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Probing Receptor Binding Activity of Interleukin-8 Dimer Using a Disulfide Trap†

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Abstract

Interleukin-8 (IL-8), a member of the chemokine superfamily, exists as both monomers and dimers, and mediates its function by binding to neutrophil CXCR1 and CXCR2 receptors that belong to the G protein-coupled receptor class. It is now well established that the monomer functions as a high-affinity ligand, but the binding affinity of the dimer remains controversial. The ~1000-fold difference between monomer-dimer equilibrium constant (μM) and receptor binding constant (nM) of IL-8 does not allow receptor-binding affinity measurements of the native IL-8 dimer. In this study, we overcame this roadblock by creating a ‘trapped’ non-dissociating dimer that contains a disulfide bond across the dimer interface at the two-fold symmetry point. The NMR studies show that the structure of this trapped dimer is indistinguishable from the native dimer. The trapped dimer, compared to a trapped monomer, bound CXCR1 with ~70-fold and CXCR2 with ~20-fold lower affinities. Receptor binding involves two interactions, between the IL-8 N-loop and receptor N-domain residues, and between IL-8 N-terminal and receptor extracellular loop residues. In contrast to a trapped monomer that bound an isolated CXCR1 N-domain peptide with μM affinity, the trapped dimer failed to show any binding, indicating that dimerization predominantly perturbs the binding of only the N-loop residues. These results demonstrate that only the monomer is a high-affinity ligand for both receptors, and also provide a structural basis for the lower binding affinity of the dimer.

Interleukin-8 (IL-8, also known as CXCL8), a member of the chemokine superfamily, plays a pivotal role in recruiting neutrophils under conditions such as tissue injury, wound healing, and bacterial infection (1). IL-8 belongs to the class of proteins now identified as ‘weak’ transient homodimers that exist as both monomers and dimers under physiological conditions (2). During active neutrophil recruitment, IL-8 is translocated from the injury site in the tissue to the vasculature, and so its concentration will vary spatially and temporally and cannot be defined by a single physiological concentration. Local concentration under these conditions could reach levels high enough so that both monomers and dimers exist. IL-8 function involves binding to neutrophil G protein-coupled receptors (GPCR)¹ and cell-surface glycosaminoglycans (GAG) (3,4). Therefore, knowledge of the relative binding affinities and functional activities of the monomer and dimer for their GPCR receptors and GAGs is critical for understanding the role of monomer-dimer equilibrium in neutrophil recruitment.

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IL-8 dimerizes at μM concentrations ($K_d \sim 10 \mu\text{M}$), and binds its receptors (CXCR1 and CXCR2) at nM concentrations ($K_d \sim 1 \text{ nM}$), suggesting that a monomer is sufficient for receptor function (4-6). An IL-8 'trapped' monomer and also monomer mutants show native binding affinities (7,8), and various other structure-function studies have also shown that mutating dimer-interface residues does not affect binding indicating that dimer-interface residues are not essential for receptor function (4,8,9). However, measuring binding affinities of the dimer is less straight forward, as the ~ 1000 -fold difference between the IL-8 monomer-dimer equilibrium constant (μM) and receptor binding constant (nM) precludes binding studies of the native IL-8 dimer. In principle, the IL-8 dimer, compared to the monomer, could bind with higher, lower or same affinity, or could be completely inactive, and also the IL-8 dimer could bind the two receptors with different affinities.

Previous studies have used the strategy of designing non-dissociating disulfide-linked IL-8 dimers, but these variants show both native-like and reduced receptor function (10-12). All these studies used the criterion of proximity in the IL-8 dimer structure for mutating a pair of residues to cysteine for introducing disulfides across the dimer interface (13). Leong *et al.* designed a single chain fusion protein by joining the C-terminus of one monomer to the N-terminus of another monomer using a flexible linker, and created four different variants by introducing a pair of disulfides across the dimer interface between Glu29 or Thr37 in one monomer and Ala69 or Ser72 in the other monomer (10). Compared to native IL-8, they observed the disulfide variants to bind CXCR1 with 6 to 21-fold lower affinities and CXCR2 with native to 4-fold lower affinities, and concluded that dimer dissociation is not essential for IL-8 biological activity. Williams *et al.* observed that a disulfide-linked dimer created by introducing a pair of disulfides (E29C/A69C) across the dimer interface, was as active as the native protein in a neutrophil Ca^{2+} release assay (11). We had reported in a preliminary study that a R26C disulfide-linked dimer, produced by introducing a single disulfide at the site of two-fold symmetry, was 15-fold less active in a neutrophil elastase activity (12). The latter two studies did not report binding affinities, and also neutrophils express both CXCR1 and CXCR2 receptors, and so reduced function could be due to reduced binding to one or both receptors. Most importantly, the consequence of introducing disulfides in these variants is not known, except Williams *et al.* have characterized their E29C/A69C mutant by nuclear magnetic resonance (NMR) spectroscopy (11). They observed chemical shift changes for a number of helical residues, including those of Phe65 and Leu66 that play an important role in stabilizing the native dimer, suggesting that the packing interactions in the dimer interface are perturbed; so its functional properties do not correspond to that of the native dimer. Whereas Arg26 is

¹Abbreviations

IL-8	interleukin-8
CXCR1	CXC chemokine receptor 1
GAG	glycosaminoglycan
GPCR	G protein-coupled receptor
ITC	isothermal titration calorimetry
NMR	nuclear magnetic resonance
N-domain	N-terminal domain

solvent exposed, residues Glu29, Thr37 and Ala69 are largely buried in the dimer structure (13); so mutating the latter set of residues for introducing new disulfides, if not optimally accommodated, would perturb the dimer interface structure, as observed by Williams *et al.* for the E29/A69 disulfide mutant. Therefore, these disulfide-linked dimers could have differential structural perturbations of the dimer interface and so have varied function, and therefore it is not clear which of these disulfide-linked variants mimic native dimer structure.

