Trimeric, Coiled-coil Extension on Peptide Fusion Inhibitor of HIV-1 Influences Selection of Resistance Pathways*

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Background: N-terminal, heptad repeat (HR1) peptides of HIV envelope glycoprotein form coiled-coil oligomers that inhibit viral entry, but the targets are unclear.
Results: An HR1 peptide stabilized as a trimer preferentially selects one resistance pathway, whereas the same unrestrained peptide selects two pathways.
Conclusion: Stabilizing the trimer affects development of resistance.
Significance: These findings inform inhibitor design and provide insights into virus entry.

Peptides corresponding to N- and C-terminal heptad repeat regions (HR1 and HR2, respectively) of viral fusion proteins can block infection of viruses in a dominant negative manner by interfering with refolding of the viral HR1 and HR2 to form a six-helix bundle (6HB) that drives fusion between viral and host cell membranes. The 6HB of the HIV gp41 (endogenous bundle) consists of an HR1 coiled-coil trimer with grooves lined by antiparallel HR2 helices. HR1 peptides form coiled-coil oligomers that may bind to gp41 HR2 as trimers to form a heterologous 6HB (inhibitor bundle) or to gp41 HR1 as monomers or dimers to form a heterologous coiled coil. To gain insights into mechanisms of Env entry and inhibition by HR1 peptides, we compared resistance to a peptide corresponding to 36 residues in gp41 HR1 (N36) and the same peptide with a coiled-coil trimization domain fused to its N terminus (IZN36) that stabilizes the trimer and increases inhibitor potency (Eckert, D. M., and Kim, P. S. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 11187–11192). Whereas N36 selected two genetic pathways with equal probability, each defined by an early mutation in either HR1 or HR2, IZN36 preferentially selected the HR1 pathway. Both pathways conferred cross-resistance to both peptides. Each HR mutation enhanced the thermostability of the endogenous 6HB, potentially allowing the virus to simultaneously escape inhibitors targeting either gp41 HR1 or HR2. These findings inform inhibitor design and identify regions of plasticity in the highly conserved gp41 that modulate virus entry and escape from HR1 peptide inhibitors.

The envelope glycoprotein (Env) of HIV-1 undergoes conformational changes that trigger fusion between the virus and host cell membranes, releasing the viral core into the cell’s cytoplasm to initiate infection. Binding of the surface subunit (gp120) of Env to receptors on target cells triggers the conformational changes that culminate in refolding of the N- and C-terminal heptad repeat regions (HR12 and HR2, respectively) in the transmembrane subunit (gp41) of Env to form a thermostable 6HB structure (endogenous bundle) (1, 2). Three HR1 helices from the trimeric Env comprise the internal coiled-coil core (endogenous coiled coil) of the 6HB with grooves that are lined by HR2 helices packing in an antiparallel manner (3–7). During gp41 refolding, each HR becomes transiently exposed and accessible to inhibitors before collapsing into the 6HB that draws the viral and host membranes together (Fig. 1A) (8–10).

Peptides corresponding to HR1 and HR2 inhibit HIV infection by interfering with formation of the endogenous bundle by a dominant negative type of mechanism (11–13). The HR2 peptide T20 (enfuviride or Fuzeon®), which is an antiretroviral drug used in the clinic, binds to the HR1 of gp41 and probably forms a peptide-gp41 6HB (inhibitor bundle) that impedes refolding of the endogenous 6HB (Fig. 1A) (14). However, resistance to T20 can develop, and escape mutations frequently map to specific residues in the N terminus of HR1 that reduce the affinity for the HR2 peptide (15–19).

HR1 peptides also inhibit HIV, but they are typically less potent than HR2 peptides unless restrained by modifications that stabilize a trimeric, coiled-coil structure (20, 21). The relatively weaker potency of unrestrained HR1 peptides compared with HR2 peptides probably results from the tendency of HR1 peptides to aggregate (22) and exhibit multiple oligomeric states (23), which would reduce the effective concentration of the inhibitory species. Modifications of HR1 peptides that stabilize trimers and reduce aggregation enhance potency, presumably by increasing the concentration of coiled-coil trimers, which can bind to the HR2 of gp41 to form an inhibitor bundle (Fig. 1A). For example, a chimeric HR1 peptide corresponding to 36 amino acids in the HR1 of gp41 with a synthetic trimeric...
coiled-coil domain at its N terminus is considerably more potent than the same peptide (N36) without the extension (20). Similarly, an inhibitor called 5-helix, which mimics the 6HB but lacks a single HR2 helix and exposes a single coiled-coil groove, is also much more potent than an unmodified HR1 peptide (21). HR1 peptides have not yet advanced to the clinic, and data on HR1 resistance are limited.

Unrestrained HR1 peptides form less stable trimers compared with restrained HR1 peptides. Although this attribute would reduce the contribution that the trimeric species could make to inhibition, other non-trimeric species that may be present in a dynamic equilibrium would also have the potential to interact with gp41 in alternative ways to inhibit HIV entry. According to the dominant negative mode of inhibition, unrestrained HR1 peptides, as monomers for example, could bind to the HR1 of gp41 by intercalating into the HR1 endogenous coiled-coil to form an inhibitor coiled-coil (Fig. 1A). In support of this model are reports showing that an engineered HR1 peptide with mutations that interfere with binding to HR2 can inhibit HIV (24), and an ectodomain fragment of gp41 from the simian immunodeficiency virus exists in an monomer-trimer equilibrium (25). Thus, HR1 could inhibit HIV by binding to either HR1 or HR2 on gp41.

