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# Differential Regulation of Cellular Tropism and Sensitivity to Soluble CD4 Neutralization by the Envelope gp120 of Human Immunodeficiency Virus Type 1

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**Using recombinant and mutant viruses generated between two human immunodeficiency virus type 1 isolates that display differences in cell tropism and sensitivity to soluble CD4 neutralization, we show that these two properties of the virus are regulated by different mechanisms. Whereas there is an association between V3 loop conformation and a particular cellular tropism, soluble CD4 neutralization sensitivity appears to be determined by amino acid differences in the C2 domain of the envelope gp120 that modulate the stability of gp120-gp41 association. Our findings further illustrate the importance of functional interactions among different regions of the envelope gp120 in regulating the biological phenotypes of human immunodeficiency virus and suggest that additional probing of the V3 loop with monoclonal antibodies may identify specific structural features of this loop that determine cell tropism.**

One of the major challenges in designing effective vaccines and therapeutic approaches for controlling AIDS is the biological heterogeneity of its causative agent, the human immunodeficiency virus (HIV). In vitro, HIV isolates differ in tropism, replicative pattern, cytopathicity, sensitivity to soluble CD4 (sCD4), and serum neutralization (for reviews, see references 22 and 45). Whereas noncytopathic macrophage-tropic HIV strains are reported to be preferentially transmitted and therefore responsible for the establishment of primary infection (23, 49, 50), fast-replicating and highly cytopathic T-cell-line-tropic strains are readily isolated from patients with advanced disease (2, 6, 42). Although all HIV isolates utilize the CD4 cell surface antigen as their primary cellular receptor (10, 22), primary macrophage-tropic isolates have been shown to be resistant to sCD4 neutralization (11). Thus, an understanding of the mechanisms controlling entry into primary macrophages and escape from sCD4 neutralization might be of value in the development of AIDS prophylaxis.

The genetic determinants for cell tropism and sCD4 neutralization sensitivity of HIV-1 have been mapped to the gp120 portion of the viral glycoprotein (5, 7, 8, 20, 28, 29, 37–39, 47). In particular, the V3 hypervariable region is reported to be a major determinant for these biological properties (8, 20, 39, 47). This region of envelope gp120 participates in postbinding events that are believed to be necessary for viral entry and infection (9, 14, 21, 27, 32, 33, 36). On the basis of structure-function analyses of the envelope glycoprotein, we have previously proposed that the overall conformation of the envelope gp120, in particular the V3 loop, determines the type and/or extent of postbinding interactions or conformational changes that the envelope glycoprotein undergoes in mediating viral entry (40).

In this study, we investigate the role of the V3 loop structure in regulating cellular tropism and the sensitivity to sCD4 neutralization of HIV-1. Our results show that, contrary to findings of another study (20), sCD4 neutralization sensitivity and cellular tropism are regulated by different regions of gp120. Whereas the structure of the V3 loop appears to associate with cellular tropism, it does not determine the sCD4 neutralization sensitivity of the viruses studied here. This latter biological property is determined by a region of gp120 outside the V3 loop that modulates the gp120-gp41 association.

## MATERIALS AND METHODS

**Viruses and cell lines.** Peripheral blood mononuclear cells (PBMCs) were obtained from donated blood received from the Irwin Memorial Blood Bank (San Francisco, Calif.) by Ficoll gradient purification. Prior to use, these cells were stimulated with phytohemagglutinin (3 μg/ml) for 3 days and then maintained in RPMI 1640 growth medium containing interleukin-2 (10%; Collaborative Research, Bedford, Mass.). Human rhabdomyosarcoma cells (RD-4 cells) and simian COS-7 cells were obtained from the American Tissue Culture Collection (Rockville, Md.) and maintained as monolayer cultures in Dulbecco modified Eagle's medium. All media were supplemented with 10% fetal calf serum and antibiotics.

The origin, isolation, molecular cloning, and sequencing of HIV-$1_{SF2}$ and HIV-$1_{SF162}$ have been reported previously (5). Molecularly cloned viruses were obtained from transfection of RD-4 cells with cloned DNA followed by cocultivation (<2 weeks) with PBMCs. High-titer culture supernatants (reverse transcriptase [RT] activity of $>10^6$ cpm/ml) were filtered through 0.45-μm-pore-size filters (Nalgene, Rochester, N.Y.) and frozen as 1-ml aliquots at −70°C for future use. The generation of recombinant and mutant DNAs and viruses has been described previously (38, 39). Briefly, viruses were recovered by cotransfection of the 3′ recombinant or mutagenized proviral DNA together with the 5′ proviral DNA into RD-4 cells followed by cocultivation with PBMCs. For repeat experiments, fresh virus preparations were again produced by transfection of RD-4 cells. Two independent clones of each mutant virus were used to control for possible effects due to secondary-site mutations generated during the mutagenesis process and to ensure reproducibility of results.

