Role of the C Terminus of the Interleukin 8 Receptor in Signal Transduction and Internalization*

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Interleukin 8 (IL-8) is a potent neutrophil chemoattractant and activator. Two IL-8 receptor subtypes, A and B, are expressed in neutrophils. In this work, we analyzed the role of the C terminus domain of the IL-8 receptor on the signal transduction and receptor internalization mechanisms. The IL-8 receptor A was tagged with an epitope corresponding to the monoclonal antibody 1D4 to monitor the localization of the IL-8 receptor on the cell surface. We demonstrated IL-8-dependent receptor internalization by monitoring the density of surface 125I-labeled IL-8 binding sites and by immunofluorescence microcopy. Truncation of the last 27 amino acids of the IL-8 receptor A severely impaired the IL-8-induced internalization of the receptor. Of importance was the observation that binding of IL-8 to receptors A and B triggered a dramatically faster rate of internalization of receptor B than receptor A, suggesting that the heterologous C termini among receptor subtypes modulate the rate of internalization of IL-8 receptors. However, substitution of the C terminus of the receptor subtype A for the C terminus of receptor B reduced the internalization rate of receptor A. Furthermore, we found that the rate of internalization of IL-8 receptor B triggered by IL-8 was faster than the one induced by the IL-8-related peptide, melanoma growth stimulatory activity. Studies with human neutrophils pretreated with 100 nM IL-8 for 5 min revealed a positive and a negative calcium response mediated by receptors A and B, respectively. In contrast, neutrophils pretreated with melanoma growth stimulatory activity showed positive calcium responses to both receptors A and B. These data suggest that the neutrophil responses mediated by IL-8 are modulated by the rate of internalization of receptors.

Interleukin 8 (IL-8) is a major mediator of transendothelial migration and activation of neutrophils at the site of inflammation (Oppenheim et al., 1991). Two IL-8 receptor subtypes, designated as type A and B, have been identified in human and rabbit neutrophils (Holmes et al., 1991; LaRosa et al., 1992; Thomas et al., 1991; Prado et al., 1994). These receptors belong to the superfamily of G-protein-coupled receptors. IL-8 receptor subtype A binds with high affinity to IL-8 and with low affinity to structurally related peptides MGSA (melanoma growth stimulating activity) and NAP-2 (neutrophil activating peptide-2) (Lee et al., 1992; LaRosa et al., 1992). In contrast, IL-8 receptor B binds with high affinity to IL-8 and MGSA and moderate affinity to NAP-2 (LaRosa et al., 1992; Lee et al., 1992). Termination of neutrophil responses at the site of injury is thought to be a key step in the regulation of the inflammatory process. The mechanisms of termination of receptor signals mediated by IL-8 are unknown. Studies with other G-protein-coupled receptors have suggested that agonist binding to receptors triggers multiple structural and temporal changes of the receptor, leading to the generation of activation signals and termination signals for the phosphorylation, down-regulation, and internalization of receptors (Sibley et al., 1987). Several reports have also suggested that these signals are generated via independent pathways. For example, substitution of phosphorylated residues of α2-adrenergic receptor by Ala abolished desensitization without affecting internalization of the receptor (Eason et al., 1995). Furthermore, it appears that different internalization pathways operate in various G-protein-coupled receptors. Thus, C termini domains are involved in internalization of angiotensin II receptor (Thomas et al., 1995), β2-adrenergic receptors (Liggett et al., 1993), parathormone receptor (Huang et al., 1995) and α1B-adrenergic receptors (Lattion et al., 1994), whereas the third intracellular loop regulates the internalization of muscarinic receptors (Pals-Rylaarsdam et al., 1995). The role of internalization in several G-protein-coupled receptors is unclear. Although internalization of the receptor into an intracellular compartment appears to play a role in the desensitization of the β2-adrenergic receptors (Barak et al., 1994), the role of the C terminus in signaling and regulation of neutrophil chemoattractant receptors is unknown. Although recently Ben-Baruch et al. (1995) has shown that truncation of the distal C terminus of the IL-8 receptor B does not affect the IL-8-dependent chemotaxis in transfected CHO cells. In this study, we characterize the internalization profile of the IL-8 receptor subtypes, the role of the C-terminal tail in internalization, and the potential role of internalization of IL-8 receptors in regulating the IL-8-dependent neutrophil responses.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were made using an automated DNA synthesizer (model 373A, Applied Biosystem, Foster City, CA). Taq polymerase was from Promega (Madison, WI). Deep Vent Polymerase and restriction enzymes were obtained from DuPont NEN. Mammalian expression vector pRc/CMV was from Invitrogen (San Diego, CA). All cell culture reagents were obtained from Life Technologies, Inc. Chinese Hamster Ovary (CHO.K1) cells (CCL 61) were from American Type Culture Collection (Rockville, MD). G418 was purchased from Sigma. IL-8, MGSA, and NAP-2 were a gift from Repligen Corp. (Cambridge,
Construction of Plasmids—The cDNAs encoding for the human and rabbit IL-8 receptors, types A and B, were subcloned into the pRC/CMV mammalian expression vector. F3RTag cDNA, encoding the rabbit IL-8 receptor A containing the 8-amino acid epitope for Mab 1D4 at the C terminus, was synthesized by PCR using the sense oligonucleotide, 5'-GAT ATC GAA TTC AAG CTT ACT GTG GCC GTA ATG GAA GTA AAC-3' (primer A) and the antisense oligonucleotide, 5'-GATATCGAA TTC TCT AGA TTA TGC AGG TGC CAC CTG AGA GGT TTC GAG ATTTGAAGGCACGTTGGT-3' (primer B). The sense oligonucleotide contained a HindIII site, whereas the antisense oligonucleotide contained a XbaI site and a sequence encoding the epitope for the Mab 1D4 (ETSQVAPA) (Mackenzie, 1984). The PCR product (F3RTag) was digested with HindIII and XbaI and ligated to pRC/CMV. The tag sequence and the fidelity of the cloned PCR were confirmed by DNA sequencing.

