We have previously shown that the first generation human immunodeficiency virus (HIV) fusion inhibitor T20 (Fuzeon) contains a critical lipid-binding domain (LBD), whereas C34, another anti-HIV peptide derived from the gp41 C-terminal heptad repeat, consists of an important pocket-binding domain (PBD), and both share a common 4–3 heptad repeat (HR) sequence (Liu, S., Jing, W., Cheung, B., Lu, H., Sun, J., Yan, X., Niu, J., Farmar, J., Wu, S., and Jiang, S. (2007) J. Biol. Chem. 282, 9612–9620). T1249, the second generation HIV fusion inhibitor, has both LBD and PBD but a different HR sequence, suggesting that these three anti-HIV peptides may have distinct mechanisms of action. Here we rationally designed a set of peptides that contain multiple copies of a predicted HR sequence (5HR) or the HR sequence plus either LBD (4HR-LBD) or PBD (PBDb-HR) or both (PBDb-3HR-LBD), and we compared their anti-HIV-1 activity and biophysical properties. We found that the peptide 5HR exhibited low-to-moderate inhibitory activity on HIV-1-mediated cell-cell fusion, whereas addition of LBD and/or PBD to the HR sequence resulted in a significant increase of the anti-HIV-1 activity. The peptides containing PBD, including PBDb-4HR and PBDb-3HR, could form a stable six-helix bundle with the N-peptide N46 and effectively blocked the gp41 core formation, whereas the peptides containing LBD, e.g. 4HR-LBD and PBDb-3HR-LBD, could interact with the lipid vehicles. These results suggest that the HR sequence in these anti-HIV peptides acts as a structure domain and is responsible for its interaction with the HR sequence in N-terminal heptad repeat, whereas PBD and LBD are critical for interactions with their corresponding targets. T20, C34, and T1249 may function like 4HR-LBD, PBDb-4HR, and PBDb-3HR-LBD, respectively, to interact with different target sites for inhibiting HIV fusion and entry. Therefore, this study provides important information for understanding the mechanisms of action of the peptic HIV-1 fusion inhibitors and for rational design of novel antiviral peptides against HIV and other viruses with class I fusion proteins.

The human immunodeficiency virus type 1 (HIV-1) transmembrane subunit gp41 plays an essential role in the virus fusion with the target cell. It consists of three essential functional regions closely related to its fusion activity as follows: N-terminal fusion peptide and two leucine zipper-like motifs called N-terminal heptad repeat (NHR or HR1) and C-terminal heptad repeat (CHR or HR2) domains. Both domains contain a number of 4–3 heptad repeats (HR), which have a tendency to form coiled coil structures (Fig. 1A). Peptides derived from the NHR and CHR regions are named N- and C-peptides, respectively (1, 2).

In the early 1990s, we and others discovered a number of N- and C-peptides with potent anti-HIV activity (3–5). One of the C-peptides, T20 (generic name, enfuvirtide; brand name, Fuzeon), which was jointly developed by Trimeris Inc. (Durham, NC) and Hoffmann-La Roche as a novel anti-HIV-1 drug, was licensed by the United States Food and Drug Administration in 2003 as the first member of a new class of anti-HIV drugs, HIV fusion inhibitors, to treat HIV-infected people with advanced disease and those who have failed to respond to current antiretroviral therapeutics, including reverse transcriptase inhibitors and protease inhibitors (www.fda.gov).

