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Infectious Properties of Human Immunodeficiency Virus Type 1 Mutants with Distinct Affinities for the CD4 Receptor

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Recent evidence suggests that primary patient isolates of T-cell-tropic human immunodeficiency virus type 1 (HIV-1) have lower affinities for CD4 than their laboratory-adapted derivatives, that this may partly result from tighter gp120-gp41 bonds that constrain the CD4 binding sites of the primary viruses, and that selection for increased CD4 affinity may be the principal factor in laboratory adaptation of HIV-1 (S. L. Kozak, E. J. Platt, N. Madani, F. E. Ferro, Jr., K. Peden, and D. Kabat, J. Virol. 71:873–882, 1997). These conclusions were based on studies with a panel of HeLa-CD4 cell clones that differ in CD4 levels over a broad range, with laboratory-adapted viruses infecting all clones with equal efficiencies and primary T-cell-tropic viruses infecting the clones in proportion to cellular CD4 levels. Additionally, all of the primary and laboratory-adapted T-cell-tropic viruses efficiently used CXCR-4 (fusin) as a coreceptor. To test these conclusions by an independent approach, we studied mutations in the laboratory-adapted virus LAV/IIIB that alter the CD4 binding region of gp120 and specifically reduce CD4 affinities of free gp120 by 85 to 98% (U. Olshevsky et al., J. Virol. 64:5701–5707, 1990). These mutations reduced virus titers to widely varying extents that ranged from severalfold to several orders of magnitude and converted infectivities on the HeLa-CD4 panel from CD4 independency to a high degree of CD4 dependency that resembled the behavior of primary patient viruses. The relative infectivities of the mutants correlated closely with their sensitivities to inactivation by soluble CD4 but did not correlate with the relative CD4 affinities of their free gp120s. Most of the mutations did not substantially alter envelope glycoprotein synthesis, processing, expression on cell surfaces, incorporation into virions, or rates of gp120 shedding from virions. However, one mutation (D457R) caused a decrease in gp160 processing by approximately 80%. The fact that several mutations increased rates of spontaneous viral inactivation (especially D368P) suggests that HIV-1 life spans may be determined by structural stabilities of viral envelope glycoproteins. All of the wild-type and mutant viruses were only slowly and inefficiently adsorbed onto cultured CD4-positive cells at 37°C, and the gradual declines in viral titers in the media were caused almost exclusively by spontaneous inactivation rather than by adsorption. The extreme inefficiency with which infectious HIV-1 is able to infect cultured susceptible CD4-positive cells in standard assay conditions casts doubt on previous inferences that the vast majority of retrovirions produced in cultures are noninfectious. Apparent infectivity of T-cell-tropic HIV-1 in culture is limited by productive associations with CD4 and is influenced in an interdependent manner by CD4 affinities of viral gp120-gp41 complexes and quantities of cell surface CD4.

A major problem in understanding human immunodeficiency virus type 1 (HIV-1) derives from the enormous diversity of HIV-1 envelope glycoproteins in patients and from resulting differences in viral replication rates, cellular tropisms, syncytium-inducing activities, and interactions with the patient's immune system (13, 14, 21, 34, 40). In addition, T-cell-tropic primary viruses newly isolated from patients by growth in peripheral blood mononuclear cells generally cannot replicate in CD4-positive leukemic cell lines (13, 34). However, rapidly growing laboratory-adapted HIV-1 variants with altered envelope glycoproteins may form in such cultures after several weeks (15, 42, 46). Laboratory-adapted HIV-1 isolates have been widely studied, but it now appears that they differ substantially from patient viruses in susceptibilities to inactivation by antibodies (39, 46) and soluble CD4 (sCD4) (7, 28).

The bases for infectivity differences between HIV-1 isolates are only partly understood. Recent evidence has indicated that different chemokine receptors collaborate with CD4 to facilitate infections by HIV-1 (2, 6, 8–10, 12). Specifically, macrophage-tropic isolates use CC chemokine receptor 5 as their coreceptor (2, 6, 8–10), whereas laboratory-adapted and primary patient T-cell-tropic viruses principally use CXCR-4 (fusin) (6, 12, 20). In addition, recent evidence has suggested that laboratory-adapted isolates of HIV-1 may have substantially higher affinities for sCD4 and for cell surface CD4 than their progenitor primary patient T-cell-tropic viruses (19, 20, 26). In agreement with this possibility, these viruses have distinct infectivities for a panel of HeLa-CD4 cell clones that differ in CD4 quantities over a broad range, with laboratory-adapted HIV-1 infecting all clones that express more than a trace of CD4 with equal efficiencies and primary T-cell-tropic viruses infecting the clones with efficiencies that are linearly dependent on cell surface densities of CD4 (19, 20). Indeed, for T-cell-tropic HIV-1 isolates, there is a close correlation between viral susceptibilities to inactivation by sCD4, their abilities to grow in CD4-positive leukemic T-cell lines, and their abilities to infect HeLa-CD4 cells that have low quantities of CD4 (15, 19, 20, 45). Although this evidence is all consistent with the idea that laboratory-adapted and primary T-cell-tropic viruses differ in affinities for CD4, gp120 molecules released from these viruses do not uniformly differ in affinities for sCD4 (42). This discrepancy could be resolved by the hypothesis that gp120-gp41 intersubunit and oligomeric bonds constrain or partially occlude CD4 binding sites of virion-associated gp120

