Probing the metastable state of influenza hemagglutinin

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Viral entry into host cells is mediated by membrane proteins in a metastable state that transition to a more stable state upon a stimulus. For example, in the influenza envelope protein hemagglutinin (HA), the low pH in the endosome triggers a transition from the metastable prefusion conformation to the stable fusion conformation. To identify probes that interfere with HA function, here we screened a library of H7 HA peptides for inhibition of H7 HA-mediated entry. We discovered a peptide, PEP87 (WSYNAELLVAMENQHTI), that inhibited H7 and H5 HA-mediated entry. PEP87 corresponds to a highly conserved helical region of the HA2 subunit of HA that self-interacts in the neutral pH conformation. Mutagenesis experiments indicated that PEP87 binds to its native region in the HA trimer. We also found that PEP87 is unstructured in isolation but tends to form a helix as evidenced by CD and NMR studies. Fluorescence, chemical cross-linking, and saturation transfer difference NMR data suggested that PEP87 binds to the neutral pH conformation of HA and disrupts the HA structure without affecting its oligomerization state. Together, this work provides support for a model in which PEP87 disrupts HA function by affecting its oligomerization state. Moreover, our observations indicate that the HA prefusion structure (and perhaps the metastable states of other viral entry proteins) is more dynamic with transient motions being larger than generally appreciated. These findings also suggest that the ensemble of prefusion structures presents many potential sites for targeting in therapeutic interventions.

The membrane envelope proteins of viruses play critical roles in the entry of diverse viruses, including Ebola, HIV, influenza, and SARS2 (1–3). In general, the entry of enveloped viruses comprises three steps: (i) receptor binding by the envelope protein, (ii) triggering of large conformational changes in the envelope protein to expose a hydrophobic region termed the fusion peptide, and (iii) insertion of the fusion peptide into the target membrane and subsequent fusion of the viral and target cell membranes promoted by the final conformation of the envelope protein. Interestingly, before attachment, the viral envelope proteins exist in a metastable prefusion conformation that transitions to a more stable fusion conformation upon a triggering event (1, 4, 5) in which the trigger is receptor binding (e.g. HIV; Ref. 2), proteolysis (e.g. SARS; Ref. 3), or pH (e.g. influenza; Ref. 4). Finally, we note that the critical nature of envelope proteins makes them attractive targets for therapeutic intervention, particularly because of their extracellular nature, multiple target conformations during the entry process, and action prior to cellular infection (2).

In the case of influenza, the envelope protein hemagglutinin (HA) is expressed as precursor HA0, a trimer of identical subunits with each subunit anchored to the membrane by a C-terminal transmembrane domain (4–6). Subsequently, HA0 is cleaved and processed to form HA1 (comprising residues 1–328) and HA2 (comprising residues 1–211), which are covalently linked by a disulfide bond between HA1 residue 14 and HA2 residue 137 (4–6). During the first step of influenza entry, HA1 binds to sialyllactose receptors on the cell surface, resulting in receptor-mediated endocytosis. Next, the low pH of the endosome triggers a large conformational change in HA2 to expose its fusion peptide. In the final stage of entry, the fusion peptide is inserted into the endosomal membrane, and further conformational changes occur in HA2 to bring the viral and target membranes in close proximity to allow for subsequent fusion. Much is known about the conformational states of HA. For example, there are X-ray structures of HA at neutral pH (i.e. before triggering) and low pH (i.e. after triggering), and strikingly a loop of HA2, which connects the N-helix to the C-helix, becomes helical at low pH and promotes the projection of the fusion peptide toward the target membrane (1, 4, 5). Importantly, the HA studies give valuable insights into the influenza entry process in particular and virus entry in general (4–6) and formed the basis for the concept of metastable states in viral proteins (1, 4, 5).

In this study, we describe efforts to screen a library of peptides from H7 HA for inhibition of H7 HA-mediated entry with the goal of discovering probes of HA function. The further characterization of one peptide inhibitor, PEP87, by virological, biochemical, and biophysical techniques indicates that the peptide binds to its native region in the HA trimer and thus disrupts HA structure and function. Binding of PEP87 to its corresponding region in HA suggests that the neutral pH conformation of HA is more dynamic with relatively large motions than previ-
ously appreciated and that HA may present unexpected sites for therapeutic intervention of influenza entry.

