Stabilization of HIV-1 envelope glycoprotein trimers to induce neutralizing antibodies

de Taeye, S.W.

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Chapter 6

HIV-1 escape from a peptidic anchor inhibitor through stabilization of the envelope glycoprotein spike

Dirk Eggink¹, Steven W. de Taeye¹, Ilja Bontjer¹, Per-Johan Klasse², Johannes P. Langedijk³, Ben Berkhout¹, Rogier W. Sanders¹,²

¹ Department of Medical Microbiology, Academic Medical Center, University of Amsterdam, Amsterdam, 1105 AZ, The Netherlands
² Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, NY 10021, USA
³ Pepscan Therapeutics BV, Lelystad, the Netherlands
* Current address: Crucell Holland BV, Leiden, the Netherlands

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Chapter 6

Abstract
The trimeric HIV-1 envelope glycoprotein spike (Env) mediates viral entry into cells by using a spring-loaded mechanism that allows for the controlled insertion of the Env fusion peptide into the target membrane, followed by membrane fusion. Env is the focus of vaccine research aimed at inducing protective immunity by antibodies as well as efforts to develop drugs that inhibit the viral entry process. The molecular factors contributing to Env stability and decay need to be understood better in order to optimally design vaccines and therapeutics. We generated viruses with resistance to VIR165, a peptidic inhibitor that binds the fusion peptide of the gp41 subunit and prevents its insertion into the target membrane. Interestingly, a number of escape viruses acquired substitutions in the C1 domain of the gp120 subunit (A60E, E64K, and H66R) that rendered these viruses dependent on the inhibitor. These viruses could infect target cells only when VIR165 was present after CD4 binding. Furthermore, the VIR165-dependent viruses were resistant to soluble CD4-induced Env destabilization and decay. These data suggest that VIR165-dependent Env proteins are kinetically trapped in the unliganded state and require the drug to negotiate CD4-induced conformational changes. These studies provide mechanistic insight into the action of the gp41 fusion peptide and its inhibitors and provide new ways to stabilize Env trimer vaccines.
Introduction
With over 35 million people currently infected, HIV-1 remains a major health problem. Although progress has been made in the development of antiviral drugs, the potential of the virus to acquire resistance remains an issue. Neither a cure nor an effective vaccine is available. HIV-1 enters target cells by using its envelope glycoprotein (Env) spikes on the virus surface. Env is the target for drugs that inhibit the viral entry process and for broadly neutralizing antibodies (bNAbs) that researchers aim to induce with Env-based vaccines.

Env is a trimeric complex consisting of three gp41 transmembrane subunits and three noncovalently attached gp120 subunits. The entry process is initiated by binding of gp120 to the CD4 receptor. This interaction induces conformational changes in Env, revealing the binding site for a chemokine coreceptor, generally CXCR4 or CCR5. Additional conformational changes in gp41 involve two heptad repeat regions (HR1 and HR2) in gp41 and the fusion peptide (FP) [1]. The FP might be partially exposed in the prefusion, native state of Env. In a complex of the Env trimer with the FP-targeting bNAb VRC34, residues 512 to 519 are in contact with the bNAb, suggesting that they might be solvent exposed. In contrast, residues 520 to 527 are buried within the protein [2]. During the conformational changes that lead to membrane fusion, a trimeric coiled coil consisting of the three HR1 domains is extended and the FP is inserted into the target cell membrane. Next, the three HR2 domains associate with the HR1 coiled coil, resulting in the formation of a stable six-helix bundle which juxtaposes the viral and cellular membranes and provides free energy for membrane fusion [3–5]. The structure of an Env trimer was recently solved, providing the first detailed images of this intricate molecular machine [6–10].

Despite the weak nature of the gp120-gp41 interactions, the presence of gp120 is crucial for maintaining gp41 in its metastable, spring-loaded state before encountering a susceptible cell. gp120 is likely to orchestrate the sequential conformational changes in gp41 that culminate in membrane fusion, and premature release or shedding of gp120 renders gp41 inactive [11–14]. A number of residues in the C1 and C5 domains of gp120 were shown to be important for the interaction with gp41 [12,13,15–18]. These domains are part of a 7-stranded β-sandwich with three loops directed toward the target cell, thereby organizing the inner domain of gp120 in three structurally mobile layers [19–21]. Layer 1 and layer 2 contribute to the noncovalent interactions between gp120 and gp41 and stabilize the binding of CD4, thereby linking receptor binding to the fusion machinery in gp41 [22]. Layer 3 helps to expose the initial site of contact with CD4 and provides the interaction between the gp120 inner and outer domains [23].

Env is the target of entry inhibitors, and two such inhibitors, namely, maraviroc, which prevents coreceptor binding, and enfuvirtide (Fuzeron), which prevents six-helix bundle formation, have been approved for clinical use. The VIRIP peptide and its derivatives (e.g., VIR165, VIR353, and VIR576) (Fig. 1A) [24] form another class of entry inhibitors, termed anchor inhibitors, that bind to the FP (the “anchor”) and prevent its insertion into the target membrane (24). Although mutations in the FP can provide resistance to the VIRIP derivative VIR165 (unpublished data) and limit the clinical benefit of the related inhibitor VIR576, escape studies with another anchor inhibitor, VIR353, selected for resistance mutations outside the binding site, in the C4 and C5 domains of gp120 and the HR1 and loop domains of gp41 [25,26].

Here we describe virus escape studies with the VIRIP derivative VIR165. We
selected resistance mutations and describe the underlying molecular mechanisms. Interestingly, we identified several escape mutations in the C1 domain of the gp120 subunit of Env (A60E, E64K, and H66R) that rendered the virus dependent on the drug for CD4-induced conformational changes and viral entry. The VIR165-dependent viruses were more thermostable and remarkably resistant to CD4-induced virus inactivation. These results have relevance for understanding HIV entry and the roles of CD4 binding and FP action, as well as for development of entry-targeting therapeutics and Env-based vaccine design.

