Mode of Action for Linear Peptide Inhibitors of HIV-1 gp120 Interactions†

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ABSTRACT: The linear peptide 12p1 (RINNIPWSEAMM) was previously isolated from a phage display library and was found to inhibit interaction of HIV-1 gp120 with both CD4 and a CCR5 surrogate, mAb 17b [Ferrer, M., and Harrison, S. (1999) J. Virol. 73, 5795–5802]. In this work, we investigated the mechanism that leads to this dual inhibition of gp120 binding. We found that there is a direct interaction of 12p1 with gp120, which occurs with a binding stoichiometry of 1:1. The peptide inhibits binding of monomeric YU2 gp120 to both sCD4 and 17b at IC50 values of 1.1 and 1.6 µM, respectively. The 12p1 peptide also inhibited the binding of these ligands to trimeric envelope glycoproteins, blocked the binding of gp120 to the native coreceptor CCR5, and specifically inhibited HIV-1 infection of target cells in vitro. Analyses of sCD4 saturation of monomeric gp120 in the presence or absence of a fixed concentration of peptide suggest that 12p1 suppression of CD4 binding to gp120 is due to allosteric inhibitory effects rather than competitive inhibition of CD4 binding. Using a panel of gp120 mutants that exhibit weakened inhibition by 12p1, the putative binding site of the peptide was mapped to a region immediately adjacent to, but distinguishable from, the CD4 binding footprint. In the case of the peptide, the effects of single-12p1 residue substitutions and various peptide truncations indicate that the side chain of Trp7 and other structural elements of 12p1 are critical for gp120 binding or efficient inhibition of binding of a ligand to gp120. Finally, 12p1 was unable to inhibit binding of sCD4 to a gp120 mutant that is believed to resemble the CD4-induced conformation of gp120. These results suggest that 12p1 preferentially binds gp120 prior to engagement of CD4; binding of the peptide to gp120 limits the interaction with ligands (CD4 and CCR5) that are generally crucial for viral entry. More importantly, these results indicate that 12p1 binds to a unique site that may prove to be a prototypic target for novel CD4—gp120 inhibitors.

The Joint United Nations Programme on HIV/AIDS (UNAIDS) estimates that 42 million people were infected with HIV-1 as of the end of 2002, with 5 million newly infected persons and 3.1 million deaths in 2002. To combat this virus, multiple approaches must be taken; the seemingly few vulnerabilities of HIV may have to be targeted simultaneously to successfully prevent or inhibit viral infection and/or replication.

A primary target is the HIV envelope glycoprotein (Env). The Env spikes on the viral membrane are composed of a gp41 transmembrane trimer and three noncovalently associated gp120 surface glycoproteins. Viral infection is initiated by the interaction of gp120 with the extracellular portion of CD4 on the target cell. The binding of these two proteins promotes a conformational change in gp120 that increases its affinity for a cell surface coreceptor, usually CCR5. This second binding event leads to further conformational changes that culminate in the fusion of the viral and target cell membranes. Blocking the interactions between gp120 and cell surface receptors, then, is an attractive goal for preventing HIV infection.

The HIV-1 Env proteins, however, exhibit unusual features that influence their feasibility as a target. While there is currently no crystal structure of gp120 in the unbound state, calorimetric methods have determined that it undergoes a
large structural rearrangement upon binding of several of its ligands, especially CD4 and antibodies directed against receptor-binding regions (1). Monomeric gp120 has been proposed to be extremely flexible prior to binding; it has been suggested that the inner and outer domains of gp120 are mobile with respect to each other, and the bridging sheet that spans the two domains is unfolded (2). Thermodynamic analyses demonstrate that the binding of CD4 to gp120 causes an unusually large decrease in entropy, indicating significant ordering of the protein (3), and that this structural stabilization is propagated to other regions of the gp120 monomer, being initiated from the CD4 binding site (1). An inhibitor that could take advantage of the unusual flexibility of gp120 might be more effective than a traditional competitive inhibitor. If, for example, a small molecule could prevent gp120 from attaining conformations critical for entry, as has been seen for some CD4 binding site antibodies (4), it could inhibit either the binding of HIV-1 to its receptors or postreceptor binding events involved in fusion of the viral and target cell membranes.

Several small peptide mimetics of CD4 have been reported thus far to inhibit the binding of gp120 to CD4 (1, 5, 6). Most of these, however, have produced conformational changes in gp120 similar to those induced by CD4 and hence have led to activation, to a state with increased coreceptor affinity. In this way, these molecules are successful CD4 competitors, but potentially could facilitate, rather than inhibit, HIV-1 entry. A more successful inhibitor might not only inhibit CD4–gp120 binding but also either physically block the gp120–coreceptor interaction or prevent the activating conformational change that gp120 must undergo to efficiently bind CCR5.

