Novel Recombinant Engineered gp41 N-terminal Heptad Repeat Trimmers and Their Potential as Anti-HIV-1 Therapeutics or Microbicides*

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Peptides derived from N-terminal heptad repeat (NHR) of the HIV-1 gp41 are generally poor inhibitors of HIV-1 entry, because they tend to aggregate and do not form a trimeric coiled-coil. In this study, we have fused portions of gp41 NHR, e.g. N36 or N28, to the T4 fibritin trimerization domain, Foldon (Fd), thus constructing novel NHR trimers, designated N36Fd or N28Fd, which could be expressed in Escherichia coli cells. The purified N36Fd and N28Fd exhibited SDS-resistant trimeric coiled-coil conformation with improved α-helicity compared with the corresponding N-peptides. They could interact with a C-peptide (e.g. C34) to form stable six-helix bundle and possessed potent anti-HIV-1 activity against a broad spectrum of HIV-1 strains. N28Fd was effective against T20-resistant HIV-1 variants and more resistant to protease K compared with T20 (enfuvirtide), a C-peptide-based HIV fusion inhibitor. Therefore, N28Fd trimer has great potentials for further development as an affordable therapeutic or microbicid for treatment and prevention of HIV-1 infection.

The first essential step of HIV type 1 (HIV-1) infection involves viral fusion and entry mediated by viral envelope glycoproteins gp120 and gp41. After gp120 binding to the cellular receptor CD4 and coreceptor, CXCR4 or CCR5, the fusion peptide at the N terminus of gp41 is exposed, enabling its insertion into the target cell membrane. A series of conformational changes in gp41 takes place, leading the protein to the transition from a pre-fusion state to a post-fusion state through different intermediates that are crucial for fusion (1, 2). The core structure of gp41 ectodomain consists of two 4-3 hydrophobic heptad repeat regions defined as N-terminal heptad repeat (NHR) and C-terminal heptad repeat (CHR) (3) (see Fig. 1). Crystallographic studies have shown that CHR can interact with NHR to form a conformation termed “trimer-of-hairpins” or “six-helix bundle” (6-HB), within which three parallel NHRs form a trimeric coiled-coil core, and three CHRs pack in an antiparallel manner into the highly conserved hydrophobic grooves along the surface of the inner coiled-coil (4–6).

Peptides derived from the NHR and CHR regions are called N- and C-peptides, respectively. Some C-peptides, such as SI-2176 (7), T20 (8), and C34 (9), are potent inhibitors of HIV infection. They interfere with 6-HB formation by binding to the viral gp41 inner NHR-coiled-coil. T20 (brand name: Fuzon; generic name: enfuvirtide) has been approved by the U.S. FDA as the first member of a new class of anti-HIV drugs: HIV fusion inhibitors. Some mutated N-peptides, e.g. N36Mut(e,g), could also inhibit HIV-1 infection by interacting with NHR to form heterotrimers that disrupt the formation of the inner NHR of the gp41 core (10).

The gp41 CHR is a key target for 5-helix, an engineered peptide inhibitor that consists of three N helices and two C helices and inhibits HIV-1 entry by binding to the viral gp41 CHR region (11). In principle, N-peptides should also block the interaction between the viral gp41 NHR and CHR and inhibit HIV-1-mediated cell-cell fusion as effectively as the C-peptides. However, the actual potency of N-peptides is 2 to 3 orders lower than C-peptides, mainly because N-peptides tend to aggregate in physiological solutions (3, 9). Nonetheless, it is proposed that a properly designed N-peptide able to fold into stable and soluble trimeric coiled-coils without aggregating should possess anti-HIV-1 efficiency just as high as that of a C-peptide (3, 11). Accordingly, several NHR trimer inhibitors have been designed and constructed by introducing intermolecular disulfide bond(s) or trimeric coiled-coil motif(s) into the N-peptide to stabilize the trimeric conformation. For example, by introducing intermolecular disulfide bonds (the CCG motif) into N-peptides N35 or N34, Louis et al. constructed three anti-HIV-1 N-peptides, NCCG-gp41, in which the N35CCG peptide was linked to a minimal thermostable 6-HB (N34/C28) (12), and N34CCG and N35CCG-N13, which consist of only NHR fragments (13). By introducing trimeric coiled-coil motifs, such as a portion of GCN4-p1, I (IQ) or IZm (IZ) into N-peptides N17 or N23, Eckert et al. (14, 15) constructed stable N-helical trimers, including IQN17, IZN23, and IZN17. All of the above-mentioned peptide constructs showed much higher anti-HIV-1 activity than their corresponding N-peptides. An even more potent NHR trimer inhibitor, (CCIZN17)3, was designed by combining these two methods, i.e. adding disulfide bridges to...
the N terminus of the IZN17 sequence (16). However, it may not be practical to develop these peptides as drugs, because they are very expensive to be synthesized and refolded into active conformation.

