Chemokine CXCL1 dimer is a potent agonist for the CXCR2 receptor*

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Background: Chemokines exist reversibly as monomers and dimers, but dimer activity remains poorly defined. Results: A disulfide-linked CXCL1 dimer is highly active, and NMR studies show that dimer binds CXCR2 like the monomer. Conclusion: The potent activity of CXCL1 dimer is novel. Significance: Chemokine dimers can be highly active to completely inactive, indicating that dimerization fine-tunes chemokine-specific in vivo functions.

SUMMARY

The CXCL1/CXCR2 axis plays a crucial role in recruiting neutrophils in response to microbial infection and tissue injury, and dysfunction in this process has been implicated in various inflammatory diseases. Chemokines exist as monomers and dimers, and compelling evidence now exists that both forms regulate in vivo function. Therefore, knowledge of the receptor activities of both CXCL1 monomer and dimer is essential to describe the molecular mechanisms by which they orchestrate neutrophil function. The monomer-dimer equilibrium constant (~20 µM), and the CXCR2 binding constant (1 nM) indicate that WT CXCL1 is active as a monomer. To characterize dimer activity, we generated a trapped dimer by introducing a disulfide across the dimer interface. This disulfide-linked CXCL1 dimer binds CXCR2 with nM affinity and shows potent agonist activity in various cellular assays. We also compared the receptor binding mechanism of this dimer to that of a CXCL1 monomer, generated by deleting the C-terminal residues that stabilize the dimer interface. We observe that the binding interactions of the dimer and monomer to the CXCR2 N-terminal domain, which plays an important role in determining affinity and activity, are essentially conserved. The potent activity of the CXCL1 dimer is novel: dimers of the CC chemokines CCL2 and CCL4 are inactive, and the dimer of the CXC chemokine CXCL8 (which is closely related to CXCL1) is marginally active for CXCR1 but shows variable activity for CXCR2. We conclude that large differences in dimer activity among different chemokine-receptor pairs have evolved for fine-tuned leukocyte function.

Since their original discovery about 25 years ago, it is now firmly established that chemokines function as crucial immune regulators, and that dysregulation in their function leads to a variety of inflammatory and infectious diseases (1,2). Humans express ~50 chemokines, and all share the
following fundamental properties: they reversibly exist as monomers and dimers, mediate function by activating G-protein-coupled receptors (GPCRs) expressed on leukocytes, and regulate leukocyte trafficking by binding to glycosaminoglycans (GAGs) on endothelial cells and the extracellular matrix.

Chemokines are broadly divided into CXC and CC classes based on whether the N-terminal conserved cysteines are contiguous or separated by an amino acid. Structures of a number of chemokines have been solved by solution NMR and X-ray crystallography, which reveal that the subfamilies dimerize using different regions of the protein. However, the monomer-dimer equilibrium constants for both CXC and CC chemokines can vary by orders of magnitude, with both CXC and CC chemokines showing weak to very strong dimerization potencies (3). Dimerization is also sensitive to solution conditions such as pH and ionic strength, with some chemokines showing dramatic changes from very high order oligomers at physiological pH to folded monomers and dimers at lower pHs (4-6). However, deciphering the functional roles of monomer and dimer is challenging, as the very process of monomer-dimer equilibrium interferes in the study of one or the other form, and so studies geared towards understanding monomer and dimer function continue to be an area of active research.

The strategy of creating non-dissociating dimers has shown that the disulfide-linked dimeric forms of the CC chemokines CCL2 and CCL4 are inactive, the CXC chemokine CXCL12 is differentially active, and CXCL8 is marginally active for CXCR1 but differentially active for CXCR2 (7-12). However, there have been no studies investigating the functional roles of the dimeric form of CXCL1 or related neutrophil-activating chemokines, which specifically and selectively activate the chemokine receptor CXCR2.

