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Stabilities of the Soluble Ectodomain and Fusion Peptide Hairpins of the Influenza Virus Hemagglutinin Subunit II Protein Are Positively-Correlated with Membrane Fusion

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Abstract

Cellular entry of influenza virus is mediated by the viral protein hemagglutinin(HA), which forms an initial complex of three HA1 and three HA2 subunits. Each HA2 includes a fusion peptide(FP), soluble ectodomain(SE), and transmembrane domain. HA1 binds to cellular sialic acids, followed by virus endocytosis, pH reduction, dissociation of HA1, and HA2 structural rearrangement into a final trimer-of-SE hairpins. pH reduction also triggers HA2-mediated virus/endosome membrane fusion. SE hairpins have an interior parallel helical bundle and C-terminal strands in the grooves of the bundle exterior. FP's are separate helical hairpins. The present study compares WT-HA2 with G1E(FP) and I173E(SE strand) mutants. WT-HA2 induces vesicle fusion at pH 5.0, whereas fusion is greatly reduced for both mutants. Circular dichroism for HA2 and FHA2=FP+SE constructs show dramatic losses in stability for the mutants, including a T_m reduced by 40 °C for I173E-FHA2. This evidences destabilization of SE hairpins via dissociation of strands from the helical bundle, which is also supported by larger monomer fractions for mutant vs. WT proteins. The G1E mutant may have disrupted FP hairpins, with consequent non-native FP binding to dissociated SE strands. It is commonly proposed that free energy released by the HA2 structural rearrangement catalyzes HA-mediated fusion. The present study supports an alternate mechanistic model in which fusion is preceded by FP insertion in the target membrane and formation of the final SE hairpin. Lower fusion by the mutants is due to loss of hairpin stability and consequent reduced membrane apposition of the virus and target membranes.

Graphical Abstract

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Supporting Information

DNA sequences corresponding to protein inserts and primers, size-exclusion chromatography of replicate samples, SDS-PAGE after cross-linking of replicate samples, circular dichroism spectra of replicate samples, circular dichroism spectra of I73E-FHA2 with or without 150 mM NaCl and at different protein concentrations, van't Hoff plots, and vesicle fusion for different protein and vesicle preparations.

Summary

The present study reports protein-induced vesicle fusion by the full-length HA2 containing the FP and the TM, and reduced fusion by the G1E and I173E mutants. These mutants also result in reduced HA-mediated cell fusion. The present and previous studies support that the HA2 final trimer-of-hairpins is fusogenic. This implies common features of the influenza virus/endosome fusion and HA2/vesicle fusion that include: (1) apposing the two fusing compartments by folded hairpin HA2 proteins and maintaining this apposition with a stable SE; (2) binding of the FP to the target membrane; and (3) HA2 aggregation at low pH and consequent clustering of membrane-perturbing FP's.

Introduction

Influenza is an enveloped RNA virus in the family *Orthomyxoviridae*, and is either spherical with ~100 nm diameter, or filamentous with ~300 nm length.¹ An initial infection step is fusion (joining) of virus and cell membranes with consequent deposition of the nucleocapsid in the host cytoplasm.^{2–4} The influenza virus hemagglutinin (HA) protein mediates virus binding to the host cell and subsequent membrane fusion. HA is translated as a single protein, and then proteolytically cleaved into the disulfide-linked subunits HA1 (328 residues) and HA2 (221 residues). Fusion is catalyzed by HA2 which contains the fusion peptide (FP), soluble ectodomain (SE), transmembrane domain (TM), and endodomain with respective lengths of ~25, 160, 25, and 10 residues (Fig 1). The initial HA complex outside the membrane includes three HA2 ectodomains and three HA1's. The complex has a mushroom-shape with stem (HA2 trimer) and head (HA1's).^{5, 6} The stem includes an interior core with a parallel coiled coil of residue 76–125 helices. The HA2 N-terminal regions include 56–75 extended loops, 38–55 helices that pack antiparallel in exterior grooves of the coiled-coil, and 1–20 irregular FP and 21–35 antiparallel β sheet structures located between the stem and the head. The C-terminal HA2 regions include 130–141 β sheet hairpins and compactly-associated 146–153 and 159–170 helices.

Host cell infection begins with HA1 binding to cell-surface sialic acids, which initiates endocytosis, and then endosome maturation with pH reduction to <6. HA1 separates from HA2 at this pH, and the HA2 SE transforms to a final-state trimer-of-hairpins structure (Fig. 1B).^{7, 8} This structure includes an interior core of parallel coiled-coil 38–105 helices, 106–109 180° turns, and 110–128 helices and 154–176 strands that both pack antiparallel in the exterior grooves of the coiled-coil. Structural differences between the initial and final SE

states include: (1) the 38–55 exterior helices and 56–75 loops become part of the interior helices; (2) the 106–128 interior helices become the 106–109 turns and 110–128 exterior helices; and (3) the 159–170 helices become part of the 154–176 strand regions of the hairpins.

Besides causing significant HA structural rearrangement, reduced pH is also the trigger for virus/endosome membrane fusion. Most mechanistic insight for fusion is based on the surrogate system of fusion between HA-expressing cells (HA-cells) and red blood cells (RBC's). The process includes a hemifusion step with outer leaflet membrane mixing, followed by breakage of the hemifusion diaphragm by pore formation, and then pore expansion.^{9–11} There isn't yet imaging that temporally correlates the HA-structural changes and membrane changes during fusion, so mechanisms are primarily based on effects of mutations on fusion.^{10, 12, 13}

The HA2 FP is sequestered in the interior of the initial HA complex, and is released during the process of HA1/HA2 separation and HA2 structural transformation into the final hairpin state. The FP is the most-conserved region of the HA sequence and is important in all steps of membrane fusion, as evidenced by both the G1E mutant (HA2 numbering), which results in no HA-cell/RBC fusion, and the G1S mutant which results in hemifusion without pore expansion.^{10, 14, 15} The NMR structure of monomeric FP in detergent-rich media (without the rest of the HA2) shows predominant N-helix/turn/C-helix structure with tight antiparallel packing of the two helices.^{16, 17} In membrane, the FP shows a mixture of this “closed” structure and a “semi-closed” structure in which the F9 ring is inserted between the two helices.^{18, 19} These monomer FP structures may be relevant for the membrane-bound FP in the final hairpin state of HA2, as EPR spectral linewidths of a HA2_{1–127} construct with trimeric SE correlate with monomeric FP domains.²⁰

HA constructs that are truncated in the HA2 TM region result in arrested HA-cell/RBC hemifusion with non-expanding pores, which evidences the importance of a membrane-spanning TM in the final fusion pore expansion step.^{21, 22} Fusion is also observed for constructs containing the HA2 ectodomain fused with the TM and endodomain from a different protein.²³ CD spectra of the HA2 TM peptide in detergent and membrane correlate with the expected α helical structure.²⁴

