Cloning, Expression, and Characterization of the Human Eosinophil Eotaxin Receptor

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Summary

Although there is a mounting body of evidence that eosinophils are recruited to sites of allergic inflammation by a number of β-chemokines, particularly eotaxin and RANTES, the receptor that mediates these actions has not been identified. We have now cloned a G protein-coupled receptor, CC CKR3, from human eosinophils which, when stably expressed in AML14.3D10 cells bound eotaxin, MCP-3 and RANTES with Kd of 0.1, 2.7, and 3.1 nM, respectively. CC CKR3 also bound MCP-1 with lower affinity, but did not bind MIP-1α or MIP-1β. Eotaxin, RANTES, and to a lesser extent MCP-3, but not the other chemokines, activated CC CKR3 as determined by their ability to stimulate a Ca2+-flux. Competition binding studies on primary eosinophils gave binding affinities for the different chemokines which were indistinguishable from those measured with CC CKR3. Since CC CKR3 is prominently expressed in eosinophils we conclude that CC CKR3 is the eosinophil eotaxin receptor. Eosinophils also express a much lower level of a second chemokine receptor, CC CKR1, which appears to be responsible for the effects of MIP-1α.

Eosinophils play prominent roles in a variety of atopic conditions including allergic rhinitis, dermatitis, conjunctivitis, and bronchial asthma (1, 2). A pivotal event in the process is the accumulation of eosinophils at the involved sites. While a number of the classical chemoattractants, including C5a, LTβ4, and PAF, are known to attract eosinophils (1), these mediators are promiscuous, acting on a variety of leukocytes including neutrophils, and are unlikely to be responsible for the selective accumulation of eosinophils. In contrast, the β-chemokines, a family of 8-10-kD secreted proteins, are more restricted in the leukocyte subtypes they target (3), and studies from a variety of laboratories have implicated several as candidates for the recruitment of eosinophils in atopic diseases. In particular, RANTES, MCP-3, MIP-1α, and most recently, eotaxin, have been shown to activate eosinophils in vitro (4–6), and RANTES and eotaxin to selectively attract eosinophils in vivo (7, 8). Moreover, eotaxin is generated during antigen challenge in the guinea pig model of allergic airway inflammation (9, 10).

While elucidation of the actions of β-chemokines on eosinophils has contributed greatly to our understanding of eosinophil biology, information regarding the cell surface receptors that mediate these effects remains sparse. The known β-chemokine receptors are members of the G protein-coupled receptor superfamily. Although two of these receptors, CC CKR1 (11–13, Daugherty, B., manuscript in preparation) and CC CKR2 (MCP-1R) (14–16) have been extensively characterized, neither has the necessary ligand selectivity or the appropriate expression patterns to mediate the effects of the β-chemokines on eosinophils. Therefore, we initiated an effort to identify and characterize eosinophil-specific chemokine receptors. In this report we describe the properties of a third β-chemokine receptor, CC CKR3, cloned from primary eosinophils, and functionally expressed in AML14.3D10 cells. This receptor has the expected ligand specificity as it binds the potent eosinophil attractants, eotaxin, RANTES, and MCP-3 with high affinity. Correlation with the binding properties of primary eosinophils provides compelling evidence that CC CKR3 is the primary endogenous receptor that mediates the effects of β-chemokines on eosinophils. Eosinophils also express a much lower level of CC CKR1, a receptor that appears to be responsible for the effects of MIP-1α.

Materials and Methods

cDNA Cloning of CC CKR3. Total human eosinophil RNA was purified and used in an RT/PCR reaction (17) with the following oligonucleotide primers designed from the human CC CKR1 and CC CKR2 cDNAs (11, 14): 5'-AACCTGGCCAT(C,T)TCTGA-(C,T)CTGC-3'; 5'-GAAC(C,T)TCTC(C,A)CTGC-3'. The remaining 5' and 3' sequence encod-
ing CC CKR3 was cloned by rapid amplification of cDNA ends (RACE) with the following primers: 5'TCTCGCTGTACA-GGCTGTGTG-3' (5'RACE) and 5'CCTGCTCTCCTCCTGTTA-CATCC-3' (3'RACE). The RACE products were sequenced and the initiation and termination codons (TAG) identified. For expression of CC CKR3, a new set of PCR primers were designed to reamplify the entire coding region: 5'ATATATTAAGCCTACCAGACACCTACTAGATAAG-3'; 5'ATA-TATCTAGAGCGGGCTTAAACACAATAGGATTCG-3'. The resultant PCR product was subcloned into the expression vector pBl/NEO (Daugherty, B., manuscript in preparation) to yield pBl/NEO/CCCKR3. Several clones were sequenced and one clone comprising the consensus sequence was chosen for expression of CC CKR3 in heterologous cells.

