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# Specificity determinants for chemokine recognition identified using eotaxin-MCP-1 chimeras

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**Abstract To identify the elements of two chemokines [monocyte chemoattractant protein-1 (MCP-1) and eotaxin] that control their differential recognition by their respective receptors (CCR2 and CCR3), we have studied the receptor interactions of MCP-1-eotaxin chimeras. Each receptor was found to exhibit a distinct binding preference for proteins containing the amino-terminal region of the cognate chemokine for that receptor. However, other elements dictating chemokine preference were different for the two receptors. In some cases, the influence of replacing a particular region was dependent on the identities of neighboring regions, indicating a complex network of cooperative and/or compensating interactions.**


*Keywords:* Chemokine; Chemokine receptor; Chimera; Eotaxin; Monocyte chemoattractant protein-1; Specificity

## 1. Introduction

Chemokines are small proteins that activate G-protein-coupled receptors to trigger the migration and accumulation of leukocytes, a central component of both normal and pathological inflammatory conditions [1–4]. In addition, activation of chemokine receptors located on tumor cells can control the tissue destinations of metastatic cancer cells [5,6] and certain chemokine receptors can act as co-receptors for infection of leukocytes by HIV [7–9]. There are 45 known human chemokines, classified into two major families ('CC' and 'CXC') and 19 known human chemokine receptors [10,11]. Most chemokines can bind and/or activate several, but not all, receptors and most receptors can bind and/or be activated by several, but not all, chemokines within one family.

In an effort to decipher the complicated chemokine-receptor recognition code, there have been numerous studies of the structures and structure-activity relationships of chemokines [12–14]. These studies have identified the roles of certain amino acid residues in mediating the interactions of specific chemokines with their receptors. However, they have provided limited information regarding the elements that allow a particular receptor to distinguish between different chemokines. In this letter, we describe an initial exploration of the features dictating chemokine-specificity using a series of chimeric chemokines containing elements from each of two natural CC chemokines, eotaxin and monocyte chemoattractant protein-1 (MCP-1). These chemokines share ~65% sequence identity. However, the receptor CCR2 binds ~130-fold more tightly to MCP-1 than to eotaxin, whereas the receptor CCR3 binds ~60-fold more tightly to eotaxin than to MCP-1 [15]; the chemokine concentrations required for half-maximal receptor activation roughly parallel the binding affinities. Thus, these structurally similar but functionally distinct proteins provide an excellent opportunity to explore specificity determinants of chemokines.

## 2. Materials and methods

### 2.1. Chemokine expression and purification

Genes encoding the six chimeric chemokines were made from synthetic oligonucleotides using recursive PCR [16], then subcloned into a pET expression vector (Novagen, Madison, WI): pET-11a (for ***EEMMM*** and ***EMMMM***) or pET-30a (for other constructs). Each construct encodes a fusion protein consisting of an N-terminal $(His)_6$-tag, a protease cleavage site, and the desired protein sequence. The protease cleavage site [Factor Xa site (IEGR) for ***MEEMM***, in which the N-terminus is that of MCP-1; modified thrombin site (LVPRGP) [17] for all other constructs] was chosen to allow proteolytic release of the correct protein sequence without any N-terminal extension.

Wild type MCP-1 was purchased from R&D Systems (Minneapolis, MN). Wild type eotaxin and chimeric chemokines were expressed and purified using the protocol previously described for wild type eotaxin [17], with the following modifications: (1) vectors derived from pET-11a were transformed into Origami® *E. coli* cells (Novagen, Madison, WI) instead of BL21(DE3) cells; (2) for expression of ***EEMEM*** and ***MEEMM***, the growth temperature was reduced to 25 °C after induction; and (3) for ***MEEMM***, the $(His)_6$-tag was removed by treatment with Factor Xa (0.05 mg/mL Factor Xa in 50 mM Tris, 100 mM NaCl, and 2 mM $CaCl_2$, pH 8.0, room temperature, 5 days). The molecular weights of all proteins were verified by MALDI-TOF mass spectrometry. Protein concentrations were determined using the extinction coefficients at 280 nm calculated from the protein sequences: ***EEMMM*** and ***EEMEM*** = 7210 $M^{-1}\,cm^{-1}$, all others = 8490 $M^{-1}\,cm^{-1}$ [18].