Results

**VIR165 escape occurs in C1 of gp120 and HR1 of gp41**

To investigate whether HIV-1\textsubscript{LAI} is able to escape VIR165 inhibition (Fig. 1A), we maintained six independent cultures of wild-type (WT) HIV-1\textsubscript{LAI} that were passaged on SupT1 cells in the presence of increasing concentrations of VIR165. The initial VIR165 concentration was 3.8 μg/ml (1.7 μM; corresponds to the IC\textsubscript{50} against WT HIV-1\textsubscript{LAI}). Over the course of 2 1/2 months (20 passages) (Fig. 1B), the VIR165 concentration was gradually increased to 250 μg/ml (110 μM), which is 64-fold higher than the IC\textsubscript{50} for WT HIV-1\textsubscript{LAI}. The complete viral env gene of the proviral DNA was subsequently sequenced. Each culture contained one or two amino acid substitutions in Env (Table 1). We mapped these mutations on the Env trimer structure (Fig. 1C and D) ([6–8,38]). Surprisingly, most acquired amino acid substitutions mapped to the C1 domain of gp120, including the V42I, A58V, A60E, E64K, and H66R substitutions. Except for the V42I mutation, the mutated residues are located in layer 1 of the inner domain of gp120 [19,20], within the disulfide-bonded loop between C54 and C74 [38,39]. It is worth noting that residue 42 is in close contact with residue 523 via extensive side chain interactions [9]. Residue 523 has been shown to be one of the contact residues of VIR165 [24]. We also observed substitutions in HR1 of gp41 (A558T and Q577R). We note that the Q577R mutation was previously linked to resistance against the 3rd-generation HR2-based fusion inhibitor T2635 [40]. All positions identified in these escape cultures are highly conserved in natural isolates, as follows: V42 is present in 99.1% of isolates and I42 in 0.17%, A58 in 99.6% and V58 in 0.02%, A60 in 87.5% and E60 not found, E64 in 99.8% and K64 in 0.05% (found in two sequences, one of which contains multiple frameshifts and stop codons), H66 in 99.9% and R66 in 0.048% (in two sequences from different subtypes), A558 in 99.9% and

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**Table 1. Mutations selected in VIR165 escape studies.**

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>Mutation at indicated Env position*</th>
<th>HR1 domain</th>
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<tbody>
<tr>
<td></td>
<td>C1 domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42 58 60 64 66 558 (47) 577 (66)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A58A/V*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>H66R</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>E64K/E*</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>A558T/A*</td>
</tr>
<tr>
<td>5</td>
<td>V42I</td>
<td>E64K</td>
</tr>
<tr>
<td>6</td>
<td>A60E/A*</td>
<td></td>
</tr>
</tbody>
</table>

\* Env numbering is according to the HXB2 sequence, and gp41 numbering is indicated in parentheses.

\* mixed sequences were detected.
T558 in 0.048%, and Q577 in 99.3% and R577 in 0.36% (Los Alamos HIV Sequence Database [http://www.hiv.lanl.gov/content/index]). We observed substitutions different from those found in escape studies with VIR353, in which resistance mutations in the C4 and C5 domains of gp120 and the HR1 and loop domains of gp41 were selected [25,26]. No escape mutations were observed in FP, consistent with the lack of such mutations in escape studies using the VIRIP derivative VIR353 [24,26], probably because FP mutations pose a large penalty to fitness [41].

Figure 1. Escape of HIV-1\textsubscript{LAI} from anchor inhibitor VIR165. (A) Sequence of the natural peptide VIRIP and the more potent and stable derivatives VIR165 and VIR353, both cyclized by a disulfide bond, and the di-peptide VIR576. The p in VIR353 indicates the introduction of a D-proline. (B) Step-wise increase of the VIR165 concentration during passage experiments. Cultures were passaged twice a week for 2½ months with increasing concentrations of VIR165. (C) Linear representation of HIV\textsubscript{LAI} gp160 showing positions of VIR165 escape mutations in C1 and HR1, as well as previously identified escape mutations to VIRIP analogue VIR353 (26). (D) The residues involved in VIR165-resistance and -dependence were mapped on the structure of the BG505 SOSIP.664 trimer structure containing the complete gp41 interactive domain (PDB accession code 5CEZ (78)) using Pymol (DeLano Scientific; http://pymol.sourceforge.net). The residues are shown as spheres on a cartoon model of one of the protomers, with gp41 in sand and gp120 in green. A surface model of the two other protomers is shown in white and grey. Residues at position 42, 58, 558 and 577 which are involved in VIR165-resistance, are indicated in red. The control position 62 is indicated in cyan, and residues 60, 64 and 66, which are involved in VIR165-dependence, are depicted in yellow. For comparison, residues involved in resistance to VIRIP analogue VIR353 are indicated in blue (26). The right panel shows a detail of the region that includes residues 58, 60, 64 and 66, all situated in layer 1 of gp120. Gp41 is depicted in orange, gp120 is in green, with layer 1 in magenta and layer 2 in blue.
Substitutions in gp41 HR1 confer VIR165 resistance

To confirm the importance of the selected substitutions in providing resistance against VIR165, we constructed molecular clones of HIVLAI with the HR1 substitution A558T or Q577R. The T20-resistant V549A mutant was included as a control [28]. Single-cycle infection experiments were performed using the TZM-bl reporter cell line in the presence of serial dilutions of VIR165 (Fig. 2A; Table 2). The HR1 A558T and Q577R mutants showed 10-fold and >10-fold VIR165 resistance, respectively, compared to that of the WT. The control V549A virus did not show a significant change in sensitivity to VIR165. In the absence of drug, the A558T and Q577R substitutions caused a drop in viral infectivity in the single-cycle infection experiments with the TZM-bl reporter cell line (35% and 20% of the WT level, respectively), indicating that resistance comes with a significant loss of Env protein function and viral fitness (Fig. 2D), consistent with what was observed upon viral escape from the fusion inhibitor T2635 [40].

