The Final Conformation of the Complete Ectodomain of the HA2 Subunit of Influenza Hemagglutinin Can by Itself Drive Low pH-Dependent Fusion

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Running Title: Fusion initiated by the Influenza HA2 ectodomain

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One of the best characterized fusion proteins, the influenza virus hemagglutinin (HA), mediates fusion between the viral envelope and the endosomal membrane during viral entry into the cell. In the initial conformation of HA its fusogenic subunit, the transmembrane protein HA2, is locked in a metastable conformation by the receptor-binding HA1 subunit of HA. Acidification in the endosome triggers HA2 refolding towards the final lowest energy conformation. Is the fusion process driven by this final conformation or, as often suggested, by the energy released by protein restructuring? Here we explored structural properties as well as the fusogenic activity of the full-sized trimeric HA2(1-185) (here called HA2*) that presents the final conformation of the HA2 ectodomain. We found HA2* to mediate fusion between lipid bilayers and between biological membranes in a low pH-dependent manner. Two mutations known to inhibit HA-mediated fusion strongly inhibit the fusogenic activity of HA2*. At surface densities similar to those of HA in the influenza virus particle, HA2* formed small fusion pores but did not expand them. Our results confirm that the HA1 subunit responsible for receptor binding as well as the transmembrane and cytosolic domains of HA2 are not required for fusion pore opening and substantiate the hypothesis that the final form of HA2 is more important for fusion than the conformational change that generates this form.

Fusion mediated by the influenza virus hemagglutinin (HA) protein is often considered as a prototype of biological fusion reactions. This fusion process is utilized by the virus to deliver its RNA into the host cell by merging the viral
envelope with the membrane of an acidified endosome of the host cell. Each monomer of homotrimeric HA consists of two disulfide-linked subunits: HA1, responsible for receptor binding, and HA2. In the native neutral pH conformation of the HA protein, the HA1 subunit confines the HA2 subunit in a metastable state which has been termed the “spring-loaded” state (1-3). However, evidence from calorimetric studies indicate that the compact folded structure of the influenza hemagglutinin protein is not a kinetically trapped metastable high-energy form (4;5). In the neutral pH structure of intact HA, the functionally important amino terminal amphiphilic region of HA2, referred to as the fusion peptide, is hidden within HA molecule. The low-pH–triggered restructuring of HA unlocks HA2 and allows refolding of the HA2 subunit towards its final, low energy conformation, which consists of a hairpin structure with the fusion peptide and the transmembrane domain at the same end of the rigid rod. While it is commonly assumed that the energy released by this restructuring drives rearrangements of membrane bilayers (6-8), one may suggest an alternative hypothesis, i.e., that the final conformation itself is fusogenic.

In our earlier work, in order to test the fusogenic properties of the final conformation of the HA2 subunit in the absence of HA1 we studied a trimeric polypeptide, HA2(1-127), representing the first 127 amino acid residues out of the 221 residues of the HA2 domain of influenza virus X-31 (9;10) (Fig. 1C). The conformation of HA2(1-127) both at neutral and at low pH shows many characteristic features of the lowest-energy hairpin form of HA2 (11-13). HA2(1-127) did not mediate content mixing between bound cells and thus did not open fusion pores connecting the cells. However HA2(1-127) induces lipid mixing between liposomes and between bound cells in a low pH-dependent manner. While finding that a major portion of the final conformation of HA has fusogenic activity was intriguing, it had to be considered that HA2(1-127) represented only two thirds of the HA2 ectodomain. Since a significant part of the central coiled coil of HA2(1-127) is not covered by the outer layer of antiparallel polypeptides of the C-terminal half of the HA2 ectodomain, one may hypothesize that hemifusion mediated by HA2(1-127) is not characteristic of the entire HA2 subunit. If this is the case, then a longer polypeptide that represents the entire HA2 ectodomain (3;13) is expected to have no fusogenic activity. Up to the present time, the ability of the entire HA2 ectodomain to promote fusion had not yet been investigated.

