Basic Residues Are Critical to the Activity of Peptide Inhibitors of Human T Cell Leukemia Virus Type 1 Entry*

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A synthetic peptide based on the leash and α-helical region (LHR) of human T cell leukemia virus type 1 envelope is a potent inhibitor of viral entry into cells. The inhibitory peptide targets a triple-stranded coiled-coil motif of the fusion-active transmembrane glycoprotein and in a trans-dominant negative manner blocks resolution to the trimer-of-hairpins form. The LHR-mimetic is, therefore, functionally analogous to the C34/A triple-stranded coiled-coil motif of the fusion-active transmembrane glycoproteins and is initiated by a trigger such as receptor binding or decreased local pH and is achieved by extensive conformational changes within the fusion protein (1). After formation, the N-terminal hydrophobic peptide is thrust into a rod-like pre-hairpin intermediate, which remains anchored at the C terminus to the viral membrane and thereby bridges the opposing membranes (1–3). The pre-hairpin intermediate then resolves to a trimer-of-hairpins or six-helix bundle in which the C and N termini of the fusion protein are drawn together to bring the interacting membranes into close proximity, promoting lipid mixing and ultimately driving the formation of a fusion pore through which the viral capsid can pass to the cytoplasm of the target cell (1, 4, 5).

The retroviral envelope complex is composed of a trimer of surface glycoproteins linked to a trimer of the transmembrane glycoproteins (TM),3 a typical class 1 fusion protein. During fusion, the TM pre-hairpin intermediate is stabilized by a triple-stranded coiled coil that is assembled from an N-terminal α-helix from each of the TM monomers (6). It is thought that during fusion the coiled coil is exposed to solvent for several minutes before resolution to the trimer-of-hairpins conformation (7). For several viruses the coiled coil is a target of exogenous synthetic peptides that are potent inhibitors of membrane fusion and viral entry (8–15). The inhibitory peptides emulate the C-terminal sequences of the trimer of hairpins and, by binding to the coiled coil, act in a trans-dominant negative manner to block resolution to the trimer-of-hairpins structure (1, 3, 9, 16). The prototypic fusion inhibitor derived from human immunodeficiency virus type 1 (HIV-1) is extensively α-helical when bound to the HIV-1 core coiled coil (2, 17). By contrast, the TM region of human T cell leukemia virus (HTLV-1) that is mimicked by inhibitory peptides is atypical in that it displays a short α-helical motif flanked by extended non-helical leash regions (Fig. 1A) (18).

A synthetic peptide (P47–400) that is based on amino acid residues 400–429 of HTLV-1 envelope and includes the leash and α-helical region (LHR) of the HTLV-1 TM is a potent and highly specific inhibitor of HTLV-1-mediated membrane fusion and viral entry (9, 19). The synthetic peptide exhibits little α-helicity in aqueous solution but has a propensity to adopt a partially α-helical configuration in non-aqueous solvent (9). The peptide binds to a recombinant HTLV-1 coiled coil but fails to interact with the six-helix bundle of TM (9, 20). Moreover, specific amino acid substitutions within the peptide impair binding to the recombinant coiled coil and disrupt the inhibitory properties of the peptide (21). Thus, the accumulated evidence supports the hypothesis that the α-helical region of HTLV-1 TM serves as an attractive candidate for therapeutic intervention in human T cell leukemia virus type 1 infections.

To infect and pass between cells, enveloped viruses are dependent upon the fusion of the viral and cellular membranes. Fusion is mediated by virally encoded homo-trimeric class I fusion glycoproteins and is initiated by a trigger such as receptor binding or decreased local pH and is achieved by extensive conformational changes within the fusion protein (1). After activation, an N-terminal hydrophobic peptide is thrust into the target cell membrane to yield a rod-like pre-hairpin inter-

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3 The abbreviations used are: TM, transmembrane glycoprotein; CD, circular dichroism; MBP, maltose-binding protein; PBS, phosphate-buffered saline; TFE, trifluoroethanol; HIV-1, human immunodeficiency virus type 1; LHR, leash and α-helical region; HTLV-1, human T cell leukemia virus.
data indicate that the inhibitory peptide faithfully mimics the extended leash and α-helical structures of the C-terminal domain of the HTLV-1 trimer-of-hairpins.

Viral replication is ongoing in HTLV-1-infected individuals, and high viral loads are frequently observed in HTLV-1-associated disease (22–24). Moreover, viral envelope is a key player in cell-to-cell transfer of HTLV-1 and in infection of dendritic cells by free viral particles (25, 26). Therefore, antagonists of envelope function are likely to be of value in the treatment of HTLV-1 infections. The peptide Pcr-400, therefore, represents an attractive lead molecule from which therapeutically relevant inhibitors of viral entry can be derived. A critical objective in the design of small peptide or peptido-mimetic compounds based on Pcr-400 is the identification of a minimal region that is required for inhibitory activity of the peptide. However, attempts to truncate Pcr-400 have produced peptides that exhibit greatly compromised activity (21). The confounding issue for such peptides is that reducing the surface of interaction results in a loss of binding affinity and may remove critical determinants of specificity. Therefore, we sought to identify features that limit the activity of short peptides and to identify a core region of Pcr-400 that retains inhibitory potency.

