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Effects of Changes in gp120-CD4 Binding Affinity on Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Function and Soluble CD4 Sensitivity

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Mutant gp120 glycoproteins exhibiting a range of affinities for CD4 were tested for ability to form syncytia and to complement an *env*-defective provirus for replication. Surprisingly, gp120 mutants that efficiently induced syncytia and/or complemented virus replication were identified that exhibited marked (up to 50-fold) reductions in CD4-binding ability. Temperature-dependent changes in gp120, which result in a seven- to ninefold increase in affinity for CD4, were shown not to be necessary for subsequent membrane fusion or virus entry events. Mutant glycoproteins demonstrating even relatively small decreases in CD4-binding ability exhibited reduced sensitivity to soluble CD4. The considerable range of CD4-binding affinities tolerated by replication-competent HIV-1 variants has important implications for antiviral strategies directed at the gp120-CD4 interaction.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS, which is characterized by a depletion of CD4-positive lymphocytes (3, 16, 17, 27, 35). HIV-1 exhibits a tropism for CD4-positive cells due to a high affinity ($K_d = 4 \times 10^{-9}$ M) interaction between the CD4 glycoprotein, which serves as the viral receptor, and the HIV-1 gp120 exterior envelope glycoprotein (13, 23, 24, 28, 30, 31). Following CD4 binding, the gp120 glycoprotein and gp41, the transmembrane envelope glycoprotein, mediate the fusion of viral and target cell membranes, which is pH independent and necessary for virus entry (18, 25a, 40). Similar events mediated by the envelope glycoproteins expressed on the surface of an infected cell result in fusion of infected cells with CD4-positive cells to form syncytia (18, 25a, 29, 39).

The gp120 molecule binds to a protruding ridge of the CD4 glycoprotein analogous to the CDR2 loop of immunoglobulins (1, 2, 6–8, 21, 26, 32, 34, 36, 42). The CD4-binding site on gp120 is discontinuous, and changes in specific residues located in the second, third, and fourth conserved gp120 regions affect CD4 binding without grossly disrupting the gp120 conformation (10, 11, 18, 25a, 33). Here, we examine the effects of changes in gp120-CD4 affinity on envelope glycoprotein functions involved in syncytium formation and virus entry. We also examine the effect of changes in CD4-binding affinity on the sensitivity of gp120 variants to soluble CD4, which has been shown to exhibit antiviral activity in vitro (4, 14, 15, 19, 38, 41).

MATERIALS AND METHODS

Envelope glycoprotein expression and CD4-binding assays. For the binding assays, COS-1 cells were transfected with pSVIIIenv DNA expressing wild-type or mutant envelope glycoproteins as previously described (12, 18, 33). For initial experiments examining the effect of temperature on binding of the wild-type gp120, supernatants of transfected cells that were radiolabeled with [^{35}S]cysteine were incubated for various lengths of time with 5×10^7 SupT1 lymphocytes at either 4 or 37°C. The cells were washed once with phosphate-buffered saline (PBS), lysed, and then precipitated with an excess of a mixture of sera derived from AIDS patients (33). The unbound fraction of the supernatants was also used for immunoprecipitation. The amount of gp120 precipitated from both bound and unbound fractions was measured by densitometry of sodium dodecyl sulfate-polyacrylamide gels. Previous studies indicated that the ratio of bound to free gp120 did not vary under these conditions over a greater than 20-fold range of wild-type gp120 concentrations. CD4-binding abilities of the mutant glycoproteins were assessed as described above, by using a 90-min incubation at either 4 or 37°C with SupT1 lymphocytes.

Syncytium-forming ability of envelope glycoproteins. To assess the syncytium-forming ability of mutant envelope glycoproteins, 5×10^5 COS-1 cells in 100-mm^2 dishes were transfected with 10 μg of the envelope expressor plasmid (pSVIIIenv). Forty-eight hours after transfection, the cells were rinsed twice in PBS and incubated with 50 mM EDTA, pH 7.5, at 37°C for approximately 40 min. Cells were removed from the plate by trituration, centrifuged, washed in 5 ml of PBS, and suspended in 5 ml of Dulbecco modified Eagle medium-10% fetal calf serum (DMEM-10% FCS). To these cells was added 2×10^7 SupT1 lymphocytes suspended in 5 ml of DMEM-10% FCS. The cells were transferred to a T25 flask and returned to 37°C in a 5% CO_2 incubator, and syncytia were scored in 6 to 8 h.