In the present work we report the fusogenic activity of a 185 amino acid residue trimeric polypeptide, HA2*, that represents the entire ectodomain of the X-31 influenza virus HA2 subunit, i.e., almost the entire subunit, lacking only the TMD and the cytoplasmic segment (Fig. 1B). HA2* mediates lipid mixing between liposomes and between cells in a low pH-dependent manner. Importantly, HA2* also mediates content-mixing between the cells and thus, in contrast to HA2(1-127) (10), it drives membrane rearrangements beyond hemifusion to the opening of a nascent fusion pore. The surface density of HA2* molecules required for fusion is close to the number of HA molecules required for HA-mediated fusion. Two point mutations in the HA2 sequence that are known to inhibit HA-mediated fusion, also inhibit HA2*-mediated fusion. Thus HA2*, which presents the final conformation of the HA2 subunit of HA, mediates a fusion process that shares
important characteristics with the fusion mediated by full-sized HA. Our findings substantiate the hypothesis that neither the TMD nor the energy released in the conformational transition of the HA2 subunit from its initial metastable form in HA towards the final hairpin conformation of HA2 in the low pH form of HA, are required for the early stages of fusion.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of HA2*:** The full-length ectodomain of the HA2 subunit of the hemagglutinin protein of influenza virus, HA2*, was subcloned into a pET-24b(+) plasmid vector (Novagen) with Nde I and Xho I/Sal I restriction sites from the plasmid pHA containing full-length HA of the X31 strain (Fig. 1). The strain used for expression of plasmids bearing HA2* or its site-specific mutant G1E was *E. coli* Rosetta (DE3)pLysS (Novagen). The amino acid sequence of HA2* is: Met-[HA2 amino acids: 1-185]-Val-Glu-His6 without the TMD and the short cytoplasmic segment on the C-terminus. The C137S mutation was introduced for expression (14). Mutants were generated by QuickChange™ site-directed mutagenesis (Stratagene) and confirmed by DNA sequencing. The cells were grown in 1.8 L of LB media with 2 g/L of glucose (25 μg/mL of kanamycin; 50 μg/mL of chloramphenicol) at 37 °C with inoculation of overnight culture (1 %, v/v). When O.D. 600 reached 0.8 - 1.0, the cells were induced with 0.5 mM IPTG, the temperature lowered from 37 °C to 22 °C and the agitation speed reduced from 200 to ~100 rpm for 6 hrs. The cells were harvested by centrifugation at 6,000xg for 10 min at 4 °C and stored at –80 °C. The cell pellet was resuspended in the 20 mM imidazole buffer (50 mM NaH2PO4; 300 mM NaCl; pH 8.0; 0.2 % (v/v) Triton X-100) with additional 0.3 % (v/v) Triton X-100 (final 0.5 %); 2 mM AEBSF; 1 mM leupeptin; 1 mM pepstatin A and broken on ice by sonication. The cell lysate was vortexed for 1 min and swirled at 4 °C for 30min with 0.5 % (w/v) n-lauroyl sarcosine. After centrifugation at 15,000xg for 15 min at 4 °C, the supernatant was swirled at 4 °C for 1 hr with 2 mL of Ni-NTA-agarose beads (Qiagen) equilibrated with the 20 mM imidazole buffer. The column was washed thoroughly with the 20 mM imidazole buffer and with 10 column volumes of 50 mM imidazole buffer. The proteins were eluted stepwise with 4 column volumes each of 100 mM imidazole, 150 mM imidazole and 200 mM imidazole buffers containing 0.2% (v/v) reduced Triton X-100. The most concentrated protein fraction was eluted with 150 mM imidazole buffer with over 95 % purity on 15 % SDS-PAGE gel. HA2(1-127) was expressed and purified as described earlier (9;10).

**Preparation of large unilamellar vesicles (LUV):** All lipids, including the fluorescently labeled lipids, were purchased from Avanti Polar Lipids (Alabaster, AL). Lipids were dissolved in chloroform/methanol, 2/1 (v/v) at the desired molar ratio. The lipid was deposited as a film on the wall of a test tube by solvent evaporation with nitrogen. Final traces of solvent were removed for 2-3 hrs in a vacuum chamber attached to a liquid nitrogen trap. The lipid films were suspended in the appropriate buffer by vortexing at room temperature. The lipid suspensions were further processed with 5 cycles of freezing and thawing, followed by 10 passes through two stacked 0.1 μm polycarbonate filters using an extruder made by the Lipex Corp. (Vancouver, B.C.). The content of lipid phosphorous was determined by the method of Ames (15).

