Identification of a Signal Transduction Switch in the Chemokine Receptor CXCR1*

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Chemokine receptors belong to the superfamily of G protein-coupled receptors, which regulate the trafficking and activation of leukocytes, and operate as coreceptors in the entry of HIV-1. To investigate the early steps in the signal transmission from the chemokine-binding site to the G protein-coupling region we engineered metal ion-binding sites at putative extracellular sites in the chemokine receptor CXCR1. We introduced histidines into sites located in the second and third putative extracellular loops of CXCR1, creating single, double, and triple mutant receptors: R199H, R203H, D265H, R199H/R203H, R199H/D265H, R203H/D265H, R203H/H207Q, and R199H/R203H/D265H. Cells expressing the double mutants R199H/D265H and R203H/D265H and the triple mutant R199H/R203H/D265H failed to trigger interleukin 8-dependent calcium responses. Interestingly, calcium responses mediated by the single mutant R203H and the double mutants R199H/R203H and R203H/H207Q were blocked by Zn(II), indicating the creation of a functional metal ion-binding site. On the other hand, cells expressing all single, double, or triple histidine-substituted CXCR1 demonstrated high affinity binding to interleukin 8 in the presence and absence of metal ions. These findings indicate that occupation of the engineered metal-binding site uncouples the chemokine-binding site from the activation mechanism in CXCR1. Most importantly, we identify for the first time elements of an early signal transduction switch of chemokine receptors before the activation of cytoplasmic G proteins.

Chemokines are chemotactic cytokines regulating the trafficking and activation of immune cells (1, 2). Their function is mediated by chemokine receptors that belong to the superfamily of G protein-coupled receptors (GPCRs).³ Chemokines and their receptors have been implicated in several immune disorders including asthma, arthritis, atherosclerosis, and in the infection and propagation of HIV-1 (3–8). Chemokines are classified according to the location and number of cysteine residues in the N terminus. The CXC chemokines contain two cysteines separated by a single residue and include IL-8, melanoma growth stimulatory activity, platelet factor 4, neutrophil activating protein-2, and stromal cell-derived factor-1. The CC chemokines consist of two adjacent cysteines and include RANTES (regulated on activation, normal T cell expressed and secreted), and macrophage inflammatory protein-1α and -1β. The C chemokines contain a lone cysteine and include lymphotactin. The CXC chemokine contains two cysteines separated by three residues and is represented by fractalkine. Structural analysis of chemokines by x-ray crystallography and NMR spectroscopy revealed that chemokines exhibit a common structural fold regardless of the degree of their sequence homology or primary function (9–13). The polypeptide chain of chemokines is folded into three antiparallel β-strands with an α-helix at the C terminus. The CXC chemokine IL-8 is regarded as the paradigm of chemokines; it is secreted by many cell types in response to an inflammatory stimulus or injury and induces chemotaxis and activation of neutrophils. IL-8 binds to several chemokine receptors including CXCR1, CXCR2, Duffy, and the virus-derived receptors KSHV GPCR and ECRF3 (14–16). Whereas the N terminus of CXCR1 is the major determinant of ligand binding affinity and specificity (17, 18), the structural determinants for receptor activation before coupling to the cytoplasmic G proteins are unknown. This is because of the difficulties in distinguishing the activation mechanism from the high affinity binding and activation of G proteins. Thus, Ala-substituted CXCR1 at positions Arg-199, Arg-203, and Asp-265 displayed negligible IL-8 binding and IL-8-induced calcium responses (19). Previously, our studies showed that the mouse homologous CXCR2 did not bind human IL-8 with high affinity, although high concentrations of IL-8 triggered calcium responses in cells expressing the mouse CXCR2 (20). This observation suggested that the mouse homologous CXCR2 conserves the activation mechanism for IL-8 but lacks the structural motifs for conferring high affinity binding, supporting the hypothesis that the mechanism for chemokine receptor activation could be uncoupled from the high affinity site (21, 22). In the present study, we have demonstrated that conserved extracellular residues Arg-199 or Arg-203 and Asp-265 of CXCR1 are important for receptor activation but are not necessary for IL-8 binding. We identify for the first time structural elements of an early step in the signal transduction of CXCR1 before the coupling of cytoplasmic G proteins.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases were purchased from New England Biolabs, Inc. (Bedford, MA). The QuickChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). Oligonucleotides were purchased from Invitrogen and Integrated DNA Technologies, Inc. (Coralville, IA). All other chemicals were reagent grade unless stated otherwise.

