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Minireview

Relationships between glycosaminoglycan and receptor binding sites in chemokines—the CXCL12 example

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Abstract—Chemokines are small proteins, promoting directional migration and activation of different cells through binding to specific receptors. Most chemokines also bind to heparan sulfate (HS), a family of complex and highly sulfated glycosaminoglycan (GAG) found at the cell surface and in the extracellular matrix. This class of molecules has recently emerged as critical regulators of many events involving cell response to the external environment. Binding to HS is thought to be functionally important. Current models suggested that HS ensures the correct positioning of chemokines within tissues and maintains haptotactic gradients of the proteins along cell surfaces, thus providing directional cues for migrating cells. On the chemokine surface, the GAG binding epitopes can be displayed on different areas, some of which overlap the receptor binding domain, while others are clearly separated. We review here some structural aspects of the interaction between GAGs or receptors and chemokines. In particular, we will address the case of CXCL12, a chemokine whose receptor binding site is distinct from the GAG binding site and whose different isoforms display different GAG binding abilities. This chemokine system thus offers an unprecedented opportunity to ascertain the importance of chemokine/GAG interaction in the regulation of cell migration.

Keywords: Glycosaminoglycans; Heparan sulfate; Chemokines; Receptors; CXCL12; Interactions; Structure

Contents

1. Introduction

Chemokines (or chemoattractant cytokines) represent a family of small proteins (8–12 kDa) comprising over fifty members. These proteins bind to and trigger the activation of G-protein coupled receptors, 20 of which

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have been identified, and ultimately promote the directional kinesis of a large array of cells. As such chemokines regulate many functions including routine immune surveillance, development, angiogenesis, neuronal patterning, hematopoiesis, wound healing, inflammation, viral infection, or metastasis.[1,2]

Chemokines are traditionally classified into four groups (CC, CXC, CX3C, and C) depending on the structure of a conserved cysteine-containing motif in the amino-terminal region of the molecule and are named accordingly. All chemokines have very similar tertiary structures, organized around a core domain with a triple-stranded antiparallel β-sheet ($\beta_{(1)}$, $\beta_{(2)}$ and $\beta_{(3)}$) overlaid by a C-terminal α-helix ($\alpha_{(C)}$). Upstream of the β-sheet is the N-terminal region, which consists of a more or less disordered and extended region of 6–10 residues, followed by a long loop (N-loop), and a short 3_{10} helix. The overall structure is stabilized by disulfide bonds involving N-terminal and core domain cysteines.[3]

It has been thought that, within tissues, concentration gradients of chemokines must be formed and maintained to establish directional signals for migrating cells, and this is believed to be controlled by a class of complex polysaccharides belonging to the glycosaminoglycan (GAG) family (Fig. 1). GAGs are covalently attached to core proteins, present on all animal cell surfaces and in the extracellular matrix.[4] They display medium to high affinity for chemokines, and it has been hypothesized that without such an immobilization mechanism, chemokine gradients would be disrupted by diffusion.[5]

GAGs can be classified into four groups: the hyaluronic acid (HA), the chondroitin/dermatan sulfate (CS/DS), the heparan sulfate/heparin (HS/HP), and the keratan sulfate (KS) type. These polysaccharides consist of a repeating disaccharide unit, composed of a hexosamine and a hexose or hexuronic acid, either or both of which may be sulfated on different positions. In HS, to which most chemokines bind,[6] the hexosamine is a glucosamine and the hexuronic acid is either a glucuronate or its C5 epimer, an iduronate. Depending on the extent and the position of the sulfate groups along the chain, as the level of glucuronic acid epimerization, GAGs can display a very large variety of structures. In HS, these modifications occur in restricted domains (called S-domain, usually of about 5–10 disaccharides) that are variable in sequence, and interspersed within poorly sulfated regions (Fig. 2). This variability is believed to be on the basis of the extended protein binding capacity featured by these polysaccharides.[7] HS molecules are indeed increasingly recognized as ubiquitous protein ligands, and, in addition to the above-mentioned chemokines, this includes cytokines, growth factors, morphogenetic proteins, adhesion molecules, enzymes, plasma proteins, proteins from the extracellular matrix, and apolipoproteins, … From a functional point of view, HS is thought to affect the local concentration, compartmentalization, stability, structure or activity of its ligands. As such it is involved in a large range of functions as diverse as cell proliferation, cell adhesion, matrix assembly, chemoattraction, inflammation, immune response, development, lipid metabolism, angiogenesis, and wound healing or pathogens attachment. Understanding how HS binds to proteins and regulates their functions is thus of general interest, however, it has been hindered by the extreme complexity and chemical heterogeneity of these polysaccharides. It is clear that

