HIV-1-induced cell fusion is mediated by multiple regions within both the viral envelope and the CCR-5 co-receptor

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Although the human hCCR-5 chemokine receptor can serve as a co-receptor for both M-tropic (ADA and BaL) and dual-tropic (89.6) strains of human immunodeficiency virus type 1 (HIV-1), the closely related mouse mCCR-5 homolog is inactive. We used chimeric hCCR-5–mCCR-5 receptor molecules to examine the functional importance of the three extracellular domains of hCCR-5 that differ in sequence from their mCCR-5 equivalents. While this analysis revealed that all three of these extracellular domains could participate in the functional interaction with HIV-1 envelope, clear differences were observed when different HIV-1 strains were analyzed. Thus, while the ADA HIV-1 isolate could effectively utilize chimeric human–mouse CCR-5 chimeras containing any single human extracellular domain, the BaL isolate required any two human extracellular sequences while the 89.6 isolate would only interact effectively with chimeras containing all three human extracellular sequences. Further analysis using hybrid HIV-1 envelope proteins showed that the difference in co-receptor specificity displayed by the ADA and BaL isolates was due partly to a single amino acid change in the V3 loop, although this interaction was clearly also modulated by other envelope domains. Overall, these data indicate that the interaction between HIV-1 envelope and CCR-5 is not only complex but also subject to marked, HIV-1 isolate-dependent variation.

Keywords: chemokine receptors/HIV-1 co-receptor

Introduction

Shortly after identification of the human CD4 glycoprotein as the primary receptor for human immunodeficiency virus type 1 (HIV-1), it became apparent that CD4 was not sufficient to mediate infection (reviewed by Planelles *et al.*, 1993; James *et al.*, 1996). In particular, it was observed that while expression of CD4 could render human cell lines susceptible to infection by laboratory-adapted HIV-1 isolates, this was not true for the majority of animal cells (Maddon *et al.*, 1986; Clapham *et al.*, 1991). Subsequent research using primary isolates of HIV-1 revealed an even more complex picture. Thus, laboratory-adapted isolates of HIV-1, also referred to as

T-cell (T) tropic isolates, proved unable to infect CD4⁺ primary human macrophages even though they could readily infect transformed human CD4⁺ T-cell lines. In contrast, primary or macrophage (M) tropic isolates were generally able to replicate efficiently in macrophages but were unable to infect most CD4⁺ T-cell lines. However, both T- and M-tropic isolates were found to replicate efficiently in CD4⁺ peripheral blood mononuclear cells (PBMC) (Gartner *et al.*, 1986; Ashorn *et al.*, 1990; O'Brien *et al.*, 1990; Schuitemaker *et al.*, 1991). On occasion, an HIV-1 isolate able to infect both macrophages and T-cell lines is observed, and these are referred to as dual tropic (Collman *et al.*, 1992; Simmons *et al.*, 1996).

Mapping of determinants of HIV-1 tropism demonstrated that these were located in the viral envelope protein and were concentrated in the short V3 loop of envelope (O'Brien *et al.*, 1990; Hwang *et al.*, 1991; Shioda *et al.*, 1991; Westervelt *et al.*, 1991). It was therefore proposed that infection by HIV-1 required a co-receptor, in addition to CD4, that was present on human but not on animal cells. In addition, it appeared that at least two distinct co-receptors must exist, one for T-tropic isolates and a second specific for M-tropic isolates. Recent evidence has validated this hypothesis and has identified the CXCR-4 chemokine receptor, also termed fusin, as the predominant T-tropic HIV-1 co-receptor and the CCR-5 chemokine receptor as the dominant M-tropic co-receptor (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996).

Although the identification of specific co-receptors for HIV-1 infection represents an important step forward, little is known as yet about how they participate in envelope-mediated fusion. Chemokine receptors are seven-membrane-spanning, G-protein-coupled receptors that play a role in the inflammatory response and in the recognition of chemotactic signals (Murphy, 1994). At present, it is not known how HIV-1 envelope proteins of different tropisms interact with the four extracellular domains found on their respective co-receptors, although it has been shown that envelope, CD4 and co-receptor can form a heterotrimeric complex on the surface of cells (Lapham *et al.*, 1996; Trkola *et al.*, 1996; Wu *et al.*, 1996). It also remains unclear why animal chemokine receptors, which are often very similar to their human equivalents, are nevertheless unable to mediate HIV-1 infection.

To address these issues, we have cloned cDNA copies of the human and mouse forms of the CCR-5 receptor and have generated a series of 14 chimeras between these two closely related molecules. As predicted, mouse CCR-5 proved unable to function as an HIV-1 co-receptor. Individual substitution of either the amino-terminal extracellular domain or the second extracellular loop of human CCR-5 into mouse CCR-5 conferred efficient co-receptor function for the M-tropic HIV-1 isolate ADA. In contrast,

infection by the M-tropic HIV-1 isolate BaL or the dual-tropic isolate 89.6 required functional interactions with multiple extracellular domains of human CCR-5. These data demonstrate that several distinct regions in human CCR-5 play a role in mediating HIV-1 infection and also suggest that HIV-1 isolates can differ in terms of which regions of the viral envelope participate in CCR-5 co-receptor recognition.

Results

Both the human and the murine CCR-5 receptor are activated by the chemokines MIP-1 α , MIP-1 β and RANTES, and both are expressed on macrophages (Boring *et al.*, 1996; Samson *et al.*, 1996). While the human CCR-5 receptor is also expressed on primary T-cells, this remains to be examined for its murine counterpart. Comparison of the predicted primary sequence of these two proteins reveals that they are 82% identical (Figure 1A). The observed differences are generally conservative and are scattered throughout the protein, i.e. they are not concentrated in either the four predicted extracellular domains, the seven transmembrane domains or the four intracellular domains.

