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Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target

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ABSTRACT **Synthetic C peptides, corresponding to the C helix of the HIV type 1 (HIV-1) gp41 envelope protein, are potent inhibitors of HIV-1 membrane fusion. One such peptide is in clinical trials. The crystal structure of the gp41 core, in its proposed fusion-active conformation, is a trimer of helical hairpins in which three C helices pack against a central coiled coil. Each C helix shows especially prominent contacts with one of three symmetry-related, hydrophobic cavities on the surface of the coiled coil. We show that the inhibitory activity of the C peptide C34 depends on its ability to bind to this coiled-coil cavity. Moreover, examining a series of C34 peptide variants with modified cavity-binding residues, we find a linear relationship between the logarithm of the inhibitory potency and the stability of the corresponding helical-hairpin complexes. Our results provide strong evidence that this coiled-coil cavity is a good drug target and clarify the mechanism of C peptide inhibition. They also suggest simple, quantitative assays for the identification and evaluation of analogous inhibitors of HIV-1 entry.**

Recent crystal structures of the envelope protein subunits gp120 (1) and gp41 (2–4) have raised hopes of structure-based drug development against HIV type 1 (HIV-1) entry, an essential step in viral pathogenesis. This step is not targeted by current combination therapies. gp41 is the transmembrane subunit that mediates fusion of viral and cellular membranes. The gp41 ectodomain core is a six-helix bundle composed of three helical hairpins, each consisting of an N helix paired with an antiparallel C helix (2–4). The N helices form an interior, trimeric coiled coil with three conserved, hydrophobic grooves; a C helix packs into each of these grooves (Fig. 1). This structure likely corresponds to the core of the fusion-active state of gp41 (2, 3) and shows similarity to the proposed fusogenic structures of envelope fusion proteins from influenza (5, 6), Moloney murine leukemia virus (7), and simian immunodeficiency virus (8, 9).

Synthetic C peptides (peptides corresponding to the C helix), such as DP178 and C34 (see Fig. 1 legend), potently inhibit membrane fusion by both laboratory-adapted strains and primary isolates of HIV-1 (8, 10–12). A Phase I clinical trial with the C peptide DP178 suggests that it has antiviral activity *in vivo*, resulting in reduced viral loads (43). The structural features of the gp41 core suggest that these peptides act through a dominant-negative mechanism, in which exogenous C peptides bind to the central coiled coil of gp41 and lead to its inactivation (2, 3, 13–15).

Within each coiled-coil interface is a deep cavity, formed by a cluster of residues in the N helix coiled coil, that has been proposed to be an attractive target for the development of antiviral compounds (2). Three residues from the C helix (Trp-628, Trp-631, and Ile-635) insert into this cavity and make extensive hydrophobic contacts (Fig. 1). Mutational analysis indicates that two of the N-helix residues (Leu-568 and Trp-571) comprising this cavity are critical for membrane fusion activity (16). Therefore, compounds that bind with high affinity to this cavity and prevent normal N and C helix pairing may be effective HIV-1 inhibitors. The residues in the cavity are highly conserved among diverse HIV-1 isolates. Moreover, a C peptide (T649) containing the cavity-binding region is much less susceptible to the evolution of resistant virus than DP178, which lacks this region (17). These observations suggest that high-affinity ligands targeting the highly conserved coiled-coil surface, particularly its cavity, will have broad activity against diverse HIV isolates and are less likely to be bypassed by drug-escape mutants.

Although this hypothesis is tempting, there is no evidence that the C34 residues projecting into the cavity are important for the potency of this inhibitor. In fact, some C peptides that lack the cavity-binding residues, such as DP178, are highly effective inhibitors of HIV-1 membrane fusion. We have performed a systematic structure-function analysis of the role of cavity contacts for the inhibitory activity of C34. Our results indicate that cavity-binding residues in C34 are important for the inhibitory activity of this peptide. Moreover, we show that there is a striking relationship between the stability of these cavity contacts and the potency of C34 in inhibiting membrane fusion.

