JOURNAL OF VIROLOGY, Jan. 1992, p. 524–533
0022-538X/92/010524-10$02.00/0

Vol. 66, No. 1

Analysis of Mutations in the V3 Domain of gp160 That Affect Fusion and Infectivity

KATHLEEN A. PAGE,[1] SUSAN M. STEARNS,[1] AND DAN R. LITTMAN[1,2]*

Department of Microbiology and Immunology[1] and Howard Hughes Medical Institute,[2] University of California, San Francisco, California 94143-0414

Received 14 August 1991/Accepted 1 October 1991

The third hypervariable (V3) domain of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein has been proposed to play an important role in mediating viral entry. Antibodies to the V3 domain block HIV-1 infection but not virus binding to CD4. At the center of the V3 domain is a relatively conserved sequence of amino acids, GPGRA. It has previously been shown that mutation of some of these amino acids reduced the ability of gp160 expressed on the surface of cells to induce fusion with CD4-bearing cells. In order to analyze the role of V3 domain sequences in mediating HIV entry, we introduced several amino acid substitution mutations in the GPGRA sequence of gp160 derived from HIV-1 strain HXB2 and in the analogous sequence of strain SF33, GPGKV. Virus was generated by cotransfecting the *env* constructs and a selectable *env*-negative HIV vector, HIV-gpt. When complemented with a retrovirus *env* gene, infectious virus capable of a single round of replication was produced. The viral particles produced were analyzed biochemically for core and envelope proteins and for infectious titer. The transfected *env*s were also analyzed for ability to bind to CD4 and mediate cell fusion. Several of the amino acid substitutions resulted in moderate to severe decreases in virus infectivity and fusion activity. Envelope glycoprotein assembly onto particles and CD4 binding were not affected. These results provide evidence that V3 sequences are involved in mediating the fusion step of HIV-1 entry.

Infection with human immunodeficiency virus (HIV) begins with binding of the virus to CD4 on cells. Following binding, the envelope of HIV fuses with cellular membranes to allow penetration of the viral core into the cytoplasm. The factors involved in mediating fusion are still incompletely defined. The HIV envelope glycoprotein, gp160, mediates viral entry and has been shown to be responsible for determining cellular tropism for several isolates of HIV. Domains of gp160 that are involved in CD4 binding and cellular tropism have been identified by analyzing the effects of specific mutations on CD4 binding and infectivity. The CD4 binding region includes sequences in the carboxy terminus of gp120 throughout parts of the V4, C5, and V5 domains (15, 20). Although it has been shown that amino acid substitutions within the CD4 binding region can affect tropism (6), residues outside this region have been shown to be more prominent in determining cellular tropism for several HIV type 1 (HIV-1) isolates. For example, a point mutation that resulted in a substitution of serine for proline in the gp120 V3 domain conferred brain cell tropism on the GUN-1 isolate of HIV-1 (28). Several studies have shown the importance of the V3 domain (amino acids 303 to 338) in controlling macrophage and T-cell tropism (2, 11, 16, 19, 23, 29, 30). The role of the V3 domain in HIV entry is also evidenced by the observation that antibodies to the V3 domain block HIV-1 infection without interfering with virus binding to CD4 (22, 25).

Syncytium formation between gp160-expressing cells and CD4-positive cells is likely to involve a mechanism similar to that of virus-cell fusion that occurs during viral entry. Mutational analysis of gp160 has revealed several regions that are important for the syncytium-inducing ability of gp160. Introduction of charged residues into the hydrophobic sequence at the amino terminus of gp41 and amino acid substitutions in its transmembrane domain resulted in the loss of syncytium formation (10, 12). Amino acid changes at amino acid positions 312 and 313 in the V3 domain also impaired syncytium formation (7). In summary, it is clear that several regions of gp160 participate in the events leading to HIV entry. Upon gp160-CD4 binding, the V3 domain appears to undergo a cell-type-specific interaction that allows membrane fusion to proceed. The hydrophobic amino terminus of gp41 and sequences within the transmembrane portion of gp41 are also essential for fusion.

The V3 domain is the site of binding of the majority of neutralizing antibodies that arise in HIV-infected individuals. It has been postulated that in the course of disease progression, variants with amino acid changes in the V3 domain appear and are able to escape neutralization by the existing host antibodies (18, 24). In the center of the V3 domain is a sequence, GPGRA, that is not subject to the kind of extensive change that the rest of the V3 loop undergoes (14). This conserved sequence is predicted to exist at the tip of an exposed, hydrophilic loop that the V3 domain forms. The conservation of this sequence despite selective pressure for variation in this region suggests that it must be involved in mediating an essential function of gp120. It is possible that the role of the conserved GPGRA sequence is to mediate a conformational change in gp120 that leads to activation of the fusion domain in gp41. It has been suggested that the GPGRA sequence is a site for gp120 proteolysis that activates fusion and viral entry. A peptide inhibitor of trypsinlike proteases with significant homology to the GPGRA sequence of the V3 domain has been reported to inhibit syncytium formation upon infection with HIV-1 (9). In addition, preparations of recombinant gp120 are sometimes found to be naturally cleaved after the arginine of the GPGRA sequence, a predicted trypsinlike cleavage site (26). Indeed, the V3 loop is highly accessible to proteases, as shown by its susceptibility to specific cleavage by trypsinlike and chymotrypsinlike enzymes (5).

* Corresponding author.

To study the role of the conserved GPGRA sequence, we analyzed the effects of several mutations on the ability of gp160 to mediate syncytium formation and virus entry. Included in this analysis were mutants that would be predicted to prevent cleavage in the GPGRA sequence by trypsinlike proteases. Since amino acid variation is a hallmark of the V3 domain, it was of interest to determine whether this region is involved in mediating viral entry in two distinct HIV-1 isolates, HXB2 and SF33. SF33 differs from most HIV-1 isolates in that it contains the sequence GPGKV within its V3 loop. HXB2 and SF33 have 56% amino acid identity throughout the V3 loop. The phenotypes displayed by the various HXB2 and SF33 envelope glycoprotein mutants provide insight into the nature of V3 interactions during virus entry.

MATERIALS AND METHODS

Cell culture. HeLaT4 and COS-7 cells were maintained in Dulbecco modified Eagle medium H21 supplemented with 10% fetal calf serum and antibiotics. HeLaT4 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, from Richard Axel. HUT78 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics.

