Human interleukin-8 receptors A (IL-8RA) and B (IL-8RB) are seven-transmembrane domain (TMD) neutrophil chemokine receptors with similar sequences (77% amino acid identity) and similar G protein selectivity, but markedly different selectivity for CXC chemokines. IL-8RB is selective for IL-8, growth-related oncogene α (GROα) and neutrophil-activating peptide-2 (NAP-2), whereas IL-8RA is selective only for IL-8. To identify selectivity determinants, we made eight chimeric receptors exchanging: 1) the three main regions of sequence divergence between IL-8RA and IL-8RB (the N-terminal segment before TMD1, the region from TMD4 to the end of the second extracellular (e2) loop, and the C-terminal tail), and 2) the N-terminal segment of CC chemokine receptor 1, which does not bind CXC chemokines. Chimeras were tested by direct 125I-NAP-2 binding, heterologous competition binding, and calcium flux assays using human embryonic kidney 293 cells stably transfected with receptor DNAs. The following results were obtained: 1) chimeric receptors had binding sites for IL-8, GROα and NAP-2 distinct from those on IL-8RA and IL-8RB; 2) IL-8, GROα and NAP-2 bound to overlapping but distinct sites that mapped differentially to multiple domains on IL-8RB; 3) high affinity radioligand binding and high agonist potency were separable functions for IL-8, GROα and NAP-2, suggesting that the determinants of high affinity binding may not be critical for receptor activation; and 4) determinants of GROα and NAP-2 selectivity were found in both the N-terminal segment before TMD1 and the region from TMD4 to the end of the e2 loop of IL-8RB, and functioned independently of each other. Stated reciprocally, the N-terminal segment of IL-8RA was not a dominant selectivity determinant. These data suggest that both narrow and broad spectrum chemokine antagonists can be developed to block functions mediated by IL-8RB.

Interleukin-8 (IL-8), growth-related oncogene α (GROα) and neutrophil-activating peptide-2 (NAP-2) are members of the CXC branch of the chemokine superfamily of leukocyte chemotactants and activating factors (1). All three molecules are relatively selective for neutrophils, binding to the same G-protein-coupled 7-transmembrane domain (TMD) receptor named interleukin-8 receptor B (IL-8RB; Refs. 2 and 3). While it is thought that IL-8, GROα, and NAP-2 act beneficially in host defense and tissue repair, there is so far little direct evidence. On the contrary, IL-8 has great pathologic potential, shown most clearly by the ability of intravenous infusions of anti-IL-8 antibodies to block lung ischemia-reperfusion injury (4) and endotoxin-induced pleurisy (5) in rabbit models. Thus, IL-8 antagonists could be useful clinically in disorders that have an acute inflammatory component, and substantial efforts are already under way to develop them. The aim of the present study was to obtain basic information about the nature of IL-8, GROα, and NAP-2 binding sites on IL-8RB that could be used to guide the development of antagonists.

By analogy with the structure of rhodopsin, the membrane-spanning segments of all 7TM receptors are thought to be arranged in a circle, causing the extracellular domains to bunch together (reviewed in Ref. 6). Very small ligands such as catecholamines bind mainly to TMD determinants, and very large glycoprotein ligands such as thyroid-stimulating hormone bind mainly to the unusually long N-terminal segment of their respective receptors (6). Small peptide ligands such as the neurokinins appear to bind to sites in the extracellular loops and TMDs (6). Chemokines are intermediate in size, ~70 amino acids, and chemokine receptors have relatively small N-terminal segments (1, 3). Since the potential cumulative binding surface of the chemokine receptor ectodomains is comparable in size to that of the ligand and since receptor binding is sensitive to truncation of both N- and C-terminal residues of IL-8 (7), it is likely that chemokine binding determinants are broadly distributed on multiple domains of the receptors. CSa is a non-chemokine chemotactic peptide for leukocytes that is similar in size to chemokines (3). It has been shown to bind to at least two sites on a specific 7TM receptor that is similar in size, organization and sequence to IL-8RB (8, 9). One site is present on the N-terminal extracellular segment before TMD1, and the second is within the TMDs.

