Conserved Residue Lys\textsuperscript{574} in the Cavity of HIV-1 Gp41 Coiled-coil Domain Is Critical for Six-helix Bundle Stability and Virus Entry*

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Yuxian He\textsuperscript{1,5}, Shuwen Liu\textsuperscript{1}, Weiguo Jing\textsuperscript{1}, Hong Lu\textsuperscript{3}, Dongmei Cai\textsuperscript{1}, Darin Jeekin Chin\textsuperscript{1}, Asim K. Debnath\textsuperscript{1,5}, Frank Kirchhoff\textsuperscript{1,5}, and Shibo Jiang\textsuperscript{1,2}

From the \textsuperscript{1}Lindsley F. Kimball Research Institute, New York Blood Center, New York, New York 10021, the \textsuperscript{4}Antiviral Research Center, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, Guangdong 510515, China, and the \textsuperscript{5}Institute of Virology, University of Ulm, 89081 Ulm, Germany

The fusion-active HIV-1 gp41 core structure is a stable six-helix bundle (6-HB) formed by its N- and C-terminal heptad-repeat sequences (NHR and CHR). A highly conserved, deep hydrophobic cavity on the surface of the N-helical trimer is important for stability of the 6-HB and serves as an ideal target for developing anti-human immunodeficiency virus (HIV) fusion inhibitors. We have recently identified several small molecule HIV-1 fusion inhibitors that bind to the gp41 cavity through hydrophobic and ionic interactions and block the gp41 6-HB formation. Molecular docking analysis reveals that these small molecules fit inside the hydrophobic cavity and interact with positively charged residue Lys\textsuperscript{574} to form a conserved salt bridge. In this study, the functionality of Lys\textsuperscript{574} has been finely characterized by mutational analysis and biochemical approaches. We found that substitutions of Lys\textsuperscript{574} with non-conserved residues (K574D, K574E, and K574V) could completely abolish virus infectivity. With a set of wild-type and mutant N36 peptides derived from the NHR sequence as a model, we demonstrated that non-conservative Lys\textsuperscript{574} substitutions severely impaired the stability and conformation of 6-HBs as detected by circular dichroism spectroscopy, native polyacrylamide gel electrophoresis, and enzyme-linked immunosorbent assay. The binding affinity of N36 mutants bearing non-conservative Lys\textsuperscript{574} substitutions to the peptide C34 derived from the CHR sequence dramatically decreased as measured by isothermal titration calorimetry. These substitutions also significantly reduced the potency of N-peptides to inhibit HIV-1 infection. Collectively, these data suggest that conserved Lys\textsuperscript{574} plays a critical role in 6-HB formation and HIV-1 infectivity, and may serve as an important target for designing anti-HIV drugs.

Entry of human immunodeficiency virus type 1 (HIV-1)\textsuperscript{3} into target cells is mediated by its envelope glycoprotein (Env), a type I transmembrane protein consisting of surface subunit gp120 and non-covalently associated transmembrane subunit gp41 (1). Sequential binding of HIV-1 gp120 to its cell receptor CD4 and a coreceptor (CCR5 or CXCR4) can trigger a series of conformational rearrangements in gp41 to mediate fusion between viral and cellular membranes (2–4). Structurally, the gp41 ectodomain contains the N-terminal heptad-repeat sequence (NHR) and C-terminal heptad-repeat sequence (CHR), which are adjacent to the fusion peptide and the transmembrane segment, respectively (Fig. 1A). Crystallographic analyses demonstrated that the NHR and CHR associate to form a stable six-helix bundle (6-HB), representing a fusion-active gp41 core structure, in which three N-helices form an interior, parallel coiled-coil trimer, whereas three C-helices pack in an oblique, antiparallel manner into the highly conserved, deep hydrophobic cavity on the surface of the N-helical trimer (Fig. 1B) (5, 6).