CXCL1 (also known as MGSA or GROα), a member of the ELR-CXC subset of chemokines, plays a crucial role in recruiting neutrophils in response to microbial infection and tissue injury. CXCL1 dimerizes at micromolar (µM) concentrations (Kd ~20 µM). Cell-based in vitro assays have shown that WT CXCL1 activates CXCR2 at nanomolar (nM) concentrations, and a non-associating monomer also shows WT-like activity, indicating that WT is active as a monomer (13-16). The structure of the WT CXCL1 dimer is known, as by necessity structure determination studies are carried out at high (mM) concentrations where CXCL1 exists as a dimer (17,18). CXCL1 is upregulated under conditions of insult; under basal conditions, its concentration is negligible (nM to pM) and it exists essentially as a monomer. However, during active neutrophil recruitment, its concentration could reach levels high enough so that dimers exist at different locations and times, indicating that both monomers and dimers orchestrate neutrophil recruitment. Therefore, knowledge of the receptor activities of the dimer, and the molecular mechanisms by which monomers and dimers activate CXCR2, is essential to describe how CXCL1 monomers and dimers regulate in vivo neutrophil function.

In order to address this missing knowledge, we have now designed a disulfide-linked CXCL1 N27C dimer and have characterized its receptor function. Because the receptor N-terminal domain (N-domain) functions as a critical docking site and plays an important role in determining affinity and activity, we also characterized the binding of the trapped dimer and a designed monomer to the CXCR2 N-domain using NMR spectroscopy.

Our data indicate that the functional characteristics of the CXCL1 dimer are distinctly novel. Together, our results also emphasize that the receptor affinities, selectivities, and activities of chemokine monomers and dimers vary for different chemokine-receptor pairs, and we propose that dimerization plays chemokine-specific differential roles for fine-tuned in vivo leukocyte function.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of CXCL1 Variants** – All variants were cloned in a pET32 Xa vector and expressed and purified as His-tag thioredoxin fusion proteins, as described previously (19,20). The CXCL1 (1-67) monomer was generated by introducing a stop codon after residue 67 in the WT sequence. The trapped N27C dimer was generated using primers in which the codon of Asn27 was replaced with Cys. PCR
amplification was carried out using the QuikChange Site-Directed Mutagenesis kit (Strategene Inc., La Jolla, CA, USA).

The CXCL1 variants were expressed in the *E. coli* BL21 (DE3) strain, and 15N-labeled proteins were expressed in minimal media containing 15NH4Cl as the nitrogen source. Transformed *E. coli* BL21 (DE3) cells were grown to an A600 of 0.8, and induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 8 h at 37°C. The purity and molecular weight of the proteins were confirmed using analytical high-pressure liquid chromatography (HPLC) and matrix assisted laser desorption/ionization mass spectrometry respectively.

**Cell Culture and Differentiation** – HL60 cells were induced to differentiate into the neutrophilic lineage as follows. Briefly, the cells were maintained in RPMI 1640 medium supplemented with L-glutamine, 10% fetal bovine serum, and 50 units/ml Pen-Strep (Invitrogen, Gaithersburg, MD). Cells were subcultured every 2–3 days and differentiated in antibiotic-free medium containing 1.25% DMSO (endotoxin-free, Sigma, St. Louis, MO) and cultured for 6–7 days.

**Receptor Affinity** – The binding affinity of WT CXCL1 and the trapped dimer was measured using HL-60 cells expressing CXCR2 (HL60-CXCR2) as described previously (21). HL60-CXCR2 cells (10⁷ cells/ml) suspended in phosphate-buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin and 20 mM HEPES (pH 7.4) were incubated at 4°C for 4–6 h in the presence of 0.15 nM ¹²⁵I-CXCL1 and increasing concentrations (10⁻¹¹ to 10⁻⁶ M) of unlabeled CXCL1 WT and trapped dimer. The radioactivity in the pellet was measured using a γ-counter, and binding affinities were calculated from at least two independent experiments, with each experiment performed in duplicate.

**Intracellular Ca²⁺ Mobilization** – Changes in intracellular Ca²⁺ were measured with a FlexStation III microplate reader (Molecular Devices) using the Calcium 5 assay kit (FLIPR No-wash kit, Molecular Devices, Sunnyvale, CA). Briefly, differentiated HL60-CXCR2 cells in HBSS were plated in a flat-bottomed black microtiter plate (2 × 10⁵ cells/well). The cells were loaded with Calcium 5 dye for 1 h, and changes in fluorescence on the addition of WT CXCL1 and the trapped dimer at various doses were monitored (λ₅₈, 485 nm, λₑm 525 nm) every 5 sec for 240 to 500 s at room temperature (RT). The agonist response was determined by expressing the maximum change in fluorescence in arbitrary units over baseline.

