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Chemokine Oligomerization and Interactions with Receptors and Glycosaminoglycans: The Role of Structural Dynamics in Function

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Abstract

The first chemokine structure, that of IL-8/CXCL8, was determined in 1990. Since then, many chemokine structures have emerged. To the initial disappointment of structural biologists, the tertiary structures of these small proteins were found to be highly conserved. However, they have since proven to be much more interesting and diverse than originally expected. Somewhat like lego blocks, many chemokines oligomerize and there is significant diversity in their oligomeric forms and propensity to oligomerize. Chemokines not only interact with receptors where different oligomeric forms can induce different signaling responses, they also interact with glycosaminoglycans which can stabilize oligomers and other structures that would not otherwise form in solution. Although chemokine monomers and dimers yielded quickly to structure determination, structural information about larger chemokine oligomers, chemokines receptors, and complexes of chemokines with glycosaminoglycans and receptors has been more difficult to obtain, but recent breakthroughs suggest that this information will be forthcoming, especially with receptor structures. Equally important and challenging, will be efforts to correlate the structural information with function.

Keywords

Chemokine structure; receptor structure; oligomerization; glycosaminoglycans; structural plasticity

Introduction

Cell migration is a complex process involving many steps where chemokines and their receptors play a central role [1, 2]. Illustrated in Figure 1 is the now established concept that upon secretion from cells, chemokines accumulate on cell surface and extracellular matrix glycosaminoglycans (GAGs) as a mechanism for their localization in specific anatomical regions, where they provide directional signals for migrating cells [3, 4]. Additionally, during inflammation, perivascular cells like tissue macrophages secrete chemokines that must be transported across the endothelium (transcytosis) and presented on the luminal cell surface in order to encounter leukocytes in the blood, and transcytosis also appears to involve chemokine:GAG interactions [5-8]. Leukocyte transmigration is subsequently

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stimulated by binding of chemokines to their receptors on leukocyte surfaces. Some chemokines first induce increased cell adhesion through receptor-mediated activation of integrins prior to leukocyte transmigration [9, 10]. Inflammatory chemokines can also trigger further cellular activation resulting in destructive processes such as lysosomal enzyme release, generation of toxic products from the respiratory burst, and T cell activation [11-13]. While the molecular players in these distinct steps of cell migration and activation are reasonably well-described, the molecular details are not. However, it is becoming evident that the complete function of the chemokine system requires dynamically changing structures and interactions including chemokine oligomer association and dissociation, binding to glycosaminoglycans and receptors, and possibly receptor oligomerization. In this review, we attempt to summarize and integrate what is currently known about the structural biology of the chemokine system, to illustrate the complexity of the system and the role of different structural states and interactions.

Chemokine structures: similar tertiary structures, but many different oligomeric states

Much is known about the tertiary structure of chemokines from NMR and crystallography [14-21]. Paradoxically, despite their diversity of functions and sequences, most chemokines have similar tertiary folds as represented by the monomer structure of MCP-1/CCL2 in Figure 2A. The high structural homology in chemokines is partially enforced by the presence of 1-3 disulfides which define and stabilize much of the architecture of these proteins. The pattern of the most N-terminal cysteines has also been used to classify chemokines into four families, and formed the logical basis for a systematic nomenclature that was established in 2002 [22]; thus there are CC and CXC chemokines which comprise the two largest families, as well as CX3C and XC chemokines which have only three members.

Chemokines typically contain a disordered N-terminal domain, which as described later, is an important signaling domain. The N-terminus is followed by an irregular loop, a 3^{10} helix, a 3-stranded β -sheet and a C-terminal helix. However, while some chemokines like MCP-3/CCL7 are monomeric [14], many chemokines dimerize in solution, and initial studies suggested a strong correlation between chemokine family and dimerization motif. CXC chemokines often dimerize into structures resembling IL-8/CXCL8 due to interactions between residues in the first beta strand, thereby forming a six stranded β -sheet structure topped by two α -helices (Figure 2C). By contrast, many CC chemokines dimerize into elongated structures like MCP-1/CCL2 with interactions nucleated by residues near the N-terminus which are largely unstructured in the monomer, but form a β -strand in the dimer (Figure 2B). Finally, under certain solution conditions, lymphotactin/XCL1, one of two highly homologous XC chemokines, can form a non-canonical dimer that has no resemblance whatsoever to the standard chemokine tertiary structure (Figure 2D) [23].

