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Adaptation of the Human Immunodeficiency Virus Type 1 Envelope Glycoproteins to New World Monkey Receptors▽

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Human immunodeficiency virus type 1 (HIV-1) infection encounters an early block in the cells of New World monkeys because the CD4 receptor does not efficiently support HIV-1 entry. We adapted HIV-1(NL4-3) and HIV-1(KB9), two HIV-1 variants with different envelope glycoproteins, to replicate efficiently in cells expressing the CD4 and CXCR4 proteins of the common marmoset, a New World monkey. The HIV-1(NL4-3) adaptation involves three gp120 changes that result in a specific increase in affinity for the marmoset CD4 glycoprotein. The already high affinity of the HIV-1(KB9) envelope glycoproteins for marmoset CD4 did not significantly change as a result of the adaptation. Instead, changes in the gp120 variable loops and gp41 ectodomain resulted in improved replication in cells expressing the marmoset receptors. HIV-1(KB9) became relatively sensitive to neutralization by soluble CD4 and antibodies as a result of the adaptation. These results demonstrate the distinct mechanistic pathways by which the HIV-1 envelope glycoproteins can adapt to less-than-optimal CD4 molecules and provide HIV-1 variants that can overcome some of the early blocks in New World monkey cells.

The primate lentiviruses include the human immunodeficiency virus (human immunodeficiency virus type 1 [HIV-1] and HIV-2) and simian immunodeficiency virus (SIV). In nature, HIV-1 and HIV-2 infect humans, HIV-1-related SIV_{cpz} viruses infect chimpanzees, and SIV variants infect African monkeys (3, 16, 31, 32). Humans infected with HIV-1 and HIV-2 and Asian macaques infected by certain SIVs and HIV-2 often develop life-threatening immunodeficiency (AIDS) due to depletion of CD4-positive T lymphocytes (3, 9, 11, 14, 16, 21).

Animal models of HIV-1 infection and disease have been used to study viral pathogenesis and to test antiviral therapies and vaccines. Current animal models of HIV-1 infection have limitations. Chimpanzees can be infected with HIV-1 and the closely related SIV_{cpz}. However, chimpanzees are an endangered species, are prohibitively expensive, and are available only in limited numbers (35). Moreover, HIV-1-infected chimpanzees rarely progress to AIDS-like disease (1, 4, 15, 37, 38, 47).

Old World monkeys can be naturally infected with various SIV strains, including close relatives of HIV-2. By contrast, the cells of most Old World Monkeys exhibit a postentry block to HIV-1 infection that targets the viral capsid and is mediated by TRIM5α (19, 39). The infection of Asian macaques by SIV exhibits many similarities to HIV-1 infection of humans, making this model useful for studies of prophylaxis and pathogenesis. However, the inability of many HIV-1-directed modalities to interfere with SIV infection has prompted the development of chimeric simian-human immunodeficiency viruses (SHIVs). SHIVs contain elements of HIV-1 (e.g., reverse transcriptase (RT) or envelope glycoproteins) in an SIV genetic background. Some SHIVs replicate and cause AIDS-like disease in macaques (20, 42). Nonetheless, because many antiviral agents and immune responses are directed against HIV-1 elements not included in the available SHIV chimerae, the development of new animal models involving infection with more complete HIV-1-like viruses is a worthy goal.

To this end, the infection of New World monkeys by HIV-1 has been investigated. The early postentry barriers to HIV-1 infection found in Old World monkeys are not present in the cells of most New World monkeys. The principal early block to HIV-1 infection in New World monkey cells occurs at the level of virus entry (19, 30, 44). HIV-1 entry involves the viral gp120 (SU) and gp41 (TM) envelope glycoproteins, which are organized into trimers on the virion surface (6, 13, 48, 52). The binding of gp120 to CD4 allows the interaction of gp120 with one of two chemokine receptors, CCR5 or CXCR4. Receptor binding triggers additional conformational changes in the HIV-1 envelope glycoproteins that promote the fusion of the viral and target cell membranes. The CXCR4 molecule of New World monkeys such as squirrel monkeys and common marmosets can act as an efficient receptor for HIV-1; by contrast, CD4 and CCR5 from these species do not effectively bind HIV-1 gp120, leading to an entry block (19, 30, 44). The discrete nature of the early replication block in these New World monkeys and the partial functionality of the common marmoset receptors raised the possibility that this barrier might be

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overcome by direct adaptation of the virus. Here, starting with two different HIV-1 isolates with distinct envelope glycoproteins, we derived and characterized virus variants able to enter cells by using common marmoset (*Callithrix jacchus)* CD4 and CXCR4 as receptors.

MATERIALS AND METHODS

Cell lines and antibodies. 293T and Cf2Th cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (DMEM-10).

To generate Cf2Th cell lines stably expressing CXCR4, cells were transfected with pcDNA3.1/Hygro(+) expressing human or common marmoset CXCR4. Clones were selected in DMEM-10 supplemented with 200 μg/ml of hygromycin B (Roche). Cells expressing high levels of CXCR4 (Cf2Th-CXCR4^{hi}) were enriched by fluorescence-activated cell sorting with the 12G5 anti-CXCR4 monoclonal antibody (Pharmingen). Cells expressing CD4 and CXCR4 (Cf2Th-CD4/CXCR4) were generated by transfecting the Cf2Th-CXCR4^{hi} cells with pZeoSV2(+) expressing human or common marmoset CD4. Clones were selected in DMEM-10 supplemented with 200 μg/ml of zeocin (Invitrogen) and 200 μg/ml of hygromycin B. Single clones of cells expressing human or common marmoset CD4 and CXCR4 (Cf2Th-HuCD4/CXCR4 or Cf2Th-CjCD4/CXCR4, respectively) were obtained by limiting serial dilution. Expression of CD4 and CXCR4 was determined by flow cytometry with the Q4120 and 12G5 antibodies, respectively.

Soluble CD4 (sCD4) and the neutralizing antibody 1121 were purchased from ImmunoDiagnostics, Inc. The 1121 antibody recognizes the third variable (V3) loop of HIV-1 gp120. The neutralizing monoclonal antibody immunoglobulin G1b12 (IgG1b12), which recognizes a conserved gp120 epitope near the CD4-binding site, was a kind gift of D. Burton (The Scripps Research Institute). The C11 antibody, which recognizes the gp41-interactive N- and C-terminal regions of gp120, was obtained from J. Robinson (Tulane Medical Center). M. Posner (Dana-Farber Cancer Institute) provided the F105 antibody directed against the CD4-binding site of HIV-1 gp120. The 2F5 antibody, which recognizes the membrane-proximal region of gp41, was supplied by H. Katinger (Vienna, Austria). The CXCR4 inhibitor AMD3100 (bicyclam JM-2987) (5, 10, 18) was obtained though the NIH AIDS Research and Reference Reagent Program.

Virus replication. Replication-competent HIV-1 viruses were generated by transfecting 20 μg of the pNL4-3 plasmid, which contains an infectious HIV-1_{NL4-3} provirus (33), or 20 μg of the pNL4-KB9 plasmid, which contains the KB9 envelope in an NL4-3 proviral background, into 2×10^6 293T cells using the calcium phosphate transfection method (Invitrogen). Forty-eight hours after transfection, supernatants containing these viruses [herein designated HIV-1(NL4-3) and HIV-1(KB9), respectively] were harvested and cleared by low-speed centrifugation. The level of virus in the supernatant was determined by measuring RT, as described previously (43).

Cells were infected with 3,000 RT units for 14 h and then washed once with phosphate-buffered saline (PBS). Every 3 or 4 days, cell supernatants were removed and used for RT assays. Cells were trypsinized, diluted 1:10 in fresh medium, and replated.

Analysis of *env* sequences. The envelope genes of the adapted viruses were amplified by PCR of genomic DNA isolated from infected cells with the QIAamp DNA blood minikit (QIAGEN). A 2.7-kb fragment containing the full *env* sequence was generated by PCR with *Pfu*Ultra High-Fidelity DNA polymerase (Stratagene) and the primers Env-forward (5′-GATA*GAATTC*AAGAGCAGA AGACAGTG-3′) and Env-reverse (5′-TTTCTAGGTCTCGAGATA*CTGCTC*-3′) with EcoRI and XhoI restriction sites (marked in italics), respectively. The 2.7-kb fragment was cloned into the pcDNA3.1/Zeo(+) plasmid (Invitrogen) in the case of HIV-1(NL4-3) adaptation or into pCR4blunt-TOPO (Invitrogen) in the case of HIV-1(KB9) adaptation. The inserts from three to five individual clones were sequenced to obtain the consensus sequence in the envelope region of the adapted virus.

