VIROLOGY 231, 105–111 (1997)
ARTICLE NO. VY978506

Role of the Amino-Terminal Extracellular Domain of CXCR-4 in Human Immunodeficiency Virus Type 1 Entry

Laurent Picard,*,[1] David A. Wilkinson,*,[2] Áine McKnight,* Patrick W. Gray,† James A. Hoxie,‡ Paul R. Clapham,* and Robin A. Weiss*

*Chester Beatty Laboratories, The Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, United Kingdom; †ICOS Corporation, 22021 20th Avenue SE, Bothell, Washington 98021; and ‡Hematology-Oncology Division, Clinical Research Building, University of Pennsylvania, 415 Curie Boulevard, Philadelphia, Pennsylvania 19104

Received December 12, 1996; returned to author for revision January 20, 1997; accepted February 18, 1997

We have studied the role of the N-terminal extracellular domain of the human immunodeficiency virus type 1 (HIV-1) coreceptor, CXCR-4, in the entry and fusion of syncytium-inducing strains of HIV-1. Progressive deletions were introduced in the N-terminal extracellular domain of CXCR-4 and the effect on infection by different isolates was tested. Infection of cells expressing the different CXCR-4 deletion mutants by HIV-1 LAI and 89.6 was reduced only about twofold. In contrast, the HIV-1 GUN-1 and RF isolates were substantially more impaired in their ability to mediate cell-free infection and cell–cell fusion. Since LAI and RF are T-cell line-tropic viruses while 89.6 and GUN-1 are dual tropic, no clear correlation between tropism and requirements for CXCR-4 N-terminal sequences emerged. We also introduced point mutations at the two N-linked glycosylation sites. The isolates tested (LAI, RF, GUN-1, and 89.6) were not affected by the removal of predicted N-linked glycosylation sites in CXCR-4. We conclude that distinct virus strains interact differently with the CXCR-4 coreceptor and that the N-terminal extracellular domain is not the sole functional domain important for HIV-1 entry.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) enters the cell following fusion of the virion and cell membranes (Maddon *et al.,* 1988; McClure *et al.,* 1988; Stein *et al.,* 1987). This process is initiated by the high-affinity binding of the gp120 subunit of the envelope glycoprotein (Env) to the CD4 molecule (Moore *et al.,* 1993; Signoret *et al.,* 1993; Weiss, 1993). For fusion to be completed additional cell surface molecules or coreceptors are required (Chesebro *et al.,* 1990; Clapham *et al.,* 1991; Dragic *et al.,* 1992; Maddon *et al.,* 1986). Depending on the virus isolates studied these coreceptors are different. The seven-transmembrane G protein-coupled chemokine receptors CXCR-4 [previously known as LCR-1, LESTR, HUMSTR, or fusin (Federsppiel *et al.,* 1993; Feng *et al.,* 1996; Herzog *et al.,* 1993; Jazin *et al.,* 1993; Loetscher *et al.,* 1994; Nomura *et al.,* 1993)] and CCR-5 (Combadiere *et al.,* 1996; Raport *et al.,* 1996; Samson *et al.,* 1996) act as coreceptors for the entry of syncytium-inducing (SI), T-cell line-adapted (TCLA) isolates (Feng *et al.,* 1996) and primary non-syncytium-inducing (NSI) macrophage-tropic strains, respectively (Alkhatib *et al.,* 1996; Deng *et al.,* 1996; Dragic *et al.,* 1996). Additional members of the chemokine receptor family can be used by some HIV-1 isolates, including CCR-3 and CCR-2b (Choe *et al.,* 1996; Doranz *et al.,* 1996). Furthermore, some isolates (TCLA or primary) were termed dual tropic because they can infect both T cells and macrophages (Collman *et al.,* 1992; McKnight *et al.,* 1995). These isolates are able to use either CXCR-4 or CCR-5 as coreceptor (Dittmar *et al.,* 1997; Doranz *et al.,* 1996; Simmons *et al.,* 1996). The exact mechanism by which CXCR-4 and CCR-5 promote the entry of HIV-1 into CD4-expressing cells is currently unknown.

In this study, we investigated the role of the N-terminal extracellular domain of CXCR-4 in HIV-1 entry. The N-terminal domain in both the CXC and the CC chemokine receptor families is likely to contain the primary high-affinity binding site for chemokine ligands (Murphy, 1994; Premack and Schall, 1996; Wells *et al.,* 1996). Moreover, chemokines can block HIV-1 infection. In particular, stromal cell-derived factor-1 blocks infection of TCLA strains that use CXCR-4 as a coreceptor (Bleul *et al.,* 1996; Oberlin *et al.,* 1996), and RANTES, macrophage inflammatory protein (MIP)-1α, and MIP-1β block infection of CCR-5-using primary viruses on $CD4^+$ $CCR\text{-}5^+$ cells (Alkhatib *et al.,* 1996; Cocchi *et al.,* 1995, 1996; Deng *et al.,* 1996; Dragic *et al.,* 1996; Jansson *et al.,* 1996). Furthermore, it has recently been shown that gp120 can compete for MIP-1α and MIP-1β binding to CCR-5 (Trkola *et al.,* 1996; Wu *et al.,* 1996). Finally, anti-serum raised against a peptide corresponding to the N-terminal part of CXCR-4 was able to block infection and cell–cell fusion of TCLA iso-

[1] To whom correspondence and reprint requests should be addressed. Fax: +44 (0) 171 352 3299. E-mail: laurent@icr.ac.uk.