Earlier studies have shown that large HA2 constructs are hyperthermostable with $T_m > 80^\circ\text{C}$. These constructs include HA2_{20–185} $\equiv$ SE $\equiv$ SHA2, HA2_{1–185} $\equiv$ FP+SE $\equiv$ FHA2, HA2_{20–211} $\equiv$ SE+TM $\equiv$ SHA2-TM, and HA2_{1–221} $\equiv$ full-length HA2.^{25, 26} Size-exclusion chromatography (SEC) and cross-linking data often correlate to a large trimer fraction in N-lauroylsarcosine (SRC) or *n*-decyl- β -D-maltopyranoside (DM) detergent at pH 7.4, with visible aggregation at pH 5.0. The circular dichroism (CD) θ_{222} values in DM at pH 7.4 correlate with ~60% helicity, which is generally consistent with a trimer-of-hairpins structure for the SE. All four constructs induce fusion of anionic vesicles at pH 5.0, with significant fusion contributions from the SE and FP. Exogenous addition of constructs like FHA2 or the shorter HA2_{1–127} induce hemifusion and pore formation between “HA0”-cells bound to RBC's, where HA0-cells express uncleavable HA's that don't induce fusion.^{27, 28}

The present study describes structural, biophysical, and functional studies for the G1E and I173E mutants of FHA2 and HA2, with comparison to their WT counterparts. Both mutants result in nearly-complete loss of HA-cell/RBC fusion and also complete loss of HA0-cell/RBC fusion mediated by exogenously-added FHA2.^{10, 13, 27, 28} The N-terminal G1E mutant is in the FP, whereas the I173E mutant is in the SE. For the hairpin structure, I173 is in the exterior strands that bind to the grooves of the interior helical bundle. Some data are consistent with shallower membrane insertion of G1E vs. WT FP.²⁹ To our knowledge, there aren't yet biophysical data for the I173E mutant.

Materials and Methods

Materials

Some purchased materials were: DNA primers – Integrated DNA Technologies, Coralville, IA; *Escherichia coli* BL21(DE3) strain – Novagen, Gibbstown, NJ; Luria-Bertani (LB) medium – Dot Scientific, Burton, MI; isopropyl β -D-thiogalactopyranoside (IPTG) – Goldbio, St. Louis, MO; Cobalt affinity resin – Thermo Scientific, Waltham, MA; n-decyl- β -D-maltopyranoside (DM) – Anatrace, Maumee, OH; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (POPG), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) (ammonium salt) dipalmitoylphosphatidylethanolamine (*N*-NBD-DPPE), *N*-(lissaminerhodamine B sulfonyl) (ammonium salt) dipalmitoylphosphatidylethanolamine(N-Rh-DPPE) – Avanti Polar Lipids, Alabaster, AL. Most other materials were purchased from Sigma-Aldrich, St. Louis, MO.

Protein constructs, expression, and purification

The plasmids containing the DNA sequences of full-length HA2_{1–221} and FHA2 $\equiv$ HA2_{1–185} ectodomain were extracted from previously sub-cloned *E. coli* cell cultures.²⁶ The G1E and I173E mutants of FHA2 and HA2 were generated by PCR using DNA primers and confirmed by DNA sequencing (Figs. S1 and S2). Each pET24a(+) plasmid was transformed into BL21(DE3) competent cells that were then streaked on an agar plate. After overnight growth in agar, a single colony was transferred to 50 mL Luria-Bertani (LB) medium and grown overnight. All media contained 50 mg/L kanamycin antibiotic, and growth cultures were shaken at 180 rpm and 37 °C. Stock aliquots for future growths were made by mixing 1 mL culture and 0.5 mL 50% glycerol, and were stored at –80 °C. An expression culture was prepared by adding 50 μ L stock to 50 mL LB, followed by overnight growth, and then addition of 1 L fresh LB and growth until OD₆₀₀ $\approx$ 0.5. Protein expression was induced by addition of 1 mM IPTG and then continued for 5 hours at 37 °C. The culture was centrifuged at 9000g and 4 °C for 10 min, followed by harvesting the cell pellet, and storage at –20 °C.

Purifications from cells expressing WT-, G1E-, and I173E-FHA2 were based on earlier work, and is denoted **protocol A**.²⁶ Buffers were prepared at 4 °C. Wet cells (5 g) were subjected to four rounds of sonication in 40 mL of buffer A (50 mM sodium phosphate at pH 8.0, 0.5% *N*-lauroylsarcosine (SRC), 300 mM NaCl, and 10 mM imidazole) in an ice bath. Each round lasted for 1 min, with 80% amplitude intervals for 0.8 s followed by 0.2 s

off. The lysate was centrifuged at 48000*g* and 4 °C for 20 min, and subsequent purification was done at ambient temperature using cold buffers. The lysate supernatant and 1 mL of Co²⁺ affinity resin were stirred for 1 h, and the protein-coated resin was then collected by pouring the suspension through a fritted column. Weakly-bound proteins were removed from the resin by washes (4×) with buffer A, with wash volumes of 1 mL (WT-FHA2) or 2 mL (G1E- and I173E-FHA2). Subsequent elutions were done with buffer B (50 mM sodium phosphate at pH 8.0, 0.5% SRC, 300 mM NaCl, and 250 mM imidazole). The elutions (4 × 0.5 mL) were individually characterized by SDS-PAGE, and those with highest purities and quantities of FHA2 were pooled.

Low yield- and low-purity protein was obtained when protocol A was applied to cells expressing WT-HA2, so a new **protocol B** was developed for HA2 that began with sonication of 5 g wet cells in 40 mL PBS (10 mM sodium phosphate and 2 mM potassium phosphate at pH 7.4, with 137 mM NaCl and 3 mM KCl) using the same sonication parameters as above, followed by removal of the supernatant. The remaining pellet was subjected to sonication in fresh PBS and removal of the supernatant, and the new pellet was also subjected to this procedure. The final pellet was then subjected to a purification protocol similar to that used for FHA2-expressing cells, including sonication in buffer A, centrifugation of the suspension, and affinity purification of the supernatant. The eluent was mixed with an equal volume of ice-cold buffer that contained 10 mM Tris at pH 8.0, 0.17% DM, 2 mM EDTA, and 1 M *L*-arginine, followed by mixing overnight at 4 °C.^{26, 30, 31}

CD, SEC, Cross-linking, and vesicle fusion

Protein samples for biophysical experiments were prepared by dialysis of FHA2 purification eluent or HA2 in buffer with 1 M *L*-arginine. For CD, SEC, and vesicle fusion, the dialysis buffer contained 10 mM Tris at pH 7.4, and for cross-linking, 20 mM HEPES at pH 7.4. The dialysis buffer also contained either 0.10% SRC or 0.17% DM, and for SEC and vesicle fusion, 150 mM NaCl. Dialysis buffer was replaced each day for the 3-day total dialysis time (5-day for CD samples). The protein concentration was then adjusted by dilution or by a concentrator.

CD spectra of protein (15 μM) in buffer with DM were acquired with a CD instrument (Chirascan, Applied Photophysics), temperature-controlled cuvette with 1 mm pathlength, 260–190 nm wavelength range, 0.5 nm wavelength increments, and 1.5 s averaging-time per wavelength point. Each displayed spectrum is the difference between the protein + buffer and buffer-only spectra.