Replication complementation assays. Complementation of the single-step replication of the HXBΔenvCAT provirus into different lymphocytes was performed as previously described (18). Equivalent amounts of reverse transcriptase activity representing recombinant virions in COS-1 supernatants were added to Jurkat, H9, or SupT1 lymphocytes, and chloramphenicol acetyltransferase (CAT) activity was measured at 72 h following infection.

Soluble CD4 inhibition of syncytium formation and virus replication. To examine soluble CD4 inhibition of syncytium formation, transfected COS-1 cells detached from tissue

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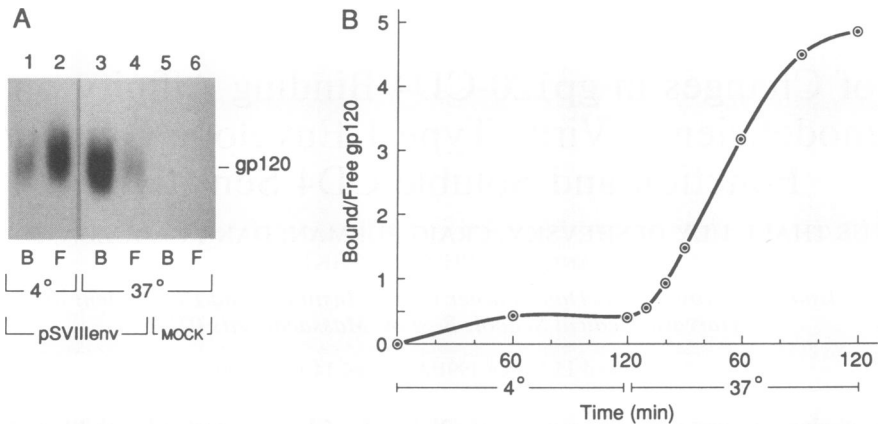

FIG. 1. Effects of temperature on relative CD4 binding. (A) Autoradiogram of wild-type gp120 glycoprotein (lanes 1 through 4) or supernatants from mock-transfected COS-1 cells (lanes 5 and 6) either bound (lanes B: 1, 3, and 5) or unbound (lanes F: 2, 4, and 6) to SupT1 lymphocytes. Experiments were conducted at either 4°C (lanes 1 and 2) or 37°C (lanes 3 through 6). (B) Graph showing the ratio of the amount of wild-type gp120 glycoprotein bound to CD4-positive SupT1 lymphocytes to the amount of unbound gp120 for various times of incubation at either 4 or 37°C. The datum points represent the mean values of data from two independent experiments.

culture dishes as described above were incubated with different concentrations of soluble CD4 (American BioTechnologies, Inc.) in 2.5 ml of DMEM-10% FCS for 1 h at 37°C. To this mixture was added 2×10^7 SupT1 lymphocytes suspended in 1 ml of DMEM-10% FCS. Cells were transferred to a T25 flask, and syncytia were scored in 8 h.

For inhibition of virus replication, recombinant HXBΔenv CAT virus produced in COS-1 cells (18) was incubated with different concentrations of soluble CD4 for 1 h at 37°C. The virus preparations were then incubated with Jurkat lymphocytes, and CAT activity was measured 48 h later as previously described (18).

RESULTS

Temperature effects on CD4 binding. The effect of temperature on the ability of radiolabeled soluble gp120 derived from the HXBc2 HIV-1 virus to bind to CD4-positive SupT1 lymphocytes is shown in Fig. 1. Binding equilibrium was achieved by approximately 2 h at either 4 or 37°C. A seven- to ninefold increase in the ratio of bound to free gp120 was observed at 37°C relative to that observed at 4°C. This observed increase at 37°C was only marginally affected by fixing the SupT1 lymphocytes in methanol prior to use (data not shown).

Effect of changes in gp120 on CD4 binding at different temperatures. To examine whether changes in the HIV-1 gp120 glycoprotein previously shown to alter its affinity for CD4 (33) affected the temperature-dependent component of the binding process, the binding of gp120 mutants to SupT1 lymphocytes was examined at both 4 and 37°C, using a 90-minute binding assay. The results presented in Table 1 and Fig. 2 demonstrate that although several of the mutants exhibited CD4-binding abilities comparable with or even better than that of the wild-type envelope glycoprotein at 4°C, significant increases in binding at 37°C were not observed for these mutants (e.g., 257 T/R, 368 D/T, 368 D/P, 368 D/E, 370 E/Q, 430 V/S, 457 D/R, and 457 D/A). The relative CD4-binding abilities of the mutants at 37°C, from a 90-min assay, were similar to those previously reported by using a 60-min binding assay (33).

