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The Structural Biology of Chemokines

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Summary

This chapter provides an overview of the literature in the field of the structural biology of chemokines. The secondary, tertiary, and quaternary structures as determined by x-ray crystallography and nuclear magnetic resonance are compared among the four chemokine families. The biological significance of chemokine structures is explored through a discussion of additional molecules that interact with the chemokines. Specific interactions of chemokines and their receptors are discussed as are interactions between chemokines and glycosaminoglycans. Additionally, a set of tables and figures summarizes the structural information available in the databases.

Key Words: Chemokines; oligomerization; GPCR; GAGs; heparin; crystallography; NMR; structure.

2.1. Introduction

Infection and tissue injury induces the migration of cells that results in the innate and/or adaptive response. The migration of these cells—macrophages, neutrophils, mast cells, basophils, natural killer (NK) cells, and lymphocytes—is initiated by a superfamily of secreted proteins known as chemokines. In addition to the proinflammatory response first described for chemokines, some chemokines and their receptors serve other important physiologic functions. These include, but are not limited to, roles in embryonic development during organ vascularization (1–3), T- and B-cell development (4,5), neuronal communication (6), and CD34 stem cell mobilization (7). Chemokines function by activating specific G protein–coupled receptors (GPCRs). In certain

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circumstances infectious agents have usurped chemokine receptors for their own benefit. Additionally, chemokines and chemokine receptors are known to be responsible for pathophysiologic or genetic diseases. A primary example of the chemokine receptors involved in pathologic roles are CCR5 and CXCR4, which are the two major HIV-1 coreceptors that allow entry of the virus into cells. Some forms of the genetic disease warts-hypogammaglobulinemia-infections-myelkathexis (WHIM) syndrome are due to mutants at the cytosolic C-terminal end of CXCR4 (8). Among many other diseases that involve chemokines and their receptors are atherosclerosis (9,10) and cancer proliferation (11–13) and metastasis (14). The physiologic and pathologic roles of chemokines and their receptors are described in greater detail in other chapters in this book.

Chemokines were initially discovered as secreted proinflammatory proteins of about 8 kD defined by four conserved cysteine residues that are disulfide bonded. The chemokine superfamily has now been extended to proteins that contain one, two, or three disulfides. The chemokine superfamily is divided into two major families containing multiple proteins and two minor families, each with only one chemokine (Table 1). The nomenclature of chemokines and their receptors was standardized in the year 2000 based on the sequence of the cysteine residues in chemokines (15). One of the two major families is defined by an intervening amino acid between the first two cysteines and is known as the CXC (or α -) chemokine family. In the CC (β -) chemokine family, the first two cysteines are adjacent to each other. The protein in the CX3C family has three amino acids between the first two cysteines. In these three families, the first cysteine forms a disulfide bond with the third conserved cysteine in the family, and the second cysteine forms a disulfide with the fourth cysteine residue. Some chemokines have six cysteines, with the two extra nonconserved cysteines forming a third disulfide. The protein in the XC family only has one disulfide. The designation of each receptor is based on the family of the chemokine(s) that activates it. For example, the receptor CXCR1 is activated by CXCL8, CCR1 is activated by numerous CCL agonists (Table 1), and CX3CR1 and XCR1 are activated by CX3CL1 and XCL, respectively. It should also be noted that two chemokines, CX3CL1 and CXCL16, are domains of single transmembrane proteins that are important for the physiologic function of these proteins. CX3CL1 is the N-terminal domain of fractalkine, a heavily glycosylated protein with a mucin stalk domain of 227 amino acids and a cytoplasmic domain of 37 amino acids (16). CXCL16 is the N-terminal domain of a 176-residue ectodomain of another protein with a 27-amino-acid cytoplasmic sequence (17).

The organization of chemokine families based on the cysteine sequence has functional significance. Some human chemokines can compete for binding and activation of receptors with other intrafamily chemokines. This raised the possibility that significant structural differences in chemokine-receptor interactions

exist among proteins in the four families that result in activation of receptors exclusively within a family. The first three-dimensional structures of CXC and CC chemokines showed remarkable differences in the quaternary arrangement of dimers that could explain the source of the family-specific binding and activation of receptors. However, subsequent experiments indicated that the interactions between chemokines and their receptors are not as straightforward as originally thought. For example, the three-dimensional structure of the CC chemokine CCL20 (18) displays the quaternary structure of a CXC chemokine. In addition, the CXC agonists of CXCR3 also function as antagonists of the CCR3 receptor (19,20), violating the hypothesis regarding competition for binding to receptors with only intrafamily chemokines. However, activation of receptors across chemokine families has not been detected. Interestingly, a herpesvirus-8 CC chemokine, viral macrophage inflammatory protein (vMIP)-II, has evolved to bind to receptors from all four families. In one case, vMIP-II functions as a CC receptor agonist (21), but in most cases it is a chemokine receptor antagonist (22–24). These observations complicate the development of a general mechanism to explain the structural basis of functional activity (agonism or antagonism) for chemokines.