SEC was done with a FPLC (DuoFlow Pathfinder 20, Bio-Rad), semi-preparative column (Tricorn Superdex 200 Increase 10/300 GL, GE Technologies), 0.3 mL/min flow rate, and *A*₂₈₀ detection. The column was equilibrated with an initial run with dialysis buffer without protein. Subsequent SEC was done with [loading protein] ≈ 1 mg/mL which corresponds to [running protein] ≈ 0.1 mg/mL.

Cross-linking between lysine ε-NH₂ groups was achieved by stirring protein (20 μM) with bis(sulfosuccinimidyl) suberate (1 mM) for 1 hour. The reaction was quenched by addition

of Tris-HCl buffer at pH 6.8, with final [Tris] = 50 mM, and products were assayed by SDS-PAGE.

Vesicles for fusion studies contained POPC, POPC:POPG (4:1), or POPC:DOTAP (4:1) lipids. PC is a common lipid headgroup of the membranes of host cells infected by influenza virus.^{32, 33} The PG lipids are included to reflect the negatively-charged lipids in the cell membrane. The DOTAP headgroup has a charge of +1 and provides an additional probe of electrostatic effects on fusion. The role of protein charge is probed by comparison of fusion at physiological pH 7.4 vs. pH 5.0, where the latter is in the 5–6 range of influenza fusion with the endosome. The protein charge is ~ -10 at pH 7.4 and $+2$ at pH 5.0, whereas the lipid charges don't change with pH. Lipids were dissolved in chloroform:methanol (9:1 v/v), followed by solvent evaporation with nitrogen gas and vacuum pumping. The resulting lipid films were suspended in 5 mM HEPES and 10 mM MES at pH 7.4 or 5.0, with 0.01% NaN_3 . Fluorescently labeled vesicles were similarly prepared, with additional 2 mole% fluorescent lipid (*N*-NBD-DPPE) and 2 mole% quenching lipid (*N*-Rh-DPPE). The lipid suspensions were subjected to 10 freeze/thaw cycles followed by multiple extrusions through a filter with 100 nm diameter pores which produced unilamellar vesicles with ~ 200 nm diameter. Labeled and unlabeled vesicles were mixed in 1:9 ratio with [total lipid] = 150 μM . HA2-induced fusion between labeled and unlabeled vesicles was detected as an increase in fluorescence due to larger average distance between fluorescent and quenching lipids. Assay conditions included vesicle stirring at 37 °C, continuous excitation with 467 nm radiation, and gated detection of fluorescence at 530 nm with 1 s time increments. The initial fluorescence of the vesicle solution is denoted F_0 , and addition of HA2 stock marks $t = 0$, with $F(t)$ then measured for ~ 600 s. The stock contained 40 μM HA2 and 0.17% DM in pH 7.4 buffer with 150 mM NaCl, and the mixed solution had HA2:lipid mole ratio = 1:300 and 0.002% DM. Subsequent addition of 12 μl of 20 % Triton X-100 detergent solubilized the vesicles and provides F_{max} . Percent vesicle fusion is calculated as $[(F(t) - F_0)/F_{\text{max}} - F_0] \times 100$. There is typical $\pm 1\%$ variation in long-time fusion extent between assay replicates, and there is negligible fusion after addition of an aliquot of stock buffer without protein. Although our conversion of fluorescence increases to percent vesicle fusion is standard practice, the calculated fusion is not a quantitative measure of percent fused vesicles. The fluorescence varies as $[1 + (R_0/R_{FQ})^6]^{-1}$, where R_0 and R_{FQ} are the Forster and average fluorophore-quencher distances, so fluorescence changes and percent lipid mixing are sensitive to the initial fluorophore and quencher concentrations. The most meaningful fusion comparisons are between different proteins with the same vesicle stock, and between different vesicle compositions using the same protein stocks.

Results

Protein preparation and folding

Fig. 2 displays SDS-PAGE of the six proteins. The purified yields of WT-, G1E-, and I173E-FHA2 are ~ 10 , 5, and 3 mg/L culture, whereas the yields of the corresponding HA2 variants are ~ 2 , 1, and 0.7 mg/L culture. High-purity FHA2 proteins were obtained by protocol A with affinity chromatography of the supernatant of the SRC-solubilized cell lysate. The proteins were well-folded after dialysis into buffer with DM, as evidenced by prominent

minima in the CD spectra at 208 and 222 nm, which are characteristic of the expected predominant α helical structure. Quantitative analysis of the $|\theta_{222}|$ values is presented in the CD results. Application of protocol A to cells that expressed HA2 resulted in low-yield and low-purity HA2. Reasonable yields of high-purity HA2 proteins were obtained by protocol B for which there was an initial step of removal of PBS-soluble and -suspendable material, followed by solubilization of the remaining cell pellet with sonication in buffer with SRC, and then affinity purification. The HA2 product was pure but not well-folded, based on a small $|\theta_{222}|$ value of the CD spectrum obtained after direct dialysis into buffer with DM. Well-folded HA2 was obtained if there was intermediate dialysis into buffer with DM and 1 M *L*-arginine, followed by dialysis into buffer with DM and no *L*-arginine. These data support folded HA2 as the lowest free-energy state in buffer with DM. The *L*-arginine may break up partially-misfolded HA2 aggregates.

We describe a model that explains the underlying bases of the different protocols to obtain pure and well-folded FHA2 and HA2, as well as the different yields. We first consider the protocol B initial sonications in PBS, with discarding of the PBS supernatants obtained after centrifugation with 48000g. The HA2-content of the initial PBS supernatant was investigated by centrifugation at 220000g, and then subjecting the pellet to protocol A. The resulting eluent contained very pure HA2 that was well-folded, as judged by the CD spectrum after direct dialysis into buffer with DM. However, the HA2 yield from this “220000g pellet” was much lower than the yield of partially-folded HA2 from the “48000g pellet” of protocol B. We interpret our results as supporting FHA2/HA2 locations both in the bacterial membrane and in inclusion bodies, with respective well-folded and partially-folded molecular structures. The protocol A supernatant from SRC sonication of cells expressing FHA2 contains mostly membrane-located FHA2, whereas the protocol B supernatant from SRC sonication of cells expressing HA2 contains mostly inclusion body HA2. The discarded supernatants from the protocol B initial sonications in PBS contain a small quantity of membrane-suspended and well-folded HA2. Overall, these data evidence that SRC is an effective detergent for both membrane- and inclusion body-FHA2/HA2, and solubilizes more rapidly the folded membrane fractions of these proteins. The latter idea is consistent with the results of protocol A for FHA2, including folded FHA2 and a substantial pellet which is not purified and contains predominantly partially-folded inclusion body FHA2. The latter point is supported by previous purification of FHA2 from this pellet, with resulting ~10 mg FHA2/L culture yield that is comparable to the protocol A yield.³¹ This inclusion body FHA2 was only well-folded when there was intermediate dialysis into buffer with 1 M *L*-arginine and DM.