**Results**

**Discovery of peptide probes**

A library of peptides, corresponding to overlapping regions of the H7 HA protein sequence, was screened for inhibition of H7 HA-mediated entry using a pseudovirus entry assay in which virus-like particles (VLPs) are prepared with the influenza proteins HA and neuraminidase in the viral membrane and the HIV core, which also contains the luciferase coding region for a readout assay of entry (7, 8). From the library of 111 peptides assayed at 100 μM, we found six peptides that exhibited 60% inhibition of virus entry and 20% inhibition of VSVG-mediated entry, an unrelated viral envelope protein that allows determination of specificity and cytotoxicity (Fig. 1, a and b). As shown in Fig. 1c, the six best inhibitory peptides are located in diverse regions of the H7 HA structure (Protein Data Bank code 4R8W). The six most potent peptide sequences are highlighted in red on one monomer of the HA trimer.

**Inhibition of H5 and H7 HA entry by PEP87**

Based on amino acid sequences and antigenic properties, HA types are divided into Groups 1 and 2 with H7 HA classified in Group 2 and H5 HA classified in Group 1 (6). Moreover, inhibitors of HA-mediated entry are generally group-specific. For example, inhibitors of Group 1 HA do not inhibit Group 2 HA, and inhibitors of Group 2 HA do not inhibit Group 1 HA (7, 9). Interestingly, further analyses of the six inhibitory peptides, which were synthesized de novo, revealed that one peptide, PEP87, inhibited H5 HA (IC_{50} ~ 120 μM) as well as H7 HA (IC_{50} ~ 84 μM) entry (Fig. 2a) with little cytotoxicity (Fig. 2b). In the native structure, the PEP87 sequence corresponds to part of the C-helix that follows the loop. Comparison of the H5 and H7 HA sequences for the region encompassed by PEP87 revealed a relatively high degree of sequence identity, particularly in the central region of PEP87 (Fig. 2c). Notably, PEP87 exhibits better inhibition of HA harboring an exact sequence match (i.e. H7 HA). Together, these observations are consistent with the notion that PEP87 inhibition occurs by binding to the corresponding region in HA.

**Mutagenesis studies of PEP87 inhibition**

Previously, we generated and characterized a number of H5 HA mutants to elucidate the inhibitory properties of MBX2329, a small-molecule entry inhibitor of Group 1 HA (8). As shown in Fig. 3a, we screened the mutational effects for PEP87 inhibition of H5 HA entry. For these experiments, we chose a PEP87 concentration of 200 μM, which resulted in 70% inhibition of wild-type H5 HA entry. Interestingly, mutants HA1-I28A, HA1-M31A, HA2-T41A, HA2-Q45A, HA2-V52A, HA2-N53A, and HA2-L99A exhibit significantly more sensitivity to inhibition by PEP87, suggesting that the mutations enhance PEP87 binding to HA. In contrast, HA2-Q42A, HA2-T49A, and HA2-M102A exhibit significantly less sensitivity to inhibition by PEP87, suggesting that the mutations disrupt PEP87 binding to HA. As noted above, this set of mutations was previously characterized for their effects on HA expression, processing, receptor binding, and entry in addition to mutational effects on entry inhibition by MBX2329, which binds to the neutral pH conformation of HA and disrupts the fusion step (8). Notably, comparison of the mutational effects on PEP87 inhibition with the mutational effects on HA function or inhibition by MBX2329 did not reveal significant differences.
any apparent correlation. In the next step, we considered the location of the mutation in the H5 HA structure with respect to the PEP87 sequence. As shown by Fig. 3b, the mutations that affect PEP87 inhibition are all located in close proximity to the PEP87 sequence (indeed, HA2-L99 and HA2-M102 are located within the PEP87 sequence) shown in yellow. Taken together, the mutational studies are consistent with the notion that PEP87 disrupts HA function by binding to its corresponding region in the HA trimer.