[2] Present address: Aaron Diamond AIDS Research Center, 455 First Ave., New York, NY 10016.

0042-6822/97 $25.00

lates of HIV-1 (Feng *et al.,* 1996) and the formation of a ternary complex between gp120, CD4, and CXCR-4 (Lapham *et al.,* 1996). We report here that N-terminal sequences required for HIV-1 infection depends on the HIV-1 isolate tested.

MATERIAL AND METHODS

Cell lines and viruses

U373-CD4-LTR*LacZ* cells, astroglioma cells stably transfected with human CD4 and a LTR*LacZ* construct, and the cat kidney CCC/CD4 cell line were all maintained in DMEM (Gibco) supplemented with 10% fetal calf serum (FCS) and antibiotics. These cell lines have all been previously described (Clapham *et al.,* 1991; Harrington and Geballe, 1993).

The T-cell line-adapted viruses RF, LAI, and GUN-1 have been previously described (Popovic *et al.,* 1984; Takeuchi *et al.,* 1991; Wain-Hobson *et al.,* 1991). HIV-1_{GUN-1} is a dual-tropic isolate (McKnight *et al.,* 1995) that can infect macrophages as well as T-cell lines. The virus stocks were prepared by cocultivation with H9 cells. The corresponding H9 chronically infected cell lines were maintained in RPMI medium supplemented with 10% FCS and antibiotics. The dual-tropic primary isolate virus 89.6 (Collman *et al.,* 1992), a gift from R. Collman, was propagated on PBMCs. All virus stocks were titrated on HeLa-CD4-LTR*LacZ* cells (Clavel and Charneau, 1994) that were a gift from M. Alizon (ICGM, Paris). For GUN-1 and 89.6, viral stocks were plated on primary macrophages to assess their dual tropism (Dittmar *et al.,* 1997; McKnight *et al.,* 1997; Simmons *et al.,* 1996).

Flow cytometry analysis of surface expression

Each of the mutated CXCR-4 constructs or pcDNA3 vector (mock) were transfected by the calcium phosphate precipitation method into U373-CD4-LTR*LacZ* cells for 16–20 hr (10 μg/100-mm petri dish). The medium was removed and the cells were incubated for another 12 hr. Cells were then trypsinized, stained with the 12G5 anti-CXCR-4 monoclonal antibody (Endres *et al.,* 1996) or with an isotype control (IgG2a; Sigma Immunochemicals), and subsequently with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin antibodies (Dako). The cells were then analyzed and the data collected on a Becton–Dickinson FACScan. To determine the percentage of positive cells, a marker was set up at a position that gives a reading of 2% of positive cells for the mock-transfected cells.

Infectivity and fusion assays

The U373-CD4-LTR*LacZ* or CCC/CD4 were plated in 12-well plates (or 60-mm petri dishes) and transfected the next day using the calcium phosphate precipitation method for 16–20 hr with 1 or 5 μg DNA per well. The transfection medium was then removed, and the cells washed and split in 96-well (or 48-well) plates in triplicate. Transfected cells were challenged 24 hr later with 500–1000 infectious units of HIV per well (as titrated on HeLa-CD4-LTR*LacZ* cells) for 24 hr. Each construct was transfected in parallel with the wild-type CXCR-4 receptor. HIV infectivity for each mutated receptor was therefore compared to infectivity estimated on the wild-type CXCR-4 receptor. Viral titers on the transfected cells were compared with titers on HeLa-CD4 cells to control for variation in transfection efficiency. Transfection efficiency was estimated by transfection of a Tat-expressing vector in LTR*LacZ* cells. For U373-CD4-LTR*LacZ* a simple X-Gal assay was performed as described previously (Dragic, *et al.,* 1995) and the blue-stained foci were counted 48 hr postinfection. In LTR*LacZ* cells, HIV-1 infection results in Tat production that stimulates the transcription of the LTR*LacZ* transgene and in the accumulation of β-galactosidase. For CCC/CD4 the cells were immunostained 3 days postinfection using an anti-p24 antibody as the primary antibody and subsequently a secondary antibody conjugated to β-galactosidase as previously described (Clapham *et al.,* 1992). The blue-stained foci were scored after a X-Gal assay. For fusion assay between U373-CD4-LTR*LacZ* and chronically infected H9 cells, the same procedure was followed.

RESULTS

In order to study the role of the N-terminal domain of CXCR-4, we used the polymerase chain reaction to generate three mutants deleted for the N-terminal sequences: LN1, LN2, and LN3 are truncated for 7, 15, and 23 amino acids, respectively (Table 1). We also used PCR and site-directed mutagenesis to mutate each of the two putative N-linked glycosylation sites in CXCR-4. One of these sites is located in the N-terminal extracellular domain (N11), the other is located in the second extracellular loop (N176). They were respectively converted to an isoleucine (LG1) and glutamine (LG2) or both were mutated (LG1G2) (Table 1). The constructs were tagged at the C-terminus with the *myc* epitope (9E10) (Evan *et al.,* 1985) and cloned into pcDNA3 (Invitrogen, The Netherlands). We also included RM3, a differentially spliced variant of CXCR-4 (in pRcCMV; Invitrogen) which has the 5 N-terminal amino acids substituted for 10 different ones (Table 1).

