HIV gp41 C-terminal Heptad Repeat Contains Multifunctional Domains

RELATION TO MECHANISMS OF ACTION OF ANTI-HIV PEPTIDES

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T20 (Fuzeon), a novel anti-human immunodeficiency virus (HIV) drug, is a peptide derived from HIV-1 gp41 C-terminal heptad repeat (CHR). Its mechanism of action has not yet been defined. We applied Pepscan strategy to determine the relationship between functional domains and mechanisms of action of five 36-mer overlapping peptides with a shift of five amino acids (aa): CHR-1 (aa 623–658), C36 (aa 628–663), CHR-3 (aa 633–668), T20 (aa 638–673), and CHR-5 (aa 643–678). C36 is a peptide with addition of two aa to the N terminus of C34. Peptides CHR-1 and C36 contain N-terminal heptad repeat (NHR)- and pocket-binding domains. They inhibited HIV-1 fusion by interacting with gp41 NHR, forming stable six-helix bundles and blocking gp41 core formation. Peptide T20 containing partial NHR- and lipid-binding domains, but lacking pocket-binding domain, blocked viral fusion by binding its N- and C-terminal sequences with gp41 NHR and cell membrane, respectively. Peptide CHR-3, which is located in the middle between C36 and T20, overlaps >86% of the sequences of these two peptides, and lacks pocket- and lipid-binding domains, exhibited marginal anti-HIV-1 activity. These results suggest that T20 and C36 contain different functional domains, through which they inhibit HIV-1 entry with distinct mechanisms of action. The multiple functional domains in gp41 CHR and their binding partners may serve as targets for rational design of new anti-HIV-1 drugs and vaccines.

HIV6 enters into a target cell in three steps: (i) HIV envelope glycoprotein (Env) surface subunit gp120 binds to CD4, the primary receptor on the target cell (1); (ii) gp120-CD4 complex interacts with a coreceptor in the target cell, CXCR4 or CCR5 (2); and (iii) the Env transmembrane subunit gp41 changes conformation and mediates membrane fusion (3). Each of these steps can serve as a target for the development of HIV entry or fusion inhibitors.

In the early 1990s, Jiang et al. discovered the first potent anti-HIV-1 peptide derived from the gp41 C-terminal heptad repeat (CHR) region, SJ-2176, which inhibited HIV-1-mediated membrane fusion at nanomolar level (4). Later, Wild et al. (5) identified another CHR-peptide, T20, which was subsequently developed into a novel anti-HIV drug, Fuzeon (or enfuvirtide) (6–8), the first member of a new class of anti-HIV drugs, namely HIV fusion inhibitors.

In 1997, Lu et al. (9) identified another CHR-peptide, C34, which covers the entire sequence of SJ-2176 and overlaps most of the T20 sequence and possesses more potent anti-HIV-1 activity than SJ-2176 and T20. They demonstrated that C34 could interact with N36, a peptide derived from the HIV gp41 N-terminal heptad repeat (NHR) region, to form a stable six-helix bundle (6-HB), representing the gp41 core structure (9). X-ray crystallographic studies have shown that the 6-HB core consists of three N36 molecules that form a trimeric core and three C34 peptides that bind to the grooves of N36 trimer in an anti-parallel manner (10). These findings suggest that the binding of gp120 to CD4 and a coreceptor, CXCR4 or CCR5, triggers the conformational changes of gp41, which include the interaction between the gp41 CHR and NHR to form a stable 6-HB core, bringing the viral and target cell membranes into close proximity. This viral fusion model has been employed for explaining the mechanism by which the CHR-peptides C34 and T20 inhibit HIV fusion, i.e. they interact with the viral NHR to form heterologous 6-HB and block the formation of the homologous 6-HB core between the viral NHR and CHR, thereby inhibiting HIV fusion. Indeed, C34 is very effective in inhibiting the gp41 6-HB formation (11, 12). However, T20 is a weak inhibitor of the gp41 core formation, suggesting that T20 and C34, although they are derived from the same region, may have different mechanisms of action (13). Based on these findings, we hypothesize that the anti-HIV-1 peptides derived from the gp41 CHR region may contain different repeat; Pepscan, peptide-scanning; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; 6-HB, six-helix bundle; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; aa, amino acid(s).
ent functional domains, through which they inhibit HIV-1 fusion with distinct mechanisms of action. Here we applied a peptide-scanning (Pepscan) strategy to define the relationship between the functional domains in the gp41 CHR region and the mechanisms of action of the CHR-peptides. We designed and synthesized five overlapping peptides that cover the entire CHR region and compared their biological activities and physical properties. We found that the CHR-peptides that contain both NHR- and pocket-binding domains (e.g. CHR-1, C36, and C34) could interact with both the viral gp41 NHR region and the hydrophobic pocket on the NHR-trimer to form stable 6-HBs and block the gp41 core formation. The CHR-peptides that contain a major part of the NHR-binding domain and a lipid-binding domain (e.g. T20) may interact with both the gp41 NHR region and lipid bilayers in the target cell membrane to mediate anti-HIV-1 activity. These functional domains in the gp41 CHR region and their binding partners may be used as targets for designing novel HIV fusion inhibitors.

