Mode of Action of an Antiviral Peptide from HIV-1

INHIBITION AT A POST-LIPID MIXING STAGE

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DP178, a synthetic peptide corresponding to a segment of the transmembrane envelope glycoprotein (gp41) of human immunodeficiency virus, type 1 (HIV-1), is a potent inhibitor of viral infection and virus-mediated cell-cell fusion. Nevertheless, DP178 does not contain gp41 coiled-coil cavity binding residues postulated to be essential for inhibiting HIV-1 entry. We find that DP178 inhibits phospholipid redistribution mediated by the HIV-1 envelope glycoprotein at a concentration 8 times greater than that of solute redistribution (the IC_{50} values are 43 and 335 nM, respectively). In contrast, C34, a synthetic peptide which overlaps with DP178 but contains the cavity binding residues, did not show this phenomenon (11 and 25 nM, respectively). The ability of DP178 to inhibit membrane fusion at a post-lipid mixing stage correlates with its ability to bind and oligomerize on the surface of membranes. Furthermore, our results are consistent with a model in which DP178 inhibits the formation of gp41 viral hairpin structure at low affinity, whereas C34 inhibits its formation at high affinity: the failure to form the viral hairpin prevents both lipid and solute from redistributing between cells. However, our data also suggest an additional membrane-bound inhibitory site for DP178 in the ectodomain of gp41 within a region immediately adjacent to the membrane-spanning domain. By binding to this higher affinity site, DP178 inhibits the recruitment of several gp41-membrane complexes, thus inhibiting fusion pore formation.

The first step in HIV-1 infection involves the binding of the viral envelope glycoproteins gp120-gp41 to CD4 (1–3) and subsequent to a co-receptor (4–8) (for recent review, see Refs. 9–11). Consequently, gp41 undergoes conformational changes that mediate the fusion between the viral and the cellular membranes or between infected and healthy cells (12, 13). Gallaher and co-workers (14, 15) postulated a model of gp41, identifying a fusion peptide followed by a leucine/isoleucine zipper-like sequence (N-helix) and an amphipathic helical segment (C-helix) in the viral glycoprotein. The indispensability of the fusion peptide for viral infection was confirmed by site-directed mutagenesis (16, 17). Furthermore, gp41 was found to contain a protease-resistant core consisting of the postulated N- and C-helices (18). Specifically, peptides corresponding to these sequences co-crystallized as a six-helix bundle in which the N- and C-helices are arranged in a three-hairpin structure (19–21). Three N peptides form a coiled-coil, and the C peptides are packed in an antiparallel manner into highly conserved, hydrophobic grooves on the surface of the coiled-coil. Recently, the solution and crystal structures of the ectodomain of the Simian immunodeficiency virus gp41, consisting of those two helices as well as the loop connecting them, confirmed the interplay of the N- and C-helices (22, 23). Remarkably, the coiled coil is a common motif found in many diverse viral membrane fusion proteins (24), as well as in proteins involved in vesicular transport (25–31).

A synthetic peptide overlapping the C-terminal amphipathic helical segment of gp41 and its tryptophan-rich sequence (32) (DP178, Fig. 1) was reported to inhibit virus infection at extremely low concentration (33). Remarkably, DP178 blocks cell fusion and viral entry at a concentration of less than 2 ng/ml in vitro and was reported to be a promising drug for treating HIV-1-infected humans (34–37). Since DP178 is a potent inhibitor of HIV-1-induced membrane fusion, elucidating its mode of action is of major importance. In essence, this means finding the stage at which DP178 inhibits the formation of the fusion-active conformation of gp41. It was suggested that the antiviral mode of action of DP178 involves interaction with the gp41 leucine/isoleucine zipper motif (38–41). However, DP178 (residues 638–673) does not contain the residues (Trp-628, Trp-631, and Ile-635) that were shown to be crucial for binding the prominent cavity in the coiled coil of gp41 (59). Weiss and colleagues (42) demonstrated that DP178 binds gp41 and inhibits envelope-mediated membrane fusion only after gp120 interacts with cellular receptors. It is currently accepted that DP178 binds to the gp41 leucine/isoleucine zipper sequence before the hairpin structure is formed (43–45), thus preventing the HIV envelope glycoprotein from adopting a fusogenic conformation. This model is supported by the results of Blumen- thal and co-workers (6, 46), suggesting that the pre-hairpin intermediate stage appears to be induced rapidly upon interaction of gp120 with CD4 and the co-receptor, but is then relatively stable for several minutes, allowing DP178 to interact with the exposed leucine/isoleucine zipper sequence.