Surface expression of the different mutants was estimated after transfection in two different cell types: cat kidney CCC cells and U373-CD4-LTR*LacZ* cells. We used two monoclonal antibodies to assess cell surface expression of the different CXCR-4 mutants. The 9E10 monoclonal antibody (MAb) recognized the C-terminal *myc*-tag by immunofluorescence of permeabilized cells (data not shown) while the 12G5 MAb, specific for CXCR-4 (Endres *et al.,* 1996), detected the CXCR-4 derivatives by flow cytometry (Fig. 1). 12G5 blocks HIV-1 infection in a cell-type and virus-specific manner

TABLE 1

Sequences of CXCR-4 N-Terminal Deletion Mutants and Glycosylation Mutants

	N-terminal extracellular domain
CXCR-4	MEGISIYTSDNYTEEMGSGDYDSMKEPCFREENANFNKIFLPTIYSIIFLTG
RM3[a]	MSIPLPPLLQIYTSDNYTEEMGSGDYDSMKEPCFREENANFNKIFLPTIYSIIFLTG
LN1	MSDNYTEEMGSGDYDSMKEPCFREENANFNKIFLPTIYSIIFLTG
LN2	MGSGDYDSMKEPCFREENANFNKIFLPTIYSIIFLTG
LN3	MKEPCFREENANFNKIFLPTIYSIIFLTG
	Glycosylation sites
LG1	N11I
LG2[a]	N176Q
LG1G2[a]	N11I and N176Q

Note. The putative first transmembrane segment is in boldface.
[a] These constructs do not contain the *myc* tag at the C-terminus.

(McKnight *et al.,* 1997). The precise location of the 12G5 epitope is currently unknown; however, the N-terminus of CXCR-4 does not appear to be required for 12G5 binding (Julie Davis Turner, personal communication). The mutated receptors were expressed at the cell surface of U373-CD4-LTR*LacZ* cells and CCC cells. Figure 1 shows that CXCR-4 was expressed on about 60% of U373-CD4-LTR*LacZ,* LN1 on 50%, LN2 on 15%, LN3 on 40%, LG1G2 on 45%, and RM3 on 15% cells as estimated by 12G5 staining. Immunofluorescence of membrane-associated staining with 9E10 correlated with 12G5 surface expression (not shown), indicating that the lower levels of expression for some constructs, as determined by flow cytometry, were not due to modification of the 12G5 epitope. The LG1 and LG2 receptors had the same level of expression as LG1G2 and wild-type CXCR-4 (not shown).

The CXCR-4 mutants were tested for sensitivity to HIV-1 infection. Each construct was transiently transfected into CCC/CD4 or into U373-CD4-LTR*LacZ* cells and challenged 24 hr later with different CXCR-4-using SI HIV-1 strains. HIV-1 strains tested were LAI, RF, GUN-1, and 89.6. LAI and RF are TCLA strains that use CXCR-4 as a coreceptor, whereas GUN-1 and 89.6 are dual-tropic strains that infect primary macrophages as well as T-cell lines (Collman *et al.,* 1992; McKnight *et al.,* 1995), using either CCR-5 or CXCR-4 (Dittmar *et al.,* 1997; Doranz *et al.,* 1996; Simmons *et al.,* 1996). Table 2 and Fig. 2 show infectivity titers for these virus strains tested on CCC/CD4 and U373-CD4-LTR*LacZ,* respectively, after transfection of appropriate plasmid DNA encoding each N-terminal deleted CXCR-4 construct. LAI infectivity was only slightly reduced when changing the first N-terminal amino acids (RM3) or when progressively truncating the

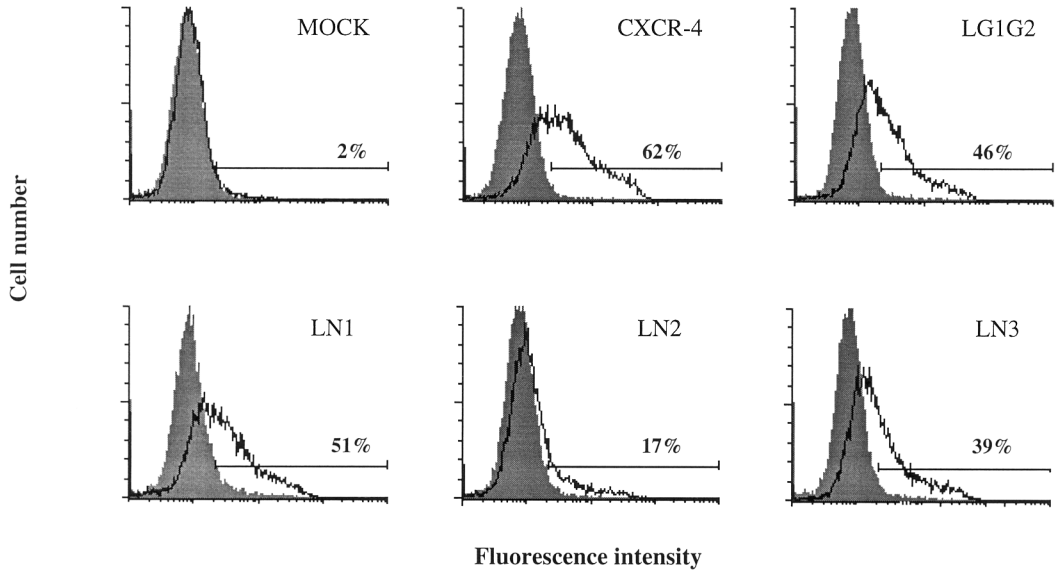

FIG. 1. Flow cytometry analysis of CXCR-4 mutant cell surface expression. Transiently transfected U373-CD4-LTR*LacZ* cells were first stained using either an isotype control (IgG2a, Kappa; Sigma Immunochemicals) (filled histogram) or the 12G5 MAb (open histogram) and subsequently with an anti-mouse FITC-conjugated secondary antibody (Dako). Data were collected and analyzed on a Becton–Dickinson FACScan.