The first experiments aimed at identifying receptor regions important for chemokine binding by IL-8RB took advantage of the fact that a second high affinity 7TM neutrophil IL-8 receptor exists (IL-8RA) that is 77% identical in amino acid sequence to IL-8RB, yet binds neither GROα nor NAP-2 (10–12). Most of the divergent residues are clustered in three regions: 1) the N-terminal segment before TMD1, 2) the region from TMD4 to the end of the second extracellular (e2) loop, and 3) the C-terminal cytoplasmic tail (Fig. 1). Using transiently transfected mammalian cells and human chemokines, both...
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LaRosa et al. (13) and Gayle et al. (14) reported that a chimeric receptor in which the N-terminal segment of rabbit IL-8RA (15) replaced the corresponding region of human IL-8RB (chimera AB1) bound $^{125}$I-IL-8 with high affinity, but GRO$\alpha$ (13, 14) and NAP-2 (13) competed weakly for binding, mimicking results for human (12) and rabbit wild type IL-8RA (15). Thus, the N-terminal segment appeared to be a dominant determinant of receptor subtype selectivity. The two groups reported conflicting results for the reciprocal chimera BA1; LaRosa et al. (13) reported high affinity $^{125}$I-IL-8 binding that was effectively competed by unlabeled GRO$\alpha$ and NAP-2 mimicking wild type IL-8RB, whereas Gayle et al. (14) reported that BA1 did not bind $^{125}$I-IL-8, even though BA1 expression on the cell surface was confirmed by detection of an epitope tag. Neither study addressed direct binding of either $^{125}$I-GRO$\alpha$ or $^{125}$I-NAP-2 or receptor activation, and the approach taken was not designed to identify the determinants of IL-8 selectivity. Moreover, the reported inability of human GRO$\alpha$ and NAP-2 to compete effectively for $^{125}$I-human IL-8 binding sites on the rabbit counterpart of IL-8RB raised the possibility that the properties of cross- and same-species IL-8 receptor chimeras could be fundamentally different (16).

In this paper we have addressed all of these concerns, by investigating both the agonist selectivity and ligand binding selectivity for wild type human IL-8RA and IL-8RB, and for a series of eight chimeric IL-8 receptors. We present a substantially expanded interpretation of the importance of the N-terminal segment in human IL-8 receptor subtype determination that is based on new findings regarding the complexity of CXC chemokine binding sites.

**Experimental Procedures**

Creation of Cell Lines Stably Expressing Wild Type and Chimeric Human IL-8 Receptors—All receptor constructs were first subcloned into Bluescript SKI$^+$ (Stratagene, La Jolla, CA) and then transferred into the NotI and Xhol sites of the hygromycin-selectable, stable episomal vector pCEF4 (Invitrogen, San Diego, CA). The sources of receptor DNA were as follows: IL-8RA, an EcoRI/HindIII genomic fragment (17); IL-8RB, the p3 cDNA (2); and CC CKR1 (also known as the MIP1$\alpha$/RANTES receptor), the p4 cDNA (18). Seven chimeric receptors were made by ligating restriction fragments of IL-8RA and IL-8RB taking advantage of conserved AccI, EcoNI, BamHl, and Ncol restriction endonuclease sites (Fig. 1). One chimera was made from IL-8RB and CC CKR1 DNA by polymerase chain reaction techniques (Fig. 1).

The numbers in the names of the first five chimeras listed denote the TMD where the switch occurs. In AB1 and BA1 all the divergent residues in the exchanged regions precede aa 37 of IL-8RA and aa 46 of IL-8RB. Thus, the switched divergent residues correspond to those studied in LaRosa et al. (13) and Gayle et al. (14). The fidelity of the chimeric expression plasmids was verified by DNA sequencing. Human embryonic kidney (HEK) 293 cells (10), grown to log phase in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, were electroporated with 20 $\mu$g of plasmid DNA as described previously (19). Multiple hygromycin-resistant colonies were picked and expanded in 150 $\mu$g/ml hygromycin.

Intracellular [Ca$^{2+}$] Measurements—HEK 293 cell transfectants (10$^6$/ml) were suspended in Hank's buffered saline solution with Ca$^{2+}$ and Mg$^{2+}$ and 10 $\mu$m Hepes, pH 7.4, containing 2.5 $\mu$m FURA-2-AM for 30 min at 37 $^\circ$C in the dark. The cells were subsequently washed twice in phosphate-buffered saline and then resuspended in Hank's buffered saline solution with Ca$^{2+}$ and Mg$^{2+}$ at 2 $\times$ 10$^5$ cells/ml. 2 ml of the cell suspension were placed in a continuously stirred cuvette maintained at 37 $^\circ$C in a MSIII fluorimeter (Photon Technology International Inc., South Brunswick, N J ). Fluorescence was monitored at $\lambda_{\text{ex}}$ = 340 nm, $\lambda_{\text{em}}$ = 380 nm, and the data presented as the relative ratio of fluorescence at 340 and 380 nm. Data were collected every 100–200 ms. To determine the agonist selectivity, the magnitude of the peak of [Ca$^{2+}$], changes elicited at varying concentrations of human CXC and CC chemokines (Peprotech, Rocky Hill, N J ) from transfected HEK 293 cells was determined. Each graphed point represents the peak response for one tracing. Each graph is from a single experiment representative of at least three separate experiments for a single done and represents four separate done tested.

