Identification of Receptor Binding and Activation Determinants in the N-terminal and N-loop Regions of the CC Chemokine Eotaxin*

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Eotaxin is a CC chemokine that specifically activates the receptor CCR3 causing accumulation of eosinophils in allergic diseases and parasitic infections. Twelve amino acid residues in the N-terminal (residues 1–8) and N-loop (residues 11–20) regions of eotaxin have been individually mutated to alanine, and the ability of the mutants to bind and activate CCR3 has been determined in cell-based assays. The alanine mutants at positions Thr7, Asn12, Leu13, and Leu20 show near wild type binding affinity and activity. The mutants T8A, N15A, and K17A have near wild type binding affinity for CCR3 but reduced receptor activation. A third class of mutants, S4A, V5A, R16A, and I18A, display significantly perturbed binding affinity for CCR3 while retaining the ability to activate or partially activate the receptor. Finally, the mutant Phe11 has little detectable activity and 20-fold reduced binding affinity relative to wild type eotaxin, the most dramatic effect observed in both assays but less dramatic than the effect of mutating the corresponding residue in some other chemokines. Taken together, the results indicate that residues contributing to receptor binding affinity and those required for triggering receptor activation are distributed throughout the N-terminal and N-loop regions. This conclusion is in contrast to the separation of binding and activation functions between N-loop and N-terminal regions, respectively, that has been observed previously for some other chemokines.

Chemokines (chemotactic cytokines) are a family of small (8–10 kDa) secreted proteins whose major function is to recruit leukocytes to sites of injury or infection (1). Several diseases, including asthma and other allergic disorders, can result from the overaccumulation of leukocytes. There are two main classes of chemokines as defined by the pattern of the first two of four conserved cysteine residues located near the N-terminus (2). When the cysteines are adjacent, the chemokines are classified as CC chemokines and typically attract monocytes, eosinophils, basophils, and T-lymphocytes. If a single residue separates the cysteines they refer to as CXC chemokines and typically attract neutrophils or lymphocytes. There are also two minor classes, which include the C chemokine lymphotactin (3) and the CXC3C chemokine fractalkine (4). The receptors for chemokines (designated CCR or CXCR, according to the class of chemokine that activates them) are seven transmembrane helix G-protein-coupled receptors located in the leukocyte cell membrane (5). Chemokines of a certain class typically bind to a subset of receptors from the corresponding receptor class (2), although the determinants of receptor specificity are not well understood. In addition to their signaling roles, chemokine receptors, particularly CCR5, CCR8, and CCR3 have been shown to be co-receptors, in conjunction with CD4, for HIV infection (6).

The CC chemokine eotaxin (7–9), and the related proteins eotaxin-2 (10, 11) and eotaxin-3 (12), are specific for the receptor CCR3, which is the most abundant chemokine receptor found on the surface of eosinophils, a terminally differentiated class of granulocytes (9, 13, 14). Through its interaction with CCR3, eotaxin facilitates the recruitment of eosinophils to the sites of parasitic infection or allergen stimulation (15). Eosinophil accumulation is a hallmark of several human diseases, including Hodgkin’s disease (15) and inflammatory diseases of the lungs (asthma), heart (hypereosinophilic syndrome), intestines (inflammatory bowel disease and gastroenteritis), and skin (atopic dermatitis). The overexpression of eotaxin in many of these diseased tissues, along with its ability to recruit eosinophils, suggests a likely role for eotaxin in these inflammatory disorders (16, 17).

The three-dimensional structures of chemokines have been reviewed recently (18). The structures of the monomeric subunits are largely conserved and consist of a single turn of helix followed by a three-stranded antiparallel β-sheet and a C-terminal α-helix (Fig. 1). The CC or CXC motif is contained in a long unstructured region (preceding the helical turn) that is covalently linked through a pair of disulfide bonds to the β-sheet and the loop connecting strands one and two (30s loop). Herein we refer to the region preceding the CC or CXC motif as the N-terminal region and the region between the CC or CXC motif and the helical turn as the N-loop region (Fig. 1). The quaternary structures of chemokines vary dramatically within the superfAMILY, with a range of monomeric, dimeric, and tetrameric structures seen, although there is convincing evidence that the monomer is sufficient for receptor activation (19, 20). The three-dimensional structures of eotaxin (21), eotaxin-2 (22), and eotaxin-3(2) have recently been determined and are all monomeric. The conserved nature of the chemokine structure has revealed little information about the mechanism of receptor specificity.