MATERIALS AND METHODS

Peptide Synthesis and Purification. Mutant peptides were synthesized by solid-phase fluorenylmethoxycarbonyl peptide chemistry as described (18) and have an acetylated amino terminus and an amidated carboxyl terminus. After cleavage from the resin, peptides were desalted with a Sephadex G-25 column (Pharmacia) and purified by reverse-phase HPLC (Waters) on a Vydac (Hesperia, CA) C18 preparative column using a linear water-acetonitrile gradient and 0.1% trifluoroacetic acid. Peptide identities were verified by matrix-assisted laser-desorption ionization mass spectrometry (Voyager Elite, PerSeptive Biosystems, Cambridge, MA), and their purities (>98%) were evaluated by analytical HPLC. Peptide concentrations were measured by tryptophan and tyrosine absorbance (at 280 nm) in 6 M guanidine HCl (19). The concentration of the Trp-631 → Nal peptide was measured by Nal absorbance using the extinction coefficient ε = 6,900 at 282 nm (20).

CD Measurements. CD measurements were performed in PBS (50 mM sodium phosphate/150 mM NaCl, pH 7.0) with an Aviv Model 62DS spectrometer as described (14). The apparent melting temperature (T_m) of each complex was estimated from the maximum of the first derivative of θ_{222} with respect to temperature.

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Abbreviation: HIV-1, HIV type 1.

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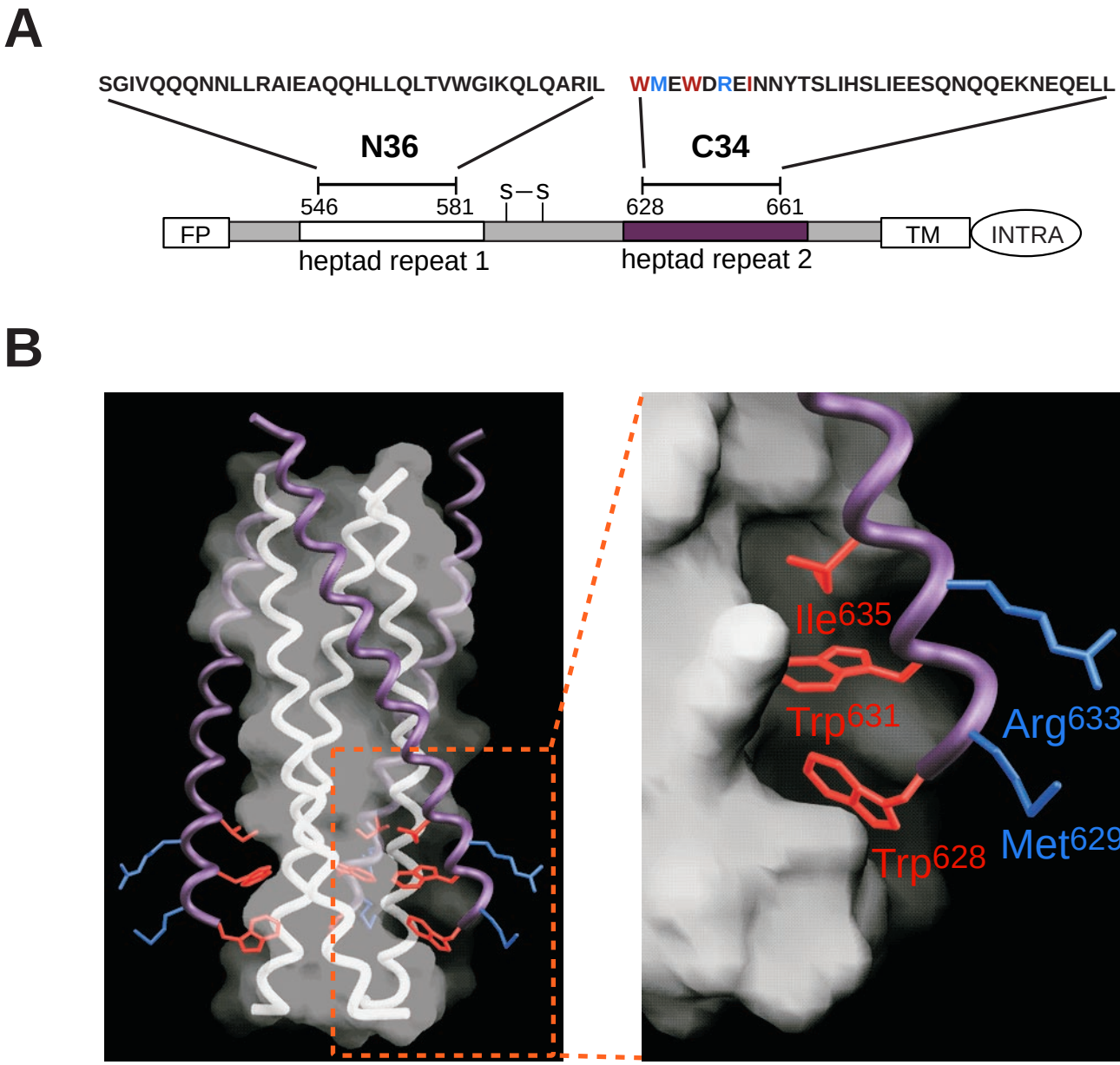