Ferrer and Harrison (7) screened a random phage library of 12-mer peptides for candidates that would bind to gp120. They discovered a sequence, RINNIPWSEAMM (12p1), that bound to gp120 and inhibited its interaction not only with CD4 but also with 17b, an antibody that recognizes an epitope overlapping the CCR5 binding site. Here we extend this study with a number of goals: (a) to quantitate the degree of co-inhibition of CD4 and 17b binding, (b) to demonstrate the direct binding of the peptide to gp120 and determine its stoichiometry, (c) to determine which residues are critical for gp120 binding and ligand inhibition, (d) to determine the site of interaction on gp120, (e) to examine interaction of 12p1 with the Env trimer, including functional virion trimers, and (f) to investigate the influence of gp120 conformation on peptide binding. A variety of modifications and truncations of the parent 12p1 peptide were made to identify residues that are critical for binding gp120. The peptides were studied both with a biosensor for effects on monomeric gp120 binding and with an ELISA for effects on binding to stable gp120 trimers.

The results confirmed that 12p1 inhibits interaction of gp120 with both CD4 and 17b, as well as CCR5, and showed that its binding exhibits 1:1 stoichiometry at inhibitory concentrations. We found that several residues, especially Trp7, are critical for 12p1 inhibition of binding of gp120 to CD4 and 17b, and that truncation of the peptide also weakens its inhibitory effectiveness. We also investigated the mode of action of 12p1. The 12p1 peptide appears to bind an unactivated (CD4-unbound) conformation of gp120, resulting in noncompetitive inhibition of the binding of ligands that induce or prefer the activated (CD4-bound) state. This suggests that limiting the ability of gp120 to assume an activated conformation may contribute to the mode of 12p1 inhibition of HIV-1 entry.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification. Peptides were initially obtained from American Peptide Co. (Sunnyvale, CA). These peptides were synthesized, purified by reverse phase HPLC (RP-HPLC), and validated by electrospray MS and amino acid analysis. We synthesized additional peptides using solid phase peptide synthesis on an Applied Biosystems 433A peptide synthesizer. Peptides were made at the 0.1 mmol scale on Rink amide resin using FastMoc synthesis, as described previously (1). Peptides were cleaved from the resin with 10 mL of trifluoroacetic acid, 0.5 mL of H2O, 0.5 mL of ethanedithiol, and 0.25 mL of thioanisole for 2 h at room temperature (RT). Resin was filtered out, and the peptide was precipitated with cold ether. Dried, solid peptide was dissolved in 10% acetic acid and purified by RP-HPLC on a C18 preparative column (Vydac). The peak with the desired peptide was verified by MALDI-MS (performed at the Wistar Institute, Philadelphia, PA). HPLC fractions were lyophilized, and the solid peptide was dissolved in phosphate-buffered saline (PBS) at pH 7.4. The peptide concentration and amino acid composition were determined by amino acid analysis (performed at the Howard Hughes Medical Institute/Keck Biotechnology Resource Laboratory, Yale University, New Haven, CT).

Biosensor Experiments. All surface plasmon resonance (SPR) experiments were performed on a BIA3000 optical biosensor (Biacore, Inc., Uppsala, Sweden), in a manner similar to that in previous studies (1). A CM5 sensor chip was derivatized by amine coupling with either the CD4 extracellular domain (sCD4), mAb 17b Fab, or gp120 YU2, using mAb 2B6R Fab (to IL-5 receptor α) as a control surface. For competition experiments, ligands were immobilized to a surface density of approximately 800–1000 RU. The indicated analytes were passed over the surfaces at a flow rate of 50 μL/min, for 2.5 min, followed by a 2 min dissociation phase. Surfaces were regenerated with 35 mM NaOH and 1.3 M NaCl for sCD4, and 10 mM HCl for 17b and 2B6R. For direct binding experiments with a YU2 gp120 surface (2000 RU), a flow rate of 5 μL/min was used, with a 5 min association phase and a 2 min dissociation phase. CD4 saturation analyses in the presence or absence of 100 μM 12p1 were performed on a YU2 gp120 surface (2400 RU). sCD4 or sCD4/12p1 analyte mixtures were injected over the YU2 gp120 surface at a rate of 5 μL/min for 5 min, followed by a 5 min dissociation phase. YU2 gp120 surfaces were regenerated with 35 mM NaOH and 1.3 M NaCl. Buffer injections and control surface binding were subtracted for all reported data. Experiments were performed twice in duplicate.