TABLE 2

Infection of Transiently Transfected CCC/CD4 Cells with Different CXCR-4 Mutants[a]

Construct	HIV-1 isolate		
	LAI	RF	GUN-1
Mock	0	0	0
CXCR-4	93 ± 12[b]	105 ± 10	54 ± 5
LN1	99 ± 4	59 ± 6	2 ± 1
LN2	45 ± 1	8 ± 2	0
LN3	34 ± 3	5 ± 2	0

[a] After transfection the CCC/CD4 cells were challenged with different isolates and immunostained for p24 accumulation 3 days postinfection (Clapham *et al.,* 1992).

[b] Number of blue-stained foci/well (mean ± SD). The experiment presented is representative of two done in triplicate.

N-terminal domain of CXCR-4. In contrast, GUN-1 titers were severely reduced on all the deletion mutants, and on CCC/CD4 cells no foci at all were observed when the deletion was as short as 15 amino acids (LN2) (Table 2 and Fig. 2A). Even minor changes in the amino-terminal region of CXCR-4 (RM3) had a marked effect on the ability of GUN-1 to enter cells, but little effect on LAI. Unlike LAI, RF infectivity was more severely reduced by the N-terminal deletions while that of 89.6 was barely influenced (Table 2 and Fig. 2B). There was no evidence that the $CD4^+$ target cell affects coreceptor function, since similar infectivity results were obtained with CCC/CD4 and U373-CD4-LTR*LacZ.* These results indicate that the CXCR-4 requirements are different for distinct HIV-1 isolates. Furthermore, these different requirements do not correlate with the cell tropism of the considered isolate: LAI and RF are T-cell tropic, whereas GUN-1 and 89.6 are dual tropic.

We tested whether cell–cell fusion was also affected by truncation of the N-terminus of CXCR-4. Cell–cell fusion was measured using U373-CD4-LTR*LacZ* cells as target cells. Syncytium formation between these cells and HIV-1-chronically infected cells results in Tat-induced transactivation of the LTR*LacZ* transgene and can be easily detected by an X-Gal assay. When we cocultivated H9 cells chronically infected with LAI or GUN-1 with transfected U373-CD4-LTR*LacZ* cells the same pattern of results was observed: H9/LAI readily formed syncytia with cells expressing CXCR-4 or the different mutants (RM3, LN1, LN2, or LN3). In contrast, and as expected from the cell-free virus infection experiments, H9/GUN-1 cells were severely affected by these N-terminal deletions and by the N-terminal substitution (RM3), losing 80–90% of their ability to fuse (data not shown). Thus, the N-terminal extracellular domain of CXCR-4 and especially the first 10 amino acids play a role in HIV-1 entry and fusion, as recently reported by others for CCR-5 (Rucker *et al.,* 1996).

Since, the N-terminal extracellular domain is likely to be glycosylated, we tested the role of N-linked glycosylation of CXCR-4 in HIV-1 entry. CXCR-4 contains two potential N-glycosylation sites, one in the N-terminal extracellular domain, the other in the second extracellular loop (Loetscher *et al.,* 1994). Berson *et al.* showed that at least one of the two sites in CXCR-4 is likely to be glycosylated (Berson *et al.,* 1996). We mutated these putative glycosylation sites, either individually (LG1 and LG2) or together (LG1G2) and studied the capacity of the resulting molecules to confer HIV-1 entry. For all the isolates tested (LAI, RF, GUN-1, and 89.6), mutation of predicted sites for N-linked glycosylation did not affect the ability of CXCR-4 to function as an HIV-1 coreceptor in either CCC/CD4 (data not shown) or U373-CD4-LTR*LacZ* (Table 3).

DISCUSSION

The chemokine receptor CXCR-4 is used as a coreceptor for entry of primary SI and T-cell line-adapted strains of HIV-1 in otherwise nonpermissive $CD4^+$ cells (Feng

