface (the desired information) or may experience any other changes that could occur during titration (buffer-condition shifts, conformational rearrangements, oligomerization, denaturation, among others). Chemokines often dimerize (or oligomerize) upon GAG binding; in fact, both binding and dimerization events can be followed as long as the CSP triggered by the dimerization can be independently characterized. In some cases, however, basic residues tightly involved in GAG binding do not (or only slightly do) experience perturbation of their backbone amides [87]. In such cases, a CSP analysis of the lysine side chain was proposed, using either an adapted NMR experiment recorded on $^{13}$C, $^{15}$N-labeled protein [87] or $^{13}$C/$^1$H-methyl spectroscopy following reductive methylation of lysine side chain, as had previously been performed to investigate a CXCL8-heparin complex [88].

To overcome the inherent limitations of mutagenesis, spectroscopic methods can be applied on wild-type chemokines to determine GAG-binding sites at the residue-to-atom scale. One recently reported approach was based on proton–deuterium exchange mass spectroscopy analysis [84]. This technique relies on the measurement of amide deuteration level differences between unbound and GAG-bound proteins. The more accessible the amide protons are, the faster they exchange to deuterium. Consequently, amide protons buried upon GAG binding experience slower exchange rates than do those of free proteins. Based on differences in the deuterium exchange rate, technique, requiring only micrograms of material and can detect binding events at physiologically relevant nanomolar concentrations (depending on the affinity of the binding partners). This is a large advantage over most of the other techniques, in particular, when using full-length GAG molecules, which at high concentrations systematically induce chemokine aggregation and precipitation. Being a label-free and real-time technique, the protein does not need to be labeled or modified, and both kinetic and thermodynamic information can be obtained. Kinetic analysis, when possible, is one of the more quantitative methods; it has been used to demonstrate, for example, that an N-terminally truncated form of CCL5, which remains monomeric in solution, dimerizes upon binding to heparin with positive cooperativity [80]. Functionally, using a model of neutrophil migration into the airspace of the lung, it has been shown that chemokines that associate and dissociate slowly from GAGs, such as CXCL2, were less effective than those characterized by rapid kinetics, such as KC (the mouse homolog of CXCL8), supporting the interpretation that the kinetics of the chemokine–GAG interaction is an important contributing factor toward understanding the role of these interactions [81]. Finally, and importantly, SPR is a surface-based technique, in which one of the binding partners (usually the GAG) is immobilized on top of a biosensor, whereas the other one (the chemokine) flows in solution over the GAG surface. For that purpose, GAGs can be biotinylated and easily captured on top of streptavidin chips, and when this is done on a single site through the reducing end of the polysaccharide, a method that has been recently improved to give high yields and stable conjugates [82], the polysaccharides are presented in an oriented manner that very closely mimics the manner of GAG chains attached to proteoglycan at the cell surface or in the matrix. In addition, the polysaccharides remain minimally modified and active compared with those covalently coupled at random sites (such as via the carboxylic groups using carbodiimide chemistry). Biolayer interferometry and quartz crystal microbalance with dissipation monitoring are also surface-sensitive techniques, providing time-resolved information, and the latter has been recently used to reconstitute a GAG layer into a well-defined surface model to which the binding of the chemokine CXCL12 was characterized [83]. These approaches have, nevertheless, some limitations, such as possible nonspecific binding to the surface. In addition, when applied to identify residues involved in GAG binding, they are usually used in conjunction with mutagenesis, where 1) only mutated residues (usually the basic ones) are challenged, 2) biochemical assays are achieved on native sequence proteins, and thereby could be biased by structural issues, and 3) cooperative behaviors can be overlooked.

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NMR is another spectroscopic method that has been extensively used to study protein–ligand complexes, especially dynamic and versatile ones, such as those involving GAGs and chemokines [79, 86]. This method needs the protein NMR signals to be assigned, which, for small and well-packed proteins, such as chemokines, is generally straightforward. The standard, and so far, the easiest way, to identify residues involved in chemokine–GAG binding is to measure the perturbations in the protein $^{13}$N/$^1$H chemical shift that results from the interaction. The CSPs are triggered by electronic environment changes around the monitored nuclei. Generally, the bigger the perturbation, the more closely the monitored residue is involved in the molecular event (for instance, the binding). Careful interpretation of the results is nevertheless needed to properly decipher the involved molecular events. Indeed, amides could either be localized at the binding interface (the desired information) or may experience any other changes that could occur during titration (buffer-condition shifts, conformational rearrangements, oligomerization, denaturation, among others). Chemokines often dimerize (or oligomerize) upon GAG binding; in fact, both binding and dimerization events can be followed as long as the CSP triggered by the dimerization can be independently characterized. In some cases, however, basic residues tightly involved in GAG binding do not (or only slightly do) experience perturbation of their backbone amides [87]. In such cases, a CSP analysis of the lysine side chain was proposed, using either an adapted NMR experiment recorded on $^{13}$C, $^{15}$N-labeled protein [87] or $^{13}$C/$^1$H-methyl spectroscopy following reductive methylation of lysine side chain, as had previously been performed to investigate a CXCL8-heparin complex [88].

To overcome the inherent limitations of mutagenesis, spectroscopic methods can be applied on wild-type chemokines to determine GAG-binding sites at the residue-to-atom scale. One recently reported approach was based on proton–deuterium exchange mass spectroscopy analysis [84]. This technique relies on the measurement of amide deuteration level differences between unbound and GAG-bound proteins. The more accessible the amide protons are, the faster they exchange to deuterium. Consequently, amide protons buried upon GAG binding experience slower exchange rates than do those of free proteins. Based on differences in the deuterium exchange rate,
proteins forming the proteoglycan family. PGs have been classified according to the nature of the polysaccharide chains that are covalently attached to the protein core and are referred to as HSPG, CSPG, or DSPG. Heparin is found attached to a single PG—serglycin—found in the secretion granules of mast cells. The core proteins usually determine the localization. The PGs can thus also be classified into 6 large families based on their placement [4], which include intracellular PGs (serglycin), cell surface or membrane PGs (such as the 4 syndecans or the 6 glypicans), the pericellular or basal lamina PGs (such as perlecan, agrin, or collagen types XV and XVIII), the extracellular PGs (aggrecan, versican, decorin, and many others), and the circulating PGs (endocan and bikunine). HS is prevalently associated with the cell surface or the basement membrane PGs and is thus in the immediate vicinity of the plasma membrane, where it can interact with signaling proteins, whereas CS and DS usually predominate within the extracellular matrix (Fig. 3).

