Receptor Recognition and Specificity of Interleukin-8 Is Determined by Residues That Cluster Near a Surface-accessible Hydrophobic Pocket

(Received for publication, October 20, 1995)

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To determine the regions of interleukin-8 (IL-8) that allow high affinity and interleukin-8 receptor type 1 (IL8R1)-specific binding of chemokines, we produced chimeric proteins containing structural domains from IL-8, which binds to both IL8R1 and interleukin-8 receptor type 2 (IL8R2) with high affinity, and from GROγ, which does not bind to IL8R1 and binds to IL8R2 with reduced affinity. Receptor binding activity was tested by competition of 125I-IL-8 binding to recombinant IL8R1 and IL8R2 cell lines. Substitution into IL-8 of the GROγ sequences corresponding to either the amino-terminal loop (amino acids 1–18) or the first β-sheet (amino acids 18–32) reduced binding to both IL8R1 and IL8R2. The third β-sheet of IL-8 (amino acids 46–53) was required for binding to IL8R1 but not IL8R2. Exchanges of the second β-sheet (amino acids 32–46) or the carboxyl-terminal α-helix (amino acids 53–72) had no significant effect. When IL-8 sequences were substituted into GROγ, a single domain containing the second β-sheet of IL-8 (amino acids 18–32) was sufficient to confer high affinity binding for both IL8R1 and IL8R2. The amino-terminal loop (amino acids 1–18) and the third β-sheet (amino acids 46–53) of IL-8 had little effect when substituted individually but showed increased binding to both receptors when substituted in combination.

Individual amino acid substitutions were made at positions where IL-8 and GROγ sequences differ within the regions of residues 11–21 and 46–53. IL-8 mutations L49A or L49F selectively inhibited binding to IL8R1. Mutations Y13L and F21N enhanced binding to IL8R1, whereas mutations L49A or L49F selectively inhibited binding to IL8R1. This region contains a second site for IL-8 receptor recognition that, in combination with the Glu4-Leu5-Arg6 region, can modulate receptor binding affinity and IL8R1 specificity.

Interleukin-8 (IL-8)1 and the related GRO proteins are members of a superfAMILY of proinflammatory cytokines that stimulate neutrophil activation and chemotaxis (see Refs. 1–3 for review). These proteins contain four conserved cysteine residues with a single intervening amino acid between the first two cysteine residues and are designated the C-X-C chemokines. IL-8 mediates the recruitment and activation of neutrophils during inflammation and has been implicated in multiple pathologic conditions involving chronic and acute inflammation and in neutrophil-mediated injury (4–11). Two receptors for IL-8 have been identified by molecular cloning and account for the observed effects of IL-8 and other C-X-C chemokines on neutrophils (12, 13). Although both are seven-transmembrane, G-protein-coupled receptors, Type 1 and Type 2 IL-8 receptors differ in ligand specificity. The Type 1 receptors (IL8R1) have restricted specificity and bind IL-8 exclusively with high affinity (14, 15). In contrast, the Type 2 receptors (IL8R2) bind IL-8 with a similarly high affinity but also recognize several other C-X-C chemokines with varying affinities (14, 15).

Structures for both the crystal and solution forms of IL-8 have been determined (16, 17). IL-8 comprises five discrete structural domains: an amino-terminal loop, three antiparallel β-sheets, and a carboxyl-terminal α-helix. The highest regions of amino acid homology among the C-X-C chemokines occur at the conserved cysteine residues and at other key structural residues, suggesting that the basic structural elements of IL-8 are conserved among family members (1–3). The solution structure for GROα (18) and crystal structure for neutrophil-activating peptide-2 (19) show similar organization.

Several studies have addressed the structure-activity relationships required for C-X-C chemokine binding to neutrophils. A conserved sequence that is essential for binding, Glu4-Leu5-Arg6 (ELR), was identified by scanning mutagenesis of IL-8 (20) and by amino-terminal truncated analogs (21). The exclusive binding of IL-8 to IL8R1 and the different affinities among C-X-C chemokines for binding to IL8R2 suggest that a second site on IL-8 determines receptor specificity. Hybrid proteins

1 The abbreviations used are: IL-8, interleukin-8; IL8R1, interleukin-8 receptor type 1; IL8R2, interleukin-8 receptor type 2; GRO, growth related protein (note that GROα is also known as melanocyte growth-stimulating activity, GRO/MGSA, and that GROα and GROγ are also known as macrophage inflammatory proteins MIP2α and MIP2β, respectively; ELR, Glu4-Leu5-Arg6 sequence; PCR, polymerase chain reaction; CHO, Chinese hamster ovary.