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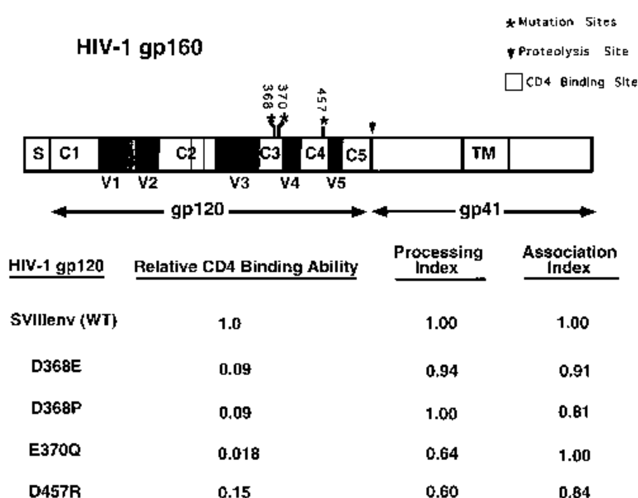
and that these bonds may be tighter in patient viruses than in laboratory-adapted isolates of HIV-1 (45). In support of this interpretation, gp120 is often spontaneously shed more rapidly from laboratory-adapted viruses (28, 39). Furthermore, virus binding to sCD4 loosens gp120-gp41 bonds (17, 27), causes exposure of previously buried epitopes in gp120 and gp41 (35, 36), and enhances shedding of gp120 from laboratory-adapted viruses (17, 26, 27, 45).

A direct approach to test these ideas would be to study the effects of mutations that reduce gp120 affinities for CD4 on infectivities and tropisms of HIV-1 in a well-controlled system. We have initiated such studies by using a set of mutations that were previously constructed by Olshevsky et al. in the CD4 binding site of gp120 in the laboratory-adapted HIV-1 isolate LAV/IIIB (29). The mutant envelope glycoproteins were expressed in virions released from COS7 cells that had been cotransfected with plasmids pSV*env* and pHIV-*gpt* (29, 30), and the infections were quantitatively detected as colonies that grew after selection in mycophenolic acid (19). In addition, the mutant *env* genes were reconstituted into replication-competent viruses. We studied effects of the mutations on viral stabilities and infectivities for the HeLa-CD4 clonal panel, on viral sensitivities to inactivation by sCD4, and on gp120 synthesis, processing, incorporation into virions, and shedding into the culture medium.



HIV-1 gp120	Relative CD4 Binding Ability	Processing Index	Association Index
SVIIIenv (WT)	1.0	1.00	1.00
D368E	0.09	0.94	0.91
D368P	0.09	1.00	0.81
E370Q	0.018	0.64	1.00
D457R	0.15	0.60	0.84

FIG. 1. HIV-1 gp160 schematic depicting mutations within the CD4 binding region. Signal peptide (S), conserved regions (C1 to C5), and variable regions (V1 to V5) of HIV-1 gp160 are shown. The proteolysis site in gp160 which is cleaved to yield gp120 and gp41 is designated by an arrow. Lightly shaded areas represent regions of gp120 important in CD4 binding (29). The transmembrane region of gp41 is denoted by TM. Point mutations were generated in J. Sodroski's laboratory. The relative CD4 binding affinity, processing, and cell surface association indices for wild-type (WT) and mutant gp120 molecules were determined by Olshevsky et al. (29).

MATERIALS AND METHODS

Cells and viruses. The panel of HeLa-CD4 clones and the COS7 cells have been described elsewhere (5, 19). The 1022 clone was the generous gift of K. Wehrly and B. Chesebro (Rocky Mountain Laboratories, Hamilton, Mont.). HeLa-CD4 clones were grown in complete medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (5 U/ml), and streptomycin (5 μg/ml). COS7 cells were grown in complete medium supplemented with glucose (4.5 mg/ml). Wild-type and mutant HIV-*gpt* virions were produced by cotransfecting (3, 16) COS7 cells with pHIV-*gpt* (30) and either pSVIII*env*, encoding wild-type LAV/IIIB gp160, or one of the following plasmids encoding mutant versions of HIV-1 gp160: D368E, D368P, E370Q, or D457R. Plasmids encoding wild-type and mutant HIV-1 gp160 were generously provided by J. Sodroski (Dana-Farber Cancer Institute, Boston, Mass.). HIV-*gpt* infections and subsequent selection of infected HeLa-CD4 colonies were performed by using cultures pretreated for 30 min with Polybrene (8 μg/ml; Sigma, St. Louis, Mo.) as previously described (19).

Replication-competent versions of the mutants specified above were constructed by placing mutant *env* sequences into the pNL4-3 provirus. Since pNL4-3 lacked convenient subcloning sites, *env* sequences were excised from pNL4-3 by double digestion with *Eco*RI and *Bam*HI and cloned into the same sites of pGem3 (Promega, Madison, Wis.), generating plasmid pG*env*, in which mutant HIV-1 envelope genes could be constructed. Plasmids encoding HIV-1 wild-type (pSVIII*env*) or mutant (D368E, D368P, E370Q, and D457R) CD4 binding sites were excised by double digestion with *Nde*I and *Bam*HI and cloned into the same sites of pG*env*. *Bam*HI-*Eco*RI *env*-containing fragments from the resulting plasmids were then ligated with *Bam*HI- and *Eco*RI-digested pNL4-3, generating plasmids encoding replication-competent viruses designated NL-*env*, NL-D368E, NL-D368P, NL-E370Q, and NL-D457R. Wild-type and mutant virions were harvested from the media 48 h after calcium phosphate transfection of HeLa cells and were used immediately or stored at −80°C. Infectivity of replication-competent viruses was measured by using a focal infectivity assay (5, 20). For sCD4 inactivation studies, viruses were preincubated with various concentrations of sCD4 at 37°C for 30 min before infection of HeLa-CD4 cells (clone HI-J). Recombinant sCD4 was obtained through the NIH AIDS Research and Reference Reagent Program (contributed by Ray Sweet).

