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Synergistic Interactions between Chemokine Receptor Elements in Recognition of Interleukin-8 by Soluble Receptor Mimics

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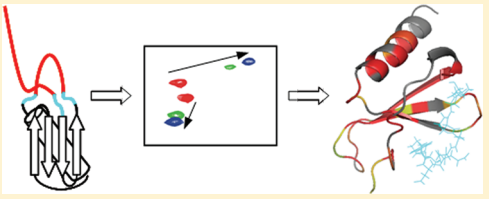
Supporting Information

ABSTRACT: Interleukin-8 (IL-8 or CXCL8), the archetypal member of the CXC chemokine subfamily, stimulates neutrophil chemotaxis by activating receptors CXCR1/IL8RA and CXCR2/IL8RB. Previous mutational studies have implicated both the N-terminal and third extracellular loop (E3) regions of these receptors in binding to IL-8. To investigate the interactions of these receptor elements with IL-8, we have constructed soluble proteins in which the N-terminal and E3 elements of either CXCR1 or CXCR2 are juxtaposed on a soluble scaffold protein; these are termed CROSS-$N^{X1}E3^{X1}$ and CROSS-$N^{X2}E3^{X2}$, respectively. Isothermal titration calorimetry and nuclear magnetic resonance spectroscopy were used to compare the IL-8 binding properties of the receptor mimics to those of control proteins containing only the N-terminal or E3 receptor element. CROSS-$N^{X2}E3^{X2}$ bound to monomeric IL-8 with the same affinity and induced the same chemical shift changes as the control protein containing only the N-terminal element of CXCR2, indicating that the E3 element of CXCR2 did not contribute to IL-8 binding. In contrast, CROSS-$N^{X1}E3^{X1}$ bound to IL-8 with ∼10-fold increased affinity and induced different chemical shift changes compared to the control protein containing only the N-terminal element of CXCR1, suggesting that the E3 region of CXCR1 was interacting with IL-8. However, a chimeric protein containing the N-terminal region of CXCR1 and the E3 region of CXCR2 (CROSS-$N^{X1}E3^{X2}$) bound to IL-8 with thermodynamic properties and induced chemical shift changes indistinguishable from those of CROSS-$N^{X1}E3^{X1}$ and substantially different from those of CROSS-$N^{X2}E3^{X2}$. These results indicate that the N-terminal and E3 regions of CXCR1 interact synergistically to achieve optimal binding interactions with IL-8.



Chemokines make up a family of secreted, soluble proteins that orchestrate the tissue localization of leukocytes by activation of G protein-coupled chemokine receptors expressed in the leukocyte membranes.[1−3] Because of the importance of leukocyte trafficking in both homeostasis and inflammatory processes, there is widespread interest in understanding the molecular and mechanistic details of these interactions. Numerous chemokine structures have been determined, and mutational studies have shown that the amino-terminal and "N-loop" regions of these proteins play critical roles in their receptor interactions. The recently determined crystal structure of receptor CXCR4[4] has confirmed that chemokine receptors consist of seven transmembrane helices, four extracellular elements (the N-terminus and three loops, herein termed E1−E3), and four intracellular elements (three loops and the C-terminus). Receptor mutants and chimeras have led to the model in which chemokine ligands interact initially with the receptor N-terminus and subsequently with a second site formed by the extracellular loops and possibly including some interactions with transmembrane helices.[5]

Interleukin-8 (IL-8 or CXCL8), the archetypal member of the CXC chemokine subfamily, activates neutrophils by binding to receptors CXCR1/IL8RA and CXCR2/IL8RB. Whereas CXCR1 is specific for IL-8, CXCR2 can also be activated by several other CXC chemokines, including CXCL1/GROα/MGSA and CXCL7/NAP-2.[6−8] IL-8, like many chemokines, is dimeric at high concentrations. Although recent results have implicated both monomeric and dimeric forms in chemotactic function,[9,10] the monomeric unit is sufficient for receptor activation.[11] The three-dimensional structures of both wild-type IL-8 and a monomeric IL-8 variant have been determined.[12,13] Mutants and synthetic variants of IL-8 have identified residues in the N-loop and 3_{10}-turn region (residues 10−22) as being important for binding to CXCR1 and CXCR2, whereas the N-terminal Glu-Leu-Arg (ELR) motif is critical for receptor activation.[14,15]

The N-terminal elements of CXCR1 and CXCR2 play a central role in the interactions of these receptors with chemokine ligands. Chimeric receptors consisting of the N-terminal element of one IL-8 receptor on a background of the other receptor displayed ligand binding selectivity very similar to that of the receptor from which the N-terminal element was derived.[16−18] In addition, site-directed mutations of several N-terminal residues caused significantly reduced binding affinity for IL-8.[19,20] Moreover, peptides corresponding to the N-terminal elements of human or rabbit CXCR1 bind to human

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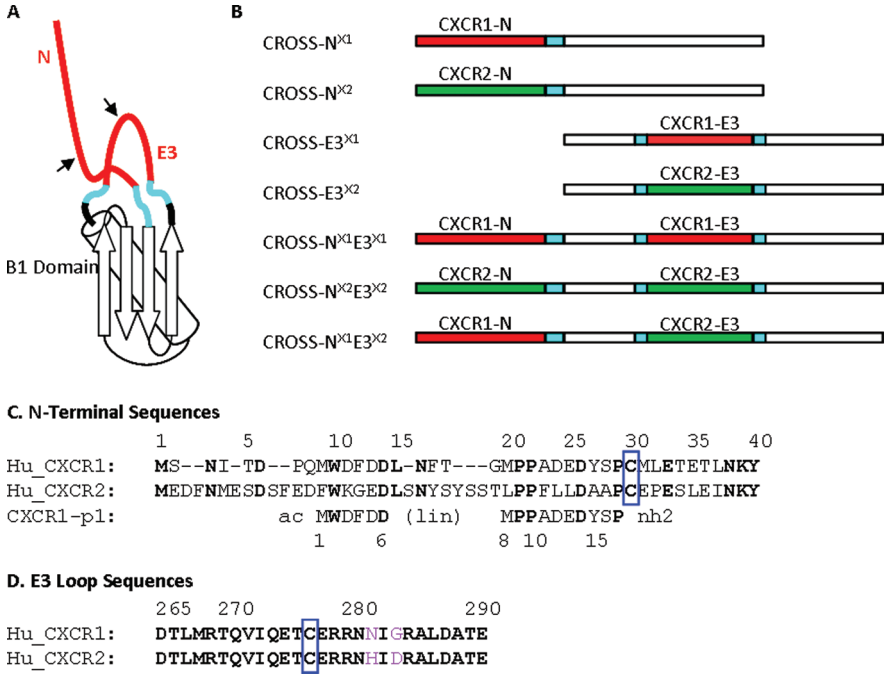

Figure 1. Schematic diagrams of CROSS proteins and sequence alignments of N-terminal and E3 elements. (A) Schematic diagram of CROSS-$N^{X1}E3^{X1}$ showing the B1 domain scaffold (black), the N-terminal and E3 loop regions of CXCR1 (red), and linkers (cyan). Arrows indicate the positions of Cys residues that form a disulfide bond in the native receptors but are mutated to Ser in the CROSS proteins used in this study. (B) Schematic representations and nomenclature of the CROSS proteins described herein, with N-terminal and E3 loop regions of CXCR1 (red) and CXCR2 (green), linkers (cyan), and B1 domain scaffold regions (black). (C) Alignment of the N-terminal sequences of CXCR1 and CXCR2 and the sequence of the CXCR1 peptidomimetic CXCR1-p1.[24] The numbering at the top corresponds to that of CXCR1, whereas the numbering at the bottom is that for CXCR1-p1. Features of CXCR1-p1 include an N-terminal acetyl group (ac), a C-terminal amide (nh2), and a hexanoic acid linker (lin). (D) Alignment of the E3 loop sequences of CXCR1 and CXCR2. The two differences between these sequences are highlighted in violet. The numbering corresponds to the sequence of intact CXCR1. In panels C and D, conserved amino acids are denoted in bold type. The Cys residues in blue boxes were mutated to Ser in the CROSS proteins used in this study.

