Chemokine-Binding Specificity of Soluble Chemokine-Receptor Analogues: Identification of Interacting Elements by Chimera Complementation

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ABSTRACT: The specificity of chemokine-receptor interactions plays a central role in the regulation of leukocyte migration in inflammatory responses. Herein, we describe a soluble mimic of CC chemokine receptor 2 (CCR2), dubbed CROSS−N_2E3_2, which incorporates the N-terminal region (N) and third extracellular loop (E3) elements of CCR2 displayed on the surface of a soluble protein scaffold. CROSS−N_2E3_2 binds to the CCR2 ligand monocyte chemoattractant protein-1 (MCP-1) with a dissociation equilibrium constant of 1.1 ± 0.1 μM but does not bind to the cognate chemokines of the receptor CCR3 (eotaxin-1, -2, and -3). Similarly, a soluble analogue of CCR3 (CROSS^5−N_3E3_3) binds to eotaxin-1, -2, and -3 but not to MCP-1. Thus, these receptor analogues have the same specificity as the natural receptors. Using soluble proteins containing N and E3 elements from different receptors (CROSS−N_2E3_3 and CROSS−N_3E3_2), we demonstrate that both receptor elements are required for optimal binding to the cognate chemokines. In addition, we report the binding affinities of all four CROSS proteins to a panel of two wild-type and six chimeric chemokines. These complementation studies indicate the regions of the chemokines that interact with each element of the receptors, allowing us to deduce the orientations of the receptor extracellular elements relative to the bound chemokines.

A central feature of inflammatory responses is the activation and migration of leukocytes to areas of inflammation. Chemokines (*1*, *2*) are small, secreted proteins that promote the chemotaxis of leukocytes from the bloodstream into inflamed tissue by activating seven transmembrane G protein-coupled receptors (GPCRs)[1] embedded in the leukocyte membrane (*2*, *3*). Chemokines are differentially expressed in a variety of inflamed or healthy tissues, and chemokine receptors are differentially expressed on various classes of leukocytes (*1*). These expression patterns along with the specificity of chemokine-receptor recognition regulate the distribution of leukocytes in tissues during inflammation and homeostasis (*1*). In addition to their role in inflammation, chemokine receptors are required by human immunodeficiency virus 1 (HIV-1) for leukocyte infection (*4*, *5*), and tumor metastasis can be influenced by activation of chemokine receptors overexpressed on tumor cells (*6*−*9*). Undoubtedly, delineating the factors that control the specificity of chemokine-receptor recognition would support efforts to develop specific anti-inflammatory, HIV-1 suppressive, or antimetastatic agents.

CC chemokine receptors 3 (CCR3) and 2 (CCR2) are the most abundant chemokine receptors expressed on the surfaces of eosinophils (*5*) and monocytes (*10*), respectively. These two receptors display differential specificity for the chemokines eotaxin-1 and monocyte chemoattractant protein-1 (MCP-1), despite the 65% sequence identity between these two chemokines. MCP-1 activates CCR2 at subnanomolar concentrations with a 50% effective concentration (EC_{50}) of 0.7 ± 0.1 nM (*11*−*14*) but activates CCR3 at much higher concentrations (*11*, *15*). In contrast, eotaxin-1 is a potent agonist for CCR3 (EC_{50} = 2.6 ± 0.2 nM) (*11*, *16*−*19*) and a much weaker agonist for CCR2 (EC_{50} ∼ 100−400 nM) (*11*, *15*). The specificity of each of these chemokine-receptor interactions leads to a myriad of differential pathologies; the CCR2−MCP-1 interaction is implicated in atherosclerosis, rheumatoid arthritis, and asthma (*20*−*24*), whereas activation of CCR3 by eotaxin-1 causes accumulation of eosinophils in allergic diseases and elicits responses to parasitic infestations (*25*−*29*).

Mutational studies have revealed many of the features of MCP-1 and eotaxin-1 required for recognition of their cognate receptors (*12*, *19*), but studies of receptor features that dictate interactions with the relevant chemokines have been less extensive. A peptide corresponding to the N-terminal region (N) of CCR3 binds weakly to eotaxin-1, whereas peptides from the three extracellular loops of CCR3 do not show detectable binding (*30*). On the other hand, cell-based studies of chimeric receptors suggest that both the N terminus and the third extracellular loop (E3) of CCR3 contribute to eotaxin-1 affinity (*31*) and that all four extracellular elements contribute to the affinity of CCR2 for MCP-1 (*14*). Detailed biochemical studies of receptor recognition are limited by the heterogeneous nature of the cell surface and the difficulty purifying and/or crystallizing these transmembrane proteins.

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[1] Abbreviations: CCR2, CC chemokine receptor 2; CCR3, CC chemokine receptor 3; N, N-terminal region; E3, third extracellular loop; MCP-1, monocyte chemoattractant protein-1; GPCR, G protein-coupled receptor; HIV-1, human immunodeficiency virus 1; EC_{50}, 50% effective concentration; CROSS, chemokine-receptor elements on a soluble scaffold; MALDI−TOF, matrix-assisted laser desorption ionization time of flight; CD, circular dichroism; IC_{50}, 50% binding inhibition concentration; E1, first extracellular loop; E2, second extracellular loop.

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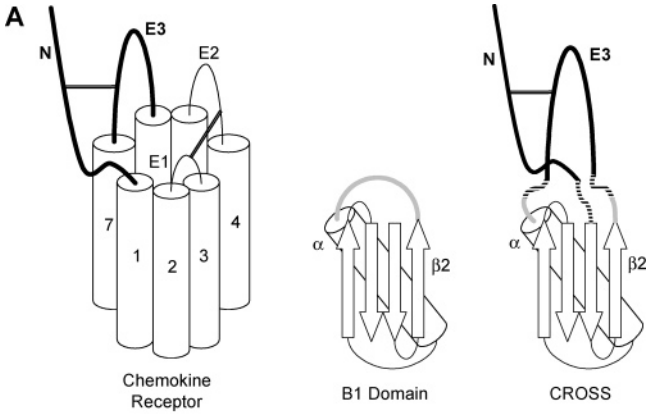

B **CROSS Protein Composition**

Receptor Mimic	N-Terminal Element From:	E3 Loop Element From:
$CROSS^5$-N_3E3_3	CCR3	CCR3
CROSS-N_2E3_2	CCR2	CCR2
CROSS-N_2E3_3	CCR2	CCR3
CROSS-N_3E3_2	CCR3	CCR2

FIGURE 1: Design of soluble receptor mimics (CROSS proteins). (A) Schematic representation of the CROSS protein design. Left, predicted topology of chemokine receptors, showing the membrane-spanning helices (cylinders), N terminus (thick black), extracellular loops [E1 and E2 (thin black) and E3 (thick black)], and disulfide bonds (double lines); Center, B1 domain of protein G (PDB code 2GB1), showing the α helix (cylinder) and β strands (arrows) and highlighting the β2−α turn (gray); Right, CROSS protein, showing the chemokine-receptor elements (thick black), displayed on the B1 domain scaffold. The N terminus of the chemokine receptor is attached at the N terminus of the B1 domain via a $(Gly)_3$ linker (dashes), whereas the E3 loop of the chemokine receptor is inserted between residues Ala-20 and Ala-23 of the β2−α turn (gray), with $(Gly)_2$ linkers (dashes) at each junction. (B) Table indicating the elements of each natural chemokine receptor (CCR2 and CCR3) contained within each of the four CROSS proteins discussed in this paper.

