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Amino Acid Substitutions in the Human Immunodeficiency Virus Type 1 gp120 V3 Loop That Change Viral Tropism Also Alter Physical and Functional Properties of the Virion Envelope

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The third variable (V3) region within the gp120 envelope glycoprotein of the human immunodeficiency virus type 1 (HIV-1) has been reported to be an important determinant of viral tropism. In this study a series of isogenic recombinant HIV-1 viruses, containing V3 regions from fresh isolates, were examined to ascertain if a relationship exists between viral tropism and specific properties of the virion-associated envelope. All of the viruses were able to infect CD4$^+$ primary lymphocytes, although with different infection kinetics. Several recombinants, however, were unable to infect a continuous CD4$^+$ T-cell line permissive for the parental virus and exhibited a marked decrease in the kinetics of virion-associated gp120 binding to a soluble form of CD4. A known macrophage-tropic HIV-1 isolate, also unable to infect the T-cell line, bound CD4 with similarly slow reaction kinetics. Although the inability to infect T-cell lines is a commonly observed property of macrophage-tropic isolates of HIV-1, the loss of T-cell line tropism by the V3 recombinants was not accompanied by a substantial infectivity for monocyte-derived macrophages, as monitored by reverse transcriptase production. Additional analyses of the recombinant virion gp120s indicated that most of the V3 substitutions increased the inherent stability of the virion gp120-gp41 envelope complex. These results indicate that V3-induced alterations in viral tropism are associated with changes in physical and functional properties of the virion envelope.

The human immunodeficiency virus type 1 (HIV-1) infects CD4$^+$ lymphocytes and monocyte-derived macrophages (14, 29). While isolates directly recovered from infected individuals or produced in vitro are able to infect primary CD4$^+$ peripheral blood mononuclear cells (PBMC), they are often restricted in their ability to establish a productive infection in either monocyte-derived macrophages or CD4$^+$ continuous T-cell lines (7, 18, 27, 44). Several studies have shown that these tropism differences are controlled by specific regions within the HIV-1-encoded surface envelope glycoprotein gp120 (5, 8–10, 21, 38, 48, 49, 54, 55) which, in association with the transmembrane envelope glycoprotein gp41, mediates virus adsorption and penetration (3, 15, 26, 32, 33, 52). More recently, amino acid changes within both gp120 and gp41 have been shown to affect HIV-1 growth in CD4$^+$ cell lines (17), while the third variable region (V3) in gp120 has been reported to be an important determinant of macrophage tropism (9, 23, 49).

The V3 region has been the focus of numerous studies (reviewed in reference 37) since it was first identified as a principal neutralizing epitope within the viral envelope (19, 31, 39, 43). It is thought that V3 is important to envelope function after gp120 binds to the cell surface HIV-1 receptor molecule CD4 (11, 25, 30, 33), since neutralizing antibodies directed against V3 block virus entry following adsorption (50) and mutations in V3 impair envelope-mediated fusion of viral and cellular membranes (3, 16). Although the molecular basis for this putative involvement of V3 in postbinding envelope function is unknown, it has been postulated that V3 may affect conformational changes which occur in the viral envelope following binding to CD4 (39, 41, 43, 45) and which are required for virus-cell fusion (12, 37). We and others have recently shown that the interaction of V3 with other regions of gp120 stabilizes the association of gp120 with virions and influences viral infectivity (51, 57, 59).

A postbinding functional role for V3 during viral entry is intriguing, given a recent analysis of the V3 amino acid sequences associated with primary isolates of HIV-1 that display distinct T-cell line or macrophage tropism (9). That study reported a high degree of amino acid homology in the V3 region among macrophage-tropic isolates compared with more extensive V3 heterogeneity among HIV-1 variants restricted to growth in T-cell lines. These findings suggest that specific structural/functional features of the HIV-1 envelope may be required to initiate productive infections in different cell types.

In the current study, we have utilized a previously published series of V3 recombinant viruses to examine if a relationship exists between viral tropism and specific properties of the virion-associated envelope. All viruses tested were able to establish productive infections in PBMCs, albeit with different infection kinetics. Several of the recombinants, however, were unable to infect a CD4$^+$ T-cell line permissive for the parental virus. This latter group of viruses exhibited a marked decrease in the kinetics of virion-associated gp120 binding to a soluble form of CD4. A known macrophage-tropic HIV-1 isolate, also unable to infect the T-cell line, bound CD4 with similarly slow kinetics. Although the inability to infect T-cell lines is a commonly observed property of macrophage-tropic isolates, the loss of T-cell line tropism by the V3 recombinants examined was not accompanied by a substantial infectivity for monocyte-derived macrophages. Additional analyses indicated that several of the V3 substitutions affected the amount of gp120 associated with virions and that most reduced the spontaneous release of gp120 from virions. These results demonstrate that V3-induced alterations in viral tropism are associated with changes in physical and functional properties of the gp120 associated with virus particles.

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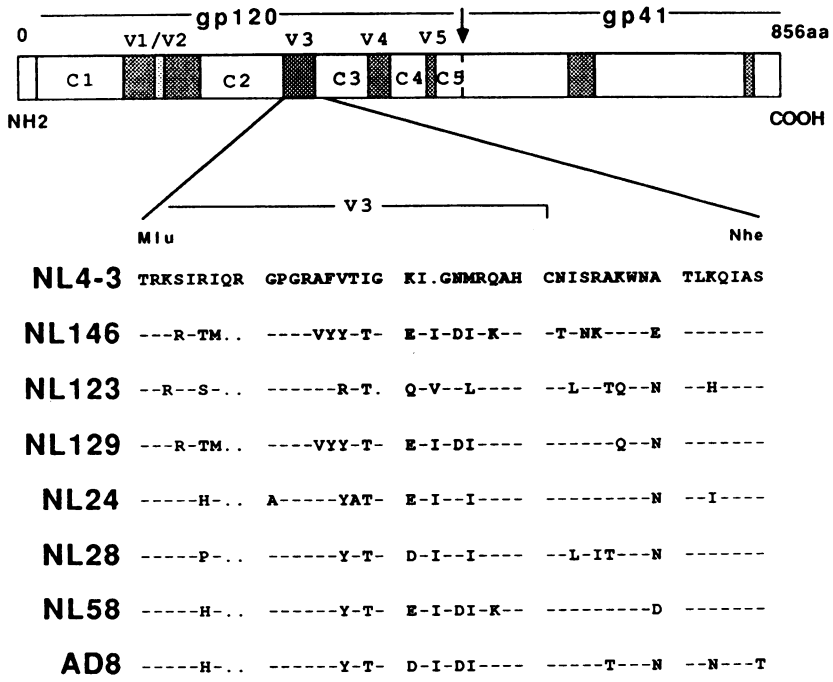

FIG. 1. Amino acid sequence differences in the parental and V3 recombinant viruses. The 856-amino-acid (aa) HIV-1 envelope precursor glycoprotein is depicted at the top, showing conserved (C1 to C5) and variable (V1 to V5) regions in gp120. Recombinant proviral plasmids were constructed by introducing PCR-amplified DNA fragments from different primary isolates of HIV-1 into the *Mlu*I and *Nhe*I sites of the pNL4-3 proviral plasmid clone of HIV-1 (9). Amino acid differences between the parental NL4-3 and V3 recombinant viruses are shown by the single-letter amino acid code, with codon deletions depicted by dots; dashes indicate amino acid identity to NL4-3. The analogous region from the AD8 macrophage-tropic isolate of HIV-1 (see text) is also shown for comparison.