**Lipid mixing assay for liposome fusion:**
The resonance energy transfer assay of Struck et al. (16) was used to monitor membrane fusion. Two populations of LUVs were prepared, one unlabeled and one labeled with 2 mol % each of N-Rh-PE and N-NBD-PE. A 9:1 molar ratio of unlabeled to labeled liposomes was used in the assay. Fluorescence was recorded at excitation and emission wavelengths of 465 nm and 530 nm, respectively; using a 490 nm cut-off filter placed between the cuvette and the emission monochromator, with 4 nm bandwidths, using an SLM Aminco Bowman AB-2 spectrofluorimeter. Measurements were made using siliconized glass cuvettes (1 cm²) with continuous stirring at constant temperature in a thermostated cuvette holder. Measurements were carried out using a buffer containing 5 mM Heps, 5 mM Mes, 5 mM citric acid, 0.15 M NaCl and 1 mM EDTA, pH 7.4. LUVs were added to 2 mL of buffer in the cuvette at 37 ºC to give a final lipid concentration of 100 μM. No lipid mixing occurs at neutral pH in the absence of protein. A small aliquot of protein solution was then added. After monitoring the slow rate of lipid mixing at neutral pH, the solution was acidified to the desired pH by the addition of 1 mM citric acid. Fluorescence was recorded for several minutes and then 20 μL of 10% Triton X-100 was added (final concentration 0.1 %). The initial residual fluorescence intensity, prior to acidification, F₀, was taken as zero. The maximum fluorescence intensity, Fₘₐₓ, was obtained by dilution of the labeled lipids with 20 μL of 10% Triton X-100. Percent lipid mixing at time t is given by: 
\[ \frac{(F_t - F_0)(F_{\text{max}} - F_0)}{100} \]
All runs were done in duplicate and were found to be in close agreement. Acidification of the LUVs in the absence of protein was used as a negative control and showed little lipid mixing.

Circular dichroism (CD): The CD spectra were recorded using an AVIV series 215 CD instrument (AVIV Associates, Lakewood, NJ). The sample was contained in a 1 mm pathlength cell that was maintained at 25 ºC in a temperature-regulated cell holder. The CD data are expressed as the mean residue ellipticity. To ensure transparency in the UV, proteins were dialyzed against phosphate buffer but containing 0.1% reduced Triton X-100. All CD runs were made with aliquots of dialyzed protein diluted several fold into 10 mM NaH₂PO₄, 0.15 M NaF, pH 7.4. The curves were analyzed for secondary structure content by the Contin and Selcon methods with the program CDPro (17). Protein concentrations were determined by the BCA assay (Pierce), using the same buffer as a blank.

Preparation of cells and cell fusion experiments: In our cell fusion experiments we used two well-characterized experimental systems: fusion between HAβ2 cells and red blood cells (RBC) and fusion between Sf9 cells. HAβ2 cells express an uncleaved precursor form of HA – HA0, which is fusion-incompetent but mediates membrane binding (10;18). Thus, in the case of fusion between HA0 –expressing HAβ2 cells (HA0-cells) and RBC, HA2* fused the cells bound by physiologically relevant interactions between HA1 and sialic acid receptors. Experiments on Sf9 cells allowed us to evaluate the fusogenic activity of the polypeptide in the absence of any full-sized HA, including fusion-incompetent HA0. RBC labeled with the fluorescent lipid PKH26 (Sigma, St. Louis, MO) and loaded with either 6-carboxyfluorescein (CF) or 10 kDa FITC-dextran (Invitrogen, Carlsbad, CA) and HA0-cells (HAβ2 cells expressing HA0 form of A/Japan/305/57 HA, subtype H2N2 (19)) were prepared for...
periments as described (18). *Spodoptera frugiperda* (Sf9) cells were grown and labeled with L-α-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Egg) (Rho-PE, Avanti Polar-Lipids, Birmingham, Alabama) as described in (20). In fusion assays, HA0-cells with 0–2 bound RBC per cell or Sf9 cells were incubated for 5 min in PBS supplemented with stated concentration of HA2*. The low-pH medium (PBS titrated with citrate to the desired acidic pH) was applied at room temperature for a stated time. Low pH application was ended by replacement of the acidic solution with PBS. We assayed the final extents of lipid and content mixing in HA0-cell/RBC fusion by fluorescence microscopy as the ratio of dye-redistributed bound RBC to the total number of bound RBC (18). We should point out that this method of measurement of contents mixing is independent of dye leakage since leakage would result in a huge (10 billion fold) dilution of the dye that would not be observable because the fluorescence intensity would be too low.