In addition to dimers, several chemokines form higher order oligomers by themselves, or upon binding to glycosaminoglycans (GAGs are described later) [24, 25]. For example, platelet factor 4 (PF4/CXCL4) forms a stable tetramer in solution [26]. The structures of MIP-1 α /CCL3, MIP-1 β /CCL4 and RANTES/CCL5, initially revealed CC chemokine-like dimers; however these structures were determined at low pH in order to destabilize the large oligomers formed by these proteins in solution [16, 27]. Recently, crystal structures of MIP-1 α /CCL3 and MIP-1 β /CCL4 were determined under more physiologically relevant pH conditions and revealed the unusual nature of these oligomers as rod-shaped, double-helical polymers (Figure 2E, F) [28]. Thus chemokines adopt a wide range of oligomeric states. Moreover, the stability of the oligomers varies quite dramatically. RANTES/CCL5, MIP-1 β /CCL4 and MIP-1 α /CCL3 are very stable as large oligomers, whereas MCP-1/CCL2 and

SDF-1/CXCL12 readily shift between monomer and dimer depending on solution conditions [29, 30], CTACK/CCL27 transitions from monomer to tetramer but only at high millimolar concentrations indicative of weak association [31], and MCP-3/CCL7 doesn't show any tendency to oligomerize [14]. These observations beg the question, what is the functional relevance of these various oligomeric forms, as well as their ability to interconvert, and the rate of these dynamic structural changes? As will be described, some insight into these question has been determined over the years, but presently, it appears that much more remains to be determined than is known.

Chemokine monomers bind receptors to cause cell migration, but oligomeric forms are functionally important

Despite the fact that many chemokines oligomerize in solution, it has been demonstrated that the monomeric form is competent to bind receptor and to induce ligand-directed cell movement (chemotaxis) *in vitro* by engineering non-oligomerizing variants [11, 30, 32, 33]; as an important distinction, here we refer to chemotaxis or cell movement as an isolated step in the overall process of cell migration illustrated in Figure 1. Amongst several engineered monomeric variants, a Proline to Alanine (P8A) mutant of MCP-1/CCL2 was shown to be incapable of oligomerization even at high millimolar concentrations, in contrast to wild type (WT) CCL2 which dimerizes at nanomolar to submicromolar concentrations depending on solution conditions. The P8A mutant also has the same affinity as WT CCL2 for its receptor, CCR2, as assessed by competitive binding assays, and it shows WT activity in the induction of chemotaxis across bare filters [30]. These and related experiments with other monomeric chemokine variants including IL-8/CXCL8 [11], RANTES/CCL5 [32] and MIP-1 β /CCL4 [33] demonstrated that the monomeric form is sufficient for receptor binding and induction of cell movement. Interestingly, in the case of CCL4, a corresponding P8A mutation as that made in CCL2, also destabilized the oligomeric form suggesting a conserved dimerization mechanism for these chemokines. Furthermore, in MCP-3/CCL7, the amino acid at the corresponding P8 position is Ser rather than Pro, suggesting that this chemokine was designed as a natural monomeric variant, and that the range of oligomerization behaviors from monomers to large oligomers likely has functional ramifications.

What then is the function of chemokine oligomers? Given the prevalence of chemokines that oligomerize, the finding that monomeric variants are fully functional in trans-filter chemotaxis assays was puzzling. However, for this industry-standard assay, a device with two chambers separated by a porous filter is used; chemokine is placed in the bottom chamber while receptor-bearing cells are placed in the top, and one measures the number of cells that migrate into the lower chamber, a scenario that does not capture the complexity of the *in vivo* situation. For example, cell surface immobilization may be required *in vivo* to prevent diffusion of chemokine in the presence of blood flow, but this is not an issue in the trans-filter assay. Furthermore, some chemokines may need to be transported across the endothelium for presentation on the correct cell surface and leukocytes may require integrin activation for arrest and firm adhesion *in vivo* (Figure 1), processes which are also not required in the trans-filter assay. To address this assay imitation, Proudfoot and colleagues examined oligomerization deficient mutants in an *in vivo* model involving recruitment into the peritoneal cavity of mice [32]. In this assay, WT or monomeric chemokines were injected into the mouse peritoneum, and the number of cells that migrated into the cavity was quantified after several hours. These studies clearly demonstrated the failure of monomeric variants of MCP-1/CCL2, MIP-1 β /CCL4 and RANTES/CCL5 to induce migration *in vivo* even though they showed robust chemotaxis *in vitro*, indicating that chemokine oligomers have a functional role. Monomeric P8A-CCL2 was also unable to induce cell migration in a thioglycollate-mediated recruitment assay, and recruitment of cells into lungs of ovalbumin sensitized mice [34]. Additionally, Luster and coworkers

engineered a monomeric variant of IP-10/CXCL10 and showed that this variant was incapable of recruiting activated T cells into airways of mice after intratracheal instillation [35]. Together these data suggest that while binding of monomeric chemokines to receptors is sufficient for inducing cell movement, that other steps in the overall process of migration must involve oligomeric forms, at least for some chemokines.

