Multiple residues in the extracellular domains of CCR3 are critical for coreceptor activity

Phong T. Ho\textsuperscript{a,1}, Benjamin E. Teal\textsuperscript{b,2}, Ted M. Ross\textsuperscript{c,*}

\textsuperscript{a}Department of Biology, East Carolina University, Greenville, NC 27834, USA
\textsuperscript{b}Department of Microbiology and Immunology, East Carolina University, School of Medicine, Greenville, NC 27834, USA
\textsuperscript{c}Department of Medicine, Division of Infectious Diseases, University of Pittsburgh, School of Medicine, Pittsburgh, PA 15261, USA

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Abstract

Human immunodeficiency virus type 1 (HIV-1) binds to the human CD4 (hCD4) and a coreceptor to enter permissive human cells. The chemokine receptors, hCCR5 and hCXCR4, are the primary coreceptors used by HIV-1 isolates in vivo, however, hCCR3 has been implicated as a coreceptor for HIV infection of the central nervous system. To determine the domains and amino acids important in hCCR3 coreceptor activity, chimeras between the permissive hCCR3 and the non-permissive rhesus macaque CCR3 (RhCCR3) were constructed and assessed for coreceptor activity for two R5 strains of HIV-1 (YU-2 and ADA) and one R5X4 strain (89.6). Even though three extracellular domains of CCR3 participated in coreceptor activity for the two R5 isolates (ECD-1, ECD-3, and ECD-4), for the R5X4 isolate, ECD-4, and to a lesser extent ECD-3, were critical for coreceptor activity. In addition, residues 13 and 20 in ECD-1, residue 179 in ECD-3, and residue in 271 in ECD-4 of CCR3 were identified for HIV-1 envelope-mediated entry for R5 isolates. In contrast, all the residues on ECD-4 appeared necessary for coreceptor activity for HIV-1\textsubscript{89.6}. Therefore, multiple residues on multiple extracellular domains of hCCR3 are important for coreceptor activity for HIV-1.

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Introduction

Although human CD4 (hCD4) is the primary receptor used by the human immunodeficiency virus type 1 (HIV-1) for infection, it is not sufficient to mediate viral entry into human cells (Ross et al., 1999). The chemokine receptor, human CXCR4 (hCXCR4), was identified as a coreceptor with hCD4 as necessary for HIV-1 entry into susceptible cells (Bleul et al., 1996; Feng et al., 1996; Oberlin et al., 1996). However, hCXCR4 mediates infection only for isolates with a specific tropism for T cells and T-cell lines, known as T-tropic or X4 isolates. The human CCR5 (hCCR5) mediates entry for isolates with specific tropism for macrophages, known as M-tropic or R5 isolates (Alkhatib et al., 1997; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). hCCR5 and/or hCXCR4 support virus entry for the vast majority of primary HIV-1 isolates (Bjornsdal et al., 1997; Connor et al., 1997; Zhang et al., 1996). However, other chemokine receptors such as CCR2, CCR3, CCR8, CCR9, or CX3CR1, as well as several orphan receptors (such as STRL33/BONZO, GPR1, GPR15/BOB and APJ) function as coreceptors for HIV-1 infection (reviewed in Philpot, 2003;
Ross et al., 1999). Each of these alternative coreceptors for HIV-1 can function to some extent in vitro for virus entry or envelope-mediated cell–cell fusion. It is yet unclear, however, how these receptors function in vivo and what impact these receptors have on the pathogenesis associated with HIV-1 infection. Potentially, the use of coreceptors other than hCCR5 or hCXCR4 could enable virus strains to infect a wider spectrum of cell types, including cells in the central nervous system, thymus, and mucosa.

