Exchanging Interleukin-8 and Melanoma Growth-stimulating Activity Receptor Binding Specificities*

(Received for publication, August 4, 1995, and in revised form, February 13, 1996)

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Interleukin-8 (IL-8), a CXC chemokine, is known to bring about chemotaxis and activation of neutrophils through high affinity binding to at least two distinct receptors, receptor-A and receptor-B. The IL-8 homolog melanoma growth stimulating activity (MGSA) is also active toward neutrophils. In contrast to IL-8, MGSA binds receptor-B with high affinity and binds receptor-A with a 400-fold lower affinity. Using the structure of IL-8 (Clore et al. (1990) Biochemistry, 29, 1689–1696; Baldwin et al. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 502-506) and the NMR-determined structure of MGSA (Fairbrother et al. (1994) J. Mol. Biol. 242, 252-270), we designed variants of both IL-8 and MGSA to investigate the basis of specificity for binding of these chemokines to the IL-8 receptors. The most outstanding structural difference between IL-8 and MGSA lies in the loop preceding the first β-strand. When the corresponding (shorter) loop from MGSA was swapped into IL-8, both receptor-A and receptor-B binding affinities were significantly (>300-fold) reduced. However, with additional mutations that affect packing interactions, an IL-8 variant specific for receptor-B binding was produced. Conversely, when the same loop from IL-8 was swapped into MGSA, receptor-B binding was maintained with only a 30-fold reduction in receptor-A affinity. Again, mutations affecting packing of the loop yielded a MGSA variant with high affinity for both receptors, like IL-8. Finally, we show, through point mutations in a monomeric IL-8 framework, that individual side chain substitutions can affect receptor specificity.

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1The abbreviations used are: IL-8, interleukin-8; Mes, 2-[N-morpholino]ethanesulfonic acid; MGSA, melanoma growth-stimulating activity; NAP-2, neutrophil-activating peptide-2; PF4, platelet factor-4.
residues are critical for binding to IL-8 receptor-A. The receptor binding properties of these mutants support the conclusion that the IL-8 N-loop region, residues 10–20 in IL-8, is significantly different, as illustrated for IL-8 and MGSA (see Fig. 2). The N-loop region of IL-8 contains a single-residue insertion relative to the sequences of MGSA and NAP-2 (see Fig. 1). Observation of this conformational difference has led to the suggestion that this region may be responsible for the receptor specificity differences observed between IL-8 and MGSA and NAP-2 (23, 25). More recent results in which the IL-8 receptor-A affinities of rabbit IL-8 and human IL-8 were interconverted by substitution of the rabbit residues His13 and Thr15 with the human residues Tyr13 and Lys15 and vice versa support this hypothesis and point to the possible importance of (human) IL-8 residues Tyr13 and Lys15 in the interaction with the IL-8 receptor-A (26).

In order to further test the hypothesis that conformational differences in the N-loop regions of IL-8 and MGSA are responsible for observed receptor specificity differences between these two CXC chemokines, we have designed mutants of IL-8 and MGSA in which the N-loop regions have been swapped. We also made point or double mutations in a monomeric variant of MGSA in which the N-loop regions have been described herein) are shown in boldface type.

**EXPERIMENTAL PROCEDURES**

**Materials—Wild-type IL-8 and MGSA were expressed and purified from Escherichia coli as described previously (27, 28).**

<sup>125</sup>l-Labeled IL-8 was obtained from New England Nuclear or prepared as described (27) with an average specific activity of 2000 Ci/mmol. Human 293 cells, stably transfected with IL-8 receptor-A (2) or IL-8 receptor-B (3, 5), were kindly provided by Dr. William Wood (Genentech, Inc.). Restriction enzymes from New England Biolabs or Life Technologies, Inc. and T7 polymerase and Sequenase® from U.S. Biochemical Corp. were used according to the manufacturers’ directions.

**Mutagenesis of IL-8 and MGSA—IL-8 variants were constructed from a starting plasmid, pPS0170, derived from phGHam-g3 (29), which contains a gene encoding IL-8 under control of the alkaline phosphatase promoter (P<sub>phoA</sub>), with an stI signal sequence for secretion into the periplasmic space of E. coli (30). MGSA variants were constructed from a similar starting plasmid pMG34 (28) or from another phGHam-g3 derivative, plasmid pH1817, which contains a gene encoding MGSA variant M1, also under control of P<sub>phoA</sub> with an stI signal sequence for periplasmic secretion (30). Each of the plasmids designed for mutagenesis also contained phage M13 origins of single-stranded DNA templates for mutagenesis and sequencing. Site-directed mutants (see Tables II and III) were produced by single-strand mutagenesis (31) using synthetic oligonucleotides containing the desired changes) in codons and verified by Sequenase® sequencing (U. S. Biochemical Corp.).

