Conserved Salt-bridge between the N- and C-Terminal Heptad Repeat Regions of HIV-1 gp41 Core Structure Is Critical for Virus Entry and Inhibition

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Abbreviations: CHR, C-terminal heptad repeat; CD, circular dichroism; CP, cytoplasmic domain; Env, envelope glycoprotein; FP, fusion peptide; N-PAGE, native polyacrylamide gel electrophoresis; NHR, N-terminal heptad repeat; Tm, thermal midpoint; TM, transmembrane domain; TMB, 3,3',5,5'-tetramethylbenzidine; TR, tryptophan-rich region; WT, wild-type.
ABSTRACT

The fusogenic HIV-1 gp41 core structure is a stable six-helix bundle formed by its N- and C-terminal heptad-repeat sequences (NHR and CHR). Notably, the negatively charged residue Asp$^{632}$ located at the pocket-binding motif in the CHR interacts with the positively charged residue Lys$^{574}$ in the pocket-formation region of the NHR to form a salt-bridge. We previously demonstrated that the residue Lys$^{574}$ plays an essential role for the six-helix bundle formation and virus infectivity and is a key determinant of the target for anti-HIV fusion inhibitors. In this report, the functionality of the residue Asp$^{632}$ has been specifically characterized by mutational analysis and biophysical approaches. We show that substitutions of Asp$^{632}$ with positively charged residues (D632K and D632R) or hydrophobic residue (D632V) could completely abolish Env-mediated viral entry while conserved substitution (D632E) retained its activity. Similar to the Lys$^{574}$ mutations, non-conserved substitutions of Asp$^{632}$ also severely impaired the α-helicity, stability and conformation of six-helix bundles as shown by N36 and C34 peptides as a model system. Furthermore, non-conserved substitutions of Asp$^{632}$ significantly reduced the potency of C34 to sequestrate six-helix bundle formation and to inhibit HIV-1-mediated cell-cell fusion and infection, suggesting its importance for designing antiviral fusion inhibitors. Taken together, these data suggest that the salt-bridge between the N- and C-terminal heptad repeat regions of the fusion-active HIV-1 gp41 core structure is critical for viral entry and inhibition.

**Key words:** HIV-1, gp41, peptide, six-helix bundle, salt-bridge, HIV-1 fusion inhibitor
INTRODUCTION

Infection of human immunodeficiency virus type 1 (HIV-1) is mediated by its envelope glycoprotein (Env), a type I transmembrane protein which is originally synthesized as a single, glycosylated, polyprotein precursor gp160 and subsequently cleaved by a cellular protease to yield gp120 and gp41 subunits. Upon binding of HIV-1 Env surface subunit gp120 to the cell receptor CD4 and subsequently a coreceptor (CCR5 or CXCR4), its transmembrane subunit gp41 is released to mediate fusion of viral and cellular membranes. Structurally, HIV-1 gp41 consists of extracellular, transmembrane (TM), and cytoplasmic (CP) domains (Fig. 1A). Its extracellular domain (ectodomain) contains four major functional regions: a hydrophobic, glycine-rich fusion peptide (FP), N-terminal heptad repeat (NHR or HR1), C-terminal heptad repeat (CHR or HR2), and a tryptophan-rich (TR) region. In the early 1990s, several peptides derived from the NHR (N-peptides) and CHR (C-peptides) were identified to have potent anti-HIV activity. Although their mechanism of action was not known at that time the unprecedented anti-HIV activity of these peptides opened a new avenue for developing antiviral drugs. A C-peptide, known as T20 (brand name: Fuzeon), has been successfully developed as a novel class of anti-HIV drug for clinical use.

The finding of anti-HIV peptides also provided important information to explore the structure of the gp41 molecule. In 1995, Lu et al. identified a stable, proteinase-resistant structure comprising two peptides, N51 and C43, derived from a recombinant protein fragment of the gp41 ectodomain by using protein dissection experiments. The N51 and C43 associate to form a stable, α-helical trimeric complex of heterodimers, with
N51 and C43 helices oriented in an antiparallel fashion. Further proteolysis of the N51/C43 complex resulted in the identification of the N36 and C34 peptides. Similarly, the N36 and C34 form a stable α-helical trimer of NHR-CHR heterodimers, whereas the N36 alone is predominantly aggregated and the C34 alone remains mostly unfolded. X-ray crystallographic studies by three independent groups confirmed that the thermostable subdomain of HIV-1 gp41 folds into a α-helical six-helix bundle, in which three NHR helices form an interior, parallel coiled-coil trimer while three CHR helices pack in an oblique, antiparallel manner into the highly conserved, deep hydrophobic grooves on the surface of the N-helical trimer. NMR analysis showed that the SIV gp41 also formed similar six-helix bundle structure in solution. The current model suggests that the six-helix bundle structure formed by NHR and CHR helices represents a core of the fusion-active conformation of HIV-1 envelope, which brings the viral and cellular membranes into close apposition to enable fusion-pore formation and virus internalization. Synthetic peptides derived from the NHR and CHR regions of HIV-1 gp41 are thought to target the pre-hairpin fusion intermediate through interacting with the counterpart regions, thus inhibiting fusion of the virus with the target cell in a dominant-negative manner.

