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Increase in Soluble CD4 Binding to and CD4-Induced Dissociation of gp120 from Virions Correlates with Infectivity of Human Immunodeficiency Virus Type 1

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Mutations in the human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins gp120 and gp41, previously shown to confer an enhanced replicative capacity and broadened host range to the ELI1 strain of HIV-1, were analyzed for their biochemical effects on envelope structure and function. The tendency of purified virions to release their extracellular gp120 component, either spontaneously or after interacting with soluble CD4 (CD4-induced shedding) was assessed. A single amino acid substitution in part of the CD4 binding site of gp120 (Gly-427 to Arg) enhanced both spontaneous and CD4-induced shedding of gp120 from virions, while a single change in the fusogenic region of gp41 (Met-7 to Val) affected only CD4-induced shedding. Although each codon change alone conferred increased growth ability, virus with both mutations exhibited the most rapid replication kinetics. In addition, when both of these mutations were present, virions had the highest tendency to shed gp120, both spontaneously and after exposure to soluble CD4. Analysis of CD4 binding to virion-associated gp120 showed that the changes in both gp120 and gp41 contributed to increased binding. These results demonstrated that the increased replicative capacity of the ELI variants in human CD4$^+$ cell lines was associated with altered physical and functional properties of the virion envelope glycoproteins.

The envelope of human immunodeficiency virus type 1 (HIV-1) has been shown to be the major determinant of cell tropism (9, 11, 13–15, 18, 39), the major viral protein against which both neutralizing and nonneutralizing antibodies are raised in infected people (6, 30), and a protein shown to elicit T-cell responses (16, 17, 66). The viral envelope is synthesized as a gp160 glycoprotein precursor that is proteolytically cleaved by host enzymes into the extracellular gp120 and the transmembrane gp41 components (2, 42, 56, 62, 65, 70). These processed envelope products are then transported to the plasma membrane and incorporated into virions, where they associate by noncovalent interactions (34, 37, 43). Interaction of HIV-1 with its cell surface receptor CD4 leads to a conformational change in the envelope (20, 33, 57), and when soluble derivatives of CD4 are used, dissociation of gp120 from gp41 occurs (3, 7, 19, 33, 36, 40, 48, 49). Fusion (41, 61) between the viral and cellular membranes leads to virus uptake. Since the amino-terminal region of gp41 contains the fusogenic domain of the HIV-1 envelope (5, 24, 27, 29, 37, 53, 60) and epitopes near the N terminus of gp41 become reactive with monoclonal antibodies after the binding of CD4 to virions (33, 57), it is possible that this CD4-induced dissociation of gp120 reflects a physiological process important to virus uptake (20).

Several lines of evidence have brought into question the biological relevance of envelope shedding. Different isolates were found to shed gp120 to various extents in response to soluble CD4 treatment, despite the fact that they are all replication competent (45, 47). Also, some mutations eliminated certain critical envelope functions such as fusion while not affecting CD4-induced gp120 shedding (4, 64); others seriously impaired CD4-induced gp120 dissociation while having less effect on virus uptake (64). Therefore, the relationship of CD4-induced release of gp120 from HIV-1 virions with virus infectivity remains unclear. Isogenic HIV-1 variants that differ from each other at only a few amino acids and exhibit distinct biological properties would be useful to relate envelope structure to biological function and to determine whether the dissociation of gp120 by soluble CD4 correlates with virus infectivity.

After prolonged culture of certain partially defective viruses, variants that have an increased replicative capacity sometimes emerge in the culture (12, 23, 54). Adaptation to growth is thought to occur by mutation and subsequent selection. We have previously reported that growth of the ELI1 strain of HIV-1 in H9 cells led to the production of variants after a delay of more than 20 days. The emerging virus not only had increased replication kinetics in H9 and other T-cell lines but also was able to establish a productive infection in U937 promonocytic cells, which are refractory to infection with the parental ELI1 virus (54). Mutations accounting for this enhanced replication capacity and extended host range were localized to two regions of the *env* gene (26). One was identified in a part of gp120 known to be involved in the binding of gp120 to CD4 (18, 38, 51, 55), and the other was identified in the fusogenic domain (5, 24, 27, 29, 53, 60) at the amino terminus of gp41.

As a first step in understanding the molecular mechanisms for the augmented infection kinetics of these adapted ELI viruses, we have investigated the consequences of the *env* mutations on the stability of the virion-associated HIV-1 external envelope glycoprotein in the absence and presence of soluble CD4. Using the set of biologically derived HIV-1_{ELI} variants, we show that mutations in the envelope that conferred an enhanced replicative capacity in CD4$^+$ cell lines increased both the binding ability of virion-associated gp120 for soluble CD4 and the amount of CD4-induced shedding of gp120 from virions. These results demonstrated that altered physical and functional properties of the virion envelope of the

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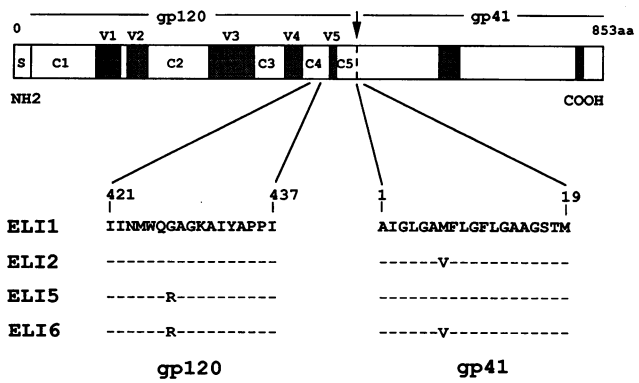


FIG. 1. Sequences of ELI mutants. Shown at the top is the 853-amino-acid (aa) HIV-1 envelope of ELI indicating the signal peptide (S), the conserved regions (C1 to C5), and the hypervariable regions (V1 to V5) of gp120 and gp41 (shaded). Predicted envelope amino acid sequences of ELI1, ELI2, ELI5, and ELI6 from residues 421 to 437 of the gp120 C4 region and residues 1 to 19 of gp41 are shown. Dashes indicate amino acid identity; letters indicate differences.

ELI variants correlated with increased infectivity in human $CD4^+$ cell lines.

MATERIALS AND METHODS

Bacterial strain, plasmids, and plasmid DNA preparation. Reagents and methods have been described previously (54).

Construction of viruses. The infectious molecular clone of $HIV\text{-}1_{ELI}$, pELI1, has been described previously (1, 54). The descriptions, sequences, and properties of viruses derived from pELI1, pELI2, and pELI4 have been published previously (26, 54). The plasmids pELI5 and pELI6 were constructed with appropriate restriction fragments by combining the CD4 binding region mutation in pELI4 (Gly-427 to Arg) either with the wild-type gp41 region in pELI1 (to produce pELI5) or with the gp41 mutation in pELI2 (Met-7 to Val) to produce pELI6. The relevant part of the envelope sequence from these viruses is shown in Fig. 1.