Transfection into AML14.3D10 Cells. Transfection into AML 14.3D10 cells (18) was performed as described (19). Stable clones were generated by selection in medium containing 2 mg/ml Geneticin for 8-10 d until individual surviving clusters appeared. Clones were derived from these clusters by limiting dilution and assayed by Western blotting and ligand-induced Ca²⁺ flux.

Purification of Eosinophils. Primary eosinophils were isolated from granulophoresis preparations (20) obtained from allergic and asthmatic donors. The granulocytes were purified (21) and subsequently treated with anti-CD16 microbeads (Miltenyi Biotech, Auburn, CA) followed by MACS separation (22). Eosinophils were typically >99% pure.

Generation of α-CC CKR3 Antisera and Immunoblotting. Polyclonal rabbit antisera was generated to CC CKR3 using the 14.3D10 cells (18) was performed as described (19). Stable clones were generated by selection in medium containing 2 mg/ml Geneticin for 8-10 d until individual surviving clusters appeared. Clones were derived from these clusters by limiting dilution and assayed by Western blotting and ligand-induced Ca²⁺ flux.

Ligand-induced Ca²⁺ fluxes in transfected AML14.3D10 cells were performed with indo-1 as described (25).

Results and Discussion

Orphan Cloning of an Eosinophil β-Chemokine Receptor. The previously characterized β-chemokine receptors, CC CKR1 (11) and CC CKR2 (14), share substantial homology in transmembrane helices II and VII. Using an RT/PCR strategy based on this homology, we cloned a novel open reading frame from total human eosinophil RNA which codes for a protein of 355 amino acids. The sequence of this protein, designated CC CKR3, is 63% and 51% identical to CC CKR1 and CC CKR2, its two closest homologues (Fig. 1). This sequence is also identical to that reported by Combadiere et al. (26) except that it contains a lysine in place of asparagine at position 107. We have confirmed our sequence by analysis of genomic clones. The discrepancy is unlikely to be due to genetic polymorphism since all α- and β-chemokine receptors analyzed to date contain lysine in that position including the recently described basophilic β-chemokine receptor (27), CC CKR1 (11), MCP-1R (14), IL-8RA and IL-8RB (28, 29), the three murine β-chemokine receptors (30, 31) as well as three human chemokine-like receptors (32-34). An unusual feature of CC CKR3, in contrast to other chemokine receptors, is the cluster of negatively charged amino acids from Amersham (Arlington Heights, IL). Binding of [125I]-labeled ligands (typically a total of 2 × 10⁶ cpm) in the presence of varying concentrations of unlabeled ligands to intact cells (typically 1.5 × 10⁶, 10⁷, or 10⁸ for experiments with labeled eotaxin, MCP-3, or MIP-1α, respectively) were performed at 32°C as described (25). Ligand-induced Ca²⁺ fluxes in transfected AML14.3D10 cells were performed with indo-1 as described (25).

