The CXC Chemokines Growth-regulated Oncogene (GRO)α, GROβ, GROγ, Neutrophil-activating Peptide-2, and Epithelial Cell-derived Neutrophil-activating Peptide-78 Are Potent Agonists for the Type B, but Not the Type A, Human Interleukin-8 Receptor*

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Interleukin-8 (IL-8), growth-related oncogene (GRO)α, GROβ, GROγ, neutrophil-activating peptide-2 (NAP-2), epithelial cell-derived neutrophil activating peptide-78 (ENA-78), and granulocyte chemoattractant protein-2 are potent neutrophil chemoattractants 40–90% identical in amino acid sequence that comprise a subgroup of human CXC chemokines defined by the conserved sequence motif glutamic acid-leucine-arginine (ELR). Two human chemotactic receptor subtypes for IL-8, named IL-8 receptors (IL8R) A and B, have been cloned. They are 78% identical in amino acid sequence, coexpressed in neutrophils, and distinguished by their different selectivities for GROα and NAP-2. Their selectivity for other ELR+ CXC chemokines has not been previously reported. By measuring calcium flux in human embryonic kidney 293 cells transfected with plasmids encoding IL8RA or IL8RB, we have now defined receptor selectivity for GROβ, GROγ, and ENA-78. The rank order of agonist potency, based on inspection of the mean effective concentration values (EC50), for IL8RB was GROγ (1 nM) > IL-8 (4 nM) > GROα (5 nM) > GROβ (4 nM) > NAP-2 (7 nM) > ENA-78 (11 nM), and for IL8RA was IL-8 (4 nM) >> ENA-78 (40 nM) > NAP-2 (45 nM) > GROα (63 nM) > GROγ (65 nM) >> GROβ. The maximal response of IL8RA to IL-8 was at least 2-fold greater than the other five chemokines. All six agonists for IL8RB competed for high affinity 125I-GROα, NAP-2, and ENA-78 binding sites at IL8RB. GROα, GROβ, GROγ, NAP-2, and ENA-78 competed weakly for the high affinity IL-8 binding site at IL8RA. Thus, IL8RA and IL8RB are both highly selective for IL-8 and have similar sequences but differ dramatically in their selectivity for all other ELR+ CXC chemokines tested. These findings have important implications for developing novel neutrophil-specific anti-inflammatory drugs directed against the CXC chemokine signaling system.

Chemokines are a superfamily of proinflammatory peptides ~70–80 amino acids long that attract and activate leukocytes by binding to G protein-coupled receptors on the cell surface (1, 2). They can be classified into one of three subfamilies, C, CC, or CXC, based on the number and arrangement of conserved cysteine residues (1, 3, 4). The CXC subfamily can be further subdivided into ELR+ and ELR− groups, based on the presence or absence of the sequence motif glutamic acid-leucine-arginine (ELR) N-terminal to the first cysteine (1). All ELR+ CXC chemokines attract and activate human neutrophils in vitro at low nanomolar concentrations and induce neutrophil recruitment in vivo, whereas the ELR− CXC chemokines are not neutrophil chemoattractants (1, 5–7). Seven human ELR+ CXC chemokines have been described, IL-8,1 GROα, GROβ, GROγ, NAP-2, ENA-78, and granulocyte chemoattractant protein-2. The GRO proteins are ~90% identical in amino acid sequence; IL-8, NAP-2, granulocyte chemoattractant protein-2, and ENA-78 are ~40–50% identical to each other and to any of the GRO proteins (1, 5).

Manipulation of the chemokine system in mice and rabbits has begun to reveal important roles in the pathogenesis of inflammation for ELR+ CXC chemokines (8–10). Antibody neutralization in vivo of rabbit IL-8 and a mouse homologue of the GRO proteins, MIP-2, markedly reduced neutrophil-mediated inflammation in several models of acute inflammation. Mice with targeted disruption of the gene for the receptor for MIP-2 had markedly reduced neutrophil accumulation after chemically induced peritonitis (11, 12). Thus, the receptors for ELR+ CXC chemokines may be good targets for developing neutrophil-specific anti-inflammatory drugs.