The crystal structure of HIV-1 gp41 core also reveals a highly conserved, hydrophobic cavity on the surface of the NHR helices. Considerable evidence suggests that the hydrophobic interaction between the cavity-forming domain in the NHR and the cavity-binding motif in the CHR is highly important for stabilization of the six-helix bundle and viral infectivity. Importantly, the deep hydrophobic cavity formed by the internal coiled-coil trimer has been proposed to be an ideal target for
designing inhibitors that can disrupt the six-helix bundle formation. Another important observation from the X-ray structure is that the positively charged residue Lys located at the cavity-forming region in the NHR interacts with the negatively charged residue Asp at the cavity-binding motif in the CHR to form a salt-bridge (Fig.1C). We have proposed that during the fusion process the formation of salt-bridge between the NHR and CHR of gp41 core, besides the hydrophobic interaction in the cavity, may play a critical role in viral infection and inhibition thereof. We previously demonstrated that the residue Lys in the NHR of gp41 is essential for the functional six-helix bundle formation and virus entry. Non-conserved substitutions of Lys could completely abolish Env-mediated HIV-1 entry and severely impaired the conformation and stability of the six-helix bundles. The mutant peptides bearing non-conserved substitutions of Lys had much less potency to inhibit HIV-1 infection. These data highlight the importance of the salt-bridge formation in the gp41 core structure. In this study, we have focused our efforts to characterize the functions of the residue Asp in the gp41 CHR region of the HIV-1 by using mutational analysis and biophysical approaches.

MATERIALS AND METHODS

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 NIH AIDS Research and Reference Reagent Program. A panel of HXB2-Env mutants (D632E, D632K, D632R, and D632V) were generated by mutagenesis using the QuickChange XL kit (Strategene, 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.

**Single-cycle infection assay.** HIV-1 pseudovirus was developed as previously described\textsuperscript{22,32}. Briefly, HEK293T cells were co-transfected with a plasmid encoding wild-type (WT) or mutant HXB2-Env and a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc.RE) by using Fugene 6 reagents (Boehringer-Mannheim, Indianapolis, IN). Supernatants containing HIV-1 pseudovirus were harvested 48 h post-transfection and clarified by centrifugation at 1000 g for 10 min and filtration through a 0.45 µm filter. The virus-containing clarified supernatants were stored in -80 ºC before use. For single-cycle infection, U87-T4-CXCR4 cells were plated at 10^4 cells/well in 96-well tissue culture plates and grown overnight. The pseudovirus were added to cells and incubated overnight. The culture was re-fed and incubated for an additional 48 h. Cells were washed with phosphate-buffered saline (PBS) and lysed using lysis reagent included in a luciferase kit (Promega, Madison, WI). Aliquots of cell lysates were transferred to 96-well Costar flat-bottom luminometer plates (Corning Costar, Corning, NY), followed by addition of luciferase substrate (Promega). Relative light units (RLU) were determined immediately in the Ultra 384 luminometer (Tecan US).

**Synthesis of wild-type and mutant peptides.** Peptides N36 (residues 546-581, SGIVQQQNLLRAIEAQHQHLQLTVWGIKQLQARIL) and C34 (residue 628-661, WMEWDREINNYTSIHLIESQNQQEKNEQELL) as well as C34 mutants (D632E, D632K, D632R, and D632V) were synthesized by a standard solid-phase FMOC method
in the MicroChemistry Laboratory of the New York Blood Center. The peptides were purified to homogeneity (>95% purity) by high-performance liquid chromatography (HPLC) and identified by laser desorption mass spectrometry (PerSeptive Biosystems, Framingham, MA). The concentration of peptides was determined by UV absorbance and a theoretically calculated molar-extinction coefficient $\varepsilon$ (280 nm) of 5500 mol/L$^{-1}$cm$^{-1}$ and 1490 mol/L$^{-1}$cm$^{-1}$ based on the number of tryptophan (Trp) residues and tyrosine (Tyr) residues (all the peptides tested contain Trp and/or Tyr), respectively$^{16}$.