**EXPERIMENTAL PROCEDURES**

Reagents—MT-2, TZM-bl, and HIV-1IIIb-infected H9 (H9/ HIV-1IIIb) cells as well as T20-resistant HIV-1 strains were obtained from the NIH AIDS Research and Reference Reagent Program. Peptides N46, CHR-1, C36, CHR-3, T20, CHR-5, C34, T20-scramble, and T20-ANAA (see the sequences in Fig. 1) as well as C34-biotin (biotin was added to the N termini of C34) were synthesized by a standard solid-phase Fmoc (N-(9-fluorenylmethoxycarbonyl) method as previously described (13). These peptides were purified to homogeneity by high performance liquid chromatography. The identity of the purified peptides was confirmed by laser desorption mass spectrometry (PerSeptive Biosystems).

**HIV-1-mediated Cell-Cell Fusion**—The inhibitory activities of the CHR-peptides against HIV-1-mediated cell-cell fusion were determined using a dye transfer assay as previously described (14, 15). Briefly, 50 μl of H9/HIV-1IIIb cells (2 × 10⁶/ml), which were pre-labeled with Calcein AM, a fluorescent dye (Molecular Probes, Eugene, Oregon), were incubated with a 50-μl peptide at a graded concentration at 37 °C for 30 min, followed by the addition of 100 μl of MT-2 cells (1 × 10⁶/ml) in a 96-well plate. After coculture 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 disc (10 × 10 mm²) and a 10× objective. Four fields per well were examined, and the average percentage of cell fusion was calculated by the following formula: fused cells/(fused + unfused cells) × 100%. The wells for positive control were added with 50 μl of culture medium, instead of peptides, and 50 μl of Calcein-labeled, HIV-infected cells. The wells for negative controls were added with culture medium and Calcein-labeled, unfixed H9 cells. The percentage of inhibition of cell fusion by a peptide was calculated as previously described (14, 15), and IC₅₀ (the concentration for 50% inhibition) values were calculated using the computer program CalcuSyn (16) kindly provided by Dr. T. C. Chou (Sloan-Kettering Cancer Center, New York, NY).

**Native-PAGE**—Native-PAGE as described previously (11) was used to detect the 6-HB formed by NHR- and CHR-peptides. In this study, NHR-peptide N46, instead of N36, was used as the CHR-peptide-binding partner, because N46 is longer than N36 by 10 amino acid residues and almost covers the entire NHR region. N46 (100 μM) was mixed with each of the CHR-peptides at a 1:1 ratio. The mixtures were incubated at 37 °C for 30 min, followed by 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).

**ELISA for Measuring Inhibitory Activity of CHR-peptides on the gp41 6-HB Formation between N46 and Biotinylated C34**—A conformation-specific monoclonal antibody NC-1 (17) was used in a modified ELISA as previously described (18) to determine the inhibitory activity of CHR-peptides on the gp41 6-HB formation between N46 and biotinylated C34 (C34-biotin). Briefly, a 96-well polystyrene plate (Costar, Corning Inc., Corning, NY) was coated with the monoclonal antibody NC-1 IgG (4 μg/ml in 0.1 M Tris, pH 8.8). A CHR-peptide at graded concentrations was mixed with C34-biotin (0.25 μM) and incubated with N46 (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 washing buffer (PBS containing 0.1% Tween 20) 6 times. Then streptavidin-labeled horseradish peroxidase (SA-HRP, Zymed Laboratories Inc., Invitrogen Immunodetection, S. San Francisco, CA) and the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma) were added sequentially. Absorbance at 450 nm (A₄₅₀) was measured using an ELISA reader (Ultra 384, Tecan, Research Triangle Park, NC). The percent inhibition by the peptides was calculated as previously described (19), and the IC₅₀ values were calculated using CalcuSyn software (16).