In previous studies, competition binding assays have been used to identify human neutrophil receptors for ELR+ CXC chemokines. NAP-2, GROα, GROβ, and GROγ all compete for 125I-IL-8 binding sites on neutrophils, although less effectively than IL-8 itself (13, 14). In contrast, IL-8, GROα, GROβ, and GROγ are all able to compete completely for 125I-GROα binding sites on neutrophils, with a rank order of IL-8 = GROα = GROγ > GROβ (13, 14). This suggested that human neutrophils express at least two ELR+ CXC receptor subtypes: at least one shared by IL-8 and other ELR+ CXC chemokines and one restricted to IL-8.

cDNAs encoding two human neutrophil IL-8 receptor subtypes, named IL8RA and IL8RB, have been cloned (15, 16). IL8RA and IL8RB are members of the rhodopsin-like 7-transmembrane domain receptor superfamily and have 78% amino acid identity (2). IL8RB binds IL-8, GROα and NAP-2 with high affinity, whereas IL8RA binds only IL-8 with high affinity (15–20). Likewise IL-8, GROα, and NAP-2 are potent agonists for IL8RB when calcium flux and chemotaxis are measured in transfected cells, whereas IL-8 is the only potent agonist found so far for IL8RA (19, 20). Neither IL8RA nor IL8RB recognize CC chemokines or ELR− CXC chemokines.

1The abbreviations used are: IL-8, interleukin-8; GRO, growth-regulated oncogene; NAP-2, neutrophil-activating peptide-2; ENA-78, epithelial cell-derived neutrophil activating peptide-78; MIP, macrophage inflammatory protein; IL8, IL-8 receptor; HEK, human embryonic kidney.
Competition binding studies with ENA-78 and granulocyte chemoattractant protein-2 have not been reported yet. Moreover, the functional receptors for GRO-β, GRO-γ, ENA-78, and granulocyte chemoattractant protein-2 have not been defined, although given the broad selectivity of IL8RB for IL-8, GRO-α, and NAP-2, IL8RB is the best known candidate. Using both competition binding assays and a calcium flux assay of receptor activation, we now show directly that IL8RB has similar selectivity for IL-8, GRO-α, GRO-β, GRO-γ, NAP-2, and ENA-78, whereas IL8RA is at least 40-fold more selective for IL-8 than for the other ELR+ CXC chemokines.

**EXPERIMENTAL PROCEDURES**

Creation of Cell Lines Expressing IL-8 Receptors—The sources of wild type human IL-8 receptor DNA were IL8RA, a ∼2 kilobase pair EcoRV-HindIII genomic fragment (21); IL8RB, the 17-kilobase pair cDNA designated as p3 (16). Receptor DNA was first subcloned into Bluescript KS+ genomic fragment and then transferred into the Ncol and Xhol sites of the hygromycin-selectable, stable episomal vector pCEP4 (Invitrogen, San Diego, CA). Human embryonic kidney (HEK) 293 cells (107) grown to log phase in Dulbecco's modified Eagle's medium with 10% fetal bovine serum were electrophorized with 20 μg of plasmid DNA as described previously (19). Multiple hygromycin-resistant colonies were picked and expanded in 150 μg/ml of hygromycin.

Preparation of Human Neutrophils—Neutrophils were purified from peripheral blood of healthy volunteers by Hypaque/Ficoll density gradient centrifugation, and hypotonic lysis of residual erythrocytes (22). The cell preparations were 95–99% pure as measured by flow cytometry.