Figure 2. HIV-1 LAI VIR165 escape variants can be resistant to or dependent on VIR165. Single cycle infection experiments were performed as described in the material and methods section. Inhibition of HIV-1 LAI variants containing (A) VIR165-resistance mutations in gp41, (B) VIR165-resistance mutations in the C1 domain, (C) VIR165-dependence mutations within C1. A D62N control virus, the T20-resistant variant V549A, and the T20-dependent virus V549A/N637K (46) were included as control viruses. (D) Infectivity of VIR165-resistant and -dependent viruses in the absence of VIR165 in a single cycle infection assay relative to WT virus. The virus mutants indicated with an (*) were VIR165-dependent, i.e. they were not infectious in the absence of VIR165. (E) VIR165-dependent virus variants are inhibited at high VIR165 concentrations. Single cycle infection experiments were performed using concentrations of VIR165 up to 300 μg/ml revealing a bell-shaped dose-response curve. (F) Maximum infectivity of VIR165-dependent mutants relative to WT in the presence of VIR165. The infectivity of VIR165-dependent viruses was obtained in the presence of VIR165 (indicated with an *) at 10 μg/ml VIR165 (A60E and E64K), or 30 μg/ml (H66R). WT infectivity was measured in the absence of VIR165 (WT) or in the presence of 10 μg/ml VIR165 (WT*).

Substitutions in gp120 C1 confer VIR165 resistance or dependence

We next evaluated the contributions of the selected C1 mutations V42I, A58V, A60E, E64K, and H66R to VIR165 resistance. A D62N mutant of C1 was generated to serve as a control.
The H66N substitution, which was previously described for a cold-resistant HIV-1 variant and shown to affect the interaction with CD4, was also included [42,43]. The V42I and A58V substitutions caused 4-fold and 8-fold VIR165 resistance (Fig. 2B; Table 2), respectively, with little impact on viral fitness (Fig. 2D). The effects of the A60E, E64K, and H66R substitutions were more intriguing. Although these substitutions were located very close to the A58V resistance mutation, they yielded a strikingly different phenotype. In the absence of VIR165, these variants were barely infectious or not infectious at all (Fig. 2D). However, they became infectious with increasing VIR165 concentrations (Fig. 2C). Thus, these mutants displayed a VIR165-dependent phenotype. Drug dependence is a relatively rare phenomenon in HIV-1 drug resistance. T20- or retrocyclin (RC101)-dependent viruses that involve a combination of substitutions in the HR1 binding site of the inhibitors and in HR2 have been described [44,45]. The T20-dependent V549A/N637K virus did not show cross-resistance to or dependence on VIR165 (Fig. 2A; Table 2). The control D62N and H66N substitutions did not confer VIR165 resistance or dependence and did not affect infectivity (Fig. 2B, C; Table 2).

Table 2. Resistance to VIR165 in single-cycle infection assays.

<table>
<thead>
<tr>
<th>Virus</th>
<th>VIR165 IC$_{50}$ (μg/ml)</th>
<th>Fold resistance to VIR165$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.7</td>
<td>1.0</td>
</tr>
<tr>
<td>C1 mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V42I</td>
<td>10.6</td>
<td>3.8</td>
</tr>
<tr>
<td>A58V</td>
<td>21.2</td>
<td>7.7</td>
</tr>
<tr>
<td>A60E</td>
<td>60$^b$</td>
<td>37</td>
</tr>
<tr>
<td>D62N</td>
<td>3.2</td>
<td>1.2</td>
</tr>
<tr>
<td>E64K</td>
<td>60$^b$</td>
<td>37</td>
</tr>
<tr>
<td>H66N</td>
<td>3.2</td>
<td>1.1</td>
</tr>
<tr>
<td>H66R</td>
<td>100$^b$</td>
<td>58</td>
</tr>
<tr>
<td>HR1 mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A522V</td>
<td>2.2</td>
<td>0.8</td>
</tr>
<tr>
<td>A558T</td>
<td>27.3$^c$</td>
<td>9.9$^c$</td>
</tr>
<tr>
<td>Q577R</td>
<td>&gt;30</td>
<td>&gt;10</td>
</tr>
<tr>
<td>A549A</td>
<td>3.5</td>
<td>1.3</td>
</tr>
<tr>
<td>A549A/N637K</td>
<td>2.4</td>
<td>0.9</td>
</tr>
</tbody>
</table>

$^a$ Values in bold represent resistances of ≥3-fold compared to that of the WT virus.

$^b$ Value for a VIR165-dependent virus; the IC$_{50}$ was estimated based on the data in Fig. 2E.

$^c$ Estimated value, as no complete sigmoidal dose-response curve was obtained.

To test whether VIR165-dependent variants could be inhibited by a high dose of VIR165, we repeated the dose inhibition experiments with VIR165 concentrations of up to 300 μg/ml (Fig. 2E). Clear drug induction was observed, with the variants reaching maximum infectivity at approximately 10 (A60E and E64K mutants) and 30 (H66R mutant) μg/ml VIR165. However, all three VIR165-dependent variants were inhibited at higher concentrations. We determined IC$_{50}$s of 60 μg/ml for the A60E and E64K mutants and 100 μg/ml for the H66R mutant, which translate to approximately 37-fold and 58-fold resistance, respectively, compared to that of the WT.
Using the optimal VIR165 concentration for stimulation of each VIR165-dependent virus variant, we reassessed the maximal infectivities of these viruses. We had already shown a nearly complete loss of infectivity of the A60E, E64K, and H66R variants in the absence of drug (Fig. 2C and D), and we next compared WT virus infectivity without VIR165 with the maximum infectivities of the dependent viruses in the presence of VIR165 (10 μg/ml for the A60E and E64K mutants and 30 μg/ml for the H66R mutant) (Fig. 2F). Infectivity of the WT virus was fully inhibited in the presence of 10 μg/ml VIR165. The E64K and H66R mutants exhibited only ~10% infectivity in the presence of VIR165, while the A60E mutant could be activated by VIR165 to achieve ~50% infectivity compared to that of the WT virus without VIR165. Thus, VIR165 dependence came with a considerable loss of viral fitness, in particular for the E64K and H66R variants.

Modeling of VIR165 occupancy explains the bell-shaped dose-response curves
The enhancement and inhibition of VIR165-dependent HIV-1 infection were modeled mathematically in order to assess whether enhancing or inhibitory effects could be explained by differential FP occupancy levels by VIR165 (1, 2, or 3 VIR165 peptides per Env trimer). Different mathematical VIR165 occupancy models were fitted to the experimentally obtained bell-shaped stimulation-inhibition data (Fig. 3). The model functions were derived from the binomial theorem as described previously [46]. They postulate incremental effects of trimer ligation by VIR165. No assumptions were made about total numbers of trimers or minimal thresholds of functional trimers required for infectivity. Discrepancies between the modeled curves and the experimental data can be attributed partly to this oversimplification [46,47].