**CD and Thermal Midpoint (Tₘ) Analysis**—CD spectroscopy was performed to determine the conformation of NHR- and CHR-peptide complexes as previously described (11). Briefly, each of the CHR-peptides dissolved in PBS (50 mM sodium phosphate and 150 mM NaCl, pH 7.2) at 10 μM was mixed with N46 in PBS at a 1:1 ratio. After incubation at 37 °C for 30 min, the samples were then cooled down to 4 °C. The CD spectra of each sample were acquired on a Jasco spectropolarimeter (Model J-715, Jasco Inc., Japan) at 4 °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 was performed at 222 nm 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 previously described (11).

**Preparation of Large Unilamellar Vesicles of POPC Liposome**—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) dissolved in chloroform was purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. 20 mg of POPC stock solution was dried under a stream of nitrogen and stored under vacuum overnight to completely remove trace amounts of organic solvent. The dried lipid film was suspended...
**gp41 Functional Domains and Anti-HIV Peptide Mechanisms**

**TABLE 1**

| CHR-peptides | IC_{50} | IC_{90} |
|--------------|--------|--------|
| CHR-1        | 17.27 ± 0.31 | 26.41 ± 0.55 |
| C36          | 4.67 ± 0.07  | 7.04 ± 0.12  |
| CHR-3        | 1.4700 ± 20.0  | 2.3800 ± 35.00 |
| T20          | 19.29 ± 0.22  | 29.23 ± 0.45  |
| CHR-5        | 2.185.0 ± 15.00  | 2.858.0 ± 6.00 |
| T20-Scramble | >20,000       | >20,000       |
| C34          | 8.20 ± 0.13  | 12.37 ± 0.26  |

### RESULTS

**Peptides CHR-1, C36, and T20 Exhibited Highly Potent Inhibitory Activities on HIV-1-mediated Cell-Cell Fusion**—To determine the functional domains in the HIV-1 gp41 CHR region involved in the process of fusion between the virus and target cell membranes, five 36-mer CHR-peptides covering the CHR region (aa 623–678), namely CHR-1, C36, CHR-3, T20, and CHR-5 (Fig. 1) were synthesized for Pepscan study. The consecutive peptides each had sequence shifts of five residues. Peptide C36 covers the entire sequence of C34. The inhibitory activities of these CHR-peptides on HIV-1-induced cell-cell fusion were compared. As shown in Table 1, the peptide C36 exhibited the most potent inhibitory activity with IC_{50} < 5 nm. The peptides CHR-1 and T20 had similar potency in inhibiting HIV-1-mediated cell-cell fusion with IC_{50} in the range of 15–20 nm, whereas the peptides CHR-3 and CHR-5 showed marginal inhibitory activity.

**Unlike T20 and CHR-5, Peptides CHR-1, C36, and CHR-3 Could Form 6-HB with N46**—CD spectroscopy was used to differentiate the secondary structures of the complexes formed between the NHR-peptide N46 (sequence shown in Fig. 1) and the CHR-peptides. N46 peptide alone in PBS formed typical α-helical structure, although its α-helicity was moderate (Fig. 2A and Table 2). The peptides CHR-1, C36, and CHR-3 interacted with N46 to form complexes with typical α-helical conformation (Fig. 2A). The α-helicity of these complexes are in the following order: C36 plus N46 > CHR-1 plus N46 > CHR-3 plus N46, in agreement with their T_{m} values (Table 2), suggesting that the α-helical complex formed by C36 and N46 is the most stable, whereas the one formed between CHR-3 and N46 is the least stable. These data are also consistent with the inhibitory activities of the respective peptides against HIV-1-induced cell-cell fusion, indicating that peptides CHR-1 and C36, like the peptide C34, inhibit HIV-1 fusion by interacting with the gp41 NHR region. Addition of the peptides T20 and CHR-5 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 (Fig. 2A and Table 2). This is in agreement with the reports by Wild et al. (21) and Lawless et al. (22), who demonstrated that T20 could severely distort the α-helical conformation and reduce α-helicity of T21, a 37-mer peptide derived from the HIV-1 gp41 NHR region. These results suggest that the peptides T20 and CHR-5, although they may interact with the gp41 NHR region to form non-helical complexes, cannot associate with the viral N-helices to form a stable heterologous gp41 core.

