N-terminal domain of eotaxin-3 is important for activation of CC chemokine receptor 3

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Introduction

Chemokines comprise a group of small structurally related molecules, with molecular weights (Mw) of 8–12 kDa, that regulate the cell trafficking of various types of leukocytes through interactions with a subset of seven transmembrane helix G protein-coupled receptors, and play fundamental roles in the development, homeostasis, and function of the immune system (Rollins, 1997; Luster, 1998; Baggioni, 1998). Some chemokines function as HIV-suppressive factors by interacting with chemokine receptors, which, together with CD4, were found to be the binding sites for HIV-1 (Garzino-Demo et al., 2000). Furthermore, some chemokines and their receptors have been shown to be involved in cancer metastasis (Müller et al., 2001). Thus, understanding of the structure–function relationships of chemokines will stimulate the development of rational therapeutic strategies against inflammatory diseases, HIV-1 infection and cancer metastasis.

Approximately 40 chemokines have been identified so far in humans, and they have been classified into at least four subfamilies on the basis of the numbers and relative positions of their cysteine residues. The CC (or β), CXC (or α), and CX3C (or fractalkine) chemokines contain four conserved cysteine residues, the first of which is separated by no, one and three amino acid residues; the C (or lymphotactin) chemokine has only the first and third conserved cysteines (Zlotnik and Yoshie, 2000). Chemokine receptors are generally specific for a particular subfamily; hence, receptors are denoted by the prefixes ‘CCR’, ‘CXCR’, etc. However, many chemokines can activate more than one receptor within the relevant subfamily, and most chemokine receptors can be activated by multiple chemokines that belong to the same subfamily (Zlotnik and Yoshie, 2000). Detailed analysis of the structure–function relationships of chemokines and receptor molecules is necessary to understand this complicated network.

The three-dimensional structures of several chemokines have been determined by nuclear magnetic resonance and/or X-ray crystallography (Baldwin et al., 1991; Lodi et al., 1994; Skelton et al., 1995; Handel and Domaille, 1996; Crump et al., 1997, 1998; Mayer and Stone, 2000; Ye et al., 2001). The structure of the monomeric unit is highly conserved, and consists of an unstructured N-terminal domain preceding the first cysteine, an irregularly structured N-loop following the second cysteine, a single turn of a helix, a backbone made of three β-strands, and a C-terminal α-helix. Studies with a series of N-terminally truncated and substituted analogs of some CC (Gong and Clark-Lewis, 1995; Gong et al., 1996; Mayer and Stone, 2001) and CXC chemokines (Clark-Lewis et al., 1991; Hebert et al., 1991; Crump et al., 1997; Proost et al., 2001) have revealed that the N-terminal domain is critical for receptor activation.

Eotaxin-3 belongs to the CC chemokine family, and is one of the eotaxin subgroup (eotaxin, eotaxin-2 and eotaxin-3), which are specific agonists for CCR3 expressed on the surface of eosinophils, basophils and T helper type 2 cells (Kitaura et al., 1999; Shinkai et al., 1999). In order to obtain information on the structural determinants of the activity of eotaxin-3, we constructed a set of N-terminal deletion mutants, and investigated their activity toward eosinophils in vitro. We found that the N-terminal region preceding the first cysteine is critical for the activation of CCR3.

Materials and methods

Materials

Recombinant human eotaxin, RANTES and MIP-1α were purchased from PeproTech (London, UK). Recombinant human 125I-eotaxin and 125I-MIP-1α were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

Construction of plasmids carrying the wild-type and various N-terminal deletion mutants of eotaxin-3

To construct pET-HVC, a plasmid for expression of the wild-type eotaxin-3, synthetic oligonucleotides (5′-TATGATCGA-
Expression and purification of recombinant eotaxin-3 proteins

Wild-type eotaxin-3 expressed in baculovirus-infected insect cells was purified from the culture medium using a heparin–Cellulofine and SP Sepharose column chromatography. Samples (2 µg each) were then analyzed by SDS–PAGE as described in Materials and methods. Lane 1, molecular markers.

