Human Immunodeficiency Virus Type 1 Coreceptors Participate in Postentry Stages in the Virus Replication Cycle and Function in Simian Immunodeficiency Virus Infection

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Primate lentiviruses use chemokine coreceptors in addition to the CD4 receptor to initiate virus infection. Simian immunodeficiency virus (SIV) productively infects human cells expressing CD4 and the human allele of the chemokine coreceptor CCR-5 as efficiently as it infects macaque cells expressing human CD4, suggesting that SIV can function with either a simian or a human coreceptor in conjunction with human CD4. In the same macaque cells expressing human CD4, the replication of human immunodeficiency virus type 1 (HIV-1) is blocked at several stages of infection; some isolates are restricted prior to reverse transcription, while others, including some macrophage-tropic and primary isolates, are restricted at a step after reverse transcription but prior to migration of the preintegration complex to the nucleus. Both blocks in HIV-1 replication can be relieved by either expression of the appropriate human coreceptor (CCR-5 or CXCR-4) or expression of SIV gene products in *cis* with the HIV-1 envelope as a chimera between SIV and HIV-1 (SHIV). Thus, a virus with a SIV core and HIV-1 envelope can efficiently infect macaque cells expressing human CD4, presumably by interacting with the simian coreceptor, whereas a virus with an HIV-1 core and an HIV-1 envelope requires expression of the human allele of the coreceptor for productive infection of these cells. These studies suggest that there are interactions among the coreceptor, the viral envelope, and another viral gene product that govern postentry steps of virus replication. These data are consistent with the hypothesis that such interactions may be required for translocation of the virus core to the nucleus. Moreover, the differential abilities of SIV and HIV-1 to function in these processes with heterologous primate coreceptors may have implications for cross-species transmission.

Primate lentiviruses, such as human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV), share a primary cellular receptor, the CD4 molecule (13, 27, 36, 38, 45). HIV-1 and SIV can each utilize either human or macaque CD4 as a receptor; however, HIV-1 and SIV have distinct infectivity requirements in CD4⁺ primate cells (7, 11, 37, 45). For example, SIV does not infect several CD4⁺ human T-cell lines which can be infected by HIV-1 (1, 28). On the other hand, HIV-1 replicates poorly in rhesus macaque peripheral blood mononuclear cells (PBMCs) (30), and it cannot establish a persistent infection in rhesus macaques (41). The reasons why HIV-1 is restricted in macaque cells are not clear. One possibility is that the differences in tropism between HIV-1 and SIV are due to cell surface restrictions, which are mediated by the ability of the viral envelope protein to interact with certain species-specific cellular entry cofactors.

Isolates of HIV-1 have generally been characterized on the basis of their replication patterns in PBMCs, primary macrophages, and immortalized human T-cell lines. All HIV-1 isolates replicate to some degree in PBMC cultures; M-tropic viruses also replicate in macrophages, whereas T-tropic viruses replicate in T-cell lines, and dual-tropic isolates replicate in all three cell types (reviewed in reference 18). Because both macrophages and the T-cell lines used in these studies express CD4, additional interactions between specific viral determi-

nants and other cellular proteins have been thought to govern M versus T tropism. Secondary cellular determinants for HIV-1 fusion and entry were recently identified on the basis of their interactions with chemokines (macrophage inflammatory protein 1 α [MIP-1 α], MIP-1 β , and RANTES) that act as potent modulators of HIV-1 infection (12). The chemokine receptors CXCR-4 (also known as fusin) and CCR-5 were shown to serve as the major HIV-1 coreceptors for T- and M-tropic HIV-1 isolates, respectively (2, 3, 10, 14–16, 19). The chemokines MIP-1 α , MIP-1 β , and RANTES specifically block infection by M-tropic HIV-1 strains because they are ligands for the CCR-5 coreceptor.

Chemokine receptors are G-protein-coupled transmembrane proteins that help direct leukocyte migration through their interactions with chemotactic cytokines (reviewed in references 23 and 43). While the precise function(s) of these proteins as viral coreceptors is unclear, the ability of either the CCR-5 or the CXCR-4 coreceptor, in combination with the appropriate viral envelope protein, to enhance syncytium formation suggests that these molecules facilitate membrane fusion (2, 3, 10, 14–16, 19). CXCR-4 has been shown to associate with the HIV-1 envelope (gp120)-CD4 complex, and it has been hypothesized that this interaction leads to viral fusion and entry (29). Because these coreceptors have been identified only recently, these processes are, as yet, ill-defined, and it is unclear if other viral or cellular proteins play a role in these early events of viral replication or if the coreceptor molecule influences later steps during replication.

There is evidence to suggest that there are factors other than cell surface restrictions that limit HIV-1 infection of nonhu-

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man primate cells. This has been demonstrated most clearly by expressing different HIV and SIV genes in *cis* to generate infectious, chimeric viruses called SHIVs. SHIV chimeras which contain the HIV-1 *env*, *tat*, and *rev* coding sequences in a background of the SIV genome have been shown to infect cultured rhesus macaque PBMCs and lead to a persistent infection in rhesus and cynomolgus macaques (31, 32, 35, 47). These results indicate that the HIV-1 envelope does not limit viral replication in macaque PBMCs and suggest that postentry restrictions may be responsible for certain blocks to HIV-1 replication in macaque cells.