IL-8[18,21] and inhibit dimerization of the chemokine.[22] NMR studies have mapped the binding sites of N-terminal peptides from human and rabbit CXCR1 on the surface of IL-8.[21,23] Skelton et al. have reported the solution structure of IL-8 bound to a peptidomimetic (CXCR1-p1) containing residues 9−14 and residues 20−29 of CXCR1 connected via a hexanoic acid linker.[24] The peptidomimetic binds into a groove between the N-loop residues and the third β-strand of IL-8. In contrast, the IL-8 binding surface for the N-terminal element of CXCR2 has not been reported.

In addition to the N-terminal element, the three extracellular loops of CXCR1 and CXCR2 are also likely to play a role in receptor binding and/or activation by IL-8. A chimera consisting of the N-terminal element of receptor CCR1 (which is not a receptor for CXCR2 ligands) on a background of CXCR2 supported both binding and activation by CXCR2 ligands.[17] Similarly, substitution of the C-terminal end (transmembrane region 5 onward) of human CXCR2 with the corresponding region of rabbit CXCR1 increases the affinity for IL-8 and for GRO/MGSA, suggesting that the third extracellular loop (E3) may contribute to the binding interaction.[18] Moreover, extensive site-directed mutagenesis has identified residues in all three extracellular loops of CXCR1 as being necessary for IL-8 binding.[19,20] In particular, alanine mutations of several residues in the E3 loop cause substantial reductions in IL-8 affinity, although most of the mutants can support full receptor activation at high ligand concentrations. Despite the importance of the E3 loop in ligand binding, there is currently no information about the region of IL-8 that interacts with this receptor element.

We have previously developed a model system for chemokine receptors in which the N-terminal and E3 regions of a receptor are attached to a soluble scaffold protein in a manner that allows them to interact simultaneously with cognate chemokines for that receptor.[25,26] These "receptor mimics" are termed CROSS proteins, for chemokine receptor elements on a soluble scaffold. In this paper, we describe the development of CROSS proteins corresponding to CXCR1 and CXCR2 and the application of these proteins in characterizing the interactions of receptor N-terminal and E3 elements with IL-8. Initial results suggested that the E3 region of CXCR1 was interacting with IL-8 whereas the E3 region of CXCR2 was not. Further characterization of a chimeric receptor mimic indicated that the difference could be attributed to the adjacent N-terminal element, indicating that the N-terminal and E3 elements of CXCR1 interact synergistically with IL-8.

EXPERIMENTAL PROCEDURES

Expression and Purification of Monomeric IL-8(1−66).

A plasmid containing the gene encoding a fusion protein between thioredoxin and IL-8(1−66)[27] was received as a kind gift from K. Rajarathnam (The University of Texas Medical Branch, Galveston, TX). The coding region for IL-8(1−66) was subcloned between the HindIII and XhoI sites of *Escherichia coli* expression vector pET11c (Novagen, Inc., Madison, WI). The resulting construct encodes an N-terminal His_6 tag, and a modified thrombin cleavage site (LVPR*SA), so that cleavage

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after the R* residue yields the native N-terminus (S^1A^2...) of IL-8; the gene and protein sequences are given in Figure S1 of the Supporting Information. Throughout this work, IL-8 refers to this IL-8(1−66) construct, which was used to avoid IL-8 dimerization, a complicating factor in interpretation of binding data. BL21(DE3) cells transformed with this construct were grown in LB medium (for unlabeled protein) or in M9 medium supplemented with 1 g/L [^{15}N]ammonium chloride (for the ^{15}N-labeled protein) at 37 °C to an OD_{600} of 0.6, induced with 1 mM IPTG, and grown for an additional 4 h at 37 °C before being harvested. Cell pellets were washed in 20 mM Tris-HCl (pH 8.0) and then resuspended in the same buffer (50 mL/L of culture). Phenylmethanesulfonyl floride (PMSF) was added to a final concentration of 200 μM, and the cells were lysed by two passes through a cell homogenizer. The lysate was centrifuged (30 min at 27000*g*), and the pellet was suspended in 20 mM Tris-HCl, 0.75 M urea, and 1% Triton X-100 (pH 8.0) (50 mL/L of culture) and then centrifuged (30 min at 27000*g*). The pellet was then solubilized in 6 M guanidine HCl, 100 mM NaH_2PO_4, and 10 mM Tris Base (pH 8.0) (50 mL/L of culture) and clarified by centrifugation (30 min at 27000*g*). The supernatant was incubated with 5−7 mL of Ni-NTA agarose resin (Qiagen, Valencia, CA), which was then poured into a benchtop column, washed successively with 6 M guanidine HCl, 100 mM NaH_2PO_4, 10 mM Tris Base (pH 8.0), and the same buffer at pH 6.3, and then eluted with the same buffer at pH 4.9. Dithiothreitol was added to a final concentration of 50 mM, and the sample was incubated for 3 h at ambient temperature. The protein was then dialyzed against 0.8 M guanidine HCl, 20 mM NaH_2PO_4, 0.5 mM oxidized glutathione, and 2.5 mM reduced glutathione (pH 6.5) for 12 h at 4 °C; 20 mM acetic acid for 8 h at 4 °C; and finally 20 mM Tris-HCl (pH 8.5) for 12 h at 4 °C. The dialysate was clarified by centrifugation (30 min at 27000*g* and 4 °C). The N-terminal His_6 tag was removed by incubation with thrombin (≈5 units/mL, Sigma, St. Louis, MO) for 45 min at ambient temperature. The cleaved IL-8 protein was then loaded onto a HiTrap SP HP cation exchange column (Amersham Biosciences, Piscataway, NJ) pre-equilibrated with 20 mM Tris-HCl and 25 mM NaCl (pH 8.5) and eluted with a 0 to 2 M NaCl gradient. The purity and molecular weight were verified by SDS−PAGE and MALDI-TOF mass spectrometry.