MATERIALS AND METHODS

Viruses. The origin of the pNL4-3 proviral molecular clone of HIV-1 has been previously reported (1). The construction of proviral plasmid derivatives of pNL4-3 containing V3 sequences from primary HIV-1 isolates has also been described (9). The amino acid differences among the viruses in the V3 region of gp120 are shown in Fig. 1. The virus designations and isolate origin for the V3 sequences are as follows: NL4-3 (wild type; see above); NL146 (isolate 13231, clone 21-14); NL123 (isolate 13539, clone 22-16); NL129 (isolate JR-CSF, mutant 129); NL24 (isolate K7, clone 24-22); NL28 (isolate K8, clone 28-32); and NL58 (isolate JR-FL, clone 58-17) (9). The HIV-1 macrophage-tropic virus AD-87(M) (42) was derived from the ADA strain of HIV-1 (19). An infectious molecular clone (AD8-2) (53a) was isolated from the unintegrated DNA present in PBMCs infected with the AD-87(M) virus. Virus stocks were prepared by transfecting HeLa cells (one 25-cm^2 flask per virus) with 25 μg of calcium phosphate-precipitated plasmid DNA as previously described (56). At 24 h posttransfection, the cells were removed by scraping, resuspended in 4 ml of fresh Dulbecco's modified Eagle's medium, and transferred to new upright 25-cm^2 flasks for an additional 24 h. Virus-containing supernatants were subjected to low-speed centrifugation (268 × *g*) and filtered through a 0.45-μm-pore-size filter to remove any cells. Virions were pelleted from the medium by ultracentrifugation (56, 58), resuspended in 500 μl of RPMI 1640 medium containing 10% fetal bovine serum (FBS), and filtered through a 0.45-μm-pore-size Spin-X filter (Costar). The virion suspensions were assayed for reverse transcriptase (RT) activity as previously described (60).

Cells and infectivity assays. HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The sources of PBMC and the $CD4^+$ lymphocyte cell line (CEM 12D7) have been described (40). A total of 5×10^5 12D7 cells or phytohemagglutinin-activated PBMCs were transferred to individual wells in 24-well tissue culture plates (Costar), and comparable amounts of virus (determined by RT activity) were added. 12D7 cells were grown in 2 ml of RPMI-FBS medium, while the PBMC cultures were maintained in 2 ml of RPMI-FBS containing 10% interleukin-2. Cell-free culture supernatants were collected every 2 days beginning on day 3 after infection for determination of RT activity, and the cells were subsequently diluted 1:2 with fresh medium. Primary monocyte-derived macrophages were prepared from elutriated monocytes (20) as previously described (28), and 7×10^4 cells were transferred to individual wells in a 96-well plate (Nunc) 24 h prior to infection. Comparable amounts of each virus were added to individual wells, and the cells were maintained in 200 μl of Dulbecco's modified Eagle's medium containing 10% human serum as described previously (28). The medium was replaced every 2 days, beginning on day 3 after infection, and cell-free supernatants were collected for determination of RT activity.

Preparation of ^{35}S-labeled virions. HeLa cells were transfected with the different proviral plasmid DNAs and labeled for 22 to 24 h with [^{35}S]methionine (58), and virions were pelleted from the medium by ultracentrifugation as previously described (56, 58). Virions were resuspended in RPMI-FBS for the different analyses (see below).

Incorporation of gp120 into virions. Labeled virion suspensions were generated following the transfection of HeLa cells (one 25-cm^2 flask per virus) and were assayed for the presence

of RT activity. Comparable amounts of virions (determined on the basis of RT activity) were lysed by the transferral of virion suspensions into 800 μl of lysis buffer containing 0.1% Triton X-100, 300 mM NaCl, and 50 mM Tris-HCl (pH7.5) followed by vortex mixing. HIV-1 proteins were immunoprecipitated (56) by using HIV-1-reactive antibodies present in a mixture of AIDS patients' sera. All precipitates were washed twice with lysis buffer. The proteins were solubilized in reducing sample buffer, separated on 10% acrylamide AcrylAide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and visualized by fluorography as previously described (56). The gp120 envelope and p66 RT proteins were quantitated with a Fujix Bas 2000 Bio-image analyzer (Fuji), and the ratio of gp120 to p66 was calculated to determine the relative amount of gp120 in the different viruses.

Pulse-chase envelope processing analysis. HeLa cells transfected with the pNL4-3, pNL146, or pNL24 proviral plasmid DNAs (two 25-cm^2 flasks for each virus) were removed by scraping 18 to 20 h posttransfection, and pulse-chase metabolic labelings were conducted using [^{35}S]methionine as previously described (56). Cell pellets and the corresponding supernatants collected after 0, 1, 2, 4, and 6 h of chase were lysed (56), and the HIV-1 proteins were immunoprecipitated (56) by using the AIDS patients' sera. The proteins were resolved by SDS-PAGE and visualized by fluorography.

Spontaneous release of gp120 from virions. Labeled virion suspensions of each virus were prepared by ultracentrifugation following the transfection and labeling of HeLa cells (one 25-cm^2 flask per virus) as described above. The virion suspensions were split into two 100-μl aliquots and were transferred to 1.5-ml Sarstedt microcentrifuge tubes. The virions in one sample were immediately pelleted in an Eppendorf microcentrifuge for 1 h as previously described (57, 58). The supernatant was removed and transferred to a separate microcentrifuge tube. Both the pellet and supernatant samples were lysed in 1 ml of lysis buffer and were immunoprecipitated by using the AIDS patients' sera as described above. The second virion aliquot was incubated at 37°C for 48 h prior to being pelleted in the microcentrifuge. The pellet and supernatant fractions were lysed and immunoprecipitated as described above. All precipitates were resolved by SDS-PAGE and visualized by fluorography. The amount of gp120 in the pellets and non-virion-associated (free) gp120 in the supernatant samples was quantitated with the Fujix Bio-image analyzer, and the percentage of gp120 released from the virions over time was determined.