The deep hydrophobic cavity (~15–16 Å long, ~7–8 Å wide, and 5–6 Å deep) is formed by a cluster of 11 residues (Leu\textsuperscript{565}, Leu\textsuperscript{566}, Leu\textsuperscript{567}, Val\textsuperscript{570}, Trp\textsuperscript{571}, Gly\textsuperscript{572}, Lys\textsuperscript{573}, Leu\textsuperscript{574}, Leu\textsuperscript{575}, and Gin\textsuperscript{577}) in the N-helix coiled-coil (2, 6). Three residues from the C-helix (Trp\textsuperscript{528}, Trp\textsuperscript{531}, and Ile\textsuperscript{635}) penetrate into the cavity causing an extensive interaction with the hydrophobic residues in the cavity. Considerable evidence imply that interhelix interaction between the central coiled-coil trimer and the C-terminal helices is an important determinant of HIV-1 infectivity and inhibition thereof (2, 5–8). Numerous studies show that mutations of the interacting residues in the NHR and CHR regions can destabilize the gp41 core structure and thereby abolish membrane fusion and virus infectivity (9–14). Synthetic peptides derived from the N- and C-helices (named N- and C-peptides, respectively) have potent antiviral activity against both laboratory-adapted strains and primary isolates of HIV-1 (5, 15–17). They inhibit the membrane fusion stage of HIV-1 infection in a dominant-negative manner by binding to the counterpart regions of gp41 (NHR or CHR), thus
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FIGURE 1. Core structure of the HIV-1 gp41 molecule. A, schematic view of the gp41 functional regions. FP, fusion peptide. The residue numbers of each region correspond to their positions in gp160 of HIV-1 \textsc{HxB2}. The corresponding positions of the anti-HIV-1 N- and C-peptides are shown above. B, crystal structure of a six-helix bundle formed by N36/C34. C, interaction between C34 and N36 (for clarity only the cavity region is shown). A salt bridge formed by Lys\textsuperscript{574} in NHR and D632 in CHR is indicated.

blocking formation of the viral gp41 core. A C-peptide from the gp41, T-20 (brand name Fuzeon), has been successfully developed as a novel peptidic anti-HIV drug for clinical use (18).

Notably, the crystal structure of the gp41 core reveals that the positively charged residue Lys\textsuperscript{574} in the NHR of HIV-1 gp41 lines in the left wall of the cavity and forms a conserved salt bridge with negatively charged residue Asp\textsuperscript{632} in the CHR (Fig. 1C) (6). The residue Lys\textsuperscript{574} is highly conserved among all HIV-1 isolates (19), and its substitution with alanine (Ala) could severely attenuate HIV-1 replication (20), highlighting its importance for the interhelical interactions between the NHR and CHR. We have recently identified several small compounds, e.g. two N-substituted pyrrole derivatives, NB-2 and NB-64, as novel HIV-1 entry inhibitors, which inhibit HIV-1 fusion and entry at low micromolar concentration by interfering with the gp41 6-HB formation and disrupting the α-helical conformation (21). The loss of anti-HIV activity in the absence of the COOH groups of NB-2 and NB-64 suggests that the acid group may play an important role in mediating antiviral activity. Computer-aided molecular docking analysis has shown that NB-2 and NB-64 fit inside the hydrophobic cavity and their COOH groups interact with the positively charged residue Lys\textsuperscript{574} to compete with the negatively charged residue Asp\textsuperscript{632} for forming the salt bridge (21). Therefore, NB-2 and NB-64 may bind to the gp41 hydrophobic cavity region through hydrophobic and ionic interactions and block the formation of the fusion-active gp41 core, resulting in inhibition of HIV-1-mediated membrane fusion and virus entry (21). This data suggests that the salt bridge between Lys\textsuperscript{574} and Asp\textsuperscript{632} may play a critical role in interhelical interactions between NHR and CHR to form a 6-HB and may serve as a key target for developing HIV-1 entry inhibitors.