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* The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; gp, glycoprotein; CMTMR, 5-(and-6)-(((4-chloromethyl)benzoyl)amino)-tetramethylrhodamine; DNS-PE, N-[5(dimethylaminonaphthalene-1-sulfonyl)-sn-glycero-3-phosphoethanolamine; FRET, fluorescence resonance energy transfer; NBD, 7-nitrobenz-2-oxa-1,3-diazole-4-yl, PC, egg phosphatidylcholine; PE, phosphatidyethanolamine; PS, phosphatidylserine; Rho, tetramethyl rhodamine; SUV, small unila- mellar vesicles.
However, DP178 was reported to inhibit redistribution of lipid and aqueous dyes as a result of HIV-1 envelope glycoprotein-mediated fusion with significantly different values of IC_{50} (46). Binding of DP178 to the leucine/isoleucine zipper sequence in the pre-hairpin intermediate should inhibit both lipid and aqueous redistribution, suggesting the existence of two target sites for inhibition by DP178. In an attempt to identify an additional target site for inhibition by DP178, we investigated potential interactions of DP178 with the membrane, and with the leucine/isoleucine zipper sequence within the membrane. We find that DP178 binds to the membrane and oligomerizes within, but does not interact with the leucine/isoleucine zipper sequence within the membrane. We suggest that by binding to its corresponding segment in gp41, DP178 inhibits fusion pore formation.

**EXPERIMENTAL PROCEDURES**

**Peptides Preparation and Fluorescent Labeling—**DP178, N36, and C34 were synthesized by using the Boc chemistry, as described previously (47, 48). Concentrations were measured by tryptophan and tyrosine absorbance (at 280 nm) in 8 M urea (49). Labeling of the N-terminus of synthetic peptides was achieved as described previously (50, 51). Briefly, resin-bound peptides, with their amino acid side chains fully protected, were treated with trifluoroacetic acid, to remove the BOC protecting group from their N-terminal amino groups, while keeping all the other reactive amine groups of the attached peptides still protected. The resin-bound peptides were then reacted with the desired fluorescein probe, cleaved from the resin by hydrogen fluoride, and finally precipitated using ether. This procedure yielded peptides selectively labeled with fluorescent probes at their N-terminal amino acids. The synthetic peptides were purified by reverse-phase high performance liquid chromatography on an analytical C_{18} Vydac column 4.6 × 250 mm (pore size of 300 Å). The column was eluted in 80 min, at a flow rate of 0.6 ml/min, using a linear gradient of 25–80% acetonitrile in water, in the presence of 0.05% trifluoroacetic acid (v/v).

**Dye Transfer Fusion Assay—**Peptide inhibition of cell-cell fusion was assayed by monitoring the redistribution of fluorescent probes, both water soluble and lipophilic, between target and effector cells upon their co-incubation with each other (46). The HIV-1 gp120-41 expressing TP228 cells (52) were labeled with either calcein or a green fluorescent probe, and C34 were synthesized by using the Boc chemistry, as described previously (59) (Fig. 1). We performed a series of new experiments to investigate inhibition of HIV-1 envelope glycoprotein-mediated fusion by DP178 and C34. The experi-
mixing while error of these experiments. The filled diamonds represent the average of two to four independent experiments. The error bars represent the standard error of these experiments. The filled diamonds represent the calcein dye or fluorescent lipid (C1-BODIPY-C12), and the CD4+CXCR4+3T3 cells were labeled with CMTMR. After 2 h of co-culture at 37 °C, the cells were examined for lipid and solute dye transfer. The amount of mixing is presented relative to the control, which is in absence of peptide. Three fields are taken of each sample and averaged. The data represent the average of two to four independent experiments.