Circular dichroism (CD) spectroscopy. A C-peptide (C34 or its mutant) was incubated with the N-peptide (N36) at 37 ºC for 30 min (the final concentrations of N-peptide and C-peptide were 10 µM 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 bandwith, 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$^2$ dmol$^{-1}$) according to the references$^{9,58}$. Thermal denaturation was monitored at 222 nm by applying a thermal gradient of 2 ºC /min in the range of 4-98 ºC. The reversibility of the peptide mixtures was measured by performing the reverse melt from 98 to 4 ºC. The melting curve was smoothened, and the midpoint of the thermal unfolding transition ($T_m$) values was calculated using Jasco software utilities as described previously$^{39}$. 
Native polyacrylamide gel electrophoresis (N-PAGE). N-PAGE was carried out to determine the six-helix bundle formation between the N- and C-peptides as described previously. Briefly, a C-peptide (C34 or its mutant) was incubated with the N36 at a final concentration of 40 µM and incubated at 37 ºC for 30 min. The mixture was loaded onto a 10 x 1.0-cm precast 18% Tris-glycine gels (Invitrogen, Carlsbad, CA) at 25 µl/per well with an equal volume of Tris-glycine native sample buffer (Invitrogen). Gel electrophoresis was carried out with 125V 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).

Detection of six-helix bundle by enzymed-linked immunosorbent assay (ELISA). A capture ELISA as previously described was used to detect the formation of six-helix bundle between the N- and C-peptides. Briefly, 2 µg/ml IgG purified from rabbit antisera developed against the N36/C34 complex was pre-coated onto wells of a 96-well polystyrene plate (Corning Costar, Acton, MA) in 0.1 M carbonate buffer (pH 9.6) at 4 ºC overnight. After blocking with 2% non-fat milk, a mixture formed by an N-peptide (N36 or its mutant) and C34 at equimolar concentrations (2 µM) was added and incubated at 37 ºC for 1 h, followed by four washes with PBS containing 0.1% Tween 20. Captured six-helix bundles were detected by sequential addition of NC-1, a mouse mAb specific for the N36/C34 six-helix bundle that was developed in our laboratory, and biotin-labeled goat anti-mouse IgG (Sigma, St. Louis, MO), and streptavidin-labeled horseradish peroxidase (SA-HRP; Zymed, South San Francisco, CA). The reaction was
visualized by addition of the substrate 3,3',5,5'-tetramethylbenzidine (TMB) and absorbance at 450 nm was measured by an ELISA plate reader (Tecan US, Research Triangle Park, NC).

**Inhibition of six-helix bundle formation by CHR peptides.** Inhibitory activity of CHR peptides (C34 and its mutants) on the six-helix bundle formation was measured by a modified ELISA-based method as previously described. Briefly, a 96-well polystyrene plate (Costar, Corning Inc., Corning, NY) was coated with a six-helix bundle-specific monoclonal antibody NC-1 IgG (4 µg/mL in 0.1 M Tris, pH 8.8). A tested peptide at graded concentrations was mixed with C34-biotin (0.25 µM) and incubated with N36 (0.25 µM) at room temperature for 30 min. The mixture was then added to the NC-1-coated plate, followed by incubation at room temperature for 30 min and washing with a washing buffer (PBS containing 0.1% Tween 20) three times. Then streptavidin-labeled horseradish peroxidase (Invitrogen) and the substrate 3,3',5,5'-tetramethylbenzidine (Sigma) were added sequentially. Absorbance at 450 nm ($A_{450}$) was measured using an ELISA reader (Ultra 384, Tecan, Research Triangle Park, NC). The percent inhibition by the peptides and the IC$_{50}$ values were calculated as previously described.

**Cell-cell fusion assay.** A dye transfer assay was used for detection of HIV-1 mediated cell-cell fusion as previously described. Briefly, H9/HIV-1$_{111B}$-infected cells were labeled with a fluorescent reagent, Calcein-AM (Molecular Probes, Inc., Eugene, OR) and then incubated with MT-2 cells (ratio = 1:5) in 96-well plates at 37 °C for 2 h in the
presence or absence of tested peptides. The fused and unfused calcein-labeled HIV-1-infected cells were counted under an inverted fluorescence microscope (Zeiss, Germany) with an eyepiece micrometer disc. The percent inhibition of cell-cell fusion and the IC₅₀ values were calculated as described before using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

**Measurement of HIV-1 infectivity.** Inhibitory activity of the peptide C34 or its mutants on infection by laboratory-adapted HIV-1 strain (IIIB) was determined as previously described. Briefly, 1 × 10⁴ MT-2 cells were infected with HIV-1 IIIB at 100 TCID₅₀ (50% tissue culture infective dose) in 200 µl RPMI 1640 medium containing 10% FBS 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 µl 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., Hercules, CA). 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 Biotech., Santa Cruz, CA), SA-HRP (Zymed), and 3,3′,5,5′-TMB (Sigma) were added sequentially. Reactions were terminated by addition of 1N H₂SO₄. 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