In this study, we describe new recombinant NHR trimer inhibitors constructed in a novel way by fusing N-peptides to the N terminus of Foldon (Fd), the natural trimerization domain of T4 bacteriophage fibritin (17, 18). The peptides, named N36Fd and N28Fd, were shown to be stable trimers and interact strongly with C-peptide to form soluble coiled-coils. These peptides, especially N28Fd trimer, were highly potent inhibitors of infection by a broad spectrum of HIV-1 strains. Compared with T20, N28Fd trimer is much more potent in inhibiting T20-resistant HIV-1 strains, much less sensitive to proteinases, and more affordable because N28Fd, which is expressed in prokaryotic Escherichia coli cells, can be put into large-scale production at reduced cost.

**EXPERIMENTAL PROCEDURES**

Peptides—The sequences of the N-peptides, N36 (aa 546–581) and N28 (aa 559–586), and the C-peptides, C34 (aa 628–661) and T20 (aa 638–673), used in this study were derived from the NHR and CHR, respectively, of the HIV-1Ibx2 gp41 (Fig. 1, A and B). These peptides and T1249 and T1144 were synthesized by a standard solid-phase Fmoc (N-(9-fluorenlymethoxycarbonyl) method using an Applied Biosystems model 433A peptide synthesizer. The N and C termini of these peptides were acetylated and amidated, respectively. The peptides were purified to homogeneity (>95% purity) by high-performance liquid chromatography 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 and 1490 mol/liter\(^{-1}\)cm\(^{-1}\) based on the number of tryptophan (Trp) residues and tyrosine (Tyr) residues (all the peptides tested contain Trp and/or Tyr), respectively (19).

Construction of Vectors Encoding N36Fd and N28Fd—N36Fd consists of N36 peptide and the Fd sequence (GYIPEA-PRDGQAYVRKDGEWVLLSTFL) that was derived from the natural trimeric motif of T4 bacteriophage fibritin, whereas N28Fd consists of N28 and Fd sequence. Fd was fused to the C terminus of the N-peptide with no linker (Fig. 1C). The DNA fragments of N36 and N28 were amplified by PCR using the Platinum PCR SuperMix High Fidelity kit (Invitrogen) from a pHBX-env/wt plasmid with a forward primer containing a BamH1 site and a reverse primer that had no restriction site. The fragment of Fd was produced by annealing a long synthetic forward primer coding the last 9 amino acids of the N-peptide (N36 or N28) and the Fd with its complement, a long reverse primer with an XhoI site. Then the two overlapping fragments were mixed and used as templates for another PCR reaction using the BamH1 forward primer and a shorter reverse primer coding the last few amino acids of the Fd with an XhoI site. The product was purified using a gel extraction kit (Qiagen, Valencia, CA), digested with BamH1 and XhoI enzymes (TaKaRa Bio, Madison, WI), and cloned into a pGEX6p-1 vector (Qiagen). The sequence was confirmed by DNA sequencing.

**ProteinExpression and Purification**—Either the N36Fd-pGEX6p-1 or N28Fd-pGEX6p-1 plasmid was transformed into E. coli Rosetta 2(DE3) (Novagen, Gibbstown, NJ). The cells were incubated at 37 °C in LB medium until the A\(_{600}\) reached 0.8–1.0. The culture was induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside and incubated at 30 °C for 4 h. Then, the cells were harvested and broken by sonication in 1% Triton PBS buffer. After the samples were centrifuged, the supernatant was loaded into a GST-bind column (Novagen). The column was rinsed, and the bound GST-fused N-Fd peptides were then cleaved onto the column with PreScission Protease (GE Healthcare) in cleavage buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) at 4 °C overnight. The cleaved peptides were eluted from the column on the next day by washing with cleavage buffer. Either N36Fd or N28Fd in this rough product was further separated from GST by a series of ultrafiltrations using Amicon Ultra-15 Centrifugal Filter Devices (Millipore, Billerica, MA). N36Fd and N28Fd performed monomeric conformation in the buffer with pH lower than 3.0, which enabled them to be collected in the centrifuge tube of the 30-kDa Ultra-15 Centrifugal Filter Device, while GST was kept in the filter unit. Finally, the N-Fd peptide was dialyzed against ddH\(_2\)O (pH 7.0) and refolded into trimers using the 10-kDa Ultra-15 Centrifugal Filter Device.

**SDS-PAGE Analysis**—Purified N36Fd and N28Fd were analyzed by SDS-PAGE as previously described (18). Briefly, 5 μl/well 100 μM N36Fd or N28Fd was mixed with 4X SDS sample buffer (Novagen). The sample was boiled for 5 min or kept at room temperature before loading onto a 10–20% Tricine-glycine gel (Invitrogen). The electrophoresis was conducted in SDS-PAGE running buffer with 125 V of constant voltage at 4 °C. The gels were stained with SimplyBlue SafeStain (Invitrogen).