Biosensor Data Analysis. The initial rate (νi) of binding of gp120 to either sCD4 or 17b in the presence or absence of peptide was determined during the period of the association phase from 6 to 21 s. The slope of that line (RU/s, νi) at each peptide concentration was calculated using Biacal 3.0 software (Biacore). The fraction of the νi of gp120 binding in the presence versus absence of peptide was
determined for each peptide concentration. For determination of the IC50 of 12p1, these fractions were plotted against the log of the 12p1 concentration. Curves were fit using SigmaPlot (SPSS, Inc.), and the 12p1 concentration at which the v1 of gp120 binding was half of that without peptide was designated the IC50. For direct binding of the peptide to gp120, Bioeaval software was used to determine R_eq at equilibrium (during the period of the association phase from 285 to 295 s) for each peptide concentration. R_eq was plotted versus 12p1 concentration, and the software calculated R_max for 1:1 binding. R_max was estimated for comparison by performing the identical experiment with sCD4 and calculating R_max for 12p1 based on its mass difference with sCD4, as R_max is proportional to mass. Sensorgrams obtained from sCD4 saturation analyses also were fit to a 1:1 binding model using Bioeaval software, and resultant R_eq values were plotted against sCD4 concentration. Saturation curves of R_eq versus sCD4 concentration. Saturation curves of R_eq at 37°C for 2 h. After being washed, the cells were reseeded at a density of 6 x 10^3 cells/well in 96-well luminometer-compatible tissue culture plates (Dynex) 24 h before infection. On the day of infection, the 12p1 peptide (0.3–300 µM) was added to recombinant viruses (10 000 reverse transcriptase units) to 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/peptide mixture for 48 h at 37 °C. The medium was removed from each well, and the cells were lysed with 30 µL of passive lysis buffer (Promega) and by three freeze–thaw cycles. An EG&G Berthold LB 96V microplate luminometer was used to measure luciferase activity of each well after the addition of 100 µL of luciferin buffer [15 mM MgSO4, 15 mM KPO4 (pH 7.8), 1 mM ATP, and 1 mM dithiothreitol] and 50 µL of 1 mM d-luciferin potassium salt (BD Pharmingen).

RESULTS

Peptide 12p1 Inhibits Binding of YU2 gp120 to both CD4 and 17β. It has been previously shown that peptide 12p1 (sequence shown in Table 1) inhibits the binding of gp120 to CD4 (by ELISA) and 17β (by SPR) (7). We sought to establish a quantitative assay for determining the extent of inhibition by 12p1 and variant peptides. To assess the inhibition of binding of gp120 to CD4 and 17β, we examined
the interactions by surface plasmon resonance (SPR) using a Biacore 3000 instrument. The analyte, YU2 gp120 (50 nM) in the absence or presence of increasing amounts of peptide, was passed over immobilized sCD4, 17b Fab, and control 2B6R Fab. Under the conditions that were used, 12p1 exhibited no direct binding to either sCD4 or 17b (data not shown). As shown in Figure 1, increasing concentrations of 12p1 significantly inhibited the binding of YU2 to both sCD4 and 17b surfaces. The fraction of the initial rate of YU2 binding in the presence or absence of peptide (taken to be 1.0) was determined for each peptide concentration and plotted as a function of the log of the peptide concentration (Figure 1C). Using SigmaPlot software, the IC50 values for 12p1 inhibition of binding of gp120 to sCD4 and 17b were determined to be 1.1 and 1.6 \(\mu M\), respectively. The value for sCD4 inhibition is more than 20-fold lower than that reported by Ferrer and Harrison for HxBc2 gp120 (as measured with an ELISA), but is in line with the values reported for strains SF2 and ADA (7).

Peptide 12p1 Binds Directly to YU2 gp120. We next used SPR to verify that 12p1 binds directly to gp120 and to determine the binding stoichiometry at the peptide’s effective concentration. Increasing concentrations of 12p1 were passed over a high-density (2000 RU) YU2 gp120 surface (Figure 2A). The binding data fit to those expected of a bimolecular binding process with a \(K_D\) of 3.7 \(\mu M\) (Figure 2B). Using sCD4 as a standard, the expected \(R_{\text{max}}\) for 12p1, assuming 1:1 binding, was 26.7 RU. Although the response for peptide binding exceeded 30 RU at the highest 12p1 concentrations, the \(R_{\text{max}}\) at 100 \(\mu M\) peptide (roughly 30-fold greater than \(K_D\)) did not exceed the expected stoichiometric value. Therefore, we believe that the effect of the peptide is a result of 1:1 peptide–gp120 binding and not of an aggregation of the peptide, or of the peptide binding to multiple sites on gp120.