**HTLV-1 LHR-mimetic Entry Inhibitors**

We now demonstrate that LHR-based peptides can be reduced in length while maintaining activity. The α-helical segment of the LHR is required but is not sufficient for peptide activity. By contrast, two charged residues are of particular functional importance and define a limit for C-terminal truncations and provide support to the view that interactions of LHR-based peptides with the extreme N terminus of the core coiled coil are vital to inhibitory function. Nevertheless, interactions between the N-terminal end of the inhibitory peptide and the coiled coil are also required for optimal activity. The implications for inhibitor design are discussed.

**EXPERIMENTAL PROCEDURES**

**Cells and Plasmids**—HeLa, 293T, and HOS cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Plasmids pHTE-1 (27), pMAL-gp21fishhook (MBP-Fishhook), pMAL-gp21hairpin (MBP-Hairpin) and pMAL-STOP (MBP-Stop) (9) and pNL4-3.Luc.R+E (28) (generously provided by Dr. Nathaniel Landau through the AIDS Research and Reference Reagent Program) have been previously described.

**Peptide Synthesis**—Peptides (Table 1) were synthesized using standard solid-phase Fmoc chemistry and have acetylated N termini and amidated C termini. The peptides were purified by reverse-phase high pressure liquid chromatography and verified for purity by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. All peptides were dissolved in DMSO. Where possible, peptide concentration was confirmed by absorbance at 280 nm in 6 M guanidine hydrochloride, and peptides were used at the final concentrations indicated. In all experiments shown each replicate was carried out using separate peptide stock solutions to allow for slight variations in peptide concentration for those peptides with no absorbance at 280 nm. In addition, far UV absorbance values were used to

**TABLE 1**

| Peptide  | Amino acid position | Sequence | MW |
|----------|---------------------|----------|----|
| Pcr-400  | gp21 400–429        | CCPLNITNSHVSLERQPLPLRVLGL | 3411 |
| Pcr-ΔN   | gp21 412–429        | I---------- | 2901 |
| Pcr-ΔC   | gp21 400–419        | C---------- | 2326 |
| Pcr-Δ8   | gp21 403–424        | L---------- | 2583 |
| Pcr-ΔCys | gp21 401–429        | C---------- | 3350 |
| Pcr-ΔT   | gp21 401–427        | C---------- | 3179 |
| Pcr-Δ5   | gp21 401–425        | C---------- | 2936 |
| Pcr-Δ16A | gp21 401–423        | C---------- | 2721 |
| Pcr-Δ22A | gp21 401–423        | C---------- | 2635 |
| Pcr-Δ17G | gp21 401–423        | C---------- | 2680 |
| Pcr-Δ18G | gp21 401–423        | C---------- | 2680 |
| C34      | gp21 627–661        | CA--------- | 4418 |
assess solubility and concentration given that peptide bonds absorb strongly between 190 and 225 nm with little variation in response between different proteins (29). For these measurements, stock peptides were dissolved in organic solvent as DMSO absorbs strongly in the far UV region. Samples were centrifuged before UV analysis to remove any insoluble peptide, and values at 310 nm were also taken to check for the possibility of light-scattering artifacts.

**Determination of Peptide Solubility**—Serial dilutions (2-fold) of stock peptide in DMSO were performed with filtered PBS to a total volume of 200 μl and a final DMSO concentration of 1.5% in duplicate wells of a 96-well microplate. Plates were incubated at room temperature for 1 h, and the relative solubility of peptides was established by measuring forward scattered light using a NEPHELOstar laser-based microplate nephelometer (BMG LABTECH). Wells containing PBS, 1.5% DMSO were used as blanks. Data analysis was carried out using ActivityBase, and peptides giving readings up to and including 3-fold higher than the average reading for the DMSO control were considered to be in solution at the concentrations specified.

**Circular Dichroism (CD) Measurements**—To examine the secondary structures of the peptides and their propensities to form α-helices, CD measurements were obtained in both aqueous buffer and buffer containing 50% (v/v) trifluoroethanol (TFE) at 20 °C on a Jasco J-600 spectropolarimeter. Spectra were recorded using peptide concentrations of 0.3 mg/ml in a 0.02-cm path length quartz cuvette. The results were analyzed using the CDSSTR procedure hosted by DICHROWEB (30, 31).

**Expression and Purification of MBP-TM Proteins**—Expression and purification of the MBP-TM peptides was carried out as previously described (9). The oligomerization status of the protein chimera was assessed by Superdex 200 gel filtration chromatography in PBS (data not shown; see Ref. 9). Protein concentration was estimated by Bradford assay, and the recombinant protein was stored at −80 °C in PBS supplemented with 20% glycerol.