Using proteolytic dissection strategies, Lu et al. (6, 7) isolated several pairs of protease-resistant N- and C-peptides from gp41, e.g. N36 and C34, which have been widely used for studying the structure and function of the HIV-1 gp41. In 1997, the x-ray crystal structure of the HIV-1 gp41 core, a six-helix bundle (6-HB), was solved. The 6-HB core consists of three N-helices, which associate to form the central trimeric coiled coil, and three C-helices, which pack obliquely in an anti-parallel configuration into the highly conserved hydrophobic grooves on the surface of the internal N-trimer (8–12). In each of the
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FIGURE 1. Schematic representation of the HIV-1 gp41 and the putative conformation of the designed C-peptides. A, functional domains in gp41 and the peptide sequences. FP, fusion peptide; NHR, N-terminal heptad repeat; CHR, C-terminal heptad repeat; TM, transmembrane domain; CP, cytoplasm domain. The pocket-binding domain (PBD) and lipid-binding domain (LBD) in C-peptides are highlighted in blue and red, respectively, whereas the pocket-forming domain (PFD) in the N-peptide is marked in green. One of the 4–3 heptad repeats (HR) in the peptide C34 and the designed C-peptides is underlined. The residues in the C-peptides and those in the gp41 NHR and CHR regions corresponding to the a, d, e, and g positions in the helical wheel, respectively, are labeled with italic letters. B, predicted interactions between the residues in N46 and those in the C-peptides or between the residues in NHR and CHR are shown by dashed lines. C, predicted α-helical wheel of the peptide PBD-3HR-LBD. The residues at the a and d positions (blue) can form the hydrophobic face to interact with hydrophobic face of the N-helices, whereas those at the b, c, and f positions (yellow) can form hydrophilic face to interact with the water in solution. Those residues from LBD (in red) may not form a helical structure, although they were put in the helical wheel. D, 6-HB formed by N46 and PBD-3HR-LBD peptides. The gp41 6-HB is formed by N46 and PBD-3HR-LBD peptides through the hydrophobic amino acid residues localized in the hydrophobic interfaces. The residues at the a position (orange) and d position (blue) in a helix can interact with the d position (blue) and a position (orange) of other N-helices, respectively, to form an N-trimer. The residues at the a and d positions (green) in a C-peptide can interact the e and g positions (red) in the N-helices, respectively, to form 6-HB. E, predicted ionic interaction between the basic and acidic amino acid residues in the peptide PBD-3HR-LBD. The acidic residues at the b and c positions (i) in a C-peptide can interact with the basic residues in the f and g positions in the same peptide (i + 4), respectively, to form intramolecular salt bridges for stabilizing the α-helical conformation and increasing solubility.

grooves, there is a highly conserved hydrophobic deep pocket, formed by the pocket-forming sequence in NHR, which plays an important role in viral fusion and maintaining the stability of the 6-HB (13–15). The thermostable 6-HB can be recognized by a conformation-specific monoclonal antibody NC-1 (16, 17).

Based on the above fusogenic core structure of gp41, the fusogenic mechanism and model of C-peptide-mediated antiviral activity were proposed (1). The entry process begins when viral envelope glycoprotein (Env) surface subunit 120 binds to the cellular receptor (CD4) and co-receptors (CXCR4 or CCR5). This causes a conformational change in the Env transmembrane subunit gp41 and activates gp41-mediated virus-cell fusion. In the presence of a C-peptide (e.g. C34) at the fusion-intermediate state, it interacts with the viral gp41 NHR region to form a heterogeneous 6-HB, thus blocking the formation of fusion-active core of gp41 and inhibiting the fusion of the virion with the target cell. Both T20 and C34 were derived from gp41 CHR region and share a common 4–3 HR sequence (amino acids 638–658, YTSLIHSLIESQNYEQEKNEQ) (5, 7). It has been proposed that T20 and C34 may have a similar mechanism of action in inhibiting the fusion between HIV and the target cell membranes because both peptides may interact, via the shared 4–3 HR sequence, with the viral gp41 NHR region to form heterologous 6-HB and block the formation of the gp41 fusogenic core (18, 19). However, our previous studies have shown that T20 and C34 may have distinct mechanisms of action because C34 contains a pocket-binding domain (PBD), through which C34 can interact with the pocket-forming sequence in the NHR to form stable 6-HB. However, T20 does not have the PBD sequence, thereby being unable to form stable 6-HB with NHR, but instead it contains a lipid-binding domain (LBD), through which T20 may interact with the

