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Molecular Anatomy of CCR5 Engagement by Physiologic and Viral Chemokines and HIV-1 Envelope Glycoproteins: Differences in Primary Structural Requirements for RANTES, MIP-1α, and vMIP-II Binding

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Molecular analysis of CCR5, the cardinal coreceptor for HIV-1 infection, has implicated the N-terminal extracellular domain (N-ter) and regions vicinal to the second extracellular loop (ECL2) in this activity. It was shown that residues in the N-ter are necessary for binding of the physiologic ligands, RANTES (CCL5) and MIP-1α (CCL3). vMIP-II, encoded by the Kaposi's sarcoma-associated herpesvirus, is a high affinity CCR5 antagonist, but lacks efficacy as a coreceptor inhibitor. Therefore, we compared the mechanism for engagement by vMIP-II of CCR5 to its interaction with physiologic ligands. RANTES, MIP-1α, and vMIP-II bound CCR5 at high affinity, but demonstrated partial cross-competition. Characterization of 15 CCR5 alanine scanning mutants of charged extracellular amino acids revealed that alteration of acidic residues in the distal N-ter abrogated binding of RANTES, MIP-1α, and vMIP-II. Whereas mutation of residues in ECL2 of CCR5 dramatically reduced the binding of RANTES and MIP-1α and their ability to induce signaling, interaction with vMIP-II was not altered by any mutation in the exoloops of the receptor. Paradoxically, monoclonal antibodies to N-ter epitopes did not block chemokine binding, but those mapped to ECL2 were effective inhibitors. A CCR5 chimera with the distal N-ter residues of CXCR2 bound MIP-1α and vMIP-II with an affinity similar to that of the wild-type receptor. Engagement of CCR5 by vMIP-II, but not RANTES or MIP-1α blocked the binding of monoclonal antibodies to the receptor, providing additional evidence for a distinct mechanism for viral chemokine binding. Analysis of the coreceptor activity of randomly generated mouse-human CCR5 chimeras implicated residues in ECL2 between H173 and V197 in this function. RANTES, but not vMIP-II blocked CCR5 M-tropic coreceptor activity in the fusion assay. The insensitivity of vMIP-II binding to mutations in ECL2 provides a potential rationale to its inefficiency as an antagonist of CCR5 coreceptor activity. These findings suggest that the molecular anatomy of CCR5 binding plays a critical role in antagonism of coreceptor activity.

Keywords: CCR5; chemokines; ligand binding; HIV-1 coreceptor; vMIP-II

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Abbreviations used: N-ter, N-terminal extracellular domain; ECL, extracellular loop; RANTES, regulated upon activation normal T cell expressed and secreted; MIP-1α, macrophage inflammatory protein 1 alpha; vMIP-II, viral macrophage inflammatory protein; HIV, human immunodeficiency virus; mAb, monoclonal antibody; 5-HT, 5-hydroxytryptamine; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CHO, Chinese hamster ovary; FEBS, fetal bovine serum, FACS, fluorenscence activated cell sorting.

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Introduction

CCR5 is a member of the serpentine receptor superfamily that binds RANTES (CCL5), MIP-1α (CCL3), and MIP-1β (CCL4)[1] and is expressed by monocytes, memory T-lymphocytes, preferentially Th1 cells and NK cells.[2–4] It acts in concert with CD4 to associate with the envelope glycoprotein of HIV-1 leading to fusion of viral and target cell membranes and subsequent viral entry.[5–7] It functions as the front line coreceptor for macrophage (M-) tropic (R5) strains of HIV-1[8,9] and individuals homozygous for a 32 bp deletion in the gene encoding CCR5 lack a functional receptor and are highly resistant to infection by commonly transmitted strains of HIV-1.[10,11] The interaction between the gp120 subunit of the HIV-1 envelope glycoprotein and CCR5 is blocked by its cognate ligands,[5,12,13] a specific, small molecule antagonist,[14,15] and a subset of monoclonal antibodies (mAbs) to the receptor.[16,17] MAbs that bind epitopes in the second extracellular loop (ECL2) have been found to block infection with greater efficacy than those directed against the N terminus (N-ter).[16,17]

The precise mechanism for the engagement of CCR5 by the CD4-activated gp120 is complex and appears to involve the contribution of multiple coreceptor domains for envelope-mediated fusion. Both the N-ter and the body, which includes the hydrophobic core and interhelical loops, of CCR5 have been shown to be sufficient, but not necessary for coreceptor activity in the context of reciprocal chimeras.[18,19] It has been established that the N-ter domain plays a key role in this association, and specific acidic and aromatic residues contribute to coreceptor activity.[20–22] Posttranslational sulfation of Y10 and Y14 plays a role in ligand binding and coreceptor activity, as well. The finding that synthetic peptides containing sulfated Y10 and Y14 residues bind gp120 at micromolar affinities provides further evidence for the direct involvement of the CCR5 N-ter domain with envelope glycoproteins.[23,24] The body of CCR5 is also sufficient to impart coreceptor activity and studies with envelope glycoproteins containing a P → A switch in the conserved G-P-G crown of the third hypervariable domain (V3) indicate that the coreceptor function of this region may require the type 2 β-hairpin turn architecture typical of the V3 loop of gp120.[25] Residues in predicted transmembrane spanning domains (TM) 4[26] and 5[27] have also been implicated in coreceptor activity, but it is not yet clear whether these hydrophobic helices interact directly with envelope glycoprotein or influence the conformation of solvent-exposed segments available at the extracellular surface. Amino acid residues required for the activity of TAK-779, a small molecule CCR5 inhibitor, have been mapped to TM1, 2, 3, and 7.[14]

The physiologic ligands of CCR5 have been shown to antagonize its utilization as a coreceptor by M-tropic envelope glycoproteins.[5,13,28,29] In contrast, a viral chemokine encoded by the Kaposi's sarcoma-associated herpesvirus (vMIP-II)[30] appears to have limited efficacy as a coreceptor inhibitor, although it is a high affinity receptor antagonist.[31] The disparity between the inhibitory efficiency of physiologic ligands of CCR5 and vMIP-II may result from differences in engagement of key receptor domains and, thus, receptor blockade. Substitution of CCR5 N-ter domain with that of CCR2 did not have a significant impact on RANTES and MIP-1α binding, indicating that sequences that impart ligand binding specificity reside in the body of the receptor.[32] In contrast, CCR5 variants with alanine scanning mutations in acidic residues in the N-ter domain (D11A and E18A) have been shown to exhibit decreased binding to MIP-1β, suggesting a role in receptor conformation.[20] Also D11A, but not E18A, exhibited a reduced ability of MIP-1α, MIP-1β and RANTES to block HIV-1 infection.[21]

To elucidate the structural basis for coreceptor activity of the body of CCR5 and gain insight into amino acid residues in these domains that are critical targets for receptor blockade, regions involved in M-tropic coreceptor activity were dissected using random chimeragenesis. Alanine scanning mutagenesis of charged residues in CCR5 revealed that the requirements for binding of MIP-1α and RANTES, effective coreceptor antagonists, are different than those of vMIP-II, an ineffective inhibitor of this function. Whereas basic residues in the distal segment of ECL2 of CCR5 implicated in coreceptor activity were required for binding of RANTES and MIP-1α, these mutations did not alter vMIP-II binding. Competition experiments with MIP-1α and mAbs provided additional evidence that vMIP-II engages CCR5 by a different mechanism from that used by the cognate ligands. These findings indicate that although vMIP-II binds to the same domain of CCR5 as RANTES and MIP-1α, binding of the viral chemokine has structural and/or conformational requirements different from those of the physiologic ligands.