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derived from IL-8 and IP10, a C-X-C chemokine that lacks the ELR sequence and that does not bind neutrophil IL-8 receptors, indicated that the ELS sequence as well as IL-8 sequences for amino acids 10–22 and 30–34 are required for neutrophil IL-8 and GRO\(\alpha\) indicated a site that enhances binding to neutrophils is within amino acids 13–29 (23). Receptor specificity was addressed directly on recombinant IL-8 receptors with trophils is within amino acids 10–22 and 30–34 are required for neutrophil receptor specificity indicated that the ELR sequence as well as IL-8 sequences for ELR sequence and that does not bind neutrophil IL-8 receptors, 

**Materials—** Recombinant human IL-8, GRO\(\alpha\) (GRO/MGSA), and 

**Derivation of Chimeric Protein**

Chimeric IL-8/GRO\(\alpha\) was produced in yeast using the pAB24 (29) and introduced into Saccharomyces cerevisiae strain MB2-1 by electroporation. Chimeric and mutant chemokines were purified from 50–200 ml of yeast culture broth by batch adsorption on 5-Sepharose FF (Pharmacia Biotech Inc.) after adjustment to pH 5.5 with 50 mm sodium acetate and eluted in 20 mm HEPEs, pH 8.3, 1 nM CaCl\(_2\) to a final concentration of 0.2–2 mg/mL. SDS-polyacrylamide gel electrophoresis on 18% Tris/glycine gels (Novex) indicated 80–95% purity. Protein concentrations were estimated by Coomassie-stained polyacrylamide gels and by BCA (Pierce) protein assays. Amino acid composition and molecular dynamics (35).

**RESULTS**

**Chimeric IL-8/GRO\(\alpha\) Proteins**—Chimeric IL-8/GRO\(\alpha\) proteins were designed to test the contribution of each structural domain of IL-8 for binding to IL8R1 and IL8R2. Four conserved amino acid residues were identified as structural domain boundaries for IL-8: His\(^{18}\), Pro\(^{22}\), Gly\(^{46}\), and Pro\(^{53}\) (Fig. 1). To maintain the overall C-X-C chemokine structure, sequences for complete structural domains between these boundaries were interchanged between IL-8 and GRO\(\alpha\) (Fig. 2). The corresponding chimeric chemokines were produced in yeast using the \(\alpha\)-factor mating pheromone secretion pathway (29) and purified to near homogeneity by a single step enrichment/purification protocol. With the exception of 132G, all proteins were expressed...

**TABLE I**

| Oligonucleotide linkers and primers for chimeric IL-8/GRO\(\alpha\) | Sequence (5’ to 3’) |
|---|---|
| mluA | ATCAAAAGACTCAGGTGATTTGG |
| accX | GAATTTCTCTTGCAGTTCTG |
| Xmu | TTTTCTCTTGCAGTTCTG |
| mluX | TTTTCTCTTGCAGTTCTG |
| bgIA | CTTTCTCTTGCAGTTCTG |
| accZ | TTTTCTCTTGCAGTTCTG |
| Zbgl | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| BglZ | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| ilprA | TTTTCTCTTGCAGTTCTG |
| gro25–32 | TTTTCTCTTGCAGTTCTG |
| ilprB | TTTTCTCTTGCAGTTCTG |
| g32iA | TTTTCTCTTGCAGTTCTG |
| g32iB | TTTTCTCTTGCAGTTCTG |
| i32gA | TTTTCTCTTGCAGTTCTG |
| i32gB | TTTTCTCTTGCAGTTCTG |
| ilpr#1 | TTTTCTCTTGCAGTTCTG |
| ilpr#2 | TTTTCTCTTGCAGTTCTG |
| mluX | TTTTCTCTTGCAGTTCTG |
| bgIA | TTTTCTCTTGCAGTTCTG |
| accZ | TTTTCTCTTGCAGTTCTG |
| Zbgl | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| BglZ | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| ilprA | TTTTCTCTTGCAGTTCTG |
| gro25–32 | TTTTCTCTTGCAGTTCTG |
| ilprB | TTTTCTCTTGCAGTTCTG |
| mluX | TTTTCTCTTGCAGTTCTG |
| bgIA | TTTTCTCTTGCAGTTCTG |
| accZ | TTTTCTCTTGCAGTTCTG |
| Zbgl | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| BglZ | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| ilprA | TTTTCTCTTGCAGTTCTG |
| gro25–32 | TTTTCTCTTGCAGTTCTG |
| ilprB | TTTTCTCTTGCAGTTCTG |
| mluX | TTTTCTCTTGCAGTTCTG |
| bgIA | TTTTCTCTTGCAGTTCTG |
| accZ | TTTTCTCTTGCAGTTCTG |
| Zbgl | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| BglZ | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| ilprA | TTTTCTCTTGCAGTTCTG |
| gro25–32 | TTTTCTCTTGCAGTTCTG |
| ilprB | TTTTCTCTTGCAGTTCTG |
| mluX | TTTTCTCTTGCAGTTCTG |
| bgIA | TTTTCTCTTGCAGTTCTG |
| accZ | TTTTCTCTTGCAGTTCTG |
| Zbgl | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| BglZ | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| ilprA | TTTTCTCTTGCAGTTCTG |
| gro25–32 | TTTTCTCTTGCAGTTCTG |
| ilprB | TTTTCTCTTGCAGTTCTG |
| mluX | TTTTCTCTTGCAGTTCTG |
| bgIA | TTTTCTCTTGCAGTTCTG |
| accZ | TTTTCTCTTGCAGTTCTG |
| Zbgl | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| BglZ | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| ilprA | TTTTCTCTTGCAGTTCTG |
| gro25–32 | TTTTCTCTTGCAGTTCTG |
| ilprB | TTTTCTCTTGCAGTTCTG |
| mluX | TTTTCTCTTGCAGTTCTG |
| bgIA | TTTTCTCTTGCAGTTCTG |
| accZ | TTTTCTCTTGCAGTTCTG |
| Zbgl | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| BglZ | TTTTCTCTTGCAGTTCTG |
| CTTTCTCTTGCAGTTCTG |
| ilprA | TTTTCTCTTGCAGTTCTG |
| gro25–32 | TTTTCTCTTGCAGTTCTG |
Determinants of IL-8 Recognition by IL8R1 and IL8R2