**Characterization of PEP87 secondary structure**

As discussed above, the region corresponding to PEP87 in HA is part of a helical trimer (Fig. 1b). We next characterized the structural properties of PEP87 by circular dichroism (CD) spectropolarimetry (Fig. 4a) and found that PEP87 exhibits non-regular secondary structure under aqueous conditions; however, in the presence of 40% TFE, a helix-stabilizing agent, PEP87 exhibits ~25% helical structure. Previously, we have characterized a peptide from a similar region of the SARS envelope protein and observed similar behavior under aqueous conditions and in the presence of 40% TFE (10). Interestingly, in the case of the SARS peptide, we observed the formation of helix under aqueous conditions at high peptide concentrations, suggesting an equilibrium between unstructured monomer and a helical trimer (10). In contrast, PEP87 secondary structure does not exhibit a concentration-sensitive transition to helix under aqueous conditions as shown in Fig. 4b. In summary, PEP87 is relatively unstructured in isolation but exhibits a propensity to form helix.

**Characterization of PEP87 tertiary structure**

NMR is a powerful technique to determine the tertiary structure of peptides under aqueous conditions and in the presence of cosolvents or detergents (10–12). Given that the PEP87 sequence is helical within the HA structure and the observation that PEP87 forms a partial helix in the presence of cosolvent, we assigned the $^1$H and $^{13}$C resonances and determined the structure of PEP87 in the presence of 40% TFE. As shown by the
secondary $^{13}$C$_o$ chemical shifts in Fig. 5a, PEP87 exhibits downfield shifts characteristic of helices (13) in the central region of the peptide. As shown by the $^1$H-$^1$H NOESY spectrum in Fig. 5b, PEP87 exhibits a significant number of short-range NOEs between $^1$H$_N$ characteristic of helices. In the next step, the NOE and chemical shift data were used to determine the PEP87 structure (data summarized in Table 1). The resulting structure consists of a continuous helix from residue Asn-4 to His-15 (Fig. 5c). Further examination of the PEP87 structure reveals a negatively charged surface comprising residues Glu-6 and Glu-12 and a hydrophobic surface comprising residues Leu-7, Leu-8, Ala-10, and Met-11 (Fig. 5d). In summary, the helical structure of PEP87 in the presence of cosolvent is similar to the structure of the corresponding region in the neutral pH structure of HA.

**Characterization of PEP87 binding by NMR**

We then characterized the interaction of PEP87 with recombinant HA, the form of HA used in crystallization studies, in which the transmembrane and C-terminal regions have been removed and a C-terminal foldon sequence has been added for stabilization (15). Previously, we as well as others have shown that saturation transfer difference (STD) NMR is a useful technique to characterize the binding of small molecules and peptides to large-molecular-weight viral proteins, including those embedded in viral membranes (7, 8, 16–18). Accordingly, we assayed whether PEP87 binds to recombinant H5 HA using STD NMR. For reference, we show the 1D NMR spectrum of PEP87 in Fig. 6a (the sample is in 100% $^2$H$_2$O, and thus the chemical shifts in Fig. 5a, the positively phased resonances of PEP87 in the STD NMR spectrum are indicative of binding to recombinant H5 HA. Previously, we have shown that STD may be used to detect binding of receptor analogs to HA located in HA1 and demonstrated that this assay could be used to discern ligands binding to the receptor-binding site (17). Accordingly, to probe whether PEP87 binds to the HA1 subunit, we used the STD experiment to show that the addition of PEP87 does not affect the reporter signals of $\alpha$2,3-sialyllactose (Fig. 6c), an HA receptor analog, suggesting that PEP87 binding does not bind to or perturb the receptor-binding site located in HA1. Notably, all of the NMR binding experiments were performed at neutral pH, and thus they present clear evidence of PEP87 binding to the prefusion, neutral pH form of HA.