The salt-bridge-forming residue Asp\textsuperscript{632} in the CHR is essential for Env-mediated HIV-1 entry. We have recently reported that the salt-bridge-forming residue Lys\textsuperscript{574} in the NHR of HIV-1 gp41 plays an essential role for HIV-1 entry into target cells \textsuperscript{23}. Here, we used a similar approach to analyze the functionality of the residue Asp\textsuperscript{632}, a partner residue in the CHR to form the salt-bridge between two heptad repeat regions of the fusogenic gp41 molecule. The negatively charged Asp\textsuperscript{632} was converted to a negatively charged residue glutamic acid (Glu) or positively charged residue lysine (Lys), arginine (Arg) or hydrophobic residue valine (Val) by mutagenesis. The point mutations were verified by DNA sequencing and the expression of HIV-1\textsubscript{HXB2} Env glycoprotein was confirmed by radioactive immunoprecipitation assay (data not shown). To determine the effects of these mutations on the Env-mediated HIV-1 entry, a panel of pseudoviruses bearing wild-type or mutant Env glycoprotein were generated and used in a single cycle complementation assay. As shown in Fig. 2, the conserved mutation of Asp\textsuperscript{632} (D632E) had no significant effect on the viral entry since the pseudovirus retained its infectivity similar to the wild-type virus. However, non-conserved substitutions (D632K, D632R, or D632V) completely abolished the infectivity of the pseudoviruses. These results suggest that the salt-bridge-forming residue Asp\textsuperscript{632} in the CHR region of HIV-1 gp41 is also essential for virus entry.
Similar to Lys\textsuperscript{574}, substitutions of Asp\textsuperscript{632} with non-conserved residues severely impair the stability of six-helix bundle. To investigate the mechanism how non-conserved substitutions of Asp\textsuperscript{632} can determine Env-mediated HIV-1 infectivity, we used CD spectroscopy to analyze the interaction of NHR peptide N36 and CHR peptide C34. The counterpart peptides N36 and C34 were included in this study since the thermostable six-helix bundle complex formed by these two peptides has been proposed to be a core structure of the fusion-active gp41\textsuperscript{5}. As expected, the equimolar mixture of wild-type C34 and N36 has a typical conformation of \(\alpha\)-helix, characterized by double minima at 208 and 222 nm (Fig. 3A). The CD spectra of all mutant C34 peptides indicated that they could interact with N36 to form six-helix bundle structures as shown by the induction of \(\alpha\)-helix signals (Fig. 3B-E). However, the \(\alpha\)-helicity induced by non-conserved C34 mutants (D632K, D632R, or D632V) significantly decreased whereas the conserved D632E mutant resulted in a slightly increased \(\alpha\)-helical signal.

The stability of each six-helix bundle structure formed by N- and C-peptides was determined by thermal denaturation analyses. The signal at 222 nm of the peptide mixture was monitored when the temperature was slowly raised from 4 to 98 °C at a scan rate of 2°C/min. The melting curves for the peptide combination were shown in Fig. 4 and their thermal unfolding transition \((T_m)\) values were calculated in Table 1. As a control, the complex of N36 and C34 had a \(T_m\) of 64 °C (Fig. 4A), consistent with our previous data \cite{24}. Similar to the residue Lys\textsuperscript{574}, the stability of six-helix bundles was significantly reduced by non-conservative Asp\textsuperscript{632} substitutions. The D632K, D632R and D632V mutations had \(T_m\) values of 54 °C, 55 °C, and 58 °C, respectively. In contrast, the six-helix bundle bearing conserved D632E mutation even had a slight increased \(T_m\) value (67 °C).
These peptide pairs were unfolding reproducible and reversible while the reverse thermal melts were monitored from 98 to 4 °C or heated to 98 °C again. These results suggest that substitutions of Asp\(^632\) with non-conserved residues severely impair the \(\alpha\)-helicity and stability of six-helix bundles formed by NHR and CHR peptides.

**Substitutions of Asp\(^632\) with non-conserved residues severely impair the conformation of six-helix bundle.** Our previous studies indicate that the six-helix bundle core structure formed by the peptides N36 and C34 can be visualized by N-PAGE \(^{24}\). In this study, we used N-PAGE to detect six-helix bundles formed by C34 bearing conservative and non-conservative mutations. Consistent with our previous data, the net positive charged peptide N36 shows no band in the native gel as it could migrate up and off the gel, while the peptide C34, which carries net negative charges, shows a specific band (Fig. 5). The conserved D632E mutant gave a band with similar migration rate to the wild-type C34, whereas the peptides with non-conserved mutations (D632K, D632R and D632V) migrated slower. When the peptide N36 was mixed with the wild-type and mutant C34 peptides, the specific bands corresponding to the six-helix bundles appeared, but they had different migration rates. Since the migration rates of the six-helix bundles formed by N36 and C34 peptides were dependent on their net charges, molecular sizes and shapes, it was possible that conservative and non-conservative C34 mutants retained their ability to fold six-helix bundle structures but the configuration of the six-helix bundles might be changed.