To test whether the murine CCR-5 receptor would be able to substitute for the human CCR-5 receptor in mediating HIV-1 envelope-induced cell fusion, we designed a transient expression assay to quantitate fusion efficiency. In this assay, one batch of cells (either human 293T or simian COS cells) was transfected with an HIV-1 proviral expression plasmid encoding, for example, the M-tropic BaL or the T-tropic IIIB envelope protein (Gartner *et al.*, 1986; Hwang *et al.*, 1991). At the same time, a second batch of cells was transfected with expression vectors encoding human CD4, a candidate co-receptor and an indicator construct (pBC12/HIV/SEAP) containing the HIV-1 long terminal repeat (LTR) linked to the secreted alkaline phosphatase (SEAP) indicator gene (Berger *et al.*, 1988). This latter construct normally produces only very low levels of SEAP activity due to the low level of transcription driven by the basal HIV-1 LTR promoter. However, if this indicator construct is activated by the HIV-1 Tat *trans*-activator, high levels of SEAP will be secreted by the transfected cells.

At 48 h after transfection, the cells transfected with the HIV-1 provirus and the cells expressing CD4 and the candidate HIV-1 co-receptors are harvested and plated together at a 1:1 ratio. During the subsequent 48 h period of co-culture, the pBC12/HIV/SEAP indicator construct could be trans-activated by Tat due to infection of receptor-expressing cells by virions released by the HIV-1 provirus-expressing cells or, more probably, due to direct fusion between cells expressing the HIV-1 provirus and cells expressing HIV-1 receptors. Work from many laboratories has established that the receptor requirements for fusion to HIV-1 envelope-expressing cells are equivalent to the requirements for fusion with HIV-1 virions (Clapham *et al.*, 1991; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996; Rucker *et al.*, 1996).

Murine CCR-5 is not a functional co-receptor for HIV-1

A representative co-receptor assay using the simian COS cell line is presented in Figure 2A. Only low, background

levels of SEAP activity were detected when COS cells transfected with the M-tropic BaL or the T-tropic IIIB provirus were mixed with COS cells transfected with pBC12/HIV/SEAP alone, with cells transfected with the indicator construct and a CD4 expression plasmid, or with cells transfected with pBC12/HIV/SEAP and plasmids expressing either wild-type or HA-tagged forms of the human CCR-5 (hCCR-5) receptor. In contrast, readily detectable levels of SEAP were observed when BaL-, but not IIIB-, expressing cells were cultured in the presence of pBC12/HIV/SEAP transfected cells expressing both CD4 and wild-type hCCR-5 (Figure 2A). Co-expression of CD4 and an amino-terminally HA-tagged form of hCCR-5 resulted in a very similar level of cell fusion, and hence activation of the SEAP indicator gene. In contrast, no activation of SEAP expression was detected in cultures expressing CD4 and an HA-tagged form of murine CCR-5 (mCCR-5) upon co-culture with either BaL- or IIIB-expressing cells. However, co-expression of CD4 and an HA-tagged human CXCR-4 (HA-hCXCR-4) chemokine receptor resulted, as predicted, in a marked activation of SEAP expression upon co-cultivation with cells expressing the T-tropic IIIB, but not the M-tropic BaL, HIV-1 isolate (Figure 2A). Very similar results were also observed using the human cell line 293T, although these cells, like most human cells, constitutively express the T-tropic CXCR-4 co-receptor so that IIIB-induced fusion is, in this context, only dependent on the addition of CD4 (data not shown). Overall, we therefore conclude that this transient assay accurately and specifically measures the ability of different chemokine receptors to function as co-receptors for different isolates of HIV-1, and further conclude that mCCR-5, despite its close similarity to hCCR-5, is nevertheless unable to mediate fusion by either M-tropic or T-tropic HIV-1 envelope proteins.

Different regions of hCCR-5 can mediate fusion by different HIV-1 isolates

To map regions within the hCCR-5 receptor that mediate HIV-1 infection, we next constructed a series of chimeras between HA-tagged forms of hCCR-5 and mCCR-5. As indicated in Figure 1, the CCR-5 receptor contains an amino-terminal extracellular tail as well as three extracellular loops, each of which could contribute to envelope binding and membrane fusion. In addition, the seven transmembrane domains and four intracellular regions of hCCR-5 could conceivably also participate in the fusion process by, for example, promoting an appropriate receptor conformation.

Inspection of the predicted sequences of hCCR-5 and mCCR-5 shows that they differ at 10 positions (including a two amino acid insertion) in the ~35 amino acid amino-terminal tail, at four positions in the ~15 amino acid first extracellular loop and at six positions in the ~31 amino acid second loop. The ~17 amino acid third loop is predicted to be identical in both molecules. To construct chimeras between hCCR-5 and mCCR-5, we took advantage of conserved *Bgl*II and *Bsa*BI restriction sites that flank the second extracellular loop and also used an *Esp*I site located at the membrane-proximal end of the amino-terminal tail of mCCR-5 (Figure 1). Using these three sites, we constructed a set of 14 chimeric molecules by

Fig. 1. Structure of hCCR-5-mCCR-5 chimeras. (A) Protein sequence comparison between human and murine CCR-5. Proposed transmembrane domains (Boring *et al.*, 1996; Samson *et al.*, 1996) are boxed and numbered. Extracellular sequences include the amino-terminal tail and three extracellular loops located between transmembrane domains II and III, IV and V and VI and VII, respectively. Conserved cysteines potentially involved in disulfide bond formation are indicated by black triangles, while a conserved glycosylation site in extracellular loop 3 is underlined. The location of restriction sites used in the production of chimeras is indicated. (B) Schematic representation of the structure of the hCCR-5-mCCR-5 chimeras analyzed in this work.

interchange of four cassettes consisting of (i) the amino-terminal tail; (ii) the first extracellular loop, the first four transmembrane domains and the first and second intracellular regions; (iii) the second extracellular loop; or (iv) the conserved third extracellular loop, the last three transmembrane domains and the third and carboxy-terminal intracellular domains (Figure 1B). These chimeras were named according to the origin of these four segments,

in order. Thus, for example, HMHM contains the amino-terminal extracellular tail and the second extracellular loop of hCCR-5 in an otherwise mCCR-5 context.