Subsequently, we investigated whether these CHR-peptides can form 6-HBs with NHR-peptide N46 using the native-PAGE method (11). Each of the CHR-peptides was incubated with N46, respectively, at 37 °C for 30 min before analysis of the mixtures in the gel electrophoresis under native conditions. As shown in Fig. 2B, the peptide N46 (lane 1) showed no band as N46 carries net positive charges, thus it may migrate up and off the gel. Peptide CHR-1 (lane 2) exhibited a band located in the lower part of the gel. The mixture of N46 and CHR-1 (lane 3) had two bands: the lower band with less intensity located at the same position as that of CHR-1, and the upper one corresponding to the position of 6-HB formed by N36 and C34 (11). This suggests that the CHR-1 peptide can interact with N46 to form 6-HB. A similar 6-HB band was revealed in the lane where the mixture of C36 plus N46 (lane 5) was loaded. The mixture of CHR-3 plus N46 (lane 7) also showed a band of 6-HB, but having a relatively lower intensity than those formed by CHR-1 plus N46 and C36 plus N46. No 6-HB bands were revealed in the lanes where the mixtures of T20 plus N46 (lane 9) and CHR-5 plus N46 (lane 11) were loaded. But notably, the lower bands in these lanes were much less intensive than the corresponding bands in lane 8 (T20 only) and 10 (CHR-5 only). These results suggest that the peptides T20 and CHR-5 cannot form stable 6-HBs with N46, but may interact with N46 to form insoluble complexes or aggregates that cannot migrate into the gel. These data are consistent with the results obtained from CD analyses, which showed that mixing of T20 with N46 resulted in formation of non-helical complexes (Fig. 2A).

Peptides CHR-1 and C36 Had Potent Inhibitory Activities on 6-HB Formation, whereas the Peptide T20 Exhibited Only Weak Inhibition—We used a modified ELISA (18) to compare the inhibitory activities of the CHR-peptides on the gp41 6-HB formation between N46 and biotinylated C34 analyzed by ELISA. Shown here are average values from triplicate tests. The results were confirmed in two independent experiments.

### Table 2

| N46 plus CHR-peptide complex | \(\theta_{222}\) \(10^3\) deg cm\(^2\) dmol\(^{-1}\) | \(T_m\) value \(°C\) |
|-----------------------------|---------------------------------|-----------------|
| N46 plus CHR-1              | 25.89                           | 76.0            |
| N46 plus C36                | 30.93                           | 93.8            |
| N46 plus CHR-3              | 16.82                           | 68.0            |
| N46 plus T20                | -9.05                           | Undetectable    |
| N46 plus CHR-5              | -12.65                          | Undetectable    |
| N46                          | -13.81                          | 70.5            |

**FIGURE 2.** A, analysis of the secondary structures of the mixtures of N46 and the CHR-peptides by CD spectroscopy. B, determination of the 6-HB formation between the NHR-peptide N46 and a CHR-peptide. The mixtures of N46 and CHR-peptides were analyzed with native-PAGE. The ratio of N46 and a CHR-peptide in a sample is 1:1.

**FIGURE 3.** Assessment of the inhibitory activities of the CHR-peptides on the 6-HB formation between N46 and biotinylated C34 analyzed by ELISA. Shown here are average values from triplicate tests. The results were confirmed in two independent experiments.
6-HB with an NHR-peptide but can partially inhibit 6-HB formation between the peptides N46 and C34-biotin.