His-scanning Mutagenesis—The QuickChange site-directed mu-
IL-8 binding and IL-8-induced intracellular calcium mobilization of HEK 293 cells expressing CXCR1 and His mutants in the absence and presence of Zn(II).

<table>
<thead>
<tr>
<th>CXCR1</th>
<th>B_{max} (pmole/10^6 cells)</th>
<th>Binding</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IL-8 IC_{50} ± S.E.</td>
<td>ZnCl_{2} IC_{50} ± S.E.</td>
</tr>
<tr>
<td>Wild type</td>
<td>1.5 ± 0.14</td>
<td>10.4 ± 1.0</td>
<td>1119.4 ± 77.0</td>
</tr>
<tr>
<td>R199H</td>
<td>2.1 ± 0.23</td>
<td>10.9 ± 1.4</td>
<td>1183.0 ± 211.0</td>
</tr>
<tr>
<td>R203H</td>
<td>3.4 ± 0.10</td>
<td>10.3 ± 1.6</td>
<td>899.5 ± 68.7</td>
</tr>
<tr>
<td>D265H</td>
<td>1.7 ± 0.26</td>
<td>7.9 ± 0.8</td>
<td>1244.5 ± 228.1</td>
</tr>
<tr>
<td>R199H/R203H</td>
<td>1.9 ± 0.25</td>
<td>20.0 ± 2.6</td>
<td>1059.9 ± 869.2</td>
</tr>
<tr>
<td>R203H/H207Q</td>
<td>3.1 ± 0.19</td>
<td>22.0 ± 2.5</td>
<td>774.5 ± 32.5</td>
</tr>
<tr>
<td>R199H/D265H</td>
<td>2.6 ± 0.31</td>
<td>7.7 ± 2.3</td>
<td>1584.9 ± 421.8</td>
</tr>
<tr>
<td>R203H/D265H</td>
<td>1.9 ± 0.20</td>
<td>11.4 ± 2.4</td>
<td>1409.3 ± 208.6</td>
</tr>
<tr>
<td>R199H/R203H/D265H</td>
<td>1.5 ± 0.10</td>
<td>8.4 ± 1.7</td>
<td>1458.5 ± 420.3</td>
</tr>
</tbody>
</table>

^a The IC_{50} for Zn(II)-inhibited calcium mobilization was extrapolated by using the standard equation, y = 100/(1 + k IC_{50}^x), where y is the percentage of the level of calcium mobilization at a given concentration of Zn(II), x is the concentration of Zn(II), k is the IC_{50}, and n is the Hill coefficient.

^b No activation.

Fig. 1. Dose-dependent curves for calcium responses of HEK 293 cells expressing CXCR1 or His mutants. Cells expressing wild type CXCR1 (●), single His mutant R199H (▲), R203H (▼), or D265H (○) or double His mutant R199H/R203H (●) or R203H/H207Q (▼) was loaded with the calcium indicator Indo-1. Calcium mobilization was monitored by fluorescence spectroscopy upon addition of IL-8 at the indicated concentrations. Values are means of three or four independent determinations, and the bars of each point represent standard deviations.

Fig. 2. Sequence alignment of IL-8 receptors encoded by genes from different species and viruses. Amino acid residues at 199, 203, and 265 are shown in the shaded area. The putative transmembrane domains (V and VI) of IL-8 receptors are highlighted by the brackets.

