Distinct but Overlapping Epitopes for the Interaction of a CC-Chemokine with CCR1, CCR3, and CCR5

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ABSTRACT: Chemokines play an important role in inflammation. The mechanism via which they bind to more than one receptor and activate them is not well understood. The chemokines are thought to interact with their receptors via two distinct sites, one necessary for binding and the other for activation of signal transduction. In this study we have used alanine scanning mutagenesis to identify residues on RANTES that specifically interact with its receptors CCR1, CCR3, and CCR5 for binding and activation. Residues within a potential receptor binding site known as the N-loop (residues 12−20) and near the N-terminus of RANTES were individually mutated to alanine. The results of this study show that, within the N-loop, the side chain of R17 is necessary for RANTES binding to CCR1, F12 for binding to CCR3, and F12 and I15 for binding to CCR5, thus forming distinct but overlapping binding epitopes. In addition, our finding that P2 is necessary for binding to CCR5 is the first to show that a residue near the N-terminus of a CC-chemokine is involved in binding to a receptor. We have also found that P2, D6, and T7 near the N-terminus are involved in activating signal transduction via CCR1, P2 and Y3 via CCR3, and Y3 and D6 via CCR5. These results indicate that RANTES interacts with each of its receptors in a distinct and specific manner and provide further evidence to support the two-site model of interaction between chemokines and their receptors.

Chemokines or *chemo*tactic cyto*kines* are thought to play a crucial role in initiating and maintaining an inflammatory response [reviewed in Bacon and Schall (1996b), Howard et al. (1996b), and Schall and Bacon (1994)]. Chemokines can be classified on the basis of a conserved cysteine motif near their N-terminus as CC-, CXC-, CX_3C-, or C-chemokines. Each chemokine is a chemoattractant for a phenotypically specific subset of cells. RANTES (Regulated upon Activation Normal T-cell Expressed and Secreted), a CC-chemokine, is a chemoattractant for monocytes, T lymphocytes, NK cells, basophils, eosinophils, and dendritic cells, all of which can be involved in inflammation (Howard et al., 1996a). The proinflammatory role of RANTES is suggested by higher than normal concentrations of this chemokine in synovial lining and in peripheral blood and synovial T cells of rheumatoid arthritis patients (Robinson et al., 1995). In vivo, blocking antibodies against RANTES inhibit macrophage but not neutrophil or lymphocyte influx into the lung in an animal model of LPS-induced endotoxemia (VanOtteren et al., 1995). Chemokines, in addition to inflammation, may play a role in modulating the infectious cycle of HIV-1 (Cocchi et al., 1995). In vitro, RANTES is able to inhibit HIV-1 from infecting peripheral blood mononuclear cells (PBMC)[1] via CCR5, one of the RANTES receptors (Cocchi et al., 1995).

RANTES, like the other CC-chemokines, binds to more than one receptor. The known RANTES receptors are CCR1, CCR3, CCR4, and CCR5, of which CCR's 3 and 5 were recently identified as coreceptors for HIV-1 (Premack & Schall, 1996). Other CC-chemokines can also bind to each of the RANTES receptors. For example, MIP-1α and MIP-1β can both compete with RANTES for binding to CCR5 and block HIV-1 entry into cells via this receptor. The binding of multiple ligands by each receptor results in a complex network in vivo, both during an inflammatory reaction and during HIV-1 infection, since cells have been shown to express more than one receptor type.

Residues that are required for receptor binding and function of a chemokine were first identified on the N-terminus of IL-8 (Clark Lewis et al., 1991; Hebert et al., 1991). In addition, a second receptor binding site has been identified within the region known as the N-loop (residues 10−20) of IL-8 (Lowman et al., 1996). The requirement of residues near the N-terminus of the CC-chemokines RANTES, MCP-1, and MCP-3 for signal transduction has been demonstrated by Gong et al. and Zhang et al. (Gong & Clark Lewis, 1995; Gong et al., 1996; Zhang et al., 1994b). They made N-terminal truncation mutants of these CC-chemokines which could bind to THP-1 cells but not cause chemotaxis or induce a calcium flux (Gong & Clark Lewis, 1995; Gong et al., 1996; Zhang et al., 1994a). In addition, a RANTES mutant lacking the first eight amino acids was able to prevent infection of activated PBMC by HIV-1 (Arenzana-Seisdedos et al., 1996). The fact that these N-terminal truncation mutants are still able to bind to chemokine receptors on THP-1 cells or PBMCs shows RANTES must interact with its receptors via residues other than those at the N-terminus.