**Peptide Competition Binding Assays**—The ability of peptides to bind to the core coiled coil was examined using a previously described competition binding assay (20). 96-Well microtiter plates (NUNC MAXI-Sorp) were coated overnight at 4 °C with MBP-Fishhook (recombinant core coiled coil; 10 μg/ml) or control protein (MBP, or MBP-Hairpin) diluted in PBS, pH 7.2. Plates were washed twice with wash buffer (PBS, 0.2% Tween 20) and blocked with blocking buffer (5% Marvel in wash buffer) for 1 h at room temperature. The biotinylated peptide Bio-Pcr-400 was prepared as a stock solution in wash buffer containing 10 mM dithiothreitol (DTT) such that the final DTT concentration in the wells would be 5 mM when an equal volume of competing peptide/buffer was added. Bio-P<sup>cr</sup>-400 was incubated with the immobilized core coiled coil in the presence or absence of peptide competitors at room temperature for 1 h. Plates were washed (×5) in wash buffer to remove unbound peptide, incubated for 1 h with 100 μl streptavidin–horseradish peroxidase conjugate (Sigma) (1:10,000 dilution) at room temperature. Plates were then washed (×5) in wash buffer to remove unbound streptavidin–horseradish peroxidase and washed again (×2) in PBS to remove residual detergent. Bound streptavidin–horseradish peroxidase and, therefore, bound Bio-P<sup>cr</sup>-400 were detected using 2,2′-azino-bis-(3-ethylbenzthiazole-

**Viral Pseudotyping Assays**—293T cells at 70% confluence were co-transfected with equal quantities of pHTE-1 and pNL4-3.Luc.R-E using transIT<sup>®</sup>-LT1 (Mirus). After 24 h the supernatant was removed, and fresh media containing 10 mM sodium butyrate was added. After 18–20 h, the supernatant was removed, and the cells were washed once with media and incubated for 48 h in fresh media without sodium butyrate. Virus-containing supernatant was collected by centrifugation at 2500 rpm (Heraeus Instruments Labofuge 400) for 5 min followed by filtration through a 0.22 μm filter to remove cell debris. 300 μl of this supernatant was then added to 5 × 10<sup>5</sup> HOS cells in the presence or absence of peptides at the concentrations specified. The cells were incubated at 37 °C for 24 h before the supernatant was removed and replaced with fresh media. The cells were incubated for a further 24 h to maximize retroviral gene expression, washed with PBS, and lysed using cell lysis buffer (Promega). Luciferase activity was determined for each lysate using the Promega luciferase assay system and a TD-20/20 luminometer (Turner Designs). Luciferase data were normalized according to lysate protein concentration as determined by Bradford assay.

**RESULTS**

**Extensive Truncation of P<sup>cr</sup>-400 Reduces Potency**—P<sup>cr</sup>-400 is a 30-amino acid synthetic peptide modeled on residues 400–429 of the HTLV-1 envelope glycoprotein. The peptide is an effective inhibitor of HTLV-1 envelope-catalyzed membrane fusion, and it fulfils this function by binding to the core coiled coil of HTLV-1 TM (9). Recently, we demonstrated that truncation of P<sup>cr</sup>-400 by 12 amino acids at the N terminus (P<sup>cr</sup>-ΔN) or alternatively by 10 amino acids at the C terminus (P<sup>cr</sup>-ΔC) produced peptides with radically reduced inhibitory properties (21). The truncated peptides failed to inhibit HTLV-1 membrane fusion and syncytium formation even at concentrations up to 10-fold higher than the IC<sub>50</sub> of the parental peptide P<sup>cr</sup>-400. The truncated peptides display substantially reduced solubility in aqueous solution but are soluble at concentrations equivalent to and 10-fold greater than the IC<sub>50</sub> of the parental peptide P<sup>cr</sup>-400 (Table 2). Therefore, it is likely that the trunc-
HTLV-1 LHR-mimetic Entry Inhibitors

cated peptides failed to inhibit due to relatively weak binding to the core coiled coil of HTLV-1 TM. Because identification of shorter bioactive peptides is an important first step in the development of novel more "drug-like" anti-HTLV-1 peptides, the inhibitory properties of the truncated peptides were examined over a considerably extended concentration range. Syncytium interference assays revealed that peptide Pcr-ΔC does retain some inhibitory activity, albeit with a projected IC_{50} > 200-fold, greater than that of Pcr-400 (Fig. 2A); by comparison, Pcr-ΔN displayed no significant inhibitory activity even at 100 μM (data not shown). These data indicate that the extended N-terminal leash and the α-helical region of the mimetic peptide possess intrinsic inhibitory activity but that amino acid residues within the C-terminal leash are essential for full activity and likely contribute to the stability of peptide binding to its target.

In an effort to design a minimal peptide that retains inhibitory potency, a short peptide that lacks residues at both the N and C terminus was synthesized. Toward the N terminus Ile-405 makes a significant contribution to the binding of Pcr-400 to a recombinant HTLV-1 coiled coil and is also required for optimum inhibitory activity (21). Moreover, examination of the structure for the HTLV-1 trimer-of-hairpins indicates that the side chains of His-409 and Asn-407 participate in hydrogen bonding of the LHR to the coiled coil. We reasoned that loss of these interactions would significantly reduce inhibitory activity. Therefore, only three amino acids from the N-terminal end of the original peptide were omitted from the truncated peptide, thereby removing two cysteines and a phenylalanine. There is no structural information after Asn-421 of the HTLV-1 trimer-of-hairpins (18). Consequently, it is not possible to predict with confidence how truncations from the C-terminal end will affect binding of the peptide to the coiled coil. However, of the amino acids after Asn-421, only Leu-424 has been shown to contribute to inhibitory activity (21). Therefore, all five residues after Leu-424 were omitted from the abridged peptide.