CD4 binding to virion-associated gp120. HeLa cells were transfected with the different proviral DNAs (two 25-cm^2 flasks per virus), and radiolabeled virion suspensions were generated as described above. Aliquots (100 μl) were transferred into four 1.5-ml Sarstedt microcentrifuge tubes and were incubated at 37°C. The kinetics of CD4 binding to the virion-associated gp120 were determined by adding 50 nM (0.23 μg) of the soluble CD4 derivative CD4-immunoglobulin G (IgG) (4, 6; gift of Genentech, Inc., South San Francisco, Calif.) to individual virion aliquots at different intervals to give incubation times of 30 min, 1 h, and 3 h. The CD4-IgG was dialyzed against water for 24 h prior to use to remove detergent which was present in the stock solution. A fourth tube lacking any added CD4-IgG and incubated for 3 h at 37°C served as an untreated control. Following the incubation period, the tubes were placed on ice for 2 min, and the virions were subsequently pelleted from the medium by being centrifuged for 1 h at 4°C in a refrigerated Eppendorf microcentrifuge. The supernatants were removed and transferred to new tubes. Detergent lysates of the viral pellets and corresponding supernatants were prepared by the addition of 1 ml of lysis buffer followed by vortex mixing. Protein A agarose beads (50 μl; Bethesda Research Laboratories) were added to the pellet lysates to recover virion-associated gp120 which was bound to CD4-IgG. The IgG portion of CD4-IgG allows it to bind to protein A (6). After 1 h of being mixed at 4°C, gp120-CD4 complexes associated with the protein A beads were recovered by pelleting the beads in an Eppendorf microcentrifuge. The remaining supernatants were transferred to a new set of protein A beads pretreated with the AIDS patients' sera, to immunoprecipitate virion gp120 which was not bound to CD4-IgG. The supernatant samples recovered after microcentrifuge pelleting of the virions were immunoprecipitated by using protein A agarose beads pretreated with the AIDS patients' sera to detect any gp120 which was released from the virions as a result of CD4-IgG binding (22, 35, 36). All sets of beads were washed twice with lysis buffer and resuspended in sample buffer, and the proteins were resolved by SDS-PAGE. The viral proteins were visualized by fluorography, and the amount of gp120 present in the different fractions was quantified with the Fujix Bio-image analyzer. The percent gp120 bound to CD4-IgG at the different time intervals was calculated as follows: [(bound + released)/(bound + released + not bound)] × 100%.

RESULTS

Infectivity of the recombinant viruses. The recombinant viruses used in this study (Fig. 1) were generated by introducing 165-nucleotide PCR-amplified DNA fragments from primary isolates of HIV-1 into the pNL4-3 (1) proviral plasmid molecular clone of HIV-1 (9). A previous study (9) had indicated that the V3 recombinant viruses NL123 and NL146, like the HIV-1_{NL4-3} parent, were able to infect immortalized $CD4^+$ cell lines, while the NL24, NL28, and NL58 V3 recombinants were restricted to growth in PBMC and monocyte-derived macrophages. The NL129 recombinant was reported to infect both $CD4^+$ cell lines and monocyte-derived macrophages (9). Virus stocks for the current study were generated by transfecting the different proviral plasmid DNAs into HeLa cells, pelleting cell-free virions from the medium in an ultracentrifuge, and resuspending the virus particles in RPMI-FBS medium. Preparing HIV-1 inocula in this manner does not impair viral infectivity (58). All viruses were tested for replication in PBMCs, in the 12D7 derivative of the CEM T-cell line, and in primary human monocyte-derived macrophages as described in Materials and Methods. Cell-free supernatants were collected during the time course of viral infections and were assayed for the presence of RT activity as an indicator of progeny virion production. As a positive control for macrophage tropism, virus (AD8-2) produced from a full-length infectious molecular clone (pAD8) of the HIV-1_{ADA} macrophage-tropic isolate of HIV-1 (53a) was included in the analysis.

As shown in Fig. 2, all of the recombinant viruses were able to establish productive infections in PBMCs; the parental HIV-1_{NL4-3} and NL123 and NL129 V3 recombinant viruses exhibited peak virus production 4 to 6 days before the others. A marked difference in the infectivity of several recombinants was observed in the 12D7 cell line: only those viruses previously reported to be replication competent in $CD4^+$ cell lines (NL4-3, NL123, NL146, and NL129) (9) were infectious (Fig. 2, middle panel), although the NL146 virus displayed delayed infection kinetics compared with the other three. As expected, the macrophage-tropic AD8 virus did not infect the 12D7 cells. In an independent experiment, the NL58 recombinant virus showed growth and tropism characteristics (data not shown) that were virtually identical to the NL24 and NL28 viruses

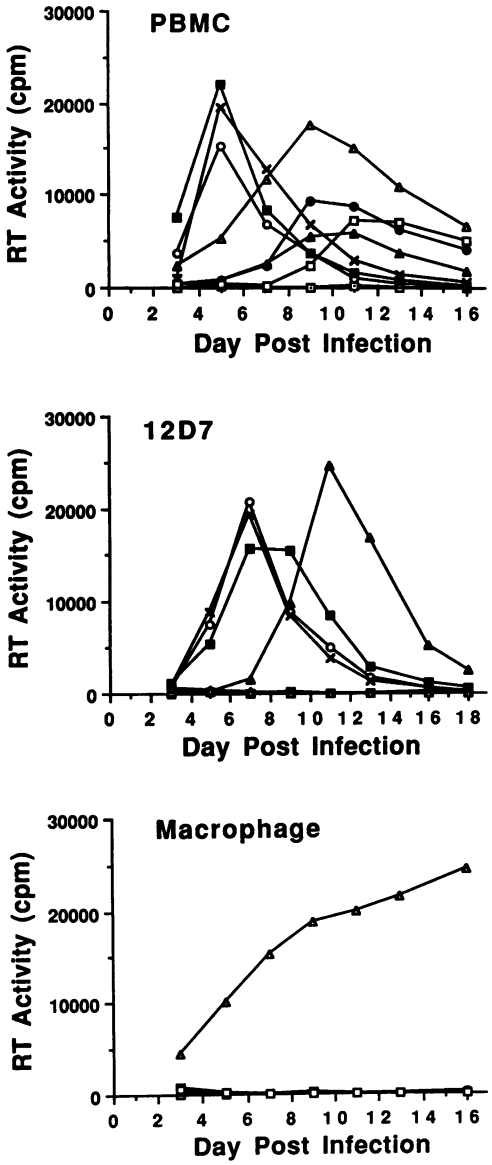

FIG. 2. Virus replication in different cell types. Virion suspensions used to infect the indicated cell types were generated by transfecting HeLa cells with the different proviral plasmid DNAs, pelleting the virions in an ultracentrifuge, and resuspending them in RPMI-FBS medium. Cell-free supernatants from infected cultures were assayed for the presence of HIV-1 RT at the specified times to indicate progeny virus production. Cell types infected were PBMC, 12D7 (CD4+ T-cell line), and primary monocyte-derived macrophages. Symbols: ⊡, mock; ○, NL4-3; ▲, NL146; ■, NL123; X, NL129; ●, NL24; □, NL28; △, AD8. It should be noted that in the PBMC and 12D7 infections, 10 μl from 2-ml cultures was sampled for RT activity compared with 10 μl from 200-μl cultures for the macrophage infections.

