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Hydrogen-deuterium exchange supports independent membrane-interfacial fusion peptide and transmembrane domains in subunit 2 of influenza virus hemagglutinin protein, a structured and aqueous-protected connection between the fusion peptide and soluble ectodomain, and the importance of membrane apposition by the trimer-of-hairpins structure

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Abstract

The influenza virus hemagglutinin (HA) protein has HA1 and HA2 subunits, which form an initial complex. HA1's bind host cell sialic acids which triggers endocytosis, HA1/HA2 separation, and HA2-mediated fusion between virus and endosome membranes. We report hydrogen-deuterium exchange-mass spectrometry(HDX-MS) on the HA2 subunit without HA1. HA2 contains fusion peptide(FP), soluble ectodomain(SE), transmembrane domain(TM) and endodomain. FP is a monomer by itself, while SE is a trimer-of-hairpins that includes an interior bundle of residue 38–105 helices, turns, and 154–178 strands packed antiparallel to the bundle. FP and TM extend from the same side of the SE hairpin, and fusion models often depict a FP/TM complex with membrane traversal of both domains that is important for membrane pore expansion. The HDX-MS data of the present study do not support this complex, and instead support independent FP and TM with respective membrane interfacial and traversal locations. The data also show low aqueous exposure of the 22–38 segment, consistent with retention of the 23–35 antiparallel β sheet observed in the initial HA1/HA2 complex. We propose the β sheet as a semi-rigid connector between FP and SE that enables close membrane apposition prior to fusion. The I173E mutant exhibits greater exchange for 22–69 and 150–191, consistent with dissociation of SE C-terminal strands from interior N-helices. Similar trends are observed for G1E mutant, as well as lower exchange for G1E FP. Fusion is highly-impaired with either mutant, which correlates with reduced membrane apposition, and for G1E, FP binding to SE rather than target membrane.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](#): Sequence coverages of the peptides observed by mass spectrometry, Tables of D% vs. tHDX for all observed peptides, HA2 structures in a single membrane, Plot comparing D% data from the present and a previous study.

Keywords

HA2; UniProtKB P03437

Introduction

Influenza is an enveloped virus enclosed by a membrane obtained during budding from an infected host cell.^{1–3} The viral protein hemagglutinin (HA) is responsible for attachment of the virus to an uninfected host cell and subsequent membrane fusion within the cell. HA is synthesized as a single polypeptide, and then proteolytically cleaved into two disulfide-linked subunits, HA1 and HA2, with respective lengths of ~330 and ~220 residues.⁴ HA2 is a single-pass integral membrane protein with an ectodomain (ED) that contains a N-terminal fusion peptide (FP) and soluble ectodomain (SE), a transmembrane domain (TM) and endodomain with respective lengths of ~25, 160, 25 and 10 residues (Fig. 1A). An initial complex is formed with a HA2 trimeric ED bundle and three HA1's bound to the outside of this bundle.^{5, 6} The HA1's bind to sialic acids on the host cell surface which triggers endocytosis. The early endosome with virus cargo migrates to the host cell nucleus and matures with reduction of its pH to <6, which induces HA1/HA2 separation and HA2 SE rearrangement into a final trimer-of-hairpins structure (Fig. 1B).^{7, 8} Reduced pH also triggers HA-mediated membrane fusion with stages that include: (1) hemifusion in which there is a single bilayer diaphragm that separates the two bodies; (2) pore formation that allows passage of <1 kDa species; and (3) pore expansion that allows deposition of the virus capsid in the cytoplasm.^{3, 9} Many functional data have been for pH-triggered fusion between HA-expressing cells (HA-cells) and red blood cells (RBC's).^{10, 11}

There are high-resolution structures for the initial complex of the proteolytically-cleaved HA ED which includes the HA2 ED and HA1. These structures typically show an organization of HA2 ED trimers with three bound HA1, but sometimes show a different organization of individual HA2/HA1 heterodimers.^{5, 6, 12, 13} The geometries of the HA2 ED/HA1 units are

very similar in either oligomeric organization, which supports the HA2 ED/HA1 heterodimer as a fundamental folding unit. The trimer has a mushroom shape with a stem region containing mostly HA2, and a globular head region containing mostly HA1. The stem includes three HA2_{76–125} helices that form an internal parallel coiled-coil pointing away from the head, and three HA2_{38–55} helices that are antiparallel to the middle of the coiled-coil. The HA2_{1–20} FP's have extended structure buried in the stem and connected to the stem by the HA2_{223–35} β sheet hairpins. The HA2_{130–141} segments are also β sheet hairpins and connect the stem to the HA2_{146–153} and HA2_{159–170} helices.

There is a topologically-distinct structure of the HA2_{33–178} SE without HA1 (Fig. 1B). This HA2 structure is hyperthermostable and likely part of the final HA2 fusion state.^{14, 15} The structure is a trimer-of-hairpins that includes HA2_{38–105} helices in an internal parallel coiled-coil, 180° turns, and HA2_{110–128} helices that are antiparallel to and in the grooves of the HA2_{90–104} region of the coiled-coil. The HA2_{130–141} β sheet hairpins connect to HA2_{146–153} helices that are perpendicular to the internal coiled-coil, and the HA2_{154–178} segments have extended structures antiparallel with the HA2_{38–78} segment of the coiled-coil. There are limited data about the transformation between the initial and final SE structures, but some data support a functional role for the final trimer-of-hairpins state.^{3, 16–19} For example, exogenously-added HA2 FP+SE constructs induce steps (1) and (2) of “HA0-cell”/RBC fusion, where HA0 is a HA-variant that binds to RBC's but does not induce fusion.^{20, 21} There are striking similarities to HA-cell/RBC fusion, including low pH requirement and greatly reduced fusion for the G1E and I173E mutants that are respectively in the HA2 FP's and SE strands. There are similar trends with pH and mutations for vesicle fusion induced by full-length HA2 in the final hairpin structure.^{14, 15} These mutants are examined in the present study. The aforementioned data support a model in which HA1 dissociation leads to FP insertion in the target membrane, formation of SE hairpin structure with consequent membrane apposition, and then membrane fusion. An alternate common mechanistic model is membrane fusion with HA2 in intermediate structures that are not the trimer-of-hairpins, with conversion of some of the free energy released during HA2 structural transformations into activation energy of fusion.^{1–3, 22} For this model, the final trimer-of-hairpins state forms after fusion is completed and is therefore a “post-fusion” state.

The FP sequence is highly-conserved across viral subtypes and its importance in fusion is evidenced by it (and the TM) as the only HA segments that are deeply inserted in the membrane after virus/vesicle fusion.^{4, 23} HA-cell/RBC fusion is also eliminated by the G1E mutation at the N-terminus of the FP.¹¹ The FP is initially in the HA2 ED/HA1 trimer in the protein interior and has extended structure, but adopts very different structures without the rest of HA2 and HA1, and in detergent-rich or lipid bilayer media.^{24–27} For example, HA2_{1–23} in detergent is a monomer and adopts “closed” helical hairpin structure at both low and neutral pH, with 1–12 helix/tight-turn/14–22 helix topology and antiparallel tight-packing of the two helices (Fig. 1B). In membrane, there is some population of the closed structure, as well as a population of a related “semi-closed” (less tightly-packed) structure, with a larger semi-closed:closed population ratio at pH 5 vs. 7, with higher fusion at the lower pH. The EPR linewidths of the spin-labeled FP segment of the trimeric HA2_{1–127} construct bound to membrane are also consistent with non-interacting monomer FP's. EPR

relaxation data correlate with tilted insertion of the FP helices in a single leaflet, with L2 and F3 ~12 Å from the membrane phosphorus plane.²⁸

The present study is focused on hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS), which is an approach to probe segmental structure and motion of proteins.^{29–32} Incubation of the whole protein in D₂O leads to exchange of backbone N-H for N-D, and is followed by pepsin digestion, and then MS-determination of percent HDX (D%) of the resultant peptides.³³ The D% of a peptide is positively-correlated with the aqueous exposure, structural disorder, and motion of the corresponding segment of the intact protein. HDX-MS has previously been applied to the HA2 ED (without TM or endodomain) in complex with HA1.³⁴ Incubation of the complex for three hours at pH 4.9 followed by HDX/MS at pH 7.4 gave D% that were generally consistent with the trimer-of-hairpins structure for the HA2 SE (Fig. 1B). For example, the expected protection of the HA2_{38–105} internal helices in this structure correlates with D% < 2% for the HA2 53–69 and 70–87 peptides for 30-minute exchange time, and exposure of HA2_{106–175} loops, helices, and strands correlates with larger D% > 70% of the HA2 102–109, 100–115, 139–148, 149–167, and 168–175 peptides. Peptides corresponding to the FP segment were not analyzed.