Cloning of Expression Constructs for CROSS Proteins. The gene and protein sequences of CROSS expression constructs used in this study (summarized in Figure 1) are given in Figure S2 of the Supporting Information. Briefly, the N-terminal and E3 regions of the human CXCR1 and CXCR2 genes (containing the native Cys codons) were synthesized from overlapping oligonucleotides using recursive polymerase chain reaction and then subcloned into the gene encoding the CCR3 mimic CROSS^5-N_3E3_3.[25,26] Subsequently, the Cys codons were modified by Quikchange (Stratagene, Inc.) site-directed mutagenesis. Each of the control constructs (see Figure 1) was obtained by one or more subcloning steps from the CROSS-$N^{X1}E3^{X1}$ and/or CROSS-$N^{X2}E3^{X2}$ constructs. The chimeric CROSS-$N^{X1}E3^{X2}$ construct was obtained from the CROSS-$N^{X1}E3^{X1}$ construct by a single round of Quikchange mutagenesis to simultaneously introduce the Asn282 → His and Gly284 → Asp mutations.

Expression and Purification of CROSS Proteins. *E. coli* BL21(DE3) cells transformed with CROSS expression constructs were grown in LB medium at 37 °C to an OD_{600} of 0.7−0.8, induced with 1 mM IPTG, and grown for an additional ≈16 h at 25 °C before being harvested. Cell pellets were resuspended in 50 mM NaH_2PO_4, 300 mM NaCl, and 5 mM imidazole (pH 8.0) (20 mL/L of culture), PMSF was added to a final concentration of 500 μM, and the cells were lysed by two passes through a cell homogenizer. The lysate was centrifuged (30 min at 27000*g*), and the filtered supernatant was loaded onto a Ni-NTA agarose column pre-equilibrated with 50 mM NaH_2PO_4, 300 mM NaCl, and 5 mM imidazole (pH 8.0), washed with the same buffer containing 20 mM imidazole, and eluted with the same buffer containing 200 mM imidazole. After dialysis against 20 mM Tris-HCl (pH 8.5) (2.5 h at ambient temperature), thrombin (≈5 units/mL, Sigma) was added and the solution was incubated for 2.5 h at ambient temperature. The cleaved CROSS protein was then loaded onto a HiTrap Q HP anion exchange column (Amersham Biosciences) pre-equilibrated with 20 mM Tris-HCl and 25 mM NaCl (pH 8.5) and eluted with a 0 to 2 M NaCl gradient. The purity and molecular weight were verified by SDS−PAGE and MALDI-TOF mass spectrometry.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) experiments were performed at 20 °C, using a VP-ITC microcalorimeter (MicroCal Inc., Northampton, MA). The CROSS and IL-8 samples were extensively dialyzed against 50 mM MOPS buffer (pH 7.0), filtered, and degassed. Protein concentrations were determined from the absorbance at 280 nm. Experiments were performed by titration of CROSS protein (0.2−0.6 mM in the injection syringe) into IL-8 (0.01−0.03 mM in the sample cell); the reference cell was filled with degassed distilled water. One injection of 3 μL followed by 36 injections of 8 μL were made, with 5 min equilibration periods between injections. The ITC data were analyzed in Origin for ITC version 5.0 (MicroCal Inc.). Data were corrected for heats of dilution, determined from the integrated peaks well beyond the inflection point of the curve, baseline corrected, and integrated. The cumulative heat evolved (Q) was fit to the single-site binding equation

$$Q = (nP_t\Delta HV_0/2)\{1 + L_t/(nP_t) + 1/(nK_aP_t) - \sqrt{[1 + L_t/(nP_t) + 1/(nK_aP_t)]^2 - 4L_t/(nP_t)}\}$$

where L_t and P_t are the total ligand (CROSS) and protein (IL-8) concentrations, respectively, n is the stoichiometry, K_a (=1/K_d) is the association equilibrium constant, ΔH is the binding enthalpy, and V_0 is the active initial cell volume. The initial 3 μL injection was excluded from the data fitting.[28] Reported parameters are the averages from three or four replicate experiments.

NMR Spectroscopy. NMR spectra were recorded at 25 °C on a Varian 600 MHz NMR spectrometer equipped with a triple-resonance Cold Probe. To monitor the binding of IL-8 to CROSS proteins, we recorded ^{15}N HSQC spectra for samples of ^{15}N-labeled IL-8 [initial concentration of 130−200 μM in 50 mM acetate, 0.1% NaN_3, and 10% D_2O (pH 5.5)] containing increasing molar ratios (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6) of unlabeled CROSS. Spectra were referenced to external DSS at 0 ppm. Data were processed using NMRPipe[29] and analyzed using Sparky version 3.113 (T. D. Goddard and D. G. Kneller, University of California, San Francisco, CA). Weighted amide chemical shift changes ($\Delta\delta_{wt}$) are defined as $\Delta\delta_{wt} = [\Delta\delta_H^2 + (\Delta\delta_N/5)^2]^{1/2}$. The amide ^{1}H and ^{15}N chemical shift assignments for IL-8(1−66) were determined previously.[27]