**Table I**

| Name              | Sequence (5' to 3')               |
|-------------------|-----------------------------------|
| Y13L              | AAGACACTGTCGGGAGACCTTCC          |
| S14Q              | AAGACAATACGAAACACCTTCC          |
| K15G              | AAGACATATCGGGAGACCTTCC          |
| F21N              | CACCCCAAAAATCAAGAAAGA           |
| F21T              | CACCCCAAAAATCAAGAAAGAA          |
| V41F              | GAATATATTCAAGACCTTCT            |
| V41K              | GAATATATTCAAGACCTTCT            |
| V41R              | GAATATATTCAAGACCTTCT            |
| D45R              | AACCTTTCGCGGAGAAGAGAG           |
| R47K              | GATGGAAGAACCTCTGGCT             |
| R47K/D52N         | GATGGAAGAACCTCTGGCT             |
| L49A              | GATGGAAGAACCTCTGGCT             |
| L49F              | GATGGAAGAACCTCTGGCT             |
| L49S              | GATGGAAGAACCTCTGGCT             |
| D52N              | GATGGAAGAACCTCTGGCT             |
| Y13L/S14Q         | GATGGAAGAACCTCTGGCT             |
| Y13L/K15G         | GATGGAAGAACCTCTGGCT             |
| S14Q/K15G         | GATGGAAGAACCTCTGGCT             |
| R47K/E48K/L49A    | GATGGAAGAACCTCTGGCT             |
| R47K/L49A/D52N**  | GATGGAAGAACCTCTGGCT             |
| E48K/L49A*        | GATGGAAGAACCTCTGGCT             |
| E48K/D52N*        | GATGGAAGAACCTCTGGCT             |
| R47K/E48K/D52N**  | GATGGAAGAACCTCTGGCT             |
| E48K/L49A/D52N**  | GATGGAAGAACCTCTGGCT             |
| 5' end sense      | GAGGGTGACCCTCTGAGATTAGGAGATA   |
| 3' end antisense  | AGACCGCTGCACTATATGGATGATGGCTAA |

*a The mutations in combination with D52N were obtained by amplifying respective mutated DNA templates with the primers for D52N mutation.

Mutations were generated by overlap PCR using sense and antisense mutated primers in combination with the respective 5' sense and 3' antisense end primers (31). Only the sense strand mutant primers are shown. The nucleotides generating the mutated amino acids are underlined. In the 5' and 3' end primers the nucleotides corresponding to the restriction site and nonhomologous to IL-8 are in italics.

**Fig. 1. Amino acid sequences of C-X-C chemokines.** Sequences for IL-8, GRO/MGSA, GROα/MIP2α, and GROβ/MIP2β are aligned for maximal homology and numbered according to the 72-amino acid form of IL-8 (Refs. 1–3 and references therein). Underlined residues are the conserved amino acids selected for domain boundaries.

pressed and recovered in yields sufficient for testing. Truncated variant proteins were observed for several of the mutants in which the amino-terminal sequences of GROα were fused with carboxyl-terminal portions of IL-8 and varied from 20 to 80% of the total chimera protein. Only the predicted amino-terminal protein sequence was detected in these samples. The carboxyl-terminal sequence of IL-8 reveals a Lys-Arg motif at residues 67 and 68 (Fig. 1). This sequence is a substrate site for the yeast diphilic protease KEX2 (36), and cleavage at this position can account for the observed reduction of approximately 5000 molecular mass units. Although previous studies have suggested that the α-helix region is not essential for IL-8 recognition by neutrophil receptors (21), the utilization of a previously silent KEX2 yeast-processing site suggests an increase in the accessibility of this region to protease attack and may indicate aberrant folding for this series of chimeric proteins.

Receptor Binding Activity of Chimeras—Receptor binding activity of chimeric proteins was measured by competition of 125I-IL-8 binding to recombinant IL8R1 and IL8R2 cell lines. GROα did not bind to IL8R1 and was 15-fold less potent than IL-8 for binding to IL8R2 (Table III). IL-8 or GROα purified in small scale by the procedure used for chimeric proteins had the same potency as that purified in large scale by multiple chromatography (data not shown). For IL-8/GROα chimeras, substitution of GROα onto the amino terminus of IL-8 (amino acids 1–18 or more) resulted in a loss of affinity to both IL8R1 and IL8R2 (Table III). Chimeric chemokines G32I, G46I, and G53I were even less potent than GROα, which may reflect improper folding since these proteins were prone to truncation as described above. In substitutions at the carboxyl terminus, replacement of the α-helix residues 53–72 of IL-8 with the corresponding region of GROα had no significant effect on receptor binding activity (Table III, 153G). In contrast, substitution of residues 46–72 of IL-8 with the corresponding GROα sequence selectively reduced binding to IL8R1 but not IL8R2 (Table III, 146G), suggesting that residue(s) within the third β-sheet (amino acids 46–53) are involved in specific binding to IL8R1 and not IL8R2.