**Sedimentation Velocity Analysis**—Sedimentation velocity measurements were performed on the Proteomelab™ XL-A/XL-1 analytical ultracentrifuge (Beckman Coulter, Fullerton, CA) equipped with a three-channel An-60 Ti rotor. N28Fd and N36Fd peptides were dialyzed against 100 mM CH\(_3\)COONa/CH\(_3\)COOH buffer (pH 6.0) and diluted to 50 μM by using the dialysis buffer. 30 μM N28Fd or N36Fd was mixed with 30 μM C34 in pH 7.4 PBS. The sample (380 μl) and corresponding buffer (400 μl) were loaded pairwise into the double sector quartz cell and run at 60,000 rpm at 20 °C. Data were collected at wavelength of 280 nm in a continuous scan mode with scanning times of 30 s. Sedimentation coefficient distribution, c(s), and molecular mass distribution, c(M), were calculated from the data by using the program SEDFIT.

**CD**—The secondary structures of N36Fd, N28Fd, and their complexes with C34 peptide were determined by CD spectroscopy. All the N-peptides were diluted in ddH\(_2\)O (pH 7.0), and all the C-peptides or the mixture of the N- and C-peptides were diluted in 50 mM sodium phosphate and 150 mM NaCl (PBS, pH 7.2) to a final concentration of 10 μM. The individual peptides and their mixtures were incubated in a 37 °C water bath for 0.5 h before testing. The spectra of each sample were acquired on a 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, and were corrected by a subtraction of the background corresponding to the solvent. The spectrum of the N-peptide portion in the individual N36Fd or N28Fd, or in the N36Fd/C34 or N28Fd/C34 mixture, was calculated by subtracting the spectrum of free Fd peptide from that of N36Fd, N28Fd, N36Fd+C34, or N28Fd+C34. The α-helicity was calculated from the CD signal by dividing the mean residue ellipticity at 222 nm by the value expected for 100% helix formation (i.e. 33,000° cm² dmol⁻¹) according to previous studies (4, 20). Thermal denaturation of the samples was monitored at 222 nm by applying a temperature gradient from 20 °C to 98 °C with a 2-degree interval, an equilibration time of 1.5 min, and an aging time of 60 s. The midpoint of the thermal unfolding transition (Tm) values was calculated using Jasco software utilities as described previously (21).

Inhibition of HIV-1-mediated Cell-Cell Fusion—HIV-1-mediated cell-cell fusion was measured by utilizing a dye transfer assay as previously described (22). Briefly, the chronically HIV-1-infected H9 (H9/HIV-1) cells were labeled with Calcein-Am (Molecular Probes, Inc., Eugene, OR). After washes, the fluorescence-labeled H9/HIV-1 cells were incubated with MT-2 cells at 37 °C for 2 h in the absence or presence of N36Fd or N28Fd at a graded concentration. The percentage of fused cells was counted under a fluorescence microscope (Zeiss, Germany), and the 50% inhibitory concentration of each drug was calculated with the CalcuSyn software program (22).

Inhibition of HIV-1 Infection—Inhibitory activities of N36Fd and N28Fd trimers on HIV-1 infection were determined as previously described (22, 23). For inhibition of infection by T-tropic HIV-1 strain IIIB (subtype B, X4) or the T20-resistant variants (HIV-1NL4-3(36G) bearing the N42S, N42T/N43K, V38E/N42S, or V38A/N42D mutations that were obtained from NIH through the AIDS Reagent Program) (24), 1 × 10⁵/ml MT-2 cells in RPMI medium 1640 containing 10% FBS were infected with HIV-1 isolates at 100 TCID₅₀ (50% tissue culture infective dose) in 200 μl of culture medium in the presence or absence of the test peptide overnight. The culture supernatants were removed, and fresh media were added on the next day. 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. For inhibition of infection by the M-tropic HIV-1 strain Bal (subtype B, R5), 100 μl of TZM-bl cells (1 × 10⁵/ml) was pre-cultured overnight and infected with Bal at 100 TCID₅₀ in the presence or absence of the test peptide overnight. The cells were harvested and lysed on the fourth day post-infection with 50 μl of lyzing reagent. The luciferase activity was analyzed using a luciferase kit (Promega, Madison, WI) and a luminometer (Ultra 386, TECAN, Durham, NC) according to the manufacturer’s instruction. The percent inhibition of luciferase activity was calculated. For inhibition of infection by the primary HIV-1 isolate 93IN101 (subtype C, R5), the peripheral blood mononuclear cells were isolated from the blood of healthy donors using a standard density gradient (Histopaque-1077, Sigma) centrifugation. After incubation at 37 °C for 2 h, the non-adherent cells were collected and resuspended at 5 × 10⁵/ml in RPMI medium 1640 containing 10% fetal bovine serum, 5 μg of phytohemagglutinin (PHA)/ml, and 100 units of interleukin-2/ml, followed by incubation at 37 °C for 3 days. The PHA-stimulated cells were infected with the primary HIV-1 isolate at a multiplicity of infection of 0.01 in the absence or presence of N-Fd peptides at graded concentrations. The supernatants were collected on the 7th day post-infection and tested for p24 antigen by ELISA as previously described (23).

