Differential Mechanisms of Recognition and Activation of Interleukin-8 Receptor Subtypes*

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We have probed an epitope sequence (His^{18}-Pro^{19}-Lys^{20}-Phe^{21}) in interleukin-8 (IL-8) by site-directed mutagenesis. This work shows that single and double Ala substitutions of His^{18} and Phe^{21} in IL-8 reduced up to 77-fold the binding affinity to IL-8 receptor subtypes A (CXCR1) and B (CXCR2) and to the Duffy antigen. These Ala mutants triggered neutrophil degranulation and induced calcium responses mediated by CXCR1 and CXCR2. Single Asp or Ser substitutions, H18D, F21D, F21S, and double substitutions, H18A/F21D, H18A/F21S, and H18D/F21D, reduced up to 431-fold the binding affinity to CXCR1, CXCR2, and the Duffy antigen. Interestingly, double mutants with charged residue substitutions failed to trigger degranulation or to induce wild-type calcium responses mediated by CXCR1. Except for the H18A and F21A mutants, all other IL-8 mutants failed to induce superoxide production in neutrophils. This study demonstrates that IL-8 recognizes and activates CXCR1, CXCR2, and the Duffy antigen by distinct mechanisms.

Interleukin-8 (IL-8) is a chemokine secreted in response to injury and infection that selectively attracts and activates neutrophils. IL-8 is a 72-79-amino acid peptide that belongs to the CXC chemokine subfamily because its first two Cys are separated by a single residue. The NMR and crystal structures of IL-8 reveal that the polypeptide chain is folded into three antiparallel β-sheets and a C terminus α-helix (1, 2). IL-8 receptors belong to the superfamily of seven transmembrane G protein-coupled receptors. Several IL-8 receptor subtypes have been identified. Two structurally homologous receptors, CXCR1 and CXCR2, are expressed in neutrophils (3-5). CXCR1 selectively binds IL-8, whereas CXCR2 binds IL-8 and the structurally related CXC chemokines Neutrophil Activating Protein-2 (NAP-2) and MGSA (6-8). The Duffy antigen of neutrophils selectively binds IL-8, whereas CXCR2 binds IL-8 and CXCR1 and CXCR2, are expressed in neutrophils (3-5). In particular, this region contains a surface-exposed hydrophobic pocket formed by Phe^{17}, Phe^{21}, Ile^{22}, and Leu^{33} which is separated by over 20 Å from the N-terminal ELR triad, and it has been suggested that residues in or adjacent to the hydrophobic pocket are major determinants for binding selectivity to both CXCR1 and CXCR2 (16, 17). This hydrophobic pocket may be essential for recognition of CXCR1. In this work, we have created IL-8 mutants in which residues corresponding to the epitope of blocking anti-IL-8 mAbs were substituted by Ala, polar, or charged residues. We determined that this epitope plays a major role in the differential recognition mechanisms of CXCR1, CXCR2, and the Duffy antigen. But most importantly, this epitope also plays a role in the activation mechanisms for CXCR1.

EXPERIMENTAL PROCEDURES

Preparation of Human Neutrophils, Red Blood Cells, and Rabbit Neutrophil Membranes—Human blood was drawn from healthy human donors and layered in a Mono-Poly Resolving Medium (ICN Biochemical, Inc., Aurora, OH). Neutrophils and red blood cells were isolated in accordance with the instructions of the manufacturer. Neutrophils were suspended in a physiological buffer containing 140 mM NaCl, 4 mM KCl, 1 mM MgCl_{2}, 1 mM CaCl_{2}, 1 mM Na,HPO_{4}, 5 mM glucose, 20 mM HEPES (pH 7.4), and 1 mg/ml bovine serum albumin. Red blood cells were stored in Alsever solution consisting of 114 mM dextrose, 27 mM sodium citrate, 71 mM NaCl (pH 6.1) at 4 °C. Rabbit neutrophil membranes were prepared as described (18).