In this report, we investigated the functions of Lys\textsuperscript{574} by mutational analysis and biophysical approaches. We found that non-conservative substitutions of Lys\textsuperscript{574} can completely abolish Env-mediated virus entry and severely impair the conformation and stability of the 6-HB modeled by N36 and C34 peptides derived from the NHR (residues 546–581) and CHR (residues 628–661), respectively. We also showed that this residue determines the potency of N-peptide (N36) to inhibit HIV-1 infection.

EXPERIMENTAL PROCEDURES

Construction of HIV-1 Env Mutants—The plasmid encoding HIV-1 HXB2-Env was obtained from Dr. Kathleen Page and Dr. Dan Littman through the National Institutes of Health AIDS Research and Reference Reagent Program. A panel of HXB2-Env mutants (K574R, K574D, K574E, and K574V) were generated by mutagenesis using the QuikChange Kit (Stratagene, La Jolla, CA) and verified by DNA sequencing. The primers used for construction of HIV-1 Env mutants were designed and synthesized according to the manufacturer’s instructions.

Generation of HIV-1 Pseudovirus—HIV-1 pseudovirus was developed as previously described (21, 22). HEK293T cells were co-transfected with a plasmid encoding wild-type or mutant HXB2-Env and a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc.RE) by using FuGENE 6 reagents (Roche Applied Science). Supernatants containing HIV-1 pseudovirus were harvested 48 h post-transfection and used for infection of U87-T4-CXCR4 cells.

Single-cycle Infection Assay—Briefly, U87-T4-CXCR4 cells were plated at 10\textsuperscript{4} cells/well in 96-well tissue culture plates and grown overnight. The supernatants containing pseudovirus were harvested at 48 h post-transfection and used for infection of U87-T4-CXCR4 cells.
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Framingham, MA). The concentration of peptides was determined by UV absorbance and a theoretically calculated molar extinction coefficient \( \epsilon \) (280 nm) of 5500 and 1490 mol/liter<sup>-1</sup> cm<sup>-1</sup> based on the number of tryptophan (Trp) residues and tyrosine (Tyr) residues (all the peptides tested contain Trp and/or Tyr), respectively (23). All peptides were dialyzed against PBS prior to CD and isothermal titration calorimetry (ITC) experiments.

Circular Dichroism (CD) Spectroscopy—An N-peptide (N36 or its mutant) was incubated with the C-peptide (C34) at 37 °C for 30 min (the final concentrations of N-peptide and C-peptide were 10 \( \mu \)M in 50 mM sodium phosphate and 150 mM NaCl, pH 7.2). The isolated N- and C-peptides were also tested. CD spectra of these peptides and peptide mixtures were acquired on Jasco spectropolarimeter (model J-715, Jasco Inc., Japan) at room temperature using a 5.0-nm bandwidth, 0.1-nm resolution, 0.1-cm path length, 4.0-s response time, and a 50-nm/min scanning speed. The spectra were corrected by subtraction of a blank corresponding to the solvent. The \( \alpha \)-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 (\(-33,000\) degrees cm<sup>2</sup> dmol<sup>-1</sup>) according to Refs. 24 and 25. Thermal denaturation was monitored at 222 nm by applying a thermal gradient of 2 °C/min in the range of 10−90 °C. To determine the reversibility, the peptide mixtures were cooled to 10 °C and kept in the CD chamber at 10 °C for 30 min, followed by monitoring of thermal denaturation as described above. The melting curve was smoothed, and the midpoint of the thermal unfolding transition (\( T_{m} \)) values was calculated using Jasco software utilities as described previously (26). Thermal denaturation was analyzed by fitting the CD data to a simple two-state model according to Equation 1 (27).

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\theta = \frac{\theta_N + m_n T + \theta_D + m_D T \exp[\Delta H_{\text{mix}}/R(1/T_m - 1/T)]}{1 + \exp[\Delta H_{\text{mix}}/R(1/T_m - 1/T)]}
\]

Where \( \theta \) is the observed signal at 222 nm, \( \theta_N \) and \( \theta_D \) are the native and denatured baseline intercepts, \( m_n \) and \( m_D \) are the native and the denatured baseline slopes, \( T \) is the temperature, \( \Delta H_{\text{mix}} \) is the van’t Hoff enthalpy, \( R \) is the gas constant, and \( T_m \) is the temperature of the transition midpoint.

