Naturally Occurring CCR5 Extracellular and Transmembrane Domain Variants Affect HIV-1 Co-receptor and Ligand Binding Function*

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Analysis of CCR5 variants in human immunodeficiency virus, type 1 (HIV-1), high risk cohorts led to the identification of multiple single amino acid substitutions in the amino-terminal third of the HIV-1 co-receptor CCR5 suggesting the possibility of protective and permissive genotypes; unfortunately, the low frequency of these mutations did not led to correlation with function. Therefore, we used analytical methods to assess the functional and structural significance of six of these variant receptors in vitro. These studies showed three categories of effects on CCR5 function. 1) Mutations in the first extracellular domain of CCR5 severely reduce specific ligand binding and chemokine-induced chemotaxis. 2) An extracellular domain variant, A298S, when co-expressed with CD4, supported HIV-1 infection whereas the others do not. 3) The transmembrane region variants of CCR5 support monotropic HIV-1 infection that is blocked by addition of some receptor agonists. Mutations in the first and second transmembrane domains increase RANTES (regulated on activation normal T-cell expressed) binding affinity but did not affect MIP1β binding affinity presumably based on differences in ligand-receptor interaction sites. Furthermore, the CCR5 transmembrane mutants do not respond to RANTES with the classical bell-shaped chemotactic response curve, suggesting that they are resistant to RANTES-induced desensitization. These data demonstrate that single amino acid changes in the extracellular domains of CCR5 can have profound effects on both HIV-1 co-receptor and specific ligand-induced functions, whereas mutations in the transmembrane domain only affect the response to chemokine ligands.

Chemokine receptors are a subclass of seven transmembrane G-protein-coupled receptors, a number of which act as co-receptors with CD4 for HIV infection (1–3). Virus infection begins with the binding of viral gp120 envelope protein to cellular CD4, initiating a conformational change. A segment of gp120 V3 loop subsequently interacts with the amino-terminal domains of the targeted chemokine co-receptor. This interaction supports the formation of a fusogenic complex between gp41 and cellular components (4). As the number of characterized chemokine receptors increases so does the number of HIV co-receptors, currently including CXCR4, CCR2b, CCR3, CCR5, CCR8, ecr9, and AXCR3 (5–9). HIV infection progresses through stages with different co-receptors being predominantly used during various stages of the infection (10, 11). HIV-1 strains that utilize the CCR5 co-receptor predominate during the initial infection. Later, the disease involves primarily lymphotropic HIV-1 strains that utilize CXCR4 as a co-receptor. The endogenous chemokine receptor ligands inhibit HIV-1 entry by blocking the formation of the fusigenic pore.

Data supporting an essential role for the chemokine receptors in HIV pathology comes from population genetic studies that have shown variants of CCR2 and CCR5 genes to reduce HIV susceptibility and/or progression to AIDS (12–15). One modification of CCR5 is a deletion in the coding region of CCR5 that leads to a truncated nonfunctional receptor, the delta 32 mutant (CCR5Δ32), protects against infection in homozygous individuals, and delays onset of AIDS in heterozygous individuals (12, 16). Another variant, characterized by a single base pair change in the CCR5 promoter, correlates with delayed onset of AIDS. These studies indicate the in vivo importance of CCR5 in HIV pathogenesis.

Additional population analyses of CCR5 for genetic variants has identified several rare alleles (17). While these variants may affect the function of CCR5, population genetic analysis cannot tell us if these are likely to be protective changes. To evaluate their role in chemokine receptor function and HIV-1 entry, we expressed individual variants in human embryonic kidney cells (HEK-293). The first and second extracellular domains of CCR5 have been shown to participate in chemokine binding and HIV-1 fusion (18–20). Thus, we have focused our studies on six codon altering allelic variants located between amino acid residues 1 and 100 of CCR5. We have investigated not only the ability of these variants to support HIV-1 infection, but also the effect of these variations on chemokine binding and chemokine-induced cell migration. Furthermore, we tested the ability of chemokines and a chemokine co-receptor-specific inhibitor, NSC 651016, to block HIV-1 infection of cells expressing these CCR5 variants.