FIGURE 1. Schematic representation of the HIV-1 gp41 and the putative conformation of the designed C-peptides. A, functional domains in gp41 and the peptide sequences. FP, fusion peptide; NHR, N-terminal heptad repeat; CHR, C-terminal heptad repeat; TM, transmembrane domain; CP, cytoplasm domain. The pocket-binding domain (PBD) and lipid-binding domain (LBD) in C-peptides are highlighted in blue and red, respectively, whereas the pocket-forming domain (PFD) in the N-peptide is marked in green. One of the 4–3 heptad repeats (HR) in the peptide C34 and the designed C-peptides is underlined. The residues in the C-peptides and those in the gp41 NHR and CHR regions corresponding to the a, d, e, and g positions in the helical wheel, respectively, are labeled with italic letters. B, predicted interactions between the residues in N46 and those in the C-peptides or between the residues in NHR and CHR are shown by dashed lines. C, predicted α-helical wheel of the peptide PBD-3HR-LBD. The residues at the a and d positions (blue) can form the hydrophobic face to interact with hydrophobic face of the N-helices, whereas those at the b, c, and f positions (yellow) can form hydrophilic face to interact with the water in solution. Those residues from LBD (in red) may not form a helical structure, although they were put in the helical wheel. D, 6-HB formed by N46 and PBD-3HR-LBD peptides. The gp41 6-HB is formed by N46 and PBD-3HR-LBD peptides through the hydrophobic amino acid residues localized in the hydrophobic interfaces. The residues at the a position (orange) and d position (blue) in a helix can interact with the d position (blue) and a position (orange) of other N-helices, respectively, to form an N-trimer. The residues at the a and d positions (green) in a C-peptide can interact the e and g positions (red) in the N-helices, respectively, to form 6-HB. E, predicted ionic interaction between the basic and acidic amino acid residues in the peptide PBD-3HR-LBD. The acidic residues at the b and c positions (i) in a C-peptide can interact with the basic residues in the f and g positions in the same peptide (i + 4), respectively, to form intramolecular salt bridges for stabilizing the α-helical conformation and increasing solubility.
lipid membrane and interfere with the viral fusion process (20, 21). A second generation HIV fusion inhibitor T1249 was designed by adding the PBD to the N terminus of T20 and replacing some of the residues with those from the corresponding region in the HIV-2 and simian immunodeficiency virus transmembrane glycoproteins (20). Compared with the first generation fusion inhibitor T20, T1249 has greater anti-HIV activity. But its mechanism of action is still unknown.

In this study, we designed and synthesized a set of C-peptides that contain multiple copies of a 7-mer sequence predicted to form 4–3 HR conformation with or without addition of the PBD and/or LBD. We compared their inhibitory activity on HIV-1-mediated cell-cell fusion and analyzed their biophysical and biochemical properties. The information is useful for understanding mechanisms of action of the first and second generation HIV fusion inhibitors and for rational design of novel antiviral peptides against HIV and other viruses with class I fusion proteins.

**EXPERIMENTAL PROCEDURES**

**Peptide Design and Synthesis**—The following four peptides were designed: 1) 5HR, which contains five copies of one predicted 4–3 HR sequence; 2) 4HR-LBD in which an LBD was added to the C terminus of 4HR, which consists of four copies of one predicted 4–3 HR sequence; 3) PBD-4HR in which a PBD was added to the N terminus of 4HR; and 4) PBD-3HR-LBD in which both LBD and PBD, respectively, were added to the C and N termini of 3HR, which consists of three copies of one predicted 4–3 HR sequence (Fig. 1A). The peptide 5HR was designed based on the following rationale. First, the optimal peptide length was required for the antiviral activity. The most potent anti-HIV-1 peptides (e.g. C34 and T20) have 34–36 amino acid residues (5, 7). Therefore, we designed the peptide 5HR with 35 residues (five copies of one predicted 4–3 heptad repeat, EYETKKI). Second, the residues at the a and d positions in the helical wheel of the C-helices are amino acids with high hydrophobicity, e.g. Ile and Tyr, whose hydrophathy indices (22) are 4.5 and −1.3, respectively. This will result in a face with high hydrophobicity to increase the interaction with the residues at the e and g positions in the helical wheel of the N-helices. Finally, the residues at the b and c positions in the helical wheel of the C-helices are acid amino acids, e.g. Glu, which have negative charges and high hydrophilicity (hydropathy index = −3.5). The residues at the f and g positions in the helical wheel of the C-helices should be basic amino acids, e.g. Lys, which have positive charges and high hydrophilicity (hydropathy index = −3.9). This will result in a face with high hydrophilicity to increase the interaction with water, to increase the solubility of the peptides. Furthermore, the acid residues at b and c positions in the helical wheel can interact with those at the f and g positions, respectively, to form intrahelical salt bridges, to increase the stability of the helical structures of the peptides (23) (Fig. 1).

Peptides C34-biotin (biotin was added to the N terminus of C34), N46, and the above designed C-peptides were synthesized on 4-methylbenzhydrylamine resin by using the Fmoc (N-(9-fluorenyl)methoxycarbonyl) strategy. They were cleaved from the resin by hydrogen fluoride. The N termini of the peptides were acetylated, and their C termini were amidated. All the peptides were purified by reverse phase high performance liquid chromatography to >90% homogeneity. The molecular weight of the peptides was confirmed by matrix-assisted laser desorption ionization time-of-flight-mass spectrometry (Autoflex III, Bruker Daltonics).

