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IL-8 single-chain homodimers and heterodimers: Interactions with the chemokine receptors CXCR1, CXCR2, and DARC

STEVEN R. LEONG,[1] HENRY B. LOWMAN,[2] JUN LIU,[3] STEVEN SHIRE,[3] LAURA E. DEFORGE,[4] BETH L. GILLECE-CASTRO,[5] ROBERT McDOWELL,[6] and CAROLINE A. HÉBERT[1]

Departments of [1]Immunology, [2]Protein Engineering, [3]Pharmaceutical Research & Development, [4]BioAnalytical Technology, [5]Protein Chemistry, and [6]Bioorganic Chemistry, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080

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Abstract

Covalent single-chain dimers of the chemokine interleukin-8 (IL-8) have been designed to mimic the dimeric form of IL-8 in solution and facilitate the production of heterodimer variants of IL-8. Physical studies indicated that use of a simple peptide linker to join two subunits, while allowing receptor binding and activation, led to self-association of the tethered dimers. However, addition of a single disulfide crosslink between the tethered subunits prevented this multimer from forming, yielding a species of dimer molecular weight. Crosslinked single-chain dimers bind to both IL-8 neutrophil receptors CXCR1 and CXCR2 as well as to DARC, as does a double disulfide-linked dimer with no peptide linker. In addition, neutrophil response to these dimers as measured by chemotaxis or β-glucuronidase release is similar to that elicited by wild-type IL-8, providing evidence that the dissociation of the dimeric species is not required for these biologically relevant activities. Finally, through construction of single-chain heterodimer mutants, we show that only the first subunit's ELR motif is functional in the single-chain variants.

Keywords: analytical ultracentrifugation; chemokine; dimerization; mutagenesis; neutrophil

Interleukin-8 (IL-8), an approximately 8.5 kDa protein, is a member of a large family of proteins known as chemokines that act as chemoattractants for and activators of leukocytes in the inflammatory response of the immune system (for reviews, see e.g., Hébert & Baker, 1993; Baggiolini et al., 1994; Howard et al., 1996). A sub-family of these proteins, the CXC chemokines, to which IL-8 belongs, are generally active towards neutrophils, whereas another sub-family, the CC chemokines, are generally active towards other leukocyte types. The CXC chemokines contain the sequence motif "Cys-X-Cys," while the CC chemokines have the motif "Cys-Cys." The activities of these molecules are mediated through their binding to specific GTP-coupled, seven-helix transmembrane receptors, of which there are two known types, CXCR1 and CXCR2 (also known as IL-8 receptors type A and type B, respectively), responding to IL-8 on neutrophils (Lee et al., 1992). IL-8 also binds to a receptor on erythrocytes, DARC, which binds a number of both CXC and CC chemokines, but whose physiological function is less clear (Horuk et al., 1993). Despite distinctions in sequence and biological function, the two chemokine sub-families share a very similar overall tertiary fold, in which a β-sheet is followed by an α-helix, illustrated in the known structures of a number of chemokines (for a review, see Fairbrother & Skelton, 1996).

Interestingly, a further similarity arises in the quaternary structures of the chemokines. In both NMR and crystallographic studies, these molecules have been found to form non-covalently associating, symmetric dimers (molecular weight approx. 17 kDa). In the case of the CXC chemokines, such as IL-8 and MGSA, the dimer interface is formed by main-chain hydrogen bonding between the β-sheet of one monomer subunit with that of the other subunit, as well as packing interactions between the helix of one subunit and the β-sheet of the other (Fig. 1; Clore et al., 1990; Baldwin et al., 1991). The CC chemokines, on the other hand, have a different but apparently conserved dimerization motif. For example, RANTES (Skelton et al., 1995) and MIP-1β (Lodi et al., 1994) undergo dimerization primarily through interactions of the N-terminal segments of each subunit. PF4, another CXC chemokine, actually forms a tetramer (Zhang et al., 1994).

The possible functional consequences of these dimerization events have thus far remained unclear. While IL-8 and other chemokines typically bind their cognate receptors with affinities on the order of 10^9 M^{-1}, in vitro studies have shown dimerization constants on

Reprint requests to: Henry B. Lowman, Department of Protein Engineering, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080; e-mail: hbl@gene.com.