**EXPERIMENTAL PROCEDURES**

Unless otherwise stated all chemicals were purchased from Sigma. The distamycin analog NSC 651016 (2,2′[4,4′[laminocarboxyl]-amino]bis[N,4′-di(pyrrrole-2-carboxamide-1,1′-dimethyl]-6,8-naphthalene-sulfonic acid) hexasodium salt; FACs, fluorescence-activated cell sorting; m.o.i., multiplicity of infection.
lendsulfonyl acid (hexadesodium salt) was provided to the National Cancer Institute by Pharmacia Upjohn/Farmaitalia. The Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program Division of Cancer Treatment, National Cancer Institute, was the immediate source of the reagent used in this study. Chemokines were purchased from the National Institutes of Health cytokine repository.

Site-directed Mutagenesis—Plasmids that direct the expression of mutated species of CCR5 were generated by overlap PCR mutagenesis and subsequent fragment replacement. Primers were designed adjacent to the mutation site. The two fragments were extended and amplified as a single fragment and cloned into the pCRII vector. After sequence confirmation, the HindIII (21) fragment was cloned into pcDNA (In-vitrogen, Carlsbad, CA). All constructs were confirmed by DNA sequence analysis of the entire open reading frame of CCR5. The receptors were named based on the mutation and its position. Individual amino acids (the corresponding codon) and the amino acid position are listed here for each mutant as follows: I(ATC)12T(ACC), C(TCG)20S(AGC), A(GCA)/29S(CTA), I(ATC)42P(TTC), L(CTG)55Q(CAG), A(GCC)73V(GTC).

Cells—HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker, Walkersville, MD) containing 10% fetal bovine serum (HyClone, Logan, UT) and 2 mM glutamine and 100 units/ml penicillin and streptomycin (Quality Biologicals, Gaithersburg, MD). Parental HEK-293 cells were transfected with linearized CCR5 mammalian expression constructs by electroporation (21). After selection in media containing 400 mg/ml Geneticin (Life Technologies, Inc.) for 2 weeks, single cell cloning was performed. Three single cell clones were isolated for each mutant. HEK-CCR5 variant clones with similar receptor numbers, determined by binding assays or fluorescence-activated cell sorting (FACS), were chosen for additional analysis.

Fluorescence-activated Cell Sorting (FACS)—Monoclonal antibodies were a kind gift from Dr. Monica Tsang of R & D Systems (Minneapolis, MN). Monoclonal antibody clone 45549.111, which binds to a carboxy-terminal extracellular domain, was used in these studies. FACS analysis was performed as described previously (21).

Binding Studies—Binding assays were performed in triplicate by adding increasing amounts of unlabeled competitor and constant radiolabeled chemokine, 0.2 ng/assay (RANTES-NEX 292 and MIP1β-NEX 299, NEN Life Science Products), to individual 1.5-ml microcentrifuge tubes (22). 200 μl/samples of cells (2 × 10⁶ cells/ml) suspended in binding media (RPMI 1640, 1% bovine serum albumin, 5 mm HEPES, pH 8.0) were added, and cultures were continued for 24 h. The cells were lysed and genomic DNA isolated by the protease K/phenol/chloroform method (23). PCR was performed on 0.5 μg of DNA using the M661/M667 primer pair to identify late reverse transcription products (24). The PCR conditions were previously reported and shown to be semi-quantitative (24). Following PCR, each sample was ethanol-precipitated and quantitatively transferred to a 2% agarose gel. The amount of proviral gag DNA was visualized by ethidium bromide staining. The results were photographically documented (UVP, Image Store, Upland, CA) at a 1:1 ratio. DNA from uninfected CEM-SS cells was used for the PCR-negative control, and DNA from HIV-1-infected CEM-SS cells was used for the PCR-positive control. Densitometry was performed using NIH Image version 1.61 (NIH shareware).

RESULTS

Receptor Constructs and Surface Expression—Previously, Carrington et al. (17) identified 12 naturally occurring allelic mutations resulting in single amino acid changes in the CCR5 coding region. They also identified a single amino acid deletion mutant, 228delK (17). Three of the altered amino acids were near the putative HIV-1 binding domains located in the first extracellular domain. Three other variants, located in transmembrane domains, were selected for their potential to modify the topography of the extracellular domains (17). Positions of the mutations are illustrated in Fig. 1. While preparing these expression constructs, the original data were reviewed, and an error was noted in the original report. The amino acid change at position 12 is isoleucine to threonine (12T), not isoleucine to leucine (12L) as was originally reported.