Cell Culture and Transfection—HEK 293 cells were seeded at 150,000 cells/well in a 6-well plate in 1:1 mixture of Dulbecco’s modified Eagle’s medium and nutrient mixture F-12 containing 10% fetal bovine serum supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. HEK 293 cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. One day after seeding, HEK 293 cells were transfected with the plasmid pcDNA 3.1 containing cDNAs encoding wild type CXCR1 and His mutants using FuGENE 6 (Roche Molecular Biochemicals). G418-resistant clones were isolated, and the expression was monitored by measuring specific binding of IL-8 to transfected cells.
sodium chloride, 4 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 1 mM sodium phosphate buffer, 20 mM HEPES, 1 mM probenecid, 0.1% bovine serum albumin, pH 7.4). HEK 293 cells were resuspended at 1 x 10^7 cells/ml and incubated with Indo-1/AM (Molecular Probes) at 37°C for 30 min. After 30 min, the HEK 293 cells were washed with physiological buffer solution and resuspended at 1 x 10^7 cells/ml. Calcium mobilization experiments were performed as described (23).

RESULTS AND DISCUSSION

His-scanning Mutagenesis of Extracellular Residues in CXCR1 Did Not Affect IL-8 Binding Affinity—Previous studies on human CXCR1 mutants, containing single or multiple Ala substitutions of extracellular residues Arg-199, Arg-203, and Asp-265, displayed low binding to IL-8 and negligible receptor activity, as demonstrated by the failure of IL-8 to induce calcium mobilization in HEK 293 cells expressing receptor mutants (19). Ala substitutions could induce long range structural changes and inactivation of the receptor or produce localized structural changes such as creating cavities at the protein-protein interface, which often results in loss of binding energy (24). To minimize the potential cavity-creating mutations we have created CXCR1 mutants with His substitutions of Arg-199, Arg-203, and Asp-265, as histidine provides a substantial steric bulk at these sites. CXCR1 mutants were stably expressed in HEK 293 cells, and Scatchard plot analysis indicated that the density of receptors in cells expressing His mutants and wild type CXCR1 varied from 1.5 to 3.4 pmol/10^6 cells (Table I). In contrast to Ala-substituted CXCR1, cells expressing His mutants did not show significant changes in IL-8 binding affinity, as demonstrated by their similar IC_{50} values derived from displacement binding studies (Table I). As the binding affinity of His-substituted CXCR1 mutants is indistinguishable from that of the wild type CXCR1 it is likely that these sites (Arg-199, Arg-203, and Asp-265) are not directly interacting with IL-8 because any modification at the interacting sites should cause a major reduction in binding affinity. For example, Glu substitution of Asp-113 in the β-adrenergic receptor, the interacting site with the amine moiety of adrenergic ligands, resulted in a dramatic reduction in the affinity of the receptor for both agonists and antagonists (25). It is therefore likely that Arg-199, Arg-203, and Asp-265 play a secondary role, possibly stabilizing the binding pocket of IL-8. We suggest that Ala substitutions at these sites create cavities at the interface of the IL-8-receptor complex, destabilizing the binding pocket in the receptor. His residues at these positions may provide the steric bulk to fill these cavities, restoring the architecture of the binding pocket. In contrast to our studies with CXCR1, His substitutions of the corresponding residues of the putative second extracellular loop in the promiscuous chemokine receptor KSHV GPCR (R208H/R212H) abolished binding to ^125I-IL-8 (26), suggesting that the mechanisms of IL-8 recognition by CXCR1 and KSHV GPCR are distinct.

His-scanning Mutagenesis of Extracellular Residues in CXCR1 Regulates IL-8-dependent Activation—In contrast to Ala-substituted mutants, CXCR1 mutants containing single His substitutions (R199H, R203H, and D265H) and the double mutants R199H/R203H and R203H/D265H mediated IL-8-dependent calcium responses with EC_{50}(s) similar to that of the wild type receptor (Fig. 1 and Table I). However, the double CXCR1 mutants R199H/D265H and R203H/D265H and the triple mutant R199H/R203H/D265H failed to trigger IL-8-dependent calcium responses, although these mutants exhibited high affinity binding to IL-8 (Table I). Sequence analysis of IL-8 receptors encoded by genes from different species and viruses reveals that Arg-199 or Arg-203 and Asp-265 sites are conserved in functional receptors (Fig. 2). However, the non-functional Duffy contains Ser and Lys at the sites corresponding to Arg-199 and Arg-203. We suggest that Asp-265 in combination with either Arg-199 or Arg-203 are important elements for transmitting the signal of IL-8 to the cytoplasmic domain of the receptor. The putative extracellular location of these residues

