The second extracellular loop of CXCR4 is involved in CD4-independent entry of human immunodeficiency virus type 2

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Human immunodeficiency virus type 2 (HIV-2) strains that infect cells in the absence of cellular CD4 emerge spontaneously in vitro after culture in CD4 T-cell lines. The HIV-2ROD/B strain can use the CXCR4 chemokine receptor for efficient entry into CD4 cells. Here we have shown that the rat homologue of CXCR4, in the absence of CD4, failed to mediate CD4-independent entry by ROD/B. Furthermore, using rat–human chimeric CXCR4 receptors we have demonstrated that the second extracellular loop (E2) of human CXCR4 is critical for HIV-2 infection of CD4 cells. E2 is also important for HIV-1 infection of CD4 cells. Our results therefore indicate that the role of E2 in HIV entry is conserved for HIV-1 and HIV-2 and for infection in the presence or absence of CD4.

Primate immunodeficiency viruses [human immunodeficiency virus types 1 and 2 (HIV-1, -2) and simian immunodeficiency virus (SIV)] enter cells by a process of membrane fusion triggered by the interaction of their envelope glycoproteins (Env) with the cellular receptor complex (reviewed in Moore et al., 1993; Weiss, 1993). CD4 acts as a receptor for the outer envelope glycoprotein (SU) and is responsible for induction of conformational changes in Env. Several seven-transmembrane domain spanning molecules belonging to the chemokine receptor family have been identified as coreceptors (or second receptors) for HIV entry (reviewed in Clapham & Weiss, 1997; D’Souza & Harden, 1996; Moore et al., 1997). Chemokine receptors CXCR4 and CCR5 are major coreceptors for T-cell-line-tropic and macrophage-tropic HIV-1 isolates respectively (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). Other seven-transmembrane receptors can also mediate HIV-1 entry, namely CCR2b, CCR3, STRL33/Bonzo/TYMSTR, GPR15/BOB and the human-cytomegalovirus-encoded US28 (Choe et al., 1996; Deng et al., 1997; Doranz et al., 1996; Farzan et al., 1997; Liao et al., 1997; Loetscher et al., 1997; Pleskoff et al., 1997). Like HIV-1, HIV-2 requires a coreceptor for entry into CD4+ cells (Clapham et al., 1991; Dragic & Alizon, 1993; McKnight et al., 1994). CXCR4 and CCR5 function as coreceptors for different strains of HIV-2 (Deng et al., 1997; Hill et al., 1997; McKnight et al., 1998; Pleskoff et al., 1997; Sol et al., 1997) as well as other members of the seven-transmembrane receptor family (Deng et al., 1997; Farzan et al., 1997; McKnight et al., 1998). Feline immunodeficiency virus (FIV) is able to fuse human cells expressing CXCR4 or its feline homologue (Willeit et al., 1997a, b), suggesting an evolutionary link between chemokine receptor utilization and lentivirus infection.

Certain HIV-2 strains, adapted in vitro, infect CD4+ cells (Clapham et al., 1992; McKnight et al., 1994) and use CXCR4 as a primary receptor (Endres et al., 1996; Potempa et al., 1997; Reeves et al., 1997), presumably by direct interaction. In addition, some HIV-2 strains can be activated by soluble CD4 (sCD4) to use other chemokine receptors, namely CCR3 and V28 as well as CXCR4, for entry into CD4+ cells, albeit less efficiently (Reeves et al., 1997).

We previously reported that the N-terminal extracellular domain and the glycosylation state of CXCR4 influence infection and fusion of certain HIV-1 and HIV-2 strains (Breton et al., 1997; Picard et al., 1997; Potempa et al., 1997; Talbot et al., 1995), and that the rat homologue of CXCR4 is not functional for CD4-dependent entry of some isolates of HIV-1 and HIV-2ROD (Pleskoff et al., 1997a). The second extracellular loop of human CXCR4 confers CD4-dependent infection for several HIV-1 strains when placed into a rat CXCR4 background (Breton et al., 1997). Here we show that the same region is also critical for CD4-independent infection by HIV-2.