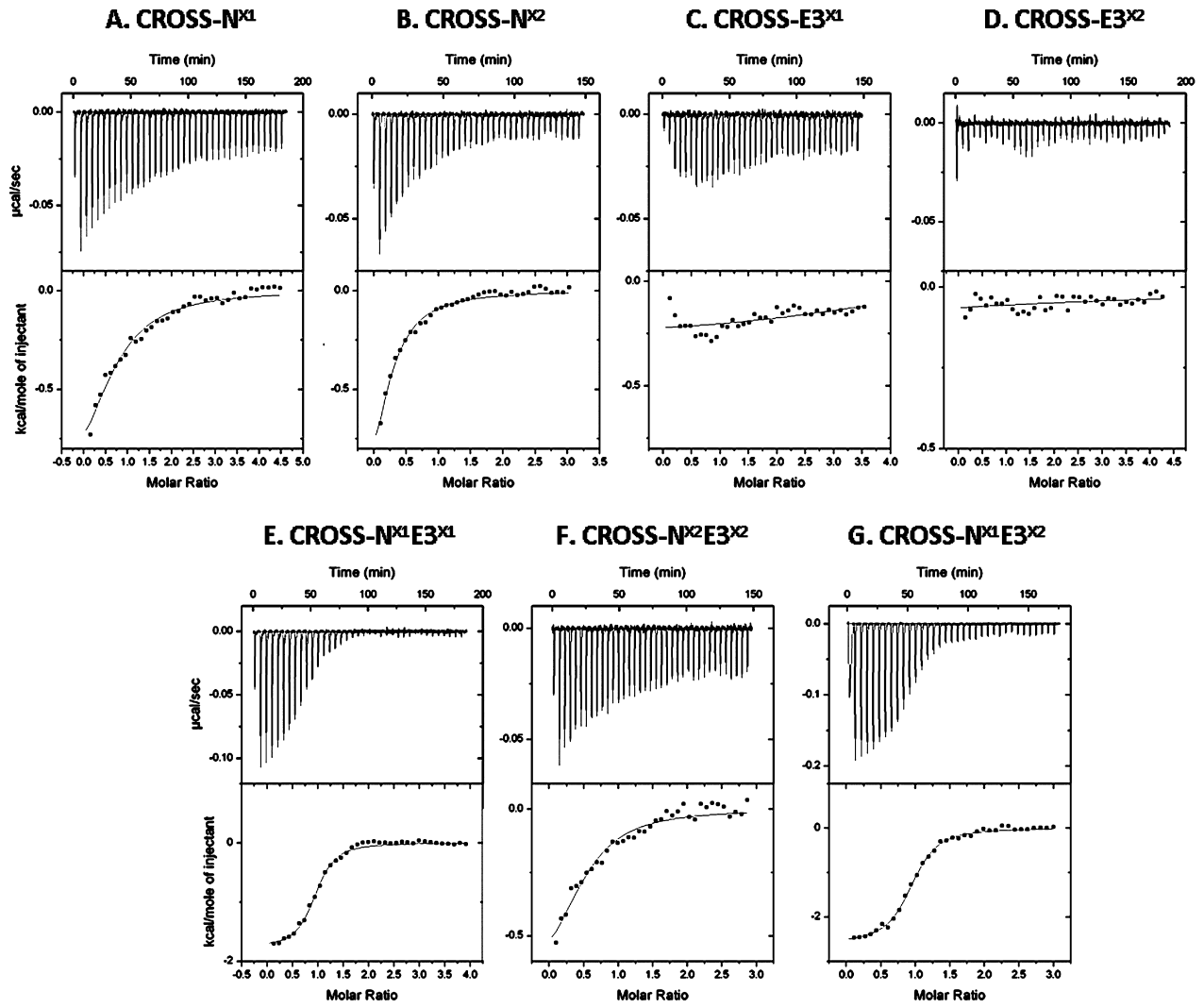

Figure 2. Isothermal titration calorimetry data for binding of CROSS proteins and controls to IL-8. Each panel shows the raw titration data (top) and the fitted binding isotherm (bottom) for a representative example from triplicate or quadruplicate experiments. Derived thermodynamic parameters are listed in Table 1.

Table 1. Thermodynamic Parameters for Binding of CROSS Proteins to IL-8[a]

protein	stoichiometry (n)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	ΔG (kcal/mol)	K_d (μM)
CROSS-N^{X1}	0.8 ± 0.1	−1.2 ± 0.3	−5.5 ± 0.4	−6.8 ± 0.1	9.6 ± 1.9
CROSS-N^{X2}	0.4 ± 0.1	−1.5 ± 0.5	−5.5 ± 0.6	−6.9 ± 0.1	7.1 ± 1.6
CROSS-E3^{X1}			ND[b]		
CROSS-E3^{X2}			ND[b]		
CROSS-N^{X1}E3^{X1}	0.9 ± 0.1	−1.8 ± 0.1	−6.4 ± 0.2	−8.2 ± 0.1	0.8 ± 0.1
CROSS-N^{X2}E3^{X2}	0.8 ± 0.2	−1.0 ± 0.5	−6.1 ± 0.5	−7.1 ± 0.2	5.7 ± 1.7
CROSS-N^{X1}E3^{X2}	0.9 ± 0.1	−2.3 ± 0.3	−5.9 ± 0.3	−8.3 ± 0.1	0.7 ± 0.1

[a]Parameters were obtained by isothermal titration calorimetry at 20 °C. The values reported are from an average of three or four experiments. [b]Binding not detected. A very weak exothermic signal was observed.

RESULTS

Development of Soluble Mimics of CXCR1 and CXCR2. In previous studies, we developed soluble proteins containing the N-terminal and E3 elements of CC chemokine receptors CCR2 and CCR3.[25,26] These "CROSS" proteins utilized a stabilized form of the B1 domain of streptococcal protein G as a soluble scaffold. The N-terminal region of the receptor was incorporated as an N-terminal extension to the scaffold protein, whereas the receptor E3 element was inserted between two secondary structure elements (the β2-strand and α-helix) of the scaffold. The two receptor elements were connected by a native disulfide bond. In this study, we initially expressed CROSS proteins in which the N-terminal and E3 elements of CXC chemokine receptor CXCR1 or CXCR2 were incorporated in a manner identical to that of the elements of CCR2 and CCR3 in the previous CROSS proteins. However, these proteins were expressed as heterogeneous, oligomeric mixtures, and attempts to refold them to the desired monomeric form were unsuccessful (data not shown). To

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prevent oligomerization, we mutated the Cys residues in the stabilized scaffold back to their native B1 domain residues and the single Cys residue in each receptor element to Ser. Notably, the latter mutations remove a native disulfide bond that plays an important role in maintaining native receptor structure.[20] The resulting CROSS proteins were expressed as soluble monomers, could be readily purified to homogeneity, and gave circular dichroism spectra consistent with their expected secondary structures (Figures S3 and S4 of the Supporting Information). Throughout the remainder of this work, all proteins described incorporate the Cys to Ser mutations. These CROSS proteins and controls are represented schematically, along with their nomenclature, in panels A and B of Figure 1. We refer to the CXCR1 and CXCR2 CROSS proteins as CROSS-$N^{X1}E3^{X1}$ and CROSS-$N^{X2}E3^{X2}$, respectively, in which the two superscripts indicate the receptors from which the N-terminal and E3 elements are derived. To identify the roles of each receptor element, we prepared four control proteins (CROSS-N^{X1}, CROSS-$E3^{X1}$, CROSS-N^{X2}, and CROSS-$E3^{X2}$), each containing only the N-terminal element or only the E3 element of CXCR1 or CXCR2 (Figure 1B).

Binding of Receptor Mimics to IL-8. The affinity and thermodynamics of binding between monomeric IL-8 and each receptor mimic or control protein were monitored by ITC (Figure 2). Binding parameters are summarized in Table 1. The two control proteins containing only receptor N-terminal elements each bound exothermically to IL-8 with equilibrium dissociation constants (K_d) of approximately 10 μM (Figure 2A,B), whereas the control proteins containing only E3 elements showed little to no detectable binding (Figure 2C,D). The two CROSS proteins differed in their binding characteristics. CROSS-$N^{X1}E3^{X1}$ bound to IL-8 with a K_d of 0.8 ± 0.1 μM, approximately 10-fold more strongly than the CROSS-N^{X1} control protein (Figure 2E). However, the affinity of CROSS-$N^{X2}E3^{X2}$ for IL-8 (K_d = 5.7 ± 1.7 μM) was not significantly different from the corresponding control [K_d = 7.1 ± 1.6 μM for CROSS-N^{X2} (Figure 2F)]. These results suggested that, in the context of these CROSS proteins, the E3 element of CXCR1 contributes to IL-8 binding but the E3 element of CXCR2 does not. The stoichiometry of binding estimated from ITC data was close to 1:1 for most CROSS proteins. However, the data for CROSS-N^{X2} indicated a stoichiometry of 0.4 ± 0.1, suggesting a possible error in the concentration of this protein; this would have an only small effect on the fitted K_d value.