### 2.2. Receptor binding and activation assays

Cell-based assays were performed using CHO cells stably transformed to express CCR2 or versions of CCR3 bearing N-terminal FLAG (MDYKDDDD) or $His_6$ (MHHHHHH) epitope-tags (Flag-CCR3 and $His_6$-CCR3, respectively) [15]. CHO-CCR2 and CHO-$His_6$-CCR3 cell lines were used to perform calcium mobilization assays, whereas competitive ligand binding data were gathered using the CHO-CCR2 and CHO-FLAG-CCR3 cell lines. Detailed procedures for cell-based assays are presented elsewhere [15]. Briefly, CCR2 and CCR3 binding assays were performed in duplicate using $^{125}I$-labeled

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MCP-1 and $^{125}$I-labeled eotaxin, respectively, and varying concentrations of cold competitor chemokines. Binding data were fit to the equation: fraction bound = $1 - 1/(1+(IC_{50}/[\text{chemokine}])^{slope})$. Calcium mobilization assays were performed in duplicate by monitoring increases in the ratio of fluorescence excitation at 340 and 380 nm ($\lambda_{em} = 510$ nm) after challenging Fura-2-loaded cells with varying concentrations of chemokines. Dose–response data were fit to the equation: observed signal = maximum signal/$(1+([\text{chemokine}]/EC_{50})^{slope})$.

## 3. Results

### 3.1. Design of chimeras

There are 26 amino acid positions that differ between eotaxin and MCP-1. Based upon an analysis of the distances between these non-conserved residues in eotaxin ($C_\alpha$ atoms for Gly; $C_\beta$ atoms for all other residues) [19], we defined five regions (labeled I–V), each representing a cluster of 4–6 spatially proximal residues, as indicated in Fig. 1. Within the same cluster, the average (±S.D.) of inter-residue distances is $9.0 \pm 3.2$ Å, whereas the corresponding value for residues in different clusters is $22.5 \pm 7.1$ Å. For example, residue Leu-45 (region III) is 5.6–9.9 Å from each other residue in region III, but 11.6–30.1 Å from each residue in other regions. One residue, Gly-32, did not naturally fall into any of the initial five regions based upon the distance analysis, but was subsequently added to region V because it is located on the same face of the chemokines as other region V residues.

There are thirty two ($2^5$) possible combinations of the five regions defined in Fig. 1. In this initial study, we have examined a subset (eight) of these proteins, six chimeras and the two wild type chemokines; these are represented schematically in Fig. 1C. Chimeras ***EEEEM***, ***EEEMM***, ***EEMMM***, and ***EMMMM*** involve successive replacement of regions V, IV, III, and II from eotaxin with the corresponding regions from MCP-1; subsequent replacement of region I gives wild type MCP-1. This set of six proteins was selected to provide five pairwise comparisons in which individual chemokines differ in a single region. The remaining two chimeras (***EEMEM*** and ***MEEMM***) allow three additional pairwise comparisons and therefore some insights into the context-dependence of binding effects (vide infra).

### 3.2. Receptor binding by chimeras

We have previously developed stable cell lines that express CCR2 and epitope-tagged versions of CCR3 (FLAG-CCR3 and $His_6$-CCR3) at very high levels [15]. The receptors in these cell lines are competent for high affinity chemokine-binding and/or for activation by chemokines. In the present study, the apparent affinities of the wild type and chimeric chemokines for CCR2 and FLAG-CCR3 were determined by competitive radioligand binding assays, using these CHO cell lines. Data for binding of wild type chemokines and representative chimeras to each receptor are presented in Fig. 2 and apparent binding affinities ($IC_{50}$ values) for each ligand-receptor combination are listed in Table 1. The two receptors have opposite specificities for the wild type chemokines. For each receptor, most chimeras display $IC_{50}$ values intermediate between those of the two natural chemokines.

### 3.3. Receptor activation by chimeras

The ability of each wild type chemokine and chimeric variant to activate CCR2 and CCR3 was evaluated by calcium mobilization assays using the CHO cell lines expressing CCR2 and $His_6$-CCR3. Dose–response curves for the wild type chemokines and representative chimeras are shown in Fig. 3. Table 1 lists the ligand concentrations required for half maximal activation ($EC_{50}$ values) and the maximum levels of wild type activity observed (relative to those for the relevant wild type chemokine). In most cases, the variations in $EC_{50}$ values follow similar trends to those observed for $IC_{50}$ values; two exceptions are discussed below. Finally, there was some variation in the peak levels of activation observed for different chemokine constructs on the same receptor. These could potentially reflect differences in the kinetics of receptor activation [20]. Alternatively, they could arise from differences in the final