We subsequently determined whether the C34 mutants affect the conformation of six-helix bundle with a gp41 core-specific mAb NC-1 \(^{29}\). Consistently, NC-1 did not react
with the isolated peptides N36 and C34, but strongly react with the six-helix bundle formed by these two peptides mixed at equal concentrations (Fig. 6). Interestingly, NC-1 recognized the six-helix bundle formed by D632E mutant similar to the wild-type C34 but non-conservative substitutions in the peptide C34 could severely damage its reactivity. These results further suggest that the six-helix bundles might undergo conformational changes after non-conservative substitutions in the CHR peptides and suggest a role of the salt-bridge in the formation of a functional conformation of six-helix bundle.

**K574D and D632K double mutations can not rescue the function of the wild-type salt-bridge.** Our data have indicated that single non-conserved substitutions of the salt-bridge-forming residues Lys\(^{574}\) and Asp\(^{632}\) can severely impair the conformation and stability of the six-helix bundles and thus inactivate the virus, suggesting the importance of the Lys\(^{574}\)-Asp\(^{632}\) salt-bridge in the gp41 core structure. It is interesting to know that whether Asp\(^{574}\)-Lys\(^{632}\) mutant can rescue the function of the wild-type salt-bridge. We therefore generated a pseudotyped HIV-1 bearing the K574D and D632K double mutations and used in single cycle complementation assay. From Fig. 7A we can see that Asp\(^{574}\)-Lys\(^{632}\) double mutations can not rescue the virus infectivity. We also tested K574D and D632K peptides by CD spectroscopy. We found that these two peptides could interact to form a six-helix bundle structure but this bundle had a significant lower \(\alpha\)-helicity (~60%) compared to the wild-type or single mutant peptides (Table 1). However, the K574D/D632K bundle had a \(T_m\) value of 62 °C, which is close to the wild-type N36/C34 bundle (64 °C). These results imply that Asp\(^{574}\)-Lys\(^{632}\) pair may form a
salt-bridge that stabilizes the six-helix bundle core but its function for virus infectivity cannot be rescued.

Non-conserved mutations of Asp\(^{632}\) dramatically impair C34 to inhibit six-helix bundle formation. The mechanism of NHR or CHR-derived anti-HIV peptides has been considered to inhibit the formation of six-helix bundle in a dominant negative fashion\(^6,62\). Considering the peptide C34 has potent anti-HIV activity, we are interested to know whether the mutations affect the potency of C34 to block the six-helix bundle formation. To do so, an ELISA-based method has been developed, in which the six-helix bundle-specific mAb NC-1 was used as a capture antibody and the peptide C34 was biotinylated (see Material and Method). Fig. 8A shows that NC-1 can detect the six-helix bundle formed by N36 and biotin-C34 in a dose-dependent manner. We then tested the inhibitory activity of wild-type and mutant C34 on the six-helix bundle formation. As shown in Fig. 8B and Table 1, the wild-type and D632E mutant could efficiently block the formation of six-helix bundle; however, this activity for the peptides bearing D642K, D632R or D632V mutation dramatically decreased. This result also suggests that the formation of a salt-bridge between the NHR residue Lys\(^{574}\) and the CHR residue Asp\(^{632}\) is critical for the six-helix bundle core structure.

Non-conservative substitutions of Asp\(^{632}\) significantly reduce C34-mediated anti-HIV activity. Our above results demonstrated that non-conservative substitutions of Asp\(^{632}\) could dramatically affect the CHR peptides to block six-helix bundle formation, implying that their anti-HIV activity may be compromised. First, we tested their anti-
HIV activity by cell-cell fusion assay. From Fig. 9A, we can conclude that the peptides with non-conservative substitutions had much reduced activity to inhibit HIV-1 mediated cell-cell fusion. The data in Table 1 shows that the wild-type C34 and D632E mutant could inhibit the fusion with IC_{50} at 5.0 and 6.6 nM, respectively. In contrast, the peptides D632K, D632R and D632V mutants only inhibited the fusion with IC_{50} of 171.1, 136.0 and 92.6 nM, respectively. Further, the infectivity of HIV-1 IIIB was measured in the presence or absence of the wild-type or mutant peptides by a p24-based assay. Consistently, the wild-type C34 and D632E peptides could inhibit HIV-1 infectivity with IC_{50} at 1.4 and 2.2 nM, respectively (Fig. 9B and Table 1), whereas the peptides with non-conservative substitutions (D642K, D632R or D632V) possessed much less antiviral activity, with IC_{50} of 35.5, 29.3, 15.2 nM, respectively. These results demonstrate that the residue Asp^{632} in the CHR peptides or its ionic interaction with the residue Lys^{574} in the NHR target is highly critical for designing anti-HIV peptides.

**DISCUSSION**

In the present study, we have continued our focus to characterize the function of the salt-bridge formed by the positively charged residue Lys^{574} in the NHR region and the negatively charged residue Asp^{632} in the CHR region of the fusion-active HIV-1 gp41 core structure. We show that the residue Asp^{632} is essential for Env-mediated HIV-1 entry. Biophysical characterization demonstrated that this residue determines the conformation and stability of the six-helix bundle structure modeled by NHR peptide (N36) and CHR peptide (C34). Further, we show that the residue Asp^{632} is also a major determinant for the anti-HIV activity of the peptides. Taken together with our previous
data on the residue Lys$^{574}$, we propose that the salt-bridge between the NHR and CHR of HIV-1 gp41 core is highly critical for the virus entry and inhibition.