Recently, we showed that macaque cells engineered to express human CD4 were highly susceptible to SIV infection but were refractory to infection by all of the HIV-1 isolates that we tested (8). This distinction between HIV-1 and SIV was consistently observed, including when SIVs and HIV-1s that were T-tropic or M-tropic strains and laboratory-adapted or primary isolates were compared. Here, we used these cells to delineate the viral and cellular determinants of infectivity that restrict HIV-1 replication in nonhuman primate cells. These studies show that primary HIV-1 isolates can enter macaque cells expressing human CD4, presumably by using a simian coreceptor for entry; however, HIV-1 requires the human coreceptor allele for productive virus infection in these cells. In contrast, SIV can productively infect human cells expressing the human CD4 and CCR-5 genes, and, thus, does not require any macaque-specific proteins for replication. A chimeric virus particle with an HIV-1 envelope complemented by SIV core, enzymatic, and other accessory proteins can productively infect macaque cells in the absence of exogenous expression of the human coreceptor. Together, these data suggest that replication is governed by an interaction between the envelope protein, a second viral gene product, and the cellular coreceptor. On the basis of the studies reported here, we hypothesize that the coreceptor not only participates in viral entry but also continues to interact with the viral core to complete translocation of the preintegration complex to the nucleus.

MATERIALS AND METHODS

Cell lines and viruses. CEMx174 cells (a cell line derived from a fusion of a human T-cell line [CEM] and a human B-cell line [721.174]) were cultured in complete RPMI (RPMI 1640 supplemented with 10% fetal bovine serum, 100 Units of penicillin and 100 μ g of streptomycin per ml, 1 mM sodium pyruvate, and 100 μ M nonessential amino acids). Human PBMCs were cultured in complete RPMI with 1% phytohemagglutinin-M and 50 U of interleukin-2 per ml. The sMAGI cell line (macaque mammary tumor CMMT cells expressing an integrated copy of human CD4 and the HIV-1 long terminal repeat [LTR] fused to the β -galactosidase [β -gal] reporter gene [8]) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 U of penicillin, 100 μ g of streptomycin, and 300 μ g of glutamine per ml plus 0.2 mg of G418 (Gibco, Grand Island, N.Y.) per ml and 50 U of hygromycin (Calbiochem, La Jolla, Calif.) per ml (complete DMEM). The HeLa-CD4-LTR- β -gal (MAGI) cell line was cultured in complete DMEM plus 0.15 mg of G418 per ml as described elsewhere (26).

HIV-1 and SHIV virus stocks were generated by infecting human PBMCs with cell-free virus, and SIV stocks were prepared by infecting CEMx174 cells with cell-free virus and culturing for 2 to 4 weeks. Virus production was monitored by a protein immunoassay specific for the appropriate viral Gag antigen following the protocol of the manufacturer, p24^{gag} (Abbott Laboratories, Abbott Park, Ill.) was used for HIV-1, and p27^{gag} (Immunotech, Westbrook, Maine) was used for SIV and SHIV. Viral stocks were collected at times of peak antigen production, typically at 2 weeks postinfection. In all cases, cell-free virus was harvested from the cultures by low-speed centrifugation for 5 min to remove the majority of cells, followed by filtration through a 0.22- μ m-pore-size filter.

The tissue culture infectious doses (TCIDs) of viral supernatants were determined by end-point dilution. The TCIDs of SIV supernatants were determined by end-point dilution of virus in the CEMx174 cell line. The TCIDs of SHIV and HIV-1 supernatants were determined by end-point dilution of virus in human PBMCs. Target cells were infected with serial threefold dilutions of virus in triplicate. Productive virus infection was detected by assaying for viral Gag antigen (SIV p27^{gag} antigen [for SIV and SHIV] and HIV-1 p24^{gag} [for HIV-1])

after 2 weeks, and the TCID was defined as the lowest dilution to yield an infected culture.

The cell lines described here will be available through the AIDS Research and Reference Reagent Program.

Construction of sMAGI-CXCR-4, sMAGI-CCR-5, and MAGI-CCR-5 cell lines. The plasmids pBabe-CXCR-4, pBabe-CXCR-4-GFP, pBabe-CCR-5, and pBabe-CCR-5-GFP have been described previously (14). These plasmids contain the 5'-LTR and sequences through the 5'-half of *gag* from Moloney murine leukemia virus (Mo-MuLV), followed by nonretroviral sequences coding for the human coreceptor genes (CXCR-4, a CXCR-4-green fluorescent protein [GFP] fusion protein, CCR-5, or a CCR-5-GFP fusion protein), the puromycin resistance gene under the control of the simian virus 40 (SV40) promoter, and the Mo-MuLV 3'-LTR. Plasmid DNAs were transiently transfected into the PA317 packaging cell line (39) by using the Stratagene transfection kit to generate amphotropic MuLV particles encapsidating these retroviral vectors.