The F3RDel cDNA, encoding the rabbit IL-8 receptor A with 27 amino acids truncated at its C terminus, was synthesized by PCR using primer A and an antisense oligonucleotide, 5'-GAT ATC GAA TTC TCT AGA TTA TGC AGG TGC CAC CTG AGA GGT TTC GCA AGC AAG CAT CTG-3' (primer C). Primer C was designed to contain a XbaI site, a nucleotide sequence encoding the Mab 1D4, and six amino acids from Arg-327 to Lys-322. The PCR product (F3RDel) was digested with HindIII and XbaI and ligated to pRC/CMV.

To construct the chimeric receptor, the 121-base pair PvuII/XbaI fragment encoding the last 39 amino acids was excised from pRC/CMV-5B1a and subcloned into the PvuII/XbaI-digested pRC/CMV-F3R (rabbit IL-8 receptor A) backbone. The fidelity of the chimeric receptor was confirmed by DNA sequencing.

Inositol Phosphate Determination—CHO cells were grown in 10-cm tissue culture dishes and incubated with 5 μCi/ml myo-[3H]inositol for 16 to 24 h in inositol-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal bovine serum. After labeling, cells were washed and detached with phosphate-buffered saline (PBS) containing 3 mM EDTA. Cells were pelleted and resuspended to a concentration of 4 x 10^5 cells/ml in Dulbecco's PBS containing 0.9 mM CaCl2, 1 mg/ml glucose, 20 mM Hepes, pH 7.4, and 20 mM LiCl. Cells were stimulated with 100 nM IL-8 for 10 and 30 min. Incubations were terminated by removing medium and adding three volumes of chloroform/methanol. Extraction of total inositol phosphates on Dowex AG1-X8 formate form resin was performed as described (Blount and Krause, 1993).

Intracellular Calcium Measurements—Cells were detached, pelleted, and resuspended in a physiological buffer solution containing 140 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 1 mM Na2HP04, 5 mM glucose, 20 mM Hepes (pH 7.4), and 1 mg/ml bovine serum albumin. Cells were loaded with 5 μM Indo-1 AM for 1 h at room temperature. Cells were washed and resuspended at a density of 1 x 10^7 cells/ml with buffer solution. Cells were stimulated with 100 nM IL-8, and fluores-
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...ence intensity was measured at 37 °C using an excitation wavelength of 330 nm and an emission wavelength of 405 nm (Grynkiewicz et al., 1985).

