Transitions to and from the CD4-Bound Conformation Are Modulated by a Single-Residue Change in the Human Immunodeficiency Virus Type 1 gp120 Inner Domain

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Transitions to and from the CD4-Bound Conformation Are Modulated by a Single-Residue Change in the Human Immunodeficiency Virus Type 1 gp120 Inner Domain

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Binding to the primary receptor CD4 induces conformational changes in the human immunodeficiency virus type 1 (HIV-1) gp120 envelope glycoprotein that allow binding to the coreceptor (CCR5 or CXCR4) and ultimately trigger viral membrane-cell membrane fusion mediated by the gp41 transmembrane envelope glycoprotein. Here we report the derivation of an HIV-1 gp120 variant, H66N, that confers envelope glycoprotein resistance to temperature extremes. The H66N change decreases the spontaneous sampling of the CD4-bound conformation by the HIV-1 envelope glycoproteins, thus diminishing CD4-independent infection. The H66N change also stabilizes the HIV-1 envelope glycoprotein complex once the CD4-bound state is achieved, decreasing the probability of CD4-induced inactivation and revealing the enhancing effects of soluble CD4 binding on HIV-1 infection. In the CD4-bound conformation, the highly conserved histidine 66 is located between the receptor-binding and gp41-interactive surfaces of gp120. Thus, a single amino acid change in this strategically positioned gp120 inner domain residue influences the propensity of the HIV-1 envelope glycoproteins to negotiate conformational transitions to and from the CD4-bound state.

Human immunodeficiency virus type 1 (HIV-1), the cause of AIDS (6, 29, 66), infects target cells by direct fusion of the viral and target cell membranes. The viral fusion complex is composed of gp120 and gp41 envelope glycoproteins, which are organized into trimeric spikes on the surface of the virus (10, 51, 89). Membrane fusion is initiated by direct binding of gp120 to the CD4 receptor on target cells (17, 41, 53). CD4 binding creates a second binding site on gp120 for the chemokine receptors CCR5 and CXCR4, which serve as coreceptors (3, 12, 19, 23, 25). Coreceptor binding is thought to lead to further conformational changes in the HIV-1 envelope glycoproteins that facilitate the fusion of viral and cell membranes. The formation of an energetically stable six-helix bundle by the gp41 ectodomain contributes to the membrane fusion event (9, 10, 79, 89, 90).  
The energy required for viral membrane-cell membrane fusion derives from the sequential transitions that the HIV-1 envelope glycoproteins undergo, from the high-energy unliganded state to the low-energy six-helix bundle. The graded transitions down this energetic slope are initially triggered by CD4 binding (17). The interaction of HIV-1 gp120 with CD4 is accompanied by an unusually large change in entropy, which is thought to indicate the introduction of order into the conformationally flexible unliganded gp120 glycoprotein (61). In the CD4-bound state, gp120 is capable of binding CCR5 with high affinity; moreover, CD4 binding alters the quaternary structure of the envelope glycoprotein complex, resulting in the exposure of gp41 ectodomain segments (27, 45, 77, 92). The stability of the intermediate state induced by CD4 binding depends upon several variables, including the virus (HIV-1 versus HIV-2/simian immunodeficiency virus [SIV]), the temperature, and the nature of the CD4 ligand (CD4 on a target cell membrane versus soluble forms of CD4 [sCD4]) (30, 73). For HIV-1 exposed to sCD4, if CCR5 binding occurs within a given period of time, progression along the entry pathway continues. If CCR5 binding is impeded or delayed, the CD4-bound envelope glycoprotein complex decays into inactive states (30). In extreme cases, the binding of sCD4 to the HIV-1 envelope glycoproteins induces the shedding of gp120 from the envelope glycoprotein trimer (31, 56, 58). Thus, sCD4 generally inhibits HIV-1 infection by triggering inactivation events, in addition to competing with CD4 anchored in the target cell membrane (63).  

HIV-1 isolates vary in sensitivity to sCD4, due in some cases to a low affinity of the envelope glycoprotein trimer for CD4 and in other cases to differences in propensity to undergo inactivating conformational transitions following CD4 binding (30). HIV-1 isolates that have been passaged extensively in
T-cell lines (the tissue culture laboratory-adapted [TCLA] isolates) exhibit lower requirements for CD4 than primary HIV-1 isolates (16, 63, 82). TCLA viruses bind sCD4 efficiently and are generally sensitive to neutralization compared with primary HIV-1 isolates. Differences in sCD4 sensitivity between primary and TCLA HIV-1 strains have been mapped to the major variable loops (V1/V2 and V3) of the gp120 glycoprotein (34, 42, 62, 81). Sensitivity to sCD4 has been shown to be independent of envelope glycoprotein spike density or the intrinsic stability of the envelope glycoprotein complex (30, 35).

In general, HIV-1 isolates are more sensitive to sCD4 neutralization than HIV-2 or SIV isolates (4, 14, 73). The relative resistance of SIV to sCD4 neutralization can in some cases be explained by a reduced affinity of the envelope glycoprotein trimer for sCD4 (57); however, at least some SIV isolates exhibit sCD4-induced activation of entry into CD4-negative, CCR5-expressing target cells that lasts for several hours after exposure to sCD4 (73). Thus, for some primate immunodeficiency virus envelope glycoproteins, activated intermediates in the CD4-bound conformation can be quite stable.

The HIV-1 envelope glycoprotein elements important for receptor binding, subunit interaction, and membrane fusion are well conserved among different viral strains (71, 91). Thus, these elements represent potential targets for inhibitors of HIV-1 entry. Understanding the structure and longevity of the envelope glycoprotein intermediates along the virus entry pathway is relevant to attempts at inhibition. For example, peptides that target the heptad repeat 1 region of gp41 exhibit major differences in potency against HIV-1 strains related to efficiency of chemokine receptor binding (20, 21), which is thought to promote the conformational transition to the next step in the virus entry cascade. The determinants of the duration of exposure of targetable HIV-1 envelope glycoprotein elements during the entry process are undefined.

To study envelope glycoprotein determinants of the movement among the distinct conformational states along the HIV-1 entry pathway, we attempted to generate HIV-1 variants that exhibit improved stability. Historically, labile viral elements have been stabilized by selecting virus to replicate under conditions, such as high temperature, that typically weaken protein-protein interactions (38, 39, 76, 102). Thus, we subjected HIV-1 to repeated incubations at temperatures between 42°C and 56°C, followed by expansion and analysis of the remaining replication-competent virus fraction. In this manner, we identified an envelope glycoprotein variant, H66N, in which histidine 66 in the gp120 N-terminal segment was altered to asparagine. The resistance of HIV-1 bearing the H66N envelope glycoproteins to changes in temperature has been reported elsewhere (37). Here, we examine the effect of the H66N change on the ability of the HIV-1 envelope glycoproteins to negotiate conformational transitions, either spontaneously or in the presence of sCD4. The H66N phenotype was studied in the context of both CD4-dependent and CD4-independent HIV-1 variants.