Isothermal Titration Calorimetry—Isothermal titration calorimetry was performed using a MicroCal high sensitivity VP-ITC instrument (Northampton, MA). Solutions were degassed under vacuum prior to use. Briefly, 0.25 mM C34 dissolved in PBS (pH 7.2) was injected into the chamber containing 15 \( \mu \)M N36 or its mutants, respectively. The time between injections was 8 min and the stirring speed was 394 rpm. The heats of dilution were determined in control experiments by injecting C34 into PBS buffer and subtracted from the heats produced in the corresponding peptide-peptide binding experiments. The experiments were carried out at 25 °C. Data acquisition and analysis were performed using MicroCal Origin software (version 7.0).

Native Polyacrylamide Gel Electrophoresis (N-PAGE)—N-PAGE was carried out to determine the 6-HB formation between the N- and C-peptides as described previously (28). Briefly, an N-peptide (N36 or its mutant) was mixed with peptide C34 at a final concentration of 40 \( \mu \)M and incubated at 37 °C for 30 min. The mixture was loaded onto a 10 × 1.0-cm precast 18% Tris glycine gels (Innogenet) at 25 \( \mu \)l per well with an equal volume of Tris glycine native sample buffer (Innogenet). Gel electrophoresis was carried out with 125 V constant voltage at room temperature for 2 h. The gel was then stained with Coomassie Blue and imaged with a FluorChem 8800 Imaging System (Alpha Innotech Corp., San Leandro, CA).

Enzyme-linked Immunosorbent Assay (ELISA)—A capture ELISA as previously described (29) was used to detect the formation of 6-HB between the N- and C-peptides. Briefly, 2 \( \mu \)g/ml IgG purified from rabbit antiserum developed against the N36-C34 complex was pre-coated onto wells of a 96-well polystyrene plate (Corning Costar) in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After blocking with 2% nonfat milk, a mixture formed by an N-peptide (N36 or its mutant) and C34 at equimolar concentrations (2 \( \mu \)M) was added and incubated at 37 °C for 1 h, followed by four washes with PBS containing 0.1% Tween 20. Captured 6-HB was detected by sequential addition of NC-1, a mouse mAb specific for 6-HB that was developed in our laboratory, and biotin-labeled goat anti-mouse IgG (Sigma), and streptavidin-labeled horseradish peroxidase (Zymed Laboratories Inc., South San Francisco, CA). The reaction was visualized by addition of the substrate 3,3′,5,5′-tetramethylbenzidine and absorbance at 450 nm was measured by an ELISA plate reader (Tecan US).

Measurement of HIV-1 Infectivity—The inhibitory activity of N-peptides on infection by a laboratory-adapted HIV-1 strain (IIIB) was determined as previously described (21). Briefly, 1 × 10<sup>4</sup> MT-2 cells were infected with HIV-1 IIIB at 100 TCID<sub>50</sub> (50% tissue culture infective dose) in 200 \( \mu \)l of RPMI 1640 medium containing 10% fetal bovine serum in the presence or absence of the peptides at graded concentrations overnight. Then the culture supernatants were removed and fresh media were added. On the fourth day post-infection, 100 \( \mu \)l of culture supernatants were collected from each well, mixed with equal volumes of 5% Triton X-100, and assayed for p24 antigen by ELISA. Briefly, the wells of polystyrene plates (Immulon 1B, Dynex Technology, Chantilly, VA) were coated with HIVIG in 0.85 M carbonate-bicarbonate buffer (pH 9.6) at 4 °C overnight, followed by washes with PBS-T buffer (0.01 M PBS containing 0.05% Tween 20) and blocking with PBS containing 1% dry fat-free milk (Bio-Rad Inc.). Virus lysates were added to the wells and incubated at 37 °C for 1 h. After extensive washes, anti-p24 mAb (183–12H-5C), biotin-labeled anti-mouse IgG1 (Santa Cruz Biotechnology), streptavidin-labeled horseradish peroxidase (Zymed Laboratories Inc.), and 3,3′,5,5′-tetramethylbenzidine (Sigma) were added sequentially. Reactions were terminated by addition of 1 N H<sub>2</sub>SO<sub>4</sub>. Absorbance at 450 nm was recorded in an ELISA reader (Ultra 384, Tecan). Recombinant protein p24 (US Biological, Swampscott, MA) was included for establishing a standard dose-response curve.