Figure 1. Amino acid sequence alignment of human β-chemokine receptors. The figure shows the predicted sequences for CC CKR3, CC CKR1 (11), CC CKR2B (14), CC CKR4 (27), and V8 (33). The positions of the seven putative transmembrane-spanning regions are designated with overlines. A minimum of three identical residues is indicated in the shaded region. The complete nucleotide sequence of CC CKR3 is available from EMBL/GenBank/DDJB under accession number U51241.
Expression of the Human CC CKR3 in AML14.3D10 Cells. AML14.3D10 was transfected with CC CKR3 and stable clones selected for neomycin resistance. To demonstrate expression of receptor protein, a Western blot was performed using antisera generated against a peptide derived from the predicted COOH-terminus of CC CKR3. As shown in Fig. 2, prominent immunoreactive bands migrating at 45-55 kD are present in primary eosinophils (lane 1) and clone 3.16 (lane 2), indicating that these cells express CC CKR3. The bands recognized by the antisera are specific since they are not present in either untransfected AML14.3D10 cells (lane 5), or in neutrophils (lane 4). Furthermore, the immunoreactive bands are absent in clone 3.49 (lane 3), indicating that this neomycin-resistant clone is a non-expressor of CC CKR3. Clone 3.49 therefore was used as a negative control in subsequent experiments. The sharp 45-kD immunoreactive band present in the 3.16 clone, but not in eosinophils, is likely to represent the non-glycosylated form of the receptor.

Table 1. Binding Affinities of Various Chemokines Comparing CC CKR3 Expressed in AML14.3D10 with Primary Eosinophils

<table>
<thead>
<tr>
<th>Competitor</th>
<th>CC CKR3</th>
<th>Eosinophils</th>
</tr>
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<tbody>
<tr>
<td>Human-eotaxin</td>
<td>0.1 ± 0.04</td>
<td>0.1 ± 0.03</td>
</tr>
<tr>
<td>Murine-eotaxin</td>
<td>0.1 ± 0.04</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>MCP-3</td>
<td>2.7 ± 1.7</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>RANTES</td>
<td>3.1 ± 0.6</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>MCP-1</td>
<td>60 ± 9</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>N.B.</td>
<td>N.B.</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>N.B.</td>
<td>N.B.</td>
</tr>
</tbody>
</table>

Competition binding experiments were carried out against the indicated iodinated ligand as described in the legend of Fig. 2 and in Materials and Methods. All results are the averages of the number of experiments shown in parentheses. Kd were calculated using LIGAND (36). N.B., no competition was observed.
Competition binding studies were also carried out against 125I-MCP-3. Again, human and murine eotaxin competed strongly with $K_d$ of 0.2 and 0.3 nM, respectively (Fig 3b, Table 1). MCP-3 and RANTES also demonstrated high affinity, with $K_d$ of 0.7 and 0.5 nM, values about fourfold lower than measured against 125I-eotaxin. MCP-1 competed weakly ($K_d = 16$ nM), and MIP-1α, and MIP-1β failed to compete at all. Thus, despite small quantitative differences, the overall ligand selectivity of the receptor is the same whether measured by competition against eotaxin or MCP-3, and the order of potency, eotaxin>MCP-3 = RANTES>>MCP-1, is identical.

**CC CKR3 Is Functionally Coupled in AML14.3D10 Cells.** To determine whether CC CKR3 was functionally coupled in AML14.3D10 cells, intracellular Ca$^{2+}$ levels were measured in response to various 13-chemokines. As shown in Fig. 4, eotaxin and RANTES induced Ca$^{2+}$-fluxes in cells expressing the receptor with $E_D_{50}$ of 0.3 and 10 nM, values consistent with their binding affinities. Surprisingly, 100 nM of MCP-3 was required to induce a response, and that response was smaller than those observed for eotaxin or RANTES (Fig 4). No response was generated by the addition of MIP-1α, MIP-1β, MCP-1 or IL-8 at concentrations as high as 1 μM (data not shown)$^1$. The responses to eotaxin, RANTES, and MCP-3 are due to the specific expression of CC CKR3 since none of these mediators induced fluxes in untransfected cells (data not shown), or in clone 3.49 (negative control; Fig 4).