sMAGI cells were infected with 1 ml of supernatant from transiently transfected PA317 cells in the presence of 4 μ g of Polybrene per ml. Cells in which gene transfer had occurred were selected by growth in complete DMEM plus 2 μ g of puromycin (ICN Biomedical, Aurora, Ohio) per ml. Pools of puromycin-resistant cells were analyzed for surface expression of CXCR-4-GFP and CCR-5-GFP by confocal microscopy. For this purpose, 24 h prior to analysis, approximately 8×10^4 cells were plated onto slides. Immediately prior to microscopy, cells were fixed in 1% formaldehyde for 5 min, washed extensively (10 times) with phosphate-buffered saline (PBS), and mounted in 10% glycerol in PBS. From the sMAGI-CXCR-4-GFP and sMAGI-CCR-5-GFP puromycin-resistant cell populations, we used fluorescence-activated cell sorting (FACS) to segregate the top 5 to 10% of cells that expressed the highest surface levels of GFP. Approximately 10^6 cells were detached from the monolayer by scraping in the presence of Versene (5 mM EDTA in PBS). The cells were washed three times in Hank's buffered saline (HBS) with 2% FCS and then resuspended in 1 ml of HBS plus 2% FCS prior to sorting for GFP expression. To examine coreceptor expression directly, sMAGI-CXCR-4 cells were sorted for high CXCR-4 surface levels with a monoclonal murine immunoglobulin G2a (IgG2a) against CXCR-4 (12G5) (17) in combination with fluorescein isothiocyanate-conjugated anti-mouse IgG2a. Approximately 10^6 cells were detached from the monolayer by scraping in the presence of Versene. After being washed once with HBS plus 2% FCS, the cells were pelleted, resuspended in 50 μ l of HBS plus 2% FCS with 0.25 μ g of 12G5, and incubated at 4°C for 30 min. The cells were washed three times, pelleted, resuspended in 40 μ l of HBS plus 2% FCS with 0.6 μ l of anti-mouse IgG2a-fluorescein isothiocyanate (Boehringer Mannheim), and incubated at 4°C for 30 min. Prior to sorting, the cells were washed three times and resuspended in 1 ml of HBS plus 2% FCS. FACS analysis was performed on a FACStar (Becton Dickinson).

The MAGI-CCR-5 cell line was generated by infecting MAGI cells with MuLV particles encapsidating a retrovirus vector encoding CCR-5 in the presence of 4 μ g of Polybrene per ml. Infected cells were grown in the presence of 1 μ g of puromycin per ml for approximately 3 weeks to isolate drug-resistant single cell clones. A puromycin-resistant colony was isolated and expanded in culture under continued selection with puromycin.

Infection of sMAGI and MAGI cells and their derivatives. MAGI and sMAGI cells and their derivatives were infected as described previously (8, 26). Some infections were performed in the presence of 100 ng of MIP-1 β (R & D Systems, Minneapolis, Minn.) per ml. The sMAGI (or MAGI) infectious dose has also been described previously (8, 26). Briefly, cells were infected with dilutions of virus supernatant in duplicate. At 2 (MAGI cells) or 3 (sMAGI cells) days, cells were stained and an infectious dose was determined by counting the number of blue nuclei in infected wells.

Detection of reverse transcription (RT) intermediates. The sMAGI cells were plated in 6-well plates at 2×10^5 cells per well in complete DMEM on the day before infection. Prior to infection, virus was treated with RNase-free DNase I at a concentration of 200 U/ml, in the presence of 10 mM MgCl₂ for 30 min, at room temperature. Cells were infected by removing media from each well and adding virus in a total volume of 500 μ l of complete DMEM. Some infections were also performed in the presence of 5 μ M azidothymidine (AZT; Sigma, St. Louis, Mo.). Twenty-four hours after infection, cells were harvested by trypsinization and then washed twice with PBS. Total DNA was prepared by lysing cells in 30 to 100 μ l of PCR lysis buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM MgCl₂, 0.1 mg of gelatin per ml, 0.45% Nonidet P-40, and 0.45% Tween 20 plus 10 μ g of proteinase K per ml) at 56°C for 1 h. Alternatively, nuclear DNA was prepared by a protocol adapted from Gallay et al. (21). Cells were lysed in 200 μ l of cold hypotonic buffer (20 mM HEPES [pH 7.8], 5 mM potassium acetate, 0.5 mM MgCl₂, 0.5 mM dithiothreitol) with several strokes of a Dounce homogenizer. Nuclear preservation after lysis was examined by phase-contrast microscopy. Cytoplasmic and nuclear extracts were separated by centrifugation at 750 $\times$ g for 5 min. The cytoplasmic extract was discarded, and nuclei were washed once in hypotonic buffer. Nucleus-associated DNA was prepared by phenol extraction, followed by ethanol precipitation.

RT intermediates were detected by using nested-PCR primer pairs that amplify viral DNA products of different stages of RT. Two *gag* primers (HIV-1 and SIV) and LTR/*gag* (HIV-1) or LTR/UTR (SIV) primers were used to monitor early and late RT products, respectively. Reaction mixes contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 0.4 mM each deoxynucleoside

triphosphate, 250 ng of each oligonucleotide primer, and 0.5 U of *Taq* polymerase (in a total volume of 25 μ l). One microliter of product from the first round of amplification was used as the template in the second round. The primer pairs HIV-gag1 (5'-GCATTATCAGAAGGAGCCACCCACAAG)/HIV-gag2 (5'-TCTGAACGGTACTAGTATTCCTGTCTA) (first round) and HIV-gag3 (5'-AGTGGGGGACATCAAGCAGCCATGCAAAT)/HIV-gag4 (5'-TGCTATGTCACCTCCCCTTGGTCTCTC) (second round) specific to HIV-1 *gag* sequences were used to amplify a 141-bp early product of RT (42). The primer pairs HIV-LTR1 (5'-TTGCCTTGAGTGCTTCAAGTAGAGTGTG)/HIV-gag2 (first round) and HIV-LTR2 (5'-TAGAGATCCCTCAGACCCCTTTAGTCAG)/HIV-gag4 (second round) specific to HIV-1 R/*gag* sequences were used to amplify an 896-bp late RT product. The primer pairs SIV-gag1 (5'-ATTAGGCTACGACCCGGCGGGAAGAAAAGTAC)/SIV-gag2 (5'-CAGCACTA GCTTGAACATCTGGGTAGCATTTTG) (first round) and SIV-gag3 (5'-GCA GAAAGCCTGTGGAGAAC)/SIV-gag4 (5'-CAGCAGTGTGAGTCATC CA) (second round) specific to SIV *gag* sequences were used to amplify an 830-bp early product of RT. The primer pairs SIV-LTR1 (5'-TTGAGCCCTG GGAGTTCTCTCCAG)/SIV-UTR2 (5'-TTCCTGCCGCCCTTACTGCCTT CAC) (first round) and SIV-LTR3 (5'-AGGTAGAGCCTGGGTGTTCC)/SIV-UTR4 (5'-GGCGCCAATCTGCTAGGGAT) (second round), which bind to SIV R (LTR-1 and LTR-3) and the untranslated region downstream of the tRNA primer binding site just 5' of the Gag start codon (UTR-2 and UTR-4), were used to amplify a 250-bp late product of RT. In this analysis, the *gag* PCR product represents an early RT product (generated during first-strand DNA synthesis), whereas the HIV-1 R/*gag* and SIV R/UTR PCR products each represent later products of RT (generated during second-strand DNA synthesis). For simplicity, we will refer to these PCR products as early and late RT PCR products, respectively.