The primary sequences of human and murine RANTES differ only at 11 residues, yet the murine protein is unable to bind to human RANTES receptors. On the basis of this observation, the known three-dimensional structure of human

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[1] Abbreviations: PBMC, peripheral blood mononuclear cells; wtRANTES, wild-type RANTES.

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RANTES (Skelton et al., 1995), and the residues known to be important for IL-8 binding to its receptor, we have identified a possible receptor binding site on RANTES. In order to test this hypothesis, we have used alanine scanning mutagenesis to determine the contribution of specific amino acid side chains of residues in the N-loop of RANTES to receptor binding affinity and signal transduction. In addition, each amino acid near the N-terminus was individually mutated to alanine to examine the effect of each mutation, within the context of the intact RANTES molecule, on binding affinity for CCR1, CCR3, and CCR5 and on chemotaxis or calcium flux.

MATERIALS AND METHODS

Mutagenesis. Alanine substitutions in RANTES were made by constructing a synthetic gene encoding the full-length protein from 60−80-mer overlapping oligonucleotides. The full-length gene was ligated into a vector for expression in *Escherichia coli*. Each gene construct was completely sequenced to verify the conversion of a single codon from wild-type residue to alanine as well as to ensure that unwanted mutations had not been introduced.

Protein Expression and Purification. Each of the RANTES mutants was overexpressed in *E. coli* as previously described (Kuna et al., 1992). Mutant protein was purified from cell paste using a modification of a previously published protocol (Skelton et al., 1995). Briefly, cell paste (1.5−3 g) was dispersed in 10 mL of 50 mM glycine and 250 mM NaCl (pH 3.0). Following cell disruption, the sonicated material was centrifuged at 10 000 rpm for 10 min and the pH of the supernatant adjusted to pH 6.0 with 1 M NaOH. Supernatant was filtered through a 0.22 μm filter and loaded onto a cation-exchange column (Resource S, Pharmacia) that had previously been equilibrated with sodium citrate buffer (20 mM, pH 6.0). Protein was eluted with a 0.25−1 M NaCl gradient. Fractions containing RANTES mutant protein were pooled and loaded onto a reverse-phase column (Resource RPC, Pharmacia) equilibrated with MilliQ H_2O containing 0.1% TFA. Protein was eluted with a 0−100% gradient of acetonitrile containing 0.1% TFA. Protein obtained after this step was pure and had the correct molecular weight as judged by mass spectrometry and gel electrophoresis.

Purification of Eosinophils. Human eosinophils were isolated as previously described (Hansel et al., 1991). Briefly, human granulocytes were isolated from heparin anticoagulated venous blood of normal or asymptomatic allergic donors. The freshly drawn blood was mixed in an equal volume of 3% dextran and allowed to sediment for 30 min at room temperature. The supernatant was fractionated on LSM (Organon Teknika Corp., Durham, NC), and the plasma, mononuclear cell, and media layers were removed. At this point, all procedures were done at 4 °C to prevent cell activation. RBC's were removed from the granulocyte pellet by hypotonic lysis. Eosinophils were purified by negative selection with anti-CD16 antibody coated beads (Miltenyi Biotec Inc., Auburn, CA) as described (Hansel et al., 1991). Eosinophil purity was 99 ± 1% as determined by microscopic examination with Kimura staining.

Chemotaxis Assay. Eosinophil chemotaxis assays were carried out in a 96-well chemotaxis chamber (Neuroprobe, Cabin John, MD) as previously described (Bacon & Schall, 1996a), with the following modifications. A filter with 5 μM pores was used with a 2 h incubation at 37 °C.

Binding Assay. Binding of chemokines to various cell lines was carried out as described previously (Van Riper et al., 1993). Briefly, cells were washed once in PBS and resuspended in binding buffer (50 mM Hepes, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% BSA) at (1.5−2.0) × 10^6/mL. Varying concentrations of unlabeled chemokine mutant protein were diluted in binding buffer and 25 μL aliquots added in duplicate to 96-well round bottom plates, followed by 25 μL aliquots (10 000−20 000 cpm) of ^{125}I-labeled RANTES (NEN DuPont, Wilmington, DE). Then 200 μL of cell suspension was added last. After 60 min incubation at room temperature, each well was resuspended and filtered through poly(ethylenimine)-treated Whatman GFC filters followed by 4 mL of binding buffer containing 0.5 M NaCl. Activity retained on the filters was counted in a γ counter. The LIGAND program (Munson & Robard, 1980) was used to calculate binding constants. Since mutant RANTES proteins were used as cold competitors in the binding assay, their ability to prevent wild-type RANTES from binding to the receptor by any mechanism but not their ability to directly bind to receptor will be measured.