The derived peptide, designated P^{P\alpha\beta}-ΔB (Table 1), was then tested for inhibition of envelope-mediated membrane fusion in syncytium interference assays. Given that all residues known to be important for inhibitory activity were retained, it was surprising that, although more potent than Pcr-ΔC, Pcr-ΔB with an estimated IC_{50} of 30 μM displayed a dramatic drop in potency (Fig. 2A) and solubility (Table 2) relative to P^{P\alpha\beta}-400, which has an IC_{50} of 0.3 μM (Table 2). However, P^{P\alpha\beta}-ΔB was less soluble in tissue culture medium than the parental peptide and began to precipitate out of solution at concentrations above ~30 μM. Examining the ability of P^{P\alpha\beta}-ΔB to bind to an immobilized coiled coil showed that it is able to compete with a biotinylated version of the parental peptide, Bio-P^{P\alpha\beta}-400, more effectively than P^{P\alpha\beta}-ΔC (Fig. 2B). However, in the buffer used in the binding assay, P^{P\alpha\beta}-ΔB started to precipitate at concentrations above 27 μM (Table 2).

The N-terminal Residue of P^{P\alpha\beta}-400 Is Dispensable for Inhibitory Activity—In view of the poor activity and solubility of P^{P\alpha\beta}-ΔB, we sought to identify residues that were critical to activity and solubility. We have demonstrated that the cysteine residues are not essential to the inhibitory properties of the peptides as a peptide with both N-terminal cysteine residues substituted with alanine retains

### TABLE 2

| Peptide     | Syncytium interference IC_{50} \( \mu M \) | Viral entry IC_{50} \( \mu M \) | Maximum solubilitya  |
|-------------|---------------------------------------------|-------------------------------|----------------------|
| P^{P\alpha\beta}-400 | 0.31 ± 0.01 | 0.18 ± 0.01 | >90.0 |
| P^{P\alpha\beta}-ΔC | >50 | ND | 11.2 |
| P^{P\alpha\beta}-ΔN | 0.33 ± 0.04 | ND | >90.0 |
| P^{P\alpha\beta}-ΔCys | 0.33 ± 0.01 | ND | >90.0 |
| P^{P\alpha\beta}-Δ3 | 0.29 ± 0.01 | ND | >90.0 |
| P^{P\alpha\beta}-Δ5 | 0.32 ± 0.01 | ND | 22.5 |
| P^{P\alpha\beta}-Δ7 | 0.28 ± 0.01 | 0.19 ± 0.01 | 22.5 |
| P^{P\alpha\beta}-R416A | 16.31 ± 1.69 | 1.55 ± 0.08 | 45.0 |
| P^{P\alpha\beta}-R422A | >50 | ~8.50b | 45.0 |
| P^{P\alpha\beta}-P417G | 0.30 ± 0.01 | ND | >90.0 |
| P^{P\alpha\beta}-P418G | 0.33 ± 0.01 | ND | >90.0 |
| P^{P\alpha\beta}-F402A | 0.29 ± 0.01 | ND | >90.0 |
| C34 | N/A | N/A | >90.0 |

a Maximum solubility in aqueous solution determined by laser nephelometry.

b These peptides did not reach maximum inhibition at soluble concentrations of peptide.

### FIGURE 2

**A** Some truncated LHR-mimetic peptides exhibit attenuated inhibitory activity. A comparison of the potency of P^{P\alpha\beta}-400, P^{P\alpha\beta}-ΔC, and P^{P\alpha\beta}-ΔB in syncytium interference assays. HeLa cells expressing HTLV-1 envelope were cocultured with non-transfected HeLa cells in the presence of peptide at the concentrations shown. Syncytia were scored. Data are the mean ± S.D. from a typical experiment performed in triplicate. C34, an irrelevant peptide inhibitor of HIV entry, was used as a negative control. **B**, the ability of the peptides used in A to compete with labeled P^{P\alpha\beta}-400 for binding to a recombinant coiled coil. Biotinylated P^{P\alpha\beta}-400 (Bio-P^{P\alpha\beta}-400) at a constant concentration was incubated with immobilized core coiled coil (MBP-Fishhook) in the presence of competing peptides at the concentrations shown. The ΔCys peptide derivative inhibits HTLV-1 envelope-mediated syncytium formation (C) and blocks binding of Bio-P^{P\alpha\beta}-400 to immobilized core coiled coil (D). All data are the means ± S.D. from assays performed in triplicate.
full potency (21). By contrast, removal of the first two cysteines and the phenylalanine from the N terminus of a similar peptide inhibitor of bovine leukemia virus envelope-catalyzed membrane fusion has a significantly detrimental effect on the ability of the bovine leukemia virus-derived peptide to inhibit (19). Examination of the HTLV-1 trimer-of-hairpins structure and reassessment of published data (9, 18, 21, 33) suggest that, although the side chains of Cys-400, Cys-401, and Phe-402 make little contribution to the binding of the LHR to the coiled coil, removal of the three N-terminal residues results in loss of two polar contacts between the main chain of the peptide and the coiled coil and also removes an intra-peptide hydrogen bond. Based on these observations, the first cysteine (Cys-400) was removed from the peptide, and this peptide, designated Pcr−H9004 Cys, was tested for solubility and activity in syncytium interference and competition binding assays. Pcr−H9004 Cys was just as potent and soluble as Pcr−400 in these assays (Fig. 2, C and D; Table 2), confirming that Cys-400 is not required for effective inhibition of HTLV membrane fusion.