Further demonstrating that different oligomeric forms of chemokines have distinct roles, and possibly adding mechanistic information, a disulfide-locked non-dissociating dimer of SDF-1/CXCL12 was shown to be incapable of inducing cell migration *in vitro*, although it could bind CXCR4, and induce calcium flux [36]. From these data one might be tempted to speculate that the dimer could serve as a stop signal at high concentrations where the dimer is favored. Similarly, a disulfide-locked non-dissociating dimer of IL-8/CXCL8 was engineered and compared to a non-associating monomeric variant as well as WT CXCL8 (which is in equilibrium between monomer and dimer) in an *in vivo* assay of cell recruitment into the lung [37]. While the different CXCL8 variants displayed distinct profiles of cell recruitment, in contrast to the above experiments, both monomer and dimer were capable of inducing migration; however the dimer showed the most robust recruitment, the monomer was less potent and showed less sustained recruitment and WT showed sustained and steady levels of recruitment somewhat intermediate to the monomer and dimer. While the ability of the locked dimer to recruit is at odds with the disulfide locked CXCL12 dimer, this study like all of the aforementioned studies lead to a consistent conclusion that chemokine structures are in dynamic equilibrium and that monomeric and oligomeric forms are required for full functional activity *in vivo*. In addition, the Rajarathnam study as well as a study of MIP-1 polymerization [28], concluded that chemotactic responses are also dependent on the steepness of the chemokine gradient which can be significantly effected by reversible oligomerization especially involving large polymers.

The mechanisms underlying the requirement for oligomerization are not completely clear at this point. However, as will be described later in this review, it is now known that oligomerization is involved in GAG binding. Along these lines, in the work by Luster and colleagues, it was shown that WT IP-10/CXCL10 but not the monomeric form, could be retained on endothelial surfaces and induce transendothelial cell migration, leading to the conclusion that oligomerization is required for endothelial cell surface presentation, presumably through GAG interactions [35]. Additionally, Weber and coworkers demonstrated the requirement for RANTES/CCL5 oligomerization for CCR1-mediated leukocyte arrest but not for CCR5-mediated transmigration on activated endothelium under flow conditions, suggesting a role of chemokine oligomers in integrin activation [38], although a study by Ren and coworkers appears to suggest the opposite [28]. In addition to migration, oligomeric forms may also be involved in other processes such as cellular activation associated with inflammatory responses. For example, WT CCL5, but not an oligomerization destabilized form is able to promote antigen-independent activation of T-cells [12, 13]

However, as noted above, some chemokines, like MCP-3/CCL7 and I-309/CCL1, show no tendency to oligomerize alone in solution, yet they are capable of inducing cell migration [32]. Thus although it is useful to attempt to define general mechanisms, every chemokine may have its own set of unique properties. For example, although RANTES/CCL5 requires oligomerization for T cell activation, monomeric IL-8/CXCL8 is capable of inducing neutrophil activation as measured by elastase release [11].

Much more study is required to fully understand the functional consequences of these different chemokine forms, and fortunately there are quite a few engineered variants that can be exploited to answer such questions. But there is also another layer of complexity: in

addition to their ability to homo-oligomerize, chemokines can also hetero-oligomerize as discussed elsewhere in this journal issue. For example hetero-oligomers between the CXC chemokine PF4/CXCL4 and the CC chemokine RANTES/CCL5 have been shown to enhance leukocyte arrest on endothelial cells compared to CCL5 alone [39].

Chemokine-receptor interactions: The N-terminal residues of chemokines modulate receptor signaling - origin (and revision?) of the two-site model of receptor activation

The earliest structure: function studies of chemokine: receptor interactions demonstrated a fact that has been born out with all chemokines studied to date: that the N-terminus is a key domain involved in receptor signaling [40-42]. While it is not possible to predict the exact effects that mutations within chemokine N-termini will have on receptor activation -- truncation, extension, or mutation of N-terminal residues almost always impact receptor signaling responses due to stabilization of different receptor conformations. Frequently, truncation results in the conversion of chemokine agonists into partial agonists or antagonists. In some cases like IL-8/CXCL8 and fractalkine/CX3CL1 [43, 44], truncation results in a significant loss of receptor binding affinity, while in other cases such as MCP-1/CCL2 [45], truncations can be identified where little affinity is lost but the ability to promote cell migration or other activities is completely abrogated. By contrast, truncation of the mature forms of other chemokines like HCC-1/CCL14, converts them into more active agonists [46]. Importantly, N-terminal processing by proteases is known to occur readily *in vivo*, linking these observations to natural regulatory mechanisms of chemokine activity [47].