Amplification conditions for each set of primers were as follows: for SIV-gag1/gag2, SIV-gag3/gag4, and HIV-1-LTR1/gag2, denaturation (4 min at 95°C) was followed by 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 60°C), and extension (2 min at 72°C); for HIV-1-gag1/gag2, denaturation (7 min at 95°C) was followed by 35 cycles of denaturation (1 min at 95°C), annealing (30 s at 45°C), and extension (1 min at 72°C); for HIV-1-gag3/gag4, denaturation (7 min at 95°C) was followed by 35 cycles of denaturation (1 min at 95°C), annealing (30 s at 60°C), and extension (1 min at 72°C); for HIV-1-LTR2/gag4, denaturation (4 min at 95°C) was followed by 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 55°C), and extension (2 min at 72°C); for SIV-LTR1/UTR2, denaturation (4 min at 95°C) was followed by 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 60°C), and extension (1 min at 72°C); and for SIV-LTR3/UTR4, denaturation (4 min at 95°C) was followed by 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 55°C), and extension (2 min at 72°C). To make this PCR assay quantitative, serial dilutions of each DNA or lysate sample were analyzed. The highest dilution at which PCR product was detected was considered to have at least one copy of the target viral DNA sequence.

RESULTS

To examine the viral determinants for the restriction to HIV-1 replication in macaque cells expressing human CD4, we tested the ability of SHIV to infect sMAGI cells. sMAGI cells express human CD4 and contain an integrated copy of the HIV-1 LTR fused to the β -gal reporter gene (8). This cell system has the power to detect productive infection of a single virus particle by exploiting the ability of the SIV or HIV Tat protein to transactivate the β -gal gene through the HIV-1 LTR promoter. Using these cells, we examined the infectivity of two SHIV constructs, SHIV_{SF33} and SHIV_{SF162} (35); these viruses contain the *env*, *tat*, and *rev* coding sequences from the HIV-1 isolates HIV-1_{SF33} (a T-tropic virus [51]) or HIV-1_{SF162} (an M-tropic virus [9]) in a background of the SIV strain SIVmac239 (25). SHIV_{SF33} is defective in *vpu*, whereas SHIV_{SF162} has an intact HIV-1 *vpu* gene. Table 1 shows the relative abilities of these SHIVs, as well as the parent HIV-1 and SIV clones and a positive control, SIVMneCL8, to infect the sMAGI cells. As we previously reported (8), the sMAGI cells were exquisitely sensitive to SIV infection, but not to HIV-1 infection; this was true of both the T-tropic (HIV-1_{SF33}) and M-tropic (HIV-1_{SF162}) HIV-1 isolates. Interestingly, the SHIV chimeras derived from these HIV-1 clones could productively infect sMAGI cells. There was a slightly lower (<1 log) titer for SHIVs in sMAGI cells than that in human PBMCs; however, in general, the infectivities of both SHIV and SIV were similar to their infectivities in other cells. Thus, SHIVs containing

TABLE 1. Quantitative analysis of viral infectious dose by sMAGI assaying versus end-point dilution

Virus tested	sMAGI titer ^a	TCID ₅₀ ^b
SIVMneCL8	4.0×10^4	4×10^4
SIVmac239	4.0×10^4	5×10^3
HIV-1 _{SF33}	<10	1×10^5
HIV-1 _{SF162}	<10	5×10^4
SHIV-1 _{SF33}	1.0×10^3	1×10^4
SHIV-1 _{SF162}	7.5×10^4	5×10^5

^a No. of blue nucleated centers per milliliter of virus.

^b TCID₅₀ was determined by end-point dilution with CEMx174 cells for SIV or with human PBMCs for HIV-1 and SHIV.

both T- and M-tropic Env can enter, integrate, and express viral proteins in the sMAGI cell line. These data suggest that both SHIV chimeras may use a macaque coreceptor molecule(s), in conjunction with human CD4, for entry and productive infection of macaque cells.

To determine where there is a block to HIV-1 infection in macaque cells expressing CD4, we used a PCR dilution assay to measure specific viral DNA intermediates in sMAGI cells infected with HIV-1. sMAGI cells were inoculated with two HIV-1 isolates, HIV-1_{SF33} and HIV-1_{SF162}, and, as a positive control for infection, SIVMneCL8, at a multiplicity of infection of 0.25. Prior to infection, virus stocks were treated with RNase-free DNase I to digest DNA loosely associated with the virion. As a control for viral DNA present in virions prior to infection, cells were also infected in the presence of the RT inhibitor AZT. Infected cells were lysed 24 h after infection, and RT intermediates in the crude cell lysates were detected by highly sensitive, nested-PCR methods that amplify viral DNA products of different stages of RT. We used PCR primers that amplified early (generated during first-strand synthesis) and late (generated during second-strand synthesis) products of RT. Because each of the PCR methods can detect a single copy of the viral template (data not shown), the copy number can be determined by diluting the sample to the point at which it no longer yields product in the PCR. By this single-copy PCR dilution assay, viral DNA was readily detected in cells infected