We recently exploited the knowledge that IL-8 binding to the CXCR1 N-domain peptide is in the same μM range as the dimer dissociation constant, and observed that in presence of both monomers and dimers, only the monomer preferentially binds a receptor N-domain peptide (14,15). We measured the binding constant of the trapped monomer for the receptor N-domain using ITC to be $\sim 5 \mu\text{M}$, and a NMR study has shown that the native dimer binds the N-domain with much lower affinity $\sim 170 \mu\text{M}$ (16). Though these studies show that the dimer has a lower binding potency, it can be argued that these results do not exclude the possibility of native dimer binding to the intact receptor with the same affinity as the monomer. Further, we could not carry out similar binding studies with a CXCR2 N-domain peptide due to experimental constraints, and so the relative binding affinity of the native dimer for CXCR2 remains unanswered. In this report, we have addressed some of these questions by studying the structural and receptor binding properties of the 'trapped' R26C IL-8 dimer containing a single disulfide at the two-fold symmetry axis. NMR studies indicate that mutating Arg²⁶ \rightarrow Cys and introducing a new disulfide does not perturb the native dimer interface, and that the structure of this disulfide-linked trapped dimer is indistinguishable from the native dimer. Therefore, the binding affinity of the trapped dimer should reflect the binding affinity of the native dimer. The R26C trapped dimer, compared to a trapped monomer, binds CXCR1 and CXCR2 receptors with ~ 70 fold and ~ 20 fold lower affinities, respectively. We could not detect any binding of the trapped dimer to a CXCR1 N-domain peptide indicating that the significantly reduced affinity of the dimer should be due to reduced binding of the N-loop residues. These results emphasize that only the monomer is a high-affinity ligand for both CXCR1 and CXCR2 receptors, and also provide a structural mechanism for the lower binding affinity of the dimer.

EXPERIMENTAL PROCEDURES

Protein Synthesis

Both the R26C disulfide-linked dimer and L25NMe monomer were synthesized using solid-phase peptide synthesis (SPPS), and purified by reversed-phase HPLC as described previously (17). Both proteins were synthesized in the 4-72 form, as previous studies have shown that the 4-72 form has the same binding affinity and function as the full-length form (4). The rabbit CXCR1 N-domain used in this study was also synthesized by SPPS, and has been extensively characterized previously (14,15,20).

IL-8 Receptor Binding Assays

The binding measurements in human neutrophils and Rat-1 cells expressing either CXCR1 or CXCR2 receptors were carried out as described previously (18). Human neutrophils were suspended at a concentration of 1×10^7 cells/ml in phosphate-buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin and 20 mM HEPES (pH 7.4) and incubated at 4 °C for 2 hours in the presence of 2 nM ¹²⁵I-IL-8 and increasing concentrations of unlabeled IL-8. The binding was terminated by centrifuging the incubation mixture after overlaying it on top of 10% (w/v) sucrose solution. Radioactivity in the pellet was measured in a γ counter. For stably transfected IL-8 receptors, cells were incubated in presence of 2 nM of ¹²⁵IL-8 and increasing concentrations of IL-8 ligand up to 1 μM for 2 hours at 4 °C. Cells were then washed twice with PBS, lysed with 0.2% SDS, and measured in a γ counter. Curve fit and IC₅₀ determination were acquired using the Sigma plot program (SPSS Science, Chicago, IL) from the non-linear

regression analysis. Binding affinities are from at least two independent experiments, and each experiment was performed either in duplicates or triplicates.

Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry experiments were performed using the VP-ITC system (MicroCal) at 25 °C. The trapped IL-8 dimer and monomer proteins were extensively dialyzed for 8-12 hours against the buffer, and in a typical experiment, ~0.6 mM IL-8 was injected into the 1.42-mL sample cell containing ~0.06 mM receptor N-domain peptide. The heats of dilution of the peptide and the buffer were small compared with the heat of binding, and were subtracted from the experimental titration results.

NMR Studies

¹H NMR NOESY (mixing time 150 ms) and TOCSY (mixing time 70 ms) spectra for chemical shift assignments and structure determination were acquired on a Varian Unity 750 spectrometer at 30 and 40 °C as described previously (19). The protein concentration was ~1 mM in a buffer containing 50 mM sodium acetate, 1 mM sodium azide, 1 mM sodium 2,2-dimethyl-2-silapentane sulfonate, pH 5.5 in 90% H₂O/10% ²H₂O (v/v).

RESULTS

Design and Structural Characteristics of Trapped R26C Dimer

In this study, we have characterized a trapped non-dissociating dimer that contains a single disulfide link (R26C) across the dimer interface (Figure 1). The native IL-8 dimer structure reveals that the Arg26 of the 1st β-strand constitutes the two-fold symmetry axis, its side chain is solvent exposed and is not involved in any interactions across the dimer interface, and its backbone amide is H-bonded to Ile40 carbonyl of the adjacent β-strand within the monomer (13). Introducing a disulfide at any other location involves mutating a pair of residues resulting in two disulfides across the dimer interface about the two-fold symmetry point. These observations suggested to us that substituting Arg with a Cys should not perturb the native structure. Further, in contrast to residues mutated in other studies such as Glu29 and Thr37 (10,11), Arg26 is the farthest from the functionally important N-terminal and N-loop residues. Previous studies have also shown that even a charge reversal mutation to Glu (R26E) had no effect on function indicating that the Arg side chain is not involved in receptor function (4).