NMR Mapping of IL-8 Residues Influenced by CROSS Binding. NMR spectroscopy was used to investigate the regions of IL-8 involved in binding to each receptor mimic and to the control proteins containing only N-terminal elements. Each protein induced linear changes in specific signals in the ^{15}N HSQC spectrum of IL-8 up to a molar ratio of 1:1, at which point no further changes were observed. These observations are consistent with 1:1 binding stoichiometry and affinity in the low micromolar range; K_d values in the range of 1−10 μM are not readily distinguishable because of the relatively high concentrations (130−200 μM) of IL-8 used in these experiments. Figure 3 shows a detailed region of the HSQC spectrum for IL-8 alone (red in each panel) and upon binding to each CROSS or control protein. The Supporting Information includes the full spectrum of IL-8 alone and IL-8 bound to CROSS-$N^{X1}E3^{X1}$ (Figure S5 of the Supporting Information) and both a list (Table S1 of the Supporting Information) and bar graph (Figure S6 of the Supporting Information) of the

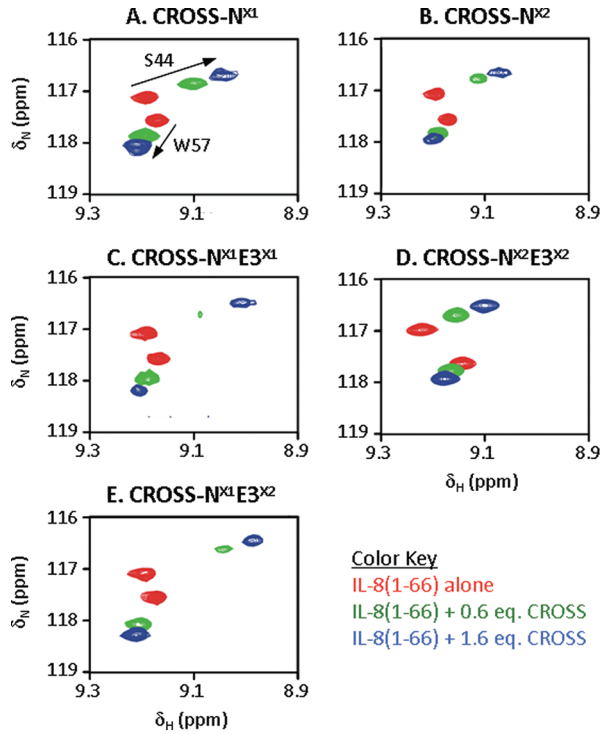

Figure 3. Two-dimensional NMR data for binding of CROSS proteins and controls to IL-8. Each panel shows the same detailed region of the ^{15}N HSQC spectrum of IL-8 alone (red), IL-8 in the presence of 0.6 molar equiv of the indicated CROSS or control protein (green), and IL-8 saturated with 1.6 molar equiv of the indicated CROSS or control protein (blue). The peak assignments and directions of peak shifts are indicated in panel A; in all other panels, the peaks shift in the same direction, although the shifts of S44 have a larger magnitude for CROSS-$N^{X1}E3^{X1}$ and CROSS-$N^{X1}E3^{X2}$.

observed chemical shift changes for each CROSS or control protein. Figure 4 and Figure S7 of the Supporting Information show scatter plots comparing the shifts observed for each pair of CROSS or control proteins; correlation coefficients and slopes are listed in Table 2.

CROSS-N^{X1} and CROSS-N^{X2} each induced substantial changes in numerous NH resonances of IL-8. Moreover, there are strong correlations (r^2 = 0.97; slope ≈ 0.7) between both the ^1H_N and $^{15}N_H$ shifts induced by these two proteins (Figure 4A and Table 2), suggesting that they interact with the same region of IL-8. The structure of IL-8 is color-coded according to the weighted NH shifts ($\Delta\delta_{wt}$) in Figure 5A. Substantial shifts are observed throughout the secondary structure elements of the chemokine.

The soluble mimic of CXCR2, CROSS-$N^{X2}E3^{X2}$, also induced changes in the HSQC spectrum of IL-8, but these changes were extremely similar to those observed for the CROSS-N^{X2} control protein (and also for CROSS-N^{X1}) as illustrated by the scatter plots in Figure 4B [$r^2 \geq 0.95$; slope ≈ 1.1 (Table 2)]. These results indicate that the E3 region of CXCR2 does not interact with IL-8 and does not alter the interaction of the N-terminal element with IL-8, which is consistent with the affinity measurements presented above.

In contrast to the results for CROSS-$N^{X2}E3^{X2}$, the chemical shift changes induced by the soluble mimic of CXCR1 (CROSS-$N^{X1}E3^{X1}$) differed substantially from those observed for the corresponding control protein (CROSS-N^{X1}) and from those observed for CROSS-$N^{X2}E3^{X2}$. This is evident from the

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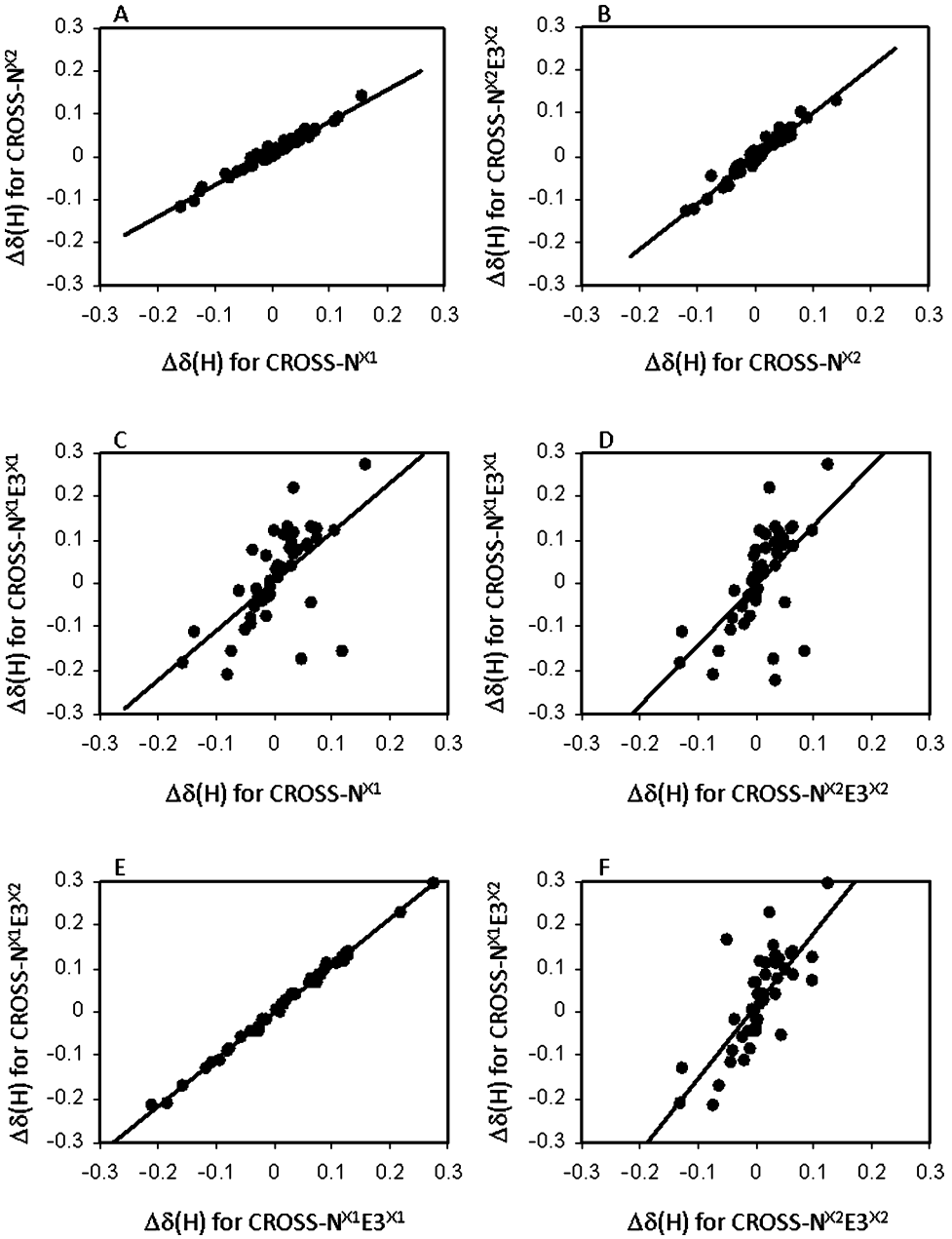