Results

vMIP-II is a high affinity CCR5 antagonist that undergoes incomplete cross competition by physiologic ligands

The binding properties of vMIP-II with wild-type CCR5 was determined by Scatchard analysis. The displacement of ^{125}I-labeled vMIP-II from CCR5 CHO transfectants by cold ligand (Figure 1(a)) corresponds to an EC_{50} of 5.5 nM. This is similar to values obtained in homologous displacement experiments with RANTES and MIP-1α. The IC_{50} values for the displacement of these chemokines by vMIP-II range from 5.0-7.0 nM. Cross-competition experiments revealed that whereas vMIP-II can efficiently displace the binding of [^{125}I]MIP-1α to CCR5 (Figure1b) and vMIP-II can displace itself to levels similar to the back-

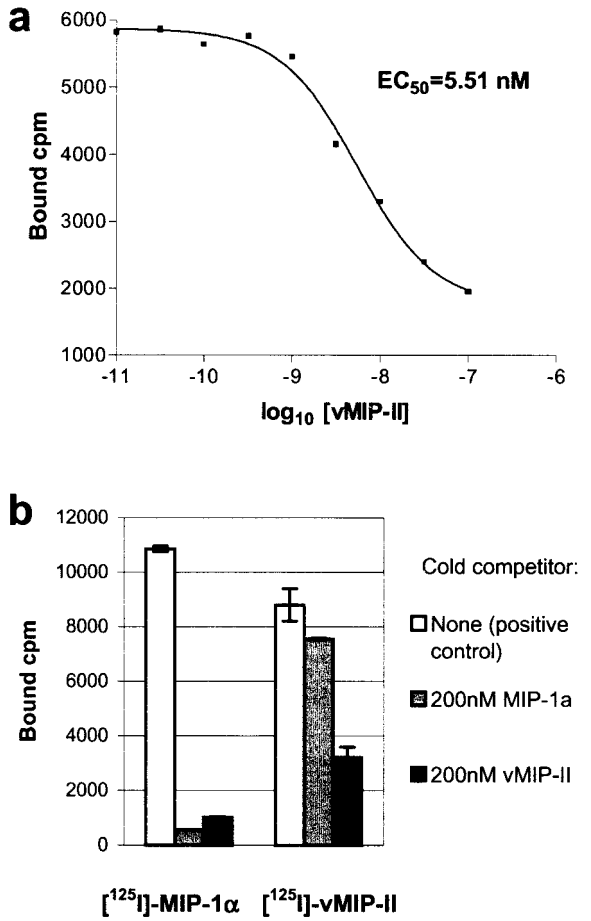

Figure 1. vMIP-II binds CCR5 at high affinity but is incompletely displaced by MIP-1α. (a) Homologous displacement of 0.1 nM [^{125}I]vMIP-II by cold ligand. Bound radioactivity was determined in duplicates for each concentration of cold vMIP-II. Values are mean ± SD of duplicates. The affinity was calculated using PRISM (GraphPad Software, San Diego, CA). (b) Cross-competition between MIP-1α and vMIP-II. Each radiolabeled chemokine (0.1 nM) was displaced by a 2000 time molar excess of the cold ligands. Each experiment was performed at least three times with similar results.

ground obtained with control CHO-K1 cells, the presence of excess MIP-1α resulted in partial displacement of [^{125}I]vMIP-II binding. Competition experiments with RANTES gave similar findings (data not shown).

Variation in the molecular anatomy of CCR5 binding by RANTES, MIP-1α, and vMIP-II

All the charged residues in the extracellular domains of CCR5 indicated in the diagram in Figure 2 were individually substituted with alanine. The panel of transfectant cell lines with stable expression of wild-type CCR5 and alanine scanning mutants listed in Figure 3(a) was tested for receptor expression by staining with mAbs to the

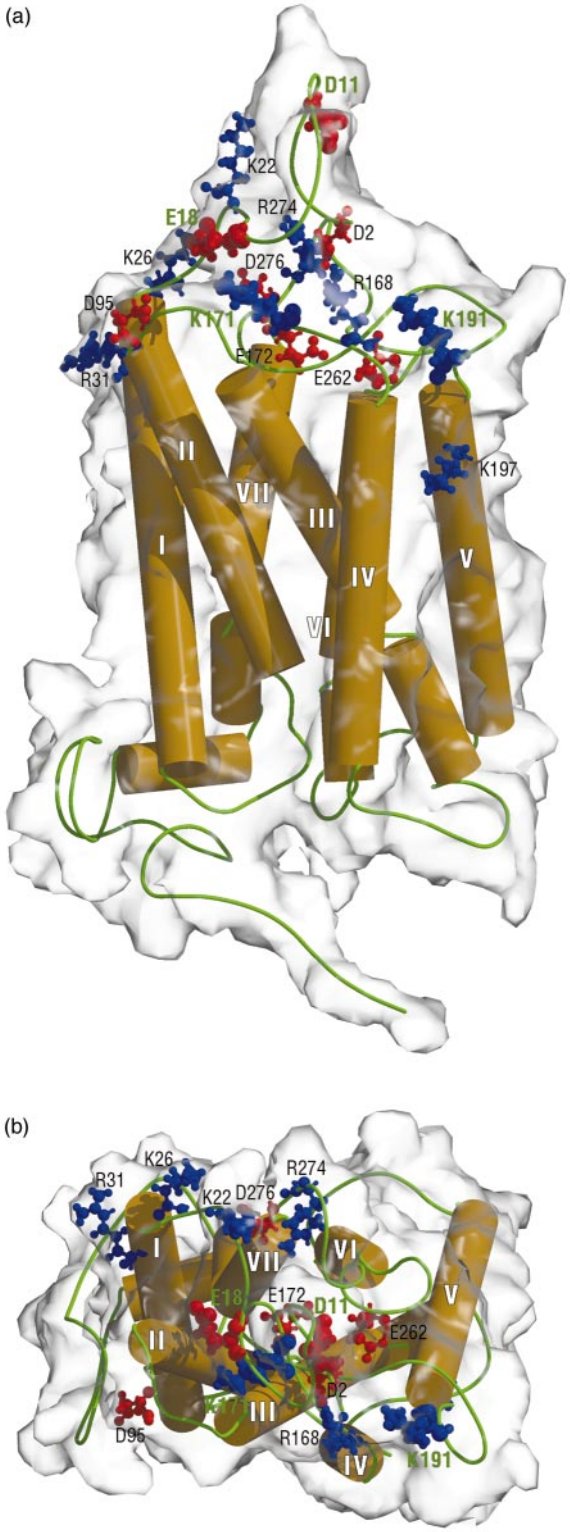

Figure 2. Schematic diagram of CCR5 based on the rhodopsin crystal structure using the Raster3D program. The shadowed area corresponds to the space filling model; helices are depicted in orange and numbered; charged amino acids in predicted extracellular domains are shown in red or blue for acidic or basic residues, respectively. Lateral (a) and extracellular (b) views are shown.