To gain insights into the Env entry mechanism and how HR1 peptide fusion inhibitors interfere with this process, we undertook a comparative study of a restrained and unrestrained HR1 peptide inhibitor (Fig. 1B). We generated multiple, independent resistance cultures to N36, an unrestrained HR1 peptide corresponding to 36 residues of the HR1 that comprises the core gp41 structure, and to the restrained HR1 peptide IZN36, corresponding to the same 36 residues of HR1 but with a synthetic trimeric coiled-coil extension at its N terminus (20). IZN36, designed by Eckert et al. (20), was previously shown to have improved coiled-coil trimer stability and greater potency than N36. We found that N36 selected for two different genetic pathways for resistance, each defined by a specific early mutation in either HR1 or HR2. This finding extends our prior study involving an overlapping peptide (35), underscoring the importance of both pathways for resistance. However, IZN36 preferentially selected the HR1 pathway, although the HR2 pathway was able to confer high level resistance. We further characterized biophysical and phenotypic properties of Env with various combinations of mutations identified in the resistance cultures. Implications for the HIV Env entry mechanism and inhibitor design are discussed.

EXPERIMENTAL PROCEDURES

Cells and Plasmids—293T cells and U87 cells expressing CD4 and CCR5 (U87-CD4+CCR5+) were provided by Dan Littman (New York University). The plasmid pRev was provided by Dr. Tom Hope (Northwestern University, Chicago, IL) (27). HeLa cells expressing various levels of CD4 and CCR5
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(RC4, RC49, and JC53) were a gift from David Kabat (28) (Oregon Health and Science University, Portland, OR). PM-1 lymphoid cells expressing CD4, CXCR4, and CCR5 receptors (29) were obtained from Michael Norcross (United States Food and Drug Administration, Bethesda, MD). Plasmids pSCTZ-α and pSCTZ-ω were gifts from Dr. Ned Landau (New York University). The proviral plasmid pLAI(JRcsf), expressing the LAI genome except with the env gene replaced with JRcsf env was provided by Keith Peden (Food and Drug Administration). The expression vector pCMV/R and the Env-deficient HIV genome plasmid pCMVAΔ2.2 and the pHR'-Luc that contains the reporter gene were provided by Gary Nabel (National Institutes of Health, Bethesda, MD). The JRcsf Env expression plasmid with wild type or selected mutations were made by inserting the env gene into the NotI and EcoRV restriction sites of the pCMV/R plasmid as described previously (35).

Reagents—Synthetic peptides N36 (corresponding to HXB2 residues 546–581; SGIVQQQNLLRAIEAQHQLTTVWKIKQLQARIILN36 (IKKEIAIIEAEKIAIKKEIIEGISIVQQQNLLRAIEAQHQLLQTVWGIKQLQARIL), C34 HXB2 (corresponding to HXB2 residues 268–661; WMEDREINNYTSILHSLIESQNNQKEKNQELL), C34 (WMWEKEIEINTNTIYTLIESQIIQQKEKNEQELL) and its mutants (T64II and E648K), and T20 (YTLIESQIIQQKEKNEQELL) were provided by Keith Peden (Food and Drug Administration). The JRcsf Env expression plasmid containing each mutation or selected combinations were constructed by restriction endonuclease digestion or mutagenesis (Stratagene, La Jolla, CA).

Pseudovirus Inhibition Assay—Pseudovirus stocks with WT or mutant Env proteins were generated and assessed for infectivity as described previously (35). Briefly, 5 × 10⁴ 293T cells in 10-cm diameter dishes were cotransfected with 0.5 μg of the Env expression vector, 4 μg of the Env-deficient viral vector (pCMVAΔ2.2), and 4 μg of reporter vector (pHR'-Luc). The supernatants were filtered and collected at 48 h posttransfection and quantified by a p24 enzyme-linked immunosorbent assay (ELISA) (NCI-Frederick), and aliquots were stored at −80 °C. Pseudovirus infectivity was determined by combining three independent pseudovirus stocks and calculating the 50% tissue culture infectivity dose by the Reed and Muench method (30). The equivalent 50% tissue culture infectivity dose inocula of pseudovirus stocks was infected to each well of U87-C4-D4 cells (2 × 10⁵ cells/well in 96-well plates) in the presence of peptide or sCD4 in a total volume of 100 μl supplemented with 8 μg of Polyperene (Sigma) per ml. Forty-eight hours after infection, the luciferase activity was measured using luciferase substrate (Promega, Madison, WI) on a luminometer (L-Max, Molecular Devices, Sunnyvale, CA) according to the manufacturer’s instructions. The HeLa cells containing various levels of CD4 and CCR5 receptors were also used for pseudovirus infection.

Cell-Cell Fusion Inhibition Assay—Five million 293T cells were transfected in a 10-cm dish with 0.5 μg of Env expression vector, 4 μg of pRev, and 4 μg of pSCTZ-α (293T-Env), and 3 × 10⁶ RC4, RC49, and JC53 cells were transfected with 6 μg of pSCTZ-ω plasmid in a 10-cm dish. Twenty-four hours later, the target cells (RC4, RC49, and JC53) were harvested with Cell Dissociation Solution (Sigma), pelleted, and resuspended in DMEM. The target cells were added in a 96-well plate at 1 × 10⁴ cells/well. After 36 h of transfection, the 293T-Env effector cells were dissociated, as described above, and 1.5 × 10⁴ cells were added into each well of target cells. After a 5-h co-cultivation at 37 °C, which we determined to be during the linear phase of

Resistance Viruses—Viral stocks of HIV type 1 (HIV-1) LAI-JRcsf were generated by transfecting the proviral molecular clone pLAI(JRcsf) into 293T cells using Fugene 6 (Roche Applied Science) and collecting filtered culture supernatants after 2 days. Virus was quantified by an HIV-1 p24 antigen capture assay (NCI-Frederick) and stored at −80 °C. HIV-1 LAI-JRcsf was passaged on PM-1 cells under increasing drug concentrations, starting at approximately the 90% inhibitory concentration (IC₉₀; 1.5 μM for N36 and 20 nM for IZN36), as described previously (26). Briefly, resistant virus was generated by infecting 10⁶ PM-1 cells with 30 ng of p24 from HIV-1 LAI-JRcsf virus stock in 4 ml of RPMI 1640 medium in the presence of each inhibitor overnight. The cells were washed once the next day by centrifugation at 200 × g for 10 min and resuspended with 4 ml of medium containing the same concentration of peptide. Three days later, half of supernatant was exchanged with fresh medium containing peptide. After the first week, half of the cells and supernatant were removed every 3 days and replaced by an equal volume of peptide-containing medium. Cell supernatants were sampled every 3 days for virus production by p24 detection. Supernatants containing the highest level of p24 were then used to establish subsequent passages, using ~30 ng of p24-containing supernatant, according to the infection protocol described above but with escalating peptide concentrations.