FIG. 1. Detection of RT intermediates by a single-copy PCR dilution assay. (A) Approximately 2×10^5 sMAGI cells were inoculated with SIVMneCL8, HIV-1_{SF162}, or HIV-1_{SF33} at a multiplicity of infection of 0.25 TCID₅₀ per cell. Twenty-four hours after infection, the cells were lysed in PCR lysis buffer. To estimate viral DNA copy number, serial dilutions of each sample were used as a template in a nested-PCR amplification reaction to detect either an 896-bp product corresponding to the HIV-1 R/*gag* sequence or a 250-bp product corresponding to SIV R/UTR sequence. (B) sMAGI cells were infected in the presence of 5 μ M AZT and subjected to an analysis similar to that described for panel A.

TABLE 2. Copy numbers of RT intermediates by DNA PCR

Virus tested	Tropism	Copy no. of late RT PCR product ^a		Copy no. of early RT PCR product ^a	
		-AZT	+AZT	-AZT	+AZT
SIVMneCL8		3×10^4	3×10^2	3×10^4	1×10^3
HIV-1 _{SF162}	M	3×10^3	1×10^2	1×10^4	3×10^2
HIV-1 _{SF33}	T	1×10^2	1×10^2	3×10^2	3×10^2
HIV-1 _{LAI}	T	1×10^2	1×10^2	3×10^2	3×10^2
HIV-1 _{ADA-M}	M	3×10^2	3×10^2	3×10^2	3×10^2
HIV-1 _{89.6}	Dual	1×10^2	1×10^2	3×10^2	3×10^2
HIV-1 _{JM1}	ND ^b	3×10^3	1×10^2	3×10^3	3×10^2
HIV-1 _{JM2}	ND	1×10^3	1×10^2	3×10^3	3×10^2
HIV-1 _{JM3}	ND	1×10^4	1×10^2	1×10^4	3×10^2
HIV-1 _{JM4}	ND	3×10^2	3×10^2	3×10^2	3×10^2

^a R/gag (HIV-1) or R/UTR (SIV) primers were used to amplify late RT products; gag primers were used to amplify early RT products. Values are total viral DNA copy numbers in lysed cells, as defined by PCR dilution assaying.

^b ND, not determined.

with SIVMneCL8 and HIV-1_{SF162} by using the PCR primers that detect later, plus-strand RT intermediates (Fig. 1A); this level of viral DNA was significantly higher (30- to 100-fold) than the amount of viral DNA detected in cells infected in the presence of AZT (Fig. 1B). The detection of newly initiated late DNA products of RT suggests that HIV-1_{SF162}, like SIVMneCL8, is able to enter and undergo RT in the sMAGI cell line. The relative amount of late HIV-1_{SF162} viral DNA was approximately 10-fold less than the amount of late SIVMneCL8 viral DNA. This may indicate that there is a partial block at the level of entry into the sMAGI cells, which is consistent with the ~10-fold-lower titer of SHIV_{SF162} in sMAGI cells compared to that in human PBMCs. In contrast to the M-tropic isolate HIV-1_{SF162}, only a very low level of amplified product (approximately 100 copies) was detected in the cells infected with HIV-1_{SF33}, a T-cell-tropic isolate (Fig. 1). This level of viral DNA was similar to what was detected in cells infected in the presence of AZT and, thus, is most likely due to viral DNA in the infecting virions (33, 50). These data suggest that HIV-1_{SF33} replication is blocked at an early stage, either at entry or some other point prior to the initiation of RT, whereas HIV-1_{SF162} replication is blocked at some later stage postentry, after RT.

To determine whether there was an association between the tropism of HIV-1_{SF162} and HIV-1_{SF33} and the stage of replication at which they were restricted in macaque cells, we infected the sMAGI cells with a variety of HIV-1 isolates and examined viral intermediates. The results of single-copy PCR dilution analyses are summarized in Table 2. The replication of most viral isolates that we tested (including HIV_{LAI}, a T-cell-tropic laboratory isolate, HIV-1_{ADA-M}, an M-tropic laboratory isolate; HIV-1_{89.6}, a dual-tropic isolate; and HIV-1_{JM4}, a primary isolate) were blocked prior to RT. These results suggest that the infection pattern observed for HIV-1_{SF33} is similar to that of other T-tropic or dual-tropic isolates but that HIV-1_{SF162} may not be representative of all M-tropic isolates. However, the replication of several primary isolates that we tested were, like HIV-1_{SF162}, blocked at a step after reverse transcription. These included HIV-1_{JM2} and HIV-1_{JM1}, uncloned viruses isolated 6 years apart from an infected North American individual prior to and after the onset of immunodeficiency, respectively, and HIV-1_{JM3}, isolated from an asymptomatic North American patient.