In addition to truncation, extension of chemokine N-termini can also modulate receptor activity. Retention of the initiating methionine in bacterially expressed RANTES/CCL5 (Met-RANTES) and MCP-1/CCL2 (Met-MCP-1) resulted in their conversion from agonists into antagonists of CCR1/CCR5 and CCR2, respectively [48, 49]. By contrast, extension of CTACK/CCL27 with a Phe converted it to a superagonist of CCR10-mediated cell migration across bare filters and through endothelial cells (Jansma, in preparation for JBC). Extensions of chemokine N-termini can also modulate the intracellular trafficking behavior of receptors. A dramatic example of the latter involves a variant of RANTES/CCL5 that has an amino-oxypentane (AOP) group appended to its N-terminus. In contrast to RANTES/CCL5, an agonist which causes receptor internalization and recycling of CCR5, AOP-RANTES is a superagonist that not only causes internalization, but it keeps the receptor off the cell surface. This finding is under exploration as a strategy for developing CCL5-based inhibitors of HIV entry, not only through direct inhibition of HIV interaction with CCR5, but by sequestering CCR5 intracellularly so that it is unavailable for viral entry [50-52].

The importance of the N-terminus has given rise to the notion that it is the key signaling trigger in chemokines, and likely interacts in a pocket formed by the receptor transmembrane helices [53], much like small molecule binding sites in other GPCRs whose structures have recently been determined [20, 54]. Beyond the N-terminal cysteine residues, the rest of the chemokine "core domain" is thought to generally contribute to binding affinity and specificity, and to interact with the N-terminus and extracellular loops of the receptor [20, 54, 55]. Several NMR studies using peptides from the N-terminal domains of receptors have demonstrated direct interactions with chemokine core domains [56-58], and the structure of an N-terminal sulfo-tyrosine containing peptide from CXCR4 bound to an SDF-1/CXCL12 dimer has been determined [36]. This apparent modularity in the binding and signaling functions has led to a "two-site" model of receptor activation with the chemokine core domain conferring the "site one" docking domain and the chemokine N-

terminus acting as the “site two” signaling trigger [55, 59]. Prior to the recent X-ray structures of CXCR4, many reports presented cartoons similar to the hypothetical model of CXCL12 bound to CXCR4 in Figure 3, in attempts to incorporate the concept of the two-site model, with the N-terminus of the receptor interacting with the chemokine core domain (site one) and the signaling domain penetrating into the receptor helical bundle (site two) [55]. Although the recent structures of CXCR4 were determined as complexes with small molecule and peptide antagonists, molecular modeling studies suggest that the orientation and stoichiometry of chemokine:receptor complex in Figure 3 may not be accurate as discussed in the next section. Nevertheless, these models have served as useful representations of the observation that binding and signaling can be decoupled in chemokines. Further, in support of the two-site model, a recent NMR study showed that the small molecule CXCR4 antagonist, AMD3100, could dislodge the CXCL12 N-terminal signaling domain from the receptor helical bundle, without displacing the chemokine core domain [60].

Structures of CXCR4 in complex with a small molecule antagonist and a peptide inhibitor reveal an unusually large ligand-binding pocket, and suggest possible interactions with CXCL12

Since 2007, there has been a relative explosion in the determination of G protein coupled receptor (GPCR) structures, with CXCR4 being one of five receptors solved besides rhodopsin, the only GPCR structure that was known until this time [61]. Compared to the other receptors, CXCR4 is the only receptor whose natural ligand is a protein, and perhaps unsurprisingly, the binding pocket is much larger and more open than the other structurally defined GPCRs that are activated by small molecules. Nevertheless, for technical reasons, CXCR4 was first solved in complex with a small molecule antagonist, IT1t, and a 16-residue cyclic peptide antagonist, CVX15 (Figure 4), both of which block SDF-1/CXCL12 binding and have activity in preventing HIV infectivity.

Both of the compounds were found to bind in the same pocket of the receptor, with IT1t localized at the base and the bulky CVX15 peptide filling most of the cavity (Figure 4). Like the SDF-1/CXCL12 N-terminus, the CVX15 peptide is highly basic, and complemented by the acidic nature of the receptor pocket. Both compounds also make contacts with residues in the receptor where mutations have been reported to disrupt CXCL12 binding and signaling, suggesting the possibility that they occupy the binding pocket of the CXCL12 N-terminal signaling trigger.