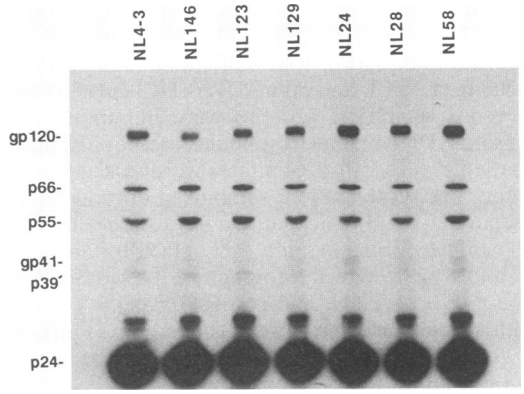

FIG. 3. Virion-associated gp120 envelope glycoproteins. HIV-1 virions present in the supernatants from transfected and metabolically labeled HeLa cells were pelleted by ultracentrifugation. Virions were lysed and the proteins were immunoprecipitated by using a mixture of AIDS patients' sera. Precipitates were resolved by SDS-PAGE and visualized by fluorography. The positions of the virion-associated gp120 and gp41 envelope glycoproteins, as well as the viral p66 RT and p55, p39, and p24 gag proteins are indicated. The origin of the protein bands above p24 is unknown. The ratios of gp120 to p66 in the different viral preparations are shown in Table 1.

(infectious only in PBMCs). Infection of monocyte-derived macrophages, as measured by RT production, was observed only in the case of the macrophage-tropic AD8 virus (Fig. 2, lower panel). Thus, this group of six V3 recombinants exhibited variable infection kinetics in PBMCs; however, only three of six were able to infect the T-cell line. The inability of the NL24, NL28, and NL58 recombinant viruses to initiate a productive infection in the 12D7 T-cell line was not accompanied by detectable infectivity for monocyte-derived macrophages.

V3 substitutions affect virion-associated gp120 levels. The growth and tropism properties of some of the recombinant viruses suggested that the V3 substitutions both affected the structure and/or function of gp120 and conferred altered biologic phenotypes on the HIV-1_{NL4-3} parent. Since it has been previously shown that the interaction of V3 with other regions of gp120 influences the association of gp120 with virions (51, 57), we initially examined the amount of gp120 present in the parental and recombinant virus particles. HeLa cells were transfected with the different proviral DNAs, and the cells were metabolically labeled with [^{35}S]methionine as described in Materials and Methods. Labeled virions were pelleted from the medium by ultracentrifugation, and virion lysates were immunoprecipitated by using HIV-1 antibodies present in a mixture of AIDS patients' sera; precipitates were resolved by SDS-PAGE and visualized by fluorography. As shown in Fig. 3, different amounts of gp120 were present in the resuspended pelleted virus particles. Since the total amount of viral proteins in some of the lanes varied, the gp120 envelope and p66 RT proteins (Fig. 3) were quantitated by phosphoimaging, and the gp120 to p66 ratio was used to assess the

TABLE 1. Relative amounts of virion-associated gp120 envelope and p66 RT proteins

Virus	Ratio of gp120 to p66[a]
NL4-3	1.9
NL146	0.8
NL123	1.7
NL129	2.1
NL24	3.6
NL28	3.1
NL58	2.5

[a] Values based on quantitation of the gp120 and p66 bands shown in Fig. 3.

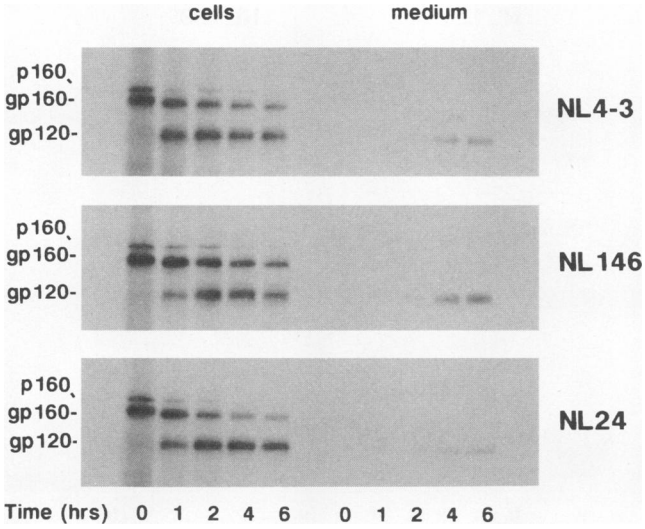

FIG. 4. Envelope processing in the parental and selected V3 recombinant viruses. HeLa cells transfected with the indicated proviral plasmid DNAs were pulse-labeled with [^{35}S]methionine for 30 min and were chased for the specified times in the absence of a radioactive label. HIV-1-specific proteins present in detergent lysates prepared from cells and the corresponding supernatant (medium) samples were immunoprecipitated by using a mixture of AIDS patients' sera and were resolved by SDS-PAGE. Only the gp160- and gp120-containing portion of the gels is shown. The band designated p160 is the HIV-1-encoded Gag-Pol fusion protein.

relative amounts of gp120 associated with the different viruses. A comparison of the gp120/p66 ratios (Table 1) among the different V3 recombinants or with the parental HIV-1_{NL4-3} indicated that the V3 substitutions in one instance (NL146) reduced the amount of gp120 present in the recombinant particles and in other instances (NL24, NL28, and NL58) increased it. Interestingly, the NL24, NL28, and NL58 recombinants contained more virion-associated gp120 than did the other viruses, yet they showed delayed infection kinetics in PBMCs and were unable to productively infect 12D7 cells (Fig. 2).

V3 substitutions do not affect envelope processing. To examine whether the observed differences in the amount of virion gp120 associated with several of the V3 recombinant viruses might simply reflect altered intracellular envelope processing, HeLa cells were transfected with the pNL4-3, pNL146, and pNL24 proviral plasmids, and processing of gp160 to gp120 was evaluated in pulse-chase experiments. The NL146 and NL24 viruses were selected for comparison because they contained the smallest and the largest amounts of virion-associated gp120, respectively, compared with NL4-3 (Table 1). The results of the pulse-chase experiment (Fig. 4) revealed no substantial differences in gp120 production among the three viruses.