C34, but 100, 200, 400, and 800 nM concentrations of DP178 were conducted on each point in both these curves to compare contents versus mixing for DP178. Mechanism of Inhibition—We detected the binding of peptides to the membrane by using fluorescence resonance energy transfer (FRET) from the polypeptide tryptophan residues to DNS chromophores incorporated into the lipid vesicles. Excitation was set at the tryptophan maximum absorbance (280 nm), and binding was detected by measuring the changes in the sensitized DNS fluorescence (518 nm). When DP178 (0.45 μM) was added to 68 μM PS/PC/cholesterol/DNS-PE (8:8:2:1, w/w) SUV (Fig. 3a), we observed a substantial increase in the DNS fluorescence, indicating that DP178 binds to the membrane. Since the DNS groups are attached to the head groups of the phospholipids, DP178 must be located near the surface of the membrane. In contrast, when C34 (0.45 μM) was added to the vesicles (Fig. 3b), there was no change in the DNS fluorescence, indicating that the tryptophans of C34 do not interact with the DNS groups, or that the interaction is too weak to be detected by this assay (see also Fig. 4). Interestingly, while treatment of HIV-1-infected cells with soluble CD4 increases binding of a monoclonal antibody (98-6) to an epitope that spans residues 644–663 (overlaps with C34 and DP178, see Fig. 1), binding of a monoclonal antibody (2F5) to an epitope that spans residues 662–667 (overlaps with DP178, but not with C34) was reduced (60). This is consistent with a model in which the conformational change of gp41 results in binding the DP178 region to the membrane, thus hiding the corresponding epitope.

The biological relevance of this membrane-binding feature is supported by the location of DP178 just upstream of the transmembrane domain. Experiments performed with either negatively charged PS/PC/cholesterol/DNS-PE (8:8:2:1, w/w) vesicles or zwitterionic PC/cholesterol/DNS-PE (16:2:1, w/w) vesicles qualitatively yielded similar results (data not shown), indicating that nonelectrostatic forces are involved in the peptide–membrane interactions.

Detection of peptide binding to the membrane. 0.45 μM DP178 (a) or C34 (b) were added to 68 μM lipid vesicles containing 5% DNS-PE. Excitation, 280 nm; emission, 518 nm.

Further checked whether interactions within the membrane may reveal another binding site.

DP178 binds to the membrane—We detected the binding of peptides to the membrane by using fluorescence resonance energy transfer (FRET) from the polypeptide tryptophan residues to DNS chromophores incorporated into the lipid vesicles. Excitation was set at the tryptophan maximum absorbance (280 nm), and binding was detected by measuring the changes in the sensitized DNS fluorescence (518 nm). When DP178 (0.45 μM) was added to 68 μM PS/PC/cholesterol/DNS-PE (8:8:2:1, w/w) SUV (Fig. 3a), we observed a substantial increase in the DNS fluorescence, indicating that DP178 binds to the membrane. Since the DNS groups are attached to the head groups of the phospholipids, DP178 must be located near the surface of the membrane. In contrast, when C34 (0.45 μM) was added to the vesicles (Fig. 3b), there was no change in the DNS fluorescence, indicating that the tryptophans of C34 do not interact with the DNS groups, or that the interaction is too weak to be detected by this assay (see also Fig. 4). Interestingly, while treatment of HIV-1-infected cells with soluble CD4 increases binding of a monoclonal antibody (98-6) to an epitope that spans residues 644–663 (overlaps with C34 and DP178, see Fig. 1), binding of a monoclonal antibody (2F5) to an epitope that spans residues 662–667 (overlaps with DP178, but not with C34) was reduced (60). This is consistent with a model in which the conformational change of gp41 results in binding the DP178 region to the membrane, thus hiding the corresponding epitope.

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Detection of the membrane binding energy of the peptides—the high sensitivity of NBD fluorescence to the polarity of its environment can be used to detect and quantify membrane binding (50, 61). When SUV (PS/PC, 1:1) were added to NBD-labeled DP178 or C34, an increase concomitant with a blue shift of the NBD fluorescence was observed, suggesting that both peptides bind to the membrane. To evaluate the biological relevance of their membrane-binding ability, we measured the energy of peptide–membrane interactions. To this end, NBD-labeled C34 (62) or DP178 (0.5 μM) were titrated with SUV. Plotting the resulting increase in the fluorescence intensities of NBD-labeled peptides as a function of lipid/peptide molar ratios yielded conventional binding curves (Fig. 4a). The binding isotherms for the peptides can be obtained by plotting $X_b$, the molar ratio of bound peptide per lipid in the
outer leaflets) versus the equilibrium concentration of free peptide in solution. The surface partition coefficient \( (K) \) is calculated from the slope of the curve. The curve for the binding of DP178 to phospholipid vesicles has two phases, suggesting positive cooperativity for this binding (Fig. 4b, filled squares). The initial slope reveals \( K \) of \( 4 \times 10^4 \text{M}^{-1} \) (\( \Delta G = -8.7 \text{ Kcal/mol} \)). Remarkably, C34 binds >10-fold weaker and with no cooperativity (Fig. 4b, open circles). Its surface partition coefficient was \( 3 \times 10^5 \text{M}^{-1} \) (\( \Delta G = -7.1 \text{ Kcal/mol} \)).