HIV-2ROD/B can infect a number of CD4+ human cell lines (Clapham et al., 1992; McKnight et al., 1994; Talbot et al.,...
Fig. 1. (a) Schematic representation of human–rat CXCR4 chimeras. Segments of human origin are open while segments of rat origin are filled. Transmembrane domains are shaded and triangles indicate junction sites. Construction of these chimeric receptors has been previously described in detail (Brelot et al., 1997). (b, c) Infectious titres of HIV-2ROD/B (b) and HIV-2ROD/A (c) on CCC cells transfected with human CXCR4, rat CXCR4 and chimeric receptors (B–N) in the presence or absence of sCD4 (5 µg/ml). Infectivity was quantified 3 days post-infection by immunostaining for expression of envelope glycoproteins. Results presented are from a representative experiment and are expressed as focus forming units/ml (f.f.u./ml) ± SD, determined on duplicate wells.

Non-human cells are generally restrictive to CD4-independent entry of ROD/B, although some, e.g. the cat kidney CCC cell line used here, are permissive for post-entry replication (Clapham et al., 1992; McKnight et al., 1994). CCC cells were seeded in 50 mm diameter dishes (10^6 cells per dish) and transfected 24 h later with 3 µg of pReCMV plasmid encoding CXCR4 receptors (Fig. 1a; Brelot et al., 1997) using Lipofectamine (Gibco BRL). Twenty-four hours post-transfection, cells were split into 48-well trays (5 × 10^4 cells per well) and infected with cell-free virus supernatant 24 h later. Infections were performed in duplicate with serially diluted virus in the presence or absence of 5 µg/ml sCD4. Virus (100 µl) was incubated with cells for 3 h at 37 °C before addition of 500 µl fresh medium. Cells were immunostained for envelope expression 3 days post-infection (Reeves et al., 1997).

Expression of human CXCR4 in CCC cells confirmed sensitivity to HIV-2ROD/B infection (Fig. 1b), indicating that CXCR4 acts as a primary receptor in these cells. Addition of sCD4 did not influence CXCR4-mediated entry of HIV-2ROD/B but activated efficient infection of the prototype HIV-2ROD/A strain (Fig. 1c). Marginal infection by this strain in the absence of sCD4 was enhanced approximately 10-fold by 5 µg/ml sCD4, consistent with previous reports (Endres et al., 1996; Potempa et al., 1997; Reeves et al., 1997). In contrast, the rat homologue of CXCR4 did not mediate entry of ROD/A or ROD/B (Fig. 1b, c). Since this molecule was expressed at
CXCR4 domains in CD4-independent HIV-2 entry

Fig. 2. (a) Infectious titres of HIV-1LAI on CCC/CD4 transiently transfected with human CXCR4, rat CXCR4 and chimeric receptors (B–N). (b) FACS data showing 12G5 binding to mock, human CXCR4 (hu CXCR4), rat CXCR4, chimera F, M and N transfected CD4− CCC cells. The shaded area represents isotype binding and the unfilled area 12G5 binding.

similar levels to human CXCR4 on transfected cells (see below), we concluded that rat CXCR4 is not functional as a receptor for HIV-2_{ROD} in CD4− cells. Human and rat CXCR4 sequences (Wong et al., 1996) are closely related (greater than 90% amino acid identity). Differences are mainly found in extracellular domains, in particular in the N-terminal extracellular domain (Nt) (11 aa differences) and in the second extracellular loop (E2) (8 aa differences), suggesting a role for these regions in HIV (co)receptor function (Brelot et al., 1997).