The overall acidic nature of the receptor extracellular domain compared to the highly basic nature of SDF-1/CXCL12, as well as the fact that the receptor structure revealed a dimer in all five structures solved (discussed further below) suggests other possible orientations and stoichiometries of the chemokine relative to the receptor, compared to the 1:1 complex illustrated in Figure 3. For example, for a migration-competent complex involving a CXCL12 monomer, one could also envision a 1:2 ligand:receptor complex, or a 2:2 complex with two CXCL12 monomers bound independently to each of the two CXCR4 subunits in the dimer. Additionally, for a 1:2 ligand:receptor complex, one could also imagine the core domain of CXCL12 binding into the pocket of one subunit of the receptor, and the signaling domain binding in trans into the pocket of the other receptor subunit (Figure 5). As mentioned, oligomeric forms of chemokines appear to be functional, and a 2:2 complex involving the CXCL12 dimer binding to the CXCR4 dimer also seems feasible. Clearly, multiple structures of CXCR4 with CXCL12 will be required to fully understand the nature of these complexes, and well designed biochemical experiments will be needed to determine their functional relevance. More generally, the CXCR4 structure represents a major

breakthrough in the chemokine field, and the technology developed to accomplish this endeavor should be applicable to the solution of many other chemokine receptor structures.

Like chemokine ligands, the receptors form homo and hetero-oligomers

Homo and heterodimerization of GPCRs has emerged as an important but incompletely understood aspect of GPCR structure and regulation, and the CXCR4 structure stands apart from other structures of GPCRs solved thus far in that it is the only one that has crystallized as a dimer [61]. Elsewhere in this journal issue, chemokine receptor homo and hetero-oligomerization is discussed and we therefore limit our discussion of this topic. However, it is important to note that CXCR4 has previously been shown not only to homodimerize *in vivo* but to heterodimerize with other chemokine receptors including CCR2 and CCR5 [62, 63].

Surprisingly, dimerization appears driven primarily from interactions between helices V and VI rather than through helix I or IV/V as previously predicted. However, as all five crystal forms revealed the same dimer interface, the result is likely real rather than an artifact of crystallization. Fortunately the structures should facilitate computational predictions of mutations that destabilize dimer formation allowing for further validation of the relevance of the observed interface. Such mutants would also serve important roles in establishing the functional relevance of CXCR4 dimerization. For example, in WHIM syndrome, CXCR4 is truncated and results in enhanced signaling due to the reduced ability of the receptor to desensitize and internalize following ligand binding. Thus dimerization with WT CXCR4 has been proposed as a mechanism to explain the dominance of mutant CXCR4 over WT in heterozygotes, where the mutated receptor could retain WT CXCR4 on the cell surface in a pseudo-heterodimeric complex [64].

It remains to be determined whether similar modes of dimerization observed in the CXCR4 homodimer are also observed in other receptor homo- and heterodimer structures. However, there is little sequence conservation in the helix V/VI interface amongst different chemokine receptors, which may suggest that there are other modes of oligomerization..... similar to chemokines? Thus, like the ligands, the receptors appear to oligomerize; the question is, what is the functional relevance and how diverse are the structures?

Chemokine interactions with glycosaminoglycans (GAGs): structural plasticity in chemokine:GAG recognition

In addition to their interactions with chemokine receptors, some if not all chemokines have essential interactions with glycosaminoglycans (GAGs) (Figure 1). Interactions with GAGs was initially anticipated as a mechanism to localize and present chemokines on cell surfaces as haptotactic gradients to guide cell migration, and to prevent diffusion of chemokines away from their site of release, especially under conditions of blood flow [24, 65, 66]. Other processes may also involve GAG interactions such as transcytosis of chemokines across cells, protection from proteolysis, co-receptor functions and even signaling [5, 6, 8]. Chemokines tend to be highly basic proteins, whereas GAGs such as heparin and heparan sulfate, have a high density of negative charge from sulfate groups, thus it was not surprising that interactions between chemokines and GAGs could be demonstrated *in vitro* [24, 67, 68]. However, in order to prove that the interaction of chemokines with GAGs is functionally relevant, chemokine variants of MCP-1/CCL2, MIP-1 β /CCL4 and RANTES/CCL5 were designed such that they maintained the ability to bind receptor and could induce cell migration in trans-well assays *in vitro*, but they lacked the ability to bind heparin [25, 69]. These mutants were then tested *in vivo* in the intraperitoneal recruitment assay, and similar to the non-oligomerizing variants described above, they failed to induce migration [32].

GAG-binding deficient mutants of other chemokines such as MCP-3/CCL7 and SDF-1/CXCL12 have also been shown to be functional *in vitro* but non-functional *in vivo* [70, 71], and together these studies clearly established the importance of glycosaminoglycan interactions in chemokine function.