Lipid mixing between Sf9 cells was quantified in a similar way as Rho-PE transfer from pre-labeled to unlabeled Sf9 cells. We also used light microscopy to measure the percentage of Sf9 cells in syncytia (the ratio of nuclei within syncytia to the total number of cell nuclei in the same field) (20). Because fusion extents for the same concentration of HA2* and pH somewhat varied from day to day, apparently as a result of variation in the numbers of the cells and the properties of RBC and RBC ghosts, we routinely started the experiments by adjusting conditions of the HA2* and low pH applications. Each set of experiments for each graph presented in the paper was repeated on at least three occasions with similar results. Presented data were averaged from the same set of experiments.

**Evaluation of cell surface concentration of HA2*: Concentrations of cell-associated polypeptide sufficient to induce cell fusion were estimated as in (10). Approximately 10⁶ Sf9 cells with bound polypeptide were lifted from the plates with EDTA/EGTA (0.5 mg/ml each) in Ca²⁺, Mg²⁺- free PBS, collected and lysed in lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 5 mM EDTA, 1.5% Triton X-100; 10% glycerol; 1 Proteinase Inhibitor Cocktail Tablet (Boehringer Mannhein) per 50 ml of buffer). After lysis, samples were diluted two fold with denaturing and reducing SDS sample buffer (0.5 M Tris-HCl, pH 6.8; 10% SDS, 20% glycerol, 20 mM dithiotreitol; 0.1% bromphenol blue) and boiled for 3 min. The lysate and known dilutions of the same polypeptide were analyzed by 4-20% gradient SDS-PAGE. For Western blotting, we used rabbit polyclonal antiserum against HA2 LOOP-36 (21), kindly provided by Dr. P. S. Kim, at 1:1000 dilution, and secondary antibodies (goat anti-rabbit IgG, 1:1000) conjugated with horseradish peroxidase (Amersham, Buckinghamshire, England). To detect protein immunoreactivity, blots were incubated in the ECL reagent (Amersham) followed by exposure to film. The amount of the bound polypeptide was found by comparison of the polypeptide band intensity with that of our standards.

**RESULTS**

Liposome lipid mixing: The HA2* exhibited considerable pH-dependent lipid mixing activity in liposomes composed of DOPC:DOPE:cholesterol (1:1:1) (Fig. 2A). There is virtually no lipid mixing activity at neutral pH, but after acidification to pH 5.0 the rate of lipid mixing is comparable to that observed
with the shorter 127 amino acid construct, HA2(1-127) (9). HA2* had about a two fold greater activity than G1E-HA2* (Fig. 2A). This mutation in the first position of the HA2 is known to abolish HA-mediated fusion (22;23). The difference between HA2* and G1E-HA2* becomes greater in the less fusogenic liposomes composed of DOPC:cholesterol (1:1) (Fig. 2B). The pH-dependence of HA2*-mediated lipid mixing for DOPC:cholesterol (1:1) liposomes is shifted to more acidic pH values than that for DOPC:DOPE:cholesterol (1:1:1) (Fig. 2C). In the case of the G1E-HA2* the pH dependence of the lipid mixing with LUVs of DOPC:DOPE:cholesterol (1:1:1) is different from that of HA2*, with some lipid mixing observed at pH 7.4 with the mutant (Fig. 2D) but not with the wild type sequence (Fig. 2C). However, for both constructs there is a marked increase in the rate at about pH 6.

In brief, HA2*-induced lipid mixing between DOPC:cholesterol liposomes depends on low pH, similarly to lipid mixing mediated by HA, and is strongly inhibited by the G1E mutation. The G1E mutation in intact HA also inhibits HA-mediated fusion (22;23). Our results indicate that studies of membrane fusion activity using liposomes that are at the brink of spontaneous lipid mixing, such as those of DOPC:DOPE:cholesterol (1:1:1), are not as discriminatory of the fusogenic activity of different protein constructs with different intrinsic fusogenic activity. This is also indicated by the fact that there is some fusion activity of the G1E-HA2* with these liposomes, but no fusion is observed with the intact G1E mutant in the experiments on cell fusion (22).

CD: To explore the effects of the single residue mutation that distinguishes G1E-HA2* from HA2*, we compared the secondary structures of the fusogenic HA2* with the fusion-incompetent G1E-HA2* using far UV CD (Fig. 3A). Secondary structure analysis is given in Table 1 and shows that HA2* has lower structure content than its G1E mutant.