Resistant Env—Genomic DNA from infected PM-1 cells was extracted by using the Qiagen DNAeasy kit. The proviral DNA from each culture was sequenced, and chromatograms were inspected to confirm the dominant mutations arising in the env gene after each passage. For selected passages, the gp160 gene from the proviral genome was amplified by PCR with the Phusion kit (New England Biolabs, Ipswich, MA) and the pair of primers Envf (ACGATCGGATATCGCCGCACTATGAGAGTAGGAGGAGAAATAC) and Envr (TCTAGAGCGCCGCGTTTATAGCAAAGCCCTTCTCAGAG). The PCR product was placed into the EcoRV and NotI sites in the Env expression plasmid pCMV/R-JRcsf-Env to replace the env gene. Each clone was verified to have the expected mutations by sequencing the entire gp160 gene. To confirm the contribution of each mutation in the HR1 or HR2 region for resistance, the mutation(s) was introduced into the JRcsf Env expression vector. The Env expression plasmids containing each mutation or selected combinations were constructed by restriction endonuclease digestion or mutagenesis (Stratagene, La Jolla, CA).
cell-cell fusion, the cells were lysed and mixed with the substrate (Galacto-Star™ system, Applied Biosystems, Bedford, MA). The light emission was measured by luminometry (L-Max, Molecular Devices). For inhibition of cell-cell fusion, the 293T-Env effector cells and HR1 peptide inhibitors at various concentrations were added into each well of target cells.

Circular Dichroism Spectroscopy—HR1 and HR2 peptides were mixed (10 μM each) in 50 mM sodium phosphate (pH 7.0) containing 150 mM NaCl and incubated at 37 °C for 30 min (final volume, 0.2 ml) prior to analysis. The isolated HR1 and HR2 peptides were also tested. Circular dichroism (CD) spectra of these peptides or peptide mixtures were acquired on a Jasco spectropolarimeter (model J-810, Jasco Inc.) at room temperature using a 1.0-nm bandwidth, 0.1 nm resolution, 0.1-cm path length, 4.0-s response time, and a 5-nm/min scanning speed. The spectra were corrected by subtraction of a blank corresponding to the solvent. The α-helical content was calculated from the CD signal by dividing the mean residue ellipticity at 222 nm by the value expected for 100% helix formation (33, 34). Thermal denaturation was monitored at 222 nm by applying a thermal gradient of 2 °C/min in the range of 4–95 °C. Reverse melt from 95 to 4 °C was also detected. The melting curve was smoothed, and the midpoint of the thermal unfolding transition (Tm) value was determined using Jasco software. The Tm averages of at least two measurements for each complex were calculated. The fraction of unfolded molecules was analyzed according to a two-state N→U mechanism (33).

Native PAGE—The 6HB formation between HR1 and HR2 peptides was detected by native PAGE, as described previously (34). The peptide pairs of N36 (or IZN36) and C34 of each variant were incubated at a final concentration of 40 μM at 37 °C for 30 min. The mixture was loaded onto a precast 18% Tris-glycine gel (Invitrogen). The gel was then stained with Coomassie Blue before imaging (Odyssey LI-COR imaging system (LI-COR, Lincoln, NE)).

Statistics—At least five independent dose-response curves, using multiple batches of each pseudovirus, were generated for each inhibitor. The 50% inhibitory concentrations (IC50 values) relative to no inhibitor for each individual pseudovirus were calculated by nonlinear regression analysis with GraphPad Prism software (La Jolla, CA). The geometric mean IC50 was determined for each pseudovirus and inhibitor. To compare across independent experiments, the -fold change in resistance relative to wild-type pseudovirus or fusion activity for each inhibitor was determined by calculating the ratios of geometric mean IC50 for mutants to geometric mean IC50 for the wild type. The IC50 for each mutant was compared with that for the wild type using Student's t test. p values of <0.05 were considered statistically significant.

RESULTS

Resistance Cultures—The hybrid virus LAI(JRcsf) consisting of the LAI proviral genome with the env gene replaced by the R5 JRcsf env gene was passaged on PM-1 cells in the presence of increasing concentrations of peptide, starting with the IC90 for each inhibitor (1.5 μM for N36 and 20 nM for IZN36). Four independent resistance cultures were established for each peptide. Two additional independent cultures that were passaged in parallel without inhibitor served as controls, along with four additional independent cultures that were previously passaged without inhibitor under similar conditions (35). After 5–8 passages, the inhibitor concentrations were increased ~10-fold (culture 1) or 20-fold (cultures 2–8) up to 30 μM for N36 and 400 nM for IZN36 (Fig. 2). Higher levels of resistance could not be achieved with additional passages of culture 1. Overall, the time to achieve a 20-fold increase in resistance tended to be
faster for IZN36 compared with N36 (Fig. 2 and supplemental Fig. S1, A and B).

Direct sequencing of the entire env gene from cellular proviral DNA revealed mutations in gp120 and gp41 in all resistance cultures (Fig. 2), but there were no mutations in gp41 in the two control cultures from this study (Fig. 2) or the four control cultures from our prior study (35). Among the four cultures under N36 selection, two patterns of gp41 mutations emerged based on acquisition of either a glutamic acid to lysine substitution at position 560 (E560K, using HXB2 numbering) in HR1 (referred to as pathway I) or a glutamic acid to lysine substitution at position 167 (D167N) or the valine to glycine substitution at position 125 (L125F) in the cytoplasmic tail of gp41 was seen in a late passage of a culture from pathway I.