Assay for Stability against Proteinase K Digestion—The stability of N28Fd or T20 against digestion by proteinase K was tested as previously described (25). Peptide (40 μg/ml) was incubated at 37 °C in PBS containing 1 microunit/ml proteinase K-Acrylic Beads (Sigma). Samples were collected at different times and centrifuged immediately. The supernatants were collected and stored at −20 °C before testing. The residual peptide concentration in each sample was then detected by ELISA as previously described (25). In brief, 50 μl of sample was coated onto wells of a 96-well polystyrene plate, followed by addition of rabbit antibodies directed against the HIV-1 gp41 N- and C-peptide mixture (22) for binding N28Fd (at 1:400 dilution) and T20 (at 1:2000 dilution), respectively. Then, biotin-labeled goat anti-rabbit IgG (Sigma), streptavidin-labeled horseradish peroxidase (SA-HRP, Zymed Laboratories Inc., South San Francisco, CA), and the substrate 3,3',5,5'-tetramethylbenzidine (Sigma) were added sequentially. The absorbance at 450 nm (A₄₅₀) was measured with an automatic ELISA reader (Ultra 384, TECAN). The remaining antiviral activity in the supernatants against HIV-1 infection was determined as described above. In both assays, the untreated N28Fd and T20 were used as controls.

RESULTS

Design of N36Fd and N28Fd Trimers—N-peptides derived from the HIV-1 gp41 NHR are commonly regarded as weak HIV-1 fusion inhibitors, because free N-peptides have a tendency to aggregate and thus cannot fold into a stable trimeric α-helical conformation in physiological solution (3, 26). In this study, we utilized the 27-mer Fd sequence to facilitate the trimerization of the N-peptides N36 and N28 by fusing the Fd sequence directly to the C terminus of N36 or N28 without any linking sequence (Fig. 1C). We first chose N36 for this study, because of its ability to form highly stable 6-HB with peptides derived from gp41 CHR (4). This peptide includes a 17-amino acid sequence that provides a critical hydrophobic pocket for the interaction between gp41 NHR and CHR (Fig. 1B, in red) (4, 27). Later, we selected N28 peptide to replace N36, because we found that the AVERY sequence (aa 582–586) sequence adjacent to the C terminus of pocket-formation sequence may also be important for gp41 6-HB formation. Specifically, the C-peptides CP-32 (aa 621–652) and CP-32M, which contain an AVERY-binding motif, QIWNNMT (aa 621–627) (Fig. 1B), are more potent than T20 in blocking 6-HB formation and inhibiting infection by HIV-1 strains, including those resistant to T20 and C34 (28, 29). N28 contains the 17-mer pocket-forming sequence (N17) plus the hexamer IEAQQH (aa 559–564) sequence and the 5-mer AVERY motif at the N and C termini of N17 sequence, respectively (Fig. 1B). Addition of the short flanking sequences at both sides of the pocket-forming sequence may improve refolding and solubility of the highly hydrophobic pocket sequence in N28. The full-length of the designed N36Fd and
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N28Fd peptides consists of 63 and 55 aa residues (Fig. 1C), respectively. Based on our experience, peptides longer than 50 aa are technically difficult to synthesize, but can be well expressed in E. coli.

N36Fd and N28Fd Trimmers Had Improved \( \alpha \)-Helical Conformation over the Corresponding N-peptides—The N36Fd and N28Fd peptides expressed in E. coli were purified and analyzed with SDS-PAGE. After boiling for 5 min in the presence of 2% SDS, N36Fd was denatured and exhibited a band in the gel corresponding to the monomer form. N36Fd treated with SDS buffer under room temperature exhibited one major band corresponding to the trimeric form and a number of minor bands with lower molecular size (Fig. 2A). This result suggests that a major portion of the N36Fd maintains trimeric conformation in SDS buffer under room temperature. A major monomer band and a minor trimer band were revealed when N28Fd was treated by boiling for 5 min, whereas only one band corresponding to the trimeric form was shown when N28Fd was treated in SDS buffer at room temperature (Fig. 2B). These results suggest that N28Fd, as a trimer, is more stable than N36Fd under SDS condition.

Sedimentation velocity analysis (SVA) provides hydrodynamic information about the size and shape of macromolecules (30). It is particularly useful for quantitatively characterizing the solution behavior such as self- or hetero-association of biomolecules (31–34). SVA was used to prove the trimer formation of N36Fd and N28Fd peptides. As shown in Fig. 3A, in 100 mM CH$_3$COONa/CH$_3$COOH buffer (pH 6.0), almost all the N28Fd appears in a sharp peak with a sedimentation coefficient of 1.55 s that corresponds to 21,811 Da, which agrees with the theoretical molecular mass for an N28Fd trimer (19,149 Da). N36Fd was also shown to mostly form trimers, as a single peak appeared at 1.60 s with an obtained molecular mass of 22,272 Da (Fig. 3B), which is in agreement with the calculated molecular mass for an N36Fd trimer (21,561 Da). Meanwhile, the populations of higher molecular weight increased in this sample, which indicates that, in comparison with N28Fd, N36Fd has a stronger tendency to aggregate in solution.