**Protein Purification—** Proteins were secreted from E. coli in 25-1000-m l shake flask cultures using minimal phosphate medium for induction of the P<sub>phoA</sub> promoter (30). Following induction for 20–24 h, cells were pelleted, frozen at −20 or −80 °C, and then thawed and resuspended in 10 mM Tris, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, pH 7.6. After 30 min on ice, the cells were again pelleted and resuspended in 300 mM NaCl, 22% sucrose, 10 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 45 mM Tris, pH 8.0. In some cases, 1–10 μg/ml (of total protein) v2-macroglobulin (Boehringer Mannheim) was added here or subsequently to further reduce proteolysis. After removal of nucleic acid by polyethyleneimine precipitation (0.25% final polyethyleneimine) or by passing over a DEAE-Sepharose column, the IL-8 and MGSA variants were purified chromatographically by NaCl gradient on an S-Sepharose column (Mes pH 6 buffer) followed by Pharmacia columns: Hi-trap heparin and then fast protein liquid chromatography Mono-S (in 10 mM Mes pH 6.0 buffer or 10 mM phosphate buffer, pH 7.6). Some mutants were further purified by hydrophobic interaction chromatography with a Pharmacia alkyl-superose column, using a reverse (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> gradient (60–0% saturation) in phosphate buffer. Concentrations were determined by amino acid analysis, by BCA assay (Pierce), or by Coomassie-stained SDS-polyacrylamide gel electrophoresis with reference to an IL-8 and MGSA amino acid analyzed standard. In some cases, all three methods were used, and the results showed agreement to within 10% of the concentration determined by amino acid analysis. Mass spectrometry was used to confirm the identity and the full-length integrity of a number of the IL-8 or MGSA variants. Samples were loaded onto a high pressure liquid chromatography system and subjected to electrospray ionization on a PE-SCiEX API III mass spectrometer.

**Binding Assays—** Stably transfected 293 cells, expressing either the IL-8 receptor-A or receptor-B, were maintained in 50:50 Ham’s F-12/Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics, with 0.5–1.0 mg/ml Geneticin (Life Technologies, Inc.) for selection, with splitting twice weekly. Cells were harvested from 150-mm tissue culture plates, washed with phosphate-buffered saline, and resuspended in ice-cold binding buffer A (0.5% bovine serum albumin (Sigma), 140 mM NaCl, 25 mM HEPES, 0.75 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.1) or buffer B (0.5% bovine serum albumin, Hanks' balanced salt solution

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with phenol red (Bio-Whittaker #10-543F)). 125I-Labeled IL-8 (0.25 nM) and suspended at a concentration of 1 x 10^7 cells/ml in assay buffer, 170 μl each of sample (25 μl) were pipetted onto 96-well microplates, and 170 μl/g/ml cytochalasin B (Sigma), 100 mM-glucuronidase activity, the method described by Dewald and Baggiolini (35) was adapted to 96-well microplates. An aliquot of sample (25 μl) was mixed with an equal volume of Hanks’ balanced salt solution (Life Technologies, Inc.) supplemented with 1% bovine serum albumin (fraction V, Sigma), 2 mM NaHCO3, and 0.01 mM HEPES, pH 7.2. Recombinant IL-8, MGSA, and mutant forms of the molecules were serially diluted in assay buffer, and aliquots were added to sterile polypropylene 96-well plates (Costar, Cambridge, MA) such that the final concentration was 1 x 10^7 cells/ml. After incubation for 1 h at 4°C, the cell suspension was layered onto 0.75 ml of an ice-cold sucrose solution (0.4% bovine serum albumin, 20% sucrose, 140 mM NaCl, 40 mM Tris, pH 7.6), and the cells were pelleted at 1500 g for 10 min at 4°C in a Sorvall RC-3B centrifuge. The upper aqueous layer and the sucrose layer were removed in two steps, and the cell pellet counted in an Isodata 120 gamma counter. The binding data were analyzed by Scatchard analysis using a 1:1 binding model with the computer program IGOR (WaveMetrics, Lake Oswego, OR) to obtain Kd.

Enzyme Release Assays—Blood was drawn from normal male donors into heparinized syringes. Neutrophils were isolated as described (33) and suspended at a concentration of 1 x 10^7 cells/ml in assay buffer, consisting of Hanks’ balanced salt solution (Life Technologies, Inc.) supplemented with 1% bovine serum albumin (fraction V, Sigma), 2 mM glucose, 4.2 mM NaHCO3, and 0.01 mM HEPES, pH 7.2. Recombinant IL-8, MGSA, and mutant forms of the molecules were serially diluted in assay buffer, and aliquots were added to sterile polystyrene 96-well plates (Costar, Cambridge, MA) such that the final concentrations ranged between 0.01 and 1000 nM. After stimulation for 15 min at 37°C with 5 μM cytochalasin B (Sigma), 100 μl of the neutrophil suspension was added to each sample well, and the plates were incubated for 3 h at room temperature. The neutrophils were pelleted by centrifuging the plates at 500 x g for 7 min, and the cell-free supernatants were stored at 4°C until analyzed for elastase and β-glucuronidase activity.

