Determinants of Human Immunodeficiency Virus Type 1 Entry in the CDR2 Loop of the CD4 Glycoprotein

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Various roles for the viral receptor, CD4, have been proposed in facilitating human immunodeficiency virus type 1 (HIV-1) entry, including virion binding to the target cell and the induction of conformational changes in the viral envelope glycoproteins required for the membrane fusion reaction. Here, we compare the structural requirements in the CDR2-like loop of CD4 domain 1, the major contact site of the gp120 envelope glycoprotein, for gp120 binding and virus entry. For every CD4 mutant examined, the level of cell surface expression and the gp120 binding affinity were sufficient to explain the relative ability to function as a viral receptor. The decrease in relative infectibility associated with decreased gp120 binding affinity was more pronounced at lower cell surface CD4 concentrations. These results imply that both receptor density and affinity determine the efficiency of HIV-1 entry and that specific structures in the CD4 residues examined are probably not required for HIV-1 entry functions other than gp120 binding.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS (4, 18). AIDS is characterized by a depletion of CD4-positive lymphocytes (16). HIV-1 infection of helper T cells and monocytes is initiated by the binding of the external subunit of the HIV envelope glycoprotein (gp120) to CD4 molecules expressed on the target cell (15, 26, 30, 31). Binding of gp120 and CD4 is followed by the fusion of the viral envelope with the host cell membrane, which leads to the entry of the virus into the host cell (46). Both the HIV-1 gp120 exterior envelope glycoprotein and the gp41 transmembrane glycoprotein are important for the membrane fusion reaction (21, 27). HIV-1 envelope glycoproteins expressed on the surface of infected cells mediate cell-cell fusion with CD4-positive target cells, resulting in the formation of syncytia (29, 44).

It has been proposed that the CD4 glycoprotein is also involved in functions in addition to gp120 binding that are important for HIV-1 entry (1, 27, 36, 41, 42). By analogy with influenza viruses, conformational changes in the HIV-1 envelope glycoproteins that allow fusogenic gp41 peptides to interact with the target cell membrane are believed to be a necessary step in the membrane fusion process (45, 53, 54). Unlike influenza virus, which initiates such conformational changes in its envelope glycoproteins following endocytosis and a decrease in pH (45, 53, 54), HIV-1 can negotiate virus-cell membrane fusion at a neutral pH (46). Binding to the viral receptor represents an attractive candidate for triggering fusion-related conformational changes in viruses, such as HIV-1, that fuse directly with the plasma membrane of the target cell (46). Indeed, CD4 binding has been shown to elicit a number of conformational changes in the HIV-1 gp120 glycoprotein (6, 19, 25, 36, 41, 42, 49). While some of these CD4-induced changes, such as those resulting in the dissociation of the gp120 and gp41 glycoproteins (19, 36), are unlikely to be necessary for virus-cell membrane fusion (7, 17, 48), the

relevance of other changes, such as the alteration of some gp120 epitopes (25, 41, 42, 49), remains to be addressed.

The CD4 molecule is a 55-kDa glycoprotein with four external immunoglobulin-like domains (domains 1, 2, 3, and 4), a transmembrane domain, and an intracytoplasmic tail. The binding site for gp120, which has been studied by mutational and structural analysis (2, 3, 9, 13, 32, 33, 38, 40, 52), is located within the N-terminal domain (domain 1). This domain shares structure features with the immunoglobulin light-chain variable region, including a framework structure with two β sheets as well as three loops (CDR1, CDR2, and CDR3) analogous to the complementarity-determining regions (CDRs) of the immunoglobulin molecules. The binding of gp120 occurs on the CDR2 loop and the supporting β strand (residues 40 to 60). Of critical importance for gp120 binding are the CD4 residues phenylalanine 43, lysine 46, and arginine 59.

Initial studies using mutagenesis, antibodies, or synthetic peptides suggested that the CDR3 region of CD4 domain 1 might also play a role in membrane fusion mediated by the HIV-1 envelope glycoproteins, independent of any contribution to gp120 binding (5, 10, 20, 24, 37, 51). Subsequent, more thorough studies, however, have failed to validate such a model. An extensive mutagenic analysis indicates that a wide variety of CDR3 sequences are compatible with a fusion-competent CD4 receptor (8). Monoclonal anti-CDR3 antibodies that block HIV-1 infection or syncytium formation have been shown to interfere with the binding of a soluble form of CD4 to the multimeric HIV-1 envelope glycoprotein complex (34). Synthetic peptides corresponding to the CD4 CDR3 sequences inhibit syncytium formation by human T-cell leukemia virus, even though membrane fusion mediated by this virus does not involve CD4 (39). Together, these results make it unlikely that the CD4 CDR3 region plays a necessary role in HIV-1 entry or cell-cell fusion.