**Chemotaxis** – Chemotaxis was measured using a 96-well chemotaxis chamber with a 3.2-µm pore diameter filter membrane (NeuroProbe; Gaithersburg, MD). Briefly, HL60-CXCR2 cells were harvested, washed, and incubated with 5 µg/ml of calcein-AM (Invitrogen, Carlsbad, CA) for 30 min at 37°C. CXCL1 WT or trapped dimer at 10 and 100 nM and the washed cells (2 × 10⁵) were added to the lower and upper chambers respectively, and the entire assembly was incubated at 37°C for 90 min. The number of migrated cells in the lower chamber was measured using a fluorescence plate reader. Data are expressed as the chemotactic index (CI), calculated as ratio of fluorescence of migrated cells due to chemoattractant and due to control medium alone.

**ERK Phosphorylation** – Cell lysates for the detection of ERK phosphorylation were obtained as described previously (22). Differentiated HL60-CXCR2 cells (5 × 10⁷ cells/ml) were stimulated for 1, 5, and 10 min with 10 nM of CXCL1 WT or trapped dimer, and lysed with equal volume of RIPA buffer (Cell Signaling, Danvers, MA) supplemented with protease and phosphatase inhibitors. Cell lysates were sonicated for 5 s (Sonifier 250, Branson Ultrasound, Danbury, CT) and centrifuged at 10,000×g for 15 min. The supernatants were subjected to SDS-PAGE, transferred onto a PVDF membrane at 100 V for 1 h, and blocked in 5% nonfat skim milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. Membranes were then immunoblotted with primary antibody overnight and then treated with secondary antibody for 1 h. Protein bands were visualized using the ChemiDoc XRS system (Bio-Rad, Hercules, CA), and quantified using Quantity One software. The rabbit anti-phospho-ERK (Thr202/Tyr204) and p44/p42 total ERK antibodies were from Cell Signaling, and secondary goat anti-rabbit IgG antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).
CXCL1 binding to CXCR2

NMR Spectroscopy and Structure – 15N-labeled CXCL1 WT, (1-67) monomer, and N27C trapped dimer were prepared in 50 mM potassium phosphate pH 5.0 or 6.0 buffer. Spectra were acquired using Varian Unity Plus 600 or INOVA 800-MHz spectrometers equipped with field gradient accessories, processed using NMRPipe (23), and analyzed using NMRView (24). Chemical shifts of the trapped CXCL1 dimer at 30°C and pH 6.0 and of CXCL1(1-67) monomer at 40°C and pH 4.5 were assigned using three-dimensional 15N-edited NOE-HSQC and TOCSY-HSQC spectra. Chemical shifts were referenced to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). The backbone chemical shifts of the CXCL1 dimer and monomer are given in the Supplementary Material (Table S1).

NMR Characterization of CXCL Binding to CXCR2 N-domain – A synthetic CXCR2 N-domain peptide (MESDFSFEWDLSNYSSSTLPFFFLLDAPSEPESELEINK) was purchased from AAPPTec (Louisville, KY). The native CXCR2 N-domain sequence has a cysteine that was mutated to a serine (underlined). Two-dimensional 1H-15N HSQC spectra were acquired at 30°C with 2048 complex points in the direct 1H dimension and 128 complex points in the indirect 15N dimension. A stock solution of CXCR2 N-domain was titrated into the CXCL1 solution to final mole ratios (peptide: CXCL1 variant) of 4.9, 7.1, and 5.2 for the CXCL1(1-67) monomer, WT, and trapped N27C dimer, respectively. Apparent dissociation constants (K_a) were determined by fitting the binding-induced chemical shift changes (Δδ) as described previously (25).

RESULTS

Design and Characterization of CXCL1 dimer and monomer – A trapped CXCL1 dimer was designed by substituting a cysteine for Asn27. The WT dimer structure shows that Asn27 of the first β-strand constitutes the 2-fold symmetry axis, its side chain is solvent-exposed and not involved in dimer interactions, and its backbone amide is H-bonded to the Ile41 carbonyl of the adjacent β-strand within the monomer (Fig. 1) (17,18,26). Gel electrophoresis, mass spectrometry, and sedimentation velocity studies show that mutating Asn27 to Cys results in the formation of a trapped non-dissociating dimer (Fig. S1). Sedimentation equilibrium experiments showed no evidence of monomer-dimer equilibrium and showed molecular weights consistent with a dimer (data not shown). NMR NOE data show characteristic sequential NOEs for the β-strand, intramolecular NOEs between β-strands, and intermolecular NOEs across the dimer interface residues, indicating that introducing the disulfide does not perturb the native structure (Fig. 2).

