Ligand Selectivity and Affinity of Chemokine Receptor CXCR1

ROLE OF N-TERMINAL DOMAIN*[S]

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Glu-Leu-Arg (“ELR”) CXC chemokines interleukin-8 (IL-8) and melanoma growth stimulatory activity (MGSA) recruit neutrophils by binding and activating two receptors, CXCR1 and CXCR2. CXCR1 is specific, binding only IL-8 with nanomolar affinity, whereas CXCR2 is promiscuous, binding all ELRCXC chemokines with high affinity. Receptor signaling consists of two events: interactions between the ligand N-terminal loop (N-loop) and receptor N-terminal domain (N-domain) residues (site I), and between the ligand N-terminal ELR and the receptor juxtamembrane domain (J-domain) residues (site II). It is not known how these interactions mediate ligand affinity and selectivity, and whether binding at one site influences binding and function at the other. Sequence analysis and structure-function studies have suggested that the receptor N-domain plays an important role in ligand selectivity. Here, we report ligand-binding properties and structural characteristics of the CXCR1 N-domain in solution and in detergent micelles that mimic the native membrane environment. We find that IL-8 binds the N-domain with significantly higher affinity in micelles than in solution (−1 μM versus −20 μM) and that MGSA does not bind the N-domain in solution but does in micelles with appreciable affinity (−3 μM). We find that the N-domain is structured in micelles and that the entire N-domain interacts with the micelle in an extended fashion. We conclude that the micellar environment constrains the N-domain, and this conformational restraint influences its ligand-binding properties. Most importantly, our data suggest that for both ligands, site I interaction provides similar affinity and that differential coupling between site I and II interactions is responsible for the observed differences in affinity.

Chemokines, or chemotactic cytokines, are small soluble proteins (70–120 residues) that play fundamental regulatory roles in native immunity, inflammation, and host defense against infection. Over 40 chemokines have been identified to date, and these can be classified as CC, CXC, C, or CX3C chemokines based on the position of conserved cysteines (1). The CXC chemokines can be further classified as Glu-Leu-Arg (“ELR”) and “non-ELR”, based on the presence or absence of this motif before the first cysteine. Chemokines carry out their function by binding and activating receptors that belong to the G-protein-coupled receptor (GPCR) superfamily. The “ELRCXC” chemokines bind two receptors, CXCR1 and CXCR2, on the neutrophil cell surface. Interleukin-8 (IL-8, also known as CXCL8) binds both neutrophil receptors with high affinity, whereas other ELRCXC chemokines, including melanoma growth stimulatory activity (MGSA, also known as CXCL1), bind only CXCR2 with high affinity (2, 3). The chemokine chemokine receptor system in general is intricately complex, with multiple ligands binding a single receptor and a single ligand binding multiple receptors within, and sometimes outside, the respective subfamily (4, 5). The structural and thermodynamic basis for the differences seen in ligand binding affinities, receptor selectivities, activation, and downstream signaling of chemokine receptors is not known. Knowledge of ligand and receptor structures, of binding-induced structural and dynamic changes in the ligand and receptor, and of the energetic basis of the ligand-receptor interaction is thus essential to understand this differential affinity and selectivity.

The structures of several chemokines, including ELRCXC chemokines such as IL-8 and MGSA, are known, and all adopt a common chemokine fold: a disordered N-terminal end, followed by a series of loops and turns (defined as the N-loop), three α-strands, and an overlying α-helix (6–11). Chemokine receptors are classified following the chemokine convention as CCR, CXCR, XCR, and CX3CR based on the kind of chemokine(s) they bind (1, 12). Although there are over 40 chemokines, there are considerably fewer receptors (6 CCR, 10 CC, 1 XCR, and 1 CX3CR). The structures of chemokine receptors are not known; indeed, the only GPCR structure solved to date is that of bovine rhodopsin (13).