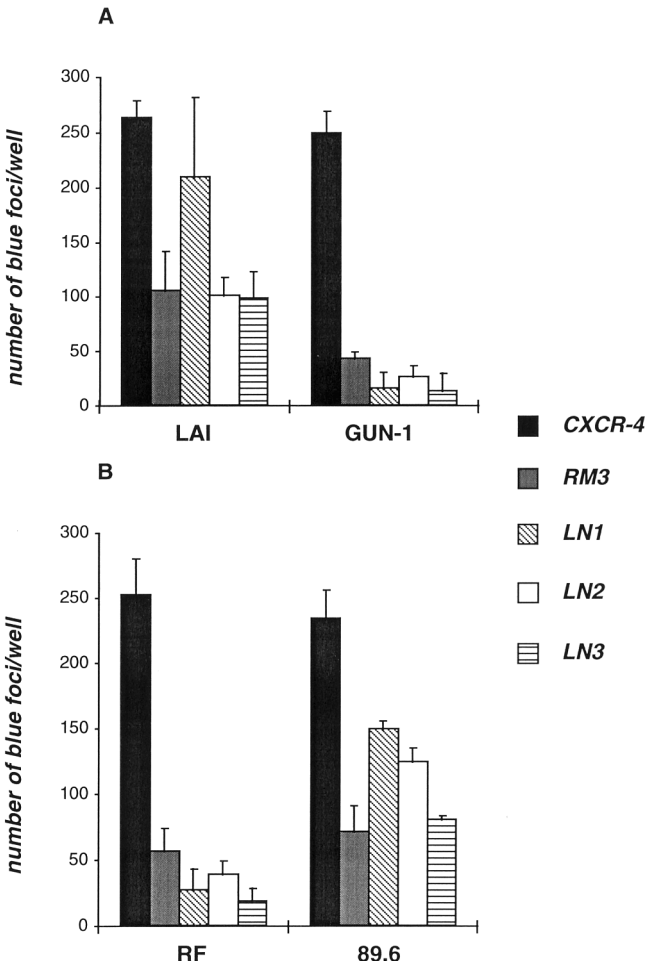

FIG. 2. Infection of cells expressing different CXCR-4 mutants. Transiently transfected U373-CD4-LTR*LacZ* cells were challenged with different HIV-1 isolates (RF, LAI, GUN-1, or 89.6) 24 hr after transfection. Blue-stained cells were scored 48 hr later after an X-Gal assay. The background foci detected on untransfected cells were substracted from the values.

TABLE 3

Infection of Transiently Transfected U373-CD4-LTR*LacZ* Cells with CXCR-4 Glycosylation Mutants[a]

Construct	HIV-1 isolate			
	LAI	RF	GUN-1	89.6
Mock	0	0	0	0
CXCR-4	252/286[b]	272/285	254/242	238/210
LG1	157/177	192/163	124/138	147/210
LG2	473/502	227/332	267/304	316/371
LG1G2	239/348	283/302	197/159	257/273

[a] After transfection U373-CD4-LTR*LacZ* cells were challenged with different isolates and blue-stained foci counted after an X-Gal assay.

[b] Results are from duplicate wells. Background foci estimated on untransfected cells were substrated from the values.

et al., 1996; Zhang *et al.,* 1996). In this study we showed that distinct HIV-1 isolates have different requirements for interaction with the CXCR-4 coreceptor.

By mutating the two potential glycosylation sites located in the N-terminal region and in the second extracellular loop, we showed that none of the four isolates tested (LAI, RF, GUN-1, and 89.6) were affected by lack of N-linked glycosylation. Glycosylation does not seem to be required for the other major coreceptor CCR-5, either (Rucker *et al.,* 1996). Binding of the 12G5 monoclonal antibody specific for CXCR-4 was not affected by loss of glycans, meaning that the glycosylation state of the receptor is not important for 12G5 recognition.

By constructing deletion mutants of CXCR-4 and testing them in cell-free virus infection and cell-fusion in two different $CD4^+$ cell lines, we found that different isolates have different dependency on N-terminal sequences. Deleting as few as 7 amino acids (LN1) or replacing the first 5 amino acids by 10 different ones abolished GUN-1 entry and syncytium-forming ability with little effect on LAI and 89.6 isolates. RF entry was less affected by these changes in CXCR-4 but was dramatically reduced by further deletion of the N-terminal extracellular sequences (LN2 and LN3). The complete removal of the N-terminal extracellular domain of CXCR-4 has not been studied in the present work but it has been recently shown that a construct deleting the 36 amino acids of the N-terminus was still functional for LAI entry and fusion while being nonfunctional for other isolates (A. Brelot, N. Heveker, and M. Alizon, personal communication). It is thus expected that this deletion mutant will not be functional for entry of GUN-1 and RF strains. Furthermore, since these deletion mutants still function for entry of some isolates, it is likely they are properly expressed, folded, and transported to the cell surface.

All the deletion mutants of CXCR-4 tested were expressed on the cell surface albeit at different levels. The lower levels of expression of some deleted receptors (especially for LN2) cannot account for the differences observed in HIV-1 infectivity, since LN2 and LN3 still mediate LAI infection at the same level. Our results indicate that different HIV-1 isolates must interact with CXCR-4 in different ways during entry into $CD4^+$ $CXCR\text{-}4^+$ cells. Interestingly, there was no correlation between T-cell or dual tropism; therefore, the differences observed here do not result from the capacity of the dual-tropic viruses to use CCR-5 as well as CXCR-4, compared to TCLA strains that use CXCR-4 but not CCR-5 (Simmons *et al.,* 1996). It has been reported that the N-terminal extracellular domain of CCR-5 is important for determining coreceptor specificity and that different isolates are dependent on different residues in the N-terminal extracellular domain (Rucker *et al.,* 1996). In particular, macrophage-tropic viruses were dependent on residues 2–5 while the dual-tropic 89.6 isolate required residues 6–9 of CCR-5 for efficient fusion. Furthermore, it indicates that 89.6 requires the N-terminal domain of CCR-5 but the equivalent region in CXCR-4 is dispensable for efficient infection.