For studies of calcium responses in neutrophils, blood was drawn from healthy human donors and layered in a Mono-Poly Resolving Medium (ICN Biomedical, Inc., Aurora, Ohio). Neutrophils were isolated in accordance to the manufacturer’s instructions, then suspended in physiological buffer solution at a concentration of 1 × 10^6 cells/ml and loaded with 5 μM Indo-1AM for 30 min at room temperature. Neutrophils were preincubated with 100 nM IL-8 or 100 nM MGSA at 37 °C for 5 min, washed with ice-cold physiological buffer, and pelleted at 1000 × g for 10 min at 4 °C. Cells were incubated at 37 °C at the indicated time points and then tested for agonist-dependent intracellular calcium rise. MGSA and IL-8 were added sequentially to the cuvette containing neutrophils to assess the responses mediated by IL-8 receptors B and A, respectively.

**Results and Discussion**

The Epitope-tagged Receptor—An 8-amino acid epitope (tag) corresponding to the Mab 1D4 was fused to the C-terminal end of receptor A to monitor the localization of the IL-8 receptors. As shown in Fig. 1a, the tagged receptor exhibited a similar binding profile as the wild-type receptor. As expected for IL-8 receptor A, high affinity binding to IL-8 and very little binding to the structurally related peptide MGSA and NAP-2 were demonstrated. Most importantly, the activation of the tagged receptor was similar to the wild-type receptor, as shown by the IL-8-dependent inositol phosphate formation or IL-8-dependent rise in intracellular Ca^{2+} assays (Fig. 1, b and c). These data indicate that the epitope tag fused at the C terminus of the receptor did not affect the binding site for IL-8 or the mechanism for receptor activation. These results are in agreement with previous studies indicating that fusion of epitope tags to the C-terminal end of the IL-8 receptor did not affect the binding to IL-8 (Gayle et al., 1993).

Internalization of the IL-8 Receptor A—Agonist binding to the IL-8 receptor A at 37 °C reduced, in a time-dependent manner, the association radioactivity was determined in a gamma counter. Data were expressed as the percentage of specific binding in the absence of IL-8 pretreatment.

**Immunofluorescence Microscopy—Immunofluorescent staining of IL-8 receptors in permeabilized CHO cells was carried out as described by Papkoff et al. (1993). In brief, CHO cells were grown on microscope slides for 24 h. Cells were then incubated at 37 °C in the presence or absence of IL-8 for various times. Cells were fixed with 3% formaldehyde in the presence of 2% sucrose and permeabilized with an ice-cold solution containing 0.5% Triton X-100, 0.3% sucrose, 0.003 M MgCl2, 0.05 M NaCl, and 0.01 M HEPES (pH 7.4) in PBS for 5 min. Permeabilized cells were incubated with 50 μg/ml of monoclonal antibody (ID4) for 30 min. Antibody binding was detected by the addition of a 1:20 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Boehringer Mannheim). Fluorescence was analyzed using a Zeiss fluorescence microscope with epi-illumination. Images were detected using a Canon camera and an ASA 400 Kodak film.

**Results**

**Internalization and localization of receptors in CHO cells.** a, internalization of wild-type rabbit IL-8 receptor A and tagged receptors expressed in CHO cells. Cells expressing wild-type (●), tagged receptors (■), and untransfected CHO cells (□) were incubated with 100 nM IL-8 at 37 °C for the indicated times. Each point was determined in triplicate. b, localization of tagged receptors in CHO cells. Untransfected CHO cells (left panel) and transfected cells with epitope-tagged receptor cDNA (middle and right panels) are indicated by arrows. Cells transfected with tagged receptor cDNA were stimulated with or without 100 nM IL-8 for 60 min and were fixed, permeabilized, and stained to allow for receptor localization.