Thus, the currently available evidence supports an interaction between the HIV-1 envelope glycoproteins and CD4 regions limited to the CDR2 loop of domain 1. Indeed, human CD4 protein sequences from residues 35 to 62, which encompass the CDR2 loop, were sufficient to allow HIV-1 entry when placed onto a rat CD4 molecule, which does not support HIV-1 binding or entry (43). Thus, it is possible that all of the

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sequence-specific functions of CD4 during HIV-1 entry are mediated by residues within the CDR2 loop. Here, we examine whether the effect of changes in the CD4 CDR2 loop on HIV-1 entry can be explained solely by changes in binding affinity or whether there exist CDR2 structural requirements for HIV-1 entry functions in addition to virus binding. We also examine the contribution of the level of CD4 expression to HIV-1 entry for CD4 glycoproteins exhibiting different affinities for the gp120 glycoprotein.

MATERIALS AND METHODS

Plasmids. The plasmid pBluescript II KS+ (Stratagene) containing the human soluble CD4 gene cloned at the *Bam*HI site was used for site-directed mutagenesis (28). For expression in Raji B lymphocytes, the *Xba*I-*Pvu*II fragment (corresponding to the 5' portion of the CD4 gene) was inserted into the pSL3.T4.hydro plasmid, in which the SL3-3 murine leukemia virus long terminal repeat (11) serves as a promoter for the full-length CD4 cDNA. The pSL3.T4.hydro plasmid also contains the Epstein-Barr virus origin of replication. For expression in HeLa cells, the *Bam*HI fragment (corresponding to the complete CD4 cDNA) from the pSL3.T4.hydro plasmid was inserted into the pcDNA1 neo vector (Invitrogen) to create the pCD4 plasmid.

Transfection. Subconfluent HeLa cells were transfected by the calcium phosphate method with 30 μ g of the pCD4 plasmid per 100-mm-diameter tissue culture dish unless otherwise indicated. The level of CD4 expressed on the cell surface was measured by flow cytometry 72 h after transfection by using the fluorescein isothiocyanate-conjugated OKT4 antibody reactive with CD4 domain 3 (31). As discussed in greater detail below, the percentage of transfected cells exhibiting fluorescence above background levels and the CD4 density in the CD4-expressing subset of cells were proportionate to each other in all of the experiments. Therefore, we arbitrarily chose the former value to represent the level of CD4 expression. HeLa cells were used for gp120 binding or virus infectivity studies at 72 h following transfection. Raji cells were transfected by the DEAE-dextran method (14), with 2 μ g of the pSL3.T4.hydro plasmid used for 5×10^6 cells. At 72 h after transfection, the Raji cells were used for virus infectivity assays.

gp120 binding studies. To provide a source of labeled gp120 glycoprotein, subconfluent COS-1 cells in 100-mm-diameter tissue culture dishes were transfected by the DEAE-dextran method with 10 μ g of the pSVIIInenv Δ 517 plasmid. This plasmid encodes a soluble form of the gp120 glycoprotein from the HXBc2 isolate of HIV-1 (27). The transfected COS-1 cells were labeled for 16 h with 100 μ Ci of [35 S]methionine per ml and 100 μ Ci of [35 S]cysteine per ml in cysteine- and methionine-free medium. The radiolabeled supernatants were incubated either undiluted or after 5- or 10-fold dilutions in RPMI 1640 plus 10% fetal calf serum with 1.5×10^6 CD4-expressing HeLa cells in a total volume of 0.5 ml. Incubation of the gp120-containing supernatants with the cells was performed for 2 h at 37°C with gentle shaking. The HeLa cells were then washed once with phosphate-buffered saline, lysed with Nonidet P-40 buffer, and used for precipitation with an excess of a mixture of sera from AIDS patients. The unbound-gp120-containing supernatants were precipitated in parallel. The amounts of precipitated gp120 glycoprotein in both fractions were estimated by densitometry of autoradiographs of sodium dodecyl sulfate-polyacrylamide gels. The reciprocals of the amounts of bound and free gp120 glycoprotein were plotted, and the dissociation constant (K_D) was determined by using the modified Scatchard equation: $1/\text{gp120}_{\text{bound}} = K_D/\text{maximum gp120}_{\text{bound}} \times 1/\text{gp120}_{\text{free}} + 1/\text{maximum gp120}_{\text{bound}}$. The slope was determined by using a linear regression program. As the concentration of labeled gp120 in these experiments was not high enough to achieve saturation of the CD4-expressing HeLa cells, flow cytometry analysis was used to estimate the level of cell surface CD4 and to approximate more accurately the maximum gp120 bound.