Because a subset of the HIV-1 isolates that we tested could complete RT in sMAGI cells, we asked whether viral DNA was

TABLE 3. Copy numbers of RT intermediates in nuclei

Virus tested	Copy no. of late RT PCR product ^a		Copy no. of early RT PCR product ^a	
	Nuclei	Total	Nuclei	Total
SIVMneCL8	3×10^4	3×10^4	3×10^4	3×10^4
HIV-1 _{SF162}	3×10^1	3×10^3	1×10^2	1×10^4

^a As described in Materials and Methods and in footnote *a* to Table 2.

localized to the nucleus in these infections. sMAGI cells were infected with a prototype of these isolates, HIV-1_{SF162}, and nuclear extracts from infected cells were assayed for viral DNA by the single-copy PCR dilution assay (Table 3). While we detected levels of nucleus-associated viral DNA in SIVMneCL8-infected sMAGI cells that are comparable to total levels of viral DNA, we were unable to detect viral DNA above background levels in the nuclei of sMAGI cells infected with HIV-1_{SF162}. This suggests that under conditions in which SIVMneCL8 viral DNA is translocated to the nucleus, the majority of HIV-1_{SF162} viral DNA is retained in the cytoplasm.

sMAGI cells that express the HIV-1 coreceptors are susceptible to HIV-1 infection. To directly examine the effects of coreceptor expression in macaque cells on HIV-1 replication, we constructed sMAGI cell lines that stably express the HIV-1 coreceptors CXCR-4 (or CXCR-4-GFP) and CCR-5 (or CCR-5-GFP) to determine whether expression of these coreceptors affected productive HIV-1 infection. Expression of CXCR-4-GFP, CCR-5-GFP, and CXCR-4 on the cell surface was confirmed by confocal microscopy and/or FACS analysis (data not shown). We tested the susceptibility of these cell lines to infection by well-characterized T- and M-tropic clade B laboratory HIV-1 isolates (Table 4). The sMAGI-CXCR-4 and sMAGI-CXCR-4-GFP cell lines could be productively infected by the two T-tropic HIV-1 laboratory isolates that we tested, HIV-1_{SF33} and HIV-1_{LAI}, and a dual-tropic isolate, HIV-1_{89.6}, but could not be infected by either of the M-tropic isolates. The infectivity titers of T-tropic HIV-1 isolates in the sMAGI-CXCR-4 cell line were similar to the TCID calculated by end-point dilution on human PBMCs, indicating that these cell lines are highly sensitive to T-tropic HIV-1 infection. The presence of a C-terminal GFP tag did not significantly inhibit the ability of the sMAGI-CXCR-4-GFP cell line to detect T-tropic HIV-1 isolates relative to the sMAGI-CXCR-4 cell line. The sMAGI-CCR-5 and sMAGI-CCR-5-GFP cell lines were sensitive to infection by the M-tropic isolates HIV-1_{SF162} and HIV-1_{ADA-M}, as well as HIV-1_{89.6}, but not any of the T-tropic isolates tested. The infectivity titers of M-tropic HIV-1 isolates on sMAGI-CCR-5 cells were 10- to 100-fold lower than the TCID determined by end-point dilution in human PBMCs, suggesting that cell surface expression of CCR-5 may be inefficient, as has been demonstrated for other cell types (10). Sorting sMAGI-CCR-5-GFP cells for high GFP expression modestly increased (by approximately twofold) the ability to detect infection by M-tropic HIV-1 isolates relative to the sMAGI-CCR-5 cell line.

We also tested the susceptibility of these cell lines to infection by three primary Kenyan isolates. HIV-1_{M8002}, a clade A virus isolated from a chronically infected individual, productively infected the sMAGI-CXCR-4 cell line, but not sMAGI-CCR-5 cells. The infectious dose of HIV-1_{M8002} on sMAGI-CXCR-4 cells was similar to the TCID determined by end-point dilution on human PBMCs, as was seen for T-tropic clade B viruses. HIV-1_{Q2850}, a clade A virus from a recently infected adult, and HIV-1_{B2059}, a clade D isolate virus from an

TABLE 4. Quantitative analysis of viral infectious dose in sMAGI-CXCR-4 and sMAGI-CCR-5 cells versus end-point dilution in human PBMCs

Virus tested	Value for:					
	sMAGI-CXCR-4 ^a	sMAGI-CXCR-4-GFP ^a	sMAGI-CCR-5 ^a	sMAGI-CCR-5-GFP ^a	Human PBMCs ^b	MAGI CCR-5 ^a
Clade B						
HIV-1 _{SF162}	<10	<10	9×10^2	2×10^3	1×10^5	5×10^4
HIV-1 _{ADA-M}	<10	<10	5×10^2	1×10^3	1×10^4	2×10^4
HIV-1 _{SF33}	7×10^4	7×10^4	<10	<10	1×10^5	1×10^5
HIV-1 _{LAI}	4×10^4	3×10^4	<10	<10	1×10^5	1×10^5
HIV-1 _{89.6}	2×10^4	2×10^4	5×10^2	7×10^2	6×10^4	5×10^4
Clade A						
HIV-1 _{M8002}	1×10^4	NT ^c	<10	NT	3×10^4	5×10^4
HIV-1 _{Q2850}	<10	NT	1×10^2	NT	1×10^3	3×10^3
Clade D						
HIV-1 _{B2059}	<10	NT	7×10^1	NT	1×10^3	5×10^3

^a No. of blue nucleated centers per milliliter of virus.^b TCID per milliliter in human PBMCs by end-point dilution.^c NT, not tested.

infected infant, replicated in the sMAGI-CCR-5 cell line but not in sMAGI-CXCR-4 cells. As was seen with clade B isolates, the infectious dose on sMAGI-CCR-5 cells was approximately 10- to 100-fold lower than the TCID determined by end-point dilution in human PBMCs. Thus, despite the fact that blocks to HIV-1 replication appear to occur subsequently to viral entry, expression of the HIV-1 coreceptors CXCR-4 and CCR-5 allows these cell lines to be infected by T- and M-tropic laboratory HIV-1 isolates, as well as phenotypically and geographically diverse primary isolates.