The present study describes HDX-MS of HA2 constructs in the absence of HA1, including HA2_{1–221}, HA2_{1–185} ≡ FP + SE ≡ “FHA2”, and HA2_{20–211} ≡ SE + TM ≡ “SHA2_TM” (Fig. 1A).^{14, 15} Studies of HA2 by itself are relevant because HA2 catalyzes fusion after separation of HA1, and as noted earlier, FHA2 and HA2 (without HA1) induce cell/cell and vesicle fusion. Other new features of the present study are inclusion of TM and endodomain, analysis of FP peptides, and comparison between functional WT and fusion-impaired G1E and I173E mutants. Our study addresses the longstanding hypothesis of a thermostable bundle containing one or more TM helices and FP helix/helices, with all helices traversing the membrane (Fig. 2). This bundle is hypothesized after HA1 dissociation and for late stages of fusion with a role in the final pore expansion step.^{1, 35–37} The bundle is potentially supported by inhibition of this final but not earlier steps of HA-cell/RBC fusion by truncation of the TM domain.³⁸ Another potentially supporting observation is full HA-cell/RBC fusion when the HA2 TM and endodomain are replaced by the TM and endodomain of a non-homologous protein.³⁹

Our study also examines the hypothesis that highly-impaired fusion for the I173E and G1E mutants is due to dissociation of C-terminal strands of the SE, with consequent destabilization of the N-terminal bundle of the SE (Fig. 2). This hypothesis is based on: (1) moderately reduced helicity of I173E vs. WT or G1E; and (2) large and comparable reductions in T_m (up to 40 °C) for I173E and G1E vs. WT, with T_m(WT) ≈ 90 °C.¹⁵ Correlation of the stability of the SE trimer-of-hairpins with fusion supports the importance of membrane apposition by the hairpin prior to fusion. The destabilizing effect of the I173E mutation may be related to loss of I173 sidechain contacts with the N-terminal bundle. G1E destabilization of the SE is surprising because the mutation site is >35 residues from the SE. We hypothesize that G1E destabilizes the FP structure, and that destabilized FP's compete with the N-helix bundle of the SE for binding with the C-terminal strands (Fig. 2). This is just a hypothesis as there aren't yet structural data for G1E.

Materials and Methods

Descriptions and commercial sources of reagents include: n-decyl- β -D-maltopyranoside (DM), Anatrace, Maumee, OH; D₂O (99.9 atom % D), Cambridge Isotope Laboratories – Andover, MA; Enzymate BEH pepsin column (2.1 $\times$ 30 mm), XBridge BEH Vanguard pre-column/trap column (2.1 $\times$ 5 mm, 2.5 μ m particle size), C18 column (2.1 $\times$ 50 mm), Waters, Milford, MA. Most other reagents were purchased from Sigma-Aldrich – St. Louis, MO. Solvents were HPLC grade.

Fig. 1A displays color-coded domain schematics and amino-acid sequences of the constructs HA2, FHA2 $\equiv$ HA2_{1–185}, and SHA2_TM $\equiv$ HA2_{20–211} (UniProtKB P03437). The sequences are from the X31 strain of influenza virus with mutation of all native Cys residues, specifically C137S, C144A, C148A, C195A, C199A, C210A, and C220A. SHA2_TM includes a N-terminal G20C mutation done for future native-chemical ligation (not part of the present study). Inter-trimer Cys cross-linking is unlikely, based on the absence of such cross-linking in a similar construct of the non-homologous HIV gp41 protein which also adopts trimer-of-hairpins structure.⁴⁰ There are non-native C-terminal regions LEG₆H₆ (HA2 and SHA2_TM) or LEH₆ (FHA2) for affinity chromatography. The G1E- and I173E-HA2 constructs were also studied. Preparation of the constructs, as well as expression, purification, and refolding protocols have been previously reported.¹⁵ Protein stocks contained 40 μ M protein in 10 mM Tris-HCl buffer at pH 7.4 with 0.17% DM.

The HDX-MS apparatus included an incubated auto-sampler (Waters 2777), external pump (Shimadzu LC-20AD), UPLC pump system (Waters Acquity Binary Solvent Manager), and mass spectrometer (Waters Xevo G2S QToF) operating with electrospray ionization in positive ion mode, and continuum-mode m/z = 50 – 2000.³³ HDX was carried out in 1.6 mL capped tubes at 25 °C. Protein stock (6 μ L) was mixed with D₂O buffer (114 μ L), where the latter contained 5 mM HEPES/10 mM MES at pD 7.4 with 0.17% DM. After initial manual mixing, individual tubes were kept without stirring in the auto-sampler for either 1, 5, 30, 60, or 120 min. A “0 min” sample was made by mixing with H₂O buffer. After the HDX time, the tube was removed from the auto-sampler, and 120 μ L of ice-cold formic acid (FA) was added, so that pH was reduced to <3 which inhibited additional HDX. Back “DHX” exchange was minimized by surrounding ice around the tube, and around other solutions, injection ports, valves, and columns.

The next set of liquid transfers was done with the external pump. Protein digestion was done in a pepsin column that had been equilibrated with flow of water with 0.1% FA (H₂O_{FA}). Protein solution (100 μ L) was transferred to the column using 0.1 mL/min flow, followed by digestion in the column for 1 min. The solution was then transferred to the trap column using 0.5 mL/min flow, followed by desalting with 1 min flow of H₂O_{FA}. Flow control was transferred to the UPLC and the flow rate set to 0.3 mL/min. The flow then went from the trap column to the C18 column, and separation of the peptides was done using mixtures of H₂OFA and acetonitrile with 0.1% FA (ACN_{FA}). The separation program was 1% ACN_{FA} (1 min), a gradient to 30% ACN_{FA} (3 min), a second gradient to 99% ACN_{FA} (3 min), and 99% ACN_{FA} (1 min). The flow from the column went to the mass spectrometer for analysis.

The above protocol was first applied to the “0 min” sample prepared by mixing with H₂O buffer. Individual m/z peak distributions in the mass spectrum were assigned to specific peptide sequences by manual comparison of the average natural-abundance mass (M_H) of the distribution with theoretical M_H 's. Assignment were confirmed by MS/MS analysis. The mass spectra of HDX samples were assigned by the same procedure. Peaks assigned to a single peptide within a user-selected m/z window were inputs for the HX-Express software, which then calculated the peak area weighted-average m/z for each t_{HDX} .⁴¹ The outputs were the average MH ($t_{HDX} = 0$) and average MD at each $t_{HDX} > 0$. For a particular peptide, the percent deuterium incorporation $D\%(t) = 100 \times [M_D(t) - M_H]/N$, where N is the number of backbone amide hydrogens in the peptide.

Results and Discussion

Fig. S1 displays sequence coverages for identified peptides from the different constructs. For all constructs, there is >90% coverage of the HA2 region of the sequence. Fig. 3 displays mass spectra vs t_{HDX} for representative peptides of WT-FHA2, SHA2_TM, and HA2, and Fig. 4 displays $D\%$ vs. $\log_{10}(t_{HDX}/\text{min})$ for all peptides of these constructs. Figs. 5 and 6 displays similar plots for WT-, G1E-, and I173E-HA2. The Fig. 3, 4 HA2 data are the same as the Fig. 5, 6 WT-HA2 data. Use of $\log_{10}(t_{HDX})$ for the horizontal axis is for clarity of presentation and is not based on a specific kinetic model of HDX. The maximum $D\%$ values are ~70% rather than 100%, which is consistent with some structure in all regions other than the HA2_{212–221} endodomain, and may also reflect some reduction in $D\%$ due to DHX back-exchange during the ~10-minute digestion and chromatographic separation steps.³¹ Back exchange is estimated using the 62% experimental exchange at 2 h for HA2_{206–217}. The 212–217 in the endodomain likely exhibits full-exchange, whereas 207–211 in the TM is estimated to have 35–40% exchange based on 48% and 31% values for HA2_{179–187} and HA2_{188–191} that include residues from the other end of the TM. The calculated exchange for 212–217 is ~80%, which implies ~20% back exchange. Informative conclusions can still be drawn from our data based on significant variations in the $D\%$ values of different peptides of the same construct, and variations in the $D\%$ values for the same peptide in different constructs.