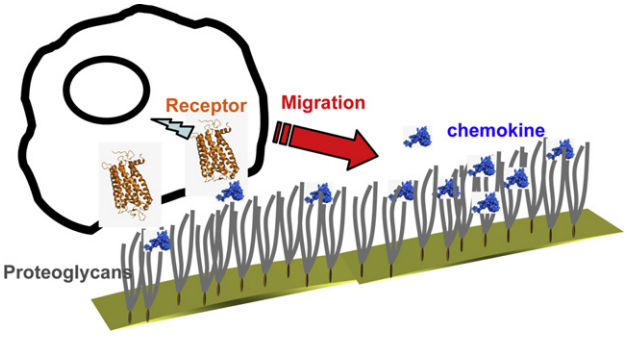

Figure 1. Schematic representation of cellular migration along a cell surface. At the cell surface, proteoglycans sequester chemokines through their GAG moieties and help maintaining chemokine gradients and/or modulate their presentation to their cognate G-coupled protein receptor.

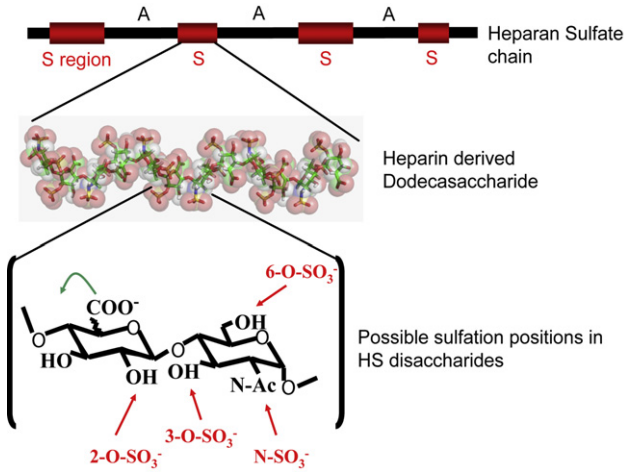

Figure 2. Heparan sulfate structural organization. HS consists of a disaccharide repeat unit composed of glucosamine and uronic acid, whose diversity stems from the differences in the number and the position of *N*- and *O*-sulfate groups along the chain (red arrows), and by the relative proportion of glucuronic versus iduronic residues (green arrow). It has been speculated that information for protein recognition resides within specific highly sulfated domains (S region) that are separated by relatively regular regions which contain predominantly N-acetylated glucosamine and glucuronic acid (A). The structure of a highly sulfated dodecasaccharide is shown (PDB:1HPN).[28]

in binding proteins, GAGs exploits the electrostatic complementarity between its anionic groups and basic amino acid sequences on the proteins on which it binds. In most cases, protein binding domains are localized within the highly sulfated S-domains, where peculiar sugar sequences and sulfation patterns may provide a certain degree of specificity.[8]

For chemokines, in addition to enhancing their immobilization and forming haptotactic gradients along cell surfaces, GAGs could influence their transport, clearance, degradation, and oligomerization. The relationships between GAGs binding and receptor binding are not well understood, and for these reasons, it has been difficult to delineate the specific contribution of GAG binding on chemokine function. Furthermore, the chemokine system is characterized by a considerable redundancy, with most chemokines binding to several different receptors and most chemokine receptors exhibiting overlapping specificity.[3] An other level of complexity is added by the fact that GAGs could potentially differentially affect chemokine function, depending on the receptor through which it signals.[9]

This review describes the current knowledge on chemokine GAG and receptor binding sites, in particular CXCL12, a chemokine in which these two binding domains are clearly non-overlapping, thus representing a simple model to investigate the mechanism by which GAGs could modulate chemokine functions.