The structure and function of HIV-1 gp41 molecule have been extensively studied. Considerable evidence suggests that during the fusion process the gp41 likely exists in at least three conformations: (1) a metastable prefusogenic state, which is stabilized by interactions with gp120 subunit; (2) a prehairpin intermediate, formed by insertion of the hydrophobic fusion peptide into the target cell membrane and concurrent folding of the N-terminal trimeric coiled-coil; and (3) the fusogenic trimer-of-hairpins form, in which two α-helical regions, NHR (adjacent to the N-terminal fusion peptide) and CHR (near the C-terminal transmembrane segment), associate to form a highly stable six-helix bundle, bringing the viral and cellular membranes into close apposition. X-ray crystallographic studies of the gp41 core reveal that the N-terminal homotrimer is packed through the interaction of residues at positions $a$ and $d$ of the characteristic 4-3 heptad repeat sequence [(abcdefg)$_n$], and its residues at the $e$ and $g$ positions lie on the outside groves to interact with the residues at the $a$ and $d$ positions of the CHR helices. A large body of evidence from mutagenesis studies suggested that the helical packing interactions between the central coiled-coil trimer and the C-terminal helix are important determinants of HIV-1 entry and inhibition. However, these studies were primarily focused on the predicted critical residues within the NHR helix or the CHR helix, especially the residues at the $a$, $d$, $e$ and $g$ positions of NHR or the residues at the $a$ and $d$ positions of CHR. To date, little attention has been paid to the residues responsible for the formation of the salt-bridge in the fusogenic gp41 core. We have recently demonstrated that the residue Lys$^{574}$,
which is located at the heptad \( b \) position of NHR, is essential for the HIV-1 entry \(^{23}\). Here, our mutational analyses indicate that the residue Asp\(^{632}\), which is located at the heptad \( e \) position of CHR, is also critical for virus infectivity. Anionic-to-cationic (D632K and D632R) or anionic-to-hydrophobic (D632V) mutations could completely abrogate Env-mediated virus entry, whereas the conserved anionic-to-anionic (D632E) mutation had no significant effect on the infectivity of the pseudoviruses. This result highlights the importance of the salt-bridge formed by the NHR and CHR during the viral fusion process.

To explore the mechanism how single non-conserved substitutions could determine the Env-mediated entry of HIV-1 into target cells, we synthesized a set of wild-type and mutant peptides and used in biophysical characterization. Similarly, the residue Asp\(^{632}\) in peptide C34 was substituted with a positively charged residue lysine or arginine, or with a hydrophobic residue valine, or with another negatively charged residue (Glu). CD spectra and thermal denaturation showed that non-conservative substitutions of Asp\(^{632}\) significantly impaired the \( \alpha \)-helicity and stability of six-helix bundle modeled by the peptides N36 and C34, whereas the conservative substitution had no such effects. Although the epitope for monoclonal antibody NC-1 may locate within the N-helical trimers \(^{12,56}\), our data here confirmed further that NC-1 only reacts with the N36/C34 complex but not with the isolated N36 (Fig. 6-7). The binding specificity of this conformation-specific antibody suggests that the conformation of the six-helix bundles bearing the non-conservative mutations might undergo an alteration. It is possible that the NHR-CHR salt-bridge confers a significant strength to the interhelical interactions and thereby stabilizes the six-helix bundles. The breakage of the salt-bridge
may disrupt the integrity of the six-helix bundles through changing their conformations or reducing their stability. The resulting destabilization and conformational change of the non-conservative substitutions observed here may be related to the fusion-defective phenotypes, this explains how single mutations can completely disrupt the viral infectivity. Further, it can be speculated that the defective phenotype of mutant Env proteins described above might be a result of the structural perturbations of gp41 caused by non-conservative substitutions.