The human chemokine receptor, CCR3 (hCCR3), was shown to serve as a coreceptor for HIV-1 binding and entry into target cells (Choe et al., 1996; He et al., 1997; Rana et al., 1997). hCCR3 is expressed predominately on eosinophils, microglial cells, basophils, and a subset of T cells (Berger et al., 1999; Philpot, 2003). This chemokine receptor has been implicated in HIV-1 infection of cells in the central nervous system (CNS) (reviewed in (Gorry et al., 2003; Martin-Garcia et al., 2002). Neurotropism by HIV-1 can cause dementia and other neurological disorders in AIDS patients (Lipton and Gendelman, 1996; Siliciano, 1999). However, only 20–30% of HIV-1-infected individuals develop AIDS dementia and the underlying causes of this complex are unclear (Gorry et al., 2003; Martin-Garcia et al., 2002). Unique to AIDS dementia, compared to other causes of encephalitis, neuronal injury is not a direct result of viral infection of neurons, but as a consequence of indirect mechanisms (Gendelman et al., 1997; Genis et al., 1992; Lipton, 1997; McArthur et al., 2003). The hCCR5 is the major M-tropic coreceptor for HIV-1, not only of macrophages in the peripheral immune system, but also for those viruses isolated in the brain (Albright et al., 1999; Choe et al., 1996; He et al., 2003; Li et al., 1999; Shieh et al., 1998; Smit et al., 2001). Both hCCR5 and hCCR3 are expressed on resident macrophages in the CNS, the microglia (He et al., 2003). In some patients, disease progression is associated with a broadening of HIV-1 tropism as a result of expanded coreceptor usage (Connor et al., 1997).

Recently, Gorry et al. (2001) demonstrated that M-tropism, regardless of coreceptor usage, is directly associated with HIV-1 infection of the brain and therefore M-tropism, rather than hCCR5 usage, predicts HIV-1 microglia infection and neurotropism.

Compared to hCCR5 and hCXCR4, the domains and amino acid residues critical for HIV-1 envelope binding and entry into cells via hCCR3 are not as well understood. Even though hCCR3 efficiently serves as a coreceptor for HIV-1 M-tropic and dual tropic isolates, the rhesus macaque homologue (RhCCR3) does not efficiently serve as a coreceptor (Sol et al., 1998). In this report, chimeras between the permissive hCCR3 and the non-permissive RhCCR3 were constructed and assayed for coreceptor activity using an R5 and R5X4 molecular clone. In addition, point mutations were engineered into the chimeras to determine important residues in the four extracellular domains (ECD) of hCCR3 necessary to mediate infection.

Results

Construction of chimeric human and rhesus macaque CCR3

This study was designed to identify regions in each of the four extracellular domains of CCR3 critical for coreceptor activity. The human CCR3, in conjunction with the hCD4, mediates HIV-1 entry into human cells (Albright et al., 1999; Choe et al., 1996; Park et al., 1999; Sol et al., 1998). In contrast, cells expressing the rhesus macaque homologue of CCR3 alone or in conjunction with hCD4 does not mediate viral entry (Sol et al., 1998). Ninety-two percent of the amino acids are identical between human and rhesus macaque CCR3. Seventeen of the 102 amino acids (17%) on the four extracellular domains, which are mostly likely necessary to mediate HIV-1 envelope binding and fusion, differ between the two CCR3 molecules (Fig. 1A). There are six residues that differ between hCCR3 and RhCCR3 in the first extracellular domain, three in the second, five in the third, and three in the fourth. Therefore, to determine the amino acids critical for coreceptor activity by CCR3, three sets of 14 chimeric hCCR3/RhCCR3 genes were constructed using unique restriction enzyme sites (Fig. 1B) and tested for the ability to mediate HIV-1 entry. Each chimeric CCR3 receptor was efficiently expressed on the surface of cells following transient transfection with plasmid DNA (Table 1).

Single extracellular domains from hCCR3 can confer HIV-1 coreceptor activity to RhCCR3