**Effects of PEP87 binding on HA tertiary and quaternary structure**

We further characterized the interaction of PEP87 with the prefusion form of recombinant H5 HA by fluorescence spectroscopy. Intrinsic tryptophan fluorescence has been shown on numerous occasions to be a useful probe of binding and accompanying structural changes due to the correlation between fluorescence and solvent exposure (19). The fluorescence spectra of H5 HA (nine tryptophans) and PEP87 (one tryptophan) in isolation are shown in Fig. 7a. Note that the PEP87 concentration must be relatively high due to its relatively modest affinity for HA. The fluorescence maximum of HA is consistent with the crystal structure in which the tryptophans are relatively unexposed to solvent. The fluorescence spectrum of PEP87 is consistent with the CD studies in which the tryptophan of the unstructured state would be highly exposed to solvent. Interestingly, the fluorescence spectrum of PEP87 added to H5 HA is very different from that expected from adding the HA and PEP87 spectra obtained in isolation, which is consistent with a binding event. Moreover, subtraction of the isolated PEP87 spectrum from the mixture (i.e. the difference spectrum) shows that the loss in fluorescence occurs in the spectral region of solvent-shielded tryptophans of HA. As shown in Fig. 7b, the change in fluorescence increases with increasing concentration of PEP87. Importantly, the decreased fluorescence of HA suggests that one or more regions containing a tryptophan are undergoing significant changes in conformation. As noted in the Introduction, HA is a symmetric trimer, and thus structural perturbations could be due to changes in tertiary and/or quaternary structure. To probe the potential perturbation of quaternary structure, we performed a chemical cross-linking assay of H5 HA. As shown in Fig. 7c, H5 HA is primarily a trimer in the absence and presence of increasing concentrations of PEP87, suggesting that peptide binding does not disrupt the HA quaternary structure. Together, the fluorescence and chemical cross-linking data indicate that PEP87 binding disrupts HA tertiary structure to an extent that presumably disrupts HA function.

**Discussion**

Peptides from the native amino acid sequence of protein targets are often screened in biological assays with the expectation that the peptide may be able to displace its native sequence to result in the disruption of activity (20–23). As presented above,
using a library of overlapping peptides corresponding to the H7 HA amino acid sequence, we discovered a peptide, PEP87, corresponding to a highly conserved helical region of HA2, that inhibits H5 and H7 HA-mediated entry. The development of influenza entry inhibitors is of high interest, particularly for the avian H5 (Group 1) and H7 (Group 2) HA strains, which exhibit high mortality rates (24, 25). Notably, small-molecules inhibitors of HA entry are generally group-specific (7, 9), and thus peptide inhibitors may be reporting different modes of action than small-molecule inhibitors.

The relatively modest inhibition of influenza entry by PEP87 (IC_{50} > 50 μM) suggests that it is more appropriate as a probe of HA structure and function than a therapeutic lead candidate. Using site-directed mutants of H5 HA, we showed that the peptide inhibitory properties are sensitive to mutations proximal to the peptide sequence, supporting the notion that the peptide displaces its native sequence. We then further characterized PEP87 using biochemical and biophysical techniques. Using CD spectropolarimetry, we showed that PEP87 is unstructured in aqueous solutions and exhibits a propensity to form helix in the presence of cosolvent. Interestingly, peptides corresponding to helical regions of viral envelope proteins often exhibit much higher propensities to form helix as previously shown for similar peptides of HIV and SARS envelope proteins (11, 26). In future studies, it will be of interest to attempt stabilization of the PEP87 helical structure, e.g. by the introduction of “staples” (27), to assay whether the preformed helix exhibits better inhibitory properties. In the next step, we determined the structure of PEP87 in the presence of cosolvent by NMR and showed that PEP87 is largely helical with electronegative and hydrophobic surfaces, mimicking the structure and interactions found for the corresponding sequence in native HA. Using STD NMR, we

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**Table 1**

| Structural statistics for the PEP87 ensemble of 15 structures |
|-------------------------------------------------------------|
| r.m.s., root mean square.                                    |
| Experimental restraints                                      |
| Interproton distance restraints                              | 29 |
| Intraresidue                                                 | 0  |
| Sequential                                                   | 21 |
| Medium range (i – j < 5)                                     | 8  |
| Long range (i – j ≥5)                                        | 0  |
| Dihedral angle restraints                                    | 45 |
| r.m.s. deviations from mean coordinate structure (Å)         |
| Backbone atoms (residues 4–15)                              | 0.55 ± 0.15 |
| All heavy atoms (residues 4–15)                             | 1.12 ± 0.20 |
| Backbone atoms (residues 1–17)                              | 1.47 ± 0.45 |
| All heavy atoms (residues 1–17)                             | 2.40 ± 0.44 |
| Ramachandran plot for the minimized mean structure           |
| Most favored region (%)                                      | 99.6 |
| Additionally allowed region (%)                              | 0.4 |