The cell-surface expression of the mutant receptors was demonstrated using FACS analysis on intact live cells stained with monoclonal antibody 45549.111, which recognizes a native epitope on an extracellular CCR5 domain. The results, in Fig. 2, compare the receptor-transfected cells to untransfected parental cells (short dashed line) stained with the same antibody.
and the rogated or severely reduced chemokine binding. We did not that changes in the first extracellular domain completely ab-
second transmembrane domain mutant, A73V.

The increase in mean log fluorescence of cells transfected with wild type CCR5 over parental HEK-293 cells was 6.8-fold. Mutants I12T, C20S, and L55Q were expressed on the cell surface with an increase in mean log fluorescence of only 1.6–1.9-fold over parental cells. Mutants A29S, I42F, and A73V were expressed on the cell surface with an increase in mean log fluorescence of 2.0–2.5-fold over parental cells. Therefore, all the mutant receptors are expressed on the cell surface but at lower levels than the wild type receptor. Two monoclonal antibody clones, 45502.111 and 45531.111, did not recognize I12T or C20S clones. These antibodies have been shown to recognize an amino-terminal epitope (25).

Ligand Binding and Binding Sites—CCR5 binds a number of ligands including RANTES, MIP1α, MIP1β, and MCP-2 (26–28). These chemokines have similar binding affinities for CCR5 when expressed in HEK-293 cells (data not shown), but they are unlikely to bind to identical sites on CCR5 (28, 29). Because of their sequence and functional differences, we chose to determine the binding capabilities of RANTES and MIP1β to these CCR5 variants (30, 31). Data, summarized in Table I, indicate that changes in the first extracellular domain completely abrogated or severely reduced chemokine binding. We did not detect specific binding of RANTES or MIP1β to cells expressing I12T or C20S. The specific binding of RANTES to A29S cells was very weak (less than 200 cpm) and that of MIP1β was not statistically significant. The low level of RANTES-specific binding was not sufficient for analysis using the LIGAND program; therefore, estimation of $K_d$ was based on the average equilibrium constant at low ligand concentrations and a nonlinear fit to the data, using a constrained monovalent receptor concentration ($2 \times 10^4$ receptors/cell). This concentration was based on the FACScan profile of A29S in comparison to I42F and A73V. In contrast, mutation of either the first or second transmembrane domain did not alter the MIP1β binding affinity. Furthermore, RANTES affinity was enhanced at least 4-fold ($t$ test $p$ value of 0.05) for the first transmembrane domain mutants I42F and L55Q and 7.8-fold ($t$ test $p$ value of 0.05) for the second transmembrane domain mutant, A73V.

Chemotaxis—We investigated the effects of these CCR5 muta-
tions on chemokine-induced cell migration. Each CCR5 vari-

The cellular clones expressing transmembrane domain vari-
anties, A29S and A73V, that did not efficiently bind ligand did not transduce a chemotactic signal in response to RANTES, MIP1α, or MIP1β. A29S transfectants also failed to show a chemotactic response to MCP-2 (data not shown).

The I42F, L55Q, and A73V transfectants transduced a chemotactic response to RANTES, MIP1α, or MIP1β but did not exhibit the usual attenuation of the response at higher ligand concentrations. Compared with expressing the wild type receptor, which produce a bell-shaped response curve, the cells expressing these transmembrane variants reached a response plateau that did not decrease with increased ligand concentration. This is particularly true for the RANTES and MIP1α response curves, which did not change with increased ligand concentration, although the 1000 ng/ml dose was 100-fold greater than the concentration at which the wild type receptor response began to decrease. In contrast, the MIP1β response curves were bell-shaped, although the attenuating dose was 10-fold higher than the dose at which the wild type receptor response began to be attenuated. The threshold dose of chemotactic response was not shifted in the I42F, L55Q, and A73V receptor variants.