We estimate >10× higher expression of FHA2 vs. HA2 based on ~5× greater purified yields of WT-, G1E-, and I173E-FHA2 via protocol A vs. their HA2 counterparts via protocol B, ~2× greater purified FHA2 yield from complete solubilization of the cell pellet vs. the protocol A partial solubilization, and detection of FHA2 but not HA2 in the washes of the Co²⁺-resin with bound protein. The lower purified yields of G1E- and I173E- vs. WT proteins probably has some contribution from different expression levels, as the ratio of purified yields for FHA2 vs. HA2 is ~5 for the WT-, G1E-, and I173E- variants. All of the reported yields are from sonications with 40 mL buffer. For protocol B, there was complete solubilization in 40 mL buffer with SRC for the pellet from cells expressing WT-HA2,

whereas 50 mL was required for the pellets from cells expressing G1E- or I173E- HA2. Use of 50 vs. 40 mL resulted in a ~0.3 mg/L greater purified yields of G1E- and I173E- HA2.

Size-exclusion chromatography and cross-linking

Fig. 3 displays (left) SEC before cross-linking and (right) SDS-PAGE after cross-linking. Data were obtained in (A) SRC and (B) DM detergents. Data for replicate samples are presented in Fig. S3. Individual SEC elution peaks and cross-linking bands are assigned to monomer, dimer, trimer, and aggregate. Data for WT proteins are similar to previous results.²⁶ For all proteins in SRC and HA2 proteins in DM, there is a large SEC peak at ~250 kDa which is assigned to trimer with ~75 kDa protein and ~175 kDa detergent contributions. This SEC assignment is consistent with earlier observations, including a ~1:120 protein:detergent mole ratio for a HA2 fusion protein trimer, and dominant trimer for the HA2 soluble ectodomain in aqueous solution.^{25, 30} Several of the SEC traces exhibit a peak at ~40 kDa which is assigned to a monomer with ~25 kDa protein and ~15 kDa detergent contributions. This is a major peak for the I173E mutants in DM and a minor peak in other traces. Some traces also exhibit a minor peak at ~150 kDa which is assigned to dimer with ~50 kDa protein and ~100 kDa detergent contributions. Both WT- and G1E- FHA2 proteins in DM have a dominant peak in the void volume corresponding to oligomers/aggregates with MW's > 1 MDa.

After cross-linking, the monomer is the most intense band for all constructs in both detergents, followed by the dimer and trimer. Higher MW bands are weaker, except for FHA2 constructs which exhibit a band for large aggregates that doesn't migrate in the gel. The cross-linking data are generally consistent with the monomer, dimer, and trimer peaks observed in SEC, with the caveat that the cross-linking reaction is not complete so that the band intensities do not reflect populations.

Circular dichroism

Fig. 4 presents CD spectra of the six constructs in DM at pH 7.4 and ambient temperature. These data were taken on the same day using the same CD spectrometer. Data for replicate samples are presented in Fig. S4 and typically exhibit $\pm 5\%$ variation in θ_{222} values among replicates. All constructs exhibit spectra characteristic of α helical structure, with minima at 208 and 222 nm. Table 1 reports average helicities calculated from θ_{222} values, with higher helicity for FHA2 vs. HA2, and for WT and G1E vs. I173E. The similar helicities of G1E and WT proteins are consistent with helical FP structure for G1E, whereas reduced helicity of I173E proteins supports some disruption of the SE structure in this mutant. There isn't NaCl in the sample buffers for the Fig. 4 CD spectra, but Fig. S5 shows that for I173E-FHA2, similar spectra are obtained in the absence vs. presence of 150 mM NaCl. Fig. S5 also presents spectra with [I173E-FHA2] = 1, 5, 10, and 20 μ M, and all the θ_{222} values correlate to a significant fraction of α helical structure.

Fig. 5 displays CD spectra obtained between temperatures of 25 and 90 °C in 5 °C increments and Fig. 6 displays plots of θ_{222} vs. temperature. All spectra for a particular construct were acquired on the same day. Thermostability of constructs are qualitatively ordered WT-HA2 > WT-FHA2 > G1E-HA2, I173E-HA2 > G1E-FHA2 > I173E-FHA2. The

θ_{222} value at each temperature data is used to calculate the fraction folded protein $f_{folded} = |\theta_{222} - \theta_{unfolded}| / |\theta_{folded} - \theta_{unfolded}|$, and then $f_{unfolded} = 1 - f_{folded}$. The model equilibrium is folded trimer $\leftrightarrow$ 3 unfolded monomers, so that $K_{eq} = (f_{unfolded})^3 / (1 - f_{unfolded})/3$. The unfolded state is likely a monomer rather than trimer because: (1) the favorable hydrophobic effect of the trimer is achieved with folded monomer units but not unfolded units; and (2) the translational entropy is greater for three independent monomers than for a trimer. The van't Hoff analysis of $\ln K_{eq}$ vs. $1/T$ is linear-fitted, and the best-fit slope and $\ln K_{eq}$ -intercept are used to calculate $H_m = -R \times \text{slope}$, $S_m = R \times \ln K_{eq}\text{-intercept}$, and $T_m = H_m / S_m$ that corresponds to $\ln K_{eq} = 0$ (Table 1). The T_m values for G1E-FHA2 and I173E-FHA2 are near the middle of the 25–90 °C experimental temperature range, and the $\ln K_{eq}$ vs. $1/T$ plots are reasonably linear over the whole range (Fig. 7). The T_m values are higher for the other proteins, and the $\ln K_{eq}$ vs. $1/T$ plots are only linear for the temperature range around T_m over which $\ln K_{eq}$ changes sign and exhibits significant changes in magnitude (Fig. S6). The numbers in parentheses in Table 1 are the fitting-associated uncertainties of the parameter values. There are also uncertainties in the θ_{folded} and $\theta_{unfolded}$ values which are chosen based on extrapolations from the respective θ_{25C} and θ_{90C} . These θ uncertainties result in uncertainties in the calculated $f_{unfolded}$, $\ln K_{eq}$, and fitted parameter values. Changes in θ_{folded} and $\theta_{unfolded}$ typically lead to <2 °C changes in T_m .

Vesicle fusion

Fig. 8A displays traces of HA2-induced PC vesicle fusion vs. time at pH 5.0, and Fig. 8B displays long-time fusion extents for PC, PC:PG (4:1), and PC:DOTAP (4:1) vesicles at pH's 5.0 and 7.4. Fig. S7 displays fusion extents from replicate assays with different protein and vesicle preparations. The trends for each protein are typically similar between the Figures for pH 5.0 vs. 7.4 and for PC:PG vs. PC:DOTAP vesicles. Influenza virus fuses with the late endosome at pH < 6 . At pH's 5.0 and 7.4, PC has a neutral zwitterionic headgroup, and PG and DOTAP respectively carry charges of -1 and $+1$. The membranes of host cells of influenza contain ~25 mole% PC and ~15 mole% anionic lipid including PG, and there are widely-varying lipid compositions of the chromatographic fractions of endosomal membranes.^{26, 32–35} The influenza virus also fuses with vesicles with a variety of lipid compositions.³⁶ Although there aren't physiological positively-charged lipids, PC:DOTAP vesicles are studied to probe the effect on fusion of the sign of lipid charge. The HA2:lipid mole ratio = 1:300 of the assay is smaller than the ~1:50 ratio of the virus.^{37, 38} HA-mediated fusion includes lipid mixing as well as pore formation, leakage, and contents mixing.^{12, 13, 36} Protein-mediated vesicle fusion exhibits faster leakage than contents mixing, and in the present study, vesicle fusion is assayed by lipid mixing, which is a common feature with HA-fusion.^{39, 40}