Using this same high-density YU2 gp120 surface, we confirmed the inhibition of gp120–CD4 binding in the reverse orientation compared with that in Figure 1A. Figure 3 shows the inhibition of binding of sCD4 to surface-immobilized YU2 gp120 by increasing concentrations of 12p1. The fraction of the initial rate of CD4 binding in the presence or absence of peptide (taken to be 1.0) was determined for each peptide concentration and plotted as a function of the log of the peptide concentration (Figure 3C). Using SigmaPlot software, the IC50 values for 12p1 inhibition of binding of gp120 to CD4 were determined to be 1.2 \(\mu M\), respectively. The value for sCD4 inhibition is more than 20-fold lower than that reported by Ferrer and Harrison for HxBc2 gp120 (as measured with an ELISA), but is in line with the values reported for strains SF2 and ADA (7).
Peptide 12p1 Inhibition Follows an Allosteric Mode. We investigated the mode of inhibition of binding of CD4 to gp120 by 12p1 via biosensor experiments. Soluble four-domain CD4 (ImmunoDiagnstics, Inc.), in the range of 0–3.5 μM, was passed over a high-density YU2 gp120 surface (2400 RU) in the presence or absence of 100 μM 12p1. The resultant sensorgrams were analyzed using Biareval 3.0 and fitted to a 1:1 binding model to obtain equilibrium binding constants $K_D$ and $K_D$. Under the conditions that were used, the presence of 12p1 had no discernible effect on the kinetics of interaction between YU2 and CD4 ($K_D$, values of 721 and 429 nM in the presence and absence of the peptide, respectively) but had a marked effect on the total amount of sCD4 bound by YU2.

In addition to buffer and control surface subtraction, the binding of peptide alone at each concentration has been subtracted from the corresponding curve plus sCD4. 12p1 clearly inhibits gp120–CD4 binding with either gp120 or sCD4 immobilized.

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**Variants of 12p1 Have Reduced Inhibitory Activity.** Ferrer and Harrison previously determined, through Ala scanning mutations of 12p1, that the peptide sequence was important for its inhibitory activity (7). Replacement of any of the interior residues (NNIPWPS) was especially detrimental to peptide inhibitory function. The effect of sequence was explored further by synthesizing a number of variants of peptide 12p1 (Table 1). Peptides were tested for their abilities to inhibit gp120 binding as described above for 12p1. All of the variations had effects on the inhibitory abilities of the peptides, to differing extents. Two peptides, [I5dA]12p1 and [W7F]12p1, showed no significant inhibitory activity toward either sCD4 or 17b binding at concentrations of up to 125 μM (data not shown). Other peptides that had truncations from either the N- or C-terminus, or that contained altered residues closer to either terminus, were still able to inhibit gp120–CD4 or -17b binding, although with reduced apparent affinities (Figure 5). Peptides 12p1(1–10), 12p1(1–8), 12p1(2–8), and [R1A]12p1 (panels A–D of Figure 5, respectively) retained inhibitory activity against 17b and sCD4–gp120 binding, but only at much higher concentrations compared with 12p1. The initial rates in the presence of each peptide were approximately half that of gp120 alone at 25, 68.9, 100, and 69 μM, respectively. In each case, the ratio of sCD4 to 17b inhibition at every peptide concentration is very close to 1, similar to the pattern of inhibition observed for 12p1. These data indicate that sequence elements spanning the entire peptide contribute to inhibitory activity.

We then investigated whether the lack of inhibition of binding of gp120 by peptides [I5dA]12p1 and [W7F]12p1 was due to an inability to bind to gp120. High concentrations of the two different peptides were used as the analyte with the high-density YU2 gp120 surface, as described above for 12p1. Both peptides were able to bind to gp120, but only at concentrations much higher than those of the other peptides, and much higher than the concentrations at which they were tested in the inhibition assays (not shown). For example, at 1.76 mM, 1000-fold higher than the 1Cp of 12p1, the $R_{max}$ for binding of [I5dA]12p1 to gp120 was only approximately half of the value expected for 1:1 stoichiometry. It is likely, then, that the peptides’ lack of inhibition of gp120 binding is due to their severely reduced affinities for gp120.