**HIV-1 infection.** Phytohemagglutinin-stimulated PBMCs

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were treated with Polybrene (2 μg/ml) for 30 min at 37°C and then exposed to 1 ml of virus-containing fluids for 2 h at 37°C. The cells were then washed once and maintained in interleukin-2-containing RPMI 1640 medium. Culture supernatants were assayed for RT activity at 3- to 4-day intervals as described previously (5, 18).

**Neutralization of infection by sCD4.** Viruses were incubated with serially diluted sCD4 (provided by Ray Sweet, SmithKline Beecham, King of Prussia, Pa.) at 37°C for 2 h, and then the mixture was added to $2 \times 10^6$ Polybrene-treated PBMCs. The amount of p24 antigen present in culture supernatants was evaluated 7 days postinfection, using an enzyme-linked immunosorbent assay (ELISA) kit (Coulter Corp., Hialeah, Fla.).

**Virus purification.** Culture supernatants from infected PBMCs with RT activity corresponding to the peak of infection were first centrifuged for 10 min at 3,000 rpm to remove cellular debris. The virus was then pelleted for 90 min at 4°C at 28,000 rpm in an SW28 rotor (Beckman Instruments, Palo Alto, Calif.) through 5 ml of a 32% sucrose cushion in 10 mM Tris–100 mM NaCl–1 mM EDTA (pH 8.0) (TNE). Two hundred microliters of TNE was added to the pelleted virus for 1 to 2 h at 4°C. Following resuspension, the virus was centrifuged overnight on a 16 to 60% sucrose gradient in an SW41 rotor (Beckman Instruments) at 40,000 rpm. Approximately 0.5-ml fractions were collected from the gradient and tested for RT activity, p24 (by ELISA), and gp120 (by Western immunoblotting and ELISA) contents. Fractions with high RT activity and high p24 and gp120 contents were pooled, occasionally checked for infectivity, and kept at 4°C until further use.

**Enzymatic cleavage of virion gp120.** Thirty microliters of sucrose-purified virus was incubated with or without sCD4 (3 μg) for 2 h at 37°C as reported previously (46). In some experiments, thrombin (3 μg) (Calbiochem Corp., La Jolla, Calif.) was added, and the incubation proceeded for an additional hour. Uncleaved viral gp120 and the cleavage products were visualized following electrophoresis on a sodium dodecyl sulfate (SDS)–polyacrylamide gel and Western blotting with goat anti-gp120 polyclonal antibodies (provided by K. Steimer, Chiron Corp., Emeryville, Calif.). The integrated optical density of each band on the Western blot was determined with a BIO IMAGE analyzer (Millipore Corp., Ann Arbor, Mich.).

**Envelope expression.** Wild-type and mutant HIV-$1_{SF2}$ *env* plasmid DNAs were constructed by cloning HIV-$1_{SF2}$ wild-type and mutant proviral *env* sequences from *Sac*I (nucleotide 6009, blunt ended) to *Xho*I (nucleotide 8914) into the *Sma*I and *Sal*I sites in the polylinker of the pSM vector (4); nucleotide numbers refer to nucleotide positions in the HIV-$1_{SF2}$ sequences entered in the Human Retroviruses and AIDS data base, Los Alamos, N. Mex. Wild-type and mutant HIV-$1_{SF162}$ *env* plasmid DNAs were constructed by cloning HIV-$1_{SF162}$ wild-type and mutant proviral *env* sequences from *Eco*RI (nucleotide 5750) to *Eco*RV (nucleotide 9214) into the *Eco*RI and *Sma*I sites in the pSM polylinker.

**Evaluation of spontaneous and sCD4-induced release of gp120.** COS-7 cells ($3 \times 10^5$) were plated in 10-cm-diameter plates and were cotransfected with wild-type or mutant *env* plasmid (10 μg) and expression vector for Rev (pREV; 5 μg) (19) by the calcium phosphate precipitation method. Following a 5.5-hour incubation, the DNA precipitate was removed and the transfected cells were refed with fresh medium; 32 h later, the supernatants were collected and the cells were recovered from the plates by trituration. The cells were washed twice, resuspended in phosphate-buffered saline and divided in half. sCD4 (0.5 μg/ml) was added to one of the two fractions. Both fractions were incubated for 2 h at 37°C. The supernatants and the cells were subsequently collected, and the amounts of gp120 were quantitated in each fraction by ELISA as described below.