GAGs all consist of a repeating disaccharide unit, comprising a hexuronic acid (glucuronic acid) linked to an N-acetylhexosamine, either or both of which may be sulfated at different positions (except for HA). In heparin and HS, the hexuronic acid is 1,4-linked to an N-acetyl-glucosamine (GlcNAc), whereas, in CS and DS, it is 1,3-linked to an N-acetyl-galactosamine (GalNAc). Based on these simple disaccharide units, which are polymerized in the Golgi apparatus, considerable diversity is created during biosynthesis by a series of interdependent, enzymatic reactions that do not occur uniformly along the chain. In heparin and HS, which typically comprise 30–400 monosaccharide units, the GlcNAc residues can be first N-deacetylated, followed or not by re-N-sulfation, 2 reactions catalyzed by NDSTs, giving rise to GlcN or GlcNS. This first modification targets 30–70% of the GlcNAc, but remarkably this mostly occurs in contiguous stretches of between 3 and 6 disaccharides, known as S domains (in green, Fig. 4A). Within these domains, GlcA can be converted into IdoA by a C5-epimerase, and the chain can be further modified by 2-, 6- or 3-OSTs catalyzing O-sulfations at some of the C6 and C3 positions of the GlcNAc and of the C2 positions of the GlcA and IdoA residues. All together, these enzyme modifications can theoretically generate up to 48 different disaccharides, 23 of which have been identified. As a result of this complex process, HS features a unique molecular design in which hypervariable, GlcNS- and IdoA-enriched and highly sulfated sequences [90], which stem from the combination of different disaccharides, are spaced apart by relatively regular regions, containing predominantly GlcNAc and GlcA residues (NA domains) and mixed NA/S regions that form the transitions between NA and S domains [91]. Thus, with a basic starting unit, enormous molecular diversity can be generated on 3 different levels: 1) the length of the HS chains, 2) the extensive variability of the sulfation and epimerization pattern within the S domains, and 3) the number and spacing of the S domains within the chain (Fig. 4). Heparin

Figure 3. GAGs are widely present at the cell–tissue interface and form a variety of molecular complexes with chemokines. (A) GAGs, which comprise HS, heparin, CS, and DS are attached to core proteins associated with the cell membrane and basal lamina (predominantly HS) or the extracellular matrix (predominantly CS/DS). With few exceptions, these highly anionic polysaccharides bind to chemokines, thereby controlling their diffusion potential and providing positional information within tissues in such forms as chemotactic gradients (red dots) along which cells can migrate directionally. (B) It is traditionally thought that chemokines bind to HSs through their C-terminal helix, as initially shown with several CXC chemokines and, as such, can be presented in a bound and active form to their cognate receptor (CKR). However, analysis of all chemokine–GAG pairs that have been biochemically studied to date (see Fig. 1) seriously challenged this view and brought to light a variety of possibilities (from left to right): chemokines can interact simultaneously with GAGs and CKR; chemokines cannot interact simultaneously with GAGs and CKRs and need to dissociate from GAGs to induce CKR-dependent signaling; chemokines undergo structural changes upon binding to GAGs (such as oligomerization), which may or may not modulate CKR activation (see “Relationships between GAG and CKR binding” paragraph); and chemokines featuring a basic C-terminal extension (cluster 5 HS-binding site) form extremely stable complexes with GAGs and can be presented to CKR as a GAG-bound form.
Regulation of GAG structure and expression

Each tissue produces specific GAG repertoires, and cells can alter their GAG expression in response to various stimuli or in pathologic states. It has thus been speculated that the extremely large structural diversity that characterizes these molecules—HS in particular—may encode specific information that could be recognized by a given set of chemokines, thereby dictating which signaling pathways will be activated in a cell- and tissue-specific manner.

Such a possibility is supported by several lines of evidence. Biochemical analysis of HS from different tissues demonstrated that the HS content, disaccharide composition, and the overall degree of N- and O-sulfation, as well as domain organization, are characteristic of each individual tissue. These structures, which are conserved within animals of the same genotype [93], thus appear to be strictly regulated. In contrast, modifications in HS structure have been observed at the cell-type level, for example, during aging, where a decline in the proportion of the trisulfated disaccharide unit UA2S-GlcNS6S has been observed in endothelial cells, thus also indicating temporal regulation. Interestingly, this modification was correlated with a reduced ability of these cells to migrate toward CXCL12 or VEGF [94], indicating that the structure of GAG influences its ability to interfere with chemokine-mediated activity. Similarly, characterization of HS extracted from 20- to 84-y-old aorta demonstrated gradual increase in the degree of O-sulfation of the GlcNS residues that markedly affect binding to platelet-derived growth factor [95].

Macrophage polarization has also alters the GAG expression level and sulfation pattern, with an increase of 2-O-sulfite containing disaccharide in M2 macrophage, when compared with M1 macrophages, which correlates with an overexpression of the biosynthetic enzyme 2-O-sulfotransferase [96] Organ specificity, with respect to average length, disaccharide composition, and expression of some low-abundance structural epitopes, was also demonstrated with a powerful approach based on the analysis of GAG mass spectra [97]. This methodology showed that leukocytes also express distinctive levels of GAGs. All leukocytes display highly sulfated HS, but with a level of expression that follows the trend of T cells/B cells > monocytes/NK cells > polymorphonuclear cells. In these cells, CS was considerably more abundant than HS, with a higher level for myeloid-lineage cells than for lymphoid and NK cells [98]. The existence of HS diversity was also shown with a panel of 10 unique and epitope-specific anti-HS antibodies that were generated with phage display technology. Each of them was found to have a topologically defined tissue distribution, indicating that HS-specific sequences do not occur...
randomly but are expressed in a controlled manner [99]. For example, the (GlcN6S-IdoA2S)\textsubscript{3} epitope, detected with such an antibody, displayed a restricted and specific distribution within tissue. In HUVECs, it is strongly up-regulated upon TNF-\textalpha stimulation and contributes to firm leukocyte adhesion [100]. Using a pan–anti-HS antibody, together with a semi-quantitative immunostaining approach on blood vessels, a striking enrichment in HS was observed in the basal lamina, when compared with luminal glycocalyx. Interestingly, skin inflammation was associated with further HS deposition around postcapillary venules and their associated pericytes. This polarized HS deposition is obviously relevant to the chemokine field because it would define a steep gradient of HS between the apical and basolateral side of the endothelium, thus favoring the extravasation of leukocytes [101].