Plasmids and site-directed mutagenesis. The HIV-1 proviral construct HIV-gpt (21) encodes all essential HIV-1 gene products except gp160 and contains a simian virus 40 origin of replication for high-level expression in COS-7 cells. A simian virus 40-based vector was chosen for expression of gp160. Vector pSM (1) was derived from the simian virus 40 expression vector pSV7d (27) by the addition of an M13 origin of replication. The M13 sequences permitted production of single-stranded plasmid DNA that was used as a template for oligo-directed mutagenesis. Plasmid HXB2-env was constructed by cloning HXB2 proviral *env* sequences from *Sac*I (nucleotide 5999) to *Xho*I (nucleotide 8896) into the *Sma*I and *Sal*I sites of the pSM polylinker (nucleotide numbers refer to nucleotide positions in sequences entered in the Human Retroviruses and AIDS data base, National Institute of Allergy and Infectious Diseases). Plasmid SF33-env was constructed by cloning SF33 proviral *env* sequences from plasmid pSF33/3′ (provided by Cecilia Cheng-Mayer and Jay Levy of the University of California, San Francisco) from the *Sac*I site upstream of the *env* coding sequences to the *Xba*I site in *nef* (nucleotide 2804) into the *Sma*I and *Xba*I sites of the pSM polylinker. In SF33-env, an ATG codon was present upstream of the *env* initiation codon. This ATG was changed to CTA by oligo-directed mutagenesis in order to enhance *env* expression. V3 domain mutations were made by using standard oligo-directed mutagenesis procedures (8, 13) with single-stranded DNA from plasmids HXB2-env and SF33-env.

Transfection and preparation of virus and cellular lysate. Virus was generated by transfection of plasmid DNA into COS-7 cells by using the calcium phosphate procedure as previously described (21). Viruses released into COS-7 culture supernatants were harvested 48 to 65 h after transfection, filtered through a 0.45-μm-pore-size membrane, and stored at −70°C. Cellular lysates were prepared 48 to 65 h after transfection by rinsing and collecting the COS-7 cells in phosphate-buffered saline (PBS) and pelleting at 1,000 × *g*. Pellets of approximately 6×10^6 cells were lysed with 100 μl of ice-cold IP buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40). The lysates were then vortexed and cleared by centrifugation at 15,000 × *g* for 5 min at 4°C. The lysates were stored at −20°C.

Isolation of viral particles. COS-7 cell supernatants containing HIV-gpt virus were inactivated by the addition of formaldehyde to 0.2%. Eight milliliters of inactivated virus was concentrated by pelleting through a 2-ml layer of 20% (wt/vol) sucrose onto a 50-μl cushion of 60% (wt/vol) sucrose at 160,000 × *g* for 1.5 h at 4°C. To recover the pelleted virus, the solution in the centrifuge tube was slowly aspirated until approximately 250 μl remained. The pelleted particles were stored at −20°C.

Western blotting. Samples of pelleted virus or cellular lysate were boiled for 4 min in reducing sample buffer (50 mM Tris [pH 6.8], 100 mM dithiothreitol, 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol) and electrophoresed on SDS–10% polyacrylamide gels. The gels were equilibrated in transfer buffer (20 mM Tris base, 150 mM glycine, 20% methanol) for 20 min and transferred to Immobilon membranes (Millipore) in a Bio-Rad Western blotting (immunoblotting) apparatus run at 90 V for 5 h. Membranes were blocked in TBST-milk (10 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Tween 20, 5% nonfat dry milk) for 30 min at room temperature and then incubated overnight at room temperature with sheep anti-gp120 serum DV-012 diluted 1:1,000 into TBST-milk. The polyclonal sheep anti-gp120 serum was obtained through the AIDS Research and Reference Reagent Program from Michael Phelan. Membranes were subsequently washed three times (10 min per wash) in TBST (10 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Tween-20). The membranes were then incubated for 2 h at room temperature with donkey anti-sheep immunoglobulin (Ig) antibody conjugated to peroxidase (Sigma) diluted 1:500 in TBST-milk. After two washes in TBST followed by two washes in TBS (10 mM Tris [pH 7.5], 150 mM NaCl), the blots were developed in peroxidase substrate solution (50 mM Tris [pH 7.6], 0.6 mg of diaminobenzidine per ml, 0.03% NiCl, 0.03% H_2O_2). Color development was stopped after 5 to 30 min at room temperature by rinsing the membranes in water.

CD4 binding assay. To detect gp160 and gp120 binding to CD4, cellular lysates were incubated with excess chimeric CD4-Ig molecule (CD4-Ig Immunoadhesin; Genentech, South San Francisco, Calif.) in IP buffer at 4°C for 1 h. Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) were then added, and the incubation was continued for an additional 1 h. gp160 and gp120 bound to the protein A beads through CD4-Ig were pelleted by centrifugation at 15,000 × *g* for 1 min. The beads were washed in IP buffer three times, resuspended in reducing sample buffer, and boiled for 5 min. The samples were then analyzed for gp160 and gp120 by Western blotting.

Quantitation of p24. An enzyme-linked immunosorbent assay (ELISA) was used to quantitate the level of p24 in each virus preparation. Reagents used to perform these assays were kindly provided by Kathelyn Steimer and Keith Higgins of Chiron Corp., Emeryville, Calif. This assay had a linear dose-response range from 0.75 to 12 ng of p24 per ml. All virus samples were assayed at multiple dilutions to ensure a reading in the linear range of the assay. Details of the ELISA procedure are available on request.

Infection and drug selection. HeLaT4 and HUT78 cells were infected with preparations of HIV-gpt as previously described (21). Virus preparations were added to monolayers of HeLaT4 and allowed to adsorb for 1 h at 37°C. Sufficient medium was then added for culture overnight.

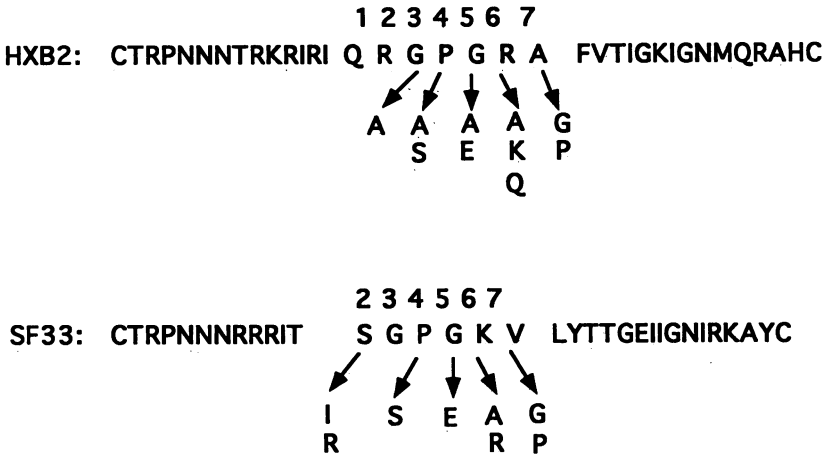

FIG. 1. Mutations in the V3 domain of gp160. Several single amino acid substitution mutations were made in the GPGRA sequence of HXB2 gp160 and in the analogous GPGKV sequence of SF33 gp160. The mutant that has a glycine-to-alanine change at position 3 (using the numbering shown) is referred to as G3A, the mutant with a proline-to-serine change at position 4 is referred to as P4S, etc. In addition to the single substitution mutations, two insertion mutations were made. INS.G3 has a three-amino-acid insertion at position 3, and INS.G5 has a four-amino-acid insertion at position 5.