To determine the relative efficiency with which these chimeric receptors could mediate HIV-1 envelope fusion, it was necessary first to demonstrate that the assay utilized was in the linear range. In fact, fusion between BaL envelope-expressing and CD4-expressing cells was

Fig. 2. Characterization of the CCR-5 co-receptor assay. **(A)** One set of COS cell cultures was mock transfected or transfected with the pBaL or pIIIB HIV-1 provirus expression plasmid. A second set was transfected with the pBC12/HIV/SEAP indicator construct and plasmids expressing CD4 and/or the indicated chemokine receptors. Some receptors were modified by addition of an amino-terminal HA tag. Cells derived from each set were co-cultivated starting 48 h after transfection, and fusion efficiency was assessed by measurement of supernatant SEAP activity 48 h later. **(B)** Dose-response curve in COS cells testing the effect of various levels of hCCR-5 expression plasmid transfection on the level of fusion activity with the BaL isolate. **(C)** Similar to **(B)**, except that the dose-response was performed in 293T cells.

observed to become increasingly efficient in both simian COS cells (Figure 2B) and human 293T cells (Figure 2C) as the level of hCCR-5 expression was increased. We therefore conclude that, under these experimental conditions, recruitment of the hCCR-5 co-receptor represents a

Fig. 3. Relative expression of hCCR-5-mCCR-5 chimeras in transfected cells. **(A)** FACS analysis of 293T cells transfected with HA-tagged forms of the indicated parental and chimeric receptors. Data are given as mean fluorescence intensity of the total cell population. Data presented are representative of three independent experiments. **(B)** Biological activity of the indicated chemokine receptors. This assay measures the agonist-dependent (100 nM MIP-1 β) ability of these receptors to inhibit forskolin-stimulated cAMP accumulation in 293T cells transfected with plasmids expressing the indicated receptors and adenylyl cyclase type V. Data are expressed as percentage inhibition of the level of cAMP accumulation seen in the absence of receptor stimulation and represent the average of three independent experiments.

rate-limiting step in the process of HIV-1 envelope-mediated cell fusion, and further conclude that the assay used here to measure hCCR-5 co-receptor function is not saturated, and is therefore capable of detecting any drop in the efficiency of co-receptor function.

A second important consideration in assessing the ability of these CCR-5 receptor chimeras to mediate HIV-1 envelope fusion is whether they are expressed at the cell surface at equivalent levels and in a native conformation. A fluorescence-activated cell sorter (FACS) analysis of cell surface expression levels for the 16 parental and chimeric CCR-5 receptors (Figure 1B), using an anti-HA tag antibody, is presented in Figure 3A. As may be observed, the parental mCCR-5 and hCCR-5 receptors, as well as 10 of the chimeric receptors, are expressed on the cell surface of transfected 293T cells at equivalent, readily detectable levels. In contrast, four of the chimeric receptors (MHMM, HHMM, HHMM and MHMM) were found to be expressed at substantially lower levels that were only slightly above the negative control.

An extensive analysis of hCCR-5 receptor function, which will be presented in detail elsewhere (I.Aramori, S.S.G.Ferguson, P.D.Bieniasz, B.R.Cullen and M.G.Caron, manuscript submitted), demonstrated that this receptor is coupled to inhibitory heterotrimeric G proteins and, as a result, is able to inhibit forskolin-stimulated cAMP accumulation effectively when activated by an appropriate chemokine. To assess whether the various chimeras retained CCR-5 receptor function, we therefore asked whether stimulation with the chemokine MIP-1 β , which is specific for the CCR-5 receptor (Boring *et al.*, 1996; Samson *et al.*, 1996), would inhibit forskolin-induced cAMP accumulation in transfected 293T cells (Salomon, 1991). As shown in Figure 3B, MIP-1 β activation of either the hCCR-5 or the mCCR-5 parental receptor inhibited cAMP production efficiently, as did binding to 10 of the 14 chimeric receptors. However, the four chimeric receptors found by FACS analysis to be expressed at low levels (Figure 3A) were also found to be entirely inactive in this assay for receptor function. Interestingly, these four chimeric receptors each have human sequences in the second cassette and murine sequences in the fourth (Figure 1B), thus suggesting that there may be some structural incompatibility between these two regions. Further analysis demonstrated that all four of these non-functional receptor chimeras, including the HHHM chimera predicted to express extracellular domains identical to the parental hCCR-5 receptor, were entirely unable to mediate HIV-1 envelope fusion (data not shown). Therefore, these four chimeras were omitted from all further analysis.

To map regions in hCCR-5 involved in mediating HIV-1 infection, we measured the ability of HIV-1 envelope proteins derived from the M-tropic BaL and ADA isolates (Hwang *et al.*, 1991; Westervelt *et al.*, 1991), the dual-tropic 89.6 isolate (Collman *et al.*, 1992) and the T-tropic IIIB isolate to fuse with COS cells expressing the two parental and 10 stable human-mouse hybrid CCR-5 receptors described in Figure 1B. These data are shown in Figure 4 and summarized in Table I.