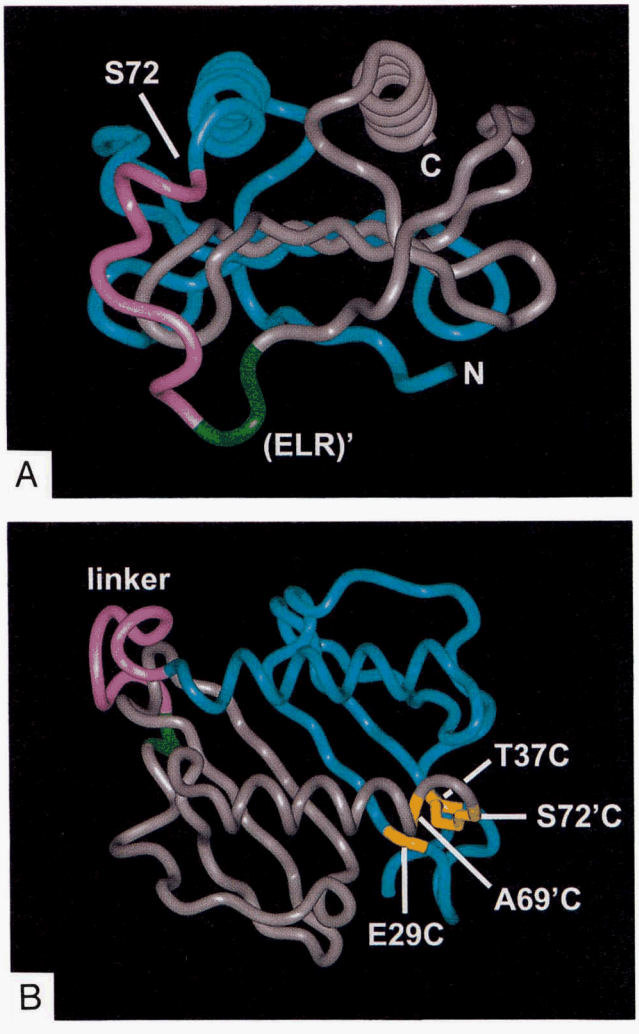

Fig. 1. Modeled structures of IL-8 single-chain dimers. **A:** Model of the engineered single-chain IL-8 dimer (scIL8) shows the added peptide linker sequence G_2S_6G (in magenta) connecting residue S72 of the first subunit (in blue) with the second subunit (in gray). Subunit-1 appears as in the X-ray structure (Baldwin et al., 1991). Subunit-2 residues S1′, A2′, K3′ (also in magenta) were modeled as part of the peptide linker. Residues E4, L5, R6 (ELR) of the first subunit are located at the N-terminus (N) of the protein, and (ELR)′, in the second, is highlighted in green. **B:** A rotated view of the modeled single-chain IL-8, showing residues targeted for possible crosslinking of the two subunits at the end of the dimer interface opposite to that where the linker peptide was added. Residues targeted (in yellow) were E29 or T37 in the first subunit, and A69′ or S72′ in the second subunit. Pairwise mutations were made to generate the possible disulfide crosslinks E29C-A69′C, E29C-S72′C, T37C-A69′C, or T37C-S72′C. The T37C-S72′C disulfide bond (as in variant d4) is shown as modeled (see Materials and methods).

the order of 10^5 M^{-1} for IL-8 (Burrows et al., 1994; Paolini et al., 1994) and for MCP-1 (Paolini et al., 1994). Thus, in solution at concentrations at which receptor binding and biological activity are observed, the predominant form by far is monomeric (Burrows et al., 1994; Paolini et al., 1994). Furthermore, stable monomers of IL-8 have been produced by synthetic (Rajarathnam et al., 1994) as well as recombinant (Lowman et al., 1997) means, and have been shown to have similar receptor-binding affinity and biological potency to wild-type IL-8 in vitro. Mutations of the ELR motif in recombinant monomers show that the ELR motif functions similarly to that of wild-type (Hébert et al., 1991) and is critical for binding both CXCR1 and CXCR2 (Lowman et al., 1996). Therefore, it seems clear that IL-8 dimerization is not required for interaction with—and activation of—its known receptors.

On the other hand, dissociation of IL-8 dimers might actually be required to render IL-8 competent for binding and activating receptor. Rajarathnam et al. (1994), considering several models of ligand:receptor interaction and stoichiometry, suggested that the introduction of an N-methyl group in the β-sheet interface between subunits, which successfully disrupted IL-8 dimer formation, might also disrupt monomer:receptor interaction if they were to occur through this site on the β-sheet. A series of IL-8 monomers produced by recombinant means (Lowman et al., 1997), introduced even more drastic charge reversal and steric changes within the dimer interface, but still had little effect on receptor binding, further arguing that the dimer interface is not a part of the receptor-binding epitope. However, a different line of experiments involved mutations at positions L25 and V27, whose side chains are essentially buried in the β-sheet:β-sheet interface between subunits. These mutations resulted in reductions of as much as 100-fold in IL-8 receptor binding (Lusti-Narasimhan et al., 1995, 1996). Such results suggest that a portion of the IL-8 receptor epitope may lie buried in the dimer interface, unable to interact with receptor, until exposed upon dissociation of the dimer.

Additionally, as an explanation for the conservation of a dimerization interface in IL-8 and homologs, it might be argued that high local concentrations of monomer immobilized on cell membranes by binding to proteoglycans (Tanaka et al., 1993; Webb et al., 1993) might favor dimerization. In fact, solution conditions in general dramatically affect the dimerization affinity of IL-8 in solution (Lowman et al., 1997). For this reason, an IL-8 dimer molecule containing only one "active" subunit, and blocked with respect to possible receptor interactions in the dimer interface, could test further whether a single subunit is active.

A few, seemingly contradictory results have been obtained for forced, disulfide-linked IL-8 homodimers. In one attempt to construct linked IL-8 dimers, two disulfides were introduced (per dimer) near the center of the β-sheet interface between the two subunits, using the mutations K23C/G31C; however, this construct lacked activity (Rajarathnam et al., 1995). Clark-Lewis and coworkers reported a single-Cys mutant, R26C, which was presumed to form a single-disulfide linked homodimer, but which was 15-fold reduced in elastase-release activity compared with wild-type (Clark-Lewis et al., 1995). More recently, it was reported that IL-8 dimers could be covalently linked through mutations E29C/A69C, which was shown to produce two inter-subunit disulfide bonds (Williams et al., 1996). While this construct was active in calcium-flux assays, receptor binding specificity and biological activity were not reported. As a platform for investigating the contributions of each subunit of the dimer to binding, these constructs do not easily permit heterodimer constructions because each subunit contains both (Cys) mutations.

We have designed variants of IL-8 to investigate whether or not IL-8 dimer dissociation is required for binding to CXCR1, CXCR2, or to DARC, to determine whether a heterodimer containing only one active subunit might still be capable of receptor binding and activation, and to provide a stable dimeric form of IL-8 in which effects of mutation in only one subunit can be further explored. We mimicked a native IL-8 dimer by constructing single-chain fusions of two IL-8 subunits linked in tandem through a flexible linker

peptide. Such a strategy has been previously used to link other dimeric structures, such as the single-chain variable domains (scFv) constructs formed by linking an antibody V_H domain to a V_L domain (for a review, see Huston et al., 1993). The addition of a single disulfide crosslink between subunits in these dimers stabilizes the dimer, and provides a molecule that cannot undergo multimerization, or additional dimer–interface interactions (e.g., with receptors).

Results

Design and construction of single-chain IL-8 dimers

In order to facilitate the construction of both homodimers and heterodimers of IL-8, we designed a peptide linker to join the C-terminal S72 (IL-8 residues are designated in single-letter code followed by residue number, with a prime when the second of two subunits is indicated) of one IL-8 subunit to the N-terminal S1′ of a second subunit (Fig. 1A). Because doing so could clearly distort the conformation of the ELR motif, critical for receptor binding (Hébert et al., 1991) in the second subunit, we examined peptides of various length by molecular modeling (see Materials and methods) to identify one that could accommodate the position of the second ELR in the crystallographic dimer (Fig. 1A; Baldwin et al., 1991). We also chose to use a linker composed primarily of Ser residues, to insure that the added peptide would not greatly increase the hydrophobicity of the dimer surface, possibly leading to aggregation of the protein. Among motifs of the form ENSGG $(S)_n$AKELRCQC (where n = 5 to 9) linking the C-terminus of the first IL-8 subunit to the N-terminus of the second, all conformations for loops in which n = 5 or 6 featured unusual backbone angles and relatively high strain energies. By contrast, the longer loops had canonical backbone torsion angles and allowed a wide range of mobility for the E4′–L5′–R6′ sequence. The shortest loop that was energetically stable, n = 7, was used as a template, with the serine N-terminal to the SAKELR sequence changed to a glycine in order to allow greater flexibility. The linker sequence $(Gly)_2$-$(Ser)_6$-Gly was chosen, and used to construct a model of a single-chain IL-8, called scIL8 (Fig. 1A).