**Ligand Binding Analysis**—To determine the ligand binding selectivity, 10$^6$ stably transfected cells were incubated in duplicate with $\sim$0.1 nm $^{125}$I-IL-8, $^{125}$I-GRO$\alpha$, or $^{125}$I-NAP-2, each having a specific activity $\sim$2000 Ci/mmol (DuPont NEN) and varying concentrations of unlabeled recombinant human chemokines in 200 $\mu$l of binding medium (RPMI 1640 with 1 mg/ml bovine serum albumin, pH 7.4). After incubation for 2 h at 4 $^\circ$C, cells were pelleted through a 10% sucrose/phosphate-buffered saline cushion and $\gamma$ emissions were counted. The data were curve-fitted with the computer program LIGAND (20) to determine the Kd and number of binding sites. To control for inter-experiment variability, the ligand binding profile of IL-8RB was analyzed concurrently on the same day that the ligand binding selectivity of an individual chimeric construct was determined. Maximum binding was determined as the percentage of total added counts that remained associated with the cell pellet, and is shown in the parentheses that follows the name of the corresponding radioligand in Figs. 2–5. The binding data depicted in Figs. 2–5 are for the done whose agonist properties are also shown. The binding data shown are representative of three separate experi-
The rank order of competition by unlabeled CXC chemokines for the radiolabeled binding sites on IL-8RB varied considerably for the three different radioligands (Fig. 2, f–h, and Table I). For example, unlabeled NAP-2 competed poorly for the $^{125}\text{I}-\text{IL-8}$-labeled sites on IL-8RB, whereas both NAP-2 and IL-8 were very effective in competing for the $^{125}\text{I}-\text{NAP-2}$-labeled sites on IL-8RB. These findings suggest that the sets of binding sites on IL-8RB for IL-8, GRO$\alpha$ and NAP-2 overlap but are not identical. The unlabeled CC chemokines MIP-1$\alpha$ and MCP-1 did not compete for binding at any of the CXC chemokine-labeled sites (Fig. 2, f–h).

These results confirm previous reports based on direct IL-8 and GRO$\alpha$ binding (11, 12) and provide new information about direct NAP-2 binding and the relative potency of all three chemokines on both receptor subtypes. To investigate the structural basis of the markedly different selectivities found for IL-8RA and IL-8RB, we made chimeric receptors in which the main regions of divergence were switched.

Multiple Domains of IL-8RB Independently Determine Selectivity for GRO$\alpha$ and NAP-2—GRO$\alpha$ and NAP-2 competed very weakly for IL-8 binding to the rabbit-human AB1 chimeras studied in LaRosa et al. (13) and Gayle et al. (14), having a rank order indistinguishable from IL-8RA, and suggesting logically that the N-terminal segment of the IL-8 receptors was a major determinant of receptor subtype selectivity. We were surprised, therefore, to find that GRO$\alpha$ and NAP-2 were both strong agonists for our human-human chimera AB1, having a rank order very similar to that of IL-8RB (Fig. 3a). The agonist selectivity of the complementary chimera BA1 was not complementary to AB1 and differed from both wild type receptors, but was much closer to IL-8RB than IL-8RA both in relative agonist potency and efficacy (Fig. 3e).

From these results we infer that independent determinants of GRO$\alpha$ and NAP-2 agonist selectivity are present in the IL-8RB-specific sequences both N-terminal and C-terminal to the switch point in TMD1. LaRosa et al. (13) and Gayle et al. (14) did not report the agonist selectivity of their chimeras. To compare more directly the properties of human-human AB1 and BA1 with those of rabbit-human chimeras, we carried out ligand binding experiments.

In fact, the rank order of competition for the $^{125}\text{I}-\text{IL-8}$ binding site on human-human AB1 was identical to that reported for rabbit-human AB1 and mimicked that for wild type human IL-8RA (compare Figs. 2b and 3b, and Table I). To address the apparent incompatibility between the indirect ligand binding and signal transduction results for GRO$\alpha$ and NAP-2, we carried out direct $^{125}\text{I}-\text{GRO}\alpha$ (Fig. 3c) and $^{125}\text{I}-\text{NAP-2}$ (Fig. 3d) binding with AB1, and found that both radioligands were able to bind with high affinity. The rank orders of competition determined with unlabeled CXC chemokines indicate clearly that the sets of binding sites for $^{125}\text{I}-\text{IL-8}$, $^{125}\text{I}-\text{GRO}\alpha$, and $^{125}\text{I}-\text{NAP-2}$ on AB1 are overlapping but distinct from each other, and distinct from the corresponding sites on IL-8RB (compare Fig. 3, panels b–d, and Fig. 2, panels f–h; see also Table I).