To determine coreceptor activity of each chimeric CCR3, different R5 and R5X4 isolates of HIV-1 were used in a single round infection assay (Ross and Cullen, 1998; Ross et al., 1998). The hCCR3 efficiently mediated viral entry, in conjunction with hCD4, with similar efficiency as hCCR5 for all the isolates tested (Fig. 2). In contrast, RhCCR3 does not serve as a coreceptor for any of the viruses (Fig. 2). The R5 isolates infected 293T cells expressing the chimeric hCCR3/RhCCR3 with the first (HRRR), third (RRHR), and fourth (RRRH) extracellular domain of hCCR3 expressed in the RhCCR3 backbone (Figs. 2A–B). For each of two R5 isolates, coreceptor activity was less efficient using RRRH compared to HRRR or RRHR. Cells expressing receptors with the second extracellular domain (RRHR) from human CCR3 were not able to mediate viral entry and infection, even though all receptors were expressed at similar levels (Table 1). Interestingly, R5X4 isolate, HIV-1_{89,6}, was able to efficiently infect cells expressing only the RRRH in conjunction with hCD4 cells (Fig. 2C). Therefore, the first, third, and to a lesser extent, the fourth extracellular domains of hCCR3 are sufficient to mediate HIV-1 infection for the R5-tropic viruses when inserted into an otherwise non-permissive RhCCR3 context. However, only the fourth domain of hCCR3 confers coreceptor activity to RhCCR3 for HIV-1_{89,6}.
Fig. 1. Panel A: primary amino acid sequences of the human and rhesus macaque CCR3. The top line represents the hCCR3 amino acid sequence and the bottom line represents the RhCCR3 amino acid sequence. Letters in bold represent the amino acids in each of the four extracellular domains of CCR3. The underlined letters indicate amino acid differences between human and rhesus macaque CCR3. Panel B: schematic of human–rhesus macaque CCR3 chimeras. The CCR3 is composed of four extracellular domains (I–IV), seven transmembrane domains, and four intracellular domains. The white circles denote amino acid sequence differences between human and rhesus macaque CCR3.
Single extracellular domains from RhCCR3 can knockout HIV-1 coreceptor activity of hCCR3

To determine if the introduction of rhesus macaque CCR3 extracellular domains into the permissive hCCR3 receptor would knockout coreceptor function, a second set of chimeric receptors was constructed to express a single domain of RhCCR3 in the hCCR3 backbone. Cells expressing hCD4 and the chimeric receptors, RHHH, HRHH, and HHHR, each served as a coreceptor for the R5 isolates. Once again, a substitution of the fourth ECD resulted in slightly lower levels of activity (Figs. 3A–B). In addition, cells expressing the third extracellular domain of RhCCR3 in hCCR3 (HHRH) did not mediate infection by R5 isolates. In contrast, only cells expressing the first ECD of RhCCR3 (RHHH) could efficiently serve as a coreceptor for HIV-189.6 (Fig. 3C). HRHH, in conjunction with hCD4,

Table 1
Expression of cell surface coreceptors

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Mean fluorescence</th>
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<tr>
<td>hCCR3</td>
<td>139.82 ± 1.5</td>
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<tr>
<td>RhCCR3</td>
<td>134.76 ± 8.5</td>
</tr>
<tr>
<td>HRRH</td>
<td>139.93 ± 0.2</td>
</tr>
<tr>
<td>RHRR</td>
<td>112.27 ± 9.1</td>
</tr>
<tr>
<td>RRRH</td>
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<tr>
<td>RHHH</td>
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<tr>
<td>HRHH</td>
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<tr>
<td>HHHR</td>
<td>140.05 ± 3.6</td>
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<tr>
<td>HHHR</td>
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<td>HHRH</td>
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<tr>
<td>HHRR</td>
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<tr>
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<tr>
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<tr>
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<td>E189Q</td>
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<td>S263T</td>
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<tr>
<td>I267V</td>
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<tr>
<td>N271L</td>
<td>136.32 ± 2.4</td>
</tr>
<tr>
<td>Vector</td>
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</tr>
</tbody>
</table>

Fig. 2. Analysis of the levels of HIV-1 coreceptor activities displayed by chimeric CCR3 expressing one human extracellular domain in a rhesus macaque CCR3 backbone. All cells expressed hCD4 and one of the CCR3 molecules. Coreceptor activity was normalized to 100% for hCCR3. Data are represented as the average of three independent experiments with standard deviations. Panel A: coreceptor activity was assayed for HIV-1YU-2. Panel B: coreceptor activity was assayed for HIV-1ADA. Panel C: coreceptor activity was assayed for HIV-189.6.
yielded approximately 50% of the activity of the activity as wild type hCCR3 for HIV-189.6 (Fig. 3C). Therefore, the presence of the third and fourth ECDs of hCCR3 is critical for coreceptor activity for all the isolates.