So how do chemokines interact with GAGs? In most studies aimed at defining the GAG binding sites on the surface of chemokines, basic residues within linear sequence motifs such as BBXB (where B is a basic residue) were mutated, and the effects of the mutations on heparin binding *in vitro* were assessed. Such studies revealed a great deal of topological diversity in the location of the binding sites: the C-terminal helix in IL-8/CXCL8, the 40s loop in MCP-3/CCL7, MIP-1 α /CCL3, MIP-1 β /CCL4 and RANTES/CCL5, the 20s loop in SDF-1/CXCL12, and the 50s cluster in I-TAC/CXCL11 [20, 72]. However, it has also been demonstrated that many chemokines oligomerize on GAGs, suggesting that the GAG-binding epitopes may be significantly larger and more distributed in the context of dimer and higher order oligomeric structures than on the surface of the monomer structures. For example MCP-1/CCL2, which is a dimer in solution (Figure 2B) was shown to form tetramers in the presence of GAG [25]. Fortuitously, a tetramer structure was captured by crystallography (Figure 6A); and when the GAG binding epitopes were mapped onto this structure, a continuous epitope enveloping the tetramer emerged, providing a compelling binding site for a linear sulfated GAG (Figure 6B) [25]. Several other tetramer structures of chemokines have now been crystallized, and similarly, it has been proposed that these structures may represent GAG-binding forms (Figure 6C-F). These findings have led to the hypothesis that oligomerization may increase the affinity of chemokines for GAGs by providing a more extensive binding surface, which may be important for cell surface presentation especially under flow. Although more speculative, it has also been suggested that different types of GAGs, which are tremendously heterogeneous, could induce different chemokine structures, which in turn could contribute to the specificity of cell migration [65]. Oligomerization may also be required for chemokines to simultaneously bind receptor and GAG when the GAG and receptor binding epitopes of the chemokine overlap, as is the case for many oligomerizing chemokines (Figure 7). Of course, this hypothesis assumes that the chemokine interactions with receptor and GAG are simultaneous as illustrated in Figure 1C, which is currently not known.

However, oligomerization on GAGs is not the only mechanism for chemokine presentation (Figure 7). Some chemokines have long disordered tails that extend beyond the chemokine domain. In the case of SDF-1 γ /CXCL12 γ , an isoform of the more widely studied α isoform the ~30 residue tail is rich in basic residues, suggesting a role in GAG binding, as was shown to be the case [73, 74]. Whereas WT CXCL12 γ bound heparin sulfate with an unusually high affinity (~0.9nM), a mutant with the C-terminal basic residues mutated to serine had reduced affinity (~10.4 nM) and a second mutant with two additional basic residues mutated in the core domain, was devoid of GAG-binding.

In contrast to chemokines that oligomerize on GAGs, those with C-terminal tails may have non-overlapping receptor binding and GAG binding epitopes so that oligomerization is not required for simultaneous binding to GAGs and receptors. It is also interesting to note that CXCL12 γ has a much higher affinity for heparin, heparan sulfate and dermatan sulfate (DS) compared to CXCL12 α which lacks the tail and in fact has no affinity for DS. As an unstructured domain that can potentially fold into different conformations to accommodate many GAG partners, such a tail could confer promiscuity in GAG recognition as the data would seem to suggest.

Perhaps the most dramatic example of structural plasticity in GAG binding relates to the XC chemokine, lymphotactin/XCL1. XCL1 can adopt two completely different conformations --

a canonical chemokine structure which binds to the lymphotactin receptor, and a novel β -sandwich dimer that binds to GAGs (Figure 2D) [23]. While each of these structures are preferentially populated at different temperatures and salt concentrations, it seems likely that *in vivo*, binding to GAGs or the lymphotactin receptor would stabilize the relevant forms. Obviously, the interactions of XCL1 with receptor and GAG must be mutually exclusive since a global conformational change is involved in recognition of the lymphotactin receptor versus GAGs. This scenario contrasts to oligomerizing chemokines where the receptor and GAG binding sites often overlap, and with chemokines like CXCL12 γ where they may be independent.

In summary, chemokines can recognize different GAGs not only through binding epitopes in the context of tertiary structures, but through changes in oligomerization state, changes in folding of unstructured domains, and even global conformational changes - all cases of structural plasticity. A challenge for the future will be to determine structures of chemokine:GAG complexes, as well as the specificity and the diversity of these structures, and their precise roles in chemokine biology. Thus far, such endeavors have been thwarted by the difficulties in working with GAGs which are heterogeneous and hard to synthesize. However new technologies in glycobiology are gradually lowering these barriers.