RESULTS

Substitutions of Lys<sup>574</sup> with Non-conserved Residues Completely Abolish Env-mediated Virus Entry—To define the function of residue Lys<sup>574</sup> for HIV infectivity, we replaced Lys<sup>574</sup>
with a conserved arginine residue (Arg) or non-conserved residue aspartic acid (Asp), glutamic acid (Glu), or valine (Val) by mutagenesis. The point mutations were verified by DNA sequencing, and the expression of HIV-1 Env glycoprotein was confirmed by radioactive immunoprecipitation assay (data not shown). We then determined the effects of these mutations on Env-mediated viral entry by using a single-cycle complementation system. As shown in Fig. 2, the pseudovirus with conservative substitution (K574R) could retain its infectivity on U87-T4-CXCR4 cells, similar to the wild-type virus. However, the pseudoviruses with non-conserved substitutions (K574D, K574E, or K574V) completely lost their capacity to infect target cells. This result suggests that residue Lys574 of HIV-1 gp41 is essential for virus entry.

Substitutions of Lys574 with Non-conserved Residues Severely Impair 6-HB Stability—The peptides N36 and C34 were initially used to solve the crystal structure of fusion-active gp41 core (6). To investigate the mechanism how non-conservative Lys574 substitutions determine Env-mediated HIV-1 infectivity, we used CD spectroscopy, a classic technique to measure the secondary structure conformation, such as α-helix, β-sheet, and random coil, of a protein or a peptide in liquid phase, to analyze the interaction of C34 with the wild-type and mutant N36. Consistently, CD spectra of the isolated N36 showed random coil structures, but the mixture of N36 and C34 at equimolar concentration resulted in formation of a conformation with high α-helicity, characterized by a typical saddle-shaped negative peak in the far UV region of CD spectrum and a significantly increased molar ellipticity (θ) at 222 nm (Fig. 3A). The CD spectra of all mutant N36 peptides indicated that they could interact with C34 to form 6-HB as shown by the induction of α-helical signals (Fig. 3B), but the α-helicity induced by K574D and K574V mutant peptides significantly decreased.

To test the stability and reversibility of each 6-HB formed by N- and C-peptides, we performed thermal denaturation analysis by monitoring the signal at 222 nm of the peptide mixture when the temperature was slowly raised from 10 to 90 °C at a scan rate of 2 °C/min, then cooled to 10 °C for 30 min and heated to 90 °C again. The melting curves for each peptide combination and their thermal unfolding transition (T_m) values are presented in Fig. 4 and Table 1. Surprisingly, whereas conservative substitution in N36 (K574R) did not significantly affect the stability of its complex with the peptide C34, the T_m values of 6-HB bearing non-conservative substitutions in N36 (K574D, K574E, or K574V) dramatically decreased. These results suggest that non-conservative substitutions of Lys574 can severely attenuate the stability of 6-HB. The majority (73–89%) of the 6-HBs formed by binding of C34 to N36 and its mutants were reversible when tested by thermal denaturation, except the 6-HB formed by C34 and K574V, which only had about 40% reversibility (Table 1). All the far-UV CD spectroscopic signals appeared to be cooperative and the transitions were relatively sharp. The reversible thermal denaturation could be fitted into a two-state model (native and denature) based on the non-linear regression analysis (Fig. 4) (27).