Expression of CXCR1 and CXCR2—CXCR1 was subcloned into the retrovirus vector MSX, and virus stocks were produced from amphotropic packaging cell lines (19). HL-60 cells were infected with these virus stocks and selected by limiting dilution and binding to ^{125}I-IL-8. Cell line HL-60 was selected for this study because it expresses a high density of CXCR1 (19) but undetectable levels of CXCR2. HL-60 cells expressing recombinant CXCR2 were kindly provided by Dr. Richard Ye (University of Illinois, Chicago).

Protein Expression and Purification—IL-8 mutants were created by site-directed mutagenesis as described previously (20). The mutant constructs were subcloned into the thioexodin-based vector GM-TRX for expression of thioexodin-IL-8 mutant fusion proteins. Escherichia coli G1724 transformed with mutant constructs was induced with 100 μg/ml isopropyl β-D-thiogalactoside for 4 h at 30 °C. The cells were lysed by French press, and the fusion protein was purified by fractionation on a QAE anion-exchange column followed by a G-75 gel filtration column. Fractions enriched with thioexodin fusion proteins were digested with enterokinase to release the IL-8 mutant protein. The digestion products were further purified by using an SP-650 cation-exchange column.

^{125}I-IL-8 Binding Assays—^{125}I-IL-8 was iodinated by the chloramine-T procedure as described (3). Rabbit neutrophil membranes were suspended at a concentration of 200 μg/ml in binding buffer (phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin and 20 mM HEPES (pH 7.4)) and incubated for 30 min at room temperature in the presence of 2 nM ^{125}I-labeled IL-8 and several concentrations of unlabeled IL-8 or IL-8 mutants. The binding reaction was terminated as...
expressing CXCR1 or CXCR2, or red blood cells. 1.4, 0.265, 0.6, or 15 nM, respectively.

Red blood cells, or HL-60 cells expressing CXCR1 or CXCR2 were suspended at a concentration of 2.5 × 10^6 cells/ml or 1 × 10^6 cells/ml in binding buffer containing 2 mM EDTA-labeled IL-8 and several concentrations of unlabeled IL-8 or IL-8 mutants and then incubated for 2 h on ice. The reaction was terminated by overlaying the incubation mixture on top of a 10% (w/v) sucrose solution followed by centrifugation. Radioactivity in the pellet was measured in a γ-counter. The Kᵢ for each IL-8 mutant was calculated according to the equation derived by Cheng and Prusoff (21): $Kᵢ = IC₅₀/(1 + [IL-8]/Kᵢ)$, where IC₅₀ is the concentration of mutant that produces 50% inhibition on IL-8 binding to the receptor subtype, [IL-8] is the concentration of radiolabeled IL-8, and Kᵢ is the dissociation constant of IL-8 binding to the receptor subtype.