Tables 1 and 2 present $\langle D\% \rangle$ for the peptides, where $\langle D\% \rangle$ is the average of all t_{HDX} data, i.e. 1, 5, 30, 60, and 120 min. The uncertainties are in parentheses with typical values of 0.5% that are calculated using the RMSD's of the $D\%$ values. Although $\langle D\% \rangle$ doesn't have a specific physical meaning, $\langle D\% \rangle$ is a convenient single number to describe each peptide. Fig. 7 displays the Fig. 2 models with colors based on the $\langle D\% \rangle$ from Tables 1 and 2.

WT structural model supports the significance of membrane apposition in fusion

Fig. 8 (left) displays a structural model for WT-HA2 that is based on integration of the HDX-MS data of the present study and high-resolution structures of segments of HA2. The FP and TM in Fig. 8 are in the different membranes that exist prior to fusion, but no significant changes in protein structure are expected when the FP and TM are in a single membrane after fusion. Fig. S2 displays the HA2 structures in a single membrane. One caveat of membrane interpretation of our HDX-MS data is that our samples are in DM

detergent. However, DM is probably a reasonable membrane mimetic, as evidenced by well-folded, hyperthermostable, and fusion-active protein in DM.¹⁵ For simplicity of presentation, we subsequently only refer to membrane. The Fig. 8 model includes the trimer-of-hairpins SE structure shown in Fig. 1B, and is supported by HDX-MS data for HA2, FHA2, and SHA2_TM (Figs. 3, 4, and 7), with typical $\langle D_{\%} \rangle < 5\%$ for 39–99 peptides from the trimer interior, and 35–50% for 110–178 peptides from the hairpin turns and trimer exterior. For clarity, Fig. 8 shows only one monomer unit of the trimer. The FP and TM in Fig. 8 are displayed as independent domains with respective membrane-interfacial and traversal locations. These locations are based on $\langle D_{\%} \rangle > 35\%$ for 1–21 and $\langle D_{\%} \rangle < 3\%$ for 192–205. The data do not support a trans-membrane FP/TM complex, for which the $\langle D_{\%} \rangle$ for the FP would be small and comparable with the TM (Fig. 2). The extensive exchange for the FP is more consistent with monomer than with oligomer FP's, whereas complete protection of the TM is consistent with either monomer or oligomer TM's that traverse the membrane. The $\langle D_{\%} \rangle = 57\%$ for the 206–217 region is consistent with a contribution from an aqueous-exposed and structurally disordered HA2_{212–221} endodomain.

Fig. 8 displays the 23–35 region as a 23–27 strand/28–30 turn/31–35 strand antiparallel β sheet. The HA2_{1–175}/HA1 structure contains this motif; by contrast, the HA2_{23–185} ED structure doesn't have clear electron density in the 23–33 region, and HA2_{1–28} doesn't exhibit NMR signals for residues 25–28. The latter observations are reasonably interpreted to support a flexible and disordered 23–35 region of HA2 with high aqueous exposure, and expected $\langle D_{\%} \rangle > 50\%$. It was therefore unexpected that $\langle D_{\%} \rangle_{22–38} = 17\%$ for HA2 and FHA2, and 13% for SHA2_TM, with 0.6% uncertainties in individual $\langle D_{\%} \rangle_{22–38}$. For comparison, $\langle D_{\%} \rangle_{39–52}$ are respectively ~4 and 8%, where 39–52 is on the interior of the trimer-of-hairpins. The small $\langle D_{\%} \rangle_{22–38}$ are consistent with a well-structured and aqueous-protected region, and are the basis for the proposed retention of the 23–35 β sheet hairpin in the final state of HA2 without HA1. The 23–35 sequence is reasonably-conserved across the subtypes of hemagglutinin and likely isn't deeply-membrane-inserted, based on few hydrophobic residues.⁴ Fig. 8 also shows a 179–185 bend region like that in one of the six monomers of the HA2_{23–185} crystal structure; the remaining monomers don't have electron density for most of these residues. The bend in all monomer units is consistent with smaller $\langle D_{\%} \rangle = 39\%$ for 179–187 vs. 50% for 172–178, where the latter is a well-structured segment on the exterior of the SE trimer-of-hairpins.

Fig. 8 provides a mechanistic basis for the observation that HA2 in its final SE hairpin state without HA1 catalyzes cell/cell and vesicle fusion, and we propose that the mechanism is also relevant for virus/endosome fusion. For this mechanism, the entire 23–185 ED provides a semi-rigid and hyperthermostable connection between the FP and TM that ensures close apposition of the two membranes prior to fusion, and compensates for much of the ~60 kT (computed) activation barrier between the initial two-membranes state and the first “stalk” fusion intermediate.³ Subsequent hemifusion and pore formation steps have much lower computed activation barriers.

We also use our Fig. 8 model to understand earlier observations that the TM is necessary for the final pore expansion step of HA-cell/RBC fusion, and that efficient fusion occurs with replacement of the HA2 TM with a TM sequence of a non-homologous protein.^{39, 42} The

HDX-MS data don't support a FP/TM complex, so the TM segments on their own might facilitate pore expansion by shielding acyl chains of lipids from water during their transition from the hemifusion diaphragm separating the two bodies to the final continuous bilayer enclosing the fused body. In addition, membrane location of both the FP and TM with the semi-rigid HA2 ED structure may lead to positive membrane curvature (see Fig. S2 for HA2 with FP and TM in a single membrane). This curvature may aid pore expansion, which typically has an associated increase in positive curvature. Deletion of TM from HA2 would probably release curvature stress and allow deeper FP membrane insertion. The latter prediction correlates with the smaller $\langle D_{\%} \rangle$ for the FP in FHA2 vs. HA2, e.g. $\langle D_{\%} \rangle_{1-8} = 45$ vs. 63% and $\langle D_{\%} \rangle_{10-21} = 54$ vs. 38%. Deletion of the FP may also result in deeper insertion of the protein which correlates with smaller $\langle D_{\%} \rangle_{22-38}$ and smaller $\langle D_{\%} \rangle_{188-191}$ for SHA2_TM vs. HA2, i.e. 13 vs. 17% and 11 vs. 15%, with typical uncertainties $\approx 0.5\%$.

Structural destabilization of I173E- and G1E-HA2 underlies their fusion impairment

Fig. 8 (center) displays a structural model for I173E-HA2 that is supported by our HDX-MS data. The I173E structure is the same as WT in the FP, 70–150 SE, TM, and endodomain regions, as supported by similar $\langle D_{\%} \rangle$ of I173E and WT for the 1–8, 70–99, 120–150, and 192–217 segments. The C-terminal strand of I173E is dissociated from the N-terminal bundle with consequent much greater aqueous exposure of the bundle, as supported by much larger $\langle D_{\%} \rangle$ of 22–69 for I173E vs. WT, e.g. $\langle D_{\%} \rangle_{22-38} = 35$ vs. 17%, $\langle D_{\%} \rangle_{39-52} = 47$ vs. 3%, and $\langle D_{\%} \rangle_{53-69} = 18$ vs. 6%. SE hairpin turns and C-terminal strands are on the exterior of the WT structure and exhibit significant $\langle D_{\%} \rangle$, but even larger $\langle D_{\%} \rangle$ for I173E in these segments supports strand dissociation and SE destabilization. For the same peptide in 100–115 and 151–191, the $\langle D_{\%} \rangle$ are always larger for I173E vs. WT, e.g. 60 vs. 50% for the 172–178 peptide that contains the mutation site. The Fig. 8 model supports that highly-impaired fusion for I173E is due to greater inter-membrane distance that results from a more flexible SE hairpin.