Extensive mutational studies of several chemokines have led to a proposed “two-step” model for receptor interaction in which

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1 The abbreviations used are: HIV, human immunodeficiency virus; MCP, monocyte chemotactant protein; R, receptor; HOS, human osteosarcoma.

2 J. Ye, K. L. Mayer, M. R. Mayer, and M. J. Stone, unpublished results.
the N-loop of the chemokine initially binds to the receptor then the N-terminal region of the chemokine docks with the receptor to induce a conformational change, resulting in receptor activation (21, 23–25). Recent papers describing the binding of receptor-derived peptides to a groove located between the N-loop and β2-β3 hairpin of their cognate chemokines (interleukin-8, fractalkine, eotaxin, and eotaxin-2) have reinforced the importance of the N-loop region for receptor binding (22, 26–28). However, the specific protein residues in the chemokine that contribute to receptor interactions vary significantly between chemokines.

Eotaxin, eotaxin-2, and eotaxin-3 differ from most chemokines in that they are specific for only one receptor, CCR3. In addition, eotaxin shares a high degree of sequence identity (~60%) with the MCP proteins, which bind a different subset of receptors. This provides us with a good model system to study chemokine/receptor interactions and the determinants of receptor specificity. To this end, we have performed alanine-scanning mutagenesis on the N-terminal and N-loop regions of eotaxin to resolve which residues in these regions are involved in receptor interactions. Herein we report the results of these studies and their interpretation in light of the available data for other chemokines.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The human osteosarcoma (HOS-CD4) cell line stably transfected with CCR3 (29, 30) was acquired from the AIDS Research and Reference Reagent Program and was maintained in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, and 1 μg/ml puromycin. The murine L1.2 pre-B cell line stably transfected with CCR3 (31) was obtained as a generous gift from Dr. Osamu Yoshi and was maintained in RPMI 1640, 10% fetal bovine serum, and 0.8 mg/ml geneticin. All cell culture materials were purchased from Life Technologies, Inc. (Rockville, MD).

**Chemokines**—Site-directed mutagenesis was performed using the QuikChange method (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. Wild type eotaxin and all eotaxin mutants were expressed and purified as described previously for wild type eotaxin (28). In brief, the pET30α-eotaxin plasmid was transformed into BL21(DE3) cells (Novagen, Santa Clarita, CA). Cells were grown at 37 °C to an A₆₀₀ = 0.7, induced with 1 mM isopropyl β-D-thiogalactopyranoside, and harvested 6 h later. Cells were resuspended in buffer (50 mM Na₂HPO₄, 500 mM NaCl, 5 mM imidazole, pH 8.0) and lysed. The protein was loaded onto a Ni²⁺-nitrilotriacetic acid-agarose affinity column (Qiagen, Valencia, CA), washed with 50 mM Na₂HPO₄, 500 mM NaCl, 30 mM imidazole, pH 8.0, and eluted with 50 mM Na₂HPO₄, 500 mM NaCl, 200 mM imidazole, pH 8.0. The (His)₆-tag was removed by treatment with thrombin (1 μg thrombin/mg eotaxin in 20 mM Tris, pH 8.0, room temperature, overnight). The cleaved protein was further purified on a Source 15 cation exchange column (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris, pH 8.5, using a 0 to 2 M NaCl gradient.

**Calcium Mobilization Assay**—HOS-CD4-CCR3 cells were washed with phosphate buffered saline, trypsinized, and resuspended in complete medium, counted, spun down, and resuspended in complete medium at a concentration of 1 × 10⁶ cells/ml. Fura-2 and pluronic-127 (both from Molecular Probes, Eugene, OR) were added to final concentrations of 2.5 μg/ml and 0.05%, respectively. The cells were incubated for 20 min in the dark at room temperature. Prior to the assay, 2 ml of cells were placed in a quartz cuvette and incubated in the fluorometer at 37 °C with stirring for 10 min. The fluorescence ratio was determined by exciting at 340 and 380 nm with emission monitored at 510 nm. Injections into the cuvette were done in a 10-μl volume. Data were collected on a SLM-AMINCO 8100 spectrophotometer (SLM Instruments).