Spontaneous release of gp120 from virions. Although no major differences in envelope processing were noted, proportionately more of the cell-associated gp120 was released into the medium from the NL146-expressing cells than from the NL4-3- and NL24-transfected cells (Fig. 4). This finding, coupled with the fact that the amount of virion-associated gp120 varied among NL146, NL24, and several of the other recombinant viruses (Fig. 3 and Table 2), suggested that V3 might be influencing the noncovalent association of gp120 with gp41 in progeny virus particles. We therefore assessed the

TABLE 2. Spontaneous loss of gp120 from virions

Virus	Amount (%) of gp120 released from virions after 48-h incubation at 37°C[a]
NL4-3	69
NL146	72
NL123	27
NL129	37
NL24	20
NL28	18
NL58	11

[a] Values are the percent free gp120 of the total gp120 (virion + free) averaged from two experiments for each virus including that shown in Fig. 3.

inherent stability of gp120 with virions by evaluating the spontaneous release of gp120 from particles over time. Radioactively labeled virion suspensions were generated following transfection of HeLa cells, separated into two aliquots, and incubated at 37°C for 0 or 48 h. Virus particles in each sample were pelleted in a microcentrifuge, and the HIV-1 proteins present in the resuspended pellet and supernatant fractions were immunoprecipitated by using the AIDS patients' sera. The amounts of virion and free gp120 were determined as described in Materials and Methods. As can be seen in Fig. 5, a substantial amount (69%; Table 2) of gp120 associated with the parent HIV-1_{NL4-3} virions was shed into the medium during the 48-h incubation period. With the exception of the NL146 recombinant, the V3 substitutions reduced by three- to sixfold the amount of gp120 spontaneously shed from virions during the 48-h incubation compared with HIV-1_{NL4-3} gp120 release (Table 2). Thus, the spontaneous shedding results indicated that most of the V3 substitutions increased the inherent stability of the virion envelope complex. However, this property did not strictly correlate with the observed changes in virus tropism. The three V3 recombinants (NL24, NL28, and NL58) with the most stable virion-associated gp120 (Table 2) were unable to initiate infections in the 12D7 T-cell line (Fig. 2), while the NL123 and NL129 HIV-1 recombinants, which exhibited more intermediate gp120 stability properties (Table 2), retained infectivity for 12D7 cells, exhibiting replication kinetics identical to those of the HIV-1_{NL4-3} parent (Fig. 2).

CD4 binding to virion gp120. We recently reported a direct correlation between the infectivity of defined variants of the HIV-1_{ELI} strain for human CD4$^+$ cell lines and the binding of virion-associated gp120 to a soluble form of CD4 (CD4-IgG) (58). Therefore, we examined CD4-IgG binding to virion gp120 in the parental HIV-1_{NL4-3} and its V3 recombinant derivatives. The assay previously used to measure the interaction between CD4-IgG and gp120 on particles (57, 58) was modified as described in Materials and Methods. Briefly, ^{35}S-labeled virion suspensions were generated from transfected HeLa cells and aliquoted (100-μl portions) into microcentrifuge tubes. Individual samples were incubated at 37°C in the presence of 50 nM (0.23 μg) CD4-IgG for 30 min, 1 h, and 3 h to monitor the kinetics of CD4-IgG binding. A fourth sample was incubated for 3 h in the absence of CD4-IgG to serve as an untreated control. Following the 37°C incubation, the virus/CD4-IgG suspensions were placed on ice for 2 min, and the virus particles were subsequently pelleted out of the reaction mixture in a microcentrifuge at 4°C. Resuspended pellet samples were assayed for the amount of gp120 bound to CD4-IgG, and the corresponding supernatants were checked for gp120 released from the virions as a result of CD4-IgG