Determination of infectibility of CD4-expressing cells by HIV-1. At 72 h after transfection, CD4-expressing HeLa or Raji cells were infected with a stock of the HXBc2-CAT virus corresponding to 20,000 cpm of reverse transcriptase activity. Approximately 10^6 HeLa cells and 5×10^6 Raji cells were incubated for 12 h at 37°C with the preparation of recombinant virus in total volumes of 8 and 3 ml, respectively. Then, medium was added to obtain final volumes of 12 ml for the HeLa cells and 10 ml for the Raji cells. The cells were cultured for another 60 h and then lysed. Equivalent amounts of protein in the cell lysates were used for determination of chloramphenicol acetyltransferase (CAT) activity.

Assessment of the effects of cell surface CD4 expression on infectibility by HIV-1. HeLa cells expressing different levels of the CD4 receptor were obtained after transfection by the calcium phosphate method with 4, 8, 13, 16, 20, and 25 μ g of the pCD4 plasmid per 100-mm-diameter tissue culture dish. Transfected cells were subjected to flow cytometry to evaluate cell surface CD4 levels as described above. Cells were assessed for infectibility by HIV-1 as described above.

TABLE 1. Abilities of mutant CD4 glycoproteins to bind gp120 and to function as HIV-1 receptors

Mutant	Relative K_D^a	Relative HIV-1 infectibility	
		HeLa ^b	Raji ^c
None (wild type)	1.0	1.00	1.00
43 F/Y	0.72	1.28	0.91
43 F/W	1.68	0.81	0.47
43 F/L	2.71	0.73	0.09
43 F/M	3.43	0.41	0.03
43 F/H	5.46	0.52	0.03
44 L/A	7.59	0.42	0.11
46 K/I	0.79	1.20	1.02
47 G/S	8.61	0.46	0.00
50 K/D	1.57	1.29	0.75
53 D/K	0.64	0.97	0.70
59 R/Q	2.90	0.88	0.24
63 D/R	0.94	0.77	0.82

^a The estimated K_D for gp120 binding of each mutant CD4 relative to that obtained for the wild-type CD4 glycoprotein is shown.

^b HeLa cells were transfected with 30 μ g of the pCD4 plasmids expressing wild-type or mutant CD4 glycoproteins. The transfected HeLa cells were assessed by flow cytometry for levels of cell surface CD4 expression and exposed to the recombinant HXBc2-CAT virus. The CAT activity observed 72 h later was compared with the CAT activity expected for a similar cell surface level of the wild-type CD4 glycoprotein to yield the relative infectibility. Values shown for relative infectibility of HeLa and Raji cells represent the means of at least two independent experiments, with variation between experiments of <20% of the mean.

^c Raji cells transfected with 2 μ g of the pSL3.T4.hydro plasmids expressing mutant or wild-type CD4 glycoprotein were exposed to the recombinant HXBc2-CAT virus. The CAT activity determined in transfected Raji cells expressing the mutant CD4 glycoprotein was compared with that observed in Raji cells transfected with equivalent amounts of a plasmid expressing the wild-type CD4 molecule to obtain the relative infectibility.

RESULTS

gp120 binding studies. In gp120 binding studies, mutant CD4 molecules containing amino acid changes in several CDR2 residues were transiently expressed in CD4-negative cells. The cell surface expression, gp120 binding ability, and ability to serve as a receptor for HIV-1 were determined for each of the mutants. The mutants analyzed are listed in Table 1 and include CD4 molecules with changes in phenylalanine 43, lysine 46, and arginine 59, residues known to be important for gp120 binding (2, 3, 9, 12, 33, 38), as well as additional CDR2 residues.