**Effects of the Peptide on Trimeric Envelope Glycoprotein Interactions.** Because gp120 exists on the virus as a trimer,
we sought to test the inhibitory effectiveness of 12p1 with this more naturally relevant form of gp120. For the ELISA-based assays, soluble, stabilized HIV-1 envelope glycoprotein trimers (HxBc2 strain) were captured on the plate via an immobilized antibody to their C-terminal C9 peptide tags. Peptide competition for the binding of biotinylated sCD4 was assessed. As shown in Figure 6A, the peptides had similar effects on envelope glycoprotein trimer-CD4 binding as they did on monomeric gp120 binding in the biosensor analyses. Peptide 12p1 was the most potent inhibitor, followed by 12p1(1–10) and the other truncated peptides. As in the biosensor assays, [I5dA]12p1 and [W7F]12p1 did not inhibit binding of CD4 to the envelope glycoprotein trimers. An additional peptide, [W7A]12p1, also did not inhibit the trimer-CD4 interaction. These results are consistent with those from the biosensor assays.

The group of peptides also was tested for their effects on sCD4 binding to a mutant envelope glycoprotein trimer with an alteration in gp120, S375W. This gp120 mutant was designed with the intention of filling the cavity that is partially occupied by Phe43 of CD4 in the gp120-CD4 complex (12, 13). The S375W change appears to promote a gp120 conformation that resembles the activated, CD4-bound, state (13). Previous studies have shown that this mutant binds sCD4 more readily than wild-type gp120 and shows a slight increase in the level of CCR5 binding in the absence of sCD4 (13). None of the peptides, including 12p1, were able to inhibit binding of sCD4 to the S375W trimeric envelope glycoprotein (Figure 6B).

Localization of Peptide Binding. In an effort to further elucidate the nature of the binding of 12p1 to gp120, we used a modification of the ELISA described above to determine peptide competition with a panel of human anti-gp120 mAbs. Peptide and antibody were added to the immobilized trimers simultaneously, and bound antibody was detected with an HRP-conjugated goat anti-human antibody. Figure 7A shows the binding of four different antibodies to the envelope glycoprotein trimers in the absence or presence of peptides 12p1 and [R1A]12p1. Antibody 2G12 recognizes a discontinuous glycosylated epitope in the gp120 outer domain (14–16). The 17b antibody, which recognizes an epitope overlapping the CCR5 binding region (17), is the same CD4-inducible antibody used in the biosensor experiments. Antibodies F105 and b12 both bind distinct epitopes overlapping the CD4 binding site (18–20). However, F105 preferentially binds an unactivated, or CD4-unbound, conformation of gp120 (13).

With wild-type envelope glycoprotein trimers, antibodies 2G12 and b12 bound well, and the peptides did not affect this binding. 12p1 inhibited the binding of both 17b, as was seen in Figure 1B, and F105. [R1A]12p1 slightly inhibited these interactions as well, but was much less effective. Identical experiments were performed with two mutant envelope glycoprotein trimers with changes in gp120. The S375W variant, as described earlier, favors the CD4-bound, “active”, conformation, while I423P disrupts the CD4-bound conformation (13). As seen in Figure 7B, each antibody in the absence of peptide bound similarly to the S375W and wt trimers, except for F105. This result is consistent with previous studies suggesting that F105 favors an “unactivated” conformation of gp120 and that the S375W mutant approximates a more “activated”, CD4-bound conformation (4, 13). Neither peptide inhibited the binding of 17b to the S375W trimer.
The I423P mutant does not bind CD4 or 17b and is thought to be inefficient at assuming the CD4-bound conformation (13). Here, I423P trimers bound 2G12 and b12 as well as the wild-type gp120, and 12p1 did not significantly affect antibody binding (Figure 7C). 17b did not bind to this “unactivated” gp120 mutant, as previously reported (13).

F105, however, was able to bind I423P trimers very well, and peptide 12p1 inhibited the interaction.

Effects of the Peptide on gp120–CCR5 Interactions. As 12p1 inhibits interactions of gp120 with both CD4 and 17b, we sought to determine if the inhibitory effect on 17b, a CCR5 surrogate, could be extended to CCR5 itself. To test this, we studied the binding of a CD4-independent gp120 (ADA ΔV1/V2) metabolically labeled with [35S]Cys/Met, in the presence or absence of peptides, to CD4-negative target CF2Th cells expressing CCR5 (21). The bound proteins were immunoprecipitated with a mixture of sera from HIV-1-infected individuals and visualized by SDS–PAGE. Figure 8 shows a hierarchy of CCR5 inhibition efficacies similar to that obtained with the biosensor and ELISA for CD4 and 17b inhibition. 12p1 strongly inhibited binding of gp120 to CCR5, with the other peptides exhibiting reduced activity.