Ca^{2+} Flux Assay. Intracellular calcium flux assays were performed as previously described with the following modifications (Leong et al., 1994). Cells at 2 × 10^5/mL were loaded with Indo-1 AM (2 μg/mL) in complete media for 30 min at 37 °C. Cells were stimulated with 100 nM RANTES or mutants of RANTES, and intracellular calcium concentration was calculated.

RESULTS

Mutagenesis of RANTES. The primary sequences of human and murine RANTES differ only at 11 residues. Inspection of the solution structure of human RANTES (Skelton et al., 1995) indicates that all of the amino acid substitutions in the murine form occur in the solvent-exposed surface locations or else may be accommodated in the protein core with only slight changes in some side-chain dihedral angles. Thus, the three-dimensional structures of murine and human RANTES are likely very similar. In spite of this, murine RANTES does not bind to any human RANTES receptors (data not shown). Interestingly, four contiguous residues (15−18) differ in the primary sequence of the human and mouse proteins, suggesting a possible involvement of the "N-loop" (residues 12−20) in receptor binding and specificity. Since the N-loop has recently been shown to be a specificity determinant for CXC-chemokines binding to CXCR1 and CXCR2 (Lowman et al., 1996), we chose the corresponding loop in human RANTES for detailed structure−function analysis. We individually mutated to alanine each amino acid between F12 and P20 (residues in pink, Figure 1), except for residues 13 and 16 which are alanines in the wild-type RANTES sequence. In addition, we individually mutated to alanine residues in the N-terminus of RANTES preceding the first two cysteines (residues in cyan, Figure 1) in order to further the structure−function analysis begun by Gong et al. (1996) and Zhang et al. (1994b). Alanine substitutions remove interactions beyond the β-carbon and should reveal the contribution to binding made by the side chain that was removed (Cunningham & Wells, 1989; Wells, 1991). Alanine scanning mutagenesis does not allow the functional analysis of residues that are alanine or very similar but was chosen as a refined method of structure−function analysis for its minimal disruption of

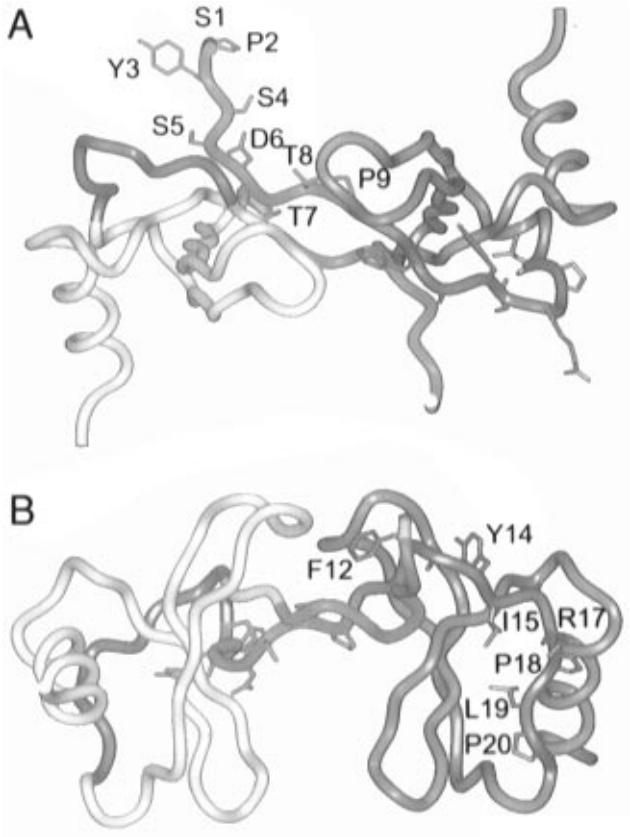

FIGURE 1: Location of residues mutated to alanine within the RANTES dimer. The backbone conformations of the monomers within the RANTES dimer (Skelton et al., 1995) (PDB accession number 1RTO) are represented by the gray and white tubes. (A) Residues near the N-terminus individually mutated to alanine are shown in cyan. (B) Residues in the N-loop individually changed to alanine are shown in pink. This view of the RANTES dimer is a 90° rotation about a horizontal axis relative to that shown in (A).

protein structure. Alanine substitutions were confirmed by sequencing the encoding DNA and by mass spectrometry.