Sixteen to 28 h after infection, the medium was replaced with Dulbecco modified Eagle medium containing 10% fetal calf serum, 250 μg of xanthine per ml, 14 μg of hypoxanthine per ml, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 μg of mycophenolic acid (Calbiochem, La Jolla, Calif.) per ml. The medium was changed every 3 to 4 days until colonies formed (11 to 14 days). Infectivity was measured by counting the number of mycophenolic acid-resistant colonies that formed per nanogram of p24 antigen in the virus preparation. To infect HUT78 cells, four- or fivefold serial dilutions of virus were added to 24-well plates containing 10^5 cells per well. Twenty-four hours after infection, RPMI 1640 medium containing the same supplements as described for the HeLaT4 cells, except with 10 μg of mycophenolic acid per ml, was added to the cultures. The cells were split 1:2 into selective medium every 3 days. Twenty days after infection, each well was assessed for outgrowth of mycophenolic acid-resistant cells.

RESULTS

Mutations in the V3 loop of HXB2 and SF33 envelope glycoproteins. In order to determine the functional consequences of alteration in the conserved portion of the V3 domain, we made several amino acid substitution mutations and two short insertion mutations in HXB2-env and SF33-env (Fig. 1). Some of the mutations are predicted to prevent tryptic cleavage at the GPGRA (HXB2-env) or GPGKV (SF33-env) sequences. In HXB2-env, these mutations are changes of arginine to alanine or glutamine (R6A and R6Q) and a change of the alanine to proline (A7P). In SF33-env, the analogous changes are lysine to alanine (K6A) and valine to proline (V7P). These mutations would specifically prevent trypsinlike cleavage but might not prevent cleavage by proteases with other specificities. In addition to mutations designed to block trypsinlike cleavage, several conservative and nonconservative amino acid substitution mutations were made to test the sensitivity of this region to minor change. Two short insertion mutations of three and four amino acids were fortuitously generated in the mutagenesis process. These mutations may disrupt the secondary structure of the V3 loop by lengthening the distance between the putative antiparallel β strands that are predicted to exist in the V3 domain (14). The V3 sequence of each mutagenized plasmid was verified by DNA sequencing.

In order to produce HIV-1 virions with wild-type and mutated envelope glycoproteins, envelope constructs were expressed in COS-7 cells along with an *env*-negative HIV-1 proviral construct, HIV-gpt (21). Translation initiation codons for *tat* and *rev* are not present in the *env* expression plasmids, and the initiation codon for *vpu* is immediately followed by a stop codon in both *env* constructs. Thus, gp160 is the only HIV gene product supplied by HXB2-env and SF33-env; all other viral proteins are made from the cotransfected HIV-gpt plasmid. HIV-gpt is a replication-defective proviral construct that carries a selectable marker gene, *SV-gpt*. When HIV-gpt is cotransfected with an *env* expression plasmid, infectious HIV particles that are capable of transmitting the selectable marker gene to infectible cells are produced. This system, shown schematically in Fig. 2, provides a quantitative measure of HIV infection, since each successful infection event leads to the outgrowth of a drug-resistant colony. We have previously shown that virus

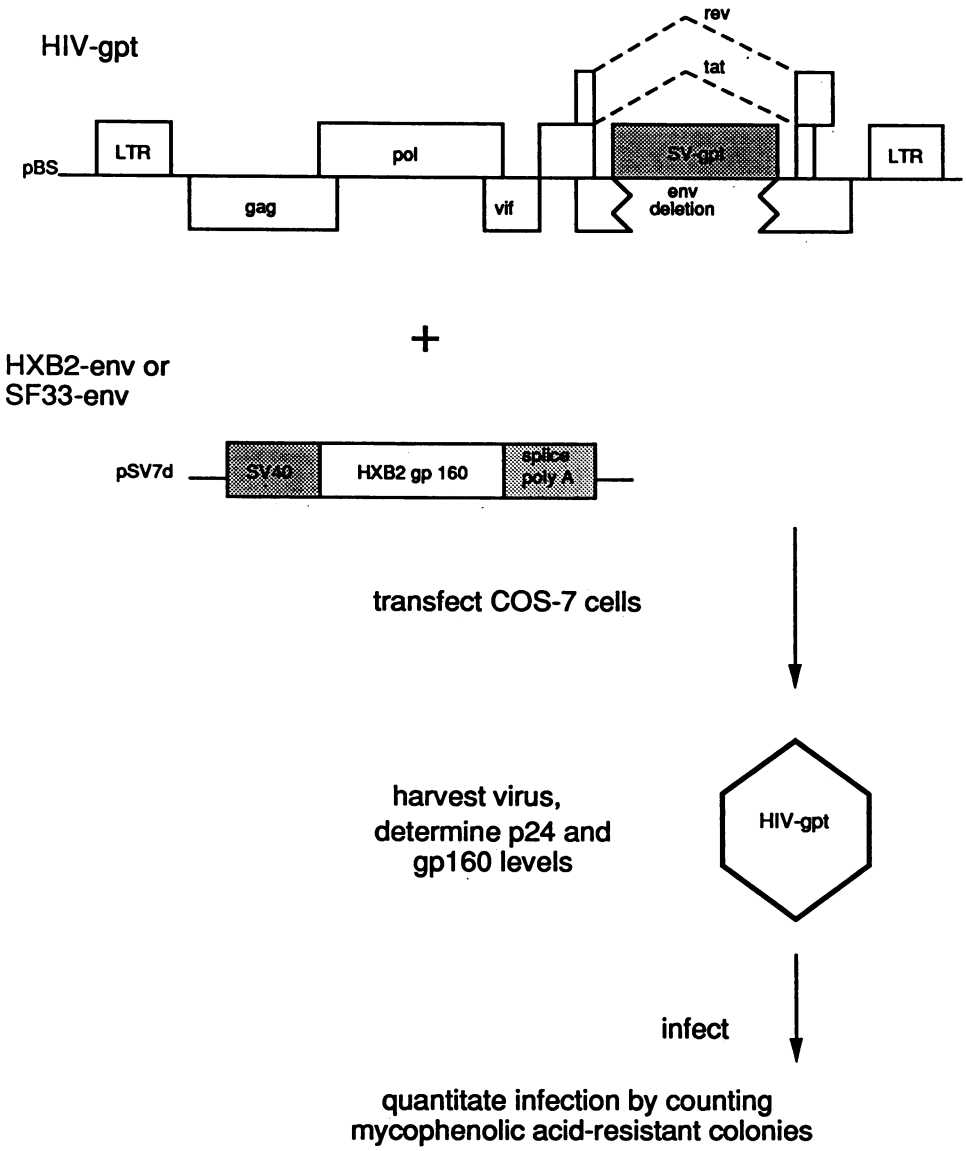

FIG. 2. Strategy for preparation and analysis of HIV-1 bearing mutant envelope glycoproteins. Virus preparations were made with HIV-gpt (21) and wild-type or mutant gp160 *env* constructs. Virus generated by cotransfection of the HIV-gpt and *env* plasmids is infectious for a single round of virus entry and transmits the *SV-gpt* selectable marker gene. LTR, long terminal repeat.

produced by HIV-gpt complemented with a gp160 expression construct has the same density, morphology, and host range as naturally isolated HIV (21). Use of HIV-gpt provides experimental advantages over work with replication-competent HIV; virus with a variety of different envelope glycoproteins can be easily prepared, and infection can be rapidly and accurately quantitated.