The one exception to the above correlation is peptide [R1A]12p1, which exhibited strong inhibition of CCR5 binding, but had levels of 17b and CD4 inhibition much lower than that of 12p1.

Peptide Inhibition of Viral Infection. To determine whether 12p1 inhibits infection of cells by HIV-1 virus, we used a single-round infection assay in which recombinant HIV-1 viruses encoding luciferase are pseudotyped with different envelope glycoproteins. The 12p1 peptide was incubated with recombinant wild-type and mutant viruses, and this virus/peptide mixture was added to target cells expressing the appropriate HIV-1 receptors. The luciferase activity in the cells provided an indication of the efficiency of infection in
the presence of the peptide. 12p1 inhibited infection of cells by both R5 (YU2, JR-CSF, and ADA) and X4 (HxBc2) HIV-1 isolates (Figure 9 and data not shown). The observed inhibition was specific to HIV, as infection with a VSV G-pseudotyped control virus was not inhibited.

The inhibition of several variants of the ADA, YU2, and JR-CSF viruses by 12p1 was examined. In CD4-CCR5-expressing target cells, 12p1 was found to have IC₅₀ values of 8 μM for wild-type ADA and 67 and 90 μM for the CD4-independent [N197S]gp120 and ADA ΔV1/V2 viruses, respectively (data not shown). The 12p1 peptide did not inhibit infection of cells by virus pseudotyped with YU2 [S375W] envelope glycoproteins (data not shown), in agreement with the trimer ELISA data (Figure 6). Finally, while cell infection by a virus pseudotyped with wild-type JR-CSF envelope glycoprotein was inhibited by 12p1, a variant in which Arg476 substituted with Ala was strongly resistant to 12p1 (Figure 10A). Similar substitutions at residues Lys97 and Glu102 showed intermediate resistance (data not shown). These gp120 residues are located near the binding site for CD4 (Figure 10B) but do not make contact with CD4 (12).

**DISCUSSION**

The overall goal of this study was to gain an improved understanding of the properties governing the interaction of peptide 12p1 (RINNIPWSEAMM) with HIV-1 gp120 and, by inference, how it is able to inhibit binding interactions of the multiple function-associated receptor with gp120. We used SPR to quantitate 12p1 binding. We showed that 12p1 inhibits YU2 gp120 binding to both CD4 and 17b with equivalent potencies, with IC₅₀ values of 1.1 and 1.6 μM, respectively (Figure 1). 12p1 inhibition of CD4 binding was found to be noncompetitive (Figure 4), possibly caused by allosteric binding of the peptide to gp120 at a site present in the CD4-unbound form of gp120 that lies adjacent to the site of the CD4 binding footprint (Figure 10B). Importantly, 12p1 inhibition of CD4 and 17b binding (Figures 6A and 7A) was found to extend to trimeric envelope glycoproteins of the HxBc2 strain, a prerequisite for inhibitors targeted to multiple and diverse viral isolates. The IC₅₀ versus CD4 estimated for the trimer using the ELISA (12.5 μM) was slightly higher than that suggested by the SPR data (not shown). The difference in IC₅₀ may reflect differences between monomeric and trimeric forms of the HIV-1 envelope glycoproteins, between the methods, or between the two gp120 strains. The possibility of strain disparity was expected, however, as 12p1 was previously reported to have slightly different efficacies against gp120s derived from different viral strains (7).

A key aim of this study was to determine the stoichiometry of binding of 12p1 to gp120, as this had not been addressed before and the potential usefulness of the peptide as an antagonist lead depends on a well-defined stoichiometry for the peptide inhibitors of HIV-1 gp120 interactions.
binding to gp120. Figure 2 shows that 12p1 indeed binds directly to gp120, and that the extent of this binding is concentration-dependent. Over the range of concentrations at which 12p1 inhibits binding of gp120 to CD4, 17b, and CCR5, the interaction appears to occur with a binding stoichiometry of \( \leq 1:1 \). Although we did see interactions at the highest concentrations of peptide that might fractionally exceed 1:1 binding, these may be due to peptide aggregating with itself. There is no evidence for a \( > 1:1 \) stoichiometry at the lowest concentrations, where inhibition is still very strong. In addition, the observation that 12p1 inhibits CD4 and 17b binding with nearly identical IC\(_{50}\) values supports a single binding site for the peptide. Considering all these data, we conclude that the binding interaction of 12p1 with gp120 is specific and leads to a 1:1 peptide–gp120 complex. 