It has been reported that the gp120 subunit of Env binds to the coreceptor in a gp120/CD4/coreceptor ternary complex on the cell surface during the entry process and can compete for MIP-1α and MIP-1β binding to CCR-5 (Lapham *et al.,* 1996; Trkola *et al.,* 1996; Wu *et al.,* 1996). Furthermore, the specificity for different coreceptors and inhibition of HIV-1 entry by CC chemokine has been mapped to a region that includes the V3 domain of gp120 (Choe *et al.,* 1996; Cocchi *et al.,* 1996; Jansson *et al.,* 1996; Trkola *et al.,* 1996; Wu *et al.,* 1996). It is therefore likely that viruses with divergent V3 loops (e.g., LAI and GUN-1) will interact with the CXCR-4 molecule differently. Furthermore, there is evidence that an antiserum against the N-terminal domain of CXCR-4 partially blocks HIV infection, syncytium formation, and ternary complex formation (Feng *et al.,* 1996; Lapham *et al.,* 1996). We anticipate that gp120 from GUN-1 and RF will be dependent upon an interaction with the N-terminal domain while for other isolates like LAI and 89.6, this interaction is either dispensable or does not take place during the entry process. A number of studies have shown that the *in vivo* NSI to SI phenotypic switch is associated with an increase in the net charge of the V3 loop (Callahan, 1994; Cheng-Mayer *et al.,* 1991; Fouchier *et al.,* 1992; Kuiken *et al.,* 1992), which also correlates with the capacity of SI viruses to use CXCR-4 instead of, or in addition to, CCR-5 (Jansson *et al.,* 1996). Since SI isolates accumulate positively charged residues in the V3 loop while gaining CXCR-4 coreceptor usage, we suggest that the V3 loop net charge of different viruses will affect their interaction with the negatively charged N-terminal extracellular domain. By progressively deleting the N-terminal domain, the net charge of the N-terminus is increased (from -6 for LN1 to 0 for LN3). Interestingly, LAI and 89.6 V3 loops are slightly more positive ($+9$ and $+7$, respectively) than RF and GUN-1 V3 loop sequences ($+6$ and $+5$, respectively). However, this simple model does not take into

account the probable role of other regions of CXCR-4 and gp120 in HIV-1 entry.

The N-terminal region studied here is not the sole domain necessary for CXCR-4 to function as a HIV-1 coreceptor. Not all the isolates studied were inhibited by N-terminal truncations indicating that another region(s) is likely to be involved as well. Furthermore, HIV-1 entry is inhibited by the 12G5 anti-CXCR-4 monoclonal antibody that binds to each of our constructs and thus must interact with an epitope away from the N-terminus. The other region(s) of CXCR-4 implicated in HIV-1 entry remains to be determined.

The precise mechanism by which HIV-1 uses CXCR-4, in conjunction with CD4, to gain entry into cells is still unknown. Other mutations and chimeras between CXCR-4 and inactive nonhuman counterparts will further define regions of the molecule used by HIV-1 to infect $CD4^+$cells.

ACKNOWLEDGMENTS

We thank Simon Talbot and Brian Willett for helpful discussion and critical reading of the manuscript. We thank our colleagues for the generous gift of virus and cell lines: Ron Collman for the 89.6 isolate, Robert Harrington and Michel Seman for the U373-CD4-LTR*LacZ* cell line, and Marc Alizon for the HeLa-CD4-LTR*LacZ* cell line. This work was supported by the Medical Research Council. L.P. was supported by an MRC-INSERM exchange fellowship.

REFERENCES

Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M., and Berger, E. A. (1996). CC CKR5: A RANTES, MIP-1α, MIP-1β receptor as fusion cofactor for macrophage-tropic HIV-1. *Science* 272, 1955–1958.

Berson, J. F., Long, D., Doranz, B. J., Rucker, J., Jirik, F. R., and Doms, R. W. (1996). A seven-transmembrane domain receptor involved in fusion and entry of T-cell-tropic human immunodeficiency virus type 1 strains. *J. Virol.* 70, 6288–6295.

Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and Springer, T. A. (1996). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* 382, 829–833.

Callahan, L. (1994). HIV-1 virion–cell interactions: An electrostatic model of pathogenicity and syncytium formation. *AIDS Res. Hum. Retroviruses* 10, 231–233.

Cheng-Mayer, C., Shioda, T., and Levy, J. A. (1991). Host range, replicative and cytopathic properties of human immunodeficiency virus type 1 are determined by very few amino acids changes in Tat and gp120. *J. Virol.* 65, 6931–6941.

Chesebro, B., Buller, R., Portis, J., and Wehrly, K. (1990). Failure of human immunodeficiency virus entry and infection in CD4-positive human brain and skin cells. *J. Virol.* 64, 215–221.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., and Sodroski, J. (1996). The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85, 1135–1148.

Clapham, P. R., Blanc, D., and Weiss, R. A. (1991). Specific cell surface requirements for the infection of CD4-positive cells by human immunodeficiency virus types 1 and 2 and by simian immunodeficiency virus. *Virology* 181, 703–715.

Clapham, P. R., McKnight, Á., and Weiss, R. A. (1992). Human immunodeficiency virus type 2 infection and fusion of CD4-negative human cell lines: Induction and enhancement by sCD4. *J. Virol.* 66, 3531–3537.

Clavel, F., and Charneau, P. (1994). Fusion from without directed by human immunodeficiency virus particles. *J. Virol.* 68, 1179–1185.

Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1995). Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by $CD8^+$ T cells. *Science* 270, 1811–1815.

Cocchi, F., DeVico, A. L., Garzino-Demo, A., Cara, A., Gallo, R. C., and Lusso, P. (1996). The V3 domain of the gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection. *Nat. Med.* 2, 1244–1247.