2. The GAG binding site

The characterization of GAG binding sites remains a complex issue. Amongst studies based on heparin binding protein sequence comparison, an early work led to the identification of two binding consensus sequences, XBBXBX or XBBBXXBX clusters, where B stands for a basic residue and X for any others.[10] However, recent analyses have challenged the universality of this paradigm. Site directed mutagenesis, structural characterization of protein/heparin complexes and the development of a new approach, which relies on the proteolytic digestion of protein/heparin complexes and the subsequent identification of the heparin bound peptides by N-terminus sequencing,[11] clearly indicates that binding sites are not exclusively composed of linear sequences, but can also include conformational epitopes comprising distant amino acids organized in a precise spatial orientation through the folding of the protein. For chemokines, amino acids involved in GAG recognition are more or less scattered along the polypeptide chain, however, they systematically form well-defined clusters at the surface of the folded protein.[6] Four of these clusters have been characterized, one for each of the CC and CX_3C type of chemokines and two within the CXC family (Fig. 3). Cluster 1, characteristic of

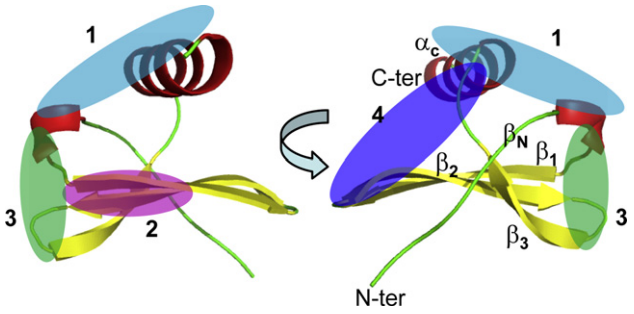

Figure 3. Localization of chemokine GAG binding sites. The localization of the four possible amino acid clusters involved in GAG binding are shown on two orientations of a chemokine ribbon structure.[6]

CXCL8 and most CXC chemokines is created by the residues of the C-terminal α-helix together with the loop connecting the extended N-terminal strand region with the first β-strand ($L_{(\beta N-\beta 1)}$). Cluster 2, which has only been observed in CXCL12, forms a crevasse at the interface between the β-strands, where three basic amino acids in both $\beta_{(1)}$ and $\beta_{(2)}$ characterize the binding site. Cluster 3 is observed in most CC chemokines and mainly consists of the loop between $\beta_{(2)}$ and $\beta_{(3)}$ strands with a typical BBXB conserved motif. Basic amino acids located at the beginning and the end of the loop connecting $\beta_{(N)}$ and $\beta_{(1)}$ also participate in the establishment of such a cluster. Finally, cluster 4 (CX_3C chemokine) comprises a flat area made up of loops between $\beta_{(1)}$ and $\beta_{(2)}$ and loop connecting $\beta_{(3)}$ and $\alpha_{(c)}$. Except for a single Lys, shared by cluster 2 and cluster 3, these GAG binding sites are not overlapping and thus represent specific binding signature of each group of chemokines. As described below, residues of these clusters may or may not be involved in receptor binding.