**Quantitation of gp120 by ELISA.** Ninety-six-well disposable ELISA plates (Corning Inc., Corning, N.Y.) were coated with D6205, an affinity-purified sheep anti-HIV gp120 polyclonal antibody (International Enzymes, Inc., Fallbrook, Calif.) at 2.5 μg/ml by overnight incubation at 4°C. This antibody was produced by immunizing sheep with a single synthetic peptide, APTKAKRRVVQREKR, corresponding to the carboxy-terminal 15 amino acids of the *env* gp120. Unbound antibody was removed by washing the wells with 144 mM NaCl–25 mM Tris (pH 7.6) (TBS); 0.25 ml of TBS containing 4% nonfat dry milk (Carnation) was added to each well for 2 h at room temperature to block unbound sites. Following washing, 0.1-ml samples containing gp120 were added and the plates were incubated for 2 h at room temperature. The plates were washed with TBS, and 100 μl of pooled sera from HIV-positive patients (1:7,500 dilution in TBS–4% milk) and 20% bovine calf serum (BCS; HyClone Laboratories, Inc., Logan, Utah) were added per well for an extra 2 h at room temperature. Following washing, 100 μl (1:7,500 dilution in TBS-milk-BCS) of goat anti-human immunoglobulin G coupled to alkaline phosphatase (GaHIgG-Ap; Zymed Laboratories, Inc., South San Francisco, Calif.) was added for 1 h at room temperature. The amount of bound alkaline phosphatase was determined with an AMPAK ELISA amplification system (Life Technologies, Inc., Gaithersburg, Md.). Optical densities at 490 nm ($OD_{490}$) were recorded with a Coulter microplate reader (Coulter Corp., Hialeah, Fla.). A standard curve ($OD_{490}$ versus nanograms of gp120 per milliliter) was constructed by using serial dilutions of recombinant HIV-$1_{SF2}$ gp120 (provided by K. Steimer, Chiron Corp.). Nonspecific binding of antibodies was controlled with the use of human sera from HIV-negative individuals. The mean $OD_{490}$ values obtained with these negative control sera were substracted from the $OD_{490}$ values obtained with the sera from HIV-positive individuals.

The sandwich was therefore built as follows: well–D6205–gp120–human serum–GaHIgG-Ap. Elimination of one or several of these components gave a background $OD_{490}$ reading.

**Quantitation of sCD4 bound to virion gp120 by ELISA.** The methodology for detection of sCD4 bound to intact virions by ELISA was performed as previously reported (25, 31). Briefly, sucrose-purified viruses (2 to 5 μl) were incubated (2 h at 4 or 37°C) with increasing concentrations (0.3 to 2,000 nM) of sCD4 in a total volume of 0.1 ml. Unbound sCD4 was separated from the virion-sCD4 complexes following centrifugation and pelleting as previously described (48). The virion-sCD4 pellet was resuspended with lysis buffer (TBS–4% milk–20% BCS–1% Nonidet P-40) and transferred to wells coated with polyclonal antibody D6205 (5 μg/ml). This incubation was carried out at 4°C for 2 h. After washing with cold TBS, 100 μl of TBS–4% milk–20% BCS–0.1% Nonidet P-40 was added per well, and the incubation proceeded for additional 2 h at 4°C. The wells were washed with cold TBS, and 100 μl of a rabbit polyclonal antiserum against sCD4 (1:7,500 dilution; provided by Ray Sweet, SmithKline Beecham) was added overnight at 4°C in TBS-milk-BCS. The amounts of rabbit serum bound were then determined by addition of goat anti-rabbit alkaline phosphatase-conjugated immunoglobulin G (GaRIgG-Ap; Zymed Laboratories), using the AMPAK amplification system. The $OD_{490}$ values from control wells (without virus but with increasing sCD4 concentrations) were subtracted from the $OD_{490}$ values of the corresponding wells containing virus and sCD4.

TABLE 1. Tropism and relative sCD4 neutralization sensitivity of HIV-$1_{SF2}$ and HIV-$1_{SF162}$ recombinant and mutant viruses

| HIV-1 | V3 region sequence[a] | Tropism[b] | sCD4 50% infectious dose (μg/ml)[c] |
|---|---|---|---|
| SF2 | | | |
| Wild type | ctrpnnntrksiYigpgrafHTtgRiigdirKahc | T | 0.3 (6.6) |
| R19[d] | ----------T-------YA--D------Q--- | M | 10 (220) |
| MU1 | ----------T-------YA--D------Q--- | M | 0.2 (4.4) |
| MU3 | ----------YA--D---------- | M/T | 0.6 (13.2) |
| MU8 | ----------D---------- | T | 0.3 (6.6) |
| Wild-type $SF_{162}$ | ctrpnnntrksiTigpgrafYAtgDiigdirQahc | M | >10 (>220) |

[a] The amino acids in the V3 loops that differ among the viruses are shown in capital letters.
[b] T, T-cell-line tropic; M, macrophage tropic; M/T, dual tropic. Dashes indicate amino acid homology.
[c] Values (mean of triplicates) represent the concentration of sCD4 resulting in 50% reduction of p24 production. Values in parentheses represent the corresponding nanomolar concentration of sCD4, assuming a molecular mass of 40 kDa.
[d] A 160-amino-acid *Stu*I-*Mst*II region of the HIV-$1_{SF2}$ gp120 was substituted with that of HIV-$1_{SF162}$.