2.2. Chemokine Structures

Tables 2 and 3 present lists of the known three-dimensional structures of human and viral chemokines. Virtually all chemokine structures, regardless of family, have the same monomeric structure. A flexible N-terminal region precedes the first cysteine and is involved in receptor activation. After the N-terminal region is the 10- to 20-residue N-terminal loop that is generally involved in receptor specificity, a short 3_{10} helix, a β -sheet composed of three antiparallel β -strands, and a C-terminal α -helix that packs against the β -sheet (Figure 1A). The loops connecting the first and second β -strand and the second and third β -strand are known as the 30s and 40s loops, respectively. One generic difference between monomers from each major family has to do with the chirality of the disulfide bonds. The chirality of the first disulfide is predominately right-handed in CXC chemokines and left-handed in the CC chemokines, whereas the second disulfide is left-handed in both families. However, both disulfides for CXCL12 stromal cell-derived factor (SDF-1 α) are left-handed. This is consistent with a hypothesis that CXCL12 may be most closely related to the evolutionary ancestor of the chemokine superfamily based on low sequence homology to both CXC and CC chemokines (25).

The oligomeric structures of chemokines vary. CXCL8 (IL-8), the prototypical CXC chemokine, is dimeric in solution (26) and in its crystalline form (due to twofold crystallographic symmetry) (27). The three-stranded β -sheet of each subunit joins to form a six-stranded β -sheet (Figure 1B). The two C-terminal

Table 1
Families of the Known Chemokines

CXCL family		Ligand	CCL family
Ligand	Corresponding CXCR		Corresponding CCR
CXCL1 (GRO- α)	CXCR2	CCL1 (I309)	CCR8
CXCL2 (GRO- β)	CXCR2	CCL2 (MCP-1)	CCR2, CCR11
CXCL3 (GRO- γ)	CXCR2	CCL3 (MIP-1 α)	CCR1, CCR5
CXCL4 (PF-4)	CXCR3B	CCL4 (MIP-1 β)	CCR5, CCR8
CXCL5 (ENA-78)	CXCR2	CCL5 (RANTES)	CCR1, CCR3, CCR4, CCR5
CXCL6 (GCP2)	CXCR1, CXCR2	CCL6 (Muc10)	CCR1
CXCL7 (NAP-2)	CXCR2	CCL7 (MCP-3)	CCR1, CCR2, CCR3
CXCL8 (IL-8)	CXCR1, CXCR2	CCL8 (MCP-2)	CCR1, CCR2, CCR3, CCR11
CXCL9 (Mig)	CXCR3	CCL9 (MIP-1 γ)	CCR1
CXCL10 (IP-10)	CXCR3	CCL10	Unknown
CXCL11 (ITAC)	CXCR3, CXCR7	CCL11 (Eotaxin)	CCR3
CXCL12 (SDF-1)	CXCR4, CXCR7	CCL12 (MCP-5)	CCR2
CXCL13 (BLC)	CXCR5	CCL13 (MCP-4)	CCR1, CCR2, CCR3, CCR11

CXCL14 (BRAK)	Unknown	CCL14 (HCC1)	CCR1
CXCL15 (Lungkine)	Unknown	CCL15 (HCC2)	CCR1
CXCL16 (SRPSOX)	CXCR6	CCL16 (HCC4)	CCR1
CXCL17 (VCC)	Unknown	CCL17 (TARC)	CCR4, CCR8
		CCL18 (PARC)	CCR3
		CCL19 (ELC)	CCR7
		CCL20 (LARC)	CCR6
		CCL21 (SLC)	CCR7
		CCL22 (MDC)	CCR4
		CCL23 (MPIF1)	CCR1
		CCL24 (Eotaxin-2)	CCR3
		CCL25 (TECK)	CCR9
		CCL26 (Eotaxin-3)	CCR3
		CCL27 (Eskine)	CCR10
		CCL28 (MEC)	CCR10
CX3CL1 (fractalkine)	CX3CR1		
XCL family			
Ligand	Corresponding XCR		
XCL1 (lymphotactin)	XCR1		

Table 2
Three-Dimensional Structures of Human Chemokines

Name	Common name	PDB code	Method	Quaternary structure	Notes
CXCL1	Gro- α	1MGS	NMR	Dimer	Complex with heparin analogue
CXCL2	Gro- β	1QNK	NMR	Dimer	
CXCL4	Platelet factor-4	1RHP	X-ray (2.4Å)	Tetramer	
CXCL4	CTAP-III	1F9P	X-ray (1.93Å)	Monomer	
CXCL7	NAP-2	1NAP	X-ray (1.9Å)	Tetramer	
CXCL8	IL-8	1ICW	X-ray (2.01Å)	Dimer	
CXCL8	IL-8	1QE6	X-ray (2.35Å)	Dimer	
CXCL8	IL-8	1ILQ	NMR	Dimer	
CXCL8	IL-8	3IL8	X-ray (2.0Å)	Dimer	
CXCL8	IL-8	1ILP	NMR	Dimer	
CXCL10	IP-10	1LV9	NMR	Monomer	With CXCR1 ¹⁻⁴⁰
CXCL10	IP-10 A,B,C,D	1O7Y	X-ray (3.0Å)	Tetramer	M-form
CXCL10	IP-10	1O7Z	X-ray (1.92Å)	Dimer	T-form
CXCL10	IP-10	1O80	X-ray (2.0Å)	Dimer	H-form
CXCL11	ITAC	1RJT	NMR	Monomer	
CXCL12	SDF-1 α	1SDF	NMR	Monomer	N33A mutant
CXCL12	SDF-1 α	1A15	X-ray (2.2Å)	Dimer	
CXCL12	SDF-1 α	1QG7	X-ray (2.0Å)	Dimer	
CXCL12	SDF-1 α	1VMC	NMR	Monomer	With CXCR4 ¹⁻²⁷
CCL1	I-309	1EL0	NMR	Monomer	Three disulfides
CCL2	MCP-1	1DOK	X-ray (1.85Å)	Dimer	P-form
CCL2	MCP-1	1DOL	X-ray (2.4Å)	Monomer	I-form, tetramer in crystal