Elastase activity was assessed using methoxy succinyl-alanyl-alanyl-prolyl-valyl-p-nitroanilide (Calbiochem, La Jolla, CA) as a substrate (34). The substrate was prepared as a 10 mM stock solution in Me2SO and stored at 4°C, and a working solution was prepared just prior to use (1.2 mM elastase substrate, 1.2 mM NaCl, 0.12 mM HEPES, pH 7.2). Samples (30 μl) were pipetted onto 96-well microplates, and 170 μl of the substrate working solution was added to each sample-containing well. The plates were placed in a 37°C tissue culture incubator for approximately 2 h. Absorbance was measured at 405 nm using a microplate reader (SLT Lab Instruments, Research Triangle Park, NC).

For measuring β-glucuronidase activity, the method described by Dewald and Baggiolini (35) was adapted to 96-well microplates. An aliquot of sample (25 μl) was mixed with an equal volume of Hanks’ balanced salt solution + 0.1% Triton X-100 in 96-well plates. Fifty microliters of substrate (10 mM 4-methylumbelliferyl-β-D-glucuronide (Calbiochem) in 0.1 mM sodium acetate, pH 4.0, 0.1% Triton X-100) was then added, and the plates were incubated in a 37°C tissue culture incubator for 90 min. The reaction was stopped by diluting 18 μl of each reaction mixture with 290 μl/well stop solution (0.05 mM glycine, 5 mM EDTA). The plates were then read in a microplate fluorometer (Cambridge Technology, Inc., Watertown, MA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

RESULTS AND DISCUSSION

Verification of Mutants—In general we found proteolysis of expressed variants from E. coli was negligible with the addition of protease inhibitors in the purification (see “Experimental Procedures”), but because the receptor binding affinity of IL-8 is sensitive to modifications at or near the amino terminus (11), it is useful to confirm by mass spectrometry that variants have not been proteolyzed. The oxidized masses (atomic mass units) of many variants were measured and found to agree with the predicted masses (Table I). Generally, protein stocks were stable for months in phosphate-buffered saline buffer at 4°C as judged by mass spectrometry.

Design of Loop Swap Variants—Superposition of the IL-8 and MGSA structures (see Fig. 2) illustrates a significant difference between these structures in the loop preceding the first β-strand, the N-loop, which is shorter by one residue in MGSA relative to IL-8 (Fig. 1). This region includes IL-8 residues 13–17, and the corresponding MGSA loop residues 15–18. We originally hypothesized that differences in this region might be responsible for the high affinity IL-8 binding and low affinity MGSA binding to IL-8 receptor A (23). If this were the case, then in the absence of any intramolecular packing or other secondary effects, exchange of a segment from the N-loop region of MGSA into IL-8 might be expected to result in loss of its high affinity receptor A binding while retaining high affinity receptor B binding. Conversely, swapping the corresponding segment from wild-type IL-8 into MGSA should confer the MGSA variant with high affinity receptor A binding activity.

Two variants of IL-8 and two variants of MGSA were initially designed to test the receptor specificity effects of mutations in the N-loop region. Seeking to make a minimal change to alter receptor specificity, we first constructed IL-8 variant “11” (see Fig. 3 and Table II for variant designations), which comprised wild-type IL-8 with the substitution of four MGSA residues Leu15, Glu16, Gln17-Leu18 for the five IL-8 residues Tyr13, Ser14, Leu15, Pro16-Phe17. The MGSA variant “M1” substituted IL-8 residues Tyr13-Ser14-Lys15-Pro16-Phe17 for the five IL-8 residues Tyr13, Ser14, Gln17-Leu18. The experimental uncertainty is ~0.01. Theoretical masses were calculated using the expected amino acid composition.

### Table I

| Variant | Theoretical mass | Experimental Mass |
|---------|----------------|-----------------|
| Loop swap variants<sup>a</sup> | | |
| I1       | 8170.6          | 8170.0          |
| I2       | 8170.5          | 8170.0          |
| I3       | 8128.4          | 8127.7          |
| I4       | 8128.4          | 8127.3          |
| I5       | 8339.7          | 8339.8          |
| I6       | 8127.5          | 8127.0          |
| I7       | 8072.4          | 8071.4          |
| M1       | 8072.4          | 8071.8          |
| M2       | 8114.5          | 8114.1          |
| M3       | 7903.3          | 7903.0          |
| IL-8 monomer IV point mutants<sup>b</sup> | | |
| IL-8 monomer IV (Q8A) | 8528.8 | 8528.2 |
| IL-8 monomer IV (T12A) | 8555.9 | 8553.3 |
| IL-8 monomer IV (Y13E) | 8551.8 | 8552.3 |
| IL-8 monomer IV (K20A) | 8528.8 | 8528.5 |
| IL-8 monomer IV (F21E) | 8567.8 | 8567.9 |
| IL-8 monomer IV (L43R) | 8628.9 | 8629.4 |
| IL-8 monomer IV (S44R) | 8655.0 | 8655.1 |
| IL-8 monomer IV (E48K) | 8584.9 | 8584.9 |
| IL-8 monomer IV (V61K) | 8614.9 | 8615.0 |

<sup>a</sup> Loop swap variants are defined in Table II.