Ligand Binding Analysis—107 stably transfected cells or neutrophils were incubated with 0.1 nM [125I]-labeled IL-8, 125I-GRO-α, or 125I-NAP-2, each having a specific activity of 2200 Ci/mmol (DuPont NEN) and varying concentrations of unlabeled recombinant human chemokines (IL-8, GRO-α, NAP-2, and ENA-78) and monocyte chemoattractant protein-1 from PeproTech, Rocky Hill, NJ; GRO-γ and GRO-β and ENA-78 from R & D; monokine inducible by γ interferon was a kind gift of Dr. J. Farber, NIH) in a total volume of 200 μl of binding medium (RPMI 1640 with 1 mg/ml bovine serum albumin, pH 7.4). After incubation for 2 h at 4°C, cells were pelleted through a 10% sucrose-phosphate-buffered saline cushion, and γ emissions were counted. The data were curve fit with the computer program LIGAND (23) to determine the dissociation constant and number of binding sites. To control for inter-experiment variability, binding experiments with each of the labeled and unlabeled CXC chemokines were performed on the same day and with the same reagents. Maximum binding was determined as the percentage of total added counts that remained associated with the cell pellet. Each concentration of unlabeled chemokine was tested in duplicate. Within each individual experiment, at each concentration of unlabeled chemokine tested, the percentage of counts that remained associated with the cell pellet was within 5–10% of the average. Between experiments, at a given concentration of unlabeled chemokine tested, the percentage of maximum total binding was within 5–15% of each other. Control competition binding experiments with HEK 293 cells transfected with IL8RA or IL8RB were included in the same experiments in which competition binding of radiolabeled chemokines to intact neutrophils was tested.

Intracellular [Ca2+] Measurements—HEK 293 cell transfectants (107/ml) were suspended in Hanks' buffered saline solution with Ca2+ and Mg2+ and 10 mM Hepes, pH 7.4, containing 2.5 μM FURA-2 for 30 min at 37°C in the dark. The cells were then washed twice in phosphate-buffered saline and then resuspended in Hanks' buffered saline solution at 2 × 106 cells/ml. M cells were placed into a continuously stirred cuvette maintained at 37°C in a MSIII fluorimeter (Photon Technology International Inc., S. Brunswick, NJ). Fluorescence was monitored at λmax = 340 nm, λaux = 380 nm, and λem = 510 nm, and the data presented as the relative ratio of fluorescence at 340 and 380 nm.

Statistical Analysis—We initially screened four independent cell lines expressing IL8RA and four independent cell lines expressing IL8RB. All four cell lines in each group gave the same chemokine specificity, which differed from those of the other group. The data presented are from one representative cell line from each group. Analysis of variance with Tukey's studentized range test was used to compare the mean of the EC50 values for the calcium flux responses for each CXC chemokine tested (SAS statistical software).

**RESULTS AND DISCUSSION**

IL-8 Receptor Agonist Selectivity—To test the functional relationship of GRO-β, GRO-γ, and ENA-78 with IL8RA and IL8RB, we created HEK 293 cell lines stably transfected with the corresponding receptor open reading frames and measured transient calcium flux responses in FURA-2-loaded cells stimulated with chemokines. This functional response is a convenient measure of receptor activation and is highly correlated with chemotaxis, degranulation, and other functional responses of leukocytes to chemokines (1). Stimulation induces a rapid concentration-dependent increase in the level of [Ca2+]i, that returns to resting values within two minutes.

Compared with IL-8, GRO-γ and ENA-78 were weak agonists for IL8RA (Fig. 1, left panel). They both induced a concentration-dependent response; however, the threshold was 25–100-fold greater than for IL-8. Moreover, the maximal responses to GRO-γ and ENA-78 were at most 40% of the maximal response to IL-8. GRO-β was even less active than GRO-γ and ENA-78 at IL8RA, requiring concentrations greater than 100 nM to induce very small calcium flux responses, less than 10% of the peak IL-8 response (Fig. 2, top left tracing). The potency and efficacy of GRO-γ and ENA-78 for inducing calcium flux in IL8RB-transfected HEK 293 cells were similar to GRO-α and NAP-2 (Table I and Fig. 2; Ref. 19). The rank order based on mean EC50 values from at least three experiments for each chemokine was IL-8 >> ENA-78 — NAP-2 > GROα = GROγ >> GROβ.