Our studies are directed toward understanding the structural basis of the ligand selectivity and binding affinity of CXCR1 for IL-8 versus MGSA. Furthermore, the specificity of chemokine and the promiscuity of CXCR2, which can bind two chemokines with similar affinities, can only be seen on a broader level in the entire chemokine-receptor system. The structural basis of IL-8 and MGSA function has been extensively characterized using both site-specific mutagenesis and chimeric constructs (14–23). These studies, and similar studies for other chemokines, show that the receptor signaling involves two distinct sites: interaction between the ligand N-terminal loop (N-loop) and the receptor N-terminal domain.
residues (site I) and between the ligand N-terminal and the receptor exoloop and transmembrane (J-domain) residues (site II; Fig. 1) (24, 25). How these interactions mediate ligand affinity, receptor selectivity, and receptor activation, and whether binding at one site influences binding/function at the other site are not known.

IL-8 binds both CXCR1 and CXCR2 with high affinity ($K_d$ 1–7 nM), whereas MGSA binds CXCR2 with high affinity ($K_d$ 3–9 nM) and CXCR1 with much lower affinity ($K_d$ 350–530 nM) (19, 26). IL-8 and MGSA have very similar biochemical properties, such as high pI, similar ratios of positive to negative charges, and similar hydrophobic surface area. Single mutations, deletions, and domain-swapping studies in IL-8 and MGSA have identified ELR and the N-loop residues as being important for receptor binding and function. IL-8 and MGSA ELR mutants show poor binding and activity, and especially mutants in which arginine has been mutated bind with 1000-fold less affinity and are completely inactive (18, 20, 27). On the other hand, mutations and domain-swapping studies indicate that the N-loop residues are involved in receptor binding and determine receptor specificity (CXCR1 versus CXCR2). These residues in IL-8 are responsible for high affinity binding to both receptors and in MGSA are responsible for high and low affinity binding to CXCR2 and CXCR1, respectively (19, 22). Comparison of the IL-8 and MGSA structures shows significant conformational differences for the N-loop region, suggesting that the differences in this region could be responsible for the receptor specificity (8). These observations suggest that the differential binding of IL-8 and MGSA to CXCR1 is due not to differences in their biochemical properties but due to differences in specific residues or structural features of the two ligands. The NMR relaxation measurements for IL-8 have also shown that the N-loop residues are more dynamic than the core structure (28). On the basis of structure-function studies on IL-8, it had been proposed that the core of chemokine ligand acts as a scaffold to which the N-terminal ELR and the N-loop residues are tethered through disulfide bonds for optimal interaction with the receptors (17, 29, 30).

The structural requirements of the receptor residues for binding and activation are less well understood than for the ligands. Studies to date indicate that specific residues from the receptor N-domain and the J-domain mediate binding and activation, but it is not clear how these interactions mediate affinity and receptor selectivity. Mutational studies have identified some of the CXCR1 and CXCR2 residues as being important for IL-8 binding, on the basis of decreased binding and receptor activity (31–34). Studies using chimeras of CXCR1 and CXCR2, and with monoclonal antibodies, suggest that the two receptors are not equivalent, and interact with IL-8 and MGSA in a differential manner (2, 35–38). These studies indicate that the N-domain plays an important role in ligand binding but suggest conflicting roles for the N-domain in determining receptor selectivity (2, 35, 37, 38). Sequence analysis of CXCR1 and CXCR2 receptors shows that the N-domain is the least conserved and most variable; the N-domains show about 40% sequence identity as opposed to 78% identity between the entire receptors. The most direct evidence for the importance of the N-domain comes from the observation that IL-8 binds the receptor N-domains with appreciable affinity and inhibits IL-8 binding to the intact receptor (35, 39–41). Understanding the structural characteristics and ligand-binding properties of the N-domain may therefore provide key insights into the structural and energetic basis of selectivity and affinity of the intact receptor.

In this study, we have used fluorescence and circular dichroism (CD) spectroscopy to characterize the structural properties of the CXCR1 N-domain and to correlate these with the ligand specificity and affinity of CXCR1 for IL-8 versus MGSA. We find that the CXCR1 N-domain is structured in detergent micelles that mimic the membrane environment and binds both IL-8 and MGSA with similar affinity. We discuss the implications of these results in the context of the structural and thermodynamic basis of ligand binding affinity and selectivity.