Finally, HS biosynthesis enzymes (i.e., glycosyltransferases involved in polymer formation and sulfotransferases and the epimerase involved in chain processing) were also subjected to strict regulation [102, 103], implying modulation of the final HS structures, although a direct link between the level of expression of these enzymes and the structure of the polymer they synthesize remains difficult to establish [104–106]. Nevertheless, taken together, these studies clearly demonstrated a dynamic extracellular environment in which defined spatial and temporal control of HS-modification patterns occur.

Despite this dynamic regulation, how such changes in HS structure or tissue distribution can affect chemokine activity has been poorly investigated. It has, nevertheless, been shown that upon renal ischemia/reperfusion, the microvasculature basement membrane HSs are modified in the renal interstitium. This modification, which is likely due to a decrease in the expression of the endosulfatase Sulf-1, enhanced the ability of the GAG chains to bind CCL2 (and also L-selectin), thus influencing the inflammatory response [107]. More recently, the importance of HS structure in the hematopoietic stem and progenitor cell migration to bone marrow has been observed in a mouse model of mucopolysaccharidosis I Hurler syndrome. This pathology, caused by genetic deficiency in iduronidase (IduA), is characterized by the accumulation of undegraded HS. In the bone marrow of IduA\textsuperscript{−/−} animals, HS was significantly increased and abnormally sulfated, and interestingly, these materials inhibited CXCL12-mediated hematopoietic stem and progenitor cell transwell migration more efficiently than normal HS did. These data suggested that highly sulfated HSs sequester CXCL12 in the bone marrow, preventing it from forming a haptotactic gradient and suggesting a mechanistic explanation for graft failure frequently observed in patients undergoing hematopoietic stem cell transplant in that pathology [108].

The structures recognized by chemokines

Although it is clear that GAG structures are regulated and that changes in sulfation or epimerization pattern have functional consequences, the GAG epitope recognized by a particular chemokine is currently unknown. Consistent with the large diversity and very high density of information that characterizes HSs, their interactions with proteins have been thought to rely on specific sequences defined by their specific sulfation and epimerization (the GlcA:IdoA ratio) pattern. In many cases, however, heparin, kilograms of which can be extracted from porcine intestinal mucosa and which is widely used in the clinic for its antithrombotic activity, has been used to investigate chemokine–GAG complexes, both structurally and functionally. As described above, however, although heparin is chemically related to HS, it is highly and regularly sulfated and, thus, is not appropriate for determining the structural features of the polysaccharides involved in chemokine recognition. Although it has been proposed that, in HS, the various disaccharides could be arranged in a sequence-specific manner to provide for selective protein binding, such epitopes would be hidden in the highly sulfated heparin. In addition, heparin is mostly found within intracellular secretory granules of mast cells, where it serves to pack intracellular proteases and is, therefore, not a physiologic ligand for chemokines.

On the other hand, GAGs isolated from natural sources, in addition to being obtained in small amounts, are only poorly suitable for evaluations of structure–activity relationships because of their extremely large polydispersity and the lack of standard analytic methods for their characterization. Thus, of the large number of GAG–protein complexes that can be experimentally demonstrated, antithrombin III, a serine protease inhibitor, is the only one for which a specific GAG sequence has been formally defined so far and reproduced by chemical synthesis. It remains plausible that many proteins do not demand specific structural features and associate with GAGs through less-specific ionic interactions. In any case, very little is known about chemokines in this respect.

An interesting methodology to investigate the importance of the oligosaccharide sequence for protein recognition is based on mass spectrometry approaches that can determine the molecular mass of a complex, and from this, the molecular formula of its composition can be deduced. A number of CC chemokines, including CCL2, CCL7, and CCL8 have been studied with this approach. Coincubated with a mixture of HS-derived octasaccharides, it was found that, within a pool of molecules with a constant charge, N-acetylation enhanced binding to CCL2. This indicated that \(\text{O}\)-sulfation was more relevant than \(\text{N}\)-sulfation for the recognition of this particular chemokine, or it could also suggest the direct involvement of an acetyl group in the binding reaction [109]. Although this result did not indicate whether there were any positional preferences for the sulfate groups along the sequence, it showed that binding does not rely solely on the net global charge.

Chemical synthesis, although notoriously difficult in the field of GAGs, is undoubtedly the most reliable source of well-defined oligosaccharides and would be of great help in interrogating the structure–activity relationships of HS-mediated processes. Building distinct sulfation sequences requires the development of complex protecting/deprotecting procedures necessary for the differential functionalization of hydroxyl groups that have very similar reactivity. Furthermore, the polymerization of the various disaccharides necessitates stereo-specific formation of \(\text{O}\)-glycosidic and \(\text{\beta}\)-glycosidic linkages. Considerable progress, however, has been made, both for HS [110–115] and for GS [116–120]. An emerging and powerful approach is the use of chemoenzymatic strategies [120], which are based on knowledge of HS biosynthesis [104] and HS biosynthetic enzymes [122–124]. This approach greatly extends the possibility of engineering GAG.
oligosaccharides having diverse and controlled sulfation patterns. The use of such tailor-made oligosaccharides has started to elucidate the structure–function relationships of HS–chemokine complexes. For example, the chemical synthesis of 3 HS-like hexasaccharides, all possessing 6 overall sulfate groups but displaying distinct placements along the chain, have been investigated for their ability to interact with various proteins, including CCL2, CCL5, CCL11, CXCL2, CXCL4, and CXCL12. The binding preferences for individual chemokines differed between the hexasaccharides. CCL2, CCL11, and CXCL2 interact with a hexasaccharide displaying a GlcNS6S–IdoA motif, but not with an otherwise similar molecule displaying a GlcNAc6S–IdoA2S motif. These studies also showed the importance of the 6-O-sulfation of the GlcN in high-affinity binding to CXCL12 [125]. Similarly, the chemical synthesis of heparin-like dodecasaccharides showed that a site-specific modification of the terminal GlcN affects a switch between inhibition of CXCL8 and CXCL12 [126].