Combination of any two extracellular domains confers CCR3 coreceptor activity for the R5-tropic viruses, but not for the R5X4-tropic isolate

The next set of chimeras (HHRR, RRHH, HRHR, HRRH, RHHR, RHRH) each contained two extracellular domains from hCCR3 and two from RhCCR3. 293T cells expressing any of these chimeric CCR3s served as a coreceptor for R5 isolates (Fig. 4). However, only the RRHH receptor efficiently mediated HIV-189.6 entry (Fig. 4). Thus, the third and fourth extracellular domains once again appear necessary and sufficient to confer coreceptor activity for HIV-189.6.

Mutational analysis of hCCR3/RhCCR3 chimeric receptors

Using an approach that our laboratory and others have used to map residues in hCCR5 important for coreceptor activity (Bieniasz et al., 1997; Chabot and Broder, 2000; Lu et al., 1997; Picard et al., 1997; Ross et al., 1998; Sol et al., 1998), a series of point mutations were engineered into the hCCR3/RhCCR3 chimeric receptors to determine which residues are critical for activity. Mutations were introduced in the chimeras expressing a single human extracellular domain in the RhCCR3 backbone. Each mutation was chosen to substitute the amino acid residue located at the same position in the RhCCR3 domain. For example, there are six amino acids and in the first extracellular domain of hCCR3 that differ with the RhCCR3 (Fig. 1A). In the HRRR chimeric receptor, the six different amino acids were individually mutated to the corresponding amino acid in the rhesus macaque CCR3 sequence (T13P, Y17D, V20M, T29V, R30G, M33I). Cells expressing the T13P or the V20M mutation completely ablated coreceptor activity in the HRRR chimeric CCR3 for both HIV-1ADA and HIV-1YU-2 (Table 2). HRRR receptors containing the other four mutations had coreceptor activity similar to HRRR and wild-type hCCR3.

The five non-homologous amino acids in the third domain of the RRHR chimeric receptor were individually mutated to the corresponding amino acid in the rhesus macaque CCR3 sequence (E173G, E176K, E179P, L186I, E189Q). Each R5 isolate infected cells expressing hCD4 and any of the mutant chimeric RRHR receptors except, E179P (Table 2). Lastly, cells expressing mutated chimeric
RRRH (S263T, I267V, N271L) were analyzed for coreceptor activity (Table 2). Cells expressing the N271L mutation did not confer coreceptor activity in RRRH for the R5 isolates (Table 2). In contrast, the S263T and I267V mutations had no effect on the ability of the RRRH to mediate entry by the R5 strains.

The HIV-189.6 isolate was also tested for the ability to use the RRRH chimera because this was the only receptor of the set that was able to confer coreceptor activity in the RhCCR3 backbone for the dual-tropic isolate (Fig. 2C). Interestingly, all three mutations knocked out the coreceptor activity of RRRH (Table 2). Once again these results indicate that the HIV-189.6 is more sensitive to changes in hCCR3 than the R5-tropic isolates.

**Discussion**

In vivo, hCCR5 and hCXCR4 are the predominant coreceptors that interact with the HIV-1 envelope, resulting in conformational changes in Env, which lead to fusion of the viral and cell membranes and thus entry of the virus into the cytoplasm (Ross et al., 1999). Several members of the chemokine receptor family can mediate coreceptor activity for HIV-1 as demonstrated by in vitro assays (Ross et al., 1999). However, their roles in HIV-1 pathogenesis in vivo remain controversial. To better define the regions on hCCR3...
necessary for HIV-1 infection, a total of 14 chimeras between hCCR3 and the non-permissive RhCCR3 were constructed (Fig. 1) and tested for coreceptor activity. Despite that, RhCCR3 and hCCR3 have 327 identical amino acids (92%) out of a total of 355, RhCCR3 was unable to mediate HIV-1 infection (Fig. 3 and (Sol et al., 1998)). The lack of RhCCR3 coreceptor activity cannot be explained by a lower cell surface expression compared to hCCR3 (Table 1).

Any chimeric CCR3 that contained two human CCR3 ECDs served as an efficient coreceptor for HIV-1-YU-2 or HIV-1ADA (Fig. 4). These M-tropic isolates were able to interact functionally with CCR3 receptors expressing the first, third, and fourth human ECD in the RhCCR3 backbone (HRRR, RRHR, RRRH) (Fig. 2), as well as RhCCR3-based receptors containing the first, second, and fourth ECD of hCCR3 (RHHH, HRHH, HHHR) (Fig. 3). In both cases, the substitution of the fourth ECD yielded activity for all three isolates, albeit at lower levels than the other active chimeras. These envelopes interacted with the extracellular domains of hCCR3 similar to the interactions with the ECDs of hCCR5 (Atchison et al., 1996; Biemiasz et al., 1997; Kuhmann et al., 1997; Picard et al., 1997; Ross et al., 1997; Speck et al., 1997).