Function of the envelope glycoprotein mutants. The ability of the envelope glycoprotein mutants to induce the formation of syncytia was examined (Table 1). For all of the mutants exhibiting less than one percent of wild-type CD4-binding ability, syncytium-forming ability was undetectable (e.g., 368 D/K, 368 D/R, 370 E/R, 427 W/S, and 427 W/V). Several of the mutants that exhibited smaller decreases in CD4 binding were capable of forming syncytia, although this occurred at a lower efficiency than that observed for the wild-type glycoproteins (e.g., 257 T/R, 368 D/T, 368 D/E, 368 D/N, 370 E/D, 430 V/S, and 457 D/A).

The ability of the mutant envelope glycoproteins to complement the entry of an *env*-defective virus into Jurkat, SupT1, and H9 lymphocytes was assessed. For the mutants exhibiting less than 1% of the wild-type CD4-binding ability, the ability to complement virus entry was less than 10% that of the wild-type envelope glycoprotein in all the target cell types examined (e.g., 368 D/K, 368 D/R, 370 E/R, and 427 W/V). The other mutants, which exhibited CD4-binding ability at 37°C of greater than 1% of the wild-type value, were able to complement virus entry into the target cells examined to various degrees. The 368 D/T, 368 D/E, and 370 E/Q mutants all allowed efficient entry into Jurkat and SupT1 lymphocytes despite approximately 3-, 20-, and 90-fold reductions, respectively, in relative CD4-binding ability. Several of the mutants that did not exhibit temperature-related affinity increases were capable of complementing virus entry into some of the target cell types (257 T/R, 368 D/T, 368 D/P, 368 D/E, 370 E/Q, 430 V/S, and 457 D/A). For these and for other mutants retaining some degree of replication competence, the efficiency of entry into the various target cells utilized in these studies exhibited the pattern Jurkat $\geq$ SupT1 $\geq$ H9 lymphocytes.

Soluble CD4 sensitivity of mutant envelope glycoproteins. The ability of syncytium induction and of virus replication to be inhibited by soluble CD4 was assessed for those mutant glycoproteins competent for these functions (Table 2 and Fig. 3 and 4). Some mutants (120/121 VK/LE, 269 E/L, and 485 K/V) that exhibited mild (less than twofold) reductions in CD4-binding ability were inhibited by soluble CD4 to the same extent as was the wild-type glycoprotein. The functional mutants that exhibited greater than twofold decreases in relative CD4-binding ability were less sensitive than the wild-type glycoprotein to soluble CD4 inhibition of syncy-

TABLE 1. Phenotypes of gp120 mutants[a]

Mutant	Relative CD4 binding at (fold increase)[b]		Syncytium formation	Replication complementation[c] in cell line		
	4°C	37°C		Jurkat	SupT1	H9
Wild type	0.14	1.00 (7.1)	100	100	100	100
257 T/R	0.12	0.06 (0.50)	5	40	25	3
368 D/T	0.31	0.30 (0.97)	16	32	23	7
368 D/P	0.18	0.10 (0.56)	<1	18	19	2
368 D/E	0.05	0.05 (1.00)	51	70	62	22
368 D/N	ND	0.22 (ND)	38	14	11	5
368 D/K	ND	<0.005 (ND)	<1	7	9	2
368 D/R	ND	<0.0004 (ND)	<1	2	2	0
370 E/D	0.14	0.42 (3.0)	73	87	81	50
370 E/Q	0.06	0.011 (0.18)	<1	33	33	3
370 E/R	ND	<0.003 (ND)	<1	1	3	1
427 W/S	ND	<0.012 (ND)	<1	ND	ND	ND
427 W/V	<0.006	<0.006 (ND)	<1	1	ND	1
430 V/S	0.41	0.65 (1.6)	80	100	ND	ND
457 D/R	0.06	0.10 (1.67)	<1	12	9	3
457 D/A	0.08	0.10 (1.25)	47	36	22	6

[a] Data reported represent the means of at least two independent experiments.