*Intracellular (Ca²⁺) Measurements—Neutrophils were suspended in physiological buffer at a density of 1 × 10⁶ cells/ml, and loaded with 5 µM Indo-1/AM (Molecular Probes, OR) for 30 min at room temperature in the dark. Neutrophils were subsequently washed once with ice-cold phosphate-buffered saline and then resuspended in physiological buffer at a density of 1 × 10⁷ cells/ml. HL-60 cells expressing CXCR1 or CXCR2 were suspended in physiological buffer at a density of 1 × 10⁶ cells/ml and loaded with Indo-1/AM for 30 min at 37 °C in the dark. HL-60 cells were subsequently washed once with ice-cold physiological buffer and resuspended in physiological buffer containing 1 mM probenecid at 1 × 10⁵ cells/ml. Neutrophils and HL-60 cells were placed in a continuously stirred cuvette maintained at 37 °C in a spectrofluorometer. Neutrophils were suspended in physiological buffer containing 130 mM NaCl, 4.6 mM KCl, 1.1 mM KH₂PO₄, 1 mM CaCl₂, 5 mM glucose, and 20 mM HEPES (pH 7.2) in the presence of IL-8 or IL-8 mutants. β-Glucuronidase assay—Human neutrophils (1 × 10⁶ cells/ml) were incubated in a Hanks’ balanced salt solution supplemented with 1% (w/v) bovine serum albumin, 2 mg/ml glucose, 4.2 mM NaHCO₃, and 10 mM HEPES (pH 7.4) at 1 × 10⁷ cells/ml. Neutralized HEPES was placed in physiological buffer containing 1 mM p-hydroxyphenylacetate, 20 units of horseradish peroxidase (HRP) (24, 25). Neutrophils were stimulated with IL-8 or IL-8 mutants. Fluorescence intensity was measured using an excitation wavelength of 330 nm and an emission wavelength of 405 nm (22). Superoxide release was monitored by addition of 2,2′-dihydroxybiphenyl-5,5′-diacetate produced from p-hydroxyphenylacetate by the enzymatic reduction of H₂O₂ by horseradish peroxidase (HRP) (24, 25). Neutrophils were suspended in physiological buffer containing 130 mM NaCl, 4.6 mM KCl, 1.1 mM KH₂PO₄, 1 mM CaCl₂, 5 mM glucose, and 20 mM HEPES (pH 7.4) at 1 × 10⁷ cells/ml. Neutrophils were placed in physiological buffer containing 1 mM p-hydroxyphenylacetate, 20 units of horseradish peroxidase and 100 µM sodium azide in a continuously stirred cuvette maintained at 37 °C in a spectrofluorometer. Neutrophils were stimulated with IL-8 or IL-8 mutants and fluorescence intensity was measured using an excitation wavelength of 334 nm and an emission wavelength of 425 nm.

**RESULTS**

*Mutations of the IL-8 Epitope Region and Receptor Recognition*

Previous studies (20) have shown that the epitope consensus sequence of the blocking anti-IL-8 mAb is H-X-K-F and corresponds to residues His¹⁸-Pro¹⁹-Lys²⁰-Phe²¹ in IL-8. These residues are in or adjacent to the surface-exposed hydrophobic pocket in IL-8 which has been previously implicated in the recognition mechanisms to the IL-8 receptors (16, 17). Moreover, binding of IL-8 to the N-terminal fragment of CXCR1 causes perturbations in the NMR chemical shifts of His¹⁸ and Phe²¹ (26), suggesting that these residues bind to the N terminus domain of the IL-8 receptors. Single and double IL-8 mutants were created by substitution of His¹⁸ and Phe²¹ by Ala, Ser, or Asp. All these mutants exhibited reduced binding to the anti-IL-8 mAb (20). In this study we expressed recombinant IL-8 mutants as fusion proteins linked to thioredoxin. IL-8 mutants were purified to near homogeneity. Fig. 1 shows the purity of the IL-8 mutants, H18D and H18A/F21A, as determined by SDS-polyacrylamide gel electrophoresis. To examine whether His¹⁸ and Phe²¹ residues play a role in the mechanisms of recognition and activation of the IL-8 receptors, we initially performed displacement of 1²⁵I-IL-8 bound to neutrophil membranes by IL-8 mutants. In Table I we showed that H18A and F21A mutants exhibited 4- and 3-fold increases in the Kᵢ, respectively, as compared with that of the wild type IL-8, suggesting a moderate reduction in binding affinity of the mutants toward IL-8 receptors. The substitution of His¹⁸ for Asp, or Phe²¹ for Ser or Asp resulted in larger increases in the Kᵢ (up to 40-fold) (Table I). To determine whether these two residues interact independently with the receptors or overlap, we examined the effect of double substitution mutants. As shown in Table I, mutants with double substitutions exhibited larger increases in the Kᵢ, up to 636-fold, than that of the wild type IL-8. Since these binding experiments were carried out with neutrophil membranes co-expressing both CXCR1 and CXCR2, we performed similar binding experiments with cells

