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_ds 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 lessor extent MCP-3, but not the other chemokines, activated CC CKR3 as determined by their ability to stimulate a Ca^{2+}-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, LTB4, 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′-AACCTGGC-CAT(C,T)TCTGA-(C,T)CTGC-3′; 5′-GAAC(C,T)TCTC(C,A)-CCAACGAAGGC-3. The remaining 5′ and 3′ sequence encoding CC CKR3 was cloned by rapid amplification of cDNA ends (RACE) with the following primers: 5′-TCTCGCTGTACAAGCCTGTGTG-3′ (5′-RACE); 5′-CCTTCTCTCTTCCTATCAATCC-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′-ATATATTAAGCTTCCACCATGACAACCTCACTAGATACAG-3′; 5′-ATATATTCTAGAGCGGCCGCTAAAACACAATAGAGAGTTCC-3′. The resultant PCR product was subcloned into the expression vector pBJ/NEO (Daugherty, B., manuscript in preparation) to yield pBJ/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 Ca^{2+} 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 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). ^{125}I-MCP-3 and ^{125}I-MIP-1α was obtained from DuPont NEN (Boston, MA) and ^{125}I-human-eotaxin from Amersham (Arlington Heights, IL). Binding of ^{125}I-labeled ligands (typically a total of 2×10^4 cpm) in the presence of varying concentrations of unlabeled ligands to intact cells (typically 1.5×10^4, 10^5, or 10^6 for experiments with labeled eotaxin, MCP-3, or MIP-1a, respectively) were performed at 32°C as described (25). Ligand-induced Ca^{2+} 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

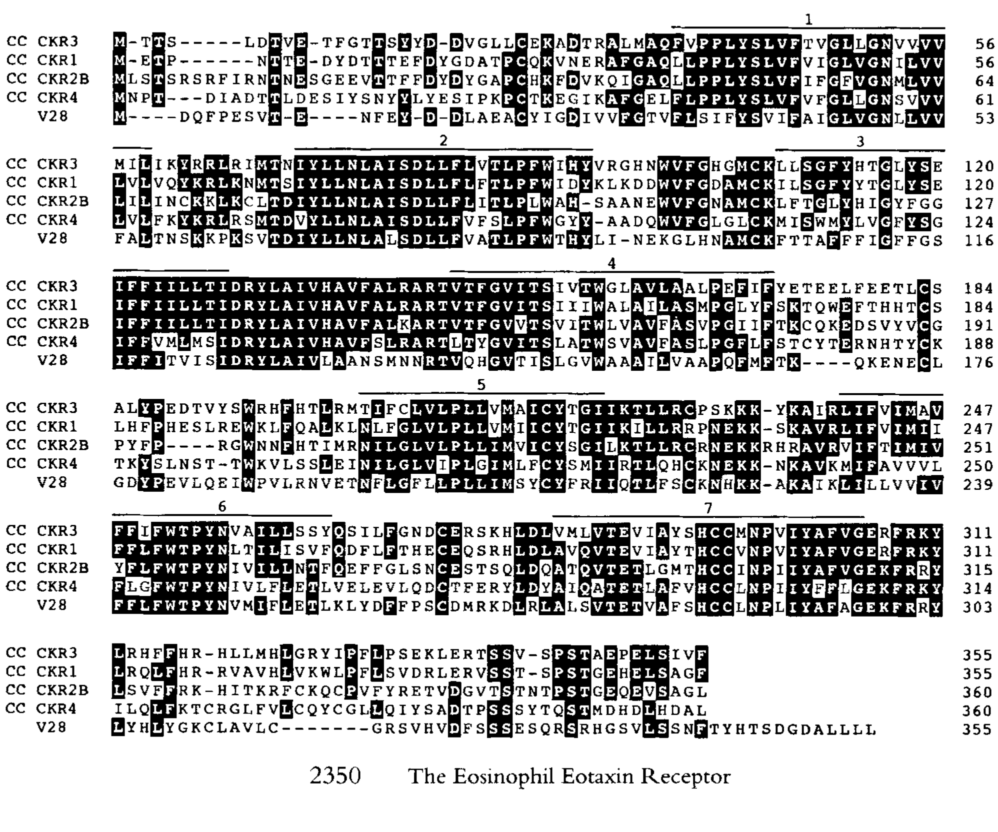

Figure 1. Amino acid sequence alignment of human β-chemokine receptors. The figure shows the predicted sequences for CC CKR3, CC CKR1 (11), CC CKR2B (14), CC CKR4 (27), and V28 (33). The positions of the seven putative transmembrane-spanning regions are designated with overlines. A minimum of three identical residues is indicated in the shaded region. The complete nucleotide sequence of CC CKR3 is available from EMBL/GenBank/DDBJ under accession number U51241.