Expression of wild-type and mutant gp160 on HIV-gpt particles. To determine whether the mutant envelope glycoproteins were properly assembled onto viral particles, virus was collected from the culture supernatant of transfected COS-7 cells and analyzed for the presence of envelope glycoproteins and p24 core antigen. p24 core antigen levels were quantitated by ELISA. Viral particles were also analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting with antibodies raised against gp120. An equivalent amount of p24 core antigen was loaded per lane for each virus preparation. As shown in Fig. 3, the wild-type and mutant *env* constructs directed expression of envelope glycoproteins that appeared on viral particles. Since each sample stained similarly for gp120, the ratio of envelope to p24 core antigen must be roughly equivalent for each sample. Therefore, none of the mutations affected the processing or appropriate expression of gp160. Figure 3 also reveals a significant amount of unprocessed precursor gp160 on the viral particles derived from COS-7 cells. This surprising finding may be related to a relatively slow rate of envelope glycoprotein cleavage in transfected COS-7 cells.

CD4 binding by wild-type and mutant gp160. The primary CD4 binding site on gp120 lies about 100 amino acids carboxy terminal to the V3 domain, and anti-V3 antibodies do not block CD4 binding. Thus, V3 mutations would not be predicted to disrupt CD4 binding. In order to determine whether the mutant *envs* were capable of binding to CD4,

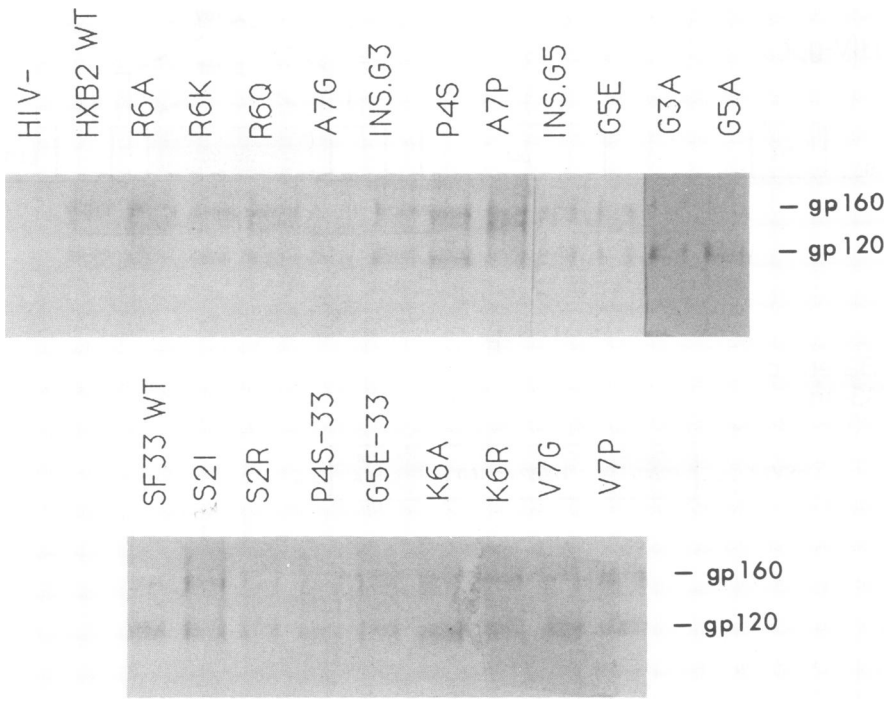

FIG. 3. Expression of mutant gp160 on HIV-gpt particles. Virus particles were generated by transfection and concentrated by pelleting onto a cushion of 60% sucrose. In order to assess the expression of gp160 on viral particles relative to the amount of $p24^{gag}$ antigen in each preparation, Western blots for gp160 were done with approximately 18 ng of p24 antigen for each wild-type and mutant virus. The virus preparations were subjected to SDS-PAGE followed by electroblotting and antibody staining for gp160 and gp120. HIV- refers to a virus preparation produced by transfection of HIV-gpt alone. These particles resemble HIV-1 in biophysical properties but lack an envelope glycoprotein and are not infectious (21).

each mutant gp160 was tested for the ability to bind a chimeric CD4-Ig molecule. Bound gp160 and gp120 were precipitated with protein A conjugated to Sepharose beads, and the precipitated envelope glycoproteins were detected on Western blots. Figure 4 shows the amount of envelope glycoprotein expression in total, unprecipitated cellular extracts and the amount precipitated with CD4-Ig. None of the V3 mutations interfered with CD4 binding. In each case, the amount of gp160 and gp120 that was immunoprecipitated corresponded to the amount present in the cellular extract used for immunoprecipitation. These results confirm previously published information that indicated that the V3 domain is not a part of the primary CD4 binding site on gp120 (20). However, it is important to note that the CD4 binding assay used in these experiments would not detect subtle alterations in the binding affinity of gp120 for CD4.

Effect of V3 mutations on HIV entry. Cotransfection of the wild-type and mutant gp160 constructs with HIV-gpt resulted in the production of virus particles that contained envelope glycoproteins capable of binding to CD4. To determine the ability of the mutant gp160s to mediate HIV entry, the virus preparations were tested for infectivity on HeLaT4 cells. Several independent transfections were performed to generate virus, whose infectious titer on HeLaT4 cells was measured. The results, expressed as means and standard deviations, are shown in Fig. 5. Several of the mutations had a moderate effect on infectivity. This indicates that, although the GPGRA sequence is conserved among HIV-1 isolates, it can be mutated without severely impairing envelope function. Many of the V3 mutations, including the HXB2 mutations G5E, R6A, R6Q, A7P, INS.G3, and INS.G5 and the SF33 mutations P4S-33, G5E-33, V7G, and V7P, resulted in a ≥100-fold reduction in infectivity. This result supports the hypothesis that the V3 domain is involved in mediating events required for HIV-1 entry into cells. Comparison of specific amino acid changes in HXB2 and SF33 envelope glycoproteins shows that some changes affect the function of the two envelopes differently. For example, a proline-to-serine change in HXB2 gp160 (P4S) resulted in a moderate reduction in infectivity, whereas the same change in SF33 gp160 (P4S-33) caused a >100-fold loss of infectivity. Similarly, change of an arginine to an alanine in HXB2 gp160 (R6A) resulted in a severe loss of infectivity, but the analogous change in SF33 gp160 (K6A) decreased infectivity only modestly. Thus, the surrounding sequence context dramatically influences the effect of specific amino acid changes. The effects of some amino acid substitutions were similar for HXB2 gp160 and SF33 gp160. For example, substitution of glutamic acid for glycine at position 5 (G5E in HXB2 and G5E-33 in SF33) and substitution of proline for alanine or valine (A7P in HXB2 and V7P in SF33) severely reduced infection in both gp160 isolates. These amino acid substitutions have been detected at a low frequency in gp160 sequences isolated by polymerase chain reaction in a survey of infected human peripheral blood mononuclear cells (14). One interpretation of this result is that these nonconservative changes could not be tolerated in the context of HXB2 and SF33 sequences but may be tolerated in the context of other *env* sequences. Alternatively, these amino acid substitutions may be present in defective envelope sequences detected by direct survey of V3 sequences in infected peripheral blood mononuclear