**Peptides Bearing Progressive Deletions of up to Six C-terminal Amino Acids Maintain Full Potency**—To retain the putative polar contacts made between the backbone of the inhibitory peptide and the interacting surface of the coiled coil, no further truncations from the N terminus of the peptide were made. Instead, based on the Pcr−H9004 Cys peptide, three additional peptides were synthesized with progressive C-terminal truncations of two, four, and six amino acids (Table 1). Given the accompanying removal of the single cysteine residue from the N terminus, these peptides were designated Pcr−Δ2, Pcr−Δ5, and Pcr−Δ7. When tested in syncytium interference assays, all three peptides were able to inhibit syncytium formation with calculated IC_{50} values essentially identical to that of the original peptide, Pcr−400 (Fig. 3A, Table 2). Therefore, all of the residues that are essential for inhibitory activity are contained within the first six C-terminal amino acids of the LHR-mimetic peptides.

**FIGURE 3.** Peptides with deletions of up to six residues maintain potency, but basic residues are essential to inhibitory activity. A, the ability of the peptides Pcr−400, Pcr−Δ3, Pcr−Δ5, and Pcr−Δ7 to inhibit HTLV-1 envelope catalyzed syncytium formation. All peptides were equally as potent as Pcr−400, and C34 was used as a negative control. B and C show the ability of the same peptides to interfere with the binding of biotinylated Pcr−400 to MBP-Fishhook. D, the peptides Pcr−Δ7 and its derivatives Pcr−R416A and Pcr−R422A bearing arginine to alanine substitutions were compared in syncytium interference assays. E, a model of the interaction of Arg-416 from the region mimicked by the peptides (yellow, sticks) with Asp-351 of the core coiled-coil (blue and red translucent space-filling model, residues-lining groove into which the C-terminal binds are shown as sticks). The bifurcated hydrogen bond is shown as a dashed line, and the arrowhead marks Leu-419. F, the ability of Pcr−Δ7, Pcr−R416A, and Pcr−R422A to block labeled Pcr−400 from binding to immobilized recombinant coiled coil was assessed.
required for maximal inhibitory activity are located within a 23-amino acid region spanning peptide residues Cys-401 to Val-423. Moreover, in competition binding assays, although Pcr-Δ3 was just as effective as Pcr-400 in competing for core-coiled-coil binding, both Pcr-Δ5 and Pcr-Δ7 were slightly less effective competitors than Pcr-400 (Fig. 3, B and C), implying that the two residues at the C terminus of Pcr-Δ3, a glycine and a tryptophan, make a modest contribution to binding of the peptide to the immobilized coiled coil. However, when deleted, the contribution of these residues to binding is insufficient to register any change in potency in assays of membrane fusion.

Basic Residues Are Critical for Potent Inhibition of Membrane Fusion—The observation that Pcr-Δ7 was able to inhibit envelope-mediated membrane fusion as effectively as Pcr-400 was surprising; the peptide Pcr-Δ5-AC is only four residues shorter at the C terminus than Pcr-Δ7, and yet Pcr-Δ5-AC is ~200-fold less potent than Pcr-400 in syncytium interference assays. Of the four C-terminal residues retained in Pcr-Δ7, one is an arginine equivalent to Arg-422 of envelope. Considering the size and charge of the arginine side chain, we elected to substitute it with alanine in the context of Pcr-Δ7 to yield Pcr-R422A. This single substitution had a dramatic effect on the potency of the peptide in syncytium interference assays, giving an increase in IC50 relative to Pcr-Δ7 of >130-fold (Fig. 3D, Table 2). In support of the syncytium interference data, Pcr-R422A also displayed a marked reduction in capacity for binding to the recombinant core coiled coil (Fig. 3F) in the competition binding assay. To examine further the role played by charged residues in the interaction of the LHR-mimetic peptide with the coiled coil, an additional peptide was synthesized in which the arginine residue equivalent to Arg-416 in HTLV-1 TM was substituted with alanine to yield Pcr-R416A. The crystal structure of the HTLV-1 trimer-of-hairpins shows that Arg-416 is at the C-terminal end of the short α-helical region, and it extends toward the coiled coil where it can form hydrogen bonds with the coiled coil residue Asp-351 (Fig. 3E). When tested for inhibitory activity in syncytium interference assays, Pcr-R416A was less potent than Pcr-Δ7 by ~60-fold (Fig. 3D). Therefore, although Arg-416 is important for potent antagonism of membrane fusion, it is somewhat less crucial than Arg-422. Concomitant with this, Pcr-R416A was more effective at competing with biotinylated Pcr-400 than Pcr-R422A (Fig. 3F). Taken together, these results show that the arginine residues located in the C-terminal region of the peptide make an enormous contribution to the affinity of the peptide for the coiled coil and, hence, the inhibitory potency of the peptide. Notably, the increase in IC50 that is observed when either of these arginine residues is substituted by alanine is dramatically more significant than the increase observed when any one of the functionally important leucine residues is substituted.