**TABLE I**

| Mutant | Neutrophils | CXCR1 | CXCR2 | Red blood cells |
|--------|-------------|-------|-------|----------------|
| Wild type | 1.2 ± 0.1 | 0.8 ± 0.1 | 3.0 ± 0.2 | 13.2 ± 0.4 |
| H18A | 4.9 ± 0.6 | 7.8 ± 1.4 | 11.3 ± 1.7 | 12.7 ± 0.5 |
| F21A | 3.7 ± 0.4 | 5.0 ± 0.3 | 17.5 ± 2.1 | 21.7 ± 1.0 |
| H18D | 44.6 ± 5.1 | 75.1 ± 5.3 | 17.6 ± 1.6 | 55.2 ± 3.4 |
| F21D | 48.1 ± 9.0 | 26.9 ± 1.3 | 71.4 ± 7.5 | 396.8 ± 41.4 |
| H18A/F21A | 52.5 ± 9.1 | 61.4 ± 3.8 | 26.1 ± 2.9 | 49.4 ± 5.6 |
| H18A/F21S | 85.5 ± 14.3 | 127.1 ± 25.9 | 41.5 ± 2.5 | 67.9 ± 5.0 |
| H18A/F21D | 763.4 ± 160.2 | 251.2 ± 127.8 | 182.2 ± 6.7 | 1712.1 ± 147.2 |
| H18D/F21D | 484.0 ± 135.6 | 344.5 ± 117.3 | 184.1 ± 15.9 | 3186.7 ± 1096.8 |

* ND, not determined.
expressing either CXCR1 or CXCR2 to determine whether the IL-8 mutants exhibit receptor-subtype selectivity. Interestingly, similar to the binding with neutrophil membranes, large increases in the \(K_i\), up to 431-fold, were observed upon binding of IL-8 mutants to cells expressing CXCR1 (Table I). In contrast, a clearly distinct binding profile with less dramatic increases in the \(K_i\), up to 61-fold, were observed upon binding of IL-8 mutants to cells expressing CXCR2 (Table I). Further studies were focused on determining the binding profile of these IL-8 mutants to other IL-8 receptors. We selected to test the binding of IL-8 mutants to the red blood cells promiscuous chemokine receptor, the Duffy antigen, that binds IL-8, MGSA, Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), and Monocyte Chemotactic Protein-1 (MCP-1) (9, 10). Binding of IL-8 to red blood cells showed a \(K_i\) of 13.2 ± 0.4 nM (Table I) that was similar to the previously reported \(K_d\) (10). We found that single and double substitution IL-8 mutants showed up to 241-fold increases in the \(K_i\) (Table I) and a distinct binding profile from those exhibited against CXCR1 and CXCR2. These findings support the idea that IL-8 recog-

FIG. 2. Dose-dependent curves for calcium responses of HL-60 cells expressing CXCR1. HL-60 cells expressing CXCR1 loaded with Indo-1 were stimulated with several concentrations of IL-8 or IL-8 mutants. IL-8 (○), H18A (●), H18D (▲), F21A (□), F21D (●), H18A/F21A (□), and H18A/F21D (●) were added at the indicated concentrations for activation of CXCR1. The percentages of intracellular calcium responses stimulated by 20 nM IL-8 is referred as 100%. Values are means of triplicate determinations, and the bars of each point represent the standard errors.

FIG. 3. Dose-dependent curves for calcium responses of HL-60 cells expressing CXCR2. HL-60 cells expressing CXCR2 loaded with Indo-1 were stimulated with several concentrations of IL-8 or IL-8 mutants. IL-8 (○), H18A (●), H18D (▲), F21A (□), F21D (●), H18A/F21A (□), and H18A/F21D (●) were added at the indicated concentrations for activation of CXCR2. The percentages of intracellular calcium responses stimulated by 20 nM IL-8 is referred as 100%. Values are means of triplicate determinations, and the bars of each point represent the standard errors.