The R26C trapped dimer was synthesized by SPPS, and its structure was characterized by solution NMR spectroscopy. The nuclear Overhauser effect (NOE) data, which is converted to semi-quantitative proton-proton distances, are the major experimental distance restraints in NMR structure calculations. The NOE data of the R26C trapped dimer showed a pattern similar to that observed for the native dimer, indicating that the structure of the R26C dimer is essentially the same as the native dimer. For example, in the native IL-8 dimer, Val27 shows NOEs to Arg26, Ile39, Leu25*, Glu24*, and Phe65* (NOEs across the dimer interface are indicated by an *); and Arg26 to Leu25, Val27, Ile40, and Val41. Figure 2 shows a region of the NOESY spectra of the R26C trapped dimer showing NOEs from Cys26 and Val27. Characteristic sequential and long-range NOEs, within the monomer and across the dimer interface observed in the native dimer, are also observed in the trapped dimer indicating that the disulfide across the dimer interface is accommodated into the protein structure without affecting the native fold.

Chemical shifts are sensitive to secondary, tertiary, and quaternary structure, and characteristic chemical shifts observed in the native dimer, including those of highly downfield and upfield shifted residues (such as Gln8, Val58, Lys15, Pro16, Phe17 and Cys50), are also observed in the trapped dimer. The exact relationship between chemical shifts and structure/dynamics is

not known, but it is well established that backbone C α H shifts are sensitive to structure and NH shifts are sensitive to both structure and dynamics. Identical backbone chemical shifts, for instance, for less structured and unstructured residues both in the native and the trapped R26C dimer would mean that they have identical structures/dynamics, and that differences in chemical shifts can be interpreted as being due to structural/dynamic changes. Therefore, we feel comparing chemical shifts provides better insight and is more sensitive into structural changes (or lack of) than comparing NOE-derived structures. Chemical shifts of the backbone NH and C α H of the trapped and native dimer, and the differences in chemical shift are plotted in Figure 3. Shifts are essentially identical, despite large variation in C α H (~ 4.5 ppm) and NH (~ 6 ppm) chemical shifts of individual residues. Slightly higher shifts for mutated residue Cys26 can be accounted by larger random coil shifts of Cys compared to Arg, and differences in Val27 and Val40 NH protons to small changes in H-bonding. Therefore, the NMR NOE and chemical shift data unambiguously indicate that introducing a disulfide does not perturb the dimer interface structure, and is essentially the same as that of the native dimer.

Receptor Binding of the R26C Trapped Dimer

We measured the binding affinity of trapped dimer and monomer in neutrophils, and observed that the trapped dimer compared to the monomer binds with ~ 25 -fold lower affinity (Figure 4, Table 1). As neutrophils co-express two IL-8 receptors (CXCR1 and CXCR2), reduced binding affinity could be due to reduced binding to one or both receptors. Therefore, we also measured the binding affinity to the individual receptors transfected in rat fibroblast cells. We observed that the trapped dimer binds the CXCR1 and CXCR2 receptors with ~ 70 - and ~ 20 -fold lower affinities respectively, indicating that only the monomeric form of the protein is a high-affinity ligand for both receptors (Figure 4, Table 1). As shown previously, the trapped monomer and the native IL-8 had similar binding affinities (data not shown).

IL-8 binds both CXCR1 and CXCR2 receptors with nM affinity (Table 1). Receptor binding involves interactions between the IL-8 N-terminal loop and the receptor N-domain residues (site-I), and between the IL-8 N-terminus and the receptor extracellular loop residues (site II). IL-8 binds the isolated CXCR1 N-domain with an affinity (μ M) similar to that for the N-domain in the intact receptor indicating that the structural requirements for site-I interaction can be studied outside the context of the intact receptor (20,21). These observations also suggest that site-I interaction provides most of the binding affinity. As IL-8 monomer-dimer equilibrium is in the same μ M range, we studied the binding of native IL-8 and trapped monomer to a CXCR1 N-domain peptide using ITC, and observed in presence of both monomers and dimers, only the monomer preferentially binds the receptor N-domain peptide (14). We have now carried out ITC studies of the trapped dimer, and failed to detect any binding to the CXCR1 N-domain peptide (Figure 5).

DISCUSSION

Design of non-associating trapped monomers and non-dissociating trapped dimers provides powerful tools for studying the structural and functional role of the individual species, and to gain insights into how monomer-dimer equilibrium regulates function. Ideal design of a trapped monomer/dimer should not perturb the native monomer/dimer structure, so that the observed function can be unambiguously attributed to the monomeric/dimeric state of the protein, and not to the structural change as a consequence of the modification. Previously, we had shown such a trapped non-associating IL-8 monomer is as active as the native protein, indicating that a monomer is sufficient for receptor function. The protein was trapped in the monomeric state by introducing a non-natural N-methyl amino acid that disrupts H-bonding interactions about the two-fold symmetry axis and functions as a β -sheet breaker (7,19). Subsequently, it has been demonstrated for various chemokines that form dimers and higher order oligomers, using N-

methyl amino acids as described here and conventional molecular biology approaches, that a monomer is sufficient for receptor function (22-26). It is now generally accepted that all chemokines are active as monomers for their receptor function, providing proof of principle that such designed trapped proteins can provide valuable information on structure-function.

Unlike designing a trapped monomer where the objective is to disrupt dimer interactions and to preserve native monomer structure, designing a trapped dimer is more challenging as the objective is to preserve the native dimer structure and not introduce new interactions. The trapped R26C dimer used in this study has a disulfide at the two-fold symmetry point, and our NMR data indicate that the dimer interface is not perturbed due to the newly introduced disulfide, and the structure of the trapped dimer is essentially the same as the native dimer; so its receptor binding characteristics reflect the binding affinity of the native dimer. Our binding studies to the intact receptors and to the isolated CXCR1 N-domain together indicate that the lower binding affinity of the dimer is due to reduced binding of N-loop residues to the receptor CXCR1 N-domain. The observation that the dimer binds CXCR1, compared to CXCR2, with lower affinity (Table 1), was also made by Leong *et al.*, though their disulfide variants had overall higher binding affinities (11). The higher binding affinities of their disulfide variants is very likely due to perturbation of the interface packing and stability as a consequence of newly introduced disulfides, and so show binding properties that do not reflect of the native dimer.