<sup>b</sup> IL-8 monomer IV point mutants are defined in Table III.

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Mass spectral analysis for IL-8 and MGSA variants

Masses for IL-8 and MGSA variants were measured using electrospray mass spectrometry. The experimental uncertainty is ~0.01. Theoretical masses were calculated using the expected amino acid composition. The experimental uncertainty is ~0.01. Theoretical masses were calculated using the expected amino acid composition. The experimental uncertainty is ~0.01. Theoretical masses were calculated using the expected amino acid composition.
Leu15-Gln16-Gly17-Ile18 in the background of wild-type MGSA. In competition binding assays with 125I-labeled wild-type IL-8 on stably expressing 293 cells, variant I1 was reduced in receptor-A affinity by a factor of at least 200-fold (Table II), as predicted, based upon the MGSA affinity. However, variant I1 was also reduced in receptor-B affinity, by a factor of 23-fold compared with wild-type IL-8 (Table II). Because both wild-type IL-8 and wild-type MGSA are able to bind receptor-B with similar high affinities, swapping this loop would not be expected to affect receptor-B affinity if the loop segments are acting as structurally and functionally independent modules. The M1 variant suffered a small loss in receptor-B affinity, but instead of gaining affinity toward receptor-A, it showed little or no improvement.

Reasoning that the “short loop” swaps contained in variants I1 and M1 might not be sufficient to act independently as receptor specificity determinants, we next tested swaps of a longer segment of this loop. Variant I2 consisted of the I1 sequence, with the additional substitutions of MGSA residues Leu12-Gln13 for IL-8 residues Ile10-Lys11. Binding assays on variant I2, however, showed that the additional substitutions not only failed to improve receptor binding but actually decreased further binding affinity of the IL-8 variant to receptor-B by about 20-fold compared with variant I1 (Table II). The reverse substitutions, IL-8 residues Ile10-Lys11 for MGSA residues Leu12-Gln13, were made in the context of variant M1 to obtain the MGSA variant M2. In contrast to variant I2, variant M2 had IL-8-like high affinity for receptor-B. However, although improved in receptor-A affinity (by at least 7-fold versus variant M1), it was still weaker by 27-fold than wild-

![Structural alignment of IL-8 and MGSA monomers.](http://www.jbc.org/Downloadedfrom)
type IL-8.

Role of Packing Residues—The structural overlap of IL-8 and MGSA sequences adjacent to the N-loop suggested that no further benefits would be obtained by extending the linear segment on either side of the segment described above. However, examination of the packing interactions between the N-loop and other residues to the interior of IL-8 or MGSA indicated differences in the underlying packing of the loops (Fig. 2B).

In particular, the side chain of Leu$^{49}$ in IL-8 packs into the “bulge” in the longer IL-8 loop, making contacts with the side chains of Tyr$^{13}$ and Phe$^{17}$. The analogous position in MGSA is occupied by an alanine (Ala$^{50}$), which is closely packed with the side chain of Leu$^{15}$. The side chain of Phe$^{21}$ in IL-8 also packs with the side chain of Phe$^{17}$. In MGSA, the analogous position is occupied by Asn$^{22}$, which makes contact with the corresponding loop residue, Ile$^{18}$. On the other side of the loop, Trp$^{57}$ in IL-8 packs against Pro$^{35}$, whereas Ile$^{28}$ in MGSA is proximal to Gly$^{17}$. The contributions of packing interactions involving Phe$^{21}$ and Leu$^{49}$ in IL-8 were tested by making point mutations in the context of the IL-8 or MGSA direct loop swap variants described above. The substitution N22F improved receptor-A affinity of the short (M1) and the long (M2) loop swap variants in MGSA by more than ~4-fold and 2-fold, respectively (Table II). Similarly, receptor-B affinity of M1 was improved by 10-fold and that of M2 was improved by 5-fold with the N22F mutation. The A50L mutation had only a small effect on receptor-A affinity, increasing M1 by 2.5-fold and reducing M2 by about 2-fold. In contrast, receptor-A affinity was improved by more than 20-fold when the A50L mutation was incorporated into M1 (variant M4) and by about 30-fold when the A50L mutation was included in M2 (variant M6) (Table II). Variant M6, an MGSA construct containing IL-8 residues Ile$^{10}$-Lys$^{11}$, Tyr$^{13}$-Ser$^{14}$-Lys$^{15}$-Pro$^{16}$-Phe$^{17}$, and Leu$^{29}$ (Fig. 3), binds both receptor-A and receptor-B with affinities indistinguishable from wild-type IL-8. Thus, in variant M6 the specificity determinants of IL-8 have been successfully swapped into the MGSA framework.