In contrast to IL8RA, GRO-β, GRO-γ, and ENA-78 were all potent agonists at IL8RB. Fig. 1 (right panel) is representative of their activity relative to IL-8. In this system, the rank order based on inspection of the mean EC50 values from three experiments for each chemokine was GRO-γ > IL-8 — GROα — GROβ — NAP-2 > ENA-78. It is important to note that the most potent agonist, GRO-γ, was only 4-fold more potent than IL-8,
and the weakest agonist, ENA-78, was only 2.5-fold less potent than IL-8. The EC_{50} value for GRO_γ was significantly different only from those for ENA-78, NAP-2, and GRO_α (p < 0.05; one-way analysis of variance test). The maximal calcium flux responses at the optimal concentration (efficacy) for GRO_β, GRO_γ, and ENA-78 were similar to that induced by IL-8. GRO_α and NAP-2 were also equipotent with IL-8 (Table 1; Ref. 19). IL8RA- and IL8RB-transfected cells did not respond to the ELR^- CXC chemokines γ interferon-inducible protein and monokine inducible by γ interferon or to the CC chemokines MIP-1α and monocyte chemoattractant protein-1 when tested at 100 nM (data not shown). Neither untransfected nor mock-transfected, hygromycin-selected HEK 293 cells responded to any of the chemokines tested (data not shown).

Thus GRO_α, GRO_β, GRO_γ, NAP-2, and ENA-78 all induce chemotaxis and calcium flux responses in neutrophils (14) and are potent inducers of calcium flux responses in IL8RB but not IL8RA transfectants, suggesting that IL8RB may be the specific receptor that mediates the neutrophil responses to these molecules. The potency order for the human neutrophil calcium flux response has not been systematically studied for all the CXC chemokines. Geiser et al. (14) have reported dose-response tracings at 1, 3, and 10 nM for GRO_α, β, and γ using calcium flux as an end point in FURA-2-loaded human neutrophils. Based on inspection of the representative tracings shown in their study, the potency order appeared to be GRO_α > GRO_β = GRO_γ, with GRO_α less than 10-fold more potent than the others. This order differs from that found for IL8RB transfectants in our study, GRO_γ > GRO_α = GRO_β; however, it is again important to note that as for the neutrophil potency order, the differences were small between the most and least potent chemokine tested. The small difference between the potency order for neutrophils and IL8RB transfectants could result from many possible sources, including differences in the sources of the GRO proteins used, cell type differences and differences in expression levels of receptors. Based on inspection of representative experiments, the potency order varied for other neutrophil functions tested by Geiser et al. (14): chemotaxis, shape change, binding competition, degranulation, and respiratory burst activation; but the EC_{50} values appeared to be consistently within 10-fold of each other as they are in our study of IL8RB. Additional studies will be needed to determine whether the CXC chemokine potency order determined for calcium flux for IL8RB is the same for other functions activated by the receptor.

Desensitization of IL-8 Receptors by ELR^- CXC Chemokines—Activation of G protein-coupled receptors typically induces a refractory period during which the receptor cannot transduce signals when stimulated a second time with the same or other agonists, a phenomenon known as desensitization. Desensitization can be homologous (same agonist, same receptor), heterologous (different agonists, same receptor), or involve the same or different agonists acting at different receptors (receptor cross-desensitization).

We and others have previously shown that when tested at equipotent concentrations, IL-8 can desensitize GRO_α and NAP-2 activation of IL8RB, whereas GRO_α and NAP-2 given first reduce but do not abolish the calcium flux signal induced by IL-8 given second (19, 24). Thus, ELR^- CXC chemokines can differ in their potency for receptor activation and heterologous desensitization.