**Prediction of the Secondary and Tertiary Structures of the Designed C-peptides**—Secondary structure prediction of designed C-peptides was carried out on Network Protein Sequence using the SOPM method (24). According to the crystal structure of the gp41 core formed by the N-peptide N36 and C-peptide C34 (8) and the predicted secondary structure of the designed C-peptides, the interaction between the C-peptides and N46 was modeled by using PyMOL program.

**HIV-1-mediated Cell-Cell Fusion**—The inhibitory activities of the four C-peptides on HIV-1-induced cell-cell fusion were determined using a dye transfer assay (25). Briefly, H9/HIV-1\textsubscript{HIV} cells were pre-labeled with a fluorescent dye, calcein AM (Molecular Probes, Inc., Eugene, OR), and incubated with a testing peptide at a graded concentration at 37 °C for 30 min in a 96-well cell culture plate. Then the CD4\textsuperscript{+} MT-2 cells were added to the H9/HIV-1\textsubscript{HIV} cells at a ratio of 10:1, followed by incubation at 37 °C for 2 h. The fused and unfused calcein-labeled HIV-1-infected cells were counted under an inverted fluorescence microscope (Zeiss, Germany) with an eyepiece micrometer disk. The percent inhibition of cell fusion by a peptide and the IC\textsubscript{50} values (the concentrations for 50% inhibition) were calculated using the software CalcuSyn.

**Native PAGE (N-PAGE)**—N-PAGE was used to detect the 6-HB formed by mixing N46 (100 μM) with an equimolar concentration of each of the 4 C-peptides (26). The mixtures were incubated at 37 °C for 30 min, followed by the addition of Tris-glycine native sample buffer (Invitrogen). The samples (20 μl) were then loaded onto Tris-glycine gels (18%; Invitrogen), which were run under 125 V constant voltage at room temperature for 2 h. The gels were stained with Coomassie Blue and then visualized with the FluorChem 8800 Imaging System (Alpha Innotech Corp., San Leandro, CA).

**CD Spectroscopy and Thermal Midpoint (Tm) Analysis**—The secondary conformational structure of the four C-peptides and their mixtures with N46 were analyzed by CD spectroscopy as described previously (26). Briefly, each of the peptides or peptide mixtures was dissolved in PBS (50 mM sodium phosphate and 150 mM NaCl, pH 7.2) at the final concentration of 10 μM and incubated at 37 °C for 30 min before the samples were then cooled down to 4 °C. The CD spectra of each sample was acquired on a Jasco spectropolarimeter (model J-715, Jasco Inc., Japan) at 25 °C using a 5-nm bandwidth, 0.1-nm resolution, 0.1-cm path length, and an average time of 5.0 s. Spectra were corrected by the subtraction of a blank corresponding to the solvent composition of each sample. Thermal midpoint analysis is used to determine the temperature in which 50% of the six-helix bundles formed by the C-peptides would decompose. It was monitored at 222 nm from 10 to 98 °C by applying a thermal gradient of 5 °C/min. The melting curve was smoothed, and the midpoint of the thermal unfolding transition (Tm) values was calculated using Jasco software utilities as described above.
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_Enzyme-linked Immunosorbent Assay (ELISA)_—ELISA was used to determine the inhibitory activity of the four C-peptides on the 6-HB core formation on N46 and biotinylated C34 (C34-biotin) with a formation-specific monoclonal antibody NC-1 (16). Briefly, a testing peptide at 0.5 μM was preincubated with an equal amount of N46 at 37 °C for 30 min, followed by the addition of C34-biotin (0.5 μM). The mixture was added to a 96-well polystyrene plate (Costar, Corning Inc., Corning, NY) coated with monoclonal antibody NC-1 IgG (2 μg/ml in 0.1 M Tris, pH 8.8) and blocked with 2% nonfat milk in PBS. The plate was then incubated for 30 min and added to horseradish peroxidase labeled with streptavidin (Zymed Laboratories Inc.). The plate was washed with the washing buffer (PBS containing 0.01% Tween 20) six times to remove any unbound peptide. The substrate 3,3',5,5'-tetramethylbenzidine (Sigma) was added sequentially. Absorbance at 450 nm (A450) was measured using an ELISA reader (Ultra 384, Tecan, Research Triangle Park, NC). The percent inhibition of 6-HB formation and the IC50 values were calculated using the CalcuSyn software.