3. The receptor binding site

Residues participating in receptor recognition are not conserved for all chemokines (due to sequence heterogeneity). However, extensive mutational, structural and functional studies have revealed that the disordered N-terminal residues (before the first cysteines) and the N-loop residues (between the second cysteine and the 3_{10} helix) are systematically involved (Fig. 4). Ligand binding and receptor activation rely on a two-step mechanism, by which affinity and binding specificity are assumed by residues in the N-loop region, which bind to the receptor N-terminus, while receptor activation and signaling are triggered by the chemokine N-terminus which interacts with the extracellular loops 2 or 3 of the receptor.[12–14]

The NMR structure of CXCL8 (which displays a cluster 1-GAG binding site) bound to a modified peptide from the N-terminus of the CXCR1 receptor has been

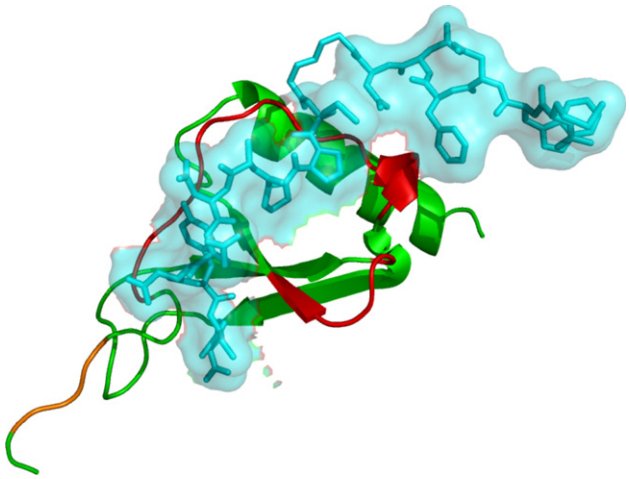

Figure 4. Localization of chemokine receptor binding sites. Complex between CXCL8 and a peptide derived from the CXCR1 receptor N-terminus (PDB:1ILP)[15] is shown. The peptide is bound in an extended conformation to chemokine residues of the N-loop, the 40s loop and the β3 strand (red). The N-terminal domain involved in signaling is displayed in orange.

reported, and illustrates the importance of the N-loop and β_3 strand which form an hydrophobic cleft where the receptor peptide binds in an extended conformation (Fig. 4).[15] The residues in this region also participate in the differential recognition of CXCR1 and CXCR2.[16] CXCL8 key signaling residues are located at the extreme N-terminus (1–6) and are thought to interact with the extracellular loops of the receptor. Binding of CCL5 (which displays a cluster 3-GAG binding site) to the N-terminus of CCR5 involves an extended surface on the N-terminus of the chemokine including the N-loop, β3 residues and the 40s loop. Mutations or acetylations of the basic residues in the 40s loop alters the binding and also the specificity of CCL5 for CCR1 and CCR5.[9,17] For CXCL12 (which displays a cluster 2-GAG binding site), the N-loop residues RFFESH (residues 12–17) are involved in binding of the chemokine to the N-terminus of CXCR4, while the first 2 residues are involved in signaling.[13]

4. Relationships between GAGs and receptor binding sites

As described above, chemokines feature a receptor and a GAG binding domain both of which are essential for proper function. In particular, GAG-binding deficient, mutated chemokines were found to be unable to induce cell migration in vivo.[18] Whereas the localization of the receptor binding site is highly conserved, chemokines residues involved in GAG binding can be displayed on four possible areas on the chemokine surface (cluster 1–4; see Fig. 3). The GAG binding clusters, depending on their positions, may or may not overlap the receptor binding domain. Thus, depending on the respective localization of the two binding domains, GAG could potentially control receptor selectivity. The differences in activity observed between a wild type and a GAG deficient binding chemokines could be either due to the lack of GAG binding or to changes in receptor affinity or selectivity. For these reasons, it has been difficult to unambiguously determine the exact role of GAGs in chemokine activity.

For three chemokines, representative of cluster 1 (CXCL8), cluster 3 (CCL5) and cluster 2 (CXCL12), the localization of the receptor binding site relative to the GAG binding site is shown in Figure 5. Cluster 4 containing chemokine will not be described here as it is only found on CX_3CL1 (Fractalkine) which, in addition, is presented at the cell surface through a transmembrane stalk. This transmembrane domain is an integral part of the protein, and the requirement of GAG binding for presentation/immobilization is, thus, not clear.