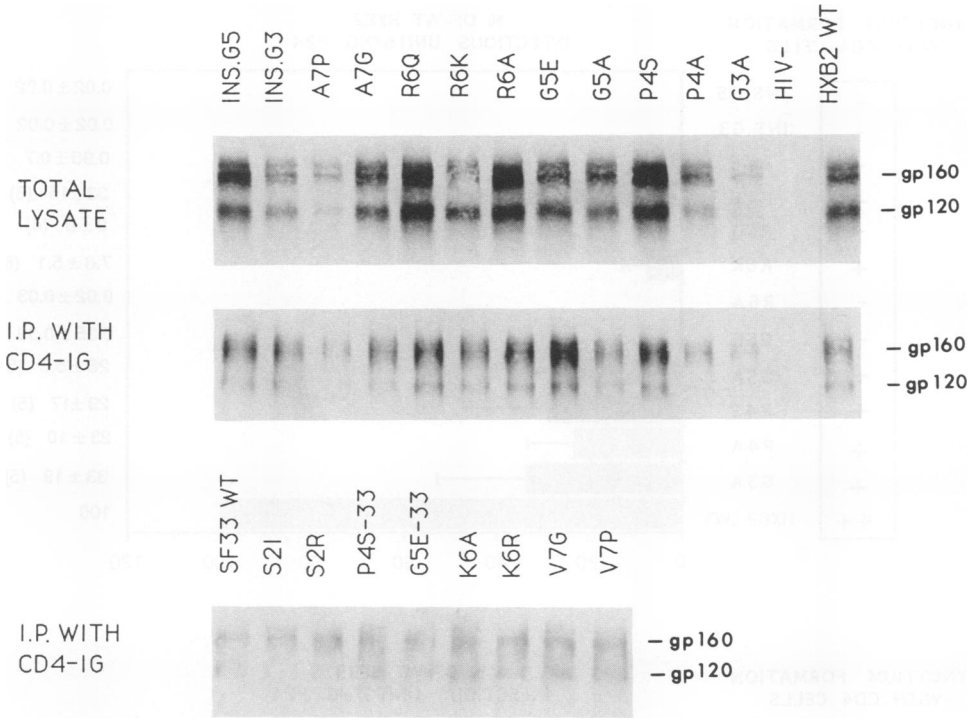

FIG. 4. CD4 binding by wild-type and mutant gp160 and gp120. COS-7 cells were transfected with HIV-gpt and wild-type and mutant *env* constructs. Cellular lysates were prepared and analyzed for gp160 expression by Western blotting. Six microliters of cellular lysate was loaded per lane for each HXB2 wild-type and mutant sample (top panel). CD4 binding ability for the HXB2 gp160 preparations was determined by incubation of 25 μl of cellular lysate with 100 ng of CD4-Ig and immunoprecipitation with protein A-Sepharose as described in Materials and Methods. The immunoprecipitated gp160 and gp120 were detected by Western blotting (center panel). For wild-type and mutant SF33 gp160, the amount of cellular lysate immunoprecipitated with CD4-Ig (bottom panel) was normalized on the basis of the level of gp160 observed on Western blots of total cellular extracts (data not shown).

cells. The two insertion mutations severely reduced viral infectivity. These mutations may have disrupted envelope glycoprotein function by disturbing the overall structure of the V3 domain.

Effect of V3 loop mutations on syncytium formation. It has previously been shown that specific mutations in the V3 domain can affect the ability of gp160 to mediate cellular syncytium formation with CD4-positive cells (7). It is likely that mutations that interfere with cell-to-cell fusion may also affect virus entry by preventing virus-cell fusion. Wild-type and mutant gp160 transiently expressed on the surface of COS-7 cells were tested for the ability to induce cellular fusion with HeLaT4 cells. Syncytium formation between gp160-expressing cells and HeLaT4 cells was observed by cocultivation of the two cell types for 6 to 16 h. As shown in Fig. 6, extensive fusion was seen with wild-type gp160. Wild-type HXB2 gp160 and wild-type SF33 gp160 exhibited similar, high levels of fusion activity. Each gp160 mutant was tested for the ability to mediate syncytium formation in three independent experiments. The results of these experiments are summarized in Fig. 5. To determine the expression level of each wild-type and mutant envelope glycoprotein, extracts of the transfected COS-7 were made and analyzed by Western blotting for gp120 (data from a representative experiment are shown in the top panel of Fig. 4). Although the level of gp120 expression varied, it was clear that even at the lowest level of expression obtained (Fig. 4, lane G3A), fusion could be observed. In addition, with the exception of mutant A7P, the gp160 mutants that were not able to mediate fusion were expressed at similar or higher levels than wild-type gp160. Thus, the V3 mutations appeared to directly affect fusion rather than expression or processing of gp160. The V3 mutations that prevented syncytium formation also severely reduced virus infectivity. These results suggest that disruption of virus entry due to mutations in the conserved GPGRA sequence is due to loss of gp160-CD4 induced membrane fusion.

Effect of V3 loop mutations on HIV-1 entry into T cells. Because the ability of particular isolates of HIV to enter cells varies according to tissue type, it was of interest to determine whether the V3 mutations that abrogated entry into HeLaT4 cells also abrogated entry into T cells. Table 1 shows the results of wild-type and mutant virus titration on HeLaT4 cells and the T-cell line HUT78. V3 mutations that severely impaired infection of HeLaT4 cells similarly impaired infection of HUT78 cells. Mutations that had a moderate effect on HeLaT4 infectivity also had moderate or no effect on HUT78 cell infectivity. Thus, the requirement for a specific V3 domain sequence is similar during virus entry into HeLaT4 and HUT78 cells. Amino acid differences in the V3 loop have been shown to influence the ability of HIV-1 to infect different cell types. In this study, we found a concordance between the V3 sequences that allow infection of HeLaT4 cells and of HUT78 cells.

DISCUSSION

The results of the mutational analysis presented here demonstrate the involvement of the V3 domain in HIV entry and gp160-mediated syncytium formation. Several of the