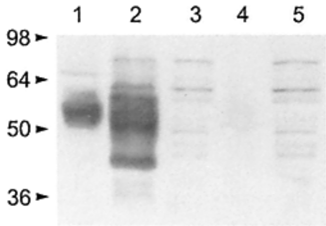

Figure 2. Expression of CC CKR3. Western blots on eosinophils (lane *1*); CC CKR3 expressing clone, 3.16 (lane *2*); nonexpressing clone, 3.49 (lane *3*); PMN (lane *4*); and untransfected AML14.3D10 (lane *5*). 10^6 cells were used in each lanes *1* and *4* and 2.5×10^5 cells were used in lanes *2*, *3*, and *5*. Preimmune sera gave no positive bands (data not shown).

(ETEELFEET) distal to transmembrane helix IV in the second extracellular loop.

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 nonglycosylated form of the receptor.

Binding to CC CKR3 on Intact AML14/CCCKR3.16 Cells. Competition binding studies were performed with ^{125}I-eotaxin on clone 3.16 in order to characterize the pharmacological properties of CC CKR3. As shown in Fig. 3 *a* and Table 1, unlabeled human and murine eotaxin both competed with K_ds of 0.1 nM. Scatchard analysis demonstrated that the eotaxins bound with a single affinity and that clone 3.16 expressed 4×10^5 receptors/cell (data not shown). This activity is due to CC CKR3 since neither nonimmunoreactive clones, such as 3.49, nor untransfected cells displayed any specific binding (data not shown). Clearly, CC CKR3 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 CKR3 with K_ds of about 3 nM (Fig. 3 *a*, Table 1). In contrast, MCP-1 competed with much lower affinity (K_d = 60 nM), and MIP-1α, MIP-1β (Fig. 3 *a*, Table 1), and the α-chemokine, IL-8 (data not shown), failed to compete at all.

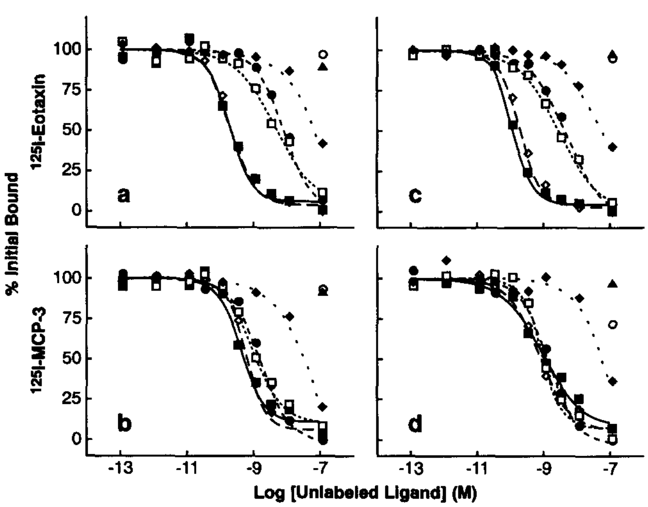

Figure 3. Equilibrium binding of β-chemokines to AML14.3D10 cells expressing CC CKR3 and to primary eosinophils. Increasing concentrations of unlabeled human eotaxin (■), murine eotaxin (◇), RANTES (□), MCP-3 (●), or MCP-1 (◆) were used to compete against fixed concentrations of either ^{125}I-human eotaxin (*a* and *c*), or ^{125}I-MCP-3 (*b* and *d*). Also shown are competition with 100 nM concentrations of MIP-1α (○), and MIP-1β (▲). The experiments were carried out either with CC CKR3 expressing clone, 3.16 (*a* and *b*), or with native eosinophils (*c* and *d*). All values are the averages of triplicate determinations. Typically, 4,000–6,000 cpm of iodinated ligand was bound in the absence of competitor with S/N ratios exceeding 15. Results are representative single experiments except data for MIP-1α and MIP-1β which are the averages of 3–7 experiments.