Site-directed mutagenesis. The sequence changes in *env* associated with viral adaptation to common marmoset receptors were introduced individually or in combination into the pSVIIIenv-NL4-3 or pSVIIIenv-KB9 plasmid expressing the full-length HIV-1(NL4-3) or HIV-1(KB9) envelope glycoprotein, respectively, by site-directed mutagenesis using the QuikChange II XL site-directed mutagenesis protocol (Stratagene). The presence of the desired mutations was determined by automated DNA sequencing.

***env* complementation assay.** Envelope complementation assays were performed using HIV-1 viruses capable of only a single round of infection, as reported previously (23). Briefly, recombinant luciferase-expressing HIV-1 viruses (17) were generated by transfecting 293T cells with 4 μg of the pSVIIIenv plasmid expressing the HIV-1 envelope glycoproteins of interest, 4 μg of the pCMVΔP1ΔenvpA packaging plasmid, and 12 μg of an HIV-1 vector plasmid, using the calcium phosphate transfection method (Invitrogen). The HIV-1 vector plasmid expresses an RNA that can be packaged into virions, reverse transcribed, and integrated into a target cell, where it encodes firefly luciferase. Forty-eight hours after transfection, supernatants containing reporter viruses were harvested and cleared by low-speed centrifugation. The amounts of virus in the supernatants were quantified by measuring RT activity.

Target cells were seeded at a density of 6,000 cells/well in 96-well luminometer-compatible tissue culture plates. Twenty-four hours later, medium was changed and 50,000 RT units of viruses were added to the cells. After 14 h of incubation, the supernatant was removed and fresh medium was added. Forty-eight hours later, the medium was removed and cells were lysed with 30 μl of passive lysis buffer (Promega). Luciferase activity was measured using an EG&G Berthold LB 96V microplate luminometer in accordance with the luciferase assay system technical bulletin from Promega. For neutralization assays, the viruses were incubated for 1 h at room temperature with the antibody or the inhibitor before being added to the target cells.

HIV-1 gp120-CD4 binding assays. The expression of HIV-1 gp120 glycoproteins was achieved in 293T cells transfected with pSVIIIenv plasmids in which a stop codon had been introduced in the region encoding the gp120-gp41 junction and a plasmid expressing the HIV-1 Tat protein. The media containing gp120 proteins were harvested 48 h after transfection, and gp120 proteins were purified by immunoaffinity chromatography using an IgG1b12-Sepharose column. The purity and concentration of gp120 proteins were determined by Coomassie staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels and measurement of absorbance at 280 nm, respectively.

Different concentrations of purified gp120 proteins were incubated for 1 h at room temperature with 2×10^6 Cf2Th cells stably expressing either human or marmoset CD4 in a total volume of 50 μl of PBS containing 5% fetal bovine serum (FBS). The cells were then washed once with PBS–5% FBS and resuspended in 50 μl of PBS–5% FBS containing 3 μg of the C11 anti-gp120 monoclonal antibody. The cell-antibody mixtures were incubated 45 min at room temperature, washed once with PBS–5% FBS, and resuspended in 20 μl of PBS–5% FBS containing 1 μl of anti-human phycoerythrin-conjugated IgG (Jackson ImmunoResearch Laboratories). After 30 min of incubation, cells were washed twice and analyzed by fluorescence-activated cell sorting using a Beckman flow cytometer.

Processing and subunit association of envelope glycoproteins. Cf2Th cells were transfected with the pSVIIIenv plasmid and a plasmid expressing the HIV-1 Tat protein using Lipofectamine 2000 (Invitrogen). Six hours after transfection, the medium was changed to a cysteine/methionine-free medium and the cells labeled with 200 μCi of [^{35}S]cysteine/methionine labeling mixture for approximately 16 h. The cell culture medium was cleared by centrifugation at 2,000 rpm for 10 min at 4°C. Cells were washed with PBS and lysed with 1 ml of lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% Igepal CA-630, and 1× protease inhibitor cocktail) for 1 h at 4°C. Cell debris was removed by centrifugation at 16,000 × *g* for 30 min at 4°C. The total amount of protein in the cleared cell lysates was normalized by measuring cpm of radioactivity. Immunoprecipitations were carried out in a total volume of 1 ml by incubating the cleared cell lysates or culture medium with 15 μl of protein A-Sepharose CL-4B beads (Amersham Biosciences) and 3 μl of a pool of sera from HIV-1-infected individuals. After two washes with 1 ml of lysis buffer, the beads were resuspended in 35 μl of 1× lithium dodecyl sulfate loading buffer with 5% β-mercaptoethanol and boiled for 5 min. Samples were analyzed on a 4 to 12% SDS-polyacrylamide gel (Bis-Tris NuPAGE; Invitrogen).

Molecular modeling and energy calculations. All protein modeling and energy calculations were performed on the Discovery Studio platform (Accelrys Software, Inc.). Protein modeling was based on protein sequence homology and alignment with proteins of known structure, using the Modeler (version 9) program in the Accelrys software package. Modeling of the NL4-3 gp120 core was based on the structure of the HXBc2 HIV-1 gp120 core (91.2% sequence identity). The V4 variable loop was specifically refined by the Loop Refinement Program in Modeler. The model of domains 1 and 2 of common marmoset CD4 was based on the structure of these domains in human CD4 (sequence identity, 72.4%). The structure of the NL4-3 gp120 core in complex with marmoset two-domain CD4 was derived by superposition of the modeled NL4-3 gp120 core and marmoset CD4 onto the HXBc2 gp120 core and human CD4, respectively, in the HXBc2 gp120 core/human two-domain CD4/17b Fab complex (1G9M). The Cα atom root mean squared deviations of the NL4-3 gp120 core and marmoset CD4 from their respective reference structures were 0.29 and 0.30 Å.

Energies were calculated using the CHARMm (Chemistry at Harvard Mac-

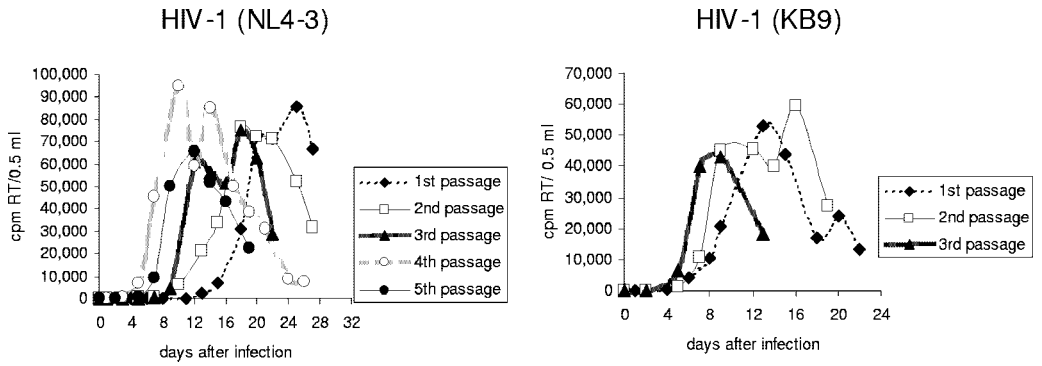

FIG. 1. Adaptation of HIV-1 for replication in cells expressing common marmoset CD4 and CXCR4. The indicated viruses were passaged in Cf2Th-CjCD4/CXCR4 cells. The RT levels in the cell supernatants are shown as a function of the time after virus-cell incubation.

romolecular Mechanics) program (32b1). The energies of interaction between gp120 core variants and marmoset CD4 were calculated after energy minimization and the introduction of solvent factors by use of the implicit of distance-dependent dielectrics model. Every altered residue underwent side-chain refinement before energy minimization. Energy minimization was achieved in two stages (the initial 1,000 steps on steepest descent, followed by an additional 2,000 steps on adopted basis NR).