[b] The relative CD4-binding ability, determined by using a 90-min binding assay as described in Materials and Methods, is shown. The relative CD4-binding ability was calculated according to the following formula:

$$\text{relative CD4 binding}_{\text{tempX}} = \left[\frac{(\text{bound mutant gp120})_{\text{tempX}}}{(\text{free mutant gp120})_{\text{tempX}}}\right] \times \left[\frac{(\text{free wild-type gp120})_{37°C}}{(\text{bound wild-type gp120})_{37°C}}\right]$$

The fold increase shown in parentheses represents the ratio of the relative CD4-binding ability of a given mutant at 37°C to that observed at 4°C.

[c] Syncytium formation and replication complementation are represented as the percentage of wild-type values observed for a given target cell. The percent activity observed for a control plasmid not expressing the HIV-1 envelope glycoproteins was less than 1% for syncytium formation and less than 2% for replication complementation in all three target cell lines.

tium formation or virus entry (e.g., 257 T/R, 368 D/T, 368 D/E, 368 D/N, 370 E/D, 370 E/Q, 457 D/A, and 457 D/N). The 430 V/S and 457 D/E mutants exhibited soluble CD4 sensitivities intermediate between that of the wild-type glycoproteins and those of mutants with larger decreases in CD4-binding ability. Mutations affecting the threonine 257 or aspartic acid 457 residues (257 T/A, 457 D/N) that did not decrease CD4-binding ability did not result in soluble CD4 resistance, whereas other changes in these residues (257 T/R, 457 D/A, 457 D/G, and 457 D/E) yielded mutants that exhibited decreased CD4 binding and resistance to soluble CD4.

DISCUSSION

The interaction of gp120 with CD4 was reduced to undetectable levels by some changes in gp120 residues 368, 370, or 427. These changes dramatically reduced the ability of the envelope glycoproteins to form syncytia or to complement an *env*-defective virus for replication, consistent with the major contribution of CD4 binding to these processes.

While the gp120 changes that reduced CD4-binding ability by 100-fold or more uniformly abrogated syncytium-forming and/or replicative functions, amino acid changes resulting in less dramatic effects on CD4 binding exerted more variable effects on these envelope glycoprotein functions. The ability of the 368 D/E, 370 E/Q, 370 E/D, and 457 D/A mutants to complement the replication of the *env*-defective virus and the ability of the 368 D/T, 368 D/E, 368 D/N, 370 E/D, 430 V/S, and 457 D/A mutants to form syncytia indicate that even 20- to 50-fold decreases in CD4-binding ability are not necessarily accompanied by abrogation of these functions. These results are consistent with reports that replication-competent HIV-1 isolates can exhibit a broad range of

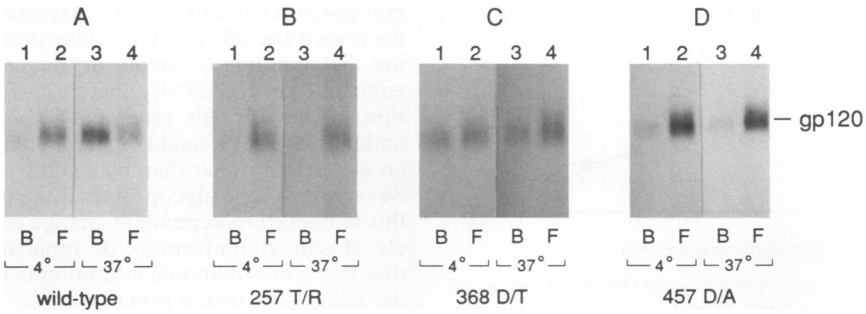

FIG. 2. Effects of amino acid changes on gp120 binding to SupT1 lymphocytes at either 4 or 37°C. The gp120 glycoprotein either bound (lanes B: 1 and 3) or unbound (lanes F: 2 and 4) following a 90-min incubation with SupT1 lymphocytes at either 4°C (lanes 1 and 2) or 37°C (lanes 3 and 4) is shown. The experiments utilized either wild-type gp120 (A) or the 257 T/R, 368 D/T, or 457 D/A mutants (B, C, and D, respectively).

TABLE 2. Soluble CD4 inhibition of envelope glycoprotein function

Mutant	Relative CD4-binding ability[a]	ID_{50} syncytium formation (μg/ml)[b]	ID_{50} virus replication (μg/ml)[b]
Wild type	1.00	1.5	0.4
113 D/A	1.1	1.5	ND
120/121 VK/LE	0.51	0.9	0.4
257 T/R	0.16	>20	>20
257 T/A	1.1	1.7	0.6
269 E/L	0.61	1.1	0.4
368 D/T	0.33	>20	1.3
368 D/E	0.09	>20	7.5
368 D/N	0.19	>20	ND
370 E/D	0.45	>20	2.5
370 E/Q	0.018	ND	>20
430 V/S	0.39	5.1	1.3
457 D/A	0.09	>20	>20
457 D/N	1.1	1.5	0.4
457 D/G	0.23	ND	>5
457 D/E	0.38	6.5	1.2
485 K/V	0.79	1.0	ND

[a] Relative CD4-binding abilities are derived from experiments in which a 60-min binding assay was used (33).

