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Human Immunodeficiency Virus Strains Differ in Their Ability To Infect CD4$^+$ Cells Expressing the Rat Homolog of CXCR-4 (Fusin)

OLIVIER PLESKOFF, NATHALIE SOL, BÉATRICE LABROSSE, AND MARC ALIZON*

INSERM U.332, Institut Cochin de Génétique Moléculaire, 75014 Paris, France

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A clade B strain of human immunodeficiency virus type 1 (HIV-1_{LAI}) could infect CD4$^+$ cells expressing human CXCR-4 (fusin) or its rat homolog with similar efficacy. By contrast, cells expressing rat CXCR-4 were not permissive to HIV-1_{NDK} (clade D), HIV-2_{ROD}, or HIV-1_{LAI} with chimeric envelope protein gp120 bearing the V3 domain from HIV-1_{NDK}. The reciprocal chimeric gp120 (HIV-1_{NDK} with V3 from HIV-1_{LAI}) could mediate infection of cells expressing either human or rat CXCR-4. Genetically divergent HIV strains have different requirements for interaction with the CXCR-4 coreceptor, and the gp120 V3 domain seems to be involved in this interaction.

Nonhuman cell lines expressing the CD4 receptor, and certain CD4$^+$ human cell lines, are resistant to human immunodeficiency virus type 1 (HIV-1) entry and to fusion with cells expressing HIV-1 envelope proteins (Env and gp120/gp41) (4, 14, 24, 31). Experiments with transient cell hybrids suggested that Env-mediated fusion and HIV-1 entry require CD4 and human-specific cofactors (7, 18, 24). An original genetic complementation approach allowed identification of a factor, named fusin, that allows infection of CD4$^+$ cells by T-cell line-adapted (TCLA) HIV-1 strains (20). Fusin, also known as LESTR, LCR1, etc. (29, 42), was an orphan G protein-coupled receptor with seven membrane-spanning domains related to chemokine receptors. Chemokines are proteins mediating leukocyte chemotaxis; they are classified into CXC (or α) and CC (or β) subgroups, according to their adjacent (CC) or nonadjacent (CXC) conserved cysteines in the amino-terminal domain (reviewed in reference 34). Fusin was later shown to be a functional receptor for stromal cell-derived factor 1 (SDF-1), a CXC chemokine (6, 35), and should now be designated CXCR-4. Several groups have confirmed the role of CXCR-4 as an entry cofactor for TCLA strains and shown that the CC chemokine receptor CCR-5 allowed entry of primary/macrophage-tropic HIV-1 strains (3, 13, 16, 17, 19).

The role of CXCR-4 as an HIV-1 entry factor was established with clade B HIV-1 strain LAI and the closely related variants HXB2 and IIIB (13, 16, 17, 19, 20). Cells expressing CXCR-4 and CD4 could also be infected by HIV-$1_{89.6}$, a dual-tropic clade B strain (13, 17) but not at all or considerably less efficiently by HIV-1_{ELI} (13). As a clade D strain, ELI has a more-than-25% amino acid difference from LAI or other clade B strains in gp120 (2). To test the ability of CXCR-4 to mediate infection by genetically divergent strains, we have used the clade D strain, HIV-1_{NDK} (46), and the even more distant strain HIV-2_{ROD}, sharing less than 40% amino acid identity with LAI in gp120 (23). Parallel experiments were performed with the rat homolog of CXCR-4, expected to be nonfunctional despite its high degree of similarity (>90% amino acid identity) to human CXCR-4 (50).

The human CXCR-4 cDNA, isolated by an expression cloning strategy similar to that of Feng et al. (20), and the rat CXCR-4 cDNA (kindly provided by R. S. Duman) were subcloned in Rc/CMV expression vectors (Invitrogen, La Jolla, Calif.). These vectors were transfected into three CD4$^+$ cell lines, human glioma cell lines U373MG and U87MG and macaque cell line sMAGI, all of which are known to be resistant to HIV-1 entry and syncytium formation with Env$^+$ cells (10, 14, 24). CD4$^+$ U87MG and sMAGI cells are naturally permissive to HIV-2 infection (10, 14). These cell lines have a stably integrated Tat-inducible *lacZ* gene (LTR*lacZ*), allowing detection of complementation for HIV entry by in situ β-galactosidase assay (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal] assay). Cells were transfected by calcium phosphate precipitation in 60-mm-diameter plates with 5 μg of DNA, seeded into 24-well plates, and infected 48 h after transfection with ~2,000 IU of HIV-1 (1,200 IU of HIV-2_{ROD}) per well.