Substitution of Conserved Proline Residues Does Not Adversely Affect Inhibitory Activity—At the end of the short α-helical segment of the LHR-mimetic peptides, there is a helix-breaking di-proline motif (Fig. 1B). Interestingly, the critical Arg-416 residue is situated immediately before the first proline, and immediately after the second proline there is a functionally important leucine, Leu-419, that makes non-polar contacts with a pocket on the core coiled coil (Fig. 1B). The presence of the di-proline motif is likely to increase the rigidity of the peptide backbone in this region and may induce a tight kink within the peptide chain. Therefore, although neither proline residue appears to make a direct contribution to binding with the coiled coil, they may facilitate accurate positioning of Leu-419 and Arg-422 relative to Arg-416 and the rest of the LHR, thereby ensuring optimum interaction with the coiled coil. To test whether these proline residues are required for docking of the peptide with the coiled coil, two peptides, Pcr-P417G and Pcr-P418G, were synthesized in which the respective prolines were individually substituted with glycine residues. Glycine substitutions were chosen rather than alanine residues to assess the effect of introducing maximum flexibility at these positions in contrast to the more constrained proline residues. When Pcr-P417G and Pcr-P418G were examined for their ability to block syncytium formation, they were both equally as potent as Pcr-Δ7 (Fig. 4A, Table 2); therefore, neither proline is essential for maximum inhibitory activity, and introducing greater flexibility at position 417 or 418 has little effect on inhibitory potency. Moreover, when the peptides were assayed for their ability to compete with Bio-Pcr-400 for binding to the recombinant core coiled coil, it was observed that Pcr-Δ7 was just as active as Pcr-Δ7, but Pcr-418G showed a slight but reproducible decrease in dose-dependent response (Fig. 4B). Therefore, a proline at residue 18 of the mimetic peptide may provide a slight advantage to peptide binding to the core coiled coil in vitro, but in the cell-based assays of envelope-mediated membrane fusion any modest impairment of peptide antagonist function due to replacement of the proline with glycine was not detectable.

The Side Chain of Phenylalanine 402 Does Not Contribute to Peptide Binding—In view of the data illustrating the importance of Arg-422 to the potency of the peptide, it was surprising...
that P<sup>cr</sup>-Δ8 was such a poor antagonist of envelope-mediated membrane fusion. P<sup>cr</sup>-Δ8 differs from the fully functional peptide P<sup>cr</sup>-Δ7 only by the removal of a cysteine (Cys-400) and phenylalanine (Phe-402) at the N terminus and by the addition of a leucine (Leu-424) at the C terminus. The two cysteine residues at the N terminus of the peptide are not necessary for optimal antagonism of membrane fusion or for coiled-coil binding, as both cysteines can be replaced with alanine without loss of peptide function (21). Moreover, for the HTLV-1 trimeric of-hairpins, the crystal structure (18) and supporting data (33) indicate that the side chain of Phe-402 faces away from the coiled coil and is unlikely to make any significant contribution to the interaction of the LHR with the coiled coil (Figs. 1B and 5A). However, given the likely orientation of Phe-402 and the hydrophobic properties of phenylalanine, it was reasoned that this residue might negatively impact on peptide activity. Therefore, in the context of the P<sup>cr</sup>-Δ7 peptide a further substitution was made replacing Phe-402 with alanine. As anticipated, the derived peptide (P<sup>cr</sup>-F402A) proved to be as effective as P<sup>cr</sup>-Δ7 in syncytium interference assays and in coiled-coil binding assays (Fig. 5, B and C), confirming that the side chain of Phe-402 is not required for binding of the peptide to the core coiled coil. However, P<sup>cr</sup>-F402A displayed significantly improved solubility characteristics in aqueous solution (Table 2). Taken together, the accumulated data are consistent with the view that the interactions between the N-terminal amino acids of the inhibitory peptide and the coiled-coil target occur not through the amino acid side chains but are instead mediated by interactions with the main peptide backbone.

Secondary Structure Analysis—We have previously demonstrated that the α-helical region of the LHR-mimetic peptide contributes to inhibitory activity (9). Because several of the peptides used in this study display reduced potency, we examined each peptide for the ability to adopt α-helical structures. CD spectroscopy revealed that, in aqueous and TFE buffers, peptides that effectively inhibit syncytium formation and viral entry invariably display spectral features similar to the prototypic peptide P<sup>cr</sup>-400, whereas poorly active peptides with large deletions had radically different profiles (Fig. 6, A–D). Analysis of P<sup>cr</sup>-400 confirmed that the peptide has a strong tendency to adopt α-helical structures in TFE-containing buffers (Fig. 6A; Ref. 9), and all of the highly active peptides including, for example P<sup>cr</sup>-Δ7, share this property (Fig. 6, A and D). Secondary structure estimates obtained using the CDSSTR algorithm on the DICHROWEB server (31) gave values for α-helix in the range of 30–35% for the bioactive peptides. Peptides P<sup>cr</sup>-ΔC and P<sup>cr</sup>-ΔN, which are significantly less effective inhibitors of syncytium formation and viral entry, exhibited reduced ability to form α-helices with typical estimates between 14 and 16%. By contrast, the α-helical content of P<sup>cr</sup>-R422A (34%) was comparable to P<sup>cr</sup>-400, and the peptide P<sup>cr</sup>-Δ8 (37%) also showed a strong tendency to form helical elements. Nevertheless, both P<sup>cr</sup>-R422A and P<sup>cr</sup>-Δ8 are ineffective inhibitors. These results highlight the importance of the arginine interaction with the coiled-coil target and indicate that the defect is not due to a radically altered peptide structure. Moreover, it is interesting to note that, in a similar manner to P<sup>cr</sup>-400, P<sup>cr</sup>-Δ8 was able to form α-helical structures in the presence of TFE; however, the spectral profile in the far UV region suggests that there are structural differences between these peptides. Such differences may preclude effective interaction of P<sup>cr</sup>-Δ8 with the coiled coil.