Fig. 8A shows much more extensive PC vesicle fusion at pH 5.0 for WT- vs. mutant- HA2's, with similar reduced fusion for both G1E and I173E. Fig. 8B shows that PC fusion is reduced at pH 7.4, but still higher for WT vs. mutant. At pH 5.0, there is lower fusion of PC:PG vs. PC vesicles, while at pH 7.4, there is similar reduced fusion for both vesicle-types. At pH 5.0, the calculated WT-HA2 charge is $+3$, so the higher PC vs. PC:PG fusion evidences that attractive HA2/vesicle electrostatic energy is not required for fusion catalysis. This conclusion is similarly supported by significant fusion of PC:DOTAP vesicles at both

pH's, even though there are respective repulsive vs. attractive HA2/vesicle electrostatic energies at pH 5.0 vs. 7.4. Detailed interpretations of the fusion extents are presented in the Discussion.

Discussion

The present study describes a structural and functional comparison between WT- full-length HA2 and the truncated construct lacking the TM, FHA2, and the G1E and I173E point mutants that are known to inhibit HA-mediated fusion. Significant findings of the present study include: (1) predominant trimer fraction in SRC detergent at pH 7.4 for all protein constructs vs. mixtures of trimer, monomer, and oligomers/aggregates in DM detergent; (2) similar helicities of WT- and G1E- proteins vs. reduced helicity of I173E- proteins; (3) hyper-thermostable WT- FHA2 and HA2 and less-stable FHA2 and HA2 mutants with respective reductions of ~40 and ~15 °C in T_m ; and (4) efficient HA2- induced vesicle fusion of neutral and anionic vesicles at pH 5.0 for WT-HA2 vs. reduced fusion with mutants.

Models of protein structure and stability

The CD spectra and analyses of Fig. 4 and Table 1 support 65% average helicity for WT- and G1E- FHA2 and 58% average helicity for WT- and G1E- HA2. The proteins are well-folded, based on reasonable agreements between these average helicities and the fractions α helical residues calculated for a model in which the only α helical residues are those in high-resolution structures of the FP in detergent and the SE in aqueous solution, and in the TM (residues 2–12, 14–22, 38–105, 110–128, 146–153, 186–210, Fig. 1B).^{8, 16} The calculated helical fractions from this model are 115/193 residues for FHA2 and 140/235 residues for HA2. The helicities of WT- FHA2 and HA2 determined in the present study are similar to the helicities reported in some earlier studies.^{26, 31, 41} However, the 65% helicity for WT- and G1E- FHA2 in the present study is higher than the respective ~25% and ~35% helicities of a 2011 study.²⁸ The origin of this discrepancy isn't known, but we note that our high vs. their low helicities correlates with the presence vs. absence of detergent in the samples. The present study also shows that the I173E mutants exhibit only 45% helicities and a model explaining this reduced helicity is presented below.

The hyperthermostabilities of WT- FHA2 and HA2 are evidenced by the CD spectra and θ_{222} vs. T plots (Figs. 5 and 6), and the accompanying van't Hoff analyses to determine T_m values (Table 1). The $T_m \approx 90$ °C for FHA2 and HA2, and the respective $T_m \approx 80$ °C for HA2_{20–185} (SE) and >85 °C for HA2_{20–211} (SE+TM) support a major contribution to stability by the SE, with smaller contributions from the FP and TM, where the latter two HA2 domains are the only ones that are deeply-inserted in the fused membrane.^{25, 26, 42} The hairpin structure of the SE in the final HA2 state allows for proximity between residues 1–37 and 177–210 that could be the basis of the higher T_m of full-length HA2 vs. truncated constructs. In previous papers, this possibility has been imagined as a bundle complex between the three FP's (e.g. HA2_{1–23}) and three TM's (e.g. HA2_{187–209}).^{2, 11}

The I173E mutation exerts significant destabilization of the SE of FHA2 as evidenced by loss of 30% of the overall helicity and reduction of T_m by 40 °C (Table 1). There is smaller

but still significant SE destabilization for I173E- vs. WT- HA2. The WT SE structure in Fig. 1B has C-terminal strands in the external grooves of the N-helix bundle, with the I173 sidechain inserted in a hydrophobic pocket of the bundle. Fig. 9 displays a structural model for the I173E SE in which there is dissociation of the strands from the bundle, with accompanying destabilization and loss of helicity near the bundle terminus. Strand dissociation is due in part to more favorable Born energy of the charged Glu sidechain in the high-dielectric aqueous environment vs. the low-dielectric protein interior. This I173E model is supported by data for shorter HA2 constructs with C-terminal truncation. The HA2₃₈₋₈₉ peptide forms a trimer of parallel helices at lower temperatures and unfolded monomers at higher temperatures, and its $T_m = 51^\circ\text{C}$ is comparable to the T_m of I173E-FHA2.⁴³ Ambient-temperature CD spectra of HA2₁₋₁₂₇ correlate with 50% helicity, which is much lower than the 85% value (106/127 residues) calculated using helical regions of the FP and SE structures.⁴⁴

G1E- and WT- FHA2 both exhibit 65% ambient-temperature helicity but the T_m for G1E-FHA2 is reduced by 32°C vs. WT-FHA2, which indicates significant destabilization of the SE by the G1E mutation. There is smaller but still significant destabilization of G1E- vs. WT- HA2. Destabilization of the SE is surprising because the G1E mutation is more than 35-residues from the SE structure. We propose that the SE destabilization for G1E is similar to that for I173E, and is a result of loss of binding of C-terminal strands with the N-terminal helical bundle (Fig. 9). This loss is due to competition with the G1E FP's for binding to the strands. Binding is not expected for the WT-FP which adopts tight helical hairpin structure that is stabilized by the favorable $\text{G1-NH}_3^+/(14-22)$ helix-dipole interaction (Fig. 1B).⁴⁵ This interaction may be disrupted for zwitterionic E1, with consequent separation of the two FP helices that can then bind with the SE strands.

G1E-FHA2, G1E-HA2, and I173E-FHA2 have respective H_m values of 49, 44, and 30 kcal/mole that are determined from van't Hoff analyses over broad temperature ranges for which there are clear changes from mostly-folded to mostly-unfolded protein (Figs. 7 and S6 and Table 1). There are likely similar unfolded monomer states for all of the proteins, so the H_m values likely reflect more favorable enthalpies for folded G1E- FHA2 and HA2 vs. I173E-FHA2. This interpretation is consistent with the previously-described direct I173E destabilization of binding between the C-terminal strands and the N-terminal helical bundle of the SE vs. less-direct G1E destabilization of the SE by weakening FP interhelical interactions, with consequent competition by the FP for binding to the strands. There are less accurate H_m values for WT- FHA2 and HA2, and I173E-HA2, because these values are determined from van't Hoff analyses from limited temperature ranges, and unfolding is sometimes incomplete at the highest temperature. The H_m values are larger for WT- vs. G1E- and I173E- FHA2 and WT- vs. G1E- HA2, which evidences more favorable enthalpies of the WT proteins. The WT H_m are similar to those of papain and trypsin, which have masses similar to the monomer HA2 constructs.⁴⁶ The S_m and H_m values are correlated in Table 1, as expected from $S_m = H_m/T_m$.