Chimeric proteins with single domains of GROα substituted into IL-8 were used to test the role of each β-sheet region. Replacement of amino acids 18–32, corresponding to the first β-sheet, reduced binding of IL-8 to both IL8R1 and IL8R2 (Fig. 3, 118G32I). Exchange of regions within amino acids 32–46 was not important for binding to either IL8R1 or IL8R2 (Fig. 3, 132G46I). Substitution for amino acids 46–53 reduced binding to IL8R1 by 10-fold without affecting binding to IL8R2 (Fig. 3, 146G53I), confirming that the third β-sheet of IL-8 participates in IL8R1 specificity.

In the reciprocal experiment, individual domains of IL-8 were substituted into GROα to examine whether any of these regions could enhance binding to IL-8 receptors. Replacement of residues 18–32, corresponding to the first β-sheet of IL-8, was sufficient to allow nearly IL-8-like activity on both IL8R1 and IL8R2 (Fig. 5, G18I32I). Two domains, corresponding to amino acids 1–18 and 32–46, slightly enhanced binding to IL8R1 (Fig. 3, 118G and G32I46I). Since the amino-terminal loop and the third β-sheet were both important for IL8R1 binding of IL-8 but had little effect when substituted into GROα...
individually, a chimera was generated to test whether simultaneous replacement of these regions of GROγ with corresponding IL-8 sequences could confer IL-8-like activity. The combined substitution of amino acids 1–18 and 46–53 allowed high affinity binding of GROγ to both IL8R1 and IL8R2 (Fig. 3, I18G46G).

Point Mutations of IL-8 to GROγ Residues within the Third β-Sheet—Since the replacement of IL-8 amino acids 46–53 with GROγ reduced binding to IL8R1 without affecting the binding to IL8R2, the role of individual amino acids in this region was examined through point mutations to substitute GROγ amino acids into IL-8. Four amino acid residues differ between IL-8 and GROγ in this region: Arg47, Glu48, Leu49, and Asp52. The GROγ amino acids were introduced into IL-8 to test each residue individually and in combinations. The mutation of IL-8 L49A, alone or in combination, selectively decreased the binding to IL8R1 (Table IV). Neither the E48K replacement nor the conservative substitution D52N affected binding to either receptor, although the double mutation of E48K/D52N decreased binding to IL8R1 by 3-fold. R47K marginally decreased the binding to both IL8R1 and IL8R2 but had no impact on receptor binding in combination mutations. The triple mutation R47K/E48K/D52N also had little effect on binding to either receptor and no selectivity for IL8R1. No protein was expressed for IL-8 mutations R47K/E48K and E48K/L49A/D52N, suggesting abnormal folding and protein degradation. IL-8 R47K/L49A did not generate yeast transformants, implying intolerance of the mutated protein by this host cell. Consistent with the chimeric substitutions in the 46–53 region, substituting Leu49 decreased the binding to IL8R1 with no significant effect on binding to IL8R2. These data indicate that Leu49 is the primary determinant within the third β-sheet for IL8R1 specificity of IL-8.

Point Mutations of IL-8 to GROγ Residues within the Amino-
terminal Loop—The chimera data demonstrated that IL-8 amino acids 1-18 of the amino-terminal loop are important for binding of IL-8 to both IL8R1 and IL8R2. Within this region there are several amino acid differences; particularly, the five amino acids of IL-8 at positions 13–17 are replaced by only four different residues in GROγ. IL-8 amino acids 13–15 were substituted with comparable amino acids of GROγ. No protein was expressed for mutants containing the K15G mutation alone or in combinations, suggesting that this alteration of the chimera structure is not tolerated. The mutation Y13L increased the binding to IL8R1 over 3-fold without affecting IL8R2 binding (Table IV). S14Q marginally decreased the binding to IL8R1, but the double mutation of Y13L/S14Q significantly decreased the binding to IL8R2 over 4-fold with little effect on IL8R1 (Table IV). These results are consistent with the amino acid 1-18 chimeric substitutions and demonstrate that Tyr13 and Ser14 contribute to the specificity of IL-8 binding to both IL8R1 and IL8R2.

Comparison of IL-8 Structure and GROγ Homology Model—Based upon the established structure of IL-8 (16, 17), residues Tyr13, Ser14, and Leu49 lie on a single face of the IL-8 molecule in a region that is unique and distant from both the conserved ELR residues previously identified as essential for IL-8 binding to IL-8 receptors and the strand of hydrophobic residues that participate in the monomer-monomer interface of the dimer form of IL-8 (Fig. 4). These residues coincide with or are adjacent to a surface-exposed hydrophobic pocket on IL-8 that consists of Tyr13, Phe17, Ile22, Val41, Leu43, Leu49, and Leu51 (Fig. 4). This slot-like hydrophobic pocket is large enough to accommodate a phenyl ring, which fits into the pocket as a coin in a slot. The entrance to this pocket is flanked by Tyr13, Lys15, Phe17, and Arg47.