Fig. 8 (right) displays a model for G1E-HA2 with a I173E-like SE structure with dissociated C-terminal strand bound to unfolded FP. This structure is in equilibrium with the WT structure, like in Fig. 2. G1E fusion is consequently impaired both by membrane apposition at greater distance and by reduced FP interaction with the target membrane. G1E may destabilize the membrane-interacting helical hairpin FP structure by reduction of the N-terminal NH_3^+ charge/12–22 helix dipole interaction.⁴³ FP/SE strand binding helps explain the G1E destabilization of the SE, where the latter observation was unanticipated because the trimer-of-hairpins structure of the SE is thermostable in the absence of the FP.¹⁴ FP/SE binding in Fig. 8 is consistent with decreased aqueous exposure of G1E FP, e.g. $\langle D_{\%} \rangle_{10-21} = 45\%$ for G1E vs. 54% for WT and $\langle D_{\%} \rangle_{12-22} = 49\%$ for G1E vs. 63% for I173E. G1E likely has the least thermostable FP, and without SE binding would exhibit higher rather than lower aqueous exposure and exchange. Partial dissociation of the C-terminal strand for G1E is consistent with $\langle D_{\%} \rangle$ values for the 151–191 region that are intermediate between the smaller WT and larger I173E values. Distinct G1E SE states similar to either WT or I173E structures are also evidenced by the bimodal behavior for 39–52, with one distribution of peaks in a m/z range similar to WT and the other in a m/z range similar to I173E (Fig. 5). For $t_{\text{HDX}} = 120$ min, there are approximately equal sums of G1E peak intensities in the two

ranges, and the calculated $D_{\%} = 16$ and 63% are similar to the 6 and 54% values for WT and I173E, respectively. Table S3 lists the $D_{\%}$ for 39–52 for all t_{HDX} . There are similar $\langle D_{\%} \rangle$ for G1E and WT for peptides in the 22–38, 53–150, and 192–217 regions.

Integration with previous studies

The high $T_m = 91$ °C of WT-HA2 correlates with the highly-ordered structural model in Fig. 8. The thermostability and rigidity of WT-HA2 are needed for the membrane apposition function of HA2 in fusion. The lower $T_m = 79$ °C for I173E and 75 °C for G1E correlate with loss of SE structure and the consequent increased inter-membrane distance and impaired fusion (Fig. 8).¹⁵

The FP and TM in Fig. 8 are displayed with membrane interfacial and traversal locations, respectively, that correlate with peptides with $\langle D_{\%} \rangle > 35\%$ in the 1–21 region and $\langle D_{\%} \rangle < 3\%$ in the 192–205 region. The low $\langle D_{\%} \rangle$ of TM are consistent with either monomer or oligomer TM that traverses the membrane. Monomer TM is consistent with low sequence conservation in this domain among HA subtypes, and with robust HA-cell/RBC fusion after substitution of the native TM and endodomain sequences with those of an unrelated protein.^{4, 39} However, cryo-electron microscopy of the HA2/HA1 initial trimer complex in detergent were interpreted to support a trimeric TM bundle.³⁶

We note that fluorescence and SDS-PAGE data for HA2 TM peptides support a fraction of oligomeric peptides.^{37, 44}

The $\langle D_{\%} \rangle = 63\%$ for 1–8 and 54% for 10–21 from the FP of HA2 are comparable with $\langle D_{\%} \rangle = 57\%$ of 206–217 which includes part of the likely unstructured and aqueous-exposed HA2_{212–221} endodomain. Thus, the FP $\langle D_{\%} \rangle$ could be consistent with unfolded FP in aqueous solution. However, we consider this scenario unlikely because of the stability of the FP helical hairpin in detergent and membrane, and the hydrophobic-effect free energy penalty associated with aqueous solubilization.^{24, 27, 45} The larger $\langle D_{\%} \rangle$ of FP in HA2 vs. FHA2 are completely inconsistent with a FP/TM complex, but we note that earlier fluorescence, EPR, and calorimetric data were interpreted to support weak association in membrane between a peptide containing the TM sequence and a peptide containing the FP sequence.^{37, 46} By contrast, there was no close contact between the FP and TM domains of a 194-residue FP+SE+TM HIV gp41 fusion protein construct.⁴⁷ HIV gp41 is non-homologous with HA2, but the SE's of the two proteins both adopt trimer-of-hairpins structures.^{40, 48–50} Finally, we note that although the preponderance of simulations and experimental data support membrane interfacial location for the FP in the absence of the rest of HA2, there are some simulations and experimental data that support membrane traversal for the isolated FP.^{24, 51–55}

The Fig. 8 and Fig. S2 models have local positive membrane curvature to achieve interfacial locations for the FP and the N-terminus of the TM. HA2-stabilized curvature could be important for fusion catalysis, as there are likely membrane intermediates during fusion with local curvature.^{1, 56} Earlier studies have provided experimental data that evidence stabilization of local membrane curvature by viral fusion peptides.^{57–60}

The $D_{\%}$ data from the present study are compared to those of an earlier study in which the HA2₁₋₁₇₅/HA1 complex which lacked TM and endodomain was incubated in a pH 4.9 solution without detergent for three hours, neutralized, and subjected to a protocol similar to the present study.³⁴ The earlier protocol included incubation in 85% D₂O for $t_{\text{HDX}} = 0.05, 1, 30,$ or 1200 min, acidification, pepsin digestion, and mass spectrometric analysis of the peptide products. Direct comparison can be made between the $D_{\%}$ for $t_{\text{HDX}} = 1$ and 30 min, which are common to both studies. Comparison is made using our data for FHA2 $\equiv$ HA2₁₋₁₈₅, as this construct is closest to the HA2₁₋₁₇₅ subunit of the complex with HA1. There are minor differences in how $D_{\%}$ was calculated in the two studies. As noted earlier, our $D_{\%}$ do not account for back-exchange and might therefore be reduced by ~ 0.8 relative to the earlier study for which back-exchange appears to have been accounted. For both studies, peptides in the 53–99 region exhibit $D_{\%} < 5\%$, which is consistent with the interior location of the 38–105 helices of the trimer-of-hairpins structure. For both studies, peptides from the 99–171 region exhibit larger $D_{\%}$, which correlates with the exterior location of most of this region in the structure. However, the $D_{\%}$ for the present study are much lower than the previous study, even with a 0.8 multiplication of the previous values. Fig. S3 provides some examples of this trend which holds for both the 22–52 and the 99–171 regions. For example, comparison of 22–38 for FHA2 vs. HA2₁₋₁₇₅/HA1 shows $D_{\%} = 14$ vs. 75% for $t_{\text{HDX}} = 1$ min and $D_{\%} = 17$ vs. 85% for $t_{\text{HDX}} = 30$ min. For 39–52, $D_{\%} = 2$ vs. 40% for $t_{\text{HDX}} = 1$ min and $D_{\%} = 5$ vs. 55% for $t_{\text{HDX}} = 30$ min. For the 110–115 peptide at either t_{HDX} , the comparison is $D_{\%} = 26$ vs. 100%. For the 151–167 peptide, the $D_{\%} = 35$ vs. 80% for $t_{\text{HDX}} = 1$ min and $D_{\%} = 37$ vs. 100% for $t_{\text{HDX}} = 30$ min. Another difference is that peptides from the FP were straightforwardly analyzed in the present study with HA2-only constructs, but not in the earlier study with low pH incubation of the HA2₁₋₁₇₅/HA1 complex because of significant broadening of the envelopes of peaks. Such broadening wasn't observed for the complex that hadn't undergone the incubation and consequent large-scale structural rearrangement. One experimental difference between the present vs. earlier study is the presence vs. absence of detergent. HA2 constructs form aggregates in the absence of detergent via the exposed FP's. Detergent is also important for FHA2 folding as evidenced by circular dichroism θ_{222} values that correlate with 65 vs. 25% helicity in the presence vs. absence of detergent.^{15, 21} The 65% helicity in detergent is consistent with folded protein as it matches the percent helicity calculated using the numbers of helical residues in the trimer-of-hairpins SE structure and in the helical hairpin FP structure in detergent. The envelope broadening of peptides from the FP and the larger $D_{\%}$ of the earlier study may therefore reflect HA2₁₋₁₇₅ proteins that are aggregated and less-folded than the HA2 constructs in detergent of the present study.

We also compare the $D_{\%}$ of the FP region of the present study with earlier NMR-measured HDX rates for HA2₁₋₂₃ in detergent.²⁴ Residues 2–6, 12–17, and 20–23 exhibit rates $> 0.1 \text{ s}^{-1}$, and would be completely exchanged even for the shortest $t_{\text{HDX}} = 1$ min of the present study. For $t_{\text{HDX}} = 1$ min, the 1–9 peptide exhibits $D_{\%} = 20\%$ for FHA2 vs. 45% for HA2, and 10–21 exhibits $D_{\%} = 35\%$ for FHA2 vs. 44% for HA2. For a model in which FP residues 2–6, 12–17, and 20–23 completely exchange and other residues don't exchange, the calculated $D_{\%} = 63\%$ for 1–9 and $D_{\%} = 73\%$ for 10–21. The FP therefore exhibits greater protection in FHA2 and HA2 vs. HA2₁₋₂₃.