Cells and media. HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (DMEM-10). The sources of peripheral blood mononuclear cells, the $CD4^+$ lymphocyte cell lines (CEM clone 12D7, HUT78 clone H9, SupT1, Jurkat clone E6-1), and the promonocyte cell line U937 have been described previously (54). The cell lines were grown in RPMI 1640 with 10% fetal bovine serum (RPMI-10) and 2 μg of Polybrene per ml (RPMI-10/2); for the peripheral blood mononuclear cells, RPMI-10/2 was supplemented with 10% interleukin 2.

Viral infectivity studies. The methods used to infect cells have been described previously (54). Virus stocks were prepared following transfection of HeLa cells with plasmid DNA (15 to 20 μg) by calcium phosphate coprecipitation methods (25, 31, 69). Reverse transcriptase (RT) activity (73) was measured 24 and 48 h after transfection. An amount of virus corresponding to 10^5 cpm of RT activity was added to 2×10^6 cells in 1 ml of RPMI-10/2. Virus adsorption was for 2 h at 37°C, after which time the cells were diluted in RPMI-10/2. At 2-day intervals, the cells were fed with RPMI-10/2 and samples were taken for determination of RT activity. Cultures were monitored daily for the presence of syncytia.

Transfections and preparation of ^{35}S-labelled virions. HeLa cells were trypsinized and transferred to 25-cm^2 flasks (3×10^6 cells per flask) 24 h prior to transfection. Calcium phosphate-precipitated uncleaved plasmid DNA (35 μg) was added to each flask (two flasks for each virus), and the cells were incubated for 3 h. The cultures were then washed once at room temperature with phosphate-buffered saline (PBS) and subjected to glycerol shock (25) for 2.5 min. Following removal of the glycerol solution, the cells were washed once with PBS and maintained in 4 ml of DMEM-10.

Transfected HeLa cells were scraped off the flasks at 18 to 20 h posttransfection, washed with methioine-free RPMI 1640, and then starved for 10 min in methionine-free RPMI 1640 medium containing 5% fetal bovine serum. The cells were pelleted, resuspended in 1 ml of labelling medium for each 25-cm^2 flask transfected, and transferred to upright 25-cm^2 flasks. Labelling medium contained 300 μCi of [^{35}S]methionine (1,100 Ci/mmol [NEN]) in 0.9 ml of methionine-free RPMI 1640 and 0.1 ml of RPMI-10. After 10 h at 37°C, an additional 1 ml of the labelling medium was added and the cells were incubated for 12 to 14 h.

The medium was clarified following the 22- to 24-h labelling period by low-speed centrifugation ($1,000 \times g$) and filtering through a 0.45-μm-pore-size cellulose acetate filter to remove any cells and cellular debris. The labelled virions were collected from the medium by centrifugation for 30 min at 35,000 rpm at 25°C in an SW55 rotor (Beckman), and the virion pellet was resuspended in RPMI-10 (200 to 500 μl) with a P1000 Pipetman (Gilson).

Microcentrifuge pelleting of HIV-1 virions. Labelled ELI1 virions were collected by ultracentrifugation as described above and resuspended in 200 μl of RPMI-10. Half of the material was transferred to a 1.5-ml polypropylene screw-cap microcentrifuge tube (Sarstedt) and centrifuged at 14,000 rpm in an Eppendorf centrifuge for 1 h at room temperature. Following centrifugation, 90 μl of the supernatant was carefully removed from the side of the tube opposite the line of centrifugal force and transferred to a new tube. The virion pellet was washed with 40 μl of PBS and centrifuged for an additional 30 min. Forty microliters of the supernatant was removed and combined with the first supernatant. The pellet fraction was resuspended in 130 μl of RPMI-10.

The efficiency of virion recovery by microcentrifugation was assessed by analyzing the resuspended pellets from the ultracentrifugation and the pellet and supernatant fractions from the Eppendorf centrifugation on sucrose gradients. Samples were layered onto 10 to 60% (wt/vol) continuous sucrose gradients (4.5 ml) and centrifuged in an SW55 rotor for 90 min at 35,000 rpm at 4°C (71). Fractions (14 by 330 μl) of the gradients were collected from the top. Each fraction was lysed by adding 700 μl of 0.5% Triton X-100 lysis buffer (300 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.5% Triton X-100) and immunoprecipitated (70, 71) with HIV-1-reactive antibodies present in a mixture of sera obtained from AIDS patients (AIDS patients' sera). Sequential immunoprecipitations demonstrated that the antibodies were in excess for the viral proteins. Immunoprecipitated proteins were solubilized by boiling for 7 min in sample buffer (2% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 1% glycerol, 65 mM Tris-HCl [pH 6.8]) and separated on 10% acrylamide–AcrylAide–SDS (FMC) gels for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were fixed in a solution of 40% methanol–10% acetic acid for 2 h at room temperature, rinsed with water, soaked in 1 M sodium salicylate for 2 h, and dried. Radioactive protein bands were visualized by fluorography.

Spontaneous shedding of gp120 from virions. Labelled virion suspensions for each virus were prepared from transfected cells by ultracentrifugation as described above. The

virion suspensions were split into two 100-μl samples and transferred to 1.5-ml Sarstedt microcentrifuge tubes. The virions in one sample from each virus were immediately pelleted in an Eppendorf microcentrifuge as described above. Both the pellet and supernatant samples were lysed in 1 ml of 0.1% Triton X-100 lysis buffer (50 mM Tris-HCl [pH 7.4], 300 mM NaCl, 0.1% Triton X-100) and immunoprecipitated with the AIDS patients' sera. The second sample of each virus was incubated in a water bath at 37°C for 48 h prior to pelleting in a microcentrifuge and immunoprecipitation; the immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. The gp120 present in the virion and supernatant fractions was quantified by using a Fujix Bas 2000 Bio-image Analyzer and summed to give the total amount of gp120. The percentage of the gp120 lost from virions over time was determined.

CD4-IgG-induced shedding of gp120 from virions. Samples (100 μl) of labelled virion suspensions from the different ELI viruses were transferred to microcentrifuge tubes and incubated for 2 h at 37 or 4°C in the absence or presence of 220 nM (1 μg) CD4-immunoglobulin G (IgG) (8, 10) (a gift from Genentech Inc., South San Francisco, Calif.). (The CD4-IgG was dialyzed against water for 24 h at 4°C prior to use to remove the detergent present in the stock solution.) The 37°C samples were pelleted at room temperature in an Eppendorf microcentrifuge, while the 4°C samples were pelleted at 4°C in a refrigerated Eppendorf microcentrifuge (model 5402) as described above. The pellet and supernatant samples were treated with 0.1% lysis buffer, immunoprecipitated with AIDS patients' sera, and analyzed by gel electrophoresis as described above. The amounts of virion-associated and free gp120 were quantified with the Fujix Bas 2000 Bio-image Analyzer, and the percentage of gp120 released from virions was calculated.