Western blot analysis of HIV-1 proteins. At 65 h posttransfection, cellular extract and conditioned medium (CM) fractions were harvested from cotransfected COS7 cells. Cell extracts were obtained by washing COS7 monolayers (approximately 5×10^6 cells per 10-cm-diameter dish) three times in phosphate-buffered saline and then lysing the cells in immunoprecipitation buffer (1% Triton X-100, 0.5% deoxycholate, 5 mM EDTA, 250 mM NaCl, 25 mM Tris-Cl [pH 7.5], 100 μM phenylmethanesulfonyl fluoride) at 2°C. Lysed cells were scraped into Eppendorf tubes, vortexed, and centrifuged at $12,000 \times g$ for 5 min to sediment insoluble material. Supernatants were reserved and used for immunoprecipitations as described elsewhere (31). Virion and free gp120 fractions were harvested from CM which had been subjected to low-speed centrifugation and filtration (0.45-μm-pore-size filter). CM was then layered over a 25% sucrose cushion (25% sucrose in 10 mM Tris-HCl [pH 7.5]–10 mM NaCl–1 mM EDTA) and centrifuged for 2 h at $100,000 \times g$. The resulting high-speed supernatant was adjusted to final detergent concentrations of 1% Triton X-100 and 0.5% deoxycholate. Pelleted virions and immunoprecipitates were dissolved in electrophoresis sample buffer (10% glycerol, 2.3% sodium dodecyl sulfate, 62.5 mM Tris-HCl [pH 6.8], 5% 2-mercaptoethanol) and were analyzed by electrophoresis and Western blotting as described below.

For immunoprecipitations, we used the following antibodies obtained through the NIH AIDS Research and Reference Reagent Program: HIV human immunoglobulin (HIVIG), donated by Alfred Prince; and a sheep HIV-1 IIIB gp120 antiserum, donated by Michael Phelan. In our assays, the HIVIG reacted very well with *gag* proteins but not with gp120. Therefore, we used the sheep anti-gp120 for detection of gp160 and gp120. Omnisorb or Pansorbin (Calbiochem, La Jolla, Calif.) was used to precipitate anti-gp120–HIV-1 gp120 or HIVIG-HIV *gag* immune complexes, respectively. Immunoprecipitated HIV-1 proteins and sedimented virions were dissolved in electrophoresis sample buffer and were then subjected to electrophoresis in 10% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate. Proteins were transferred to nitrocellulose membranes and subjected to Western blotting. HIV-1 proteins were detected by incubation with HIVIG or sheep anti-gp120 (both diluted 1:1,000 in 0.5% Tween 20–20 mM Tris-HCl [pH 7.5]). Protein A-conjugated horseradish peroxidase (HRP) (1:10,000 dilution) or protein G-conjugated HRP (1:5,000 dilution) was added to membranes incubated with HIVIG or sheep anti-gp120, respectively. Both protein A- and protein G-conjugated HRP were purchased from Bio-Rad (Hercules, Calif.). Antibody binding was detected by incubation with chemiluminescence reagents as recommended by the manufacturer (New England Nuclear, Boston, Mass.).

RESULTS

Infectivities of viruses with mutations in the CD4 binding site of gp120. Figure 1 shows a map of HIV-1 gp160 including the variable and constant regions of gp120 and the discontinuous sites in C2, C3, C4, and C5 that are important for CD4 binding by isolated gp120. The C3 and C4 sites of the mutations are brought into proximity by disulfide bonding and are believed to form a CD4-binding pocket (29). Also included in Fig. 1 are summaries of previous data concerning the processing of precursor gp160 into gp120 and gp41, the association of gp120 with gp41, and the CD4 affinities of the free gp120s as determined by Olshevsky et al. (29). They also examined other mutations, but we selected the group shown because they retained 2 to 15% of the CD4 binding activity of wild-type LAV/

TABLE 1. Differences in infectivities of wild-type and mutant HIV-*gpt* on HeLa-CD4 clones

Cell line[a]	CD4 level[b]	HIV-*gpt* infection (no. of colonies/dish)[c]				
		SVIII*env*	D368E	D368P	E370Q	D457R
HI-R	14	2,020	66	0	0	3
HI-Q	25	1,660	123	0	0	9
HI-F	30	2,345	179	3	0	7
HI-A	52	2,936	561	1	0	16
HI-K	71	3,108	444	1	0	10
HI-G	71	2,168	529	1	0	10
HI-P	128	2,178	647	6	4	26
HI-J	234	2,619	1,192	24	6	53
HI-L	300	1,199	1,118	32	2	41
1022	479	1,558	1,027	65	22	35

[a] HeLa-CD4 lines were created as previously described (12).