Dependence of $D_{\%}$ on t_{HDX}

For the present study, peptides from the exterior of the SE often exhibit significant $D_{\%}$ for $t_{\text{HDX}} = 1$ min and then small increases in $D_{\%}$ for larger t_{HDX} up to the maximum $t_{\text{HDX}} = 2$ h. $D_{\%}$ is a measure of aqueous exposure, as supported by negligible exchange of the N-helix in WT-FHA2 and HA2, and negligible exchange of TM in all HA2 proteins. Peptides from structured regions on the protein exterior have some residues facing outward with aqueous exposure and some facing inward without exposure. Even modest exposure results in complete exchange at $t_{\text{HDX}} = 1$ min, as supported by the calculated complete exchange in 1 min for the slowest NMR-measured exchange rate for HA2₁₋₂₃ described above. Thus, the $D_{\%}$ value at $t_{\text{HDX}} = 1$ min for peptides from the outside of the HA2 SE likely reflects their fraction of aqueous-exposed residues. The small increases in $D_{\%}$ with larger t_{HDX} for the WT proteins likely reflects their structural stability and small unfolded population, which is consistent with their $T_m > 85$ °C. The bimodal data for G1E-HA2₃₉₋₅₂ are also consistent with this model, with one population with low exchange from protein that remains folded and a second population with high exchange from protein that has transiently unfolded. The latter fractional population increases with t_{HDX} . Similar trends of $D_{\%}$ vs. t_{HDX} over the 1 min – 2 h range has also been observed for other proteins.³³

Summary

HDX-MS has been applied to subunit 2 of the influenza virus hemagglutinin protein that mediates fusion between the virus and endosome membranes. High aqueous exposure of the fusion peptide and high aqueous protection of the transmembrane region are consistent with separate domains that have respective membrane interfacial and traversal locations. The HDX-MS data are also consistent with a trimer-of-hairpins structure for the soluble ectodomain. High aqueous protection of the 22–38 peptide support a well-structured segment and are consistent with retention of the 23–35 antiparallel β sheet found in the initial HA2/HA1 complex. The β sheet provides a semi-rigid connection between the fusion peptide and soluble ectodomain, and aids close apposition of the two membranes prior to fusion. HDX-MS was also done on the G1E and I173E mutants that result in highly-impaired fusion. I173E shows much greater aqueous exposure vs. WT of the 22–69 and 150–191 regions, which correlates with dissociation of the C-terminal strands of the trimer-of-hairpins. G1E has aqueous exposure of these regions intermediate between WT and I173E, as well as increased protection of the fusion peptide. These data are consistent with binding of the G1E fusion peptide to the dissociated C-terminal strands. The HDX-MS data support a model in which reduced fusion by the mutants is due to larger distance between the apposed membranes, and for G1E, also loss of fusion peptide contact with the target membrane.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACN	acetonitrile
CD	circular dichroism
DM	decylmaltoside
FA	formic acid
FP	fusion peptide
HA	hemagglutinin
HDX	hydrogen-deuterium exchange
LB	Luria-Bertani
LC	liquid chromatography
MS	mass spectrometry
PBS	phosphate-buffered saline
RBC	red blood cell
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SE	soluble ectodomain
SHB	six-helix bundle
TM	transmembrane domain
WT	wild-type

References

- [1]. White JM, Delos SE, Brecher M, and Schornberg K (2008) Structures and mechanisms of viral membrane fusion proteins: Multiple variations on a common theme, *Crit. Rev. Biochem. Mol. Biol* 43, 189–219. [PubMed: 18568847]
- [2]. Harrison SC (2015) Viral membrane fusion, *Virology* 479, 498–507. [PubMed: 25866377]
- [3]. Boonstra S, Blijleven JS, Roos WH, Onck PR, van der Giessen E, and van Oijen AM (2018) Hemagglutinin-mediated membrane fusion: A biophysical perspective, *Ann. Revs. Biophys* 47, 153–173. [PubMed: 29494252]
- [4]. Nobusawa E, Aoyama T, Kato H, Suzuki Y, Tateno Y, and Nakajima K (1991) Comparison of complete amino acid sequences and receptor binding properties among 13 serotypes of hemagglutinins of influenza A viruses, *Virology* 182, 475–485. [PubMed: 2024485]
- [5]. Wilson IA, Skehel JJ, and Wiley DC (1981) Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution, *Nature* 289, 366–373. [PubMed: 7464906]

- [6]. Ekiert DC, Kashyap AK, Steel J, Rubrum A, Bhabha G, Khayat R, Lee JH, Dillon MA, O'Neil RE, Faynboym AM, Horowitz M, Horowitz L, Ward AB, Palese P, Webby R, Lerner RA, Bhatt RR, and Wilson IA (2012) Cross-neutralization of influenza A viruses mediated by a single antibody loop, *Nature* 489, 526–532. [PubMed: 22982990]
- [7]. Lakadamyali M, Rust MJ, Babcock HP, and Zhuang XW (2003) Visualizing infection of individual influenza viruses, *Proc. Natl. Acad. Sci. U. S. A* 100, 9280–9285. [PubMed: 12883000]
- [8]. Chen J, Skehel JJ, and Wiley DC (1999) N- and C-terminal residues combine in the fusion-pH influenza hemagglutinin HA2 subunit to form an N cap that terminates the triple-stranded coiled coil, *Proc. Natl. Acad. Sci. U.S.A* 96, 8967–8972. [PubMed: 10430879]
- [9]. Blijleven JS, Boonstra S, Onck PR, van der Giessen E, and van Oijen AM (2016) Mechanisms of influenza viral membrane fusion, *Sem. Cell Dev. Biol* 60, 78–88.
- [10]. Chernomordik LV, Frolov VA, Leikina E, Bronk P, and Zimmerberg J (1998) The pathway of membrane fusion catalyzed by influenza hemagglutinin: Restriction of lipids, hemifusion, and lipidic fusion pore formation, *J. Cell Biol* 140, 1369–1382. [PubMed: 9508770]
- [11]. Qiao H, Armstrong RT, Melikyan GB, Cohen FS, and White JM (1999) A specific point mutant at position 1 of the influenza hemagglutinin fusion peptide displays a hemifusion phenotype, *Mol. Biol. Cell* 10, 2759–2769. [PubMed: 10436026]
- [12]. Cho KJ, Lee J-H, Hong KW, Kim S-H, Park Y, Lee JY, Kang S, Kim S, Yang JH, Kim E-K, Seok JH, Unzai S, Park SY, Saelens X, Kim C-J, Lee J-Y, Kang C, Oh H-B, Chung MS, and Kim KH (2013) Insight into structural diversity of influenza virus haemagglutinin, *J. Gen. Virol* 94, 1712–1722. [PubMed: 23636824]
- [13]. Xiong X, Corti D, Liu J, Pinna D, Foglierini M, Calder LJ, Martin SR, Lin YP, Walker PA, Collins PJ, Monne I, Suguitan AL Jr., Santos C, Temperton NJ, Subbarao K, Lanzavecchia A, Gamblin SJ, and Skehel JJ (2015) Structures of complexes formed by H5 influenza hemagglutinin with a potent broadly neutralizing human monoclonal antibody, *Proc. Natl. Acad. Sci. U.S.A* 112, 9430–9435. [PubMed: 26170284]
- [14]. Ratnayake PU, Ekanayaka EAP, Komanduru SS, and Weliky DP (2016) Full-length trimeric influenza virus hemagglutinin II membrane fusion protein and shorter constructs lacking the fusion peptide or transmembrane domain: Hyperthermostability of the full-length protein and the soluble ectodomain and fusion peptide make significant contributions to fusion of membrane vesicles, *Protein Expression Purif.* 117, 6–16.
- [15]. Ranaweera A, Ratnayake PU, and Weliky DP (2018) The stabilities of the soluble ectodomain and fusion peptide hairpins of the Influenza virus hemagglutinin subunit II protein are positively correlated with membrane fusion, *Biochemistry* 57, 5480–5493. [PubMed: 30141905]
- [16]. Chlanda P, Mekhedov E, Waters H, Schwartz CL, Fischer ER, Ryham RJ, Cohen FS, Blank PS, and Zimmerberg J (2016) The hemifusion structure induced by influenza virus haemagglutinin is determined by physical properties of the target membranes, *Nature Microbiology* 1, Art. No. 16050.
- [17]. Gui L, Ebner JL, Mileant A, Williams JA, and Lee KK (2016) Visualization and sequencing of membrane remodeling leading to influenza virus fusion, *J. Virol* 90, 6948–6962. [PubMed: 27226364]
- [18]. Das DK, Govindan R, Nikic-Spiegel I, Krammer F, Lemke EA, and Munro JB (2018) Direct visualization of the conformational dynamics of single influenza hemagglutinin trimers, *Cell* 174, 926–937. [PubMed: 29961575]
- [19]. Eddy NR, and Onuchic JN (2018) Rotation-activated and cooperative zipping characterize class I viral fusion protein dynamics, *Biophys. J* 114, 1878–1888. [PubMed: 29694865]
- [20]. Leikina E, LeDuc DL, Macosko JC, Epand R, Shin YK, and Chernomordik LV (2001) The 1–127 HA2 construct of influenza virus hemagglutinin induces cell-cell hemifusion, *Biochemistry* 40, 8378–8386. [PubMed: 11444985]
- [21]. Kim CS, Epand RF, Leikina E, Epand RM, and Chernomordik LV (2011) The final conformation of the complete ectodomain of the HA2 subunit of Influenza Hemagglutinin can by itself drive low pH-dependent fusion, *J. Biol. Chem* 286, 13226–13234. [PubMed: 21292763]