To evaluate the gp120 binding ability of the CD4 mutants, the absorption of radiolabeled gp120 glycoprotein to HeLa cells transiently expressing the wild-type or mutant CD4 molecules was measured (Fig. 1). The level of cell surface expression of each mutant CD4 glycoprotein was determined by flow cytometry 72 h after transfection, using the OKT4 monoclonal antibody, which recognizes a CD4 epitope in domain 3 (31). Although some experimental variation in the level of cell surface expression of the CD4 mutants was observed, no consistent differences between the steady-state cell surface levels of expression for any of the mutants and that of the wild-type CD4 were noted (data not shown). Labeled gp120 glycoprotein was incubated with the transfected HeLa cells at different concentrations, and the measurements of the levels of bound and unbound gp120 glycoprotein were used to calculate the relative dissociation constant of each mutant CD4 as described in Materials and Methods.

The dissociation constants of the mutant CD4 molecules relative to that of the wild-type CD4 are listed in Table 1. Substitution of aromatic amino acids for phenylalanine 43

FIG. 1. Analysis of gp120 binding ability of CD4 mutants. HeLa cells transiently expressing wild-type and mutant CD4 glycoproteins were incubated with different amounts of labeled gp120 glycoprotein, and the amounts of bound and unbound gp120 glycoprotein were measured by immunoprecipitation. The precipitated gp120 glycoprotein bound to the HeLa cells (A) or unbound (B) after incubation with 10-fold-diluted (lanes 1:10), 5-fold-diluted (lanes 1:5), or undiluted (lanes 1) preparations of the gp120 glycoprotein are shown for the mock-transfected cells (Δ KS) and the cells expressing the wild-type CD4 (w.t.) or the 50 K/D or 47 G/S mutant.

resulted in CD4 molecules with gp120 binding affinities close to that of the wild-type glycoprotein. Replacement of phenylalanine 43 by leucine, methionine, or histidine resulted in more-significant decreases in gp120 binding affinity. Among the other substitutions in the CD4 CDR2 loop, the changes in the 44 L/A (leucine-to-alanine change at position 44), 47 G/S, and 59 R/Q mutants resulted in the more-significant decreases in gp120 binding ability.

Effect of changes in CD4 expression and gp120 binding affinity on infectibility by HIV-1. To investigate the effect of the CDR2 loop amino acid changes on the ability of CD4 to function as a receptor for HIV-1, transfected HeLa or Raji cells expressing the mutant CD4 glycoproteins were incubated with an infectious HIV-1 (HXBc2-CAT) encoding the bacterial CAT gene (47). The ability of this recombinant virus to infect cells can be precisely measured by determining the amount of CAT expression in the target cells.

To control for the level and distribution of CD4 expression in this system, flow cytometry analysis with the OKT4 antibody was performed 72 h after transfection. While the level of cell surface CD4 expressed in the transfected Raji cells was too low to be detected by flow cytometry, the surface expression of the wild-type and mutant CD4 molecules in the transfected HeLa cells was readily measured. By using mock-transfected HeLa cells as a negative control, a threshold for CD4 density, above which a cell was considered CD4 positive, was established. In the HeLa cells transfected with the pCD4 plasmid expressing the wild-type CD4 glycoprotein, two populations of cells, one CD4 positive and the other CD4 negative, could be distinguished. In these cultures, both the percentage of CD4-positive cells and the mean CD4 density on the CD4-expressing subset of cells could be determined. In all experiments, these two parameters were found to be proportionate to one another

FIG. 2. Relationship between the amount of transfected DNA, cell surface CD4 expression, and infectibility in HeLa cells. (A) HeLa cells expressing different levels of the wild-type CD4 glycoprotein were obtained after transfection with 4 to 25 μ g of the pCD4 plasmid. (B) The CAT activity following exposure to the recombinant HXBc2-CAT virus was plotted versus the level of CD4 expression on the cell surface. As discussed in Results, the latter parameter is proportionate both to the percentage of CD4-expressing cells and to the CD4 density on the CD4-expressing subset of cells and is expressed in arbitrary units.