RANTES Interaction with CCR1. Primary leukocytes express more than one chemokine receptor, so we used recombinant CCR1 expressed on 293 (human embryonal kidney) cells to analyze the interaction beween RANTES and CCR1. Native 293 cells do not bind RANTES. Each alanine scan mutant was used as a competitor in a binding assay with ^{125}I-labeled wild-type RANTES (wtRANTES) to measure binding affinity. The results of each assay were plotted as displacement curves to obtain a dissociation constant for each mutant, which are shown as a ratio (Figure 2A) of the dissociation constant of mutant to that of wtRANTES. With the exception of R17A, in which the alanine substitution caused a >1000-fold decrease in binding to CCR1, all the other mutants bound as well as or had no more than a 7-fold reduction in binding than wtRANTES. These results indicate a requirement for contact between the side chain of residue R17 on RANTES and CCR1 for the binding of RANTES to this receptor.

In addition, each mutant was used as an agonist in a calcium flux assay with CCR1 transfected 293 cells. The maximum change in free Ca^{2+} concentration stimulated by each mutant was plotted as a ratio to that stimulated by the same concentration of wtRANTES (Figure 2B). A ratio of 0.5 or less, indicating a 50% lower Ca^{2+} flux than wtRANTES, was chosen arbitrarily as a significant reduction. The mutant R17A which showed a >1000-fold decrease in binding to

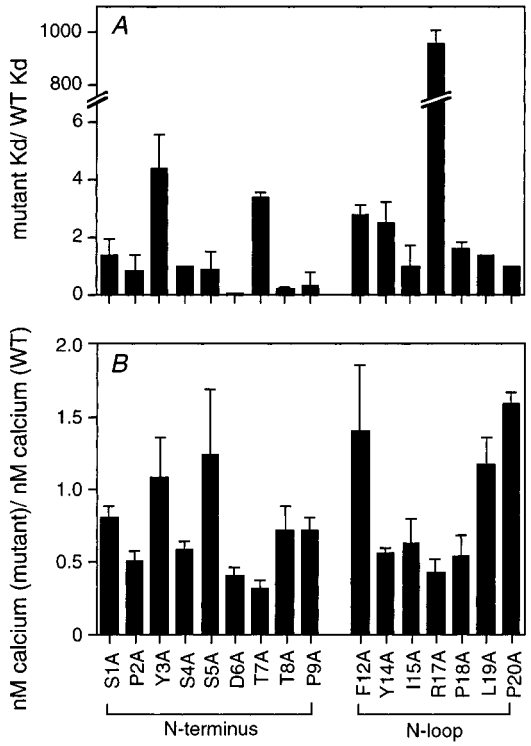

FIGURE 2: RANTES interaction with CCR1. CCR1 expressed on 293 cells was used in binding and calcium flux experiments. RANTES binds to CCR1 with a K_d of 0.9 nM (A). Each alanine scan mutant was used as a cold competitor with ^{125}I-wtRANTES in at least two different binding assays. The results were analyzed to obtain a binding affinity for each mutant which was then divided by the K_d of wtRANTES to obtain the ratio plotted in the figure; error bars represent the standard deviation from the mean value. (B) 293 cells expressing CCR1 were loaded with indo-1AM and stimulated with either wtRANTES or alanine scan mutants to obtain a calcium flux. The data were used to calculate the ratio of the maximum change in intracellular calcium concentration caused by each mutant to that caused by wtRANTES. Each bar represents the mean value of three or four experiments with the standard deviations shown as error bars.

CCR1 was a poor receptor agonist of CCR1 as judged by a calcium flux response that was only 40% of the response elicited by wtRANTES. This indicates that although the binding affinity was too low to be measured in the competition binding assay, enough of the mutant protein was able to bind to the receptors on a cell to cause a slight calcium flux. Of the N-terminal alanine scan mutants, P2A, D6A, and T7A caused a change in free Ca^{2+} that was 50% or less than that of the response to wtRANTES. In addition, the calcium flux response stimulated by S4A was not significantly different from that caused by P2A. The activation of a calcium flux response via CCR1 thus requires interaction between residues P2, S4, D6, and T7 near the N-terminus of RANTES and CCR1. The other changes to alanine stimulated a calcium flux response that was 60−100% of the response to wtRANTES.