**Competitive Radioligand Binding Assay**—The competitive binding assay was performed using previously reported procedures with slight modifications (31). Briefly, 4 × 10⁶ L1.2-CCR3 cells/ml in 25 mM HEPES, 1 mM CaCl₂, 5 mM MgCl₂, 120 mM NaCl, 0.5% bovine serum albumin (pro tease-free; Sigma), pH 7.6, were incubated with ~0.15 nM

**FIG. 1.** A ribbon structure of eotaxin (Protein Data Bank accession number 1EOT) showing the locations of the N-terminal and N-loop regions. Side chains are shown as dark gray cylinders for the residues mutated in the current study. Several of the residues that were found to be important for binding or activation are labeled. The two disulfide bonds are displayed as light gray cylinders.

**RESULTS**

**Selection of Residues for Alanine-scanning Mutation**—To assess the contribution of each amino acid side chain in the N-terminal and N-loop regions of eotaxin (residues 1–20) to receptor binding and activation, we individually mutated each residue to alanine. The only residues omitted from the scanning mutagenesis were glycine, proline, and alanine (because of their unique structural properties) and the two conserved cysteines residues (because of the requirement for the structural integrity of the protein). Thus, the following twelve residues were individually converted to alanine in this study: Ser⁴, Val⁵, Thr⁷, Thr⁸, Phe¹¹, Asn¹², Leu¹³, Asn¹⁵, Arg¹⁶, Lys¹⁷, Ile¹⁸, and Leu¹⁹ (Fig. 1). All of the mutants were expressed in *Escherichia coli* and purified using the protocol we have developed for wild type eotaxin with no modifications needed (28). The mutants expressed at approximately wild type levels (~5 μg/liter of culture).

**Activity of Eotaxin and Mutants**—The ability of wild type eotaxin and each mutant to activate CCR3 was determined using a calcium mobilization assay performed on HOS cells expressing CCR3 on the surface. The observed calcium flux for wild type eotaxin is shown in Fig. 2A, and the corresponding dose-response curve is presented in Fig. 2B. The concentration required for half-maximal eotaxin activity is ~7 nM, and maximum activity is seen for concentrations exceeding ~30 nM.

The activity of each mutant protein was determined at a concentration of 100 nM at which wild type eotaxin has maximal activity; the results are listed in Table I. Substantial losses of activity (~30% wild type activity) resulted from the substitutions at Val⁵ and Phe¹¹, with F11A showing a near complete loss of activity (within experimental error) at 100 nM concen-
Receptor Binding and Activation Determinants of Eotaxin

Previous chemokine mutational studies, and their interpretation according to the prevailing two-step model for chemokine receptor activation, have suggested a separation of function between the N-loop and N-terminal regions of chemokines (21, 23–25). The N-loop is proposed to be important for providing the initial binding energy, whereas the N-terminus is suggested to be required for triggering receptor activation subsequent to binding. As discussed below, the current calcium mobilization and radioligand binding results for eotaxin mutants suggest that the functions of the N-terminal and N-loop regions of eotaxin are not separated in a straightforward manner.

The following discussion is based on the premise that reduc-

FIG. 2. Calcium flux activity assay for wild type eotaxin. A, a typical calcium transient observed upon addition of 100 nM eotaxin to Fura-2-labeled HOS-CCR3 cells. B, a dose-response curve showing the concentration dependence of the observed calcium flux signal. Data points and error bars are the average and standard deviation, respectively, of triplicate data, normalized to the average of the signals for the three highest concentrations.

Receptor Binding of Eotaxin and Mutants—The losses in activity observed in the calcium mobilization assay could potentially result from a decreased affinity for the receptor. Alternatively, the mutants might have wild type binding affinity but be unable to induce the receptor conformational change required for transmembrane signaling. To distinguish between these two possibilities, we measured the ability of each mutant protein to inhibit binding of wild type eotaxin to CCR3 using a competitive radioligand binding assay performed using murine pre-B cells (L1.2) expressing CCR3 on their surface.

A representative competition binding curve for wild type eotaxin is shown in Fig. 3. The concentration required for 50% inhibition of 125I-labeled wild type eotaxin binding (IC50) is 1.2 ± 0.2 nM. Eotaxin mutants in which the residue Thr7, Thr8, Asn12, Leu13, Asn15, Lys17, or Leu20 was replaced by alanine competed with labeled wild type eotaxin with IC50 values in the range 0.5 to 3.5 nM (Table I), i.e. within a factor of four of wild type eotaxin. A curve for N15A is shown as an example in Fig. 3. In contrast, a second group of mutants with alanine substitution at Ser4, Val5, Phe11, Arg16, or Ile18 had IC50 values in the range 8.6 to 24.5 nM (Table I), i.e. a decrease of more than 7-fold compared with wild type eotaxin. The curve for F11A is shown in Fig. 3. The receptor binding and activation data are summarized in Table I.