In view of the large diversity of possible HS sequences, carbohydrate microarrays have become an interesting tool for investigating complex glycan molecules [127, 128]. Because microarrays enable the parallel screening of closely related compounds, which can be coupled to real-time detection systems [129], they are particularly useful for studying protein binding in libraries of natural and synthetic GAGs characterized by subtle structural differences. Examination of the interaction of 8 chemokines with a heparin microarray displaying 12 synthetic oligosaccharides revealed bindings that were both sequence and chemokine dependent [130].

In addition to the diversity within the HS S-domains, the general organization of the chain, specifically the alternation of these S-domains with the much less-charged NA-domains along the polymer, creates distinct topologies (Fig. 4), referred to as SAS motifs (for sulfated-acetylated-sulfated sequences). This particular aspect is certainly relevant for chemokines. Because many chemokines are dimers, or form dimers upon binding to HS, they can recognize composite SAS binding sites comprising 2 S-domains separated by several N-acetylated disaccharide units. Thus, the general topology of the chain, beyond the oligosaccharide sequence itself, could affect chemokine recognition, as shown by distinct SAS-like molecules interacting differently with CXCL12 and CCL5 [80].

Finally, in principle, resolving the structure of a chemokine–HS complex, as determined by X-ray crystallography, for example, should enable the identification of which sulfate groups are actually engaged at the interface and required for binding. Such approaches have, however, met with only limited success (see below) and, in addition, would probably not be able to exclude the possibility that different HS sequences engage in similar contact with a given protein through conformational adaptation to its ligand. NMR spectroscopy, which provides insight into the conformational and dynamic properties of molecules in solution, appears to be more suited for investigating the positioning and dynamics of key, interacting groups within a protein–GAG complex. Another common approach is saturation transfer difference analysis. This method has the advantages of both requiring a relatively dilute, unlabeled protein sample and good sensitivity because it relies on the signal of free GAGs, which, in turn, are used at high concentrations. In saturation transfer difference, protein-specific signals are irradiated to extinguish their magnetization, and this saturation is transferred in a distance-limited manner (proportional to r^−6) to the part of the ligand in close vicinity to the protein. The bound GAG bulk exchanges with the free GAG, and thereby, signal attenuation is transferred. The differences between 1-dimensional [3H] spectra, with or without irradiation, allow for the determination of which protons of the GAG molecule are tightly involved in binding [131, 132]. CSP analysis of GAGs, upon binding to chemokines, can also be used as long as [13C]-labeled GAG is available. A method to produce [15N, 13C]-labeled GlcA-GlcNAc polysaccharide, which chemical sulfation and controlled depolymerization yielded a well-defined (GlcA6S-GlcNS6S)₆ has been reported. Using this HS mimic, along with [15N]-labeled CXCL12, CSPs were recorded on both the chemokine and the HS side, from which a structural model of the complex was proposed [132]. Combined with chemoenzymatic strategies to generate various oligosaccharides featuring high affinities for their chemokine ligand, this approach should be helpful in obtaining well-resolved intermolecular and intramolecular nuclear Overhauser effects and thus in defining the molecular interactions that drive complex formation.

Several chemokines have also been shown to recognize chondroitin/dermatan sulfate [133], including CCL5 (see below) and, in particular, chemokines featuring a “cluster 5” GAG binding site, such as CXCL12y [46] and CCL21 [134]. CCL21 binds to CS containing IdoA(2S)-GalNAc4S (CS-B) or GlcA-GalNAc4S6S (CS-E) motifs but not to GlcA2S-GalNAc6S (CS-D), indicating the requirement of peculiar structural determinants for chemokine recognition. Interestingly, CS-B inhibits CCL21-induced α₅β₁ integrin activation and intracellular Ca⁴⁺ influx, whereas HS has the opposite effect [133], thus suggesting that the chemokine may be negatively or positively regulated, depending on the GAG structure to which it binds.

The 3D structures of chemokine–GAG complexes

Attempts to crystallize GAG–chemokine complexes—a difficult issue—only yielded limited data during the past decade, with only the structure of CCL5 [135] and CXCL12 [136] being solved, in complex with heparin-derived disaccharides. Heparin oligosaccharides with a higher degree of polymerization form high-order oligomers and/or precipitate the complex, preventing any analysis. In addition, highly sulfated molecules are likely to display multiple binding modes, causing difficulties in obtaining stable, well-defined structures on which structural methods can be applied. CCL5 was cocryrstallized with 2 heparin-derived disaccharides, GlcA2S-GlcNS6S and GlcA2S-GlcNS [135]. It showed the interaction of both disaccharides with the 40s-loop BBXB motif, which had already been described as the main HS-binding site of CCL5 (Fig. 5). The interactions of Arg-44 with the 2-0-sulfate, His-23 and Lys-45 with the carboxyl group, and Lys-45 with the 6-O-sulfate are involved in stabilizing the interaction with heparin, as previously reported. In contrast, Arg-47, which was known to be important for heparin binding [50], including in other chemokines, such as CCL2, CCL3, and CCL4 [48, 49, 53], was not involved in this particular structural model. The closest part of the ligand interacting with the BBXB domain was the
nonnative unsaturated uronic acid C4–C5 bond that resulted from the heparinase digestion used to prepare the disaccharides. Consequently, the reported interaction of His-23 with the negatively charged carboxyl, whose orientation depends on the orbital geometry of carbon C5, might be questionable. Native geometry would shift the carboxyl closer to Lys-45 than to His-23. Moreover, as shown in Fig. 5, the orientation of the disaccharide would not be compatible in the context of a longer heparin fragment. Molecular dynamics simulations performed with tetrasaccharides and dodecasaccharides, also suggested that the HS–CCL5 binding interface differs according to the structure and degree of polymerization of the GAG fragments [137]. In the crystal structure, the disaccharide also interacts with both the 30s loop (Ser-31, Gly-32, and Lys-33) and the N terminus (Tyr-3). However the mutation of the latter did not affect the binding affinity in solution, and NMR titration data did not show backbone amide perturbation for the 30s loop residues [50]. These results may suggest that these interactions between the disaccharide and CCL5 are a consequence of crystal packing, although belonging to the asymmetric unit.