As a complementary approach to the study of intact chemokine receptors, we have recently developed a soluble protein that mimics the interactions of two extracellular elements of CCR3 with eotaxin-1 (*32*). This receptor mimic consists of the N terminus and the E3 loop of CCR3 juxtaposed on a soluble scaffold, as illustrated in Figure 1 and is referred to herein as $CROSS^5-N_3E3_3$, where CROSS stands for chemokine-receptor elements on a soluble scaffold, the subscripts indicate that the N and E3 elements are derived from receptor CCR3, and the superscript indicates that this was the fifth variant prepared [$CROSS^5$ in the previous paper (*32*)]. $CROSS^5-N_3E3_3$ binds to eotaxin-1 with ∼3 μM affinity, inhibits binding of eotaxin-1 to CCR3, and interacts with many of the same elements of eotaxin-1 that are recognized by the natural receptor (*32*). Thus, $CROSS^5-N_3E3_3$ and similar proteins provide an opportunity to explore the interactions of extracellular receptor elements with the cognate chemokines.

In this paper, we describe a soluble mimic of CCR2, dubbed $CROSS-N_2E3_2$, and demonstrate that both $CROSS^5-N_3E3_3$ and $CROSS-N_2E3_2$ bind to chemokines with the same selectivity as the corresponding natural receptors. We then report measurements of the binding of these two receptor mimics and two additional CROSS proteins with a series of wild-type and chimeric chemokines. These data are analyzed to identify the receptor elements that interact with each of several regions in the cognate chemokines. On the basis of these conclusions, we develop a low-resolution model representing the orientation of the bound chemokines on the surfaces of the receptors.

MATERIALS AND METHODS

Protein Preparation. Chemokines were expressed in *Escherichia coli* and purified following similar procedures to those published previously (*29*, *30*, *33*−*35*). Fluorescein-labeled eotaxin-1 and MCP-1 for anisotropy measurements were obtained by reaction of single cysteine mutants with 5-idoacetamidofluorescein (Molecular Probes, Eugene, OR); mutations were near the C termini and did not influence affinities for receptors or CROSS proteins. Construction of $CROSS^5-N_3E3_3$ was described previously (*32*). The genes encoding $CROSS-N_2E3_2$, $CROSS-N_3E3_2$, $CROSS-N_2E3_3$, $CROSS-N_2$, and $CROSS-E3_2$ were obtained by modification of the $CROSS^5-N_3E3_3$ construct. In the $CROSS-N_2$ and $CROSS-E3_2$ sequences, cysteine residues in the receptor elements were replaced with serine residues to prevent protein oligomerization. Each CROSS protein was expressed as an N-terminal His_6-tagged fusion protein in BL21(DE3) *E. coli* and then purified by (1) nickel-affinity chromatography, (2) thrombin cleavage to remove the His_6 tag, and (3) anion-exchange chromatography, as described (*32*). For $CROSS-N_2$, thrombin treatment did not result in removal of the His_6 tag; therefore, data are reported for the purified fusion protein. For each protein, the matrix-assisted laser desorption ionization time-of-flight (MALDI−TOF) mass spectrum was consistent with the predicted monomeric molecular weight.

Circular Dichroism (CD) Spectra. CD spectra were recorded on a Jasco J-715 spectropolarimeter as described previously (*32*). Each spectrum is the sum of five scans recorded at 20 nm/min and a resolution of 1 nm using a 0.1 cm cuvette at 4 °C. Data for control scans, recorded using the same parameters, were subtracted from the scans of the protein samples to eliminate the background signal resulting from the buffer.

Fluorescence Anisotropy. Anisotropy measurements of fluorescein-labeled chemokines were recorded at 4 °C on a Perkin−Elmer LS-50B fluorometer using excitation and emission wavelengths of 494 and 518 nm, respectively. Samples were dissolved in 10 mM Hepes, 150 mM NaCl, and 3 mM EDTA at pH 7.4. For CROSS protein binding to fluorescein-labeled wild-type chemokines, duplicate affinity measurements were made by titration of CROSS proteins into 100 nM solutions of the labeled chemokines, and data were fit to a single-site binding isotherm as described (*32*). The binding affinities between unlabeled chemokines and CROSS proteins were determined by competitive fluorescence anisotropy binding assays at 4 °C, as described in detail elsewhere (*36*). For binding to $CROSS^5-N_3E3_3$ or $CROSS-N_3E3_2$, fluorescein-labeled eotaxin-1 was premixed with the CROSS variant to 75% saturation and increasing amounts of unlabeled chemokine variant were added. For binding to $CROSS-N_2E3_2$ or $CROSS-N_2E3_3$, fluorescein-labeled MCP-1 was premixed with the CROSS variant to 75% saturation and increasing amounts of unlabeled chemokine variant were added. Displacement curves were fit as de-

Table 1: Binding Affinities of Chemokines for CROSS Proteins and Natural Receptors

protein	eotaxin-1	eotaxin-2	eotaxin-3	MCP-1
CROSS^5−N_3E3_3 (K_d μM)	3.6 ± 0.8	9.8 ± 0.8	5.1 ± 0.5	>200
CROSS−N_2E3_2 (K_d, μM)	137 ± 62	>200	>200	1.1 ± 0.1
CROSS−N_3E3_2 (K_d, μM)	25 ± 10	51 ± 10	41 ± 16	93 ± 36
CROSS−N_2E3_3 (K_d, μM)	68 ± 33	>200	>100	7.6 ± 3.4
CCR3 (IC_{50}, nM)	2.1 ± 0.1	9.7 ± 0.8	1.2 ± 0.2	130 ± 20
CCR2 (IC_{50}, nM)	20 ± 2	580 ± 80	3.0 ± 0.1	0.15 ± 0.03