Collman, R., Balliet, J. W., Gregory, S. A., Friedman, H., Kolson, D. L., Nathanson, N., and Srinivasav, A. (1992). An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of human immunodeficiency virus type 1. *J. Virol.* 66, 7517–7521.

Combadiere, C., Ahuja, S. K., Lee Tiffany, H., and Murphy, P. M. (1996). Cloning and functional expression of CC CKR5, a human monocyte CC chemokine receptor selective for MIP-1α, MIP-1β, and RANTES. *J. Leukocyte Biol.* 60, 147–152.

Deng, H., Liu, R., Elmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R., and Landau, N. R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381, 661–666.

Dittmar, M. T., McKnight, Á., Simmons, G., Clapham, P. R., Weiss, R. A., and Simmonds, P. (1997). HIV-1 tropism and co-receptor usage. *Nature* 385, 495–496.

Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. G., and Doms, R. W. (1996). A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* 85, 1149–1158.

Dragic, T., Charneau, P., Clavel, F., and Alizon, M. (1992). Complementation of murine cells for human immunodeficiency virus envelope/CD4-mediated fusion in human–murine heterokaryons. *J. Virol.* 66, 4794–4802.

Dragic, T., Hazan, U., and Alizon, M. (1995). Detection of cell fusion mediated by the envelopes of human retroviruses by transactivation of a reporter gene. *In* "Viral Gene Techniques: Methods in Molecular Genetics" (K. W. Adolph, Ed.), Vol. 7, pp. 218–236. Academic Press, Orlando.

Dragic, T., Litwin, V., Allaway, G., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P., and Paxton, W. A. (1996). HIV-1 entry into $CD4^+$ cells is mediated by the chemokine receptor CC-CKR5. *Nature* 381, 667–673.

Endres, M. J., Clapham, P. R., Marsh, M., Ahuja, M., Davis Turner, J., McKnight, À., Thomas, J. F., Stoebenau-Haggarty, B., Choe, S., Vance, P. J., Wells, T. N. C., Power, C. A., Sutterwala, S. S., Doms, R. W., Landau, N. R., and Hoxie, J. A. (1996). CD4-independent infection by HIV-2 is mediated by Fusin/CXCR-4. *Cell* 87, 745–756.

Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 5, 3610–3616.

Federsppiel, B., Melhado, I. G., Duncan, A. M. V., Delaney, A., Schappert, K., Clark-Lewis, I., and Jirik, F. R. (1993). Molecular cloning of the cDNA and chromosomal localization of the gene for a putative seven-transmembrane segment (7-TMS) receptor isolated from human spleen. *Genomics* 16, 707–712.

Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996). HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane G-protein coupled receptor. *Science* 272, 872–877.

Fouchier, R. A. M., Groenik, M., Kootstra, N. A., Tersmette, M., Huisman, H. G., Miedema, F., and Schuitemaker, H. (1992). Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. *J. Virol.* 66, 3183–3187.

Harrington, R., and Geballe, A. P. (1993). Cofactor requirements for

human immunodeficiency virus type 1 entry into a CD4-expressing human cell line. *J. Virol.* 67, 5939–5947.

Herzog, H., Hort, Y. J., Shine, J., and Selbie, L. A. (1993). Molecular cloning, characterization, and localization of the human homolog of the reported bovine NPY3 receptor: Lack of NPY binding and activation. *DNA Cell Biol.* 12, 465–471.

Jansson, M., Popovic, M., Karlsson, A., Cocchi, F., Rossi, P., Albert, J., and Wigzell, H. (1996). Sensitivity to inhibition by β-chemokines correlates with biological phenotypes of primary HIV-1 isolates. *Proc. Natl. Acad. Sci. USA* 93, 15382–15387.

Jazin, E. A., Yoo, H., Blomqvist, A. G., Yee, F., Weng, G., Walker, M. W., Salon, J., Larhammar, D., and Wahlestedt, C. (1993). A proposed bovine neuropeptide Y (NPY) receptor cDNA clone, or its human homologue, confers neither NPY binding sites nor NPY responsiveness on transfected cells. *Regul. Pept.* 47, 247–258.

Kuiken, C. L., de Jong, J. J., Baan, E., Keulen, W., Tersmette, M., and Goudsmith, J. (1992). Evolution of V3 envelope domain in proviral sequences and isolates of human immunodeficiency virus type 1 during the transition of the viral biological phenotype. *J. Virol.* 66, 4622–4627.

Lapham, C. K., Ouyang, J., Chandrasekhar, B., Nguyen, N. Y., Dimitrov, D. S., and Golding, H. (1996). Evidence for cell-surface association between fusin and the CD4-gp120 complex in human cell lines. *Science* 274, 602–605.

Loetscher, M., Geiser, T., O'Reilly, T., Zwahlen, R., Baggiolini, M., and Moser, B. (1994). Cloning of a human seven-transmembrane domain receptor, LESTR, that is highly expressed in leucocytes. *J. Biol. Chem.* 269, 232–237.

Maddon, P. J., Dalgleish, A. G., McDougal, J. S., Clapham, P. R., Weiss, R. A., and Axel, R. (1986). The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell* 47, 333–348.

Maddon, P. J., McDougal, J. S., Clapham, P. R., Dalgleish, A. G., Jamal, S., Weiss, R. A., and Axel, R. (1988). HIV infection does not require endocytosis of its receptor, CD4. *Cell* 54, 865–874.

McClure, M. O., Marsh, M., and Weiss, R. A. (1988). Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism. *EMBO J.* 7, 513–518.