CCL2	MCP-1	1DOM	NMR	Dimer	D27A (10 structures) Monomeric variant
CCL3	MIP-1 α mutant	1B53	NMR	Dimer	
CCL4	MIP-1 β mutant	1JE4	NMR	Monomer	
CCL4	MIP-1 β	1HUM	NMR	Dimer	
CCL5	AOP-RANTES	1B3A	X-ray (1.6Å)	Dimer	Complex with heparin I-S Complex with heparin III-S
CCL5	RANTES	1HRJ	NMR	Dimer	
CCL5	RANTES	1RTO	NMR	Dimer	
CCL5	RANTES	1U4L	X-ray (2.0Å)	Dimer	
CCL5	RANTES	1U4M	X-ray (2.0Å)	Dimer	Complex with heparin I-S Complex with heparin III-S
CCL7	MCP-3	1BO0	NMR	Monomer	
CCL8	MCP-2	1ESR	X-ray (2.0Å)	Monomer	
CCL15	HCC-2	2HCC	NMR	Monomer	
CCL17	TARC	1NR2	X-ray (2.18Å)	Monomer	Three disulfides CCR3 ¹⁻³⁵ CCR3 ¹⁻³⁵ With CX3CR1 ²⁻¹⁹ Novel 4° structure
CCL17	TARC	1NR4	X-ray (1.72Å)	Dimer	
CCL20	MIP-3 α	1HA6	NMR	Monomer	
CCL20	MIP-3 α	1M8A	X-ray (1.7Å)	Dimer	
CCL23	MPIF	1G9I	NMR	Monomer	Three disulfides CCR3 ¹⁻³⁵ CCR3 ¹⁻³⁵ With CX3CR1 ²⁻¹⁹ Novel 4° structure
CCL24	Eotaxin-2	1EIG	NMR	Monomer	
CCL26	Eotaxin-3	1G2S	NMR	Monomer	
CX3CL	Fractalkine	1B2T	NMR	Monomer	
CX3CL	Fractalkine	1F2L	X-ray (2.0Å)	Tetramer	Novel 4° structure
XCL1	Lymphotactine	1J9O	NMR	Monomer	

Table 3
Three-Dimensional Structures of Viral Chemokines

Common name	PDB code	Method	Quaternary structure
vMIP-I	1ZXT	X-ray (1.7Å)	Dimer
vMIP-II	1CM9	X-ray (2.1Å)	Dimer
vMIP-II	1HF6	NMR	Dimer
vMIP-II	1HHV	NMR	Monomer
vMIP-II and vMIP-II (1–10)	1HFG	NMR	Monomer
vMIP-II	1VMP	NMR	Monomer

α -helices are packed against the β -sheet and provide interactions that stabilize the dimeric structure. This structure resembles the major histocompatibility complex (MHC) with the exception that CXCL8 is much smaller and compact and does not contain a major groove as is present between the MHC α -helices, which provides the antigen binding site. The oligomeric structures of specific CXC chemokines, as well as CC chemokines, are not always the same in nuclear magnetic resonance (NMR) and crystal structures. The solution structure of CXCL10 interferon inducible protein (IP-10) is monomeric (28), and CXCL12 exists as a monomer or dimer depending on solution conditions (29,30), yet the oligomeric states in the crystal structures are different: CXCL12 is a dimer (31), whereas CXCL10 was crystallized as both a dimer and tetramer (32). The tetrameric form of CXCL10 is a dimer of dimers formed by β -sheet sandwiching of two AB-type dimers (Figure 1C) similar to CXCL4 (platelet factor 4) (33), the first chemokine structure to be determined.