The occupancy of VIR165 on a protomer was expressed by the formula \( p = \frac{C}{K_d} \left( \frac{1 + C/K_d}{1 + C/K_d} \right) \), where \( C \) is the VIR165 concentration and \( K_d \) its dissociation constant, on the simplifying assumption that neither positive nor negative cooperativity between the three protomers of a trimer occurs upon VIR165 binding. For the WT virus, an inhibition model was applied that describes the infectivity, \( I \), as being proportional to the fraction of completely unoccupied trimers, i.e., \( I = A(1 - 3p + 3p^2 - p^3) \), where \( A \) is a constant reflecting...
the maximum amplitude, i.e., the maximum infectivity. An alternative model, \( I = A p^3 \), which assumes that inhibition occurs only when three VIR165 molecules are bound per trimer, was also fitted to the data for comparison.

For the VIR165-dependent viruses, the experimental data were fitted to three models. The first model posits that VIR165 occupancy of one or two protomers per trimer allows infection, whereas occupancy of all three or none does not, i.e., \( I = A(3p - 3p^2) \). The second model assumes the induction of infection by exactly one VIR165 molecule per trimer, i.e., \( I = A(3p - 6p^2 + 3p^3) \), and the third postulates that infectivity requires exactly two VIR165 molecules per trimer, i.e., \( I = A(3p^2 - 3p^3) \). The models were fitted to the inhibition data by nonlinear regression.

The models postulating complete inhibition of WT Env function by a single VIR165 peptide or by three peptides per trimer both fit the experimental data well (\( R^2 = 0.964 \) and \( R^2 = 0.961 \), respectively). The model for induction of infectivity of the VIR165-dependent mutants by one VIR165 peptide per trimer fit the experimental data better than the other two models did (\( R^2 = 0.937 \) to 0.993 for enhancement by one VIR165 peptide per trimer, \( R^2 = 0.934 \) to 0.953 for enhancement by one or two VIR165 peptides per trimer, and \( R^2 = 0.887 \) to 0.984 for enhancement by two VIR165 peptides per trimer). The models that best fit the experimental data are shown in Fig. 3.

In conclusion, mathematical modeling strongly supports a mechanistic scenario in which partial occupancy of VIR165-dependent Env trimers by VIR165 enables infection, whereas occupancy of all three binding sites, which is reached at high VIR165 concentrations, blocks infection.

VIR165-resistant and -dependent viruses are more sensitive to T20
Since all selected escape mutations are located outside the putative VIR165 binding site, it is possible that the kinetics of one or more steps of the entry process are affected, thereby limiting the window of opportunity for the drug to act [48–52]. To probe whether and how the entry process was affected by the VIR165 resistance and dependence substitutions, we analyzed the sensitivity to reagents that interfere with well-defined entry steps. As the A60E, E64K, and H66R mutants were not infectious in the absence of drug, VIR165 (10 \( \mu \)g/ml) was always present when these mutants were evaluated. First, we tested for sensitivity to polyclonal Ig from HIV-positive individuals (HIVIg). Neither VIR165-resistant nor VIR165-dependent viruses showed altered sensitivity to HIVIg compared to the WT virus, indicating that these viruses did not have a generally altered sensitivity to antibodies. Furthermore, the presence of VIR165 during infection of the dependent variants did not alter the overall neutralization sensitivity (Table 3).

The windows of opportunity for VIR165 and HR2-based inhibitors overlap, and some mutations that confer resistance to HR2-based inhibitors, e.g., the Q577R mutation, confer cross-resistance to VIR165. To investigate whether the reverse was also true, we tested all VIR165-resistant viruses (V42I, A58V, A558T, and Q577R) and two VIR165-dependent viruses (E64K and H66R) for T20 sensitivity. We did not observe cross-resistance to T20. On the contrary, all VIR165-resistant and -dependent variants were more susceptible to the fusion inhibitor T20 (3-fold to 8-fold), except for the Q577R mutant (Table 3), which was not unexpected because this mutant was also selected in fusion inhibitor resistance studies [40]. The control T20 resistance mutation V549A in HR1 [53] gave 54-fold resistance
to T20, and the D62N change did not affect T20 sensitivity. Interestingly, similar observations were made in a previous study, in which a combination of VIR353 resistance mutations also resulted in enhanced sensitivity to T20 [26]. These data suggest that the T20-sensitive step in entry (transition from a prehairpin intermediate to a six-helix bundle) is probably slower in VIR165-resistant and -dependent virus variants, resulting in an increased window of opportunity for HR1-targeting fusion inhibitors. These data confirm that VIR165 and T20 inhibit nonidentical viral entry steps.

Table 1. Sensitivities of VIR165-resistant and -dependent mutants to a series of antibodies and entry and fusion inhibitors

<table>
<thead>
<tr>
<th>Virus</th>
<th>HIVg IC50 (µg/ml)</th>
<th>Fold change in resistance&lt;sup&gt;a&lt;/sup&gt;</th>
<th>sCD4 IC50 (µg/ml)</th>
<th>Fold change in resistance&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CD4-IgG2 IC50 (µg/ml)</th>
<th>Fold change in resistance&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AMD3100 IC50 (µg/ml)</th>
<th>Fold change in resistance&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T20 IC50 (µg/ml)</th>
<th>Fold change in resistance&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>WT</td>
<td>2.40</td>
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<td>2.29</td>
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<td>1.85</td>
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<td>54.9</td>
<td>1.0</td>
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<td>Mutant viruses</td>
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</tbody>
</table>

<sup>a</sup> Because VIR165-dependent mutants are not infectious in the absence of VIR165, 10 µg/ml VIR165 was present for the A60E, E64K, and H66R mutants.

<sup>b</sup> Compared to that of the WT. Values in bold represent ≥3-fold more resistance than that of the WT; values in bold italics represent ≥3-fold more sensitivity than that of the WT.