**MATERIALS AND METHODS**

**Selection of heat-resistant virus.** The pNL4.3-KB9 plasmid, which contains the KB9 (36) envelope glycoproteins in the background of the NL4.3 (1) provirus, was used to generate infectious HIV-1. 293T cells were transfected with 25 μg of the pNL4.3-KB9 plasmid by using a calcium phosphate transfection kit (Invitro...
Transient expression of radiolabeled HIV-1 envelope glycoproteins and measurement of sCD4 binding and sCD4-induced shedding. 293T cells grown to 70% confluency in 100-mm dishes were transfected with 3 μg of an envelope glycoprotein-expressing plasmid DNA and 1 μg of an HIV-1 Tat-expressing plasmid DNA with Effectene transfection reagent (Qiagen). One day later, the medium was removed, the cells were washed once with 10 ml of phosphate-buffered saline (PBS), and 9 ml of labeling medium (10% heat-inactivated, dialyzed fetal bovine serum [FBS]; 10 μg/ml penicillin-streptomycin solution; 50 μg/ml 35S-Express protein labeling mix [Perkin Elmer, Waltham, MA]; and 2 mM L-glutamine in Dulbecco’s modified Eagle medium [DMEM]) was added to the transfected cells. The cells were incubated at 37°C for another day before the medium and cells were harvested. Cells were detached from culture dishes by incubation with 2 ml of 5 mM EDTA in PBS for 5 min and washed with PBS before equal amounts were aliquoted in duplicate to separate tubes. To one duplicate set of tubes, sCD4 was added to final concentrations of 200 and 1,000 nM; to the control set of tubes, medium without sCD4 was added. The tubes were incubated at 37°C for 1 h. The cell culture medium was harvested, and gp120 glycoproteins shed into the culture media were quantified by immunoprecipitation.

The cells were used to prepare lysates for the analysis of envelope glycoprotein expression and processing. Cells were suspended in 1 ml cell lysis buffer (0.5 M NaCl, 10 mM Tris, pH 7.5, 0.5% [vol/vol] NP-40, and a cocktail of protease inhibitors) and incubated at 4°C for 30 min, with gentle agitation. The lysates were cleared by centrifugation at 14,000 x g for 30 min at 4°C. The envelope glycoproteins were precipitated from the supernatants with a mixture of sera from HIV-1-infected individuals.

To measure sCD4 binding, approximately 5 x 10⁵ 293T cells transiently expressing the HIV-1 envelope glycoproteins were incubated for 1 h at room temperature in 1 ml of serial dilutions of a mixture immunoglobulin (Ig) in fluorescence-activated cell sorter (FACS) buffer (PBS-5% FBS). The cells were washed three times with FACS buffer and then incubated for 1 h at room temperature with either OKT4, a fluorescein isothiocyanate-conjugated anti-CD4 antibody (eBioscience, San Diego, CA) (for sCD4), or phycoerythrin-conjugated rabbit anti-human IgG (Sigma, St. Louis, MO) (for CD4- Ig). The cells were then washed three times with FACS buffer and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and FLOWJO software (FlowJo, Ashland, OR).

Immunoprecipitation of envelope glycoproteins. Radiolabeled HIV-1 gp120 glycoproteins shed into supernatants after incubation of envelope glycoprotein-expressing cells with sCD4 were detected by precipitation with a mixture of sera from HIV-1-infected individuals. Approximately 250 μl of cell culture medium was added to 80 μl of 50% protein A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ), 5 μl of 10% bovine serum albumin, and a mixture of sera from HIV-1-infected individuals; DMEM with 10% FBS was added to bring the total volume to 500 μl. For precipitation of envelope glycoproteins from the lysates of transfected 293T cells (see above), 900 μl of cell lysate was incubated with 100 μl of 50% protein A-Sepharose beads, 10 μl of 10% bovine serum albumin, and 5 μl of a mixture of sera from HIV-1-infected individuals. The mixture was incubated at 4°C for 2 to 3 h and then washed three times with 1 ml of wash buffer (0.1% NP-40, 0.3 M NaCl, 50 mM Tris-HCl [pH 7.5]) and once with 1 ml of PBS. The beads were mixed with gel loading buffer and boiled for 5 min. Following the removal of the beads by centrifugation, the supernatants were loaded on a 10% bis-Tris sodium dodecyl sulfate (SDS)-polyacrylamide gel. The gel was enhanced with AutoBioh (National Diagnostics, Atlanta, GA) for 45 min, dried at 80°C for 2 h, and exposed to film or phosphor screen. The amounts of radiolabeled HIV-1 envelope glycoproteins precipitated were measured either by densitometric analysis of autoradiograms using Quantity One, v4.6.3, software (Bio-Rad, Philadelphia, PA) or by use of a Storm PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

HIV-1 neutralization assay. Neutralization of virus infectivity was assessed by incubating sCD4 and antibodies with recombinant, luciferase-expressing virus at 37°C for 1 to 2 h and then measuring entry into target cells. The different virus mutants, at a concentration of 150 RT units/μl, were incubated with serial dilutions of sCD4 (final concentrations ranging from 0 to 500 nM) in a total volume of 650 μl at 37°C before being added to target cells to measure infectivity. After 2 to 3 days in culture, the cells were lysed and assayed for luciferase activity.

The sensitivity of HIV-1 to inhibition by the CD4-mimetic compound NBD-556 (52, 74, 103) was examined. The compound was dissolved in dimethyl sulfoxide to create 10 to 20 mM stock solutions and stored in aliquots at −20°C. It was then diluted to 1 mM in serum-free DMEM for use in assays. On the day of infection, NBD-556 (1 to 100 μM) was added to recombinant viruses (10,000 RT units) in a final volume of 50 μl and incubated at 37°C for 30 min. The medium was then removed from the target cells, which were then incubated with the virus-drug mixture for 48 h at 37°C. The medium was then removed from each well, and virus infection was assayed by lysing cells and measuring luciferase activity in the cell lysate.

sCD4 activation and stability of the CD4-bound intermediate state. The effect of sCD4 on activating the infectivity of HIV-1 was measured with C27Th-CCR5 cells. Recombinant HIV-1 at a concentration of 150 RT units/μl was incubated with different concentrations of sCD4 for 1 to 2 h at 37°C and added to C27Th-CCR5 cells. After 2 to 3 days in culture, the cells were lysed and assayed for luciferase activity.

To measure the effect of the H66N change on the stability of the CD4-bound intermediate state of the HIV-1 envelope glycoproteins, 7.5 x 10⁵ RT units/ml of recombinant HIV-1 was divided into two 1-ml aliquots. To one aliquot, sCD4 was added to a final concentration of 200 nM; the other aliquot was left untreated. All virus preparations with and without sCD4 treatment were incubated for 1 h at 37°C. After incubation, unbound sCD4 was removed by pelleting the virus three times at 41,000 rpm in an SW-41 rotor for 30 min at 4°C in a Beckman-Alegra centrifuge, followed by resuspension in DMEM with 10% FBS. The supernatants of virus-antibody complexes were incubated at 37°C. At various time points, aliquots were added to C27Th-CCR5 cells. Virus infectivity was measured by the luciferase assay, as described above.

Binding of HIV-1 gp120 to CCR5. 293T cells were transfected with plasmids expressing HIV-1 envelope glycoproteins with deletions of the gp41 cytoplasmic tail. Cells were radiolabeled as described above. The supernatants were harvested and cleared by centrifugation (200 x g for 5 min at 4°C).