A Core Peptide Is a Potent Inhibitor of Viral Entry and Requires Basic Residues for Activity—Cell-based syncytium interference assays provide a robust, effective, and semiquantitative method of determining the inhibitory activity of the synthetic LHR-mimetic peptides. To support the inhibition of membrane fusion assays, the effectiveness of the peptides in blocking entry of free virus particles into cells was examined. To this end luciferase-transducing HIV-1 particles produced from pNL4.3R<sup>E</sup> luc-transfected 293T cells and pseudotyped with the envelope from HTLV-1 were used to infect target HOS cells in the presence or absence of the test peptides. The transduced luciferase activity in cell lysates was subsequently determined.

Importantly, in these assays the core peptide, P<sup>cr</sup>-Δ7, was just as effective as the parental peptide P<sup>cr</sup>-400 in blocking viral entry into cells (Fig. 7, Table 2). Moreover, the peptides in which specific arginine residues were replaced by alanine were profoundly impaired in their ability to inhibit viral entry as was the highly truncated peptide P<sup>cr</sup>-Δ8. The relative potency of the peptides in antagonizing viral entry correlated with their activity in the syncytium interference assays, indicating that disruption of membrane fusion is a reliable indicator of the peptide capacity to block viral entry (Fig. 7). Notably, peptides that displayed impaired activity in syncytium interference assays tended to do significantly better in the viral entry assay, such that P<sup>cr</sup>-R416A, P<sup>cr</sup>-Δ8, and P<sup>cr</sup>-R422A were ~5–10-fold more...
HTLV-1 LHR-mimetic Entry Inhibitors

**FIGURE 6.** Bioactive peptides adopt \( \alpha \)-helical structures. CD analysis of typical active and inactive peptides used in this study: A, P\(^{19-400}\); B, P\(^{19-400} \Delta N\); C, P\(^{19-400} \Delta C\); D, P\(^{19-400} \Delta 7\); E, P\(^{19-400} \Delta 8\); F, P\(^{19-400} \Delta 8\). In each case the solid curve represents the far UV spectroscopic profile of the peptide in aqueous buffer, whereas the dashed line is the curve for the peptide in TFE-containing (50% v/v) buffer.

**FIGURE 7.** A core LHR-mimetic peptide efficiently blocks entry of viral particles. Comparison of the capacity of selected peptides to block entry of luciferase-transducing HTLV-1 envelope-pseudotyped virus particles is shown. HOS cells were infected with virus in the presence of peptides at the concentrations specified. The cells were incubated for 48 h, the HOS cells were washed and lysed, and luciferase assays were performed. The data are the means ± S.D. from a triplicate assay. AU, arbitrary units.

effective in antagonizing entry of pseudotyped viral particles (Table 2). Moreover, the data derived from the coiled-coil binding data correlate more closely with the viral entry assays than they do with the syncytium formation assays. Taken together, the data reveal that a core region of the LHR-mimetic peptide is sufficient to antagonize viral entry and that infection by free particles is more susceptible to inhibition than syncytium formation. Furthermore, basic residues in or adjacent to the C-terminal leash are critical determinants of peptide potency for both envelope-mediated membrane fusion and viral entry.

**DISCUSSION**

Envelope-mediated membrane fusion is beginning to be understood in molecular detail for many viruses, and a key objective is to use the emerging information to develop therapeutically practical inhibitors of viral entry. For entry inhibitors based on the C-terminal region of the trimer of hairpins, smaller, soluble, more bio-available peptide inhibitors or peptide-mimetics are highly desirable. As a step toward this objective we have identified a compact region of the HTLV-1 LHR-mimetic peptide that exhibits highly effective inhibition of fusion, and we identify features that currently limit the nominal
size of peptide antagonists. Moreover, we provide further evidence that the inhibitory capacity of these peptides is governed principally by interactions with a relatively small membrane-proximal region of the core coiled coil.

Although shorter by only 3 residues at the N terminus and 5 residues at the C terminus, the peptide derivative P35-Δ8 was ~100-fold less potent than P35-400. This loss of activity is due to removal of critical amino acid residues at each end of the peptide. The N-terminal cysteine is not required for inhibitory activity, and peptides in which both cysteines are replaced with alanine are functional. Nevertheless, removal of these three conserved N-terminal residues from a related peptide inhibitor of bovine leukemia virus entry inactivates the peptide (19).

Analysis of the HTLV-1 trimer-of-hairpins structure reveals that the loss of three residues from the N terminus of the LHR-mimetic peptide results in loss two possible polar contacts between the main chain of the peptide and the coiled coil as well as an intra-peptide hydrogen bond (Fig. 5A). Therefore, the interaction of the three N-terminal residues of the peptide is not sequence-specific but is instead dependent only on interactions between main chain and coiled coil. Such interactions may help to orientate the peptide relative to the coiled coil, and loss of these interactions may adversely influence the structure of the peptide (Fig. 6).