RANTES Interaction with CCR3. NIH 3T3 (mouse embryonic fibroblast) cells transfected with CCR3 were used to analyze the binding interaction between CCR3 and RANTES mutants as described above; the data are shown in Figure 3A. Untransfected NIH 3T3 cells do not bind RANTES. Within the N-loop, an alanine substitution for Y14 caused a 16-fold reduction and F12A caused a >5000-fold reduction in binding to CCR3 compared to wtRANTES. For the alanine substitutions near the N-terminus, S4A bound

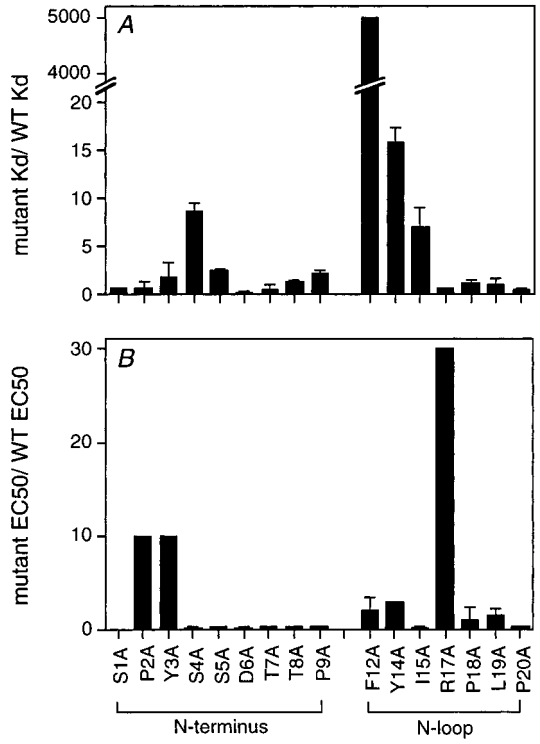

FIGURE 3: RANTES interaction with CCR3. RANTES binds to CCR3 with a K_d of 0.3 nM (A). RANTES binding to CCR3 expressed on NIH 3T3 cells was examined as described in Figure 2A for CCR1. Each bar represents the mean value of at least two different binding experiments, and the error bars represent the standard deviation from mean values. (B) Each RANTES mutant was used as a chemoattractant for eosinophils that mainly express CCR3. Eosinophils from two different donors in experiments with a series of 3-fold dilutions of each mutant were used to calculate EC_{50} values. The mean ratio of mutant EC_{50} to wild-type EC_{50} is shown; error bars represent standard deviation from mean values.

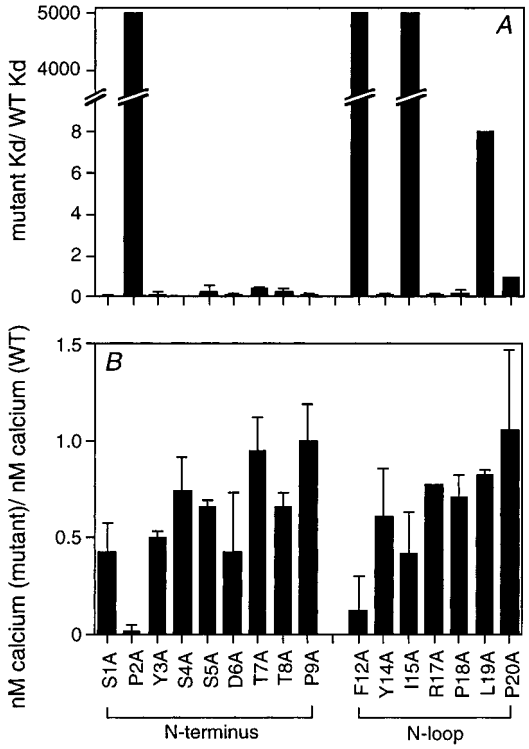

FIGURE 4: RANTES interaction with CCR5. This interaction was analyzed exactly as described for CCR1 in Figure 2, except that NIH 3T3 cells expressing CCR5 were used. RANTES binds to CCR5 with a K_d of 1.5 nM. (A) Each bar represents the mean ratio of at least two experiments comparing mutant K_d to wild-type K_d. The error bars represent standard deviation from mean values. (B) The maximum change in calcium flux stimulated by each mutant to that stimulated by wtRANTES in at least two separate experiments is shown as a ratio with standard deviations as error bars.

with a 8.8-fold lower affinity than wtRANTES. The other mutants bound as well as or had no more than a 5-fold lower binding affinity than wtRANTES.