TABLE 1. Infection of CD4$^+$ human and simian cell lines expressing human or rat CXCR-4 with HIV-1 and HIV-2 strains

CD4$^+$ cells and CXCR-4[a]	No. of blue-stained cells/well[b]			
	HIV-1_{LAI}	HIV-$1_{NL4.3}$	HIV-1_{NDK}	HIV-2_{ROD}
U373MG				
Human	450, 370	580, 550	380, 510	320, 340
Rat	310, 280	480, 500	0, 1	0, 2
None	2, 1	1, 2	2, 3	2, 5
U87MG				
Human	260, 330	440, 380	210, 160	NT[c]
Rat	195, 240	290, 350	1, 3	NT
None	8, 6	3, 5	3, 4	NT
sMAGI				
Human	380, 320	480, 390	280, 340	NT
Rat	240, 290	320, 350	1, 2	NT
None	4, 2	6, 2	4, 5	NT

[a] Cells were transfected with Rc/CMV expression vectors for human or rat CXCR-4 or with pUC19, split into 24-well plates, and infected 48 h after transfection.

[b] Values for duplicate wells (independent transfections) are shown. Cells were fixed and stained with X-Gal 36 h after infection.

[c] NT, not tested.

* Corresponding author. Mailing address: INSERM U.332, Institut Cochin de Génétique Moléculaire, 22 rue Méchain, 75014 Paris, France. Phone: 33-1-40 51 64 86. Fax: 33-1-40 51 77 49. E-mail: alizon@cochin.inserm.fr.

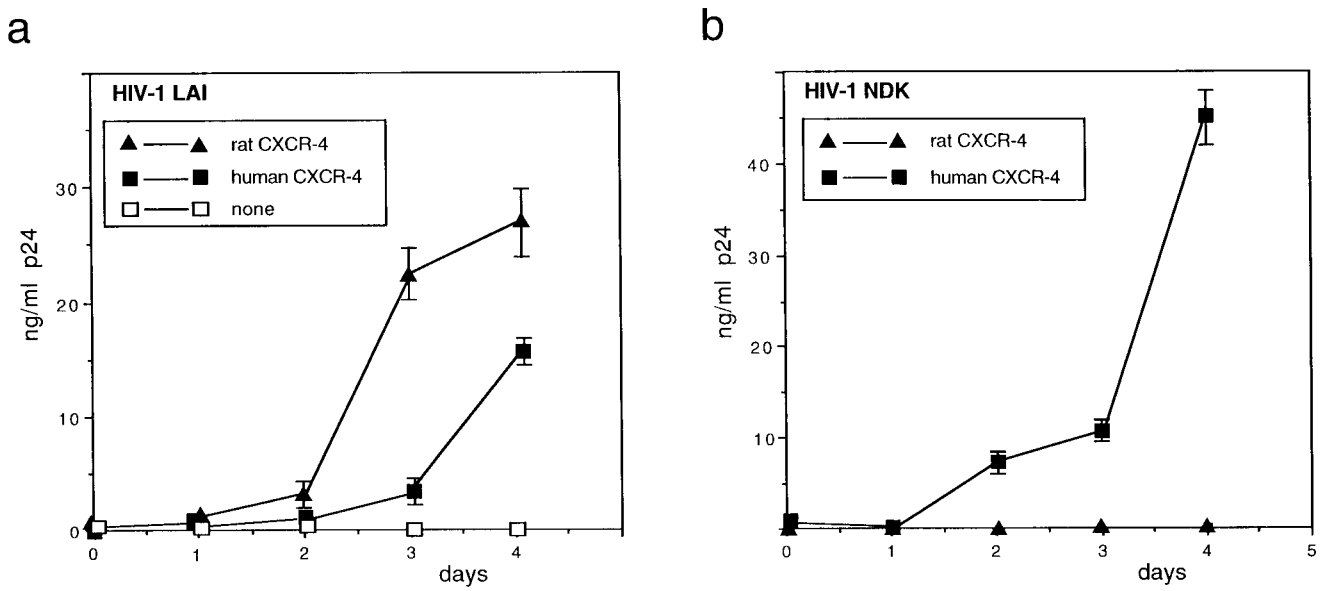

FIG. 1. Propagation of HIV-1_{LAI} and HIV-1_{NDK} in $CD4^+$ U373MG cells expressing human or rat CXCR-4. Cells transfected with expression vectors for human CXCR-4, rat CXCR-4, or pUC19 were seeded into 96-well plates and infected with HIV-1_{LAI} (a) or HIV-1_{NDK} (b) (10 ng of p24 per well) 48 h after transfection. Virus production was monitored by measuring the p24 antigen concentration in supernatants by immunoassay. Results shown are mean values of triplicate wells. When not shown, the standard error of the mean was less than 5%.