Monomers and trimers

The SEC data for all constructs in SRC show predominant trimers, with minor populations of monomers and dimers (Figs. 3A and S3). The relative populations of different species are more varied between the constructs in DM, and the monomer fractions are generally higher, particularly for I173E- FHA2 and HA2 (Figs. 3B and S3). SEC was done with [protein] $\approx$ 4 μ M and with [NaCl] = 150 mM, and CD spectra of I173E-FHA2 evidence well-folded protein for these conditions and also for different protein concentrations and no NaCl (Figs. 4 and S5). The greater monomer fraction of I173E- vs. WT- proteins is correlated with the lower stability of the I173E trimer. To our knowledge, folded monomer HA2 has not been previously reported, but there are reports of folded monomer HA1+HA2 ectodomain, including complexes with antibodies, and folded monomer gp41.^{47–57} Interestingly, both HA2 and HIV gp41 SE constructs can form folded hyperthermostable monomers and trimers, with similar structures for the monomers and monomer subunits of the trimers. Monomer fraction is correlated with inter-protein electrostatic repulsion, as evidenced by higher monomer fractions for HA2 constructs at neutral pH (charge $\approx$ –10) and for gp41 constructs at low pH (charge $\approx$ +10). There are higher populations of trimer and larger oligomers for HA2 at low pH and gp41 at neutral pH, with respective charges of $\sim$ +2 and –2. Another similarity between HA2 and gp41 is predominant trimers in anionic detergents, SRC and SDS, respectively, and higher monomer fractions in neutral detergents, DM and DPC, respectively. We hypothesize that the protein trimer may carry less than three times the monomer charge because of protonation of the Asp and Glu side chains in the trimer interior. The trimer may therefore be favored in anionic detergent because the electrostatic repulsion between the trimer and micelle is smaller than between three monomers and micelles.

Although the SEC data for I173E-FHA2 in DM show predominant monomers, the cross-linking data are consistent with a significant fraction of trimers. This difference may be due to the presence (SEC) vs. absence (cross-linking) of NaCl, as other proteins also exhibit smaller oligomer and greater monomer fractions with increased [NaCl].^{58, 59} This behavior is sometimes attributed to disruption of salt bridges between monomer units of the oligomer. The HA1+HA2 ectodomain of influenza strains A/Korea/01/2009 (KR01), A/California/04/2009 (CA04) and A/Darwin/2001/2009 (DA01) are monomers in a pH 8.0 solution with 200 mM NaCl, but only the latter two are trimers in their crystal structures, whereas KR01 shows a V-shaped arrangement of two monomers connecting through the HA1 globular head domains.^{49, 54} The monomer structure is similar in all three strains. The sequence of KR01 differs at only three residues from those of CA04 and DA01, and the crystallization conditions only differ in 100 mM NaCl for KR01 vs. no NaCl for the strains that adopt trimer structures. The presence vs. absence of NaCl may therefore favor folded monomer vs. folded trimer HA2.

Correlations between WT-HA2 fusion and electrostatic energies

Fig. 8B displays comparative vesicle fusion extents for WT- vs. mutant- HA2's, pH 5.0 vs. 7.4, and vesicle compositions of zwitterionic PC lipids vs. mixtures of PC and anionic PG lipids, or PC and cationic DOTAP lipids. We first focus on WT-HA2 fusion under different conditions, and discuss how differences in fusion extents correlate with electrostatic energies. The charge of WT-HA2, excluding the tag, is $\sim$ +3 at pH 5.0 and $\sim$ –9 at pH 7.4,

whereas the lipid charges are pH-invariant, and are 0 for PC, -1 for PG, and +1 for DOTAP. Higher PC fusion at pH 5.0 vs. 7.4 correlates with smaller inter-trimer electrostatic repulsion at pH 5.0 vs. 7.4. This also correlates with visible aggregation of the HA2 constructs at pH 5.0 that may contribute to fusion via increased local membrane concentrations and perturbations of the FP and perhaps TM.²⁶ These effects are also evidenced by higher PC:PG fusion at pH 5.0 vs. 7.4, with an additional contribution from attractive vs. repulsive HA2/vesicle electrostatic energies. This latter effect is also evident from higher PC:DOTAP fusion at pH 7.4 vs. 5.0, and corresponding attractive vs. repulsive HA2/vesicle electrostatic energies. This energy contribution is also manifested by higher fusion of PC:DOTAP at pH 7.4 vs. PC:PG at pH 5.0, and the corresponding greater attraction between the -9 charge HA2 and DOTAP vs. the +3 charge HA2 and PG. Similarly, higher PC:DOTAP fusion at pH 5.0 vs. PC:PG fusion at pH 7.4 correlates with smaller HA2/DOTAP vs. larger HA2/PG electrostatic repulsion. Vesicle/vesicle electrostatics are manifested by higher fusion for PC vs. PC:PG at pH 5.0 and the corresponding neutral vs. repulsive inter-vesicle electrostatic energies. In addition, higher PC:DOTAP fusion at pH 5.0 vs. PC fusion at pH 7.4 correlates with the previously-described fusion contribution of HA2 aggregation at pH 5.0.

Reduced fusion with mutants

G1E or I173E- HA-cells do not fuse with RBC's (i.e. negligible hemifusion and pore formation), with very similar losses-of-function of cell/RBC fusion induced by exogenous FHA2 with either of these mutations.^{10, 13, 28} There are qualitatively-similar but less dramatic effects of these mutants on HA2-induced PC and PC:PG vesicle fusion (Fig. 8). The ~4-fold reduction in fusion extent for G1E- vs. WT- HA2 with PC vesicles matches well with the reduction previously observed for G1E- vs. WT- FHA2 with PC:cholesterol vesicles.²⁸ Some mutant HA2-induced vesicle fusion vs. no mutant HA- cell/RBC fusion is ascribed to the ~10× higher density of HA2-trimers/vesicle vs. HA-trimers/cell.^{37, 38} The importance of HA density for fusion is supported by the correlation between comparable fusion of G1S- and WT- virions with vesicles vs. reduced G1S- relative to WT- HA-cell/RBC fusion, and the ~50× higher density of HA-trimers/virion vs. HA-trimers/cell.⁶⁰

For PC:DOTAP vesicles at pH 7.4, there is reduced fusion of mutant- vs. WT- HA2, which is qualitatively-similar to the PC and PC:PG results. The opposite trend is observed for PC:DOTAP at pH 5.0, with respective comparable and higher fusion extents of G1E- and I173E- vs. WT- HA2. This trend correlates with mutant- vs. WT- HA2 total charges of +2 vs. +3, and corresponding smaller mutant- vs. WT- HA2/DOTAP electrostatic repulsion.