WT CXCL1 dimerizes at micromolar concentrations (K_d ~20 µM), and is therefore a dimer at the mM concentrations used in NMR studies (16). We designed a CXCL1 monomer by deleting the last six C-terminal helix residues, as the WT CXCL1 dimer structure showed that these residues are involved in packing interactions with residues of the other monomer across the dimer interface (Fig. 1) (17,18). Sedimentation velocity showed that the CXCL1 (1-67) deletion mutant is a monomer (Fig. S1). Further, the characteristic intermolecular NOEs of dimer interface residues were absent; for instance, dimer-interface NOEs from Val28 to Ser25 and Val26 were not observed, indicating that the deletion mutant is a monomer (Fig. 2).

CXCR2 Receptor function – It is well established that CXCL1 binds and triggers various receptor activities at nM concentrations, where WT essentially exists as a monomer (13,14,27). The binding affinity of the CXCL1 disulfide-linked dimer and WT were measured by competitive binding to the CXCR2 receptor stably expressed on HL-60 cells (Fig. 3A). The trapped CXCL1 dimer effectively competed with the binding of 125I-CXCL1 like the WT (K_d of WT, 1.0 ± 0.2 nM; dimer, 1.6 ± 0.3 nM). Our measured receptor affinity of 1 nM for the WT is similar to those reported in other studies (13,14,27).

We characterized the receptor activity of the disulfide-linked dimer by measuring Ca2+ release, chemotaxis, and ERK phosphorylation (Fig. 3B-3D). The calculated EC50 values for Ca2+ release of 9 ± 2 nM for WT and 29 ± 9 nM for the trapped dimer, and similar activities for chemotaxis and ERK phosphorylation indicate that the CXCL1 dimer is a potent CXCR2 agonist.
Structural Characterization of CXCL1 monomer and dimer – The HSQC spectrum of the disulfide-linked dimer showed excellent dispersion and a single set of peaks, indicating a well folded and structured protein (Fig. 4). The backbone CαH shifts are exquisitely sensitive to secondary, tertiary, and quaternary structure. The chemical shift difference plot between the trapped dimer and WT dimer reveals that the shifts are very similar with some localized differences in residues around the site of mutation, indicating that the structure of the disulfide-linked dimer is essentially similar to the WT dimer. Analysis of NOEs also indicated that introducing the disulfide does not perturb the packing interactions between the strands within the monomer and across the dimer interface (Fig. 2).

The HSQC spectrum of CXCL1(1-67) showed the characteristic shifts of a folded protein (Fig. S2). Moreover, functional data indicated that it binds the receptor with similar affinity as the WT (Kd 1.1 ± 0.1 nM) and is highly active in the Ca2+ release assay (Fig. S3). Interestingly, the NMR spectrum of the monomer showed two peaks for several residues indicating the presence of two conformers. Reducing the pH minimized heterogeneity, and the spectrum at pH 4.5 indicated essentially a single major conformer (Fig. S2). Analytical ultracentrifugation studies showed that the CXCL1 deletion mutant is a monomer at all pH values indicating that the conformational heterogeneity is not due to dimerization. The effect of temperature at pH 4.5 showed that higher temperatures (40 vs. 20°C) further minimized heterogeneity (data not shown). These data show that the differences in conformational dynamics are unlikely to play a role in function; whether it plays some other in vivo role remains to be determined.

Mechanism of CXCL1 Dimer and Monomer Binding – Receptor activation by chemokines involves two interactions – between the ligand N-loop and receptor N-terminal domain residues (defined as Site-I) and between the ligand N-terminal and receptor extracellular loop residues (defined as Site-II) (28-30). All neutrophil-activating chemokines share a highly conserved ELR motif, and mutational studies have shown that these residues are critical for receptor activation at Site-II. The N-loop residues are less conserved, conformationally flexible, play a major role in determining receptor affinity, and are intimately coupled to receptor activation at Site-II (13,29).

The structure of the CXCR2 receptor is not known, but structures of CXCR1 and CXCR4 have shown that the receptor N-domain is unstructured (31,32). Sequence analysis and structure-function studies for various chemokines have shown that binding studies with isolated receptor N-domain can capture the structural characteristics of Site-I interactions in the context of intact receptor (12,20,25,33-40).