FIG. 1. HIV-1 gp41 structure and mutant peptides. (*A*) Schematic of HIV-1 gp41 showing the N36 and C34 peptides, located within two regions containing 4,3 hydrophobic heptad repeats (white and purple boxes). The residues corresponding to the peptides discussed in this paper are as follows: C34, 628–661; DP178, 638–673; T649, 628–663. All of these peptides are acetylated at the amino terminus and amidated at the carboxy terminus. Colored residues in C34 were mutated in this study. Red residues project into the N36 cavity, whereas blue residues do not. FP, fusion peptide; S-S, disulfide bond; TM, transmembrane region; INTRA, intraviral region. (*B*) The N36/C34 crystal structure of the HIV-1 gp41 ectodomain core (*2*). (*Left*) The trimeric N36 coiled coil is represented by white three helices overlaid with a semitransparent white molecular surface. Three C34 helices (shown in purple with selected side chains) pack against this coiled coil surface. The bottom of the N36 surface contains three symmetry-related cavities (one is outlined by the box), each of which accommodates three hydrophobic residues (red) from a C34 helix. In contrast, the blue residues project outward and do not make contacts with the coiled coil. (Right) A close-up of the cavity region with the C34 residues labeled. Here, the surface of the central coiled coil is represented by an opaque molecular surface. This figure was made with the program GRASP (42).

Peptide Inhibition Assays. The potency of C34 peptides in inhibiting viral infection was determined using recombinant luciferase-expressing HIV-1 as described (8). To produce virus, 293T cells were co-transfected with the envelope-deficient HIV-1 genome NL43LucR-E- (21) and the HXB2 gp160 expression vector pCMVHXB2 gp160 (D.C.C. and P.S.K., unpublished data) using calcium phosphate. Viral supernatants were cleared of cellular debris by low-speed centrifugation and used to infect HOS-CD4/Fusin cells (N. Landau, National Institutes of Health AIDS Reagent Program) in the presence of varying concentrations of peptide, ranging from 0 to 200 nM. Cells were harvested 48 hr postinfection, and luciferase activity was measured in a Wallac (Gaithersburg, MD) AutoLumat LB953 luminometer. The IC_{50} is the peptide concentration that results in a 50% decrease in activity relative to control samples lacking peptide. For each peptide, data from three experiments were fit to a Langmuir equation [$y = k/(1 + ([\text{peptide}]/IC_{50})]$, where y = luciferase activity and k is a scaling constant] to obtain the IC_{50} values.