Putting it all together

Overall, the structural biology of the chemokine system is much more complicated than initially expected, despite the apparent simplicity of the ligands. Oligomerization, a hallmark of many chemokines, is at the heart of this complexity, as monomers through oligomers are essential for the full repertoire of chemokine induced functions in ways that we are only beginning to understand. These various forms are intimately tied to their required interactions with receptors and GAGs. Based on the findings presented above, one can attempt to suggest some (possibly incorrect) generalizations about interactions that may be operative during the various steps in migration: (i) First, since many chemokines oligomerize on GAGs, oligomeric chemokine:GAG complexes are likely involved in transcytosis and presentation on endothelial surfaces as shown in Figure 1A and B. There are possible exceptions: chemokines like MCP-3/CCL7 may not oligomerize, and SDF-1 γ /CXCL12 γ may use its C-terminal GAG hook. (ii) Next chemokines interact with receptors on leukocytes. Here it is unclear whether chemokines immobilized on GAGs are simultaneously presented to receptors as illustrated in Figure 1C, or if chemokines dissociate from GAGs first and then bind receptors. Nevertheless, given the prior step, the first structure that the receptor encounters could be chemokine in an oligomerized form (Figure 1C) with the noted exceptions of non-oligomerizing and CXCL12 γ -like chemokines. (iii) The structures involved in receptor-mediated leukocyte arrest are a bit less clear with some evidence in favor of oligomerization [38] and other evidence suggesting the opposite [28]. However, this discrepancy may be a consequence of chemokine-specific functions such that oligomeric and monomeric forms are both involved, depending on chemokine. (iv) There is substantial evidence that monomeric forms of chemokines bind receptor and induce cell migration/extravasation; if previously oligomerized, they must disassociate (Figure 1D). (v) Once extravasated into beds of high chemokine concentration, oligomeric forms could again come into play to halt migration (as perhaps suggested by dimeric SDF-1/CXCL12) [36], or to induce leukocyte activation as described for RANTES/CCL5 [12, 13] (Figure 1E). Obviously the whole process is likely more complicated and issues like the affinity of oligomers and the rate of dissociation may be critically important. What seems indisputable however is the importance of dynamic exchange between interacting partners and that these different structure have roles in the discrete steps of cell migration.

Concluding remarks: Implications of chemokine structure and interactions for drug development

As implied above, there is much to learn in order to understand how chemokines truly function at the molecular level. However, even with the information at hand, it appears that there are many opportunities for disease intervention, as interfering with any step of the overall process of cell migration may be sufficient for blocking disease-related chemokine activities. The most conventional approach for interfering with chemokines involves targeting receptors with small molecule antagonists. With the recent breakthrough in the structure determination of CXCR4, more receptors are likely to yield to structure determination which may significantly accelerate progress in the development of such molecules. Typically small molecules are targeted towards binding pockets in the transmembrane helices. However, a novel approach was recently demonstrated, targeting the binding sites on the ligand for the receptor N-terminus [75].

Less conventional approaches that capitalize on interfering with other interactions besides the receptor, have also shown success. For example a GAG-deficient chemokine variant of RANTES/CCL5 was shown to significantly reduce clinical severity in a murine model of multiple sclerosis (experimental autoimmune encephalomyelitis, EAE) [76], and it also inhibited the progression of established atherosclerosis in mice [77]. As the mutant is crippled in its ability to bind GAGs and to form large oligomers, it was concluded that the inhibitory activity was primarily due to the formation of heterodimers with WT CCL5, resulting in improper presentation on endothelial cell surfaces and impaired leukocyte arrest. Additionally, the oligomerization-deficient chemokine P8A-CCL2 showed significant disease amelioration in a rat model of adjuvant-induced arthritis (AIA) [78], and in a murine model of EAE [34]. While the mechanism is not entirely clear, in the EAE study, P8A-CCL2 prevented leukocyte adhesion while WT CCL2 had no effect, suggesting that the monomeric form may block the induction of integrin activation, and in the AIA model it reduced the production of inflammatory cytokines. These and other compelling examples [70, 71] suggest that as we learn more about the molecular mechanisms of chemokine-mediated cell migration, additional novel strategies for therapeutic intervention may emerge.

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Figure 1.

Cartoon depicting the steps in cell migration where various chemokine structures and interactions may come into play. (A) Chemokines secreted into the extravascular space bind to GAGs and are transcytosed to the luminal side of the endothelium where (B) they are presented on the endothelial surface to chemokine receptors on leukocytes in the blood. Many chemokines oligomerize on GAGs although there are other mechanisms for transport and presentation as described in the text (see Figure 7). (C) Chemokines bind to receptors, in some cases causing leukocyte arrest and firm adhesion, and there is some evidence that oligomeric forms of chemokines are involved in this process. (D) The monomeric forms of chemokines cause cell movement, which is now well-established. (E) Following extravasation, oligomerized chemokines may provide stop signals as possibly suggested by a disulfide locked dimer of SDF-1/CXCL12 or they may cause activation of leukocytes as demonstrated by RANTES/CCL5.

Figure 2.