**EXPERIMENTAL PROCEDURES**

**Design and Synthesis of N-domain Constructs**—The rabbit CXCR1 (rCXCR1) receptor has been shown to bind human IL-8 with affinity and specificity similar to that of the human CXCR1 (hCXCR1) (35, 37). The human and rabbit receptor N-domains show high sequence identity, and both bind IL-8 with similar affinity (35). The CXCR1 N-domain sequences are presented below in Sequence 1; residues identified as being important for ligand binding are in boldface.

**bCXCR1 N-domain:**
MSNYPDFQWMDFIDOLNYMPAPQMDYPSMCLELETITLTK
rCXCR1 N-domain:
MBNWMNDLDLWTVEDE-FAANATMGMPVGRDSVLTVQTTLK

**Sequence 1**

We initially screened various peptides representing fragments of both human and rabbit CXCR1 N-domains and found that the rabbit peptides were easier to synthesize and were more soluble. We have therefore used fragments of the rCXCR1 N-domain for our structural and binding studies. The entire N-domain was observed to be sparingly soluble in solution, and therefore we used two constructs, comprising 23 and 34 residues of the full-length N-domain. Both constructs lacked 10 amino acids on the N-terminal end, and these have been shown not essential for binding (39). In addition, the 34-mer construct contained 11 membrane proximal residues on the C terminus of the N-domain. The original sequence for the 34-mer has a cysteine that was mutated to serine (underlined in Sequence 2) to minimize complications due to disulfide bond formation.

**Sequence 2**

23mer: LWTWFEDFANAGMPYVEKDSYP
34mer: LWTWFEDFANAGMPYVEKDSYPSSLVYQTTLK
scrambled 34mer: DYPFLSATAKETWAQPQYMLFLFPMNYWYPF

We also synthesized a scrambled 34-mer peptide for use as a negative control to ensure that the binding to the native sequence is specific.

N-domain peptides were synthesized either in the peptide core laboratory, University of Texas Medical Branch, or at Biomedical Research Center, Vancouver, Canada. The peptides were purified by reverse-phase high-performance liquid chromatography on a C18 preparative column using a gradient of 0.1 trifluoroacetic acid, water, and acetonitrile, and masses were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

**Expression and Purification of Human IL-8 and MGSA**—Wild-type human IL-8 was bacterially expressed as a fusion protein from plasmid vector pET32 Xa/LIC containing the IL-8 gene cloned at the ligation-
independent cloning site (a kind gift from Dr. Patty Liwong, Texas A&M University). The plasmid was transformed into *Escherichia coli* BL21(DE3)pLysS cells (OneShot™ Chemically Competent E. coli cells, Invitrogen). Transformants were grown in LB media in the presence of ampicillin to an optimal OD₆₀₀ of 0.5 and induced with 1 mM isopropyl-2-thiogalactopyranoside for 4 h at 37 °C. The cells were spun down and lysed by four freeze-thaw cycles followed by sonication. The supernatant containing the fusion protein was passed through a nickel-nitrilotriacetic acid column (Sigma-Aldrich) and eluted using 250 mM imidazole. The eluate was dia lyzed into cleavage buffer (20 mM phosphate, 50 mM sodium chloride, 2 mM calcium chloride, pH 7.4) and cleaved using Factor Xa (Novagen, EMD Biosciences, Madison, WI). The cleaved protein was purified using reverse-phase high-performance liquid chromatography column and eluted by a gradient of acetonitrile in 0.1% trifluoroacetic acid. The fractions containing the protein were pooled, lyophilized, and stored at −20 °C until further use. Wild-type MGSA was cloned into the same vector (pET32 Xa/LIC) and was transformed, expressed, and purified as outlined above.