We next analysed receptor chimeras between rat and human CXCR4 (Fig. 1a) for the ability to mediate HIV-2 infection following transfection into CCC cells (Fig. 1b, c). Introduction of the Nt and transmembrane (TM) region 1 (construct B) or a fragment encompassing the Nt and the extracellular loop 1 (E1) (construct E) of human CXCR4 into the non-functional rat homologue did not restore receptor function. Conversely, replacement of the Nt and TM1 fragment of human CXCR4 by its rat counterpart did not abrogate entry (not shown). Thus, although the Nt is essential for ROD/B cell–cell fusion and modulates cell-free infection of HIV-2_{ROD/B} to a lesser extent (Potempa et al., 1997), sequence differences between rat and human E2 were not detrimental to the receptor function of CXCR4. We next exchanged a fragment that included both E2 and E3 extracellular loops between rat and human CXCR4. Introduction of this fragment of human CXCR4 into rat CXCR4 (construct F) created a
functional receptor for HIV-2<sub>ROD/B</sub> entry and for HIV-2<sub>ROD/A</sub> entry following sCD4 activation (Fig. 1b, c). The reciprocal chimera (construct G) did not function as a receptor. These results indicated that the region(s) important for CXCR4 receptor activity are within the I2-to-E3 fragment. The two human extracellular domains of this fragment (E2 and E3) were therefore introduced individually into rat CXCR4 (constructs M and K respectively). E3 alone did not restore receptor activity to rat CXCR4 (construct K). Conversely, replacing human E3 with rat E3 did not affect receptor activity of human CXCR4 (not shown). In contrast, replacement of rat E2 by human E2 created a functional receptor for HIV-2 (construct M), whereas the reciprocal construct (N) was unable to mediate HIV-2 entry into CCC cells. The second extracellular loop of CXCR4 is thus a crucial determinant for its ability to mediate HIV-2 entry in the absence of cellular CD4. Interestingly, infection via chimeras F and M was approximately 2- and 3-fold higher, respectively, than infection mediated by human CXCR4, perhaps suggesting differences in surface expression of parental and chimeric receptors. However, this seemed not to be the case when we went on to examine cell-surface expression of CXCR4 receptors (see below).

Since monoclonal antibodies that recognize both rat and human CXCR4 have not yet been identified, we assessed cell-surface expression of parental and chimeric CXCR4 receptors in two ways. First, we tested whether chimeric receptors, expressed after transfection, supported HIV-1<sub>LAI</sub> infection on CCC/CD4 cells. HIV-1<sub>LAI</sub> is able to use human CXCR4, rat CXCR4 and rat–human chimeras for efficient entry into CD4<sup>+</sup> cells (Brelo<sub>t</sub> et al., 1997). All the chimeric receptors supported HIV-1<sub>LAI</sub> infection (Fig. 2a) when transiently transfected into CCC/CD4 cells. The levels of infection were similar between the different receptors, indicating that sufficient amounts of each receptor were expressed on CD4<sup>+</sup> CCC cells to allow LAI infection. Infections and staining were performed as described for CCC cells with the exception that p24 antigen expression (instead of Env) was determined (Simmons et al., 1996). Secondly, we assessed cell-surface expression of the rat–human chimeras on CCC cells using monoclonal antibody 12G5, which binds to human but not rat CXCR4, and recognizes a conformational epitope, including the E2 domain (Brelo<sub>t</sub> et al., 1997; Endres et al., 1996; Lu et al., 1997). Cell-surface expression of CXCR4 constructs was determined by fluorescence-activated cell sorting (FACS), as previously described (Potempa et al., 1997), 2 days post-transfection. FACS analysis confirmed that the epitope of 12G5 includes E2 of human CXCR4, with receptor constructs F and M recognized by the 12G5, albeit less efficiently (Fig. 2b). Since constructs F and M are at least as effective as human CXCR4 as a primary receptor for HIV-2 (Fig. 1b, c) and as a coreceptor for CD4-dependent HIV-1<sub>LAI</sub> (Fig. 2a), the reduced 12G5 signal may reflect a lower 12G5 affinity to F and M and not a reduced surface expression. Receptors containing rat E2 were not recognized by 12G5 (Fig. 2b and data not shown).