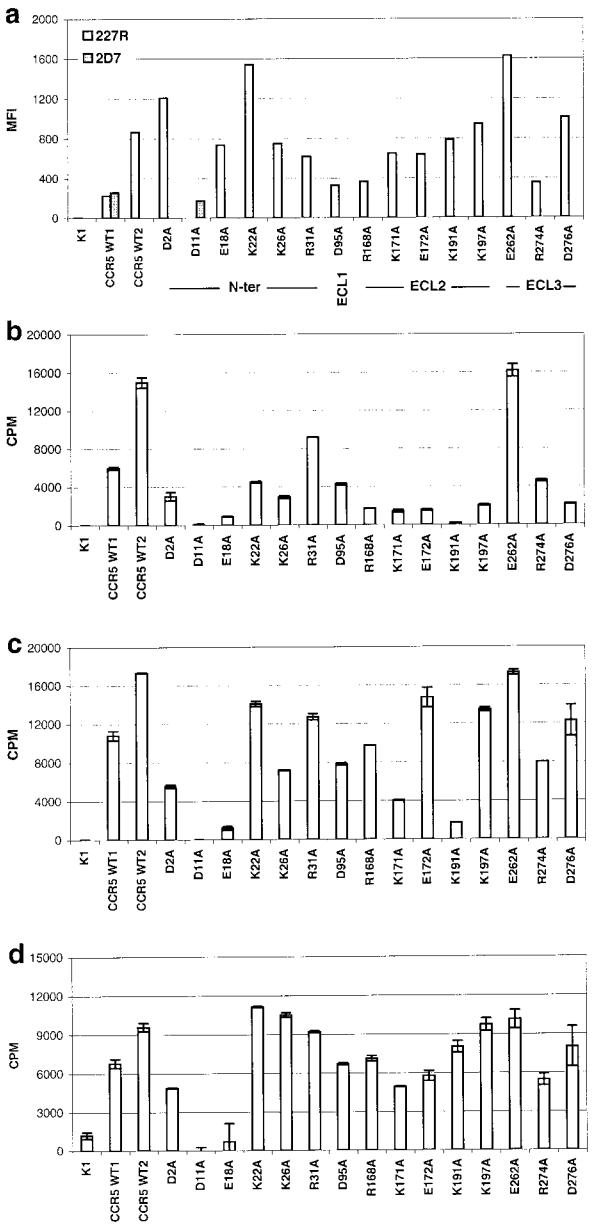

Figure 3. Binding of radiolabeled MIP-1α, RANTES and vMIP-II to stable transfectants of CHO cells expressing point mutants of CCR5. (a) A panel of 15 alanine scanning mutants were tested for their expression of the receptor by flow cytometry using mAbs to the N-ter (227R) or ECL2 (2D7). The expression level is expressed as the mean fluorescence intensity (MFI). These mutants were tested simultaneously for their ability to bind radiolabeled (b) MIP-1α, (c) RANTES and (d) vMIP-II. The specific binding was determined for each mutant by adding an excess of cold ligand. Values are mean ±SD of duplicates obtained in one of two separate experiments with similar results.

N-ter (227R) or ECL2 (531 or 2D7). The latter reagents were required for the analysis of the D11A mutant, because the epitope recognized by 227R was previously shown to include D11.[27] Flow cytometric analysis revealed significant levels of expression for all of the mutants tested, which in each case had a mean fluorescence intensity that was at least 200 units greater than that observed by staining control CHO-K1 cells with the anti-CCR5 mAbs and transfectants with isotype-matched control immunoglobulins (Figure 3(a)). Independent CCR5 transfectants with low and high levels of expression were subjected to subsequent binding analyses in order to further ensure that observed differences were not due to variation in receptor content on the cell surface. Several CCR5 variants containing alanine scanning mutations (D2A, K22A, K191A, K197A, E262A, and D276A) were expressed on the cell surface of transfectants at levels with mean fluorescence intensities higher than the CHO clone with stable high level expression of the wild-type receptor. CCR5(D11A) was expressed at levels slightly lower than the reference CCR5 transfectant with lower level expression. All of the other CCR5 variants with alanine scanning mutations were expressed on the cell surface at intermediate levels with mean fluorescence intensity values between the wild-type CCR5 reference transfectants with low and high level of expression.

The binding of RANTES and MIP-1α to the array of CCR5 alanine scanning mutants was compared with that of vMIP-II to gain insight into the molecular anatomy of the binding site for each of these ligands. Specific binding was determined for each radioligand. In many cases, the binding window was too small to calculate an EC_{50} accurately, hence only the binding window is reported. The values for binding of [^{125}I]MIP-1α, RANTES and vMIP-II are shown in Figure 3(b), (c) and (d), respectively. Mutation of charged residues in the N-ter domain of CCR5 resulted in decreased binding to these ligands, as shown for MIP-1β.[20] The D11A mutant lacked the ability to bind all three chemokines and E18A demonstrated a significantly diminished capacity to bind these ligands. The D2A mutant also had evidence of decreased binding, principally to [^{125}I]RANTES and MIP-1α. The binding of MIP-1α was also sensitive to the conversion of K22 and K26 to alanine and there was some decrease in the binding of RANTES to these mutants. In contrast, these conversions did not have a significant effect on the binding of vMIP-II to CCR5.

CCR5 variants containing alanine scanning mutations in charged residues predicted to occur in extracellular loops 1-3 were also analyzed for binding to RANTES, MIP-1α, and vMIP-II. None of the alanine scanning mutants in the extracellular loops of CCR5 significantly altered the binding of vMIP-II to the receptor variants. In contrast, the binding of RANTES and MIP-1α were affected by alanine scanning conversions in ECL2, with the latter showing significant sensitivity to receptor mutations. Substitution of alanine for D95, the only charged amino acid residue in ECL1, resulted in a slight decrease in the binding of MIP-1α, but did

not alter the binding of RANTES significantly. Two alanine scanning mutations in ECL2 had a dramatic effect on RANTES and MIP-1α binding. CCR5(K191A), although expressed on the cell surface at a mean fluorescence intensity greater than the wild-type receptor, showed a 50-75 % reduction in RANTES binding and negligible levels of interaction with MIP-1α. The CCR5 variant K171A showed decreased binding to RANTES and MIP-1α. Whereas tranfectants stably expressing CCR5(E172A) had increased binding to RANTES, parallel experiments showed that this variant had decreased interaction with MIP-1α. Mutants R168A and K197A showed diminished binding to MIP-1α, but unchanged levels of interaction with RANTES. None of the alanine scanning mutations in ECL3 had a significant impact on RANTES binding, but conversion of D276 to alanine resulted in a substantial decrease in the level of MIP-1α binding.