[b] Expressed as mean fluorescence intensity in arbitrary relative units as determined by fluorescence-activated cell sorting analysis (12).

[c] HeLa-CD4 clones were infected with HIV-*gpt* virions which contained wild-type (SVIII*env*-encoded) or mutant (D368E-, D368P-, E370Q-, or D457R-encoded) HIV-1 gp120 (produced as described in Materials and Methods), and the colonies were counted.

IIIB gp120, and we presumed that their virions might have measurable infectivities.

We produced virions bearing wild-type or mutant (D368E, D368P, E370Q, or D457R) *env* glycoproteins by cotransfecting pHIV-*gpt* and pSV*env* plasmids into COS7 cells as described previously (19), and we quantitatively analyzed infectivities on the HeLa-CD4 clonal panel by selecting mycophenolic acid-resistant colonies (19). Table 1 shows the colony counts for a representative experiment, and Fig. 2 is a plot with logarithmic axes used to show all of the data on a single graph. The results demonstrate that the mutant viruses all infect the panel with a high degree of CD4 dependency, whereas wild-type virus infects cells independently of their levels of CD4 expression above a low trace threshold. No infection occurs in control HeLa cells that lack CD4 (data not shown). Although these results imply that infectivities are highly dependent on CD4 affinities of the viruses, the relative infectivities of the mutants for the HeLa-CD4 panel differ by several orders of magnitude and clearly do not correlate with the relative CD4 affinities of the free gp120s. For example, mutant D368E is at least 20 to 40 times more infectious than D368P, although their released gp120s both bind CD4 approximately 9% as well as wild-type gp120 (29). Similarly, D457R is much less infectious than D368E, but its free gp120 has a higher affinity for CD4 (29). Similar results were obtained with many independent preparations of these viruses. The titers of all HIV-*gpt* isolates were linearly proportional to their concentrations, suggesting that they were not contaminated by inhibitors.

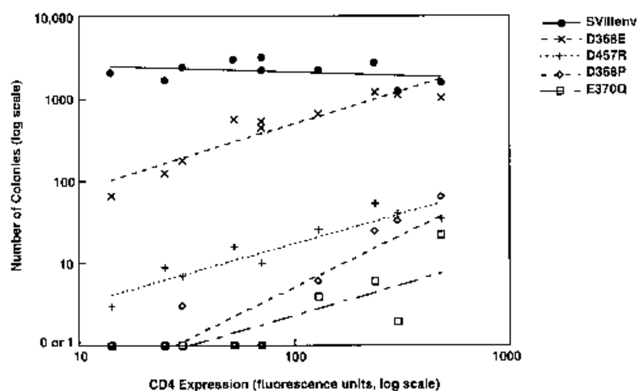


FIG. 2. Analysis of infectivities of HIV-*gpt* virions complemented with wild-type or mutant HIV-1 gp120. HeLa CD4 clones were infected with either wild-type or mutant HIV-*gpt* virions, and colonies were selected as described previously (29). HeLa-CD4 clones and CD4 expression levels in fluorescence units were as follows: 1022, 479; HI-L, 300; HI-J, 234; HI-P, 128; HI-G, 71; HI-K, 71; HI-A, 52, HI-F, 30; HI-Q, 25; and HI-R, 14.

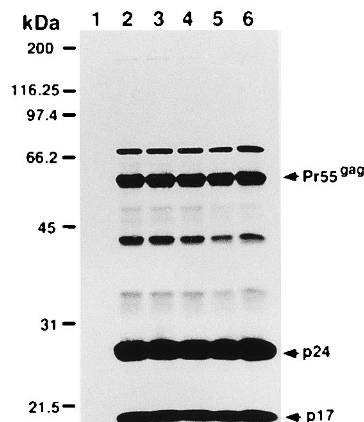


FIG. 3. Expression of HIV-1 virions by cotransfected COS7 cells. At 65 h posttransfection, virions were pelleted from cleared CM harvested from cotransfected COS7 cells by centrifugation at 100,000 × *g* for 2 h (see Materials and Methods for additional details). Virion pellets were solubilized in sample buffer and fractionated on 10% polyacrylamide gels. HIV-1 proteins were detected by Western blotting using HIVIG pooled human sera from HIV-positive donors. Lane 1, mock transfected; lane 2, SVIII*env*; lane 3, D368E; lane 4, D368P; lane 5, E370Q; lane 6, D457R.

Analyses of mutant and wild-type viral proteins. To determine whether the HIV-*gpt* preparations described above contained similar numbers of virions, we pelleted the virion-containing CM from transfected COS7 cells and analyzed the proteins by electrophoresis and Western blotting, using a preparation of immunoglobulin G from infected patients that reacts primarily with viral *gag* proteins. As shown in Fig. 3, all of the sedimented virus samples contained approximately equal amounts of HIV-1 *gag* proteins (lanes 2 to 6), whereas these components were absent in the culture medium of mock-transfected COS7 cells (lane 1).

We then analyzed the HIV-1 envelope glycoproteins by using a sheep antiserum to gp120, which works well in Western blots for detection of gp120 and gp160 but also reacts nonspecifically with several proteins that are produced by COS7 cells. A Western blot of transfected COS7 cell extracts is shown in Fig. 4A. In contrast to the extract of mock-transfected COS7 cells (lane 1), which contained only nonspecific components, all of the extracts from virus-producing cells contained large amounts of gp160 and gp120 (lanes 2 to 6). However, cells synthesizing mutant D457R reproducibly had a substantially lower ratio of gp120 relative to gp160 (three- to sixfold less) than the cells making the other viruses (lane 6 versus lanes 2 to 5). This result suggests either that D457R gp160 is processed less efficiently to form gp120 or that the resulting gp120 is shed relatively rapidly from cell surfaces.