The binding of sCD4 to monomeric gp120 was determined as follows. Amounts of gp120 in supernatants of transfected COS-7 cells were determined with the use of sera from HIV-positive patients. Appropriate dilutions of the supernatants were made so as to contain equal concentrations of gp120, and these were captured onto wells as described above. Increasing amounts of sCD4 were then added for 2 h at room temperature. The amount of sCD4 bound to gp120 was then determined as described above.

The sandwich was prepared as follows: well–D6205–gp120–sCD4–rabbit polyclonal anti-sCD4 serum–GaRIgG-Ap.

**Relative binding of anti-V3 loop human MAbs to monomeric gp120.** Anti-V3 loop human monoclonal antibodies (MAbs) 257D, 268D, and 391-95D, provided by Susan Zolla-Pazner (New York University, New York, N.Y.), were used. The epitopes recognized by these antibodies have been mapped to sequences KRIHI (257D), HIGPGR (268D), and RKRIHIG PGRAFYTT (391-95D) within the V3 loop of the MN isolate (15). For binding studies, equal amounts of wild-type, recombinant, and mutant gp120 proteins obtained from supernatants of COS-7-transfected cells were incubated for 2 h at room temperature with the MAbs. The mixture was then added to ELISA wells coated with D6205. The presence of gp120-bound anti-V3 loop MAbs was evaluated by using GaHIgG-Ap as described above. The $OD_{490}$ values obtained with supernatants of mock-transfected COS-7 cells were subtracted from the $OD_{490}$ values obtained from wells containing gp120.

In this case, the sandwich was prepared as follows: well–D6205–gp120–human anti-V3 MAb–GaHIgG-Ap.

## RESULTS

**Neutralization by sCD4.** We previously reported that the acquisition of a 0.49-kb *Stu*I-*Mst*II fragment of gp120 from macrophage-tropic HIV-$1_{SF162}$ by T-cell-line-tropic HIV-$1_{SF2}$ conferred to the recombinant virus (R19) the ability to replicate in primary macrophages and at the same time abolished its ability to productively infect T-cell lines (38) (Table 1). Three amino acid changes in the V3 loop of HIV-$1_{SF2}$ alone are sufficient to confer to the mutant virus (MU3) the same ability as R19 to grow in primary macrophages. However, this virus retains its ability to infect HUT-78 cells, albeit with a decrease in the rate of infection (39). Single amino acid change in the V3 loop (e.g., MU8) did not have any effect on cell tropism of the virus (39). To examine if, as previously reported (20), a correlation exists between sensitivity to sCD4 neutralization and tropism, the susceptibility of the wild-type, recombinant, and mutant viruses to neutralization by sCD4 was determined (Table 1). We observed that the two T-cell-line-tropic viruses (HIV-$1_{SF2}$ and MU8) and the dual-tropic virus MU3 were approximately 100 times more sensitive to neutralization by sCD4 than the wild-type macrophage-tropic virus, HIV-$1_{SF162}$ (Table 1). The recombinant virus R19 showed an sCD4 neutralization sensitivity similar to that of HIV-$1_{SF162}$. However, MU1, which contains the entire V3 loop of HIV-$1_{SF162}$ in the SF2 background and which is a macrophage-tropic virus, was as sensitive to sCD4 neutralization as wild-type T-cell-line-tropic HIV-$1_{SF2}$. These results show that although the V3 loop contains a major determinant for tropism, a determinant(s) for sCD4 neutralization sensitivity maps outside of the V3 loop but within the *Stu*I-*Mst*II fragment of the envelope gp120.

**Binding of sCD4 to soluble and virion-associated gp120.** To investigate whether the differences in sCD4 neutralization sensitivity were due to differences in virion-sCD4 binding affinities, we determined the amounts of sCD4 bound to virion-associated gp120 by ELISA. The results showed that the half-maximal sCD4 binding at 4°C for HIV-$1_{SF2}$ was 20 nM and that of HIV-$1_{SF162}$ was 42 nM (Fig. 1). The half-maximal binding of viruses MU1, MU3, and MU8 were similar to that of HIV-$1_{SF2}$, whereas recombinant virus R19 displayed a binding affinity toward sCD4 intermediate between those of the two wild type (Table 2). In agreement with results reported by others (31), the affinity toward sCD4 of all virions was higher at 37 than at 4°C (6 nM for SF2 and 12 nM for SF162). Furthermore, no difference was observed for the binding of soluble monomeric gp120 of HIV-$1_{SF2}$ and HIV-$1_{SF162}$ to sCD4 (1, 3, 24, 25, 44), half-maximal binding values for both being 2 nM (Fig. 1).