CD spectroscopy was used to study the secondary conformation of N36Fd and N28Fd trimers. Free Fd peptide and N-peptides presented little \( \alpha \)-helicity. Unexpectedly, the chimeric N36Fd and N28Fd did not exhibit typical \( \alpha \)-helical spectra either, but rather a mixture of \( \alpha \)-helix and \( \beta \)-sheet conformation (Fig. 4, A and B). Indeed, after subtraction of the spectra of free Fd peptide, which consists of \( \beta \)-sheet structure (18), from those of N36Fd or N28Fd, a typical \( \alpha \)-helical spectrum was revealed (Fig. 4, A and B). These results indicate that the Fd domain in the N36Fd and N28Fd greatly facilitated N-peptide folding into trimeric \( \alpha \)-helical conformation, mimicking the conformation of NHR trimer in the fusion intermediate state of the HIV-1 gp41. The \( \alpha \)-helicity of the N36 or N28 portion in N36Fd or N28Fd was 42 and 31%, respectively.

The frictional coefficient \((f/f_0)\) measures the diffusional boundary spread during the sedimentation analysis. This value relates to the s value of species in solution and can be extracted from the experimental data of SVA using SEDFIT program. It indicates the molecular shape asymmetry and the degree of salvation of the protein (35). As shown in Table 1, the \((f/f_0)\) values...
of N28Fd trimer and N36Fd trimer are both in the interval between 1.6 and 1.9, which indicates the moderately elongated molecular shape of these oligomers (32). The results matched the expectation that these oligomers consisting of multiple coiled-coil structures.

Interaction of N36Fd and N28Fd Trimers with C34 Peptide

Resulted in Formation of Stable 6-HB

—CD spectroscopy was used to study the interaction of N36Fd or N28Fd trimers with C-peptides. When the molar ratio of N36Fd and C34 was 1:1, N36Fd interacted with C34 to form a complex that had significantly increased $\alpha$-helicity (93% after subtracting the spectra of Fd) in comparison with N36 and C34 (72% $\alpha$-helicity) (Fig. 5A). The $T_m$ value of N36Fd trimer + C34 (80.9 °C) is 15° higher than that of N36 + C34 (65.5 °C). Notably, free N28 peptide could not form a complex with C34, but fusion with Fd promoted N28 to bind to C34 and fold into a conformation with 46% $\alpha$-helicity (Fig. 5B) and 62.2 °C of $T_m$ value. This suggests that the Fd-based NHR trimer is more effective than free N-peptide to interact with C-peptides to form stable complex.

In SVA, when N28Fd was mixed with C34 in 1:1 molar ratio in PBS (pH 7.4), the major species was shown to have a sedimentation coefficient of 2.03 s with a molecular mass of 30,706 Da (Fig. 3A). This measured molecular mass corresponds to the calculated value for a hexamer formed by three single N28Fd and three C34 peptides (molecular mass = 31,893 Da). Given that CD analysis has proved the $\alpha$-helical conformation of the complex of N28Fd + C34, this observation verifies that the N28Fd trimer associates with C34 to form a typical 6-HB. Unexpectedly, the size of species of N36Fd + C34 distributed

![FIGURE 3. Sedimentation velocity analysis of N28Fd and the N28Fd/C34 mixture (A), and N36Fd and the N36Fd/C34 mixture (B). The buffer was 100 mcm CH₃COONa/CH₃COOH buffer (pH 6.0) for N28Fd and N36Fd alone, and PBS (pH 7.4) for the mixtures. The sedimentation coefficient (s) and molecular mass (kilodaltons) of each peak are indicated.](image)

![FIGURE 4. Secondary structure of the peptides N36 and N36Fd (A), and N28 and N28Fd (B), as determined by CD spectroscopy. The spectra of (N36Fd)-Fd (A) or (N28Fd)-Fd (B) were calculated by subtracting the spectra of Fd peptide (A and B) from those of N36Fd (A) or N28Fd (B), respectively. The final concentration of each peptide in water was 10 μM.](image)

### TABLE 1

Summary of the SVA results of N28Fd and N36Fd and their complexes with C34

<table>
<thead>
<tr>
<th>Complex</th>
<th>Sedimentation coefficient (s)</th>
<th>Observed molecular mass (Da)</th>
<th>Calculated molecular mass (Da)</th>
<th>$f/f_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N28Fd trimer</td>
<td>1.55</td>
<td>21,811</td>
<td>19,149</td>
<td>1.85</td>
</tr>
<tr>
<td>N36Fd trimer</td>
<td>1.60</td>
<td>22,272</td>
<td>21,561</td>
<td>1.68</td>
</tr>
<tr>
<td>N28Fd + C34 6-HB</td>
<td>2.03</td>
<td>30,706</td>
<td>31,893</td>
<td>1.80</td>
</tr>
<tr>
<td>N36Fd + C34 6-HB</td>
<td>2.46</td>
<td>46,965</td>
<td>34,305</td>
<td>2.11</td>
</tr>
<tr>
<td>N28Fd + C34 6-HB</td>
<td>3.73</td>
<td>87,827</td>
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</tbody>
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much broader than that of N28Fd/H11001 C34, with two major peaks occurring at 2.46 and 3.73 s, which correspond to the molecular masses of 46,965 and 87,827 Da, respectively (Fig. 3B). Given that the calculated molecular mass of a hexamer consisting of three N36Fd monomers and three C34 peptides is 34,305 Da, the two species may be monomeric and dimeric 6-HB, respectively, formed by N36Fd and C34.