The culture medium from the cells used in Fig. 4A was also centrifuged to sediment the virions away from gp120 that had been shed. As shown in Fig. 4B, the sedimented virions all contained gp120. However, densitometry suggested that the mutant D457R virions (lane 6) contained only approximately 10% as much gp120 as the wild-type virions (lane 2). Similarly,

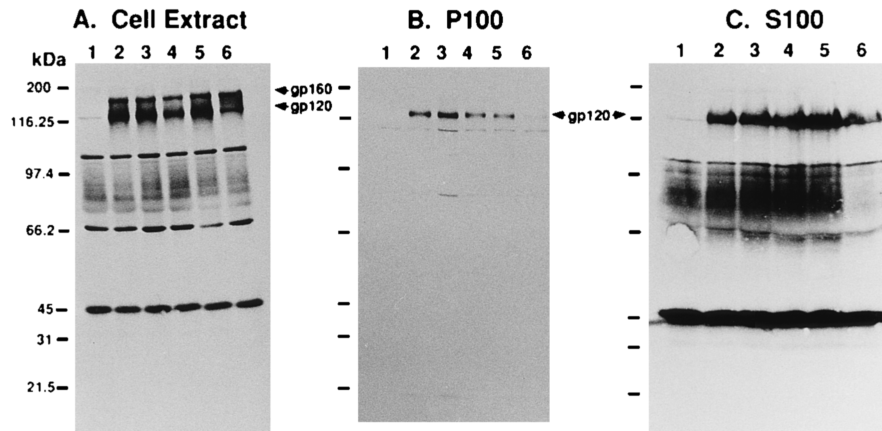


FIG. 4. Detection of HIV-1 gp120 in cotransfected COS7 cells. At 65 h posttransfection, cell extract, virion, and high-speed supernatant fractions were prepared from COS7 cells cotransfected with pHIV-*gpt* and HIV-1 *env*-encoding plasmids (SVIII*env*, D368E, D368P, E370Q, and D457R). Samples were subjected to Western blot or to immunoprecipitation-Western blot analysis (as described in Materials and Methods). HIV-1 proteins electrotransferred to nitrocellulose membranes were probed with sheep anti-gp120 serum, and antibody binding was detected by incubation with chemiluminescence reagents. (A) Immunoprecipitation-Western blot of transfected COS7 cell extracts. Lane 1, mock transfected; lane 2, SVIII*env*; lane 3, D368E; lane 4, D368P; lane 5, E370Q; lane 6, D457R. (B) Western blot of virion-associated gp120. P100, pelleted virion fraction. Lanes 1 to 6 are as in panel A. C. Immunoprecipitation-Western blot of secreted, non-virion-associated HIV-1 gp120. High-speed supernatant fractions (S100) generated from CM harvested from transfected COS7 cells were subjected to the immunoprecipitation-Western blotting procedure. Lanes 1 to 6 are as in panel A.

the mutants D368P (lane 4) and E370Q virions (lane 5) had only approximately 60% as much gp120 as the wild-type virions. Figure 4C shows analysis of the medium supernatants that remained after sedimentation of the virions. These supernatants contained large amounts of gp120 that had been shed from cell surfaces and from virions, and we estimate that the virion-bound gp120 comprised only approximately 5% of the total gp120 that was in the uncentrifuged culture medium. The amounts of gp120 released by the cells were similar for all mutants except D457R, which produced only approximately 20% of the wild-type amount. Together, these results suggest that D457R gp160 is processed to form gp120 only approximately 10 to 20% as efficiently as the wild-type glycoprotein and that the resulting virions have a correspondingly reduced amount of gp120. The other mutations do not substantially interfere with the efficiency of gp160 processing or with gp120 incorporation into released virions.

Additional factors that limit infectivities of wild-type and mutant HIV-1 in cell cultures. One possible explanation for the large differences in relative infectivities of the wild-type and mutant HIV-1, which do not correlate with affinities of their soluble gp120s for CD4 (Table 1 and Fig. 1 and 2), could be that some of the mutant virions are relatively unstable in culture medium. To address this, we incubated freshly harvested virus-containing media in empty flasks at 37°C for different times prior to analyzing residual infectivities by 2 h incubations with a clone of HeLa-CD4 cells (clone HI-J) that has a high CD4 content. Such inactivation assays can be complex because infected cells release noninfectious particles that become converted to mature infectious virions during the first hours of incubations and because rates of spontaneous HIV-1 inactivation are highly dependent on experimental conditions and are not strictly first order (23, 24). Nevertheless, based on multiple assays, we inferred that the wild-type and D368E viruses were inactivated with similar half-lives of approximately 6 to 8 h, whereas the D368P mutant was inactivated two to three times more rapidly. Results obtained with the E370Q and D457R mutants appeared to be intermediate, but these measurements were less accurate due to the low infectivities of these mutants in several of our preparations.