**Spontaneous and sCD4-induced release of gp120 from the surface of transfected cells.** Although there was more than a 100-fold difference in sCD4 sensitivity to neutralization between HIV-$1_{SF2}$ and HIV-$1_{SF162}$ (Table 1), there was only a 2-fold difference in their sCD4 binding affinities (Table 2). We therefore examined if other factors contribute to the neutralization profiles of these viruses. The release of gp120 from the viral surface upon binding to its soluble receptor (sCD4) has been shown to be one mechanism by which sCD4 neutralizes HIV infection (24–26, 31). The overall stability of the gp120-gp41 association, in the presence or absence of sCD4, was therefore examined by quantitating the relative amounts of gp120 present in the supernatants and in the cell lysates of COS-7 cells transiently expressing the different envelope glycoproteins. The percentage of gp120 that was released in the culture medium during the 32-h transfection period was evaluated, and the results are presented in Table 2. HIV-$1_{SF2}$ and MU8 showed a significantly higher amounts of spontaneous

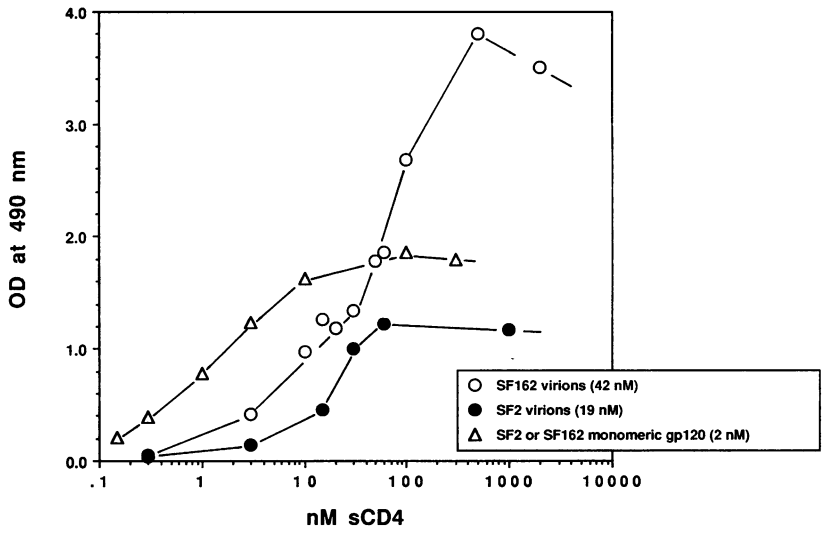


FIG. 1. Binding kinetics of sCD4 to HIV-$1_{SF2}$ and HIV-$1_{SF162}$ virion-associated or soluble gp120. Sucrose-purified viruses or soluble monomeric gp120 from transiently transfected COS-7 cells were incubated with increasing concentrations of sCD4 for 2 h at 4°C. The virion-sCD4 complexes were separated from unbound sCD4 by centrifugation. The pelleted viruses were then lysed with Nonidet P-40 and added to ELISA plates coated with D6205. The amounts of gp120-bound sCD4 were determined by ELISA as described in Materials and Methods. Numbers in parentheses indicate the average half-maximal binding values from three independent experiments.

gp120 release than HIV-$1_{SF162}$ and R19, while MU1 and MU3 showed intermediate amounts. Incubation of the transfected cells with sCD4 (0.5 μg/ml) resulted in an increased amounts of gp120 present in the medium in the cases of HIV-$1_{SF2}$, MU1, MU3, and MU8 but not in the cases of HIV-$1_{SF162}$ and R19. These observations suggest that differences in the stability of the gp120-gp41 association of envelope glycoproteins are responsible for the variability in sensitivity to sCD4 neutralization of the viruses tested here. Furthermore, the acquisition of the *Stu*I-*Mst*II fragment of HIV-$1_{SF162}$ by HIV-$1_{SF2}$ appears to stabilize the gp120-gp41 association of the recombinant virus.

TABLE 2. sCD4 binding and gp120-gp41 association of HIV-$1_{SF2}$ and HIV-$1_{SF162}$ recombinant and mutant viruses

| HIV-1 | Tropism[a] | Half-maximal binding (nM)[b] | gp120 shedding[c] | |
|---|---|---|---|---|
| | | | Spontaneous[d] | sCD4 induced[e] |
| SF2 | | | | |
| Wild type | T | 19 | +++ | 3 |
| R19[f] | M | 30 | + | 0 |
| MU1 | M | 20 | ++ | 2 |
| MU3 | M/T | 20 | ++ | 2 |
| MU8 | T | 20 | +++ | 3 |
| Wild-type $SF_{162}$ | M | 42 | + | 0 |

[a] T, T-cell line tropic; M, macrophage tropic; M/T, dual tropic.