The amount of gp120 envelope glycoprotein variants was quantitated by immunoprecipitating serial dilutions of the media; precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by densitometry. The gp120 concentrations in the supernatants were equalized by dilution with DMEM. To one set of samples, sCD4 was added to a final concentration of 200 to 400 nM; the other set of samples was left untreated. All samples were incubated at 37°C for 1 h and then added to 5 x 10⁵ to 6 x 10⁵ C27Th-CCR5 cells or kept at 4°C. The mixtures of supernatant and cells were incubated at 4°C for 2 h. Cells were washed twice with PBS to remove unbound gp120 and lysed with 1 ml cell lysis buffer. The bound gp120 was precipitated by a mixture of sera from HIV-1-infected individuals and analyzed by SDS-PAGE and densitometry. The input amount of gp120 in the CCR5-binding assay was estimated in parallel by immunoprecipitation of an aliquot of cleaved 293T cell supernatant that was not incubated with C27Th-CCR5 cells but was instead kept at 4°C.

Production and purification of HIV-1 gp120 glycoproteins. The wild-type and mutant gp120 variants with C-terminal His, epitope tags were expressed from plasmids containing a codon-optimized env gene (GeneScript Corp., Piscataway, NJ). The plasmids were transfected into 293F cells by using 293fectin reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Seven days later, the supernatant containing the gp120 envelope glycoproteins was harvested and filtered using 0.45-μm filters. The supernatant was concentrated two- to threefold by use of Centricron plus-80 filters (Amicon, Billerica, MA). Ni-nitrito-triacetic acid beads (Qiagen) were added to the concentrated supernatant and incubated overnight at 4°C with gentle shaking. The supernatant/bead mixture was poured into a small column, washed with 20 mM imidazole in buffer A (150 mM NaCl, 20 mM Tris-HCl, pH 7.4), and eluted by gravity flow with 200 mM imidazole in buffer A. The eluant was concentrated with Centriprep-30 (Amicon) and dialyzed with a 10,000-molecular-weight-cutoff dialysis cassette (Pierce, Rockford, IL) in 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl. The gp120 glycoproteins were stored at −80°C.

Isothermal titration microcalorimetry. Isothermal titration calorimetry experiments were performed using a high-precision VP-ITC titration calorimetry system from MicroCal, Inc. (Northampton, MA). The calorimetric cell (~1.4 ml) containing one of the gp120 membrane-proximal fragments dissolved in 50 mM Tris-HCl (pH 7.4, 150 mM NaCl, 500 mM KCl) (Amersham Pharmacia Biotech GmbH), pH 7.4, with 2% dimethyl sulfoxide, was titrated with sCD4 or NBD-556 dissolved in the same buffer. The concentration of gp120 was ~4 μM, and the concentration in the injection syringe was ~20 μM of CD4 or between 120 and 150 μM of NBD-556. All experiments were conducted at 25°C. The heat evolved
upon injection of sCD4 or NBD-556 was obtained from the integral of the calorimetric signal. The heat associated with the binding reaction was obtained by subtracting the heat of dilution from the heat of reaction. The individual heats were plotted against the molar ratio, and the values for the enthalpy change ($\Delta H$) and the association constant ($K_a = 1/K_d$, where $K_d$ is the dissociation constant) were obtained by nonlinear least-squares regression of the data.

SPPR. Surface plasmon resonance (SPR) experiments were performed with a Biacore 3000 biosensor system at 25°C. The gp120 ligand, either sCD4 or the 17b antibody, was immobilized on research-grade CM5 sensor chips by the recommended standard amine coupling. Wild-type or mutant gp120 variants at different concentrations were injected and allowed to bind the ligand captured on the surface of the sensor chip. To examine sCD4-induced binding of gp120 to the 17b antibody, each gp120 variant at a concentration of 500 nM was incubated with 4 μM sCD4 at 25°C for 1 h. The mixture was then injected and allowed to bind the 17b antibody on the sensor chip. All binding experiments were carried out with HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20). During the association phase, analytes were passed over the buffer-equilibrated chip surface at a rate of 50 μl/min. After the association phase, bound analytes were allowed to dissociate for 5 min. The chip surface was then regenerated by two injections of 10 mM glycine HCl (pH 2.5) at a flow rate of 50 μl/min for 30 s. Association and dissociation values were calculated by numerical integration and globally fit to a 1:1 interaction model using BiaEvaluation 3.0 software (Biacore).

Molecular modeling of the H66N gp120 mutant. The X-ray crystal structure of the HIV-1 envelope glycoprotein, when in the CD4-bound conformation, exhibited altered temperature sensitivity emerged, the parallel on 1G5 cells without heat selection. Once viruses that were obtained by nonlinear least-squares regression of the data.

RESULTS

To identify stabilizing changes in the HIV-1 envelope glycoproteins, we passaged a molecularly cloned HIV-1, NL4.3-KB9, while intermittently incubating the viruses at elevated temperatures. The NL4.3-KB9 virus is derived from the NL4.3 KB9, while intermittently incubating the viruses at elevated temperatures. The NL4.3-KB9 virus was obtained from the NL4.3 virus but has its envelope glycoproteins replaced by those of the pathogenic simian-human immunodeficiency virus KB9. The NL4.3-KB9 starting virus stock was incubated at a given temperature for different periods of time, cooled to room temperature, and added to 1G5 cells. The 1G5 cells are Jurkat T cells that contain an integrated luciferase gene under the control of the HIV-1 long terminal repeat (2). Viruses that survived the most extreme temperatures were propagated and reexposed to increased temperature, and the process was repeated. A control stock of NL4.3-KB9 virus was passaged in parallel on 1G5 cells without heat selection. Once viruses that exhibited altered temperature sensitivity emerged, the env genes of these viruses and the control viruses were PCR amplified from infected cells. Each amplified fragment was cloned and sequenced. Comparison of the env sequences from heat-selected and control viruses revealed the presence of specific mutations in the former. Studies reported elsewhere (37) revealed that the observed differences in temperature sensitivity of the HIV-1 envelope glycoproteins resulted from a single change of histidine 66 to asparagine (H66N).

Due to immune selective pressure, the HIV-1 gp120 glycoprotein exhibits significant interstrain variation, particularly within the surface-exposed variable loops (V1 to V5) (55, 75, 78, 80, 93). The more conserved gp120 segments have been designated C1 to C5. Histidine 66 is located in the first conserved (C1) region of gp120, within a short disulfide loop bounded by cysteines 54 and 74. Although the sequences within this disulfide loop differ considerably between the HIV-1 and HIV-2/SIV lineages, there is a high degree of conservation of certain residues within each of these lineages. Histidine 66, for example, is invariant in all of the HIV-1 and SIVcpz sequences currently deposited in the Los Alamos National Laboratory HIV Sequence Database (46) (see Fig. S1 in the supplemental material).

Effect of the H66N change on sensitivity to neutralization by sCD4. To investigate the effect of the H66N change on sensitivity of HIV-1 to neutralization by sCD4, this change was introduced into the envelope glycoproteins derived from several HIV-1 strains, i.e., KB9, ADAa, YU2, JR-FL, and HXBc2. The HIV-1KB9 dual-tropic envelope glycoproteins were derived from a pathogenic simian-human immunodeficiency virus passaged in monkeys (36, 68). The HIV-1LADaD, HIV-1BYU2, and HIV-1JR-FL viruses are primary, CCR5-using isolates, HIV-1HXBc2 is a TCLA, CXCR4-using isolate.