Another key aim of this investigation was to determine whether the peptide binds in a manner similar to that of CD4 or by an alternative mechanism. The two most critical gp120 binding residues on CD4 are Phe43 and Arg59 (12, 22). The presence of the large aromatic residue Trp, along with the Arg at the N-terminus of 12p1, led us to consider the possibility that the peptide mimics CD4 binding. Making this possibility more likely is the presence of Pro6, which could promote formation of a turn at the Trp7 position that would be analogous to the \( \beta \)-turn containing the critical Phe43 of CD4. The first logical peptide variation, then, was to replace Trp7 with Phe. Ferrer and Harrison had previously reported that Ala could not substitute for Trp in the peptide (7), but we found also that Phe cannot replace the Trp residue. [W7F]12p1 did not inhibit CD4 or 17b binding (Figure 6 and data not shown). Direct binding studies showed that [W7F]12p1 was able to bind to gp120, but the level of binding was very low at high peptide concentrations (not shown). Since Phe is not a surrogate for Trp, it is likely that the peptide does not bind to gp120 in the same manner as CD4. Nonetheless, a binding site near the CD4 site remains possible and even likely (see below).

Our data also confirm that the entire sequence of 12p1 is important for its action. A change of Arg1 to Ala or Lys caused a reduction in inhibitory activity, but not complete loss of inhibition (Figures 5 and 6). The various truncations of 12p1, from either the N- or C-terminal end, also reduced the efficacy of the peptides (Figures 5 and 6). In these cases, the truncations did not seem to affect the mode of action of the peptides, but seemed instead to have reduced their affinities for gp120, as demonstrated by direct binding experiments with these peptides (data not shown). A peptide smaller than residues 2–8 was not tested, as it was previously shown that these internal residues were critical for peptide activity (7). The other peptide besides [W7F]12p1 with a mutation in one of these interior residues, [15dA]12p1, also lost all inhibitory activity toward gp120 binding (Figure 6). The d-Ala residue was meant to disrupt the backbone structure of the peptide, perhaps interfering with a possible turn, indicated by the presence of the neighboring Pro residue. It is not known if indeed a turn is formed by the peptide after it binds to gp120, but the d-Ala in place of Ile5 likely would alter the conformation of the original 12p1 peptide. The importance of peptide conformation is unclear, however, as replacement of Ile5 with L-Ala also reduced inhibitory activity (7). We conclude from the available sequence variation data that many of the interior 12p1 sequence elements, and possibly peptide conformation, are critical for 12p1 inhibition of gp120 binding interactions, while the more distal residues may enhance peptide binding affinity. 

A noteworthy property of 12p1 elucidated in the original report of Ferrer and Harrison (7) is the concomitant inhibition of binding of gp120 to 17b and CD4. No other peptide inhibitors of gp120–CD4 binding that have been identified so far inhibit binding of both ligands (1, 5, 6). In the current work, we demonstrated that the inhibitory effect of the peptide extends to the coreceptor CCR5, therein showing that the peptide suppresses binding to both envelope receptors of the host cell. Assays of binding of gp120 to cells expressing CCR5 on their surface in the absence of CD4 showed that 12p1 could indeed inhibit this interaction (Figure 8). Only \( \sim 10\% \) of the gp120 was able to bind to CCR5 on the cells in the presence of 12p1, compared with binding in the presence of [W7F]12p1. The other peptide variants were much less able to inhibit this binding, except for [R1A]12p1, which did show significant inhibition. These data demonstrate that the effects of 12p1 are not confined to in vitro receptor surrogates. The additional ability of 12p1 to inhibit viral infection of cells (Figure 9) supports its possible value as a therapeutic lead.

The demonstration that 12p1 inhibits recognition of both host cell receptors by the envelope protein, combined with elucidation of both 1:1 stoichiometry and a non-CD4 character of peptide sequence usage, evokes the enticing possibility of designing unique antagonists of the HIV-1 fusion process. Toward that objective, it will be important to delineate the mechanism of action of 12p1. Several types of data obtained in this work provide key starting points in achieving this goal. CD4 saturation competition binding analysis (Figure 4) suggests that the inhibition of CD4 binding by 12p1 is noncompetitive. This argues that 12p1 is not binding at the same binding site as CD4. The findings with escape variants (for example, Figure 10A) give structural support to this latter conclusion. These results identify residues 97, 102, and 476 as being potentially important in 12p1 binding. None of these residues are CD4 contacts (7), although they are near the CD4 binding site (Figure 10B). That residues 97, 102, and 476 involve both the inner and outer domains of gp120 leaves open the possibility that 12p1 inserts into an interdomain interface on unliganded gp120. Of note here is also the fact that residues E102 and R476 are conserved among all HIV-1 isolates (12), which implies that peptide 12p1, or derivatives thereof, may prove to be effective against a broad range of viral isolates across multiple clades.