AGGTCGTACAC-3’, 5’-GTGTACGACCTCGATCA-3’) encoding Met–Ile–Glu–Gly–Arg–Thr1, and a PmaCl–NolI 390 bp fragment of pVL-HVC (Shinkai et al., 1999) encoding 2–71 amino acids of eotaxin-3 were cloned under the T7 promoter (Ndel–NolI site) in the Escherichia coli expression vector pET21a(+) (Novagen, Madison, WI). To construct plasmids for expression of the Δ1-, Δ2-, Δ3-, Δ4-, Δ5- and Δ6-eotaxin-3 genes, a Ndel–EcoRI 95 bp fragment of pET-HVC, which encodes 1–27 amino acids of mature eotaxin-3, was cloned into pET-HVC. After the desired fragment had been removed by PCR encoding Met–Ile–Glu–Gly–Arg–Thr1, Arg–Thr2 and Met–Thr2, the recombinant eotaxin-3 protein was purified from a heparin–Cellulofine (Chisso Corp., Tokyo, Japan) column as described previously (Shinkai et al., 1999). For purification of the wild-type, Δ1-, Δ2-, Δ3-, Δ4-, Δ5- and Δ6-eotaxin-3 proteins, the partially purified proteins were dialyzed against a buffer comprising 20 mM Tris–HCl, pH 8.0, 1 mM oxidized form of thioglutathione, 0.1 mM reduced form of glutathione, and then gently mixed for 12 h at 4°C to reconstitute the eotaxin-3 protein. After insoluble materials had been removed by centrifugation at 18 000 g for 30 min, the remaining solution was removed with a buffer comprising 0.1 M Tris–HCl, pH 8.0, 0.6 M guanidine–HCl, 1 mM dithiothreitol at a protein concentration of 1 mg/ml. After insoluble materials had been removed by centrifugation at 18 000 g for 30 min, the remaining solution was diluted 20 times with a buffer comprising 0.1 M Tris–HCl, pH 8.0, 1 mM oxidized form of thioglutathione, 0.1 mM reduced form of glutathione, and then gently mixed for 12 h at 4°C to reconstitute the eotaxin-3 protein. After insoluble materials had been removed by centrifugation at 18 000 g for 30 min, the remaining solution was diluted 20 times with a buffer comprising 0.1 M Tris–HCl, pH 8.0, 0.6 M guanidine–HCl, 1 mM dithiothreitol at a protein concentration of 1 mg/ml. After insoluble materials had been removed by centrifugation at 18 000 g for 30 min, the remaining solution was diluted 20 times with a buffer comprising 0.1 M Tris–HCl, pH 8.0, 1 mM oxidized form of thioglutathione, 0.1 mM reduced form of glutathione, and then gently mixed for 12 h at 4°C to reconstitute the eotaxin-3 protein. After insoluble materials had been removed by centrifugation at 18 000 g for 30 min, the remaining solution was diluted 20 times with a buffer comprising 0.1 M Tris–HCl, pH 8.0, 1 mM oxidized form of thioglutathione, 0.1 mM reduced form of glutathione, and then gently mixed for 12 h at 4°C to reconstitute the eotaxin-3 protein. After insoluble materials had been removed by centrifugation at 18 000 g for 30 min, the remaining solution was diluted 20 times with a buffer comprising 0.1 M Tris–HCl, pH 8.0, 1 mM oxidized form of thioglutathione, 0.1 mM reduced form of glutathione, and then gently mixed for 12 h at 4°C to reconstitute the eotaxin-3 protein. After insoluble materials had been removed by centrifugation at 18 000 g for 30 min, the remaining solution was diluted 20 times with a buffer comprising 0.1 M Tris–HCl, pH 8.0, 1 mM oxidized form of thioglutathione, 0.1 mM reduced form of glutathione, and then gently mixed for 12 h at 4°C to reconstitute the eotaxin-3 protein. After insoluble materials had been removed by centrifugation at 18 000 g for 30 min, the remaining solution was diluted 20 times with a buffer comprising 0.1 M Tris–HCl, pH 8.0, 1 mM oxidized form of thioglutathione, 0.1 mM reduced form of glutathione, and then gently mixed for 12 h at 4°C to reconstitute the eotaxin-3 protein. After insoluble materials had been removed by centrifugation at 18 000 g for 30 min, the remaining solution was diluted 20 times with a buffer comprising 0.1 M Tris–HCl, pH 8.0, 1 mM oxidized form of thioglutathione, 0.1 mM reduced form of glutathione, and then gently mixed for 12 h at 4°C to reconstitute the eotaxin-3 protein. After insoluble materials had been removed by centrifugation at 18 000 g for 30 min, the remaining solution was diluted 20 times with a buffer comprising 0.1 M Tris–HCl, pH 8.0, 1 mM oxidized form of thioglutathione, 0.1 mM reduced form of glutathione, and then gently mixed for 12 h at 4°C to reconstitute the eotaxin-3 protein.
Mass spectrometry
Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was carried out using a mass spectrometer (REFLEX; Bruker Daltonik GmbH, Bremen, Germany) with pulsed ion extraction and a 377 nm nitrogen laser. The spectrum was obtained in the linear mode with an accelerating voltage of 19 kV, and 50 individual laser shots were collected and averaged. Sinapinic acid (Fluka, Tokyo, Japan; MALDI grade) was prepared as a saturated solution in a 2:1 (v/v) mixture of 0.1% trifluoroacetic acid/acetonitrile, and used as a matrix. A 1 µl aliquot of a 1:1 matrix/sample mixture (v/v) was deposited onto a plate and then dried at room temperature. Horse heart cytochrome c (Aldrich, Tokyo, Japan) was used to calibrate the spectrum.