The receptor-B binding affinity of IL-8 variant I1, containing the short version of the MGSA N-loop, was also improved (about 2-fold) to within 3-fold of wild-type MGSA by the substitution L49A (variant I3). Binding to receptor-A remained weak, about 300-fold weaker than wild-type IL-8 (Table II).

### Table II

| Variant | Protein background | Mutations | Kd (mutant) | Kd (IL-8) | Specificity |
|---------|-------------------|-----------|-------------|-----------|-------------|
| **IL-8** |                   |           |             |           |             |
| I1      | IL-8              | LQGI      | >600        | >260      | 1.2         |
| I2      | I1                | I10L/K11Q | >2300       | >1000     | 27          |
| I3      | I1                | L49A      | 700±160     | 300       | 8           |
| I4      | I2                | L49A      | 220±52      | 94        | 6           |
| I5      | IL-8              | L49A      | 8.2±28     | 3.6       | 14         |
| I6      | IL-8              | P16G      | 11±3       | 5.0       | 6           |
| I7      | I3                | E48K      | >700        | >300      | 1.6         |
| **MGSA** |                   |           |             |           |             |
| M1      | MGSA              | LQGI      | >460        | >200      | 2.1         |
| M2      | M1                | L12I/Q13K | >6          | 27        | 1.5         |
| M3      | M1                | N22F      | 128±28      | 56        | 3.5         |
| M4      | M1                | A50L      | 19±1       | 8.2       | 1.6         |
| M5      | M2                | N22F      | 29±11      | 13        | 0.7         |
| M6      | M2                | A50L      | 21±0.5     | 0.9       | 1.2         |
| M7      | MGSA              | A50L      | 93±4       | 40        | 1.4         |

*Values represent means of two or more curves (mean ± S.D.).
Thus in variant I3, an IL-8 construct with MGSA residues Leu15-Gln16-Gly17-Ile18 and Ala30, the specificity determinants of MGSA have been swapped into the IL-8 framework, yielding a molecule that binds receptor-B with \( K_d \approx 8 \text{ nM} \) and receptor-A with \( K_d \approx 700 \text{ nM} \). The substitution L49A in the longer loop swap variant of IL-8, 12, improved receptor-A binding but weakened receptor-B binding, yielding a molecule, I4, with receptor specificity \( K_d(rA)/K_d(rB) \) similar to the parental wild-type IL-8 (Table II). The reason for this difference in the effect of the L49A mutation in the context of the longer MGSA loop is not clear.

The successful exchange of receptor specificities by incorporation of the A50L mutation in the background of MGSA variant M2 and of the L49A mutation in the background of IL-8 variant I1 suggests a critical role for Leu49 in binding to IL-8 to receptor-A. Analysis of the native IL-8 structure (Fig. 2B) indicates the Leu49 may be required for packing of the N-loop residues in a conformation optimal for receptor-A binding. In order to test that Leu49 is not solely responsible for the observed specificity differences, we constructed and tested point mutants of this residue in the background of the wild-type proteins, IL-8(L49A) (variant I5) and MGSA(A50L) (variant M7) (Table II). IL-8 variant I5 reduced receptor-A binding by only 4-fold and had a negligible effect on receptor-B binding relative to wild-type IL-8, whereas MGSA variant M7 resulted in a 4- to 6-fold improvement in binding to both receptors relative to wild-type MGSA. The modest loss of receptor-A affinity for variant I5 is likely due to the loss of packing interactions between Leu49 and Tyr13 in IL-8. Nevertheless, the resulting receptor specificities for the variants I5 and M7 do not differ greatly from the respective wild-type proteins, indicating that IL-8 residue Leu49 is not the sole receptor specificity determinant. In addition, the effects of these mutations in the background of the wild-type proteins differ somewhat from the equivalent mutations in the loop swap variants (i.e. M6 to M2 is not equivalent with IL-8 to I5 and I3 to I1 is not equivalent with MGSA to M7). Such discordance is most likely due to other packing interactions that differ between the different background proteins. For instance, the conformation of the MGSA N-loop is unlikely to be maintained in the context of the IL-8 background (e.g. I1) due to steric overlap with the side chain of Trp57 (Fig. 2B; in MGSA and variant M7 this residue is Ile). In the case of the loop swap variants, however, exchanging Leu49 and Ala30 appears sufficient to optimize the conformations of the transposed N-loops to give complete exchange of receptor specificities.

Within the N-loop region, as noted above, there is an insertion of one residue in IL-8 relative to MGSA. In the alignment shown in Fig. 1, this may be interpreted as a substitution of a single residue (Gly) in MGSA for the two residues Lys15 and Pro16 in IL-8. This interpretation is supported by superposition of the IL-8 and MGSA structures (Fig. 2B). Because proline imposes a severe conformational constraint on the peptide backbone, it might be supposed that this substitution could contribute to the observed receptor specificities. Clark-Lewis et al. (16) reported that P16G and Lys15-Pro16-Gly caused a 1.7- and a 40-fold decrease, respectively, in IL-8 binding to neutrophils but did not report receptor-A/B specificities. We constructed P16G for specificity testing in 293 cell binding assays. The mutation P16G had little effect (1.6-fold decrease) on receptor-B affinity and caused a modest 5-fold decrease in receptor-A affinity (Table II).