RESULTS

Adaptation of HIV-1 for replication in cells expressing common marmoset receptors. With the ultimate goal of obtaining an HIV-1 virus able to infect New World monkey cells, we have adapted two different HIV-1 isolates, HIV-1(NL4-3) and HIV-1(KB9), to use common marmoset CD4 and CXCR4 for virus entry. HIV-1(NL4-3) has envelope glycoproteins derived from an X4 HIV-1 isolate that was passaged in tissue-cultured cell lines. HIV-1(KB9) is identical to HIV-1(NL4-3) except that its envelope glycoproteins are those of SHIV(KB9). SHIV(KB9) is an R5X4, neutralization-resistant virus derived by passage of SHIV(89.6) in monkeys (22, 42). The adaptation of HIV-1(NL4-3) and HIV-1(KB9) was achieved in canine Cf2Th thymocytes stably expressing human or common marmoset CD4 and CXCR4; efficient HIV-1 replication in Cf2Th cells requires expression of primate chemokine receptors (7, 25, 30). To adapt HIV-1 to replicate in Cf2Th cells expressing marmoset receptors, we utilized a protocol previously employed to adapt HIV-1 to replicate in CD4-negative cells (25). First, a 50:50 mixture of Cf2Th cells expressing human or marmoset CD4 and CXCR4 receptors was infected with a stock of either HIV-1(NL4-3) or HIV-1(KB9) prepared by transfection of 293T cells (see Materials and Methods). Viral replication was determined by measuring RT activity in the supernatant of the Cf2Th cultures every 2 to 3 days. In both cases, a peak of RT activity was observed 8 to 10 days after infection, followed by cell death and a drop in RT activity in the supernatant. However, a few days later, the cells started to recover and the RT levels in the supernatants increased. RT activity reached a new peak around 26 to 30 days after infection. Culture supernatants at the time of these second peaks of RT activity were collected and used to infect Cf2Th-CjCD4/CXCR4 cells. In the case of viruses derived from the HIV-1(NL4-3) infection, the RT counts in the supernatant of these cultures remained at background levels for several days; the RT counts started to rise at day 13 after infection and reached a peak at about day 26. The virus stock from this day will be referred to as the first passage of the adapted HIV-1(NL4-3) virus. Adaptation of the HIV-1(KB9) viruses to cells expressing the marmoset receptors occurred more rapidly than the adaptation of the HIV-1(NL4-3) viruses. By 4 days after infection, the RT levels in the culture started to increase, reaching a peak at about day 13. The virus stock from this day will be referred to as the first passage of the adapted HIV-1(KB9) viruses. To attempt to obtain more-robust HIV-1 replication in cells expressing marmoset receptors, we continued passaging these adapted viruses in Cf2Th-CjCD4/CXCR4 cells. As shown in Fig. 1, the kinetics of virus replication improved after five and three passages of the HIV-1(NL4-3) and HIV-1(KB9) viruses, respectively.

Analysis of *env* sequences of the adapted viruses. Virus adaptation to the Cf2Th cells expressing marmoset receptors could involve multiple changes throughout the HIV-1 genome; because we were specifically interested in understanding adaptation to the heterologous CD4 and CXCR4 proteins, we focused on the changes in the envelope glycoproteins of the adapted viruses. The *env* sequences of the adapted viruses were examined by PCR amplification of the genomic DNA of cells from the day when the supernatant RT activity reached a maximum. The amplified *env* DNA from each passage was cloned in a sequencing vector, and three to five clones derived from each passage were sequenced. The Env amino acid changes associated with adaptation to the common marmoset receptors CD4 and CXCR4 are summarized in Table 1. In addition to the Env changes shown in Table 1, a few other changes were observed in some individual clones but were not maintained in subsequent passages; hence, these changes are not likely to be important to the adaptation.

After the first passage of HIV-1(NL4-3), all of the sequenced clones exhibited a conversion of serine 334 to asparagine and serine 398 to asparagine. These two changes were maintained in successive passages. Serine 334 is located at the base of the V3 loop of gp120; the S334N change eliminates an N glycosylation site at asparagine 332. Serine 398 is located in the base of the V4 variable loop of gp120, and its change to asparagine does not modify the glycosylation state of gp120. During the second passage of HIV-1(NL4-3), a new mutation that changes valine 242 in gp120 to isoleucine was introduced into the viral genome. In the third passage, we observed an additional change of alanine 281, a gp120 residue that makes

TABLE 1. Amino acid changes in HIV-1 envelope glycoproteins associated with adaptation to common marmoset receptors[a]

Virus	Predicted amino acid change(s) in Env	Env region	Frequency of change in passage of adapted virus				
			1st	2nd	3rd	4th	5th
HIV-1(NL4-3)	V242I	C2 (gp120)	0/5	1/3	5/5	5/5	5/5
	A281V	C2 (gp120)	0/5	0/3	4/5	5/5	5/5
	S334N	V3 base (gp120)	5/5	3/3	5/5	5/5	5/5
	S398N	V4 base (gp120)	5/5	3/3	5/5	5/5	5/5
	A612T	Loop (gp41)	0/5	0/3	0/5	1/5	5/5
HIV-1(KB9)	E151K/E172K	V1/V2 (gp120)	3/5	2/5	2/5		
	F176Y	V1/V2 (gp120)	2/5	2/5	3/5		
	A561T	HR1 (gp41)	3/5	5/5	5/5		

[a] The frequency of the change is expressed as the number of clones in which the change is found per total number of clones sequenced. Amino acid residue numbers are reported based on alignment with the prototypic HXBc2 sequence, as per current recommendations (26).

direct contact with CD4 (28, 29), to valine. Finally, alanine 612, in the gp41 ectodomain, was converted to threonine during the fourth passage. These five changes (S334N, S398N, V242I, A281V, and A612T) were present in all clones of the HIV-1(NL4-3) virus derived from the fifth passage.

The Env changes identified in the adaptation of HIV-1(KB9) (Table 1) were located in the V1 and V2 variable loops of gp120 and in heptad repeat 1 (HR1) of gp41; notably, the location of these changes on the envelope glycoproteins was very different from that of the changes found upon adaptation of HIV-1(NL4-3). After the first passage, three of the five sequenced clones contained the double changes E151K and E172K and two other clones contained the change F176Y in the V1 and V2 loops. These changes were preserved in subsequent passages, with the exception of one clone derived from the second passage that contained the double change E153K/I154M, also located in the V1/V2 loop (data not shown). The change A561T in HR1 of gp41 was observed in three of five clones derived from the first passage; all of the sequenced clones exhibited this change following an additional passage of the virus.

Influence of envelope glycoprotein changes in the adapted viruses on entry into cells expressing human or marmoset CD4 and CXCR4. The contribution of the observed envelope glycoprotein changes to the ability of the virus to utilize marmoset CD4 and CXCR4 for entry into cells was examined using an envelope complementation assay. The *env* changes that arose during the adaptation were introduced alone or in various combinations into pSVIIIenv plasmids that express the wild-type NL4-3 or KB9 envelope glycoproteins. Recombinant HIV-1 viruses encoding firefly luciferase and pseudotyped with these envelope glycoprotein variants were incubated with Cf2Th cells expressing human or marmoset CD4 and CXCR4. Measurement of relative luciferase units in the target cells provided an indication of the efficiency with which a single round of infection occurred. Thus, the relative efficiency with which different envelope glycoprotein variants utilize either human or marmoset receptors to mediate virus entry can be assessed. Because of potential quantitative and qualitative differences between human and marmoset CD4/CXCR4 expression in the two target cell lines, we refrain from comparing the