The second reported crystal structure was that of CXCL12 in complex with either (A) a heparin-derived disaccharide GlcA2S-GlcNS6S, or (B) a CS-derived hexasaccharide (GlcA-GalNAc4S)3 and the crystal structure of CXCL12 in complex with 2 GlcA2S-GlcNS6S (C and D) are represented. The chemokine structures are shown as gray ribbons, and both residues of interest and oligosaccharides are shown as sticks. The hydrogen bonds are represented as yellow, dotted line. The figures were prepared with the PyMol software using the data available at the protein data bank, identifiers IU4L (A) and 2SWG (C and D) or from the supplementary material in Deshauer et al. [131].

The critical analysis of these 2 chemokine–disaccharide complexes highlights the difficulty of using such small oligosaccharides to obtain insight into actual chemokine–GAG complex structures. Heparin size-dependent increase of both affinity [47, 73, 80] and CSP spreading over the chemokine surface [138] illustrate the presence of peripheral binding residues that, in addition to the major binding site, affect the binding geometry and presumably could allow distinct binding geometries.

Recently, however, the NMR structures of a dimeric CCL5 mutant E66S, in complex with CS-derived hexasaccharides were reported [131]. Thanks to the relatively simple sulfation pattern of CS (compared to that of HS), 2 well-defined hexasaccharides were prepared from natural sources by partial depolymerization. One hexasaccharide, termed “CS444”, contained 3 GlcA-GalNAc4S units, and the other one, termed “CS644” was identical, except for the nonreducing end disaccharide, which contained a GalNAc6S instead of a GalNAc4S. Both chemical shift perturbation and paramagnetic relaxation enhancement experiments demonstrated that the CS644 has multiple binding modes, whereas the CS444 appears to have a more well-defined one. These results illustrate how a switch of only one sulfate position can make oligosaccharides behave differently and further emphasize the complexity of structural studies. Importantly, nuclear Overhauser effects between the CCL5 mutant and the hexasaccharide were observed, thereby defining a valuable distance restraint set for structure-resolution purposes. The structural model showed the first 2 disaccharide units from the reducing end interacted with the BBXB domain of 1 chemokine monomer, and the last disaccharide unit lying upstream along the N-terminal loop up to the N-terminal end of the second protomer (Fig. 5). All the basic residues of the BBXB domain were involved in the binding as well as Arg-17 and Tyr-3 of the other protomer, which is in agreement with NMR titration data. Interestingly, the measured proton–proton distances between aliphatic protons of the protein (Tyr31H6, Ile15H6, Leu19H6, and Lys45H6; yellow residues in Fig. 5B) and glycosidic protons also suggested that hydrophobic contacts participated to stabilize this complex.

Figure 5. Crystal structures of chemokine-GAG complexes. The structures of CCL5 in complex with either (A) a heparin-derived disaccharide GlcA2S-GlcNS6S, or (B) a CS-derived hexasaccharide (GlcA-GalNAc4S)3 and the crystal structure of CXCL12 in complex with 2 GlcA2S-GlcNS6S (C and D) are represented. The chemokine structures are shown as gray ribbons, and both residues of interest and oligosaccharides are shown as sticks. The hydrogen bonds are represented as yellow, dotted line. The figures were prepared with the PyMol software using the data available at the protein data bank, identifiers IU4L (A) and 2SWG (C and D) or from the supplementary material in Deshauer et al. [131].
THE MANY WAYS GAGS AFFECT GRADIENTS: CHEMOTAXIS, HAPTOTAXIS, DUROTAXIS

Gradients, whatever their nature, are central to directional cell migration. Oriented movement is guided by the ability of cells to sense short-range chemical or physical differences, providing them with positional information. Gradients of chemotactic agonist ligands can be either soluble or substrate-immobilized (and referred to as chemotactic or haptotactic signals). Similarly, directional cell migration is referred to as chemotaxis or, in adhesion-dependent migration, haptotaxis. In addition to these chemical cues, cells can also sense physical gradients, such as substratum stiffness.

The source–sink model—how GAGs can shape soluble gradients and chemotaxis

In its simplest mechanism, a chemotactic gradient can be formed by passive diffusion and maintained between a location of secretion (source) and a location of degradation or removal (sink), where the balance determines the gradient profile and, therefore, the responsiveness of the migrating cells. Such soluble gradients can be modulated at both sites (the source and the sink) by GAGs. It was first shown that, in CD8+ cytotoxic T lymphocytes, CCL3 and CCL5 are localized in the cytolytic granules, from which they are released upon antigen-specific activation as a macromolecular complex associated with GAGs [139]. Such a mechanism has been further investigated in CCL5-secreting breast tumor cells, in which it was demonstrated that the chemokine secretion necessitated the trafficking of CCL5-containing vesicles on microtubules from the endoplasmic reticulum to the post-Golgi, where GAG synthesis occurs. The 40s loop of CCL5, which contains the GAG binding site, was indispensable for this traffic and for the chemokine secretion. Consistently, mutated CCL5, which displays a reduced affinity for GAGs, had a reduced propensity for colocalization with GAGs in the Golgi compared with wild-type chemokines, and its secretion in cell lines deficient for GAG expression was reduced [140]. Similarly, cells deficient in GAG expression display a modified CCL2 trafficking from the Golgi toward secretion, and a CCL2 mutation that reduced GAG binding abolished CCL2 secretion [141]. Finally, in human umbilical endothelial cells, CXCL1 was found to colocalize with serglycin, an intracellular proteoglycan found in secretion vesicles. In that system, abrogating proteoglycan synthesis reduced serglycin secretion and the number of vesicles in which serglycin and CXCL1 colocalize. Consequently, the level of CXCL1 in the apical medium, where most serglycin is secreted in polarized cells, was also reduced [142]. In addition to these intracellular trafficking issues, it has also been shown that exogenously added heparin or HS-derived disaccharides can inhibit the secretion of CXCL8 from epithelial cells at concentrations as low as a few nanograms per milliliter, and interestingly, this effect was dependent on the sulfation pattern of the molecules [143]. As such, disaccharides are generated by heparanase, an enzyme used by several cells, including activated T cells, to degrade the extracellular matrix and gain access to tissues [144], this could be viewed as a mechanism to modulate the gradient profile and control cellular migration during inflammation.