HIV-1 Ba-L Infection—We investigated the ability of these CCR5 variants to act as co-receptors with CD4 for HIV-1 infection. These results are summarized in Fig. 4. The parental HEK-293 cells express a small amount of CXCR4 which, when co-expressed with CD4, supports T-cell tropic virus infection at the very high multiplicities of infection (m.o.i.) used here. As shown in Fig. 4, all of the cellular clones were infected by the T-cell tropic virus HIV-1 Ba-L. These results indicate that the transfected cells express sufficient CD4 to be infected by HIV-1. Infection of HEK-293 cells by HIV-1 Ba-L requires co-expression of CD4 and CCR5. Since the extracellular domains of CCR5 are essential for HIV-1 infection, we expected that the variants that did not bind ligand or transmit a chemotactic signal would not function as co-receptors for HIV-1 Ba-L. This is what we observed for I12T and C20S. In contrast, even though the A29S CCR5 variant demonstrated marked functional impairment it was an effective co-receptor and supported HIV-1 Ba-L infection. The cellular clones expressing transmembrane domain variants, I42F, L55Q, and A73V, were also infected by HIV-1 Ba-L. Dextran sulfate, a charged polymer known to inhibit HIV-1 attachment to the cell, was used to show the degree of specific virus infection (gp120-CD4 mediated) in both Figs. 4 and 5. Thus, dextran sulfate-treated samples are representative of experimental background.

The ability of RANTES and MIP1β to block the HIV-1 Ba-L, infection of these CCR5 variant cells was tested, and these data are shown in Fig. 5. The relative band densities of the virus alone, virus plus RANTES, or virus plus MIP1β minus the density of the dextran sulfate band (nonreceptor mediated infection) are shown in Fig. 5B. Treatment of CCR5 wild type cells with 1 µg/ml (125 nm) of RANTES or MIP1β inhibited HIV-1 Ba-L, infection. RANTES (1 µg/ml) inhibited HIV-1 Ba-L, infection of L55Q and A73V but had little effect on the infection of A29S and I42F. In contrast, MIP1β (1 µg/ml) inhibited HIV-1 Ba-L, infection of A29S and I42F but had little effect on the infection of L55Q and A73V.

Previously we had shown that a ureido analogue of distamy-
cinc, NSC 651016, inhibited HIV-1 infection in vitro and in vivo (32). This compound inhibits both X4 and R5 tropic virus infection by blocking the HIV-1 fusion event (23). NSC 651016, at 10 µM, inhibited infection of the cells expressing any of the CCR5 variants and wild type CCR5, irrespective of the ability of exogenous chemokines to inhibit viral entry.
DISCUSSION

Population genetic studies have shown that CCR5 is an important component of AIDS pathology. Furthermore, individuals heterozygotic for CCR5<sup>D32</sup> have less inflammation and less severe disease associated with rheumatoid arthritis (33). In addition, mice with homologous deletion of functional CCR5 show reduced resistance to bacterial challenge and reduced T-cell function (34). These studies indicate that it is important to identify variations in the CCR5 receptor gene and characterize the resulting changes in receptor function. Earlier, Carrrington et al. (17) reported several rare allelic variants that might alter the function of the CCR5 receptor, but the sample size (700–5,000 depending on the cohort) was too small to study their epidemiological effect. Previous mutational analysis suggested that the amino terminus of CCR5 may be essen-

**TABLE I**

**Ligand binding properties of CCR5 variants**

Binding studies were performed as described under “Experimental Procedures” with at least three independent determinations for each variant. Scatchard analysis was performed using the LIGAND program except for variant A29S where additional least squares analysis was used.

| Mutant stably expressed in HEK-293 cells | RANTES<sup>1</sup><sup>2</sup><sup>3</sup> in nM ± S.E. | RANTES binding sites/cell ± S.E. | MIP1α<sup>4</sup> K<sub>d</sub> in nM ± S.E. | MIP1α binding sites/cell ± S.E. |
|------------------------------------------|---------------------|-------------------------------|--------------------------|-------------------------------|
| II2T                                     | None                | None                          | None                     | None                          |
| C20S                                     | –20–200             | 2 × 10<sup>4</sup>             | 2.2 ± 0.1 × 10<sup>4</sup> | None                          |
| A29S                                     | 0.57 ± 0.12         | 1.6 ± 0.4 × 10<sup>4</sup>     | 3.5 ± 0.69               | 3.0 ± 0.8 × 10<sup>4</sup>    |
| L55Q                                     | 0.62 ± 0.13         | 4.7 ± 1.7 × 10<sup>4</sup>     | 2.5 ± 0.86               | 3.5 ± 0.7 × 10<sup>4</sup>    |
| A73V                                     | 0.32 ± 0.08         | 2.1 ± 0.6 × 10<sup>4</sup>     | 1.5 ± 0.51               | 2.0 ± 0.7 × 10<sup>4</sup>    |
| Wild type                                 | 2.5 ± 0.85          | 2.4 ± 0.8 × 10<sup>5</sup>     | 3.9 ± 1.9                | 2.0 ± 0.7 × 10<sup>5</sup>    |