_Isothermal Titration Calorimetry (ITC)_—ITC was executed to detect the peptide-lipid binding activity of the four C-peptides as described previously (12). Briefly, peptide solutions were degassed under vacuum prior to use. To titrate peptide solutions, we used the large unilamellar vesicles (LUVs) of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposome, which was prepared by drying 20 mg of the POPC stock solution of any organic solvent under a stream of nitrogen gas and a vacuum oven. The dried lipid film was suspended in 2 ml of PBS for 30 min. After incubation, the lipid suspension was freeze-thawed for five cycles and extruded through two stacks of polycarbonate filters (0.1 μm) 13 times using an Avanti mini-extruder for use. LUVs of POPC were then injected into the cell containing the peptide solution. The heats of dilution were obtained by subtracting the heats generated in the control experiments, in which lipid vesicles were injected into the buffer-only cell, from the heats produced in the corresponding peptide-lipid binding experiments. The binding affinities of the four C-peptides to POPC LUVs were measured by using the Microcal VP-ITC MicroCalorimeter. Data acquisition and analyses were processed using Origin software (version 7.0, Microcal).

**RESULTS**

_Prediction of the Secondary and Tertiary Structures of the Designed C-peptides_—The secondary structure of the designed C-peptides were predicted with the NPS@ web server using HNN secondary structure prediction method (27). Like the C-peptides T20 and C34, the four designed C-peptides had 86–92% of the amino acid residues in α-helix conformation and about 6–14% of the residues in random coil conformation (Table 1). All the amino acids in the random coil conformation were localized in the N- or C-terminal regions (data not shown). These results suggest that all these designed C-peptides have a tendency to form α-helical structure. About 67–76% of the amino acid residues in the four designed C-peptides exposed with more than 16% of their surface, a little lower than those in the T20 (88%) and C34 (86%), indicating that these designed C-peptides have good solubility in water.

The tertiary structures of the designed C-peptides and their interactions with the N-peptide N46 were modeled by using the PyMOL program based on the crystal structure of the 6-HB formed by N36 and C34 (8). Like N36, the peptide N46 may also form internal N-trimer via the interaction between the amino acid residues at the a position in one N46 molecule (N46-A) and those at the d position in another N46 peptide (N46-B). C-peptides (e.g. C34) can bind to the hydrophobic grooves on the surface of the N-trimer through the interaction of the residues at the a and d positions in a C-peptide with those at the e and g positions in the adjacent two N46 molecules (Table 2). For example, five amino acid residues located at the d positions (magenta) in 5HR (Tyr-3, Tyr-10, Tyr-17, Tyr-24, and Tyr-31) may interact with the five residues at the g positions (yellow) in N46 (Gly-572, Leu-565, Ala-558, Gln-551, and Leu-544), respectively (Fig. 2B). Notably, the binding between the residues of 5HR and N46 is less complementary than that of the residues between C34 and N46 (Fig. 2).

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**TABLE 1**

| Peptides | No. residues | Secondary structure (%) | Solvent accessibility (%) |
|----------|--------------|-------------------------|--------------------------|
|          | H            | E            | L            | e         | b         |
| 5HR      | 35           | 85.71        | 0            | 14.29     | 71.43     | 28.57     |
| 4HR-LBD  | 36           | 91.67        | 0            | 8.33      | 66.67     | 33.33     |
| PBD-4HR  | 36           | 86.11        | 0            | 13.89     | 72.22     | 27.78     |
| PBD-3HR-LBD | 37   | 86.49        | 0            | 13.51     | 75.68     | 24.32     |
| C34      | 34           | 85.29        | 0            | 14.71     | 88.24     | 11.76     |
| T20      | 36           | 91.67        | 0            | 8.33      | 86.11     | 13.89     |