Point Mutations in Monomeric IL-8—We also tested point mutations in IL-8 for specificity effects in receptor-A versus receptor-B binding. Although a number of IL-8 mutants have been described (10–13, 16), the binding and activity of these variants generally have been described in terms of assays on neutrophils, rather than in terms of their specific receptor-A and receptor-B affinities.

We chose to make point mutations in the background of an engineered “monomeric” IL-8 variant known to have a weak dimerization constant, \( K_d = -2 \text{ nM} \). A monomeric analog of IL-8, in which the amide nitrogen of Leu25 was methylated to selectively block formation of hydrogen bonds between monomer subunits, has previously been shown to be fully functional in vitro (36), despite the fact that wild-type IL-8 forms dimers under the conditions used for structural studies (21, 22). Indeed, recent investigations of the IL-8 monomer-dimer equilibrium have shown a dimer dissociation constant of \( -20 \mu\text{M} \) (37, 38); thus, at the solution concentrations typically used for in vitro assays (<1 \mu M), IL-8 exists substantially as a monomer. Nevertheless, cross-linking of both monomer and dimer forms of IL-8 to receptor has been observed (39). We therefore chose to use the monomeric form for point mutations, in order to avoid any ambiguity about the dimerization state. The monomeric IL-8 variant selected for the present study, E24R/I28R/T37E/F65H/L66E/A69E/S72E, referred to as monomer version IV, 2 binds both receptors with similar affinities and specificity as wild-type IL-8 (Table III).

The NH2-terminal domains of the IL-8 receptors have been implicated in the binding interaction with IL-8 and have been shown to play a role in determining chemokine specificity (6, 18–20, 26). As an initial screen for specificity variants of IL-8, we chose to mutate residues that could be predicted to interact with the NH2-terminal regions of the receptors. Possible interacting residues had been identified previously by observation of specific IL-8 NMR chemical shift perturbations following formation of a complex between 15N-labeled IL-8 and a peptide comprising the 40 NH2-terminal residues of IL-8 receptor-A (40). In particular, chemical shift changes were observed for residues Gln8, Thr12, Lys15, Phe17, His18, Lys20, Phe21, Ser44, Glu48, Leu49, Cys50, and Val61. Of these, Phe17, Leu49, and Cys50 have the lowest degree of side chain exposure to solvent. We chose not to mutate Phe17 or Leu49 but note that a F17L mutation had no effect on neutrophil binding (16) and that Leu49 was required to maintain optimal packing interactions in the MGSA loop swap variant M6 (discussed above) and was also required to improve IP10 activity to near that of IL-8 (15). The mutation L49A in the background of wild-type IL-8 had only a small effect on receptor binding (see discussion above; Table II). Removal of the disulfide bond, Cys5-Cys50, by substitution with \( \alpha \)-aminobutyric acid has been shown to greatly reduce binding (by 8000-fold) to neutrophils (16). We mutated both of these cysteines to Ala or Ser and found similar decreases in affinity; no differences in specificity could be determined (data not shown). The results of these changes are very likely indirect and due to long range disruptive effects on the protein structure; the precise role of the Cys side chains is therefore difficult to establish.

The remaining residues described above were mutated in the context of monomer-IV, and each was tested for binding affinity on IL-8 receptors-A and -B. In general, alanine was chosen to substitute charged side chains because charge-to-Ala mutagenesis brings about a large side chain change (both steric and charge) with limited possibilities for disruption of protein fold-
though the overall specificity was not significantly changed.

(Fig. 1). Because both rabbit and human IL-8 bind receptor-B, only residues 13 and 15 differ between human and rabbit IL-8. Convert the MGSA receptorspecificity to that of IL-8 (Table II), binding to the same high affinity binding as the human protein from human IL-8, converted the rabbit low affinity receptor-A into a receptor-B with specificity to receptor-B 200-fold. The converse experiment, in which the rabbit IL-8 residues from rabbit IL-8 (His13 and Thr15) results in a 200-fold reduction in the binding affinity for IL-8 receptor-A, making it equivalent to wild-type rabbit IL-8 for binding to this receptor. The converse experiment, in which the rabbit IL-8 residues His13 and Thr15 were replaced with the corresponding residues from human IL-8, converted the rabbit low affinity receptor-A binding to the same high affinity binding as the human protein (26). Affinities of these mutants for IL-8 receptor-B were not reported, but we note that of the IL-8 residues required to convert the MGSA receptor specificity to that of IL-8 (Table II), only residues 13 and 15 differ between human and rabbit IL-8 (Fig. 1). Because both rabbit and human IL-8 bind receptor-B with high affinity, it might be reasonable to expect that the mutant proteins will also. The double Al a mutant Y13A/K15A, however, reduced binding to both receptors by 10-20-fold, although the overall specificity was not significantly changed.