McKnight, Á., Weiss, R. A., Shotton, C., Takeuchi, Y., Hoshino, H., and Clapham, P. R. (1995). Change in tropism upon immune escape by human immunodeficiency virus. *J. Virol.* 69, 3167–3170.

McKnight, Á., Wilkinson, D., Simmons, G., Talbot, S., Picard, L., Ahuja, M., M., M., Hoxie, J. A., and Clapham, P. R. (1997). Inhibition of HIV fusion by a monoclonal antibody to a co-receptor (CXCR4) is both cell type and virus strain dependent. *J. Virol.* 71, 1662–1696.

Moore, J. P., Jameson, B. A., Weiss, R. A., and Sattentau, Q. J. (1993). The HIV–cell fusion reaction. *In* "Viral Fusion Mechanisms" (J. Bentz, Ed.), pp. 233–289. CRC Press, Boca Raton, FL.

Murphy, P. M. (1994). The molecular biology of leucocyte chemoattractant receptors. *Annu. Rev. Immunol.* 12, 593–633.

Nomura, H., Nielsen, B. W., and Matsushima, K. (1993). Molecular cloning of cDNAs encoding a LD78 receptor and putative leukocyte chemotactic peptide receptors. *Int. Immunol.* 5, 1239–1249.

Oberlin, E., Amara, A., Bachelerie, F., Bessia, C., Virelizier, J.-L., Arenzana-Seisdedos, F., Schwartz, O., Heard, J.-M., Clark-Lewis, I., Legler, D. F., Loetscher, M., Baggiolini, M., and Moser, B. (1996). The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* 382, 833–835.

Popovic, M., Sarngadharan, M. G., Read, E., and Gallo, R. C. (1984). Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* 224, 497–500.

Premack, B. A., and Schall, T. J. (1996). Chemokine receptors: Gateways to inflammation and infection. *Nat. Med.* 2, 1174–1178.

Raport, C. J., Gosling, J., Schweickart, V. L., Gray, P. W., and Charo, I. F. (1996). Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1β, and MIP-1α. *J. Biol. Chem.* 271, 17161–17166.

Rucker, J., Samson, M., Doranz, B. J., Libert, F., Berson, J. F., Yi, Y., Collman, R. G., Broder, C. C., Vassart, G., Doms, R. W., and Parmentier, M. (1996). Regions in β-chemokine receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity. *Cell* 87, 437–446.

Samson, M., Labbe, O., Mollereau, C., Vassart, G., and Parmentier, M. (1996). Molecular cloning and functional expression of a new human CC-chemokine receptor gene. *Biochemistry* 11, 3362–3367.

Signoret, N., Poignard, P., Blanc, D., and Sattentau, Q. J. (1993). Human and simian immunodeficiency viruses: Virus–receptor interactions. *Trends Microbiol.* 1, 328–333.

Simmons, G., Wilkinson, D., Reeves, J. D., Dittmar, M. T., Beddows, S., Weber, J., Carnegie, G., Desselberger, U., Gray, P. W., Weiss, R. A., and Clapham, P. R. (1996). Dual-tropic, primary, syncytium-inducing HIV-1 strains that can use either Lestr or CCR5 as co-receptors for virus entry. *J. Virol.* 70, 8355–8360.

Stein, B. S., Gowda, S. D., Lifson, J. D., Penhallow, R. C., Bensch, K. G., and Engleman, E. G. (1987). pH-independent HIV entry into CD4-positive T cells via envelope fusion to the plasma membrane. *Cell* 49, 659–668.

Takeuchi, Y., Akutsu, M., Murayama, K., Shimuzu, N., and Hoshino, H. (1991). Host-range mutant of human immunodeficiency virus type 1: Modification of cell tropism by a single point mutation at the neutralization epitope in the *env* gene. *J. Virol.* 65, 1710–1718.

Trkola, A., Dragic, T., Arthos, J., Binley, J. M., Olson, W. C., Allaway, G. P., Cheng-Meyer, C., Robinson, J., Maddon, P. J., and Moore, J. P. (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature* 384, 184–187.

Wain-Hobson, S., Vartanian, J.-P., Henry, M., Chenciner, N., Cheynier, R., Delassus, S., Martins, L. P., Sala, M., Nugeyre, M. T., Guetard, D., Klatzmann, D., Gluckmann, J.-C., Rosenbaum, W., Barré-Sinoussi, F., and Montanier, L. (1991). LAV revisited: Origins of the early HIV-1 isolates from Institut Pasteur. *Science* 252, 961–965.

Weiss, R. A. (1993). Cellular receptors and viral glycoproteins involved in retrovirus entry. *In* "The Retroviridae" (J. A. Levy, Ed.), Vol. 2, pp. 1–108. Plenum Press, New York.

Wells, T. N., Power, C. A., Lusti, N. M., Hoogewerf, A. J., Cooke, R. M., Chung, C. W., Peitsch, M. C., and Proudfoot, A. E. (1996). Selectivity and antagonism of chemokine receptors. *J. Leukocyte Biol.* 59, 53–60.

Wu, L., Gerard, N. P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardoso, A. A., Desjardin, E., Newman, W., Gerard, C., and Sodroski, J. (1996). CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* 384, 179–183.

Zhang, L., Huang, Y., He, T., Cao, Y., and Ho, D. D. (1996). HIV-1 subtype and second-receptor use. *Nature* 383, 768.