After pre-activation with 150 nM GRO_β, GRO_γ, or ENA-78, the responses of IL8RB transfectants to 10 nM IL-8 given second were reduced at most 50% (Fig. 2). Cells stimulated first with 10 nM IL-8 did not respond to 50 nM ENA-78 given second but exhibited a full response to 150 nM ENA-78 given second. When IL8RB transfectants were sequentially stimulated with chemokines at 10 nM, the following results were obtained (Fig. 2). IL-8 given first abolished the response to a second stimulation with GRO_β, GRO_γ, and ENA-78 (Fig. 2 and data not shown). GRO_β and GRO_γ markedly reduced the response to a second stimulation with IL-8, whereas ENA-78 was much less effective, consistent with its lower agonist activity (Fig. 2).

IL-8 Receptor Ligand Selectivity—We next examined the ability of 125I-IL-8, 125I-GRO_α, 125I-NAP-2, and 125I-ENA-78 to bind directly to intact HEK 293 cells expressing IL8RA or...
IL8RB and the ability of unlabeled IL-8, GRO\textsubscript{a}, GRO\textsubscript{b}, GRO\textsubscript{g}, NAP-2, and ENA-78 to compete for each of the binding sites found (Fig. 3). Only \textsuperscript{125}I-IL-8 bound with high affinity to IL8RA transfectants (IC\textsubscript{50} for IL-8 = 7 ± 2 nM) (Fig. 3, a–d, and Table II). Consistent with their weak agonist activity, binding of GRO\textsubscript{a}, GRO\textsubscript{b}, GRO\textsubscript{g}, NAP-2, and ENA-78 to IL8RA could be inferred by their ability to compete partially for \textsuperscript{125}I-IL-8 binding; total binding was reduced by only 5–30% when cold chemokines were present in 5000-fold molar excess over \textsuperscript{125}I-IL-8 (Fig. 3, a). The CC chemokines MIP-1\textalpha and monocyte chemoattractant protein-1 did not compete for IL-8 binding (data not shown; Ref. 19).

\textsuperscript{125}I-IL-8, \textsuperscript{125}I-GRO\textsubscript{a}, \textsuperscript{125}I-NAP-2, and \textsuperscript{125}I-ENA-78 each bound with high affinity to IL8RB transfectants (Fig. 3, e–l). In general, the hierarchy for binding competition was similar to the hierarchy for signal transduction; however, we noted three anomalies. First, cold IL-8, GRO\textsubscript{a}, GRO\textsubscript{b}, GRO\textsubscript{g}, and ENA-78 competed with similar potency and efficacy for each of the four binding sites, but NAP-2 was consistently much less potent and effective than the others at competing for IL-8 and GRO\textsubscript{a} binding but equally effective at competing for NAP-2 and ENA-78 binding (Fig. 3, e–h). Second, all six unlabeled ELR\textsuperscript{+} CXC chemokines consistently competed; 20–50-fold more potently for the ENA-78 binding site than for the IL-8, GRO\textsubscript{a}, and NAP-2 binding sites (Fig. 3, e–l). Third, ENA-78 was consistently as potent and effective a competitor as IL-8, GRO\textsubscript{a}, GRO\textsubscript{b}, and GRO\textsubscript{g} for each of the four labeled sites, yet it was consistently 4-fold less potent than the others as an agonist (Fig. 1–3 and Tables I and II). These anomalies could result if the binding sites for NAP-2 and ENA-78 at IL8RB differ from the binding sites for IL-8, GRO\textsubscript{a}, GRO\textsubscript{b}, and GRO\textsubscript{g} or if the iodinated chemokines have significantly different binding properties from the uniodinated chemokines. Scatchard analysis of the binding data for all four radioligands indicated a single class of high affinity binding sites for IL-8, GRO\textsubscript{a}, and NAP-2 at IL8RB and 1–2 \times 10\textsuperscript{5} sites per cell, suggesting that
There is only one functional subtype of IL8RB in these cells. ELR\(^+\) CXC Chemokine Binding Sites on Human Neutrophils—When \(^{125}\text{I}\)-IL-8 was used to label intact human peripheral blood neutrophils, 1000-fold molar excess of cold IL-8 consistently reduced binding by 85%, whereas 1000-fold molar excess of cold IL-8, GRO\(\alpha\), GRO\(\beta\), GRO\(\gamma\), NAP-2, or ENA-78 consistently reduced binding by at most 30% (Fig. 4). When \(^{125}\text{I}\)-GRO\(\alpha\) was used to label neutrophils, excess cold IL-8, GRO\(\alpha\), GRO\(\beta\), GRO\(\gamma\), NAP-2, and ENA-78 consistently reduced binding by at least 35% (Fig. 4). This pattern confirms and extends the competition binding pattern described by others for IL-8 and GRO\(\alpha\) binding sites on neutrophils (13, 14), and mimics that for IL8RA and IL8RB tested in transfected cells (Fig. 4). The pattern is consistent with two neutrophil receptor subtypes for the ELR\(^+\) CXC chemokines tested.