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**TABLE 2**

| N46-A | N46-B | C34 | T20  | 5HR | 4HR-LBD | PBD-4HR | PBD-3HR-LBD |
|-------|-------|-----|------|-----|---------|---------|-------------|
| Gln-577 (e) | Gly-572 (g) | Trp-628 (a) | Trp-631 (a) | Trp-638 (a) | Trp-639 (a) | Trp-642 (a) | Trp-643 (a) | Trp-644 (a) |
| Val-570 (e) | Ile-635 (a) | Ile-642 (a) | Tyr-683 (d) | Tyr-684 (d) | Tyr-685 (d) | Tyr-686 (d) | Tyr-687 (d) | Tyr-688 (d) |
| Gln-563 (e) | Asp-652 (a) | Tyr-730 (d) | Tyr-731 (d) | Tyr-732 (d) | Tyr-733 (d) | Tyr-734 (d) | Tyr-735 (d) | Tyr-736 (d) |
| Leu-556 (e) | Ser-649 (a) | Ile-652 (a) | Ser-653 (d) | Ser-654 (d) | Ser-655 (d) | Ser-656 (d) | Ser-657 (d) | Ser-658 (d) |
| Val-549 (e) | Gly-544 (a) | Leu-545 (a) | Glu-659 (d) | Glu-660 (d) | Glu-661 (d) | Glu-662 (d) | Glu-663 (d) | Glu-664 (d) |
| Arg-542 (e) | Leu-537 (g) | Leu-538 (g) | Leu-539 (g) | Leu-540 (g) | Leu-541 (g) | Leu-542 (g) | Leu-543 (g) | Leu-544 (g) |

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(a, g) and (a, d) indicate the positions of the corresponding residues in the N- and C-helix wheels, respectively (see details in Fig. 2).
Inhibitory Activities of the Four Designed C-peptides on HIV-1-induced Cell-Cell Fusion—The inhibitory activities of the four C-peptides on HIV-1-mediated cell-cell fusion were determined using a dye transfer assay (25). As shown in Fig. 3 and Table 3, the peptide containing five copies of HR (5HR) showed weak-to-moderate anti-HIV-1 activity. The inhibitory activity of the peptide containing four copies of the HR sequence and the LBD (4HR-LBD) or the PBD (PBD-4HR) was improved about 2- and 6-fold, respectively, as compared with 5HR. Strikingly, the peptide with three copies of the HR sequence plus both LBD and PBD (PBD-3HR-LBD) is almost 33-fold more potent than 5HR. Therefore, the potency of the designed C-peptides against HIV-1-mediated cell-cell fusion is in the following order: PBD-3HR-LBD > PBD-4HR > 4HR-LBD > 5HR.

Ability of the C-peptides to Form a Helix Bundle with N46—We then determine whether these C-peptides could interact with the N-peptide N46 to form complex by using N-PAGE as described previously (26). As shown in Fig. 4, N46 exhibited no band (lane 1) because it carries net positive charges and may migrate off the gel, as another N-peptide N36 (26). Each of the C-peptides displayed a band at different positions in the gel dependent on the net negative charge and the molecular size of the peptides (Fig. 4, lanes 2, 4, 6, and 8). When each of the C-peptides was mixed with N46, the bands of 5HR and 4HR-LBD remained at the same position (Fig. 4, lanes 3 and 5, respectively), whereas the bands of PBD-4HR and PBD-3HR-LBD shifted upwards in the gel (Fig. 4, lanes 7 and 9, respectively), indicating that unlike the 5HR and 4HR-LBD peptides, the PBD-4HR and PBD-3HR-LBD peptides are able to interact with N46 to form complexes in PBS.

Ability of the C-peptides to Form an α-Helical Complex—Subsequently, we used CD spectroscopy to analyze the secondary structure of the designed C-peptides and the complexes formed by these C-peptides with the N-peptide N46. As shown in Fig. 5A, N46 peptide alone in PBS formed typical α-helical structure, although its α-helicity was moderate (21). Like the native C-peptide C34 (20, 21), all the designed C-peptides exhibited random coil structure in PBS. In agreement with our previous study (20, 21), the peptide C34 could interact with N46 to form complexes with typical α-helical conformation, characterized by the CD spectra with two minima at 208 and 220, but addition of the peptide T20 to N46 did not enable the formation of typical α-helical complexes, but rather distorted the α-helical conformation and reduced the α-helicity of N46, in agreement with our previous report (20, 21).

Like C34 (20), both PBD-4HR and PBD-3HR-LBD peptides could interact with N46 to form typical α-helical complexes.
Like T20, the peptides 5HR and 4HR-LBD distorted the α-helical conformation of N46 (Fig. 5A). The α-helicity of these complexes is in the following order: PBD-3HR-LBD N46 > PBD-4HR N46 > 4HR-LBD N46 > 5HR+C46 (Table 3), consistent with the cell-cell fusion inhibitory activity (Fig. 3).