**FIG. 3.** RANTES, MIP1α, and MIP1β-induced chemotaxis of HEK-293 cells expressing CCR5 variants. Individual chemotaxis assays were performed in triplicate with three independent determinations. A representative experiment is shown for each variant. The CCR5 variant being tested is noted at the top of individual graphs. The binding medium control is shown in open bars at the far left of each graph (M). RANTES-induced chemotaxis is shown by gray bars. The MIP1α-induced chemotaxis is shown by black-hatched white bars, and the MIP1β-induced chemotaxis is shown by white-hatched black bars. The number of cells/high powered field is graphed on the y axis, and the chemokine concentration in ng/ml is graphed on the x axis.

**FIG. 4.** HIV-1 infection of CD4<sup>+</sup> + CXCR4/HEK or CD4<sup>+</sup> + CCR5 variant/HEK cells. CD4<sup>+</sup> + CXCR4/HEK and CD4<sup>+</sup> + wild-type CCR5/HEK cells were exposed to HIV-1<sub>RF</sub> virus stocks (m.o.i. of 0.1–1.0). CD4<sup>+</sup> + CCR5 variant/HEK cells were exposed to HIV-1<sub>Ba-L</sub> virus stocks (m.o.i. of 0.1–1.0). Proviral DNA was detected at 24 h by PCR amplification of 0.5 µg of cellular DNA using M661/M667 primers. Dextran sulfate (D.S.) is an inhibitor of HIV-1 gp120-CD4 binding and is used as a control for receptor-mediated HIV infection in these assays. The receptor variants are correspondingly labeled. Transfection followed by HIV-1 infection and detection by PCR was performed at least three times. The PCR negative (−) control was performed with DNA isolated from uninfected CEM-SS cells. The PCR positive (+) control was performed with DNA isolated from HIV-1<sub>RF</sub>-infected CEM-SS cells. The receptor name is shown above the photographed PCR bands, and the virus type and treatment is shown below. The presence of a PCR band indicates HIV-1 infection.

**TABLE I**

*Ligand binding properties of CCR5 variants*

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| Mutant stably expressed in HEK-293 cells | RANTES<sup>1</sup><sup>2</sup><sup>3</sup> in nM ± S.E. | RANTES binding sites/cell ± S.E. | MIP1α<sup>4</sup> K<sub>d</sub> in nM ± S.E. | MIP1α binding sites/cell ± S.E. |
|------------------------------------------|---------------------|-------------------------------|--------------------------|-------------------------------|
| II2T                                     | None                | None                          | None                     | None                          |
| C20S                                     | –20–200             | 2 × 10<sup>4</sup>             | 2.2 ± 0.1 × 10<sup>4</sup> | None                          |
| A29S                                     | 0.57 ± 0.12         | 1.6 ± 0.4 × 10<sup>4</sup>     | 3.5 ± 0.69               | 3.0 ± 0.8 × 10<sup>4</sup>    |
| L55Q                                     | 0.62 ± 0.13         | 4.7 ± 1.7 × 10<sup>4</sup>     | 2.5 ± 0.86               | 3.5 ± 0.7 × 10<sup>4</sup>    |
| A73V                                     | 0.32 ± 0.08         | 2.1 ± 0.6 × 10<sup>4</sup>     | 1.5 ± 0.51               | 2.0 ± 0.7 × 10<sup>4</sup>    |
| Wild type                                 | 2.5 ± 0.85          | 2.4 ± 0.8 × 10<sup>5</sup>     | 3.9 ± 1.9                | 2.0 ± 0.7 × 10<sup>5</sup>    |
the premise that natural variants might provide uniquely informative data beyond that already determined by domain swapping and alanine scanning studies.

The three amino-terminal variants, I12T, C20S, and A29S, are expressed on the cell surface but do not respond to ligand, most likely due to alteration of the ligand binding site. One of the allelic variants identified by Carrington et al. (17) encoded a change from isoleucine to threonine suggesting that this mutation could result in increased polarity of an already strongly polar domain. The isoleucine at amino acid position 12 is common to both mouse and human CCR5 but not other CC receptors. The functional change was dramatic; the I12T mutant did not bind ligand nor did it act as an HIV-1 co-receptor.