Recombinant HIV-1 encoding luciferase was pseudotyped with the wild-type and H66N variants of the above-described envelope glycoproteins and used to infect C12T3 canine thymic epithelial cells expressing human CD4 and the appropriate human chemokine receptor. The infections were carried out after the viruses were incubated at 37°C for 1 h without treatment or with increasing concentrations of sCD4. The basal levels of infection in the absence of sCD4 treatment were similar for viruses with the wild-type and H66N envelope glycoproteins (see Fig. S2 in the supplemental material).

As expected (55, 75, 78, 80, 93), viruses with the wild-type HXBc2 envelope glycoproteins exhibited the greatest sensitivity to sCD4, with a 90% inhibitory concentration (IC90) of approximately 1.2 nM (Fig. 1A). Recombinant HIV-1 viruses with the wild-type envelope glycoproteins from the R5 primary isolates were significantly more resistant to sCD4, with IC90 values ranging from 14 to 80 nM (Fig. 1B to D). Viruses with the dual-tropic KB9 envelope glycoproteins exhibited intermediate levels of sCD4 sensitivity (IC90 of 3 nM) (Fig. 1E). Thus, as previously reported (5, 16, 57, 82), viruses with envelope glycoproteins derived from TCLA and primary HIV-1 strains differ in sCD4 sensitivity.

Viruses with the H66N envelope glycoproteins were more resistant to sCD4 than viruses bearing the wild-type HIV-1 envelope glycoproteins. Depending on the particular envelope glycoproteins, the degree of sCD4 resistance varied from approximately 7-fold to 33-fold. Thus, the H66N change confers some degree of sCD4 resistance to viruses with a variety of
HIV-1 envelope glycoproteins, in the context of infection of CD4-positive cells.

Effect of the H66N change on HIV-1 infection of CD4-negative cells. To examine the effect of the H66N change on HIV-1 infection of CD4-negative, CCR5-expressing cells, the recombinant viruses with wild-type or H66N R5 envelope glycoproteins were incubated with Cf2Th-CCR5 cells along with different concentrations of sCD4. In the absence of sCD4, the level of infection of the Cf2Th-CCR5 cells by any of the viruses was near the background for the assay (Fig. 2). The addition of sCD4 resulted in enhanced infection by viruses with the wild-type JR-FL envelope glycoproteins but not by viruses with the wild-type ADAfl or YU2 envelope glycoproteins. The lack of enhancement of the viruses with the wild-type ADAfl or YU2 envelope glycoproteins was not due to a lack of sCD4 binding to the envelope glycoproteins, because the infectivity of these viruses for CD4-positive target cells was markedly inhibited by incubation with sCD4 in this same concentration range (Fig. 1B and C).

Recombinant viruses bearing the H66N envelope glycoproteins exhibited greater enhancement by sCD4 than viruses with the respective wild-type envelope glycoproteins. As the H66N and wild-type viruses were normalized by RT measurements and demonstrated nearly equivalent levels of infectivity with Cf2Th-CD4/CCR5 cells (see Fig. S2A in the supplemental material), we conclude that the H66N change results in an increase in sCD4 enhancement of infection of CD4-negative, CCR5-expressing target cells.

Effects of H66N on gp120 ligand binding and induction of conformational changes. The above-described results suggest that the H66N change facilitates the enhancing effects of sCD4 binding on HIV-1 infection, diminishes the negative consequences of sCD4 binding on infection, or both. To investigate the first possibility, we used isothermal titration microcalorimetry to assess the effect of H66N on sCD4 binding to gp120 and on the ability of sCD4 to induce conformational changes in gp120. We studied the binding of sCD4 to the wild-type and H66N gp120 glycoproteins from HIV-1KB9 (Table 1). The H66N mutant gp120 exhibited a slight (~2-fold) decrease in affinity for sCD4 compared with that of the wild-type gp120.

<table>
<thead>
<tr>
<th>gp120 protein</th>
<th>Kd (nM)</th>
<th>ΔG (kcal/mol)</th>
<th>ΔH (kcal/mol)</th>
<th>−TΔS (kcal/mol)</th>
</tr>
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<tbody>
<tr>
<td>KB9</td>
<td>11</td>
<td>−10.8</td>
<td>−40.9</td>
<td>+30.1</td>
</tr>
<tr>
<td>KB9-H66N</td>
<td>21</td>
<td>−10.5</td>
<td>−39.6</td>
<td>+29.1</td>
</tr>
<tr>
<td>ADA N197S</td>
<td>25</td>
<td>−10.4</td>
<td>−28.0</td>
<td>+17.6</td>
</tr>
<tr>
<td>ADA N197S-H66N</td>
<td>116</td>
<td>−9.5</td>
<td>−19.0</td>
<td>+9.5</td>
</tr>
</tbody>
</table>

*ΔG, free energy change.
*ΔH, enthalpy change.
*T, temperature; ΔS, entropy change.
TABLE 2. SPR measurements of the effect of the H66N change on gp120-sCD4 binding

<table>
<thead>
<tr>
<th>gp120 protein</th>
<th>$K_{on}^a$ (M$^{-1}$ s$^{-1}$)</th>
<th>$K_{off}^b$ (s$^{-1}$)</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB9</td>
<td>1.1 x 10$^4$</td>
<td>1.9 x 10$^{-4}$</td>
<td>17</td>
</tr>
<tr>
<td>KB9-H66N</td>
<td>1.1 x 10$^4$</td>
<td>2.8 x 10$^{-4}$</td>
<td>24</td>
</tr>
<tr>
<td>ADA N197S</td>
<td>9.3 x 10$^4$</td>
<td>6.4 x 10$^{-4}$</td>
<td>69</td>
</tr>
<tr>
<td>ADA N197S-H66N</td>
<td>7.3 x 10$^4$</td>
<td>1.1 x 10$^{-3}$</td>
<td>140</td>
</tr>
</tbody>
</table>

$^a$ $K_{on}$, on-rate constant.  
$^b$ $K_{off}$, off-rate constant.

Small decreases in the magnitudes of the entropic and enthalpic changes associated with sCD4 binding were seen for the wild-type HIV-1K$_{B9}$ gp120 glycoproteins, compared with the values observed for the wild-type HIV-1K$_{B9}$ gp120. The slight reduction in affinity for sCD4 associated with the H66N change was corroborated by SPR analysis (Table 2). Of note, the slight difference in sCD4 affinity between the wild-type and H66N HIV-1K$_{B9}$ gp120 glycoproteins arises entirely from a difference in off-rate of binding.

We wished to examine the effect of the H66N change on affinity for sCD4 associated with the H66N change was corroborated by SPR analysis (Table 2). Compared with the values observed for the wild-type HIV-1K$_{B9}$ gp120, the magnitudes of the entropic and enthalpic changes associated with sCD4 binding to the ADA N197S gp120 envelope glycoproteins to bind CCR5 and to mediate infection of CCR5-expressing cells without prior interaction with CD4 (44). Similarly to our observations with the CD4-dependent KB9 envelope glycoprotein, the H66N change in the ADA N197S gp120 glycoprotein resulted in a reduction in affinity for sCD4 (Tables 1 and 2). Compared with the values observed for ADA N197S gp120, the magnitudes of the entropic and enthalpic changes associated with sCD4 binding to the ADA N197S-H66N gp120 were decreased. As was observed for KB9 gp120, the reduction in affinity for sCD4 resulting from the H66N change in ADA N197S gp120 was primarily due to an increased off-rate. Therefore, in both CD4-dependent and CD4-independent envelope glycoproteins, the H66N change slightly decreases affinity for CD4, apparently by destabilizing the gp120-CD4 complex and allowing more-rapid dissociation of gp120 and CD4.