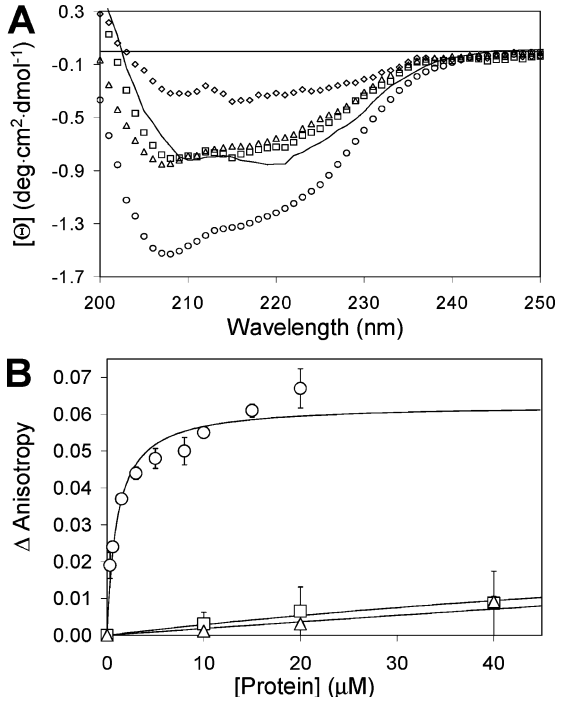

FIGURE 2: Physical characterization of CROSS proteins. (A) Far-UV CD spectra of the B1 domain (—), CROSS^5−N_3E3_3 (○), CROSS−N_2E3_2 (□), CROSS−N_3E3_2 (△), and CROSS−N_2E3_3 (◇). All spectra were recorded using 20 μM protein in 20 mM NaH_2PO_4 and 150 mM NaCl at pH 8.0 and 4 °C. Spectra are presented in units of molar ellipticity; if spectra are viewed in terms of residue molar ellipticity, the B1 domain (56 residues) shows a larger negative ellipticity than the CROSS proteins (116−124 residues) (B) Titration of fluorescein-labeled MCP-1 with CROSS−N_2E3_2 (○), CROSS−N_2 (□), and CROSS−$E3_2$ (△). The B1 domain does not bind detectably to MCP-1.

scribed (*36*) to yield the K_d of the CROSS variant for each unlabeled chemokine.

RESULTS

A Soluble Mimic of CCR2 (CROSS−N_2E3_2). In our previous paper, we reported that juxtaposition of the N and E3 elements of CCR3 on a soluble scaffold (a stabilized variant of the protein G B1 domain) yielded a protein, CROSS^5−N_3E3_3, that binds to eotaxin-1 with ~3 μM affinity (*32*). To test whether the corresponding elements of CCR2 are also adequate to mimic the ligand binding of this receptor, we prepared an analogue of CROSS^5−N_3E3_3 in which the N and E3 elements of CCR3 are replaced by those of CCR2 (Figure 1). After purification, the CCR2 analogue, dubbed CROSS−N_2E3_2, had the expected molecular weight (13 403.7 Da found; 13 400.7 Da calculated). The CD spectrum (Figure 2A) shows that CROSS−N_2E3_2 has secondary structural features similar to those expected for the B1 domain scaffold but reduced secondary structure content relative to CROSS^5−N_3E3_3. Nevertheless, CROSS−N_2E3_2 binds to MCP-1 with a dissociation equilibrium constant (K_d) of 0.9 ± 0.2 μM, as demonstrated by the change in fluorescence anisotropy of fluorescein-labeled MCP-1 (Figure 2B and Table 1).

To determine whether the interaction of CROSS−N_2E3_2 with MCP-1 utilizes both the N and E3 regions, we prepared the control proteins CROSS−N_2 and CROSS−$E3_2$, which incorporate only the N or E3 element of CCR2, respectively. As shown in Figure 2B, the affinity of each control protein for MCP-1 was at least ~100-fold lower (K_d > 100 μM) than the affinity of CROSS−N_2E3_2 for MCP-1. The simplest explanation of these results is that both receptor elements interact directly with MCP-1 in the CROSS−N_2E3_2:MCP-1 complex. However, an alternative possibility is that the interactions of one element with MCP-1 are dependent on structural constraints (such as the disulfide bond) imposed by the other element. Very similar results were obtained for the interactions of eotaxin-1 with CROSS^5−N_3E3_3 and control proteins lacking each CCR3 element (*32*).

Chemokine Specificity of CROSS Proteins: Comparison with Natural Receptors. We have previously reported the affinities of the natural chemokine receptors CCR2 and CCR3 for the chemokines MCP-1, eotaxin-1, eotaxin-2, and eotaxin-3 (*11*). For comparison with the CROSS proteins, the concentrations (IC_{50} values) required to half-maximally inhibit binding of ^{125}I-labeled MCP-1 or eotaxin-1 to CCR2 or CCR3, respectively, are listed in Table 1 and shown graphically in Figure 3A. In cases for which others have reported similar affinity measurements, they are consistent with our data (*15*). CCR2 displays a strong preference for MCP-1 over the eotaxin group chemokines, whereas CCR3 displays the opposite selectivity.

To test whether the specificities of the CROSS proteins mimic those of the corresponding natural receptors, we used competitive fluorescence anisotropy assays to measure the affinities of CROSS^5−N_3E3_3 and CROSS−N_2E3_2 for eotaxin-1, eotaxin-2, eotaxin-3, and MCP-1. In these assays, CROSS^5−N_3E3_3 or CROSS−N_2E3_2 was bound to a fluorescein-labeled chemokine (eotaxin-1 or MCP-1, respectively), and the chemokine of interest was titrated into the mixture causing displacement of the labeled chemokine and a consequent decrease in fluorescence anisotropy. Data are presented in Figure 4, and K_d values are listed in Table 1. CROSS^5−N_3E3_3 binds to all three eotaxin group chemokines with low micromolar affinity but does not bind detectably to MCP-1 (K_d > 200 μM) (Figure 4A). In contrast, CROSS−N_2E3_2 binds to MCP-1 with a K_d of 1.1 ± 0.1 μM in the competition assay but to all three eotaxin group chemokines with much lower affinities (K_d > 100 μM) (Figure 4B). Thus, the two CROSS proteins display parallel specificities to the natural chemokine receptors (parts A and B of Figure 3), increasing confidence that the interactions of CROSS^5−N_3E3_3 and CROSS−N_2E3_2 with the relevant chemokines