Interestingly, the R5X4 isolate, HIV-1$_{89.6}$, was more sensitive to changes in the CCR3 extracellular domains than the R5 viruses. The third and fourth ECDs of CCR3, together or independently, were required to confer coreceptor activity for the R5X4 strain (Fig. 4). These results confirm and extend previous findings (Alkhatib et al., 1997; Sol et al., 1998). In addition, using a different set of chimeras (hCXCR4/hCXCR2), Lu et al. (1997) demonstrated that, for HIV-1$_{89.6}$, ECD-4, and to a lesser extent ECD-3, of hCXCR4 are essential for coreceptor activity. The sensitivity of this envelope to changes in coreceptor domains may be due, in part, to constraints on its flexibility imposed by its ability to use both hCXCR4 and hCCR5.

Curiously, there were some inconsistencies that demonstrated the complexity of the interactions between the various extracellular domains. For example, both hCCR3 (HHHH) and HRRH efficiently served as coreceptors for both R5 isolates (Table 2), however, the HHRH receptor had significantly impaired coreceptor activity (Fig. 3). In contrast, the RRRH receptor is fully active (Fig. 2). Therefore, even though the RhCCR3 (RRR) is unable to efficiently serve as a coreceptor for HIV-1, the RhCCR3 ECDs, in the context of the chimeras described in this study, can participate in coreceptor activity.

The hCCR3/RhCCR3 chimeras were constructed using unique restriction enzyme sites that resulted in not only the exchange of the extracellular domains, but also the exchange of transmembrane and cytoplasmic regions. Even though the chimeric receptor strategy has been described in several previous studies to identify critical regions in ECDs necessary for coreceptor activity (Bieniasz et al., 1997; Chabot and Broder, 2000; Lu et al., 1997; Picard et al., 1997; Ross et al., 1998; Sol et al., 1998), the introduction of amino acid changes in non-ECD regions may have played a role in coreceptor function, possibly by contributing to either (1) the overall conformational structure of the receptor or (2) the ability to bind to the envelope (Doranz et al., 1999). However, this may not be as large a concern in this study, compared to studies using chimeras composed of non-homologous coreceptors (i.e., hCCR1/hCCR3 (Alkhatib et al., 1997) or CCR5/CCR2b (Atchison et al., 1996)) due to the limited number of amino acid differences in non-ECDs of the two CCR3 molecules. Eleven of the 28 amino acids that differ between hCCR3 and RhCCR3 are in non-extracellular regions (Fig. 1A) and most are quite conservative amino acid changes.

It is important to understand how M-tropic and dual-tropic isolates interact with M-tropic receptors (hCCR5 and hCCR3), however, a direct mutational approach may not be productive due to the redundancy in the use of the four extracellular domains by HIV-1 Env, as described above. Therefore, mutations were introduced into the hCCR3/RhCCR3 chimeras containing a single human ECD in a manner similar to hCCR5/mCCR5 chimeras (Ross et al., 1998) and assayed for coreceptor activity. The residues that were mutated were selected because they differed in the ECDs between the two CCR3 homologues and therefore, we expected that these amino acid residues must contribute to hCCR3 receptor function. None of the mutations affected cell surface expression of the chimeric CCR3 molecules (Table 1).

Interestingly, at least one residue in three of the ECDs was identified as important for coreceptor activity for the two R5 viruses tested (Table 2). Two individual substitutions in the first human ECD (T13P and V20M) knocked out coreceptor function. In addition, one change in the third ECD (E179P) and one change in the fourth ECD (N271L) markedly affected coreceptor activity for R5 strains. For HIV-1$_{89.6}$, only the mutated RRRH receptor was used for analysis because this chimeric receptor was the only one of the set that efficiently served as a coreceptor. Interestingly, mutating any of the three amino acids (S263T, I267V, N271L) disrupted coreceptor activity (Table 2).