To extend these results and to obtain information about the abilities of these viruses to adsorb onto HeLa-CD4 cells, we serially incubated the virus-containing culture media for 2 h periods with four replica cultures of HeLa-CD4 cells (clone HI-J) and assayed the numbers of infected cells in each culture by selection with mycophenolic acid. The results, which are plotted in Fig. 5A, indicate that the titer of the D368P mutant declines approximately three times faster than the titers of the wild-type and D368E viruses and that the titers of the E370Q and D457R mutants decline at intermediate rates. These conclusions were fully supported by four independent repeats of this serial transfer analysis. The apparent difference in decline rates for the wild-type and D368E mutant viruses were not reproducible in our different assays. To control for the spontaneous rates of virus inactivation in identical cell culture conditions, samples of each virus-containing medium were also transferred serially at 0, 2, and 4 h onto replica cultures of control HeLa cells that lack CD4, and the resulting media were then assayed at 6 h for residual titers by 2-h incubations with HeLa-CD4 cells (clone HI-J). These control titers are indicated in Fig. 5A by the letter "c." These results (Fig. 5A) show that the residual titers were identical within experimental error for virus samples that had been serially incubated for 6 h with HeLa-CD4 cell cultures or with control HeLa cultures. Therefore, we infer that the losses of infectious virions from the culture media must have been almost exclusively due to virus inactivation in the conditions of our cell cultures rather than due to specific adsorption onto HeLa-CD4 cells. This conclusion is consistent with a previous study which suggested that HIV-1 with other envelope glycoproteins was only inefficiently adsorbed onto HeLa-CD4 cells or CD4-positive T cells during a 2-h incubation at 37°C (19). Identical losses of infectious virions during incubations with HeLa-CD4 compared with HeLa control cultures were also obtained in assays using replication-competent laboratory-adapted and primary patient HIV-1 isolates (data not shown).

In another control for the analysis in Fig. 5, we incubated the virus samples in empty flasks for 6 h before analyzing their residual infectivities. The titers in this case were approximately 30% higher than the residual titers in the media that had been

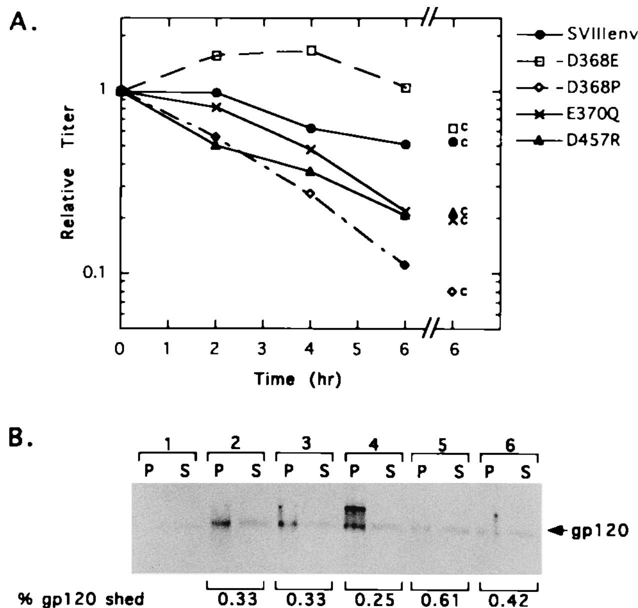


FIG. 5. Stabilities of wild-type and mutant HIV-*gpt* virions. (A) Inactivation of wild-type and mutant viruses. Wild-type and mutant viruses freshly harvested from transfected COS7 cells were incubated sequentially on replicate cultures of high-CD4-expressing clone HI-J which had been pretreated for 30 min with Polybrene (8 μg/ml) (Table 1). Incubation periods were for 2 h at 37°C in a humidified atmosphere containing 5% CO_2. Sequential infections were initiated at 0 h, with subsequent infections implemented by transferring the virus-containing culture media onto fresh HI-J cultures at 2, 4, and 6 h. At 48 h postinfection, HI-J cells were placed under mycophenolic acid selection. Viral titers were determined by counting the number of drug-resistant colonies and were normalized relative to the initial titers for each virus sample. Relative titers of wild-type and mutant viruses were plotted on a logarithmic scale versus time. Data points represent the means of duplicate assays. Data points labeled c denote controls in which virus samples were sequentially incubated with cultures of Polybrene (8 μg/ml)-pretreated (30 min) HeLa cells that lack CD4 at 0, 2, and 4 h and were then incubated an additional 2 h with a culture of HI-J cells. (B) Shedding of gp120 from wild-type and mutant HIV-*gpt* virions. HIV-*gpt* virions were harvested as described in Materials and Methods. Viral pellets were resuspended in complete medium and incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. At 48 h, virions were pelleted in a microcentrifuge at top speed for 1 h. Pellet (P) and supernatant (S) fractions were collected, solubilized in sample buffer, and subjected to electrophoresis and Western blotting. Relative contents of gp120 in gel bands for pellet and supernatant fractions were determined with an Agfa Studioscan II image analyzer and NIH Image version 1.57 imaging program and are displayed as percent gp120 shed. Samples analyzed: 1, control medium from mock-transfected cells; 2, wild type; 3, mutant D368E; 4, mutant D368P; 5, mutant E370Q; 6, mutant D457R.

serially incubated with the HeLa-CD4 and HeLa cultures. We do not consider this small difference significant because these control samples were not transferred serially between different empty flasks and they were not exposed to Polybrene or to the same periodic removals from the incubators. Serial transfers onto the cell cultures also involved unavoidable dilutions by media that were adherent to each monolayer. Consistent with these considerations, there was no reproducible or significant difference between the titers of HIV-1 virions that had been incubated without Polybrene for 6 h at 37°C in single flasks that were empty or that contained HeLa cells.