A previous study that removed the tyrosines at amino acid positions 10, 14, and 15 and replaced them with alanines showed that the polarity of this region is important for HIV-1 infection (18). Taken together, these data suggest that changes in the number of polar residues in the amino terminus of CCR5 would result in markedly altered receptor function. It is possible that the C20S variant resulted in the interruption of disulfide bond formation between the first and fourth extracellular domain, which has been predicted to be essential for receptor function (37). There is a cysteine at this position in all the characterized CC receptors (38). When Rabut et al. (18) replaced Cys-20 with alanine there was a striking decrease in HIV-1 co-receptor function. However, Hill et al. (25) failed to observe a similar effect. In our study, the C20S mutant did not bind chemokines nor did it act as an HIV-1 co-receptor, indicating that the cysteine at amino acid position 20 is necessary for receptor function. Analysis of data from AIDS patients suggests that C20S heterozygosity may delay progression to AIDS (17). Our data predict that the C20S allele may be protected against AIDS and, by inference from the CCR5A32 studies, may also reduce the severity of arthritis.

The A29S variant encoded a change from alanine to serine suggesting that this mutation could result in increased amino-terminal polarity. However, previous mutational analysis showed that amino acid changes neighboring Ala-29 did not greatly affect HIV-1 co-receptor activity (18, 38). Since A29S was infected by HIV-1Ba-L, our results were consistent with these earlier findings. However, the minimal specific binding of CCR5 ligands to this mutant receptor was unexpected. Despite being unable to induce A29S-mediated chemotaxis, MIP1β nevertheless blocked HIV-1 infection (Fig. 5). In contrast, RANTES had no effect of the HIV-1 infection of A29S. A29S has HIV-1 co-receptor activity but reduced interaction with CCR5 ligands, suggesting that HIV-1 entry might not be effectively blocked by chemokines. Therefore, A29S is likely to be HIV-1 permissive and could be associated with more rapid progression to AIDS.

These observations appear contradictory until the nature of the interaction between chemokines and their receptors is considered. Pakianathan et al. (39) showed that RANTES interacts differently with each of its receptors. The epitopes on RANTES that bind individual receptors are overlapping but distinct. Although the tertiary structures of the CC chemokine are quite similar, the primary amino acid sequences are not, suggesting that the interaction between individual chemokines and specific receptors may be distinct. Data derived from mutational analysis of CC receptors suggest that the second and third extracellular domains, in addition to the first extracellular domain, may participate in ligand function and HIV-1 entry (28, 36). Alkhateeb et al. (36) showed that the third extracellular domain of CCR3 was essential for cellular fusion with monocryptic HIV-1. Although both RANTES and eotaxin are established ligands for CCR3, only eotaxin efficiently blocked CCR3
co-receptor activity, whereas RANTES only partly blocked CCR3 co-receptor activity. These data show that inhibition of HIV-1 infection and receptor binding can be uncoupled. Studies of CCR2b-CCR5 chimeras demonstrate that ligand binding, HIV-1 co-receptor activity, and receptor-mediated functional responses are not necessarily located in the same domains (19, 28). A chimeric receptor, 2255 (composed of the amino terminus of CCR2 through the second extracellular domain linked to the carboxyl-terminal half of CCR5), had no HIV-1 co-receptor activity, but MIP1β could activate inositol phosphate release by this receptor (19). Ligand binding assays were not performed in this study (19). However, another group studying CCR2b and CCR5 chimeras showed that 2255 bound MIP1α, although at a 12-fold lower affinity than wild type receptor (28). These data indicate that the HIV-1 co-receptor activity is located in the amino-terminal half of CCR5 and that a functional ligand interaction site is located in the carboxyl-terminal half of CCR5. In addition, analysis of a 5525 chimera showed no specific MIP1α or MCP-1 binding. Unfortunately, no MIP1β binding assays were performed in this study. Neither MIP1α nor RANTES induced increased metabolic activity of the 5525 chimera. However, MIP1β and MCP-1 induced metabolic activity in the 5525 chimera-transfected cells by 10 and 40%, respectively, demonstrating that detectable specific ligand binding and functional responses are not necessarily linked to each other. These studies are strengthened by our observations, suggesting that MIP1β blocks HIV-1 by interacting with A29S at a co-receptor activity domain distinct from both specific ligand binding and functional response domains. Furthermore, RANTES does not appear to share this site. Our results are consistent with the earlier observation that HIV-1 infection occurs independently of co-receptor activation (40). These data support a model of distinct HIV-1/CCR5 and chemokine/CCR5 interaction sites and further suggest that the MIP1β/CCR5 and RANTES/CCR5 interaction sites are also distinct from each other.