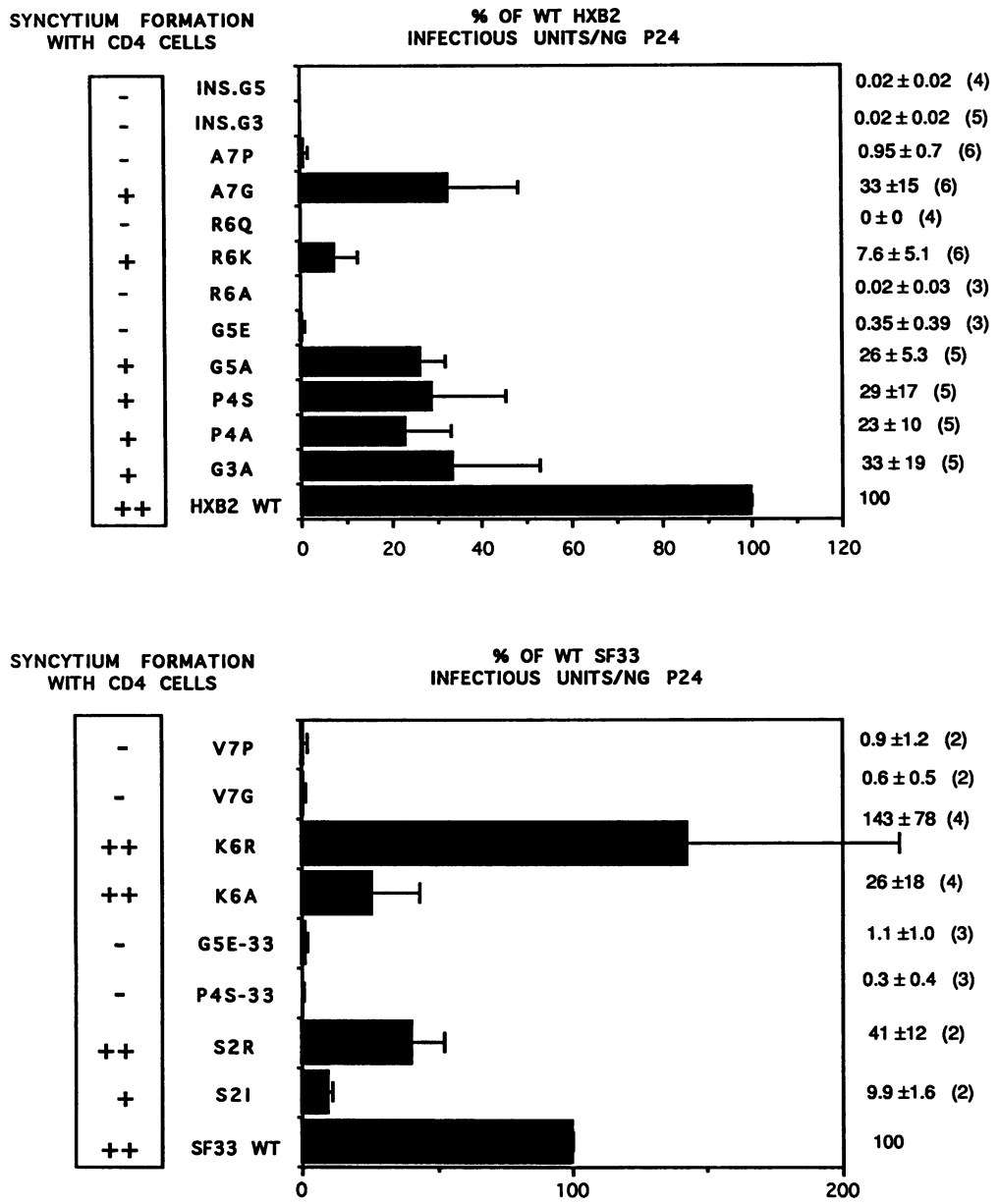

FIG. 5. Effect of V3 mutations on infectivity and syncytium formation. Titers of viruses prepared by cotransfection of COS-7 cells with HIV-gpt and *env* constructs were determined for infectivity on HeLaT4 cells and analyzed for p24 level by ELISA. Infectious titer was normalized for p24 content and compared with wild-type virus that was prepared in parallel. Each envelope construct was tested in several independent transfection experiments. The means and standard deviations, expressed as percent of wild-type (WT) infectious units per nanogram of p24, are shown. The numbers in parentheses refer to the numbers of independent transfections included in the calculation of the data. HIV-gpt prepared with wild-type HXB2 *env* gave a mean titer of 156 ± 90 infectious units per nanogram of p24 (n = 7), and HIV-gpt prepared with wild-type SF33 *env* gave a mean titer of 18 ± 12 infectious units per nanogram of p24 (n = 5). COS-7 cells transfected with HIV-gpt and *env* plasmids were cocultivated with HeLaT4 cells and assessed for syncytium formation after 6 to 16 h. Each *env* construct was tested for the ability to mediate syncytium formation in three independent experiments. The results of the three experiments were consistent and are summarized as follows: ++, high number of syncytia (see Fig. 6); +, moderate number of syncytia; and −, rare or absent syncytia.

single amino acid substitution mutations and both insertion mutations resulted in a 100- to 5,000-fold loss of infectivity and abrogated syncytium-forming ability. These mutations did not affect the expression of gp160 on viral particles or CD4 binding ability. Therefore, it is likely that the loss of infectivity was due to a loss of fusion activity after CD4 binding. It is clear from these results that the V3 domain actively participates in HIV entry subsequent to CD4 binding.

Many of the conservative amino acid changes that were made resulted in only a moderate decrease in infectivity and syncytium formation. For example, the glycine-to-alanine changes (G3A and G5A) and the alanine-to-glycine change (A7G) in HXB2-env decreased infectivity three- to fourfold.