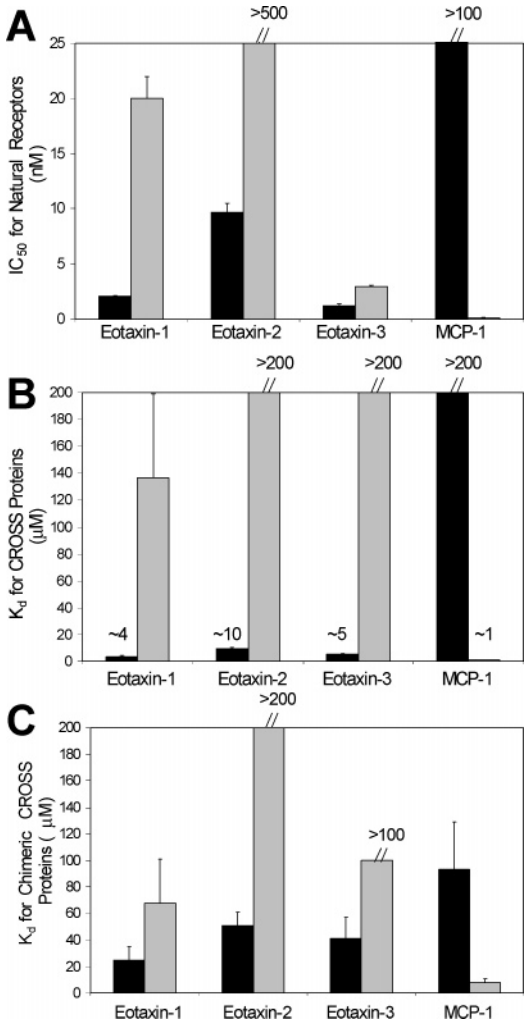

FIGURE 3: Comparison of chemokine-binding affinities of CROSS proteins and natural receptors. (A) IC_{50} values determined for binding of CCR3 (black bars) and CCR2 (gray bars) to eotaxin-1, eotaxin-2, eotaxin-3, and MCP-1 (*11*). (B) K_d values determined for binding of CROSS$^5-N_3E3_3$ (black) and CROSS$-N_2E3_2$ (gray) to eotaxin-1, eotaxin-2, eotaxin-3, and MCP-1. (C) K_d values determined for binding of the chimeric CROSS proteins CROSS$-N_3E3_2$ (black) and CROSS$-N_2E3_3$ (gray) to eotaxin-1, eotaxin-2, eotaxin-3, and MCP-1. Data values and error bars are the averages and standard errors of duplicate binding affinity measurements.

faithfully mimic the interactions of the natural receptors with these chemokines.

Chimeric CROSS Proteins: Binding to Wild-Type and Chimeric Chemokines. To understand the differential specificities of chemokine receptors, it is necessary to identify the features of the receptors that interact with specific regions on the chemokines. This question can be addressed by studying the chemokine binding of mutant or chimeric receptors. However, interpretation of such experiments can be complicated by variations in receptor expression levels and nonspecific binding of chemokines to the cell surface. Considering that the CROSS proteins display parallel specificities to the natural receptors, they provide a simplified context for investigating the molecular basis of chemokine-receptor specificity. Therefore, we undertook a series of experiments to determine the interacting elements of CROSS proteins and chemokines. Our approach was to compare the affinities of wild-type chemokines and six chimeric chemokines for the two "wild-type" CROSS proteins (CROSS$^5-$

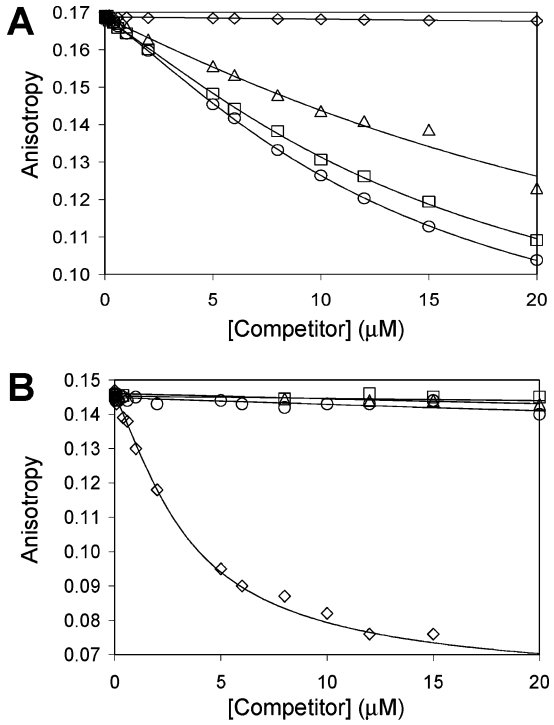

FIGURE 4: Binding of wild-type chemokines to (A) CROSS$^5-N_3E3_3$ and (B) CROSS$-N_2E3_2$ measured by competitive fluorescence anisotropy (see the Material and Methods for details). Data for are shown for eotaxin-1 (○), eotaxin-2 (△), eotaxin-3 (□), and MCP-1 (◇). Solid lines are fits to theoretical binding isotherms (*36*). Standard errors in anisotropy values are comparable to the sizes of the symbols shown.

N_3E3_3 and CROSS$-N_2E3_2$) described above and two additional "chimeric" CROSS proteins. The chimeric CROSS proteins contain N and E3 elements from different receptors (CCR2 and CCR3) and are therefore named CROSS$-N_3E3_2$ and CROSS$-N_2E3_3$ (Figure 1B). The chimeric CROSS proteins were expressed and purified similarly to the other CROSS proteins. Using the CD spectra (Figure 2A) as a qualitative indicator of secondary structure, CROSS$-N_3E3_2$ has similar secondary structure content to CROSS$-N_2E3_2$, whereas CROSS$-N_2E3_3$ has reduced secondary structure content.

The affinities of CROSS$-N_3E3_2$ and CROSS$-N_2E3_3$ for eotaxin-1, eotaxin-2, eotaxin-3, and MCP-1 were measured by competitive fluorescence anisotropy assays; K_d values are listed in Table 1 and represented graphically in Figure 3C. Replacement of the N element of CROSS$^5-N_3E3_3$ with the corresponding element from CCR2 (to give CROSS$-N_2E3_3$) reduced the affinity for each eotaxin group chemokine by ~20-fold or more, whereas substitution of the E3 element (to give CROSS$-N_3E3_2$) reduced the affinities by ~5−8-fold. Similarly, a comparison of MCP-1 binding to CROSS$-N_2E3_2$ and the two chimeric CROSS proteins indicates that the N-terminal element contributes ~90-fold to specificity, whereas the E3 element contributes ~7-fold to the binding preference of MCP-1 for the CCR2 mimic (relative to the CCR3 mimic). These results demonstrate that both receptor elements contribute to the receptor specificity of the chemokines, although the contribution of the N element is larger.