FIG. 4. H18A/F21D is a full agonist of CXCR2 but a partial agonist of CXCR1. HL-60 cells expressing CXCR1 (A) or CXCR2 (B) were loaded with Indo-1. IL-8 (20 nM) was added to HL-60 cells expressing CXCR1 or CXCR2 to induce maximal calcium responses. A, H18A/F21D (2 μM) was added to HL-60 cells expressing CXCR1. B, H18A/F21D (500 nM) was added to HL-60 cells expressing CXCR2. These are representative records of three independent determinations.

Fig. 5. IL-8 mutants released β-glucuronidase. Human neutrophils were treated with several concentrations of IL-8 or IL-8 mutants, and degranulation of β-glucuronidase was assayed as described under "Experimental Procedures." IL-8 (○), H18A (●), H18D (▲), F21A (□), F21D (●), H18A/F21A (□), H18A/F21D (●), H18A/F21S (□), and H18D/F21D (●) were added at the indicated concentrations. The percentages of β-glucuronidase release in the presence of 1 μM IL-8 is referred as 100%. Values are means of triplicate determinations, and the bars of each point represent the standard errors.
nizes CXCR1, CXCR2, and the Duffy antigen by distinct mechanisms.

**Activation of IL-8 Receptors**

Activation of IL-8 receptors were evaluated by measuring the agonist-dependent rise in intracellular calcium in neutrophils and HL-60 cells expressing either CXCR1 or CXCR2. All single and double mutants triggered maximal calcium responses in neutrophils (data not shown), suggesting that the activation mechanisms on the IL-8 mutants are preserved. However, neutrophils co-express both CXCR1 and CXCR2, and it is possible that some mutants selectively activate either CXCR1, CXCR2, or both. Mobilization of intracellular calcium were examined with HL-60 cells expressing either CXCR1 or CXCR2. We found that mutants with single substitutions, and the double mutant H18A/F21A, exhibited calcium responses in a dose-dependent fashion (Figs. 2 and 3). Maximal calcium responses were achieved at concentrations of IL-8 mutants near their $K_i$, indicating that full receptor occupancy is not necessary for maximal calcium response as previously observed with the fMLP chemoattractant receptor (27). Furthermore, because these mutants elicited maximal calcium responses as those of the wild type IL-8, it is likely that these mutant sites are major determinants for the binding affinity to the receptors but not for the activation mechanisms of the receptors. On the other hand, double mutants containing either polar and charged or two charged residues showed a unique activation profile. For example, the mutant H18A/F21D triggered a calcium response in HL-60 cells expressing CXCR2 in a dose-dependent fashion and a maximal calcium response as IL-8 did (Figs. 3 and 4B). In contrast, this mutant elicited a weak calcium response in HL-60 cells expressing CXCR1 (Figs. 2 and 4A). This observation indicates that double mutants containing one or two charged residues are full agonists of CXCR2 but are partial agonists of CXCR1. These data suggest that IL-8 activates CXCR1 and CXCR2 by distinct mechanisms.

**Neutrophil Responses**

**Release of β-Glucuronidase**—Except for double mutants H18A/F21D and H18D/F21D, all single and double mutants triggered release of β-glucuronidase in neutrophils (Fig. 5). This finding suggests that the rise in intracellular calcium mediated by binding of H18A/F21D or H18D/F21D to CXCR2 is not sufficient to elicit neutrophil degranulation.