**Peptides T20 and CHR-5 Interact with Lipid Bilayers—**
Previous studies have shown that the HIV-1 gp41 6-HB interacts with lipid bilayers with substantial binding affinity (23), perhaps participating in the formation of a fusion pore. Because the CHR helices are located in the outer layer of the 6-HB, the CHR-peptides derived from the CHR region may bind to the lipid bilayers. We thus used the POPC LUV liposome system to detect the potential interaction of the CHR-peptides with lipid bilayers. Because all five CHR-peptides contain tryptophan residues, the tryptophan fluorescence emission spectra of the CHR-peptides may change if they interact with the lipid bilayers. Significant blue shift of the fluorescence spectra was observed when the peptides T20 and CHR-5 were presented in PBS (Fig. 4a), compared with their presence in PBS (Fig. 4a), whereas other CHR-peptides did not show remarkable fluorescence spectra shift. These data suggest that both T20 and CHR-5 peptides may interact with the host cell membranes.

We also used an ITC assay to determine the interaction of CHR-peptides with lipid bilayers. As shown in Fig. 4c, the highest heats released from binding were seen in the titration of POPC LUVs into a solution containing CHR-5. The binding constant of CHR-5 to POPC LUVs was 1.66 \times 10^5 \text{ M}^{-1}. T20 ranked second with a binding constant of 5.41 \times 10^4 \text{ M}^{-1}. The bindings of CHR-1, C36, and CHR-3 to POPC LUVs were very weak, and the binding constant could not be calculated by the Origin software (Fig. 4d). These results confirm that, unlike the other three CHR-peptides, the peptides T20 and CHR-5 can interact with lipid bilayers.

Then, we determined which part of the T20 is critical for its interaction with lipid bilayers. It has been reported that a WNWF motif at the C terminus of T20 is critical to its inhibitory activity. Replacement of WNWF with ANAA results in the significant loss of T20-mediated anti-HIV activity (22, 24). In the present study, we compared the lipid-binding activity of T20 with its mutant, T20-ANAA. As shown in Fig. 5, unlike T20, T20-ANAA could not bind to POPC LUVs as determined by both fluorescence spectroscopy and ITC assays. These results suggest that the WNWF motif at the C terminus of T20 is important for both its anti-HIV and lipid-binding activities.

**DISCUSSION**

After the determination of the x-ray crystal structure of the HIV-1 gp41 6-HB core consisting of the NHR- and CHR-peptides in 1997 (10, 27), a model was proposed to elucidate the mechanism of the gp41-mediated membrane fusion and to explain the mechanism of action of the anti-HIV peptides derived from the gp41 CHR region (3). It has been widely believed that T20 and C34, which share a 26-amino acid sequence (>72% of each peptide sequence), may have the same mechanism of action, i.e. interacting with the viral gp41 NHR region to form heterologous 6-HB and block viral fusion (6–8, 28). The major supporting evidence came from the studies showing that both T20 and C34 can bind to the fusion-intermediate conformation of gp41 induced by CD4 molecule, and the determinants responsible for resistance to T20 were localized in the gp41 NHR region (26, 29, 30). To challenge this assumption, we have applied a Pepscan strategy to define the potential relationship between the functional domains and the
anti-HIV-1 activity of the CHR-peptides. We reasoned that if T20 and C34 share a common functional domain and possess the same mechanism of action, a peptide like CHR-3, which is located in the middle of these two peptides and having their major sequences, should have similar anti-HIV-1 activity as C34 and T20. But to our surprise, CHR-3 had only marginal anti-HIV-1 activity, almost 100-fold less potent than that of C34 and T20, suggesting that C34 and T20 indeed have distinct mechanisms of action through different functional domains in these two peptides.

As shown in Fig. 6A, and Table 4 the gp41 CHR contains three functional domains: (i) a pocket-binding domain (aa 628–635), (ii) an NHR-binding domain (aa 628–666), and (iii) a lipid-binding or tryptophan-rich domain (aa 666–673). The peptides CHR-1 and C36 contain both the NHR-binding domain and the pocket-binding domain, which plays a critical role in stabilization of the gp41 core (31, 32). Therefore, these two peptides can interact with the viral NHR to form stable heterologous 6-HBs, resulting in inhibition of fusion between the viral and target cell membranes (Fig. 6B). The peptide T20 contains the entire sequence of lipid-binding domain and two-thirds of the NHR-binding domain but not the pocket-binding domain. Its N-terminal fragment may interact with the viral NHR region to form a non-helical complex, and its C-terminal lipid-binding domain may bind to lipid bilayers in the target cell membranes to maintain itself in a position to block the fusion-active gp41 core formation between the viral NHR and CHR regions and gp41-mediated membrane fusion (Fig. 6B). Therefore, both the NHR- and lipid-binding domains are required for T20-mediated inhibition of membrane fusion.