[b] Values shown are the averages of the results of three independent experiments. Values represent the concentration of soluble CD4 required to inhibit syncytium formation or virus entry by 50%. ND, not determined.

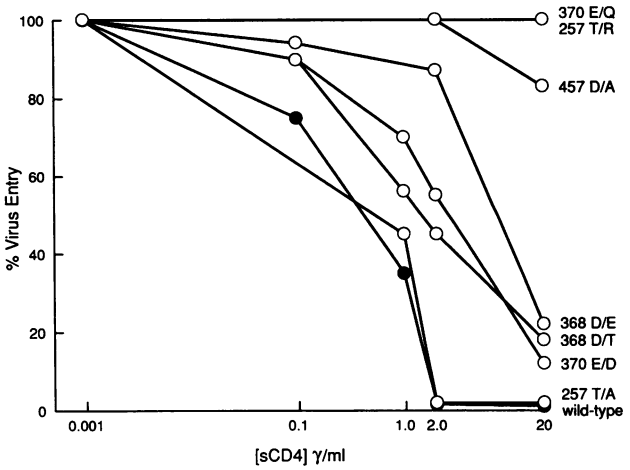

FIG. 4. Effects of gp120 amino acid changes on sensitivity to soluble CD4 inhibition of replication complementation. The effect of different soluble CD4 concentrations on the CAT activity transferred to Jurkat lymphocytes by recombinant virions carrying the wild-type or mutant envelope glycoproteins is shown. Each value represents the percentage of CAT activity observed for a given mutant in the presence of soluble CD4 relative to the activity observed for the mutant in the absence of soluble CD4.

CD4-binding abilities (20). The decreases in syncytium-forming or replicative abilities for some of the mutants (e.g., 257 T/R, 368 D/P, and 457 D/R) that exhibit less than 50-fold reductions in CD4-binding ability are likely to result from the effect of the changes on envelope glycoprotein functions other than receptor binding.

For some of the mutants (257 T/R, 368 D/P, 370 E/Q, and 457 D/R), syncytium formation is affected more dramatically than is the ability to complement virus replication in the same target cells. A similar result has been seen for amino acid changes in the HIV-1 gp41 amino terminus that affect the membrane fusion process (25). These results probably reflect the requirement for a greater number of successful

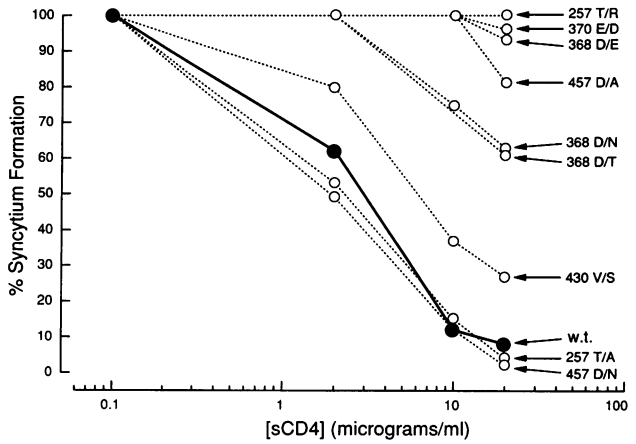

FIG. 3. Effects of amino acid changes in gp120 on inhibition of syncytium formation by soluble CD4. The percentage of the number of syncytia scored in the absence of soluble CD4 is shown for increasing concentrations of soluble CD4. Values are shown for a typical experiment with the wild-type (w.t.) or mutant envelope glycoproteins.

envelope glycoprotein-receptor interactions in the process of cell-cell fusion than in the process of virus entry.

Decreases in CD4-binding ability exerted greater effects on virus entry into some target cells than they did in other cell types. For all of the mutants examined, however, the relative efficiency of entry into various target cells followed the pattern Jurkat ≥ SupT1 ≥ H9 lymphocytes. Thus, the efficiency of entry of the mutant virus into these cell types appears to be governed by quantitative factors, one of which may be the level of target cell CD4 expression.