Thermal denaturation of the α-helical complexes formed by the C-peptide and N46 was monitored, and their $T_m$ values were calculated. As shown in Fig. 5B, the $T_m$ values of N46/5HR and N46/4HR-LBD mixtures were undetectable, whereas the mixtures of N46/PBD-4HR and N46/PBD-3HR-LBD exhibited high $T_m$ values (82.4 and 82.8 °C, respectively), suggesting that the α-helical oligomer formed by the N-peptide N46 and the C-peptides PBD-4HR and PBD-3HR-LBD are highly stable in phosphate buffer.

Inhibitory Activity of the C-peptides in 6-HB Formation—The ability of these four C-peptides to block 6-HB formation between N46 and C34-biotin was assessed by using ELISA. As shown in Fig. 6, 5HR exhibited no significant inhibition in 6-HB formation at a concentration as high as 120 g/ml, whereas 4HR-LBD showed a moderate inhibitory activity with an IC$_{50}$ value of 117.67 ± 2.54 µg/ml. Both PBD-4HR and PBD-3HR-LBD peptides exhibited highly potent inhibitory activity against 6-HB formation with IC$_{50}$ values of 2.79 ± 0.28 and 0.58 ± 0.06 µg/ml, respectively.

Interaction of the C-peptides with Lipid Bilayers—HIV-1 gp41 has the ability to bind to cell lipid bilayers with high binding affinity. Here we sought to determine the interaction between each of the C-peptides and the lipid membrane by using the POPC LUV liposome system to measure the heat generated from the peptide-lipid association in an ITC assay. As shown in Fig. 7, there was a huge release of heat when POPC LUVs were added into solutions containing 4HR-LBD and PBD-3HR-LBD. Their binding constants were 6.80 × 10$^4$ and 1.27 × 10$^5$ M$^{-1}$, respectively, suggesting that unlike the peptides 5HR and PBD-4HR, both PBD-3HR-LBD and 4HR-LBD peptides possess lipid binding activity.

DISCUSSION

T20, a 36-mer peptide, is the first HIV fusion inhibitor approved by the United States Food and Drug Administration.
for clinical use. T1249, a 39-mer peptide, was developed as the second generation HIV fusion inhibitor. However, their mechanisms of action have not been well defined so far. It has been proposed that because T20 contains a 4–3 HR sequence shared by C34 and T20, it may function like C34 to interact with the viral gp41 NHR region. It is known that all the class I fusion proteins must have a unique HR sequence in the CHR region. The HR sequence may also serve as a structural common feature, i.e. containing 4–3 HR sequences in their CHR (or HR2) regions that can interact with HR sequences in the viral gp41 NHR region. The functional domains, e.g. PBD in C34 and LBD in T20, are also important for the antiviral activity of these C-peptides because they need to interact with the corresponding target sites via their functional domains. To test this assumption, we thus designed a set of C-peptides that mimic the structures of T20, C34, and T1249, respectively, as probes to study the mechanism(s) of action of the peptidic HIV fusion inhibitors.

The first peptide designed is 5HR that contains five copies of a non-native 4–3 HR sequence (EYETKKI), in which the residues at the d and a positions in the helical wheel of the C-helices are hydrophobic amino acids (Tyr and Ile) to allow the C-peptide to interact with the hydrophobic amino acid residues at the corresponding position in the NHR to form a helical complex, and at the i and i + 4 positions (b, c and f, and g positions, respectively, in the helical wheel) are pairs of acidic and basic amino acid residues (e.g. Glu and Lys) that can form intrahelical salt bridges to increase the stability and solubility of the C-peptides (Fig. 1). Surprisingly, this engineered peptide with substantially different sequence from those of anti-HIV C-peptides derived from the native CHR sequence displayed antiviral activity, although at a low-to-modest level, against HIV-1-mediated cell-cell fusion (Fig. 3), suggesting that this non-natural HR sequence may interact with 4–3 HR sequence in the viral gp41 NHR region. The peptide 5HR is predicted to be mainly α-helical structure (Table 1), and its hydrophobic amino acids (Tyr and Ile) at the d and a positions have a tendency to interact with the amino acid residues (mainly hydrophobic) at the g positions (e.g. Leu-565 and Leu-544, etc.) in one N46 peptide and those at the e positions (e.g. Val-570 and Leu-556, etc.) in another N46 molecule, respectively (Table 2 and Fig. 2). Our result suggests that the native HR sequence in the C-peptides can be replaced by non-native sequence as long as it can form a coiled coil structure that is required for interaction of the C-peptide with the HR sequence in the viral gp41 NHR region. The HR sequence may also serve as a structural domain to maintain an appropriate length and conformation of the C-peptides to allow the functional domains (e.g. PBD and LBD) to interact with their corresponding target sites.