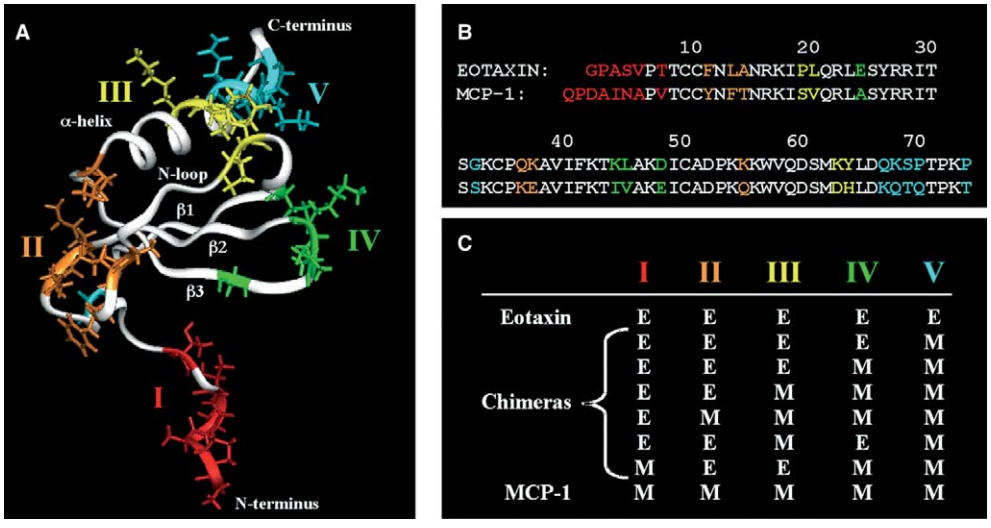


Fig. 1. (A) 3D ribbon structural model of eotaxin (PDB code 1EOT; [23]) with the non-conserved residues (with respect to MCP-1) shown as cylinders. Color coding indicates the composition of the five spatially defined regions (I, red; II, orange; III, yellow; IV, green; and V, cyan). (B) Amino acid sequence alignment of eotaxin and MCP-1, with numbering corresponding to that of eotaxin. Non-conserved residues are colored as in (A). (C) Composition of the two wild type and six chimeric chemokines, indicating which regions are derived from eotaxin (E) and MCP-1 (M) in each protein.

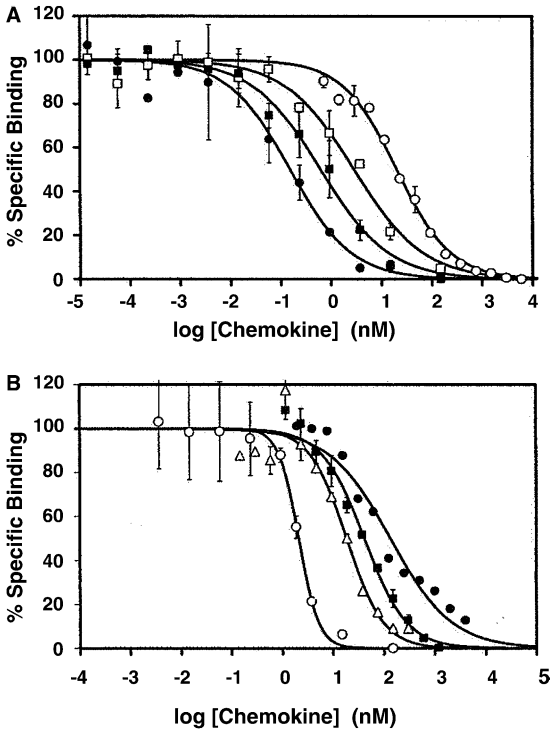


Fig. 2. Competitive radioligand-binding assays. Shown are competition data for displacement of (A) $^{125}$I-MCP-1 from CHO-CCR2 cells and (B) $^{125}$I-eotaxin from CHO-FLAG-CCR3 cells using increasing concentrations of wild type chemokines [MCP-1 (●) and eotaxin (○)] and selected chimeras [***EEMMM*** (□), ***MEEMM*** (△), and ***EMMMM*** (■)]. Data and error bars are averages and standard errors from duplicate independent data sets. The solid lines are fits to the equation given in Section 2.