Chimera BA1 also had binding properties distinct from both wild type receptors. Despite the fact that IL-8 was its most potent agonist, similar in potency to wild type IL-8RB (EC$_{50}$ = 10 and 5 nM, respectively; Figs. 2e and 3e), we could not demonstrate specific binding of $^{125}\text{I}-\text{IL-8}$ to it (Fig. 3f). This matches the report of Gayle et al. for rabbit-human BA1 (14) but differs from that of LaRosa et al. (13). In contrast, $^{125}\text{I}-\text{GRO}\alpha$ (Fig. 3g) and $^{125}\text{I}-\text{NAP-2}$ (Fig. 3h) both bound to BA1 with high affinity. As for AB1, competition binding analysis with unlabeled CXC chemokines distinguished the binding sites for $^{125}\text{I}-\text{GRO}\alpha$ and $^{125}\text{I}-\text{NAP-2}$ on BA1 from those on IL-8RB (compare panels g
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Table I

| Construct | Agonist Potency EC<sub>50</sub> (nM) | [<sup>125</sup>I]-CXCR Chemokine Binding Affinity (K<sub>i</sub>, nM) |
|-----------|------------------------------------|----------------------------------------------------------|
|           | IL-8 | GRO<sub>x</sub> | NAP-2 | IL-8 | GRO<sub>x</sub> | NAP-2 | IL-8 | GRO<sub>x</sub> | NAP-2 |
| IL8RA     | 3    | 50     | 40    | 3.6  | ND     | ND     | 1.4  | 9.9    | 67    |
| IL8RB     | 5    | 8      | 9     | 1.4  | 9.9    | 67    | 0.8  | 1      | 1.9   |
| AB1       | 2    | 9.5    | 2     | 3.8  | ND     | ND     | 0.8  | 13     | ND    |
| BA1       | 10   | 20     | 50    | No specific binding | No specific binding | No specific binding | 3.4  | 0.7    | 8.4   |
| CC CKR1/IL8RB | 10 | 10     | 11    | No specific binding | No specific binding | No specific binding | 21   | ND     | ND    |
| ABA       | 0.5  | 6      | 10    | 2.2  | 17     | 53     | 0.05 | 1      | 0.5   |
| AB5       | 1    | 10     | 7     | No specific binding | No specific binding | No specific binding | No specific binding | No specific binding |
| BSA       | 3    | ND     | 11    | No specific binding | No specific binding | No specific binding | No specific binding | No specific binding |
| AB7       | 10   | 11     | 3     | No specific binding | No specific binding | No specific binding | 4.7  | 0.5    | 5     |
| AB8       | 8    | ND     | ND    | 1.8  | ND     | ND     | 1.5  | 0.3    | 1.2   |

Fig. 3. Chemokine selectivity of chimeric receptors switched in TMD1. HEK 293 cells stably transfected with the following chimeras, AB1 (a–d), BA1 (e–h), or CC CKR1/IL-8RB (i–l), were analyzed for their agonist selectivity (a, e, and i), and ligand binding selectivity to [<sup>125</sup>I]-IL-8 (b, f, and j), [<sup>125</sup>I]-GRO<sub>x</sub> (c, g, and k), and [<sup>125</sup>I]-NAP-2 (d, h, and l). At the top of each column of panels is the name of the corresponding chimeric receptor and a schema illustrating its sequence composition (white, IL-8RA sequence; black, IL-8RB sequence; diamond, CC CKR1 sequence). The code for the symbols used is given at the upper left of the figure. The average number of binding sites per cell calculated using either [<sup>125</sup>I]-IL-8 and unlabeled IL-8 or [<sup>125</sup>I]-NAP-2 and unlabeled NAP-2 was between 1 and 2 × 10<sup>5</sup> for AB1 and CC CKR1/IL-8RB, but 40,000 for BA1. Specific binding of [<sup>125</sup>I]-MIP-1α or [<sup>125</sup>I]-RANTES was not detected for CC CKR1/IL-8RB.

and h in Figs. 2 and 3; see also Table I).

To test further the importance of IL-8RB regions C-terminal to TMD1 for GRO<sub>x</sub> and NAP-2 selectivity, we replaced residues 1–50 of IL-8RB with the corresponding residues of the CC chemokine restricted receptor CC CKR1 (amino acids 1–34), making chimeric CC CKR1/IL-8RB. The switched residues comprise the N-terminal segments of the respective receptors (2, 18). The sequence of residues 1–34 of CC CKR1 differs markedly from the corresponding sequence of IL-8RA and IL-8RB, but all three have a high content of acidic residues and several invariant residues are present (Fig. 1). This chimera is also useful for mapping determinants of IL-8 selectivity in IL-8RB, which cannot be meaningfully analyzed in chimeras made from the two IL-8 receptors.