The inhibitory activity of DP178 can be partially explained by its ability to bind to the gp41 leucine/isoleucine zipper sequence before binding to the membrane (43–45). Here, we examined whether the same sequence may also be the target for inhibition within the membrane.

The Leucine/Isoleucine Zipper Sequence Is Not a Target for Inhibition by DP178 within the Membrane—DP178 interacts with the leucine/isoleucine zipper sequence (represented by N36, see Fig. 1) in aqueous solution (45). Contrary, NBD-Rho FRET experiments that used NBD-DP178 and Rho-N36 (Fig. 5, open squares) or NBD-N36 and Rho-DP178 (Fig. 5, open circles) revealed efficiencies of energy transfer that were close to the calculated values assuming random distribution of donor and acceptor monomers (57), and \( R = 51 \text{ Å} \) (58), is given for comparison (dashed line). Excitation, 467 nm; emission, 500–600 nm.

Membrane-bound DP178 Forms Homo-oligomers—The ability of DP178 to oligomerize within the membrane was also measured by NBD-Rho FRET. When Rho-DP178 (a final concentration of 0.035–0.105 \( \mu \text{M} \)) was added to a mixture of NBD-DP178 (0.11 \( \mu \text{M} \)) and SUV (303 \( \mu \text{M} \), a dose-dependent quenching of the donor’s emission, which is consistent with energy transfer, was observed (Fig. 5, filled squares). Note that the acceptor peptide was added only after the donor peptide was already bound to the membrane, thus preventing any association in solution. The lipid/peptide ratio in these experiments was kept high to create low surface density of donors and acceptors to reduce the energy transfer between unassociated peptide monomers. To confirm that the observed energy transfer was due to peptide aggregation, we compared the transfer efficiencies observed in the experiments with the energy transfer expected for randomly distributed membrane-bound donors and acceptors (Fig. 5, dashed line). The results reveal that membrane-bound DP178 forms homo-oligomers.

DISCUSSION

The striking similarities between structural motifs in various viral envelope glycoproteins led to the notion that the native conformation of gp41 is metastable and it is stabilized by gp120 (63). Upon binding of gp120 to its receptors, gp41 is free to form the more energetically favorable hairpin structure (Fig. 6, a-c). 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. The N-helices form an interior, trimeric coiled coil with three conserved, hydrophobic grooves; a C-helix packs into each of these grooves. This structure is believed to correspond to the core of the fusion-active state of gp41 and shows similarity to the proposed fusogenic structures of envelope fusion proteins from influenza (64), Moloney murine leukemia virus (65, 66), simian parainfluenza virus 5 (67), Ebola virus (68), and simian immunodeficiency virus (22, 23, 69). Synthetic C peptides (peptides corresponding to the C-helix), such as DP178 and C34, potently inhibit membrane fusion by both laboratory adapted strains and primary isolates of HIV-1 (33). The structural features of the gp41 core suggest that an intermediate, in which the leucine/isoleucine zipper sequence is not associated with the C-heptad repeat, exists before the formation of the hairpin structure, and is the target for inhibition by C-peptides (43–45). However, no direct evidence for the existence of this pre-hairpin intermediate exists, and an alternative for the mechanism of inhibition exerted by the C-peptides, based on the observed monomer-trimer equilibrium of the simian immunodeficiency virus gp41 has been postulated (22, 70). According to the alternative model, gp41 exists in equilibrium between monomer and trimer. In the presence of excess inhibitory peptide, the equilibrium is driven from homotrimeric gp41 to a heterotrimer of gp41 and C-peptide. Since the peptides are only effective upon gp120 dissociation, the inhibitory state is the heterotrimer of peptide and the target binding site on the trimeric core.

**Fig. 4.** a, increase in the fluorescence of NBD-DP178 (filled squares) or NBD-C34 (empty circles) (0.5 \( \mu \text{M} \) each) upon titration with PS/PC (1:1) phospholipid vesicles. b, binding isotherms derived from panel a by plotting \( X_b \) (molar ratio of bound peptide per lipid in outer leaflet) versus \( C_f \) (the equilibrium concentration of free peptide in solution).