We then used an ITC to determine the binding affinity of the representative N-peptides to C34. As shown in Fig. 5 and Table 2, whereas the binding constant of wild-type N36 and its conserved mutant (K574R) was $3.06 \times 10^6$ and $1.86 \times 10^6$ M$^{-1}$,
respectively, non-conserved K574D and K574V mutants had reduced binding affinity ($4.86 \times 10^3$ and $1.45 \times 10^4$ M$^{-1}$, respectively), consistent with their thermal stability measured by CD spectroscopy. Unexpectedly, the titration experiments revealed the stoichiometry of the binding of C34 with N36 and its mutants is 1:0.3–0.4, rather than 1:1. It was reported that N-peptides have the tendency to aggregate in phosphate solutions (5, 31). Therefore, N36 and its mutants may be present in phosphate buffers in multiple forms, e.g. monomers, trimers (32–34), and aggregates. The kinetic transition of these forms (monomers $\leftrightarrow$ trimers $\leftrightarrow$ aggregates) is dependent on the peptide concentration, i.e. the peptide solution with a higher concentration has a greater chance of forming aggregates. To reduce the aggregation, we prepared the peptide stock solutions at the lowest possible concentrations (e.g. 0.5 mg/ml). Furthermore, the stock solutions were centrifuged at high speed (10,000 $\times$ g) and the supernatants were collected for measuring the peptide con-

FIGURE 4. The stability of the $\alpha$-helical complexes formed by C34 with N36 (A) and its mutants, K574R (B), K574E (C), K574D (D), and K574V (E). Thermal denaturation was monitored at 222 nm by applying a thermal gradient of 2 °C/min in the range of 10–90 °C. The solid lines are plotted with the actual experimental data, whereas the dotted lines were plotted based on the predicted numbers from a non-linear regression analysis according to the two-state model.
centration and used for the CD and ITC experiments. Although the major aggregates in the peptide solutions were removed by high speed centrifugation before measuring the peptide concentration, there may still be some microaggregates present, which may not be able to interact with C34. Therefore, the actual concentration of the monomeric N36 peptide in solution that can interact with C34 is lower than expected. This may explain why the stoichiometry of the binding of C34 with N36 and its mutants from the titration experiments is not 1:1.

### TABLE 1

**The α-helicity and thermal stability of six-helix bundles formed between N- and C-peptides**

| Peptide complex | $\theta_{222}$ (°C) | Helix content (%) | $T_m^a$ (°C) | Enthalpy $^b$ (kcal mol$^{-1}$) | Reversibility (%) |
|-----------------|---------------------|-------------------|--------------|-------------------------------|------------------|
| Wild-type       | 29.467              | 89.3              | 63.8         | 25.5                          | 88.5             |
| Mutants         |                      |                   |              |                               |                  |
| K574R/C34       | 31.097              | 94.2              | 66.2         | 26.3                          | 88.9             |
| K574D/C34       | 16.176              | 49.0              | 43.8         | 12.9                          | 73.2             |
| K574E/C34       | 24.006              | 72.7              | 50.5         | 20.3                          | 86.7             |
| K574V/C34       | 13.480              | 40.8              | 53.2         | 11.4                          | 40.3             |

$^a$ The apparent $T_m$ values were estimated from the thermal dependence of CD signal at 222 nm.

$^b$ The reversibility was calculated by comparing the enthalpy of the first and the second heating scans at a scan rate of 2 °C/min from 10 to 90 °C.

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**FIGURE 5. ITC assay.** A, the titration traces determined when 0.25 mM C34 dissolved in PBS was injected into a solution containing 15 μM N36. The binding affinity of C34 to the wild-type N36 (B) and its mutants, K574R (C), K574D (D), and K574V (E) were calculated using MicroCal Origin software.
less anti-HIV-1 activity, with IC₅₀ at 2.86, 2.25, and 52.55 μM, respectively. This result suggests that Lys₅⁷₄ is critical for peptide-mediated antiviral activity.