Mutations in gp120 were present in all resistance and control cultures. Many of these mutations arise in regions of gp120 that have been associated with receptor interactions and probably reflect, at least in part, adaptation to growth on the PM-1 cells, which have low levels of CD4 and CCR5 receptors. For example, the leucine to phenylalanine substitution at position 125 (L125F), present in cultures 3, 4, 5, and 6, was seen in one of the prior control cultures (35) and has been associated with CD4 receptor interactions (36). We also noted previously that the L125F mutation frequently occurs with the E560K mutation but not the E648K mutation (35). On the other hand, mutations in V3 occurred in both cultures with the E648K mutation, confirming the pattern of V3 mutations clustering with the E648K mutation, which was seen previously (35) Mutations in the V1/V2 loop, such as the aspartic acid to asparagine substitution at position 167 (D167N) or the valine to glycine substitution at position 169 (V169G) occurred in both cultures with the E648K mutation (pathway II) and in one culture selected with IZN36 (culture 7, pathway I), respectively. The D167N mutation, as well as a few other point mutations in V1/V2, was observed in control cultures from our previous study (35) suggesting that they are adaptive mutations that improve virus growth in the culture.

Effect of Mutations on HR1 Peptide Resistance—Envs with all or selected gp41 mutations from each of the resistance cultures were incorporated into pseudoviruses for assessing their contributions to resistance. All Envs examined were efficiently expressed, processed, and incorporated into pseudovirus (supplemental Fig. S2). The IC50 for each inhibitor was determined on target cells (Table 1), which express high levels of CD4 and CCR5 target cells and limits of the single-cycle infection assay that cannot amplify the phenotype through repeated rounds of infection. We also analyzed selected mutations alone and in combinations for their contributions to resistance in the
pseudovirus assay (Fig. 3B). Only multiple mutations showed high level resistance, and Q577R appeared to synergize with E560K or E648K in conferring resistance. Combinations of gp41 mutations appeared to account for almost all of the resistance.

To further address how levels of receptors affects resistance, we analyzed the resistance mutations in a cell-cell fusion assay (37) that gives a robust readout of Env-mediated fusion in cells with low levels of receptors. For example, the Env from culture 1 under N36 selection, which did not show significant resistance in our pseudovirus assay, indeed showed higher levels of resistance in cells with lower levels of receptors (RC4) compared with cells with high levels of receptors (JC53) (Fig. 4A).

Both wild-type and mutant Env showed reduced sensitivity to N36 inhibition using cells with high receptor levels compared with low receptor levels (Fig. 4A). In all cases involving the cell-cell fusion assay using target cells with low levels of receptors, Envs from the resistance cultures show significant resistance against N36 (Fig. 4B) and IZN36 (Fig. 4C).

Effect of Mutations on Sensitivity to Other Inhibitors—We next examined the effect of the mutations on the susceptibility to inhibition by T20 or sCD4 (Fig. 5 and Table 1). Compared with wild-type Env, the pseudovirus with Env from cultures 1, 2, 3, and 7 showed significant resistance to T20 (Fig. 5A). As before, these assays were performed on U87-CD4\(^+\) CCR5\(^+\) cells that support efficient entry of pseudoviruses, so resistance levels may be higher in cells with lower levels of receptors. Individual or combined gp41 mutations showed that most of the resistance could be attributed to mutations in HR2 or HR2 mutations with Q577R (Fig. 5B). Significantly, these mutations have not been previously reported as primary resistance mutations to T20.

All Envs from the resistance cultures showed dramatic sensitivity to sCD4, with IC\(_{50}\) values generally reduced by approx-
approximately 2 logs (Fig. 5C and Table 1). Whereas greatly enhanced sensitivity to sCD4 would be consistent with gp120 adaptive mutations, we found that the Envs with mutations in HR1 and no gp120 mutations were also highly susceptible to sCD4 inhibition, although they did not account for all of the enhanced sensitivity (Fig. 5D). The isolated HR2 mutations did not confer enhanced sensitivity to sCD4, and HR1 was dominant over HR2 in conferring the sCD4-sensitive phenotype.

Effect of Mutations on HR1-HR2 Interactions—Resistance mutations in the HR that would directly interfere with inhibitor binding to gp41 would also probably interfere with the ability of the HR1 and HR2 in gp41 to self-assemble into the 6HB that drives HIV entry. To model HR1-HR2 interactions between the N36 or IZN36 inhibitor and HR2 of gp41 (inhibitor bundle) or between HR1 and HR2 of gp41 (endogenous bundle), we made HR1 and HR2 synthetic peptides with and without the resistance mutations (Fig. 1B) and analyzed their self-assembly by circular dichroism spectroscopy in thermal denaturation studies (Fig. 6 and Table 2). The endogenous bundle, referring to the 6HB that is formed by self-assembly of the HR1 and HR2 (based on JRcsf sequences) from the virus, was modeled by the N36 JRcsf + C34 peptides. The inhibitor bundle, referring to the 6HB formed by the HR1 inhibitors (N36 or IZN36, based on HXB2 sequences) binding to HR2 of the virus, was modeled by the N36 or IZN36 + C34 peptides.

All mixtures of HR1 and HR2 peptides demonstrated high helical content (supplemental Fig. S3) and displayed relatively high transition midpoints ($T_m$) in their thermal denaturation curves (ranging from 43.5 to 76 °C), consistent with the formation of 6HB structures (Table 2 and Fig. 6). Remarkably, each mutation increased the $T_m$ of peptide mixtures representing the endogenous bundle, and combinations of mutations increased the $T_m$ values further (Fig. 6, A–C, and Table 2A). Both E560K and E648K mutations, representing the anchor mutations defining escape pathways I and II, respectively, each increased the $T_m$ by 5.5 °C ($\Delta T_m$), whereas the Q577R mutation contributed a further gain of 8.5 °C and 7 °C in thermal stability in pathways I and II, respectively (Fig. 6, A and B, and Table 2A, $\Delta T_m$ column), in agreement with findings in our prior study (35) and another study using a different HR1 peptide inhibitor (38). The combination of E560K, Q577R, and T641I mutations, seen in culture 7, provided the greatest net gain in the $T_m$ (21.5 °C, Table 2A, $\Delta T_m$ column). Thus, increasing thermal stability of the 6HB is a theme among all the resistance cultures for both inhibitors.