We also analyzed the rates of spontaneous gp120 shedding from the wild-type and mutant virions. This was done by incubating a suspension of purified virions for 48 h at 37°C and by then centrifuging the virions and analyzing the gp120 contents of the pelleted virions and supernatant fractions. As shown in Fig. 5B, the E370Q and D457R mutants shed a slightly higher proportion of gp120 into the soluble fraction than the other viruses. These differences in rates of gp120 shedding were small. Although D368P virions were rapidly inactivated at 37°C (Fig. 5A), they did not rapidly shed gp120 (Fig. 5B).

TABLE 2. Infectivities of wild-type and mutant replication-competent HIV-1 isolates on HeLa-CD4 clones[a]

Cell clone	CD4 level	Virus titer (no. of foci/well)[b]					
		NL4-3	NL-*env*	NL-D368E	NL-D368P	NL-E370Q	NL-D457R
HI-Q	25	1,320	1,920	510	1	0	16
		1,290	1,435	450	3	0	12
HI-J	234	960	760	950	155	2	310
		955	1,240	1,340	190	6	450

[a] HeLa-CD4 cell clones and their relative CD4 levels are described in Table 1, footnotes *a* and *b*.

[b] The replication-competent viruses were produced and assayed as described in Materials and Methods. The foci were counted in duplicate wells, and the results from both wells are shown. NL4-3 is the standard wild-type virus. NL-*env* is wild-type virus reconstructed with a wild-type laboratory-adapted *env* gene encoded by pSVIII*env*. The other viruses were reconstituted by using *env* genes that contained the indicated mutations.

Infectivities and sCD4 sensitivities of replication-competent viruses that contained wild-type and mutant envelope glycoproteins. The wild-type and mutant *env* genes used for the foregoing studies were reconstituted into replication-competent proviral genomes, and virions were then obtained from the culture media of transiently transfected HeLa cells (see Materials and Methods). As shown in Table 2, the relative infectivities and CD4 dependencies of the resulting viruses were nearly identical to the results obtained for HIV-*gpt* isolates (Table 1).

Because these replication-competent viruses could be readily titered in small wells that contained little medium, it was feasible to analyze their sensitivities to inactivation by sCD4. All of the mutations that reduced CD4 affinities of gp120 also reduced viral susceptibilities to inactivation by sCD4. As shown in Fig. 6, susceptibilities to inactivation correlated closely with the relative infectivities of the viruses but not with the relative CD4 affinities of their free gp120s. Thus,

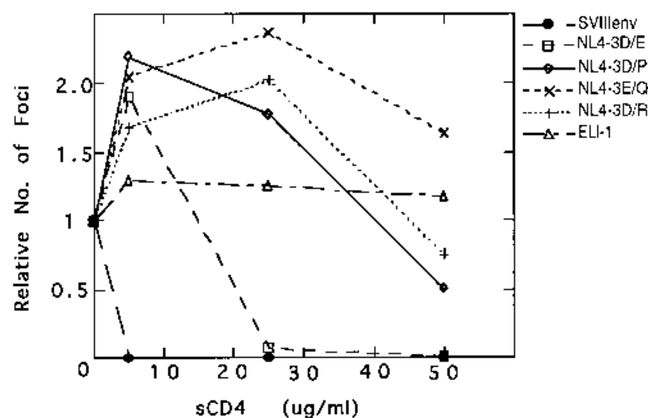


FIG. 6. Effects of sCD4 on infectivities of wild-type and mutant HIV-1 virions. The wild-type and mutant *env* genes used in this study were reconstituted into replication-competent HIV-1 isolates as described in Materials and Methods, and the virions were incubated for 30 min at 37°C with various concentrations of sCD4. The viral titers were then measured by the immunoperoxidase focal infectivity method (5) and are expressed as the relative number of foci, with the number of foci in the absence of sCD4 equal to 1. The reconstituted viruses used were wild-type (NL-*env*), NL-D368E, NL-D368P, NL-E370Q, and NL-D457R; ELI1 (20) was used to illustrate resistance of a primary patient T-cell-tropic virus.

in agreement with the data in Tables 1 and 2, the wild-type virus was highly susceptible to inactivation whereas mutant D368E was slightly susceptible. In contrast, the other mutants were highly resistant. In the same study, the primary patient T-cell-tropic virus ELI1 (1) was also resistant to inactivation. Consistent with previous data (39), the mutant viruses were even slightly activated by the sCD4.

DISCUSSION

These results support other evidence that infections of cultured cells by HIV-1 are extremely sensitive to CD4 affinities of the viral gp120s (Table 1 and Fig. 1 and 2) (18, 19, 26, 41). Previously, we reported that laboratory-adapted isolates of HIV-1, which are defined by their enhanced abilities to replicate in CD4-positive leukemic cell lines, infect all clones of our panel of HeLa-CD4 cells with equal efficiencies, whereas primary patient T-cell-tropic isolates have extremely low titers on cell clones with low amounts of CD4 and proportionately greater titers on clones with more CD4 (19). Recently, we found that single mutations in the *env* gene of a T-cell-tropic primary patient virus (ELI1) that cause different degrees of laboratory adaptation all cause corresponding enhancements in viral sensitivities to sCD4 and in infectivities for HeLa-CD4 clones that have only low amounts of CD4 (20). Moreover, laboratory adaptation of the ELI1 virus occurred without a change in coreceptor specificity (20). The latter results suggested that increased viral affinity for CD4 may be the primary factor involved in laboratory adaptation of HIV-1 (20). By using an inverse approach, this study demonstrates that mutations that specifically reduce CD4 affinities of gp120 in a laboratory-adapted strain LAV/IIIB convert viral infectivities from CD4 independence to the strong CD4 dependence previously described for T-cell-tropic primary patient isolates (Tables 1 and 2 and Fig. 2).