As for AB1, IL-8, GRO<sub>x</sub>, and NAP-2 all had high equipotent agonist activity for CC CKR1/IL-8RB (Fig. 3i). In contrast, the agonists for CC CKR1 (MIP-1α, RANTES, and MCP-3) lacked agonist activity for CC CKR1/IL-8RB (Fig. 3i). The agonist rank order was IL-8 = GRO<sub>x</sub> > NAP-2 (EC<sub>50</sub> ~ 10 nM in each case), similar to that for wild type IL-8RB and chimera AB1.
This further suggested that IL-8RB sequence C-terminal to TMD1 contains determinants of GRO\textsubscript{a} and NAP-2 agonist selectivity that can operate independently of GRO\textsubscript{a} and NAP-2 determinants in the N-terminal segment of IL-8RB. Furthermore, it indicated that the same region of IL-8RB contains determinants of IL-8 agonist selectivity.

As we found for AB1 and BA1, all of the binding sites on CC CKR1/IL-8RB were distinct from those on the wild type receptors (Fig. 3, j–l). The least unusual was the NAP-2 site (Fig. 3l). \(^{125}\text{I}-\text{NAP-2}\) bound to CC CKR1/IL-8RB with high affinity (\(K_i = 1 \text{ nM}\)). However, the rank order of competition for \(^{125}\text{I}-\text{NAP-2}\) binding was NAP-2 > GRO\textsubscript{a} > IL-8 for CC CKR1/IL-8RB (Fig. 3l; Table I) as compared to IL-8–GRO\textsubscript{a} > NAP-2 for IL-8RB (Table I). Binding of \(^{125}\text{I}-\text{GRO}\textsubscript{a}\) to CC CKR1/IL-8RB was much more anomalous. A high percentage of total radioligand added bound to cells expressing CC CKR1/IL-8RB; however, unlabelled GRO\textsubscript{a} did not compete for binding even when present in 500-fold molar excess (Fig. 3k). Paradoxically, unlabelled IL-8 competed for the \(^{125}\text{I}-\text{GRO}\textsubscript{a}\) site on CC CKR1/IL-8RB, but not very effectively (Fig. 3k). NAP-2 did not compete for \(^{125}\text{I}-\text{GRO}\textsubscript{a}\) binding, even though \(^{125}\text{I}-\text{NAP-2}\) bound with high affinity (Fig. 3, k and l). The IL-8 binding site on CC CKR1/IL-8RB was even more unusual and was reminiscent of the IL-8 binding site on BA1. Even though IL-8 was a potent agonist for CC CKR1/IL-8RB, \(^{125}\text{I}-\text{IL-8}\) binding was not detectable (Fig. 3, i and j). However, a binding interaction of IL-8 with CC CKR1/IL-8RB could be inferred by its ability to compete for the \(^{125}\text{I}-\text{GRO}\textsubscript{a}\) and \(^{125}\text{I}-\text{NAP-2}\) sites (Fig. 3, k and l).

Taken together, the results suggest that the determinants of high affinity \(^{125}\text{I}-\text{IL-8}\) binding differ from the determinants of high agonist activity for IL-8, and that the N-terminal segment of IL-8RB contains determinants of high affinity \(^{125}\text{I}-\text{IL-8}\) binding. The latter conclusion is consistent with the results of Suzuki et al. (21), who reported that a chimeric receptor composed of the N-terminal segment of rabbit IL-8RA and the sequence C-terminal to TMD1 of the mouse IL-8 receptor homologue bound \(^{125}\text{I}-\text{human IL-8}\) with high affinity, whereas the wild type mouse IL-8 receptor homologue did not (22, 23). The former conclusion was not tested by Suzuki et al. (21).

The most direct test of the importance of the N-terminal segment in chemokine selectivity and receptor function is to remove it; however, experiments with truncated receptors have been non-informative so far (Ref. 14).\(^2\) Mutagenesis of the IL-8RB-specific residues in chimera BA1 will be needed to locate more precisely the GRO\textsubscript{a} and NAP-2 selectivity determinants in this region. Additional mutagenesis of the invariant residues and acidic residues in this region may also provide important insights for the structural basis of chemokine selectivity.