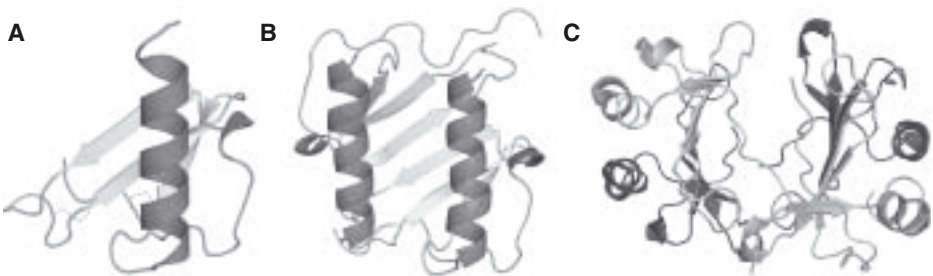


Fig. 1. Oligomerization of CXC chemokines. (A) Monomeric form of CXCL8 (IL-8) with disulfide bridges shown as black sticks. (B) Dimeric form of CXCL8 showing six-stranded β -sheet. (C) Tetrameric form of CXCL10 (IP-10).

The structure of CC chemokines also comes in a variety of oligomeric forms that include monomers, dimers, and tetramers. The monomeric structure is similar to that of CXC chemokines (Figure 1A). However, the dimeric and tetrameric chemokine structures are significantly different. The first identified structure of a CC chemokine was that of CCL4 (MIP-1 β), which, like CXCL8, was also dimeric. The difference in the dimeric structure of CCL4 relative to CXCL8 was unexpectedly striking (Figure 2A). The subunit interface of CCL4 involves mostly the N-terminal region and parts of a loop connecting β -strands 1 and 2. In contrast with most CXC chemokines, the N-terminal regions of some CC chemokines are not as flexible due to their role in forming the dimeric interface. These interactions lead to an elongated protein with each helix at opposite ends of the molecule. The structure of CCL2 monocyte chemoattractant protein (MCP-1) determined by x-ray crystallography was tetrameric (34). In this case, one of the dimers within the tetramer has similarities with CXC dimers due to crystallographic symmetry (Figure 2B). Whether this tetramer is due only to the symmetry present in crystals or is physiologically important remains to be determined. It is important to note that in the presence of a heparin octasaccharide, a tetrameric CCL2 is observed in sedimentation equilibrium studies, but the three-dimensional configuration of this tetramer is unknown (35). Another CC chemokine, CCL20, crystallizes as a CXC-type dimer (Figure 2C) (18), although an equilibrium between monomer and dimer was observed by NMR (36) and analytical ultracentrifugation (18).

The structure of fractalkine, the CX3C chemokine consisting of residues 1 to 76 of a full-length single transmembrane protein, was determined by both NMR (37) and x-ray crystallography (38). The solution form of the chemokine domain of fractalkine is monomeric (Figure 3A), but the crystal form is tetrameric (Figure 3B)—a dimer of dimers where the interactions between the dimers are mediated by water molecules. Although this tetrameric structure is unique, it is likely to be an artifact of crystallization. The *in vivo* oligomerization of fractalkine, either as part of, or proteolytically cleaved from, the full-length protein is unknown. Glycosaminoglycan (GAG) binding to the fractalkine chemokine is weak and therefore improbable in inducing dimerization. However, electrostatic analysis of the fractalkine chemokine domain indicates the dimeric interface is relatively uncharged (in contrast with the opposite site, which is fully charged) and could form an interface as part of the full-length protein (38).

The dimeric form of fractalkine (Figure 3C) resembles a compact CC chemokine compared with the elongated form seen in Figure 2A. The first disulfide of fractalkine forces the N-terminal region to remain close to the core of the molecule (38). The interface between subunits is asymmetrical and involves a β -strand from residues Cys-8 to Thr-11 from one monomer and residues Thr-11