Other binding data in this work further clarify the nature of the 12p1 binding site. Because 12p1 is effective on trimeric and functional envelope glycoproteins, as well as gp120 monomers, we can rule out interactions with the portions of gp120 that are inaccessible on the trimer, primarily the inner domain (23). The 2G12 antibody recognizes a largely glycosylated (mostly mannose) gp120 epitope specified by the C3 and V4 regions (14–16). 2G12 can bind
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**FIGURE 11:** Scheme showing a proposed allosteric model for the mode of inhibition of gp120 by 12p1. In this model, gp120 is assumed to exist in at least two distinct forms: (1) a CD4 binding efficient form and (2) a CD4 binding inefficient form. gp120 in both states can bind to the 12p1 inhibitor, but the latter binds preferentially to the inefficient form (left side of the diagram). The binding of 12p1 to gp120 is proposed to "trap" a conformational state that CD4 cannot access and that exhibits slow refolding kinetics back to a CD4 binding efficient conformation.

gp120 in multiple conformations. The 12p1 peptide had no effect on 2G12 binding, and thus, the peptide is not interacting with that glycosylated epitope. The interrelationship of 12p1 and Ab b12 is slightly unusual. Since b12 is a CD4 binding site (CD4BS) Ab and the peptide does not compete with it, we might conclude that the peptide does not bind to the CD4 binding site. The b12 epitope, however, seems to be dependent on the gp120 strain and/or oligomerization state. Xiang et al. (13) found that b12 did not bind to monomers of S375W from the YU2 strain, while here we found robust binding to HXBc2 trimers and mutants. This may be due to the importance of the V1 and V2 loops of gp120, which vary among strains, for b12 recognition, and to differences between the monomer and trimer. Whatever the b12 epitope, it does not appear to overlap significantly with the binding site of the 12p1 peptide. Further support for this assumption comes from a recent study (24), which shows that b12 is only moderately affected by alanine substitutions of E102 and R476. The ability of 12p1 to inhibit binding of F105 to another CD4BS Ab, might be due to epitope overlap, the common effect of unactivated state binding, or both.

That 12p1 appears to bind to a site different from that of CD4 leads us to the hypothesis that this peptide functions allosterically and inhibits envelope interactions with its receptors by stabilizing a conformational state closer to that of unliganded gp120 rather than the activated, CD4-bound form (Figure 11). Because gp120 in its unliganded form is highly flexible (1), it is likely that it exists in a range of conformations between its unliganded state and activated state. In accordance with this view, peptide 12p1 could either stabilize a nonproductive intermediate conformation of gp120 or prevent the transition to an active conformation of gp120. Several types of data obtained in this work are consistent with the view described above. 12p1 does not inhibit binding of CD4 to the gp120 variant S375W, which is believed to be in a ligand-activated state (4, 13), suggesting that 12p1 cannot access this latter state. In contrast, 12p1 inhibits binding of F105 to the I423P variant of envelope trimers, suggesting that 12p1 can access the unactivated state that this envelope mutant assumes and hence could stabilize an unactivated state in the HIV-1 envelope. These data are consistent with the possibility that the HIV-1 envelope may not be able to convert to a conformation that is appropriate for high-affinity receptor binding once 12p1 is bound.

In this work, we determined that peptide 12p1 inhibits the interaction of gp120 with both CD4 and 17b with similar IC50 values. The inhibition extends to different strains of gp120, to both monomers and trimers, and to cellular coreceptor CCR5. 12p1 also is able to inhibit viral infection of cells. Several sequence elements, as well as the length of the peptide, are critical for its effectiveness. Truncations of the peptide and changes in residues 2–8 all caused reduction in inhibitory activity. 12p1 appears to bind preferentially to an unactivated conformation of gp120 to which CD4 and 17b/CCR5 are unable to bind, thereby inhibiting viral infection. Dual inhibition of CD4 and coreceptor binding and inhibition of viral infection evoke the enticing possibility of using 12p1 as an HIV/AIDS therapeutic lead. In this regard, the data obtained in this work show clearly that 12p1 binds in a 1:1 manner at a unique site on gp120 that can be distinguished from that of CD4 or the coreceptor. This now raises the possibility of determining the structure of 12p1 in the bound state and using that structure to design higher-affinity antagonists. Since 12p1 itself already has a substantial (low micromolar Kd) affinity even without the conformational constraint and alternative scaffolding that could be employed in follow-ups, the results of this work allow the chance of utilizing this peptide system in the drug discovery process.

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REFERENCES

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