DISCUSSION

A moderate loss of activity (<80% wild type activity) was seen for substitutions at positions Ser4, Thr8, Asn15, Arg16, and Lys17, whereas substitution of alanine at residues Thr7, Asn12, Leu13, Ile18, and Leu20 produced mutant proteins with near wild type activity at 100 nM.

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The following discussion is based on the premise that reduc-
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Tations in receptor binding affinity or activation upon mutation of a particular residue imply a role for that residue in the receptor interaction. As in all mutational studies, an alternative possibility is that the mutations induce a structural change in the protein, detrimentally affecting its ability to interact with the receptor. Although we have not experimentally excluded this possibility in the current study, it appears relatively unlikely considering that the mutations have all been made in regions of the protein that are known to be conformationally flexible (32).

A graphical representation of the influence of alanine mutations on the receptor binding and activity of eotaxin (Fig. 4) illustrates the classification of mutants into four categories (groups 1–4 in Fig. 4). The alanine mutants at positions Thr4, Asn12, Leu13, and Leu20 (group 1) show near wild type binding and activity suggesting that their side chains do not play a major role in the mechanism of receptor activation. It is important to note that small reductions in activity (less than ~5-fold increases in EC50 values) would not necessarily be detected by measuring calcium flux at 100 nM concentration, so it remains possible that these mutations have subtle influences on receptor activation.

The mutants T8A, N15A, and K17A (group 2, Fig. 4) all have near wild type binding affinity for CCR3 yet have reduced activation as measured by calcium mobilization. These results are interesting, because they not only confirm the predicted importance of the N-terminus (Thr5) for receptor activation but also implicate residues in the N-loop (Asn15 and Lys17) in activation of CCR3 by eotaxin. In a recent NMR study of binding between eotaxin and peptides derived from the N-terminus of CCR3, residues Asn15 and Lys17 of eotaxin underwent chemical shift changes upon addition of peptide (28). These combined results suggest that the N-terminus of CCR3 interacts with the N-loop residues Asn15 and Lys17 during the events that lead to receptor activation.

A third class of mutants, S4A, V5A, R16A, and I18A (group 3, Fig. 4), each possess significantly perturbed binding affinity for CCR3 while retaining the ability to activate or partially activate the receptor. The mutation at Ile18 is interesting, because it shows a 7-fold reduced binding affinity yet has wild type activity at 100 nM. This can be rationalized by noting that even with its weakened binding affinity, significant I18A should be able to bind CCR3 at the 100 nM concentration used in the activity assays (>90% predicted receptor occupancy), allowing the production of a near wild type signal. As noted above for the group 1 mutants, it remains possible that the I18A mutation has a small effect on the EC50 for receptor activation. Importantly, two of these four group 3 mutants with reduced binding affinity are located in the N-loop (R16A and I18A), whereas the other two are located in the N-terminus (S4A and V5A). This observation again challenges the hypothesis of binding and activation functions being partitioned between these two structural elements. The importance of the N-terminus in receptor binding is supported by the observation that eotaxin truncated by two N-terminal residues displays reduced receptor binding affinity and activation (33). NMR binding studies using receptor-derived peptides (28) support the involvement of the two N-loop residues in receptor binding; Ile18 is located at the base of the binding groove proposed in that study, and Arg16 is at the hydrophilic edge of the same groove.

In addition to their weakened receptor binding affinity, mutants at positions Ser4, Val5, and Arg16 also show reduced receptor activation in the calcium flux assay. It would not be unexpected for a mutation that affects receptor binding to also affect receptor activation. However, it is also possible for a mutation to affect both the initial binding step and the receptor-triggering event separately. Although it is difficult to interpret the influence of mutations at these positions on the receptor activation step, it is noteworthy that the three mutants all show approximately the same range of reduced binding (S4A, V5A, I18A) and activation (R16A, S4A, V5A).