CCR3 transfected 3T3 cells when activated with wtRANTES showed a small but consistent calcium flux response. However, the magnitude of this response was not enough to detect differences in the calcium flux stimulated by alanine scan mutants. Therefore, each mutant was also used as a chemoattractant in an eosinophil chemotaxis assay, using primary peripheral blood eosinophils which are known to mainly express CCR3 (Ponath et al., 1996a,b). The EC_{50} concentration for chemotaxis of eosinophils was calculated for each mutant and plotted as a ratio to wild-type EC_{50} calculated from the same experiment (Figure 3B). Of the mutations in the N-loop, mutant Y14A had a 1.5-fold higher EC_{50} concentration required for chemotaxis than wtRANTES but a 16-fold decrease in binding to CCR3. However, R17A which bound to CCR3 as well as wtRANTES showed a 30-fold increase in EC_{50} for chemotaxis of eosinophils. This may be explained by the expression of CCR1 by eosinophils (Daugherty et al., 1996), which is a receptor that requires contact with the side chain of R17 (Figure 2). In addition, F12A binds to eosinophils with the same affinity as wtRANTES (data not shown), which corresponds to its EC_{50} value in the chemotaxis assay. Alanine substitutions near the N-terminus, at positions P2 and Y3, resulted in EC_{50} values 10-fold higher than wtRANTES, indicating the importance of these residues for the activation of a chemotactic response via CCR3.

RANTES Interaction with CCR5. The interaction between RANTES and CCR5 was analyzed using NIH 3T3 cells transfected with CCR5. The analysis was performed exactly as described above for CCR1, using the same binding and calcium flux assays as these cells show a significant calcium flux response to RANTES. The data are shown as a ratio of mutant K_d to wild-type K_d for binding (Figure 4A) and calcium flux stimulated by mutant to that stimulated by the same concentration as wtRANTES (Figure 4B). All the alanine substitution mutants of RANTES bound to CCR5 as well as wtRANTES, with the exception of P2A, F12A, and I15A, which had binding affinities that were >5000-fold lower than wtRANTES. These results indicate that residues P2, F12, and I15 of RANTES are required for binding to CCR5.

In the calcium flux assay, I15A caused a calcium flux that was 40% of the wild-type response while P2A and F12A only stimulated a 5−10% response. This could be due to the low-binding affinities of P2A, F12A, and I15A for CCR5, which result in a reduction in the number of receptors being occupied by these mutants. Of the alanine substitution mutants near the N-terminus, S1A, P2A, Y3A, and D6A induced a calcium flux response that was less than 50% that of the response to wtRANTES, while all the other mutants stimulated one that was 60−100% that of the response to wtRANTES. Overlapping error distributions for mutants S4A, S5A, and T8A with D6A are observed; however, they have 60−70% of wild-type activity and are thus not

considered to have a major effect on calcium flux responses. These results indicate that residues S1, Y3A, and D6 have side chains whose contact with CCR5 is required for activation of a calcium flux response.

DISCUSSION

Although mutagenesis studies have identified residues within the extracellular portions of some chemokine receptors that are required for ligand binding (Leong et al., 1994; Monteclaro & Charo, 1996), the mechanism by which chemokines bind and activate their receptors is not well understood. There are believed to be two or more sites of interaction between the CC- and CXC-chemokines and their receptors (Lowman et al., 1996; Monteclaro & Charo, 1996). In this study we have identified specific residues within the N-loop and near the N-terminus of RANTES whose side chains are involved in binding to the CC-chemokine receptors CCR1, CCR3, and CCR5. In addition, our study has found that the side chains of different residues near the N-terminus of RANTES are involved in the functional activation of each of the receptors CCR1, CCR3, and CCR5.

All of the single alanine substitution mutants were as active as wtRANTES in at least one assay, which argues against gross conformational changes in the structure of the protein caused by alanine mutations. However, the side chain of F12 is buried at the dimer interface, and the side chain of I15 is predominantly buried in the core of the monomer. Changes in receptor binding and activation when these residues are mutated to alanine may reflect perturbations of the dimer geometry or local N-loop conformation, rather than disruption of direct receptor contact. The position of I15 suggests that it plays a local structural role in the monomer and dimer, either in the backbone conformation or in the orientation of other key side chains. However, the side chain of F12 appears considerably more solvent exposed in the context of the monomer, and in this context, it would appear reasonable to postulate a direct interaction of this residue with the receptor. The other residues that are required for RANTES binding to its receptors (P2, Y14, and R17) are all solvent exposed and therefore probably interact with the receptor via their side chains.