Involvement of residues in the CCR5 N terminus and ECL-2 in signal transduction

The decreased ability of CCR5 point mutants to bind RANTES and MIP-1α was further correlated to signal transduction capacity. Calcium flux assays were performed using concentrations of RANTES and MIP-1α ranging from 1-100 nM. Mutants binding 20 % or less of the wild-type showed a reduced or absent response to 1 nM of each agonist (data not shown). The highest concentration (100 nM), being two- to tenfold greater than the saturating dose for the wild-type receptor, was used to determine if the conformational changes triggering signal transduction could be induced in mutant receptors with diminished binding capability. As shown in Figure 4, stimulation of CHO transfectants stably expressing CCR5 alanine scanning mutants in D2, E18, or K191 with 100 nM RANTES or MIP-1α resulted in a mobilization of

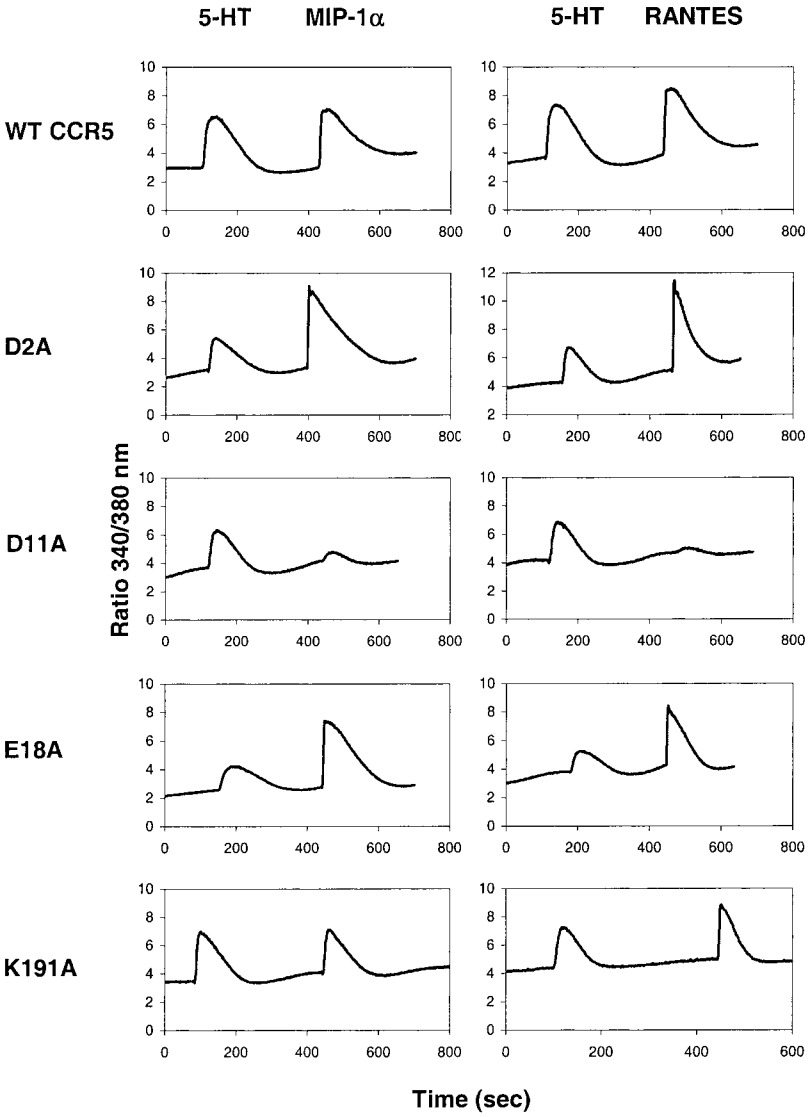

Figure 4. Signal transduction in point mutants of CCR5 by calcium flux. The ability of the mutants with undetectable or very reduced binding of MIP-1α and RANTES were tested for their ability to respond to a high concentration of ligand (100 nM). A release of calcium in the cytoplasm was observed in all Fura-2 loaded mutants except D11A. 5-HT was used as a control of the cell reactivity.

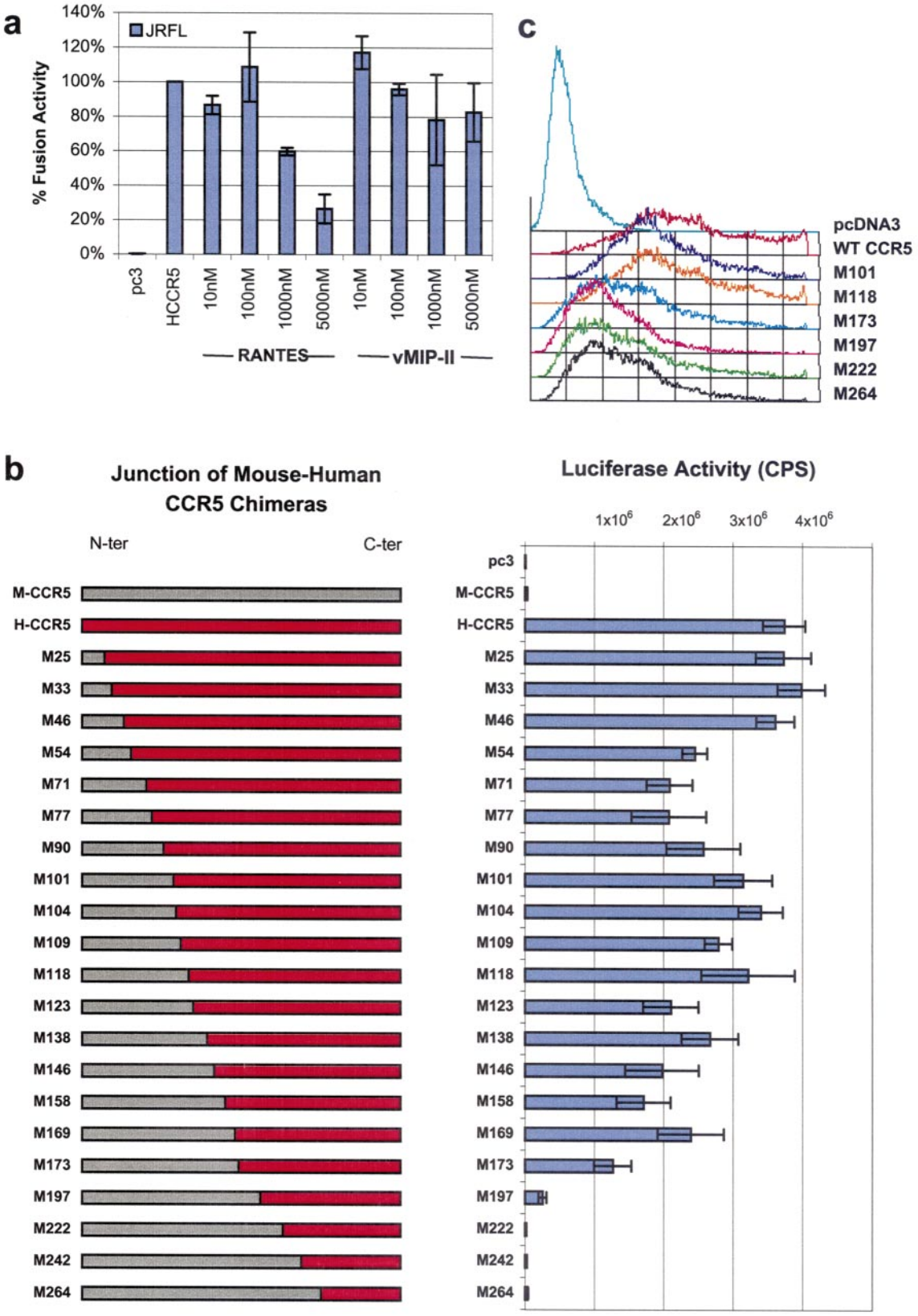

Figure 5 (*legend opposite*)

cytosolic calcium ions similar to that seen in transfectants expressing wild-type CCR5. In contrast, exposure of a transfectant cell line stably expressing the CCR5(D11A) mutant to these ligands did not induce a significant calcium flux. Exposure of all the CHO transfectants to 5-hydroxytryptamine (5-HT) yielded an increase in free cytosolic calcium ions mediated by the endogenous cognate receptor, confirming appropriate loading with the calcium sensitive fluorochrome.