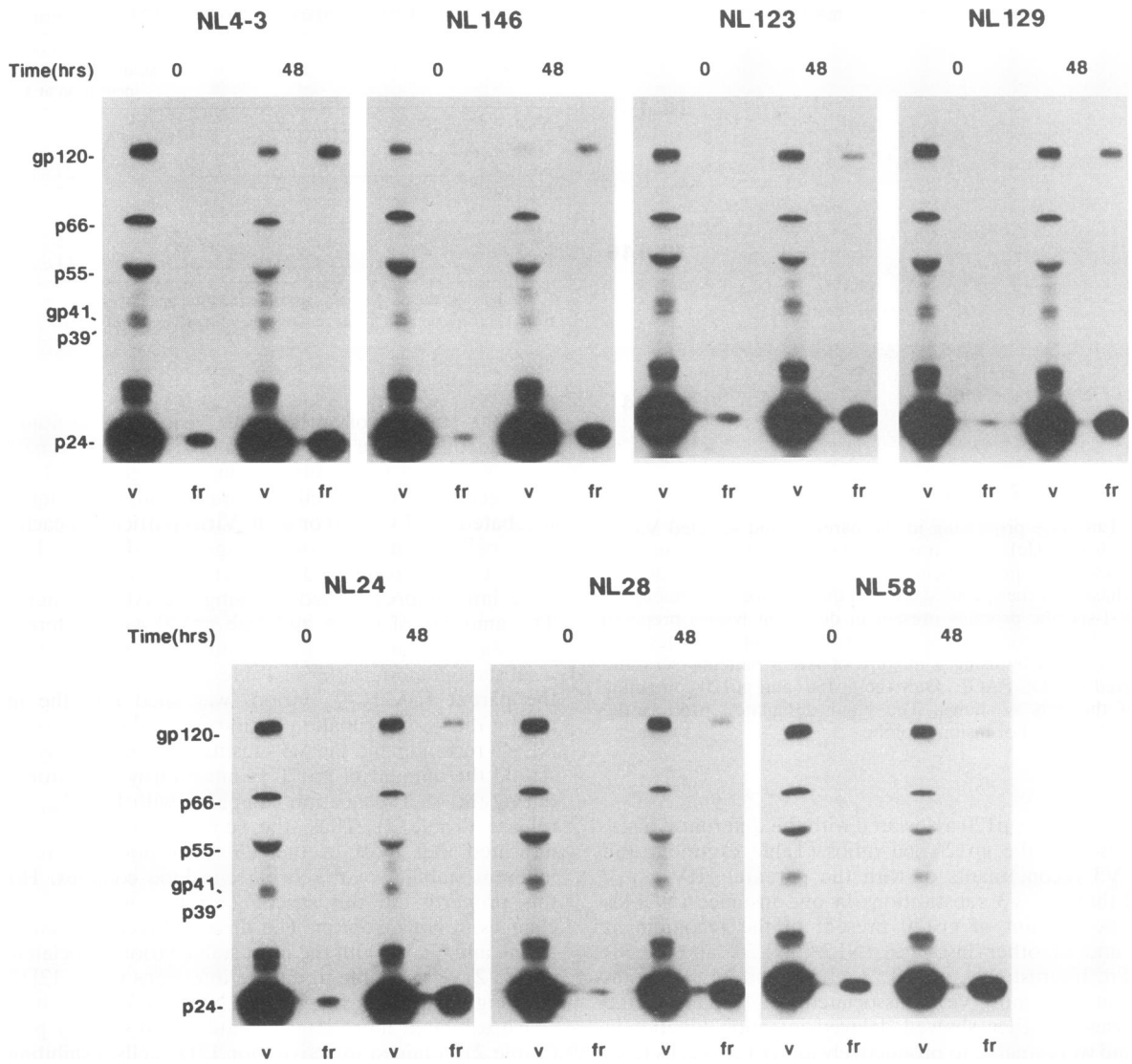

FIG. 5. Spontaneous release of gp120 from virions. Metobolically labeled virion suspensions were generated as described in Materials and Methods. Aliquots (100 μl) of the different suspensions were incubated at 37°C for 0 and 48 h, and the virion-associated (v) and free (fr) viral proteins were separated by pelleting the virions in a microcentrifuge. Pellet and supernatant fractions were lysed, immunoprecipitated with a mixture of AIDS patients' sera, and resolved by SDS-PAGE. Times of incubation are shown across the tops of the gels. The HIV-1-encoded proteins are indicated on the left and defined in the legend to Fig. 3. The amounts of gp120 released from the virions are quantitated in Table 2.

binding. The amount of gp120 associated with CD4-IgG in the virion pellet plus any gp120 released from the virions because of binding were divided by the total gp120 (bound + released + not bound) to calculate the proportion of gp120 bound to CD4-IgG following different periods of incubation.

As shown in Fig. 6, two groups of V3 recombinant viruses became readily apparent, on the basis of the kinetics of particle-associated gp120 binding to CD4. The rate at which CD4-IgG bound to the NL24, NL28, and NL58 virion gp120s was substantially slower than that observed for the parental NL4-3 and the three other V3 recombinants. This difference was most evident at the 30-min time point, at which only 16 to 22% of the NL24, NL28, and NL58 gp120s had associated with CD4-IgG, compared with 63 to 72% for the NL4-3, NL146, NL123, and NL129 viruses (Fig. 6B). The percent gp120 bound to CD4-IgG in the NL146 virus might actually be slightly less than what is depicted in Fig. 6B, since we did not account for the small amount of gp120 which was spontaneously lost from the NL146 virions during the 3-h incubation in the absence of CD4-IgG (see NL146 lanes in Fig. 6A). Nonetheless, the rate of CD4-IgG binding in NL146 was still comparable to the NL4-3, NL123, and NL129 viruses. The cumulative results from the binding experiments indicate that several of the V3 substitutions influenced the interaction of virion-associated gp120 with CD4. Of significance was the consistently reduced CD4 binding of the NL24, NL28, and NL58 HIV-1 recombinants, the same three V3 recombinants that had lost infectivity for the 12D7 T-cell line (Fig. 2).

Physical and functional properties of a macrophage-tropic virus envelope. Although several of the V3 recombinant viruses selected for this study possessed particle-associated gp120s with physical and functional properties conferring replicative and tropic properties that differed from the HIV_{NL4-3} parent, none were able to establish detectable

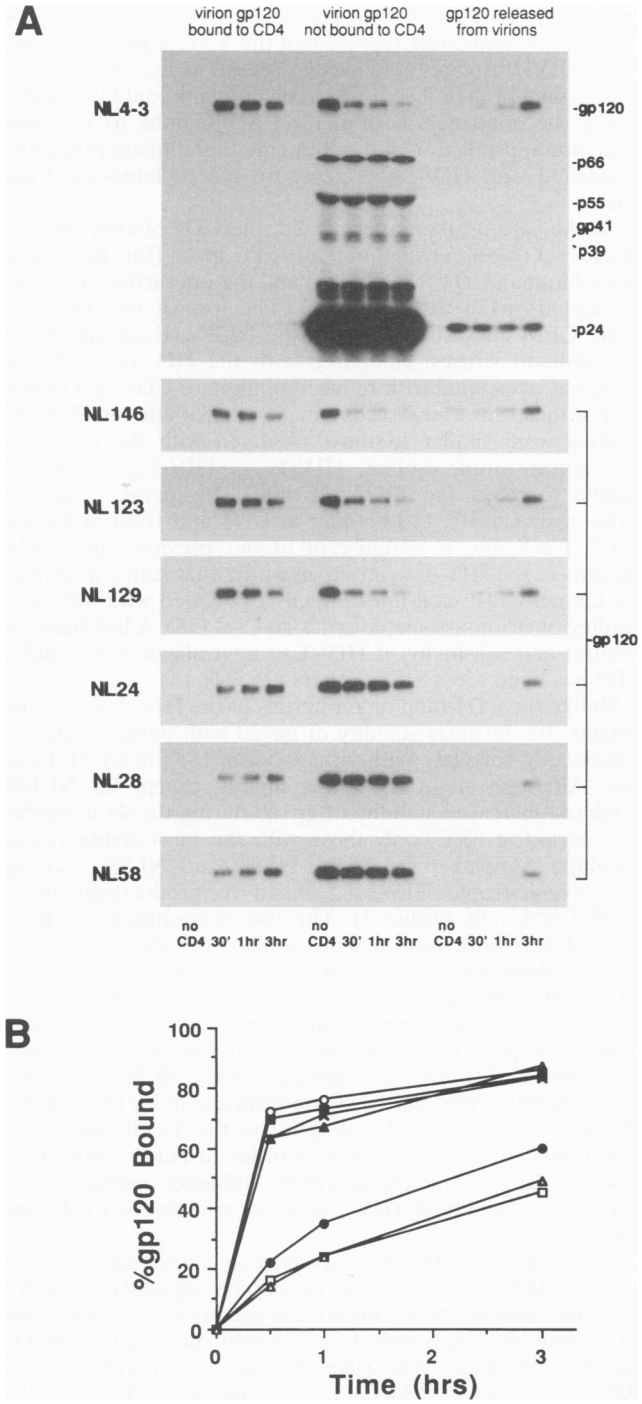

FIG. 6. CD4 binding to virion-associated gp120. Aliquots (100 μl) from metabolically labeled virion suspensions were transferred into microcentrifuge tubes and incubated in the absence or presence of 50 nM CD4-IgG at 37°C for 30 min, 1 h, and 3 h. The virions were pelleted in a microcentrifuge, and the pellet and supernatant fractions were treated according to the protocol outlined in Materials and Methods. The proteins were resolved by SDS-PAGE, and the gp120 bands were quantitated to determine the amount of gp120 bound to CD4-IgG at the different time points. (A) The top gel shows the distribution of the NL4-3 HIV-1 proteins in the different fractions after incubation of the virions in the presence of CD4-IgG and following pelleting of the virions by microcentrifugation. The remaining gels show only the gp120 portions of the gels. Fractions containing virion gp120 bound to CD4-IgG, virion gp120 not bound to CD4-IgG, and gp120 released from virions are indicated at the top; times of incubation are shown at the bottom of the gels. Untreated sample lanes (no CD4) are indicated. (B) Percent gp120 bound to CD4-IgG at the different time points, based on quantitation of the gp120 bands shown in the upper panel. Symbols: ○, NL4-3; ▲, NL146; ■, NL123; X, NL129; ●, NL24; □, NL28; and △, NL58.