**Superoxide Production**—Previous studies have indicated that MGSA, in contrast to IL-8, is a poor activator of superoxide production in neutrophils (28). We have tested the effect of single and double mutants on superoxide production. IL-8 mutants at concentrations ranging from 100 nM to 2 μM failed to trigger superoxide production. The H18A and F21A mutants were only partially active (Fig. 6). These data indicate that activation of calcium responses mediated by both IL-8 receptor subtypes in neutrophils is not sufficient to trigger superoxide production.
DISCUSSION

The results of this work have indicated that His^{18} and Phe^{21} are involved in the mechanisms of recognition to anti-IL-8 mAb (20), CXCR1 and CXCR2, and the Duffy antigen of red blood cells. The data show that IL-8 recognizes CXCR1, CXCR2, and the Duffy antigen by different mechanisms. Furthermore, despite the high degree of sequence homology of CXCR1 and CXCR2, they appear to be differentially activated by IL-8. This is consistent with our previous work indicating that IL-8 binding to IL-8 receptor subtypes causes a higher rate of internalization of CXCR2 than that of CXCR1 (29), suggesting that agonist binding to IL-8 receptor subtypes trigger different internalization signals. Finally, this work shows that the transient rise of intracellular calcium is not sufficient to trigger neutrophil responses including release of β-glucuronidase and superoxide production.

His^{18} and Phe^{21} are adjacent or are in the surface-exposed hydrophobic pocket that has been argued to drive the association of IL-8 to the receptor by hydrophobic interactions (17). In our present work, substitution of His^{18} and Phe^{21} for Ala caused modest changes in binding affinity, calcium responses, and release of β-glucuronidase. These observations indicate that the hydrophobic nature of these residues does not play a major role in the mechanisms of recognition and activation of the IL-8 receptors. Although, these single Ala mutants are poor activators of superoxide production, suggesting that these mutants are not full agonists in terms of production of superoxide. Single or double substitutions of His^{18} and Phe^{21} by polar or charged residues produce major changes in binding affinity to CXCR1 and the Duffy antigen. In contrast, minor changes were observed with CXCR2. These findings support the idea that this epitope plays a role in determining binding selectivity among IL-8 receptors.

Substitution of His^{18} and Phe^{21} by Ala, polar, or charged residues may lead to localized, long range, or gross structural changes. In our studies, the mutants do not appear to show gross structural changes because all the mutants trigger full calcium responses in neutrophils, and they exhibit different binding affinities toward CXCR1, CXCR2, and the Duffy antigen. In addition, NMR chemical shifts of the mutant F21A are indistinguishable from the wild type, suggesting that this mutation causes a localized change (17). His^{18} and Phe^{21} are in close proximity to each other on the protein (Fig. 7). Whether these two residues overlap or interact independently with the IL-8 receptor subtypes remain to be established.

In summary, our binding data generated with this set of IL-8 mutants strongly argue that distinct mechanisms operate in the recognition of IL-8 to CXCR1, CXCR2, and the Duffy antigen. Binding of IL-8 to the N terminus fragment of CXCR1 causes perturbations in the NMR chemical shifts of His^{18} and Phe^{21} (26). This finding plus our binding data suggest that the selective recognition of IL-8 receptor subtypes by IL-8 is mediated by the interaction of His^{18} and Phe^{21} with the variable N terminus domains of IL-8 receptors (6).

On the basis of the patterns of calcium responses elicited with this set of IL-8 mutants, we can distinguish at least two sets of mutants. One set composed of H18A, F21A, H18D, F21D, F21S, H18AF21A, and H18A/F21S exhibit wild-type calcium responses and release of β-glucuronidase. The other set composed of H18A/F21D and H18D/F21D are full agonists of CXCR2 but are partial agonists of CXCR1. This finding suggests that the chemical nature of both residues 18 and 21 in IL-8 is a major determinant for the calcium responses mediated by CXCR1 but not for the calcium responses mediated by CXCR2. It is likely that IL-8 contains distinct motifs for activation of IL-8 receptor subtypes. Interestingly, H18A and F21A release β-glucuronidase as the wild type IL-8 but are poor activators of superoxide production in neutrophils. This finding supports the idea that the single Ala mutants are partial agonists in terms of superoxide production. This study provides the framework to further elucidate the activation motifs in IL-8 that trigger the biological responses in neutrophils including superoxide production, phagocytosis, and degranulation.

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