In CXCL8, the GAG and the receptor binding sites do not share common residues. However, they are in close proximity, with K20 and F21 in particular, both in the 3_{10} helix, being involved in GAG[19] and receptor binding,[14] respectively. In contrast, CCL5 shows a significant overlap between the receptor and GAG binding surfaces. The three basic residues in the 40s loop, forming a typical BBXB GAG binding motif, appeared to contribute to receptor binding affinity and specificity. In particular, mutation of these residues into alanines

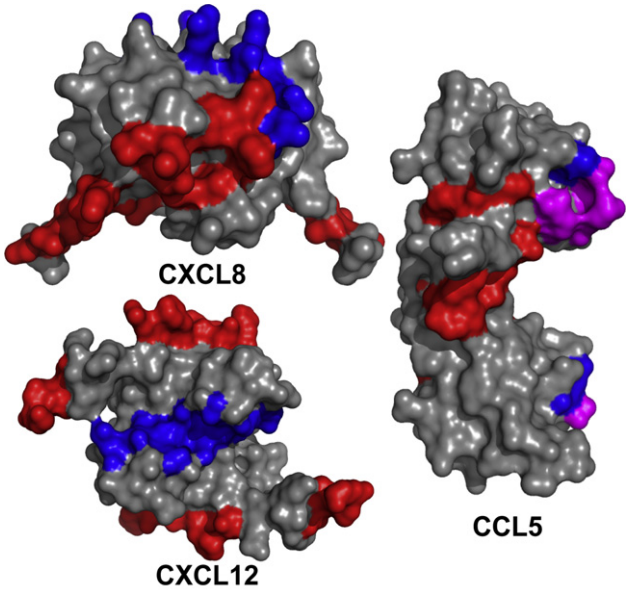

Figure 5. GAGs and receptor binding sites for CXCL8, CCL5 and CXCL12. CXCL8 (PDB:1IL8),[12,15,16,19,29] CCL5 (PDB:1B3A),[9,17,30,31] and CXCL12 (PDB:1A15)[13,20,24,32] are displayed in their dimeric form on which their GAG and receptor binding domains are, respectively, in blue and red. For CCL5, the overlap between the GAG and the receptor binding sites is shown in purple.

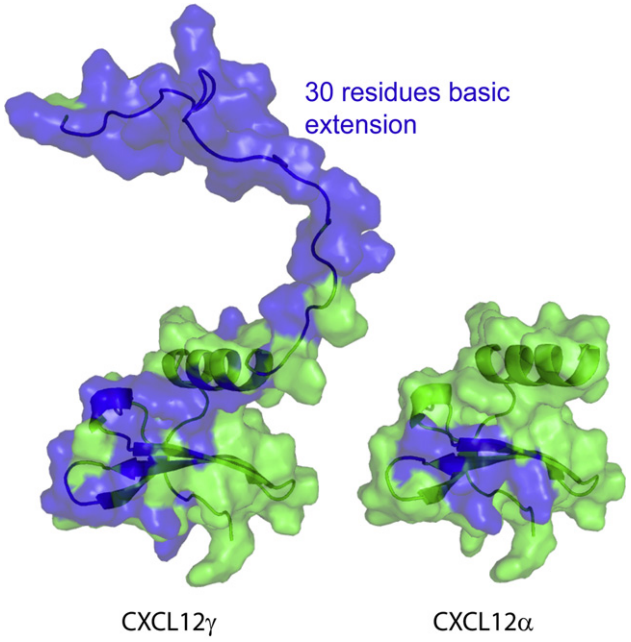

Figure 6. GAG binding surfaces of the CXCL12 α and γ isoforms. The GAG binding sites on both CXCL12α (PDB:1VMC) and CXCL12γ are shown in blue.[20,22] The γ isoforms are characterized by an extended GAG binding surface which provides the chemokine with an unprecedented high affinity for both HS and DS.

results in a significant decrease of CCR1 binding while the affinity for CCR5 remains unaffected.[9] CXCL12, which possesses a unique GAG binding cluster (cluster 2), shows a very clear separation between receptor (N-loop) and GAG (β1 and β2 strands) binding sites, which are localized on the opposite sides of the molecule. Mutation of the essential GAG binding residues does not affect receptor signaling,[20] and in vivo data showed that CXCL12, in complex with GAG-like molecules, remained active.[21] Splice variants of CXCL12 have been recently described and they were found to mostly differ in their affinity for HS and DS.[22] The existence of these different CXCL12 isoforms, thus, represents an interesting model to study the importance of GAGs in regulating the activity of chemokines.