What could be the structural basis for the differential binding of the monomers and dimers? The solution structure of the trapped IL-8 monomer has been determined by NMR spectroscopy, and is observed to be essentially similar to that of a monomer in the dimer structure except that the C-terminal helical residues are unstructured in the monomer and structured and helical in the dimer (13,19). N-terminal and N-loop residues mediate receptor binding, and these residues are located away from the dimer interface. Binding to the receptor N-domain peptide shows that the reduced binding affinity of the dimer is due to the altered binding properties of the N-loop residues. Structure and the topology of the N-loop residues are preserved between the monomer and dimer, and so the reduced binding of the dimer is not due to structural changes. On the other hand, NMR amide exchange experiments reveal that the dimer is conformationally more constrained compared to the monomer, suggesting that the overall reduced conformational flexibility inhibits binding of the N-loop residues to the receptor N-domain (7,19). Greater loss of binding to CXCR1 is consistent with the observation from structure-function studies that have shown mutating IL-8 N-loop residues results in greater loss of CXCR1 binding indicating that properties of N-loop residues is more stringent for CXCR1 than CXCR2 binding (27).

IL-8 function involves binding to GPCRs on target cells evoking signaling events such as cell shape change and Ca^{2+} release, and binding to endothelial cell surface syndecans which are heparan sulfate proteoglycans for rolling, adhesion, and extravasation of the target cells (28). Various studies have shown that IL-8 promotes neutrophil recruitment at low concentrations, but actually attenuates recruitment at high concentrations, suggesting high IL-8 concentrations negatively regulate receptor function (29-31). Therefore, it is possible at high concentrations, IL-8 exists as a dimer, and the observed lower activity is due to lower receptor affinity of the dimer. A variety of *in vitro* and *ex vivo* studies using both native and monomeric mutants of IL-8, also suggest that cell surface proteoglycan-bound IL-8 dimer plays an essential role for neutrophil recruitment (4,32-34). Under conditions of active neutrophil recruitment, self-association could provide a mechanism by which *in vivo* IL-8 dimerization negatively regulates receptor binding and positively regulates GAG binding, and that this regulation plays an important role in the neutrophil recruitment process. The observation that dimerization results in more pronounced loss of binding affinity for CXCR1 compared to CXCR2 also provides a tantalizing possibility of selective *in vivo* activation of CXCR2 over CXCR1. There is recent

evidence that both CXCR1 and CXCR2 can form homodimers and heterodimers (35,36), but the relevance of this observation for binding of monomer and dimer ligands remains to be studied.

Chemokines, the largest subfamily of cytokines with more than 40 members identified to date, play very fundamental and diverse roles from immune surveillance, leukocyte trafficking, to organogenesis. Structural and functional studies indicate that all chemokines share a common protein-fold, all bind their receptors with the same two-site mechanism, and also that all bind GAGs. Structure determination and solution characterization indicate that dimerization is a fundamental property shared by all chemokines. The dimerization potency varies from weak (mM) to strong (nM), and is sensitive to solution conditions such as pH and ionic strength (6,8,37). Even chemokines observed as monomers in solution NMR studies at mM concentration, are observed as dimers under crystallization conditions (38,39). Nevertheless, structure-function data consistently indicate, including for chemokines such as CCL4 and CCL5 that form strong dimers, that a monomer is sufficient for receptor binding, and a dimer or tetramer formation is coupled to GAG binding (23,40,41). In CC chemokines, the dimer interface and the receptor-binding interface overlap, and there is evidence that dimerization in these chemokines also reduces receptor binding potency (23). Recently, various chemokines including IL-8 have been shown to form heterodimers with other chemokines, and such heterodimers show distinctly different *in vitro* activities compared to monomers indicating that heterodimerization could also play a role *in vivo* (42). Therefore, our results on the role of IL-8 dimerization are directly relevant for all chemokines, and we propose that the fundamental property of chemokines to form reversible homodimers plays a crucial role in mediating *in vivo* function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Murphy PM. Neutrophil Receptors for Interleukin-8 and Related CXC Chemokines. *Sem. Hematology* 1997;34:311–318.
2. Nooren IMA, Thornton JM. Structural characterisation and functional significance of transient protein-protein interactions. *J. Mol. Biol* 2003;325:991–1018. [PubMed: 12527304]
3. Lau EK, Allen S, Hsu AR, Handel TM. Chemokine-receptor interactions: GPCRs, Glycosaminoglycans and viral chemokine proteins. *Adv. Prot. Chem* 2004;68:351–391.
4. Clark-Lewis I, Dewald B, Loetscher M, Moser B, Baggiolini M. Structural Requirements for IL-8 Function Identified by Design of Analogs and CXC Chemokine Hybrids. *J. Biol. Chem* 1994;269:16075–16081. [PubMed: 8206907]
5. Burrows SD, Doyle ML, Murphy KP, Franklin SG, White JR, Brooks I, McNulty DE, Scott MO, Knutson JR, Porter D, Young PR, Hensley P. Determination of the monomer-dimer equilibrium of interleukin-8 reveals it is a monomer at physiological concentrations. *Biochemistry* 1994;33:12741–12745. [PubMed: 7947677]
6. Rajarathnam K, Kay CM, Clark-Lewis I, Sykes BD. Characterization of Quaternary Structure of Interleukin-8 and Functional Implications. *Methods Enzymol* 1997;287:89–105. [PubMed: 9330317]
7. Rajarathnam K, Sykes BD, Kay CM, Dewald B, Geiser T, Baggiolini M, Clark-Lewis I. Neutrophil activation by monomeric interleukin-8. *Science* 1994;264:90–92. [PubMed: 8140420]