**DISCUSSION**

Triggered by gp120 binding to CD4 and a coreceptor, HIV-1 gp41 undergoes a conformational shift from a native prefusogenic state to a fusogenic state, in which the NHR and CHR regions associate to form a 6-HB, representing the fusion-active gp41 core (6, 36). The helical packing interactions in the HIV-1 gp41 core have been extensively explored by mutagenesis and it was shown that a number of mutations were able to destabilize the 6-HB and abolish viral infectivity (14, 37, 38). With mutational analysis, we have investigated the role of the residue Lys₅⁷₄ located in the NHR cavity. Markedly, non-conservative substitutions of Lys₅⁷₄ (K₅⁷₄D, K₅⁷₄E, and K₅⁷₄V) could completely abrogate Env-mediated viral entry, whereas the conservative substitution of Lys₅⁷₄ (K₅⁷₄R) had no significant effect on the infectivity of the pseudoviruses. This result indicates that Lys₅⁷₄ plays an essential role in viral infectivity.

Structurally, the coiled-coil domain of HIV-1 gp41 shares a characteristic 4-3 heptad repeat sequence, (abcdefg)ₙ with hydrophobic residues at the first (a) and fourth (d) positions (6, 36). The N-terminal homotrimer is packed against each other through the interaction of residues at the a and d positions. Its residues at the e and g positions lie on the outside of the central coiled-coil and create extensive interactions with the residues at the a and d positions of the C-helices. Importantly, each of the grooves on the surface of the N36 trimer has a particularly deep cavity that accommodates three hydrophobic residues from each C34 helix (Ile₆₃⁵, Trp₆₃₁, and Trp₆₂₈) (5, 6, 36). The positively charged residue Lys₅⁷₄ in the NHR cavity forms a conserved salt bridge with the negatively charged residue Asp₆₃₂ in the CHR. It is possible that this salt bridge confers a significant strength to the interhelical interactions and thereby stabilizes the fusion-active six-helical bundles. We, therefore, synthesized a set of wild-type and mutant peptides to study the effects of single-point substitutions of the Lys₅⁷₄ on viral infectivity. The residue Lys₅⁷₄ in peptide N36 was substituted with a negatively charged residue aspartic acid or glutamic acid, or with a hydrophobic residue valine, or with another positively charged residue (Arg). Consistently, non-conservative substitutions of Lys₅⁷₄ significantly impaired the conformation and stability of six-helical bundle modeled by peptides N36 and C34 as shown by CD spectra, ITC assay, N-PAGE, and ELISA with mAb NC-1, whereas the conservative substitution (K₅⁷₄R) had no effect on 6-HB stability. K₅⁷₄V substitution induced the most dramatic change in the CD spectra, consistent with its significantly reduced thermal stability and binding affinity as well as the lowest anti-HIV-1 activity. K₅⁷₄D and K₅⁷₄E substi-
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Substitutions also resulted in significant changes in the CD spectra, thermal denaturation, binding affinity, and antiviral activity. The inability of NC-1 to recognize the gp41 6-HB{s} bearing the non-conservative substitutions may be attributed to the conformational changes induced by the mutations, indicating the importance of the positive charge for antibody binding and sheds light on the actual binding site of this antibody, which has been shown to bind also to the extended assembled trimer of the N-helix (39).

The resulting conformational change and destabilization effect of the non-conservative substitutions observed here may be related to the fusion-defective phenotypes, explaining how single substitutions can completely abolish the viral infectivity. Previous studies indicate that the residues located at the heptad $a$, $d$, $e$, or $g$ position in the cavity region are critical for the interhelical interactions between the NHR and CHR. Here we show, for the first time, that residue Lys$^{574}$, which is located at the heptad $b$ position, determines the stability and conformation of the gp41 core structure. Furthermore, it is believed that the structure of the HIV-1 gp120-gp41 complex exists as a trimer and that the highly conserved 4-3 hydrophobic repeat in the N-terminal portion of gp41 plays a critical role in oligomer formation and stability (14, 40). Single substitutions of isoleucine or leucine with proline within the NHR abolished Env-mediated membrane fusion activity, but did not interfere with Env oligomerization (41, 42), suggesting that the putative coiled-coil domain is required for virus entry. The defective phenotype of mutant Env proteins described here might be a result of the structural perturbations of gp41 caused by the non-conservative substitutions. Recently, there is a growing evidence that HIV-1 membrane fusion may start prior to 6-HB formation and that the N-helix directly participates in the fusion (39, 43, 44). This raises the possibility that the non-conserved mutations could abolish the fusogenic activity of the mutated N-helices. This assumption is supported by the fact that although all the non-conserved mutations fully abolished virus-cell fusion, some of them still bound to the C-helix with relatively strong affinity.