Because the designed linker peptide had the potential for flexibility, and because the scIL8 construct had no constraints between the subunits at the end of the helical axes opposite the linker (Fig. 1A), we were concerned that the scIL8, although covalently linked, might still be able to "flex," opening about a hinge at the linkage site. To restrict this type of motion, we introduced Cys residues in a series of mutants, at sites which could potentially result in disulfide crosslinks between the two subunits, based upon spacing of less than 6 Å between Cα coordinates in the dimer (Fig. 1B). The mutations E29C or T37C were combined with A69′C or S72′C to yield the homodimer variants d1 [scIL8(E29C-A69′C)], d2 [scIL8(E29C-S72′C)], d3 [scIL8(T37C-A69prC)], and d4 [scIL8(T37C-S72′C)]. To test the effect of disulfide constraints, independent of possible effects from the peptide linker, we also constructed a "linkerless" homodimer variant, IL-8(T37C/S72C), designed to form two inter-subunit disulfide bonds.

Physical analysis of single-chain dimers

Each of the single-chain variants was successfully expressed and secreted in *E. coli*, and was recoverable using techniques previously described (Hébert et al., 1991; Lowman et al., 1996). Yields were lower than that obtained for wild type, with all variants expressing about 1 mg/liter of culture, except variant d2, which expressed at a level of about 0.2 mg/liter. Following purification by ion-exchange and heparin-affinity chromatography, SDS-PAGE confirmed that each of the single-chain variants, as well as IL-8 (T37C/S72C), had an apparent molecular weight consistent with that of the 17 kDa dimer (data not shown). The precise molecular weights of several mutants were determined using mass spectrometry. In each case, the experimental mass of the mutant was found to be within experimental error of the respective theoretical mass, calculated with the assumption of oxidized disulfides between the subunits. The observed and predicted masses, respectively were 17,443.8 and 17,443.2 for scIL8; 16798.5 and 16,795.7 for IL-8(T37C/S72C); 17,454.4 and 17,455.2 for variant d4; 17,271.0 and 17272.0 for d5 17,270.0 and 17,272.0 for d6; and 17,084.0 and 17,086.8 for d7.

The quaternary structures of scIL8 and one crosslinked version, d4, were evaluated in solution using gel filtration. Both variants eluted earlier than wild-type IL-8 from a Superdex-75 column; however, scIL8 showed an apparent molecular weight of 23 kDa, while d4 showed an apparent molecular weight, as expected, of about 17 kDa (data not shown). This distinction was further examined by analytical ultracentrifugation of the two single-chain variants. Equilibrium sedimentation experiments (Fig. 2) indicated that variant d4, had an apparent molecular weight of 17.3 kDa ± 0.4, with no apparent self-association. On the other hand, scIL8 (lacking the crosslink) did undergo self-association, with an apparent dimerization (for formation of a dimer of dimers; $(scIL8)_2$, MW approx. 35 kDa) constant corresponding to K_d = 5.7 ± 3 μM. Thus, the addition of a disulfide crosslink was necessary to maintain an intact dimer and prevent multimer formation.

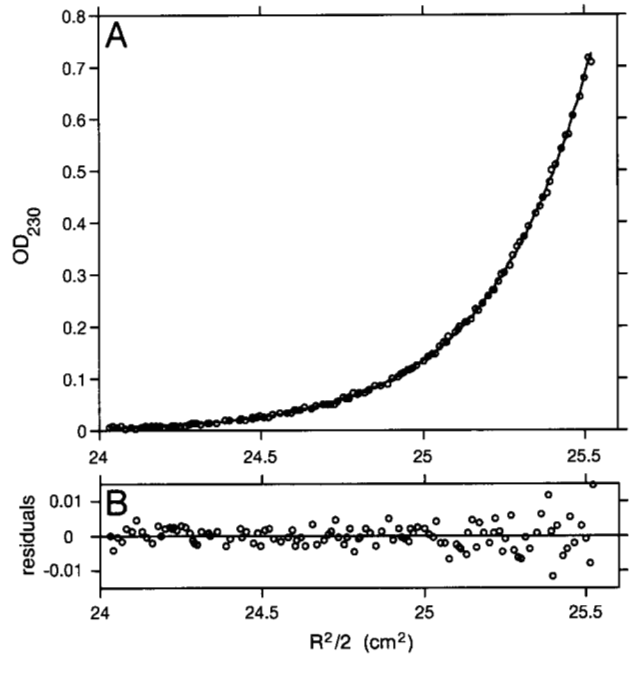

Fig. 2. Analysis of variant d4 by analytical ultracentrifugation. **A:** Optical density (OD_{280}) is plotted as a function of one-half the radial distance (R) squared. The fit corresponds to a an apparent single-species of molecular weight 17.3 ± 0.4 kDa. Under these conditions, IL-8 dimerized with K_d = 4.0 μM, and scIL8 dimerized with K_d = 5.7 μM. **B:** Residual plot showing the difference between the data and the fitted curve.

Design of heterodimer variants

To express dimers of IL-8 specifically mutated in one or both subunit moieties in an *E. coli* periplasmic secretion system, we designed a strategy for site-directed mutagenesis in which the gene encoding each subunit could be mutated in separate plasmids, pIL8 or pIL8′ (see Materials and methods). In this way, the same IL-8 cDNA, and in some cases the same mutagenic oligonucleotides, could be used to produce a given mutation in one or both subunits of the dimer. Because of the critical role of the ELR motif in neutrophil receptor binding (Hébert et al., 1991) as well as in DARC receptor binding (Hesselgesser et al., 1995), mutation of these residues to AAA was used to disable one or both subunits for binding to these receptors. Heterodimer variants d5 [scIL8(E4A/L5A/R6A/T37C-S72′C)] and d6 [scIL8(E4′A/L5′A/R6′A/T37C-S72′C)] were prepared by site-directed mutagenesis. A homodimer "knock-out" variant, d7 [scIL8(E4A/L5A/R6A/E4′A/L5′A/R6′A/T37C-S72′C)], was also constructed to provide a negative control for binding and activity analysis.