**Fluorescence Spectra in Solution and Micelles**—All fluorescence spectra were measured on a Spex Fluoromax™ fluorimeter, with excitation at 295 nm and emission measured from 310 to 400 nm at 23 °C in 50 mM Tris (pH 8.0), 50 mM sodium chloride buffer. Receptor peptide was used at a concentration of 2–5 μM. Micelles were prepared by dissolving dodecylphosphocholine (DPC, Avanti Polar Lipids, Alabaster, AL) in a 1-cm path-length cuvette. Fluorescence spectra of the blank IL-8 or MGSA solutions were subtracted or without a fixed concentration (2 mM) of N-domain peptide. Fluorescence of the N-domain becomes quenched on adding IL-8 while remaining unchanged upon adding MGSA to either peptide in solution. These fluorescence studies show that the 23-mer N-domain peptide binds IL-8 with a dissociation constant \( K_d \) of ~37 μM (Fig. 4A). Although both N-domain peptides contain residues important for ligand binding (Tyr-32, Pro-26, and Pro-27), the 34-mer has additional hydrophobic residues on the C terminus that might hinder binding in solution either sterically or by increasing the entropy of the peptide in solution. The slightly lower binding of the 34-mer fragment to IL-8 in solution could be due to this reason. In contrast to IL-8, MGSA does not show detectable binding to either peptide in solution (Fig. 4, B and D). Previous studies have shown various N-domain fragments of hCXCR1 and rCXCR1 bind IL-8 in solution with \( K_d \) ~ 20 μM (35, 39, 41). Our binding affinities of the 23-mer and 34-mer for IL-8 in solution are comparable to these binding studies.

We also measured ligand-binding affinities of the N-domain
peptides in the presence of DPC micelles (Fig. 4). Trypophan fluorescence of the N-domain fragments was again quenched on addition of IL-8. In the presence of micelles, both the 23-mer and 34-mer peptides bound IL-8 with \( K_d \) values of \( \sim 1 \mu M \) (Table I). This affinity is 5- to 25-fold higher than the observed affinity of the N-domains free in solution. Furthermore, MGSA did not bind the 23-mer N-domain in micelles (Fig. 4D) but did bind the 34-mer N-domain in micelles with appreciable affinity (\( K_d \sim 3 \mu M \)) (Fig. 4B). We also measured the binding of IL-8 and MGSA to a scrambled 34-mer peptide both in buffer and micelles and observed no interaction (see Supplemental Material) indicating that binding characteristics to the native peptide is specific.

These results confirm that the 34-mer N-domain does bind MGSA in micelles, as observed in our CD experiments, indicating that the micellar or native membrane environment influences the properties of the membrane-proximal N-domain. Further, the measured \( K_d \) of \( \sim 3 \mu M \) is similar to that measured for IL-8 indicating that site I interaction is not the major determinant of selectivity in CXCR1.

Structure of N-domain in Membrane Environment—In the intact receptor, the N-domain is proximal to the membrane environment, and this might cause it to be less flexible and more constrained than in solution. Possible structures adopted by the N-domain in the native membrane environment are shown in Fig. 5. For, simplicity, only helical secondary structure is represented. The N-domain could be unstructured (model A), partially structured (e.g., having secondary structural elements but no tertiary structure, models B and C), exist as a molten globule (model D), or possess a well defined tertiary structure (model E).

Such conformational constraints might influence the ligand binding properties, as was observed in our ligand binding studies. To understand the effect of the membrane environment on structural characteristics of the N-domain, we set out to identify which portion, if any, of the N-domain interacts with the native membrane interface. Hydrophobicity calculations showed that the C-terminal (membrane-proximal) residues are more hydrophobic than the N-terminal residues (data not shown). Therefore, it is possible that these C-terminal residues “anchor” the N-domain to the membrane interface and could be responsible for any structure induced in the N-domain in the presence of the membrane environment. It is also possible that the entire N-domain interacts with the membrane environment.

To gain insights into native conformation of the rCXCR1 N-domain, we studied the structural characteristics of the 23-mer and 34-mer peptide constructs in micelles. Micelles have been extensively used as mimics of the membrane environment in the characterization of membrane proteins. By virtue of their properties of amphipathicity, self-association, and variety of head groups with different properties, detergents have successfully been used in the structural and functional characterization of membrane proteins (42). A variety of studies using