A homology-based model of GROγ was constructed to compare the relative positions of the corresponding residues. The predicted structure is shown in the same relative orientation as the structure of IL-8 (Fig. 4). In IL-8, one side of the hydrophobic pocket is formed by the Tyr13–Ile22 strand. In GROγ, little of this stretch of residues is conserved, and there is a deletion of one residue. Tyr13 and Phe17 are conservatively substituted with isoleucine in GROγ, and Ile22 is conserved. Thus, the residues contributing to the hydrophobic core appear to be conserved. However, the deletion corresponding to Lys15 in IL-8 reduces the length of the Tyr13–Ile22 strand, effectively shrinking the hydrophobic pocket in GROγ. The substitution of Pro19 by a leucine in GROγ affects the structure of this strand and its contribution to the hydrophobic pocket. According to our homology model, the hydrophobic pocket in GROγ is much smaller than that in IL-8 and cannot accommodate a phenyl ring. In addition, none of the gateway residues of IL-8 are conserved in GROγ.

IL-8 Mutations within the Hydrophobic Pocket Region—Additional point mutations of IL-8 were designed to test the role of the key hydrophobic pocket and flanking residues in the recognition of IL-8 by IL8R1 and IL8R2. Mutations were introduced at positions Phe21, Val41, Asp45, and Leu49 corresponding to residues that are unique in IL-8. F21N increased the binding affinity by 5.5- and 2.4-fold to IL8R1 and IL8R2, respectively (Fig. 5). Mutation D45R had no effect on IL8R1 binding but decreased the binding to IL8R2 over 4-fold with little effect on IL8R1 (Table IV). These results are consistent with the amino acid 1-18 chimeric substitutions and demonstrate that Tyr13 and Ser14 contribute to the specificity of IL-8 binding to both IL8R1 and IL8R2.

### Table III

| Chemokine | IC50 Valuea | IL8R1 μg/ml | IL8R2 μg/ml |
|-----------|-------------|-------------|-------------|
| IL-8      | 0.040 ± 0.006 | 0.020 ± 0.002 |               |
| GROγ      | 0.29 ± 0.06  | 0.21 ± 0.04  |               |
| I13G      | 25.6 ± 8.0   | 0.21 ± 0.04  |               |
| I32G      | ND           | ND           |               |
| I46G      | 2.85 ± 0.87  | 0.047 ± 0.014|               |
| I53G      | 0.071 ± 0.021| 0.021 ± 0.004|               |
| G18I      | 9.8 ± 1.7    | 0.43 ± 0.08  |               |
| G32I      | —            | 8.1 ± 1.3    |               |
| G46I      | —            | 3.5 ± 1.1    |               |
| G53I      | —            | 3.2 ± 1.0    |               |

a Competitive binding assays were performed on CHO-IL8R1 and CHO-IL8R2 cells with 0.2 nm 125I-IL-8 and 0.001-30 μg/ml test proteins. Data are the mean ± S.E. of at least three experiments.

b No significant inhibition at 30 μg/ml.

c ND, not determined.

| Chemokine | IC50 Valuea | IL8R1 μg/ml | IL8R2 μg/ml |
|-----------|-------------|-------------|-------------|
| IL-8      | 0.040 ± 0.006 | 0.020 ± 0.002 |               |
| GROγ      | 0.29 ± 0.06  | 0.21 ± 0.04  |               |
| I13G      | 25.6 ± 8.0   | 0.21 ± 0.04  |               |
| I32G      | ND           | ND           |               |
| I46G      | 2.85 ± 0.87  | 0.047 ± 0.014|               |
| I53G      | 0.071 ± 0.021| 0.021 ± 0.004|               |
| G18I      | 9.8 ± 1.7    | 0.43 ± 0.08  |               |
| G32I      | —            | 8.1 ± 1.3    |               |
| G46I      | —            | 3.5 ± 1.1    |               |
| G53I      | —            | 3.2 ± 1.0    |               |

a Competitive binding assays were performed on CHO-IL8R1 and CHO-IL8R2 cells with 0.2 nm 125I-IL-8 and 0.001-30 μg/ml test proteins. Data are the mean ± S.E. of at least three experiments.

b No significant inhibition at 30 μg/ml.

c ND, not determined.

**Fig. 3.** Relative receptor binding activity of IL-8/GROγ chimeras with substitutions of individual structural domains. Left panels, individual domains of GROγ inserted into IL-8. Right panels, individual domains of IL-8 inserted into GROγ. Competitive binding assays were performed on CHO-IL8R1 (upper panels) and CHO-IL8R2 cells (lower panels) with 0.2 nm 125I-IL-8 and 0.001-30 μg/ml test proteins. The IC50 values for IL-8 were 0.037 ± 0.006 μg/ml and 0.023 ± 0.005 μg/ml for IL8R1 and IL8R2, respectively. Relative potency was calculated as the ratio of IC50 values for IL-8 versus the IC50 value of each chimera. Data are the mean ± S.E. of at least three independent experiments.
Leu⁴⁹. insertion of the aromatic residue in L49F selectively decreased binding to IL8R1 by 6.2-fold with no significant effect on IL8R2. In contrast the relatively conservative substitution of L49S had little effect on IL-8 receptor activity. Taken together with the observations for L49A and for Y13L, these mutations reveal that key residues for determining receptor-specific binding of IL-8 are clustered around the surface-accessible hydrophobic pocket and suggest that increased access to the pocket by removal of the aromatic residues at Tyr¹³ and Phe⁶¹ can enhance binding to IL8R1.