Receptor-binding affinity and specificity of variants

As an initial screening for the functionality of variants, single-chain IL-8 dimers were tested for binding to receptors on neutrophils or to the DARC receptor on erythrocytes by competitive displacement assays using ^{125}I-labeled IL-8. The results (Table 1) indicated that scIL8, IL-8(T37C/S72C), and the disulfide cross-linked single-chain dimer variants d1, d2, d3, and d4, all bound neutrophils with affinities similar (within threefold) to that of wild-type IL-8. Similarly, when these variants were tested for binding to erythrocytes, all had similar affinities (within twofold) to that of wild-type for DARC (Table 1). Substitution of the critical ELR motif with AAA in both subunits of the d4 variant (i.e., variant d7), on the other hand, reduced neutrophil binding at least 1100-fold, and DARC binding at least 50-fold (Table 1). Similarly, when the first ELR motif of variant d4 was mutated to AAA (variant d5), binding to both neutrophils and erythrocytes was reduced 220-fold and 130-fold, respectively. However, the covalent heterodimer d6, in which only the second (linked) ELR motif had been mutated was only about fourfold reduced in affinity for neutrophils compared with IL-8 (sevenfold compared with d4) and only fourfold reduced in affinity (threefold compared with d4) for DARC (Table 1).

To test for receptor-specific effects, we measured binding affinities of IL-8 variants in ^{25}I-IL-8 competition assays using 293 human embryonic kidney cells stably transfected (Lee et al., 1992) with either CXCR1 or CXCR2. In these assays, the binding affinity of scIL8 was five- to sixfold weaker than that of wild-type on both neutrophil receptor types (Table 2). The remaining scIL8 variants (d1, d2, d3, and d4) were similar in affinity (within twofold) to that of wild-type for binding CXCR2, but somewhat reduced in affinity (6- to 20-fold) for binding CXCR1. The linkerless homodimer variant, T37C/S72C similarly had sixfold reduced affinity for CXCR1 and about twofold improved affinity for CXCR2. The binding of d5 and d7 to CXCR1 and CXCR2, reflecting the neutrophil binding results above, was very weak (K_d > 700 nM). Binding of the d6 variant also reflected the neutrophil binding results. The specificity effect seen for d4 was retained in this variant, which showed 13-fold reduced affinity for CXCR1 compared with wild-type (twofold compared with d4) and 1.4-fold reduced affinity (threefold compared with d4) for CXCR2 (Table 2).

Biological activity of dimeric variants

The biological activity of the single-chain IL-8 dimers was directly tested on human neutrophils in vitro. ScIL8, d1, d2, and d3 showed no significant difference in chemotactic response compared with wild-type IL-8 (Fig. 3A). The d4 variant showed somewhat enhanced chemotactic response in comparison to with that of wild type. An approximately twofold enhanced amplitude of response (number of neutrophils migrating) was observed over a concentration range of 2.5–10 nM with this protein. This effect was reproducible for variant d4, but not observed with the linkerless T37C/S72C homodimer variant (Fig. 3B). As expected based upon receptor binding data, variant d6 showed wild-type–like chemotactic activity, while d5 and d7 showed no chemotactic activity compared with buffer control (Fig. 3B) up to protein concentrations of 50 nM (data not shown). In the case of each variant, chemotaxis was shown to be IL-8 dependent through successful

Table 1. *Binding affinities of IL-8 variants to human neutrophil and erythrocyte receptors*[a]

Cells	Neutrophils		Erythrocytes	
IL-8 variant	K_d (nM)	K_d(mut)/K_d(IL-8)	K_d (nM)	K_d(mut)/K_d(IL-8)
IL-8	0.89 ± 0.13	1	19 ± 2.4	1
IL-8(T37C/S72C)	1.1 ± 0.3	1.3	38 ± 5.4	2.0
scIL8	1.5 ± 0.36	1.7	16 ± 4.1	0.83
d1 = scIL8(E29C-A69′C)	2.2 ± 0.56	2.5	24 ± 1.9	1.3
d2 = scIL8(E29C-S72′C)	n.d.[b]		18 ± 1.0	0.95
d3 = scIL8(T37C-A69′C)	0.90 ± 0.31	1.0	15 ± 2.3	0.79
d4 = scIL8(T37C-S72′C)	0.60 ± 0.24	0.67	27 ± 3.2	1.4
d5 = d4+(E4A/L5A/R6A)	190 ± 13	220	2,400 ± 260	130
d6 = d4+(E4′A/L5′A/R6′A)	3.9 ± 3.5	4.4	69 ± 9.4	3.7
d7 = d6+(E4A/L5A/R6A)	>1,000	>1,100	>1,000	>50

[a]Apparent receptor binding K_ds were determined in parallel and normalized to wild-type controls, representing an average of duplicates or triplicates (±standard deviation).

[b]n.d., not determined.

Table 2. *Binding affinities of IL-8 variants to transfected 293 cells*[a]

Receptor	CXCR1		CXCR2	
IL-8 variant	K_d (nM)	K_d(mut)/K_d(IL-8)	K_d (nM)	K_d(mut)/K_d(IL-8)
IL-8	2.3 ± 1.5[a]	1	0.84 ± 0.42[a]	1
IL-8(T37C/S72C)	14 ± 2.2[b]	6.0	0.46 ± 0.30[b]	0.55
scIL8	15 ± 5.1	6.4	3.8 ± 2.0	4.5
d1 = scIL8(E29C-A69'C)	48 ± 13	21	1.4 ± 0.18	1.6
d2 = scIL8(E29C-S72'C)	24 ± 5.7	10	1.3 ± 0.12	1.5
d3 = scIL8(T37C-A69'C)	13 ± 3.9	5.8	0.95 ± 0.33	1.1
d4 = scIL8(T37C-S72'C)	14 ± 1.9[b]	6.0	0.38 ± 0.04[b]	0.45
d5 = d4+(E4A/L5A/R6A)	1,800 ± 180[b]	770	790 ± 230[b]	940
d6 = d4+(E4'A/L5'A/R6'A)	29 ± 8.5[b]	13	1.2 ± 0.31[b]	1.4
d7 = d6+(E4A/L5A/R6A)	>1,000	>430	>1,000	>1,100

[a]Apparent receptor binding K_ds were determined in parallel and normalized to wild-type controls, representing an average of duplicates or triplicates (±standard deviation), except as noted.
[b]Values for wild-type IL-8 are from Lowman et al., 1996.
[c]Value represents the fit of a single displacement curve ± standard error of fit.

blocking of the chemotactic response by the addition of a monoclonal anti-IL-8 antibody (data not shown).