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**Figure 5. NMR characterization of PEP87 in the presence of cosolvent.** a, secondary chemical shift of 13C. Random coil chemical shifts are taken from Merutka et al. (14). b, 1H–1H NOESY spectrum for the H_N region. Cross-peaks are denoted by PEP87 residue numbers. c, minimized mean structure of PEP87. d, electrostatic profile of the minimized mean structure of PEP87. Experimental conditions were 200 μM PEP87 in 20 mM phosphate, pH 7.5, 50 mM NaCl, 40% TFE-d, 10% 2H₂O at 25 °C.
showed that PEP87 binds to recombinant H5 HA. To our knowledge, this is the first observation of peptides from envelope helical regions binding to their native envelope conformation. For example, similar peptides from HIV and SARS disrupt virus entry by binding to late-stage fusion conformations of their envelope protein (11, 26), which may suggest that HA is somewhat less stable and more dynamic than other envelope proteins in the native or prefusion conformation. In addition, using STD NMR competition assays, we showed that PEP87 does not disrupt binding to a receptor analog, which is consistent with the notion that PEP87 binds to HA2 and not to HA1.

Finally, using fluorescence and chemical cross-linking experiments, we demonstrated that PEP87 binding to HA results in a large increase in solvent exposure of tryptophan groups, suggesting significant structural perturbation of the HA tertiary structure without disruption of the HA quaternary structure.

In summary, our studies are consistent with the notion that PEP87 inhibits influenza entry by binding to the HA prefusion metastable state and disrupting HA-mediated function. Interestingly, binding of PEP87 to the native site in HA would seem to imply a relatively large displacement of one or more HA2 subunits (Fig. 1c), perhaps suggesting that HA undergoes relatively large motions with the presence of transient states in this region that have not been characterized to date. Moreover, the low propensity of the PEP87 sequence to become helical may be functionally relevant in that the HA conformational change requires large-scale motions, and thus the PEP87 region of the HA trimer may be intrinsically metastable and dynamic. Importantly, our work suggests that the prefusion metastable state of HA may consist of an ensemble of structures that comprise transiently exposed target sites, which potentially could be exploited by small-molecule therapeutics.

**Experimental procedures**

**Peptides and proteins**

The library of 111 17-mer peptides corresponding to the H7 HA sequence was obtained from BEI Resources (catalogue number NR-44011). Peptides used in biophysical studies were synthesized by solid-phase peptide synthesis with unmodified N- and C-terminal groups. The peptides were purified by HPLC, and the mass was verified by MALDI-TOF mass spectrometry. Recombinant H5 HA was prepared as described previously (18). Briefly, the extracellular domain of H5 HA, with the addition of a C-terminal foldon domain added for stability and polyhistidine sequence added for subsequent purification, was expressed by baculovirus infection of Sf9 insect cells and purified by Ni-affinity and size-exclusion chromatography.

**Preparation of wild-type and mutant VLPs**

VSVG and wild-type and mutant HA VLPs were prepared as described previously (7, 8). Briefly, HEK 293T cells were cotransfected by plasmids pVSVG (bearing VSVG), pH7HA (bearing wild-type H7 HA), or pH5HA (bearing wild-type or mutant H5 HA); pN1NA (bearing N1 influenza neuraminidase); and pNL4–3.Luc.R-E using PEI (Polysciences, Inc.). Forty-eight hours post-transfection, the medium was harvested and filtered through a 0.45-μm filter to make the VLP stocks, which were subsequently quantified by p24 content.
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Viral entry assays

Viral entry assays using VLPs were performed as described previously (7, 8). Briefly, VLP stocks, normalized to p24 content, were added to HEK 293T cells for ~6 h. VLPs were removed, the cells were incubated for 24 h, and subsequent viral entry was quantified by luciferase activity. For the initial inhibition screening, the peptide concentrations were 100 μM. Cytotoxicity was determined using a CellTiter-Glo kit (Promega).