One of the more interesting mutant chimeric CCR3s assessed was the V20M mutation. This mutation is a relatively conservative change, since both amino acids are non-polar and hydrophobic. It is unclear why this point mutation had an effect on coreceptor function, but it is possible that the methionine at position 20 was more efficiently used as a start codon than the natural ATG, despite the lack of a good Kozak consensus sequence. Therefore, a significant portion of the expressed receptors may have had the first 20 amino acids in CCR3 truncated. The N-terminal eight residues are critical for the natural ligand of CCR3, eotaxin, to bind and activate hCCR3 (Shinkai et al., 2002). The N-terminus of hCCR5 is contacts the envelope and mediates subsequent interactions (Rabut et al., 1998; Trkola et al., 1996). In addition, the naturally occurring Δ32/hCCR5 receptor is unable to serve as a coreceptor for M-tropic isolates (Huang et al., 1996).
Introduction into CCR3 of the T13P mutation in ECD-1 or the E179P mutation in ECD-3 most likely disrupts protein conformation. Using alanine scanning, residues in these same regions in hCXCR4 were identified as necessary for coreceptor activity (Chabot and Broder, 2000). A similar region was identified in hCCR5 (Doran et al., 1999; Dragic et al., 1996; Rabut et al., 1998; Ross et al., 1998) and this region appears critical for determining T and M-tropism (Chabot and Broder, 2000). Negatively charged residues in the amino terminal domain of hCCR5 and hCXCR4 are critical for coreceptor activity (Chabot and Broder, 2000; Doranz et al., 1999; Dragic et al., 1996; Kuhmann et al., 1997, 2000) and that substitutions of these residues affect the ability of gp120 to bind efficiently to hCCR5, probably by reducing the affinity of the gp120-coreceptor interactions. The aspartic acid residues in the hCCR3/RhCCR3 chimeras were not mutated in this study, however, four of the five residues mutated in the third domain of the RRHR receptor were negatively charged glutamic acids (Fig. 1). The only mutation of a glutamic acid residue in ECD-3 that interfered with coreceptor activity was E179P, which can be explained by the disruption of the conformational structure. Therefore, the charged residues in ECD-3 of hCCR3 may not play a significant role in binding of the HIV-1 Env to this coreceptor.

Lastly, the only residue in the fourth extracellular domain identified as critical for R5 viruses was N271L. The substitution of this hydrophobic amino acid for a polar amino acid may disrupt the integrity or affinity of Env binding to the molecule, which results in an imbalance in the net charge of ECD-4. The other mutations (I267V and S263T) are relatively conservative changes, and not surprisingly, did not affect CCR3 coreceptor activity. However, mutation of any of the three residues in ECD-4 knocked out coreceptor activity for HIV-1st. Once again demonstrating the high level of sensitivity of this dual-tropic envelope to changes in the coreceptor sequence.

This report indicates that similar to its interactions with hCXCR4 and hCCR5, the HIV-1 envelope uses multiple extracellular domains of hCCR3 to enter into human cells. The hCCR3 is one of many identified coreceptors for HIV-1 in vitro (Philpot, 2003; Ross et al., 1999). Controversially, hCCR3 has been associate with in vivo infection and has been implicated in HIV-1 infection in the CNS and AIDS (Gorry et al., 2003; Martin-Garcia et al., 2002). A thorough understanding of the interaction between HIV-1 envelope and hCCR3, in addition to hCCR5 and hCXCR4, may be needed to prevent viral spread.

Material and methods

Cell culture

Human embryonic kidney cells, 293T, were maintained in complete Dulbecco Modified Eagle Medium (DMEM) supplemented to contain 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA, USA), 4 mM L-glutamine, and 0.4 mg/l gentamicin. Cells were incubated at 37 °C in a humidified 5% CO2 incubator.