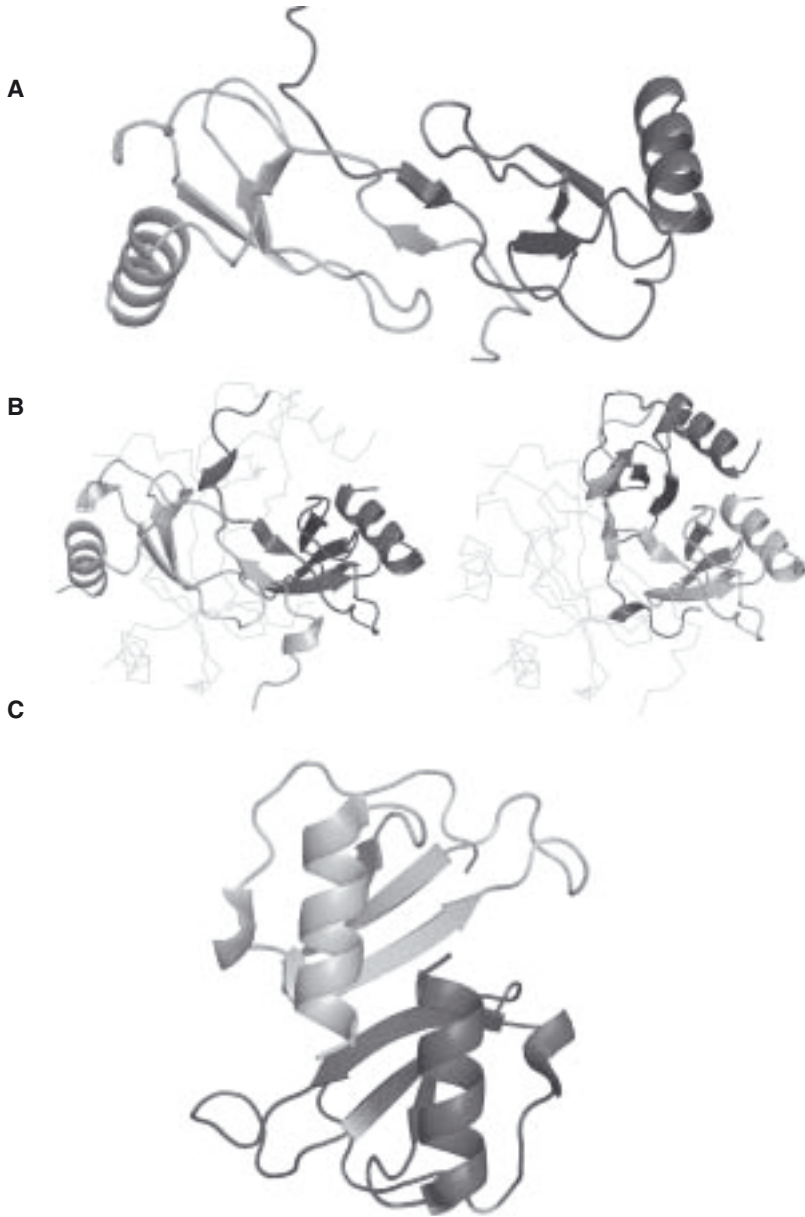


Fig. 2. Oligomerization of CC chemokines. (A) Dimeric form of CCL4 (MIP-1 β). (B) Tetrameric form of CCL2 (MCP-1) highlighting both CC dimerization (left) and CXC dimerization (right). (C) CCL20 forms liver and activation regulated chemokine (LARC) a CXC dimer.

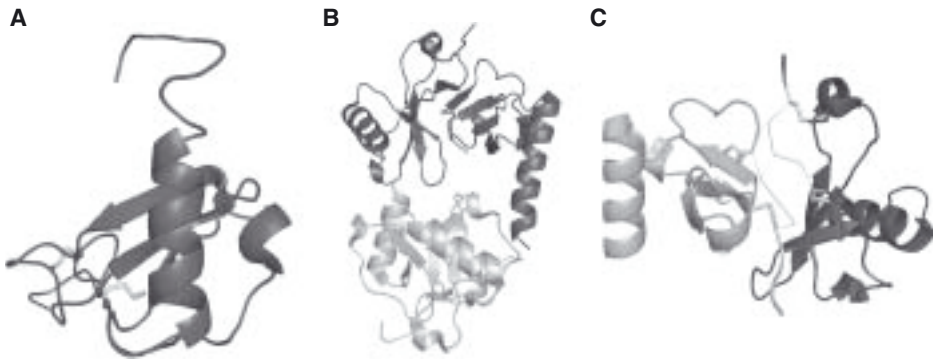


Fig. 3. Oligomerization of fractalkine. (A) Monomeric form of CX3CL1 showing disulfide bonds as sticks. (B) Tetrameric form of CX3CL1 in which a dimer of dimers is formed through water contacts. (C) Dimer of CX3CL1 with the three residues between the first two conserved cysteines shown as a backbone trace.

to Lys-14 from the other monomer to form a two-stranded β -sheet. It is not surprising that fractalkine forms a CC-type dimer given that the sequence homology of fractalkine is closest to CC chemokines.

Lymphotactin (XCL1) also displays unique structural properties. It lacks one of the two disulfides that is present in all other chemokines and has an extended C-terminal sequence that is required for biological activity (39,40). At low temperature (10°C) in the presence of 200 mM NaCl and 20 mM phosphate buffer (pH 6.0), lymphotactin possesses the typical monomeric chemokine fold with a disordered C-terminal extension (that follows the C-terminal α -helix) (41). The NMR chemical shifts are dramatically different at higher temperature and lower ionic strength. The experimental data indicate that lymphotactin undergoes a structural transition leading to an equilibrium of tertiary and quaternary structures that are not observed with any other chemokine (42). The three-stranded β -sheet is refolded into a four-stranded β -sheet, with the loss of the C-terminal helix. Analytical ultracentrifugation indicates that at 40°C and 100 mM NaCl, the K_d decreases from 1.2 mM (at low temperature and 200 mM NaCl) to $\sim 40 \mu\text{M}$, significantly increasing the amount of dimeric lymphotactin present in solution. Although this K_d may appear too high given that nanomolar levels of chemokines are detected in the plasma, the actual concentration in the presence of glycosaminoglycans at the surface of endothelial cells or in tissues where cells react to a haptotactic gradient is probably large enough for the formation of dimers or higher molecular weight complexes. The presence of a single disulfide, formation of a four-stranded β -sheet in the monomer without an α -helix, and the possibility that the four-stranded β -sheet forms a dimer makes lymphotactin unique among all chemokines.