Preparation of human eosinophils
Human granulocytes were separated from venous blood of healthy donors by Percoll (1.085 g/ml) gradient centrifugation at room temperature as described (Hansel et al., 1991). After removing red blood cells by hypotonic lysis, CD16-positive cells were enriched by means of an immunomagnetic beads technique as described (Hansel et al., 1991). The content of eosinophils determined by analysis of a Diff-Quik-stained cytocentrifugation preparation (International Reagents, Kobe, Japan) was 95%.

Competition binding assaying of $^{125}$I-chemokines
Human eosinophils were mixed with 0.1 nM of either $^{125}$I-eotaxin or $^{125}$I-MIP-1α for 1 h at 37°C in 200 µl of binding buffer (50 mM HEPES, pH 7.5, 1 mM CaCl₂, 5 mM MgCl₂, 0.5% BSA, 0.02% sodium azide) containing various concentrations of the respective unlabeled chemokine. After the cells had been washed with a washing buffer (binding buffer plus 0.5 M NaCl), the γ-radiation of the cells was counted as described previously (Shinkai et al., 1999).

In vitro chemotaxis assay
Chemotaxis of eosinophils was assessed in 96-well microplate chambers (Neuro Probe, Cabin John, MD). Aliquots (350 µl) of chemokines were placed in the wells of the lower compartment, and 200-µl aliquots of an eosinophil suspension (1×10⁵ cells/ml) were placed in the upper wells of the chamber as described (Shinkai et al., 1999). After incubation at 37°C for 1 h under humidified air containing 5% CO₂, the migrated cells were counted by measuring eosinophil peroxidase activity as described previously (Shinkai et al., 1999).

Other methods
Protein concentrations were determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard. SDS-PAGE was performed with a ready-made 15% (w/w) polyacrylamide gel (ATTO Corp., Tokyo, Japan) according to the method of Laemmli (Laemmli, 1970), and the gel was stained with Coomassie brilliant blue R-250. N-terminal sequence analysis was performed with a protein sequencer (PPSQ-10; Shimadzu, Tokyo, Japan).