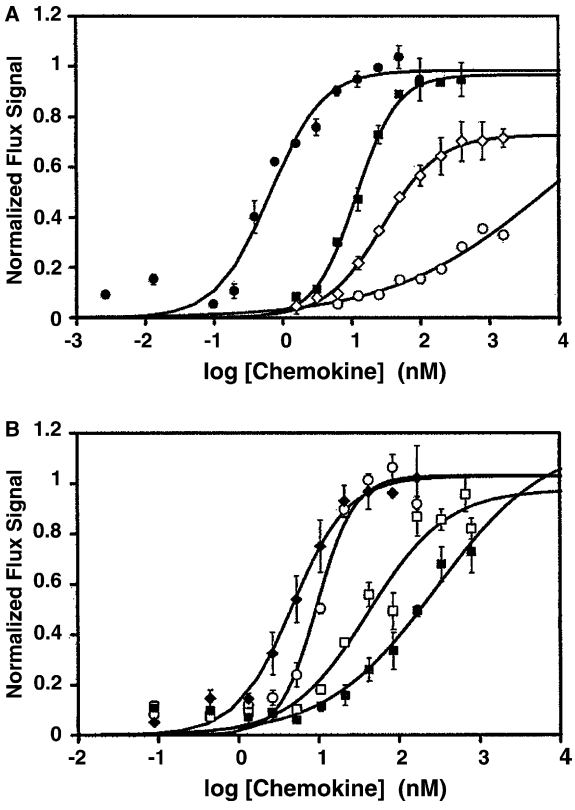


Fig. 3. Receptor activation by chemokines. Shown are dose–response curves for calcium mobilization in (A) CHO-CCR2 cells and (B) CHO-$His_6$-CCR3 cells treated with increasing concentrations of wild type chemokines [MCP-1 (●) and eotaxin (○)] and selected chimeras [***EEMMM*** (□), ***EEEMM*** (◆), ***EEEEM*** (◊), and ***EMMMM*** (■)]. Data and error bars are averages and standard errors from duplicate independent data sets. Solid lines are fits to the equation given in Section 2.

conformational state of the receptor induced by binding to different chemokines [21].

## 4. Discussion

### 4.1. Specificity of chemokine binding by receptors

Fig. 4 displays the changes in receptor binding affinities ($IC_{50}$ ratios) observed for each of the eight pairs of chemokines that differ only in a single region; for regions I, III, and IV, two independent pairwise comparisons can be made, labeled 'a' and 'b'. $IC_{50}$ ratios significantly greater than one indicate that the relevant receptor interacts more favorably with the swapped region of the cognate chemokine than the corresponding region of the non-cognate chemokine.

The data for binding to CCR2 (Fig. 4, solid bars) show that replacement of region III or IV of MCP-1 by the corresponding region of eotaxin has little effect on CCR2-affinity, whereas replacement of region II or V reduces CCR2 affinity about 5-fold. The role of region I is less obvious. Comparison

Table 1
Receptor binding and activation data for wild type chemokines and chimeras

| Receptor | CCR2 | | | CCR3 | | |
|---|---|---|---|---|---|---|
| Chemokine | $IC_{50}$ (nM) | $EC_{50}$ (nM) | % Wild type activity[a] | $IC_{50}$ (nM) | $EC_{50}$ (nM) | % Wild type activity[a] |
| **Eotaxin** | 20.2 ± 1.5 | 100–400 | 73 | 2.1 ± 0.1 | 10 ± 2 | 100 |
| ***EEEEM*** | 4 ± 1 | 28 ± 2 | 73 | 1.8 ± 0.7 | 11 ± 3 | 133 |
| ***EEEMM*** | 1.7 ± 0.7 | 9.1 ± 0.1 | 57 | 0.7 ± 0.1 | 4.8 ± 0.6 | 100 |
| ***EEMMM*** | 2.9 ± 0.5 | 8.9 ± 0.9 | 73 | 26 ± 3 | 40 ± 20 | 95 |
| ***EMMMM*** | 0.6 ± 0.1 | 11.7 ± 0.7 | 97 | 41 ± 4 | >150 | 113 |
| ***EEMEM*** | 3.1 ± 0.7 | 22 ± 5 | 55 | 2 ± 1 | 16 ± 5 | 117 |
| ***MEEMM*** | 1.1 ± 0.4 | 50 ± 10 | 88 | 18 ± 3 | [b] | 0 |
| **MCP-1** | 0.15 ± 0.03 | 0.7 ± 0.2 | 100 | 130 ± 20 | [b] | 0 |

[a] "% Wild type activity" is the magnitude of the calcium flux signal observed with the chemokine relative to that with the wild type chemokine on the same receptor (MCP-1 on CCR2 or eotaxin on CCR3).
[b] No flux detected at 200 nM.