Involvement of ECL2 residues vicinal to K191 in HIV-1 coreceptor activity

Whereas physiologic ligands, especially RANTES, have been shown to be effective antagonists of CCR5 coreceptor activity, vMIP-II appears to have limited efficacy.[31] These differences of efficacy between CCR5 ligands were confirmed in a cell-cell fusion assay (Figure 5(a)). One potential explanation is that vMIP-II binding does not effect occupation of residues critical to coreceptor activity. To identify residues in ECL2 of CCR5 that may be involved in this function, an array of mouse-human CCR5 hybrids was generated by random chimeragenesis, as described for human-mouse hybrids,[27] and tested for coreceptor activity in a cell-cell fusion assay. This approach yielded 21 different chimeras with incremental amounts of mouse sequences extending from the N terminus. The boundaries between mouse and human sequences of these hybrids spanned the domains of the receptor (Figure 5(b)). The coreceptor activity of the battery of mouse-human CCR5 chimeras with the JRFL envelope glycoprotein is shown in Figure 5(b). Chimeras with N-ter mouse sequences extending to residue 169, which is predicted to be at the beginning of ECL2, demonstrated coreceptor fusogenic activity similar to that of wild-type human CCR5 with the JRFL envelope. The reporter gene values obtained with this subset of chimeras were no less than 60% of those obtained using the human receptor. The mouse-human CCR5 chimera containing residues 1-197 from the mouse ortholog and 198-352 from the human receptor consistently had marginal coreceptor activity, with reporter gene activities less than 5% of wild-type CCR5. The junction of this chimera (M197) is at the interface of ECL2 and TM5. All of the chimeras with greater than 197 amino acid residues of mouse CCR5 had minimal coreceptor activity which was similar to that of wild-type mouse CCR5.

To test the cell surface expression of these chimeras, the mouse sequence $D^{15}YGM$ in the N-ter was replaced by the corresponding human sequence $N^{13}YYT$ by directed mutagenesis in order to recreate the epitope recognized by the mAb 227R.[27] The coreceptor activity of these mutated chimeras was identical to that of the chimeras with the mouse N-ter sequence (not shown). Staining with the mAb 227R and FACS analysis revealed that all the chimeras are expressed at relatively high level on the membrane when transfected in QT6 cells (Figure 5(c)). Furthermore, cell-cell fusion assays performed with serial dilutions of the plasmid coding for human CCR5 in target cells showed that variations of the expression level of the coreceptor in that range had limited influence on the fusion activity (not shown).

mAbs to ECL2, but not the N terminus, block ligand binding

In order to gain further insight into the role of the N-ter domain and ECL2 of CCR5 in ligand interactions, the ability of domain-specific anti-CCR5 mAbs to block the binding of radiolabeled RANTES, MIP-1α, and vMIP-II to the receptor was determined (Figure 6). A high molar excess (125 nM) of the 2D7 and 531 antibodies, which recognize epitopes on ECL2 of CCR5,[16,33] efficiently blocked the binding of 0.1 nM of [^{125}I]RANTES and [^{125}I]MIP-1α to this receptor, as already reported for MIP-1β.[16] These reagents also inhibited [^{125}I]vMIP-II binding. In contrast, two mAbs that interact with epitopes contained within the N-ter, 227R and 5C7,[12,33] did not alter the binding of these radioligands.

Figure 5. Analysis of CCR5 coreceptor inhibition by chemokines and molecular anatomy of this function. (a) Inhibition of CCR5 coreceptor activity in cell-cell fusion assay by RANTES and vMIPII as described in Materials and Methods. Results are the mean values of two (RANTES) and three (vMIP-II) independent experiments performed in quadruplicate. (b) Coreceptor activity of mouse/human CCR5 chimeras generated by random chimeragenesis. The content of M-CCR5 in each chimera is indicated (left panel). This includes mouse residues that are aligned with those in the human ortholog, thus the two-residue insertion in the N terminus of M-CCR5 is not counted. Coreceptor activity of these M/H CCR5 chimeras, analyzed in the cell-cell fusion assay, is shown in the right panel. Effector cells were programmed to express JRFL (M-tropic) envelope glycoprotein and T7 polymerase. Target cells were transiently transfected with hCD4, a candidate coreceptor chimera and a plasmid containing the luciferase gene under the control of a T7 polymerase promoter. Luciferase activity was measured eight hours after mixing effector and target cells. The results shown are from a representative experiment repeated in three independent assays. The values are the mean ± SD of quadruplicates. (c) Membrane expression of mouse-human CCR5 chimeras. QT6 cells transfected with CCR5 or a panel of mouse-human chimeras mutated to contain the sequence NYYT in their N-ter were stained with mAb 227R and analysed by flow cytometry.

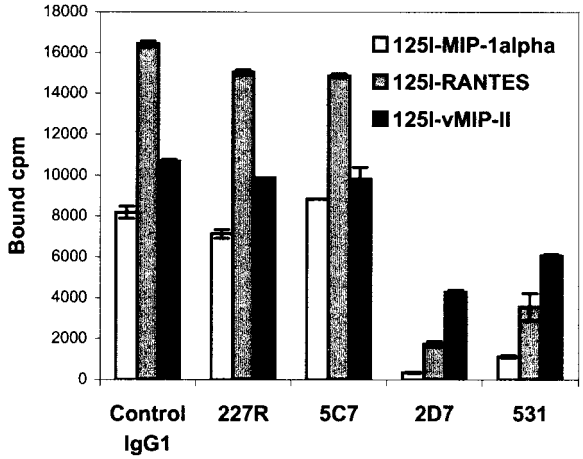

Figure 6. Blocking of radiolabeled chemokines with mAbs anti-CCR5. Cells were incubated with BSA alone (no competitor) or mAbs against the N-ter (227R and 5C7) or ECL2 (2D7 and 531) of CCR5 at saturating concentrations (20 μg/ml, 125 nM), then 0.1 nM of ^{125}I-labeled chemokines was added. After two hours of incubation on ice, the bound radioactivity was counted. Results are mean ±SD of duplicates from one experiment representative of at least two similar experiments.