Mapping of Differential Determinants of GRO\textsubscript{a} and NAP-2 Selectivity for IL-8RB—To locate more precisely the GRO\textsubscript{a} and NAP-2 selectivity determinants in the portion of IL-8RB C-terminal to TMD1, we created the chimeras ABA, AB5, and AB7 (Fig. 4). IL-8, GRO\textsubscript{a} and NAP-2 were all strong agonists for ABA, suggesting that the collection of IL-8RB-specific residues in the region from TMD4 to the end of the e2 loop contains GRO\textsubscript{a} and NAP-2 agonist selectivity determinants (Fig. 4a). IL-8 was actually 10-fold more potent for chimera ABA than for wild type IL-8RB (Fig. 4a and Table I; EC\textsubscript{50} for

\(^2\) S. K. Ahuja and P. M. Murphy, unpublished data.
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[Ca²⁺], transients 0.5 nm versus 5 nm), and 10–20-fold more potent than GROα and NAP-2 on ABA.

IL-8 was a potent agonist for chimera AB7, whereas GROα and NAP-2 were not agonists, suggesting that the IL-8RB-specific residues present in the C-terminal cytoplasmic tail do not determine GROα and NAP-2 agonist selectivity (Fig. 4e).

The region from the BamHI site to the Ncol site contains 10 amino acid differences between IL-8RA and IL-8RB that were not selectively tested in any of the chimeras presented so far (Fig. 1). In chimera AB5 this region was switched together with the C-terminal cytoplasmic tail (Fig. 4i). As we observed for ABA and AB7, IL-8 was a strong agonist for AB5 but GROα completely lacked agonist activity. However, NAP-2 remained a strong agonist (Fig. 4i).

Taken together, the results of these functional tests suggest that independent determinants of NAP-2 agonist selectivity are present in three separate regions of IL-8RB: 1) the N-terminal segment, 2) the region from TMD4 to the end of the e2 loop, and 3) among the 10 IL-8RB-specific amino acids from the BamHI and Ncol sites, but are not present in the C-terminal cytoplasmic tail. In contrast, independent determinants of GROα agonist selectivity are present in the first two of these three regions.

When binding experiments were carried out, the results for ABA and AB7 were very much in line with those expected based on the signal transduction results. In the case of ABA, 125I-IL-8, 125I-GROα, and 125I-NAP-2 all bound with high affinity (Fig. 4, b–d, and Table I). The apparent affinities of 125I-GROα and 125I-NAP-2 on ABA were similar to each other and to the corresponding affinities on IL-8RB, and correlated well with the relative potency of GROα and NAP-2 as agonists for both ABA and IL-8RB (Table I). The apparent affinity of ABA for 125I-IL-8 was ~200-fold greater than for 125I-GROα and 125I-NAP-2 on ABA and for 125I-IL-8, 125I-GROα, and 125I-NAP-2 on wild type IL-8RB, which correlated well with the high agonist activity of IL-8 for ABA relative to IL-8RB (Table I).

In the case of AB7, only 125I-IL-8 binding was detectable and the apparent affinity was similar to that observed for wild type IL-8RA and IL-8RB (Fig. 4f); unlabeled GROα and NAP-2 competed very ineffectively for 125I-IL-8 binding to AB7 (Fig. 4, g and h). This also correlated well with the signal transduction results for AB7 (Fig. 4e).

In contrast, even though NAP-2 was a potent agonist for AB5, we were unable to detect 125I-NAP-2 binding to it (Fig. 4i). A binding interaction of NAP-2 with AB5 consistent with its agonist activity could be inferred by the ability of unlabeled NAP-2 to compete for 125I-IL-8 binding to AB5 (Fig. 4i). This is the third chimera of those presented so far in which direct binding of a radiolabeled agonist was not demonstrable. 125I-GROα binding to AB5 was not detectable, nor did unlabeled GROα compete significantly for 125I-IL-8 binding (Fig. 4, j and k). These results were concordant with GROα’s lack of agonist activity for AB5 (Fig. 4i).

The results presented so far can be summarized as follows. 1) Complementary chimeras do not have complementary functional properties; 2) chimeric receptors have sets of chemokine binding sites that are distinct from those on wild type IL-8RA and IL-8RB; 3) high affinity 125I-chemokine binding and high chemokine potency are separable functions in chimeric receptors; 4) multiple independent regions of IL-8RB can determine agonist selectivity for GROα and NAP-2; and 5) the sets of binding sites for IL-8, GROα, and NAP-2 on IL-8RB are overlapping but distinct.