Results
Expression and purification of the wild-type and mutant eotaxin-3 proteins
Wild-type eotaxin-3 was expressed in E.coli as a fusion protein comprising mature eotaxin-3 preceded by Met–Ile–Glu–Gly–Arg, which can be removed by factor Xa. Since the fusion protein was expressed as an insoluble form, it was dissolved in a buffer containing 6 M guanidine–HCl, and diluted 20 times by the addition of a buffer containing the oxidized and reduced forms of glutathione for reconstitution. Then, the fusion protein was purified by heparin–Cellulofine column chromatography, followed by treatment with factor Xa to cleave the N-terminal extra five amino acids, and further purified on an SP Sepharose column, resulting in >95% purity on SDS-PAGE (Figure 2). The N-terminal amino acid of the purified protein was Thr, and the Mw of the protein determined from the MALDI-TOF MS spectrum was 8393.3. These results indicate that the protein was the entire mature eotaxin-3. The E.coli-produced eotaxin-3 caused induction of chemotaxis of eosinophils from human venous blood to the same extent as that expressed by baculovirus-infected insect cells and purified from the culture medium without refolding steps (Figure 3A).
Furthermore, the E.coli-produced eotaxin-3 as well as that produced by baculovirus-infected insect cells was a potent inhibitor of eotaxin binding to eosinophils, suggesting that both eotaxin-3 proteins recognize CCR3 (Figure 3B). We inferred that the reconstituted eotaxin-3 folded like that expressed in eukaryotic cells as a secretory protein.

In order to determine the function of the N-terminal region of eotaxin-3, a set of N-terminal amino acid deletion mutants was constructed (Figure 1). As in the case of recombinant expression of the wild-type eotaxin-3, we designed each deletion mutant as a fusion protein, with Met–Ile–Glu–Gly–Arg as the N-terminal of each mutant. The Δ1-, Δ2-, Δ3-, Δ4-, Δ5- and Δ6-eotaxin-3 proteins, which lack the N-terminal 1, 2, 3, 4, 5 and 6 amino acids of the eotaxin-3 protein, respectively, were obtained by means of the same procedure as for the wild-type protein with >95% purity (Figure 2). We confirmed that these proteins had the desired primary structures by analyzing the N-terminal amino acid sequences and MALDI-TOF MS spectra of the proteins, respectively (data not shown). Considering the N-terminal amino acid sequences, and the Mw determined from the MALDI-TOF MS spectra of the Δ7-, Δ8-, Δ9- and Δ10-eotaxin-3 proteins, the N-terminal Met–Ile–Glu–Gly–Arg was not cleaved on treatment with factor Xa when the deletion extended for more than seven amino acids (data not shown). Therefore, in the case of these proteins, we designed the desired N-terminus immediately following the initiating methionine. These mutant proteins were also purified using heparin–Cellulofine and SP Sepharose, with >95% purity for the Δ7-, Δ8- and Δ9-eotaxin-3 proteins, and ~50% purity for the Δ10-eotaxin-3 (Figure 2). The Mw of the purified Δ7-, Δ8-, Δ9- and Δ10-eotaxin-3 proteins determined from the MALDI-TOF MS spectra and by N-terminal amino acid sequence analysis indicate that the N-terminal methionine residues of all these mutant proteins except Δ7-eotaxin-3 had been removed, presumably through the activity of methionine aminopeptidase in the host cells.

**Competitive binding activity of the mutant eotaxin-3 proteins toward eosinophils**

The binding activity of the mutant eotaxin-3 toward eosinophils was analyzed by means of competitive binding studies. Eotaxin and MIP-1α have been shown to recognize CCR3 and CCR1 on eosinophils, respectively (Daugherty et al., 1996; Ponath et al., 1996). The Δ1-, Δ2-, Δ3-, Δ4-, Δ5- and Δ6-eotaxin-3 proteins were almost as potent inhibitors of 125I-eotaxin-3 binding to eosinophils as wild-type eotaxin-3, the ID₅₀ values being ~10 nM (Figure 4A and B). The ID₅₀ values of the Δ7-, Δ8- and Δ9-eotaxin-3 proteins were ~30 nM. The competitive binding activity of Δ10-eotaxin-3 was dramatically reduced (Figure 4C). Although the purity of the Δ10-eotaxin-3 was lower than those of the other eotaxin-3 proteins, the contaminated proteins in the Δ10-eotaxin-3 hardly affected the binding of wild-type eotaxin-3 toward eosinophils (see below, Figure 6). Therefore, the low binding affinity of the Δ10-eotaxin-3 was not derived from the impurity. The ID₅₀ values of the wild-type and mutant eotaxin-3 proteins were summarized in Figure 1. 125I-MIP-1α binding to eosinophils was not inhibited by the addition of each mutant eotaxin-3 (data not shown), indicating none of the truncated mutants binds to CCR1 like wild-type eotaxin-3 does (Shinkai et al., 1999).