[b] Aliquots (2 to 5 μl) of sucrose-purified viruses were incubated with increasing concentrations of sCD4 for 2 h at 4°C in a total volume of 0.1 ml. The amounts of sCD4 bound to the virions were determined by ELISA as described in Materials and Methods.

[c] COS-7 cells transiently expressing the various gp120 glycoproteins were used to quantitate the amount of gp120 present in the cell lysates and cell supernatants as described in Materials and Methods.

[d] Results are based on the percentage of gp120 present in the culture supernatants compared with the total amount of gp120 found in both the supernatants and the cell lysates. The equation used to determine this percentage was (gp120 in medium/gp120 in medium + in cell lysate) × 100. +++, 30 to 50%, ++, 15 to 30%; +, <15%.

[e] Expressed as fold increase in gp120 release in the cell supernatants induced by sCD4 following a 2-h incubation of the cells with sCD4 (0.5 μg/ml) compared with the release of gp120 in the absence of this agent.

[f] A 160-amino-acid *Stu*I-*Mst*II region of the HIV-$1_{SF2}$ gp120 was substituted with that of HIV-$1_{SF162}$.

**Tropism is not determined by the extent of proteolytic cleavage of virion-associated envelope gp120.** Our findings so far indicate that tropism is not regulated by receptor binding affinities or gp120 release. Certain cellular proteases, e.g., thrombin, are known to cleave the external envelope glycoprotein specifically at the tip of the V3 loop (GPGRA) to generate two fragments of 50 and 70 kDa upon binding of gp120 to sCD4 (9, 27, 36). It has been reported that T-cell-line-tropic viruses undergo this type of cleavage whereas macrophage-tropic viruses do not, leading to the proposal that gp120 cleavage is involved in determining tropism (13, 16). We therefore examined the susceptibility to cleavage by thrombin of virion-associated gp120 in the presence or absence of sCD4 (Fig. 2 and Table 3). Incubation of virions with thrombin alone did not result in gp120 cleavage. As reported previously (46), incubation of HIV-$1_{SF2}$ virions with sCD4 resulted in limited cleavage of virion-associated gp120 molecules. The combination of sCD4 with thrombin resulted in substantial gp120 cleavage in the case of HIV-$1_{SF2}$ but not in the cases of HIV-$1_{SF162}$ and R19. Of note is the observation that the envelope gp120 of MU8, a T-cell-line-tropic virus, was resistant to cleavage, while that of MU3, a dual-tropic isolate, was susceptible to cleavage. Thus, susceptibility to proteolytic cleavage of gp120 at the V3 loop does not appear to correlate with a particular cellular tropism.

**Relative binding of anti-V3 loop human MAbs to soluble monomeric gp120.** Although proteolytic cleavage of gp120 by thrombin per se does not correlate with cellular tropism, the results from the cleavage experiments suggest that the structures and/or exposures of the V3 loop of HIV-$1_{SF2}$ and HIV-$1_{SF162}$ are different. To further examine whether structural differences in the V3 loops might be involved in determining tropism, the relative binding of three conformational anti-V3 loop human MAbs to soluble gp120 of the wild-type, recombinant, and mutant viruses was determined. The

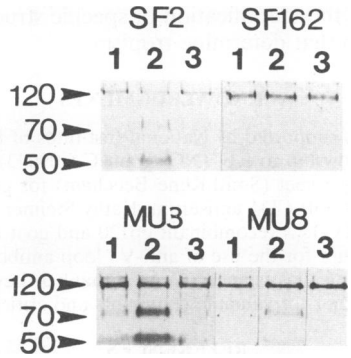


FIG. 2. Cleavage of virion-associated gp120 by sCD4 and thrombin. Sucrose-purified viruses (30 μl) were incubated in the absence or presence of 3 μg of sCD4 for 2 h at 37°C. In certain cases, thrombin (3 μg) was added for an extra hour. The mixtures were then lysed and loaded on an SDS–10% polyacrylamide gel. Visualization of the proteins was achieved with the use of a sheep anti-gp120 serum. Lanes: 1, sCD4 alone; 2, sCD4 and thrombin; 3, thrombin alone. Sizes are indicated in kilodaltons.