**Fig. 5.** Theoretically and experimentally derived percentage of energy transfer. Transfer efficiencies between donor and acceptor-DP178 (filled squares), donor-DP178 and acceptor-N36 (open squares), and donor-N36 and acceptor-DP178 (open circles) are plotted versus the bound-acceptor/lipid molar ratio. A theoretical plot showing energy transfer efficiency as a function of the surface density of the acceptors, assuming random distribution of donor and acceptor monomers (57), and \( R = 51 \text{ Å} \) (58), is given for comparison (dashed line). Excitation, 467 nm; emission, 500–600 nm.
It follows that the absence of fusogenic activity displayed by the gp41-peptide heterotrimers is due to the fact that the heterotrimers can no longer present a sufficient number of fusion peptides to the target membrane for effective fusion to take place. Another explanation could be that the homotrimer structure is required for further oligomerization. The data also suggest that the homotrimeric state is stabilized by gp120 such that heterotrimers (which would be expected to bind less tightly to gp120 since they do not possess a trimeric loop structure) cannot be formed in the presence of bound gp120 (42). Anyway, the peptides act through a dominant-negative mechanism, in which exogenous C peptides bind to the leucine/isoleucine zipper sequence (N-helix) of gp41 and block further conformational changes needed for fusion. 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. Chan et al. (59) showed 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, they observed a linear relationship between the logarithm of the inhibitory potency and the stability of the corresponding helical-hairpin complexes. A single mutation in the C34 peptide from Trp-631Gly increased the IC_{50} for cell fusion by about two log units. Reported inhibitory concentrations of DP178 and
C34 appear to be quite similar (we find that the IC_{50} for solute mixing is about 4-fold higher for DP178 as compared with C34). Since DP178 lacks all three residues from the C helix (Trp-628, Trp-631, and Ile-635) which insert into the cavity, we would expect the IC_{50} to be at least 2 orders of magnitude higher for DP178.

This conundrum has led us to postulate that there are two targets for inhibition by DP178: (i) DP178 inhibits the formation of the heterotrimeric coiled coil by interacting with the leucine/isoleucine zipper sequence in the aqueous solution; (ii) fusion pore formation is inhibited by the interaction of DP178 with the membrane-bound state of its corresponding sequence in gp41 which will enhance melting of the coiled coil soluble form. Accordingly, a mutagenesis study, which revealed that the tryptophan-rich region (overlaps with DP178, see Fig. 1) must function in proximity to the membrane (71, 32) support the notion that this region binds to the surface of the membrane. For clarity, it is worth noting that three segments in the cytoplasmic tail of gp41 may associate with the membrane (48, 72–80), whereas both Rabenstein and Shin (81) and our results suggest that segments in the ectodomain bind to the membrane. These studies suggest that segments both upstream and downstream of the transmembrane domain of HIV-1 gp41 associate with the membrane.

Our results are consistent with the notion that C34 inhibits HIV-1-induced membrane fusion by blocking heterotrimeric coiled coil formation only. The relative low affinity of C34 to the membrane (Figs. 3 and 4) does not allow it to interfere with steps that occur within the membrane. DP178 interacts with this leucine/isoleucine zipper sequence when in aqueous environment (38, 45), but this interaction cannot take place in the membrane environment (Fig. 5, open squares and open circles). On the other hand, DP178, which was found to be monomeric in aqueous solution when its concentration was less than 10 μM (38), forms oligomers even at 100 times lower concentration in the membrane (Fig. 5, filled squares). However, we cannot rule out the possibility that an increase in its membrane local concentration might shift gp41 closed heterotrimeric (soluble)–gp41 open bundle (membrane-bound) equilibrium toward the closed bundle soluble form. In other words, DP178 can prevent melting of the heterotrimeric hairpin complex. The fact that DP107 (a shifted version of N36) inhibited (although at higher concentrations) HIV-1-mediated cell fusion with no differences in cell and tissue tropism suggests that the BODIPY protocol, and Dr. A. Puri for assistance.

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9. Discussion—We are grateful to Dr. Zdenka Jonak for a gift of the HIV-1-induced membrane fusion by blocking heterotrimeric coiled coil, but before the formation of the fusion pore. Indeed, it is believed that the recruitment of several oligomeric units of the fusion proteins is needed for complete fusion to occur (83–88). Accordingly, by binding to its corresponding segment in the membrane, DP178 inhibits the formation of fusion pores (Fig. 6, the transition from c to d). Further studies are needed to understand the mechanism by which DP178 inhibits this process.

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