**In vitro chemotactic activity of the mutant eotaxin-3**

Wild-type eotaxin-3 caused the most efficient chemotaxis at concentrations of 20–80 nM (Figure 5A). Δ1-, Δ2- and Δ3-eotaxin-3 showed almost the same chemotactic activity as wild-type eotaxin-3 (Figure 5A). The chemotactic activity gradually decreased with further extension of the N-terminal deletion (Figure 5A and B), and when the deletion extended to Lys8, the activity was not detected up to 1 μM (Figure 5C). The relative activity of the mutant eotaxin-3 proteins compared to the wild-type was summarized in Figure 1.

**Inhibition of chemokine-induced migration by non-chemotactic eotaxin-3 mutants**

The effects of the non-chemotactic mutants, the Δ8-, Δ9- and Δ10-eotaxin-3 proteins, on the chemotactic activity of wild-type eotaxin-3 toward eosinophils were investigated (Figure 6A and B). We analyzed the effects on 25 and 50 nM wild-type eotaxin-3, which are within the optimal concentration range (Figure 5A). The Δ8- and Δ9-eotaxin-3 proteins inhibited the chemotaxis caused by both 50 and 25 nM wild-type eotaxin-3 in a dose-dependent manner. In contrast, Δ10-eotaxin-3 showed hardly any such inhibitory activity. Next, we analyzed the effects of the mutant eotaxin-3 on eotaxin
Fig. 5. In vitro chemotactic activity of the wild-type and truncated eotaxin-3 proteins. A transwell assay was performed to measure the chemotaxis. The cells were placed in the upper wells, whereas chemokines were placed in the lower wells. Migrated cells were collected and counted by measuring the eosinophil peroxidase activity. (A) The profiles of wild-type (●), Δ1- (○), Δ2- (×), Δ3- (■) and Δ4-eotaxin-3 (□) at concentrations of 0–80 nM. (B) The profiles of wild-type (●), Δ5- (▲), Δ6- (△), Δ7- (▽), Δ8- (◁) and Δ10-eotaxin-3 (◀) at concentrations of 0–80 nM. (C) The profiles of wild-type (●), Δ7- (▽), Δ8- (◁), Δ9- (◆) and Δ10-eotaxin-3 (◀) at concentrations of 0–1000 nM. Three independent experiments were performed, respectively, and the results were expressed as the mean ± SD. C represents nM chemokine. The relative activity of each eotaxin-3 is shown in Figure 1.

Fig. 6. The effects of the truncated eotaxin-3 proteins on the chemotactic activity of wild-type eotaxin-3. A transwell assay was performed to measure the chemotaxis. Peripheral blood eosinophils were placed in the upper wells, whereas chemokines were placed in the lower wells. Migrated cells were collected and counted by measuring the eosinophil peroxidase activity. Δ8- (●), Δ9- (○) or Δ10-eotaxin-3 (□) was added to 25 (A) and 50 nM (B) wild-type eotaxin-3.

Fig. 7. The effects of the truncated eotaxin-3 proteins on the chemotactic activity of eotaxin and RANTES. A transwell assay was performed to measure the chemotaxis. Peripheral blood eosinophils were placed in the upper wells, whereas chemokines were placed in the lower wells. Migrated cells were collected and counted by measuring the eosinophil peroxidase activity. Δ8- (●), Δ9- (○), or Δ10-eotaxin-3 (□) was added to 5 nM eotaxin (A) and RANTES (B).
and RANTES that also activate CCR3. Since these chemokines caused the most efficient chemotaxis at an approximately 10 times lower concentration than that of eotaxin-3 (Shinkai et al., 1999; data not shown), we analyzed the effects on these chemokines at the concentration of 5 nM. As a result, basically the same effects were observed on the chemotaxis caused by eotaxin and RANTES (Figure 7A and B). These results are consistent with the observations described above, i.e. ∆8- and ∆9-eotaxin-3 lost chemotactic activity toward eosinophils while keeping the wild-type-like binding activity toward the cells, and both activities of ∆10-eotaxin-3 were dramatically reduced.