<sup>c</sup> ND, not done.

**VIR165-dependent viruses are resistant to inhibition by soluble CD4 but more sensitive to inhibition by AMD3100.**

Soluble CD4 (sCD4) induces an activated Env state that is able to mediate virus entry for a limited time, after which Env rapidly decays into a functionally inactive form [12,54,55]. Alterations in layer 1 of the C1 domain (e.g., H66A and W69L) have been shown to affect this decay process and are thought to stabilize the sCD4-activated Env intermediate, slowing its transition to a functionally inactive Env form [42,43,55,56]. We wondered whether VIR165 resistance and dependence mutations affected the interaction of Env with sCD4. We therefore first performed neutralization studies with sCD4 as well as the CD4 mimetic CD4-IgG2. Both sCD4 and CD4-IgG2 can effectively neutralize WT HIV-1 infection (Table 3). Most VIR165-resistant viruses showed sensitivities to sCD4 and CD4-IgG2 similar to those of the WT and the D62N variant. The V42I virus was slightly less sensitive to CD4-IgG2 (3-fold) but not to sCD4, while the A558T virus was slightly more sensitive to sCD4 but not to CD4-IgG2. Interestingly, the VIR165-dependent E64K and H66R mutants were both less sensitive to neutralization by sCD4 (4-fold and 13-fold, respectively) and CD4-IgG2 (3-fold and 6-fold, respectively).

The effect of mutations in either C1 or HR1 on the conformational changes associated with CD4 binding may also have an effect on the affinity for the coreceptor. We therefore tested the sensitivity of the WT virus and its variants to inhibition by the CXCR4 antagonist AMD3100 (Table 3). Whereas VIR165 resistance mutations in C1 had no effect on sensitivity to AMD3100 inhibition, resistance mutations within gp41 caused a small increase in AMD3100 sensitivity. The VIRIP dependency mutations E64K and H66R rendered the virus
3- to 5-fold more sensitive to AMD3100, suggesting an enhanced dependence on CXCR4 for proceeding with conformational changes toward fusion.

**VIR165-dependent viruses are less prone to CD4-induced decay**

We hypothesized that VIR165-dependent viruses might have an altered Env stability and require VIR165 to trigger conformational changes following CD4 binding. Soluble CD4 induces an activated Env state that is short-lived in the absence of a coreceptor and target membrane [55,56]. Substitutions at position 66 have been reported to decrease this CD4-induced decay [42,43,56]. We therefore tested the decay rates of WT, VIR165-resistant, and VIR165-dependent viruses by incubating them at physiological temperature (37°C) for different intervals, followed by assessment of their remaining infectivity on TZM-bl cells. For the VIR165-dependent viruses, VIR165 was added before the transfer to TZM-bl cells (Fig. 4A and B; Table 4). WT HIV-1LAI decayed with a half-life ($t_{1/2}$) of 11.3 h, which is in the range of values measured for JR-CSF ($t_{1/2}$ = 18.9 h) and ADA ($t_{1/2}$ = 9.0 h) [57]. The VIR165-resistant virus was less stable (A58V $t_{1/2}$ = 8.2 h), and so were the VIR165-dependent viruses (A60E $t_{1/2}$ = 9.4 h, E64K $t_{1/2}$ = 9.5 h, and H66R $t_{1/2}$ = 8.2 h).

We next tested viral decay in the presence of sCD4. To avoid interference with the subsequent infection, a subneutralizing concentration of sCD4 was used. Again, VIR165 was added to the VIR165-dependent viruses before transfer of the viruses to TZM-bl cells. In the presence of sCD4, the WT virus decayed twice as quickly (1.9-fold; $\Delta t_{1/2} = −5.2$ h), which is indicative of CD4-induced deactivation (Fig. 4A and C; Table 4). The VIR165-resistant virus (A58V), which was among the least stable viruses in the absence of sCD4, also decayed more rapidly in the presence of sCD4 (1.5-fold; $\Delta t_{1/2} = −2.6$ h). In contrast, the VIR165-dependent variants, in particular the H66R variant, remained relatively stable in the presence of sCD4 (1.2-fold change for the E64K variant [$\Delta t_{1/2} = −1.5$ h], 1.1-fold change for the H66R variant [$\Delta t_{1/2} = −0.3$ h], and 1.3-fold change for the A60E variant [$\Delta t_{1/2} = −2.4$ h]). Because an H66N

![Figure 4. VIR165-dependent viruses are less prone to CD4-induced decay.](image-url)

(A) Representative experiment of at least three independent experiments in which VIR165-resistant and VIR165-dependent viruses were incubated at physiological temperature (37°C) in the absence of sCD4, for different time intervals, followed by assessing the remaining infectivity on TZM-bl cells in the absence of VIR165 for WT virus or in the presence of sCD4 for the dependent variants. (B) Half life ($t_{1/2}$) for WT and VIR165 resistant and dependent mutants, measured in at least three independent experiments performed in duplo. (C) The difference in $t_{1/2}$ when incubated in the absence or presence of sCD4 during incubation at 37°C. Statistical significance is indicated with asterisks: * P < 0.05; ** P < 0.005.
mutant was reported to be resistant to virus inactivation by cold (42, 43), we repeated these experiments at 0°C. None of the VIR165-dependent viruses were resistant to cold inactivation, but the VIR165-dependent viruses were more resistant to sCD4-induced decay at 0°C (data not shown), similar to what we observed at 37°C. We therefore concluded that VIR165-dependent Env variants are less prone to CD4-induced decay.

Table 4. Stability of VIR165-resistant and -dependent mutants in the absence or presence of sCD4

<table>
<thead>
<tr>
<th>Virus</th>
<th>Decay at 37°C</th>
<th>Thermostability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t1/2 (h)</td>
<td>t1/2 (+sCD4) (h)</td>
</tr>
<tr>
<td>WT</td>
<td>11.3 (0.9)</td>
<td>6.1 (0.3)</td>
</tr>
<tr>
<td>Mutant viruses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A58V</td>
<td>8.2 (1.1)</td>
<td>5.6 (0.6)</td>
</tr>
<tr>
<td>A60E</td>
<td>9.4 (0.5)</td>
<td>7.0 (0.4)</td>
</tr>
<tr>
<td>E64K</td>
<td>9.5 (1.4)</td>
<td>8.0 (1.2)</td>
</tr>
<tr>
<td>H66R</td>
<td>8.2 (1.3)</td>
<td>7.9 (1.1)</td>
</tr>
</tbody>
</table>

*Average values are shown for at least three independent experiments, each performed in duplicate, with standard errors of the means (SEM) shown in parentheses.