The first three chimeras tested (HMMM, MMHM and MMMH) largely examine whether either the first or third extracellular sequence within hCCR-5 is sufficient to mediate fusion when expressed in the context of mCCR-5 (Figure 1B). As shown in Figure 4A, both the HMMM chimera and the MMHM chimera indeed proved able efficiently to mediate fusion with cells expressing the M-tropic ADA envelope. In contrast, no fusion was observed with the HMMM chimera, and minimal levels with the MMHM chimera, when the M-tropic BaL isolate was tested (Figure 4B). As expected, neither M-tropic isolate was able to interact functionally with the MMMH chimera, which is predicted to have extracellular sequences identical to the parental mCCR-5 receptor. The dual-tropic 89.6 envelope (Figure 4C) and the T-tropic IIIB envelope each failed to interact with any of these three chimeras when expressed in COS cells. Indeed, the IIIB envelope, as expected (Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996), failed to interact functionally with any chimeric CCR-5 receptor, and these data are therefore simply summarized in Table I. Overall, these first three chimeras demonstrate that either the amino-terminal tail or the second extracellular loop of hCCR-5 are fully

Fig. 4. Analysis of the level of co-receptor activity displayed by hCCR-5-mCCR-5 chimeras. Co-receptor activity was assayed in COS cells as described in the text using the indicated viral envelope proteins. These data represent the average of three independent experiments, with the standard deviation indicated. Observed SEAP activities are given relative to the parental hCCR-5 co-receptor, which was arbitrarily designated as 100. No significant differences in the level of observed SEAP activity were observed when these three virus isolates were tested with wild-type hCCR-5 (data not shown).

sufficient to mediate fusion by the ADA envelope protein when expressed in an otherwise entirely mCCR-5 context on the surface of CD4⁺ cells. In contrast, these two hCCR-5 sequences are clearly not sufficient individually to mediate efficient fusion by the M-tropic BaL or the dual-tropic 89.6 envelope when presented in the mCCR-5 context.

The next set of three chimeras (MHHH, HHMH and HHHM) test whether any single extracellular sequence in hCCR-5 is essential for fusion (Figure 1B). As shown in Figure 4A and B, both the ADA and the BaL envelope proved able to fuse efficiently to cells expressing receptor

Table I. Analysis of the ability of different HIV-1 envelope proteins to functionally interact with different parental or chimeric chemokine receptors

Candidate co-receptor	Viral envelope used					
	ADA	BaL	89.6	IIIB	V3-ADA	V3-BaL
hCCR-5	++	++	++	–	++	++
mCCR-5	–	–	–	–	–	–
hCXCR-4	–	–	++	++	–	–
HMMM	++	–	–	–	+	–
MMHM	++	±	–	–	++	++
MMMH	–	–	–	–	–	–
MHHH	++	++	+	–	++	++
HMHH	++	++	+	–	++	++
HMHM	++	++	–	–	++	++
MMHH	++	+	–	–	++	++
HMHM	++	++	–	–	++	++
MHMH	+	–	–	–	±	±
HMMH	++	+	–	–	++	+

This summary of the ability of different parental or chimeric chemokine receptors to mediate fusion with cells expressing different cloned HIV-1 envelope proteins is drawn from Figures 4 and 5. The data for ADA and BaL represent the average of data obtained using both COS and 293T cells, while the data for the other viruses are based entirely on experiments using COS cells.

++ = >50% of hCCR-5; + = 25–50% of hCCR-5 while ± indicates marginal activity.

chimeras in which any of the first three extracellular sequences of hCCR-5 were substituted with the equivalent mCCR-5 sequence. We therefore conclude that, for these two M-tropic viruses, no single extracellular loop in hCCR-5 is critical for fusion. In contrast, the dual-tropic 89.6 envelope interacted only inefficiently with the MHHH and HMHH chimeras and not at all with HMMH (Figure 4C). We therefore conclude that all three of these extracellular sequences in hCCR-5 play a role in mediating 89.6 fusion, with the second extracellular loop being especially critical.

The next set of four chimeras each contains two regions from hCCR-5 and two from mCCR-5 (Figure 1) and includes three chimeras (MMHH, MHMH and HMMH) in which two of the first three extracellular domains of hCCR-5 are substituted with murine sequences while the fourth cassette, containing the invariant third extracellular loop (Figure 1A), remains of human origin. As predicted by the earlier observation that either the amino-terminal tail or the second extracellular loop of hCCR-5 is sufficient to mediate efficient fusion by the ADA envelope, the three chimeras MMHH, HMMH and HMHM were each found to be essentially as effective as parental hCCR-5 in mediating ADA envelope fusion (Figure 4A). The observation that the MHMH chimera also gave a low but significant level of activity with ADA clearly demonstrates that the first extracellular loop of hCCR-5 also participates in the ADA envelope fusion process, although less effectively than the amino-terminal tail or the second extracellular loop. Analysis of BaL envelope fusion revealed that the HMHM chimera also mediated the activity of this viral protein efficiently. Given that the MMHM and HMMM chimeras earlier were found to be inefficient or inactive in mediating BaL envelope fusion (Figure 4B), it was surprising that the similar MMHH and HMMH chimeras gave detectable, albeit low, levels of fusion with BaL, as these chimeras are predicted to share identical extracellular sequences. However, the MHMH chimera, which gave some activity with ADA, was found to be entirely inactive with the BaL envelope (Figure 4B). It is of interest that the 89.6 dual-tropic envelope was

unable to utilize any of these four chimeric CCR-5 receptors, although 89.6, unlike either ADA or BaL, did prove able to fuse with cells expressing the T-tropic hCXCR-4 co-receptor (Figure 4C).