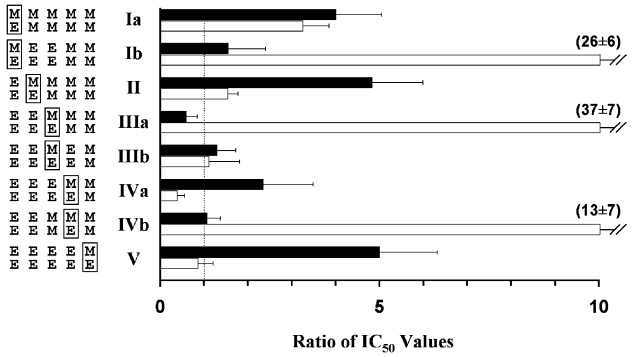


Fig. 4. Bar graph showing the ratios of $IC_{50}$ values for pairwise combinations of chemokines and chimeras. Solid and open bars represent ratios of $IC_{50}$ values for CCR2 and CCR3, respectively. Each pair of bars is labeled (Roman numerals) according to the region that differs between the two chemokines; for regions I, III, and IV, the two independent pairwise comparisons are labeled 'a' and 'b'. For each pairwise comparison, the compositions of the two chemokines/chimeras are represented schematically on the left following the convention of Fig. 1C, with boxed letters indicating the regions that differ between each pair. Ratios of $IC_{50}$ values were calculated for CCR2 by dividing the $IC_{50}$ for the bottom protein of each pair by the $IC_{50}$ for the top protein of the pair, while those for CCR3 were calculated by dividing the $IC_{50}$ for the top protein by the $IC_{50}$ of the bottom protein.

Ia, between ***EMMMM*** and MCP-1, indicates that replacement of region I causes a ~4-fold reduction in affinity. However, comparison Ib (between ***EEEMM*** and ***MEEMM***) suggests that region I plays a very minor role. Thus, the contribution of region I is dependent on the identity of other structural elements.

The data for binding to CCR3 (Fig. 4, open bars) also indicate that replacement of region I can cause a substantial reduction in receptor affinity, although again the magnitude of this effect (~3-fold for comparison Ia and ~26-fold for comparison Ib) depends on the identity of other regions. However, replacement of region II or V of eotaxin by the corresponding region of MCP-1 has little or no effect on CCR3 affinity. On the other hand, replacement of region III or IV can have a substantial effect on receptor affinity, although, once again, the effects are dependent on the identity of neighboring regions. In summary, CCR2 appears to utilize interactions with regions I, II, and V, whereas CCR3 appears to utilize interactions with regions I, III, and IV in order to distinguish between the two chemokines.

The current data do not indicate precisely which amino acid residues within each region are responsible for the observed binding preferences. However, previous mutational studies have provided evidence for the importance of some of the swapped residues (Y13 of MCP-1 [22,23]; S4, V5, and F11 of eotaxin [14]) in binding to CCR2 and CCR3. It seems likely that some of these residues, in addition to providing affinity for the cognate receptor, also contribute to the chemokine-specificity of the receptors. On the other hand, there are also several residues that are identical in both MCP-1 and eotaxin yet whose mutation decreases affinity for the cognate receptors; these include R24, K35, and K49 of MCP-1 [22,23] and R16 and I18 of eotaxin [14]. These residues are less likely to contribute to the binding specificity of the receptors, except in cases where their interactions are dependent on those of neighboring non-conserved amino acids.

### 4.2. Context-dependent effects on binding

Substitution of region I causes a 4-fold reduction in CCR2 binding affinity for comparison Ia (MCP-1 versus ***EMMMM***), in which all other regions correspond to those of MCP-1, but only a ~1.5-fold reduction in comparison Ib (***MEEMM*** versus ***EEEMM***), in which regions II and III both correspond to those of eotaxin. In contrast, substitutions Ia and Ib cause ~3.3-fold and ~26-fold reductions in CCR3 binding affinity, respectively. Thus, the effect on CCR3-binding is much greater when regions II and III correspond to those of eotaxin, whereas the effect on CCR2-binding is somewhat higher when regions II and III correspond to those of MCP-1. This pattern is indicative of positive cooperativity for the interactions of region I and region(s) II and/or III of a particular chemokine with the cognate receptor for that chemokine. Considering that region I is closer to region II than to region III (minimum $C_\beta$–$C_\beta$ distances of 11.3 and 22.8 Å, respectively; Fig. 1A), we propose that the cooperativity is between regions I and II. Notably, previous mutational data indicate that residues in region II of both chemokines and residues in region I of eotaxin contribute to receptor binding affinity [14,22–24].