Although our evidence strongly suggests that weak binding of the mutant viruses to cell surface CD4 limits their infections of HeLa-CD4 cells, there is a poor correlation between relative infectivities of the mutant viruses (Table 1 and Fig. 2) and relative CD4 affinities previously measured for their free gp120s (29). Mutant D368E is approximately 40 times more infectious than D368P, yet both of their gp120s bind cell surface CD4 9% as strongly as wild-type gp120. Similarly, D457R is 20 times less infectious than D368E, but its free gp120 has a higher affinity for cell surface CD4. Although these discrepancies could partially be explained by the relatively low gp120 content of D457R virions (Fig. 4) and by the two- to threefold decrease in half-life for D368P virions (Fig. 5A), we believe that these factors could not fully account for the large differences in infectivities observed. For virions of equal infectivities produced at the same rates (see Fig. 3), the steady-state titers should be inversely proportional to their rates of inactivation. Therefore, the threefold increase in inactivation rate of the D368P mutant could not by itself explain its extremely low infectivity compared with that of mutant D368E. Our results are compatible with previous evidence (26, 45) that CD4 affinities of virus-associated gp120s differ from those of free gp120s, in part due to constraints imposed on the CD4 binding sites by oligomeric and intersubunit interactions between envelope glycoproteins. The relative sCD4 sensitivities of these viruses (Fig. 6) correlated with their infectivities (Tables 1 and 2) rather than with the sCD4 affinities of their free gp120s (Fig. 1).

Based on previous evidence, it seems likely that CD4 affinities of virions could influence infectivities at several steps of the virus entry pathway. First, CD4 affinity could influence the rate of forming a monovalent virus-receptor bond during the initial step of cellular adsorption. If the bond were weak or rapidly reversible, the virus might often dissociate from the cell surfaces. In addition, there is evidence that HIV-1 infections may require diffusion of additional CD4 molecules into the site of attachment (11, 22, 33), thereby reducing virus dissociation and facilitating the membrane fusion reaction. Multivalent virus-receptor complexes may also be required for infections by other enveloped viruses (4, 32, 37, 38, 44). Weak affinity of HIV-1 for CD4 would be expected to reduce the rate and efficiency of forming a competent multivalent complex.

Rates of HIV-1 inactivation in culture medium at 37°C were substantially altered by mutations in gp120 (e.g., Fig. 5A). Therefore, instabilities in the folding of envelope glycoproteins rather than in other viral components can limit the life spans of infectious HIV-1 in cell culture conditions. Additional work will be required to determine whether this is generally true for HIV-1 or whether it is correct only for the mutants that we analyzed. Previous studies are consistent with the former possibility (24, 25).

It is evident that titers of HIV-1 measured in standard assay conditions are not absolute estimates of the numbers of infectious virions in any sample. For the mutant viruses (Table 1 and Fig. 2) and for primary patient T-cell-tropic isolates (19, 20), the titers measured after 2-h incubations with HeLa-CD4 cell cultures were highly dependent on CD4 contents of the cells. In addition, the titers of each infectious virus became only slowly and equally reduced in culture media that were serially incubated for 2 h with multiple cultures of HeLa-CD4 cells or with control HeLa cells (Fig. 5A). Moreover, the rates of infectious virus depletion that occurred during these serial transfer and infection assays were not significantly different than the rates of depletion that occurred when the virus-containing media were incubated in controlled conditions in empty flasks (see Results). Consequently, the slow depletion of infectious HIV-1 from the media in cell cultures at 37°C must be almost exclusively caused by processes that lead to virus inactivation rather than by specific adsorption in a pathway that leads to infection. The inactivating processes presumably include spontaneous inactivation as well as nonproductive adsorptions onto the walls of the flasks and onto the surfaces of cells or extracellular matrices. Similar evidence for slow and inefficient adsorption was obtained in assays using diverse replication-competent isolates of HIV-1 incubated with CD4-positive T cells (reference 19 and unpublished results).

The extremely slow and inefficient adsorption of infectious HIV-1 onto CD4-positive cells in standard conditions for infection of cell cultures has several major implications. It was previously inferred from quantitative comparisons of viral titers with virion particle counts that only minute proportions of HIV-1 virions are infectious (24). However, our results have shown that titers of retroviruses measured in standard conditions severely underestimate the numbers of infectious virions in the samples (19, 20, 37, 43). Indeed, as suggested by the data in Fig. 5A, standard conditions for infection of cell cultures result in no significant loss of infectivity from the media other than that caused simply by inactivation or nonspecific adsorptions. Consequently, we propose that retrovirus particles released from cells may be uniformly infectious. In addition, these results strongly imply that even laboratory-adapted viruses such as LAV/IIIB infect cultured cells only inefficiently.

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