The HIV-1 co-receptor functions similarly in different cell contexts

While the hCCR-5 co-receptor appears able to mediate fusion with HIV-1 envelope-expressing cells when co-expressed with CD4 on the surface of cells derived from several tissues and species (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996), it remains possible that cellular context could affect fusion efficiency. To test this hypothesis, we repeated the experiments shown in Figure 4A and B using a different cell line, i.e. human 293T cells instead of simian COS cells. As shown in Figure 5, this difference in cell surface environment had little effect on the efficiency of cell fusion observed with the various hCCR-5–mCCR-5 chimeras and the ADA and BaL envelope proteins. However, the MHMH chimera did seem to function somewhat more efficiently with the ADA envelope protein, and the HMMH chimera somewhat more effectively with the BaL envelope, when expressed on 293T cells (Figure 5) when compared with COS cells (Figure 4). While the basis for these minor differences is not clear, FACS analysis does indicate that 293T cells express somewhat higher levels of chemokine receptors on their surface after transfection (data not shown).

The V3 loop of HIV-1 envelope influences co-receptor usage

The data presented in Figures 4 and 5, and summarized in Table I, demonstrate that three different HIV-1 isolates capable of utilizing hCCR-5 as a co-receptor, i.e. ADA, BaL and 89.6, nevertheless each have clearly distinct requirements for hCCR-5 binding, as revealed by the above analysis of hCCR-5–mCCR-5 chimera function. Previously, we and others have demonstrated that the V3 loop, an ~35 amino acid disulfide-bonded protein domain within envelope gp120, is a major determinant of HIV-1

Fig. 5. Analysis of the hCCR-5-mCCR-5 co-receptor function in 293T cells. These assays were performed essentially as described in Figure 4 and are presented using the same format.

cell tropism. In particular, substitution of the V3 loop sequence from any one of several M-tropic viruses into the T-tropic IIIB envelope results in an M-tropic hybrid envelope that can interact functionally with the hCCR-5 co-receptor (Hwang *et al.*, 1991; Westervelt *et al.*, 1991; Choe *et al.*, 1996). It is of note that the V3 loop sequences in the M-tropic BaL and ADA isolates are very similar, differing only by a single conservative Phe→Leu change. In contrast, the T-tropic IIIB and the dual-tropic 89.6 isolates contain V3 loop sequences that diverge extensively from both the M-tropic ADA and BaL isolates and from each other (Planelles *et al.*, 1993).

To examine whether minor differences in V3 loop sequences would affect the envelope-CCR-5 interaction, we next examined the ability of M-tropic envelope chimeras, consisting of the ADA and BaL V3 loops substituted into the IIIB envelope context (Hwang *et al.*, 1992), to mediate fusion with the various hCCR-5-mCCR-5 receptor chimeras (Figure 6, Table I). Because the results using COS and 293T cells showed no significant cellular context effect on efficiency of fusion (Figures 4 and 5), these studies were performed exclusively in COS cells. The IIIB-V3-ADA chimera behaved in these assays largely indistinguishably from the ADA envelope itself. In particular, the IIIB-V3-ADA envelope, like the ADA envelope, gave rise to readily detectable levels of fusion with the HMMM and MMHM chimeras that each contain only a single hCCR-5 extracellular domain in an otherwise entirely mCCR-5 context. In contrast, the IIIB-V3-BaL chimera gave rise to an activity pattern that, while generally similar to the patterns seen with the ADA and IIIB-V3-

Fig. 6. The V3 loop of HIV-1 envelope can modulate the CCR-5 co-receptor-envelope interaction. Co-receptor activity was assayed in COS cells as described in the text using the indicated, IIIB-derived V3 loop substitution mutants. Data are presented as in Figure 4.

ADA envelopes, was nevertheless distinct in that little if any fusion activity was observed with the HMMM chimera, and relatively low levels with the similar HMMH chimera. It is of interest that, while the parental BaL envelope also gave no detectable activity with the HMMM chimera and only low levels with HMMH, both the ADA and the IIIB-V3-ADA envelope gave readily detectable levels of fusion with both the HMMM and the HMMH co-receptor chimeras (Figures 4 and 6, Table I). It appears, therefore, that the sequence of the V3 loop not only confers, on the T-tropic IIIB envelope, the ability to interact with hCCR-5, but also that very minor changes in the V3 loop can modulate this interaction in specific ways.

Discussion

While the cell surface CD4 glycoprotein represents the primary receptor for HIV-1, CD4 alone is not sufficient to mediate infection. Instead, fusion of the HIV-1 envelope with target cells also requires a functional interaction with a co-receptor that belongs to the chemokine receptor family of seven-membrane-spanning, G-protein-coupled receptors (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996). Two chemokine receptors, the CCR-5 co-receptor for M-tropic HIV-1 isolates and the CXCR-4 co-receptor for T-tropic isolates, have now been shown to represent by far the most prevalent co-receptors for HIV-1 (Zhang *et al.*, 1996), although two isolates able to utilize CCR-3 and one capable of utilizing CCR-2b have also been described (Choe *et al.*, 1996; Doranz *et al.*, 1996).

While little is known as yet about how CCR-5 or CXCR-4 mediate virion entry, evidence has been presented suggesting that the co-receptor forms a ternary complex with CD4 and gp120 on the surface of target cells (Lapham *et al.*, 1996; Trkola *et al.*, 1996; Wu *et al.*, 1996).

Here we have attempted to answer two questions relevant to understanding the functional interaction between CCR-5 and M-tropic HIV-1 envelopes. These are (i) which extracellular regions of the hCCR-5 receptor contribute to this interaction? and (ii) do different M-tropic HIV-1 envelope proteins interact with hCCR-5 in the same way? Our approach to these questions involved generating a set of chimeras between the permissive human CCR-5 receptor and the very similar, yet non-permissive, murine CCR-5 molecule (Figure 1). We then devised a rapid, quantitative assay that measures the ability of these chimeric receptor molecules to mediate fusion with cells expressing natural or artificial M-tropic (ADA, BaL, IIIB-V3-ADA, IIIB-V3-BaL), dual-tropic (89.6) or T-tropic (IIIB) HIV-1 envelope proteins.