These amounts were determined by titration in the HeLa P4 cell line ($CD4^+$ LTR*lacZ*) as previously described (15). The viral stocks were supernatants of acutely infected CEM cells, excepted for LAI, produced by transfection of HeLa cells with a cloned provirus (38), and ROD, produced by chronically infected CEM cells (8). Cells were fixed, and X-Gal assays were performed 36 h after infection, as previously described (18).

The transfection of human CXCR-4 allowed infection of the three cell types by HIV-1_{LAI}, HIV-$1_{NL4.3}$ (a recombinant provirus with LAI *env* [1]), or HIV-1_{NDK} (Table 1) and infection of $CD4^+$ U373MG cells by HIV-2_{ROD}. High levels of p24 antigen production were also detected in supernatants of $CD4^+$ U373MG cells transfected with human CXCR-4, at days 3 and 4 after infection by HIV-1_{LAI} or HIV-1_{NDK} (Fig. 1). These experiments indicate that human CXCR-4 is an entry cofactor for genetically divergent HIV-1 and HIV-2 strains.

Unexpectedly, expression of rat CXCR-4 in $CD4^+$ U373MG, U87MG, or sMAGI cells allowed their infection by HIV-1_{LAI} or HIV-$1_{NL4.3}$ with an efficiency close to that of human CXCR-4 (Table 1). By contrast, rat CXCR-4 did not allow infection by HIV-1_{NDK} or HIV-2_{ROD} (Table 1) or propagation of HIV-1_{NDK} in $CD4^+$ U373MG cells (Fig. 1). Syncytium formation assays were also performed with $CD4^+$ U373MG cells transfected with human or rat CXCR-4 and H9/IIIB cells (40) chronically infected with a LAI variant or CEM cells infected with HIV-1_{NDK} or with clade B HIV-1 strains NY5 and GUN-1 (obtained from R. Weiss). Cells were fixed and stained with X-Gal after a 24-h coculture. In this experiment, blue-stained foci were essentially due to the fusion of LTR*lacZ* cells with Tat^+ cells and, to a lesser extent, to their infection. Rat CXCR-4 allowed fusion with H9/IIIB cells and with CEM cells infected with HIV-1_{NY5} or HIV-1_{GUN}, while a number of fusion events close to the background were observed with cells infected with HIV-1_{NDK} (Table 2). The three clade B strains tested were therefore able to use rat CXCR-4, but with markedly different efficiencies.

The third variable domain (V3) of HIV-1 surface envelope protein gp120 is thought to participate in post-CD4 binding steps of HIV-1 entry (5, 22, 26, 37) and to play a role in the cell tropism of HIV-1 (12, 25, 36, 49). We have previously created unique *Mlu*I and *Sma*I restriction sites in the *env* gene of LAI, allowing substitution of the V3 domain, and observed that chimeric *env* with V3 from strain NDK or ROD mediated HIV-1 entry into $CD4^+$ cell lines (39). When stably expressed

TABLE 2. Syncytium formation between $CD4^+$ U373MG cells expressing human or rat CXCR-4 and HIV-infected cells

CXCR-4	No. of blue-stained foci/well[a]			
	H9/IIIB cells	HIV-1_{NDK}-infected CEM cells	HIV-1_{GUN}-infected CEM cells	HIV-1_{NY5}-infected CEM cells
Human	260	400	310	225
Rat	190	12	28	80
None	5	6	5	10

[a] The X-Gal assay was performed after a 24-h coculture of transfected $CD4^+$ U373MG cells (subconfluent monolayer) with HIV-infected cells ($\sim 5 \cdot 10^5$ per well) on 24-well plates.

TABLE 3. Syncytium formation between $CD4^+$ U373MG cells expressing human or rat CXCR-4 and HeLa cells stably expressing wild-type or chimeric HIV-1_{LAI} Env

CXCR-4	No. of blue-stained foci/well[a]		
	Wild-type Env	Chimeric V3-NDK	Chimeric V3-ROD
Human	425, 385	180, 165	125, 140
Rat	230, 260	0, 1	0, 0
None	0, 1	0, 0	0, 0

[a] The X-Gal assay was performed after a 24-h coculture of transfected U373MG cells with HeLa-Env cells (1:1 ratio) on 24-well plates. Values are shown for duplicate wells (independent transfections).