Soluble gradients are also shaped by the removal (including by decay receptor; see [145, 146]) or the degradation of the chemokines, many of which have been identified as substrates for numerous proteolytic enzymes usually present in inflammatory sites, including matrix-metalloproteinases (MMP) 1, 2, 3, 8, 9, 13 and 14, CD26/DPP-IV, peptidylarginine deiminase, CD15/aminopeptidase N, cathepsin G and L, elastase, proteinase-3, urokinase plasminogen activator, chymase, plasmin, thrombin, carboxypeptidase N and furin [147–150]. Proteolytic processing, which can occur either at the N- or the C-terminal ends of chemokines, can either activate or inhibit chemokines, and interestingly, GAGs have been found to interfere with such processes. Cathepsin B processing of various chemokines was almost completely abolished in the presence of CSC. This result is consistent with the cleavage site of this enzyme being in the carboxy-terminal end of the chemokine, where the basic residues involved in GAG binding are found. GAGs may sterically hinder the C-terminal interaction between cathepsin B and chemokines. The cleavage of chemokines by cathepsins L, S, and K is also affected by GAGs, giving a more complex picture: the processing by cathepsin L and S occurs in the N-terminal part of the chemokines and was preserved in the presence of GAGs, whereas that of cathepsin K increased in the presence of GAGs [151]. Similarly, it has been found that heparin protects CCL11 from direct proteolysis by plasmin and from indirect proteolysis by cathepsin G and elastase [152]. CD26/DPP-IV, a serine protease that codistributes with CXCR4, the CXCL12 receptor, at the cell surface, mediates the selective removal of the N-terminal dipeptide of CXCL12. HS, as heparin, specifically prevents the processing of the chemokine by DPP-IV expressed by Caco-2 cells. The level of processing increases with the level of differentiation of these cells, which correlates with an increase in DPP-IV activity [153]. This result appeared to be specific to DPP-IV because GAGs did not prevent CXCL12 processing by several other proteases, including several matrix metalloproteinases [154]. In contrast to the above experiments, proteolytic degradation of CCL16 in its extended carboxy-terminus by MMP is not affected by GAGs, but gives rise to a chemokine form displaying enhanced GAG-binding affinity [148]. In addition to degrading soluble chemokines, proteases may also interfere with the function of GAG-bound chemokines. Dendritic cells, for example, have been shown to degrade part of the long and highly basic C-terminal extension that provides CCL21 with high affinity for HS, thereby releasing it from its solid phase. The truncated chemokine, which thus lacks GAG-binding domains, but remains functional for CCR7 activation, forms a soluble gradient and triggers chemotactic migration [58]. Finally, proteolytic enzymes can also cleave the core protein of proteoglycans, releasing a soluble form of the GAG chains and its potentially associated chemokines. MMP-7, for example, induces syndecan-1 shedding, generating a gradient of KC (CXCL8) that has been shown to promote neutrophil influx in a mouse model of bleomycin-injured lung [155].

Immobilized chemokines and haptotaxis

Chemokine binding to GAGs is thought to restrict their diffusion along the cell surface or within tissues, thereby providing a mechanism to localize and concentrate them in specific
compartments. GAG-mediated chemokine oligomerization was thought to have a key role in this process, presumably by sequestering the chemokines at high concentration, near their production site. Chemokines, which all have a clear structural basis for dimerization with both the N terminus and the β-sheet providing scaffolds for quaternary interactions [13], display much larger GAG-binding surfaces in their dimeric form, thus showing greater affinity for the polysaccharide than do their corresponding monomeric form [19].

Direct evidence for the involvement of chemokine–GAG interactions in generating immobilized gradients that direct the oriented migration of leukocytes has been found in recent studies. First, following the introduction of a CXCL2-containing agarose gel into the cremaster of a mouse at a controlled distance (400 μm) from a postcapillary venule, a chemokine gradient immobilized along the endothelium was visualized by confocal microscopy. Neutrophils crawling along this gradient were recruited toward the chemokine source, compared with a situation in which CXCL2 was homogeneously administered. In heparanase (a HS-degrading enzyme)-overexpressing mice, which feature shorter HS chains, the CXCL2 staining of the venular endothelium was strongly reduced, and neutrophils were found to adopt a random migration pattern, resulting in fewer emigrated cells [15]. A GAG-dependent chemokine gradient has also been visualized using a zebrafish larvae model into which a local source of CXCL8 was induced by microtransplanting cells ectopically expressing the chemokine. It was observed that, within a distance of 100 μm from the transplant, the proportion of migrating cells was greater and the cells had less-distracted trajectories when moving toward CXCL8-expressing cells than they did when moving toward mock cells. Immunohistochemistry revealed an extracellular gradient of the chemokine that decayed within a distance of 50–100 μm. In contrast, a transplant expressing a CXCL8 mutant that was unable to bind to HS but remained biologically active did not give rise to any observable gradient around the site of secretion and was less robust in recruiting neutrophils [156]. Finally, quantitative imaging of CCL21, using explanted mouse ears as an ex vivo model, revealed depots of the chemokine in lymphatic endothelial cells that decayed steeply within the perilymphatic interstitium. Interestingly, these gradients match the migratory patterns of exogenously added dendritic cells that rapidly enter the interstitium and migrate directionally toward vessels from a distance of 90 μm. In that model, the exogenous addition of the chemokine, which diffusely localized within the tissue and thereby masked and/or flattened the endogenous CCL21 gradients, resulted in a misguided dendritic cell migration that remained scattered and remote from the vessels. In contrast, addition of a truncated form of CCL21 that does not bind to GAG did not modify the migratory pattern of the cells. Furthermore, as observed in the previously mentioned studies, pretreatment of the explant with HS-degrading enzymes severely changed the CCL21 pattern and dendritic cell migration [157].