The effects of substituting regions III and IV on binding to CCR3 are also dependent on the context of the substitutions. The four chimeras ***EEMMM***, ***EEEMM***, ***EEMEM***, and ***EEEEM*** all contain regions I and II of eotaxin and region V of MCP-1, but they differ in regions III and IV (underlined), covering all four possible substitution patterns in these regions. For ***EEEMM***, ***EEMEM***, and ***EEEEM***, in which region III and/or region IV correspond(s) to that/those of eotaxin, the apparent affinity for CCR3 is in the range 0.7–1.8 nM, whereas for ***EEMMM***, in which both regions correspond to those of MCP-1, the $IC_{50}$ increases dramatically to 26 nM. This indicates that optimal binding to CCR3 requires some element of eotaxin region III or some element of eotaxin region IV, but does not require both.

### 4.3. Receptor activation

Induction of transmembrane signals by chemokine receptors is proposed to proceed via a two-step mechanism, involving first binding to the cognate chemokine, then a conformational

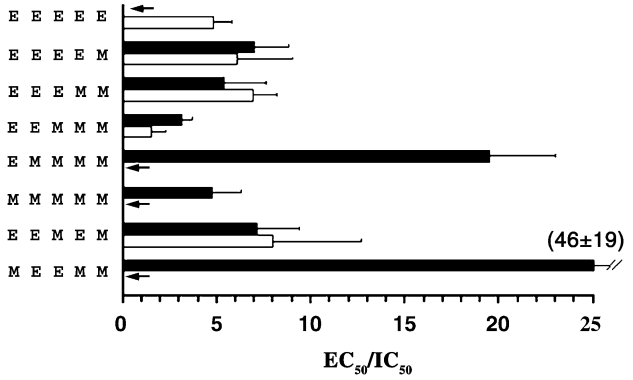


Fig. 5. Bar graph showing the ratios of $EC_{50}$ values to $IC_{50}$ values for each wild type chemokine and chimera, assayed against CCR2 (solid bars) and CCR3 (open bars). The composition of each chemokine is shown schematically at the left, following the convention of Fig. 1C. Arrows indicate cases in which $EC_{50}$ values could not be determined (see Table 1).

rearrangement coupled to G-protein activation [13,23]. Consistent with the model that binding precedes, but is not sufficient for, receptor activation, $EC_{50}$ values are typically somewhat higher than $IC_{50}$ values. For MCP-1, eotaxin, and most of the chimeras studied herein, the $EC_{50}/IC_{50}$ ratios are in the range ~2–10 (Table 1 and Fig. 5). Thus, reductions in the ability to activate the receptors are generally correlated with decreased binding affinity. However, for ***MEEMM*** and ***EMMMM***, concentrations ~44-fold and ~20-fold higher than the $IC_{50}$ values are required for half-maximal activation of CCR2, suggesting that these chimeras can bind avidly to the inactive state of the receptor but are deficient in inducing the conformational change to the activated state. Among all the chemokines studied herein, ***MEEMM*** and ***EMMMM*** are the only two in which regions I and II are from different chemokines. Thus, we speculate that the ability to induce CCR2 activation may depend upon cooperative interactions of these two regions with the receptor.

### *4.4. Concluding remarks*

The studies of chimeric chemokines described herein have begun to reveal some of the elements of MCP-1 and eotaxin that control differential chemokine recognition by their respective receptors. The main conclusions are as follows. (1) Each receptor has a distinct preference to interact with region I (the N-terminus) of its cognate ligand over region I of the other chemokine; this preference is most pronounced when region II is also from the cognate chemokine. (2) CCR2 utilizes interactions with regions II and V to enhance specificity for MCP-1, whereas CCR3 uses mutually compensating interactions with regions III and/or IV to increase specificity for eotaxin. (3) The contributions of each region to specificity are sensitively dependent on the identities of nearby regions. Thus, a thorough model to account for the observed recognition will require, at a minimum, a consideration of the intrinsic contributions of each region as well as the cooperativity between each pair of regions. Finally, the data suggest that cooperativity between regions may also be important in controlling receptor activation following an initial binding event.

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