Subsequently, we designed the second and third peptides by adding the PBD or LBD to the N or C terminus of the 4–3 HR sequence (PBD-4HR or 4HR-LBD, respectively), mimicking the sequence of C34 and T20, respectively. These two peptides exhibited significantly improved anti-HIV-1 activity, compared with 5HR (Fig. 3). Interestingly, PBD-4HR functions like C34, i.e. forming highly stable 6-HB with N46 and blocking the gp41 core formation between N46 and biotinylated C34 (Figs. 4–6), whereas 4HR-LBD acts like T20 by interacting with the lipid membrane (Fig. 7). Notably, the anti-HIV-1 activity of 4HR-LBD and PBD-4HR is lower than that of T20 and C34, perhaps because the non-native 4–3 HR sequence in these designed peptides could not interact complementarily and tightly with the native HR sequence in the viral gp41 NHR region. It is known that all the class I fusion proteins of the enveloped viruses (e.g. HIV, simian immunodeficiency virus, SARS-CoV, and Ebola virus) share a common feature, i.e. containing 4–3 HR sequences in their CHR or HR2 regions that can interact with HR sequences in the corresponding NHR or HR1 region in a dominant-negative manner (1, 29). However, each of the class I fusion proteins must have a unique HR sequence in the CHR region to specifically bind to the HR sequence in the NHR region of the same virus, rather than to that of other virus. A virus that
maintains its unique protein sequence can also avoid the attack by immune mediators elicited by proteins of other viruses in the same host. That is why the highly potent anti-HIV peptides (e.g. C34) are unable to inhibit SARS-CoV infection, and vice versa, although both anti-HIV and anti-SARS-CoV peptides contain 4–3 HR sequences (30). Therefore, when designing a peptidic fusion inhibitor against a virus, one must select an HR sequence that can specifically interact with high binding affinity with the HR sequence in the NHR region of the class I fusion protein of the same virus.

Finally, we designed the fourth peptide (PBD-3HR-LBD) by adding the PBD and LBD to the N and C termini of the 3HR sequence, respectively, mimicking the sequence of T1249. This peptide has further improved anti-HIV-1 activity, compared with the peptides 4HR-LBD and PBD-4HR (Fig. 3), consistent with the fact that T1249 has greater anti-HIV-1 activity than T20 and C34 (31, 32). Strikingly, this peptide could interact with N46 to form stable 6-HB and block the gp41 core, like C34, and also bind to the lipid membrane, like T20 (Figs. 4–7). Given that the 4–3 HR sequence between PBD and LBD may be too short to allow both domains to interact with the hydrophobic pocket in NHR helices and lipid membranes of the target cell simultaneously, we suspect that a part of peptide PBD-3HR-LBD binds to the pocket while the remaining part of the peptide interacts with another target, the host cell membrane. T1249 may act in a manner similar to PBD-3HR-LBD, i.e. T1249 inhibits HIV fusion by interacting either with the pocket region of the viral gp41 NHR or with the target cell membranes.

Based on the above findings, we propose the putative modes of action of the peptides 4HR-LBD, PBD-4HR, and PBD-3HR-LBD, which mimic the structure of the peptidic HIV fusion inhibitors T20, C34, and T1249, respectively. The peptides containing PBD (e.g. PBD-4HR and C34) inhibit HIV fusion by binding to the HR sequence and pocket region in NHR to form stable trim-mer-of-heterodimer and block the gp41 six-helix bundle core formation. The peptides with LBD (e.g. 4HR-LBD and T20) inhibit HIV infection by interacting with the HR sequence in NHR and the target cell membrane. The peptides consisting of both PBD and LBD (e.g. PBD-3HR-LBD and T1249) may use either of the above action models to inhibit HIV infection (Fig. 8). Therefore, this study provides important information for understanding the role of each functional domain in the anti-HIV C-peptides and for further elucidating the mechanism(s) of action of the HIV fusion inhibitors. This information will be useful for designing novel peptidic antiviral therapeutics against HIV and other viruses with class I fusion proteins.

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