Discussion

The three-dimensional structure of eotaxin-3 has been determined by nuclear magnetic resonance spectroscopy (Ye et al., 2001). The structure consists of an unstructured N-terminal domain before the first cysteine residue, an irregularly structured N-loop following the second cysteine, a single turn of a 3_10-helix, a three-stranded anti-parallel β-sheet, an α-helix, and an unstructured C-terminal tail. These structural features are conserved among the chemokine molecules of which the structures have been determined so far (Baldwin et al., 1996; Crump et al., 1997, 1998; Mayer and Stone, 2000). In order to gain an insight into the structure–function relationship of eotaxin-3, we constructed a set of N-terminal deletion mutants and investigated their activity toward peripheral blood eosinophils. Based on the results of competitive binding studies and chemotaxis assay, we concluded that the first nine amino acid residues, which correspond to the unstructured N-terminal domain of the three-dimensional structure, were not critical for binding to CCR3 on eosinophils, and that the first eight residues were essential for activation of the receptor. The mutant eotaxin-3 with this region deleted showed antagonist activity toward CCR3. These results support the previous hypothesis that a flexible N-terminal region of a chemokine is supported by the core of the protein and is presented to a receptor in a manner that optimizes binding and activation (Crump et al., 1997, 1998). ∆10-Eotaxin-3 displayed only weak binding affinity. According to the three-dimensional structure of eotaxin-3, Cys10 is not inside the flexible region but structured (Ye et al., 2001), therefore, the ∆10-eotaxin-3 may not be completely folded. The importance of the N-terminal flexible domain of a CC chemokine for activation of a receptor has also been shown through truncation mutants of MCP-1 (Gong and Clark-Lewis, 1995), MCP-3 and RANTES (Gong et al., 1996). The primary sequence of the N-terminal domain is not conserved among these chemokines. The numbers of amino acid residues comprising the N-terminal domains of MCP-1, MCP-3 and RANTES are 10, 10 and nine, respectively, in which the 1–6 region of MCP-1, 1–7 region of MCP-3, and 1–5 region of RANTES are critical for activation of their receptors. Thus, the sequence and number of amino acid residues essential for receptor activation differ among the chemokines. ∆1-, ∆2- and ∆3-eotaxin-3 exhibited wild-type-like chemotactic activity, and the activity gradually decreased with further extension of the N-terminal deletion. All the N-terminal truncated eotaxin-3 constructed in this study, except ∆10-eotaxin-3, bound to eosinophils through CCR3 to almost the same extent as wild-type eotaxin-3. Thus, the ability of receptor activation depends on the length of the N-terminal domain. In the case of MCP-1, a deletion mutant as to the N-terminal pyroglutamate residue exhibits greatly reduced activity compared with that of the wild-type, indicating this residue is important for the function (Gong and Clark-Lewis, 1995). Deletion of the next residue leads to a total loss of activity, and analogs with N-terminal three- and four-residue deletions are active (Gong and Clark-Lewis, 1995). A chemokine has been thought to bind to the N-terminal part of a receptor molecule (Monteclaro and Charo, 1997; Pease et al., 1998), and the N-terminal flexible domain of a chemokine may activate the receptor. However, the precise mechanism of the activation of the receptor may differ among chemokines.

Infiltration of eosinophils, basophils and T helper type 2 cells has been thought to cause parasite- and allergen-induced inflammation (Weller, 1991; Teixeira et al., 1995). CCR3 is specifically expressed on these cell types and plays a central role in their infiltration (Luster, 1998). Therapies that block the function of CCR3 may be of benefit in allergic diseases such as asthma, dermatitis and sinusitis. Furthermore, CCR3 is a co-receptor for several strains of HIV-1 (Choe et al., 1996); thus, a CCR3 antagonist can also be possibly used for anti-HIV-1 therapy. Several peptide or non-peptide CCR3 antagonists have been reported so far (Nibbs et al., 2000; White et al., 2000; Ye et al., 2000). The CCR3 antagonists obtained in this study, which can be easily produced in E.coli, are also good candidates for such therapeutic applications.

References


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