Neutrophil degranulation activity was evaluated by measuring β-glucuronidase release (Lowman et al., 1996) of IL-8 and variants T37C/S72C, d4, and d6. Each of these variants was active in this assay, with somewhat higher EC_{50} values (effective concentration for half-maximal response) than that of wild type (Fig. 4): [EC_{50}(mutant)/EC_{50}(wild type)] ≤ 5 in each case. Each of these variants also produced an apparently lower plateau of β-glucuronidase activity than wild type at high protein concentrations (Fig. 4; see Discussion).

Discussion

Dimerization is a prevalent structural feature among ligands of both the CXC and the CC chemokine sub-families, although the known dimerization affinities are considerably lower than those of the respective ligand:receptor interactions. The role of chemokine dimerization in their biological function is therefore not obvious. One CXC chemokine, IL-8, has been extensively studied with respect to its structure, receptor-binding epitope, and dimerization in vitro. The latter indicates that the dimer dissociation constant of IL-8 under physiological conditions (in solution) is on the order of 10–20 μM (Burrows et al., 1994; Paolini et al., 1994), although solution conditions can drastically alter this affinity (Lowman et al., accompanying submission). Previous studies have demonstrated that IL-8 mutants having even weaker dimerization affinities than that of wild-type IL-8 were nevertheless able to bind and activate neutrophils with similar potency to that of wild-type (Rajarathnam et al., 1994; Lowman et al., 1997).

Even though the single chain presented here was predicted to allow formation of a native-like IL-8 dimer, such dimers could still "flex," or dissociate into a non-native dimer conformation in solution, with only the peptide linker tethering the two subunits together. Indeed, sedimentation results showed that the wild-type IL-8 dimers could self-associate in a concentration range similar to that of wild-type IL-8, namely about 6 μM. Although this behavior occurs at concentrations much higher than those at which receptor binding and activity are observed (about 1 nM IL-8), we could not be sure whether the native interface was being formed at low (~ nM) concentrations of scIL8. A similar example of dimerization of single-chain–linked proteins has been observed among scFvs, in which two linked dimers of V_H and V_L domains can associate to form a "diabody" (Perisic et al., 1994). Based upon the observed K_d for dimerization of IL-8 (Burrows et al., 1994; Paolini et al., 1994; Lowman et al., 1997), and that reported here for scIL8, we predict the behavior in solution of these variants to be similar: one [i.e., $(IL\text{-}8)_2$)] or two [i.e., $(scIL8)_2$)] native-like dimer interfaces (Fig. 1) form as the concentration of protein is raised into the μM range.

In contrast, the addition of a single disulfide crosslink effectively prevents the formation of a $(scIL8)_2$ species based upon sedimentation results (Fig. 2), maintaining the native dimer interface over a wide concentration range. In fact, four different disulfide crosslinks appeared to yield similar results. These molecules were well expressed in *E. coli* and appeared to form disulfide bonds between the peptide-linked subunits, based upon mass spectrometry, SDS-PAGE, gel filtration, and sedimentation analysis.

The use of a peptide-linker strategy, combined with intersubunit disulfides, has the advantage of allowing facile construction of single-chain heterodimers. Here, we also describe heterodimers in which either the first or the second subunit has been disabled toward receptor binding by specifically changing one of the ELR motifs to AAA. Interestingly, mutation of the second subunit's ELR results in a heterodimer variant (d6), which binds the IL-8 neutrophil receptors with near wild-type affinity (Table 2), and activates these receptors on neutrophils (Figs. 3 and 4). Binding to the DARC receptor on erythrocytes was also of essentially wild-type affinity (Table 1). We conclude that only one subunit in the single-chain dimer need be functional to confer binding and activity to each of the known IL-8 receptors.

The heterodimer variant d5 in which the first ELR was mutated to AAA, leaving only the peptide-linked ELR of the second subunit intact, had greatly reduced affinity towards DARC as well as towards CXCR1 and CXCR2 (Tables 1 and 2), comparable to that of the homodimer (d7) control variant. This suggests that the ELR motif of IL-8 cannot function in receptor binding with a long N-terminal extension (i.e., another IL-8 subunit) and/or with con-