Labelled virion suspensions were prepared for each ELI virus, and 100-μl samples were either untreated or treated with 10-fold dilutions of CD4-IgG (ranging from 0.5 to 500 nM [2.3 ng/100 μl to 2.3 μg/100 μl]) to evaluate their effect on gp120 shedding. All samples were incubated for 2 h at 37°C, and virion and supernatant fractions were collected following centrifugation in a microcentrifuge at room temperature. Samples were treated with 0.1% Triton X-100 lysis buffer, immunoprecipitated, and analyzed by SDS-PAGE as described above. The amount of gp120, both released and remaining virion associated after treatment of the virions with various dilutions of CD4-IgG, was quantified with the Fujix Bas 2000 Bio-image Analyzer.

Binding of CD4-IgG to virions. HeLa cells were transfected with pELI1, pELI2, pELI5, or pELI6, and the cells were labelled for 24 h as described above. Radioactively labelled virions of ELI1, ELI2, ELI5, and ELI6 were purified by ultracentrifugation, and the virion pellets were resuspended in 400 μl of RPMI-10. Samples (100 μl) were transferred into four 1.5-ml screw-cap Sarstedt microcentrifuge tubes. CD4-IgG was added to three tubes to give final concentrations of 5, 50, and 500 nM, with a fourth tube serving as an untreated control. The samples were incubated on ice for 1 h, and the virions were pelleted at 14,000 rpm for 1 h at 4°C in a refrigerated Eppendorf microcentrifuge. The supernatants were carefully removed and transferred to new tubes. Detergent lysates of the viral pellets and corresponding supernatant samples were prepared by adding 1 ml of 0.1% Triton X-100 lysis buffer to each tube followed by vortex mixing. Protein A-agarose (50 μl [Bethesda Research Laboratories]) was added to the viral pellet lysates to recover any gp120 associated with CD4-IgG. The IgG portion of the CD4-IgG molecule allows it to bind to protein A (8, 10). After 1 h of mixing at 4°C, the gp120-CD4-protein A-agarose complexes were collected by centrifugation, and the supernatants were transferred to tubes containing protein A-agarose that had been pretreated with the AIDS patients' sera for a second round of immunoprecipitation to determine the amount of gp120 not bound to CD4. The supernatant samples recovered after pelleting the virions were also immunoprecipitated with protein A-agarose beads treated with the AIDS patients' sera to detect any gp120 released from the virions as a result of CD4 binding. All precipitates were washed twice with lysis buffer and resuspended in sample buffer. The proteins were resolved by SDS-PAGE, visualized by fluorography, and quantified as described above. The relative binding properties of the different gp120 proteins were determined by calculating the percentage of gp120 that had bound to CD4-IgG as [(bound + released)/(bound + released + not bound)] × 100.

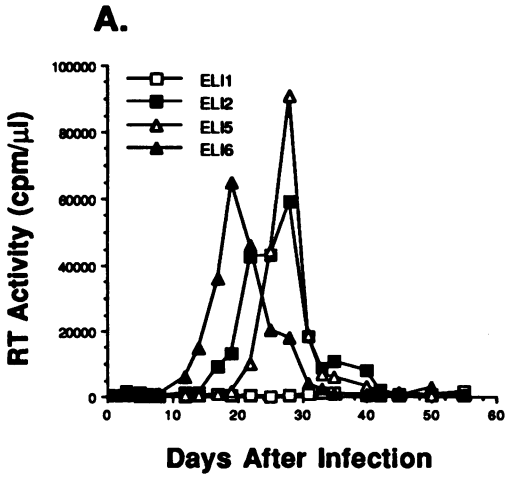
RESULTS

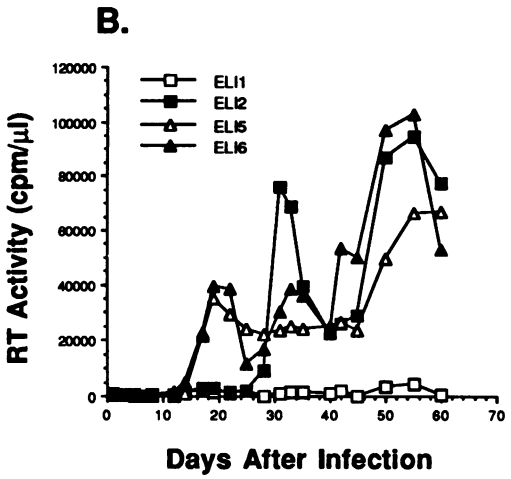
Construction of ELI5 and ELI6. Adaptation of ELI1 to replicate in the H9 T-lymphocytic cell line involved the acquisition of mutations in the amino-terminal region of gp41 at amino acids 7 (Met to Val) and 49 (Glu to Gly). The virus with the single Met-7-to-Val change was called ELI2, and the one with both Met-7 to Val and Glu-49 to Gly was called ELI3. In addition, variant ELI4 emerged during passage in H9 cells, and this virus had both of the gp41 mutations plus a mutation at residue 427 (Gly to Arg) of gp120 in a region known to be involved in the binding of gp120 to the virus receptor molecule CD4. All three viruses exhibited enhanced infection kinetics on T-cell lines compared with ELI1, while ELI4 had the additional property of being able to infect the promonocytic cell line U937 (26).

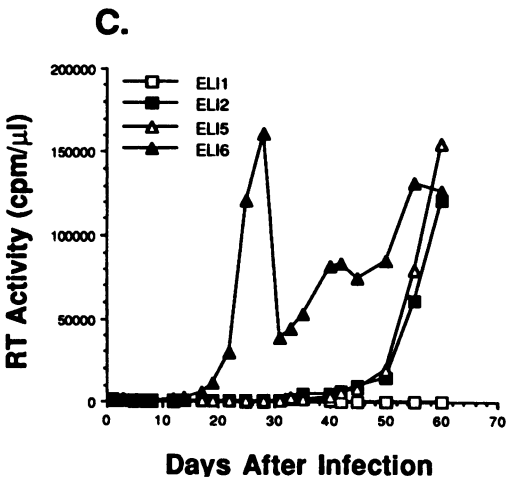
To investigate the contributions of the individual mutations to the phenotype of the viruses, the mutation in the CD4 binding region was introduced into the parental ELI1 gp41 background (with Met at position 7 of gp41) to produce ELI5 and the mutation was also introduced into the background of the biological variant ELI2 (Val-7 gp41) to produce ELI6 (Fig. 1). Subsequent work has demonstrated that the predominant variant that replicated in U937 cells (26) was in fact ELI6 (26a).