The hCCR-5 and mCCR-5 receptors differ significantly in the amino-terminal extracellular tail and in the first and second extracellular loop, but are predicted to have an identical third extracellular loop (Figure 1). Therefore, while these chimeras can shed light on the contribution of the three variable extracellular domains to hCCR-5 co-receptor function, they do not address whether the fourth extracellular domain plays any important role in this process. In considering these data, it is also important to remember that mCCR-5 derived sequences may well participate in the interaction with HIV-1 envelope when in the context of an hCCR-5-mCCR-5 chimera. Thus, while a given hCCR-5 sequence may be sufficient to mediate cell fusion when introduced into the otherwise non-permissive mCCR-5 receptor context, this does not necessarily imply that the introduced human sequence is sufficient to mediate the observed phenotype. Of the original 14 hCCR-5-mCCR-5 receptor chimeras, 10 were found to be expressed efficiently on the surface of transfected cells and also retained the ability to be activated by the chemokine MIP-1 (Figure 3). Data obtained with these 10 hybrid molecules, as well as with the wild-type hCCR-5, mCCR-5 and hCXCR-4 receptors, are presented in Figures 4–6 and summarized in Table I.

At least three extracellular domains of hCCR-5 are involved in co-receptor function

The M-tropic ADA HIV-1 envelope protein proved able to interact functionally with every chimera tested, with the exception of MMMH, which is predicted to be identical in its extracellular sequence to the parental mCCR-5 receptor (Table I). Thus, ADA interacted very efficiently with chimeric receptors in which any one of the first three extracellular domains of hCCR-5 was replaced with murine sequences (MHHH, HMHH and HHMH) and also interacted very effectively with mCCR-5-based receptors containing only the first or the third extracellular domain of the human protein (HMMM and MMHM). Unfortunately, the MHMM chimera proved unstable and therefore could not be tested. However, the MHMH chimera, which differs from the non-permissive mCCR-5 and MMMH proteins, in extracellular terms, only in the first loop, did give rise to significant levels of fusion with ADA envelope-

expressing cells (Table I). Therefore, it is apparent that the first and third extracellular domains of hCCR-5 are each fully sufficient to induce ADA envelope-induced cell fusion when expressed in the otherwise non-permissive mCCR-5 context, while the second extracellular domain of hCCR-5 is at least partially active.

Analysis of the BaL M-tropic envelope produced a significantly different set of data (Table I). Although the BaL envelope, like the ADA envelope, was able to interact efficiently with chimeras in which any single human sequence was substituted with that of mouse (i.e. MHHH, HMHH and HHMH), BaL was not able to interact effectively with any of the three chimeras containing single human extracellular domain substitutions (HMMM, MMHM and MHMH) that were utilized effectively by the ADA envelope. Curiously, the MMHH and HHMH chimeras, which should present extracellular sequences identical to MMHM and HMMM, did give a modest level of fusion with the BaL envelope, although this was again lower than seen with ADA. This suggests that the additional human sequences present in these two chimeras may either promote a conformation favorable to envelope binding when present *in cis* or that they promote steps subsequent to binding that permit more efficient fusion to occur. A final important point is that the HHMH chimera containing the first and third extracellular domain of hCCR-5 did promote efficient fusion with the BaL envelope (Table I). Overall, these data suggest that, while no single extracellular domain of hCCR-5 is critical for BaL envelope fusion, no domain is sufficient either. Instead, these data demonstrate that any two of the first three human CCR-5 extracellular domains are required to confer co-receptor function on mCCR-5 when challenged with the BaL envelope protein.

Perhaps the most surprising data in this study were obtained with the dual-tropic 89.6 envelope protein, which is able to use the very different CCR-5, CXCR-4, CCR-3 and CCR-2b molecules as co-receptors and has, as a result, been termed a 'remarkably promiscuous' envelope protein (Rucker *et al.*, 1996). In contrast to this plasticity, 89.6 proved intolerant of almost any change in the hCCR-5 receptor (Table I). In particular, and unlike ADA and BaL, the 89.6 envelope interacted poorly with chimeras containing either the first or second mCCR-5 extracellular domain inserted into the hCCR-5 receptor (MHHH and HMHH) and failed entirely to interact with a chimera containing the second mouse extracellular loop (HHMH). Therefore, it is apparent that the efficient interaction of the 89.6 envelope with the hCCR-5 co-receptor is dependent on the functional integrity of all three of the extracellular domains of hCCR-5 examined here.

Overall, the data discussed thus far indicate that all three of the envelope proteins examined, i.e. ADA, BaL and 89.6, functionally interact with not only the hCCR-5 amino-terminal tail but also with the first and second extracellular loop. For the ADA envelope, the amino-terminal tail and the second extracellular loop are fully sufficient, in the mCCR-5 context, to mediate efficient fusion, while the first extracellular loop can mediate partial activity. In contrast, for the 89.6 envelope, all three regions must be of human origin for efficient fusion to occur. Finally, the BaL envelope is intermediate in that no single hCCR-5 extracellular domain is sufficient, yet any

combination of two is fully active. Thus, BaL requires any two extracellular hCCR-5 sequences, ADA requires any one sequence and 89.6 can only fuse efficiently if all three hCCR-5 extracellular domains are present.