Neutrophil Chemotaxis—The functional activation of IL-8 receptors was assessed in neutrophil chemotaxis assays. GRO-Y was less potent than IL-8 and had lower efficacy at optimal chemotaxis concentrations (Fig. 6). Chimeric IL-8/GRO-Y proteins demonstrated neutrophil chemotactic activity consistent with relative receptor binding activity (Fig. 6 and additional data not shown). Proteins with characteristic binding properties of GRO-Y, i.e. an absence of binding to IL8R1 and a reduced affinity binding to IL8R2, displayed GRO-Y-like chemotactic activity. An IL-8-like chemotactic efficacy correlated with recognition by IL8R1, and the relative potency paralleled the affinity for IL8R1 observed in competitive binding assays (Fig. 6).

**TABLE IV**

| Mutation      | IL8R1  | IL8R2  |
|---------------|--------|--------|
| IL-8          | 1.0    | 1.0    |
| Y13L          | +3.4 ± 1.0 | +1.4 ± 0.2 |
| S14Q          | -2.6 ± 0.6 | -1.8 ± 0.7 |
| R47K          | -2.2 ± 0.4 | -2.0 ± 0.9 |
| E48K          | 1.0 ± 0.3 | +1.4 ± 0.3 |
| L49A          | -6.6 ± 0.7 | +1.6 ± 0.1 |
| D52N          | 1.0 ± 0.5 | 1.0 ± 0.1 |
| Y13LS14Q      | -1.7 ± 0.6 | -4.3 ± 0.8 |
| R47KD52N      | 1.0 ± 0.04 | +1.5 ± 0.2 |
| E48KL49A      | -5.0 ± 1.0 | -1.3 ± 0.5 |
| E48KD52N      | -2.9 ± 0.3 | -1.3 ± 0.4 |
| R47K/E48KD52N | -2.0 ± 0.2 | -1.8 ± 0.5 |

**DISCUSSION**

By construction of IL-8/GRO-Y chimeras we have demonstrated that the amino-terminal loop (amino acids 1–18) and the first β-sheet (amino acids 18–32) of IL-8 contain residues that are essential for high affinity, IL-8-like binding to both IL8R1 and IL8R2. These domains encompass the regions identified by IL-8/IP₁₀ hybrids (22) and by IL-8/N51 chimeric proteins (23) as necessary for maximal binding to neutrophils. The third β-sheet of IL-8 contains residues that confer IL8R1-specific binding and are not required for binding to IL8R2. Complete analysis of this region by point mutations indicated that L49A substitution is responsible for the specific reduction of IL8R1 binding. Heinrich et al. (23) observed that a chimeric IL-8/N51 protein containing this substitution (corresponding to I34N50i) exhibited neutrophil binding properties similar to IL-8. That result may reflect the participation of both IL8R1 and IL8R2 receptors in neutrophil binding.

Schauflesstatter et al. (24) have reported that the carboxyl terminus of IL-8/GRO-Y chimeric proteins affects specificity of binding to IL8R2 but not to IL8R1. Chimeric 151G₅α behaved like IL-8 in its binding to IL8R1 but had 5-fold lower affinity for IL8R2 (24). The present study demonstrates that the carboxyl terminus of IL-8 beyond amino acid residue 53 does not define
receptor specificity for IL8R1 or IL8R2. Exchanges of the carboxyl terminus did not affect neutrophil binding of chimeric IL-8/IP10 (22) or IL-8/N51 (23), and with truncated analogs of IL-8 the removal of the terminal α-helix reduced but did not prevent neutrophil receptor binding (21). We have identified several variants of IL-8 with specifically altered binding affinity for IL8R1 or IL8R2. Mutations at Tyr13, Phe21, and Leu49 modified IL8R1 binding selectively. Recent studies by Schraufstatter et al. (39) identified Tyr13 as well as Lys15 as determinants of the differential IL8R1 affinity for human and rabbit IL-8. These key residues that affect binding to IL8R1 surround a unique hydrophobic pocket on the surface of IL-8. The increase in binding to IL8R1 by the replacement of Tyr13 with leucine indicates that the removal of the aromatic ring provides increased access for receptor docking. Y13H also decreased binding to IL8R1 (39), and other mutations at this position altered binding to neutrophils (22). The increased binding to IL8R1 of F21N can also be explained as facilitating the receptor interaction by the replacement of the aromatic group with smaller amino acids. F21L had reduced binding to neutrophils (22). Substitutions at Leu49 are not as clear-cut. L49F decreases binding to IL8R1, which could be interpreted as the large aromatic ring of phenylalanine obstructing the receptor docking. L49A also decreases R1 binding, which might suggest that this is due to an alteration of the hydrophobic pocket itself. The replacement with an intermediate sized residue, L49S, moderately increased binding. The double mutation Y13L/S14Q decreases binding to IL8R2 selectively, indicating that IL8R2 recognition involves different determinants than IL8R1 but is also localized near the hydrophobic pocket on IL-8. Thus this region contributes to IL8R2 binding, and the pocket itself may be essential for binding to IL8R1.