Growth properties of ELI1, ELI2, ELI5, and ELI6. Stocks of each virus were prepared by transfecting HeLa cells with pELI1, pELI2, pELI5, and pELI6 and were used to infect peripheral blood mononuclear cells and various cell lines. While all viruses productively infected peripheral blood mononuclear cells with indistinguishable growth kinetics (data not shown), differences were observed following infection of $CD4^+$ cell lines. On CEM cells, ELI6 induced the most rapid infection, with peak virus production occurring about day 19 after infection. Virus production of ELI2 preceded that of ELI5 by about 5 days, and there was no evidence that ELI1 generated progeny virions even after 56 days in culture (Fig. 2A). Virus production for ELI5 and ELI6 occurred contemporaneously in H9 cells, with peak activity on days 19 and 20, and before that of ELI2, which peaked on day 31. Again, ELI1 failed to grow in these cells (Fig. 2B). Therefore, each of the mutations separately conferred to $HIV\text{-}1_{ELI}$ the capacity to infect T-cell lines, although ELI6, containing both the Gly-427-to-Arg change in gp120 and the Met-7-to-Val change in gp41, had the fastest replication kinetics.

The increased infectivity of ELI6 compared with the other viruses was more pronounced with U937 cells, where the peak of ELI6 production occurred on about day 30 after infection (Fig. 2C). Little virus was detected with ELI2 and ELI5 before







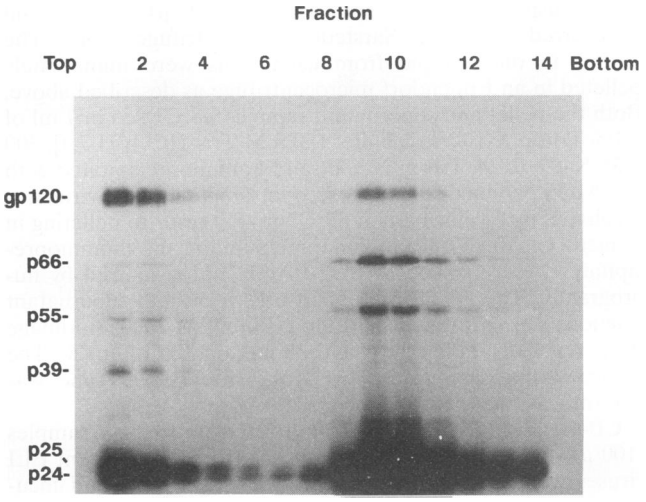


FIG. 3. Sucrose gradient fractionation of the supernatant from transfected HeLa cells. The supernatant (200 μl) recovered from HeLa cells transfected with pELI1 and labelled with [^{35}S]methionine was centrifuged through a 10 to 60% (wt/vol) sucrose gradient. Viral proteins were detected in the gradient fractions by immunoprecipitation with AIDS patients' sera and SDS-PAGE. Fraction numbers are given across the top, and the positions of the major HIV-1 proteins visible are indicated: gp120 envelope, p66 RT, and p55, p39, p25, and p24 Gag.

day 50. Thus, efficient replication of ELI in U937 cells required both the gp120 and the gp41 mutations. In fact, from the sudden increase in virus production so late after infection of U937 cells with ELI2 and ELI5, it is likely that additional mutations had occurred to allow replication.

Analysis of gp120 present in transfection supernatants. A major focus of this study was to investigate the consequences of the mutations in gp120 and gp41 on the structure and function of the virion-associated HIV-1 envelope. Because substantial amounts of non-virion-associated gp120 had been observed in the medium of HeLa cells transfected with other HIV-1 molecular clones (unpublished results), it was first necessary to determine the distribution of free and virion-associated gp120 in virus preparations obtained following transfection. HeLa cells were transfected with pELI1, and the cells were labelled metabolically with [^{35}S]methionine as described in Materials and Methods. Virus particles were separated from free proteins in the clarified transfection medium by centrifugation on a 10 to 60% sucrose gradient as described previously (71), and the HIV-1 proteins in each fraction were identified by immunoprecipitation with AIDS patients' sera and gel electrophoresis analysis (Materials and Methods). As shown in Fig. 3, most (ca. 75%) of the gp120 was found to be free (fractions 1 and 2) rather than in the part of the gradient (fractions 9 to 11) shown previously to contain virions (71). Therefore, for all subsequent biochemical work, virions were pelleted in the ultracentrifuge to remove the free gp120 present in transfection

FIG. 2. Replication of ELI1, ELI2, ELI5, and ELI6 in CEM (A), H9 (B), and U937 (C) cells. Approximately 2×10^6 cells were infected with 5×10^5 cpm of RT from virus stocks generated following transfection of HeLa cells with the different ELI proviral molecular clones. Cultures were monitored daily for the appearance of syncytia, and every 2 days the cultures were fed and samples were taken for RT determination.

supernatants (see below). Importantly, pelleting the virus under these conditions does not impair infectivity (unpublished results).

Fractionation of free gp120 from virion-associated gp120. We wanted to examine the dissociation of gp120 from virions of the different ELI variants. While free envelope can be separated from virion-associated envelope by pelleting HIV-1 in the ultracentrifuge (69a), this procedure is not particularly amenable to the processing of multiple samples. Therefore, we tested whether a microcentrifuge could be used to pellet virions instead, since earlier work (59) had suggested that this was possible. Labelled virions were first collected by ultracentrifugation and resuspended in RPMI-10 (200 μl), and half of this material was fractionated by sucrose gradient centrifugation. The remainder was centrifuged for 1 h at 14,000 rpm at room temperature in an Eppendorf microcentrifuge, and the resulting supernatant and resuspended pellet were separated by sucrose gradient centrifugation. Fractions from the gradients were immunoprecipitated with AIDS patients' sera and analyzed by SDS-PAGE. Because the HIV-1 proteins obtained from the pellet samples from both the ultracentrifuge (Fig. 4A) and the microcentrifuge (Fig. 4B) were predominantly associated with particles (fractions 9 to 12) and were not in the upper part of the gradient, which contained free proteins (Fig. 3), we conclude that little disruption of the virions had occurred during the pelleting and resuspension. Furthermore, since no virion-associated gp120 was detected in the supernatant following pelleting in the microcentrifuge (Fig. 4C), this method can be used to separate free gp120 from virion-associated gp120.

Differential spontaneous gp120 shedding from virions of the ELI variants. It has been reported that HIV-1 virions spontaneously lose gp120 over time (28, 44) and that the extent of the loss varies according to the isolate (44). Since the spontaneous dissociation of gp120 is most likely a reflection of the inherent physical properties of the virion-associated envelope, it may be a useful parameter for evaluating envelope structure and function. Therefore, we examined the spontaneous release of gp120 from virions of the ELI variants to determine whether specific physical properties of the envelopes correlated with the viral replication characteristics. Suspensions of pelleted ^{35}S-labelled virions were prepared from HeLa cells transfected with the four ELI proviral molecular clones. Equal volumes (100 μl) of each virion suspension were incubated at 37°C for 0 or 48 h, and the released and particle-associated gp120 components were separated by centrifugation in the microcentrifuge. The amounts of gp120 in the pellet and supernatant fractions were quantified following immunoprecipitation and gel electrophoresis.