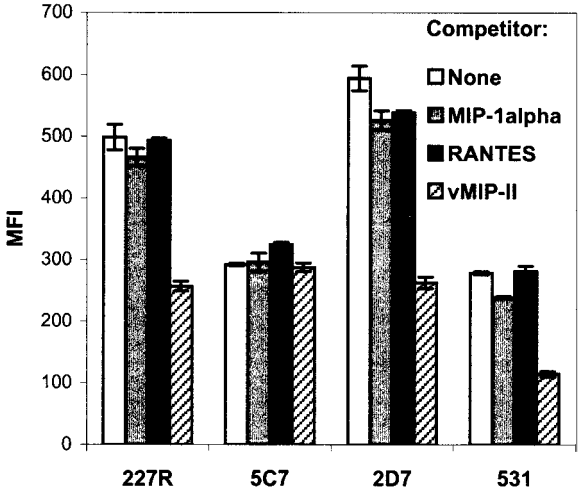

Figure 7. Blocking of mAbs against CCR5 by chemokines. Cells were incubated with BSA alone (no competitor) or 500 nM of MIP-1α, RANTES or vMIP-II used as competitors, then with mAbs 227R, 5C7, 2D7 at 1 μg/ml (6 nM) or 531 at 5 μg/ml (30 nM, final concentrations). These incubations were performed on ice to prevent any internalization of the receptor. After one hour, cells were washed in cold PBS-BSA and antibody binding was detected by secondary staining with an anti-mouse IgG antibody labeled with PE, followed by FACS analysis. The mean fluorescence intensity (MFI) of 2×10^4 cells was measured for each sample. Results are the mean ±SD of duplicates and are from one experiment representative of two with similar results.

Engagement of CCR5 by vMIP-II, but not RANTES or MIP-1α, blocks anti-receptor antibody binding

The engagement of CCR5 by RANTES and MIP-1α results in G-protein-mediated signal transduction and ultimately receptor internalization. vMIP-II binds CCR5 at high affinity without promoting down-modulation of the receptor (not shown) and efficiently cross-competes with these physiologic ligands without evidence of the involvement of residues in ECL2 that are critical for their interaction with this receptor. To ascertain whether the engagement of CCR5 by RANTES and MIP-1α is different from receptor binding by vMIP-II, reverse blocking experiments were performed to determine whether these ligands could interfere with the interaction of mAbs specific for CCR5 domains (Figure 7). Preincubation of vMIP-II with CCR5 transfectants at 4 °C blocked the subsequent binding of a battery of mAbs, including one that binds an epitope in the N-ter spanning from D11 to E18 (227R), and those that recognize epitopes that involve ECL2 (2D7 and 531). In contrast, preincubation with RANTES and MIP-1α at 4 °C had no effect on the binding of these mAbs to epitopes mapped to the CCR5 N-ter or ECL2. None of the ligands could block 5C7, a mAb mapped to the most proximal end of the N-ter.

The role of the N terminus in ligand binding is not receptor specific, but structurally required

Whereas analysis of alanine scanning mutants demonstrated the importance of implicated residues in the N-ter of CCR5 to ligand binding, the failure of mAb 227R, recognizing an epitope including the critical residue D11, to displace any ligand raises questions regarding the mechanism for involvement of this region. To determine whether the CCR5 N-ter plays a receptor-specific or a generic/structural role in ligand binding, CCR5 variants with truncation or substitution of this domain were tested for the ability to bind RANTES, MIP-1α, and vMIP-II. Transfectants stably expressing a CCR5 truncation mutant lacking the first 15 amino acid residues of the N-ter region bound mAbs to epitopes involving ECL2, but did not show significant binding of RANTES, MIP-1α or vMIP-II (data not shown). Similarly, transfectants expressing a construct directing the expression of the N-ter domain of CCR5 tethered to the plasma membrane by a glycosyl phosphatidyl inositol linkage bound a mAb mapped to this region, but did not bind these ligands. In contrast, transfectants stably expressing a chimeric receptor (B555) in which the 20 amino acid residues of the distal N-ter domain were substituted with the corresponding region of CXCR2 (IL-8RB), thus preserving the four conserved cysteine residues and the integrity of the receptor, showed binding of mAbs to the N-ter of CXCR2, to ECL2 of CCR5, and to both MIP-1α and vMIP-II. Scatchard analysis revealed that the EC_{50} value for MIP-1α binding

was 1.4 nM, in comparison to 0.7 nM for the wild-type receptor (Figure 8).

Discussion

Here we demonstrate the importance of residues in ECL2 of CCR5 in (1) coreceptor activity with the M-tropic envelope glycoprotein JRFL by analyzing an array of mouse-human CCR5 chimeras, and (2) binding RANTES and MIP-1α, two physiologic ligands that can blockade this function. In contrast, the binding of vMIP-II, a high affinity antagonist of CCR5 ligand engagement, but not coreceptor function, was not affected by two alanine scanning mutations in ECL2 Lys residues that conferred loss of binding to RANTES and MIP-1α. Additional evidence for the disparate molecular anatomy of CCR5 interactions with vMIP-II and these physiologic ligands was based on partial cross-competition between vMIP-II and MIP-1α and the finding that engagement by vMIP-II, but not RANTES and MIP-1α, blocked the binding of mAbs to this receptor. Whereas alanine scanning mutations of D11 and E18 and truncation of the N-ter domain decreased the binding of RANTES, MIP-1α, and vMIP-II, substitution of this segment with the corresponding region of CXCR2 did not alter the binding of these ligands significantly, indicating that intact conformation of the N-ter is required for ligand binding, but this segment does not impart the specificity of this interaction. The finding that a mAb mapped to this region did not block chemokine binding suggests that it may play a structural role in determining receptor conformation.

Studies with receptor chimeras containing CCR5 domains have demonstrated that ECL2 plays a role in determining the repertoire of chemokine binding. mAbs mapped to this region effectively inhibit ligand binding and HIV-1 coreceptor function, as well. This overlap in structure-function relationships of ligand binding and coreceptor antagonism suggests that the site of CCR5 engagement by ligands may determine the efficacy of blockading interactions with envelope glycoproteins. Our findings with mouse-human CCR5 hybrids generated by random chimeragenesis indicate that residues between G173 and K197 subserve a critical function in the interaction with envelope glycoproteins. This segment contains six residues that are divergent between human CCR5 and the mouse homolog (human CCR5: L174F, S180P, Y184H, S185T, Q188H, N192S) and it is likely that a subset is important to coreceptor function. The two K residues in ECL2 at positions 171 and 191, that were found to be important for the binding of RANTES and MIP-1α are conserved in the mouse receptor, as are all of the other charged residues analyzed, except E18. Alanine scanning mutations of these residues did not influence the binding of vMIP-II, which was found to be a high affinity antagonist of ligand binding, but not coreceptor function. Thus, although vMIP-II association with CCR5 involves ECL2, our data indicate that the mechanism for this interaction has structural requirements that are