Similar to the values of N28Fd trimer and N36Fd trimer, the $f/f_0$ of N28Fd + C34 is also between 1.6 and 1.9 (Table 1), which is in agreement with the moderately elongated shape of a 6-HB. In contrast, the $f/f_0$ value of the complex of N36Fd + C34 was shown in the range of 2–3. If there is only one species of molecules in solution, the value of 2.11 accounted for a very elongated shape (32). However, multiple peaks appeared in the result of SVA of N36Fd + C34, suggesting that several species with different molecular shapes may exist in the mixture. In such cases, fitting data using the measured $f/f_0$, the weight average of the frictional ratio of molecular species present in solution, may lead to considerable errors (34). A possible explanation for the deviation of the obtained molecular weights of the two major peaks of N36Fd + C34 from their calculated weights is the presence of multiple species, such as monomeric and dimeric 6-HB, in the solution.

N36Fd and N28Fd Trimers Exhibited Highly Potent Anti-HIV-1 Activities—Subsequently, we compared the inhibitory activity of the N36Fd and N28Fd trimers with that of N36 and N28 on HIV-1-mediated cell-cell fusion. As shown in Fig. 6, N36Fd trimer was $\sim$15-fold more potent than N36, whereas N28Fd trimer was $>138$-fold more effective than N28 in inhibiting HIV-1-induced cell-cell fusion. The N28Fd trimer displayed an IC$_{50}$ value of 21 nM, which is as potent as the clinically used peptide anti-HIV-1 drug T20 (enfuvirtide, IC$_{50}$ = 24 nM), but it is less effective than T1249 and T1144, the second and third generations of the peptidic HIV fusion inhibitors (36, 37). As a control, Fd peptide alone showed no inhibitory activity at the concentration up to 5000 nM (Fig. 6). This result suggests that the Fd-fused N-peptides function as potent HIV-1 fusion inhibitors. Similarly, both N36Fd and N28Fd trimers could also effectively inhibit HIV-1$_{HIV}$ replications with IC$_{50}$ values of 99 nM and 39 nM, respectively. N36 peptide exhibited moderate inhibitory activity (IC$_{50}$ = 1033 nM), whereas Fd and N28 peptides showed no inhibitory activity at a concentration as high as 3000 nM (Fig. 7A). Strikingly, N28Fd displayed higher antiviral activity than N36Fd in both assays.

Besides the laboratory-adapted subtype B strain HIV-1$_{HIV}$ that uses the coreceptor CXCR4 (X4), we also tested N36Fd and N28Fd trimers against primary HIV-1 isolates using the coreceptor CCR5 (R5) and T20. N28Fd trimer blocked the infection by HIV-1 Bal (subtype B, R5) with an IC$_{50}$ of 9.8 nM (Fig. 7B). N36Fd trimer had lower antiviral activity against Bal, with an IC$_{50}$ of 182.9 nM. Both N36Fd and N28Fd trimers displayed low nanomolar inhibitory activity against the infection by HIV-1 93IN101 (subtype C, R5). The IC$_{50}$ values were 1.36 nM for the N36Fd trimer and 1.27 nM for the N28Fd trimer (Fig. 7C). For comparison, T20 showed IC$_{50}$ values of 3.4 nM against Bal and 1.75 nM against 93IN101 in these two assays. These results suggest that N36Fd and N28Fd trimers, like T20, have a broad spectrum antiviral activity against HIV-1, irrespective of coreceptor usage.
Mutations in the NHR region of HIV-1 virus NL4–3 cause genetic resistance to T20 (24). The inhibitory activity of N36Fd and N28Fd trimers on the replication of T20-resistant strains was determined. As shown in Table 2, T20 could potently inhibit the T20-sensitive strain HIV-1NL4–3(36G) N42S (IC50 = 30 nM), but it was much less effective against the T20-resistant strains HIV-1NL4–3(36G) N42T/N43K (IC50 = 374 nM), HIV-1NL4–3(36G) V38A/N42D (IC50 = 2297 nM). However, N36Fd and N28Fd trimers had similar high potency against both T20-resistant and -sensitive strains (IC50 = 27–59 nM).

N28Fd Was Much More Resistant Than T20 to Proteinase K Digestion—One of the principal disadvantages of the current gp41 CHR-based peptide drug T20 is its short half-life in vivo and high sensitivity to the proteolytic enzymes in blood (38). We speculated that the well folded secondary conformation of N28Fd trimer might gain resistance against proteolytic enzymes. Therefore, the stability of N28Fd trimer under the digestion of the broad-spectrum serine proteinase, proteinase K, was determined. After treatment with 1 microunit/ml proteinase K in PBS for 3 h, the N28Fd trimer maintained 84% of the original amount detected by ELISA and 97% of the original antiviral activity against HIV-1IIIB infection (Fig. 8, A and B). Under the same condition, T20 retained only 17% of the original amount (Fig. 8A) and completely lost its anti-HIV-1 activity after 3-h treatment with proteinase K (Fig. 8B). These results suggest that the N28Fd trimer is considerably more resistant to proteinase K than T20.