As shown in Fig. 5, there was no free gp120 present in the 0-h incubation samples of ELI1, ELI2, ELI5, or ELI6. Only a small proportion of the gp120 was lost from the parental ELI1 and the mutant ELI2 virions during the 48-h incubation (16 and 19%, respectively [Fig. 5]). In contrast, the Arg-427 substitution in gp120 of ELI5 increased the amount of gp120 spontaneously released from virions to 35% during the same period (Fig. 5). While ELI5 replicated better than both ELI1 and ELI2 in H9 cells, the presence of the less stable ELI5 envelope did not correlate with better growth in CEM cells (Fig. 2). When both the Arg-427 mutation in gp120 and the Val-7 mutation in gp41 were present (ELI6), 48% of the gp120 was lost spontaneously from virions after incubation at 37°C for 48 h (Fig. 5). This virus had the highest replicative capacity in T-cell lines and was the only variant able to infect the promonocytic U937 cell line (Fig. 2). These results demonstrated that the gp120 mutation alone or in combination with the change in gp41 altered the inherent stability of the particle-associated envelope. However, an increase in the spontaneous release of gp120 did not consistently correlate with the ability of a particular variant to replicate in different cell lines.

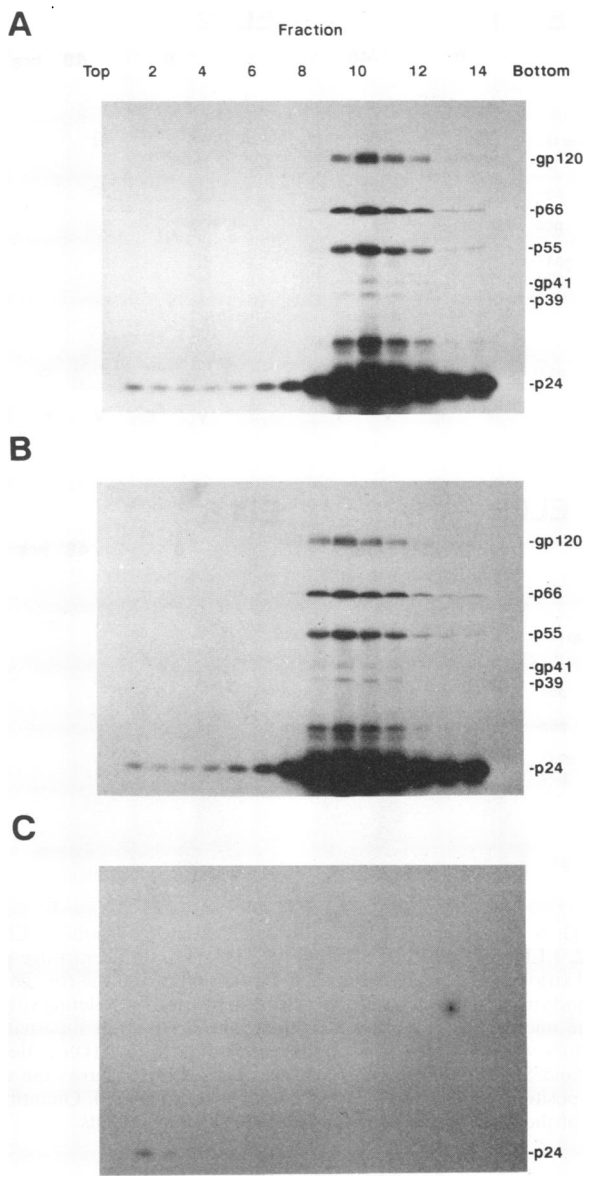


FIG. 4. Sucrose gradient centrifugation analysis of ELI1 virions purified by ultracentrifugation and microcentrifugation. [^{35}S]methionine-labelled ELI1, prepared from transfected HeLa cells, was pelleted in the ultracentrifuge and resuspended. Half was fractionated by sucrose gradient centrifugation (A). The remainder was pelleted in the Eppendorf microcentrifuge, and both the pellet (B) and supernatant (C) fractions were centrifuged on sucrose gradients. Gradient fractions were immunoprecipitated with AIDS patients' sera and analyzed by SDS-PAGE. Fraction numbers are given across the top, and the positions of the major HIV-1 proteins visible are indicated (see legend to Fig. 3).

CD4-induced shedding of gp120. The binding of soluble CD4 to virions is known to induce the release of gp120, and this dissociation has been suggested to reflect events that occur

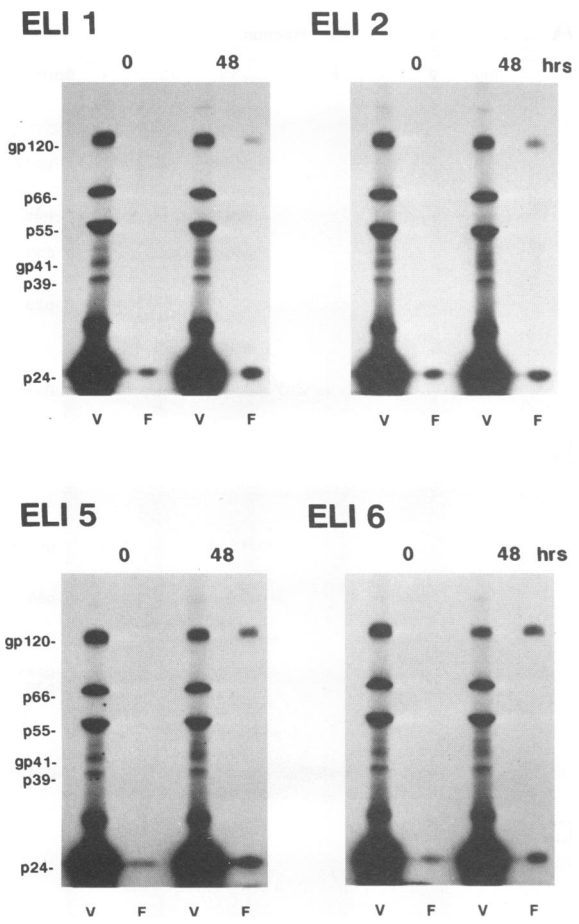


FIG. 5. Spontaneous shedding of gp120 from the virions of ELI1, ELI2, ELI5, and ELI6. [^{35}S]methionine-labelled, ultracentrifuge-pelleted virions were incubated at 37°C for 0 or 48 h, and the free gp120 (F) and virion-associated gp120 (V) were separated by pelleting virions in the microcentrifuge at room temperature. Pellet and supernatant fractions were analyzed by immunoprecipitation with AIDS patients' sera and SDS-PAGE. Times of incubation are given across the top. The positions of the major HIV-1 proteins are indicated. Quantification of the gp120 released from virions is given in Results.