To identify the regions on the chemokines with which the two receptor elements interact, we compared the affinities of the four CROSS proteins for eight chemokines: eotaxin-

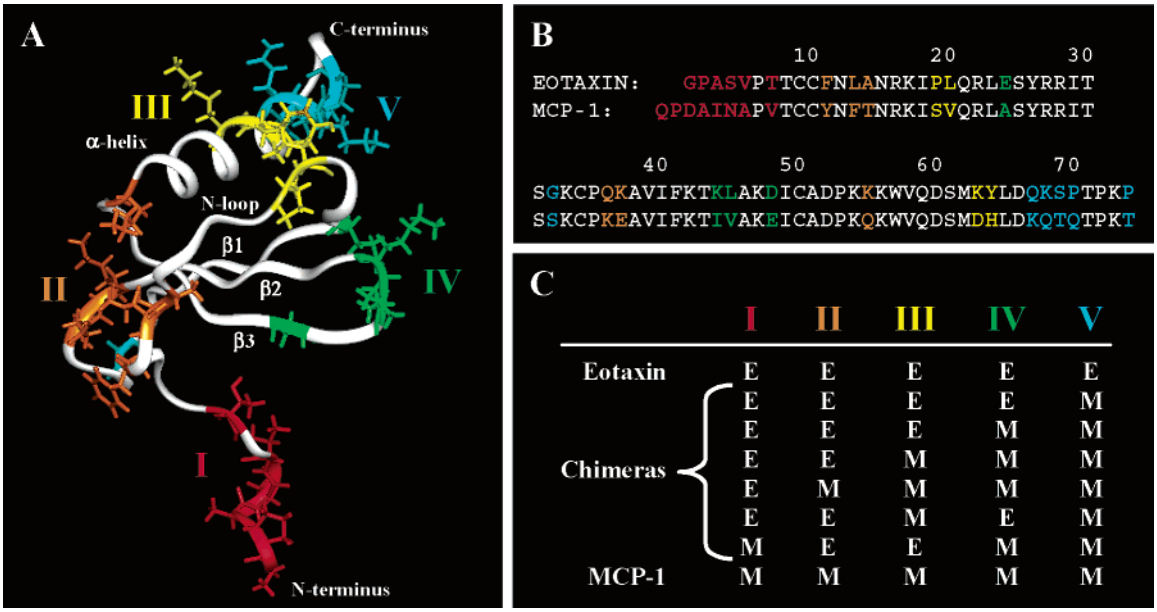

FIGURE 5: (A) Three-dimensional ribbon structure of eotaxin-1 (PDB code 1EOT; ref *39*) with the nonconserved residues (with respect to MCP-1) shown as cylinders. Color coding indicates the composition of the five spatially defined regions (I, red; II, orange; III, yellow; IV, green; and V, cyan). (B) Amino acid sequence alignment of eotaxin-1 and MCP-1, with numbering corresponding to that of eotaxin-1. Nonconserved residues are colored the same as in A. (C) Composition of the two wild-type and six chimeric chemokines, indicating which regions are derived from eotaxin-1 (E) and MCP-1 (M) in each chimeric chemokine.

Table 2: Binding Affinities of CROSS Proteins for Wild-Type and Chimeric Chemokines[a]

chemokine	CROSS^5−N_3E3_3	CROSS−N_2E3_2	CROSS−N_3E3_2	CROSS−N_2E3_3
eotaxin-1	3.6 ± 0.8	137 ± 62	25.1 ± 9.7	67.6 ± 32.9
EEEEM	4.0 ± 1.1	16.5 ± 4.1	8.8 ± 2.3	22.2 ± 5.6
EEEMM	5.4 ± 1.4	24.6 ± 6.3	3.1 ± 0.9	49.2 ± 12.3
EEMMM	7.8 ± 2.0	19.8 ± 4.2	4.5 ± 1.2	31.0 ± 7.8
EMMMM	40.0 ± 10.2	1.5 ± 0.4	40.3 ± 11.2	7.4 ± 1.8
EEMEM	19.6 ± 5.0	13.7 ± 3.4	15.7 ± 4.0	34.5 ± 8.8
MEEMM	>200	4.2 ± 1.2	>100	2.0 ± 0.5
MCP-1	>200	1.1 ± 0.3	92.7 ± 24.2	7.6 ± 2.0

[a] Values listed are dissociation equilibrium constants (K_d) in micromolars.

1, MCP-1, and six chimeras in which various combinations of five spatially defined regions (I−V) have been swapped between eotaxin-1 and MCP-1. These chimeras are represented schematically in Figure 5 and are named according to the identities of the various regions; for example, EEMMM is the chimera in which regions I and II are derived from eotaxin-1 and regions III−V are derived from MCP-1. The preparation of these chimeric chemokines has been described in a previous report (*35*). Among the eight chemokines, there are eight pairs in which the two chemokines differ in only a single region, allowing us to probe the role of that region in CROSS protein recognition. The affinities of the four CROSS proteins for the chimeric chemokines were measured using the competitive fluorescence anisotropy assays described above. The experimental data are presented in Figure 6, and the K_d values obtained by fitting these data to a competitive binding equation are listed in Table 2. A detailed interpretation of these data is presented in the Discussion.

DISCUSSION

CROSS Proteins Mimic Chemokine Recognition by Natural Receptors. The data presented above indicate that CROSS−N_2E3_2, a soluble protein containing two extracellular elements of CCR2 displayed on the stabilized B1 domain scaffold, is capable of binding with ~1 μM affinity to MCP-1, the cognate ligand of CCR2. Removal of either the N or E3 element or replacement of either element with the corresponding element from CCR3 decreases the affinity for MCP-1, indicating that both of these receptor regions are involved in MCP-1 recognition by CROSS−N_2E3_2. Similarly, CROSS^5−N_3E3_3, a soluble mimic of CCR3, utilizes both N and E3 elements in recognition of all three eotaxin group chemokines. Moreover, the chemokine specificities of the two CROSS proteins parallel those of the corresponding natural receptors. All of these lines of evidence suggest that the CROSS proteins include two of the most important ligand-recognition elements of the natural receptors and that analyzing the interactions of these elements in the soluble model system will provide structural insights that are relevant to the recognition of chemokines by intact receptors. Furthermore, the approach of displaying receptor extracellular elements on a soluble scaffold may be generally applicable to other chemokine receptors and possibly also to other members of the GPCR family in which the primary ligand-binding site is in the extracellular domain rather than the transmembrane helical bundle.

Although the CROSS proteins utilize both the N and E3 elements for chemokine binding, the affinities of both CROSS^5−N_3E3_3 and CROSS−N_2E3_2 for the relevant chemokines are substantially lower than the apparent affinities of