![CD spectra of 23-mer and 34-mer CXCR1 N-domain in complex with IL-8 and MGSA in solution. All solutions contain 25 \( \mu M \) CXCR1 N-domain \( \pm 25 \mu M \) ligand in 50 mM sodium phosphate, 50 mM sodium chloride buffer. Predicted and observed spectra are represented by solid and dotted lines, respectively. Non-superimposable predicted and observed spectra indicate ligand binding. A, predicted and observed far-UV spectra of 23-mer plus IL-8. B, predicted and observed far-UV spectra of 34-mer plus IL-8. C, predicted and observed far-UV spectra of 23-mer plus MGSA. D, predicted and observed far-UV spectra of 34-mer plus MGSA. E, predicted and observed near-UV spectra of 23-mer plus IL-8. F, predicted and observed near-UV spectra of 34-mer plus IL-8.](image)
model peptides have shown that multiple factors, including hydrophobic and electrostatic interactions and preferential partitioning of aromatic residues such as tryptophans to the lipid/aqueous interface, influence the partitioning, the structure, and conformation of the peptides in micelles (45). Detergent micelles also have correlation times short enough to be used in solution NMR studies of membrane proteins (43). We have used dodecylphosphocholine (DPC), a medium length side-chain detergent with an uncharged head group, which has been found to be extremely useful in membrane protein reconstitution and characterization (44). The 23-mer and 34-mer N-domain constructs each contain two tryptophan residues, toward the N terminus. An increase in fluorescence intensity and blue shift in the emission maximum show that the environment of these tryptophans becomes considerably more apolar in DPC micelles (Fig. 6). CD spectra of the peptides in solution and in micelles were also collected to assess structural changes in the N-domain (Fig. 7). The far-UV CD spectra of both peptides in solution are characteristic of unstructured proteins, whereas spectra in DPC micelles show evidence of secondary structure, namely, helices and/or turns. Near-UV CD spectra show differences in the environment of the aromatic residues in buffer and in micelles for both peptides (Fig. 7, C and D). One interpretation is that the changes seen in the tryptophan environment in the 23-mer arise from partitioning of the tryptophans into the micelle, whereas the changes seen in both tryptophans and the tyrosine in the 34-mer arise from partial induction of tertiary structure. It is also possible that there is no induction of tertiary structure and that all changes seen are due to the differential partitioning of aromatic residues into the micelle arising from differences in length and hydrophobicity in the two peptides. Our future studies using high resolution NMR will shed further light on these observations. We further observed, using both CD and fluorescence, that the peptides assume secondary structure only above the critical micelle concentration of DPC (see Supplemental data), indicating specific interaction of the peptides with the micellar surface and not with detergent monomers. We also observed that the scrambled peptide showed much more random coil nature in solution, and much less induction of structure in micelles than was observed for the N-domain peptides (see Supplemental data).

Fluorescence and CD spectra of the N-domain in solution and in micelles thus suggest that the N-domain is structured in micelles and, further, that this structure influences the ligand binding properties of the N-domain. Comparison of the CD spectra of the 23-mer and 34-mer throws further light on the conformation of the N-domain in the context of the membrane. Both domain fragments show almost the same degree of mean residue ellipticity in the presence of micelles. This indicates that interaction with the micelle is not limited to the hydrophobic membrane-proximal residues alone, because their ab-
sence in the 23-mer does not affect the degree to which this fragment interacts with the micelle. The spectral characteristics thus indicate that the entire N-domain interacts with the micelle in an extended fashion (Fig. 5C). This model of the N-domain in the micellar environment agrees with the observed induction of secondary structure and lack of well defined tertiary structure in micelles. Further, preliminary NMR experiments in our laboratory also showed that the 34-mer fragment is structured in the presence of DPC micelles (data not shown).

### Structural Basis for Binding to CXCR1 N-domain

The tertiary structural changes seen in the near-UV CD spectra in the ligand binding studies are in the wavelength range of 270–290 nm, indicating the involvement of tyrosine residues on the ligand or receptor, or both. This is in agreement with previous observations that Tyr-13 in the N-loop of IL-8 makes contacts with Pro-21, Pro-22, and Tyr-27 in the N-domain of hCXCR1 (21, 41, 46). These two tyrosines have been shown to make specific contacts to each other, and Tyr-27 of hCXCR1 has been shown to bind in a cleft in the N-loop of IL-8 formed by a cluster of hydrophobic residues that include Tyr-13 (41). The rabbit CXCR1 has two prolines and a tyrosine (Pro-26, Pro-27, and Tyr-32, respectively, corresponding to Pro-21, Pro-22, and Tyr-27 of hCXCR1). As the changes in the observed near-UV CD spectra are in the wavelength range of tyrosine absorption, it is very probable that the changes seen are from either Tyr-13 of IL-8, or Tyr-32 of the rCXCR1 N-domain, or both. Thus, our CD experiments afford some insight into specific intermolecular interactions of rCXCR1 and IL-8.