The thermal denaturation of HA2* at neutral pH measured by CD, exhibits a marked loss of secondary structure over a broad temperature range from about 55 to 90 °C (Fig. 3B). There are two components of the thermal transition, one around 65 °C and the other at about 85 °C. The lower temperature transition is similar to that seen with the intact protein (24;25). The transitions are partially reversible on cooling. Similar behavior is observed with G1E-HA2* (Fig. 3C), although in this case the two components are less distinct and the reversibility is less marked.

The thermal denaturation of HA2* at pH 5 was irreversible and showed initial loss of structure at 25 °C (Fig. 3D). The thermal denaturation of G1E-HA2* and HA2(1-127) at pH 5 could not be carried out using the buffer conditions required for CD due to precipitation of the proteins. However, at pH 7.4 the behavior of HA2(1-127) (Fig. 3E) was similar to that of the G1E-HA2* and HA2* proteins.

We also studied the unfolding of HA2* by DSC. The major endotherm occurred at 86.8 °C, much higher than the transition observed by CD. We have no explanation for this discrepancy but it should be noted that CD and DSC measure different things and the denaturation is irreversible and therefore influenced by kinetic factors. It is not possible to have identical heating protocols for both methods, but evaluation of this issue was not pursued.

Our data indicate that replacing a single amino acid residue in G1E-HA2* results in a notable increase in the total
alpha-helical content in the secondary structure of HA2*, rather than just in a local change in the immediate vicinity of the fusion peptide.

Cell fusion; HA2* mediates lipid and content mixing but does not expand fusion pores: The ability of HA2* to fuse biological membranes was explored using two experimental systems. To test whether the HA2* is capable of mediating fusion of membranes bound by physiologically relevant interactions between HA1 and sialic acid receptors, we studied fusion of red blood cells (RBC) with HA0-cells. The HA0 form of HA is competent for receptor-binding but incompetent for fusion, if it is not activated by trypsin-cleavage into the fusogenic HA1-HA2 form. In the second experimental system, to test whether the HA2* fuses cells in the absence of any wild type HA, we also used Sf9 cells, which spontaneously establish physiological contacts and thus do not require any artificial means of bringing cells into contact.

Application of HA2* to HA0- cells with bound RBC followed by lowering the pH resulted in lipid mixing between the cell membranes detected as a rapid redistribution of the fluorescent lipid PKH26 from the RBC to the HA0-cell membranes. The extent of lipid mixing depended upon HA2* concentration (Fig. 4A) and pH (Fig. 4B). Since HA2* was added to cells in 0.05% Triton X-100, we verified that low pH application to the cells in the presence of HA2*-free 0.05% Triton X-100 yielded no measurable lipid mixing (data not shown).

In many fusion processes, merger of contacting leaflets of the membranes that allows lipid mixing precedes merger of the distal leaflets to form a fusion pore that allows content mixing. In contrast to HA2(1-127) that mediates only lipid mixing (10), HA2* formed fusion pores large enough to pass CF. These pores did not expand to sizes large enough to allow redistribution of a larger aqueous probe, 10 kDa FITC-dextran (Fig. 4C). Neither lipid mixing nor syncytium formation was observed in the control experiments where either low pH or HA2* application was omitted (not shown). Both the fusogenic activity of HA2* and its inability to form the expanding fusion pores that we observed using HA0-cell/RBC complexes, were confirmed with Sf9 cells where application of HA2* resulted in lipid mixing between these cells without the formation of syncytia (not shown).

We verified the important difference between fusogenic properties of HA2* and HA2(1-127) in side-by-side comparison (Fig. 4D). While these two constructs induced similar extents of lipid mixing, the longer construct - HA2*, was much more effective in inducing contents mixing.

The dependence on low pH is not the only feature of HA2*-mediated fusion that is in common with fusion mediated by full-sized HA. We demonstrate that the same mutations that reduce the fusogenic activity of full-sized HA also cause the loss of fusogenic activity of HA2* (Fig. 4A,B and Fig. 5A). A single amino acid substitution at position 173 (I173E), detrimental for HA-mediated fusion (26), significantly inhibited the fusogenic activity of HA2*. Similarly, substitution of glutamic acid for the glycine in the amino-terminus (G1E) of HA2, that is known to inhibit HA-mediated fusion (22), resulted in an almost complete loss of the fusogenic activity of the HA2*. Even at concentrations more than 3-fold higher than the concentrations at which wild type HA2* mediated effective fusion, G1E-HA2* mediates no CF redistribution.
and produces almost no lipid mixing (Fig. 5A).

As fusion mediated by full size HA (27), fusion mediated by HA2* is blocked by lowering the temperature to 4°C (Fig. 5B).