DISCUSSION

When stabilized into a trimeric coiled-coil, it was previously proved that N-peptides become efficient HIV-1 fusion inhibitors (13, 14, 16). In this study, we took advantage of the self-trimerization domain, Fd of T4 fibrin, to construct a series of novel gp41 NHR-based trimers. Fd is more advantageous over other trimerization motifs, such as IQ or IZ, because Fd-based peptide or protein trimer is more stable than IZ-based trimeric molecules, especially under reducing conditions (17). We found, for instance, that the peptide consisting of IZ and N28 (IZN28) had less anti-HIV-1 potency than N28Fd.4 The two recombinant engineered polypeptides N36Fd and N28Fd were expressed in prokaryotic E. coli cells and purified from the supernatants of the cell lysates. Both polypeptides emerged as SDS-resistant -helical trimers. Compared with the synthetic monomeric N36 and N28 peptides, N36Fd and N28Fd trimers exhibited significantly enhanced antiviral activity against broad HIV-1 strains, especially the primary isolates Bal (subtype B, R5) and 93IN101 (subtype C, R5). CD spectroscopy demonstrated that the helicity of the complex formed by the N-peptide portion of N-Fd trimer with C34 was greatly enhanced compared with the complex formed by the corresponding free N-peptide with C34, indicating a trimerization-promoted binding of the gp41 portion of N-Fd trimer with C34 was greatly enhanced compared with the complex formed by the corresponding free N-peptide with C34, indicating a trimerization-promoted binding of the gp41 portion of N-Fd trimer with C34 was greatly enhanced compared with the complex formed by the corresponding free N-peptide with C34, indicating a trimerization-promoted binding of the gp41 portion of N-Fd trimer with C34 was greatly enhanced compared with the complex formed by the corresponding free N-peptide with C34, indicating a trimerization-promoted binding of the gp41 portion of N-Fd trimer with C34 was greatly enhanced compared with the complex formed by the corresponding free N-peptide with C34, indicating a trimerization-promoted binding of the gp41 portion of N-Fd trimer with C34 was greatly enhanced compared with the complex formed by the corresponding free N-peptide with C34, indicating a trimerization-promoted binding of the gp41 portion of N-Fd trimer with C34 was greatly enhanced compared with the complex formed by the corresponding free N-peptide with C34, indicating a trimerization-promoted binding of the gp41 portion of N-Fd trimer with C34 was greatly enhanced compared with the complex formed by the corresponding free N-peptide with C34, indicating a trimerization-promoted binding of the gp41 portion of N-Fd trimer with C34 was greatly enhanced compared with the complex formed by the corresponding free N-peptide with C34, indicating a trimerization-promoted binding of the gp41 portion of N-Fd trimer with C34 was greatly enhanced compared with the complex formed by the corresponding free N-peptide with C34, indicating a trimerization-promoted binding of the gp41 portion of N-Fd trimer with C34.
at the transit fusion-intermediate state, thus having influences on its binding efficiency (39, 40). Based on this theory, downsizing the inhibitor should result in increased potency. However, Eckert et al. reported that the IC50 of IQN23 was lower than that of both IQN17 and IQN36 (14). In our attempt to express N17Fd, which was constructed by fusing the 17-aa pocket portion to Fd, inclusion bodies were formed because of its high hydrophobicity (data not shown). N36Fd also aggregated at the concentration of 40 μg/ml PBS in the test of resistance against protease K. At the same time, N28Fd dissolved well in PBS at the concentration as high as 200 μg/ml. SVA also showed that N36Fd has more tendency to aggregate in comparison with N28Fd; similarly, the complex formed by N36Fd and C34 is more prone to aggregation than the complex of N28Fd + C34. Given that the inhibition takes place in physiological buffer, the solubility is another important factor that affects the potency of the NHR trimers. Because of these factors, the production of N28Fd from the supernatant is higher, and the purification is easier when compared with that of N36Fd. Therefore, among all the N-Fd polypeptides we attempted to express, N28Fd is the most suitable for development of a new anti-HIV-1 agent.

Although T20, the first peptidic HIV-1 fusion inhibitor used in clinics, possesses doubtless potent antiviral activity, it has two critical drawbacks: high cost to produce and short half-life in vivo (38). A patient needs more than $20,000 per year for the drug and two injections per day (41). In contrast, more than 6 mg of N28Fd trimer could be obtained from 1 liter of bacteria culture using the laboratory scale protein expression and purification procedures in a few steps. Under these conditions, the cost of producing N28Fd on a large-scale platform would be extremely low. Moreover, results showed that N28Fd was much more resistant to proteinase digestion than T20, prolonging the existence of N28Fd in the human circulatory system. Finally, N36Fd and N28Fd potently inhibit a series of T20-resistant strains, although we cannot exclude the possibility that these NHR trimers may lose antiviral activity against the HIV-1 variants with compensatory mutations in the CHR region (42–44). Because N28Fd and T20 target different regions of gp41, combining them in clinical practice should help to avoid generating viruses otherwise resistant to either one alone. With all these features, N28Fd is demonstrated to be a promising basis for a novel NHR-based anti-HIV-1 agent.