Our measured binding affinities in micelles are significantly higher than in solution, indicating that the micellar environment reduces the entropic penalty of the N-domain peptides in binding to ligand. This conclusion is further corroborated by the fact that, although the 34-mer exhibited slightly lower ligand binding affinity in solution than the 23-mer, both peptides bound ligand in the presence of micelles with approximately the same high affinity (~1 μM; Table I). The entropic penalty paid by the longer peptide (34-mer) was reduced in micelles, allowing it to bind ligand with the same affinity as the 23-mer. Our observation that both fragments exhibit the same degree of conformational constraint in micelles (in terms of mean residue ellipticity) lends further credence to this premise. Unlike the 23-mer, the 34-mer N-domain did bind MGSA with significant affinity (~3 μM) in micelles, suggesting that MGSA binding requires additional residues that are not present on the 23-mer for its interaction with the N-domain. The fact that

### Table I

Binding affinities of N-domain to IL-8 and MGSA in buffer and micelles

| Ligand   | Receptor | Kd in buffer | Kd in micelles |
|----------|----------|--------------|---------------|
| IL-8 23-mer | 7.7 ± 1.5 | 1.6 ± 0.8 |
| IL-8 34-mer | 37.1 ± 3.2 | 1.5 ± 0.6 |
| IL-8 Scrambled | ND | ND |
| MGSA 23-mer | ND | ND |
| MGSA 34-mer | 3.0 ± 0.9 |

**a** Reported Kd is a representative value of three to six independent binding measurements using at least two different samples.

**b** ND, not detected.

At the maximum value (367 nm for buffer, 350 nm for DPC) was plotted against ligand concentration.

![Binding affinities of N-domain to IL-8 and MGSA in buffer and micelles](image-url)
both IL-8 and MGSA bound the N-domain in micelles with similar affinity indicates that the N-domain is not the primary determinant of ligand selectivity in the intact receptor.

Structure-Function Relationships—Chemokine binding and function involves two interactions: between the receptor N-domain and ligand N-loop residues (site I) and between receptor exoloops and/or the transmembrane (J-domain) and ligand N-terminal residues (site II). The two-site interaction in receptors that bind protein and peptide ligands is believed to have evolved from the simple one-site interaction, involving the receptor exoloops/transmembrane residues, seen in 7-transmembrane receptors that bind small molecules. Such a two-site model affords far greater opportunities for fine-tuning of regulation, by modulating the binding affinity and selectivity of the receptor through differential communication between the two sites in different ligand-receptor pairs.

The binding affinity can be expressed in terms of free energy ($\Delta G$), and two possible models are considered. In the simplest model (Model I), the two interactions are simply additive,

$$\Delta G_{\text{binding}} = \Delta G_{\text{siteI}} + \Delta G_{\text{siteII}}$$

Model I

where $\Delta G_{\text{binding}}$ is the free energy change associated with the overall binding interaction, $\Delta G_{\text{siteI}}$ is the free energy change associated with the ligand N-loop and receptor N-domain interaction, and $\Delta G_{\text{siteII}}$ is the free energy change associated with the ligand ELR and receptor J-domain interaction.

A model in which the binding events are coupled is represented below (Model II),

$$\Delta G_{\text{binding}} = \Delta G_{\text{siteI}} + \Delta G_{\text{siteII}} + \Delta G_{\text{coupling}}$$