The variant E48K is noteworthy because it also significantly changed the specificity of IL-8. In contrast to Y13E, however, receptor-A binding was only slightly reduced, whereas receptor-B binding was enhanced. The improvement in receptor-B binding seen for IL-8 E48K is consistent with the results of a previous mutagenesis study, which suggested that Lys49 of MGSA forms a favorable interaction with receptor-B; the substitution K49A in MGSA caused a significant reduction in receptor-B binding affinity (15). Interestingly, the side chain of Lys49 points away from the N-loop region in MGSA. These results prompted us to introduce the E48K mutation in the background of IL-8 variant I3 (Table I) in an attempt to attain full MGSA-like receptor-B binding affinity. As predicted, the receptor-B binding affinity of variant 17 improved (95-fold) relative to I3 and is identical, within the experimental uncertainty, to that of wild-type MGSA (Table II).

In contrast to E48K, the mutation H18A lessened the specificity toward receptor-B due to a 7-fold reduction in affinity compared with only a 2-fold reduction for receptor-A binding. The results for IL-8 H18A on receptor-B are also reminiscent of those found upon substitution of the corresponding residue in MGSA, where the mutant H19A was found to reduce receptor-B binding by 13-fold (15). Effects were less dramatic at other sites in the IL-8 monomer, but K20A, F21E, and L43R each reduced both receptor-A and receptor-B binding by about 10-fold or more, suggesting that these residues come into contact with both types of receptor or that structural perturbations resulting from these substitutions affect the binding to both receptors.

Substitutions at Thr12, Ser44, and Val61 had less than 10-fold effects on receptor-A binding and little effect on specificity. This is rather convincing evidence that these side chains are not required for receptor binding, especially because some of the mutations involve drastic changes of the steric and hydrophobic properties (e.g., S44R and V61K).

Overall, the results of these point mutations in the context of an engineered IL-8 monomer are consistent with the results described above for IL-8/MGSA loop swap variants and the variant of rabbit/human IL-8 described by Schraufstätter et al. (26). However, we have found some significant effects on receptor-B as well as receptor-A binding upon mutation of certain residues (for example, Y13E and F21E), suggesting that receptor-B contacts may also occur in this region.

| Protein | Mutations | Receptor-A | Receptor-B | Specificity |
|---------|-----------|------------|------------|-------------|
|         |           | Kd       | Kd (mutant) | Kd (monomer IV) | Kd (mutant) | Kd (monomer IV) | Kd (receptor-A) | Kd (receptor-B) |
| IL-8    |           | 2.3 ± 1.5 | 0.16       |               | 0.84 ± 0.42 | 0.22          |               | 2.7          |
| monomer IV (see legend) | 15 ± 2 | 1 |               |               | 3.9 ± 1.4 | 1 |               | 3.8          |
| monomer IV QA | 11 ± 6 | 0.71 |               |               | 2.9 ± 1 | 0.73 |               | 3.8          |
| monomer IV T12A | 30 ± 4 | 2.0 |               |               | 3.1 ± 0.7 | 0.81 |               | 9.7          |
| monomer IV Y13E | 2300 ± 1010 | 150 |               |               | 170 ± 27 | 43 |               | 14           |
| monomer IV Y13A/K15A | 160 ± 40 | 11 |               |               | 87 ± 29 | 22 |               | 1.8          |
| monomer IV H18A | 34 ± 8 | 2.3 |               |               | 28 ± 11 | 7.1 |               | 1.2          |
| monomer IV K20A | 140 ± 38 | 9.3 |               |               | 73 ± 12 | 19 |               | 1.9          |
| monomer IV F21E | 560 ± 10 | 37 |               |               | 250 ± 90 | 64 |               | 2.2          |
| monomer IV L43R | 390 ± 120 | 26 |               |               | 220 ± 50 | 56 |               | 1.8          |
| monomer IV S44R | 15 ± 0.2 | 0.98 |               |               | 7.6 ± 1.6 | 2.0 |               | 2.0          |
| monomer IV E48K | 26 ± 2 | 1.8 |               |               | 1.6 ± 0.3 | 0.41 |               | 16           |
| monomer IV V61K | 26 ± 3 | 1.8 |               |               | 17 ± 2 | 4.3 |               | 1.5          |

* Values represent means of two or more curves (mean ± S.D.).
Bioactivity of Specificity Variants—In order to confirm that the engineered variants of IL-8 were biologically active, some of the variants produced here were tested for neutrophil degranulation by measuring either elastase or β-glucuronidase release (Fig. 4). The EC_{50} for activation in these assays correlated well with K_{d} of binding. Some differences were observed, however, in the maximal response seen at high doses (1 μM) of certain variants. Comparison of IL-8 and MGSA activities on neutrophils shows that MGSA has a higher EC_{50} than IL-8 for neutrophil degranulation (Fig. 4). This is most easily understood by considering the higher affinity of IL-8 for receptor-B, compared with a 3-fold lower affinity of MGSA for the same receptor. MGSA binds receptor-B but not receptor-A with high affinity, whereas IL-8 binds both receptors with high (K_{d} < 3 nM) affinity. The fact that both receptors are generally present and can signal on neutrophils may explain the approximately 3-fold higher plateau of response with IL-8 compared with MGSA. Similarly, when the MGSA loop was swapped into IL-8, yielding a molecule with MGSA-like specificity (I3), the EC_{50} shifted, and the plateau fell to a level comparable with that of MGSA itself (Fig. 4).