Both HIV-1 and SIV infect a MAGI-CCR-5 cell line. Our data suggested that HIV-1 cannot optimally function with a macaque coreceptor. SIV, on the other hand, cannot infect human HeLa-CD4 (MAGI) cells. MAGI cells can be infected by T-tropic HIV-1 isolates, but not M-tropic HIV-1 (26), suggesting that HeLa cells may not express CCR-5 but do express CXCR-4 or another protein that functions as a T-tropic HIV-1 coreceptor. To determine if either human coreceptor could relieve the block to SIV infection in MAGI cells, we engineered the cells to express human CCR-5. The MAGI-CCR-5 cell line was susceptible to all HIV-1 isolates that we tested, including M-tropic and primary HIV-1 strains (Table 4). Moreover, the infectivity titers of all HIV-1 isolates on the MAGI-CCR-5 cell line were similar to the TCIDs calculated by end-point dilution on human PBMCs and were approximately 10- to 100-fold higher than results with the sMAGI-CCR-5 cells. This could reflect the fact that the MAGI-CCR-5 cell line was generated from a single cell clone that expresses high levels of CCR-5, whereas the sMAGI-CCR-5 cell line was generated from a pool of cells; on the other hand, it could indicate that other species-specific differences affect CCR-5 expression or function. Regardless, the key result observed here was that the MAGI-CCR-5 cell line was highly susceptible to SIV infection. The titer of SIVMneCL8 in MAGI-CCR-5 cells was 2×10^4 , which was similar to the titers determined with sMAGI cells and the TCID calculated by end-point dilution on CEMx174 cells, which were 5×10^4 . SIVMneCL8 did not infect the parent MAGI cell line, indicating that the human CCR-5 allele can serve as an SIV coreceptor.

Effects of MIP-1 β on viral infection. MIP-1 β , a β -chemokine that binds to CCR-5 (44), competitively inhibits the ability of M-tropic HIV-1 isolates to infect cells expressing CCR-5 (12, 14, 16), presumably by interfering with the ability of virus to interact with the coreceptor. We tested the ability of MIP-1 β to inhibit the infection of M-tropic HIV-1 isolates in two CCR-

5-expressing cell lines, the sMAGI-CCR-5-GFP cell line and the MAGI-CCR-5 cell line. Consistent with earlier published reports, MIP-1 β partially inhibited (by approximately 50 to 65%) infection of both CCR-5-expressing cell lines by the macrophage-tropic isolate HIV-1_{SF162} (Fig. 2). No inhibition

FIG. 2. Inhibition of viral infectivity by the β -chemokine MIP-1 β . sMAGI cells (A), sMAGI cell expressing human CCR-5 (B), or MAGI cells expressing CCR-5 (C) were infected with SIV, SHIV, or HIV-1 in the presence or absence of 100 ng of MIP-1 β per ml. The cells were infected with approximately 250 sMAGI, sMAGI-CCR-5, or MAGI-CCR-5 infectious viral particles, respectively, and the numbers of infected cells in macaque sMAGI cells (A and B) or human MAGI cells (C) were assayed. Error bars show standard deviations.

of infection was observed when MAGI-CCR-5 cells were infected with the T-tropic isolate HIV-1_{SF33} in the presence of MIP-1 β , indicating that MIP-1 β inhibition is specific to M-tropic HIV-1 isolates that utilize CCR-5 as a coreceptor. While MIP-1 β inhibited SHIV-1_{SF162} infection in the MAGI-CCR-5 cell line, it had no effect on the ability of SIVMneCL8 to infect these cells, suggesting that SIV interacts with CCR-5 in a manner different from that of viruses that express the envelope protein from HIV-1_{SF162}. Significantly, MIP-1 β had no effect on the replication of SIVMneCL8 or SHIV-1_{SF162} in the sMAGI cell line, which might indicate that SIVMneCL8 and SHIV-1_{SF162} use the same coreceptor in this macaque cell line.

DISCUSSION

A rhesus macaque cell line that expresses the human CD4 molecule is highly susceptible to SIV infection but cannot be productively infected by HIV-1 (8). Our studies show that at least a subset of HIV-1 isolates can enter macaque cells expressing human CD4 (sMAGI cells) but are blocked at later stages in replication. The postentry restriction in HIV-1 replication can be relieved by expression of the appropriate human coreceptor gene.

HIV-1 infection of the sMAGI cell line was blocked at two distinct stages of the virus replication cycle, depending on the isolate tested. We were unable to detect newly reverse transcribed viral DNA in sMAGI cells infected with virus from the majority of HIV-1 proviral clones and one of four primary isolates that we tested, indicating that the replication of these viruses was blocked at a step prior to RT. Our experiments do not distinguish between a block at entry or immediately after entry with these isolates. However, previous reports showed that the replication of T-tropic HIV-1 isolates in monkey PBMCs is restricted at an early step after virus fusion but prior to RT (22, 48). In contrast to the results with most laboratory-adapted isolates, late DNA intermediates of RT were detected in sMAGI cells infected with one M-tropic isolate, HIV-1_{SF162}, and three primary isolates cocultured from individuals at various stages of disease. This indicates that some HIV-1 isolates can enter and initiate RT in the sMAGI cell line. However, we did not detect nuclear viral DNA in cells infected with these HIV-1 isolates, suggesting that these viruses were restricted in their ability to migrate to the nucleus. These data for sMAGI cells are similar to the blocks to replication described for some T-tropic HIV-1 and SIV clones in macrophages (24, 40, 46). In the context of the studies presented here, these restrictions to replication may be explained by an impaired or suboptimal interaction between the cellular coreceptor and envelope that is influenced by a second viral determinant.