To test these conclusions further, we made two additional chimeras, BA5 and BAB, the complements of AB5 and ABA, respectively. IL-8, GROα, and NAP-2 were equipotent agonists for both chimeras, supporting conclusion 4 (Fig. 5, a and d). The agonist selectivities of BAB and BA5 support conclusion 1; the agonist selectivity of BAB was identical to that of ABA (Figs. 4a and 5a), whereas the agonist selectivity of AB5 and ABA were also similar except for the inability of AB5 to respond to GROα (Figs. 4i and 5e). The rank order of competition for each of the labeled sites on BA5 was distinct from IL-8RB, supporting conclusion 2 (Fig. 5, f–h, and Table I). Binding of radiolabeled IL-8, GROα, and NAP-2 was not detectable for BAB, even though all three unlabeled proteins were highly potent and effective agonists (Fig. 5, a–d), supporting conclusion 3. It is important to note that BAB contained IL-8RB-specific sequence in both the N-terminal segment and the region from TMD4 to the end of the e2 loop, i.e. in two of the regions that contain independent determinants of GROα and NAP-2 agonist selectivity shown in chimeras BA1, BAB, and ABA. The combined chemokine binding and agonist selectivity found for BA5 is the most like wild type IL-8RB of the eight chimeras tested (compare panels f–h in Figs. 2 and 5, and Table I). Gayle et al. (14)
reported competition binding with unlabeled IL-8 and GRO\textsubscript{a} and \textsuperscript{125}I-IL-8 to a rabbit-human BA5, made from rabbit IL-8RA and human IL-8RB, that had properties similar to those shown for human-human BA5 in Fig. 5. \textsuperscript{125}I-GRO\textsubscript{a} and \textsuperscript{125}I-NAP-2 binding and receptor activation were not reported in their study (14).

CXC Chemokine Binding Sites on Wild Type and Chimeric IL-8Rs Are Functionally Distinct—To further dissect ligand-receptor relationships we analyzed the [Ca\textsuperscript{2+}]\textsubscript{i} transients from the stable HEK 293 cell lines expressing IL-8RB, BA5, and CC CKR1/IL-8RB, in response to sequential stimulation with chemokines (Fig. 6). When cell lines expressing these three receptors were sequentially stimulated with 10 nM IL-8 and GRO\textsubscript{a} and 25 nM NAP-2, the following results were obtained (Fig. 6). For IL-8RB, IL-8 completely cross-desensitized the response to GRO\textsubscript{a} (Fig. 6) and NAP-2 (data not shown). These results confirm and extend those previously reported by Schraufstetter et al. (24) for human IL-8RB. In contrast, in BA5 transfectants, IL-8 only partially cross-desensitized the response of GRO\textsubscript{a}, whereas in CC CKR1/IL-8RB transfectants IL-8 did not alter the subsequent response to a second stimulation with GRO\textsubscript{a}. GRO\textsubscript{a} had little or no effect on a second stimulation with IL-8 from the three transfectants. NAP-2, which was a potent agonist for all three transfectants, did not alter the response to a second stimulation with IL-8 from both IL-8RB and BA5; however, it markedly attenuated the subsequent response of IL-8 from CC CKR1/IL-8RB (Fig. 6), and AB1 transfectants (data not shown). Thus, even though the agonist selectivity of BA5, CC CKR1/IL-8RB, and AB1 matched that of IL-8RB, the differences in cross-desensitization clearly indicated that the CXC chemokine binding sites were functionally distinct.

**DISCUSSION**

The present work illustrates the complexity of CXC chemokine interactions with IL-8RB. The major findings are as follows. 1) IL-8, GRO\textsubscript{a}, and NAP-2 selectivity determinants appear to be broadly but differentially distributed on multiple domains of IL-8RB; and 2) low affinity IL-8, GRO\textsubscript{a}, and NAP-2 binding sites appear to be capable of mediating efficient receptor activation.

The first conclusion is best illustrated by two examples. First, the efficacy of competition by unlabeled NAP-2 varied considerably for the \textsuperscript{125}I-IL-8-, \textsuperscript{125}I-GRO\textsubscript{a}-, and \textsuperscript{125}I-NAP-2-labeled sites on IL-8RB; second, three separate regions of IL-8RB can independently confer NAP-2 agonist activity to IL-8RA-IL-8RB chimeric receptors (BA1, ABA, AB5, and AB7). Furthermore, two of these three regions can independently confer GRO\textsubscript{a} selectivity to IL-8RA-IL-8RB chimeric receptors. Thus, in contrast to two previous reports suggesting that the N-terminal segment of the IL-8 receptors is a dominant determinant of receptor subtype selectivity (13, 14), our data would indicate that the N-terminal segment of IL-8RB, but not IL-8RA, is dominant.