One might expect that the MGSA variant recruited to bind with IL-8 affinity and specificity (M6) might behave conversely, because its affinity for both receptors was found to be indistinguishable from that of wild-type IL-8 (Table II). Although the EC_{50} of this variant improved relative to wild-type MGSA, the maximal degranulation response was only about 50% of wild-type IL-8 (Fig. 4). The reason for this lower plateau is not clear. Unknown receptor type(s), which bind IL-8 but not MGSA and certain other variants, may exist on neutrophils. If this were true, then the higher plateau of activity for IL-8 might be explained by its binding to three (or more) classes of receptor as compared with binding of the IL-8 or MGSA variants reported here through two and with binding of wild-type MGSA to only one.

Conclusions—By measuring separately the binding affinities of IL-8 and MGSA variants for IL-8 receptors-A and -B, we have shown that the N-loop regions of IL-8 and MGSA are responsible for the receptor specificity difference between these chemokines. An IL-8 variant specific for receptor-B binding (I3) was produced by substitution of the one-residue shorter loop from MGSA, along with mutations of a packing residue, L49A, into IL-8. Conversely, when the N-loop from IL-8 was swapped into MGSA, high affinity receptor-B binding was maintained and including A50L "rescued" this variant (M6), yielding both receptor-A and receptor-B affinities within 2-fold of wild-type IL-8. Both of these variants are biologically active in degranulation assays on human neutrophils. Point mutations in the background of a monomeric IL-8 show the functional importance of certain side chains in specificity determination. These residues form a subset of those for which NMR chemical shift data suggested interaction with an NH_{2}-terminal peptide from IL-8 receptor-A (40).

The conformation of the N-loop is critical for receptor-A binding, based upon the need for Leu^{49} (which packs with N-loop residues Tyr^{13} and Phe^{17}) in the MGSA variant M6 and for Ala^{50} in the IL-8 variant I3. Single substitutions within the loop often have rather small effects (Table III and Ref. 16). One likely explanation is that main chain atoms within the N-loop may be important as direct contacts to receptor. The three-dimensional arrangement of these atoms would in turn be affected by the multiple mutations between IL-8 and MGSA in this region. Improvement in neutrophil binding was demon-
strated when the ELR motif was substituted at the NH₂ terminus of PF4 (13); however, the receptor specificity of the chimeric protein ELR-PF4 was not reported. Because the IL-8 N-loop residues are critical for binding to receptor-A and PF4 has a short N-loop like MGSA (Fig. 1), we predict that the neutrophil activity reported for ELR-PF4 results from interactions with receptor-B and that binding of this protein to receptor-A is weak.

Combination of the above results with the observation of receptor NH₂-terminal peptide interactions at or near the N-loop suggests strongly that this region of IL-8 may be a secondary binding site distinct from the ELR motif. We speculate that there is a one-to-one interaction of this secondary site on IL-8 with a portion of the receptor NH₂-terminal domain and that the ELR residues interact with a region of charged residues identified in receptor extracellular loops 3 and 4 (20). From the recruitment of rabbit IL-8 to bind human receptor-A it was suggested that the NH₂ terminus of receptor-A but not receptor-B may contact the N-loop area of IL-8, including residues Tyr¹³ and Lys¹⁵ (26). However, our recruitment experiments with MGSA showed that disruption of the packing interactions of this loop could decrease both receptor-A and receptor-B binding affinity, suggesting that receptor-B also contacts IL-8 in this region. This does not, however, exclude the possibility of other receptor-B contacts on IL-8 or MGSA. For example, in the IL-8 E48K variant the introduced lysine likely represents a contact residue for receptor-B but not receptor-A.

Such variants reveal the importance of a structurally independent site on IL-8 distal to the ELR residues that serves as a specificity determinant for IL-8 receptor-A/receptor-B binding and may be useful as probes of receptor specific activities of IL-8 and MGSA.

Acknowledgments—We thank Daniel Yansura for MGSA plasmids and construction of the M1 and M2 variants; Alan Padua and Bill Henzel for amino acid analysis; Mark Vassar, Peter Ng, and Parkash Jhumani for synthetic oligonucleotides; and Joshua Theaker for modifications to the program ALIGN.

Note Added in Proof—In a recent publication, Williams and co-workers (42) demonstrated using NMR spectroscopy that the mutation L49A in the background of wild-type IL-8 induces some structural cations to the program ALIGN.