(data not shown), and therefore we have arbitrarily utilized the former measurement to indicate the level of CD4 expression. Figure 2A shows that when HeLa cells were transfected with increasing amounts (from 4 to 25 μ g) of the pCD4 plasmid, increasing levels of CD4 expression on the cell surface were obtained. Further increases in cell surface CD4 expression were not observed when more than 30 μ g of the pCD4 plasmid was used for transfection (data not shown). These HeLa cells were exposed to equivalent amounts of the HXBc2-CAT virus, and the CAT activity in the target cell lysates, normalized for protein content, was measured. As expected, transfected HeLa cell populations containing a higher percentage of CD4-positive cells and a higher mean CD4 density on the CD4-expressing cells also exhibited a greater ability to be infected by the HXBc2-CAT virus (Fig. 2B).

HeLa and Raji cells expressing the mutant CD4 molecules were assessed for the ability to be infected by the HXBc2-CAT virus. The CAT activity measured in the HeLa cells expressing a CD4 mutant was compared with that predicted by Fig. 2B for HeLa cells expressing an equivalent cell surface level of the wild-type CD4 glycoprotein. The CAT activity in the Raji cells expressing a CD4 mutant was compared directly with that observed in Raji cells transfected with equal amounts of a

FIG. 3. Relationship between relative K_D for gp120 binding and infectivity by HIV-1. (A) Transfected HeLa cells were assessed by flow cytometry for CD4 expression level and exposed to the recombinant HXBc2-CAT virus. The relative K_D and the relative infectivity were determined as described in the footnotes to Table 1. The relative infectivity of HeLa cells expressing the 43 F/V mutant CD4, which exhibits at least a 23-fold reduction in gp120 binding ability, was 0.11 in this assay (data not shown). (B) Transfected Raji cells were exposed to the recombinant HXBc2-CAT virus. The relative infectivity was determined as described in Table 1, footnote c, and was plotted versus the relative K_D , which was determined for HeLa cells as described in Materials and Methods.

plasmid expressing the wild-type CD4 glycoprotein. Thus, a determination of the relative infectivity of cells expressing each mutant CD4 compared with that of cells expressing the wild-type CD4 glycoprotein was made (Table 1). The relative infectivity is compared with the relative K_D for gp120 binding in Fig. 3. The exponential curves obtained for HeLa and Raji target cells are similar, although the effects of moderate changes in relative K_D on the efficiency of virus infection are more pronounced in the Raji cells than in the HeLa cells. For none of the CD4 mutants examined was the relative infectivity significantly lower than that predicted by taking into account the relative K_D and experimental variations in cell surface expression.

The more pronounced effect of decreases in gp120 binding affinity on the relative infectibility of Raji cells than on that of HeLa cells might be a consequence of the lower levels of CD4 expressed in the Raji cells. Thus, we investigated the effect of the level of cell surface CD4 expression on the relationship between gp120 binding affinity and relative infectivity by HIV-1. HeLa cells were used in these experiments, since the cell surface expression of CD4 could be easily measured and since the use of a single target cell type eliminates other

FIG. 4. Effect of cell surface expression of wild-type and mutant CD4 glycoproteins on infectivity by HIV-1. HeLa cells were transfected with various amounts (4 to 25 μ g) of pCD4 plasmid DNA expressing the wild-type (Δ), 43 F/L ($\square$), 43 F/W ($\bullet$), or 43 F/H ($\circ$) CD4 glycoprotein and exposed to the recombinant HXBc2-CAT virus. The CAT activities determined in HeLa cells transfected with plasmids expressing various levels of the mutant CD4 glycoproteins were divided by the CAT activities observed in HeLa cells transfected with equivalent amounts of the wild-type-CD4-expressing plasmid to obtain the relative infectivity.

cell-type-specific variables that might influence entry. HeLa cells were transfected with various amounts of the pCD4 plasmid expressing the wild-type, 43 F/L, 43 F/W, or 43 F/H CD4 glycoprotein. The amounts of mutant and wild-type CD4 expressed on the transfected cell surface, assessed by flow cytometry, increased with increasing amounts of plasmid DNA transfected (data not shown). The transfected HeLa cells were exposed to the HXBc2-CAT virus, and the CAT activity was determined. Figure 4 shows that as the level of CD4 expressed in the HeLa cells decreased, a decrease in the infectivity of the mutant CD4-expressing cells greater than that of the cells expressing the wild-type CD4 was observed.