The surface-accessible hydrophobic pocket on IL-8 is sufficiently large to accommodate an aromatic ring structure and could accept a phenylalanine or tyrosine side chain from IL8R1. The region of the hydrophobic pocket, and particularly of residues 12–18 within the amino-terminal loop, corresponds to the site of greatest structural differences between IL-8 and other C-X-C chemokines (GROα and NAP-2) that bind to IL8R2 but not to IL8R1 (18, 19). The solution structure of GROα indicates a hydrophobic pocket smaller than that on IL-8 (18). For GROγ, homology-based structural modeling predicted that the hydrophobic pocket is much smaller and more restricted in access, consistent with the lack of IL8R1 binding and reduced IL8R2 binding.

Interleukin-8 interacts with two sites on IL-8 receptors; conserved charged residues in extracellular domains 3 and 4 are essential for IL-8 binding to IL8R1 or IL8R2 (40), and the amino-terminal extracellular domains confer the ligand specificity profiles characteristic of each receptor type (41, 42). Our data indicate that the hydrophobic pocket and/or surrounding residues contribute to the binding of C-X-C chemokines to IL-8 receptors and serve as the second binding site on IL-8 to modify receptor recognition in conjunction with the ELR sequence. Thus, receptor extracellular domains 3 and 4 provide the primary interaction with ELR (residues 4–6) of IL-8, and receptor amino-terminal domains interact with a secondary binding site.
localized in or near the surface hydrophobic pocket on IL-8 bounded by residues Tyr$_{13}$, Lys$_{25}$, Phe$_{21}$, and Arg$_{47}$.

Acknowledgments—We thank Lauri Goda for DNA synthesis, Joan Joh and Guillin Wang for DNA sequencing, Susan Hilt for providing CHO-IL8R1 and CHO-IL8R2 cells, Kathryn Collins for 125I-IL-8, Lawrence Caouens for purification of GRO$\alpha$, Frank Masiarz and Scott Chamberlain for protein sequencing, and Terry Calarco for preparation of graphics.