Sequences both inside and outside the V3 loop affect the envelope-co-receptor interaction

The finding that different HIV-1 envelope proteins can interact with the CCR-5 co-receptor in ways that are apparently quite different in molecular detail raises the questions of which regions of envelope participate in the hCCR-5 interaction. It is well established that the ~35 amino acid V3 loop of the envelope is the major, albeit not the only, determinant of HIV-1 tropism (O'Brien *et al.*, 1990; Hwang *et al.*, 1991; Westervelt *et al.*, 1991; Boyd *et al.*, 1993). We therefore asked how the V3 loop would affect the interaction of envelope proteins with the chimeric receptors described here and, in particular, if the V3 loops of ADA and BaL, which differ by only a single phenylalanine to leucine substitution (Planelles *et al.*, 1993), would exert a detectably different phenotype when inserted into an identical, IIIB-derived envelope context.

As shown in Figure 6 and summarized in Table I, this was indeed shown to be the case. Thus, while the IIIB-V3-ADA envelope protein was able to utilize the HMMH chimera efficiently, and the similar HMMM with some effectiveness, the IIIB-V3-BaL envelope proved entirely unable to interact functionally with HMMM and gave only partial activity with HMMH. Importantly, the level of activity observed for IIIB-V3-BaL is identical to the pattern seen with these two co-receptor chimeras upon challenge with the parental BaL envelope (Table I). As IIIB-V3-ADA and IIIB-V3-BaL differ by only a single amino acid, it appears that this point mutation has specifically compromised the ability of the IIIB-V3-BaL envelope to interact functionally with the amino-terminal extracellular domain of hCCR-5. It is of interest that the IIIB-V3-BaL virus is, however, similar to ADA and to IIIB-V3-ADA in being able to interact efficiently with both the MMHM and the MMHH chimera, both of which are used relatively poorly by BaL (Table I). It would appear, therefore, that the effectiveness of the interaction between HIV-1 envelope proteins and the third extracellular domain of hCCR-5 is regulated, at least in part, by sequences outside of the V3 loop.

In conclusion, we have shown that the first, second and third extracellular domains of hCCR-5 all participate in the functional interaction with HIV-1 envelope and have further shown that the relative contribution of each of these hCCR-5 sequences to the process of membrane fusion is HIV-1 isolate dependent. Clearly, the existence of multiple, functionally redundant gp120 contact sites on CCR-5, as well as the observed plasticity of the envelope-CCR-5 interaction, could have important consequences *in vivo*. In particular, while sequence variation in the envelope clearly influences the way in which M-tropic envelopes interact with the wild-type human CCR-5 co-receptor, this variation does not appear to compromise this interaction significantly. Thus, the potential exists for HIV-1 mutants to arise *in vivo* that have gained the ability to use alternative co-receptors, yet still retain their ability to use CCR-5. Indeed, recent evidence suggests that most freshly isolated CXCR-4-utilizing virus strains, unlike

their laboratory-adapted counterparts, also retain the ability to use CCR-5 as a co-receptor (Simmons *et al.*, 1996; Zhang *et al.*, 1996). In the same way, sequence divergence in the envelope protein in response to selective pressures imposed by anti-viral immune responses could be facilitated by the flexible nature of the functional interaction between envelope and the CCR-5 co-receptor (McKnight *et al.*, 1995). This might be of particular importance to the V3 loop, which is not only a major target for neutralizing antibody but also, as shown here, specifically involved in regulation of the envelope-CCR-5 interaction.

Two recent reports, one published immediately prior to submission of this manuscript (Rucker *et al.*, 1996) and one shortly after (Atchison *et al.*, 1996), have also addressed the ability of chimeric chemokine receptors to function as co-receptors for HIV-1. Rucker *et al.* (1996) examined chimeras generated between hCCR-5 and human CCR-2b, a distinct chemokine receptor which has been shown to be functional with the dual-tropic 89.6 isolate but not with M-tropic HIV-1 isolates. That study also documented the importance of both the amino-terminal domain and the first extracellular loop of CCR-5 in infection by both M- and dual-tropic HIV-1 envelopes and, most importantly, was able to show that these different isolates interacted with different sub-regions of the CCR-5 receptor during the infection process. However, Rucker *et al.* (1996) did not observe any differences in the ability of different M-tropic HIV-1 isolates to use their panel of hCCR-5-hCCR-2b chimeras, and also did not report any evidence to support the critical role for the second extracellular loop documented by our data (Table I). While the basis for these differences is unclear, these two studies examined chimeras constructed using a very different partner for hCCR-5 (mCCR-5 in this report, hCCR-2b in the earlier report) and also used different HIV-1 isolates.

The recent study by Atchison *et al.* (1996) examined a limited panel of not only hCCR-5-hCCR-2b but also hCCR-5-mCCR-5 chimeras, and is similar to the current study in that multiple extracellular domains of hCCR-5 were shown to contribute to efficient infection by M-tropic HIV-1. However, these two studies differ in that chimeras equivalent to our HMMM and MMHM constructs were each reported by Atchison *et al.* (1996) to mediate relatively efficient infection by HIV-1 BaL, the only isolate they examined. In contrast, while we observed efficient utilization of these chimeric receptor constructs by the ADA HIV-1 envelope, the BaL envelope proved ineffective (Figure 4A and B). This discrepancy clearly could result from differences in the assay system used in these two studies, in that Atchison *et al.* (1996) measured infection by cell-free virus, while here we quantified the level of envelope-mediated cell membrane fusion. However, evidence from several studies (Clapham *et al.*, 1991; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996; Rucker *et al.*, 1996) has shown that the receptor requirements for HIV-1 envelope-mediated virion-cell fusion are equivalent to the requirements for cell-cell fusion. More probably, this difference results from the fact that Atchison *et al.* (1996) utilized an uncloned, and probably heterogeneous, stock of the BaL HIV-1 isolate, which has been in culture for >10 years (Gartner *et al.*, 1986), while we utilized a cloned BaL envelope gene. In any event, this minor discrepancy does not affect, and

even serves to confirm, the finding that the interaction between the HIV-1 envelope and chemokine receptors is complex.