We observed that mutant F12A binds >5000-fold less well to CCR3 than wtRANTES but stimulates an eosinophil chemotaxis as well as wtRANTES. Furthermore, R17A binds to CCR3 as well as wtRANTES, yet has an EC_{50} that is 30-fold higher than wtRANTES in the eosinophil chemotaxis assay. Primary peripheral blood eosinophils were used in this functional assay for CCR3 since they mainly express CCR3. However, this assay was used with the caveat that the cell surface expression of other chemokine receptors by eosinophils has not been conclusively proven or disproven since antibodies against CC-chemokine receptors other than CCR3 are not available. We have found that mutant F12A does bind to eosinophils with the same affinity as wtRANTES (data not shown). Daugherty et al. (1996) have proposed the expression of CCR1 on eosinophils on the basis of a functional response of eosinophils to MIP-1α. The expression of CCR1 by eosinophils could account for the difference between binding and functional data for R17A and F12A, since R17A has a low binding affinity for CCR1 but F12A binds and stimulates CCR1 to a level comparable to wtRANTES. The chemotactic response of eosinophils to F12A could also be explained by the expression of a still unknown chemokine receptor that binds RANTES.

Each of the alanine substitution mutants R17A, F12A, and I15A, despite >5000-fold reductions in binding to CCR1 or CCR5, retain 15−40% of wild-type activity in a calcium flux assay. A reduction in binding affinity would result in fewer receptors being occupied by mutant ligand. The present data would argue that this reduced receptor occupancy is nonetheless sufficient for the signaling of a calcium flux event, albeit 15−40% of the response to wtRANTES.

Previous work by two independent groups using N-terminal truncation mutants of MCP-1, RANTES, and MCP-3A (Gong & Clark Lewis, 1995; Gong et al., 1996; Zhang et al., 1994b) showed that the mutants could still bind to receptors on THP-1 cells but not activate a calcium flux or chemotaxis response. Truncation mutants, however, do not address the question of specific interactions between residues in the ligand and in the receptor. Residues other than those near the N-terminus are thought to contribute most of the binding energy while the amino acids preceding the first two cysteines are required for functionally activating the receptor (Gong & Clark Lewis, 1995; Gong et al., 1996; Zhang et al., 1994b). Alanine substitutions at positions P2, S4, D6, and T7 result in mutant proteins that bind to CCR1 with a K_d comparable to that of wtRANTES but stimulate a calcium flux that is 30−50% of the wild-type response (Figure 2). Changes to alanine of residues P2 and Y3 do not affect binding to CCR3, but these residues do have side chains that are required for interaction with CCR3 to cause the chemotaxis of eosinophils (Figure 3). In addition, S1, Y3, and D6 when mutated to alanine bind to CCR5 with a K_d comparable to that of wtRANTES but only stimulate a calcium flux response that is 40−50% of the response to wtRANTES. The two-site model of interaction between ligand and receptor suggests that mutations near the N-terminus need not result in a decrease in receptor binding but can cause a decrease in functional activation of the receptor. The data described above generally support a two-site model of interaction between the CC-chemokines and their receptors. The exception to this generalization is the change P2A near the N-terminus, which is required both for binding to and for the activation of CCR5.

Our data emphasize the importance of N-loop residues in the binding of RANTES to its receptors. We have found that the following side chains on RANTES are required for receptor binding: residue R17 for binding to CCR1 (in pink, Figure 5A), residues F12 and Y14 for binding to CCR3 (in cyan, Figure 5A), and residues P2, F12, and I15 for binding to CCR5 (in blue, Figure 5A). Residues I15 and R17 are leucines in murine RANTES and may explain why murine RANTES does not bind to human RANTES receptors. Our results also show for the first time that mutation to alanine of an N-terminal residue (P2) of a CC-chemokine dramatically affects binding to CCR5. Within one monomer, P2 is spatially removed from the N-loop residues involved in receptor binding. However, in the context of the RANTES dimer, the N-terminus of one monomer packs against the N-loop of the other monomer. This brings P2′ and F12, I15, and R17, the residues important for receptor binding and function, into close proximity on the surface of the dimer (Figure 5A). Whether the monomer or the dimer form of RANTES is the active species for receptor binding and