A major functional consequence of CD4 binding to the HIV-1 gp120 glycoprotein is the induction of a conformation capable of binding the chemokine receptor CCR5 or CXCR4 (33, 50, 86, 94). To examine whether the H66N changes in affinity for CCR5-expressing cells lacking CD4 (43, 44). The loss of a glycosylation site at asparagine 197 has been shown to allow the ADA N197S gp120 glycoprotein to spontaneously assume the CD4-bound conformation more efficiently than the H66N counterpart. The H66N change apparently destabilizes the CD4-bound conformation in the absence of CD4 but does allow efficient CCR5 binding following CD4 engagement.

Effect of the H66N change on CD4-independent HIV-1 infection. We wished to examine the effect of the H66N change on infection by CD4-independent HIV-1. Recombinant HIV-1 viruses bearing the ADA N197S or ADA N197S-H66N envelope glycoproteins were compared with respect to their abilities to infect CD4-independent HIV-1. Recombinant HIV-1 viruses expressing the ADA N197S or ADA N197S-H66N envelope glycoproteins was measured using C2Th-CD4/CCR5 or C2Th-CCR5 cells (Fig. 4A). Both viruses efficiently infected C2Th-CD4/CCR5 cells. Conformation more efficiently than the H66N counterpart. The H66N change apparently destabilizes the CD4-bound conformation in the absence of CD4 but does allow efficient CCR5 binding following CD4 engagement.

**FIG. 4. Effect of the H66N change on CD4-independent CCR5 binding and infection.** (A) The infectivity of recombinant, luciferase-expressing viruses with the ADA N197S CD4-independent envelope glycoproteins or the ADA N197S-H66N envelope glycoproteins was measured using C2Th-CD4/CCR5 or C2Th-CCR5 cells. The means and standard deviations of infectivity, reported as relative luciferase units (RLU), from a representative experiment performed in triplicate are shown. (B) Radiolabeled ADA N197S or ADA N197S-H66N gp120 envelope glycoproteins were incubated with control C2Th cells or C2Th-CCR5 cells in the presence (+) or absence (−) of 200 nM sCD4. Bound gp120 and input gp120 were immunoprecipitated and separated by SDS-PAGE.
consistent with the results obtained with CD4-dependent HIV-1 envelope glycoproteins, the H66N change exerted only a small effect on the efficiency of infection of cells expressing CD4 and a chemokine receptor. In contrast, although viruses with the ADA N197S envelope glycoproteins efficiently infected C127h-CCR5 cells, as expected (44), no infection of these CD4-negative cells by viruses with the ADA N197S-H66N envelope glycoproteins was detected. Thus, the H66N change eliminates the ability of otherwise CD4-independent envelope glycoproteins to mediate infection of target cells lacking CD4, even though the ability to infect CD4-expressing cells is retained.

The gp120 glycoproteins of CD4-independent HIV-1 variants can efficiently bind the chemokine receptor, without prior interaction with CD4 (44). To examine the effect of the H66N change on this process, radiolabeled ADA N197S and ADA N197S-H66N gp120 glycoproteins were incubated with C127h-CCR5 cells in the absence or presence of sCD4. Studies using CCR5 ligands have demonstrated that gp120 binding to C127h-CCR5 cells is dependent upon CCR5 (86, 94). Consistent with this, no gp120 binding to C127h cells that do not express CCR5 was detected in either the presence or the absence of sCD4 (Fig. 4B). In the absence of sCD4, the ADA N197S gp120 glycoprotein, but not the ADA N197S-H66N gp120 glycoprotein, bound the C127h-CCR5 cells. Both gp120 glycoproteins bound the C127h-CCR5 cells efficiently following incubation with sCD4, with a mild decrease in binding for the ADA N197S-H66N gp120 compared with that for the ADA N197S glycoprotein. These results suggest that the H66N change decreases the ability of the ADA N197S gp120 glycoprotein to bind CCR5 spontaneously but does not prevent CCR5 binding once CD4 is engaged.

The change in asparagine 197 in the ADA N197S mutant removes a glycan from the V1/V2 stem, thereby increasing the flexibility of the gp120 V1/V2 variable loops and contributing to CD4 independence (44). A CD4-independent phenotype can also be achieved by deleting the V1 and V2 variable loops of the ADA gp120 glycoprotein (43). To examine whether the phenotype of H66N is dependent on the integrity of the gp120 V1/V2 loops, the H66N change was introduced into an HIV-1 envelope glycoprotein, ADA ΔV1/V2, in which the gp120 V1 and V2 loops were deleted. As shown in Fig. S3A in the supplemental material, viruses with the ADA ΔV1/V2 envelope glycoproteins efficiently infected C127h-CCR5 cells, as expected (43). However, the infectivity of viruses with the ADA ΔV1/V2-H66N envelope glycoproteins was significantly lower using these cells. We conclude that the H66N change decreases the ability of CD4-independent HIV-1 envelope glycoproteins to infect CD4-negative cells expressing CCR5 and that this phenotype does not depend on the presence of the gp120 V1 and V2 variable loops.

**Effect of the H66N change on antibody recognition and neutralization.** Distinct conformations of the HIV-1 gp120 glycoproteins are recognized by particular groups of monoclonal antibodies. For example, CD4i antibodies recognize the CD4-bound conformation of HIV-1 gp120 (13, 84, 96), whereas most CD4 binding site antibodies recognize conformations of gp120 other than that induced by CD4 binding (97). Because antibody binding to the HIV-1 envelope glycoprotein leads to neutralization (40, 65, 69, 88, 98), we assessed the conformational state of the ADA N197S and ADA N197S-H66N envelope glycoproteins by measuring the sensitivities of viruses with these envelope glycoproteins to neutralization by several monoclonal antibodies. Viruses with the ADA N197S and ADA N197S-H66N envelope glycoproteins exhibited similar degrees of sensitivity to the carbohydrate-directed antibody 2G12 (72, 87) and to F105, an antibody directed against the CD4 binding site of gp120 (67, 85) (Fig. 5). The viruses with the ADA N197S envelope glycoproteins were slightly more sensitive to neutralization by the 2F5 antibody than viruses with the ADA N197S-H66N envelope glycoproteins. The 2F5 antibody recognizes a membrane-proximal epitope in the gp41 ectodomain (9, 15, 60).

In contrast to the results with the antibodies described above, the viruses with the ADA N197S-H66N envelope gly-
coproteins were significantly more resistant to neutralization by three CD4i antibodies, 48d, 412d, and 17b (Fig. 5). Because of steric constraints, HIV-1 neutralization by CD4i antibodies must occur prior to the engagement of CD4 on the target cell by virus (49, 83). Thus, our results suggest that, in the absence of bound ligand, the ADA N197S-H66N envelope glycoproteins on the virus are recognized less efficiently than the ADA N197S envelope glycoproteins by CD4i antibodies.