- [22]. Carr CM, and Kim PS (1993) A spring-loaded mechanism for the conformational change of influenza hemagglutinin, *Cell* 73, 823–832. [PubMed: 8500173]
- [23]. Durrer P, Galli C, Hoenke S, Corti C, Gluck R, Vorherr T, and Brunner J (1996) H⁺-induced membrane insertion of influenza virus hemagglutinin involves the HA2 amino-terminal fusion peptide but not the coiled coil region, *J. Biol. Chem* 271, 13417–13421. [PubMed: 8662770]
- [24]. Lorieau JL, Louis JM, and Bax A (2010) The complete influenza hemagglutinin fusion domain adopts a tight helical hairpin arrangement at the lipid:water interface, *Proc. Natl. Acad. Sci. U.S.A* 107, 11341–11346. [PubMed: 20534508]
- [25]. Ghosh U, Xie L, and Weliky DP (2013) Detection of closed influenza virus hemagglutinin fusion peptide structures in membranes by backbone ¹³CO-¹⁵N rotational-echo double-resonance solid-state NMR, *J. Biomol. NMR* 55, 139–146. [PubMed: 23329392]
- [26]. Du TP, Jiang L, and Liu ML (2014) NMR structures of fusion peptide from influenza hemagglutinin H3 subtype and its mutants, *J. Peptide Sci* 20, 292–297. [PubMed: 24677267]
- [27]. Ghosh U, Xie L, Jia LH, Liang S, and Weliky DP (2015) Closed and semiclosed interhelical structures in membrane vs closed and open structures in detergent for the Influenza Virus hemagglutinin fusion peptide and correlation of hydrophobic surface area with fusion catalysis, *J. Am. Chem. Soc* 137, 7548–7551. [PubMed: 26039158]
- [28]. Macosko JC, Kim CH, and Shin YK (1997) The membrane topology of the fusion peptide region of influenza hemagglutinin determined by spin-labeling EPR, *J. Mol. Biol* 267, 1139–1148. [PubMed: 9150402]
- [29]. Marcisin SR, and Engen JR (2010) Hydrogen exchange mass spectrometry: what is it and what can it tell us?, *Anal. Bioanal. Chem* 397, 967–972. [PubMed: 20195578]
- [30]. Pirrone GF, Iacob RE, and Engen JR (2015) Applications of hydrogen/deuterium exchange MS from 2012 to 2014, *Anal. Chem* 87, 99–118. [PubMed: 25398026]
- [31]. Koshy SS, Eyles SJ, Weis RM, and Thompson LK (2013) Hydrogen exchange mass spectrometry of functional membrane-bound chemotaxis receptor complexes, *Biochemistry* 52, 8833–8842. [PubMed: 24274333]
- [32]. Koshy SS, Li X, Eyles SJ, Weis RM, and Thompson LK (2014) Hydrogen exchange differences between chemoreceptor signaling complexes localize to functionally important subdomains, *Biochemistry* 53, 7755–7764. [PubMed: 25420045]
- [33]. Chodavarapu S, Jones AD, Feig M, and Kaguni JM (2016) DnaC traps DnaB as an open ring and remodels the domain that binds primase, *Nucl. Acids Res* 44, 210–220. [PubMed: 26420830]
- [34]. Garcia NK, Guttman M, Ebner JL, and Lee KK (2015) Dynamic changes during acid-Induced activation of influenza hemagglutinin, *Structure* 23, 665–676. [PubMed: 25773144]
- [35]. Tamm LK (2003) Hypothesis: spring-loaded boomerang mechanism of influenza hemagglutinin-mediated membrane fusion, *Biochim. Biophys. Acta* 1614, 14–23. [PubMed: 12873762]
- [36]. Benton DJ, Nans A, Calder LJ, Turner J, Neu U, Lin YP, Ketelaars E, Kallewaard NL, Corti D, Lanzavecchia A, Gamblin SJ, Rosenthal PB, and Skehel JJ (2018) Influenza hemagglutinin membrane anchor, *Proc. Natl. Acad. Sci. U.S.A* 115, 10112–10117. [PubMed: 30224494]
- [37]. Chang DK, Cheng SF, Kantchev EAB, Lin CH, and Liu YT (2008) Membrane interaction and structure of the transmembrane domain of influenza hemagglutinin and its fusion peptide complex, *BMC Biology* 6:2. [PubMed: 18197965]
- [38]. Armstrong RT, Kushnir AS, and White JM (2000) The transmembrane domain of influenza hemagglutinin exhibits a stringent length requirement to support the hemifusion to fusion transition, *J. Cell Biol* 151, 425–437. [PubMed: 11038188]
- [39]. Melikyan GB, Lin SS, Roth MG, and Cohen FS (1999) Amino acid sequence requirements of the transmembrane and cytoplasmic domains of influenza virus hemagglutinin for viable membrane fusion, *Mol. Biol. Cell* 10, 1821–1836. [PubMed: 10359599]
- [40]. Liang S, Ratnayake PU, Keinath C, Jia L, Wolfe R, Ranaweera A, and Weliky DP (2018) Efficient fusion at neutral pH by Human Immunodeficiency Virus gp41 trimers containing the fusion peptide and transmembrane domains, *Biochemistry* 57, 1219–1235. [PubMed: 29345922]
- [41]. Weis DD, Engen JR, and Kass IJ (2006) Semi-automated data processing of hydrogen exchange mass spectra using HX-Express, *J. Am. Soc. Mass Spectr* 17, 1700–1703.