8. Lowman HB, Fairbrother WJ, Slagle PH, Kabakoff R, Liu J, Shire S, Hebert CA. Monomeric variants of IL-8: Effects of side chain substitutions and solution conditions upon dimer formation. *Protein Sci* 1997;6:598–608. [PubMed: 9070442]
9. Horcher M, Rot A, Aschauer H, Besemer J. IL-8 derivatives with a reduced potential to form homodimers are fully active in vitro and in vivo. *Cytokine* 1998;1:1–12. [PubMed: 9505139]
10. Leong SR, Lowman HB, Liu J, Shire S, Deforge LE, Gillece-Castro BL, McDowell R, Hebert CA. IL-8 single-chain homodimers and heterodimers: interactions with chemokine receptors CXCR1, CXCR2, and DARC. *Protein Sci* 1997;3:609–617. [PubMed: 9070443]
11. Williams G, Borkakoti N, Bottomley GA, Cowan I, Fallowfield AG, Jones PS, Kirtland SJ, Price GJ, Price L. Mutagenesis studies of interleukin-8. Identification of a second epitope involved in receptor binding. *J. Biol. Chem* 1996;271:9579–9586. [PubMed: 8621632]
12. Clark-Lewis I, Kim K-S, Rajarathnam K, Gong J-H, Dewald B, Moser B, Baggiolini M, Sykes BD. Structure-activity relationships of chemokines. *J. Leukoc. Biol* 1995;57:703–711. [PubMed: 7759949]
13. Clore GM, Appella E, Yamada M, Matsushima K, Gronenborn AM. Three-dimensional structure of interleukin 8 in solution. *Biochemistry* 1990;29:1689–1696. [PubMed: 2184886]
14. Fernando H, Chin C, Rösgen J, Rajarathnam K. Dimer Dissociation is Essential for Interleukin-8 (IL-8) Binding to CXCR1 Receptor. *J. Biol. Chem* 2004;279:36175–36178. [PubMed: 15252057]
15. Rajagopalan L, Rösgen J, Bolen WD, Rajarathnam K. Novel use of an osmolyte to dissect thermodynamic linkages between receptor N-domain folding, ligand binding, and ligand dimerization in a chemokine-receptor system. *Biochemistry* 2005;44:12932–12939. [PubMed: 16185062]
16. Clubb RT, Omichinski JG, Clore GM, Gronenborn AM. Mapping the binding surface of IL-8 complexed with an N-terminal fragment of the type 1 human IL-8 receptor. *FEBS Lett* 1994;338:93–97. [PubMed: 8307164]
17. Clark-Lewis I, Vo L, Owen P, Anderson J. Chemical synthesis, purification, and folding of C-X-C and C-C chemokines. *Methods Enzymol* 1997;287:233–250. [PubMed: 9330326]
18. Suetomi K, Lu Z, Heck T, Wood TG, Prusak DJ, Dunn KJ, Navarro J. Differential mechanisms of recognition and activation of interleukin-8 receptor subtypes. *J. Biol. Chem* 1999;274:11768–11772. [PubMed: 10206993]
19. Rajarathnam K, Clark-Lewis I, Sykes BD. ¹H NMR Solution Structure of an Active Interleukin-8 Monomer. *Biochemistry* 1995;34:12893–12990.
20. Rajagopalan L, Rajarathnam K. Ligand Selectivity and Affinity of Chemokine Receptor CXCR1: Role of N-terminal Domain. *J. Biol. Chem* 2004;279:30000–30008. [PubMed: 15133028]
21. Gayle RB, Sleath PR, Srinivasan S, Birks CW, Weerawarna KS, Cerretti DP, Kozlosky CJ, Nelson N, Bos TV, Beckmann MP. Importance of the Amino Terminus of the Interleukin-8 Receptor in Ligand Interactions. *J. Biol. Chem* 1993;268:7283–7289. [PubMed: 8463264]
22. Paavola CD, Hemmerich S, Grunberger D, Polsky I, Bloom A, Freedman R, Mulkens M, Bhakta S, McCarley D, Wiesent L, Wong B, Jarnagin K, Handel TM. Monomeric monocyte chemoattractant protein-1 (MCP-1) binds and activates the MCP-1 receptor CCR2B. *J. Biol. Chem* 1998;273:33157–33165. [PubMed: 9837883]
23. Laurence JS, Blanpain C, Burgner JW, Parmentier M, LiWang PJ. CC chemokine MIP-1 beta can function as a monomer and depends on Phe13 for receptor binding. *Biochemistry* 2000;39:3401–3409. [PubMed: 10727234]
24. Rajarathnam K, Kay CM, Dewald B, Wolf M, Baggiolini M, Clark-Lewis I, Sykes BD. Neutrophil activating peptide-2 (NAP-2) and melanoma growth stimulatory activity (MGSA) are functional as monomers for neutrophil activation. *J. Biol. Chem* 1997;272:1725–1729. [PubMed: 8999852]
25. Booth V, Keizer DW, Kamphuis MB, Clark-Lewis I, Sykes BD. The CXCR3 binding chemokine IP-10/CXCL10: structure and receptor interactions. *Biochemistry* 2002;41:10418–10425. [PubMed: 12173928]
26. Czaplewski LG, McKeating J, Craven CJ, Higgins LD, Appay V, Brown A, Dudgeon T, Howard LA, Meyers T, Owen J, Palan SR, Tan P, Wilson G, Woods NR, Heyworth CM, Lord BI, Brotherton D, Christison R, Craig S, Cribbes S, Edwards RM, Evans SJ, Gilbert R, Morgan P, Hunter MG, et al. Identification of amino acid residues critical for aggregation of human CC chemokines macrophage