At 4°C, the HIV-1 gp120 glycoprotein bound to CD4-positive cells with an affinity that was approximately seven- to ninefold lower than is seen at 37°C. Following a shift to 37°C, a gradual increase in the association of the wild-type gp120 glycoprotein with CD4-positive cells was observed. This phenomenon cannot be simply explained by endocytosis of cell surface gp120-CD4 complexes, since methanol fixation of the target cells did not eliminate the observed increase. If the effect of low temperature is to limit conformational changes in gp120 or CD4, the results would suggest that induced changes in gp120 and/or CD4 contribute to a higher affinity interaction at 37°C. Mutations affecting several gp120 residues (Thr-257, Asp-368, Glu-370, or Asp-457) decreased the efficiency of these induced changes, suggesting that multiple elements in the protein-protein interface contribute to the affinity increase. The gp120-CD4 interaction, as is true for binding of some antibodies to their antigens (5, 9, 37), might be more appropriately described by an induced fit rather than by a static "lock-and-key" model. Several envelope glycoprotein mutants that did not undergo this temperature-dependent change exhibited significant levels of syncytium-forming or replicative ability, indicating that this conformational alteration is not a necessary step in the membrane fusion process.

Relatively small decreases in CD4-binding ability were associated with envelope glycoprotein mutants resistant to inhibition by soluble CD4. A good correlation was observed between mutants that exhibited a lower affinity for CD4 and

resistance to soluble CD4. This correlation was also observed for mutations affecting the same gp120 amino acid but resulting in different effects on CD4 binding. For example, the 257 T/R, 457 D/A, 457 D/G, and 457 D/E mutants exhibited decreased CD4 binding and were resistant to soluble CD4, whereas the 257 T/A and 457 D/N mutants exhibited approximately wild-type CD4-binding abilities and remained sensitive to soluble CD4. While these results do not rule out other mechanisms for the generation of soluble CD4-resistant HIV-1 variants, they strongly imply that changes in affinity for CD4 represent one such mechanism. Since fresh patient isolates of HIV-1 are often less sensitive to soluble CD4 than are HIV-1 isolates passaged extensively in T-lymphocyte cell lines (12a), some of these primary isolates might exhibit small but consequential decreases in binding affinity for CD4. The sixfold-lower CD4-binding affinity reported for the primary monocyte-propagated Ba-L isolate compared with the T-cell-passaged BH10 isolate might contribute to soluble CD4 resistance (20). HIV-2, which is less sensitive to soluble CD4 than is HIV-1, has been reported to have a 25-fold-lower affinity for CD4 (32a). Such changes in affinity apparently result in major effects on soluble CD4 sensitivity, while exerting only minor effects on virus entry or syncytium formation. This difference may result from the higher cooperativity of envelope glycoprotein-receptor interactions during the latter processes compared with the interaction of soluble CD4 and the envelope glycoproteins. Similar results have recently been reported for the inhibition of polio virus replication by soluble receptor molecules, in which replication-competent viruses resistant to soluble receptor resulted from altered receptor affinity (22).

For some of the mutants exhibiting small changes in relative CD4-binding ability (e.g., 368 D/T and 370 E/D), the 50% inhibitory dose (ID_{50}) for soluble CD4 inhibition of syncytium formation is affected more than the corresponding ID_{50} for virus replication. Soluble CD4 has been reported to act both as a competitive inhibitor and as an irreversible inhibitor, the latter activity resulting from dissociation of the gp120 and gp41 subunits (22a, 32b). Since the soluble CD4-induced subunit dissociation is more efficient for envelope glycoproteins on the virions than on the cell surface (32b), the observations for the mutants could be accounted for by differences in the contributions of reversible and irreversible inhibitory activities of soluble CD4 in the syncytium formation or virus entry process.

Our results have implications for antiviral therapies directed at the gp120-CD4 interaction. The net result of either a reduction in gp120-CD4 affinity or the presence of a competitive inhibitor of the gp120-CD4 interaction is to decrease the ratio of $gp120_{bound}/gp120_{free}$. One would expect that the effects of an identical reduction in this ratio on virus replication would be less for a competitive inhibitor than for a mutant exhibiting decreased affinity, since in the former case, the bound gp120 retains wild-type function. Thus, our results imply that competitive inhibitors of the gp120-CD4 interaction will need to be extremely effective to significantly abrogate virus replication, especially in target cells expressing high levels of CD4.

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