We focus on PC and PC:PG vesicle fusion at pH 5.0 because these lipid compositions and this pH reflect some of the conditions of HA-mediated virus/endosome fusion. Relative to WT-HA2, G1E- and I173E- HA2 exhibit similar reductions in fusion that correlate with their lower SE hairpin stabilities. A more-open hairpin could reduce fusion via the larger separation of vesicles bound to the terminal FP and TM segments. In addition, the mutant HA2's may have less FP/membrane interaction due to higher FP interaction with the dissociated C-terminal SE region (Fig. 9). Reduced I173E-HA2 fusion may also be associated with its larger monomer:trimer ratio (Figs. 3 and S3). Relative to trimer,

monomer protein has lower local concentrations of the FP and TM in the membrane and consequently less membrane perturbation. This hypothesis is supported by earlier observations of reduced vesicle fusion for monomer vs. trimer hairpin constructs of the HIV gp41 fusion protein.^{47, 52, 61} Gp41 is non-homologous with HA2 but the two proteins share some structural similarities, including a final hairpin SE state. Reduced fusion of the G1E FP of HA2 may also be correlated to shallower membrane insertion of the N-terminal FP.²⁹

Common enveloped virus fusion model with significant functions for hairpin trimers and monomers

We integrate the present and earlier results into a common model for HA-mediated virus/endosome fusion, as well as fusion by other enveloped viruses. The initial trimeric HA1/HA2 complex is the lowest free-energy state at pH 7.4, whereas at pH 5–6 of the mature endosome, the complex is metastable.⁶² The HA1 subunits move away from the HA2 subunits, which then rearrange to the final trimer-of-hairpins state, which is the predominant structural state of the WT-HA2 in our experiments. There are accompanying membrane intermediates during fusion that include the hemifusion diaphragm, pore formation, and pore expansion. In some contrast, there hasn't yet been experimental identification of the HA2 structural intermediates between the initial complex and the final trimer-of-hairpins. It is sometimes proposed that some of the free energy released during the HA2 transformation to the final hairpin state is converted to activation energy and perhaps also thermodynamic energy needed for membrane intermediate states.^{2, 4} The pictures usually show a HA2 “pre-hairpin intermediate” with fully-extended SE and with FP inserted in the target membrane that then folds into the final hairpin state, with membrane intermediate formation time-correlated with the evolution of hairpin closure. The final step of fusion pore expansion is sometimes pictured as requiring a transmembrane bundle of three TM segments and sometimes an additional three FP segments. The SE hairpin is commonly referred to as the “post-fusion” state which implies that fusion is completed before the hairpin is formed. This mechanical picture of coupling of HA2 structural changes to lipid rearrangements is visually-appealing, but is disfavored by the entropic penalties for such a coordinated mechanism.

We propose an alternate model in which FP binds to the target membrane during the HA2 structural transformation to the final hairpin state, and subsequent membrane fusion occurs with HA2 in this final state. The SE hairpin maintains membrane apposition during the ~1 minute fusion time, and clustering of hairpin trimers and therefore FP domains in the target membrane perturbs this membrane and lowers the activation barrier to achieve hemifusion and pore formation.^{11, 26, 47, 52} For this model, the SE hairpin is a fusogenic state rather than a post-fusion state. This model is supported by the WT-HA2-induced vesicle fusion at pH 5.0, which is observed with <100 HA2 trimers/vesicle, less than the ~400 HA trimers/influenza virion.^{37, 38} The SE hairpins may be more stable at pH 5.0 vs. 7.4, based on the higher T_m for the HA2_{38–89} peptide at pH 5.0 vs. 7.0, and greater HA2 clustering at low pH is evidenced by visible aggregation of the SE constructs.^{26, 43} The fusion relevance of the final hairpin state is also supported by observation of cell/RBC fusion after addition of exogenous FHA2. Such fusion is very similar to HA-cell/RBC fusion, with both fusion types requiring low-pH and exhibiting nearly-identical losses-of-function for the G1E mutant and

for the I173E mutant.^{10, 28, 63} These mutants also show reduced vesicle fusion (Fig. 8). Reduced cell/cell and vesicle fusion for I173E- and G1E- vs. WT- proteins correlates with lower SE hairpin stability for the mutants and consequent impaired membrane apposition. There may also be less membrane perturbation by the mutants because of FP binding with the C-terminal strand of the SE, and for G1E, because of shallower insertion of the FP in the target membrane.

This model can also describe gp41-mediated fusion between the HIV membrane and the plasma membrane of a host-cell. Gp41 forms an initial trimeric gp160 complex with a receptor-binding protein (gp120) that is analogous with the trimeric HA1/HA2 complex.^{64, 65} Fusion occurs via gp120- binding to host-cell receptors, separation of gp120 from gp41, and structural transformation of gp41 into a final trimer-of-hairpins state.^{66, 67} A FP +SE+TM gp41 construct in this final state catalyzes fusion at pH 7.4 between vesicles composed of PC:Cholesterol or PC:PG:Cholesterol.⁵² This is the pH of HIV/host-cell fusion, and there are significant fractions of PC and anionic lipids in the plasma membranes of cells infected by HIV.⁶⁸ Gp160-cells also fuse with receptor-bearing cells, and the V2E mutation at the N-terminus of gp41 results in highly-impaired V2E- vs. WT- gp160-cell/receptor-cell fusion.⁶⁹ This observation correlates with highly-impaired G1E- vs. WT- HA-cell/RBC fusion.

A monomer fraction for HA2 in SEC correlates with monomer fractions of the initial HA1/HA2 complex under some conditions, as well as large monomer fractions for gp41 under some conditions.^{47, 48, 50, 52, 56, 57, 70} For both HA2 and gp41, there is a significant structural rearrangement between the initial trimeric HA1/HA2 or gp160 complex, and the final trimer-of-hairpins without the receptor proteins. Transient dissociation of HA2 and gp41 into monomers may be functionally important because monomer rearrangement into a hairpin followed by association into a trimer-of- hairpins may be topologically more straightforward than a concerted rearrangement of trimeric protein. Monomer intermediates have also been postulated for the structural transformations of class II and class III enveloped virus fusion proteins.^{3, 70} These hypotheses are evidenced by: (1) the initial class II protein complex is a single heterodimer of E1+E2 subunits, whereas the final state is a trimer- of-hairpins of E1 subunits; and (2) one class III structure contains two different monomer units, with one monomer similar to the initial state and the other similar to the final state. Monomer gp41 may also be the binding target of peptides which inhibit gp160-mediated fusion and HIV infection, and whose sequences correspond to segments of the C-terminal region of the gp41 SE.^{52, 63}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CD	circular dichroism
DM	decylmaltoside
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DPC	dodecylphosphocholine
FP	fusion peptide
HA	hemagglutinin
IPTG	isopropyl β -D-1-thiogalactopyranoside
LB	Luria-Bertani
N-NBD-DPPE	N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (ammonium salt) dipalmitoylphosphatidylethanolamine
N-Rh-DPPE	N-(lissamine rhodamine B sulfonyl) (ammonium salt) dipalmitoylphosphatidylethanolamine
PAGE	polyacrylamide gel electrophoresis
POPC (PC)	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPG (PG)	1-palmitoyl-2-oleoyl-sn-glycero-3-[phosphorac-(1-glycerol)] (sodium salt)
RBC	red blood cell
SDS	sodium dodecylsulfate
SE	soluble ectodomain
SEC	size-exclusion chromatography
SRC	N-lauroylsarcosine
TM	transmembrane
WT	wild-type