2.3. Chemokine-Receptor Interactions

The identification of GPCRs as chemokine receptors led to the expectation that small-molecule antagonists (or agonists) would be easier to identify and develop into drugs for diseases caused by dysregulation of these proteins (43). This expectation was complicated by the realization that chemokine receptors display a great deal of redundancy, with different receptors having overlapping activities. Moreover, multiple chemokines can activate the same receptor. The possibility was suggested that chemokines bind to distinct but overlapping regions of GPCRs (44), which may add to the complexity of identifying a single receptor antagonist that blocks activation by all activating chemokines. The cocrystallization of a chemokine receptor bound to its ligand as a means for drug design or for understanding the properties necessary to induce or inhibit activation also presents a serious obstacle. To date, the only experimentally determined structure of a GPCR is that of bovine rhodopsin (45). Rhodopsin is unique among GPCRs as it constitutes greater than 70% of the proteins in the membranes of the rod outer segments of the retina allowing milligram quantities to be purified from a single bovine eye. Recombinant GPCRs expressed in various cells may produce higher expression levels of proteins than in endogenous cells, but they are not as stable during purification and are difficult to crystallize. Nonetheless, the determination of crystal structures of other GPCRs is inevitable. For the moment, alternative approaches have been used to study the structural basis of chemokine-receptor interactions.

The N-terminal region of chemokine receptors is known to interact with specific chemokines (37,46–49). One of the approaches to study chemokine-receptor interactions involves NMR studies of chemokines with peptides derived from the N-terminal region of chemokine receptors. These peptides have dissociation constants for their respective chemokines that are in the micromolar to millimolar range compared with the picomolar to nanomolar range for the full-length receptor. Such studies have been carried out for two CXC chemokines, two CC chemokines, and the CX3CL chemokine fractalkine. One of the limiting factors in these studies is the inability to discern whether these peptides interact with the monomeric or dimeric forms of the chemokines. Another interesting approach that is still being optimized for structural studies involves using a soluble protein scaffold to present multiple extracellular regions from receptors to chemokines (50,51). Although the studies with receptor peptides or protein scaffolds are important starting points to increase our understanding of how chemokines interact with receptors, it is important to interpret the initial results with caution. For example, it is not known whether the peptides or protein scaffolds correctly mimic the interactions that occur *in vivo*. Roles for receptor glycosylation or sulfation (52–55) as well as functions of other

molecules such as GAGs, which are known to be important for chemokine interactions, are ignored in these studies. The results from these studies will provide models for interactions that need to be verified by other methods.

CXCL8 has been a major focus in structural studies of chemokine-receptor interactions (46,47). An initial study used the peptide consisting of CXCR1 residues 1 to 40 (CXCR1¹⁻⁴⁰). Measuring chemical shift changes as CXCR1¹⁻⁴⁰ was titrated into a solution containing ¹⁵N-labeled CXCL8, a dissociation constant between 170 and 340 μM (depending on whether the CXCL8 dimer was interacting with one or two peptides) was determined (46,47). The residues with the greatest ¹H and ¹⁵N chemical shifts for CXCL8 were the side chain of Gln-8, and the backbone atoms of Thr-12, Lys-15, Phe-17, His-18, Lys-20, Phe-21, Ser-44, Glu-48, Leu-49, Cys-50, and Val-61. Assuming the largest chemical shifts are associated with interacting amino acids, the receptor peptide forms interactions with CXCL8 residues from (i) the N-terminal loop, (ii) the 40s loop, (iii) β-strand 3, and (iv) the α-helix. Of interest is that there is no evidence that Glu-4, Leu-5, Arg-6 (the ELR motif) of CXCL8 interacts with the N-terminus of the CXCR1 peptide. The ELR sequence had been identified in earlier studies as important for CXCR1 and CXCR2 receptor activation (56,57). In a more detailed crystallographic characterization of the interactions between CXCL8 and a receptor-based peptide, the CXCR1 N-terminal region was reduced to 21 amino acids corresponding with residues Met-9 to Pro-29 and was further modified by replacing residues Leu-15 to Gly-19 with a 6-amino hexanoic acid linker (47). CXCL8 residues most affected by addition of the peptide containing the linker were similar to those described in the NMR study above. These residues form the boundary of a cleft containing solvent-exposed hydrophobic groups that binds only a small segment of the CXCR1⁴⁻²⁹ peptide, as the cleft is not large enough to accommodate either of the CXCR1¹⁻⁴⁰ or the modified CXCR1⁹⁻²⁹ peptides (Figure 4A and B, see color plate). These studies partly agree with a mutagenesis study of 27 residues of CXCL8 that identified a hydrophobic groove composed of residues Phe-17, Phe-21, Ile-22, and Leu-43 (58). This groove is more than 20 Å from the ELR motif in either subunit of the CXCL8 dimer, suggesting that the CXCL8 hydrophobic groove binds to the CXCR1 N-terminus and the CXCL8 ELR motif binds to an uncharacterized site of the receptor.