Together, these studies strongly support a role for HS in establishing chemokine gradients that are more robust and spatiotemporally stable than soluble gradients. These data raised the question of the structure and the binding capacity of individual HS molecules and the regulation of this process. As described above, HS oligosaccharide sequences are tightly controlled, particularly during disease or inflammation, and such regulation is likely to modify gradient characteristics, in terms of shape, the nature of the chemokines recruited and the kinetics of the interaction. To our knowledge, these aspects of chemokine functions have not yet been studied, and it will certainly be a coming challenge for future experiments to better understand the close relationship between GAG binding and directed cellular migration.

**Mechanical forces and durotaxis**

In addition to chemical factors, migrating cells also sense physical forces, such as gradients in substrate stiffness, a process called durotaxis [158]. GAGs, in interacting with most of the other molecules that compose the extracellular matrix and the basement membrane, are key molecules for the assembly of these structures, modulating their biophysical properties and stiffness in a tissue-specific manner [159]. As cells migrate toward areas of higher extracellular matrix rigidity, GAGs likely contribute by promoting oriented cell migration through regulating substrate rigidity. Interestingly, using biophysical techniques, such as quartz crystal microbalance and fluorescence recovery after photobleaching, various HS-binding cytokines, including chemokines, rigidify HS and that this effect, which depends on the architecture of the chemokines’ HS-binding sites, was due to protein cross-linking of the GAG chains [160]. The ability of chemokines to physically change matrix organization and mechanical properties suggests that the functions of chemokines may not be restricted simply to cellular receptor activation. In the present case, in binding to HS, chemokines induced both chemical and physical gradients in the solid phase to guide migrating cells.

**MANIPULATING THE GAG–CHEMOKINE INTERFACE TO INVESTIGATE THE ROLE OF CHEMOKINE–GAG INTERACTIONS IN VIVO**

Strategies used to analyze the functional importance of chemokine–GAG interactions in vivo are either pharmacologic or genetic. The former is based on the injection of molecules (either glycanic or peptidic) that disrupt chemokine–GAG complexes or on the injection of wild type or non-GAG–binding mutant chemokines, whose biologic activities are compared. The latter method relies on genetic manipulations to either modify HS structure or to generate mice expressing a chemokine that displays altered HS-binding properties.

In a pioneering assay, attempts were made to demonstrate the role of GAG-binding functions in the biologic activity of the chemokines CCL2, CCL4, and CCL5. Derivatives of these proteins with reduced heparin binding were made by mutating residues in a conserved BBXB cluster in the 40s loops, [14AA15] and [14AA48] for CCL4 and CCL5, respectively, and in basic residues located in the extended strand β(N), [18AA48], for CCL2. Injected into mice peritoneum, these chemokines failed to promote significant leukocyte migration, even when injected at in vivo supraoptimal concentrations, whereas the wild-type counterpart elicited a robust increase in the number of recruited...
cells to a level of 2- to 3-fold over the control [16]. These data suggested that, in vivo, these chemokines needed to be localized through their interactions with GAGs, a process that involves, or is facilitated by, oligomerization to maintain a stable haptotactic gradient. It should be noted, however, that the mutated residues in the GAG-disabled mutants were also involved in receptor binding. For example, the CCL5 (14AA15) mutant retains a high affinity for CCR5, but displayed a 200-fold reduction in CCR1 binding, whereas the CCL2 (18AA19) showed a 20-fold decrease for CCR2. These mutants also showed a reduced potency to induce chemotaxis in an in vitro and normally GAG-independent assay [16]. Therefore, the substantial reduction of chemotaxis activity displayed by the GAG-disabled mutants observed in vivo remains difficult to interpret in terms of the structure–activity relationship of the chemokine–GAG interaction. Perhaps more convincingly, experiments carried out with a derivative of CXCL12β carrying a mutated BBXB cluster in the β1 strand of the protein, displaying strongly reduced HS binding, demonstrated a lack of chemotactrant properties in vivo while showing intact CXCR4 agonist capacity [161]. Similarly, the CXCL12y isoform, which displayed sustained binding to HS when compared with CXCL12α (K_D = 1.5 vs. 200 nM), promotes intraperitoneal leukocyte accumulation and angiogenesis in matrigel plugs with much higher efficiency than does CXCL12α. Mutants of both these isoforms, selectively devoid of HS-binding capacity, but fully functional for CXCR4 binding and activation, failed to promote significant cell recruitment in vivo [17].

Next, indirect evidence supporting a biologic role of GAG binding in the functionality of chemokines was obtained from experiments in which HS mimics were injected i.v. to outcompete endogenous, HS-associated chemokines. Under these experimental conditions, performed in both mice and monkeys, a reduction in the level of CXCL12 in the bone marrow was observed, together with a concomitant increase in the blood stream. Coinciding with the disruption of the gradient between the bone marrow and the peripheral blood, the number of circulating leukocytes increased dramatically, a finding consistent with the role of this chemokine in the regulation of leukocyte settling in bone marrow and trafficking [162]. In a similar assay, i.v. injection of HS-like octasaccharides was also shown to induce a rapid mobilization of B and T lymphocytes as well as hematopoietic progenitor and stem cells, strengthening the role of HS in the retention of cells within the bone marrow through chemokine sequestering [163]. In a different strategy, but pursuing the same purpose, it has been shown that injection of synthetic peptides encompassing the HS-binding sites of chemokines can compete with chemokines for binding and immobilization on GAGs, thereby preventing the formation of a gradient and impeding the functionality of the displaced chemokine. For example, i.v. injection of the carboxy-terminal CXCL9 peptide (residues 74–103), a highly positively charged domain comprising a cluster 5 HS-binding sites (see Fig. 1), diminished the neutrophil extravasation induced by a simultaneous injection in the joint of CXCL8 [57].

Genetic evidence also supported a role for GAG–chemokine interactions in vivo. Mutant mice deficient in the endothelial expression of Ext1 (Fig. 4), the enzymes that polymerize the disaccharide units of HS, displayed reduced chemokine (CCL21 and CCL2) presentation at the endothelial cell surface. This phenotype results in both a pronounced decrease in lymphocyte adhesion to high endothelial venules and in the recruitment of resident dendritic cells through lymphatic vessels to the lymph nodes. Mutant animals show severely impaired lymphocyte homing and a compromised contact-hypersensitivity response [14, 164]. Genetic inactivation of NDST1, the enzyme that converts GlcNAc into GlcNS residues, which is thus key to the biosynthesis of the HS-binding sites (S-domains, see Fig. 4) in endothelial cells and leukocytes, led to impaired neutrophil infiltration in various inflammation models. Decreased neutrophil infiltration was partially due to altered rolling velocity correlated with weaker binding of L-selectin to endothelial cells, but chemokine transcytosis across endothelial cells and presentation on the cell surface were also reduced, resulting in decreased neutrophil firm adhesion and migration [165].