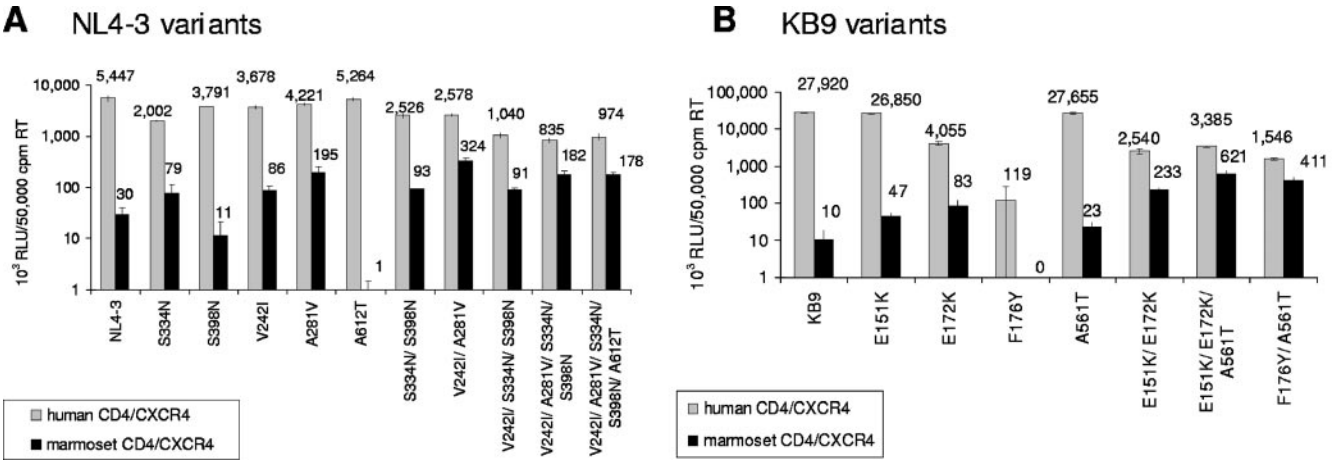

FIG. 2. Ability of wild-type and variant envelope glycoproteins to complement virus entry in cells expressing human CD4/CXCR4 (gray bars) or marmoset CD4/CXCR4 (black bars). HIV-1 vectors encoding luciferase and pseudotyped with the indicated envelope glycoproteins derived from HIV-1(NL4-3) (A) or HIV-1(KB9) (B) were incubated with cells for 14 h at 37°C. After washing and 48 h of culturing, the cells were lysed and assayed for luciferase activity. RLU, relative luciferase units. The results shown are representative of three independent experiments. The values shown are the means of three data points from a single experiment, and the error bars represent the standard deviations.

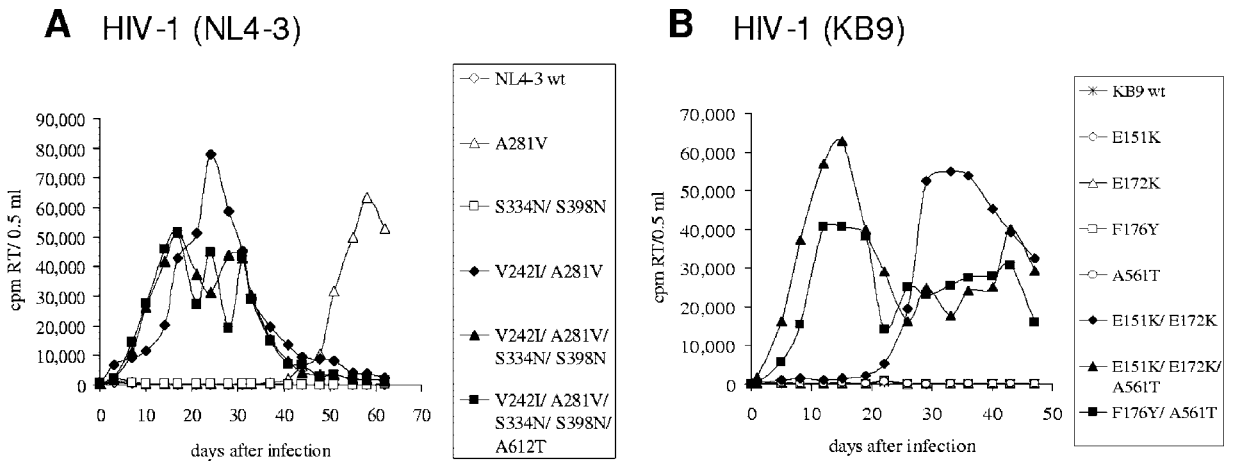

FIG. 3. Replication of adapted viruses in cells expressing common marmoset CD4 and CXCR4. Cf2Th-CjCD4/CXCR4 cells were infected with 40,000 cpm of RT of either HIV-1(NL4-3) (A) or HIV-1(KB9) (B) viruses carrying the indicated envelope glycoprotein variants. The RT activity in the culture supernatants was determined at the indicated times. The results shown are representative of two independent experiments. The RT counts were at background levels for the NL4-3 wild-type (wt) and S334N/S398N virus variants and for the KB9 wt, E151K, E172K, F176Y, and A561T virus variants (open symbols).

relative efficiencies with which human and monkey receptors support the entry of any particular virus variant.

Figure 2A shows the luciferase activity in cells incubated with recombinant HIV-1(NL4-3) variants. Some NL4-3 variants, particularly those containing the S334N change, supported slightly decreased entry into cells expressing human receptors compared with that of the wild-type NL4-3 envelope glycoproteins. The S398N and A612T changes did not increase the efficiency of entry into cells expressing the marmoset receptors. However, the S334N change, which appeared during the first passage, and the V242I change, which appeared during the second passage, enhanced the efficiency of entry into Cf2Th-CjCD4/CXCR4 cells by two- to threefold. The A281V change raised the efficiency of entry into cells expressing the marmoset receptors by about sixfold compared to that of the wild-type NL4-3 envelope glycoproteins. The highest efficiency of entry using the marmoset receptors (~11-fold increase) was observed with the V242I/A281V double mutant. However, virus entry was less efficient for envelope glycoproteins in which these two changes were combined with some of the other Env changes.

The results obtained with HIV-1(KB9) variants are shown in Fig. 2B. The ability of the envelope glycoproteins with the single changes E151K and E172K to support entry into cells expressing marmoset receptors was increased over that of the wild-type KB9 envelope glycoproteins. Entry of the E151K/E172K double mutant in Cf2Th-CjCD4/CXCR4 cells was more than 23-fold greater than that observed for the wild-type KB9 virus. Though the A561T change alone only minimally increased entry into cells expressing the marmoset receptors, when it was combined with the E151K/E172K or F176Y change, severalfold increases in the efficiency of entry resulted. This was especially striking in the case of the F176Y variant, which on its own exhibited low levels of entry into cells expressing either human or marmoset receptors. These results suggest that a subset of the amino acid changes acquired in the envelope glycoproteins as a consequence of adaptation to cells expressing marmoset CD4 and CXCR4 increase the ability of the virus to enter cells by using the marmoset receptors. Several of the observed changes (E172K, F176Y, and E151K/E172K) also decreased the efficiency with which viruses infected cells expressing human receptors.

Replication of adapted viruses in cells expressing marmoset CD4 and CXCR4. To determine whether the adaptation-associated envelope glycoprotein changes were sufficient to allow the virus to replicate efficiently in Cf2Th cells expressing marmoset CD4 and CXCR4, we introduced the *env* changes into replication-competent HIV-1 proviruses containing wild-type HIV-1(NL4-3) and HIV-1(KB9) *env* genes. Viruses generated by transfection of these proviruses were used to infect Cf2Th-CjCD4/CXCR4 cells (Fig. 3). The HIV-1(NL4-3) and HIV-1(KB9) parental viruses were not able to infect the Cf2Th-CjCD4/CXCR4 cells, although they were able to infect Cf2Th-HuCD4/CXCR4 cells efficiently (not shown). All of the variant viruses tested, with the exception of the F176Y variant, also replicated efficiently in cells expressing the human receptors. Several of the HIV-1(NL4-3) and HIV-1(KB9) variants supported very effective replication in Cf2Th-CjCD4/CXCR4 cells. These variants corresponded to those that exhibited a high level of complementation of the single-round infection of *env* deletion viruses in the assay described above. Thus, some combinations of the observed envelope glycoprotein changes are sufficient to confer an improved ability to support infection of cells expressing marmoset CD4 and CXCR4.