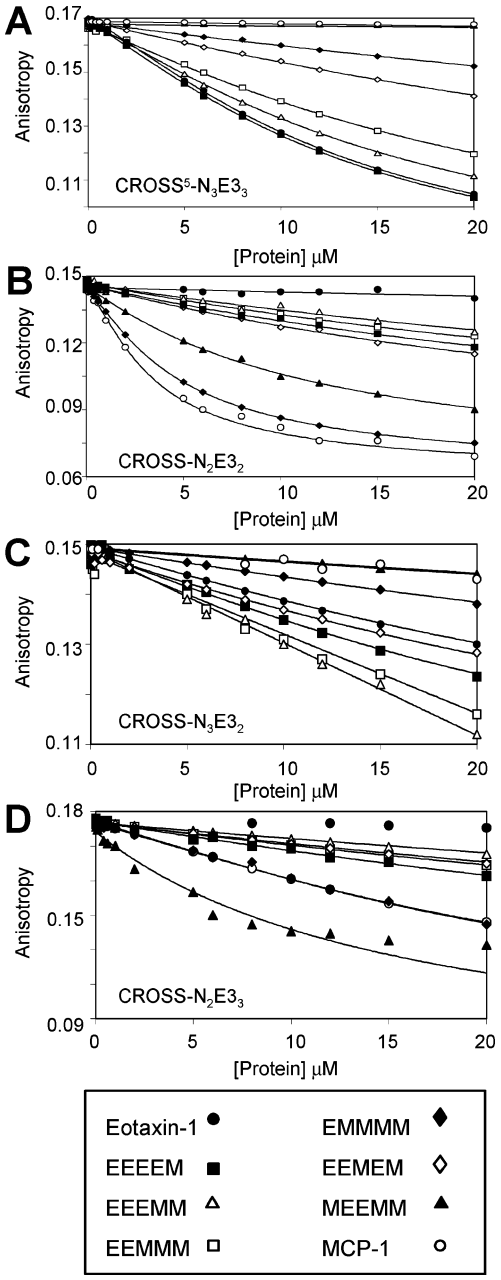

FIGURE 6: Chemokine binding by CROSS proteins. Competitive binding of (A) CROSS^5−N$_3$E3$_3$, (B) CROSS−N$_2$E3$_2$, (C) CROSS−N$_3$E3$_2$, and (D) CROSS−N$_2$E3$_3$ to fluorescein-labeled chemokines with unlabeled chemokines. For binding to CROSS^5−N$_3$E3$_3$ and CROSS−N$_3$E3$_2$, the labeled chemokine was eotaxin-1, whereas for binding to CROSS−N$_2$E3$_2$ and CROSS−N$_2$E3$_3$, the labeled chemokine was MCP-1. Increasing concentrations of unlabeled chemokines displace the labeled chemokine from the CROSS protein, hence decreasing the observed anisotropy. In each panel, data are shown for unlabeled eotaxin-1 (●), MCP-1 (○), EEEEM (■), EEEMM (△), EEMMM (□), EMMMM (◆), EEMEM (◇), and MEEMM (▲). Solid lines are fits to theoretical binding isotherms (see the Materials and Methods). Errors in points are approximately the size of the symbols shown.

the corresponding natural receptors for the same chemokines (parts A and B of Figure 3 and Table 1). We have previously discussed several factors that may contribute to the ∼1000-fold greater eotaxin-1 affinity of CCR3 compared with that of CROSS^5−N$_3$E3$_3$, including (1) the additional receptor elements (E1 and E2), (2) post-translational modifications (such as sulfation) of the natural receptor, (3) structural and/or dynamic differences, and (4) the role of the cell membrane and/or surface proteins or proteoglycans (*32*). Clearly, the same factors could explain the ∼10 000-fold greater affinity of MCP-1 for CCR2 compared with that for CROSS−N$_2$-E3$_2$, although the greater reduction in binding affinity in the latter case suggests that one or more of these factors may play a more significant role in MCP-1 binding by CCR2.

Identification of Interacting Elements in Chemokines and CROSS Proteins. Each of the four CROSS proteins studied herein displays a wide range of affinities for the two wild-type chemokines and the six chimeric chemokines. By comparison of the various K_d values, it is possible to deduce which receptor elements are the primary interaction sites for each region of the chemokines. Our approach is to compare the K_d values for each pair of chemokines that differ only in a single region. There are eight such pairs, and the logarithmic plot in Figure 7 shows eight corresponding clusters of four data bars each (one bar for each CROSS protein). Each data bar represents a "K_d ratio", defined as the K_d value for binding of the relevant CROSS protein to the chemokine containing the MCP-1 element divided by the K_d value for the binding of the same CROSS protein to the chemokine containing the eotaxin-1 element. Thus, a K_d ratio greater than 1 (positive bar in Figure 7) indicates that the CROSS protein interacts more tightly with the substituted region of eotaxin-1, whereas a K_d ratio less than 1 (negative bar in Figure 7) indicates that the CROSS protein interacts more tightly with the substituted region of MCP-1. Below, we first use the K_d ratios for the two "wild-type" CROSS proteins to deduce which regions of the chemokines are recognized specifically by these two CROSS proteins. Next, we compare these conclusions to similar comparisons for the intact receptors. Finally, we use the data for the chimeric CROSS proteins to draw conclusions regarding the receptor elements that interact with each region of the chemokines.

For comparisons, Ia, Ib, II, IIIb, and IVb, the K_d ratios for CROSS^5−N$_3$E3$_3$ (open bars in Figure 7) are large (positive bars), indicating that this CROSS protein interacts more strongly with each region (I−IV) of eotaxin-1 than with the corresponding region of MCP-1. The corresponding K_d ratio for comparison V is not significantly different from one, indicating that the CCR3 elements have no preference for region V of either chemokine, whereas the data bars for comparisons IIIa and IVa are small and positive, supporting the preferential interactions of CROSS^5−N$_3$E3$_3$ with regions III and IV of eotaxin-1 but indicating that the extent of these preferences is dependent on the identity of neighboring regions in the chemokine. The K_d ratios for CROSS−N$_2$E3$_2$ (solid bars in Figure 7) are very small (negative bars) for comparisons Ib, II, and V, indicating that this CROSS protein interacts more strongly with regions I, II, and V of MCP-1 than with the corresponding regions of eotaxin-1. Comparison Ia shows a smaller preference for MCP-1. Once again, comparisons for regions III and IV show variable (generally weak) preferences, indicative of context-dependent binding.

We have previously reported the apparent binding affinities (IC_{50} values) for binding of the same wild-type and chimeric chemokines to the natural receptors CCR2 and CCR3 (*11*). The major conclusions of that study were that (1) each receptor has a distinct preference to interact with region I of its cognate ligand over region I of the other chemokine; (2) CCR2 utilizes interactions with regions II and V to enhance specificity for MCP-1; (3) CCR3 uses mutually compensating