We have now characterized the Site-I binding interactions of the CXCL1 WT, designed monomer, and disulfide-linked dimer by titrating unlabeled CXCR2 N-domain into 15N-labeled CXCL1 variants. Essentially the same N-loop residues were perturbed in the monomer and in the disulfide-linked and WT dimers, indicating that the same structural interactions mediate the binding of the monomer and dimer (Fig. 5). In particular, G17 and K21 show the largest chemical shift perturbations in both the monomer and dimer. The WT and trapped dimer bind the isolated CXCR2 N-domain with ~5 fold lower affinity compared to the CXCL1 monomer (Fig. 6). The affinity of the trapped dimer to the intact receptor was at most ~2-fold lower compared to the monomer (Fig. 3A), indicating that similar Site-I and Site-II interactions mediate monomer and dimer binding interactions.

NMR studies facilitate the description of how individual amino acids in CXCL1 mediate the binding process. We observe that essentially the same residues mediate receptor binding in the monomer and dimer. Binding induced significant chemical shift changes for the N-loop, second β-strand, 40s turn, and third β-strand, and the C-terminal helical residues (Fig. 5). Significantly perturbed N-loop residues include Thr14, Leu15, Gln16, Gly17, Ile18, and Lys21, and to a lesser extent Leu12, Gln13, His19, and Asn22. The structure shows that these residues span the entire length of the N-loop (Fig. 7), that Leu15 and Gly17 are packed against the protein core, and that the side chains of Ile18 and Asn22 are partially buried and in close proximity to each other. As the N-loop is conformationally dynamic, it is possible that the side chains of Leu15, Gly17, and Ile18
become exposed and engage in binding or indirectly influence the binding of the solvent-exposed residues. Consistent with our observations, mutational studies have shown that Leu12, His19, and Lys21 are critical for receptor binding and activation (14,41).

The binding data also show significant perturbation of residues in the second and third β-strands and the C-terminal helix (Fig. 5). In the case of third β-strand residues that are perturbed, Arg48 and Lys49 are solvent exposed (ASA ~0.7), and Ala50, Leu52, and Asn53 are largely buried and involved in packing interactions against the N-loop residues. Similarly, the significantly perturbed C-terminal residues Ile62 and Ile63 are also involved in packing interactions (Fig. 7).

DISCUSSION

NMR and X-ray structure determination of a large number of CXC and CC chemokines have shown that they share a common structural fold at the monomer level, and biophysical measurements have shown that chemokine dimerization constants can vary by orders of magnitude from very weak (Kₐ ~mM) to very strong (Kₐ ~nM) (3-5, 42). The monomer structure consists of three antiparallel β-strands followed by a C-terminal α-helix. However, the dimer structures are distinctly different between CC and CXC chemokines. CXC chemokines dimerize using the 1st β-strand and α-helix forming a globular structure, and the dimer interface is located away from the receptor binding N-terminal and N-loop regions (Fig. 1). CC chemokines dimerize using their N-loop residues and form an extended structure, and so their dimerization and receptor binding domains overlap.

No less than seven CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8) mediate neutrophil function by activating two receptors, CXCR1 and CXCR2 (27). These chemokines are characterized by a highly conserved N-terminal ELR motif, and also share the property of reversible monomer-dimer equilibrium. CXCL8 alone is active for both CXCR1 and CXCR2 and the others only for CXCR2. CXCL8 monomer is highly active for both CXCR1 and CXCR2. However, CXCL8 dimer is marginally active for CXCR1 and differentially active for CXCR2, with the dimer being as active in some functional assays and less active in others (10). Nothing is known regarding the role of dimerization for CXCR2-specific chemokines.

In this study, we show that the CXCL1 dimer is a potent agonist and activates CXCR2 with potencies comparable to that of the WT monomer. Sequence comparison and structure-function studies have shown that the chemokine N-loop residues determine receptor selectivity. Comparison of the CXCL1 and CXCL8 structures and dynamic characteristics suggest that differences in N-loop conformational dynamics between monomers and dimers and between CXCL1 and CXCL8 must be responsible for the observed functional differences. We have shown previously that dimer interface interactions disfavor the binding of the CXCL8 dimer to the CXCR1 N-domain. On the other hand, our current NMR studies indicate that the dimer interface interactions play no role in the binding of the CXCL1 dimer, providing a structural basis for the similar CXCR2 receptor binding affinities of the monomer and the dimer.