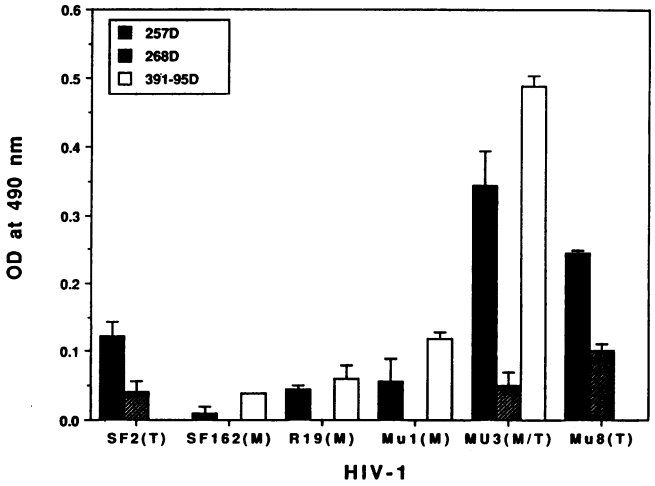


FIG. 3. Relative binding of conformational human anti-V3 loop MAbs to soluble gp120 molecules. Equal amounts of gp120 obtained from supernatants of COS-7 cells transiently expressing the various envelope glycoproteins were incubated with the three antibodies (1:100 dilution) and then added to ELISA plates coated with D6205. Detection of bound antibodies was carried out as described in Materials and Methods. The $OD_{490}$ values obtained with supernatants from mock-transfected COS-7 cells were substracted from values obtained with supernatants containing the different gp120 glycoproteins. T, T-cell line tropic; M, macrophage tropic; M/T, dual tropic.

epitopes recognized by these MAbs have been reported elsewhere (15). Furthermore, radioimmunoprecipitation, immunoblot, and ELISA analyses conducted with these MAbs indicate that they recognize conformational epitopes on gp120 (12, 15, 41).

The relative binding of these antibodies to the various gp120s examined in this study is presented in Fig. 3. We observed that of the three MAbs used, MAb 391-95D bound preferentially to macrophage-tropic viruses (HIV-$1_{SF162}$, R19, and MU1) and to the dual-tropic virus MU3 but not to T-cell-line-tropic isolates (HIV-$1_{SF2}$ and MU8). Differences in relative binding of 391-95D to the macrophage-tropic viruses were noted, with MU3 showing the highest binding affinity. In contrast, MAb 268D did not bind to the macrophage-tropic viruses (HIV-$1_{SF162}$, R19, and MU1), while it bound to HIV-$1_{SF2}$ and MU8 and to the dual-tropic virus MU3. MAb 257D bound to all gp120 molecules but with consistently higher $OD_{490}$ readings in the cases of HIV-$1_{SF2}$, MU8, and MU3 compared with HIV-$1_{SF162}$, R19, and MU1. Since equal amounts of gp120s were used, the different amounts of MAb bound probably reflect differences in binding affinities.

These results of differential MAb binding suggest an association between conformation of the V3 loops and cell tropism. Acquisition of the V3 loop sequences of HIV-$1_{SF162}$ by HIV-$1_{SF2}$ conferred on this mutant virus (MU1) a V3 loop conformation similar to that of macrophage-tropic HIV-$1_{SF162}$ and a macrophage-tropic phenotype. Amino acid substitutions in the V3 loop that confer dual tropism on HIV-$1_{SF2}$ (MU3) resulted in a V3 loop structure that appears to have partial features of both of the two parental isolates (HIV-$1_{SF2}$ and HIV-$1_{SF162}$); the gp120 from MU3 binds both MAb 268D and MAb 391-95D. A single amino acid change in the V3 loop (MU8) that did not affect tropism of HIV-$1_{SF2}$ also did not alter the conformation of the V3 loop, as measured by the binding of these MAbs.

TABLE 3. Cleavage of virion-associated gp120 in the presence of sCD4 and thrombin[a]

| HIV-1 | Tropism[b] | % gp120 cleavage[c] | |
|---|---|---|---|
| | | In the presence of sCD4 | In the presence of sCD4 and thrombin |
| SF2 | | | |
| Wild type | T | 45 | 80 |
| R19[d] | M | 0 | 19 |
| MU1 | M | 3 | 27 |
| MU3 | M/T | 12 | 58 |
| MU8 | T | 0 | 26 |
| Wild-type SF162 wt | M | 19 | 22 |

[a] Incubation of virions with sCD4 and thrombin was performed as described in Materials and Methods. The viral lysates were then loaded on an SDS–10% polyacrylamide gel. Following electrophoresis, the protein bands were visualized by Western blotting (Fig. 2). The integrated optical density (IOD) of each band was determined.

[b] T, T-cell-line tropic; M, macrophage tropic; M/T, dual tropic.

[c] Determined by using the following equation: $(IOD_{70\ kDa} + IOD_{50\ kDa}/IOD_{120\ kDa} + IOD_{70\ kDa} + IOD_{50\ kDa}) \times 100$.