(A) Monomeric and (B) dimeric structures of MCP-1/CCL2 (PDB ID 1DOM) [79]. (C) Dimer Structure of IL-8/CXCL8 (PDB ID 1IL8) [80]. (D) Non-canonical dimer of lymphotactin/XCL1 (PDB ID 2JP1). This non-canonical structure predominates at high temperature and low ionic strength (40°C, no salt) and binds to GAGs, whereas the canonical chemokine fold is stabilized by low temperature and high salt concentrations (10°C, 200 mM NaCl), and binds the lymphotactin receptor [81]. (E) and (F) polymeric form of MIP-1α/CCL3 from the side and down the helical axis (PDB ID 2X69) [28].

Figure 3.

Model of CXCR4 with SDF-1/CXCL12 depicting how chemokines were roughly hypothesized to interact with chemokine receptors until the recent crystal structure of CXCR4 was determined. The homology model of CXCR4 was generated based on the β 2-adrenergic structure as a template; the conformation of the receptor N-terminus in complex with SDF-1 was inherited from the complex solution structure (PDB ID 2K05) [36]. Based on the results of mutagenesis studies, a restraint was imposed to ensure the spatial proximity of the CXCR4 transmembrane pocket residue, Glu288, with the N-terminal Lys1 of SDF-1. The model illustrates the so called “two-site model of receptor activation” involving two hypothetical interactions: the interaction of the NT signaling domain of the ligand with the receptor helical bundle, and the interaction of the “core domain” of the ligand with the N-terminus and extracellular loops of the receptor. In this model, SDF-1/CXCL12 is shown in pastel colors (blue, pink and red); K1 and K68 are the N and C-terminal residues of the ligand. The receptor is shown in orange where the N-terminus, ending in M1, is wrapped around the chemokine core domain. The figure was prepared using ICM software (www.Molsoft.com).

Figure 4.

Structure of CXCR4 with the small molecule antagonist IT1t (left) and with the cyclic peptide CVX15 (right); both ligands are shown as yellow space filling models (PDB ID 3ODU and 3OEO) [61]. The receptor is colored according to electrostatic potential from red (negative) to blue (positive), in order to highlight the acidic nature of the binding pocket, which is shown in red. The structure of the receptor is clipped in order to visualize the pocket and the helices are shown as white ribbons. Only one subunit of the receptor dimer is illustrated. Figures were prepared using ICM software (www.Molsoft.com).

Figure 5.

Structures of the CXCR4 dimer and the CXCL12 monomer and dimer (PDB ID 2J7Z) colored according to their electrostatic potential from red (negative) to blue (positive), in order to highlight the charge complementarity of these proteins. On the left, the CXCR4 structure is shown in two orientations -- on the top looking into the ligand binding pocket and on the bottom, from the side of the dimer. The top right shows the monomer and dimer of CXCL12; the bottom right shows the structure of the CXCR4 dimer, clipped, in order to illustrate the binding pocket and that multiple stoichiometries and orientations of the CXCL12: CXCR4 seem feasible, as described in the text (no orientations are implied in the figure). Figures were prepared using ICM software (www.Molsoft.com).

Figure 6.

Structures of chemokine tetramers. (A) The MCP-1/CCL2 tetramer is illustrated in a space filling representation and (C) with a ribbon diagram, where each subunit is color coded differently (PDB ID 1DOL) [79]. (B) The MCP-1/CCL2 tetramer rotated by 90 degrees from the view in (A), highlighting the GAG-binding epitopes in light blue to illustrate how the GAG binding site wraps around the tetramer. (D) The tetramer of fraktalkine/CX3CL1 (PDB 1D 1F2L) [82]. (E) The tetramer of human IP-10/CXCL10 (PDB 1D 107Y) [83]. (F) The tetramer of murine IP-10/CXCL10 (PDB 1D 2R3Z) [84].

Figure 7.

Different mechanisms for presentation of chemokines on GAGs to receptors on leukocytes. Note that it is not known whether chemokines interact simultaneously with GAGs and receptors as implied in the figure, or if the interactions are mutually exclusive. (A) Presentation of oligomerized chemokines on GAGs; this mechanism may be operative when the GAG and receptor binding sites overlap as in the case of MCP-1/CCL2 and many other chemokines. While only an hypothesis, oligomerization would permit binding of some chemokine subunits to the GAG while other subunits would be available for receptor binding. (B) Some chemokines like SDF-1 γ , have long unstructured C-terminal tails that can be used as GAG “hooks”; in this case the GAG binding and receptor binding epitopes may be independent allowing for simultaneous interaction of chemokines with GAGs and receptors. (C) Although not discussed in the text, it is worth noting that non-signaling seven transmembrane receptors can act as presentation molecules; for example, CCRL2 acts as presentation scaffold for the chemoattractant chemerin [85]. (D) Furthermore, fractalkine/CX3CL1 and CXCL16 are attached to long mucin-like stalks which are tethered to the membrane by single transmembrane helixes. In addition to providing a presentation mechanism for these chemokines, the tethered chemokines act directly as adhesion molecules [86].