Peptides derived from the NHR and CHR regions of HIV-1 gp41 (e.g. N36 and C34) can interact with the counterpart regions and interfere with the 6-HB formation, thus inhibiting fusion of the virus with the target cell. T-20 is the first and only entry inhibitor approved for clinical use. It is a 36-amino acid peptide derived from the gp41 CHR sequence and can bind to the NHR to prevent formation of the 6-HB by dominant negative fashion (16). T-20 therapy shows safety, potent antiretroviral activity, and immunological benefit in patients but its clinical application is limited by resistance development, the requirement for parenteral delivery, and the high cost of chemical synthesis (46, 47). Therefore, development of novel fusion inhibitors remains a priority to curb the global epidemic of HIV-1 infection. The small molecule compounds NB2 and NB64 have potent activity in blocking 6-HB formation and HIV-1 entry. Using a competition assay based on specific interaction of the D10-p5-2k peptide with the gp41 cavity modeled by peptide IQN17 (48), we have demonstrated that NB-2 and NB-64 significantly block the interaction of D10-p5-2k with IQN17 (21), suggesting that these two compounds specifically bind to the gp41 cavity. More interestingly, NB177 and -178, which have identical structures as NB-2 and NB-64, respectively, but lack the COOH group, possess no inhibitory activity against 6-HB formation and HIV-1 replication (21, 49–53), indicating that the acid group in NB-2 or NB-64 is critical for their interaction with a basic residue near the cavity region, like Lys$^{574}$. Further analysis with a computer-aided molecular docking method suggests that the COOH group of NB-2 and NB-64 may interact with positively charged residue Lys$^{574}$ to compete with the negatively charged residue Asp$^{632}$ (21). Recently, we have found that these compounds can bind to the wild-type N36 but not K574D mutant (data not shown), further suggesting that Lys$^{574}$ is a key determinant of the target. Therefore, the salt bridge formed by Lys$^{574}$ and Asp$^{632}$ might play a critical role in peptide and non-peptide-mediated antiviral activity (21, 30, 45). Co-crystallization of an inhibitor with its target protein is the best approach to elucidate in detail the key residues in the protein and chemical groups in the compounds involved in the interaction. We and others have made repeated attempts to crystallize the cavity-containing N-helix coiled-coil domain in complex with the small molecule HIV-1 fusion inhibitors, but we have not been successful so far because of the difficulty to generate a stable and soluble complex with the gp41 cavity exposed. This reveals the importance of specific approaches (mutagenesis and biophysical analysis) to study the role of basic residue(s) located near the hydrophobic cavity, like Lys$^{574}$, in the 6-HB formation and virus-cell fusion.

Significantly, the non-conservative substitutions of Lys$^{574}$ also impaired the capacity of N-peptides to inhibit HIV-1 infection, suggesting that the N-peptide mutants cannot form stable heterologous 6-HBs with the viral CHR, thus are unable to block the formation of the gp41 core between the viral NHR and CHR. It is speculated that disruption of the salt bridge can reduce the binding affinity between NHR and CHR helices, resulting in destabilization of the 6-HB and reduction of HIV-1 infectivity. These results provide important information for designing anti-HIV-1 drugs, considering that the charged residues within the cavity region and cavity-binding site between the NHR and CHR helices may serve as a target for development of HIV-1 entry inhibitors.

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