In an attempt to rescue the function of the Lys$^{574}$-Asp$^{632}$ salt-bridge, we generated a pseudotyped HIV-1 bearing Asp$^{574}$-Lys$^{632}$ double mutations but this virus had no infectivity (Fig. 7). Biophysical analyses with the mutant peptides (K574D and D632K) suggest that Asp$^{574}$-Lys$^{632}$ may form a salt-bridge that stabilizes the six-helical bundle but the $\alpha$-helicity of this core structure can not be rescued and thereby result in virus inactivation. Obviously, our present data can not rule out that the residues Lys$^{574}$ and Asp$^{632}$ have roles other than forming a salt-bridge. From the crystal structure of the gp41 core, the interaction of the NHR and CHR helices, especially within the cavity, is largely dependent on hydrophobic contacts but supplemented by electrostatic interactions$^{2,5,45,60,63}$. The exposed surface of the C-terminal helix is predominantly negative, whereas that of the N-terminal helix is mainly neutral. Therefore, the Asp$^{574}$-Lys$^{632}$ double mutations may change the electrostatic potential within the six-helical bundle core and thus abolish its functionality. Further, the residue Lys$^{574}$ is located at the left wall of the cavity and is critical for the cavity formation, but non-conserved substitutions of Lys$^{574}$ may change the configuration of the cavity region. Also, it is possible that non-conserved substitutions of Lys$^{574}$ or Asp$^{632}$ can severely damage the $\alpha$–helicity of the peptide itself.
Taken together, we speculate that although the possible Asp$^{574}$-Lys$^{632}$ salt-bridge can strengthen the stability of the six-helix bundle, it can not maintain the normal conformation of the bundle and rescue its function for virus fusion.

We started to characterize the function of the salt-bridge of gp41 six-helix bundle since our finding of two small molecule-based anti-HIV fusion inhibitors, NB-2 and NB-64. These two N-substituted pyrrole derivatives were selected from a drug-like chemical library, and they could inhibit infection by both laboratory-adapted and primary HIV-1 strains with distinct genotypes (clades A-G and group O) and phenotypes (R5, X4 and R5X4) at low micromolar levels. Computer-aided molecular docking has shown that these compounds fit inside the deep cavity and their acid groups interact with the positively charged residue Lys$^{574}$ to form a salt-bridge, which may compete off the residue Asp$^{632}$ and thus block the six-helix bundle formation. Therefore, the salt-bridge-forming residue Lys$^{574}$ may serve as a key determinant of the target by the compounds and the breakage of the salt-bridge formed by Lys$^{574}$ and Asp$^{632}$ may be their mechanism of action. Recently, Jacobs et al designed a covalent inhibitor that specifically targets the residue Lys$^{574}$. Using a temperature-arrested state prime wash in vitro assay they provided evidence for the trapping of a pre-six-helix bundle fusion intermediate conformation by a covalent reaction of the Lys$^{574}$ with the specific anti-HIV peptide composed of a chemical spacer and a reactive moiety positioned strategically to facilitate covalent attachment. Therefore, the salt-bridge between the NHR and CHR of the gp41 core is critical for the conformation and stability of the six-helix bundle structure and thereby can serve as a potential target for designing anti-HIV peptides and small molecules.
Previous studies demonstrate that the prototypic peptides derived from the CHR region of HIV-1 gp41, such as C34 and T20, have potent antiviral activity \(^{11,17,40}\). It is generally accepted that the peptide C34 has better activity than T20 in inhibiting HIV-1-mediated cell-cell fusion and infection. Our studies suggest that these two CHR-derived peptides may work by different anti-HIV mechanisms \(^{38,39}\). Although they are largely overlapped by their NHR-binding domain, C34 contains the salt-bridge residue Asp\(^{632}\) at its N-terminal pocket-binding motif whereas T20 lacks this residue. It is possible that the salt-bridge interaction may be together with the hydrophobic interactions within the pocket to facilitate the NHR binding activity of the peptide C34 and thereby benefit its anti-HIV activity. In line with this hypothesis, C34 with non-conserved mutations (D632K, D632R and D632V) have much less activity to block the formation of six-helix bundle (Fig. 8) and to inhibit HIV-1-mediated cell-cell fusion and infection (Fig. 9), while C34 with conserved mutation (D632E) possesses a similar anti-HIV activity with the wild-type C34 possibly because it maintains its ability to form a salt-bridge with the residue Lys\(^{574}\). It has been demonstrated that introduction of the ion pair interactions or salt-bridges into the peptides can increase the \(\alpha\)-helicity and stabilize the coiled-coils and thereby improve the peptides for their pharmacokinetic profiles and anti-HIV activity \(^{24,34,49,59}\). Although the intrahelical salt-bridges are important for anti-HIV peptides, our data presented here suggest that formation of such ionic interactions between a fusion inhibitor (peptide or small molecule) and its target sequence (NHR or CHR) should also be considered.

In summary, the significance of our present studies is threefold. First, we demonstrate that the residue Asp\(^{632}\) in the CHR of HIV-1 gp41 is critical for six-helix
bundle formation and viral entry, which further highlights the importance of the salt-bridge of the gp41 core. Second, we propose that the salt-bridge of the gp41 core can serve as a molecular target for anti-HIV peptides and small molecules. Third, the formation of a salt-bridge with the residues within the NHR or CHR is also important for designing novel anti-HIV fusion inhibitors.