REFERENCES

1. Oppenheim, J. J., Zachariae, C. O. C., Mukaida, N., and Matsushima, K. (1991) Annu. Rev. Immunol. 9, 617–648
2. Baggiolini, M., Dzedal, B., and Moser, B. (1994) Adv. Immunol. 55, 97–179
3. Miller, M. D., and Kranget, M. S. (1992) Crit. Rev. Immunol. 12, 17–46
4. Endo, H., Akahoshi, T., Takagishi, K., Kashiwazaki, S., and Matsushima, K. (1991) Lymphokine Cytokine Res. 10, 245–252
5. Miller, E. J., Cohen, A. B., Nages, S., Griffith, D., Maurer, R. J., Martin, T. R., Weiner-Kronish, J. P., Sticherling, M., Christophers, E., and Matthay, M. A. (1992) Am. Rev. Respir. Dis. 146, 427–432
6. Mahida, Y. R., Ceska, M., Effenberger, F., Kurlak, L., Lindley, I., and Hawkey, C. J. (1992) Clin. Sci. 82, 273–275
7. Izzo, R. S., Witkon, K., Chen, A. I., Hadiyane, C., Weinstein, M. I., and Pellicchfa, C. (1992) Am. J. Gastroenterol. 87, 1447–1452
8. Broadus, V. C., Boytan, A. M., Hoiffe, J. M., Kim, K. J., Sadick, M., Chuntafara, A., and Hébert, C. A. (1994) J. Immunol. 152, 2960–2967
9. Mulligan, M. S., Jones, M. L., Bolanowski, M. A., Baganoft, M. P., Deppeler, C. L., Meyers, D. M., Ryan, U. S., and Ward, P. A. (1993) J. Immunol. 150, 5380–5385
10. Sekido, N., Mukaida, N., Harada, A., Nakashishi, I., Watanabe, Y., and Matsushima, K. (1993) Nature 365, 654–657
11. Harada, A., Sekido, N., Akahoshi, T., Wada, T., Mukaida, N., and Matsushima, K. (1994) J. Leukocyte Biol. 56, 559–564
12. Holmes, W. E., Lee, J., Kuang, W.-J., Rice, G. C., and Wood, W. I. (1991) Science 253, 1278–1280
13. Murphy, P. M., and Tiffany, H. L. (1991) Science 253, 1280–1283
14. Lee, J., Horuk, R., Rice, G. C., Bennett, G. L., Camerato, T., and Wood, W. I. (1992) J. Biol. Chem. 267, 16283–16287
15. Cerretti, D. P., Kozlofský, C. J., Vandenlow, T., Nelson, N., Gearing, D. P., and Beckmann, M. P. (1993) Mol. Immunol. 30, 359–367
16. Baldwin, E. T., Weber, I. T., St. Charles, R., Xuan, J.-C., Appella, E., Yamada, M., Matsushima, K., Edwards, B. F. P., Clare, G. M., Gronenborn, A. M., and Wladawer, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 502–506
17. Clare, G. M., Appella, E., Yamada, M., Matsushima, K., and Gronenborn, A. M. (1990) Biochemistry 29, 1689–1696
18. Fairbrother, W. J., Reilly, D., Colby, T. J., Hasselgesser, J., and Horuk, R. (1994) J. Mol. Biol. 242, 252–270
19. Makowski, M. G., Wu, J. Y., Lazar, J. B., Johnson, P. H., and Edwards, B. F. P. (1995) J. Biol. Chem. 270, 7077–7078
20. Hébert, C. A., Vantant, R. V., and Baker, J. B. (1991) J. Biol. Chem. 266, 18989–18994
21. Clark-Lewis, I., Schumacher, C. Baggiolini, M., and Moser, B. (1991) J. Biol. Chem. 266, 23128–23134
22. Clark-Lewis, I., Dowad, B., Loetscher, M., Moser, B., and Baggiolini, M. (1994) J. Biol. Chem. 269, 16075–16081
23. Heinrich, J. N., O’Rourke, E. C., Chen, L., Gray, H., Dorfman, K. S., and Bravo, R. (1994) Mol. Cell. Biol. 14, 2849–2851
24. Schauffstatter, I. U., Barratt, D. S., Ma, M., Oades, Z. G., and Coughran, C. G. (1993) J. Immunol. 151, 6418–6428
25. Hammond, M. E. W., Laportite, G. R., Feucht, P. H., Hilt, S., Gallegos, C. A., Gordon, C. A., Giedlin, M. A., Mullenbach, G., and Tekamp-Olson, P. (1995) J. Immunol. 155, 1428–1433
26. Higuchi, R. (1990) in PCR Protocols (Innis, M. A., Gelfand, D. H., Sminsky, J. J., and White, T. J., eds) pp. 177–183, Academic Press, Inc., San Diego, CA
27. Miller, E. J., Cohen, A. M., Carr, F. K., Hayashi, S., Chiu, C. Y., Lee-Ng, C. T., and Mullenbach, G. (1995) Protein Expression Purif. 6, 357–362
28. Tekamp-Olson, P., Gallegos, C., Bauer, D., McClain, J., Sherry, B., Fabre, M., van Deventer, S., and Cerami, A. (1990) J. Exp. Med. 172, 911–919
29. Brake, A. (1990) Methods Enzymd. 185, 408–421
30. Shyamala, V., and Ames, G. F.-L. (1993) Methods in Molecular Biology 15, 339–348
31. Shyamala, V., and Ames, G. F.-L. (1991) Gene (Amst.) 97, 1–6
32. Leatherbarrow, R. J. (1991) GraFit version 2.10, Erithacus Software Ltd., Staines, UK
33. Needleman, S. B., and Wunsch, C. D. (1970) J. Mol. Biol. 48, 443–453
34. Levitt, M. (1992) J. Mol. Biol. 226, 507–533
35. Leong, S. R., Kabakoff, R. C., and Hébert, C. A. (1994) J. Biol. Chem. 269, 19343–19348
36. Wu, D., LaRosa, G. J., and Simon, M. I. (1993) Science 261, 101–103
37. Shyamala, V., Moulethop, T. H. M., Stratton-Thomas, J., and Tekamp-Olson, P. (1994) Cell & Mol. Biol. Res. 40, 285–296
38. Schauffstatter, I. U., Ma, M., Oades, Z. G., Barratt, D. S., and Coughran, C. G. (1995) J. Biol. Chem. 270, 14028–14031
39. Wu, D., LaRosa, G. J., and Simon, M. I. (1993) Science 261, 101–103
40. Leong, S. R., Kabakoff, R. C., and Hébert, C. A. (1994) J. Biol. Chem. 269, 19343–19348
41. Wu, D., LaRosa, G. J., and Simon, M. I. (1993) Science 261, 101–103
42. Shyamala, V., Moulethop, T. H. M., Stratton-Thomas, J., and Tekamp-Olson, P. (1994) Cell & Mol. Biol. Res. 40, 285–296
43. Schauffstatter, I. U., Ma, M., Oades, Z. G., Barratt, D. S., and Coughran, C. G. (1995) J. Biol. Chem. 270, 14028–14031
44. Wu, D., LaRosa, G. J., and Simon, M. I. (1993) Science 261, 101–103
45. Leong, S. R., Kabakoff, R. C., and Hébert, C. A. (1994) J. Biol. Chem. 269, 19343–19348
46. Wu, D., LaRosa, G. J., and Simon, M. I. (1993) Science 261, 101–103
47. Shyamala, V., Moulethop, T. H. M., Stratton-Thomas, J., and Tekamp-Olson, P. (1994) Cell & Mol. Biol. Res. 40, 285–296
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J. Biol. Chem. 1996, 271:8228-8235.
doi: 10.1074/jbc.271.14.8228

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