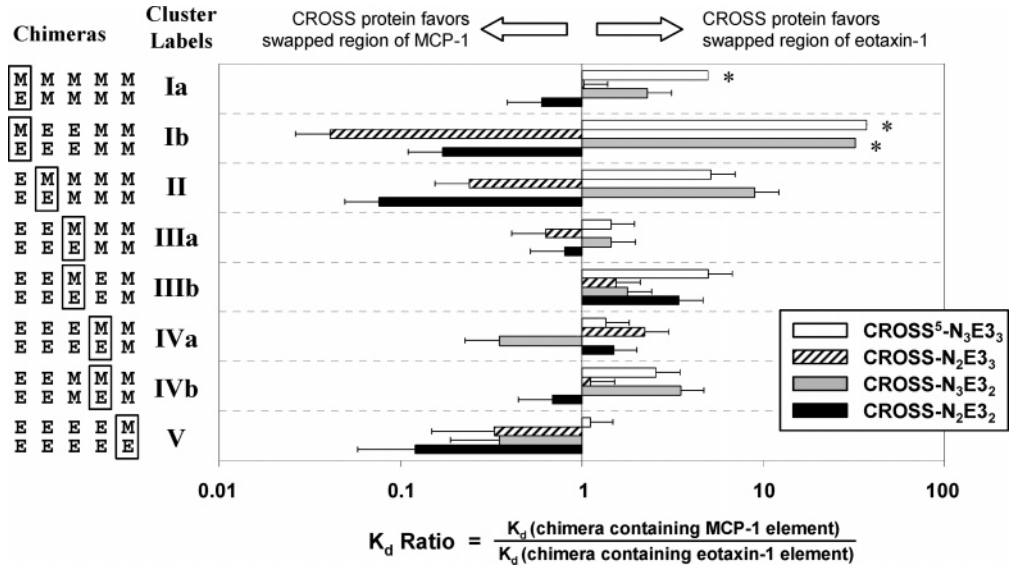

FIGURE 7: Ratios of K_d values for the eight possible pairwise comparisons of chemokines or chimeras that differ in a single region. The graph shows the effects of chemokine substitutions on binding to receptor mimics (CROSS$^5-N_3E3_3$ and CROSS$-N_2E3_2$) and chimeras (CROSS$-N_3E3_2$ and CROSS$-N_2E3_3$) using several clusters of four bars each. Each cluster represents a comparison between two chemokines that differ in only a single region, represented schematically on the left. Each data bar represents the change in K_d value for a particular CROSS protein (as indicated in the key) when the relevant chemokine region is changed from its identity in eotaxin-1 to its identity in MCP-1. Asterisks indicate that the value shown is a lower limit.

interactions with regions III and/or IV to increase specificity for eotaxin-1; and (4) the contributions of each region to specificity are dependent on the identities of nearby regions. The conclusions reported herein for CROSS$-N_2E3_2$ and CROSS$^5-N_3E3_3$ are entirely consistent with conclusions (1), (2), and (4) for the natural receptors. The roles of regions III and IV (conclusion 3) are less obvious in the CROSS system; perhaps this should not be surprising considering that the context-dependent effects observed for the natural receptors are rather subtle. Finally, in addition to the effects seen for the natural receptor, the CROSS data indicate that the N and/or E3 elements of CCR3 utilize interactions with region II to enhance specificity for eotaxin-1. The absence of this effect in the intact CCR3 data may indicate that some feature of CCR3 that is not present in CROSS$^5-N_3E3_3$ actually interacts preferentially with region II of the non-cognate ligand. Nevertheless, the overall similarity between the data for CROSS proteins and intact receptors supports the contention that much of the specificity of the receptors resides in the N and E3 elements and argues that the specific interactions discussed below are likely to be relevant to the natural chemokine-receptor interactions.

In cases for which one or both of the "wild-type" CROSS proteins display(s) a preference for a particular region of the cognate chemokines, the data for the chimeric CROSS proteins allow us to differentiate the contributions of the two receptor elements to this recognition. For example, consider the four data bars for comparison II in Figure 7. The positive bar for CROSS$^5-N_3E3_3$ indicates that this CROSS protein has a preference for region II of eotaxin-1 over that of MCP-1. The negative bar for CROSS$-N_2E3_2$ indicates that this CROSS protein has the opposite preference. The K_d ratio for CROSS$-N_3E3_2$ (gray bar) is almost identical to that for CROSS$^5-N_3E3_3$, indicating that the E3 element does not influence the binding specificity. In contrast, the K_d ratio for CROSS$-N_2E3_3$ (striped bar) is similar to that for CROSS$-N_2E3_2$, indicating that swapping the N element (relative to CROSS$^5-N_3E3_3$) is sufficient to swap the specificity. Thus, we conclude that the N element of each CROSS protein interacts preferentially with region II of the cognate chemokine. The cluster of K_d ratios for comparison Ib is very similar to that for comparison II; therefore, we can conclude that the N element of each CROSS protein also interacts preferentially with region I of the cognate chemokine. Although the changes in K_d values are less dramatic for comparison Ia, they are also consistent with this proposal. Comparison IIIa shows similar, albeit less pronounced, K_d changes to comparisons Ib and II, and in comparison IVb, substitution of the CROSS N element again has a much greater influence on the K_d ratio compared with substitution of the CROSS E3 element. Although these data for comparisons IIIa and IVb support the interaction of these two regions with the receptor N termini, they differ significantly from the data for comparisons IIIb and IVa, respectively. The latter are not interpretable by this simple approach, but the differences provide further evidence that the binding thermodynamics of regions III and IV are each dependent on the identity of the other region. Finally, comparison V indicates that CROSS$^5-N_3E3_3$ has no obvious preference but that CROSS$-N_2E3_2$ has a strong preference for interacting with region V of MCP-1. The two chimeric CROSS proteins both display decreased preferences for region V of MCP-1. We therefore conclude that region V of MCP-1 interacts with both the N and E3 elements of CCR2.

Implications for the Structures of Chemokine-Receptor Complexes. On the basis of the conclusions of the previous section, in combination with other available data, we can propose a crude structural model for the chemokine-receptor complexes. We make the assumption that the eotaxin-1:CCR3 and MCP-1:CCR2 complexes are structurally very similar, which is consistent with the data reported herein and previous mutational and biophysical data (*12*, *19*, *30*, *37*, *38*); although we have noted some significant differences in