VIR165-dependent viruses have increased thermostability in the presence of sCD4

We also tested the thermostability of WT, VIR165-resistant, and VIR165-dependent viruses by incubating them for 1 h each at escalating temperatures, followed by testing their residual infectivity on TZM-bl reporter cells (in the presence of VIR165 for the dependent variants). The WT virus had a midpoint of thermal denaturation (Tm) of 44.6°C (Fig. 5A and B; Table 4), which is in the same range as those reported for HIV-1ADA (Tm = 40.5°C), HIV-1JR-CSF (Tm = 44.6°C), and HIV-1LAI (Tm = 42.0°C) ([57,58]; D. Leaman and M. Zwick, personal communication). The VIR165-resistant virus had a Tm similar to that of the WT virus (A58V Tm = 44.8°C), as did the VIR165-dependent viruses (A60E Tm = 44.7°C, E64K Tm = 44.6°C, and H66R Tm = 44.7°C) (Fig. 5A and B; Table 4). Thus, we did not observe major differences in the thermostability of the test viruses.

To study whether the addition of sCD4 would affect thermostability, we also performed these experiments in the presence of (subneutralizing concentrations of) sCD4. The addition of sCD4 destabilized WT HIV-1LAI and the VIR165-resistant virus considerably (WT ΔTm = −2.7°C and A58V ΔTm = −2.7°C) (Fig. 5A and C; Table 4). In contrast, the VIR165-dependent viruses were less affected by the addition of sCD4 (A60E ΔTm = −1.4°C, E64K ΔTm = −0.9°C, and H66R ΔTm = −0.5°C) (Fig. 5C; Table 4). We concluded that VIR165-dependent viruses, in particular the H66R variant, are more thermostable and less prone to undergo CD4-induced conformational changes that destabilize the Env spike.

Discussion

VIRIP and VIRIP derivatives are members of a class of peptide fusion inhibitors termed anchor inhibitors and are thought to inhibit the HIV-1 entry process by binding to the hydrophobic
6

Figure 5. VIR165-dependent viruses show increased thermostability in the presence of sCD4. (A) The thermostability of WT, VIR165-resistant and VIR165-dependent viruses was tested by incubating them for 1 h at escalating temperatures, followed by testing the remaining infectivity on TZM-bl reporter cells in the absence of VIR165 (WT) or the presence of 10 or 30 μg/ml VIR165 (dependent variants). A representative experiment is show of at least three independent experiments. (B) The midpoints of thermal denaturation (\(T_m\)) of WT and mutant viruses measured in at least three independent experiments performed in duplo. (C) Changes in \(T_m\) in the presence of sCD4. Statistical significance is indicated with asterisks: ** P < 0.005; *** P < 0.0005.

The fusion peptide of gp41 (Fig. 6A and B). A previous study used the VIRIP analogue VIR353 to select resistance mutations in the HR1 and loop domains of the gp41 subunit and substitutions in the C4 and C5 domains of the gp120 subunit [26]. In the present study, we performed in vitro HIV-1 evolution experiments to select resistance against the VIRIP analogue VIR165, one of the most potent VIRIP derivatives. Again, escape mutations were not selected in the putative FP binding site of the peptide but rather in the HR1 domain of gp41 and the C1 domain of gp120. Moreover, we found that some C1 mutations conferred drug dependence.

How can we mechanistically explain these escape mutations that occur outside the putative binding site of VIR165? An escape mechanism alternative to “decreased drug affinity” is “decreased drug opportunity” [44,48,59]. In this scenario, the drug-target affinity per se is unaltered, but the opportunity for the drug to access the target site is restricted, for example, because the target site is exposed for a shorter time. This mechanism has been described for HIV-1 fusion inhibitors [48,60]. We think that such a window mechanism can also account for the VIR165 resistance mutations. Part of the binding site for VIR165 (residues 513 to 518) might be partially exposed in the prefusion state. It is likely that CD4- and possibly coreceptor-induced conformational changes allow additional contact residues 520 to 523 and 525 to 531 to become available for VIR165 binding [2,9,24]. We propose that the C1 and HR1 substitutions change the kinetics of Env conformational changes, thereby decreasing the time that the FP is accessible. The observation that a resistance mutation against the third-generation HR2-based fusion inhibitor T2635 that did not occur in the drug binding site (Q577R) provided cross-resistance to VIR165 is consistent with this hypothesis [40]. In fact, the Q577R substitution was selected in both T2635 [40] and VIR165 escape studies (Table 2). Since VIR165 and HR2-based inhibitors target overlapping, but not identical, intermediate Env states, it makes sense that some mutations can provide cross-resistance by changing the kinetics of this entry step. We have not studied the C1 resistance mutations V42I and A58V in much detail, but the A58V mutant was less stable and more
prone to CD4-induced decay, suggesting that it is more metastable than the WT Env spike. This might be consistent with enhanced fusion kinetics and shortened FP exposure. Residue 42 is in close contact with residue 523, which is one of the residues of Env contact with VIR165. Alterations of the side chain interactions between residues 42 and 523 might affect the exposure of the critical VIR165 contact residue 523. The selection of VIR353 resistance mutations in the C4 and C5 domains of gp120 and the HR1 and loop domains of gp41, i.e., outside the inhibitor binding region, might be explained by a similar mechanism, particularly as these substitutions also resulted in enhanced sensitivity to the fusion inhibitor T20 [25,26].

Figure 6. Hypothetical model of the inhibiting and enhancing modes of action of VIRIP-derived peptides of WT and VIR165-dependent viruses.