We next assessed the effect of the resistance mutations on the inhibitor bundle formed by the inhibitors (N36 or IZN36) binding to the HR2 peptides (C34) with or without the resistance mutations (Fig. 6, A–C, and Table 2B). Because the inhibitor cannot mutate, HR1 mutations acquired by the virus would only affect the endogenous bundle, not the inhibitor bundle. HR2 mutations, on the other hand, could affect both the endogenous and inhibitor bundles.

First, we found that the $T_m$ for the inhibitor bundle formed by mixing the N36 peptide inhibitor with the wild-type C34 peptide was 50 °C, considerably higher than the endogenous bundle formed by mixing the endogenous HR1 peptide (N36 JRcsf) with wild-type C34 peptide (43.5 °C) (Fig. 6A). This find-

![FIGURE 5. Inhibition of pseudoviruses with various mutations by T20 and sCD4. The IC50 values for T20 and sCD4 were determined for the indicated pseudoviruses. All mutations in each resistance culture are shown in A and C. Pseudoviruses with the indicated gp41 mutations are shown in B and D. Values were normalized to the IC50 of the wild-type pseudovirus (WT). The averages and S.D. values (error bars) of at least three independent experiments are shown. Cultures 4 and 5 have the same mutations. C, culture. *, $p < 0.05$ compared with WT.](#)
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![Graphs showing thermal denaturation studies](image)

FIGURE 6. Thermal denaturation studies of the α-helical complexes formed with HR1 and HR2 peptides. Unfolding was recorded at 222 nm by circular dichroism spectroscopy at the indicated temperatures. The calculated $T_m$ values are shown. A, complexes formed by mixing C34 with IZN36 or the indicated N36 peptide. B, complexes formed by mixing C34 with or without the mutation at position 568 with IZN36 or the indicated N36 peptide. C, complexes formed by mixing IZN36 or N36 with or without the mutation with C34 with or without the mutation at position 641. D, HR1 peptides, with and without resistance mutations, and N36 and IZN36 inhibitors based on HXB2 HR1 sequences. Results shown are representative of two experiments.

Unfolding was recorded at 222 nm by circular dichroism spectroscopy at the indicated temperatures. The calculated $T_m$ values are shown. A, complexes formed by mixing C34 with IZN36 or the indicated N36 peptide. B, complexes formed by mixing C34 with or without the mutation at position 568 with IZN36 or the indicated N36 peptide. C, complexes formed by mixing IZN36 or N36 with or without the mutation with C34 with or without the mutation at position 641. D, HR1 peptides, with and without resistance mutations, and N36 and IZN36 inhibitors based on HXB2 HR1 sequences. Results shown are representative of two experiments.

The inhibitor bundle formed by mixing the more potent IZN36 peptide and the wild-type C34 peptide has an even higher $T_m$ (66.3 °C) (Fig. 6A). When the inhibitors were mixed with HR2 peptides with the resistance mutations, the $T_m$ values of the bundles formed with the inhibitors increased further (Fig. 6, B and C, and Table 2B). Although the $T_m$ values of inhibitor bundles formed with wild-type HR2 peptide (C34) were higher than the $T_m$ of the endogenous bundle, the E648K resistance mutation nonetheless conferred a slightly greater net increase in $T_m$ for the endogenous bundle ($\Delta T_m$, 5.5 °C) compared with the inhibitor bundles formed with the N36 ($\Delta T_m$, 3 °C) or IZN36 ($\Delta T_m$, 3.7 °C) (Fig. 6, A and B, and Table 2B). The addition of the Q577R mutation in HR1, which was seen in six of the eight selection cultures in this study, contributed an additional gain of 8.5 °C to the endogenous bundles only (Fig. 6B and Table 2A, $\Delta T_m$ column).

The 6HB formed by mixing the HR1 and HR2 peptides was also visualized by native PAGE (Fig. 7). The HR1 peptides, which have net positive charges, showed no bands because they migrated out of the native gel, whereas the C34 peptides carrying a net negative charge migrated into the gel and showed a single specific band (Fig. 7, A and B). The HR2 peptides migrated with slight differences due to the varying mutations; the peptides with K or R mutations migrated more slowly than wild type due to their greater net positive charge.

The specific bands resulting from mixing the HR1 and HR2 peptides migrated much more slowly, consistent with the formation of the 6HB (Fig. 7, A and B). Again, the slightly different rates of migration were a result of the differences in net charges and molecular masses. The bands seen for the IZN36 and C34 peptide mixtures also migrated more slowly than N36 and C34 pairs due to the larger molecular mass of IZN36 compared with N36 (Fig. 7A, right lanes). In agreement with the CD analyses, the native PAGE studies showed that the peptide mixtures, even with resistance mutations in the C34 peptides, did not show a reduction in bands corresponding to the 6HB, confirming that the mechanism of resistance does not depend on direct impairment of inhibitor binding to contact residues in the gp41 HR. Rather, all mutations in HR1 and HR2 appeared to improve formation of the endogenous bundle, especially N36 JRcsf-560K577R-C34, N36 JRcsf-560K577R-C34–641I, and N36 JRcsf-577R-C34–648K (Fig. 7).

Finally, we investigated the potential impact of the HR1 mutations on the ability the endogenous HR1 to form a coiled coil in the absence of HR2 peptides (Fig. 6D and Table 2C), which may reflect a structure present in an early fusion intermediate conformation of Env (Fig. 1A). Unrestrained HR1 peptides do not form homogeneous solutions (39) and may exist as multiple oligomeric species with some aggregation; however, our data indicate that these HR1 peptides have helical content, and the thermal denaturation curves were reversible. Surprisingly, we found that the E560K mutation alone increased the $T_m$
The Q577R mutation alone increased the $T_m$ by only 1 °C and partially offset the effect of E560K on the $T_m$, resulting in a net increase of 8 °C (Fig. 6A and Table 2C, $/H9004 T_m$ column). These findings raise the intriguing possibility that the E560K mutation in the context of the trimeric Env may have an effect on stabilizing or enhancing formation of the endogenous coiled-coil trimer, which could improve competitiveness of the resistant virus compared with wild-type virus against the trimeric IZN36 inhibitor.