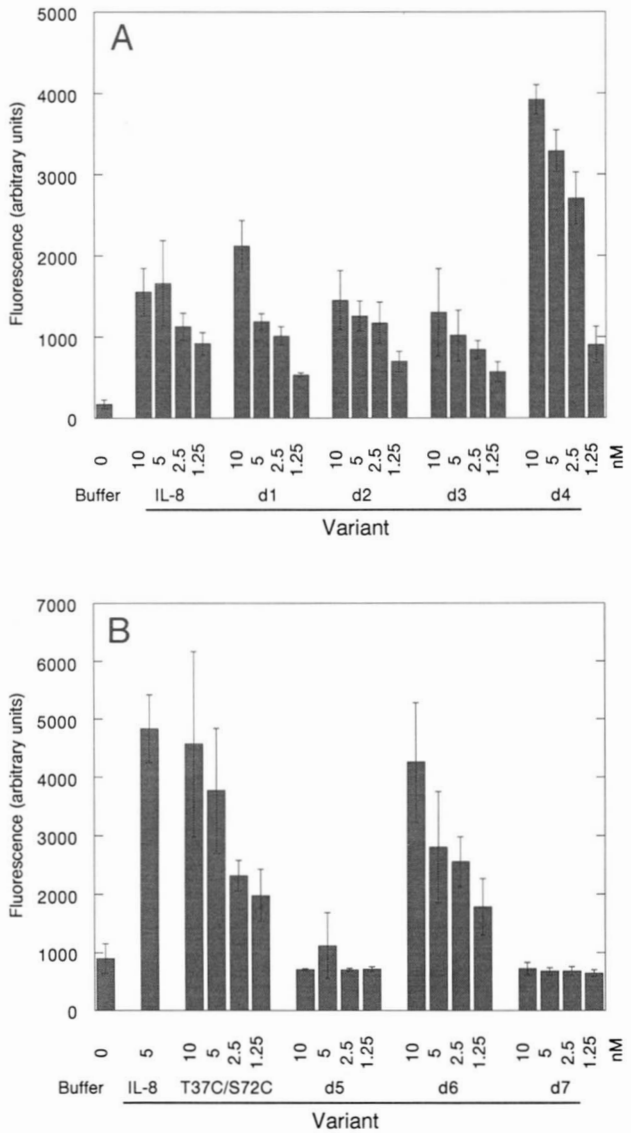

Fig. 3. Chemotaxis of neutrophils measured in response to single-chain IL-8 variants, compared with wild-type IL-8. The fluorescence signal (in arbitrary units) is plotted as a function of the concentration of IL-8 used. See Table 1 and text for nomenclature of single-chain variants. **A:** Comparison of wild-type IL-8 with single-chain variants having four different disulfide linkages: d1, d2, d3, and d4. **B:** Comparison of wild-type IL-8, the linkerless homodimer IL-8(T37C/S72C), and the ELR mutants of the single chain d4 variant: d5, d6, and d7.

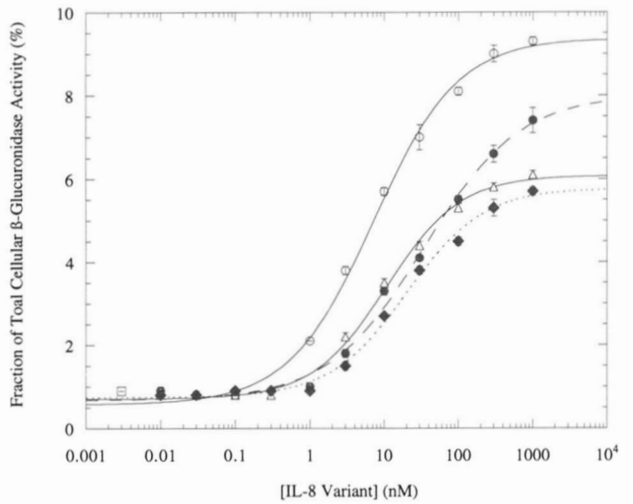

Fig. 4. Degranulation activity of IL-8 variants was evaluated by measuring release of β-glucuronidase by human neutrophils. The data show β-glucuronidase activity ($\pm$SD of triplicate measurements) as a function of the concentration of IL-8 (open circle), IL-8 (T37C/S72C) (open triangle), variant d4 (closed circle), or variant d6 (closed diamond). EC_{50} values calculated from the fit curves were 7.1 nM, 11 nM, 34 nM, and 20 nM, respectively. A control in which no IL-8 was added is also shown (open square).

formational constraint. Similarly, even a short N-terminal extension on another CXC chemokine, PF4, led to markedly reduced activity (Clark-Lewis et al., 1993). The flexibility of the ELR motif in wild-type IL-8 has been noted (Grasberger et al., 1993), and the peptide linker in scIL8 may restrict flexibility in a way that prohibits a productive conformation of ELR for receptor binding. Further structural and functional characterization of these variants may provide useful information for the rational design of ELR-based IL-8 antagonists.

The homodimers bind with similar affinity (Table 1) as wild-type IL-8 to both neutrophils as well as to the erythrocyte DARC receptor. Some specificity differences are seen in binding to the transfected neutrophil receptors, CXCR1 and CXCR2, on neutrophils (Table 2). The greatest difference is seen for the d4 variant, i.e., scIL8(T37C-S72′C), which is twofold improved in CXCR2 affinity but sixfold reduced in CXCR1 affinity, yielding a 12-fold increase in specificity towards CXCR2 as compared with wild type. This is likely an indirect effect of the mutations, rather than a direct result of dimer formation, as CXCR1 binding is characteristically sensitive to small structural perturbations (Lowman et al., 1996), and the effect is not large enough to be ascribed to steric blocking of the interface in the dimeric form. In fact, each of the four disulfide constrained single-chain variants (d1, d2, d3, and d4) described here, as well as the linkerless homodimer T37C/S72C, display a receptor specificity shift, with weaker affinity, by 6- to 20-fold, for CXCR1 and equal or slightly tighter affinity for CXCR2. It is not obvious why these variants are reduced in CXCR1 affinity. However, a common feature of all is that they contain a Cys mutation at either the start (E29) or end (T37) of a loop that is in contact with the ELR region in the crystal structure of wild-type IL-8. Indeed, mutations of G31 and P32 within this loop have been shown to affect receptor binding (Clark-Lewis et al., 1994). We, therefore, suggest that the reduction in CXCR1 binding affinity results from indirect perturbations of the ELR.

Single-chain dimers are biologically active. However, some reduction in maximal β-glucuronidase activity is seen with both a disulfide-linked IL-8 homodimer (i.e., T37C/S72C) and with scIL8 variants d4 and d6 (Fig. 4). The reduced maximal degranulation response is consistent with previous observations that defects in CXCR1 (receptor-A) binding affinity can reduce maximal degranulation response, even when CXCR2 (receptor-B) binding affinity is high, as found among receptor-specific variants of IL-8 and MGSA (Lowman et al., 1996). The single-chain disulfide-linked IL-8 dimers are also active in neutrophil chemotaxis, with one variant, scIL8(T37C-S72′C) showing somewhat enhanced chemotactic response (Fig. 3) compared with wild type. The reason for this enhancement is not clear.

Elution of single-chain IL-8 variants from a heparin column (see Materials and methods) occurred at an ionic strength similar to that at which wild-type IL-8 elutes. Elution of IL-8 from heparin has been used as a relative measure of proteoglycan-binding affinity (Webb et al., 1993). The affinity of the single-chain variants for proteoglycans therefore appears to be similar to that of the wild type.