**Binding Properties of Primary Eosinophils.** The selectivity of CC CKR3 for the various 13-chemokines mirrors the effectiveness of these ligands as eosinophil chemoattractants suggesting that CC CKR3 is the primary mediator of chemokine-induced eosinophil chemotaxis. To provide additional pharmacological evidence we conducted binding studies on primary eosinophils. When measured by competition against 125I-eotaxin, unlabeled human eotaxin gave a $K_d$ of 0.1 nM, a value identical to that obtained on cloned CC CKR3 (Fig. 3c, Table 1). Scatchard analysis showed a single binding affinity, and $4 \times 10^4$ sites/cell averaged over three donors (data not shown). The affinities for RANTES and MCP-3 were indistinguishable from those measured on CC CKR3, and as with CC CKR3, MIP-1α and MIP-1β did not exhibit any ability to compete with radiolabeled eotaxin (Fig. 3, a and c, Table 1). Similarly, the $K_d$ obtained by competition against 125I-MCP-3 on eosinophils were within twofold of those measured against cloned CC CKR3 (Fig. 3, b and d, Table 1). All of the observations and measurements, taken together with the Western blots (Fig. 2) showing expression of CC CKR3, verify that CC CKR3 is the eosinophil eotaxin receptor, and appears to be largely responsible for mediating the effects of most 13-chemokines on eosinophils.

**Eosinophils Also Express CC CKR1 at Low Levels.** One difference between data obtained with eosinophils and that with cloned CC CKR3 is that MIP-1α partially inhibited the binding of 125I-MCP-3 on eosinophils (Fig 3d). To investigate the nature of the site responsible for these effects, detailed studies were carried out by competition against 125I-MIP-1α. As shown in Fig. 5, MIP-1α, MCP-3, and RANTES all competed strongly with $IC_{50}$ of 0.3, 0.7, and 0.9 nM, respectively. In contrast, human and murine eotaxin competed with relatively low affinity, showing $IC_{50}$ of 45 and 11 nM, respectively, while the affinity of MCP-1 is even lower with an $IC_{50}$ of 120 nM. These pharmacological characteristics are clearly distinct from those of CC CKR3, but are identical to those we have reported for CC CKR1 expressed in RBL2H3 cells (Daugherty, B., manuscript in preparation). Scatchard analysis shows 0.5–2 $\times 10^4$ sites/cell, only 1–5% the level of CC CKR3 (data not shown).

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1 Combadiere et al. (26) have reported cloning a receptor that differs by only one amino acid from the sequence reported in the present communication. While their very preliminary functional characterization differs greatly from ours, they were unable to demonstrate any specific binding to cells putatively expressing the receptor, and their functional data have now been retracted (35).
The properties of CC CKR3 and CC CKR1 can account for the reported effects of \( \beta \)-chemokines on eosinophils. As discussed above, the data strongly support the conclusion that CC CKR3 is the eotaxin receptor. While the properties of the two receptors indicate that either is capable of mediating the activity of RANTES and MCP-3, CC CKR3 is probably the primary transducer since it is expressed at 20–80 times the level of CC CKR1 (4 \( \times \) 10^5 vs. 0.5–2 \( \times \) 10^4 sites/cell), a difference that more than compensates for the greater affinity of CC CKR1 for the two chemokines. MIP-1\( \alpha \) must act through CC CKR1 as it binds strongly to and activates this receptor (11, Daugherty, B., manuscript in preparation), but does not bind to CC CKR3. The identification of the two \( \beta \)-chemokine eosinophil receptors is consistent with predictions made from heterologous desensitization experiments. Based on these studies Dahinden et al. (4) postulated the existence of two receptors, one that is activated by RANTES and MCP-3, and a second that is activated by MIP-1\( \alpha \), RANTES, and by MCP-3. Although these studies predate the discovery of eotaxin, the properties of the first receptor are consistent with CC CKR3, and those of the second with CC CKR1.

CC CKR3 is the third \( \beta \)-chemokine receptor to be extensively characterized, and like CC CKR1 and CC CKR2 it binds and is activated by multiple ligands. The selectivities of the three receptors overlap, but are not identical: CC CKR1 binds MCP-3, RANTES, and MIP-1\( \alpha \) (11–13, Daugherty, B., manuscript in preparation), CC CKR2 binds MCP-1 and MCP-3 (14, 16), and CC CKR3 is selective for eotaxin, RANTES and MCP-3. While there is little correlation between overall sequence homology of the \( \beta \)-chemokines and the receptors they target, local motifs must exist which control specificity. Elucidation of those motifs should significantly advance structurally based approaches to develop selective antagonists for the different receptors.

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