The N36 inhibitor, based on the HXB2 sequences, proved to have an even higher $T_m$ compared with the endogenous coiled coil (43 versus 21 °C, respectively) (Fig. 6D and Table 2D). Nonetheless, an HR1 mutation in the virus that stabilizes a coiled-coil intermediate would still favor the virus over the inhibitor because the inhibitor does not have the mutation. In contrast to E560K, the Q577R mutation alone appears to do little to improve potential coiled-coil stability and instead appears to have its greatest impact on stabilizing the endogenous bundle (Fig. 6, A and D, and Table 2, A and C). The curve for the IZN36 inhibitor did not show a plateau, so a $T_m$ was not calculated (Fig. 6D). Together, these studies indicate that the mutations confer a greater net thermodynamic advantage to the virus compared with the inhibitors. Both pathways favor formation of the endogenous bundle, and the E560K anchor

### TABLE 2

Thermal denaturation studies of HR1 and HR2 peptides

| A. Endogenous bundle | HR1 peptide | HR2 peptide | $T_m^a$ (°C) | $\Delta T_m^b$ (°C) |
|----------------------|-------------|-------------|--------------|------------------|
| N36 JRcsf           | C34         | 43.5        | -            |
| N36 JRcsf           | C34-648K    | 49.0        | 5.5          |
| N36 JRcsf-577R      | C34         | 52.0        | 8.5          |
| N36 JRcsf-577R      | C34-648K    | 56.0        | 12.5         |
| N36 JRcsf-560K      | C34         | 49.0        | 5.5          |
| N36 JRcsf-560K577R  | C34         | 57.5        | 14.0         |
| N36 JRcsf           | C34-641I    | 53.5        | 10.0         |
| N36 JRcsf-560K577R  | C34-641I    | 65.0        | 21.5         |

| B. Inhibitor bundle | HR1 peptide | HR2 peptide | $T_m^a$ (°C) | $\Delta T_m^b$ (°C) |
|---------------------|-------------|-------------|--------------|------------------|
| N36                 | C34         | 50.0        | -            |
| N36                 | C34-641I    | 58.0        | 8.0          |
| N36                 | C34-648K    | 53.0        | 3.0          |
| IZN36               | C34         | 66.3        | -            |
| IZN36               | C34-641I    | 76.0        | 9.7          |
| IZN36               | C34-648K    | 70.0        | 3.7          |

| C. Endogenous HR1 peptide | HR1 peptide | $T_m^a$ (°C) | $\Delta T_m^b$ (°C) |
|----------------------------|-------------|--------------|------------------|
| N36 JRcsf                  | 21.0        | -            |
| N36 JRcsf-560K             | 34.0        | 13.0         |
| N36 JRcsf-577R             | 22.0        | 1.0          |
| N36 JRcsf-560K577R         | 29.0        | 8.0          |

| D. Inhibitor HR1 peptide | HR1 peptide | $T_m^a$ (°C) | $\Delta T_m^b$ (°C) |
|--------------------------|-------------|--------------|------------------|
| N36 JRcsf                | 43.0        | -            |
| IZN36                    | ND          | -            |

$^a T_m$, midpoint of the thermal unfolding transition.

$^b \Delta T_m$, change in $T_m$ due to the mutation compared with the peptide without mutation.

$^c$ ND, not determined; --, not applicable.

by 13 °C (Fig. 6D and Table 2C), even more than it increased the $T_m$ of the 6HB (5.5 °C) (Fig. 6A and Table 2A). The Q577R mutation alone increased the $T_m$ by only 1 °C and partially offset the effect of E560K on the $T_m$, resulting in a net increase of 8 °C (Fig. 6A and Table 2C, $\Delta T_m$ column). These findings raise the intriguing possibility that the E560K mutation in the context of the trimeric Env may have an effect on stabilizing or enhancing formation of the endogenous coiled-coil trimer, which could improve competitiveness of the resistant virus compared with wild-type virus against the trimeric IZN36 inhibitor.

The N36 inhibitor, based on the HXB2 sequences, proved to have an even higher $T_m$ compared with the endogenous coiled coil (43 versus 21 °C, respectively) (Fig. 6D and Table 2D). Nonetheless, an HR1 mutation in the virus that stabilizes a coiled-coil intermediate would still favor the virus over the inhibitor because the inhibitor does not have the mutation. In contrast to E560K, the Q577R mutation alone appears to do little to improve potential coiled-coil stability and instead appears to have its greatest impact on stabilizing the endogenous bundle (Fig. 6, A and D, and Table 2, A and C). The curve for the IZN36 inhibitor did not show a plateau, so a $T_m$ was not calculated (Fig. 6D). Together, these studies indicate that the mutations confer a greater net thermodynamic advantage to the virus compared with the inhibitors. Both pathways favor formation of the endogenous bundle, and the E560K anchor

### Figure 7

Complexes formed after mixing HR1 and HR2 peptides shown in native gel electrophoresis. $A$, both panels are from the same gel, but lanes with irrelevant peptides were removed. $B$, both panels are from the same gel, but lanes with irrelevant peptides were removed. HR1 peptides N36JRcsf and IZN36 peptides migrated off the gel due to the net positive charge. A shift in mobility indicates interactions between the peptides.
mutation in pathway I may possibly also favor formation of the endogenous coiled coil.

**Modeling Resistance Mutations on Six-helix Bundle**—To gain insight into how the mutations might increase thermal stability of the 6HB and the resistance phenotype, we analyzed atomic interactions in the vicinity of the resistance mutations in HR1 and HR2 using available high resolution models of the 6HB. We first located the position of the resistance mutations across the length of the gp41 ectodomain in the HIV model derived from the NMR restraints from the SIV gp41 ectodomain (40) and noted that the mutations cluster in two distinct regions (Fig. 8A). Both anchor mutations defining pathways I and II (highlighted in red) reside in the layer that is located closer to the transmembrane region. The other layer, located closer to the loop region between HR1 and HR2, contains the common Q577R mutation (highlighted in purple) as well as other mutations (highlighted in yellow), which emerged in later passages in some of the cultures (35). The Q577R mutation helps create the hydrophobic pocket (3) and was previously found to contribute significantly to 6HB stability in peptide models (35).