Role of the C Terminus in Receptor Internalization—The C terminus domain of various G-protein-coupled receptors has been implicated in the mechanisms of G-protein activation, receptor desensitization, and internalization (Dohman et al., 1991). To examine the role of the C terminus of the IL-8 receptor, CHO cells were transfected with a cDNA construct encoding the rabbit IL-8 receptor A deleted of its last 27 amino acids. All potential phosphorylation sites (Ser and Thr) at the C terminus were removed in the truncated receptor. CHO cells expressing the truncated receptor exhibited a binding profile similar to the wild-type receptor (Fig. 3a), showing that truncation of the last 27 amino acids did not affect the binding domain of the IL-8 receptor. The IL-8-dependent inositol phosphate production and the IL-8-dependent calcium rise were similar in both the wild-type and the mutant receptor (Fig. 3, b and c), indicating that the mechanisms of receptor activation were unaffected by deletion of the last 27 amino acids of the IL-8 receptor A. These data are consistent with recent findings indicating that deletion of the distal region of the C terminus of
the IL-8 receptor did not affect the IL-8-dependent chemotaxis of transfected cells (Ben-Baruch et al., 1995). However, the IL-8-induced internalization of the truncated IL-8 receptor A was severely impaired (Fig. 4a). This result suggests that the distal C terminus domain is a major component of the internalization pathway of the IL-8 receptor. Since the homologous IL-8 receptor subtypes A and B exhibit high sequence diversity in their distal C termini, we examined whether IL-8 receptors show different internalization profiles. Indeed, we found that the rate of internalization of receptor subtype B was dramatically faster than the receptor subtype A (Fig. 4c). After a 5-min exposure to 100 nM IL-8, more than 95% of IL-8 receptors B and less than 40% of receptors A were internalized. Of importance is that similar rates of IL-8-dependent internalization were observed among human and rabbit receptors of the same subtype. These results are in good agreement with a recent study in human neutrophils indicating that the IL-8-dependent internalization of receptor B is faster than receptor A (Chuntharapai and Kim, 1995). These results suggest that binding of IL-8 and MGSA to receptor B induces different structural changes of the receptor, leading to the generation of distinct internalization pathways.

To determine whether the type of ligand dictates the internalization profile of the receptors, we examined the MGSA versus IL-8-dependent internalization of the IL-8 receptor B. MGSA and IL-8 bind with similar high affinity to IL-8 receptor B, and both ligands are equally potent in inducing a Ca\(^{2+}\) response in cells transfected with IL-8 receptor B (LaRosa et al., 1992; Lee et al., 1992). As shown in Fig. 5, MGSA triggered a slower rate of internalization of receptor B than IL-8. These data strongly support the idea that binding of IL-8 and MGSA to receptor B induces different structural changes of the receptor, leading to the generation of distinct internalization pathways.
IL-8 and MGSA-mediated Ca$^{2+}$ Responses in IL-8 and MGSA-treated Neutrophils—The functional significance of internalization of IL-8 receptor subtypes is unknown. Previous studies with the $\beta_2$-adrenergic receptor system have suggested that internalization or sequestration is required for receptor resensitization (Barak et al., 1994). To examine the role of internalization of receptors in neutrophils, we monitored the agonist-dependent Ca$^{2+}$ internalization of all of the IL-8 receptors (B, Fig. 4b). On the other hand, MGSA induced internalization of less than 40% of IL-8 receptor A (Fig. 4b). The response to IL-8 is probably mediated by IL-8 receptor A that did not get completely internalized by IL-8 (Fig. 4b). Consistent with this idea, MGSA-pretreated neutrophils, in which MGSA induces a slow rate of internalization of receptors B (Fig. 5), responded quickly to both MGSA and IL-8 (Fig. 6d). This study strongly supports the hypothesis that internalization of the IL-8 receptor is a major mechanism for modulating the neutrophil responses elicited by IL-8 and its functionally related peptides such as MGSA.

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Fig. 6. Ca$^{2+}$ responses mediated by IL-8 receptors A and B in human neutrophils. Neutrophils were loaded with 5 $\mu$M Indo-1AM as described under “Experimental Procedures.” Neutrophils were pre-treated with vehicle alone (a), with 100 nM IL-8 (b and c), or 100 nM MGSA (d) at 37°C for 5 min and then washed with ice-cold PBS. Pretreated neutrophils were incubated at 37°C for the indicated times and then challenged with 100 nM MGSA or 100 nM IL-8 to activate IL-8 receptors B and A, respectively.