Chimeric SHIVs containing the *env*, *tat*, and *rev* reading frames from T- and M-tropic HIV-1 isolates readily infected sMAGI cells. These same SHIV chimeras can productively infect PBMCs from rhesus macaques (22, 35). Together, these results indicate that the HIV-1 envelope can function to allow a productive infection of macaque cells and, thus, that the HIV-1 envelope presumably interacts with a macaque coreceptor. Interestingly, infection of sMAGI cells with M-tropic SHIV, like SIV, is not inhibited by the β -chemokine MIP-1 β under conditions that inhibit interactions between HIV-1 and the human CCR-5 allele. Because both sMAGI cells and macaque PBMCs are highly susceptible to SIV infection, one candidate for the coreceptor used by HIV-1 in these cells is a SIV coreceptor. SHIV chimeras containing HIV-1 envelopes conferring T-cell and macrophage tropism productively infected the sMAGI cell line, indicating that either HIV-1 envelope

is competent to interact with putative macaque-SIV coreceptor(s).

HIV-1 could productively infect sMAGI cells expressing the human allele of the HIV-1 coreceptors. The sMAGI-CXCR-4 cell line was susceptible to infection by T-tropic HIV-1 isolates, and the sMAGI-CCR-5 cell line was susceptible to infection by M-tropic HIV-1 isolates. Both cell lines were permissive to infection by clade A, B, and D HIV-1 isolates, indicating that phenotypically and geographically diverse HIV-1 isolates replicate in these cell lines when the appropriate coreceptor is expressed. Significantly, CCR-5 or CXCR-4 expression alleviated both the pre- and post-RT replication blocks that we had observed upon HIV-1 infection of sMAGI cells. Thus, our experiments show that the restriction against HIV-1 replication in macaque cells could be overcome in either of two ways: expression of the human coreceptor or complementation of HIV-1 envelope with SIV gene products. Taken together, these data provide the first indication that the coreceptor functions at postentry steps in the virus replication cycle in a manner that is apparently dependent on a second viral protein.

Interestingly, although the macaque coreceptor in sMAGI cells does not support productive infection of HIV-1 in the absence of SIV proteins, the human allele of CCR-5 can function as a coreceptor for SIV infection in the absence of HIV-1 proteins. Expression of human CCR-5 in a human HeLa-CD4 cell line that is otherwise not susceptible to SIV infection rendered these cells highly sensitive to SIV infection. Moreover, human cells expressing the human CCR-5 gene are susceptible to infection by a variety of SIV isolates, including both T- and M-tropic variants (32a), suggesting that the macaque CCR-5 homolog may function as a dominant coreceptor for SIV infection. The differences in the ability of SIV and HIV-1 to function with other primate lentiviral coreceptors may be noteworthy in terms of the potential of these viruses for cross-species transmission. Our data, which show that SIV can efficiently infect human cells expressing the human HIV-1 receptor and coreceptor, suggest that SIV is a less species-restricted virus than HIV-1, which does not function optimally with the macaque SIV coreceptor.

MIP-1 β does not block SIV infection in human cells expressing human CCR-5 under conditions in which HIV-1 infection can be inhibited in these cells. These data suggest that the binding sites on the human coreceptor may be different for SIV and HIV-1. The mechanism(s) by which the β -chemokines function to inhibit HIV-1 interactions with the CCR-5 coreceptor is unclear. It is possible that these polypeptides directly compete with an HIV-1 protein for binding sites on their receptors; alternatively, binding of the chemokines to the receptors could downregulate receptor cell surface expression. This second model is inconsistent with our data demonstrating that when M-tropic HIV-1 infection is inhibited by chemokines in MAGI-CCR-5 cells, SIV infection is not affected.

Previous studies have demonstrated a role for the chemokine coreceptors in HIV-1 fusion, suggesting these proteins participate in viral entry (2, 3, 10, 14–16, 19). The specificity of the T- and M-tropic isolates for different coreceptor proteins further suggests a role for the coreceptor in envelope-mediated entry. The studies presented here, which demonstrate that coreceptor expression can alleviate restrictions in postentry events in the HIV-1 replication cycle, suggest that entry may be coupled to later events in virus replication by the coreceptor. Our data with SHIV chimeras suggest that this cascade involves interactions between the coreceptor, envelope protein, and a second viral protein. Previous studies of SHIVs have suggested that the restriction to replication in macaque cells maps to a region spanning the 3' half of the LTR, *gag*, and *pol*

(48). Thus, specific interactions between 5' viral and cellular elements, much like those previously defined with strains of MuLV which are restricted by the Fv-1 locus (4, 5) or with cyclophilins and HIV-1 (20, 34, 49), could be responsible for blocks to viral replication. Because the SHIV data indicate that Gag is one candidate for the viral determinant that restricts HIV-1 replication, an intriguing possibility for a protein that interacts with the coreceptor to allow productive HIV-1 infection is the matrix protein, which has been shown to be important for nuclear translocation of the HIV-1 preintegration complex to the nucleus (6).

The ability of the coreceptor to alleviate postentry blocks in HIV-1 replication is consistent with a model in which the coreceptor continues to interact with the viral core after fusion. A second hypothesis to explain the data presented here is that there is a signaling event(s) triggered by interaction between viral components and chemokine receptors that influences later steps in viral replication, such as nuclear localization. The ability of SIV proteins to complement the HIV-1 envelope in this process provides a powerful tool to define the second viral gene involved in these pathways. Identification of the species-specific viral determinant for HIV-1 coreceptor function will allow biochemical analysis of the specific interactions between this protein, envelope, and coreceptor that are required for HIV-1 infection. Such studies will be important for elucidating coreceptor function in the virus replication cycle and for designing strategies to inhibit HIV-1 replication at both entry and later stages of infection.

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