Materials and methods

Construction of molecular clones

cDNA clones encoding human CCR-5 and CXCR-4, as well as the murine CCR-5 homolog, were obtained by PCR using a human T-cell cDNA library or mouse genomic DNA as templates. In each case, the 5' primer introduced an *NcoI* restriction site underlying the translation initiation codon. Subsequently, a sequence encoding the nine amino acid influenza HA-derived epitope tag (NH₂-YPYDVPDYA-COOH) (Wilson *et al.*, 1984) was inserted between CCR-5 or CXCR-4 amino acid residues 2 and 3 by PCR. The 3' PCR primers introduced unique *XhoI* sites. The PCR products were inserted as *NcoI*-*XhoI* fragments into the expression plasmid pBC12/CMV (Cullen, 1986) and their complete nucleotide sequences verified.

To generate chimeras, the two receptors were divided arbitrarily into four cassettes separated by unique *EspI*, *BglIII* and *BsaBI* restriction sites (Figure 1). Cassettes 1 and 3 contain only the 1st and 3rd extracellular sequences, respectively, whereas cassettes 2 and 4 contain the 2nd and 4th extracellular sequences as well as all the various transmembrane and intracellular sequences. Chimeric receptors (Figure 1), which represent all possible combinations of these four cassettes, were constructed by exchange of restriction fragments or *Pfu*-amplified PCR products. All chimeras subsequently were transferred as *HindIII*-*XhoI* fragments into the expression plasmid pCMV5 (Andersson *et al.*, 1989). Similarly, a pCMV5 CD4 expression plasmid was generated by insertion of a *HindIII*-*XhoI* fragment CD4 cDNA from pBC12/CMV/CD4 into pCMV5. pC5/HIV/SEAP was constructed by deletion of the CMV promoter from pCMV5 and insertion of an *Acc651*-*XhoI* fragment from pBC12/HIV/SEAP (Berger *et al.*, 1988) containing the HIV-1 LTR linked to the SEAP indicator gene.

The HIV-1 proviral clones pIIIB, pBaL, pIIIB/V3-BaL and pIIIB/V3-ADA have been described previously (Hwang *et al.*, 1992). Complete envelope gp120-coding sequences from the HIV-1 ADA and 89.6 strains of HIV-1 were PCR amplified from published proviral clones (Westervelt *et al.*, 1991; Collman *et al.*, 1992), using primers targeted to a *SalI* site located within the first coding exon of HIV-1 Tat and a *BamHI* site located within envelope gp41 sequences. These DNA fragments were then used to generate chimeric infectious virus clones pADA and p89.6 by replacement of the corresponding *SalI*-*BamHI* fragment of pIIIB (Hwang *et al.*, 1992).

Cell fusion assay for co-receptor function

COS cells and 293T cells were maintained as described previously (Hwang *et al.*, 1992). Transient virus indicator cells were generated by co-transfection of COS cells using lipofectamine with 800 ng of pBC12/CMV/CD4-, 800 ng of pBC12/HIV/SEAP- and 800 ng of pBC12/CMV-based plasmids expressing one of the parental or chimeric receptors. The total amount of plasmid DNA in each transfection was maintained at 2.4 µg by inclusion of the requisite quantity of a pBC12/CMV 'filler' plasmid. Simultaneously, virus-producing cells were generated by transfection of COS cells with 2 µg of each HIV-1 proviral construct. At 48 h post-transfection, producer and indicator cells were harvested by trypsinization, and equal numbers (~5 × 10⁴) co-cultivated in 48-well plates. After 48 h, culture supernatants were harvested and SEAP activity determined as described previously (Berger *et al.*, 1988).

Similar assays were performed using 293T-based indicator and producer cells. Procedures were identical except that indicator cells were transfected with 400 ng of pCMV5/CD4-, 400 ng of pC5/HIV/SEAP- and 50 ng of pCMV5-based co-receptor expression plasmids. The total amount of plasmid DNA in each transfection was maintained at a constant level (1.8 µg) by inclusion of the pCMV5 parental plasmid.

Analysis of CCR-5 expression levels

293T cells were transfected with 2 µg of pCMV5 based co-receptor expression plasmids and, 72 h later, unfixed cells were stained with a murine monoclonal antibody (12CA5, Boehringer Mannheim) specific for the HA epitope tag (10 µg/ml), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma, 1:200). Surface expression of the chimeric receptors was then quantitated by FACS analysis (Barak *et al.*, 1994) and expressed as the mean fluorescence intensity of the total cell population.

Assay for cAMP production

Human 293T cells were transfected with pCMV5-based parental or chimeric CCR-5 expression plasmids and with a pCMV5-based plasmid expressing adenylyl cyclase type V (Ishikawa *et al.*, 1992). At 48 h after transfection, cultures were labeled with 1 µCi/ml of [2,8-³H]adenine, in minimal essential medium supplemented with 5% fetal bovine serum. The cells were then washed with fresh medium containing 10 mM HEPES (pH 7.4) and 0.2% bovine serum albumin (BSA), treated with 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 15 min, and then stimulated with 1 µM forskolin, alone or with 100 nM MIP-1β. After incubation for 30 min at 37°C, the medium was aspirated, and the reaction was terminated by addition of 2.5% perchloric acid, 0.1 mM cAMP and 4 nCi/ml of [¹⁴C]cAMP. The cAMP levels were then quantitated as described previously (Salomon, 1991).

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