CXCL12 interactions with CXCR4 have been studied by a similar NMR-based approach (49). CXCR4 N-terminal peptides 1-27, 1-17, 7-27, and 18-27 were initially screened for binding to ¹⁵N-labeled CXCL12. The dissociation constants determined by NMR titration experiments for CXCR4¹⁻²⁷ and CXCR4¹⁻¹⁷ were 45 μM and 133 μM, respectively. The magnitude of the change in chemical shifts of ¹⁵N-CXCL12 upon addition of CXCR4⁷⁻²⁷ was too small to determine a dissociation constant and CXCR4¹⁸⁻²⁷ provided no

evidence for binding. The largest chemical shifts were produced by the presence of the CXCR4¹⁻²⁷ peptide for CXCL12 residues Val-23, His-25, and Ala-40. Additional changes in chemical shifts are present for (i) the N-terminal loop residue Phe-13, (ii) Lys-24 of the first β -strand, (iii) Gln-37, Val-39, Arg-41, and Leu-42 of the second β -strand, (iv) 40s loop residue Asn-45, (v) Gln-48 and Val-49 of the third β -strand, and (vi) Ile-58 and Tyr-61 of the α -helix. Many of these residues contribute to a shallow groove in the top half of the β -sheet (Figure 4C). Again, it is interesting to note that there are no interactions between the N-terminal-based peptide of CXCR4 and the flexible N-terminus of CXCL12, which is responsible for receptor activation, providing evidence for the two-stage binding and activation model of chemokine-receptor interaction (59).

Interactions between a receptor-based peptide and CC chemokines are available for CCR3 with CCL11 and CCL24 (48,60). The study with CCR3 underscores the complexity of chemokine-receptor interactions. The two chemokines have a sequence identity of about 35% and exclusively bind to CCR3. CCL2 shares 65% identity with CCL11, functions as an agonist for CCR2 and CCR4, but has no affinity for CCR3. These observations highlight that the interactions between chemokines and receptors are subtle and require high-resolution information concerning chemokines, receptors, and other molecules that participate in these interactions.

In the CCL11 study, a variety of peptides based on the predicted extracellular domains of CCR3 were screened for binding to ¹⁵N-labeled CCL11. These peptides included the linear sequence of the N-terminal region (CCR3¹⁻³⁵) and each of the three extracellular loops connected to the transmembrane domains. The three extracellular loops were also synthesized with a Cys-Gly-Gly at the N-terminus and a Gly-Gly-Cys at the C-terminus. In an attempt to mimic constraints for each loop linked to the transmembrane helices, each peptide was oxidized to form an intramolecular disulfide bond between the cysteine at the termini. Of the seven CCR3 peptides screened by NMR, only CCR3¹⁻³⁵ induced concentration-dependent changes in the chemical shifts of CCL11. The linear and cyclic peptides from loops 2 and 3 did not induce any changes in the heteronuclear single quantum correlation (HSQC) spectrum of CCL11, whereas the linear and cyclic peptides of loop 1 caused precipitation and could not be used for further experiments. Titration of CCR3¹⁻³⁵ affected the chemical shift of residues mainly in the N-loop (residues 13 to 18), the ₁₀ helix (residues 20 to 22), and β -strand 3 (residues 46 to 49, 51). Other residues with significant changes in chemical shift included Arg-28 (β -strand 1), Gln-36 and Ala-38 (30s loop), and Lys-55 and Trp-57 (residues at the N-terminus of the α -helix) (Figure 4D). CCL24 interactions with CCR3 were studied with the same receptor-based peptide approach (60). In addition to CCR3¹⁻³⁵ concentration-dependent chemi-

cal shift changes, the line shape of other residues—Ser-5, Cys-7, Cys-8, Phe-11, Ile-16, Lys-34, Ala-35, and Val-37—was altered by broadening and/or distortion. The peaks of Arg-15 and Glu-64 disappeared from the spectrum in the presence of CCR3 peptide. Despite the low similarity in primary sequence between CCL11 and CCL24, the major changes occurred in a conserved area with CCL11 consisting of a hydrophobic base surrounded by charged or hydrophilic residues (Figure 4E). The electrostatic potential of CCL2, CCL11, and CCL24 was calculated and analyzed in an attempt to explain the difference between CCR3-binding properties of CCL11 and CCL24 and the lack of binding by CCL2. Significant differences were found in the CCR3^{1–35} binding region of CCL11 and CCL24 with respect to the same location on CCL2.

The interaction of the chemokine domain of fractalkine (CX3CL1) with a CX3CR1 peptide was also characterized. Titration of the CX3CR1^{2–19} peptide into a sample of ¹⁵N-labeled fractalkine resulted in changes in chemical shifts for residues 6 to 17 (N-terminal region and N-loop), Gln-31 and Gly-35 (the 30s loop), and residues Ile-40, Gln-45, and Leu-48 (Figure 4F) (37). This is the only chemokine that has a change in chemical shift for the N-terminal region, but it is important to reemphasize that these changes may have nothing to do with direct interactions.