Table 1. *Binding Affinities of Various Chemokines Comparing CC CKR3 Expressed in AML14.3010 with Primary Eosinophils*

Competitor	CC CKR3		Eosinophils	
	Kd (nM)			
	^{125}I-human eotaxin			
Human-eotaxin	0.1 ± 0.04	(4)	0.1 ± 0.03	(3)
Murine-eotaxin	0.1 ± 0.04	(3)	0.1 ± 0.01	(2)
MCP-3	2.7 ± 1.7	(5)	3.0 ± 0.2	(2)
RANTES	3.1 ± 0.6	(5)	2.6 ± 0.3	(2)
MCP-1	60 ± 9	(3)	41 ± 2	(2)
MIP-1α	N.B.	(4)	N.B.	(2)
MIP-1β	N.B.	(4)	N.B.	(2)
	^{125}I-MCP-3			
Human-eotaxin	0.2 ± 0.1	(4)	0.2 ± 0.1	(2)
Murine-eotaxin	0.3 ± 0.1	(2)	0.2 ± 0.1	(3)
MCP-3	0.7 ± 0.4	(4)	1.1 ± 0.6	(10)
RANTES	0.5 ± 0.3	(4)	0.9 ± 0.4	(8)
MCP-1	16 ± 2	(3)	61 ± 13	(2)
MIP-1α	N.B.	(4)	See text	
MIP-1β	N.B.	(4)	N.B.	(2)

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 parenthesis. K_ds 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_ds of 0.2 and 0.3 nM, respectively (Fig 3 *b*, Table 1). MCP-3 and RANTES also demonstrated high affinity, with K_ds of 0.7 and 0.5 nM, values about fourfold lower than measured against ^{125}I-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 AML14.3010 cells, intracellular Ca^{2+} levels were measured in response to various β-chemokines. As shown in Fig. 4, eotaxin and RANTES induced Ca^{2+}-fluxes in cells expressing the receptor with ED_{50}s 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 β-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 ^{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. 3 *c*, Table 1). Scatchard analysis showed a single binding affinity, and 4 × 10^5 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_ds 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 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 β-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 ^{125}I-MCP-3 on eosinophils (Fig. 3 *d*). To investigate the nature of the site responsible for these effects, detailed studies were carried out by competition against ^{125}I-MIP-1α. As shown in Fig. 5, MIP-1α, MCP-3, and RANTES all competed strongly with IC_{50}s of 0.3, 0.7, and 0.9 nM, respectively. In contrast, human and murine eotaxin competed with relatively low affinity, showing IC_{50}s 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 × 10^4 sites/cell, only 1–5% the level of CC CKR3 (data not shown).

[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).

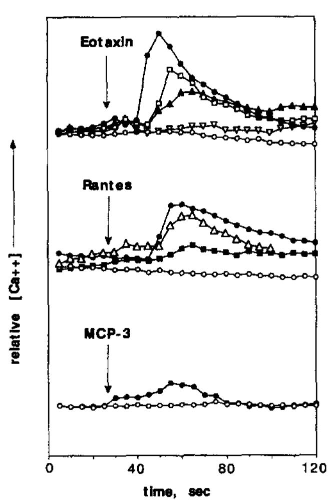

Figure 4. Human eotaxin, RANTES and MCP-3 are agonists for CC CKR3. Ca^{2+}-fluxes were induced in CC CKR3-expressing clone 3.16 by addition of eotaxin, RANTES, or MCP-3 at 0.03 nM (∇); 0.3 nM (▲); 3 nM (□); 10 nM (■); 30 nM (Δ); and 100 nM (●). Responses of the non-expressing clone 3.49 to 100 nM of each chemokine (O) are used as controls.

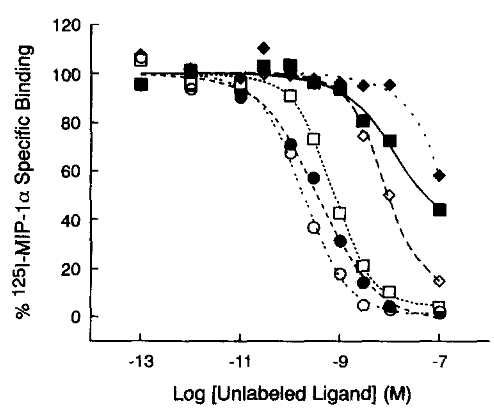

Figure 5. Pharmacology of the MIP-1α binding site on eosinophils. Increasing concentrations of unlabeled human eotaxin (■), murine eotaxin (◇), RANTES (□), MCP-3 (●), MCP-1 (◆), or MIP-1α (O) were used to compete against a fixed concentration of ^{125}I-MIP-1α as described in Fig. 3.

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 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 (4×10^5 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-1α 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 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 CKR3, and those of the second with CC CKR1.

CC CKR3 is the third β-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-1α (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 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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