during viral entry (20). To determine whether the envelopes of the four ELI variants had different susceptibilities to soluble CD4-induced shedding, labelled virions were incubated with the soluble CD4 derivative CD4-IgG (220 nM) at 4 or 37°C for 2 h. The released gp120 was separated from the virion-associated gp120 by microcentrifugation, immunoprecipitated, and quantified (Fig. 6 and Table 1). In the presence of CD4-IgG, no gp120 was released from the virions of ELI1 at 4°C. Mutations in either gp41 or gp120 conferred an increased sensitivity to CD4-induced gp120 shedding. For example, with the Val-7 change in gp41, ELI2 lost 25% of its gp120 at 4°C. Thus, while ELI1 and ELI2 behaved similarly in the spontaneous release of their gp120 components (Fig. 5), differences between them were revealed after treatment with CD4-IgG. Substituting Arg at position 427 of gp120 had a marked effect on CD4-induced gp120 shedding, with the loss of 40% of gp120 from ELI5 virions at 4°C. When both the gp120 and gp41 mutations were present (ELI6), 63% of the gp120 was dissociated by CD4-IgG at the same temperature. At 37°C, the effect was more pronounced, with CD4-IgG inducing a 14% loss of virion-associated gp120 from ELI1 and a 90% loss from ELI6, the most sensitive variant (Fig. 6 and Table 1). These results demonstrated that mutations in both gp120 and gp41 can influence the interaction of soluble CD4 with the virion. They also showed that increased sensitivity to CD4-induced release of gp120 from virions directly paralleled the ability of the viruses to replicate in CD4$^+$ cell lines.

It was possible that the differences among the ELI variants in their CD4-induced gp120 shedding properties were due to the concentration of CD4-IgG used in the assay. To test this, virions of the ELI variants were incubated at 37°C for 2 h with 0, 0.5, 5, 50, and 500 nM CD4-IgG, and the amounts of free and virion-associated gp120 were determined. These results (Fig. 7) demonstrated that the release of gp120 from the virions of the four viruses depended on the concentration of CD4-IgG. Considering the quantities of CD4-IgG required to release about 20% of the gp120 from virions, there appears to be 10-fold less CD4-IgG necessary to induce this amount of gp120 dissociation from ELI2 compared with the amount necessary for ELI1 and about 1,000-fold less CD4-IgG required for ELI5 and ELI6 compared with the amount required for ELI1.

Binding of CD4-IgG to virions. The results from the experiments described above demonstrated that single amino acid substitutions in both gp120 and gp41 affected the ability of CD4-IgG to induce the release of gp120 from virions. One reason for the differential effects on CD4-IgG-induced shedding of gp120 is that the four ELI viruses have different abilities to bind to CD4-IgG, since to induce gp120 release CD4-IgG must first bind to the virions. Therefore, we developed an assay to measure the binding of CD4-IgG to virions (72). To minimize the shedding of gp120, the assay was carried out on ice and is described in Materials and Methods.

Labelled virions of the four ELI viruses were incubated for 1 h at 0°C in the presence of CD4-IgG at 0, 5, 50, and 500 nM, after which the virus particles were collected by centrifugation. To ascertain the amount of gp120 released from virions by treatment with CD4-IgG, the supernatant fractions were lysed and immunoprecipitated with AIDS patients' sera. The pellet fractions were similarly lysed, and protein A-agarose was added to precipitate the gp120 associated with CD4-IgG on the virions, since the IgG portion of CD4-IgG binds to protein A. After pelleting the protein A-agarose beads, which contained the gp120 complexed with CD4-IgG, the supernatant fraction was immunoprecipitated with AIDS patients' sera to recover the gp120 not bound to CD4-IgG. The amount of gp120 in the bound-plus-released fractions divided by the total (bound plus released plus not bound) gives the proportion of gp120 bound to CD4-IgG in the virion.

The results from this experiment demonstrated that different amounts of CD4-IgG bound to the four HIV-1_{ELI} variants, with ELI1 binding the least and ELI6 binding the most (Fig. 8); results from replicate experiments are quantified in Table 2. Only at the highest concentration of CD4-IgG (500 nM) could any binding to ELI1 virions be demonstrated (6%). The Val substitution for Met in gp41 of ELI2 allowed more binding of CD4-IgG (5% at 50 nM and 29% at 500 nM). The single change of Arg for Gly at position 427 in gp120 (ELI5) also allowed more CD4-IgG binding (8% at 50 nM and 34% at 500 nM) compared with ELI1. When the gp120 and gp41 mutations were both present (ELI6), the highest level of CD4-IgG binding was found (20% at 50 nM and 56% at 500 nM).