(A) The FP becomes fully exposed upon binding of the CD4 receptor and co-receptor and is inserted into the target membrane, followed by subsequent fusion of the two membranes. (B) VIR165 binds to the FP in the short-lived intermediate state that is induced by CD4-binding, thereby inhibiting insertion of the FP into the target membrane. (C) In VIR165-dependent viruses a hyperstable gp120-gp41 interaction cannot be sufficiently weakened by CD4-binding and CD4-induced conformational changes in the gp120-gp41 complex and subsequent entry steps are blocked. (D) VIR165 in sub-saturating amounts acts as a “wedge” between gp120 and gp41, destabilizing the gp120-gp41 interaction and facilitating CD4-induced conformational changes that allow subsequent entry steps. We do not know whether in this scenario VIR165 remains associated with the one or two FP(s) that is occupies and only the free FP(s) insert into the cell membrane, or whether it dissociates to allow all three FPs to insert. (E) High concentrations of VIR165 result in occupancy of all three FPs of the Env trimer, effectively inhibiting infection.
The substitutions that confer VIR165 dependence might be explained by a mechanism of “decreased drug opportunity.” Drug dependence can be classified as a special form of resistance, since the virus is able to replicate in the presence of the drug but the resistance mutations cause a severe replication defect in the absence of drug, which can only be overcome by addition of the drug. This phenomenon has been described for only a limited number of cases, although drug stimulatory effects and drug dependence were reported for several HIV-1 inhibitors targeting the Gag, protease, and Env proteins [44,45,61–63]. For Env, enhanced fusion kinetics can render HIV-1 resistant to fusion inhibitors (decreased opportunity), but “hyperfusogenic” Env variants can become dependent on the drug to avoid a premature HR1-HR2 collapse in the absence of a target membrane [44,45,59]. In such cases, the drug acts as a safety pin to prevent premature conformational changes, but drug dissociation is eventually needed to allow such conformational changes to occur in the presence of a target cell, leading to membrane fusion and virus entry [44]. VIR165 dependence is mechanistically distinct from T20 dependence. The presence of VIR165 is required not during virus production but during an intermediate step in viral entry.

We speculate that VIRIP-dependent viruses have a hyperstable gp41-C1 interaction that cannot be weakened sufficiently by CD4 binding, and as a consequence, CD4-induced conformational changes in the gp120-gp41 complex are blocked (Fig. 6C). Since the FP is located at the interface of gp120 and gp41, it is conceivable that binding of a peptide to the FP affects the gp120-gp41 interactions. We propose that the dependent viruses require VIR165 to act as a “wedge” between gp120 and gp41 to facilitate the CD4-induced conformational changes that allow the formation of the complete HR1 (Fig. 6D) [1], followed by coreceptor binding and, ultimately, gp41-C1 dissociation and membrane fusion. In this scenario, VIR165 might bind to the exposed contact residues in the N-terminal part of the FP and “pull out” the rest of the fusion peptide, thereby forcing the required conformational changes that allow continuation of the entry process. In agreement with this, the VIR165-dependent viruses are more stable and resistant to sCD4-induced decay. Recently, we used the E64K and H66R substitutions to stabilize recombinant soluble (SOSIP) trimers in the closed prefusion state. These substitutions facilitated the stabilization of SOSIP trimers from 4 different isolates: BG505 (clade A), B41 (clade B), AMC008 (clade B), and ZM197M (clade C). Hydrogen-deuterium exchange (HD-X) and other studies demonstrated that these substitutions stabilize SOSIP trimers by blocking CD4-induced conformational changes, consistent with the virological observations described here [64]. Whereas T20 dependence involves enhanced fusion kinetics that need to be tempered by T20 from the moment Env appears at the cell/virus surface [48,51,59], VIR165 dependence involves reduced fusion kinetics, necessitating the presence of VIR165 as a catalyst for Env conformational changes following CD4 binding.

If one assumes that this resistance phenotype involves a decreased window of opportunity, then this might represent a paradox for delayed fusion kinetics. How can reduced fusion kinetics decrease the window of opportunity for VIR165 to inhibit? The fact that a VIR165-dependent virus is more sensitive to HR2-based fusion inhibitors that target a similar stage during entry also appears to be paradoxical. This can be explained only if the steps in which VIR165 and HR2-based inhibitors act are separated in time. CD4 binding induces a short-lived activated state that probably also exposes the FP. The FP is probably fully exposed only very shortly, after CD4 binding and until the FP is inserted into the target
membrane. VIR165 exclusively targets this step, whereas HR2-based fusion inhibitors that may also act during this stage will remain active when the FP is inserted into the target cell membrane, up to the time that the six-helix bundle is formed. The windows of opportunity for VIR165 and HR2-based inhibitors are therefore overlapping but not identical.

Inhibition of VIR165-dependent viruses requires a high dose of inhibitor. The bell-shaped activation-inhibition curves can be explained mathematically by a model in which VIR165 occupancy of one or two FPs per Env trimer allows for entry, while occupation of all three FPs results in inhibition (Fig. 6E). It has indeed been proposed that not all three FPs are required for fusion ([65]; M. J. Root, personal communication). This is consistent with a model in which VIR165-dependent Env is activated when one or two VIR165 molecules separate gp120 from gp41, while the remaining unbound FP(s) can insert into the target membrane and mediate membrane fusion.

There is an interest in generating stable soluble mimics of the native Env trimer for vaccine and structural studies. Efforts to generate such mimics have been hampered by the metastable nature of the native Env spike. We have reported a number of stabilization tricks resulting in the stable trimer mimic BG505 SOSIP.664 gp140 [18,66–71]. Additional modifications that kinetically trap BG505 SOSIP.664 trimers in the unliganded state were recently described [9,72,73]. We have shown that the VIR165 dependence mutations are useful for further stabilization of different recombinant Env trimers to lock them in the unliganded ground state and improve their properties as immunogens [64].

Materials and methods

Reagents

Reagents were obtained as gifts or purchased from the following sources. William Olson (Progenics Pharmaceuticals) provided soluble CD4 (sCD4) and CD4-IgG2. AMD3100 was a generous gift from D. Schols (Rega Institute, Leuven University). Peptides VIR165 (LEAIPCSIPCAFKNPKFVF) and T20 (YTSLHSLIESQNQKNEQELLELDKWASLWNWF) were synthesized as described previously [27,28].