To obtain more quantitative information on the effect of the H66N change on recognition by CD4i antibodies, we used an SPR assay to measure the binding of gp120 glycoproteins to the 17b antibody captured on a Biacore chip. In the experiment with results shown in Fig. S4A in the supplemental material, the same concentrations of ADA N197S and ADA N197S-H66N gp120 glycoproteins were incubated in the absence or presence of sCD4 prior to exposure to the captured 17b antibody. In the absence of sCD4, the binding of the ADA N197S-H66N gp120 glycoprotein to the 17b antibody was much lower than that of the ADA N197S glycoprotein. The H66N change reduced the affinity of the ADA N197S gp120 glycoprotein for the 17b antibody by more than 60-fold (see Fig. S4B in the supplemental material). This decreased affinity resulted from a slower association between gp120 and 17b as well as a faster dissociation of the complex. Similar results were obtained for the wild-type and H66N variants of the KB9 gp120 glycoprotein, which is a CD4-dependent envelope glycoprotein (see Fig. S4C in the supplemental material). Thus, in the absence of sCD4, HIV-1 gp120 with the H66N change is recognized only weakly by the 17b antibody. In the presence of sCD4, however, the ADA N197S-H66N gp120 glycoprotein bound the 17b antibody nearly as efficiently as the ADA N197S glycoprotein (see Fig. S4B in the supplemental material).

In summary, the H66N change decreases the efficiency with which unliganded HIV-1 gp120 samples the CD4-bound conformation recognized by CCR5 and the CD4i antibodies.

**Effects of H66N on sensitivity of CD4-independent envelope glycoproteins to sCD4.** The sensitivity of CD4-independent viruses to neutralization by sCD4 was examined. These experiments were conducted with both CD4-positive and CD4-negative target cells.

The basal levels of infectivity of viruses with the ADA N197S and ADA ΔV1/V2 envelope glycoproteins and their H66N counterparts were comparable with C2Th-CD4/CCR5 target cells (see Fig. S2B in the supplemental material). Both two-domain and four-domain sCD4 proteins inhibited the ADA N197S-H66N viruses less efficiently than the ADA N197S viruses (Fig. 6A and data not shown). In fact, mild enhancement of the virus with the ADA N197S-H66N envelope glycoproteins was evident at low concentrations of sCD4. Likewise, the viruses with the ADA ΔV1/V2-H66N envelope glycoproteins were significantly more resistant to sCD4 than the viruses with the ADA ΔV1/V2 envelope glycoproteins (see Fig. S3B in the supplemental material). Thus, H66N confers a degree of sCD4 resistance to both CD4-dependent and CD4-independent HIV-1 viruses in the course of infection of CD4-expressing target cells.

In CD4-negative target cells expressing only CCR5, the basal levels of infection of the viruses with the ADA N197S and ADA ΔV1/V2 envelope glycoproteins were significantly higher than those of viruses with the H66N-modified envelope glycoproteins on the virus are recognized less efficiently than the ADA N197S envelope glycoproteins by CD4i antibodies.
glycoproteins (Fig. 4A; also see Fig. S3C in the supplemental material). Incubation with sCD4 neutralized the viruses with the ADA N197S and ADA ΔV1/V2 envelope glycoproteins but enhanced the infection of viruses with the ADA N197S-H66N and ADA ΔV1/V2-H66N envelope glycoproteins (Fig. 6B; also see Fig. S3C in the supplemental material). Two-domain sCD4 and four-domain sCD4 exhibited similar effects in this assay (data not shown). In the setting of CD4-independent infection, the potential inhibitory effects of sCD4 resulting from competition with membrane-bound CD4 for gp120 binding are eliminated. Thus, the observed sCD4 inhibition of the CD4-independent viruses with the ADA N197S and ADA N197S-H66N envelope glycoproteins results solely from detrimental effects of sCD4 binding on these envelope glycoproteins. The H66N change apparently prevents or diminishes these detrimental effects and reveals the activating effects of sCD4 binding on virus entry. The sCD4 activation of infection of CD4-negative cells by CD4-independent viruses with the H66N change is reminiscent of that seen for viruses with natural HIV-1 envelope glycoproteins modified by H66N (Fig. 2).

Stability of the sCD4-induced state of the viral envelope glycoproteins. The above-described studies support the existence of both positive and negative effects of sCD4 binding on HIV-1 entry; the phenotypes of the H66N variants provided an opportunity to examine the stability of the sCD4-induced envelope glycoprotein states. Recombinant viruses with the ADA N197S or ADA N197S-H66N envelope glycoproteins were incubated with sCD4 for 1 h at 37°C, and then the viruses were pelleted and washed repeatedly to ensure removal of sCD4 from the medium. The resuspended viruses were incubated at 37°C and, at different times, added to Cf2Th-CCR5 cells. The resultant levels of infection are shown in Fig. 6C. In agreement with the above-described observations, sCD4 enhanced the infection of viruses with the ADA N197S-H66N envelope glycoproteins by 10-fold but inhibited infection by viruses with the ADA N197S envelope glycoproteins by more than 40-fold. The activated state of the ADA N197S-H66N envelope glycoproteins persisted for at least 3 h, the duration of the experiment. The inhibitory effect of sCD4 on viruses with the ADA N197S envelope glycoproteins occurred by the time of the first measurement of infection and persisted thereafter. Thus, the sCD4-activated state of the ADA N197S-H66N envelope glycoproteins and the sCD4-induced, functionally inactivated state of the ADA N197S envelope glycoproteins are both long-lived.

Effect of the H66N change on sCD4 binding to the envelope glycoprotein trimer and induction of gp120 shedding. The above-described results suggest that the major effect of the H66N change on the resistance of HIV-1 to sCD4 neutralization is mediated by stabilization of a sCD4-induced activated form of the envelope glycoproteins, minimizing the probability of transitions to less functional states. To examine this possibility further, we studied the binding of sCD4 to HIV-1 envelope glycoprotein trimers and the sCD4-induced, functionally inactivated state of the ADA N197S envelope glycoproteins are both long-lived.

![FIG. 7. Effect of H66N on sCD4 binding and induction of gp120 shedding. (A) 293T cells transiently expressing cytoplasmic-tail-deleted ADA N197S, ADA N197S-H66N, KB9, or KB9-H66N envelope glycoproteins were incubated at 37°C for 1 h with sCD4 at the indicated concentrations. After the cells were washed, the bound sCD4 was detected by FACS analysis with OKT4, a fluorescein isothiocyanate-conjugated anti-CD4 antibody. The mean fluorescence intensity (MFI) values at different sCD4 concentrations are shown. Mock-transfected cells (Mock) were included as a negative control. (B) 293T cells transiently expressing cytoplasmic-tail-deleted KB9 or KB9-H66N envelope glycoproteins or ADA N197S or ADA N197S-H66N envelope glycoproteins were radiolabeled and incubated either in the absence of sCD4 or with 200 or 1,000 nM sCD4 at 37°C for 1 h. The gp120 shed into the supernatant was precipitated by a polyclonal mixture of sera from HIV-1-infected individuals and analyzed by SDS-PAGE.](https://example.com/fig7.png)
counterparts yielded similar results (see Fig. S5 in the supplemental material).