[d] A 160-amino-acid *Stu*I-*Mst*II region of the HIV-$1_{SF2}$ gp120 was substituted with that of HIV-$1_{SF162}$.

## DISCUSSION

Using a set of recombinant and mutant viruses generated between two molecularly cloned HIV-1 isolates, HIV-$1_{SF2}$ (a T-cell-line-tropic virus) and HIV-$1_{SF162}$ (a macrophage-tropic virus), we show that on the genomic background of HIV-$1_{SF2}$, regions outside the V3 loop (but within the *Stu*I-*Mst*II region) control the sensitivity to sCD4 neutralization, while the V3 loop alone can confer to the virus a macrophage-tropic phenotype. Our observations differ from those reported by others (20) and suggest that these two biological properties of HIV-1 can be regulated by different mechanisms. Since a particular domain of the envelope gp120 probably affects the HIV-1 biological phenotype in conjunction with other envelope regions, this discrepancy in results most likely reflects the difference in viruses used in the studies.

Kinetic analyses of sCD4 binding to sucrose-purified intact virions indicate that viruses which are sensitive to sCD4

neutralization have an affinity for sCD4 that is only twofold higher than that of viruses which are resistant. However, we observe that the ratios of p24 to gp120 for HIV-$1_{SF2}$ and HIV-$1_{SF162}$ are 60 and 6.5, respectively (data not shown) and that the gp120-gp41 association of the envelope glycoproteins is stronger for the sCD4 neutralization-resistant viruses than for the sensitive viruses. Taken together, our data are in agreement with those of others (24, 31, 43) and show that the higher sensitivity of viruses to sCD4 neutralization is the result of a decreased stability of the gp120-gp41 association, resulting in a smaller number of gp120 molecules per virion.

A region outside the V3 loop, but within the 0.49-kb *Stu*I-*Mst*II fragment of gp120, influences the sCD4 binding affinity of the virus and to a greater extent the stability of its gp120-gp41 association. The *Stu*I-*Mst*II fragment of gp120 encompasses the second conserved domain, in which amino acids critical for binding of gp120 with CD4 have been localized (30). Furthermore, mutations in this region can disrupt the gp120-gp41 association (17, 40, 48). Thus, it is likely that amino acid differences in the second conserved domains of HIV-$1_{SF2}$ and HIV-$1_{SF162}$ are responsible for the differences in sCD4 neutralization sensitivity of these viruses.

Enzymatic cleavage studies of the envelope gp120 indicate that the susceptibility of the V3 loop to proteolytic cleavage upon binding of virion-associated gp120 to sCD4 does not correlate with tropism. This finding is contrary to results published by others, which suggested a correlation between protease susceptibility of recombinant envelope proteins and cell-specific tropism properties (13, 16). Nevertheless, in one of these studies (16), the gp120 of a mutant virus that displays both macrophage and T-cell line tropism is easily cleaved with thrombin. Thus, there is a lack of a strict correlation between tropism and enzymatic cleavage of the V3 loop.

Our immunochemical analyses of the envelope gp120, however, indicate that the structure of the V3 loop appears to play a major role in determining cell tropism. Similar to observations made by others (13), the conformation of the V3 loop, as probed by its reactivity with anti-V3 conformational antibodies, differs between T-cell-line-tropic and macrophage-tropic isolates. Acquisition of the V3 loop of a macrophage-tropic isolate by a T-cell-line-tropic isolate confers macrophage tropism to the virus and at the same time confers a V3 loop structure similar to that of the macrophage-tropic strain. Furthermore, we observed that dual-tropic viruses have a V3 loop structure of intermediate configuration. These differences in V3 loop conformation may affect postbinding events that are required for viral entry, among which could be the interaction of the V3 loop with cell-specific secondary receptors. Further studies are needed to examine the extent to which our observations can be applied to other HIV-1 strains that display macrophage or T-cell line tropism.

The V3 loop structures that we examined are those present on native monomeric gp120, yet they appear to correlate with the function of the oligomeric, virion-associated gp120 in determining cell tropism. The V3 loop becomes more exposed upon binding of virion or cell-associated gp120 to soluble CD4 (34–36) (Table 3). Although we observed, upon interaction of the native monomeric gp120s with soluble CD4, a slight increase in the binding of some of the anti-V3 MAbs used (data not shown), no significant changes in the overall reactivity patterns were observed, indicating that the V3 loop of the soluble monomeric gp120 molecules undergoes limited postbinding conformational changes. Further probing of the V3 loop structures of these and other recombinant and mutant soluble gp120s and of virion gp120 with additional antibodies should lead to the identification of specific structural features of the V3 loop that determine tropism.

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