We went on to examine inhibition of ROD infection by 12G5 and SDF-1α. The anti-CXCR4 12G5 MAb is able to neutralize CD4-independent Env-induced cell fusion of HIV-2 (Endres et al., 1996; McKnight et al., 1997). Fig. 3(a) shows that HIV-2<sub>ROD</sub> infection, mediated by CXCR4 in the absence of cellular CD4, was inhibited in a dose-dependent manner by 12G5. HIV-2 ROD/A and ROD/B infection through human CXCR4 was inhibited approximately 65–85% at 10 μg/ml of 12G5. For infection via construct M, however, 12G5 inhibition was much weaker, with only approximately 23–33% of infection inhibited at 10 μg/ml 12G5. This indicates that rat sequences outside E2 must influence the recognition by 12G5 and its capacity to inhibit infection. This would be consistent with a reduced detection of chimera M by 12G5 in FACS being due to a reduced affinity, rather than surface expression. Thus, although E2 must contain part of the 12G5 conformational epitope, other region(s) of the receptor are likely to influence its affinity.

The CXCR4 ligand, SDF-1α, blocked HIV-2<sub>ROD/A</sub> and HIV-2<sub>ROD/B</sub> entry in a dose-dependent manner on CCC cells expressing CXCR4 (Fig. 3b). Unlike 12G5, SDF-1α inhibition of HIV-2<sub>ROD</sub> infection was equally efficient on cells expressing chimeric receptor M (human E2 in rat CXCR4) or human CXCR4. This shows that rat sequences outside E2 are not detrimental to human SDF-1α binding. The CC chemokine RANTES, which does not bind to CXCR4, had no effect on CD4-independent CXCR4-mediated HIV-2<sub>ROD</sub> infection. These results confirm that CD4-independent entry is specifically mediated by CXCR4 and emphasize the essential role played by the second extracellular loop of CXCR4. We also recently showed that the N-terminal 23 aa of CXCR4 were involved in HIV-2<sub>ROD</sub> infection of CD4<sup>+</sup> cells (Potempa et al., 1997). Therefore both E2 and N-terminal sequences are likely to be involved in the HIV-2 Env–CXCR4 interaction.

Mutations in ROD/B Env (SU and TM) responsible for its CD4-independent phenotype have been determined (Reeves & Schulz, 1997). One amino acid change from the ROD/A genotype resides in the TM subunit, two flank the V4 loop and the fourth change, which enhances a minimal CD4-independent phenotype, is in the V3 loop. It has been proposed that the initial CD4 binding, which occurs in CD4-dependent entry, induces conformational changes in the Env proteins, exposing a region involved in binding to a second receptor or coreceptor (reviewed in Moore et al., 1993, 1997). The mutations in ROD/B may alter the Env conformation so that the binding site for CXCR4 is exposed in the absence of CD4, and the activation threshold required for HIV-2-induced fusion of CD4<sup>+</sup> cells is reduced (Reeves & Schulz, 1997).

Our results show that the interaction of HIV-2<sub>ROD/B</sub> with CXCR4 is similar to that of CD4-dependent HIV-1 isolates, suggesting that the Env–coreceptor interaction is conserved between CD4-dependent and -independent viruses, as well as between HIV-1 and HIV-2.

Although primary CD4-independent HIV-1 or HIV-2
isolates have not yet been identified, there is evidence of infection of CD4− cells in HIV-infected humans (Cohen, 1995; Housset et al., 1993; Lee et al., 1997; Livingstone et al., 1996; Wiley et al., 1986). It is, however, unclear if infection of CD4− cells influences virus pathogenesis. Nevertheless, any therapy targeted at HIV entry should take into account the possibility of CD4-independent infection in vivo, since the potential is obvious from the emergence of such strains in vitro. Our studies into the mechanism of CD4-independent infection will therefore assist assessment of the suitability of such therapeutics as they are developed.

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