Deletions at the extreme C terminus are relatively well tolerated, and the residues LTGWGL do not contribute significantly to inhibitory activity. However, although P35-Δ7 is able to inhibit syncytium formation, P35-ΔC has an IC50 calculated to be >150-fold higher than that of P35-Δ7. At the C terminus these peptides differ by only four residues (ENRV); of these, only two are resolved in the crystal structure of the HTLV-1 trimer-of-hairpins (18). Glu-420 makes a hydrogen bond with Lys-344 at the membrane-proximal end of the coiled coil, but the side chain of Asn-421 projects out into solvent and is unlikely to contribute to binding of LHR-derived peptides. Of the remaining residues, the large size, charge, and therefore potential for hydrogen binding suggested that Arg-422 would play a significant role in stabilizing the interaction of the peptide with the coiled coil. Indeed, in the context of the LHR-mimetic, substitution of Arg-422 with alanine produced an inhibitor that was >150-fold less potent than P35-400 and bound poorly to the core coiled coil. Thus, Arg-422 plays a critical role in stabilizing the binding of the peptide and, therefore, the LHR with the coiled coil. However, this single substitution did not reduce peptide function as much as the truncation in P35-ΔC, suggesting that to a lesser extent Glu-420 and Val-423 also contribute to binding.

Our data reveal that another basic residue (Arg-416) also contributes substantially to inhibitory activity and to the stability of peptide binding. Arginine 416 is situated at the C-terminal end of the short α-helix within the region mimicked by our peptides, and it makes a bifurcated hydrogen bond with Asp-351 of the core coiled coil immediately adjacent to the deep pocket into which Leu-419 of the peptide binds (Fig. 3E) (18, 21). We observed a 60-fold increase in IC50 associated with the R416A substitution in syncytium interference assays. Given that the highest single drop in potency observed when specific leucine residues are substituted is ~25-fold (21), it is clear that the interactions of charged residues Arg-416 and Arg-422 are more critical to the binding of the peptide to the coiled coil. Currently, Arg-422 is not resolved in the structure of HTLV-1 TM (18), and it is, therefore, difficult to predict with any accuracy the way in which this residue interacts with the coiled coil. However, a clear understanding of this interaction is likely to be critical to the success of future antiviral strategies that target the coiled coil of HTLV-1 TM.

A key issue in the design of smaller more bioactive peptides is whether the α-helical segment of the LHR contributes to the activity of the peptide. Our data demonstrate that peptides that are unable to form stable α-helical elements are poor inhibitors of envelope function. However, the ability to form such α-helices is not sufficient for inhibitory activity, as one peptide P35-Δ8 has a strong tendency to form an α-helix but displays little or no activity. Furthermore, the data reveal that the failure of peptides with substitutions of basic residues is not due to radical changes in peptide structure, as CD analysis indicates that such peptides display identical spectroscopic profiles to the highly active parental peptide P35-400.

The presence of two proline residues in the C-terminal leash of TM seems to function to break the short α-helix and allow Leu-419 to dock in the correct orientation. However, we found that substitution of either proline with a glycine residue has little impact on peptide potency. Although such substitutions can be accommodated in the peptide inhibitors, this may reflect an intrinsic flexibility of the short peptides, allowing the peptide to dock with the target site through an induced fit process. If this is indeed the case, atypical peptide inhibitors that display functionally important non-helical segments may be particularly amenable to incorporation of non-natural amino acids or adducts to rationally improve the biological and pharmacological properties of the inhibitors. Further work to test these ideas is currently in progress.

Transmission of HTLV-1 involves both cell-to-cell transfer of viral particles via virally induced synapses and direct entry of free viral particles into dendritic cells (25, 26, 34). Intriguingly, we have found that peptides that are poor inhibitors of syncytium formation are substantially more effective at blocking entry of viral particles. This may be due to the difficulty of directing sufficient concentrations of relatively low affinity peptides to the points of intimate cell contact and high envelope density that are likely found on cells undergoing syncytium formation. Notably, in cells undergoing synaptic transfer of viral particles, the membranes of the interacting cells are also closely apposed, and high levels of envelope can be detected in the area bounding the synaptic cleft (25, 34). It will, therefore, be of significant interest to determine whether virions undergoing synaptic transfer exhibit reduced susceptibility to peptide inhibitors compared with infection by free virus.

HTLV-1 infections have a significant clinical impact. HTLV-1 causes the aggressive lymphoproliferative disorder, adult T cell leukemia, and also a progressive neurodegenerative disease known as HTLV-1-associated myelopathy or tropical spastic paraparesis (35–37). The persistence of viral replication in these diseases suggests that compounds inhibiting the entry and cell-cell transfer of HTLV-1 may be of therapeutic utility. Our study emphasizes the significance of contacts mediated by
the C-terminal leash and α-helical regions of a peptide inhibitor of HTLV-1 envelope-mediated membrane fusion and identifies two basic residues that are critical to inhibitory activity. Understanding the way in which these residues contribute to inhibitory peptide function may lead to pharmacologically relevant inhibitors of viral entry. Furthermore, the current LHR-based peptide inhibitors require contacts mediated by the main chain of the peptide for optimal inhibition. We suggest that structure-assisted design of more potent peptides that do not require these contacts may be a viable objective on the route to a small molecule inhibitor of HTLV-1 entry.

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