Biological Implications of Two Neutrophil IL-8 Receptors—More than 30 human chemokines have already been discovered, making the chemokine superfamily the largest known group of cytokines (1). Their receptors also comprise a large family of related molecules, and differences in receptor distribution have major implications regarding the molecular mechanisms of leukocyte subset recruitment in different inflammatory diseases (2, 25–31). It is interesting to note that although the number of known human ELR\(^+\) CXC chemokines is similar to the number of known human CC chemokines, many more human CC than ELR\(^+\) CXC receptors have been found, six versus two. The promiscuity of ligand recognition found for IL8RB is also found for the CC receptors, and most of them share at least one chemokine agonist with at least one other receptor (2, 19, 25–31).

Regardless of whether additional ELR\(^+\) CXC chemokine receptors exist, a central question arises as to the biological roles of IL8RA and IL8RB. Do they function redundantly, or do they mediate separate parts of the inflammatory response? Our results imply that the answer to this question will depend in part on the bioavailability at irritated body sites of IL-8 versus the other ELR\(^+\) CXC chemokines specific for IL8RB. In vitro, ELR\(^+\) CXC chemokines have overlapping actions on neutrophils, including chemotaxis, shape change, calcium flux, degranulation, and respiratory burst activation. Both IL8RA and IL8RB are able to activate the same set of neutrophil functions, and both human receptors are able to activate the chemotactic pathway independently in transfected Jurkat cells (1, 20). However, the receptors appear to be regulated differently in human neutrophils, at least in vitro, suggesting that they could operate during different phases of the inflammatory response (32, 33).

Although the selective pressures that broadened the selectivity of IL-8 for both IL8RB and IL8RA and restricted the selectivity of other ELR\(^+\) CXC chemokines to IL8RB are totally obscure, the structural determinants accounting for the selectivity differences have been partially defined (19). Analysis of chimeric IL8RA-IL8RB receptors in which divergent regions were exchanged has suggested that IL-8, GRO\(\alpha\), and NAP-2 bind to distinct sites on multiple extracellular domains of IL8RB (19). Both the N-terminal segment before transmembrane domain 1 and the region from transmembrane domain 4 to the end of the second extracellular loop are dominant determinants of GRO\(\alpha\) and NAP-2 selectivity when tested separately, but not in A-B chimeric receptors. Site-directed mutagenesis has
identified multiple residues of IL8RA spread over all four extracellular domains that, when mutated to alanine, affect the binding affinity for IL-8 (34, 35).

In conclusion, we have shown that IL8RB has high selectivity for all six ELR+ CXC chemokines tested, whereas IL8RA has high selectivity only for IL-8. Additional work will be needed to define more precisely the receptor binding sites for these chemokines. The selectivity that we have defined for IL8RA and IL8RB and the emerging work linking ELR+ CXC chemokines to inflammatory pathology will be useful in future work aimed at developing antagonists.

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