Construction of molecular clones and mutants

Mammalian expression plasmids encoding the hCD4, hCCR3, hCCR5, and hCXCR4 have been described previously (Ross and Cullen, 1998; Ross et al., 1998). The rhesus macaque CCR3 (RhCCR3) gene was PCR amplified and isolated from the peripheral blood mononuclear cells (PBMC) of a healthy donor rhesus macaque (kind gift from Francis Novembre, Emory University). Each chimera was constructed using unique restriction sites (Fig. 1). For example, the chimera HRRR was expressed using a fusion of the human CCR3 sequence (nucleotides 1–228) and the rhesus macaque CCR3 (nucleotides 229–1065). The first set of chimeric gene sequences encoded for one extracellular domain from the hCCR3 and three ECDs from the RhCCR3. Each chimera was given a designation based upon the composition of the human and rhesus macaque extracellular domains. For example, the chimera construct, HRRR, contains the gene sequences from the first human extracellular domain in the backbone of rhesus macaque CCR3. Due to the relative position of the restriction enzyme sites, HRRR contained, in addition to the first extracellular domain, the human first and second transmembrane domains and the first intracellular domain of hCCR3. A similar strategy was employed to generate single RhCCR3 extracellular domain expressing plasmids, as well as plasmids that expressed CCR3 proteins containing two domains from each homologue. Each construct was verified using unique diagnostic restriction enzyme sites and sequencing analysis.

All wild-type and mutant chimeras were expressed using the pBC12/CMV mammalian expression vector (Bieniasz et al., 1997; Ross and Cullen, 1998; Ross et al., 1998). In addition, the hemagglutinin (HA) epitope tag was cloned between the codons coding for amino acid residues 2 and 3 of the each protein. Mutations were introduced into the coreceptor chimeras by using synthetic oligonucleotide primers and the Quick Change mutagenesis kit (Stratagene, La Jolla, CA, USA) and were verified by DNA sequencing. Point mutants were named according to the nature and location of the mutation. For example, the Y16D mutation encodes aspartic acid in place of tyrosine at residue 16 of the HRRR chimera. The expression plasmids pBC12/CMV/CD4 and pBC12/HIV/LUC, and the HIV-1 proviral clones containing the YU-2, ADA, and 89.6 envelope genes, have been described previously (Ross and Cullen, 1998; Ross et al., 1998).

The plasmids were amplified in *Escherichia coli* strain-DH5α, purified using an endotoxin-free, anion-exchange resin columns (Quiagen, Valencia, CA, USA) and stored at −20 °C in dH2O. Plasmids were verified by appropriate
restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at 260 and 280 nm and therefore, each DNA plasmid preparation had >50 fg/µg of endotoxin.

Infection assay

Two sets of 293T cells were plated at a concentration of approximately 5.0 × 10^5 cells/well in a 6-well plate. Cells were allowed to acclimate for 24 h at 37 °C plus 5% CO₂. The first set of 293T cells was transfected with 2 µg of the proviral DNA plasmid (Bieniasz et al., 1997; Ross and Cullen, 1998; Ross et al., 1998) using 12% lipofectamine according to the manufacturer’s guidelines (Life Technologies, Grand Island, NY, USA). A second set of 293T cells was transfected with plasmids expressing surface receptors and/or a luciferase reporter plasmid (pHV-1 LTR-Luc), which drives expression of the firefly luciferase gene from the HIV-1 LTR promoter (Ross and Cullen, 1998). Each set of transfected cells was incubated at 37 °C plus 5% CO₂. The total amount of DNA was maintained at 3 µg using pBC12/CMV plasmid as filler. The medium from receptor expressing cells was aspirated, thoroughly washed, and overlayed with supernatant from cells transfected with proviral DNA. Overlayed cells were incubated an additional 48 h at 37 °C plus 5% CO₂. Cell lysates were prepared by adding 300 µl of 1% Triton X-100 lysis buffer (Sigma, St. Louis, MO, USA). Plates were rocked until cells were completely detached from the plate. Cells were mixed and each cell lysate was collected and stored at −80 °C. The lysates were assayed for luciferase activity according to manufacturer’s instructions (Promega, Madison, WI, USA).

Analysis of CCR3 expression levels

293T cells were transfected with 2 µg of the coreceptor expression plasmids, and 72 h later cells were stained with a murine monoclonal antibody (12CA5, Boehringer Mannheim) specific for the HA epitope tag (10 µg/ml), followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (1:200) (Sigma). Cells were fixed and by fluorescein isothiocyanate-conjugated goat anti-mouse

Statistics

For statistical analysis, the average ± standard deviation of three experiments was determined. A Student’s t test was employed and differences were considered statistically significant when P < 0.05.

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