Figure 4. Correlations between chemical shift changes for binding of IL-8 to CROSS proteins. Each panel shows the ^{1}H chemical shift change (in parts per million) for binding of IL-8 to one CROSS protein or control protein (*x*-axis) plotted vs the ^{1}H chemical shift change for another CROSS protein or control protein (*y*-axis). Each point represents a different residue of IL-8. Data are shown for all residues excluding L43, C50, and L51, which show intermediate exchange behavior in spectra of IL-8 bound to CROSS-N^{X1}E3^{X2}. Solid lines show linear regression fits to the data. Similar graphs for ^{15}N chemical shift changes are presented in Figure S7 of the Supporting Information. Correlation coefficients and slopes for the fits are listed in Table 2.

Table 2. Correlations between Chemical Shift Changes for Binding of CROSS Proteins to IL-8

	$\Delta\delta(^{1}H)$		$\Delta\delta(^{15}N)$	
protein	r^2	slope	r^2	slope
CROSS-N^{X1} vs CROSS-N^{X2}	0.97	0.74	0.97	0.73
CROSS-N^{X2} vs CROSS-N^{X2}E3^{X2}	0.95	1.05	0.99	1.09
CROSS-N^{X1} vs CROSS-N^{X1}E3^{X1}	0.40	1.13	0.58	1.33
CROSS-N^{X2}E3^{X2} vs CROSS-N^{X1}E3^{X1}	0.38	1.39	0.58	1.26
CROSS-N^{X1}E3^{X1} vs CROSS-N^{X1}E3^{X2}	1.00	1.08	0.99	1.12
CROSS-N^{X2}E3^{X2} vs CROSS-N^{X1}E3^{X2}	0.59	1.69	0.83	2.29

scatter plots in panels C and D of Figure 4 [$r^2 \approx 0.4$–0.6 (Table 2)]. These comparisons indicate that the E3 region of CXCR1 either interacts with IL-8 or alters the interaction of the N-terminal element with IL-8, both of which would be consistent with the higher affinity of IL-8 for CROSS-N^{X1}E3^{X1} compared to those of other proteins (vide supra). Notably, the structural color map for binding of CROSS-N^{X1}E3^{X1} to IL-8 (Figure 5B) and the difference map (Figure 5C) indicate that the same regions of IL-8 are influenced by binding to both CROSS-N^{X1} and CROSS-N^{X1}E3^{X1} (although the shifts at each specific position were different). Thus, the

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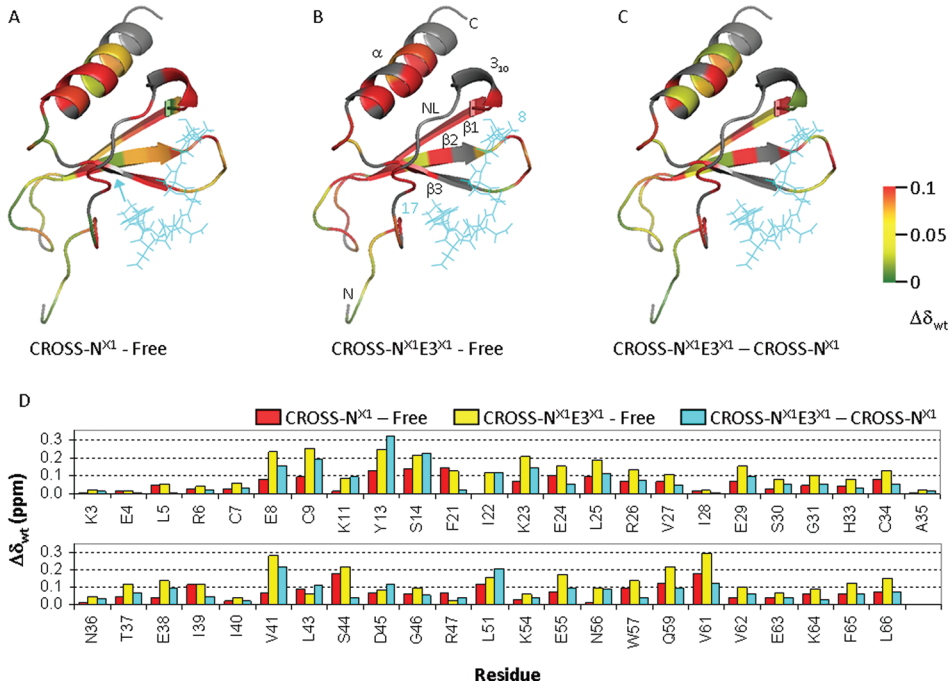

Figure 5. Chemical shift mapping of binding of IL-8 to CROSS proteins. The structure of IL-8 bound to the peptidomimetic CXCR1-p1 is color-coded according to the weighted chemical shift changes ($\Delta\delta_{wt}$) observed for binding to (A) CROSS-N^{X1} and (B) CROSS-$N^{X1}E3^{X1}$ and (C) the weighted difference in chemical shift between IL-8 bound to CROSS-N^{X1} and CROSS-$N^{X1}E3^{X1}$. The continuous color scale is shown in the color bar; residues with a $\Delta\delta_{wt}$ of >0.1 are colored red, and residues for which data were not obtained are colored gray. The structurally well-defined region of CXCR1-p1 is shown as cyan sticks. The termini of CXCR1-p1 and several structural elements of IL-8 are labeled in panel B (NL, N-loop; 3_{10}, 3_{10}-turn). The cyan arrow in panel A indicates the direction in which CXCR1 residues following Pro17 may extend to interact with strands β1 and β2. Panels A–C were generated using PyMol. (D) Bar graph showing the weighted differences in chemical shifts between (red) free IL-8 and IL-8 bound to CROSS-N^{X1}, (yellow) free IL-8 and IL-8 bound to CROSS-$N^{X1}E3^{X1}$, and (cyan) IL-8 bound to CROSS-N^{X1} and CROSS-$N^{X1}E3^{X1}$.

data did not clearly indicate a specific binding site on IL-8 for the E3 element of CXCR1.

Analysis of a Chimeric Receptor Mimic. The E3 regions of CXCR1 and CXCR2 differ by only two amino acids (Figure 1D). Therefore, it was surprising that the addition of the E3 element enhanced IL-8 binding for the CXCR1 mimic but not for the CXCR2 mimic. It was possible that the two variable amino residues were responsible for this difference. However, an alternative hypothesis was that the E3 region of CXCR2 behaved differently from that of CXCR1 only because of its position adjacent to the N-terminal region of CXCR2 rather than to the N-terminal region of CXCR1. To distinguish between these two possibilities, we prepared and characterized a chimeric receptor mimic (CROSS-$N^{X1}E3^{X2}$) containing the N-terminal region of CXCR1 and the E3 region of CXCR2 (Figure 1B).