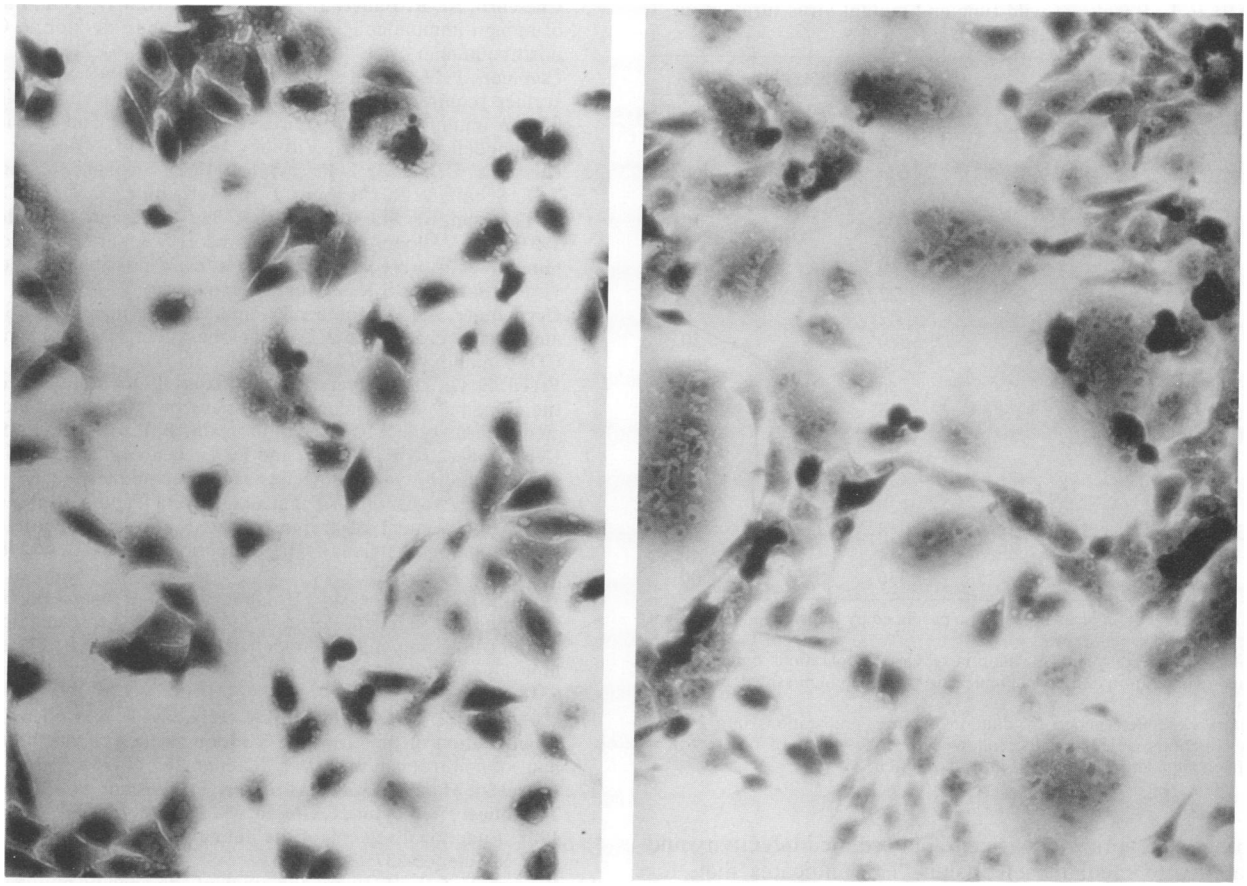

FIG. 6. Syncytium formation between COS-7 cells expressing wild-type gp160 and HeLaT4 cells. (Left panel) COS-7 cells transfected with HIV-gpt alone were cocultivated with HeLaT4 cells for 14 h. (Right panel) COS-7 cells transfected with wild-type SF33 *env* and HIV-gpt were cocultivated with HeLaT4 cells. Equal numbers of cells were cocultivated for 14 h, fixed in 10% formalin, and stained with Giemsa stain.

This indicates that, although the GPGRA sequence is conserved among HIV-1 isolates, it can be mutated and still retain biologic activity. The presence of a relatively conserved sequence within the principal neutralizing determinant of gp160 has prompted the development of peptide-based vaccines that include the GPGRA sequence. Antibodies raised against this sequence could conceivably provide protection against a broad spectrum of HIV isolates. The ability of HIV-1 to accommodate amino acid changes within the GPGRA sequence may significantly influence the efficacy of such vaccinations. While the results of the present study are confined to in vitro-generated mutations and in vitro infections, they may provide some indication of the ability of HIV-1 to tolerate change even within the relatively conserved sequences of the principal neutralizing determinant.

The molecular events that occur during HIV entry are not well understood. Binding of HIV to CD4 does not appear to be sufficient to initiate infection. Mouse cells that express human CD4 bind gp160 but cannot be infected (17). Similarly, human cells that express CD4 but are not infectible with HIV have been described previously (3, 4). These cells either lack an essential factor required for infection or contain an inhibitor of infection. Several strains of HIV-1 are restricted in their tissue specificity for primary T cells or macrophages. In several cases, a portion of the envelope gene including the V3 region was found to play a role in controlling tissue specificity. It is possible that the V3 domain interacts with a surface molecule that differs between T cells and macrophages. This interaction may be essential for virus entry to occur. In a study using the GUN-1 isolate of HIV-1, a single amino acid substitution of serine for proline in the GPGRA sequence resulted in a dramatic increase in infection of a brain tumor cell line without significant loss of ability to infect T cells (28). In the present study, the substitution of serine for proline in HXB2-env resulted in a moderate decrease in infection of HeLaT4 and HUT78 cells whereas the same substitution mutation in SF33-env resulted in an almost complete loss of infectivity. While we have not tested the ability of these mutant viruses to infect the brain tumor cells used in the analysis of the GUN-1 variant, it is likely that the complete V3 sequence, as well as individual amino acids, will be important for determining tissue tropism. It is also important to consider that sequences outside the V3 domain may influence the ability of HIV-1 to enter into cells.

The GPGRA sequence is a potential cleavage site for trypsinlike proteases. It has been proposed that cell surface proteases may cleave the V3 loop after gp160 binding to CD4, thus altering the conformation of gp160 and activating fusion. Consistent with this hypothesis, the HXB2-env mutants R6A, R6Q, and A7P and the SF33-env mutant V7P have lost the putative tryptic cleavage site and were virtually noninfectious. However, the SF33-env mutant K6A also lost

TABLE 1. Results of wild-type and mutant virus titration on HeLaT4 and HUT78 cells

Virus prepn[a]	p24 (ng/ml)	Titer (infectious units/ml) on:	
		HeLaT4 cells[b]	HUT78 cells[c]
HIV(−)	36	0	0
HXB2 WT	25	8,000	200
G3A	27	1,540	50
P4A	25	1,200	10
P4S	23	1,660	50
G5A	27	2,160	200
G5E	32	22	0
R6A	55	42	0
R6K	56	580	50
A7G	44	3,720	200
A7P	31	50	0
INS.G3	29	4	0
INS.G5	22	4	0
SF33 WT	78	840	1,000
S2R	43	220	50
P4S-33	104	8	0
G5E-33	21	4	0
K6A	64	336	200

[a] HIV(−) refers to a virus preparation produced by transfection of HIV-gpt alone. WT, wild type.

[b] Titers were determined by dilution of virus preparations onto HeLaT4 cells and enumeration of mycophenolic acid-resistant colonies that arose after selection.

[c] Titers were determined by dilution of virus preparations into 24-well plates containing HUT78 cells, followed by assessment of outgrowth of mycophenolic acid-resistant cells at each dilution.

the putative tryptic cleavage site but retained infectivity and the ability to mediate cell fusion. This indicates that, for SF33, tryptic cleavage at the GPGKV sequence is not a requirement for infectivity. The role of the V3 domain in HIV entry may be to mediate a conformational change in gp160 that results in exposure of the hydrophobic sequence at the amino terminus of gp41. It is possible that the V3 domain can utilize a variety of different types of interactions that cause a conformational change in gp160. In some cases, proteolytic cleavage of the V3 loop may activate infectivity, whereas in other cases, a binding interaction with a cell surface molecule may be required. The remarkable variability of the V3 domain suggests that any functional property of this domain must work in a flexible manner.

ACKNOWLEDGMENTS

We gratefully acknowledge the generosity of Kathelyn Steimer and Keith Higgins of Chiron Corp. for providing reagents and methods needed to perform p24 ELISAs. We also thank Rebecca Ward of Genentech for the gift of CD4-Ig Immunoadhesin and Cecilia Cheng-Mayer and Jay Levy for the SF33 DNA. We thank Richard Locksley for use of his biocontainment laboratory.

This work was supported by Public Health Service grants 1F32-AI 0890 to K.A.P. and AI 24286 to D.R.L. from the National Institutes of Health. D.R.L. is an investigator of the Howard Hughes Medical Institute.