infections in monocyte-derived macrophages characteristic of prototypic macrophage-tropic HIV-1 isolates such as HIV-1_{Ba-L}, HIV-1_{JR-CSF}, or HIV-1_{ADA} (18, 19, 27). We have recently obtained an infectious molecular proviral DNA clone (53a) from PBMCs infected with HIV-$1_{AD-87(M)}$ (42), a derivative of the HIV-1_{ADA} isolate (19). This cloned proviral DNA (pAD8-2) was used to prepare ^{35}S-labeled virions from transfected HeLa cells, and the physical and functional properties of the virion-associated gp120 of this macrophage-tropic strain (Fig. 2) were examined as described for the V3 recombinants. As shown in Fig. 7, the AD8 virion gp120-gp41 envelope complex was extremely stable, since virtually no gp120 (approximately 1%) was spontaneously released from the virions following the 48-h incubation at 37°C (Fig. 7A). The binding of the AD8 virion gp120 to CD4-IgG was reduced (Fig. 7B and C) compared with that of the T-cell line-tropic viruses (NL4-3, NL146, NL123, and NL129; Fig. 6), but it was similar to that observed for the NL24, NL28, and NL58 V3 recombinant viruses (Fig. 6) which, like AD8, were unable to productively infect the 12D7 T-cell line (Fig. 2). Thus, although our analysis of HIV-1_{AD8} further demonstrated a relationship between CD4 binding and tropism for the T-cell line, it failed to delineate unique envelope properties specific for macrophage tropism.

DISCUSSION

The biological classification of HIV-1 isolates has typically focused on properties such as replication rate, syncytium-inducing capacity, and cell tropism (2, 7, 13, 46, 53). Since the method of choice for the isolation of virus from seropositive individuals invariably employs a cocultivation technique utilizing activated recipient human PBMCs, virtually all of the HIV-1 isolates obtained to date are able to infect the CD4$^+$-positive peripheral blood lymphocytes contained within the larger PBMC population. A subset of fresh virus isolates is able to infect monocyte-derived macrophages (as well as CD4-positive peripheral blood lymphocytes) but typically cannot induce syncytium formation or initiate infections in immortalized human T-cell lines such as CEM or H9 (7, 18, 27, 44). Other isolates, particularly those recovered during the symptomatic phase of an HIV-1 infection, are able to replicate well in both PBMCs and immortalized CD4$^+$ T-cell lines but are not infectious for monocyte-derived macrophages (46, 47, 53). Several groups have mapped the viral determinants of these two extremes of HIV-1 cell tropism to specific domains within the viral envelope glycoproteins, particularly the V3 region of gp120 (9, 23, 49). However, the intrinsic genetic variability of the gp120s associated with different HIV-1 isolates makes it difficult to predict how a specific amino acid substitution(s) or the structure of a specific *env* domain might modulate a particular baseline tropic phenotype.

As a first step toward understanding the relationship between V3 determinants of macrophage and T-cell line tropism and concomitant V3-induced changes in physical and functional properties of the virion-associated gp120, we examined a group of previously reported recombinant viruses (9) containing a 40-amino-acid V3 replacement for residues normally