5. A new isoform of CXL12, CXCL12γ displays unusually high GAG binding affinity

CXCL12, a chemokine highly conserved among mammalian species, was initially identified from bone marrow stromal cells and characterized as a pre-B-cell stimulatory factor. It is constitutively expressed within tissues during organogenesis and adult life. Its biological activities are mainly mediated by the G-protein coupled receptor CXCR4, to which the chemokine binds and triggers cell signaling.[23] Most data on this chemokine have been obtained from two isoforms (CXCL12α and β), arising from the alternative splicing of a single gene. CXCL12α binding to HS critically involves amino acids K24 and K27, which together with R41 form the essential part of the HS-binding site and are distinct from those required for binding to CXCR4 (Fig. 5).[20,24] A novel isoform, CXCL12γ, has been identified recently,[25] and is characterized by a distinctive 30 amino acids long C-terminal peptide (see Fig. 6). This peptide contains as much as 18 basic residues (B) nine of which are clustered into three putative 'BBXB' HS-binding domains. As shown by NMR spectroscopy, this carboxy-terminal peptide (residues 69–98) is characterized by an important flexibility, and was highly disordered in solution, while the first 68 residues of CXCL12γ have a structure very similar to that of CXCL12α with a typical chemokine fold.[22] Biacore based binding assays in which reducing end biotinylated HP, HS or DS were captured on top of a streptavidin coated sensorchip, showed that CXCL12γ interacts not only with HP and HS (as the α and β isoforms) but also with DS. Titration experiments of ^{15}N-CXCL12γ with different HP derived di-(dp2), tetra-(dp4) and octa-(dp8) saccharides showed that several resonances both in the core domain and in the γ extension were highly perturbed upon interaction, and reveal two binding domains on the protein. On the CXCL12γ core region, the most perturbed residues form a continuous surface, from R20 to R41. On the C-terminal extension, most of the residues were perturbed by the interaction in particular residues 83–97, demonstrating that the carboxy-terminal extension was strongly involved in GAG recognition, and specific mutations within these domains were found to decrease the binding reaction. Kinetic analysis also showed that the residence time on HP of CXCL12γ was equal to 525 s, but only 9 s for CXCL12α. These data demonstrated that the C-terminus which characterized the γ isoform functions in stabilizing the formed complex. This, in combination with the HP binding site of the core domain, provides the chemokine with the highest affinity for HP ever observed: $K_d = 0.9$ nM for CXCL12γ (93 nM for CXCL12α). Specific mutations within both the core domain and the carboxy-terminal sequence were found necessary to inhibit the binding reaction.

6. Conclusion

GAGs are usually highly expressed at the cell surface and constitute an abundant source of binding sites for chemokines. If it has been shown that chemokine binding to GAGs is required to promote cellular recruitment in vivo, the understanding of the mechanism by which this occurs is not yet completely clear. The simplest explanation is that GAGs provide a mechanism for retention at the site of production, and as such maintain directional signals for migrating cells. However, it

became clear that GAGs could be involved in several other aspects, including promoting oligomerization[26,27] or contributing to receptor selectivity.

The biochemical characterization of the CXCL12-HS complex showed that, in that particular system, the GAG binding domain and the receptor binding site are spatially distant, and any mutations that prevent binding to one ligand did not affect the recognition of the other. In addition, the observation that CXCL12 α and γ mostly differ by their ability to bind GAG, provides a simple system of investigation. The comparison of the activity of these two naturally occurring variants, without the need of using mutant forms, of the chemokine should give rise to a better understanding of the importance of GAGs in mediating chemokine localization in vivo.

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