Binding of gp120 glycoproteins from adapted viruses to human and marmoset CD4. To determine if the improved ability of some envelope glycoproteins from the adapted viruses to infect cells expressing the marmoset receptors was due to an increase in the affinity of gp120 for marmoset CD4, we studied the binding of soluble gp120 variants to human or marmoset CD4 expressed on the surfaces of cells. Bound gp120 was detected by the C11 anti-gp120 antibody, which recognizes an epitope involving the N and C termini of gp120, and antihuman phycoerythrin-conjugated IgG. None of the adaptation-associated changes in the HIV-1 envelope glycoproteins af-

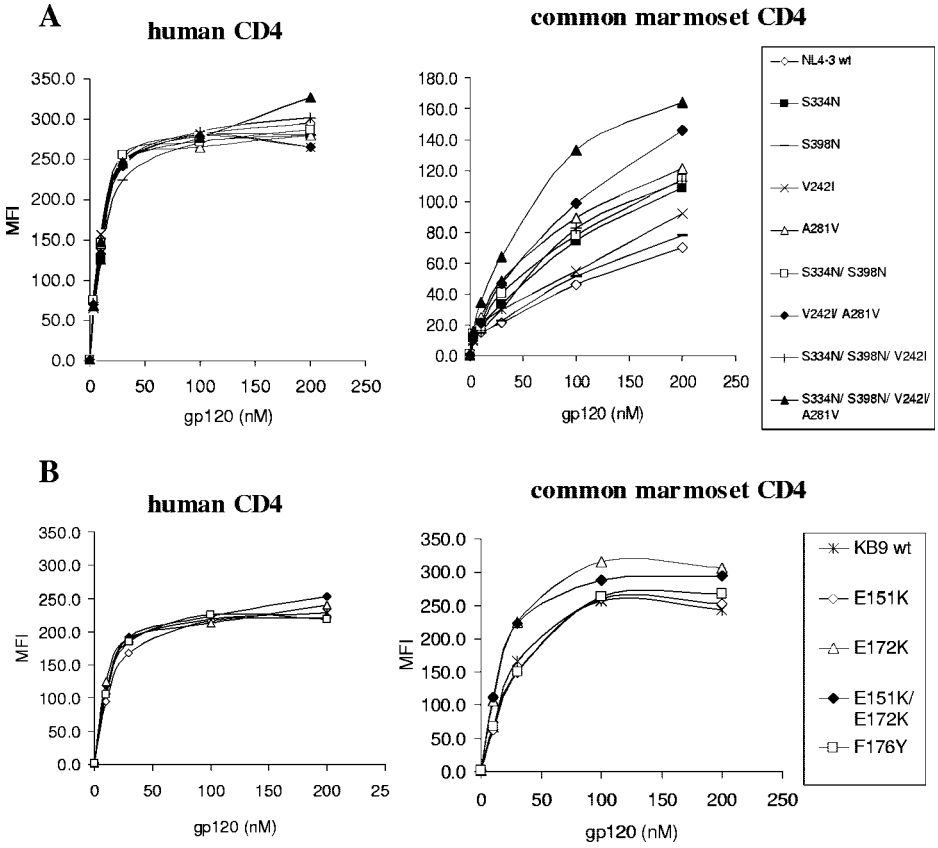

FIG. 4. Binding of wild-type and variant gp120 glycoproteins to human and marmoset CD4 expressed on the surface of Cf2Th cells. The binding of soluble gp120 derived from HIV-1(NL4-3) (A) or HIV-1(KB9) (B) variants to the CD4-expressing cells was studied by using flow cytometry, as described in Materials and Methods. MFI, mean fluorescence intensity. The results shown are representative of two or more independent experiments.

fected recognition by the C11 antibody (data not shown). The binding of the wild-type HIV-1(NL4-3) and HIV-1(KB9) gp120 glycoproteins to control CD4-negative Cf2Th cells was at the background of the assay, close to the value (mean fluorescence intensity = 1 to 2) observed with Cf2Th-CD4 cells when gp120 was not included in the assay (data not shown). Figure 4A shows the binding of HIV-1(NL4-3) gp120 variants to human (left panel) or marmoset (right panel) CD4, and Fig. 4B shows the results for HIV-1(KB9) gp120 variants. In all cases, the affinity for human CD4 was similar for the wild-type and variant gp120 glycoproteins. In the case of NL4-3 variants, we observed a good correlation between binding of gp120 to marmoset CD4 and entry into cells expressing the marmoset receptors. The wild-type and S398N NL4-3 gp120 glycoproteins exhibited a relatively low binding affinity for marmoset CD4. S334N/S398N/V242I/A281V gp120 exhibited the highest binding affinity for marmoset CD4, followed by the V242I/A281V double mutant. The single changes A281V and S334N improved the binding to marmoset CD4 by about twofold over binding of wild-type NL4-3 gp120.

The binding of wild-type KB9 gp120 to common marmoset CD4 was surprisingly efficient. This robust ability of KB9 gp120 to bind marmoset CD4 might be unusual, since the gp120 from HIV-1(89.6), the parent of HIV-1(KB9), doesn't bind efficiently to marmoset CD4 (data not shown). The E151K and F176Y gp120 glycoproteins bound marmoset CD4 comparably to binding of wild-type KB9 gp120, whereas the E172K and E151K/E172K gp120 glycoproteins exhibited a slight but consistent increase in binding to marmoset CD4 relative to that of KB9 gp120. Thus, the adaptation of HIV-1(NL4-3) involves an increase in the low affinity of the gp120 glycoprotein for marmoset CD4, whereas the already efficient KB9 gp120 undergoes less of an increase in affinity for marmoset CD4 during HIV-1(KB9) adaptation.

Processing and subunit association of envelope glycoproteins. The expression of wild-type and variant envelope glycoproteins was examined. The processing of the gp160 envelope glycoprotein precursor of all of the HIV-1(NL4-3) variants was efficient (Fig. 5, left panels). The processing of most of the HIV-1(KB9) envelope glycoproteins was efficient, with the exception of the F176Y and F176Y/A561T envelope glycoproteins, the processing of which was markedly reduced compared to that of the wild-type glycoprotein. Evaluation of the ratio of gp120 associated with the cells and gp120 in the medium (Table 2) demonstrated that slight decreases in gp120-gp41 association were observed for a few mutants, which might explain the lesser ability of these variants to complement virus entry in Cf2Th-HuCD4/CXCR4 cells.

Neutralization sensitivities of Env mutants. Sensitivity to inhibition by soluble CD4, neutralizing antibodies, and small-

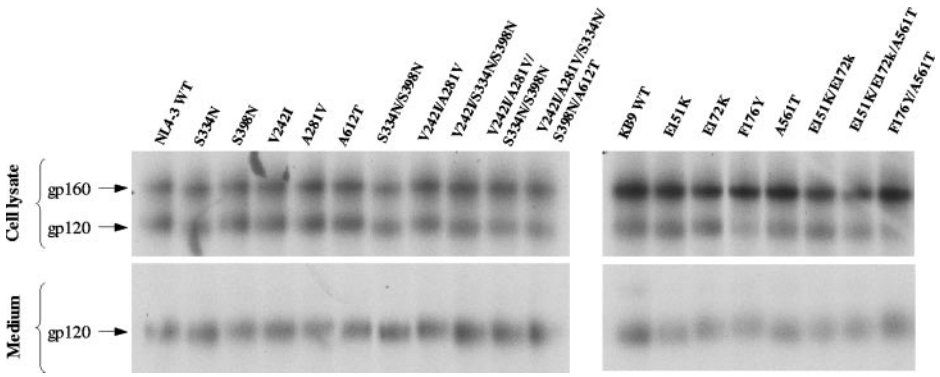

FIG. 5. Processing and subunit association of the wild-type and variant envelope glycoproteins. Cf2Th cells transfected with plasmids expressing the indicated envelope glycoprotein variants were radiolabeled with [^{35}S]cysteine/methionine for approximately 16 h. The cell supernatants and cell lysates were immunoprecipitated with a pool of sera from HIV-1-infected individuals and analyzed by 4-to-12%-gradient SDS-polyacrylamide gel electrophoresis. Similar results were obtained with 293T cells (data not shown).

molecule ligands of chemokine receptors can provide an indication of changes in the receptor-binding regions of the HIV-1 envelope glycoproteins (24, 41). We tested the sensitivities of the envelope glycoprotein variants from the adapted viruses to neutralization by different antibodies and compounds. Table 3 shows the concentration of antibody or inhibitor that reduces the infection of Cf2Th-HuCD4/CXCR4 cells by 50%. The HIV-1(NL4-3) variants were generally more sensitive to neutralization by antibodies and by sCD4 than the HIV-1(KB9) variants. All of the HIV-1(NL4-3) variants exhibited similar levels of sensitivity to neutralization by antibodies and sCD4, with the mutants containing the V242I change exhibiting slightly greater (~2-fold) sensitivity. As previously reported (22), wild-type HIV-1(KB9) is very resistant to neutralization by antibodies that bind gp120 (IgG1b12, F105, and 1121). The changes in the HIV-1(KB9) envelope glycoproteins associated with adaptation to marmoset receptors generally increased the sensitivity to antibody and sCD4 neutralization. Thus, adapta-

TABLE 2. Phenotypes of HIV-1 envelope glycoprotein variants from viruses adapted to common marmoset receptors CD4 and CXCR4