Our NMR studies also indicate that similar direct and indirect interactions mediate the binding of both the CXCL1 dimer and monomer to the receptor N-domain, and that these interactions mediate receptor activation. We had previously proposed a fly-casting mechanism for the binding of CXCL8 binding to the CXCR1 N-domain on the basis that both the N-loop and the N-domain residues are conformationally dynamic (25,43). We observe similar interactions for the CXCL1/CXCR2 axis, and propose that the fly-casting mechanism best describes the binding of CXCL1 monomers and dimers to the CXCR2 N-domain, and that coupling between Site-I and Site-II mediates receptor activation.

It has been previously shown that disulfide-trapped CCL2 and CCL4 dimers are inactive (7,8). The inactivity of CC chemokine dimers is not surprising as dimerization occludes the receptor-binding N-terminal and N-loop residues. On the other hand, a disulfide-trapped CXCL12 dimer elicits robust ERK phosphorylation but weak Ca²⁺ release and fails to elicit chemotaxis (11,12). CXCL12, like CXCL8, is a CXC chemokine and therefore dimerization does not limit receptor
accessibility. We propose that dimerization perturbs the distribution of the conformational substates, which in turn differentially affects the activation of various downstream signaling pathways.

Designed monomers of CXCL10, CCL5, CCL4, were shown to be inactive in mouse models (44,45). However, a recent in vivo study has shown that a designed CCL2 monomer has WT-like activity in contrast to a previous study that had reported CCL2 monomer was inactive (45,46). The differences have been attributed to differences in experimental design including the dosage, time point, and the organ studied (46). On the other hand, both the CXCL8 monomer and dimer are active in the mouse lung and peritoneum models (47,48). It has been proposed in the case of CXCL8 that the monomer-dimer equilibrium and receptor and cell surface GAG interactions together regulate in vivo recruitment, and that differential GAG interactions of the monomer and dimer play crucial roles in defining gradient formation which in turn regulate the kinetics, flux, and duration of the neutrophil recruitment into the target tissue. Animal model studies using the full-length trapped monomer similar to those used in the case of CXCL8, and the trapped dimer, are necessary to better understand how CXCL1 monomer-dimer equilibrium regulates neutrophil trafficking.

In summary, our current study on the disulfide-linked CXCL1 dimer and previous studies for various chemokines collectively indicate that the role of dimerization varies from chemokine to chemokine, and that differential interactions of the dimer for their receptors and GAGs, as well as the monomer-dimer equilibrium, dictate in vivo function. We propose that chemokines have evolved to exploit the property of reversibly existing as monomers and dimers as a means to regulate a wide variety of physiological functions.

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FOOTNOTES

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The abbreviations used are: MGSA/CXCL1, melanoma growth stimulatory activity/CXC ligand 1; IL-8, interleukin-8; CXCR, CXC chemokine receptor; GPCR, G-protein-coupled receptor; N-domain, N-terminal domain; NMR, nuclear magnetic resonance; WT, wild type; HSQC, heteronuclear single quantum coherence; ASA, accessible surface area

FIGURE LEGENDS

Figure 1. Design of a CXCL1 dimer and CXCL1 monomer. (A) The structure of the WT CXCL1 dimer (PDB id: 1MGS) (17). The individual monomers in the dimer are shown in red and blue. (B) The spatial proximity of the solvent-exposed Asn27 side chain (in gold) in the dimer structure is highlighted. (C) A single monomeric subunit of the WT CXCL1 dimer is shown. The CXCL1(1-67) monomer was designed by deleting the last six C-terminal helix residues (shown in magenta); the functionally important N-loop and N-terminal regions are also highlighted.