An examination of the panels in Figure 4 (see color plate) indicates that the N-terminal peptides from CXCR1, CXCR4, CCR3, and CX3CR1 all interact with the similar regions of their respective agonists. The similarity in the N-terminal receptor binding region for chemokines from three different families strongly suggests a common mechanism of initial binding. Site-directed mutagenesis has identified some of the subtleties of chemokine-receptor interactions (56,61). The absence of any direct interactions between the receptor peptides and the activating N-terminal sequence of chemokines from these structural studies (with the exception of CX3CL1) support the proposed two-step model of receptor binding and activation for most chemokines. The initial binding will certainly involve the chemokine regions shown in Figure 2–4 with the N-terminal region of the receptor followed by activation due to interactions of the N-terminal sequence of the chemokine with the receptor.

2.4. Chemokine-Glycosaminoglycan Interactions

The activation of a chemokine receptor is more complex than the traditional agonist-receptor paradigm. For example, chemokine activity is mediated by GAGs (heparin, heparan, and heparin sulfate; chondroitin sulfate; and dermatan sulfate) at various sites during the chemotactic process. Chemokines released by tissue injury, infection, or inflammation activate adjacent endothelial cells and induce rolling and extravasation of leukocytes. These interactions between

endothelial cells and leukocytes involve selectins, integrins, and intracellular adhesion molecules (ICAMs). Chemokines are also involved in the interactions between the two cell types. Endothelial cells are active in transcytosing chemokines to their luminal surface, where they interact with GAGs, are presented for interaction to chemokine receptors on leukocytes, are involved in integrin activation, and induce firm attachment to the endothelium prior to extravasation (62). The GAG-chemokine interaction may also be important in the connective tissue and extracellular matrix. GAGs induce the oligomerization of chemokines (63) and may be responsible for creating haptotactic gradients through the release of chemokines from the GAG-polychemokine complex necessary for the migration of leukocytes (64). On the cell surface, the GAG-induced oligomerized chemokines increase the local concentration of chemokines and promote interactions with the cell surface receptor (63,65).

The importance of GAGs in the biological function of chemokines is highlighted by a number of studies. Mutants of CCL2, CCL4, and CCL5 deficient in GAG binding retain chemotactic activity *in vitro* but are unable to induce migration of cells when injected intraperitoneally in mice compared with wild-type controls (66). In the same study, mutant monomers of CCL2, CCL4, and CCL5 were chemotactic *in vitro* but lost this activity *in vivo*, suggesting that the oligomerization of chemokines *in vivo* by GAGs is necessary for mediating a chemotactic response. A variant of CCL5 (with a sequence from residues 44 to 47 of AANA) that does not bind heparin inhibits the *in vivo* activity of endogenous CCL5, presumably by the formation of inactive heterodimers with endogenous wild-type CCL5 (67). These important regulatory roles for GAGs in various aspects of chemokine activities suggest that the GAG-binding site of a chemokine might be a more specific drug target than the receptor binding pocket (67).

Three-dimensional structures of chemokine-GAG complexes are difficult to determine because of the propensity of GAGs to induce the precipitation of chemokines at the high concentrations required for structural work. Nuclear magnetic resonance studies have been used to study the affinity, oligomerization, and/or GAG binding site of CXCL4 (68), CXCL8 (69), CXCL12 (Murphy et al., unpublished results), and CCL5 (65,70). The crystal structures of CCL5 and two heparin-derived disaccharides have been determined by x-ray crystallography (Figure 5A; see color plate) (71). The interaction between chemokines and the disaccharides are largely electrostatic, yet specific. However, these studies may reveal a potential template for structure-based drug optimization that increases both the affinity and selectivity for disaccharide-based derivatives (67). Interestingly, there is variation in the GAG interaction site within a chemokine family. Nuclear magnetic resonance studies of CXCL4 (68) indicate the GAG binding site is located at two loops and the C-terminal helix (Figure

5B; see color plate) that in the tetramer forms a ring of positive charges (not shown) as predicted by Stuckey et al. (72). Nuclear magnetic resonance studies of CXCL8 (69) (Figure 5C; see color plate) indicate that only the C-terminal helix is involved in binding to GAGs. In contrast, structural data on CXCL12 (Murphy et al., unpublished results) verify the results of mutagenesis experiments that heparin disaccharide I-S binds to basic residues within the subunit interface at the first and second β -strands (Figure 5D; see color plate) (73). Mutagenesis of murine CXCL10 reveals a heparin binding site that is similar to that of CXCL4 (Figure 5E compared with B; see color plate) (74). This location involves N-terminal loop residues Arg-20, Arg-22, and Ile-24, β -strand 1 residue Lys-26, and 40s loop residues Lys-46 and Lys-47 (Figure 5E; see color plate).

2.5. Future Directions

The three-dimensional structures of chemokines have been straightforward to determine. This is due to a number of factors. The small size of these proteins allows for either chemical synthesis or production by recombinant methods. The physiochemical properties of these proteins are generally well-suited for NMR or crystallization. As a consequence, we have learned a lot from these structures. It is much more challenging to structurally characterize complexes of chemokines with GAGs or chemokine receptors. The future of structural biology for chemokines now depends on developing methods to overcome the difficulties associated with determining structures of chemokine-GAG and chemokine-receptor complexes. Once these structures are determined, our knowledge of how the chemokine activity is regulated and how receptors are activated will increase dramatically.

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