We also observed that the resistance mutations in the lower layer are found in helical positions with the side chains oriented away from the 6HB core (Fig. 8B), a somewhat unexpected finding given their contributions to thermal stability and resistance. In the context of the gp41 crystal structure (4), it appears that E560K and E648K would have the potential to interact with the adjacent helix containing the lysine mutation (Fig. 8C, middle and bottom). Although there are no negatively charged residues close by that could make good salt bridge partners with the lysine mutation, there is an asparagine and arginine residue that could help establish hydrogen bond interactions. In fact, rearrangement of hydrogen bond networks has previously been reported to increase thermal stability of the 6HB with a glutamic acid to glutamine mutation at position 648, which was identified by another group after N36 selection of the pNL4-3 strain of HIV (38). The Q577R mutation, in contrast, is oriented toward the 6HB core and is putatively hydrogen-bonded with tryptophan residues 628 and 631 (Fig. 8C, top). Substitutions in this position are therefore likely to affect interhelical interactions. Overall, selection with the peptides has identified several non-core 6HB positions that can have a significant effect on 6HB assembly and function.

**DISCUSSION**

Peptide fusion inhibitors corresponding to the HR regions of viral fusion proteins offer new opportunities to study Env struc-
turance-function relationships and develop novel inhibitors. Much has been learned about resistance to T20, corresponding to HR2 sequences, but little is known about resistance to inhibitors corresponding to HR1 sequences. We undertook a comparative analysis of resistance to a restrained and unrestrained HR1 peptide inhibitor to investigate the target sites of HR1 inhibitors and shed light on the mechanism of Env entry and how HR1 peptides disrupt the entry process.

Resistance Pathways—Notably, the unrestrained N36 peptide selects two different genetic pathways with seemingly equal probability, each defined by the mutation of E560K in HR1 (pathway I) or E648K in HR2 (pathway II). Each of these mutations is further associated with gp120 adaptive mutations; the L125F occurring with HR1 resistance mutations in pathway I and in some control cultures has been linked to CD4 utilization (36), whereas mutations in the base of V3 that are found in association with HR2 resistance mutations in pathway II may be involved in co-receptor use. These results, together with our prior study involving selection with a longer HR1 peptide that had a point mutation near its N terminus (N44) (35), strongly suggest that there are two equally fit pathways for HIV to escape an unrestrained HR1 peptide, and they provide further support for a functional link between the resistance mutations in HR1 and HR2 and specific regions of gp120 that may influence CD4 and chemokine receptor use, respectively.

In contrast to N36, resistance to the restrained IZN36 inhibitor showed a preference for the genetic pathway defined by the HR1 mutation (pathway I). All four of the first resistance cultures selected for pathway I. We further confirmed the preference for pathway I by establishing four additional IZN36 resistance cultures and found that three cultures took pathway I, whereas one culture took pathway II (supplemental Fig. S1C). High level resistance to IZN36 also emerged more slowly in pathway II compared with pathway I (supplemental Fig. S1), implying that viruses from pathway I grew better under IZN36 pressure than viruses from pathway II in the replicating cultures, although this difference in resistance was not evident in the pseudovirus assays. Other attributes in the replicating virus cultures, such as multiple rounds of spreading infection or differences in receptor levels on target cells, may also have affected selection of escape mutations. The pseudovirus assays further showed that each pathway conferred cross-resistance to both N36 and IZN36, extending results from our prior study (35), and that resistance against IZN36 was consistently higher than N36, even in the less preferred pathway II.

That IZN36 strongly selected for pathway I, whereas N36 selected equally for pathways I and II, although both pathways confer cross-resistance to both inhibitors, raises many interesting questions regarding the mechanisms of resistance and the gp41 target of each inhibitor. According to the dominant negative model for inhibition, N36 as a trimer could bind to the HR2 in gp41 to interfere with formation of the endogenous bundle, whereas N36 presumably as a monomer or dimer could bind to the HR1 in gp41 to interfere with the endogenous coiled coil (Fig. 1A). Could pathways I and II reflect selection by these different inhibitory species, namely that the trimer selects for pathway I and the monomer selects mostly for pathway II?

The presence of multiple inhibitory species in a dynamic equilibrium would potentially have important implications for the resistance mechanism. If the HR1 peptide inhibitors have more than one target on gp41, such as HR1 and HR2, then escape through mutation of a contact residue for one form of the inhibitor might not impair binding of another form of the inhibitor to a different site. This challenge could be overcome by finding pathways that could confer cross-resistance to both inhibitors.

Biophysical Studies—Our biophysical data reveal an inhibitory mechanism that confers cross-resistance to both inhibitors. The thermal denaturation (Fig. 6 and Table 2A) and binding studies of mixtures of HR1 and HR2 peptides with and without resistance mutations (Fig. 7) indicate that these mutations enhance stability of the endogenous bundle and, in the case of E560K, possibly the coiled coil itself (Table 2C). Increasing the propensity of the endogenous HR to collapse into the fusogenic 6HB would improve the ability of the resistant virus to compete against an inhibitor targeting the fusion intermediate, which may explain the cross-resistance not only to N36 and IZN36 but also to the HR2 peptide T20.

The thermostability studies also introduce an intriguing possibility for why IZN36 preferentially selects pathway I and N36 selects both pathways. Pathway I, defined by the early acquisition of the E560K mutation, may enhance formation of the endogenous coiled coil. This pathway could allow the endogenous trimer to compete better against the trimeric inhibitor. Pathway II, defined by early acquisition of the E648K mutation, appears to give slightly more benefit to the endogenous bundle compared with the inhibitor bundle (Table 2, A and B), and this mutation would have no direct effect on formation of the isolated, endogenous coiled coil.