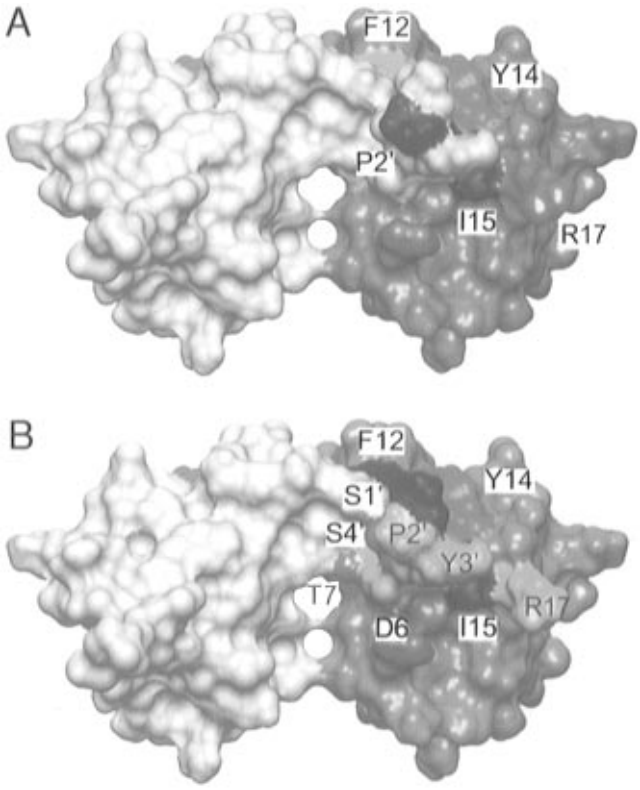

FIGURE 5: Dimer structure of RANTES as a solvent-accessible surface. The orientation is the same as that shown in Figure 1A. (A) Residues required for receptor binding are shown in pink (CCR1, R17), cyan (CCR3, Y14), blue (CCR5, P2′ and I15), and yellow for overlapping residues (CCR3 and CCR5, F12). (B) Residues required for signal transduction are shown in pink (CCR1, S4′ and T7), cyan (CCR3, Y14), blue (CCR5, S1′, F12, and I15), and yellow for overlapping residues (CCR1 and CCR5, D6; CCR1 and CCR3, R17; CCR3 and CCR5, P2′ and Y3′).

activation is unknown. Our observation suggests the possibility that the dimeric rather than the monomeric form of RANTES may be important for activity in vitro and in vivo.

In summary, this study shows that RANTES interacts with each of its receptors in a distinct way. Our data show that specific residues within the N-loop and P2 near the N-terminus of RANTES are critical for its binding to the receptors CCR1, CCR3, and CCR5. We propose a model of distinct but overlapping binding epitopes on RANTES to explain the specific binding of the same ligand to three different receptors (overlapping residues in yellow, Figure 5A). Mutating these residues to alanine also affects receptor activation since the mutant proteins do not bind as well as wild-type RANTES. In addition, we have identified discrete residues near the N-terminus of RANTES that, in the context of an intact molecule, are responsible for the activation of the receptors CCR1, CCR3, and CCR5 (Figure 5B). The residues near the N-terminus form overlapping activation epitopes for each receptor, distinct from the binding region formed by the N-loop and P2. The residues that are required by more than one receptor form a central core (in yellow, Figure 5B) surrounded by residues involved in receptor-specific signaling. Thus the binding epitopes in the N-loop of RANTES appear to be distinct from the receptor activation epitopes near the N-terminus with the exception of residue P2.

These results also provide further evidence that the CC- and CXC-chemokines interact with their receptors differently. The ELR motif near the N-terminus of IL-8, a CXC-chemokine, is important for binding to and activation of both its receptors (Clark Lewis et al., 1991; Hebert et al., 1991), while we have not found a consensus sequence in RANTES that affects binding to all of its receptors. Receptor specificity for IL-8 is determined by residues in the N-loop (Lowman et al., 1996); however, as we show in this study with the CC-chemokine RANTES, receptor binding and activation are not as discretely localized as for IL-8.

RANTES binding to its receptors is the first step in an inflammatory response. It has been argued that a general chemokine antagonist would be an effective antiinflammatory molecule. Conversely, being able to target an antagonist to a receptor involved in attracting a particular subset of inflammatory cells to a site of inflammation would be more specific and therefore may be therapeutically useful. In addition, a CCR5-specific antagonist may have utility as a therapeutic for HIV infection. The results of this study will be useful for designing receptor-specific antagonists by aiding in the understanding of the specific molecular interactions between RANTES and three of its receptors.

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