Envelope variant	gp160 processing[a]	Subunit association[a]	Relative entry in Cf2Th-CjCD4/CXCR4 cells[b]	Virus infectivity in Cf2Th-CjCD4/CXCR4 cells[c]	Binding to CjCD4[d]
Wild-type NL4-3	++++	++++	1	−	+
NL4-3 variants					
S334N	++++	+++	3	ND	++
S398N	++++	++++	0.38	ND	+
V242I	+++	+++	3	ND	+
A281V	+++	++++	6	55	++
A612T	+++	++++	0.03	ND	NA
S334N/S398N	++++	++	3	−	++
V242I/A281V	+++	+++	11	26–29	+++
V242I/S334N/S398N	+++	++	3	ND	++
V242I/A281V/S334N/S398N	+++	++	6	26–29	+++
V242I/A281V/S334N/S398N/A612T	+++	++	6	26–29	NA
Wild-type KB9	++	+++	1	−	++++
KB9 variants					
E151K	++	+++++	5	−	++++
E172K	++	++++	8	−	+++++
F176Y	+	+++	0.02	−	++++
A561T	++	++++	2	−	NA
E151K/E172K	++	+++++	23	29–39	+++++
E151K/E172K/A561T	++	++++	62	12–15	NA
F176Y/A561T	+	++	41	12–15	NA

[a] The efficiency of processing and subunit association are reported as follows: +++++, greater than 110% of level for wild-type NL4-3; ++++, 90 to 110% of level for wild-type NL4-3; +++, 70 to 90% of level for wild-type NL4-3; ++, 50 to 70% of level for wild-type NL4-3; +, less than 50% of level for wild-type NL4-3.

[b] The entry efficiency was determined as described in the legend to Fig. 2 and in Materials and Methods. The results are reported as *n*-fold increases with respect to the levels of entry supported by the respective wild-type NL4-3 and KB9 envelope glycoproteins.

[c] The infectivities of the viruses were determined as described in the legend to Fig. 3 and in Materials and Methods. The days when the RT activity level reached a maximum are indicated. Lack of detectable virus replication is indicated by a minus sign. ND, not determined.

[d] The binding of soluble gp120 to CjCD4 was determined as described in the legend to Fig. 4 and in Materials and Methods. More-efficient binding is expressed as an increasing number of plus signs, with the "+" symbol indicating a binding affinity comparable to that of wild-type NL4-3 gp120 for CjCD4 and "+++++" indicating a binding affinity comparable to that of wild-type NL4-3 gp120 for human CD4. NA, not applicable.

TABLE 3. Neutralization of pseudotyped HIV-1 viruses by antibodies and entry inhibitors

Envelope variant	IC_{50} (μg/ml) of[a]:					
	IgG1b12 on CD4BS gp120	F105 on CD4BS gp120	1121 on V3 loop gp120	2F5 on gp41	sCD4 on CD4-binding site gp120	AMD3100 on CXCR4
Wild-type NL4-3	0.618 ± 0.06	0.547 ± 0.26	0.124 ± 0.09	1.310 ± 0.89	0.536 ± 0.11	0.012 ± 0.005
S334N	0.443 ± 0.03	0.504 ± 0.26	0.074 ± 0.05	0.728 ± 0.68	0.328 ± 0.07	0.007 ± 0.002
S398N	0.473 ± 0.03	0.868 ± 0.56	0.144 ± 0.15	1.290 ± 1.32	0.599 ± 0.08	0.014 ± 0.003
V242I	0.362 ± 0.05	0.219 ± 0.08	0.089 ± 0.06	0.387 ± 0.25	0.287 ± 0.17	0.015 ± 0.006
A281V	0.456 ± 0.16	0.609 ± 0.22	0.227 ± 0.16	1.474 ± 1.01	0.415 ± 0.14	0.010 ± 0.002
A612T	0.451 ± 0.04	0.958 ± 0.88	0.273 ± 0.37	1.257 ± 0.68	0.490 ± 0.16	0.014 ± 0.002
S334N/S398N	0.505 ± 0.30	0.304 ± 0.15	0.062 ± 0.03	1.268 ± 0.22	0.371 ± 0.16	0.007 ± 0.001
V242I/A281V	0.290 ± 0.15	0.206 ± 0.12	0.086 ± 0.05	0.257 ± 0.15	0.243 ± 0.11	0.010 ± 0.006
V242I/S334N/S398N	0.327 ± 0.10	0.306 ± 0.09	0.068 ± 0.04	0.354 ± 0.21	0.223 ± 0.10	0.011 ± 0.009
V242I/A281V/S334N/S398N	0.290 ± 0.17	0.246 ± 0.07	0.045 ± 0.02	0.309 ± 0.23	0.270 ± 0.13	0.012 ± 0.011
V242I/A281V/S334N/S398N/A612T	0.204 ± 0.10	0.384 ± 0.34	0.059 ± 0.03	0.287 ± 0.18	0.222 ± 0.06	0.008 ± 0.002
Wild-type KB9	>25	>10	>10	6.33 ± 5.2	1.13 ± 0.36	0.021 ± 0.012
E151K	>25	>10	>10	7.44 ± 3.6	0.819 ± 0.53	0.013 ± 0.004
E172K	10.79 ± 3.0	>10	1.93 ± 1.7	0.287 ± 0.12	0.262 ± 0.12	0.004 ± 0.000
F176Y	ND	ND	ND	ND	ND	ND
A561T	>25	>10	>10	0.702 ± 0.31	1.08 ± 0.65	0.006 ± 0.005
E151K/E172K	1.38 ± 0.97	~10	0.400 ± 0.55	0.575 ± 0.60	0.225 ± 0.06	0.005 ± 0.002
E151K/E172K/A561T	5.29 ± 2.9	~10	0.509 ± 0.74	0.469 ± 0.40	0.287 ± 0.26	0.007 ± 0.004
F176Y/A561T	6.54 ± 0.02	3.62 ± 0.40	0.025 ± 0.03	0.268 ± 0.12	0.194 ± 0.09	0.008 ± 0.002

[a] Each IC_{50} represents the concentration of antibody or inhibitor resulting in a 50% reduction in a single-round infection of Cf2Th-HuCD4/CXCR4 cells. The values shown are means from three independent experiments done in duplicate ± standard deviations. ND, not determined. The target on gp120, gp41, or CXCR4 and the antibody/inhibitor are indicated. CD4BS, CD4-binding site epitope on gp120.

tion to marmoset receptors resulted in an increase in sensitivity to neutralization by antibodies and sCD4 for HIV-1(KB9) but not for HIV-1(NL4-3).

The low affinity of X4 or R5X4 gp120 glycoproteins for CXCR4 can make accurate assessment of CXCR4-binding ability difficult. Since differences in chemokine receptor-directed binding affinity result in different sensitivities of virus infection to chemokine receptor-directed ligands (41), we investigated the effect of AMD3100, a CXCR4 ligand, on the infection of cells by viruses with the variant envelope glycoproteins. Viruses with all of the HIV-1(NL4-3) envelope glycoprotein variants exhibited similar sensitivities to AMD3100 (Table 3).

Infection of Cf2Th-HuCD4/CXCR4 cells by viruses with the wild-type KB9 envelope glycoproteins was inhibited by AMD3100 at concentrations similar to those observed for infection by viruses with the NL4-3 envelope glycoproteins (Table 3). The adaptation-associated changes in the KB9 gp120 V1/V2 variable loops and gp41 ectodomain resulted in a two- to fivefold increase in sensitivity to AMD3100. Increased AMD3100 sensitivity was observed even for changes, such as E172K and A561T, that individually were not sufficient to allow efficient replication in cells expressing the marmoset receptors. Thus, increased AMD3100 sensitivity was associated with HIV-1(KB9) virus adaptation but was insufficient to explain the increased ability to utilize marmoset receptors.

The above experiments were conducted using target cells that express human CD4 and CXCR4. Although human and marmoset CXCR4 proteins are 98% similar, HIV-1 adaptation to cells expressing marmoset receptors might be specific for marmoset CXCR4. Therefore, we tested the effect of AMD3100 on the infection of cells expressing human CD4 and marmoset CXCR4 (Table 4). Approximately 10-fold-lower concentrations of AMD3100 were required to inhibit infection of these cells by the viruses with the variant envelope glycoproteins compared with cells expressing human CD4 and human CXCR4. This result is consistent with lower affinities of the envelope glycoproteins of the parental and adapted viruses for the marmoset CXCR4 protein, relative to those for human CXCR4. No increases were observed in the concentrations of AMD3100 required to inhibit viruses with the adaptation-associated envelope glycoproteins compared with those required to block infection of viruses with the parental envelope glycoproteins. We conclude that an increase in the affinity of the HIV-1 envelope glycoproteins for marmoset CXCR4 is not necessary for virus adaptation to cells expressing the marmoset receptors.