Additional resistance mutations in both pathways further contribute to the stabilization of the endogenous bundle. It is tempting to speculate that the distribution of the pathways selected by the two inhibitors reflects, at least in part, the dynamic equilibrium of the inhibitor species in the cultures. The restrained IZN36 peptide, which is shifted more toward trimers, selected pathway I in seven of eight cultures. The unrestrained N36 peptide, relative to IZN36, would be expected to be shifted more toward monomers. N36 monomers or dimers could bind to the HR1, making a heterologous coiled coil that would interfere with formation of the endogenous 6HB (Fig. 1A). Such a mechanism has been reported by Bewley et al. (24) for an HR1 peptide inhibitor with mutations that impair binding to the HR2. The E648K mutation, by stabilizing assembly of the endogenous bundle, could favor formation of the endogenous 6HB in the presence of an inhibitor that reversibly binds to HR1. The presence of both trimeric and non-trimeric inhibitory forms of N36 could conceivably account for a more balanced selection of the two pathways.

Although pathways I and II would appear to be a good resistance mechanism for inhibitors with multiple forms that could target HR1 and HR2, it is also possible that N36 targets a single site. In this case, the bundle-stabilizing mutations may have been selected because the alternative solution of mutating residues that would directly impair HR1 inhibitor binding would be too detrimental to the endogenous bundle and impair virus entry. The balanced distribution of pathways I and II resulting from N36 selection might therefore simply reflect two equally
Resistance Pathways Selected by HIV Fusion Inhibitors

...good sets of mutations that permit viral escape from an inhibitor that binds to a single site on Env.

Although the peptide models indicate that thermodynamic parameters contribute to resistance, other factors not directly attributable to thermodynamics also appear to contribute to the selection of particular resistance pathways. We note that the absolute $T_m$ values of the inhibitor bundles are higher than the endogenous bundle, although the resistance mutations improve the thermal stability of the endogenous bundle more than the inhibitor bundle and favor the resistant virus over the wild-type virus (Fig. 6 and Table 2). Therefore, the peptide models, removed from the context of the larger envelope glycoprotein and its interactions with membranes, do not take into account all factors affecting inhibition by the peptides. For example, steric factors relating to refolding of Env intermediates in pathway II may limit access of the larger and more rigid IZN36 compared with N36. Previously, Hamburger et al. (41) reported that increasing the size of a cargo protein fused to an HR2 peptide decreased inhibitor potency.

Additionally, our thermodynamic measurements do not take into account kinetic factors that could play an important role in selection of resistance pathways. Steger and Root (42) have reported that association kinetics correlates with inhibitor potency for a designed protein with an exposed HR1 coiled-coil face. Importantly, the current and prior studies (35) involving three different HR1 inhibitors and more than 12 resistance cultures consistently show that the mechanism of resistance involves enhancement of the thermostability of the endogenous 6HB rather than direct impairment of inhibitor binding.

The comparative resistance studies involving different HR1 inhibitors in the context of otherwise identical culture conditions with clonal viruses also offer insights for inhibitor design. High level resistance tended to emerge faster to IZN36 than to N36 (Fig. 2 and supplemental Fig. S1). Stabilization of the trimer might make the inhibitor more rigid and less able to adapt to subtle structural alterations that are introduced as resistance mutations are acquired. The trimeric extension itself could also impose extra steric constraints that could hinder access to the target site and restrict selection of a particular pathway. Thus, modifications that increase inhibitor potency may not necessarily retard the development of resistance. In addition, inhibitors exhibiting a dynamic equilibrium with multiple inhibitory species that target Env in different ways could create extra hurdles for the virus to escape, because each pathway must resist all forms present at inhibitory concentrations.

Structural Modeling—Finally, we turned to high resolution models of the 6HB to provide clues about how the virus escapes inhibition while maintaining Env function. We observed that the Q577R mutation, located near the hydrophobic pocket adjacent to the loop connecting HR1 and HR2, greatly contributes to the 6HB stability and is often coupled with one or more pathway-defining resistance mutations that reside in the layer closer to the membrane (Fig. 8A). These hot spots of resistance mutations point to potential long range interactions across the length of the gp41 core that appear to synergistically contribute to resistance (Fig. 3B). A similar finding was also seen by Izumi et al. (38) using N36 selection and a different HIV strain. More recently, Welch et al. (43) reported that the E560K mutation along with valine to isoleucine substitution at position 570 emerged after selection with a D-peptide inhibitor targeting the hydrophobic pocket in HR1. Stabilization of the core at a site closer to the membrane could also be important for escape, but the mechanistic details of how these mutations affect Env refolding require further study.

It is also remarkable that the mutations defining each resistance pathway are located at the edges of the packing interface between HR1 and HR2 (Fig. 8B), despite their effect on increasing stability of the core structures. The location at the periphery of the core might allow greater flexibility in tolerating changes, without disrupting the function of the core. In fact, residue 648 is a rare site of polymorphism in the otherwise conserved gp41 core (44). Nonetheless, the resistance mutations do appear to enhance the thermostability of the fusogenic 6HB. Perhaps the gp120 mutations that are associated with each pathway modulate gp120 interactions with receptors so that they are coordinated with changes in gp41 refolding due to the coiled-coil and bundle-enhancing resistance mutations. In this regard, the mutations identified in these resistance studies may be altering the threshold of Env for triggering or enhancing its intrinsic reactivity (45) in response to receptor activation.

CONCLUSION

In summary, we found that an HR1 peptide fusion inhibitor with a trimeric coiled-coil extension (IZN36) that increases inhibitory potency (20) preferentially selected a single resistance pathway, whereas the unrestrained HR1 peptide selected two different pathways with equal probability, although both pathways conferred cross-resistance to each peptide through a mechanism that appears to involve stabilization of the endogenous 6HB. These findings indicate that HR1 peptides pressure the virus to escape inhibition by adopting mutations that enhance the formation of fusogenic conformations of Env to compete better against the inhibitors. This mechanism of resistance sharply contrasts with the mechanism of resistance seen for T20, an HR2 peptide inhibitor, which typically involves mutation of residues that contact the inhibitor. These studies inform inhibitor design and further highlight regions of plasticity in gp41 and gp120 that coordinate to modulate virus entry and escape inhibition by HR peptides.

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