- inflammatory protein (MIP)-1alpha, MIP-1beta, and RANTES. Characterization of active disaggregated chemokine variants. *J. Biol. Chem* 1999;274:16077–16084. [PubMed: 10347159]
27. Lowman HB, Fairbrother WJ, Slagle PH, Kabakoff R, Liu J, Shire S, Hebert CA. Monomeric variants of IL-8: effects of side chain substitutions and solution conditions upon dimer formation. *Protein Sci* 1997;6:598–608. [PubMed: 9070442]
 28. Middleton J, Neil S, Wintel J, Clark-Lewis I, Moore H, Lam C, Auer M, Hub E, Rot A. Transcytosis and surface presentation of IL-8 by venular endothelial cells. *Cell* 1997;91:385–395. [PubMed: 9363947]
 29. Wiekowski MT, Chen S-C, Zalamea P, Wilburn BP, Kinsley DJ, Sharif WW, Jensen KK, Hedrick JA, Manfra D, Lira SA. Disruption of Neutrophil Migration in a Conditional Transgenic Model: Evidence for CXCR2 Desensitization In Vivo. *J. Immunol* 2001;167:7102–7110. [PubMed: 11739532]
 30. Simonet WS, Hughes TM, Nguyen HQ, Trebasky LD, Danilenko DM, Medlock ES. Long-term impaired neutrophil migration in mice overexpressing human interleukin-8. *J. Clin. Invest* 1994;94:1310–1319. [PubMed: 7521886]
 31. Feniger-Barish R, Yron I, Meshel T, Matityahu E, Ben-Baruch A. IL-8-Induced Migratory Responses through CXCR1 and CXCR2: Association with Phosphorylation and Cellular Redistribution of Focal Adhesion Kinase. *Biochemistry* 2003;42:2874–2886. [PubMed: 12627953]
 32. Kuschert GSV, Coulin F, Power CA, Proudfoot AEI, Hubbard RE, Hoogewerf AJ, Wells TNC. Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. *Biochemistry* 1999;38:12959–12968. [PubMed: 10504268]
 33. Goger B, Halden Y, Rek A, Mösl R, Pye D, Gallagher J, Kungl AJ. Different affinities of glycosaminoglycan oligosaccharides for monomeric and dimeric interleukin-8: a model for chemokine regulation at inflammatory sites. *Biochemistry* 2002;41:1640–1646. [PubMed: 11814358]
 34. Frevert CW, Kinsella MG, Vathanaprida C, Goodman RB, Baskin DG, Proudfoot A, Wells TNC, Wight TN, Martin TR. Binding of interleukin-8 to heparan sulfate and chondroitin sulfate in lung tissue. *Am. J. Respir. Cell Mol. Biol* 2003;28:464–472. [PubMed: 12654635]
 35. Trettel F, Di Bartolomeo S, Lauro C, Catalano M, Ciotti TM, Limatola C. Ligand-independent CXCR2 dimerization. *J. Biol. Chem* 2003;278:40980–40988. [PubMed: 12888558]
 36. Wilson S, Wilkinson G, Milligan G. The CXCR1 and CXCR2 receptors form constitutive homo- and heterodimers selectively and with equal apparent affinities. *J. Biol. Chem* 2005;280:28663–28674. [PubMed: 15946947]
 37. Veldkamp CT, Peterson FC, Pelzek AJ, Volkman BF. The monomer-dimer equilibrium of stromal cell-derived factor-1 (CXCL 12) is altered by pH, phosphate, sulfate, and heparin. *Protein Sci* 2005;14:1071–1081. [PubMed: 15741341]
 38. Crump MP, Gong J-H, Loetscher P, Rajarathnam K, Arenzana-Seisdedos F, Virelizier J-L, Baggiolini M, Sykes BD, Clark-Lewis I. Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J* 1997;16:6996–7007. [PubMed: 9384579]
 39. Dealwis C, Fernandez EJ, Thompson DA, Simon RJ, Siani MA, Lolis E. Crystal structure of chemically synthesized [N33A] stromal cell-derived factor 1alpha, a potent ligand for the HIV-1 “fusin” coreceptor. *Proc. Natl. Acad. Sci. USA* 1998;95:6941–6946. [PubMed: 9618518]
 40. Proudfoot AE, Handel TM, Johnson Z, Lau EK, LiWang P, Clark-Lewis I, Borlat F, Wells TN, Kosco-Vilbois MH. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc. Natl. Acad. Sci. USA* 2003;100:1885–1890. [PubMed: 12571364]
 41. McCormack MA, Boren DM, LiWang P. Glycosaminoglycan Dissacharide Alters the Dimer Dissociation Constant of the Chemokine MIP-1β. *Biochemistry* 2004;43:10090–10101. [PubMed: 15287737]
 42. Nesmelova IV, Sham Y, Dudek AZ, van Eijk LI, Wu G, Slungaard A, Mortari F, Griffioen AW, Mayo KH. Platelet factor 4 and interleukin-8 CXC chemokine heterodimer formation modulates function at the quaternary structural level. *J. Biol. Chem* 2005;280:4948–4958. [PubMed: 15531763]