- [42]. Markosyan RM, Cohen FS, and Melikyan GB (2000) The lipid-anchored ectodomain of influenza virus hemagglutinin (GPI-HA) is capable of inducing nonenlarging fusion pores, *Mol. Biol. Cell* 11, 1143–1152. [PubMed: 10749920]
- [43]. Lorieau JL, Louis JM, and Bax A (2011) Helical hairpin structure of Influenza Hemagglutinin fusion peptide stabilized by charge-dipole interactions between the N-terminal amino group and the second helix, *J. Am. Chem. Soc* 133, 2824–2827. [PubMed: 21319795]
- [44]. Tatulian SA, and Tamm LK (2000) Secondary structure, orientation, oligomerization, and lipid interactions of the transmembrane domain of influenza hemagglutinin, *Biochemistry* 39, 496–507. [PubMed: 10642174]
- [45]. Han X, and Tamm LK (2000) A host-guest system to study structure-function relationships of membrane fusion peptides, *Proc. Natl. Acad. Sci. U.S.A* 97, 13097–13102. [PubMed: 11069282]
- [46]. Lai AL, and Freed JH (2015) The interaction between Influenza HA fusion peptide and transmembrane domain affects membrane structure, *Biophys. J* 109, 2523–2536. [PubMed: 26682811]
- [47]. Lakomek NA, Kaufman JD, Stahl SJ, Louis JM, Grishaev A, Wingfield PT, and Bax A (2013) Internal dynamics of the homotrimeric HIV-1 viral coat protein gp41 on multiple time scales, *Angew. Chem. Int. Ed* 52, 3911–3915.
- [48]. Caffrey M, Cai M, Kaufman J, Stahl SJ, Wingfield PT, Covell DG, Gronenborn AM, and Clore GM (1998) Three-dimensional solution structure of the 44 kDa ectodomain of SIV gp41, *EMBO J.* 17, 4572–4584. [PubMed: 9707417]
- [49]. Yang ZN, Mueser TC, Kaufman J, Stahl SJ, Wingfield PT, and Hyde CC (1999) The crystal structure of the SIV gp41 ectodomain at 1.47 Å resolution, *J. Struct. Biol* 126, 131–144. [PubMed: 10388624]
- [50]. Sackett K, Nethercott MJ, Epand RF, Epand RM, Kindra DR, Shai Y, and Weliky DP (2010) Comparative analysis of membrane-associated fusion peptide secondary structure and lipid mixing function of HIV gp41 constructs that model the early pre-hairpin intermediate and final hairpin conformations, *J. Mol. Biol* 397, 301–315. [PubMed: 20080102]
- [51]. Panahi A, and Feig M (2010) Conformational sampling of Influenza fusion peptide in membrane bilayers as a function of termini and protonation states, *J. Phys. Chem. B* 114, 1407–1416. [PubMed: 20043654]
- [52]. Baylon JL, and Tajkhorshid E (2015) Capturing spontaneous membrane insertion of the Influenza virus Hemagglutinin fusion peptide, *J. Phys. Chem. B* 119, 7882–7893. [PubMed: 25996559]
- [53]. Jia LH, Liang S, Sackett K, Xie L, Ghosh U, and Weliky DP (2015) REDOR solid-state NMR as a probe of the membrane locations of membrane-associated peptides and proteins, *J. Magn. Reson* 253, 154–165. [PubMed: 25797012]
- [54]. Victor BL, Lousa D, Antunes JM, and Soares CM (2015) Self-assembly molecular dynamics simulations shed light into the interaction of the influenza fusion peptide with a membrane bilayer, *J. Chem. Inform. Model* 55, 795–805.
- [55]. Worch R, Dudek A, Krupa J, Szymaniec A, and Setny P (2018) Charged N-terminus of influenza fusion peptide facilitates membrane fusion, *Int. J. Mol. Sci* 19.
- [56]. Kuzmin PI, Zimmerberg J, Chizmadzhev YA, and Cohen FS (2001) A quantitative model for membrane fusion based on low-energy intermediates, *Proc. Natl. Acad. Sci. U.S.A* 98, 7235–7240. [PubMed: 11404463]
- [57]. Epand RM, Epand RF, Martin I, and Ruyschaert JM (2001) Membrane interactions of mutated forms of the influenza fusion peptide, *Biochemistry* 40, 8800–8807. [PubMed: 11467940]
- [58]. Gabrys CM, Yang R, Wasniewski CM, Yang J, Canlas CG, Qiang W, Sun Y, and Weliky DP (2010) Nuclear magnetic resonance evidence for retention of a lamellar membrane phase with curvature in the presence of large quantities of the HIV fusion peptide, *Biochim. Biophys. Acta* 1798, 194–201. [PubMed: 19616505]
- [59]. Tristram-Nagle S, Chan R, Kooijman E, Uppamoochikkal P, Qiang W, Weliky DP, and Nagle JF (2010) HIV fusion peptide penetrates, disorders, and softens T-cell membrane mimics, *J. Mol. Biol* 402, 139–153. [PubMed: 20655315]

- [60]. Yao HW, and Hong M (2013) Membrane-dependent conformation, dynamics, and lipid Interactions of the fusion peptide of the paramyxovirus PIV5 from solid-state NMR, J. Mol. Biol 425, 563–576. [PubMed: 23183373]

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A Protein constructs **HA2**

1 GLFGAIAGFI ENGWEGMIDG WYGFRHQNSE GTGQAADLKS TQAAIDQING KLN RVIEKTN
 61 EKFHQIEKEF SEVEGRIQDL EKYVEDTKID LWSYNAELLV ALNQHTIDL TDSEMNKLFE
 121 KTRRQLRENA EEMGNSGFKI YHKADNAAIE SIRNGTYDHD VYRDEALNNR FQIKGVELKS
 181 GYKDWILWIS FAISAFLLAV VLLGFIMWAA QRGNIRANIA IGGGGGGLEH HHHHH

FHA2

1 GLFGA IAGFI ENGWEGMIDG WYGFRHQNSE GTGQAADLKS TQAAIDQING KLN RVIEKTN
 61 EKFHQIEKEF SEVEGRIQDL EKYVEDTKID LWSYNAELLV ALNQHTIDL TDSEMNKLFE
 121 KTRRQLRENA EEMGNSGFKI YHKADNAAIE SIRNGTYDHD VYRDEALNNR FQIKGVELKS
 181 GYKDWLEHHH HHH

SHA2_TM

20 C WYGFRHQNSE GTGQAADLKS TQAAIDQING KLN RVIEKTN
 61 EKFHQIEKEF SEVEGRIQDL EKYVEDTKID LWSYNAELLV ALNQHTIDL TDSEMNKLFE
 121 KTRRQLRENA EEMGNSGFKI YHKADNAAIE SIRNGTYDHD VYRDEALNNR FQIKGVELKS
 181 GYKDWILWIS FAISAFLLAV VLLGFIMWAA QGGGGGGLEH HHHHH

B Ribbon diagrams of the soluble ectodomain and fusion peptide **Figure 1.**

(A) Schematic diagrams and sequences of full-length HA2 and truncated constructs with domains colored: fusion peptide (FP), pink; soluble ectodomain (SE), blue; transmembrane domain (TM), green; and endodomain (Endo), orange. This color-coding is also used in Figures 2 and 8. Amino acid sequences are from the X31 strain which is a H3 subtype. The C-terminal regions in black include a non-native H₆ tag for affinity chromatography. The G1E and I173E mutation sites are underlined in the HA2 sequence. (B) Ribbon diagrams of the SE in the final trimer-of-hairpins structure (PDB 1QU1) and the FP with closed helical hairpin structure (PDB 2KXA). A monomer subunit is highlighted for the SE, as well as the terminal residues of regular secondary structure elements, and residue 173.

Figure 2.

Structural models for WT and mutant HA2 constructs that are based on residue 1–22 FP closed helical hairpin (PDB 2KXA), 36–175 SE hairpin (PDB 1QU1), 186–211 TM continuous α helix that traverses the membrane, and 212–221 endodomain that is disordered. Residues 23–35 and 176–185 are represented as squiggly lines, because there isn't electron density for these residues in PDB 1QU1 for all or some of the monomers of the HA2_{23–185} construct. The WT SE is the trimer-of-hairpins like in Fig. 1B, but a single monomer SE is displayed for clarity. Two WT-HA2 models are shown that either have a FP with helical hairpin structure at the membrane-interface, or FP with continuous α helical structure in complex with TM and with membrane traversal. I173E-HA2 is shown with dissociated C-terminal SE strands and reduced helicity in the N-terminal SE. This model is

consistent with reduced overall helicity at ambient temperature and reduced T_m for I173E vs. WT. G1E-HA2 is shown as an equilibrium between the WT structure and the I173E structure with unfolded FP's that bind the C-terminal SE strands. This model is based on similar helicity at ambient temperature and reduced T_m for G1E vs. WT.

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Figure 3.

HDX-MS data for selected peptides of FHA2, SHA2_TM, and HA2 proteins in DM detergent. Plots of mass spectral signal intensity vs. m/z are displayed for all $t_{\text{HDX}} \equiv$ the incubation time of the protein in buffer with D_2O . The identity and charge of each peptide is given in the left column, with identities confirmed by MS/MS analysis. The shifts of the distributions to larger m/z with t_{HDX} reflect increasing H $\rightarrow$ D substitution in the segment of the protein corresponding to the peptide.

Figure 4.

Plots of percent deuterium incorporation ($D\%$) vs. $\log_{10}[t_{\text{HDX}}/\text{min}]$ for all analyzed peptides of FHA2, SHA2_TM, and HA2. Peak intensities vs. m/z (Fig. 3) are inputs for the HX-Express software, which calculates peak area weighted-average m/z , denoted MH for dilution into H_2O buffer, or $M_D(t)$ for dilution into D_2O buffer for duration $t \equiv t_{\text{HDX}}$. The $D\%(t) = 100 \times [M_D(t) - M_H]/N$, where N is the number of backbone amide hydrogens in the peptide. Each point in a plot is the average of triplicate measurements, with typical RMSD of 1%. Tables S1 list all $D\%(t)$ and associated replicate uncertainties.

Figure 5.