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(A) FHA2 sequence

1 GLFGAIAGFIENGWEGMIDGWYGRHQNSEGTGQAADLKSTQAAIDQING
 51 KLN RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLV
 101 AL ENQHTIDLT DSEM NKLFEKTRRQLRENAEEMGNGSFKIYHKADNAAIE
 151 SIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWLEHHHHHH

HA2 sequence

1 GLFGAIAGFIENGWEGMIDGWYGRHQNSEGTGQAADLKSTQAAIDQING
 51 KLN RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLV
 101 AL ENQHTIDLT DSEM NKLFEKTRRQLRENAEEMGNGSFKIYHKADNAAIE
 151 SIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWILWISFAISAFLLAV
 201 VLLGFIMWAAQRGNIRANIAIGGGGGGLEHHHHHH

(B) Fusion peptide and soluble ectodomain structures **Figure 1.**

(A) Amino acid sequences of the FHA2 and HA2 constructs with domains colored: fusion peptide (FP), pink; soluble ectodomain (SE), blue; transmembrane domain (TM), green; and endodomain, orange. The non-native C-terminal regions in black include a H₆ tag for affinity chromatography. The underlined residues denote the sites of the G1E and I173E mutations. (B) Ribbon diagrams of the FP in the closed conformation (PDB ID 2KXA) and trimeric SE in the final hairpin conformation (PDB ID 1QU1).

Figure 2.
SDS-PAGE of the purification eluents. The $MW_{FHA2} = 22.4$ kDa and $MW_{HA2} = 26.7$ kDa.

SEC before cross-linking

SDS-PAGE after cross-linking

(A) 0.10% SRC detergent

(B) 0.17% DM detergent

Figure 3.

SEC traces prior to cross-linking (left) and SDS-PAGE after cross-linking (right) and in (A) SRC and (B) DM detergents. SEC parameters include [loading protein] = 1 mg/mL, [protein running] $\approx$ 0.1 mg/mL, and A_{280} detection. Arrows mark the elution volumes of the void (V_0) and MW standards in kDa. Cross-linking conditions include [protein] = 0.5 mg/mL, fifty-fold molar excess of bis(sulfosuccinimidyl) suberate cross-linking agent, and 1 hour time. The gels were made using 12% acrylamide. See Fig. S3 for data on replicate samples.

Figure 4. CD spectra of the (A) FHA2 and (B) HA2 proteins at ambient temperature. Samples contained 15 μ M protein in 10 mM Tris buffer at pH 7.4 with 0.17% DM. See Fig. S4 for spectra of replicate samples.

Figure 5.
Temperature-series CD spectra. Protein and buffer conditions are the same as Fig. 4.

Figure 6.
Plots of θ_{222} values vs. temperature.

Figure 7. Van't Hoff plots of the unfolding $\ln K_{eq}$ vs. $1/T$ of the (A) G1E-FHA2 and (B) I173E-FHA2 proteins based on θ_{222} data for the temperature range around T_m . Best-fit parameters are given with uncertainties in parentheses.

Figure 8.

HA2-induced vesicle fusion with protein:lipid = 1:300. (A) Time-courses of POPC vesicle fusion at pH 5.0. (B) Percent vesicle fusion at 600 s after addition of protein for different lipid compositions and pH's. Each bar represents the average of three replicates. There is typically $\pm 1\%$ variation in percent vesicle fusion among the replicates. See Fig. S7 for data using different preparations of proteins and vesicles.

Figure 9.
Structural model for a monomer unit of the SE of mutant HA2's. There is partial dissociation of the C-terminal strand and partial unfolding of the N-terminal helix.

Table 1.

Analysis of CD spectra

Construct	Average percent helicity ^a	$T_m^{b,c}$ (°C)	H_m (kcal/mole)	S_m (cal/mole-K)
WT-FHA2	64	86.8(0.2)	186(11)	517(29)
G1E-FHA2	65	55.2(1.3)	49.1(1.0)	149.6(3.0)
I173E-FHA2	44	45.4(2.7)	30.1(1.3)	94.4(3.8)
WT-HA2	58	90.7(1.2)	126(36)	345(99)
G1E-HA2	58	75.0(2.4)	43.6(3.6)	125(11)
I173E-HA2	45	79.2(1.2)	165(17)	469(48)

^aCalculated using the θ_{222} of ambient temperature CD spectra, with 100% helicity taken as $\theta_{222} = -33000$ degrees-cm²-dmole⁻¹.

^bThe T_m , H_m , and S_m parameters are calculated using van't Hoff analysis of $\ln K_{eq}$ vs. $1/T$, where $K_{eq} = (f_{unfolded})^3 / (1 - f_{unfolded})^3$, $f_{folded} = |\theta_{222} - \theta_{unfolded}| / |\theta_{folded} - \theta_{unfolded}|$, $f_{unfolded} = 1 - f_{folded}$, and θ_{folded} and $\theta_{unfolded}$ values are based on extrapolations from the respective θ_{25C} and θ_{90C} . Fitting is done over the temperature range for which $\ln K_{eq}$ changes sign and exhibits significant changes in magnitude, and over which $\ln K_{eq}$ vs. $1/T$ is reasonably linear. The $H_m = -R \times \text{slope}$, $S_m = R \times \ln K_{eq}$ -intercept, and $T_m = H_m / S_m$. Specific θ_{folded} and $\theta_{unfolded}$ in degrees-cm²-dmole⁻¹, and fitting temperature ranges in °C are: WT-FHA2, -18000, -2000, 80-90; G1E-FHA2, -17000, -1500, 25-85; I173E-FHA2, -21000, -2700, 25-90; WT-HA2, -19000, -2000, 80-90; G1E-HA2, -19500, -500, 55-90; I173E-HA2, -15000, -900, 70-90.

^cThe numbers in parentheses are fitting-associated uncertainties. The uncertainties in the H_m and S_m parameters are based on statistically-reasonable variations in the sum of residuals $\sum_j (\ln K_j^{exp} - \ln K_j^{calc})^2$ where $exp \equiv$ experimental, $calc \equiv$ calculated, and j is the data index. Variations in H_m and S_m are highly correlated with one another. The uncertainty in $T_m \equiv \delta T_m$ was calculated using the variation in $1/T \equiv \delta 1/T$ associated with the average residual $\equiv \delta \ln K$. The $\delta 1/T = \delta \ln K / (H_m/R)$ and the $\delta 1/T / (1/T_m) = \delta T_m / T_m$ were the basis for the calculated $\delta T_m = \delta 1/T \times T_m^2$.