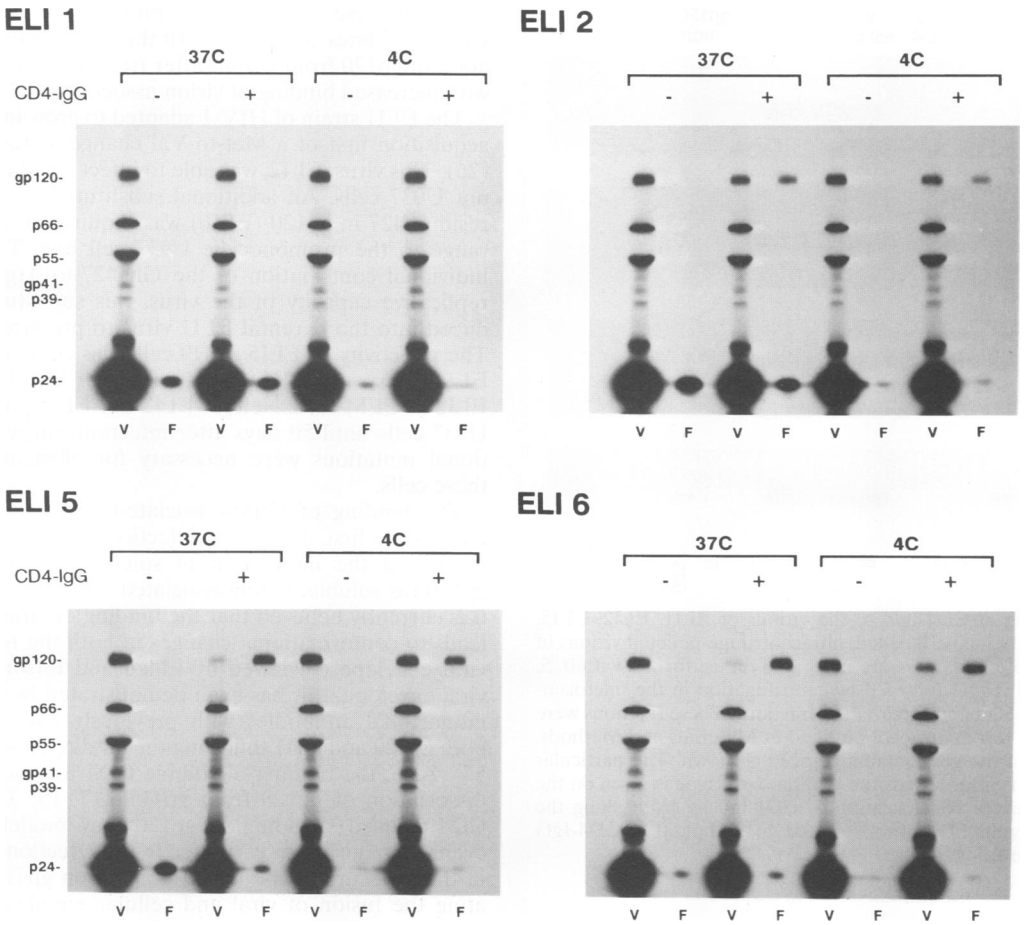


FIG. 6. CD4-induced shedding of gp120 from the virions of ELI1, ELI2, ELI5, and ELI6. [^{35}S]methionine-labelled, ultracentrifuge-pelleted virions were incubated at 37 or 4°C for 2 h in the absence or presence of 220 nM CD4-IgG, and the free gp120 (F) and virion-associated gp120 (V) were separated by pelleting virions in the microcentrifuge. Pellet and supernatant fractions were analyzed by immunoprecipitation with AIDS patients' sera and SDS-PAGE. Across the top of each panel are indicated the temperature of the incubation and whether the incubation was done in the presence (+) or absence (−) of CD4-IgG. The positions of the major HIV-1 proteins are indicated. Quantification of the gp120 released from virions is shown in Table 1.

DISCUSSION

Determinants of HIV-1 tropism reside predominantly in the viral envelope, and within this protein, specific regions that govern the ability of a virus to infect different cell types have been identified. For example, the V3 region of gp120 has been shown to be a major determinant of macrophage tropism for HIV-1 (9, 11, 14, 15, 35, 51, 58, 67, 68), although other

TABLE 1. CD4-IgG-induced gp120 shedding from virions

Virus	% of gp120 released at[a]:			
	4°C		37°C	
	−	+	−	+
ELI1	0	0	4	14
ELI2	1	25	9	40
ELI5	1	40	10	86
ELI6	3	63	12	90

[a] Values are the percentages of free gp120 from total gp120 and are calculated from the results shown in Fig. 6. −, without CD4-IgG incubation; +, with CD4-IgG incubation.

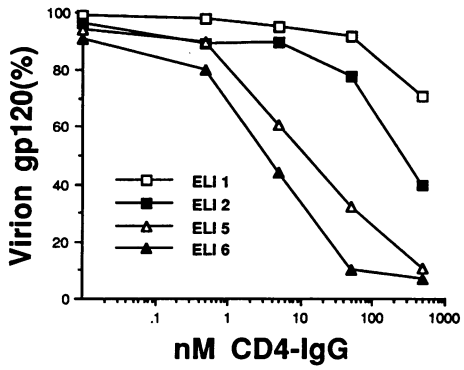


FIG. 7. Titration of CD4-induced shedding of gp120. [^{35}S]methionine-labelled, ultracentrifuge-pelleted virions of ELI1, ELI2, ELI5, and ELI6 were incubated at 37°C with 0, 0.5, 5, 50, or 500 nM CD4-IgG for 2 h followed by centrifugation in the Eppendorf centrifuge to obtain pellet and supernatant fractions. After immunoprecipitation with AIDS patients' sera and SDS-PAGE, the levels of free and virion-associated gp120 were determined by quantifying the amount of gp120 remaining on virions with a Fujix Bas 2000 Bio-image Analyzer. The percentage of virion-associated gp120 was plotted against the log of the CD4-IgG concentration.

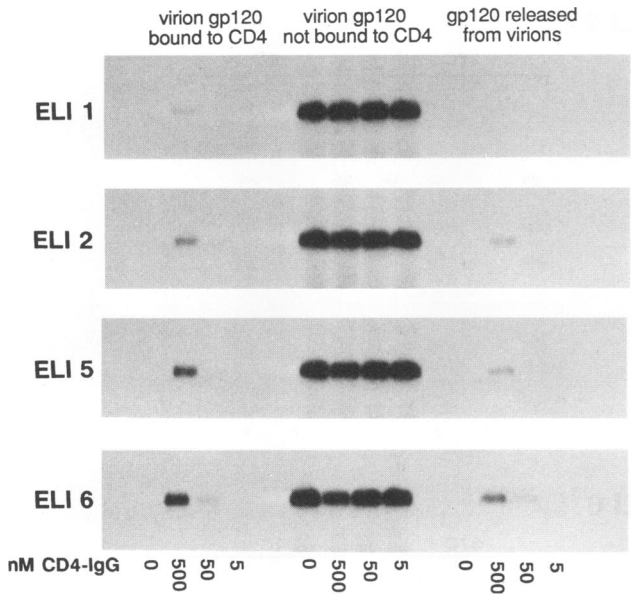


FIG. 8. Binding of CD4-IgG to the virions of ELI1, ELI2, ELI5, and ELI6. [^{35}S]methionine-labelled, ultracentrifuge-pelleted virions of ELI1, ELI2, ELI5, and ELI6 were incubated on ice for 1 h with 0, 5, 50, or 500 nM CD4-IgG followed by centrifugation in the microcentrifuge to obtain pellet and supernatant fractions. These fractions were treated according to the protocol outlined in Materials and Methods. Only the region of the gel containing gp120 is shown. The particular fraction analyzed is given across the top, the virus type is given on the lefthand side, and the concentration of CD4-IgG is given along the bottom. The amounts of virion-associated gp120 bound to CD4-IgG are given in Table 2.

envelope domains can augment this activity (32, 68). While the regions of envelope that confer the ability of a virus to infect CD4$^+$ cell lines have not been delineated as precisely as those for macrophage tropism (9), the locations of mutations in the *env* gene that allowed HIV-1$_{ELI}$ to replicate in such cell lines mapped to a region of gp120 involved in CD4 binding and to the fusogenic domain in gp41 (26). In the current study, we have investigated the mechanism of the enhanced replicative capacity of HIV-1$_{ELI}$ variants that arose during adaptation to growth in H9 and U937 cells. We have used these biologically derived mutants to examine how the amino acid changes affect envelope structure and function by evaluating the tendency of purified virions to release their gp120 components either spontaneously or after exposure to the CD4-IgG form of soluble CD4. The results of these studies showed that the ability of these variants to establish a productive infection in CD4$^+$ cell lines correlated with their sensitivity to the dissociation of gp120 from virions after treatment with CD4-IgG and with increased binding of virion-associated gp120 to CD4-IgG.