TABLE 4. Neutralization of pseudotyped HIV-1 viruses by the entry inhibitor AMD3100 in cells expressing human CD4 and common marmoset CXCR4

Envelope variant	IC_{50} (μg/ml) of AMD3100 with cells expressing CXCR4[a]
Wild-type NL4-3	0.0012 ± 0.0004
V2421/A281V	0.0013 ± 0.0002
S334N/S398N/V242I/A281V	0.0009 ± 0.0003
S334N/S398N/V242I/A281V/A612T	0.0006 ± 0.0002
Wild-type KB9	0.0022 ± 0.0007
E151K/E172K	0.0009 ± 0.0002
E151K/E172K/A561T	0.0010 ± 0.0004
F176Y/A561T	0.0013 ± 0.0008

[a] IC_{50}s represent concentrations of AMD3100 resulting in a 50% reduction in a single-round infection of Cf2Th-HuCD4/CjCXCR4 cells. Values shown are means from four independent experiments done in duplicate ± standard deviations.

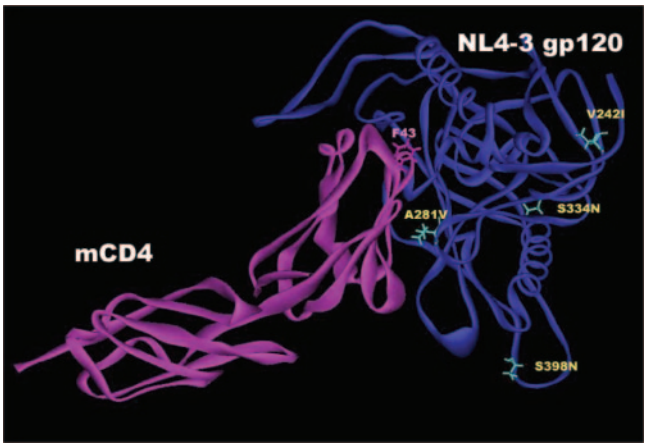

FIG. 6. Modeling the interaction of the adapted HIV-1(NL4-3) gp120 core with marmoset CD4. The modeled NL4-3 gp120 core (blue) bound to two-domain marmoset CD4 (magenta) is shown, with the gp120 residues that changed during the adaptation indicated. Also shown is phenylalanine 43 of CD4, which makes critical contributions to gp120 binding (28, 29).

DISCUSSION

In this work, we adapted two different HIV-1 isolates, HIV-1(NL4-3) and HIV-1(KB9), to replicate in cells using the common marmoset receptors CD4 and CXCR4. The analysis of the envelope glycoproteins of the adapted viruses revealed that in both cases a small number of changes that appeared during the adaptation allowed the viruses to use the marmoset receptors to gain entry into the cells. Probably because of differences in the phenotypes of the parental viruses, HIV-1(NL4-3) and HIV-1(KB9) adapted to the marmoset receptors by distinct mechanistic pathways. The genotypic and phenotypic properties of the adapted viruses are summarized in Table 2.

The changes that allow the HIV-1(NL4-3) virus to enter cells expressing marmoset CD4 and CXCR4 involve gp120 residues 281, 334, and 242. The changes that appeared in these residues during the adaptation increase the gp120 binding affinity for marmoset CD4 without a detectable effect on the affinity for human CD4. In X-ray crystal structures of the HIV-1 gp120-CD4 complex, alanine 281 makes direct contact with CD4 (Fig. 6) (28, 29). Molecular modeling predicts that the larger side chain of valine that was associated with adaptation makes better contact with CD4 and increases the gp120 binding affinity for marmoset CD4 (Table 5). The major differences between human and marmoset CD4 that impact gp120 binding involve residues 48 and 59 in CD4 domain 1 (30). In human CD4, proline 48 contributes to a β-turn that results in an antiparallel orientation of the C″ and D strands of CD4, each of which makes important contacts with gp120 (29, 49). The replacement of proline 48 with a glutamine residue in common marmoset CD4 might alter the β-turn and consequently affect the relationship of the C″ and D strands. The S334N change in gp120 has been previously seen in an HIV-1 isolate adapted to replicate efficiently in cells expressing a human CD4 mutant (K46D) with a low affinity for gp120 (8). Residue 334 is located near the base of the V3 loop and appears to be too far from the CD4 binding site to alter CD4 affinity directly; however, the resulting loss of a carbohydrate at asparagine 332 might change the conformation of the unliganded gp120 and thus indirectly increase CD4-binding ability (Table 5). The V242I change alone increases the binding affinity of soluble gp120 for marmoset CD4 only minimally but does contribute to marmoset CD4 binding in the context of the A281V change. Valine 242 projects into a groove in gp120 that has been proposed to make contact with gp41 and regulate fusion efficiency (51). It is possible that the adaptation-associated change in valine 242 amplifies the impact of CD4 binding on subsequent conformational changes required for virus entry. Consistent with this, the viruses containing the V242I change exhibited a slight increase in sensitivity to neutralization by different antibodies (Table 3), possibly reflecting a more activation/deactivation-prone state. In summary, most of the HIV-1(NL4-3) adaptation-associated changes result in specific increases in binding to marmoset CD4, providing a natural explanation of the adaptation.

The changes that evolved in the HIV-1(KB9) envelope glycoproteins as a consequence of the adaptation to cells expressing marmoset receptors differ in location and mechanistic consequences from those observed in the case of HIV-1(NL4-3). One likely explanation for these differences lies in the already high level of efficiency with which the parental KB9 gp120 glycoprotein binds marmoset CD4. One obvious reason for this high binding affinity is that KB9 gp120 already has a valine at residue 281, which the above studies with the NL4-3 gp120 variants demonstrate contributes to marmoset CD4 binding. Of interest, the conversion of alanine 281 to valine occurred during the in vivo passages of SHIV-89.6 in rhesus monkeys that led to the generation of SHIV-KB9 (22). Previous work, which we confirmed here, indicated that the KB9 gp120 glycoprotein binds human and rhesus monkey CD4 comparably to binding of the 89.6 gp120 glycoprotein; however, KB9 gp120

TABLE 5. Modeling predictions of binding energies between HIV-1(NL4-3) gp120 core variants and two-domain marmoset CD4[a]

HIV-1 gp120 core variants	Total interaction energy (kcal/mol)	van der Waals energy (kcal/mol)	Electrostatic energy (kcal/mol)
Wild-type NL4-3	−320.2	−104.5	−215.7
V242I	−318.5	−105.1	−213.4
A281V	**−326.8**	**−107.1**	**−219.7**
S334N	−323.5	−105.9	−217.7
S398N	−324.4	−106.4	−218.0
V242I + A281V	−326.7	−107.3	−219.4
S334N + S398N	−323.3	−106.2	−217.1
S334N + S398N + V242I	−323.8	−106.4	−217.4
S334N + S398N + V242I + A281V	−329.1	−108.1	−221.0
Wild-type NL4-3 + marmoset CD4 F43A[b]	−305.7	−92.3	−213.4

[a] As described in Materials and Methods, the interaction of the indicated HIV-1(NL4-3) gp120 core with two-domain marmoset CD4 was modeled based on a known structure of the HIV-1 gp120 core/human two-domain CD4 complex (29). After energy minimization, the total interaction energy and the contributions of van der Waals and electrostatic interactions were calculated. Boldface indicates the most favorable energy changes associated with the alteration of a single residue.

[b] As a control, the interaction of the wild-type NL4-3 gp120 core and the F43A mutant of marmoset CD4 was modeled and used for the calculation of energy. Changes in phenylalanine 43 of human CD4 significantly affect gp120 binding (29).