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FIGURE LEGENDS

Fig. 1. Structure and function of the HIV-1 gp41 core. (A) Schematic view of the gp41 functional regions. FP, fusion peptide; NHR, N-terminal heptad repeat; S-S, disulfide bond loop; CHR, C-terminal heptad repeat; TM, transmembrane domain; CT, cytoplasmic tail. The residue number of each region correspond to their positions in gp160 of HIV-1<sub>HXB2</sub>. (B) Crystal structure of the six-helix bundle modeled by the peptides N36 and C34. The N36 helices are colored green, whereas the C34 helices are red. (C) The salt-bridge formed by the residues Lys<sup>574</sup> in the NHR and Asp<sup>632</sup> in the CHR is indicated.

Fig. 2. Infectivity of HIV-1 pseudoviruses. Single-cycle infection of the corresponding wild-type (WT) or mutant HIV-1<sub>HXB2</sub> pseudovirus on U87-T4-CXCR4 cells was measured by a luciferase-based assay. Each sample was tested in triplicate and data are presented as mean ± standard deviation (SD).

Fig. 3. The α-helical conformation of the complex formed by N- and C-peptides analyzed by CD spectroscopy. (A) N36, C34 and their mixture. (B) N36, D632E and their mixture. (C) N36, D632K and their mixture. (D) N36, D632R and their mixture. (E) N36, D632V and their mixture. Final concentration of each peptide in PBS is 10 µM.

Fig. 4. The thermostability of the α-helical complexes formed by N- and C-peptides determined by thermal denaturation analyses. (A) N36+C34. (B) N36+D632E. (C)
N36+D632K. (D) N36+D632R. (E) N36+D632V. The unfolding temperature of each complex was scanned at 222 nm by CD spectroscopy, and their $T_m$ values were calculated. Inserted is the first derivative of the curve against temperature, which was used to determine the $T_m$ value. (F) The reversibility of the complexes formed by N- and C-peptides.

Fig. 5. Visualization of six-helix bundles formed between N36 and C34 bearing conserved or non-conserved substitutions by N-PAGE.

Fig. 6. Detection of six-helix bundles formed between N36 and C34 bearing conserved or non-conserved substitutions by ELISA using the gp41 core conformation-specific mAb NC-1.

Fig. 7. Rescue analysis of salt-bridge by Asp$^{574}$-Lys$^{632}$ double mutations. (A) Infectivity of HIV-1 pseudovirus bearing K574D and D632K double mutations. (B) The $\alpha$-helicity of K574D and D632K peptide complex. (C) The thermostability of K574D and D632K peptide complex.

Fig. 8. Inhibition of six-helix bundle formation by wild-type and mutant C34 peptides. (A) A dose-dependent curve of binding of six-helix bundle formed by N36 and biotin-C34 to the mAb NC-1 coated on the plate. Insert: binding to NC-1 of the isolated N36 (250 nM), biotin-C34 (250 nM), and equimolar mixture of N36 and
biotin-C34. (B) Inhibition of wild-type and mutant C34 on the formation of six-helix bundles.

Fig. 9. Anti-HIV activity of wild-type and mutant C34 peptides. (A) Inhibition on HIV-1_{IIIB}-mediated cell-cell fusion. (B) Inhibition on HIV-1_{IIIB}-mediated infection of MT-2 cells. Sample was tested in quadruplicate (for A) or triplicate (for B). The results are presented as mean ± SD.
Fig. 1
Fig. 2

HIV-1 pseudoviruses

Wild-type

D632E

D632K

D632R

D632V

Luciferase activity (RLU)

$10^1$ $10^2$ $10^3$ $10^4$ $10^5$ $10^6$
Fig. 4
Fig. 6

NC-1 binding (A450)

N36

C34

N36+C34

N36+D32E

N36+D32K

N36+D32R

N36+D32V
Fig. 7

A

HIV-1 pseudoviruses

Luciferase activity (RLU)

Wild-type  K574D+D632K

B

Wavelength (nm)

K574D  D632K  K574D+D632K

C

Temperature (°C)

K574D+D632K  $T_m=62^\circ C$

\[\theta\] (10$^3$ deg cm$^2$ dmol$^{-1}$)

\[\frac{d\theta}{dT}\]

\[1000\quad 500\quad 0\quad -500\quad -1000\]

\[2000\quad 1500\quad 1000\quad 500\quad 0\]

\[0\quad 20\quad 40\quad 60\quad 80\quad 100\]

\[\theta\] (10$^3$ deg cm$^2$ dmol$^{-1}$)

\[\frac{d\theta}{dT}\]

\[1000\quad 500\quad 0\quad -500\quad -1000\]

\[2000\quad 1500\quad 1000\quad 500\quad 0\]

\[0\quad 20\quad 40\quad 60\quad 80\quad 100\]
Table 1. Biophysical properties and anti-HIV activity of wild-type and mutant C34

<table>
<thead>
<tr>
<th>Peptide</th>
<th>6-HB formation</th>
<th>Inhibition (IC₅₀, nM)</th>
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<tbody>
<tr>
<td></td>
<td>[θ]₂₂₂</td>
<td>α-helicity (%)</td>
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<tr>
<td>C34</td>
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<tr>
<td>D632E</td>
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