The ITC data in Figure 2G and the thermodynamic parameters in Table 1 indicate that CROSS-$N^{X1}E3^{X2}$ binds to IL-8 with affinity, enthalpy, and stoichiometry indistinguishable from those of CROSS-$N^{X1}E3^{X1}$. Moreover, CROSS-$N^{X1}E3^{X2}$ induces changes in the IL-8 HSQC spectrum (Figure 3E) that are highly correlated with those of CROSS-$N^{X1}E3^{X1}$ [$r^2 \approx 0.99$; slope $\approx$ 1.1 (Figure 4E and Table 2)] but relatively poorly correlated with those of CROSS-$N^{X2}E3^{X2}$ [$r^2 \approx 0.6$–0.8; slope $\approx$ 1.7–2.3 (Figure 4F and Table 2)]. Again, the comparisons of NMR shifts are consistent with the variations of binding affinity observed by ITC. These data strongly suggest that CROSS-$N^{X1}E3^{X2}$ has the same IL-8 binding interactions as CROSS-$N^{X1}E3^{X1}$.

DISCUSSION

Interactions of Receptor N-Terminal Elements with IL-8. In this study, we have used a soluble model system to investigate the interactions of monomeric IL-8 with the N-terminal and E3 elements of both IL-8 receptors CXCR1 and CXCR2. We have shown that the N-terminal elements of both receptors, when attached to the N-terminus of the stabilized B1 domain, bind to IL-8 with affinities of 9.6 ± 1.9 and 7.1 ± 1.6 μM, respectively, and that binding of both peptides is characterized by a small favorable enthalpy change and a larger favorable entropy change. The affinities of these interactions are very similar to that previously reported for the binding of monomeric IL-8 to a peptide derived from the N-terminus of rabbit CXCR1 (K_d = 8.6 μM).[27] A similar affinity was also found for binding between the rabbit CXCR1 peptide and wild-type IL-8, although in the latter case peptide binding was coupled to dissociation of the IL-8 dimer.[22] Rabbit CXCR1 peptide was used in the previous studies because of practical difficulties in working with peptides derived from the N-terminal region of human CXCR1.[27] The use of the B1 domain scaffold in this study provides a way to overcome this impediment.

The NMR studies reported herein provide information about the regions of IL-8 that are influenced by interaction with the receptor N-terminal elements. Changes observed in amide chemical shifts could potentially result from direct interactions of the affected residues with the receptor elements or from indirect structural changes induced by these interactions. The amide chemical shift changes observed upon binding of CROSS-N^{X1} are mapped onto the structure of IL-8 in Figure

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5A. There are pronounced chemical shift changes in the N-loop (Cys9, Ile10, Tyr13, Ser14, and Phe17), the 3_{10}-turn (Lys20 and Phe21), and strand β3 (Glu48, Leu49, and Lys51) of IL-8. These residues form a contiguous surface that is similar to the IL-8 binding surface for the N-terminal peptidomimetic (designated CXCR1-p1) observed previously by Skelton et al.[24] In addition, we observed substantial chemical shift changes at the N-terminal end of the α-helix (Asn56, Trp57, Gln59, Arg60, and Val61) and for some residues in the β2−β3 turn (Leu43 and Ser44) and strands β1 and β2 (Glu24, Leu25, and Ile39) of IL-8. Changes in the α-helix are not surprising as the affected region interacts directly with the N-loop. Similarly, changes in the β2−β3 turn could be indirect effects related to the interactions of nearby residues in strand β3. The causes of the changes in strands β1 and β2 are less obvious but may be an indication that residues of CXCR1 following Pro17 continue to wrap around under the β-sheet as indicated by the cyan arrow in Figure 5A.

The interaction surface on IL-8 for the N-terminal element of CXCR2 has not been described previously. The correlation shown in Figure 4A indicates that binding of CROSS-N^{X2} induces amide chemical shift changes of IL-8 almost identical to those observed upon binding of CROSS-N^{X1}. Thus, the N-terminal element of CXCR2 interacts with the same surface of IL-8 as that discussed above for the N-terminus of CXCR1. The observation that the two receptor N-terminal elements bind to the same region of IL-8 and that they do so with very similar thermodynamics is surprising considering that there are numerous differences between the amino acid sequences of these two elements. In the alignment shown in Figure 1C (which includes numerous gaps), there are only 16 amino acid identities among the 40 residues of CXCR1 (or 49 residues of CXCR2). Nevertheless, it is noteworthy that the CXCR1-p1 peptidomimetic that binds to IL-8 incorporates six of these identical residues, and four of them (Pro9, Pro10, Asp14, and Pro17) lie within the region of residues 8−17 that is well-defined in the structural ensemble (Figure 1C).[24] Among these, the three proline residues form numerous hydrophobic interactions with the binding groove, and Asp14 is in the proximity of IL-8 residue Lys11. In summary, our data suggest that the N-terminal element of CXCR2 uses the few conserved amino acids to interact with IL-8 in a manner very similar to that observed for the CXCR1 peptidomimetic.

Possible Interactions of Receptor E3 Loops with IL-8. The CROSS proteins described herein provide an opportunity to explore the interactions of the E3 loop regions of CXCR1 and CXCR2 with IL-8. In the absence of the receptor N-terminal elements (i.e., for CROSS-$E3^{X1}$ and CROSS-$E3^{X2}$), we did not observe any interaction of receptor E3 elements with IL-8. Similarly, the protein containing both N-terminal and E3 elements of CXCR2 (CROSS-$N^{X2}E3^{X2}$) bound to IL-8 with an affinity identical to that for CROSS-N^{X2}, which contains only the N-terminal element, suggesting that the E3 element of CXCR2 was not interacting with IL-8. This conclusion is supported by the excellent correlation between IL-8 chemical shift changes induced by CROSS-N^{X2} and CROSS-$N^{X2}E3^{X2}$ (Figure 4B). In contrast, the protein containing both N-terminal and E3 elements of CXCR1 (CROSS-$N^{X1}E3^{X1}$) displayed a 12-fold higher affinity than CROSS-N^{X1} for IL-8, and there was a much weaker correlation between chemical shift changes observed for these two proteins (Figure 4C). Initially, this result suggested that the E3 element of CXCR1 was interacting with IL-8. In an effort to identify a region of IL-8 that interacts with the E3 element of CXCR1, we have color-coded the IL-8 structure with the chemical shift changes observed for binding of CROSS-N^{X1} (Figure 5A) and CROSS-$N^{X1}E3^{X1}$ (Figure 5B). Although the binding of CROSS-$N^{X1}E3^{X1}$ induces larger changes in amide chemical shift (more red in Figure 5B than in Figure 5A), there is no region of IL-8 that is affected by binding to CROSS-$N^{X1}E3^{X1}$ and not affected by binding to CROSS-N^{X1}. Further illustrating this point, panels C and D of Figure 5 show the chemical shift differences between IL-8 bound to CROSS-N^{X1} and CROSS-$N^{X1}E3^{X1}$. The regions of IL-8 showing the greatest differences are the same as those displaying the greatest changes upon binding of CROSS-N^{X1}. Thus, these data do not allow us to identify a specific binding surface on IL-8 for the E3 loop of CXCR1.