DISCUSSION

In this study, the effects of amino acid changes in the CD4 CDR2 loop, the major binding site for the HIV-1 gp120 envelope glycoprotein (2, 3, 9, 12, 13, 32, 33, 38, 40, 52), on virus binding and infectivity were measured. While numerous studies have examined the effect of amino acid changes in CD4 on HIV-1 gp120 binding, only a few attempts to evaluate the effects of these changes on HIV-1 entry have been made. This is in part because of the difficulty of precisely quantitating the infectivity of cells by HIV-1 while simultaneously controlling for variables such as level of CD4 expression, which can change after HIV-1 infection (22). In this study, by using a recombinant HIV-1 carrying a marker gene, the efficiency of HIV-1 replication was quantitated soon after the initial infection. This allowed precise relationships between cell surface CD4 expression, gp120 binding affinity, and efficiency of virus infectivity to be deduced for a panel of mutant CD4 molecules.

The level of cell surface CD4 expression was one parameter that influenced the efficiency of HIV-1 infection. The effect of a decrease in gp120 binding affinity on relative infectivity was greater when levels of cell surface CD4 were lower (Fig. 4). Changes in the percentage of CD4-positive cells in the transfected population would proportionately affect the infectivity of cells expressing wild-type and mutant CD4 glycoproteins. Therefore, the changes in relative infectivity seen in Fig. 4 must be a consequence of changes in CD4 density. Higher concentrations of the CD4 molecule on the target cell are thus

able to compensate, at least in part, for a lowered affinity of the monomeric gp120-CD4 interactions.

The contribution of the surface CD4 density to the effect of changes in gp120-CD4 binding affinity on the infectibility of cells is consistent with previous data obtained on the infectivity of HIV-1 gp120 mutants with lowered CD4 binding affinity (50). In that study, it was observed that some HIV-1 gp120 mutants with considerable (up to 20-fold) decreases in CD4 binding affinity were still able to infect some established T-cell lines. The relative infectivity of the mutant viruses compared with that of the wild-type HIV-1 depended upon the target cell used. The decreases in relative infectivity were more substantial for cells, such as H9 lymphocytes, that express lower CD4 levels than for cells, such as Jurkat or SupT1 lymphocytes, that express higher CD4 levels. Thus, decreases in the monomeric binding affinity resulting from changes either in gp120 or in CD4 are better tolerated with respect to HIV-1 entry when higher levels of CD4 are expressed on the target cell. These results are also consistent with those observed for primary HIV-1 isolates, which exhibit a lower oligomeric affinity for CD4 than laboratory-passaged isolates (35) and also exhibit a greater dependence than the latter viruses on levels of cell surface CD4 expression (23). Sequestration of these primary viruses in lymph nodes, where the local CD4-positive-lymphocyte density may be high, might help to compensate for the effects of lowered CD4 binding affinity on viral replication.

This study focused on a number of CDR2 loop residues of CD4 that are positioned proximal to the gp120 binding site (2, 3, 9, 12, 33, 41). Since in every case examined cell surface expression and gp120 binding affinity could explain the receptor capability of the mutant CD4 molecule, no evidence for a particular structural requirement in these residues for non-binding functions in HIV-1 entry was obtained. If, as proposed previously (1, 27, 36, 41, 42), CD4 binding is critical for the induction of conformational changes in HIV-1 envelope glycoproteins that promote the membrane fusion process, regions of CD4 outside the major gp120 binding site may play a role. Extensive mutagenic analysis of the CD4 CDR3 region makes a role for this region in either gp120 binding or fusion-related processes unlikely (8). Structures in the immunoglobulin-like framework of CD4 domain 1 could conceivably trigger fusion-related conformational changes in the HIV-1 envelope glycoproteins. Such structures, however, would have to be conserved in rat CD4, since the CD4 chimera in which the human CDR2 sequences are fused with rat framework sequences functions as an efficient receptor for HIV-1 (43). Alternatively, in lieu of specific structures, steric interactions involving large surfaces of CD4 and gp120 could serve to trigger fusion-related events. Future studies will be directed at documenting the complete role of CD4 in virus entry and defining structural elements associated with entry functions.

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