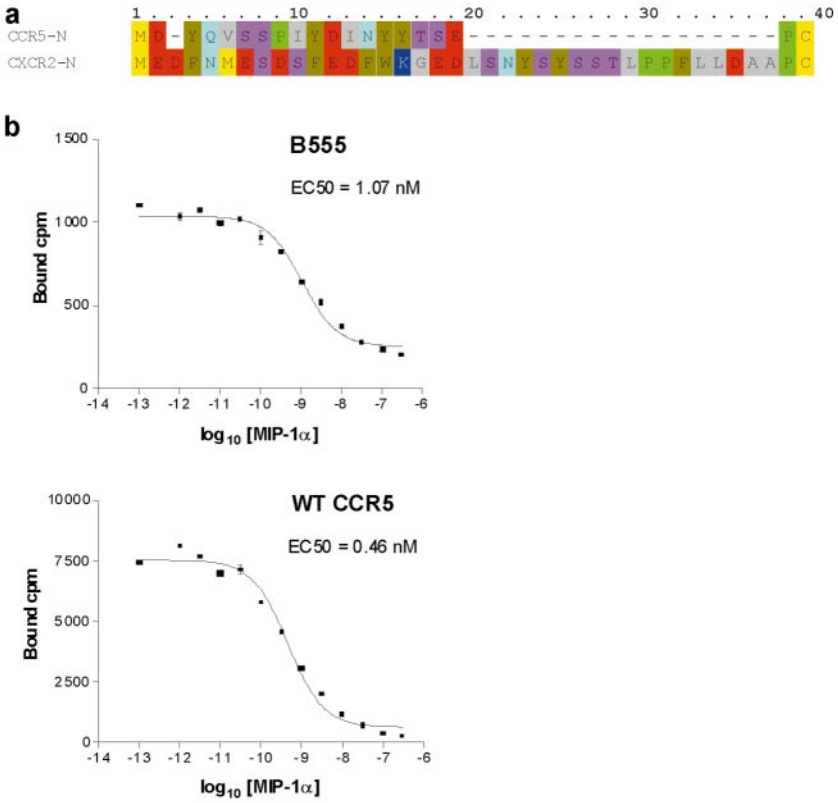

Figure 8. Binding of MIP-1α on CCR5 and the chimeric receptor B555. Radiolabeled MIP-1α was displaced by cold ligand on each cell line as described above and the EC_{50} value was calculated using PRISM.

different from the binding of RANTES and MIP-1α to this domain. The recognition that the primary structure of the distal segment of CCR5-ECL2 is important to coreceptor function, and that vMIP-II binding to this segment is different from CCR5 ligands that are effective coreceptor antagonists provides further evidence for the importance of this domain as a target for blockade of gp120 binding.

The analysis of CCR5 alanine scanning mutants in binding and signaling experiments suggest that RANTES and MIP-1α employ a similar mechanism for interacting with this receptor. vMIP-II has been shown to be an antagonist of a wide spectrum of chemokine receptors, including CCR5 and CXCR4. This is the first report that directly characterizes the binding of labeled vMIP-II, demonstrating that it engages CCR5 differently from these physiologic ligands. Whereas RANTES and MIP-1α show complete cross-competition, MIP-1α incompletely displaced the binding of [^{125}I]vMIP-II, suggesting that they engage sites that partially overlap. The ability of vMIP-II, but not RANTES or MIP-1α, to inhibit the binding of mAbs to CCR5 also supports the interpretation that it engages the receptor differently. The displacement of all ligands of CCR5 by mAbs to ECL2 emphasizes the importance of the physical accessibility of this domain for ligand binding. The fact that only vMIP-II is able in turn to displace the same macromolecules without having an affinity for CCR5 higher than MIP-1α or RANTES suggests that vMIP-II interacts with sites partially different from the natural ligands, or, alternatively, that the binding of vMIP-II induces a conformation of CCR5 that does not allow mAb binding. Similarly, the mutation K171 has been reported to abolish the binding of the mAb 2D7.[16] We report that the same mutation profoundly affects the binding of MIP-1α and RANTES. However, high concentrations of MIP-1α and RANTES could not displace 2D7. This illustrates the fact that the effects of mutations like K171or K191A may be interpreted as a consequence on the conformation of ECL2. These findings provide the first molecular anatomic basis for the finding that vMIP-II lacks efficacy as an inhibitor of CCR5 coreceptor activity. Insight into the regions of vMIP-II that are involved in CCR5 binding may provide genetic engineering approaches for optimizing the antagonist activity of this unique ligand.

The N-ter domain of CCR5 has been shown to play a key role in ligand binding and coreceptor activity. Mutation of critical acidic (D11 and E18) and aromatic (Y15) residues[20–22] and N-ter truncations[20,34] impair these functions. Substitution of this domain with the corresponding region of receptors with different ligand binding repertoires and lacking coreceptor activity reveals that it is not necessary for determining the specificity of interactions with chemokines or envelope glycoproteins.[32,33] Antibodies to the N-ter of CCR5 lack efficacy in blocking coreceptor activity as well as binding of MIP-1β and RANTES.[16,17] We extend this observation by demonstrating that these reagents do not have a significant impact on the binding of MIP-1α and vMIP-II. The 19 residues distal to the Cys residue in the N-ter domain of CCR5 were substituted with 38 amino acid residues that form this segment of CXCR2 (IL-8 receptor B) in the B555 chimera. The difference in length complicates the interpretation of the alignment of these segments, and the identity between the two N-ter domains is limited (Figure 8(a)). The CXCR2 segment has acidic residues at positions 2, 3, 7, 9, 12, 13, 18 and 19, thus the spacing of negatively charged amino acids from the N-ter is similar to that of CCR5. This chimera was designed to insert an ''N-terminal module'' sufficient to retain some intrinsic structural relationship from a genetically divergent chemokine receptor into CCR5 in order to contribute an architectural scaffold without providing ligand specificity. The preserved functionality of this chimera suggests that the N-ter domain of CCR5 may exert an influence on receptor conformation, but not play a direct role in the engagement of physiologic and pathologic ligands.

We report findings to support three concepts: (1) amino acid residues in ECL2 are involved in the binding of physiologic ligands as well as in coreceptor activity of CCR5 with M-tropic envelope glycoproteins; (2) the molecular anatomy of CCR5 binding by RANTES and MIP-1α is different from that employed by vMIP-II, although they bind at similar affinities; and (3) the N-ter domain of CCR5, while critical to ligand interactions, does not determine the specificity of chemokine binding and may participate indirectly in this process. The difference in the efficacy of coreceptor antagonism between the cognate ligands of CCR5 and vMIP-II may result, at least in part, from the involvement of critical K residues in ECL2 in binding the former, but not the viral chemokine, which is a high affinity receptor antagonist. Understanding the three-dimensional structure of G protein-coupled receptors in active and inactive state has been the focus of many studies. The development of a computational molecular modeling of CCR5 based on molecular biology data like ours will help to rationalize these findings, defining in particular the effect of mutations on the conformation of the extracellular domains of the receptor, and could allow to design small molecules that would specifically antagonize the chemokine receptor or the HIV-1 coreceptor activity.