Synergistic Interactions of N-Terminal and E3 Elements. The E3 elements of CXCR1 and CXCR2 have highly similar sequences (Figure 1D), with only two differences in 27 residues. It was therefore surprising that the E3 element of CXCR1 enhanced IL-8 binding affinity whereas the E3 element of CXCR2 did not. The simplest possible explanation of these observations is that the E3 element of CXCR1 interacts directly with IL-8 but that the two variant residues play critical roles so the E3 element of CXCR2 cannot interact. To test this hypothesis, we constructed the chimeric protein (CROSS-$N^{X1}E3^{X2}$) containing the N-terminal element of CXCR1 and the E3 element of CXCR2. Both the affinity data and the chemical shift perturbation data for this variant were identical to those for CROSS-$N^{X1}E3^{X1}$. Thus, the ability of the E3 element to enhance IL-8 binding affinity is not reliant on the identities of the two variant residues but is dependent on the presence of the CXCR1 N-terminal element. This is a clear indication of synergistic interactions between the N-terminal and E3 elements of the CXCR1 receptor.

There are two general structural models that could account for the observation of synergistic interactions. One possibility is that the E3 element (of CXCR1 or CXCR2) interacts directly with IL-8 but that this interaction can occur (at least in this model system) only in the presence of the N-terminal element of CXCR1, not in the presence of the N-terminal element of CXCR2. An alternative explanation is that the E3 element does not interact directly with IL-8 but instead is able to support additional or stronger interactions with IL-8 by the N-terminal element of CXCR1 (but not by the N-terminal element of CXCR2). It is not possible on the basis of our data to differentiate unequivocally between these two models. For example, both models would be consistent with the observation that the enhanced affinity upon addition of the E3 element has both enthalpic and entropic components ($\Delta\Delta H$ = −0.6 kcal/mol, and $-T\Delta\Delta S$ = −0.9 kcal/mol). However, as noted above, there is no evidence from chemical shift mapping to indicate a specific binding surface for the E3 loop. Therefore, these data provide some implicit support for the latter model. Structural analysis of CROSS-$N^{X1}E3^{X1}$ bound to IL-8 could potentially reveal the structural details of the interactions. However, unfortunately we have not been able to identify appropriate conditions for either crystallization or NMR structural analysis of CROSS-$N^{X1}E3^{X1}$ and its complex with IL-8.

These results have possible implications for the widely accepted two-step model for chemokine−receptor binding and activation.[5] According to the two-step model, in the second stage of receptor interaction the N-terminal region of IL-8 interacts with the extracellular loops and/or the transmembrane

helices of the receptor. Because we could not identify a specific binding surface for the E3 loop on IL-8 and the E3 region induces little if any chemical shift perturbation in the N-terminal region of the chemokine, including the functionally critical ELR sequence, our data suggest that the E3 region alone is not sufficient to form the second binding site in the two-step model. Thus, the receptor site involved in the second step of chemokine binding is likely to include regions of the receptor that are not part of the CROSS proteins described here. This conclusion is consistent with the previous observation that mutations within extracellular loop 2 of CXCR1 reduce binding affinity and eliminate activation by IL-8.[20]

Irrespective of the specific structural model, our observation of synergistic interactions suggests that the N-terminal and E3 elements are in the proximity of each other in the IL-8 complex of CROSS-$N^{X1}E3^{X1}$, suggesting that they are also adjacent to each other in the intact receptor. This is consistent with expectations based on previous information. In one study of CXCR1, mutation of E3 residue Asp265 to Ala resulted in weakened staining with an antibody raised against the receptor N-terminal element, suggesting that the E3 loop is adjacent to the N-terminus.[19] On the basis of chemokine receptor sequence comparisons and the CXCR4 structure,[4] the N-terminal and E3 elements are expected to be adjacent to each other and linked by a conserved disulfide bond; mutation of the participating cysteine residues renders CXCR1 almost completely inactive.[20] It is interesting that our data suggest interactions between these two receptor elements, even in the absence of this disulfide bond, which was excluded form the CROSS proteins described here to allow isolation of homogeneous proteins. Thus, previous structural and functional data are consistent with a model in which N-terminal and E3 residues interact with each other and/or with the same regions of the chemokine ligand.

It is curious that synergistic interactions between the N-terminal and E3 elements occur in the CXCR1 model protein but not in the CXCR2 model protein. However, this difference may reflect true differences between the structures and interactions of the intact receptors, which have previously been suggested by studies of receptor internalization rates. In one study, equal doses of IL-8 caused CXCR1 to be 70% internalized in 30 min whereas CXCR2 was 95% internalized within 5 min.[10] Studies of chimeric and mutant receptors clearly demonstrated that the differences in the rate and extent of internalization could be attributed to differences between the receptor extracellular elements,[10,30] suggesting that IL-8 interacts differently with the extracellular regions of CXCR1 and CXCR2, thereby inducing distinct conformational changes and different rates of internalization. The existence of different activated conformations of the receptors is supported by the observation that saturating doses of different CXCR2 ligands (or mutants of natural ligands) also stimulate receptor internalization at different rates.[30]

Concluding Remarks. The structural basis of G protein-coupled receptor activation remains incompletely understood. However, the picture emerging from recent structural and pharmacological data is that these receptors have remarkable structural plasticity allowing different ligands to induce different activated states and thereby to stimulate different signaling pathways. The studies of CXCR1 and CXCR2 internalization rates discussed above have previously suggested that these receptors can be induced to access different structural states depending on the chemokine agonist used and that the same chemokine (IL-8) can induce different structural states of the two receptors. This study shows that IL-8 forms very similar interactions with the isolated N-terminal region of each receptor. However, the adjacent E3 region of the receptor enhances these interactions for the N-terminal region of CXCR1 but not for the corresponding region of CXCR2. These new observations are consistent with the inference from previous studies that IL-8 binds to CXCR1 and CXCR2 differently and induces different conformational changes of these two receptors. In summary, the data reported herein enhance our understanding of the structural interactions underlying differential CXCR1 and CXCR2 activation by IL-8.

■ ASSOCIATED CONTENT

Supporting Information

A table listing chemical shift changes of IL-8 upon binding to CROSS proteins, figures showing the gene and protein sequences of the IL-8 and CROSS expression constructs, SDS−PAGE gels and circular dichroism spectra of purified CROSS-$N^{X1}E3^{X1}$ and CROSS-$N^{X2}E3^{X2}$, the ^{15}N HSQC spectrum of monomeric IL-8 alone and bound to CROSS-$N^{X1}E3^{X1}$, chemical shift changes of IL-8 upon binding to CROSS proteins, and correlations between ^{15}N chemical shift changes for binding of IL-8 to CROSS proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

$\Delta\delta_{wt}$, weighted amide chemical shift change; CCR, CC chemokine receptor; CROSS, chemokine receptor elements on a soluble scaffold; CXCR, CXC chemokine receptor; COSY, correlation spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt; HSQC, heteronuclear single-quantum coherence; IL-8, interleukin-8; IPTG, isopropyl β-D-thiogalactopyranoside; ITC, isothermal titration calorimetry; K_d, dissociation equilibrium constant; MALDI-TOF, matrix-assisted laser desorption ionization; MGSA, melanoma growth stimulatory activity; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NAP, neutrophil-activating protein; NMR, nuclear magnetic resonance; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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