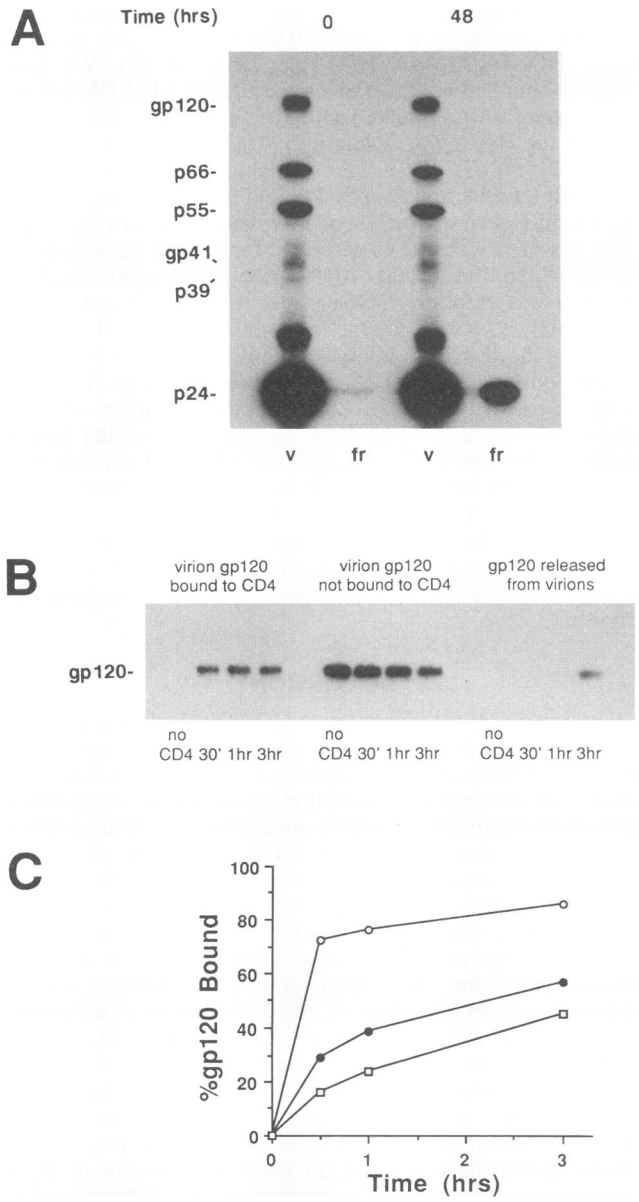

FIG. 7. Physical and functional properties of a macrophage tropic HIV-1 envelope. (A) Spontaneous release of gp120 from virions. Metabolically labeled virion suspensions were generated following transfection of HeLa cells with the pAD8-2 macrophage-tropic proviral plasmid clone of HIV-1, and 100-μl virion suspension aliquots were incubated at 37°C for 0 and 48 h. Virion-associated (v) and free (fr) viral proteins were separated by pelleting the virions in a microcentrifuge. Viral pellet and supernatant fractions were lysed, immunoprecipitated with a mixture of AIDS patients' sera, and resolved by SDS-PAGE. The different HIV-1 proteins are indicated on the left and are defined in the legend to Fig. 3. (B) CD4 binding to virion-associated gp120. Aliquots (100 μl) from metabolically labeled AD8 virion suspensions were incubated in the absence or presence of 50 nM CD4-IgG at 37°C for 30 min, 1 h, and 3 h. The virions were pelleted in a microcentrifuge, and the pellet and supernatant fractions were treated according to the protocol outlined in Materials and Methods. The proteins were resolved by SDS-PAGE. The panel shows only the gp120 portion of the gel. Fractions containing virion gp120 bound to CD4-IgG, virion gp120 not bound to CD4-IgG, and gp120 released from virions are indicated; times of incubation are shown at the bottom of the panel. Untreated sample lanes (no CD4) are indicated. (C) Percent AD8 gp120 bound to CD4-IgG (●) at the different time points, based on quantitation of the gp120 bands shown in panel B. Binding data for the NL4-3 (○) and NL28 (□) viruses from Fig. 6 are included for comparison.

present in the T-cell line-tropic isolate, HIV-1_{NL4-3}. Because the inserted sequences represented the V3 regions of different fresh HIV-1 isolates and were present within the isogenic background of HIV-1_{NL4-3}, the recombinants could be used to assess the contribution of natural V3 regions to envelope structure and function and potentially to delineate properties associated with HIV-1 infectivity for macrophages or T-cell lines.

Of the various assays performed, the CD4 binding analysis revealed a direct correlation between tropism (for the immortalized human $CD4^+$ T-cell line) and the interaction of virion-associated gp120 with CD4-IgG. The loss of infectivity for 12D7 CEM cells observed with the NL24, NL28, and NL58 recombinant viruses, compared with the HIV-1_{NL4-3} prototype, was associated with reduced binding to CD4-IgG (Table 3). For these three viral recombinants, the kinetics of CD4-IgG binding were similar to those observed with the prototype macrophage-tropic isolate, HIV-1_{AD8}. HIV-1_{AD8} was also unable to infect the 12D7 cell line. This parallel between virion-associated gp120 binding to CD4 and tropism for the 12D7 T-cell line is reminiscent of our previous analysis of variants of the HIV-1_{ELI} strain, in which augmented infectivity for human $CD4^+$ cell lines directly correlated with increased binding of virion-associated gp120 to CD4 (58). A link between tropism and sensitivity of HIV-1 to neutralization by soluble CD4 has been reported by others (24, 34).

Unlike the CD4-binding properties of the HIV-1 V3 recombinants, the intrinsic stability of gp120 with particles did not consistently correlate with virus tropism for the CEM T-cell line. Although all of the recombinants, except for NL146, exhibited increased stability of gp120 during the 48-h incubation period at 37°C, only those with the most stable virion-envelope complex (viz., NL24, NL28, and NL58) and the macrophage-tropic HIV-1_{AD8} failed to productively infect 12D7 CEM cells (Table 3). The two recombinants with increased yet more intermediate levels of particle-associated gp120 stability (NL123 and NL129) were able to infect 12D7 CEM cells with kinetics identical to the parental HIV-1_{NL-4-3}. We cannot rule out the possibility, however, that the inherent physical properties of the virion envelope, in combination with the reduced CD4 binding observed with the NL24, NL28, and NL58 recombinants and AD8, are responsible for the inability of these viruses to productively infect the T-cell line. Additional studies will be required in order to better understand how specific envelope characteristics influence envelope function and the ability of HIV-1 to grow in different $CD4^+$ cell types.

As noted earlier, this study focused on a group of previously reported HIV-1 recombinants containing segments of the V3 envelope domain that conferred a spectrum of tropism for $CD4^+$ human cells. Some of the recombinants, such as NL146 and NL123, were able (like the isogenic parental virus, HIV_{NL4-3}) to infect PBMCs and continuous cell lines such as CEM and HeLa 1022, whereas a second group were classified as macrophage-tropic (9). A subset of the latter group was included in our analysis. In contrast to other macrophage-tropic isolates of HIV-1 previously examined (19, 27), the subset evaluated in the present study failed to generate significant levels of progeny particles in monocyte-derived macrophages (as monitored by RT production) compared with a

TABLE 3. Comparative properties of V3 recombinants versus prototypical T-cell line and macrophage-tropic viruses

Virus designation	Infectivity (day of peak RT)			Stability of gp120 with virions	Kinetics of CD4 binding
	PBMC	T-cell line	Macrophage		
NL4-3 (T)[a]	5	7	ND[b]	Low	Rapid
NL146	9–11	11	ND	Low	Rapid
NL123	5	7	ND	Intermediate	Rapid
NL129	5	7–9	ND	Intermediate	Rapid
NL24	9	ND	ND	High	Slow
NL28	11	ND	ND	High	Slow
NL58	9	ND	ND	High	Slow
AD8 (M)[c]	9	ND	11–16	High	Slow

[a] Prototypical T-cell line virus.
[b] No detectable RT activity.
[c] Prototypical macrophage-tropic virus.

prototypical macrophage tropic isolate, HIV-1_{AD8}. When virus production in monocyte-derived macrophages was measured by using an antigen capture assay to detect the HIV-1 capsid protein p24, up to fivefold more NL24, NL28, and NL58 progeny virions could be detected (data not shown) compared with HIV-1_{NL4-3} (22 pg/ml). In the same experiments, the prototype macrophage-tropic isolate HIV-1_{AD8} directed the synthesis of 7×10^5 pg of p24 per ml. The low levels of progeny virus production observed for the three recombinant viruses may reflect the selection of three V3 recombinants exhibiting only modest infectivities, as well as differences in our monocyte-derived macrophage culture system compared with that employed in the previous study (9). It also seems likely that since the substituted V3 regions in the NL24, NL28, and NL58 recombinant viruses were not in the context of their native envelope glycoproteins, they would not necessarily be expected to affect envelope function in an identical manner while in the background of the NL4-3 envelope. On the other hand, the designation of the HIV-1_{Lai} or HIV-1_{NL4-3} and HIV-1_{Ba-L}, HIV-1_{JR-CSF}, or HIV-1_{AD8} as prototypes of T-cell line- and macrophage-tropic isolates, respectively, may merely reflect a tissue-culture adaptation phenomenon attending their isolation, rather than the replicative properties of all HIV-1 strains in vivo. The relatively low level of infectivity for monocyte-derived macrophages observed for the NL24, NL28, and NL58 viral recombinants might simply be indicative of a tropism continuum which exists among different isolates of HIV-1.

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