One detrimental effect of sCD4 binding is the shedding of gp120 from the HIV-1 envelope glycoprotein trimer (22, 26, 31, 56, 58, 59). To test whether the H66N change affects the propensity of the HIV-1 envelope glycoproteins to shed gp120 in response to CD4 binding, we expressed radiolabeled functional envelope glycoproteins on the surface of 293T cells. The gp41 cytoplasmic tail was removed from these envelope glycoproteins to enhance cell surface expression (28, 101). Cells expressing KB9, KB9-H66N, ADA N197S, and ADA N197S-H66N envelope glycoproteins were incubated with different concentrations (200 and 1,000 nM) of sCD4 at 37°C for 1 h. Media were harvested and examined for shed gp120 by immunoprecipitation with a mixture of sera from HIV-1-infected individuals. The addition of sCD4 resulted in an increase in the shedding of gp120 into the medium. The H66N change in KB9 and ADA N197S envelope glycoproteins decreased the amount of shed gp120 (Fig. 7C). Expression levels and processing efficiencies of the various envelope glycoproteins were equivalent (see Fig. S6 in the supplemental material).

These results suggest that the H66N change allows efficient sCD4 binding to the HIV-1 envelope glycoprotein trimer but decreases the degree of gp120 shedding consequent to sCD4 binding.

Effects of the H66N change on susceptibility to a small-molecule CD4 mimic. NBD-556 is a low-molecular-weight compound that can induce conformational changes in the HIV-1 gp120 glycoprotein similar to those that occur upon CD4 binding (52, 74, 103). NBD-556 can enhance HIV-1 infection of CD4-negative, CCR5-positive cells and weakly inhibits infection of CD4-expressing cells (52, 74, 103).

The effects of NBD-556 on the infectivity of viruses with the wild-type KB9 or KB9 H66N envelope glycoproteins were examined using C127-Th-CD4/CCR5 and C127-Th-CCR5 target cells. NBD-556 inhibited the infection of viruses with the KB9 envelope glycoproteins in C127-Th-CD4/CCR5 cells but did not significantly affect the infectivity of viruses with the KB9-H66N envelope glycoproteins (Fig. 8A). With the CD4-negative C127-Th-CCR5 cells, NBD-556 dramatically enhanced infection of viruses bearing the wild-type KB9 envelope glycoproteins but did not affect the infectivity of viruses with the KB9-H66N envelope glycoproteins (Fig. 8B). These results indicate that the H66N change renders the HIV-1 envelope glycoproteins resistant to both the negative and the positive effects of NBD-556 on viruses with the corresponding wild-type envelope glycoproteins.

One explanation for the above-described observations is that the H66N change interferes with the binding of NBD-556 to the gp120 glycoprotein. To test this, isothermal titration microcalorimetry studies were conducted with NBD-556 and the wild-type and H66N gp120 glycoproteins from the KB9 HIV-1 strain (Table 3). The H66N change in gp120 reduced the NBD-556 binding affinity to below the detection limits of the assay, explaining the resistance of the virus with the KB9 H66N envelope glycoproteins to the effects of NBD-556. This result is also consistent with the observations made using another virus with the CD4-dependent ADA envelope glycoproteins, in which the H66N change rendered the virus resistant to the effects of NBD-556 (Fig. 8C and D). In contrast, for the CD4-independent ADA N197S envelope glycoproteins, where removal of a sugar in the V1/V2 stem allows CCR5 binding without prior binding to CD4 (43), NBD-556 binding and entry enhancement were not eliminated by the H66N change (Fig. 8E and F; Table 3). Thus, histidine 66 promotes the transition of free gp120 into the CD4-bound conformation, a process that contributes to the ability of NBD-556 to bind gp120 and induce this conformation. The CD4-independent gp120 glycoprotein has a greater propensity to make the transition into the CD4-bound conformation.
bound state than the CD4-dependent envelope glycoprotein. As a likely consequence, the H66N change is less effective in preventing the sampling of the CD4-bound conformation by the CD4-independent envelope glycoprotein.

DISCUSSION

The H66N gp120 variant of HIV-1<sub>kEB5</sub> was derived by repeated selection of viruses by extremes of temperature; the effect of the change in histidine 66 on the temperature stability of HIV-1 has been described elsewhere (37). Here, we demonstrate that the H66N change in gp120 affects several of the biological properties of HIV-1 related to the induction of the CD4-bound state and the functional consequences of CD4 binding. The phenotypic effects of the H66N change in the envelope glycoproteins derived from several different HIV-1 strains were similar. This observation and the highly conserved nature of histidine 66 (see Fig. S1 in the supplemental material) support the generality of the results and suggest that HIV-1 strains have conserved mechanisms whereby changes in receptor binding trigger conformational transitions. Some of the CD4-induced conformational transitions, such as those that allow CCR5 binding or result in the exposure of the HR1 groove in the gp41 ectodomain, play positive roles in infection (12, 27, 45). Other CD4-induced transitions lead to inactivation of the HIV-1 envelope glycoprotein trimer and, in extreme cases, involve the shedding of gp120 (30, 31, 56).

For most wild-type, primary HIV-1 envelope glycoproteins, competence for CCR5 binding and priming of the gp120-gp41 interaction occur only rarely in a spontaneous fashion; thus, CD4 binding is required to allow these events, and virus entry, to proceed (17). CD4-independent HIV-1 variants generated in the laboratory negotiate these transitions spontaneously, thus bypassing the need for CD4 binding (43, 44). The H66N change reverts the CD4-independent envelope glycoproteins back to envelope glycoproteins with CD4-dependent phenotypes. This reversion is evident in a decrease in spontaneous gp120 binding to CCR5; in reduced infection of CD4-negative, CCR5-expressing cells; and in decreased sensitivity to CD4i antibodies, which recognize the CD4-bound conformation of gp120 (13, 84, 96). These properties suggest that the unliganded CD4-independent envelope glycoproteins with asparagine 66 spontaneously sample the CD4-bound conformation less than their counterparts with histidine 66. This implies that, in the absence of sCD4, the H66N change either destabilizes the CD4-bound conformation or stabilizes unliganded gp120 conformations other than the CD4-bound state. We favor the former model for two reasons: (i) it is more consistent with our observation that the H66N change selectively decreases sensitivity to neutralization by CD4i antibodies and does not affect sensitivity to the F105 CD4 binding site antibody, and (ii) it proposes a positive function for the highly conserved histidine 66, i.e., stabilization of the spontaneously sampled CD4-bound conformation. Such stabilization could assist the flexible, unliganded HIV-1 gp120 glycoprotein to make secondary contacts with CD4 after the initial, weak contact of the two molecules (27, 45, 77, 92). This explanation is consistent with the observation that alteration of histidine 66 primarily increases the dissociation of gp120-sCD4 complexes, with no apparent effect on the rate of association of the two proteins.