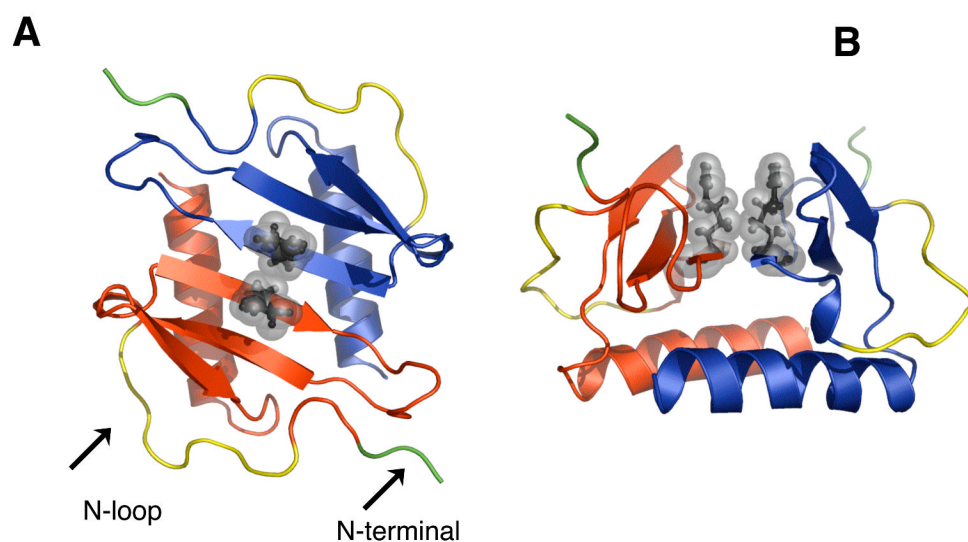
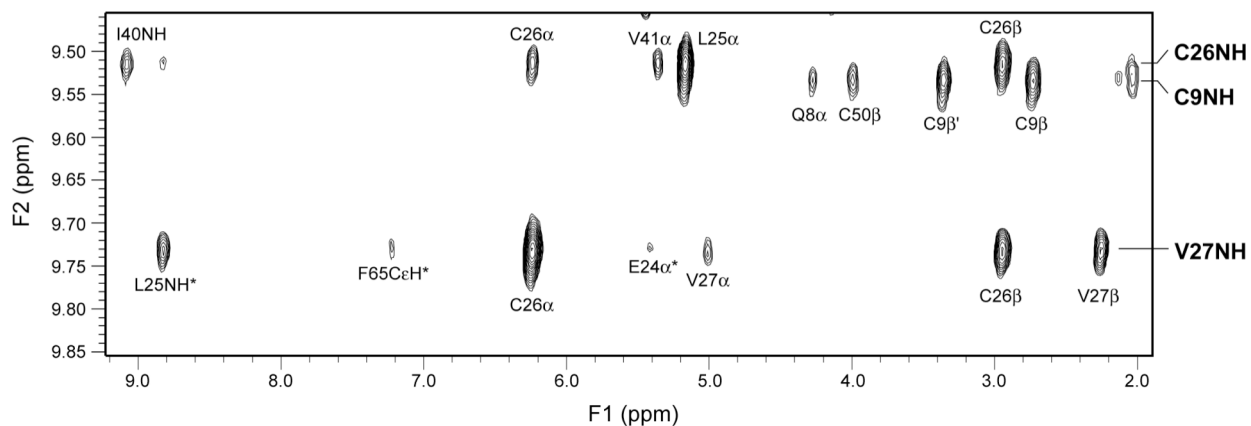
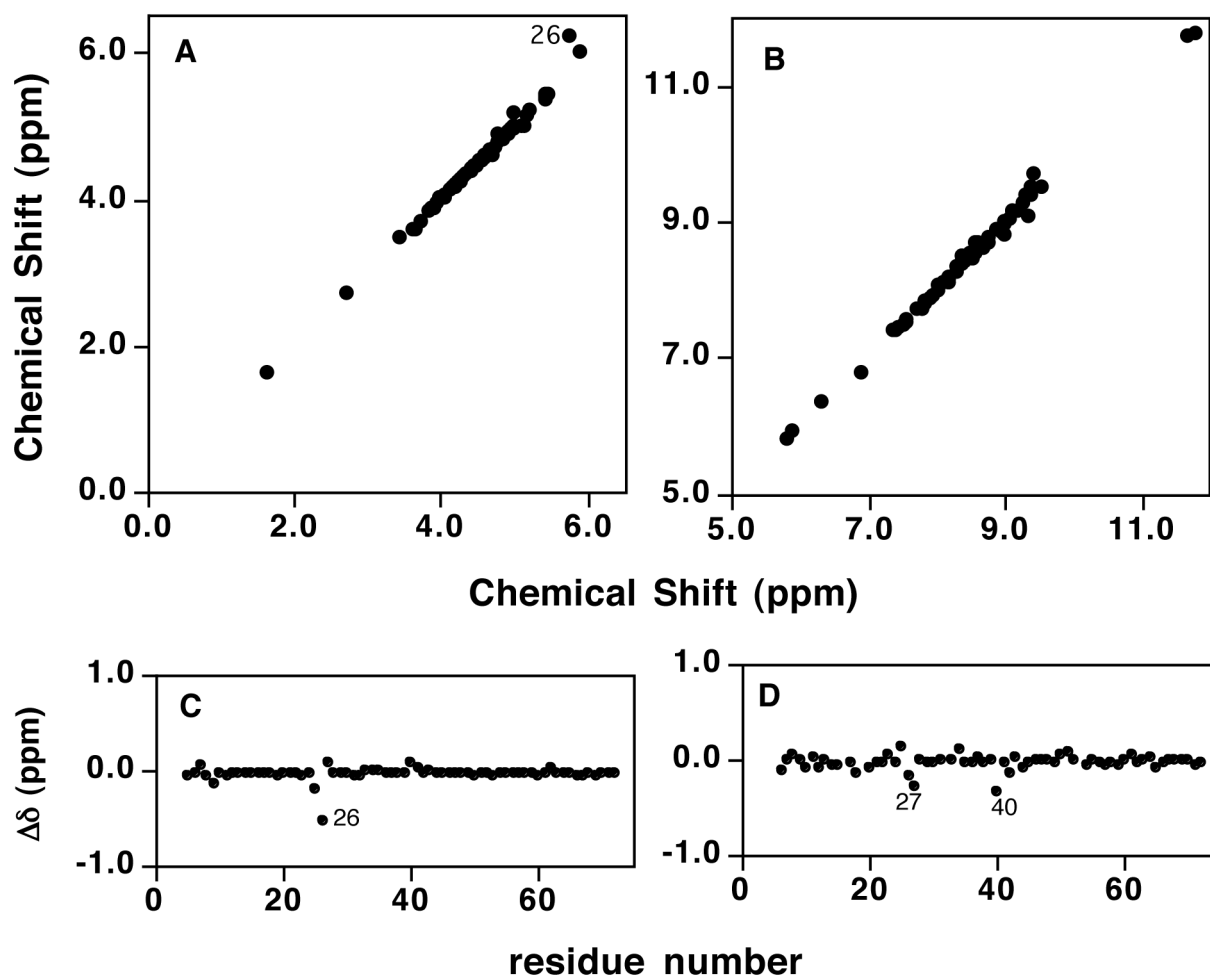


FIGURE 1.