HDX-MS data for selected peptides of WT-, G1E-, and I173E-HA2 proteins in DM detergent. Plots of mass spectral signal intensity vs. m/z are displayed for all $t_{\text{HDX}} \equiv$ the incubation time of the protein in buffer with D_2O . The identity and charge of each peptide is given in the left column, with identities confirmed by MS/MS analysis. The shifts of the distributions to larger m/z with t_{HDX} reflect increasing H $\rightarrow$ D substitution in the segment of the protein corresponding to the peptide.

Figure 6.

Plots of percent deuterium incorporation ($D\%$) vs. $\log_{10}[\text{HDX time/min}]$ for all analyzed peptides of WT-, G1E-, and I173E-HA2. Peak intensities vs. m/z (Fig. 5) are inputs for the HX-Express software, which calculates peak area weighted-average m/z , denoted M_H for dilution into H_2O buffer, or $M_D(t)$ for dilution into D_2O buffer for duration $t \equiv t_{\text{HDX}}$. The $D\%(t) = 100 \times [M_D(t) - M_H]/N$, where N is the number of backbone amide hydrogens in the peptide. Each point in a plot is the average of triplicate measurements, with typical RMSD of 1%. Tables S2 list all $D\%(t)$ and associated replicate uncertainties.

Figure 7.

Presentation of peptide $\langle D_{\%} \rangle$ from Tables 1 and 2 using color-coding and the structural models of Fig. 2. The colors for WT-HA2 are based on the $\langle D_{\%} \rangle$ of peptides 1–9, 10–21, 22–38, 39–52, 53–69, 70–87, 86–91, 92–98, 99–110, 110–115, 120–138, 139–141, 142–150, 151–167, 168–171, 172–178, 179–187, 188–191, 192–197, 198–202, 200–205, and 206–217. The colors for other constructs are based on the $\langle D_{\%} \rangle$ of subsets of these peptides with a few substitutions that include 3–9 for G1E-HA2 and 1–8 and 12–22 for I173E-HA2.

Figure 8.

Structural models for WT-, I173E-, and G1E-HA2 developed using the HDX-MS data of the present study. The proteins are bound to two membranes prior to fusion using color coding of domains like in Figs. 1 and 2. WT has trimer-of-hairpins structure for the SE as in Fig. 1B, but only one monomer is shown for clarity. The approximate positions of specific residues are indicated by the numbers. The WT model combines residue 1–22 closed FP helical hairpin (PDB 2KXA), 23–35 antiparallel β sheet of the HA2_{1–175}/HA1 complex (PDB 2HMG), 36–178 hairpin SE structure (PDB 1QU1), 186–211 continuous α helix for TM, and 212–221 unstructured endodomain. The 179–185 extended structure is found in one monomer in PDB 1QU1, but there isn't electron density for these residues in the other two monomers. The TM traverses the virus membrane, the FP is interfacially-bound to the target membrane, and the membranes are held in close apposition by the fully-structured and semi-rigid 23–185 SE. The close apposition reduces the activation energy for fusion. The HDX-MS data support new features in Fig. 8 vs. 2 that include the 23–35 β sheet, 179–185 structure, and respective membrane interfacial and traversal locations of the FP and TM rather than the FP/TM complex in Fig. 2. The Fig. 8 structure is retained in the final fused membrane which has local positive curvature that accommodates the FP and TM locations (see Fig. S2) and may be important for fusion pore expansion. The I173E model shows dissociation of the C-terminal strands from the trimer-of-hairpins and reduced helicity for the N-helices, with resulting larger average distance between the membranes, and impaired fusion catalysis. The G1E model shows binding of the FP to the C-terminal strands, which

results in more-distant membrane apposition and weaker FP binding to the target membrane, and consequent impaired fusion catalysis. The displayed G1E structure is in equilibrium with the WT structure, like in Fig. 2.

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Table 1.

 $\langle D_{\%} \rangle$ for peptides from FHA2, SHA2_TM, and HA2^{a,b}

Peptide	Protein		
	FHA2	SHA2_TM	HA2
1–8	44.88 (0.58)		63.02(0.52)
1–9	46.82(0.40)		63.03(0.94)
10–21	38.43(0.68)		53.88(0.31)
4–11	34.67(0.76)		
22–38	17.19(0.61)	13.13(0.55)	17.43(0.66)
39–52	4.74(0.35)	8.12(0.13)	3.26(0.23)
53–69	3.33(0.34)	5.05(0.29)	5.50(0.23)
70–87	1.66(0.78)	3.47(0.15)	2.01(0.06)
86–91	1.81(0.42)	4.16(0.55)	3.02(0.54)
92–98	0.82(0.06)	0.74(0.12)	1.09(0.39)
92–99	1.73(0.29)	3.44(0.18)	2.18(0.25)
99–110	16.10(0.35)	19.05(0.52)	17.21(0.22)
110–115	28.04(0.70)	23.19(0.65)	32.87(0.36)
120–138	41.29(0.54)	41.40(0.96)	42.81(0.87)
133–138	35.72(0.54)	41.17(0.31)	42.20(0.51)
139–141	42.43(0.49)	47.03(0.55)	43.88(0.96)
142–150	46.68(0.83)	49.13(0.54)	47.04(0.04)
151–162	37.14(0.65)		37.95(0.38)
151–167	37.41(0.76)	41.21(0.66)	37.50(0.56)
163–167	43.33(0.71)	44.20(0.68)	45.43(0.34)
163–171	35.26(1.07)	35.38(0.83)	34.27(0.83)
168–171	52.98(0.73)	54.26(0.55)	48.97(0.87)
172–178	50.03(0.45)	49.36(0.98)	49.85(0.67)
179–187		39.10(0.47)	39.07(0.54)
188–191		11.27(0.54)	15.31(0.13)
192–197		2.59(0.32)	2.19(0.17)
198–202/199–203		1.19(0.28)	1.24(0.42)
200–202		0.40(0.24)	0.13(0.11)
200–205			0.89(0.13)
206–217			57.41(1.04)

^a $\langle D_{\%} \rangle$ is the average $D_{\%}$ for $t_{\text{HDX}} = 1, 5, 30, 60$, and 120 min (see Fig. 4 and Table S1).^bThe numbers in parentheses are the calculated uncertainties.

Table 2. $\langle D_{\%} \rangle$ for peptides from WT-, G1E-, and I173E-HA2^{a,b}

Peptide	Protein		
	WT-HA2	G1E-HA2	I173E-HA2
1–8	63.02(0.52)		53.89(0.74)
3–9		55.09(1.00)	55.33(1.45)
10–21	53.88(0.31)	44.68(0.70)	
12–22		49.79(1.14)	63.18(1.19)
22–38	17.43(0.66)	17.29(0.57)	35.23(0.42)
39–52	3.26(0.23)		47.00(0.87)
53–69	5.50(0.23)	4.12(0.51)	18.44(0.41)
70–87	2.01(0.06)	2.93(0.41)	1.75(0.11)
86–91	3.02(0.54)	3.16(0.54)	0.90(0.22)
92–98	1.09(0.39)	4.12(0.21)	4.64(0.12)
92–99	2.18(0.25)	1.92(0.59)	2.74(0.58)
99–110	17.21(0.22)	19.32(0.53)	21.40(0.92)
110–115	32.87(0.36)	31.43(0.77)	38.17(0.98)
120–138	42.81(0.87)	39.47(0.44)	46.49(0.77)
133–138	42.20(0.51)	43.02(0.39)	36.69(0.40)
139–141	43.88(0.96)	38.90(0.46)	40.22(1.05)
142–150	47.04(0.04)	50.58(0.29)	47.77(0.61)
151–167	37.50(0.56)	39.77(0.16)	44.26(0.85)
163–167	45.43(0.34)	46.64(0.41)	48.70(0.71)
163–171	34.27(0.83)	35.99(0.90)	40.11(0.71)
168–171	48.97(0.87)	54.33(1.51)	56.21(0.82)
172–178	49.85(0.67)	50.34(0.53)	60.05(0.81)
179–187	39.07(0.54)	42.79(0.96)	49.78(0.94)
188–191	15.31(0.13)	27.00(1.10)	32.11(1.11)
192–197	2.19(0.17)	1.07(0.17)	1.31(0.10)
198–202/199–203	1.24(0.42)	2.13(1.12)	1.62(0.85)
200–202	0.13(0.11)	0.00(0.00)	0.13(0.06)
206–217	57.41(1.04)	56.56(0.45)	55.63(1.00)

^a $\langle D_{\%} \rangle$ is the average $D_{\%}$ for tHDX = 1, 5, 30, 60, and 120 min (see Fig. 6 and Table S2).^bThe numbers in parentheses are the calculated uncertainties.