REFERENCES

1. **Brodsky, M. H., M. Warton, R. M. Myers, and D. R. Littman.** 1990. Analysis of the site in CD4 that binds to the HIV envelope glycoprotein. J. Immunol. **144**:3078–3086.
2. **Cheng-Mayer, C., M. Quiroga, J. W. Tung, D. Dina, and J. A. Levy.** 1990. Viral determinants of human immunodeficiency virus type 1 T-cell or macrophage tropism, cytopathogenicity, and CD4 antigen modulation. J. Virol. **64**:4390–4398.
3. **Chesebro, B., R. Buller, J. Portis, and K. Wehrly.** 1990. Failure of human immunodeficiency virus entry and infection in CD4-positive human brain and skin cells. J. Virol. **64**:215–221.
4. **Clapham, P. R., D. Blanc, and R. A. Weiss.** 1991. Specific cell surface requirements for the infection of CD4-positive cells by human immunodeficiency virus types 1 and 2 and by simian immunodeficiency virus. Virology **181**:703–715.
5. **Clements, G. J., M. J. Price-Jones, P. E. Stephens, C. Sutton, T. F. Schulz, P. R. Clapham, J. A. McKeating, M. O. McClure, S. Thomson, M. Marsh, J. Kay, R. A. Weiss, and J. P. Moore.** 1991. The V3 loop of the HIV-1 and HIV-2 surface glycoproteins contain proteolytic cleavage sites: a possible function in viral fusion? AIDS Res. Hum. Retroviruses **7**:3–16.
6. **Cordonnier, A., L. Montagnier, and M. Emerman.** 1989. Single amino acid changes in HIV envelope affect viral tropism and CD4 binding. Nature (London) **340**:571–574.
7. **Freed, E. O., D. J. Myers, and R. Risser.** 1991. Identification of the principal neutralizing determinant of human immunodeficiency virus type 1 as a fusion domain. J. Virol. **65**:190–194.
8. **Geisselsoder, J., F. Witney, and P. Yuckenberg.** 1987. Efficient site-directed *in vitro* mutagenesis. BioTechniques **5**:786.
9. **Hattori, T., A. Koito, K. Takatsuki, H. Kido, and N. Katunuma.** 1989. Involvement of tryptase-related cellular protease(s) in human immunodeficiency type 1 infections. FEBS Lett. **248**:48–52.
10. **Helseth, E., U. Olshevsky, D. Gabuzda, B. Ardman, W. Haseltine, and J. Sodroski.** 1990. Changes in the transmembrane region of the human immunodeficiency virus type 1 gp41 envelope glycoprotein affect membrane fusion. J. Virol. **64**:6314–6318.
11. **Hwang, S. S., T. J. Boyle, H. K. Lyerly, and B. R. Cullen.** 1991. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science **253**:71–74.
12. **Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and J. Sodroski.** 1987. Functional regions of the human immunodeficiency virus type 1. Science **237**:1351–1355.
13. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotype selection. Proc. Natl. Acad. Sci. USA **82**:448.
14. **LaRosa, G. J., J. P. Davide, K. Weinhold, J. A. Waterbury, A. T. Profy, J. A. Lewis, A. J. Langlois, G. R. Dreesman, R. N. Boswell, P. Shadduck, L. H. Holley, M. Karplus, D. P. Bolognesi, T. J. Matthews, E. A. Emini, and S. D. Putney.** 1990. Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. Science **249**:932–935.
15. **Lasky, L. A., G. Nakamura, D. H. Smith, C. Fennie, C. Shimasaki, E. Patzer, P. Berman, T. Gregory, and D. J. Capon.** 1987. Delineation of a region of the HIV-1 gp120 glycoprotein critical for interaction with the CD4 receptor. Cell **50**:975–985.
16. **Liu, Z.-Q., C. Wood, J. A. Levy, and C. Cheng-Mayer.** 1990. The viral envelope gene is involved in macrophage tropism of a human immunodeficiency virus type 1 strain isolated from brain tissue. J. Virol. **64**:6148–6153.
17. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47**:333–348.
18. **Nara, P. L., L. Smit, N. Dunlop, W. Hatch, M. Merges, D. Waters, J. Kelliher, R. C. Gallo, P. J. Fischinger, and J. Goudsmit.** 1990. Emergency of viruses resistant to neutralization by V3-specific antibodies in experimental human immunodeficiency virus type 1 infection of chimpanzees. J. Virol. **64**:3779–3791.
19. **O'Brien, W. A., Y. Koyanagi, A. Namazie, J. Q. Zhao, A. Diagne, K. Idler, J. A. Zack, and I. S. Y. Chen.** 1990. HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. Nature (London) **348**:69–73.
20. **Olshevsky, U., E. Helseth, C. Furman, J. Li, W. Haseltine, and J. Sodroski.** 1990. Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. J. Virol. **64**:5701–5707.

21. **Page, K. A., N. R. Landau, and D. R. Littman.** 1990. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. J. Virol. **64**:5270–5276.
22. **Rusche, J. R., K. Javaherian, C. B. McDanal, J. Petro, D. L. Lynn, P. J. Fischinger, D. P. Bolognesi, S. D. Putney, and T. J. Matthews.** 1988. Antibodies that inhibit fusion of HIV-1 infected cells bind a 24 amino acid sequence of the viral envelope, gp120. Proc. Natl. Acad. Sci. USA **85**:3198–3202.
23. **Shioda, T., J. A. Levy, and C. Cheng-Mayer.** 1991. Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. Nature (London) **349**:167–169.
24. **Simmonds, P., P. Balfe, C. A. Ludlam, J. O. Bishop, and A. J. L. Brown.** 1990. Analysis of sequence diversity in hypervariable regions of the external glycoprotein of human immunodeficiency virus type 1. J. Virol. **64**:5840–5850.
25. **Skinner, M. A., A. J. Langlois, C. B. McDanal, J. S. McDougal, D. P. Bolognesi, and T. J. Matthews.** 1988. Neutralizing antibodies to an immunodominant envelope sequence do not prevent gp120 binding to CD4. J. Virol. **62**:4195–4200.
26. **Stephens, P. E., G. Clements, G. T. Yarranton, and J. Moore.** 1990. A chink in HIV's armour? Nature (London) **343**:219. (Letter.)
27. **Stuve, L. L., S. Brown-Shimer, C. Pachl, R. Najarian, D. Dina, and R. L. Burke.** 1987. Structure and expression of the herpes simplex virus type 2 glycoprotein gB gene. J. Virol. **61**:326–335.
28. **Takeuchi, Y., M. Akutsu, K. Murayama, N. Shimizu, and H. Hoshino.** 1991. Host range mutant of human immunodeficiency virus type 1: modification of cell tropism by a single point mutation at the neutralization epitope in the *env* gene. J. Virol. **65**:1710–1718.
29. **Westervelt, P., H. E. Gendelman, and L. Ratner.** 1991. Identification of a determinant within the human immunodeficiency virus 1 surface envelope glycoprotein critical for productive infection of primary monocytes. Proc. Natl. Acad. Sci. USA **88**:3097–3101.
30. **York-Higgins, D., C. Cheng-Mayer, D. Bauer, J. A. Levy, and D. Dina.** 1990. Human immunodeficiency virus type 1 cellular host range, replication, and cytopathicity are linked to the envelope region of the viral genome. J. Virol. **64**:4016–4020.