Materials and Methods

Preparation of transfectants

Stable transfectants of CCR5 mutants were prepared in CHO-K1 cells by transfection with cDNA encoding alanine scanning mutants in pcDNA3 (Invitrogen, Carlsbad, CA) using lipofectamine (GIBCO, Rockville, MD) and selection in neomycin (G418 500 μg/ml; GIBCO). Resistant cells were incubated with mAb 227R (ICOS, Seattle, WA) or, for mutant D11A, 531 (clone RD45531; R&D, Minneapolis, MN) and transfectants expressing

high levels of each mutant of CCR5 were magnetically sorted with paramagnetic beads and a MiniMACS system (Miltenyi Biotec, Auburn, CA).

The expression of CCR5 on the surface of each transfectant was measured by flow cytometry. The cells were sequentially incubated for 45 minutes at 4 °C with saturating concentrations of the primary mAb (227R or 531 for mutant D11A) and of the phycoerythrin-labeled goat F(ab')2 anti-mouse IgG (Jackson Immunoresearch Laboratory, West Grove, PA). After being washed twice, the cells were analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA) using the same instrument settings for every mutant. MAbs 2D7 and 5C7[12,33] (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health), specific for ECL2 and N-ter of CCR5, respectively, were also used for competitive binding experiments.

Radiolabeling of vMIP-II

vMIP-II was produced as a recombinant soluble protein in *Pichia pastoris*. The protein secreted in the supernatant was purified by cation exchange and reversed phase HPLC. The purified vMIP-II was iodinated on tyrosine residues with ^{125}INa using chloramine T. Briefly, 1.25 μg (0.15 nmol) of vMIP-II was diluted in 7.5 μl of 100 mM sodium phosphate buffer (pH 7.0). Following addition of 5 μl of ^{125}INa (500 μCi, IMS30; Amersham, Piscataway, NJ), the labeling reaction was initiated by addition of 1.25 μl of chloramine T (1 mg/ml in water) (Sigma, St Louis, MO) as oxidative agent. The reaction was stopped after 90 seconds with 3.25 μl of sodium metabisulfite (2 mg/ml in water) and quenched with 3.25 μl of KI (10 mM in water). The reaction volume was brought to 500 μl with PBS containing 1 mg/ml BSA and the radiolabeled protein was separated from the free ^{125}I by size exclusion chromatography using a PD10 column (Pharmacia, Piscataway, NJ) equilibrated with PBS supplemented with 1 mg/ml BSA. Fractions of 500 μl were collected, the fraction containing the elution peak was adjusted to 10 mg/ml BSA and aliquots were frozen at −20 °C. The specific activity of [^{125}I]vMIP-II was consistently between 2000 and 2400 Ci/mmol and free ^{125}I was always less than 1 %, as assessed by protein precipitation with trichloroacetic acid.

Ligand binding

Radiolabeled MIP-1α and RANTES were purchased from Amersham. CHO transfectants were harvested by treatment with trypsin-EDTA, allowed to recover in complete growth medium (MEM-α, 100 μ/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 10 % (v/v)) for four to five hours and then washed in cold binding buffer (PBS containing 2 mg/ml BSA). For ligand binding, the cells were resuspended in binding buffer at 1×10^7 cells/ml, and 100 μl aliquots were incubated with 0.1 nM of the radioactive ligands for two hours on ice under constant agitation. Free and bound radioactivity were separated by centrifugation of the cells through an oil cushion and bound radioactivity was measured with a gamma-counter (Cobra, Packard, Downers Grove, IL). For each mutant and the wild-type receptor, the non-specific binding was obtained by displacing each radioligand with an excess of 200 nM of MIP-1α (a generous gift from British Biotech Pharmaceuticals Ltd, Oxford, UK) (for [^{125}I]MIP-1α) or vMIP-II (for [^{125}I]RANTES or [^{125}I]vMIP-II). That value was subtracted from the total count to obtain the specific binding.

Competitive binding between chemokines and mAbs to CCR5

In each of these experiments, the competitors were used in large molar excess to the measured ligands. The mAbs used in these experiments include 5C7 (proximal N-ter, including D2), 227R (N-ter, residues D11 to T16), 2D7 (proximal ECL2, residues K171 and E172) and 531 (distal ECL2, residues Y184 to F189).[12,16,27,33] For the displacement of radiolabeled chemokines by mAbs directed to CCR5, 1×10^6 cells were incubated with 125 nM of each of the mAbs for 30 minutes on ice, then 0.1 nM of ^{125}I-labeled chemokines was added and incubated for another two hours on ice. Cells were separated from the free ligand and the radioactivity bound to the cells was counted. For the displacement of the antibodies by chemokines, 1×10^6 cells in 100 μl of PBS-BSA were incubated with 500 nM of each of the chemokines for 30 minutes, then non-saturating concentrations of each antibody (1 μg/ml or 6 nM for 5C7, 227R and 2D7 and 5 μg/ml or 30 nM for 531) were added for another hour. The antibodies bound to the cells membrane were then detected by a PE-labeled secondary antibody and the cells were analyzed by (FACS).

Signal transduction

For calcium flux experiments, cells were harvested and allowed to recover as described earlier. Then the cells were washed in PBS containing 1 mg/ml BSA at room temperature and resuspended at 3×10^6/ml in PBS containing 10 mg/ml BSA. Fura-2 AM dissolved at 2 mg/ml in 80 % (v/v) dimethyl sulfoxide/20 % (v/v) Pluronic F-127 (Molecular Probes, Eugene, OR) was added to a final concentration of 2 μg/ml and cells were loaded for 30 minutes at 37 °C in the presence of 4 mM probenecid (Sigma). Then cells were washed twice in PBS-BSA and finally resuspended at 1×10^6/ml in Hank's Balanced Salt Solution (HBSS; GIBCO). Aliquots (300 μl) were loaded in a microcuvette and the response of the cells at 37 °C after exposure to MIP-1α or RANTES (PeproTech, Rocky Hill, NJ) was recorded by a spectrofluorometer (F2500; Hitachi, San Jose, CA) using excitation wavelengths of 340 nm and 380 nm and monitoring the fluorescence at 510 nm. Cell responsiveness was assessed by stimulating the endogenous serotonin receptor expressed by CHO cells with 10 μM of 5-HT (Sigma).

Analysis of coreceptor activity of CCR5 chimeras

A panel of 21 mouse/human CCR5 chimeras was generated by random chimeragenesis using a strategy previously described.[35] Fusogenic activity of CCR5 chimeras was analyzed using a luciferase-based reporter gene assay in the Japanese quail fibrosarcoma cell line, QT6.[18,35] Briefly, target QT6 cells were cotransfected by calcium phosphate precipitation with vectors encoding CD4, CCR5 or CCR5 chimeras, and luciferase under the transcriptional control of a T7 promotor. Effector cells were prepared by infecting QT6 cells with recombinant vaccinia viruses that directed expression of JR-FL ENV on the cell surface, and T7 polymerase in the cytoplasm (vTF1.1). Eighteen hours posttransfection, effector cells were mixed with target cells for eight hours. Following

the eight hour incubation period, the medium was aspirated, and detergent lysates were assayed for luciferase activity with a LucLite Luciferase Reporter Gene Assay Kit (Packard) using a Top Count Luminometer (Packard). Inhibition of CCR5 M-tropic coreceptor activity by chemokines in the fusion assay was performed by preincubating chemokines at various concentrations with target cells for 30 minutes at 37 °C prior to the addition of effector cells.

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