TABLE 4. Infection of $CD4^+$ U373MG cells expressing human or rat CXCR-4 with HIV-1 expressing wild-type or chimeric Env

CXCR-4	No. of blue-stained cells/well[a]			
	$HIV\text{-}1_{LAI}$ expressing V3-LAI	$HIV\text{-}1_{NDK}$ expressing V3-NDK	$HIV\text{-}1_{LAI}$ expressing V3-NDK	$HIV\text{-}1_{NDK}$ expressing V3-LAI
Human	175, 192	224, 240	218, 113	115, 138
Rat	153, 178	7, 4	5, 7	97, 106

[a] Transfection of U373MG cells and infections were performed as described in Table 1, footnote *a*. Values for duplicate wells (24-well plates) are shown.

in HeLa cells as previously described (43), this chimeric *env* could mediate fusion with $CD4^+$ U373MG cells transfected with human CXCR-4 but not with its rat homolog (Table 3). Both the human and rat forms of CXCR-4 allowed fusion with HeLa cells stably expressing wild-type LAI Env (43). The V3 domain of LAI seems, therefore, to be necessary for functional interaction with rat CXCR-4. The *Mlu*I and *Sma*I restriction sites could be introduced into NDK *env* by site-directed mutagenesis without modification of the amino acid sequence of gp120, and chimeric NDK/V3-LAI *env* was inserted into an NDK provirus (46). Like their parental strains, chimeric LAI and NDK, produced by transfection of proviruses into HeLa cells, could infect $CD4^+$ U373MG cells transfected with human CXCR-4 (Table 4). Only wild-type LAI and chimeric NDK/V3-LAI could infect cells expressing rat CXCR-4 (Table 4). Substitution of the V3 domain from LAI into NDK gp120 was therefore sufficient to alter the phenotype of $HIV\text{-}1_{NDK}$ and confer the ability to use both the rat and human forms of CXCR-4.

After a decade of unsuccessful attempts, the concept of a human-specific HIV-1 entry cofactor, or coreceptor, was seemingly validated by the identification of CXCR-4 (fusin). The finding that its rat homolog mediates $HIV\text{-}1_{LAI}$ infection with similar efficiency was therefore unexpected, even if one rat cell line (Y3) was previously found to be permissive to fusion with Env^+ cells (4) and to HIV-1 entry (44). The CXCR-4 mRNA was not detected by Northern blotting in U87MG, U373MG, and NIH 3T3 cells (20, 45) but is apparently abundant in mouse cell lines A20 and BW5147 and in mouse lymphocytes (41), all of which are resistant to Env-mediated fusion (4) and HIV-1 entry (30, 47). In contrast to rat CXCR-4, mouse CXCR-4 may not be a HIV-1 coreceptor. It can also be envisioned that this function requires posttranslational modifications of CXCR-4 or cooperation with other factors, for example, some form of association with CD4 that is less efficient in a nonhuman cell context. This could explain the failure of previous attempts at genetic complementation with murine cells. Expression of high levels of CXCR-4 with the vaccinia virus/T7 RNA polymerase system (20) probably allowed bypass of this restriction.

The mechanism by which chemokine receptors are involved in the process of HIV-1 entry is not fully understood. Recent reports suggest a direct interaction of gp120 with CXCR-4 or CCR-5 that is markedly increased in the presence of CD4 (28, 48, 51). The genetic divergence of the gp120 proteins of $HIV\text{-}1_{LAI}$, $HIV\text{-}1_{NDK}$, and $HIV\text{-}2_{ROD}$ does not prevent their interaction with CD4, although with less affinity in the case of $HIV\text{-}2_{ROD}$ (32). However, CD4 binding is mediated essentially by conserved domains of gp120 (33). Here we have observed that a hypervariable domain of gp120 (V3) from $HIV\text{-}1_{LAI}$ was required for the use of rat CXCR-4 and sufficient to confer this property to another HIV-1 strain, suggesting that V3 plays a direct role in the gp120–CXCR-4 interaction. Also, the V3 domain of macrophage-tropic HIV-1 strains was required for the interaction of their gp120 proteins with CCR-5 (48, 51). The extreme variability of the V3 loops of strains such as LAI, NDK, and ROD is difficult to reconcile with their use of the same coreceptor. The interaction of CXCR-4 with V3 could be relatively nonspecific, for example, mediated by electrostatic interactions with basic amino acids known to accumulate in the V3 loop of TCLA strains (9, 11, 21, 27). This type of interaction, together with CD4 binding, could be sufficient to initiate the conformational changes that activate the fusogenicity of Env. Alternatively, the initial recognition of V3 could allow a subsequent interaction of CXCR-4 with a more conserved domain or spatial structure of Env. The ability of LAI, but not NDK and ROD, to use rat CXCR-4 as an entry cofactor suggests that these strains have different requirements for functional interaction with human CXCR-4. The domains required for this interaction could be defined by using chimeric receptors constructed with the human and rat forms of CXCR-4.

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