![Graph](http://www.jbc.org/)

**Fig. 3.** Zn(II)-inhibited IL-8-stimulated calcium responses in HEK 293 cells expressing His mutants R203H and R203H/H207Q. a, IL-8-induced calcium mobilization in cells expressing wild type CXCR1 (A), mutant R203H (B), and mutant R203H/H207Q (C) in the absence and presence of increasing concentrations of ZnCl2 (1–300 μM for mutants and 50–1000 μM for wild type CXCR1). These traces are representative records of three or four independent determinations. b, cells expressing wild type CXCR1 (●), mutant R203H (▼), or mutant R203H/H207Q (■) were loaded with the calcium indicator Indo-1. The maximum fluorescence induced by 50 nM IL-8 in the absence of ZnCl2 is referred to as 100% response. Values are means of three or four independent determinations, and the bars of each point represent standard errors.
Activation of CXCR1—We probed whether metal ions modulate the functional properties of His-substituted mutants of CXCR1, as Zn(II) coordinates readily with the surface-exposed His residue. This approach is based on the thought that binding of Zn(II) to His-substituted receptors should probe the proximity and orientation of His residues and regulate the functional properties of receptors in a metal-dependent manner (27, 28). For instance, binding of Zn(II) to His-substituted rhodopsin mutants blocked the activation of G proteins (29). This finding suggested that binding of Zn(II) restrains the structural changes involved in the transfer of the light signal to the G protein (transducin).

To determine whether a Zn(II) site has been created in His-substituted CXCR1 mutants we monitored the binding of Zn(II) by measuring the Zn(II)-inhibited IL-8 binding or activation of cells expressing wild type CXCR1 and mutants. Zn(II) produced negligible inhibition of IL-8 binding (Table I) of all single, double, and triple His-substituted CXCR1 mutants. These results are consistent with the view that these sites are not directly interacting with IL-8. Furthermore, Zn(II) did not inhibit the IL-8-triggered calcium responses of cells expressing wild type or single His-substituted CXCR1 (R199H or D265H). In contrast, Zn(II) dramatically inhibited the IL-8-induced calcium responses mediated by the single His-substituted CXCR1 (R203H) with an IC_{50} of 38.8 ± 5.0 μM (Table I and Fig. 3). Zn(II) also inhibited the calcium responses mediated by the double mutant R199H/R203H, although with a high IC_{50} of 337.5 ± 110.4 μM. This finding suggests that the introduction of His at position 203 created a Zn(II) site, which upon binding to the metal ion immobilizes this site, thereby preventing the transfer of the IL-8 signal to the cytoplasmic domain of CXCR1.

The fact that Zn(II) does not affect binding to IL-8 argues that the Zn(II) binding does not denature the receptor. We conclude that Zn(II) binding uncouples the binding of the ligand from the signal transmission. Our findings are in agreement with previous studies indicating that Zn(II) blocks the constitutive activation of the related CXCR1 receptor, KSHV GPCR (R208H/R212H) (26). A key unresolved question is, what is the nature of the Zn(II) coordination site? His-X_{2}-His on an α-helix creates the simplest Zn(II)-binding site (30). Modeling this site in CXCR1 suggested that the native residue His-207 is one helical turn from the introduced His-203, thus creating at least a consensus His-X_{2}-His-binding site for Zn(II). Alternatively, His-203 may have created a tetrahedral Zn(II)-binding site delineated by His-203, His-207, Asp-265, and an unidentified residue or water. We created a double mutant R203H/H207Q to determine whether His-207 is a part of the coordination site for Zn(II). Cells expressing the R203H/H207Q mutant exhibited IL-8 binding and IL-8-induced calcium mobilization as the wild-type receptor (Table I); however, this mutant was 3.6-fold less sensitive to Zn(II) than R203H mutant (Fig. 3 and Table I), suggesting that H207Q is a component of the coordination site for Zn(II). In conclusion, our studies identify for the first time the sites Arg-199 or Arg-203, His-207, and Asp-265 as important motifs in the early steps of the activation mechanism of CXCR1.

REFERENCES