Figure 2. NMR structural characterization of the designed monomer and dimer. A strip plot showing NOEs from Val28, a dimer-interface residue for CXCL1(1-67) (A) and CXCL1 N27C variants (B). Characteristic intermolecular dimer interface NOEs (in red) are observed for the N27C variant indicating that it is a dimer; these NOEs are absent (location indication by open circles) for the CXCL1(1-67) variant, indicating that it is a monomer.
Figure 3. Binding affinity and receptor activity of the CXCL1 trapped dimer. (A) The binding affinities of CXCL1 WT (●) and trapped dimer (○) were calculated by measuring the inhibition of binding of $^{125}$I-CXCL1 to the CXCR2 receptor. (B) Ca$^{2+}$ release activity of CXCL1 WT and trapped dimer as a function of different doses. The calculated EC$_{50}$ values indicate that the trapped dimer is a potent agonist. (C) ERK phosphorylation activity of 10 nM CXCL1 WT and trapped dimer at 1, 5, and 10 min. Results from densitometric analysis of western blots (n=3) are expressed as mean ± SD of the phosphorylated ERK normalized to total ERK for each sample. (D) Chemotaxis activity of WT and trapped dimer at 10 and 100 nM concentrations were measured using a Boyden chamber type assay. The data were collected in quadruplicate and the results are expressed as mean ± SD, and are representative of three independent experiments.

Figure 4. Structural Characterization of CXCL1 trapped dimer. (A) $^{15}$N-1H HSQC 600 MHz NMR spectrum of the CXCL1 dimer at pH 6.0 and 30°C. (B) Plot of the $^1$H NMR $C\alpha$ chemical shift differences between the WT dimer and the disulfide-linked trapped dimer.

Figure 5: Binding of CXCL1 dimer and monomer to CXCR2 N-terminal domain. Histograms of the binding-induced chemical changes ($\Delta\delta$, ppm) for the CXCL1 (1-67) monomer (A) and the N27C dimer (B), respectively. Change in chemical shifts was calculated as $\Delta\delta = \sqrt{(\Delta\delta_H)^2 + (\Delta\delta_N/8)^2}$. Chemical shifts of Gly17 in the monomer and Lys21 in the monomer and dimer disappear during the titration due to line broadening, and are represented as open bars. The chemical shift of Gly17 (*) in the dimer is perturbed by 1.1 ppm, but is plotted on a scale to 0.5 ppm in line with panel A. The open bars are shown at the same height as Ile18 in the monomer and Gly17 in the dimer, which show maximal chemical shift changes. Monomer Gln10 (*) is not observed, and residues 20, 31, 33, 54, and 57 are prolines.

Figure 6. Binding affinities of the CXCL1 variants to CXCR2 N-domain. (A-C) CXCR2 N-domain binding-induced chemical shift changes of Gln16 in the CXCL1 monomer, WT dimer, and the trapped dimer. The peaks are color-coded according to peptide:protein mole ratios: for the monomer, 0 (black), 0.2 (green), 0.7 (blue), and 4.9 (red); for the WT dimer, 0 (black), 0.6 (green), 1.1 (blue), and 4.7 (red); for the trapped dimer, 0 (black), 0.6 (green), 1.2 (blue), and 5.3 (red). (D-F) Binding profiles for calculating binding constants. The calculated binding affinities (K$_d$) for the CXCL1 monomer, WT dimer, and trapped dimer are 21 ± 3 µM, 90 ± 16 µM, and 86 ± 18 µM, respectively.

Figure 7. Molecular basis of CXCL1-CXCR2 interactions. Molecular plot of CXCL1 highlighting the residues that are significantly perturbed on CXCR2 N-domain binding. The N-loop residues are shown in red, the second $\beta$-strand and the 40s loop residues are shown in purple, and the third $\beta$-strand residues are shown in blue.
Figure 1
Figure 2
Figure 3

A

MGSA WT  
MGSA dimer

125I bound (%)  
Log [Conc], M

B

WT  
Dimer

Ca²⁺ release  
log [Conc], M

C

Control  
WT  
Dimer

Ratio (pERK/ERK)  
Time (min)

D

Chemotactic index  
WT CXCL1  
Dimer  
WT CXCL1  
Dimer

10 nM  
100 nM
Figure 5

A

B

\[ \Delta \delta \text{ (ppm)} \]

Residue Number

\( \beta_1 \)  \( \beta_2 \)  \( \beta_3 \) \( \alpha \text{ Helix} \)

\[ \Delta \delta \text{ (ppm)} \]

Residue Number

\( \beta_1 \)  \( \beta_2 \)  \( \beta_3 \) \( \alpha \text{ Helix} \)
Figure 6

A

B

C

D

E

F

\( \Delta \delta \) (ppm)

MGSA monomer

Mole Ratio

WT MGSA dimer

Mole Ratio

MGSA trapped dimer

Mole Ratio
Figure 7
Chemokine CXCL1 dimer is a potent agonist for the CXCR2 receptor
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