![Graph](image-url)  

**Fig. 3.** Competitive radioligand binding data for wild type eotaxin (circles), the N15A mutant (triangles), and the F11A mutant (squares). Data for the N15A and F11A mutants are presented as typical examples of mutants with binding affinity within 4-fold of wild type and those with binding affinity reduced by greater than 7-fold, respectively.

| Mutant activity/ wild type activity | IC50 (nM) |
|------------------------------------|----------|
| Wild Type                          | 1        |
| S4A                                | 0.76 ± 0.01 |
| V5A                                | 0.28 ± 0.02 |
| T7A                                | 0.97 ± 0.02 |
| T8A                                | 0.71 ± 0.03 |
| F11A                               | 0.07 ± 0.1 |
| N12A                               | 1.11 ± 0.06 |
| L13A                               | 1.03 ± 0.04 |
| N15A                               | 0.45 ± 0.11 |
| R16A                               | 0.61 ± 0.02 |
| K17A                               | 0.55 ± 0.06 |
| I18A                               | 1.11 ± 0.01 |
| L20A                               | 0.86 ± 0.02 |

*Table I*
V5A, and R16A have IC\textsubscript{50} values of 12.5, 9.8, and 12.2 nM, respectively) whereas V5A has a much greater effect than the other two mutations on eotaxin activity (23% of wild type for V5A versus 76% for S4A and 61% for R16A). This comparison suggests that the mutation at Val\textsuperscript{5} is likely to affect both the initial binding and the receptor-triggering steps separately.

Finally, the mutant Phe\textsuperscript{11} has little detectable activity and 20-fold reduced binding affinity relative to wild type eotaxin, the most dramatic effect observed in both assays. The substantial loss of receptor interactions at this position is not surprising as several mutational studies on other chemokines have implicated an aromatic residue at this position as being key for receptor binding and activation (19, 34–37). Of some interest in the case of F11A in eotaxin is that the magnitude of the change in binding affinity is smaller than is seen in several other chemokines. For the CC chemokines MCP-1 and macrophage inflammatory protein-1b, mutation of the comparable aromatic position to alanine resulted in significant loss of activity for the proteins and 100- and 1000-fold weaker receptor binding affinity, respectively (34, 36). For the CC chemokine RANTES (regulated on activation normal T cell expressed and secreted) mutation of the aromatic residue to alanine resulted in 5000-fold weaker binding affinity for CCR3 (37). In all studies, the aromatic residue was shown to provide very important protein/receptor interactions.

Alanine-scanning mutagenesis has been performed previously on the CC chemokines MCP-1 and RANTES (34, 35, 37). Results from the RANTES experiments are difficult to compare, as the protein is able to bind and activate several receptors, and experiments were performed using cells that may have expressed multiple receptors. In the case of MCP-1, no individual residues in the N-terminus were identified as being important for binding or activation of the receptor CCR2b. However, N-terminal truncation resulted in a complete loss of ability for the truncated protein to activate the receptor while still retaining the ability to bind CCR2b (19). Thus, it was suggested that, for the case of MCP-1, the exact nature of the side chains in the N-terminus is unimportant for activation of the receptor and that the mere presence of a polypeptide chain is sufficient for receptor activation once the vital binding step has occurred (35). The only residue in MCP-1 found to be important for binding in either the N-terminal or N-loop regions was Tyr\textsuperscript{13} (corresponding to Phe\textsuperscript{11} in eotaxin) (34). These results contrast with those shown here for eotaxin, in which individual residues in the N-terminus are linked to binding and activation of the receptor, and several residues in the N-loop, in addition to Phe\textsuperscript{11}, show involvement in receptor activation and binding. Thus, eotaxin displays a relatively diffuse or delocalized set of contributions to receptor binding and activation throughout the N-terminus and N-loop regions whereas MCP-1 has a more important single residue (Tyr\textsuperscript{13}). These contrasting results for eotaxin and MCP-1, proteins similar in sequence that are specific for different receptors, provide some clues about the different mechanisms by which receptor specificity may be achieved.

Conclusions—Several residues important for the binding and activation of CCR3 by eotaxin have been identified. The location of these residues demonstrates that the N-terminus and N-loop of eotaxin are each involved in both signaling and receptor binding. Thus, the results of this study demonstrate that, at least for some chemokines, the N-loop and N-terminus do not have separable receptor binding and activation functions. The varying distributions of functionally important residues in different chemokines are likely to play an important role in controlling chemokine/receptor specificity.

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