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 $K_d$ 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.

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′-AACCTGGCAT(C,T)TCTGTA-(C,T)CTTGC-3′; 5′-AAAC(G,T)TCTC(C,A)-CCACGAGGC-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'-TTCGCTGTACAGCTTGGTGT-3' (5'-RACE); 5'-TCTCTTCCTCTCTCATTCCAATCC-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'-ATATATTTAGCTTCCACATGACACCTACTAGATAAG-3'; 5'-ATATATTAGCTCAGGGCCGTATAAAACATAGAGAGTTCC-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 granulopheresis 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 COOH-terminal decapeptide sequence TAEPELSIVF (23). SDS PAGE (24) was carried out with whole cells on 4–20% gels (Novex, San Diego, CA), and immunoblotting was performed as described (Novex).

Assays. Recombinant chemokines were obtained from Peprotech (Rocky Hill, NJ). 125I-MCP-3 and 125I-MIP-1α was obtained from DuPont NEN (Boston, MA) and 125I-human-eotaxin from Amersham (Arlington Heights, IL). Binding of 125I-labeled ligands (typically a total of 2 × 104 cpm) in the presence of varying concentrations of unlabeled ligands to intact cells (typically 1.5 × 104, 103, or 102 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).

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 CKR2B, 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 × 104 cpm) in the presence of varying concentrations of unlabeled ligands to intact cells (typically 1.5 × 104, 103, or 102 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).

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Expression of the Human CC CRK3 in AML14.3D10 Cells. AML14.3D10 was transfected with CC CRK3 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 CRK3. 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 CRK3. 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 CRK3. 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.

Binding to CC CRK3 on Intact AML14/CCCRK3.16 Cells. Competition binding studies were performed with 125I-eotaxin on clone 3.16 in order to characterize the pharmacological properties of CC CRK3. As shown in Fig. 3 and Table 1, unlabeled human and murine eotaxin both competed with Kd of 0.1 nM. Scatchard analysis demonstrated that the eotaxins bound with a single affinity and that clone 3.16 expressed 4 x 10^5 receptors/cell (data not shown). This activity is due to CC CRK3 since neither nonimmunoreactive clones, such as 3.49, nor untransfected cells displayed any specific binding (data not shown). Clearly, CC CRK3 is a high affinity receptor for eotaxin. Cross-competition studies with other β-chemokines known to be potent eosinophil chemoattractants, MCP-3 and RANTES, demonstrated that they bound to CC CRK3 with Kd of about 3 nM (Fig. 3, Table 1). In contrast, MCP-1 competed with much lower affinity (Kd = 60 nM), and MIP-1α, MIP-1β (Fig. 3, Table 1), and the α-chemokine, IL-8 (data not shown), failed to compete at all.

Table 1. Binding Affinities of Various Chemokines Comparing CC CRK3 Expressed in AML14.3010 with Primary Eosinophils