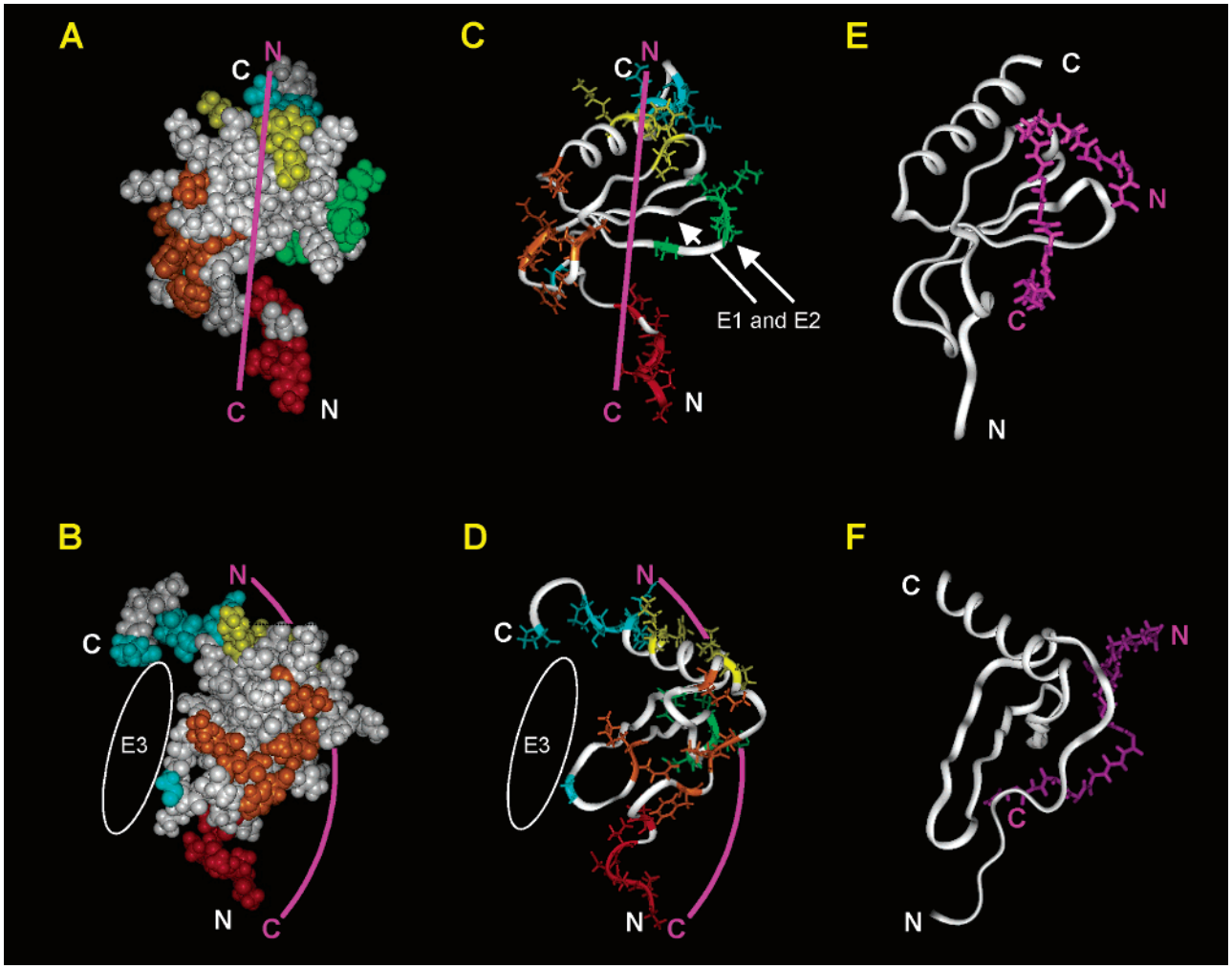

FIGURE 8: Structural interpretation of the chimera complementation data. (A and B) Space-filling models and (C and D) ribbon representations showing the 3D structure of eotaxin-1 (PDB code 1EOT; ref *39*), color-coded the same as in Figure 5 to highlight the five-substituted regions; the chemokine N and C termini are labeled in white. The "side view" of B and D is rotated 90° around the vertical axis relative to the "front view" of A and C. The deduced approximate positions of the receptor N element is shown as a purple line or curve, whereas those of the extracellular loop elements (E3 and E1/E2) are indicated in white. (E and F) Three-dimensional structure of the complex between IL-8 and a peptide mimetic related to the N terminus of the IL-8 receptor CXCR1 (PDB code 1ILQ; ref *38*), oriented to match the two views of eotaxin-1. The peptide mimetic is shown in purple.

the relative energies of certain interactions, these could easily occur without major structural changes. The above chimera complementation analysis indicates that the N elements of one or both receptors interact with regions I, II, and V of the cognate chemokine. In addition, there is weaker evidence that the receptor N termini interact with regions III and IV of the chemokines in a manner that is sensitive to the identities of nearby regions. When these observations are taken together, they suggest that the receptor N elements bind, in an extended conformation, to the front face of the chemokine, as shown in parts A and C of Figure 8. This conclusion is consistent with our previous observations that binding to an N-terminal peptide from CCR3 causes chemical-shift changes localized to this face of eotaxin-1 (*30*) and eotaxin-2 (*34*). Furthermore, Skelton and co-workers have reported the NMR structure of the CXC chemokine interleukin-8 (IL-8) bound to a peptide mimetic similar to the N-terminal region of the IL-8 receptor (CXCR1) (*38*), shown in parts E and F of Figure 8. The receptor peptide lies primarily on the front face of the chemokine (Figure 8E) and interacts with residues corresponding to regions II−IV. Although the peptide does not contact region V, a slightly different conformation of the N terminus could easily bring the peptide into contact with region V, as suggested by our data for CROSS−N_2E3_2 and CCR2 binding to MCP-1. The peptide used by Skelton et al. terminates at residue 16. However, expansion of this peptide to its full length (40 residues), in an extended conformation, would bring it into contact with the amino-terminal region of the chemokine (region I), again consistent with our observations. Consequently, we propose that the N elements of CCR3 and CCR2 occupy the approximate position indicated in parts A−D of Figure 8.

The complementation data for the E3 receptor element suggest that this element interacts with region V but not with the other regions surveyed in this study. These data suggest that the primary interaction site of the E3 element is close to the back face of the chemokine in the view of parts A and C of Figure 8 (left side in view of parts B and D of Figure 8). The orientation of the receptor N terminus proposed above would imply that the transmembrane region of the receptor is located toward the bottom (N terminus) of the chemokine, as shown in parts A−D of Figure 8. Assuming that the overall topology of chemokine receptors

is similar to those of bacteriorhodopsin (*21*, *22*) and rhodopsin (*23*, *24*), the arrangement of extracellular elements (N to E1 to E2 to E3) progresses in an anticlockwise direction when these proteins are viewed from the extracellular side of the membrane. Therefore, if the receptor N and the E3 elements interact with the front and back faces of the chemokine, respectively (view of parts A and C of Figure 8), the other two extracellular elements of the receptor (E1 and E2) must be located near the right-hand side of the chemokine. We therefore speculate that E1 and E2 make contact with the exposed side of the β sheet and/or the $\beta2-\beta3$ hairpin turn, as indicated in Figure 8C.

Concluding Remarks. We have demonstrated that juxtaposing two extracellular elements of CCR2 on a soluble scaffold yields a receptor mimic ($CROSS-N_2E3_2$) that binds to MCP-1 with low micromolar affinity. This receptor analogue and the corresponding mimic of CCR3 ($CROSS^5-N_3E3_3$) display the same chemokine-binding specificities as the natural receptors, suggesting that they are good models of these receptors. We have utilized the CROSS protein system, along with chimeric chemokines, to dissect the relative contributions of the two receptor elements to the chemokine-binding specificities of CCR2 and CCR3 and to identify the regions of the cognate chemokines with which these receptor elements interact. These data have been interpreted to provide a low-resolution working model of the chemokine-receptor complex that can serve as a guide for future structure−activity studies.

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