Syncytia formation was assayed by coculturing the HXB2 envelope-expressing cell line Chinese hamster ovary [HIVe](clone 7d2) (22) with the CD4-expressing cell line HeLa-CD4-LTR-Beta-gal (M. Emerman, National Institutes of Health AIDS Reagent Program) in the presence of varying

Table 1. Stability of mutant N36/C34 complexes and the inhibitory potency of C34 mutants

Peptide	$[\theta]_{222}$, 10^3 deg cm^2 dmol^{-1}	T_m, °C	IC_{50}, viral entry, nM	IC_{50}, cell fusion, nM
Wild-type C34	−31.7	66	2.1 ± 0.31	0.55 ± 0.03
Cavity-binding				
Trp-628 → Ala	−27.0	53	10 ± 2.0	3.8 ± 0.33
Trp-631 → Ala	−24.9	37	61 ± 16	15 ± 0.82
Ile-635 → Ala	−25.9	55	4.1 ± 0.91	0.96 ± 0.12
Control residues				
Met-629 → Ala	−32.0	66	2.0 ± 0.27	0.74 ± 0.03
Arg-633 → Ala	−30.7	65	2.6 ± 0.89	0.76 ± 0.07

Mutant C34 peptides (10 μM) were complexed with the N36 peptide (10 μM) in phosphate-buffered saline (pH 7.0) for CD measurements. The apparent (T_m) were estimated from the thermal dependence of the CD signal at 222 nm. Inhibition of viral entry was measured in a cell-culture infection assay using recombinant luciferase-expressing HIV-1. Inhibition of cell-cell fusion was measured in a syncytium assay. The means and standard errors were from triplicate trials.

concentrations of peptide, ranging from 0 to 200 nM. Cell fusion results in expression of nuclear β-galactosidase from the HeLa-CD4-LTR-Beta-gal indicator cell line. Fifteen hours after coculture, monolayers were stained with the colorimetric substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside, and syncytia formation was quantitated by counting multinucleated cells containing at least three β-galactosidase-positive nuclei. For each peptide, data from three experiments were fit to a Langmuir equation to obtain the IC_{50} values.

RESULTS

Cavity-Binding Residues of C34 Stabilize Its Interaction with N36. To determine the role of cavity contacts in inhibitory activity, we performed structure-based mutagenesis on C34. The core of the gp41 ectodomain (Fig. 1) was reconstituted with two synthetic peptides called N36 and C34 (2, 23). Variants of the C34 peptide with single alanine substitutions were synthesized, and the helical content and thermal stability of mutant N36/C34 complexes were quantitated by circular dichroism. As expected, mutation of C34 residues (Met-629, Arg-633) that do not contact the N36 coiled coil had little effect on mean residue ellipticity at 222 nm (θ_{222}, a measure of helical content) or stability of N36/C34 complexes (Table 1). However, mutation of any of the three residues (Trp-628 → Ala, Trp-631 → Ala, or Ile-635 → Ala) that project into the cavity of the N36 coiled coil resulted in N36/C34 complexes with substantially decreased mean residue ellipticity and stability (Table 1). It should be noted, however, that in the case of the Trp-628 → Ala and Trp-631 → Ala mutations, the decrease in θ_{222} is likely to overestimate the actual reduction in helical content. The removal of tryptophan residues from model helices has been reported to significantly reduce the absolute value of θ_{222} even when there is little change in helical content (24). The greatest destabilization was observed with the mutant Trp-631 → Ala, which formed N36/C34 complexes with an apparent T_m of 37°C, compared with 66°C for wild type. These results demonstrate that C34 residues making contacts with the N36 cavity are important for stabilizing the helical-hairpin structure of the gp41 ectodomain core.

Cavity-Binding Residues of C34 Are Important for Inhibition of Fusion. To determine the importance of these residues in the ability of C34 to inhibit membrane fusion, we tested the activity of these C34 peptides in HIV-1 viral entry and syncytium assays (Table 1). Wild-type C34 showed IC_{50} values of ≈2.1 nM and ≈0.55 nM for inhibition of viral entry and syncytia formation, respectively. For comparison, the reported IC_{50} of DP178 is ≈20 nM for HIV entry (10) and ≈0.4 nM for syncytia formation (12). Mutations that had little effect on the stability of the N36/C34 complex (Met-629 → Ala and Arg-633 → Ala) also had little effect on the inhibitory activity of C34. However, mutation of the strictly conserved Trp-628 or Trp-631 to alanine resulted in a substantial decrease in activity: ≈5-fold and ≈30-fold, respectively (Table 1). Mutation of the less well-conserved Ile-635 resulted in only a ≈2-fold decrease in inhibitory activity.