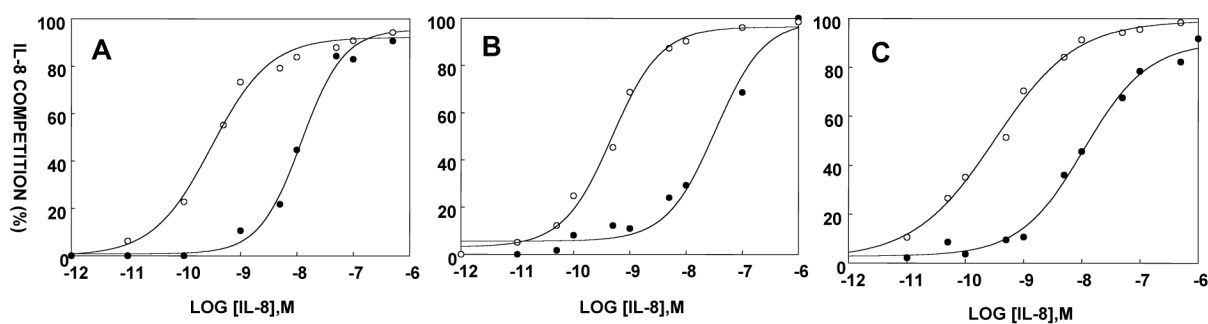
A schematic of the native IL-8 dimer. Structure is shown in two different orientations to highlight the orientation of the solvent exposed Arg26 side chain. The individual monomers in the dimer structure (PDB ID 1IL8; ref. 13) are shown in red and blue. The structure reveals that the Arg26 side chains (shown in black) in the individual monomers are solvent exposed and are spatially proximal to each other. The functionally important N-terminal region (green) and the N-loop region (yellow) are highlighted, and labeled in one of the monomers.

**FIGURE 2.**

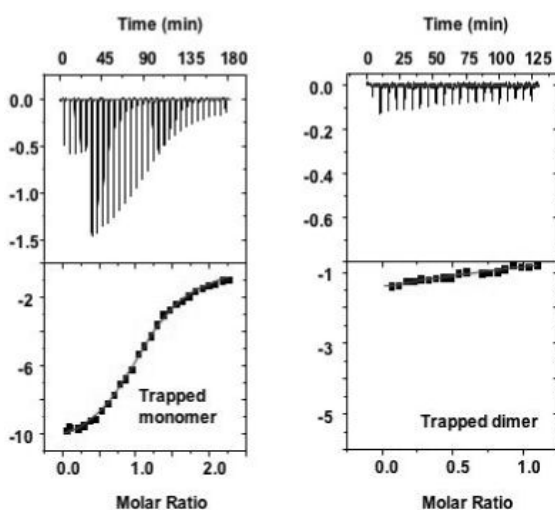
NMR NOESY data of the trapped IL-8 dimer. A section of the 750-MHz 2D NOESY ^1H NMR spectrum of the R26C trapped IL-8 dimer showing NOEs from Cys26, Val27 and Cys9 amide protons. The NOE pattern is similar to that observed in the native dimer. NOEs across the dimer interface are indicated by an *.

**FIGURE 3.**

Plots of the ^1H NMR (A) C_αH and (B) NH chemical shifts of the native and the trapped IL-8 dimer. The chemical shifts of the native protein are plotted along the y-axis. The chemical shift differences between the native and the R26C dimer for C_αH and NH chemical shifts are shown in panels C and D, respectively. Residues that show relatively large shift differences are labeled.

**FIGURE 4.**

Binding of the trapped IL-8 dimer for CXCR1 and CXCR2 receptors. Plots of the competitive binding curves of the trapped IL-8 dimer (●) and trapped monomer (○) to neutrophils expressing both receptors (A), and to Rat-1 fibroblast cells expressing either CXCR1 (B) or CXCR2 receptors (C). The results shown are representative of two to five independent experiments each performed in duplicates or triplicates.

**FIGURE 5.**

Binding of the trapped IL-8 dimer and monomer to CXCR1 N-domain peptide. The isothermal titration calorimetry (ITC) titration profiles for the trapped dimer and trapped monomer are shown in panels A and B, respectively. The data were collected at 25 °C in 50 mM Hepes, 50 mM NaCl, pH 8.0. The upper panels represent the ITC thermograms and the lower panels represent the fitted binding isotherms.

Table 1
Binding affinities of trapped CXCL8 dimer and monomer^a

Ligand	Neutrophils K _d (nM)	Fold	CXCR1 K _d (nM)	Fold	CXCR2 K _d (nM)	Fold
Monomer ^b	0.4 ± 0.1	1	0.8 ± 0.3	1	0.5 ± 0.3	1
Dimer ^b	10.4 ± 1.2	26	55.0 ± 6.7	69	8.5 ± 2.0	17

^a Fold is the relative dissociation constant of the dimer compared to the monomer(K_d, dimer/K_d, monomer)

^b monomer and dimer correspond to the trapped L25NNMe monomer and trapped R26C dimer