We observed changes in chemokine binding and chemokine-induced chemotaxis in the three transmembrane variants. These variants had a 4–7.8-fold greater affinity for RANTES than the wild type receptor. The seemingly smallest change, A73V, had the greatest effect on ligand binding. The chemotaxtractant signal transduced by the transmembrane variants was also modified. Seven transmembrane G-protein-coupled receptors typically respond to increasing concentrations of ligand by first increasing the response and then attenuating the response, yielding a bell-shaped response curve. These variants do not induce the typical response in the HEK-293 cells, rather the response remains elevated. The decrease in response at higher ligand concentrations is presumably based on homologous desensitization. Although our data do not directly measure desensitization, it is interesting to consider the factors that might participate in desensitization. Although several groups have examined this phenomenon, the essential signaling components responsible for desensitization have not been completely identified (41–43). Phosphorylation of the chemokine receptor carboxyl terminus, which is indicative of desensitization, was not found to be necessarily associated with either decreased G-protein-mediated signal or receptor internalization (43). The agonist-dependent phosphorylation of homologous desensitization is likely to be regulated by G-protein receptor kinases (44, 45). Whether G-protein receptor kinases phosphorylate the receptor and/or some component of the receptor endocytosis pathway remains to be determined. Our study indicates that single amino acid changes in the transmembrane domains can result in alteration of the receptor activity profile, presumably based on reduced receptor phosphorylation.

Analysis of the transmembrane variants, I42F, L55Q, and A73V, susceptibility to HIV-1 infection, revealed that they have co-receptor function (Fig. 4). However, even though the RANTES binding affinity was increased for I42F, RANTES did not efficiently block HIV-1 infection of I42F-expressing cells (Fig. 5, A and B). Furthermore, although the binding affinity of L55Q and A73V for MIP1β was not significantly changed, inhibition of HIV-1 infection by MIP1β was reduced. This again supports the conclusion that ligand binding and HIV-1 co-receptor activity are not necessarily linked. These data show that alteration of the first and second transmembrane domains did not prevent the HIV-1 fusion event but decreased the ability of ligands to inhibit HIV-1 infection, suggesting that these would also be HIV-permissive variants resulting in a potentially rapid progression to AIDS. The transmembrane domains of the chemokine receptors are not thought to interact directly with ligands; however, based on our data it is not unreasonable to suggest that modification of transmembrane domain amino acids would alter the membrane position and interaction of receptor domains. It is tempting to speculate that altered membrane position and receptor domain interaction could result in changed ligand affinity, like that observed for RANTES. Furthermore, these same receptor modifications could alter the ability of individual ligands to sterically block the HIV-1 fusion event depending upon location of ligand interaction sites on specific receptor domains. Proof of these suppositions requires that the tertiary structures of individual chemokine receptors be determined.

Additionally, we compared the in vitro antiviral efficacy of a small molecule negative agonist, NSC 651016, to that of the chemokines on these CCR5 variants. Previously we have reported that NSC 651016 inhibited HIV-1 infection in vitro and in vivo by blocking, at a minimum, the second extracellular domain of the chemokine co-receptors (23, 32). In the studies presented here, we show that there are variants of CCR5 that support HIV-1 infection but do not efficiently bind natural ligands. Therefore, HIV-1 infection of these variants is not likely to be effectively inhibited by natural ligands. In case of A29S or I42F, one would predict that ligand-based anti-virals such as AOP-RANTES and Met-RANTES would not block HIV-1 infection (46, 47). In contrast, HIV-1 infection of cells expressing these CCR5 variants was efficiently blocked by NSC 651016 regardless of ligand responsiveness. This suggests that NSC 651016 and other small molecule inhibitors of HIV-1 infection might provide a broader spectrum of antiviral therapeutics than ligands.

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