Although the backbone structures of chemokines are very similar, differences in the side chains and/or in the composition of the unordered N-terminal sequence prior to the first conserved cysteine must account for the complexity of binding to IL-8RB, as well as for the dramatic and unpredictable differences in chemokine selectivity observed for the CC chemokine receptors\textsuperscript{3} (3, 18, 25). Given the likelihood that 7TM receptors probably lack independently folding domains, it should not be surprising that “artificial” receptors such as the chimeric receptors we tested have ligand binding sites that are in some cases strikingly and unpredictably different from those of the wild type receptors. Nevertheless, all of the chimeric receptors tested were restricted to interactions with CXC chemokines and failed to interact with CC chemokines, a characteristic shared with wild type IL-8RA and IL-8RB.

In five instances we observed that chimeric receptors could be efficiently activated by one or more CXC chemokines, whereas direct binding to the chimera by the corresponding iodinated protein could not be demonstrated. The examples include IL-8 in the case of the chimeras BA1 and CC CKR1/IL-8RB; NAP-2 in the case of chimera AB5; and IL-8, GRO\textsubscript{a}, and NAP-2 in the case of chimera BAB. The extent to which chemical modification of the ligands by iodination, and changes in on rates and off rates, account for these surprising results has not been examined.

\textsuperscript{3}C. Combadiere, S. K. Ahuja, H. L. Tiffany, and P. M. Murphy, submitted for publication.
been determined yet. Based on the Kᵣ values determined by heterologous competition binding that were available for chimeras BA1 and CC CKR5-LIL-8RB, we estimate that the affinity in each of these cases is reduced at least 20-fold relative to the corresponding affinity determined on IL-8RB. This implies that the determinants of high affinity binding for 125I-IL-8, 125I-GROᵦ, and 125I-NAP-2 by the wild type receptors may differ from those responsible for high agonist potency, at least for calcium mobilization, the function that we tested directly. It is possible that high affinity binding may be essential for some other function of IL-8RB, such as neutrophil chemotaxis.

Functionally significant low affinity chemokine binding sites have been proposed for two wild type chemokine receptors. First, endothelial cells migrate and proliferate in response to IL-8 presumably by binding to a specific receptor (reviewed in Ref. 1); however, 125I-IL-8 binds to endothelial cells with low affinity (26). Second, the CC chemokines MIP-1α, RANTES, and MIP-β are strong agonists for a CC chemokine receptor named CC CKR5, but direct binding of the corresponding radioligands has not been demonstrated.3

Our findings imply that distinct antagonists could be developed that block either high affinity binding but not receptor activation, or receptor activation but not high affinity binding, or both high affinity binding and receptor activation. The most useful antagonists clearly would be those that block receptor activation. Since the sets of binding sites for IL-8, GROᵦ, and NAP-2 on IL-8RB are overlapping but distinct, both narrow and broad spectrum chemokine antagonists could conceivably be developed that act specifically at IL-8RB or more broadly at both IL-8RA and IL-8RB.

Our results provide a broad foundation for future investigations of the structural basis of chemokine selectivity for IL-8RB as well as the mechanism of receptor activation. Site-directed mutagenesis of IL-8RB will be important for identifying functionally critical residues both within the set of residues that are different between IL-8RA and IL-8RB as well as within the set of conserved residues, as these could have different functional significance in different sequence contexts. Extensive site-directed mutagenesis studies for IL-8RA, testing both IL-8 binding and IL-8-induced calcium flux responses have been reported (27, 28). Both functions were insensitive to alanine substitution for each of the amino acids in the N-terminal segment, with the exception of the cysteine at position 30 (27, 28). The C30A phenotype probably reflects an important role of this cysteine in the overall folding of the receptor. It would be surprising if the highly acidic nature of the N-terminal segment of the IL-8 receptors were not functionally important, however evidence is currently lacking. Systematic alanine-scanning mutagenesis of all the amino acids in the ectodomains of IL-8RA has shown that amino acids Cys-187, Arg-199, Arg-203, Asp-265, and Cys-277 in the predicted second and third extracellular loops were sensitive to mutation for both IL-8 binding and signal transduction (28). All of these positions are conserved in the sequence of IL-8RB and therefore are unlikely to discriminate receptor subtype selectivity.

In summary, our results illustrate the complex nature of CXC chemokine binding sites and show that the inferences drawn from them for the properties of chimeric chemokine receptors can differ for direct or indirect binding or signal transduction data sets. IL-8RB subtype selectivity is not simply determined by the N-terminal segments, but instead is determined by multiple regions on IL-8RB that can function independently in IL-8RA/IL-8RB chimeric contexts. Future studies using CXC chemokine mutants, the chimeric receptors we have described and site-directed mutagenesis may help to infer a more detailed model of chemokine binding to IL-8 receptors, which can be tested by more direct physical and chemical methods.

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