Collectively, these findings support a role for chemokine–GAG interactions in vivo, but not without ambiguous experimental setups. For example, the administration of sulfated glycans or basic peptides does not induce competitive displacements of chemokine from HS in a selective manner. Similarly, genetic interference selectively inhibiting the role of critical HS biosynthetic enzymes has broad effects and would affect, beyond chemokine binding, the interaction of GAG with a plethora of other factors. In other cases, and as a consequence of the partial overlapping or spatial proximity of chemokine domains involved in receptor-mediated cell signaling and GAG attachment, GAG-binding mutant chemokines displayed reduced receptor binding and/or agonist capacity. Exogenous administration of GAG-binding mutant chemokines with reduced agonist capacity thus makes the interpretation and significance of results tricky regarding the importance of the binding to GAGs.

Unambiguously elucidating the functionality of GAG–chemokine interactions in vivo would require the intactness of both the receptor-binding and cell-signaling properties of mutant derivatives devoid of HS binding, using an assay in which the investigated chemokine is physiologically expressed. To this aim, a transgenic mouse knock-in model, expressing a cxcl12 gene carrying mutations that selectively preclude interactions with HS while not affecting CXCR4-dependent cell signaling of all of the α, β, and γ CXCL12 isoforms was engineered. Among all the chemokines whose interaction with both HS and their signaling receptors have been biochemically and functionally characterized, CXCL12 appeared to be unique in terms of the spatial separation between these 2 binding sites, which are localized on the opposite sides of the molecule [17, 45–47], thus permitting the investigation of each domain independently. The mutant mice, which express normal levels of isoform-specific cxcl12 mRNA, showed enhanced serum levels of free CXCL12 and more circulating leukocytes and CD34+ hematopoietic cells [18]. This phenomenon could be explained by reduced cell attraction/retention in lymphoid tissues and supports the existence, in normal mice, of a pool of functional HS-sequestered CXCL12. The inability of the mutant CXCL12 to adhere to the cell surface and to the surrounding extracellular matrix of CXCL12-producing stromal cells in the bone marrow niches, could facilitate its egress from the bone marrow. Alternatively, the increased numbers of these cells in the periphery could be
ascribed to the gradient generated by the blood accumulation of free HS-binding–disabled CXCL12 proteins, that would also inhibit the return of leukocytes to the bone marrow. Interestingly, although it has been shown that progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of CXCL12 [166], these mice display dramatically reduced capacities to regenerate vascular growth after acute ischemia, which is recovered by expression of wild-type CXCL12 [18]. This indicates that bound to HS structures on the apical surface of endothelial cells, CXCL12 could be determinant for vascular targeting of circulating progenitor and inflammatory cells and the induction of both firm adhesion and transendothelial migration of infiltrating bone marrow–derived cells. This finding thus also strongly supports the role of HS interactions in the functions of CXCL12, in vivo, both in homeostasis and physiopathologic settings.

**SUMMARY AND OUTLOOK**

The essential role of protein–GAG interactions in the regulation of various physiologic processes has been now been recognized for several decades and many studies have revealed how these interactions could determine protein functions [3]. Regarding chemokines, it has been traditionally thought that GAGs capture these proteins through their C-terminal helix and functionally present them to CKR, thereby forming and/or maintaining the haptotactic gradient of concentrations along which cells can migrate directionally. If indeed GAGs provide a mechanism for establishing haptotactic gradients, we described here that, beyond immobilizing and patterning their ligands, GAGs can interfere with chemokine activities through a large variety of processes and binding geometries. These interactions can affect chemokine intracellular or extracellular storage, secretion, transport, stability, structure, the ability to form homo-oligomers or hetero-oligomers, and finally, CKR recognition and downstream signaling.

Importantly, we also wished to highlight that GAGs are characterized by fascinating structural diversity and an array of spatial and temporal regulations. Based on a simple disaccharide repeat, these biopolymers acquire an extensive range of primary sequences during biosynthesis, thereby providing a large variety of docking sites for their numerous protein ligands.

The structural heterogeneity of GAGs and the lack of appropriate tools to study their fine structures have obviously limited the investigations into their roles in chemokine regulation. Nevertheless, it is established that 1) biochemically, chemokine binding depends on the degree and pattern of HS modifications, and 2) altering chemokine–HS interactions through manipulating HS structures in vivo has important biologic consequences.

In that context, a central question is thus how HS structures are defined, controlled, and modified during pathophysiologic regulation (i.e., development, inflammation, or tissue repair) and how that would affect chemokine activities. Addressing these important questions would undoubtedly require a better understanding of the GAG biosynthetic machinery, that is, determining how the expression of HS biosynthetic enzymes is regulated, how HS biosynthetic enzymes are organized, how available their substrates are within the Golgi apparatus, and how specific combinations of these factors define specific HS motifs along the growing polymer. A second related and challenging aspect to address is to understand the extent to which the interactions between HS and chemokines are specific, keeping in mind that, similar to other HS-binding proteins, chemokine–HS complexes may be characterized by a certain degree of structural plasticity. This would require the production of structurally defined HS oligosaccharides that could be used both for resolving the structures of chemokine–HS complexes and for performing functional assays for which the results could be unambiguously correlated to the features of the oligosaccharide.

GAGs pervade the intercellular space of many tissues and establish complex networks at the periphery of virtually all cells in organisms as primitive as planaria up to human [167], supporting their involvement in very fundamental processes and implying a tight control of their structures. Clearly, chemokines, similar to many other signaling proteins, do not escape from this regulatory GAG network. Efforts to better understand the structure–activity relationships of chemokine–GAG interactions would undoubtedly open new avenues in chemokine biology.

**AUTHORSHIP**

Y.M., F.A.S., and H.L.J. wrote the text. Y.M. and H.L.J. designed the figures. H.L.J. supervised the project.

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**DISCLOSURES**

The authors declare no conflicts of interest.

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