Selection of VIRIP-resistant HIV-1 variants

For the selection of VIR165-resistant viruses, 1 × 10⁶ SupT1 cells were infected with wild-type (WT) HIV-1_LAI (the equivalent of 1.5 ng CA-p24). Six independent cultures were started to initiate evolution with 3.8 μg/ml VIR165, which corresponds to the 50% inhibitory concentration (IC₅₀) for WT HIV-1_LAI. Cultures were split twice weekly, and when HIV-induced cytopathic effects and/or increased CA-p24 production were apparent, virus replication was maintained by passage of cell-free culture supernatant onto uninfected SupT1 cells with a 2-fold increased VIR165 concentration. Initially, we started with passing of 100 μl of cell-free supernatant onto fresh cells. We gradually decreased the volume of supernatant in subsequent passages, from 100 μl in the second passage to a minimum of 10 μl. Cell and supernatant samples were taken at regular time points and stored at −80°C. Cell culture, transfections, and CA-p24 determinations were performed as previously reported [29]. After 2 1/2 months of culture, DNA was extracted from infected cells by use of a QIAamp DNA minikit (Qiagen, Valencia, CA), and the complete proviral env gene was PCR amplified using primers...
1 (5′-ATAAGCTTAGCAGAAGACAGTGGCAATG-3′) and 2 (5′-GCAAAATCCTTTCCAAGCCC-3′) and then sequenced. Amino acid numbering is based on the HXB2 isolate.

**Construction of HIV-1\textsubscript{LAI} molecular clones**

The full-length molecular clone of HIV-1\textsubscript{LAI} (pLAI) [30] was used to produce WT and mutant viruses. The plasmid pRS1 was used to introduce mutations as described previously [27,31], and the entire env gene was verified by DNA sequencing. Mutant env genes in pRS1 were cloned back into pLAI as SalI-BamHI fragments. Each virus variant was transiently transfected into C33A cells by calcium phosphate precipitation as previously described [32] or by transfection into HEK293T cells by use of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The virus-containing supernatant was harvested at 3 days posttransfection and stored at −80°C, and the virus concentration was quantitated by a CA-p24 enzyme-linked immunosorbent assay (ELISA) as described previously [29].

**Infectivity and IC\textsubscript{50} determination**

The TZM-bl reporter cell line [33,34] stably expresses high levels of CD4 and the HIV-1 coreceptors CCR5 and CXCR4 and contains luciferase and β-galactosidase genes under the control of the HIV-1 long terminal repeat promoter. The TZM-bl cell line was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (via John C. Kappes, Xiaoyun Wu, and Tranzyme Inc. [Durham, NC]). One day prior to infection, 17 × 10\textsuperscript{3} TZM-bl cells per well were plated in a 96-well plate in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin-streptomycin (both at 100 U/ml) and then incubated at 37°C with 5% CO\textsubscript{2}. Virus (5.0 ng CA-p24) was preincubated for 30 min at room temperature with serial dilutions of an antiserum or entry inhibitor (polyclonal Ig from HIV-positive individuals [HIVIg], sCD4, CD4-IgG2, AMD3100, T20, or VIR165). This mixture was added to the cells in the presence of 400 nM saquinavir (Roche, Mannheim, Germany) to block secondary rounds of infection and of 40 μg/ml DEAE in a total volume of 200 μl. DEAE was added to enhance HIV-1 infection of TZM-bl reporter cells. This did not affect the sensitivity to the inhibitors tested ([35–37]; data not shown). At 2 days postinfection, the medium was removed and cells were washed once with phosphate-buffered saline (PBS; 50 mM sodium phosphate, pH 7.0, 150 mM NaCl) and lysed in reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using a luciferase assay kit (Promega, Madison, WI) and a Glomax luminometer (Turner BioSystems, Sunnyvale, CA) according to the manufacturers' instructions. All infections were performed in duplicate in at least two independent experiments. Uninfected cells were used to correct for background luciferase activity. The infectivity of each mutant without an inhibitor was set at 100%. Nonlinear regression curves were determined using sigmoidal dose-response curves (variable slope; bottom constraint = 0), and 50% inhibitory concentrations (IC\textsubscript{50}s) were calculated using Prism software, version 5.0. When full inhibition was not yet reached at the highest concentration, sigmoidal curves were modeled using extrapolation. Fold differences compared to the WT were calculated, and viruses were considered resistant when the calculated IC\textsubscript{50} was >3-fold higher than that for the WT [35,36]. The infectivities of mutant viruses were calculated relative to the infectivity of HIV-1\textsubscript{LAI} (set at 100%). Infections were performed in quadruplicate, and luciferase activity in the absence of an inhibitor was measured and corrected for background luciferase activity.
Chapter 6

Virus thermostability experiments
WT and mutant viruses were incubated for 1 h in the presence or absence of 0.5 μg/ml sCD4 over the temperature range of 37°C to 50°C by use of a thermocycler before testing of their residual infectivity on TZM-bl reporter cells as described above. Because the A60E, E64K, and H66R mutants were not infectious in the absence of drug, VIR165 (10 or 30 μg/ml) was added after incubation in the presence or absence of sCD4 and was present during infection of TZM-bl reporter cells. The midpoint of thermal denaturation ($T_m$) was defined as the temperature at which 50% residual infectivity was observed.

Virus decay experiments
WT and mutant viruses were incubated at 37°C in the presence or absence of 1.0 μg/ml sCD4 (final concentration during infection, 0.5 μg/ml) for 0, 2, 4, 8, 16, 24, 32, and 96 h before testing of their infectivity on TZM-bl reporter cells as described above. Because the A60E, E64K, and H66R mutants were not infectious in the absence of drug, VIR165 (10 or 30 μg/ml) was added during infection of TZM-bl reporter cells. Nonlinear regression curves were determined and half-lives ($t_{1/2}$) calculated using Prism software, version 5.0.

Statistical analysis
All statistical comparisons were performed by the unpaired t test (two-tailed), using Prism software, version 5.0, and statistical significance is indicated by asterisks in the figures, as described in the figure legends.

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Escape from HIV-1 anchor inhibitor VIRIP165 through Env stabilization