The most remarkable peptide is CHR-3, which contains >85% sequence overlapping with the C- and N-terminal sequences of C36 and T20, respectively. However, it does not contain the pocket-binding sequence, the most important functional domain at the N terminus of C36, nor the lipid-binding sequence located at the C terminus of T20. Therefore, CHR-3 may not be able to utilize the mechanism of action of either C36 or T20 to mediate inhibition of HIV-1 fusion. Indeed, CHR-3 could not block the 6-HB between N46 and C34-biotin nor bind with the lipid bilayers. This may explain why CHR-3 possesses only marginal anti-HIV-1 activity, ~100-fold less potency than C36 and T20. The peptide CHR-5, although it contains the lipid-binding domain, consists of only half of the NHR-binding sequence. Therefore, its interaction with the viral NHR domain may be too weak to block the gp41 6-HB formation for inhibition of viral fusion with the target cell.

The above conclusions are further supported by the following evidence: (i) the peptides CHR-1 and C36 interacted with the NHR-peptide N46 to form stable 6-HBs, and could block the gp41 core formation, but could not interact with lipid bilayers; (ii) the peptide T20 was capable of interacting with the LUVs of POPC. The peptide-lipid interaction was abolished when the C-terminal WNWF motif in T20 was replaced by ANAA. T20 could not form stable 6-HB with N46 but might interact with N46 to form non-helical insoluble complexes. T20 was capable of partially blocking the 6-HB formation between

**TABLE 3**

Comparison of inhibitory activity of T20 and C36 on infection by T20-resistant HIV-1 variants

| HIV-1 variants (mutations in gp41) | Catalog number | Phenotype (for T20) | IC<sub>50</sub> T20 | IC<sub>50</sub> C36 |
|-----------------------------------|----------------|---------------------|----------------------|----------------------|
| pNL4-3 gp41 D36G (parental NL4-3) | #9489          | Sensitive           | 10.73 ± 0.64         | 3.39 ± 1.45         |
| pNL4-3 gp41 (36G) V38A            | #9490          | Resistant           | 76.97 ± 16.87        | 0.31 ± 0.12         |
| pNL4-3 gp41 (36G) N38E, N42S      | #9496          | Resistant           | 1236.53 ± 187.34     | 10.34 ± 1.59        |
| pNL4-3 gp41 (36G) N38A, N42T      | #9498          | Resistant           | 1682.43 ± 87.49      | 24.71 ± 11.34       |

**FIGURE 5.** Comparison of the liposome-binding activity of T20 and T20-ANAA. The fluorescence spectra of T20 and T20-ANAA presented in PBS (a) or POPC LUVs (b) were measured as described above. The binding activity of T20 and T20-ANAA to lipid bilayers was compared using ITC. c, determination of the titration traces after 10 mg/ml POPC LUVs were injected into a solution containing 40 μM of T20. d, assessment of binding affinity of T20 and T20-ANAA to POPC vesicles. The binding activity of T20-ANAA to POPC LUVs was undetectable.
FIGURE 6. Involvement of the functional domains in the gp41 CHR region in the membrane fusion process. A, localization of the functional domains in the gp41 CHR region. The gp41 CHR contains three functional domains: 1, NHR-binding domain (aa 628–666, in blue); 2, pocket-binding domain (aa 628–635, in orange); and 3, lipid-binding domain (aa 666–673, in red). During the viral fusion process, NHR-binding domain in CHR region interacts with the NHR region (aa 536–581, in green) to form a hairpin structure. Three molecules of hairpins associate with each other to form a 6-HB. Interaction of the pocket-binding domain in CHR with the pocket-forming domain in NHR (aa 565–581, in brown) is critical for stabilization of the 6-HB (31, 32). The dashed lines between the NHR and CHR regions indicate the interaction between the residues located at e and g positions in NHR and a and d positions in CHR, respectively. The peptide C34, CHR-1, and C36 contain pocket-binding sequence (in orange) but not the lipid-binding sequence. The peptide T20 contains the lipid-binding (or tryptophan-rich) sequence (in red) and partial NHR-binding domain but does not have the pocket-binding sequence. B, a model of the HIV-1 gp41 mediated membrane fusion and mechanism of action of CHR-peptide-mediated anti-HIV-1 activity. Upon gp120 binding to CD4 and a coreceptor on the target cell membrane, gp41 changes conformation by inserting its fusion peptide (FP) into the target cell membrane. Then the NHR-binding domain in the N-terminal portion of CHR interacts with NHR to form a 6-HB, which brings the viral and cellular membranes into close proximity. The lipid-binding domain in the C-terminal portion of CHR may participate in fusion pore formation by interacting with lipid bilayers. In the fusion-intermediate state, the peptide CHR-1, C36, and C34 may bind to the viral NHR to block 6-HB formation, resulting in inhibition of membrane fusion. The peptide T20 may also interact with the viral NHR through its N-terminal portion and bind to the cell membrane via its C-terminal WNWF motif, leading to inhibition of HIV-1 fusion. These functional domains in gp41 CHR and NHR regions may act at different stages of viral fusion.
N46 and C34-biotin; (iii) C36 was effective against T20-resistant HIV-1 strains; (iv) CHR-3 interacts with N46 to form unstable 6-HB, but was unable to block the 6-HB formation. Unlike T20, it could not interact with the POPC LUVs; and (v) CHR-5 can bind to LUVs of POPC but could not interact with N46 to form 6-HB nor block the gp41 core formation.