The Potency of C34 Is Directly Related to Its Ability to Bind the Cavity. To clarify the relationship between the potency of mutant C34 peptides and the stability of mutant N36/C34 complexes, we took advantage of the greatly destabilizing effect of the Trp-631 mutation to construct a series of N36/C34 complexes with a gradation of stabilities. By using the Trp-631 position as a "guest site," we systematically substituted the tryptophan with natural and unnatural amino acids representing a broad range of hydrophobic bulk. In order of increasing hydrophobic bulk, the substitutions were: glycine, alanine, L-α-aminobutyric acid, valine, leucine, phenylalanine, the wild-type residue tryptophan, and L-β-(1-naphthyl) alanine. This approach resulted in a set of C34 peptides that form N36/C34 complexes with T_m values ranging from 35°C to 66°C (Table 2). In HIV-1 infection and syncytium assays, these peptides show potencies that correlate with the T_m values of the corresponding N36/C34 complexes (Fig. 2). The potency order of these mutants is wt ≈ Nal > Phe > Leu > Val ≈ Abu

Table 2. Substitution of Trp-631 with a series of hydrophobic amino acids

Peptide	$[\theta]_{222}$, 10^3 deg cm^2 dmol^{-1}	T_m, °C	IC_{50}, viral entry, nM	IC_{50}, cell fusion, nM
Wild-type C34	−31.7	66	1.5 ± 0.2	0.55 ± 0.03
Trp-631 → Nal	−32.0	62	1.4 ± 0.3	0.79 ± 0.08
Trp-631 → Phe	−26.3	59	3.6 ± 0.8	1.6 ± 0.05
Trp-631 → Leu	−26.7	50	5.3 ± 1.0	3.2 ± 0.1
Trp-631 → Val	−23.9	43	13 ± 2.8	4.5 ± 0.09
Trp-631 → Abu	−23.2	43	16 ± 4.8	6.9 ± 0.4
Trp-631 → Ala	−24.9	37	40 ± 4.3	15 ± 0.8
Trp-631 → Gly	−17.1	35	38 ± 6.1	25 ± 3.8

Values were determined as in Table 1. The means and standard errors for the inhibition assays were from triplicate trials.

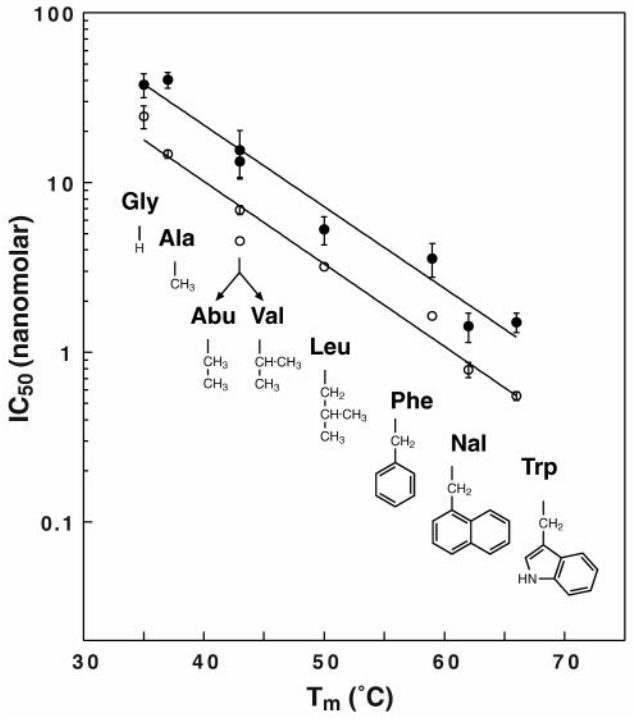

FIG. 2. Correlation of C34 inhibitory potency with N36/C34 stability. C34 peptide variants containing substitutions at position Trp-631 were tested for inhibition of viral entry (●) and cell-cell fusion (○). IC_{50} values (Table 2) are plotted on a logarithmic scale against the T_m of the corresponding N36/C34 complex. The identities and chemical structures of the substitutions are indicated under the corresponding data points. Bars = SD from triplicate experiments.