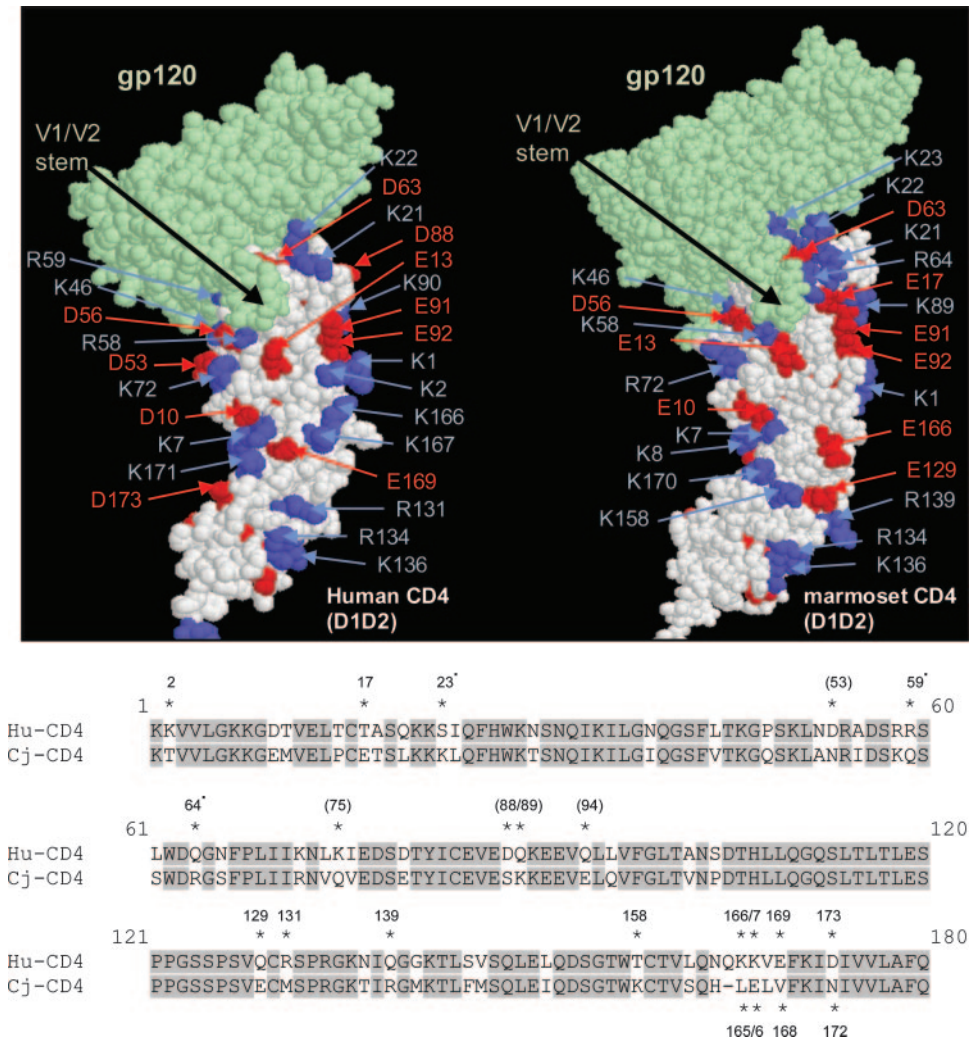

FIG. 7. Differences between human and common marmoset CD4. The primary amino acid sequences of the amino-terminal two domains of human (Hu) and common marmoset (Cj) CD4 are aligned. Identical residues are shaded. Amino acid residues that exhibit differences in charge between the two CD4 proteins are designated with an asterisk. Asterisk-marked residues buried in the HIV-1 gp120 core-CD4 interface are dotted; asterisk-marked residues on the CD4 surface opposite to the expected positions of the gp120 V1/V2 loops are placed in parentheses. The image shows the structure of the HIV-1 gp120 core (green) in complex with two-domain (D1D2) human CD4 (left) (28, 29). The location of the V1/V2 stem is indicated. The D1D2 CD4 basic surface residues are blue, and the acidic surface residues are red. The image on the right depicts the modeled structure of HIV-1 gp120 complexed with marmoset two-domain CD4. The color scheme is identical to that used for the gp120-human CD4 complex.

binds marmoset CD4 more efficiently than 89.6 gp120 (data not shown). Thus, the differences between the 89.6 and KB9 gp120 glycoproteins, including the alanine-valine substitution at residue 281, contribute to an increased binding affinity of KB9 gp120 for common marmoset CD4 but not for human or rhesus monkey CD4.

The adaptation-associated changes in the HIV-1(KB9) envelope glycoproteins involve the gp120 V1 and V2 variable loops and the gp41 HR1 region. Presumably because the KB9 gp120 glycoprotein starts off with a relatively high affinity for marmoset CD4, the adaptation-associated changes in the HIV-1(KB9) envelope glycoproteins only minimally affect marmoset CD4 binding. Only the V2 loop change, E172K, is associated with a slight but specific increase in the affinity of gp120 for marmoset CD4. How then do the observed adaptation-associated changes exert their dramatic impact on the ability of the HIV-1(KB9) envelope glycoproteins to utilize marmoset receptors? One possibility that we considered is that the adapted KB9 gp120 glycoprotein binds marmoset CXCR4 more efficiently. Although the gp120 V1/V2 and gp41 changes are not expected to affect the CXCR4-binding region of gp120 directly, they could hypothetically exert indirect effects. An increase in affinity for CXCR4 predicts a decrease in sensitivity to the CXCR4 ligand, AMD3100, which we did not observe. In fact, the adaptation-associated changes resulted in a slightly increased sensitivity to AMD3100 for infection of cells bearing human or marmoset CXCR4. Thus, an increase in affinity for CXCR4 is an unlikely explanation for the HIV-1(KB9) adaptation to marmoset receptors.

An alternative explanation that is more consistent with the

observations recognizes the high degree of resistance to neutralizing antibodies that the HIV-1(KB9) envelope glycoproteins achieved as a result of in vivo SHIV propagation in monkeys. Resistance to neutralization by antibodies is often accompanied by an increase in HIV-1 dependence on CD4 for entry (27, 40, 45). Thus, as a result of in vivo requirements to avoid neutralizing antibodies, the HIV-1(KB9) envelope glycoproteins may less efficiently undergo the requisite conformational changes for virus entry in response to nonoptimal CD4. Even though the KB9 envelope glycoproteins bind marmoset CD4 efficiently, minor differences in the interaction of CD4 and the gp120 variable loops, for example, could impact the efficiency of entry. CD4 binding has been shown to reposition the gp120 V1/V2 loops, promoting subsequent events in the virus entry process (46, 50). These events may include chemokine receptor binding and gp41 conformational rearrangements. Although the structural details of V1/V2 loop interaction with CD4 are unknown, the surface of CD4 domains 1 and 2, which contains an abundance of charged residues, is likely involved. In this light, it is intriguing that both of the KB9 V1/V2 alterations that contribute to marmoset CD4 adaptation involve a change in charge, from acidic glutamic acid residues to basic lysine residues. Because at least 10 surface-exposed residues potentially able to contact the V1/V2 gp120 loops differ in charge between human and marmoset CD4 domains 1 and 2 (Fig. 7), the charge changes in the adapted KB9 gp120 V1/V2 loops may allow attractive or repulsive electrostatic forces to drive the appropriate loop movements in a marmoset CD4-specific fashion. A salt bridge between lysine 172 and an acidic residue on the marmoset CD4 surface might additionally contribute to the slight increase in binding affinity observed for this variant. The decreased ability of KB9 envelope glycoproteins with some of V1/V2 loop changes to support entry into cells expressing human CD4 is consistent with the above model. The adaptation-associated V1/V2 loop changes, particularly E172K, also appear to disrupt interactions between elements on the unliganded KB9 trimer that govern neutralization resistance, since the adapted virus envelope glycoproteins are much more susceptible to neutralization by antibodies and sCD4.

The A561T change in the gp41 HR1 region contributes to a two- to threefold improvement in the efficiency of infection of cells expressing the marmoset receptors by HIV-1(KB9) viruses. This change did not alter the sensitivity of the virus to antibodies or sCD4. The mechanism by which the A561T change contributes to adaptation to marmoset receptors presumably involves promotion of entry-related conformational events in the setting of nonoptimal receptor interactions. Of interest, changes in the adjacent residue 560 have been associated with SIV macrophage tropism (34, 36), which involves the ability to use the low levels of CD4 on the macrophage surface more efficiently (2, 12). Both residues 560 and 561 are located in the b and c positions, respectively, within the gp41 heptad repeat; the predicted surface exposure of these b and c residues would allow interactions with other envelope glycoprotein elements either prior to or after formation of the gp41 six-helix bundle thought to represent the fusogenic conformation.

The derivation of HIV-1 envelope glycoprotein variants that can efficiently use the common marmoset receptors CD4 and CXCR4 to enter cells advances efforts to overcome the major early block to HIV-1 in these New World monkeys. The use of these viruses should allow exploration of any additional blocks to HIV-1 in these monkeys. Understanding and circumventing these blocks may lead to novel animal models for study of HIV-1 pathogenesis.

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