Efforts could be taken to design Fd-fused NHR trimers with higher efficiency. Because hydrophobicity and molecular size influence inhibitory activity, site mutations to polar amino acids could be induced to the gp41 portion to help the folding and dissolving of the N-Fd trimer, combined with deletions to shorten the length of the polypeptide. It was reported that particular N-terminal substitutions promoted gp140 trimerization (45), and shifts of a few amino acids in the peptide sequence caused significant changes in potency (28). Thus, these strategies may also be used for optimization of the N-Fd trimers with increased anti-HIV-1 activity. It is possible that using different fragments of the NHR would lead to better N-Fd inhibitors.

One of the disadvantages of peptide and protein-based drugs for systemic application is the potential of the peptide or protein to induce antibody response against itself, which may suppress the efficacy of the peptide or protein drugs (46, 47). During the early stage of T20 development, there was a big concern
that the anti-T20 antibodies may inhibit T20-mediated anti-
HIV-1 activity. But later, this concern was resolved, because the
high levels of anti-T20 antibody in the patients treated with T20
did not impair the efficacy or safety of T20 (48). The monono-
clonal antibody D50 can strongly bind T20 (49), but it shows no
effect on the antiviral activity of T20 (50). In the present study,
we investigated the potential effect of antibody response
directed against N28Fd on the N28Fd-mediated anti-HIV-1
activity. We found that a low titer (1:2500) of anti-N28Fd anti-
odies was detected in the sera of mice receiving three intrave-
nous injections of N28Fd (10 μg/mouse) at a 10-day interval,
but the antisera had no significant effect on the anti-HIV-1
activity mediated by N28Fd (data not shown), resolving the
concern regarding the potential negative effect of antibody
response induced by N28Fd when it is used for treatment.

Furthermore, N28Fd-based antiviral agent may also be used
for stem cell therapy of HIV infection/AIDS. Hutter et al. have
recently shown that transplantation of CD34+ stem cells from a
donor who was homozygous for CCR5 delta32, which can pro-
vide resistance against HIV-1 acquisition, to an HIV-1-infected
patient leads to reduction of viral load to an undetectable level in
the recipient for >20 months (51), suggesting the potential of
transplanting self-protected stem cells for treatment of HIV
infection. Hildinger M et al. demonstrated that T cells trans-
duced with a retrovirus vector expressing membrane-anchored
T20 on the cell surface became highly resistant to HIV-1 infec-
tion (52). Considering the advantages of N28Fd over T20 as an
HIV fusion inhibitor, we speculate that stem cells from umbil-
cial cord blood or bone marrow could be transduced with a vector
encoding such N-peptide trimers for treatment and pre-
vention of HIV infection.

Heterosexual transmission is the primary route for women to
acquire HIV/AIDS, and development of anti-HIV microbicides
for prevention of sexual transmission of HIV is urgently needed
(53). The ideal microbicide should be highly effective, safe, sta-
ble, and affordable (54, 55). T20 and T-1249 peptides have been
developed as candidate microbicides (56, 57). While safe and
effective as HIV fusion inhibitors, they are, however, not stable
in the vaginal environment where a variety of proteolytic
enzymes reside. In addition, because of the high cost of peptide
synthesis, neither T20 nor T-1249 is affordable to high risk
populations living in developing countries. Given that N28Fd is
more resistant to proteolytic enzyme and less expensive than
T20 peptide, N28Fd has a good potential to be developed as an
anti-HIV microbicide for preventing HIV sexual transmission.

Fusing Fd domain to the C-terminal of an N-peptide offers a
new way to construct stable NHR trimer exhibiting a structure
that mimics the native conformation of NHR at the gp41
fusion-active intermediate state. Therefore, this kind of chi-
meric NHR trimers can be utilized as a stable recombinant mol-
ecule target for screening of peptidic or non-peptidic HIV-1
fusion/entry inhibitors targeting the hydrophobic pocket in the
gp41 NHR trimer using ELISA, fluorescence-linked immuno-
absorbance assay, and fluorescence resonance energy transfer
techniques (15, 58–63). This construct may also be used for
screening of gp41 pocket-binding antibodies with HIV-1-neu-
tralizing activity, like the monoclonal antibody 5D (64). Fur-
thermore, Louis et al. reported that the antibodies elicited by
the covalent N35-CCG-N13 trimer showed neutralizing activity
(13). This suggests that N36Fd and N28Fd trimers can also be
used for screening of HIV-1-neutralizing antibodies or as
immunogens to eliciting neutralizing antibodies targeting the
fusion intermediate of gp41.

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