Model II

where $\Delta G_{\text{coupling}}$ is the coupling energy between the sites. This coupling energy could be positive or negative; in the former case, binding at site I/II increases the free energy required for binding at site II/I, and in the latter, binding at one site decreases the free energy for binding at the other site.
Substitution and deletion of the ELR residues both in IL-8 and MGSA result in up to 1000-fold reduction in binding (18, 20, 27). These mutations minimize but cannot completely eliminate the contribution of these residues to site II interaction, and the measured binding affinity cannot be exclusively assigned to site I interaction (26, 32). Thus, to understand the individual components of the two-site interaction, as well as to understand the coupling between these sites, we need to measure the individual interactions under conditions similar to those seen in the intact receptor. CXCXR1 N-domain fragments inhibit IL-8 binding to the intact receptor with an inhibition constant ($K_i$) of $\sim 20 \mu M$ (35, 39). Because the N-domain is proximal to the membrane interface, we measured the direct binding of the ligand to the N-domain in micelles that mimic the native membrane environment. Our data indicate that both IL-8 and MGSA bind CXCXR1 N-domain in micelles with fairly high affinity ($K_d \sim 1–3 \mu M$) underscoring the importance of measuring direct ligand binding to individual domains in micelles that reflect a native-like environment. Measuring $\Delta G$ is not trivial, because multiple receptor exoloop residues mediate site II interaction (31, 32). In principle, $\Delta G_{\text{all}}$ can be obtained from measuring the binding affinity of a peptide the containing ELR motif to the intact receptor. Such studies could not detect any binding, suggesting that the binding is either weak or undetectable in solution due to conformational entropy (27). Thus, in CXCXR1, the N-domain is a major contributor to affinity, binding IL-8 and MGSA with a $K_d$ of $\sim 1–3 \mu M$. The observations that the ELR sequence is conserved and ELR deletion mutants for both ligands result in similar reduction in binding indicate that free energy of binding for site II interaction is similar (18, 20, 27). These observations suggest $\Delta G_{\text{coupling}}$ dictates ligand specificity. We propose that $\Delta G_{\text{coupling}}$ is positive for MGSA, and hence binding at site I increases the energy required for binding to site II (negative cooperativity). Coupling between the two sites could arise due to a conformational change in either the ligand or receptor, or both. Both ELR and N-loop residues are adjacent to each other, and further are connected to the core structure via disulfide bonds. Mutations in N-loop residues such as His-18 in MGSA result in high affinity binding but lower activity, whereas a similar mutation in IL-8 results in a marginal decrease in both binding and function (14, 23). Mutations remote from the binding surface also effect binding to different extent in IL-8 and MGSA. For instance, mutation of Glu-38 in MGSA results in 10-fold reduction in binding and function, whereas a similar mutation has no effect on IL-8 binding and function (18, 47). These observations suggest that the ligand structural features play a role in coupling the two-site interactions. The role of receptor N-domain and the exoloops in determining binding affinity and ligand selectivity have been studied by swapping these domains between CXCXR1 and CXCXR2. Some of the receptor chimeras show binding affinities and function that cannot be attributed simply for switched domains of the parent receptors, suggesting that receptor domains also play a role in coupling the two-site interactions (2, 35).

In summary, our studies indicate that the CXCXR1 N-domain adopts a defined conformation in a micellar environment, and direct ligand binding measurements carried out using isolated receptor domains in micelles capture the functional characteristics of that domain in the intact membrane-bound receptor. The ligand-binding affinities shown by the N-domain peptides were significantly higher in micelles than in solution, and the structural and energetic basis of IL-8 and MGSA interaction with the receptor N-domain were different. They share common binding regions, but MGSA requires additional sites at the

**Fig. 7.** CD spectra of 23-mer and 34-mer rCXCXR1 N-domain in solution and micelles. All measurements were carried out in 50 mM sodium phosphate, 50 mM sodium chloride buffer (pH 8.0), with or without 50 mM DPC. The far-UV CD spectra of 23-mer (A) and 34-mer (B), and near-UV CD spectra of 23-mer (C) and 34-mer (D) in buffer and micelles are shown. Solid lines represent spectra in buffer, whereas dotted lines represent spectra in micelles.
C-terminal region, and the observation that MGSAs binds to the CXCR1 N-domain only in micelles will establish a new approach to looking at the energetic and structural basis of ligand binding, selectivity, and specificity in this system. Chemokines and their receptors have been implicated in a number of inflammatory diseases, including cancer (48, 49). Therefore, knowledge of the structural and thermodynamic basis for receptor binding is essential for designing receptor-specific and broad spectrum inhibitors.

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