> Ala ≈ Gly, in close agreement with the hydrophobic bulk of the substitution and the stability of N36/C34 complexes (Table 2). There is a striking linear relationship when the IC_{50} is plotted on a logarithmic scale as a function of the T_m (Fig. 2). Because $\Delta G = -RT\ln K$ (ΔG, change in free energy; R, gas constant; T, absolute temperature; and K, equilibrium constant) and ΔT_m ($T_{m,\,wt} - T_{m,\,mutant}$) is proportional to $\Delta\Delta G$ ($\Delta G_{wt} - \Delta G_{mutant}$) (25), the observed relationship strongly suggests that the potency of the C34 variants is directly related to their affinity for the N helix coiled coil, as predicted by a dominant-negative mode of inhibition.

DISCUSSION

Our results provide strong support for the proposal that the cavity on the gp41 coiled coil is an attractive drug target (2). Conserved residues projecting into the hydrophobic cavity clearly play a major role in the ability of C34 to inhibit HIV-1 infection, indicating that this inhibitor works by forming a high-affinity complex with the N helix coiled coil. Although the importance of cavity contacts in the potency of C34 is clear, it remains a challenge to isolate small molecules that bind to the cavity with sufficient affinity to inhibit HIV-1 infection. Short peptides consisting of only the cavity-interacting portion of C34 lack inhibitory activity (D.C.C., D. M. Eckert, C.T.T., and P.S.K., unpublished results). However, modification of C peptides by covalent linkages has been reported to increase the helicity and potency of shortened C peptides (26). Moving beyond traditional peptides, mirror-image phage display techniques (27) and combinatorial chemistry (28–30) may be useful in identifying D peptides, peptidomimetics, and small molecules that bind with high affinity to the cavity. The close correlation between N36/C34 stability and C34 inhibitory potency suggests that the effectiveness of such compounds will depend critically on the strength of their cavity contacts. Indeed, our results suggest that candidate compounds can be tested for the ability to form a stable complex with N36, thereby providing a basis for rapid, quantitative screens to identify and evaluate potential inhibitors of HIV-1 entry.

These studies on the mechanism of C peptide action also support the hypothesis that the trimeric hairpin structure of the gp41 core (2–4) corresponds to the fusion-active state of gp41. Our studies show that the inhibitory potency of C34 depends on its ability to bind to the N helix coiled coil of gp41. Kinetic studies suggest that C peptides do not act on the native conformation of gp41 (31) but can act after the onset of rapid receptor-activated conformational changes (32, 33). Because the hairpin structure of gp41 is extremely stable, with a melting temperature in excess of 90°C (14), it is unlikely that nanomolar concentrations of C34 can disrupt this structure once it has formed, especially given the high effective concentration of the N and C helices within an intact gp41 molecule. Rather, C peptides likely act before the formation of the gp41 hairpin by binding to a transient pre-hairpin intermediate, in which the central coiled coil is exposed (13). Binding of C peptides to this pre-hairpin intermediate inactivates gp41 and prevents its conversion to the fusion-active hairpin structure. This model is supported by immunoprecipitation experiments indicating that the C peptide DP178 binds to receptor-activated gp41 (31). In addition, viruses escaping DP178 inhibition show mutations in the central coiled-coil region of gp41 (17).

Finally, our findings on HIV-1 gp41 are likely to be relevant to other membrane fusion events. The gp41 hairpin structure is similar to fusion proteins from several virus families, including retroviruses (7–9), orthomyxoviruses (5), and paramyxoviruses (34–37). Moreover, these similarities extend to v-SNARE and t-SNARE protein complexes involved in vesicle fusion (38–41), suggesting that formation of hairpin helices may be involved in a wide range of membrane fusion reactions.

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