Both CD4-dependent and CD4-independent HIV-1 envelope glycoproteins with the H66N change exhibited decreased susceptibility to the detrimental effects of sCD4 on virus infection. Some part of this resistance to sCD4 inhibition may arise as a consequence of the small differences in ability to bind CD4 between wild-type and H66N gp120 glycoproteins. If the ~2-fold difference in CD4-binding affinity between monomeric wild-type and H66N gp120 glycoproteins applies, the levels of occupancy of the envelope glycoprotein trimers should be similar at high CD4 concentrations. Indeed, the levels of binding of sCD4 to wild-type and H66N envelope glycoprotein complexes expressed on cell surfaces were indistinguishable. These and other results suggest that the H66N envelope glycoproteins differ from their unaltered counterparts in the consequences of CD4 binding. The CD4-bound HIV-1 envelope glycoproteins represent metastable intermediates in the virus entry process and make further transitions that either promote virus entry (activation pathway) or lead to nonfunctional states (inactivation pathway[s]) (30, 31, 56). The variables that determine which of these pathways predominates are still being elucidated but presumably include the availability and binding affinity of the chemokine receptor and the intrinsic strength of the interactions that stabilize the CD4-bound state of the envelope glycoprotein trimer. It is noteworthy that when CD4 and CCR5 were present at high concentrations on the target cells, there were minimal differences in infectivity of viruses with unmodified and H66N envelope glycoproteins. In contrast, with CD4<sup>−</sup>CCR5<sup>−</sup> target cells, when sCD4 was first incubated with virus so that the time between CD4 binding and chemokine receptor binding was prolonged, dramatic differences in infectivity between the wild-type and H66N viruses were evident. Whereas the wild-type viruses were inactivated by sCD4 under these circumstances, infection by viruses with the H66N envelope glycoproteins was enhanced. This observation suggests that the H66N change may decrease the efficiency of envelope glycoprotein transitions to less functional states, thus favoring transitions to membrane-fusogenic states promoted by chemokine receptor binding. In other studies, wild-type HIV-1 envelope glycoproteins induced to assume the CD4-bound conformation at a distance from a target cell were shown to undergo a short-lived activation followed by irreversible inactivation (30). By limiting the ability of the HIV-1 envelope glycoproteins to assume the full CD4-bound state, the H66N change may decrease the likelihood of negotiating transitions into inactive states. This model is consistent with the H66N envelope glycoprotein being selected on the basis of improved temperature stability of the virus (37). Thus, resistance to generic destabilizing influences, such as temperature extremes, can be achieved by avoidance of the labile CD4-bound conformation.

The other interesting phenotype associated with the H66N change in CD4-dependent envelope glycoproteins is resistance to the CD4-mimetic compound NBD-556. This drug inhibits virus entry into cells by competitive inhibition of CD4 binding (103) and by prematurely triggering metastable activated envelope glycoprotein states that decay into inactive forms (30). Binding of NBD-556 to gp120 induces conformational changes similar to those associated with CD4 binding, albeit to a lesser extent (66). When introduced into the gp120 glycoprotein of a CD4-dependent virus, the H66N change completely eliminated
binding to NBD-556, whereas this change only slightly reduced binding affinity for CD4 itself. Apparently, the contribution of histidine 66 to the achievement of the CD4-bound state is critical for the ability of a small molecule like NBD-556 to induce this state in a CD4-dependent envelope glycoprotein. Presumably, the greater number of gp120 contacts made by CD4 compensates more effectively for the loss of the histidine 66 contribution to the gp120 transition to the CD4-bound state.

Of interest, the H66N derivative of a gp120 glycoprotein from a CD4-independent HIV-1 bound NBD-556 efficiently. Apparently, the gp120 change (N197S or ΔV1/V2) that allows CD4 independence compensates for the lack of histidine 66 and allows NBD-556 to bind gp120 and drive it into the CD4-bound conformation. The magnitudes of the NBD-556-induced entropic and enthalpic changes in CD4-independent gp120 are lower than those observed for CD4-dependent gp120, consistent with a model in which the former gp120 glycoproteins have spontaneously negotiated a portion of the conformational transition to the CD4-bound state.

What is the structural explanation for the observed effects of the H66N change? As the H66N alteration can exert phenotypes independently of the gp120 V1/V2 variable loops, insights into histidine 66 function should be possible without consideration of the V1/V2 loops, which have been deleted from the “gp120 core” proteins for which structures are currently available (11, 47, 48). Although CD4-bound structures of the HIV-1 gp120 core and unliganded structures of the SIV gp120 core have been determined, the gp120 N-terminal segment containing histidine 66 was deleted from these constructs to allow crystallization (11, 48). Recently, however, the structure of the HIV-1 gp120 glycoprotein with intact termini has been solved in the CD4-bound state (Pancera et al., unpublished). The disulfide-bonded loop in which histidine 66 resides is part of the gp120 inner domain (48, 95) (Fig. 9). In the envelope glycoprotein complex, this disulfide-bonded loop is thought to be located near the trimer axis and thus may be proximal to the gp41 ectodomain. Although some elements of the gp120 inner domain are thought to contact gp41 (Pancera et al., unpublished), the histidine 66 side chain is not surface exposed in the CD4-bound conformation. Rather, histidine 66 projects from the α0 helix in the disulfide-bonded loop into the interior of the inner domain, contacting residues phenylalanine 210 and proline 212 (Fig. 9). Modeling of the wild-type and H66N gp120 glycoproteins suggests that histidine 66 makes stacking interactions with phenylalanine 210 and proline 212 that are predicted to stabilize the CD4-bound conformation (see Fig. S7 in the supplemental material). These interactions are lost upon substitution of an asparagine residue at position 66, resulting in destabilization of the CD4-bound state. The modeling predictions are consistent with multiple observations, discussed above, that suggest that the H66N gp120 spontaneously samples the CD4-bound conformation less efficiently than the wild-type gp120 glycoprotein. Examination of the gp120-CD4 complex structure shown in Fig. 9 rules out any direct contact between histidine 66 and CD4; based on recent mapping of the NBD-556 binding site (52), histidine 66 is not likely to contact this compound either. Thus, the effects of the H66N change on CD4/NBD-556 interactions with the HIV-1 envelope glycoproteins likely arise indirectly, by influencing associations within the inner domain that promote the attainment of the CD4-bound state. Of interest, the H66N change does not affect the on-rate of the CD4 interaction with gp120, which is thought to involve contacts primarily between CD4 and the gp120 outer domain (54). Rather, H66N increases the dissociation rate of the gp120-CD4 complex. This observation is consistent with a model in which the folding of the gp120 inner domain and bridging sheet stabilizes the complex with CD4. Thus, histidine 66, by contributing to interactions within the gp120 inner domain, helps gp120 to achieve the CD4-bound conformation from its previously unliganded state.

How might the H66N change prolong the stability of the activated state induced on the viral envelope glycoproteins by sCD4? The longevity of this activated state of infectivity has recently been shown to be correlated with the duration of sCD4-induced exposure of gp41 ectodomain sequences (30). Stabilization of this activated intermediate would presumably require modification of the gp120-gp41 interactions involved in governing conformational transitions in the envelope glycoproteins. Histidine 66 is strategically positioned between the putative gp41-interactive surface of gp120 and the binding site for CD4 and is thus potentially available to influence the consequences of CD4 binding on gp41 conformation. However, in the CD4-bound state, the histidine 66 side chain is completely buried and therefore cannot contact gp41. It is possible that the interactions of histidine 66 with other inner domain residues could indirectly influence the spatial relationship of gp120 and gp41. Alternatively, in the unliganded gp120 glycoprotein
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REFERENCES