The two-binding-site model of T20 proposed here is also supported by findings of others. Chang and colleagues (33) have reported that the LLSGIV motif in the N-terminal region of the gp41 NHR, a determinant of the HIV-1 resistance to T20, is a critical binding site for T20. Shai and colleagues have shown that T20 can interact with a long NHR-peptide N54 (34), but it inhibits the formation of the gp41 viral hairpin structure at a much lower affinity than C34 (35). They have also reported that the ability of T20 to inhibit membrane fusion at a post-lipid mixing stage is correlated with its activity to bind and oligomerize on the membrane surfaces (35). It was proven that the C-terminal WNWF motif of T20 is important for its anti-HIV-1 activity, because deletion of this motif or substitution of WNWF with ANAA results in loss of anti-HIV-1 activity, and octylation of ANAA mutant rescues T20-mediated fusion inhibitory activity (22, 24, 36). von Laer and co-workers (37) have demonstrated that the wild-type T20 and the ANAA mutant T20 have similar anti-HIV-1 activities when both peptides are anchored to the target cell membrane by fusion to a transmembrane domain. Veiga et al. (38) have shown that T-1249, an analog peptides of T20, and its C-terminal peptide inhibit HIV-1 fusion by interacting with lipid bilayers. The tryptophan-rich (or lipid-binding) domain in the membrane proximal region of gp41 was shown to play important roles in HIV-1 fusion (22, 36, 39), because it may bind to the membrane surface (40) and participate in oligomerization of gp41 to form fusion pores in the membrane (23, 35, 41, 42). Shnaper et al. (43) have shown that the ordered and cholesterol-rich membranes can be destabilized by the gp41 CHR region. We previously demonstrated that a short hydrophobic peptide derived from the membrane-spanning region of gp41 blocked T20-mediated membrane fusion inhibitory activity (13). This information suggests that the lipid-binding domain in the C-terminal portion of CHR may play a part in fusion pore formation by binding to the lipid bilayers of target cell membranes.

Because C36 contains different functional domains and inhibits HIV-1 infection through distinct mechanism of action from T20, this peptide may also be developed as a new anti-HIV-1 drug, which may have complementary and/or synergistic effects with T20. For example, C36 remains highly effective against T20-resistant viruses (26), suggesting that C36 may be used for treatment of patients who are infected by HIV-1 isolates resistant to T20.

In conclusion, we have defined the relationship between the three functional domains in the HIV-1 gp41 CHR region and the distinct mechanisms of action of the CHR-peptides with anti-HIV-1 activity. Further studies of these functional domains will provide valuable information for understanding the undefined phenomenon of gp41-mediated membrane fusion. These functional domains and their binding partners may serve as targets for rational design of novel anti-HIV therapeutics and vaccines.

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