These variants provide direct evidence that dissociation of the IL-8 dimer structure is not required for binding to the known IL-8 receptors, nor for neutrophil bioactivity. Therefore, it is likely that mutations at sites buried in the dimer interface, such as L25 and V27 (Lusti-Narasimhan et al., 1995), which influence receptor binding, do so indirectly, through structural perturbations within the monomer. It remains to be seen whether the dimeric form of these ligands has a unique functional role in the biology of IL-8 and other chemokines. Additional single-chain heterodimers may be useful for testing the possible effects of mutating either site-1 (i.e., the "ELR" region) or site-2 (see Lowman et al., 1996; Williams et al., 1996) in each subunit in the context of a stable, biologically active dimer, such as the d4 variant described here.

Materials and methods

Molecular modeling

Coordinates for the IL-8 monomer were taken from the X-ray structure (3il8; Baldwin et al., 1991); dimer coordinates were obtained by symmetry transformations based on the unit cell. Potential loops that could link the two monomers were evaluated using a model building approach. Peptide fragments were constructed based on the following sequence: ENSGG(S)$_n$AKELRCQC (n = 5–9). For each fragment, distance geometry was used to generate 20 conformations that: (a) superimposed the backbone atoms of the N-terminal ENS residues with the corresponding atoms of E70-S72 of monomer 1, (b) superimposed the C-terminal CQC backbone atoms with the C7-C9 backbone of monomer 2, and (c) contained no unfavorable contacts with the rest of the dimer. Each of these conformations was "spliced" into the X-ray structure of the dimer and refined by restrained molecular dynamics and minimization. The resulting conformations were evaluated using Ramachandran analysis.

A model of scIL8(T37C-S72′C) was constructed from the lowest energy conformer of the scIL8 dimer, where n = 7 (see above). The distance between the Sγ atoms of the modified side chains was minimized by adjusting the ψ and χ_1 angles of C72′. The structure was refined using restrained minimization in which all atoms of residues A35–I39 and E70′–C72′ were allowed to move. Minor changes in the backbone conformation (primarily in residues N71′ and C72′) were required in order to normalize the disulfide bond geometry.

Distance geometry calculations were performed using DGEOM (J.M. Blaney, G.M. Crippen, A. Dearing, and J.S. Dixon, DGEOM, *QCPE # 590*). Molecular dynamics simulations were conducted with DISCOVER (Molecular Simulations Inc., San Diego) using the 1991 AMBER forcefield (Weiner et al., 1984, 1986). In the restrained molecular dynamics calculations, all atoms derived from X-ray data were held rigid. Following an initial minimization of the distance geometry coordinates, each model was allowed to heat from 50 K to 700 K over a 70 ps interval, followed by 60 ps equilibration and subsequently cooled to 50 K over an additional 70 ps. The final structure was minimized using conjugate gradients to a maximum gradient of 0.001 kcal mol^{-1}. A linear dielectric of 4.0*r was used to partially compensate for the lack of explicit solvent.

Construction of IL-8 variants

Restriction enzymes, T4 DNA polymerase, and T4 DNA ligase (Gibco-BRL, Bethesda, MD; or New England Biolabs, Beverly, MA), were used according to the manufacturer's directions. An expression plasmid for periplasmic secretion of scIL8 from *E. coli* was produced through a series of restriction-digestion, ligation, and mutagenesis steps using standard techniques (Sambrook et al., 1989; Kunkel et al., 1991), beginning with a secretion plasmid for wild-type IL-8, called pAPSTII.IL8.72 (Hébert et al., 1991). The gene encoding the first subunit of IL-8 in the dimer was obtained by digesting pAPSTII.IL8.72 (5093 bp) with XbaI and EcoRI, and isolating the 299 bp DNA fragment, which contained the stII secretion-signal sequence followed by the IL-8 (residues 1–70) gene. For the second subunit of IL-8, another sample of pAPSTII.IL8.72 was digested with NsiI and blunt-ended at the codon for residue A2′ (i.e., the second codon of the second subunit). Next, this sample was digested with XbaI, and the 5008 bp DNA fragment, containing the gene for the second subunit, was isolated. The 299 bp XbaI-EcoRI fragment was then ligated to the 5008 bp blunt-XbaI fragment using a linker (encoding N71–S72 of the first subunit (underlined), and the GGSSSSSSG linker) that consisted of synthetic oligodeoxynucleotides 5′-AAT TCA GGT GGA TCC TCT TCT TCT TCT TCT GGC-3′ and 5′-GCC AGA AGA AGA AGA AGA GGA TCC ACC TG-3′, creating a new Bpu1102I. To facilitate mutagenesis and DNA sequencing, we assembled a plasmid containing ssDNA and dsDNA origins of replication by digesting this construct with XbaI and EcoRV to excise the entire stII-scIL8 fragment, which was ligated with the 4125 bp fragment from an XbaI/EcoRV digestion of pB0720, a derivative of pB0475 in which the BamHI sites have been destroyed using a linker consisting of synthetic oligodeoxynucleotides 5′-CGA TGG TAC CGA T-3′ and 5′-A TCG GTA CCA T-3′. This plasmid, designated pSCIL8, allows for preparation of single-stranded DNA (Kunkel et al., 1991), as well as periplasmic secretion of scIL8.

To facilitate the independent mutagenesis of each subunit within scIL8, a separate IL-8 expression plasmid was constructed by again digesting pB0720 with NsiI and EcoRV and isolating the large (4125 bp) fragment. Next, the IL-8 (single-subunit) gene was amplified from pAPSTII.IL8.72 by PCR using Taq polymerase (Perkin-Elmer Corp.) with primers containing NsiI and ClaI sites. After digestion with NsiI and ClaI, this fragment was ligated with the 4125 bp NsiI-EcoRV(blunt) pB0720 fragment, using a linker consisting of synthetic oligodeoxynucleotides 5′-CGA TGG TAC CGA T-3′ and 5′-A TCG GTA CCA T-3′, to yield pIL8. This construct allows for mutagenesis of each subunit of IL-8 independently, after which the dsDNA can be digested and recombined to reconstruct pSCIL8.