<table>
<thead>
<tr>
<th>Competitor</th>
<th>CC CRK3</th>
<th>Eosinophils</th>
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<tr>
<td><strong>Kd (nM)</strong></td>
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<tr>
<td>125I-human eotaxin</td>
<td>0.1 ± 0.04 (4)</td>
<td>0.1 ± 0.03 (3)</td>
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<tr>
<td>Murine-eotaxin</td>
<td>0.1 ± 0.04 (3)</td>
<td>0.1 ± 0.01 (2)</td>
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<tr>
<td>MCP-3</td>
<td>2.7 ± 1.7 (5)</td>
<td>3.0 ± 0.2 (2)</td>
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<tr>
<td>RANTES</td>
<td>3.1 ± 0.6 (5)</td>
<td>2.6 ± 0.3 (2)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>60 ± 9 (3)</td>
<td>41 ± 2 (2)</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>N.B. (4)</td>
<td>N.B. (2)</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>N.B. (4)</td>
<td>N.B. (2)</td>
</tr>
<tr>
<td>125I-MCP-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human-eotaxin</td>
<td>0.2 ± 0.1 (4)</td>
<td>0.2 ± 0.1 (2)</td>
</tr>
<tr>
<td>Murine-eotaxin</td>
<td>0.3 ± 0.1 (2)</td>
<td>0.2 ± 0.1 (3)</td>
</tr>
<tr>
<td>MCP-3</td>
<td>0.7 ± 0.4 (4)</td>
<td>1.1 ± 0.6 (10)</td>
</tr>
<tr>
<td>RANTES</td>
<td>0.5 ± 0.3 (4)</td>
<td>0.9 ± 0.4 (8)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>16 ± 2 (3)</td>
<td>61 ± 13 (2)</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>N.B. (4)</td>
<td>See text</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>N.B. (4)</td>
<td>N.B. (2)</td>
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</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 
\(^{125}\)I-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 \(^{125}\)I-eotaxin. MCP-1 com-
peted weakly (\(K_d = 16\) nM), and MIP-1\(\alpha\) and MIP-1\(\beta\) 
failed to compete at all. Thus, despite small quantitative dif-
ferences, 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 \(\beta\)-chemokines. As shown 
in Fig. 4, eotaxin and RANTES induced Ca\(^{2+}\)-fluxes in 
cells expressing the receptor with ED\(_{50}\) 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-1\(\alpha\), MIP-1\(\beta\), MCP-1 or IL-8 at concen-
trations as high as 1 \(\mu\)M (data not shown). The responses to 
eotaxin, RANTES, and MCP-3 are due to the specific 
expression of CC CKR3 since none of these mediators in-
duced fluxes in untransfected cells (data not shown), or in 
clone 3.49 (negative control; Fig. 4).

**Binding Properties of Primary Eosinophils.** The selectivity 
for CC CKR3 for the various \(\beta\)-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 phar-
macological evidence we conducted binding studies on pri-
mary eosinophils. When measured by competition against 
\(^{125}\)I-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 bind-
ing affinity, and \(4 \times 10^5\) sites/cell averaged over three do-
nors (data not shown). The affinities for RANTES and 
MCP-3 were indistinguishable from those measured on CC 
CKR3, and as with CC CKR3, MIP-1\(\alpha\) and MIP-1\(\beta\) did 
inhibit any ability to compete with radiolabeled eotaxin 
(Fig. 3, a and c, Table 1). Similarly, the \(K_d\) obtained by 
competition against \(^{125}\)I-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 mea-
surements, 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 \(\beta\)-chemokines 
on eosinophils.

**Eosinophils Also Express CC CKR1 at Low Levels.** One dif-
ference between data obtained with eosinophils and that 
with cloned CC CKR3 is that MIP-1\(\alpha\) partially inhibited 
the binding of \(^{125}\)I-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 
\(^{125}\)I-MIP-1\(\alpha\). As shown in Fig. 5, MIP-1\(\alpha\), 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 eot-
taxin 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 pharma-
co- logical 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., manu-
script 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 commu-
nication. 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 β-chemokines on eosinophils. As discussed above, the data strongly support the conclusion that CC CR3 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 CR3 is probably the primary transducer since it is expressed at 20–80 times the level of CC CR2. MCP-1α must act through CC CR1 as it binds strongly to and activates this receptor (11, Daugherty, B., manuscript in preparation), but does not bind to CC CR3. The identification of the two β-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-1α, RANTES, and by MCP-3. Although those studies predate the discovery of eotaxin, the properties of the first receptor are consistent with CC CR3, and those of the second with CC CR1.

CC CR3 is the third β-chemokine receptor to be extensively characterized, and like CC CR1 and CC CR2 it binds and is activated by multiple ligands. The selectivities of the three receptors overlap, but are not identical: CC CR1 binds MCP-3, RANTES, and MIP-1α (11–13, Daugherty, B., manuscript in preparation), CC CR2 binds MCP-1 and MCP-3 (14, 16), and CC CR3 is selective for eotaxin, RANTES and MCP-3. While there is little correlation between overall sequence homology of the β-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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