TABLE 2. Amount of virion-associated gp120 bound to CD4-IgG

Virus	% of binding at CD4-IgG concn of[a]:		
	5 nM	50 nM	500 nM
ELI1	0	0	6 ± 2
ELI2	0	5 ± 2	29 ± 8
ELI5	0	8 ± 3	34 ± 4
ELI6	0	20 ± 9	56 ± 13

[a] Values are calculated from the amount of gp120 bound plus gp120 released divided by the total gp120 (bound plus released plus not bound) from experiments similar to and including that shown in Fig. 8. The values for ELI2 are the averages of four experiments, and those for ELI1, ELI5, and ELI6 are the averages from three experiments; standard deviations are given.

The ELI1 strain of HIV-1 adapted to grow in H9 cells by the acquisition first of a Met-to-Val change at residue 7 in gp41 (26). This virus, ELI2, was able to infect CEM and H9 cells but not U937 cells. An additional substitution of Arg for Gly at residue 427 in gp120 (ELI6) was required to extend the host range to the promonocytic U937 cell line. To ascertain the individual contribution of the Gly-427-to-Arg change to the replicative capacity of the virus, this substitution was introduced into the parental ELI1 virus to produce ELI5 (Fig. 1). The infectivity of ELI5 in H9 cells was comparable to that of ELI6; however, ELI5 exhibited slower growth kinetics than ELI2 in CEM cells. Neither ELI2 nor ELI5 produced virus on U937 cells until 50 days after infection, suggesting that additional mutations were necessary for efficient replication in these cells.

The binding of virion-associated gp120 to cell-associated CD4 is the first step in the infection process. On the basis of studies of the interaction of soluble CD4 derivatives with gp120 (as soluble, virion-associated, or cell-associated forms), it is currently believed that the binding of virus to its receptor leads to conformational changes in both the receptor and the viral envelope (reviewed by Eiden and Lifson [20]). For the viral envelope, this has been demonstrated by the reactivity of monoclonal antibodies with previously masked epitopes on both gp120 and gp41 following the binding of soluble CD4 (33, 57). Also, the binding of soluble CD4 to virions leads to the dissociation of gp120 from gp41 (3, 7, 19, 33, 40, 49). This CD4-induced shedding of gp120 may model a postbinding event that, during an early step in the infection process, results in the exposure of the fusion domain on gp41, thereby mediating the fusion of viral and cellular membranes and subsequent virus entry.

In this study, we investigated the CD4-induced gp120 dissociation properties and the relative binding abilities of virion-associated gp120 for soluble CD4 exhibited by the four ELI variants. Our analyses showed that a correlation existed between the sensitivities of the different ELI viruses to CD4-induced gp120 dissociation and their ability to infect CD4$^+$ cell lines. The increased sensitivity to CD4-induced gp120 dissociation paralleled an increase in the binding of the virion-associated gp120 to CD4-IgG; a similar correlation between soluble CD4 binding and CD4-induced shedding has been reported (64). Because the gp41 substitution alone or in combination with the gp120 change increased CD4-IgG binding, we conclude that the overall conformation adopted by gp120 depends on both the gp120 and gp41 envelope components. Importantly, no differences in CD4 binding were apparent when CD4-IgG was titrated against non-virion-associated gp120 from ELI1 and ELI2 (Gly at position 427 of gp120) or from ELI5 and ELI6 (Arg at position 427) (unpublished results). Therefore, the effect of some mutations on the binding of gp120 to soluble CD4 may be revealed only by studying the interactions in the context of the intact envelope in virions. A similar conclusion has been reached by Moore et al. (47) for HIV-1 and by Mulligan et al. (50) for HIV-2.

Primary isolates of HIV-1 are much less prone to CD4-induced gp120 shedding (47, 52) and have a lower capacity to bind to soluble CD4 (47) than strains adapted to growth in established cell lines. Since primary isolates do not usually replicate well in CD4$^+$ cell lines (21, 22, 63), it is possible that the capacity for growth in such cells and the ability of the viral envelope to bind to CD4 and dissociate the gp120 component

are functionally linked. The results described in this paper support this. In fact, ELI1 has soluble CD4 binding and CD4-induced gp120 shedding properties similar to those associated with primary isolates, while the analogous properties of ELI6 are characteristic of laboratory-adapted viruses.

The amount of gp120 released from virions after treatment with soluble CD4 most likely involves at least two steps: (i) the association of CD4 with virions, which is determined by the ability of the virions to bind to CD4, and (ii) the capacity of the envelope to undergo postbinding conformational rearrangements that result in the dissociation of the gp120 from gp41. It appears that the mutations conferring $CD4^+$ cell line tropism to $HIV\text{-}1_{ELI}$ affected both processes. If, as proposed by others (46, 47), an initial low-affinity interaction between the virion and CD4 is converted to a high-affinity interaction by a CD4-induced conformational rearrangement of the envelope leading to CD4-induced gp120 dissociation, then the two processes may be linked. An alteration in the physical properties of the virion-associated envelope, as manifested by an increased spontaneous release of gp120 in the absence of CD4, could influence such a conformational rearrangement and therefore affect viral infectivity. In the ELI5 and ELI6 variants, spontaneous dissociation of gp120 from virions was increased by the presence of Arg at position 427 of gp120, either with the parental Met-7 gp41 (ELI5) or with the mutant Val-7 gp41 (ELI6). The Val-7 change alone did not markedly alter the spontaneous shedding of gp120 from virions (ELI1 versus ELI2), although the mutation did increase the infectivity of ELI2. Since there was no strict correlation between the tendency of a particular virus to shed its extracellular envelope component spontaneously and its replicative capacity, the significance of this property to envelope function is not yet clear.

The results from our study demonstrated that increased sensitivity of the ELI variants to CD4-induced gp120 dissociation paralleled their ability to infect $CD4^+$ cell lines. Moore et al. (45) have made similar observations by using primary isolates that had adapted to grow in cell lines. However, the functional significance of CD4-induced gp120 shedding remains unclear and has recently been questioned. For example, some mutations that reduced the amount of CD4-induced gp120 shedding had only moderate effects on virus uptake (64), and some others that eliminated the ability of the viral envelope to fuse with the cellular membrane had no effect on soluble CD4-induced dissociation of gp120 (4, 64). Our finding that the ELI variants also displayed an increase in CD4 binding to virion-associated gp120 raises the possibility that this property alone accounts for the replication phenotypes of the adapted viruses. However, while it is likely that increased CD4 binding to virions is the major component that accounts for the enhanced infectivity, we think that other factors such as the inherent physical properties of the virion may contribute as well. Additional studies are needed to elucidate the parameters that relate envelope function to viral growth.

Finally, since the ELI variants were derived by passage in cell lines, it is not clear whether they reflect the types of viruses that arise during virus replication in vivo. However, we feel that the diversity among $CD4^+$ cell populations in different tissues and at different stages of T-cell and monocyte and/or macrophage development raises the possibility that similar selection pressures are exerted on the virus for expanded growth potential and tropism during HIV-1 replication in an infected individual.

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