Site-directed mutants of scIL8 were produced by single-strand mutagenesis (Kunkel et al., 1991), and verified by double-strand DNA sequencing using Sequenase® (US Biochemical). For example, uracil-containing ssDNA template derived from the scIL8 construct was used to perform site-directed mutagenesis of T37C of the first IL-8 subunit (plasmid pIL8) and S72′C in plasmid pIL8′ (in the second subunit; prime indicates the second subunit sequence numbers). The mutagenesis of each subunit was thus car-

ried out independently. The conversion of the wild-type codon sequence to that of cysteine (TGC) was confirmed by DNA sequencing. The plasmid pIL8 containing the T37C mutation in the subunit adjacent to the signal peptide was digested with Bpu1102I and AflIII to remove the wild-type IL-8 subunit immediately following the peptide linker connecting the IL-8 monomers. The plasmid pIL8′ containing the S72′C mutation was also digested with the same enzymes to isolate the 2050 bp fragment carrying the second subunit, which was subsequently ligated to the 2633 bp fragment carrying the T37C mutation, to reconstitute a covalent single-chain dimer of IL-8 containing a putative disulfide bond between C37 of the first subunit and C72′ of the second.

A construct expressing IL-8(T37C/S72C) homodimers (i.e., without the linker peptide) was produced by digesting the scIL8(T37C-S72′C) construct with SstII, removing the second half of the first subunit and the first half of the second. The larger fragment from this digestion was isolated using agarose gel electrophoresis and re-ligated to produce this linkerless homodimer.

Mutation of the ELR motif of each subunit of IL-8 to AAA was accomplished by site-directed mutagenesis, using the scIL8(T37C-S72′C) construct as template, with the oligodeoxynucleotide 5′-T GCC TAT GCA TCA GCT AAA GCT GCA GCT TGT CAG TGC ATA-3′ for E4A/L5A/R6A (i.e., mutating the first subunit's ELR, underlined), or with the oligodeoxynucleotide 5′-TCT GGC TCA GCT AAA GCT GCA GCT TGT CAG TGC ATA-3′ for E4′A/L5′A/R6′A (mutating the second subunit's ELR), or both oligodeoxynucleotides (for the homodimer variant).

Protein purification

IL-8 variants were produced by secretion into the periplasmic space of *E. coli* using a plasmid encoding wild-type or mutant IL-8 genes under the control of the P_{phoA} promoter (Chang et al., 1987). Purification of IL-8 and single-chain dimer variants followed procedures described previously (Hébert et al., 1990; Lowman et al., 1996). Briefly, variants were purified chromatographically by NaCl gradients on an S-sepharose column (pH 6 buffer) followed by Pharmacia FPLC columns: Mono-S (pH 6; or phosphate buffer, pH 7.6), and then alkyl superose, using a reverse $(NH_4)_2SO_4$ gradient. In some cases, α2-macroglobulin was added to reduce proteolysis. Further purification was obtained by desalting and running the samples on Hitrap-heparin columns in 10 mM phosphate (pH 7.2) buffer, eluting with a linear gradient of 0–1 M NaCl. Each of the scIL-8 variants described here were eluted from Hi-trap heparin columns within a relatively narrow range of salt concentration of 0.47 to 0.57 M, as compared with 0.54 M NaCl for wild-type IL-8 (data not shown). Samples were desalted by gel filtration into PBS buffer (137 mM NaCl, 2.7 mM KCl, 7.9 mM Na_3PO_4, and 1.14 mM K_3PO_4, pH 7.2).

Size exclusion chromatography

IL-8 or variant samples (10 μM) were run in PBS buffer on a Pharmacia Sephadex-75 FPLC column at a flow-rate of 0.35 mL/min, with detection by optical density at 280 nm. The chromatograms were compared with those for molecular weight standards (Pharmacia): bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). Data were plotted as the logarithm of the molecular weight (MW) versus the volume (V) of elution at which a peak was observed. The fitted curve for the standards, $\log(MW) = 6.15 - (0.150)(V)$, with $R^2 = 0.985$), was used to calculate the apparent molecular weights of IL-8, scIL8, and variant d4, as 12.1 kDa, 25.9 kDa, and 16.9 kDa, respectively.

Analytical ultracentrifugation

Equilibrium sedimentation analysis was carried out in a Beckman XL-A analytical ultracentrifuge, in PBS buffer (see above) at 20 °C. Samples (0.11 mL) of IL-8 wild-type were run at 0.5 mg/mL and 0.1 mg/mL; samples of scIL8 and variant d4 were run at 0.5 mg/mL, 0.1 mg/mL, and 0.072 mg/mL. Optical density (280 or 230 nm) was used to measure concentration as a function of radial distance at 25,000–35,000 RPM, and data were fit to a single-species model or to a simple monomer-dimer model to obtain dimer dissociation constants (Johnson et al., 1981). Wild-type IL-8 shows K_d (dimer dissociation) of about 10 μM under these conditions (Burrows et al., 1994; Paolini et al., 1994; Lowman et al., 1997).

IL-8 receptor binding

Neutrophils were prepared from normal human donors as described (Leong et al., 1994). Binding assays were carried out on neutrophils, or on 293 cells expressing CXCR1 or CXCR2 receptor as described (Leong et al., 1994; Lowman et al., 1996). Binding to the DARC receptor on erythrocytes was performed as described (Hesselgesser et al., 1995). Cells were incubated with ^{125}I-labeled IL-8 (0.25–0.5 nM) and serial dilutions of cold competitor at 4 °C for one hour. Thereafter, cells were layered onto a sucrose solution and pelleted. The supernatant was removed and the cells counted on a gamma counter. The data was analyzed by Scatchard analysis (Munson & Rodbard, 1983) using the program Igor (WaveMetrics, Lake Oswego, OR) to determine the ratio of mutant IL-8 affinity to wild-type IL-8 affinity, $K_d(\text{mut})/K_d(\text{wt})$.

Neutrophil bioactivity assays

Neutrophils were prepared from human donors (Leong et al., 1994), labeled with 2 μM calcein-AM, washed, and resuspended in Hank's balanced salt solution without Ca^{2+} or Mg^{2+}. Chemotaxis (average of 4 wells ± SD) was measured in MBB96 Neuro Probe chambers, with incubation for 40 to 60 min at 37 °C in 5% CO_2. The number of neutrophils migrating in response to IL-8 variants or receptor antibodies was measured by fluorescence intensity (λ = 485 ± 20 nm for excitation, 530 ± 25 nm for emission).

For assays of neutrophil degranulation, neutrophils were prepared and assayed as described (Lowman et al., 1996), by measuring β-glucuronidase release in response to IL-8 or variants.

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