Circular dichroism

CD spectra were measured on a Jasco-710 spectropolarimeter. Wavelength spectra were recorded from 190 to 260 nm at peptide concentrations of 100 μM (0.2 mg/ml) unless otherwise noted in cells of 0.5-mm path length. For all experiments, the spectra were corrected with the subtraction of a blank corresponding to the buffer of that experiment. Buffer conditions were 400 μM HA in 50 mM phosphate, 50 mM NaCl, pH 8.2. Experimental conditions were 0–250 μM PEP87 ± 2.5 μM HA in 50 mM phosphate, 50 mM NaCl, pH 8.2.

NMR

For the assignment and structure determination, NMR experiments, which included 2D 1H total correlation spectroscopy (1H NOESY mixing time, 200 ms), and 13C heteronuclear single quantum coherence, were performed as described previously (28) on a Bruker 900-MHz spectrometer equipped with a triple-resonance cryoprobe. Experimental conditions were 400 μM PEP87 in 50 mM phosphate, pH 7.5, 150 mM NaCl, 40% TFE-δ in 10 or 60% 2H2O at 25 °C in 3-mm NMR tubes. STD NMR was performed as described previously (17, 29) on a Bruker 800-MHz spectrometer equipped with a triple-resonance room temperature probe. Experimental conditions were 10 μM HA ± 200 μM PEP87 ± 3 mM α,β,γ-sialyllactose in 50 mM phosphate, pH 8.2, 50 mM NaCl in 100% 2H2O at 25 °C in 5-mm NMR tubes. In these experiments, protein 1H were saturated with a train of 50-ms Gaussian-shaped pulses at 100-Hz power for 1 s with “on” resonance saturation at 2.5 ppm and “off” resonance saturation at 30 ppm (the relaxation delay was 2.5 s before the saturating pulses). Spectra were processed by NMRPipe with a 5-Hz line-broadening function and analyzed by NMRDraw (30).

Structure calculations

The PEP87 structure was calculated with the program CNS (31) as described previously (28). The protocol consisted of four steps: (a) high-temperature torsion angle molecular dynamics starting from an extended conformation, (b) slow-cooling torsion angle molecular dynamics, (c) slow-cooling Cartesian dynamics, and (d) conjugate gradient minimization. The φ, ψ, and χ1 dihedral angles were set to the values derived from 1H and 13C chemical shifts using the program PREDICTOR (32). For the structure calculation, errors of 30° were used for the dihedral restraints. NOEs were classified as strong (<2.7 Å), medium (<3.3 or <3.5 Å for amide protons), weak (<5.0 Å), and very weak (≤6.0 Å). A correction of 0.5 Å was added to proton distances involving methyl groups. The assigned chemical shifts, experimental restraints, final group of 15 low-energy structures, and minimized mean structure were deposited as Biological Magnetic Resonance Bank (BMRB) entry SMS21079. The electrostatic map and structural figures were generated by the molecular graphics program PyMOL (33).

Fluorescence

Fluorescence titrations were performed with a PTI fluorescence system using a 0.1-cm path length at 25 °C with excitation at 280 nm. Emission was recorded between 300 and 420 nm. Experimental conditions were 0–250 μM PEP87 ± 2.5 μM HA in 50 mM phosphate, 50 mM NaCl, pH 8.2.

Chemical cross-linking

Cross-linking experiments were performed by first mixing 2.25 μg of recombinant H5 HA with increasing concentrations of PEP87 in PBS, pH 8.2 buffer at 37 °C (total volume = 9 μl). After 30 min, 9 μl of MES-buffered saline or cross-linker (2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 0.02 M Sulfo-NHS) in MES-buffered saline was added for 90 min at room temperature. The reaction was quenched by adding 9 μl of 2 M glycine, pH 7.4, for 10 min at room temperature and analyzed by SDS-PAGE.

Author contributions—C. N. K. performed the screening, CD, STD NMR, and fluorescence experiments. A. A., H. P.-H., and M. D. prepared recombinant protein and mutations. M. C. and B. R. performed the NMR assignment and structure calculations. H. P.-H. performed the cross-linking experiments. C. N. K., M. C., and A. L. designed the experiments and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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