As many other viral fusogens, full size HA mediates fusion when expressed in only one of two fusing membranes and inactivates if treated with low pH in the absence of the target membrane. Likewise, we found HA2* to fuse membranes when added to only one of them and to inactivate if acidified prior to application of the target cells – RBCs (Fig. 5C).

Importantly, we found HA2* to fuse cells in the experiments where application of HA2* to HA0-cell/RBC complexes (Fig. 5C) or to Sf9 cells (not shown) was followed by washing the cells with HA2*-free medium prior to acidification. This tightness of the HA2* -cell surface binding allowed us to estimate the surface density of HA2* required to observe HA2*-mediated fusion of Sf9 cells. We treated the cells with HA2*, washed unbound polypeptide away, lifted and lysed the cells with bound polypeptide. We then used quantitative Western blotting to compare the HA2* band in a cell lysate with the bands obtained with known amounts of the free polypeptide. The total surface area of the plasma membrane of cells that is available for HA2* binding was evaluated as a product of the known number of the cells and the average area of cell surface, estimated on the basis of the capacitance of Sf9 cells reported in electrophysiological studies (~16 pF (28)) using 1 μF/cm² as the specific capacitance of a membrane bilayer. According to this rough estimate, 4 μM concentration of HA2* at which HA2* effectively fuses Sf9 cells corresponds to a surface concentration of ~5,000 trimers/μm². This concentration is comparable to surface concentrations of full-sized HA required for membrane fusion, which varies from ~2,000 in HA-expressing cells (29), to ~15,000 trimers/μm² for the viral envelope (30). Thus, it appears that HA2* and full-sized HA form fusion pores at comparable surface densities.

**DISCUSSION**

In this study we explored the fusogenic activity of the complete 185 amino acid ectodomain of HA2 that represents the final conformation of the HA2 subunit of HA. As previously found with the shorter 127 amino acid construct, HA2(1-127) (9;10), HA2* induces lipid mixing between liposomes (Fig. 2) and between cells (Figs. 4, 5). However, in contrast to HA2(1-127), HA2* drives fusion beyond hemifusion to fusion pore opening (Fig. 4C, D). Thus, the addition of residues 128-185 allows the peptide to more closely resemble the full-sized HA with regard to its ability to form a fusion pore. Like HA, both HA2(1-127) as well as HA2* mediate lipid and content mixing only after application of acidic pH. In the case of the intact HA, acidification causes dissociation of the HA1 and HA2 subunits, resulting in a large conformational change in HA2 (1;12;21). However, with HA2(1-127) and HA2* there is no HA1 subunit and little conformational change in these constructs upon acidification. Nevertheless, the fusion results with the HA2 constructs indicate that there must be functionally important low pH-dependent interactions between the final conformations of HA2 subunits and/or between these conformations and the membranes. It has been suggested that protonation of HA leads to a loosening of the structure of HA as a result of electrostatic repulsion (31;32).
The nature of the low pH-dependent stage of HA2*-mediated fusion is not completely resolved. The pH dependence of fusion of the native virus is complex. The kinetics shows a lag time as well as a subsequent rate of fusion that depends on the temperature, the pH, and on the nature of the target membrane. In addition, the kinetics involve both a binding step as well as the fusion step (33). The rate of the fusion step is not directly given by the overall rate of fusion. Furthermore, additional effects that are subtle to detect may influence viral protein-promoted processes. For example, a small difference in the pH dependence of hemolysis between an intact influenza virus and the isolated hemagglutinin protein has been observed (34). The detailed study of the pH dependence of fusion by Remeta et al. shows that at 35 ºC the pH required for half maximal activity for lipid mixing with erythrocyte ghosts was 5.50 (24). The pH required for half maximal promotion of heterokaryon formation with cells expressing the influenza virus X-31 hemagglutinin protein is 5.2 (35), while the pH required for half maximal hemolytic activity is 5.75 (36). Our results for the pH dependence of the HA2* is in the range of those observed for other pH-dependent functions of the HA of the X-31 strain of influenza virus. Curiously, the pH dependence of HA2* more closely resembles that of the shorter 90 amino acid fragment of HA2 (37) than it does the longer 127 amino acid HA2(1-127) (9). This relative comparison also holds for the G1E mutation, in that it is less inhibitory for both the 90 and 185 amino acid fragments than it is for the 127 one. This amino acid substitution in the intact hemagglutinin (22;38) or in HA2(1-127) (37) results in the virtually complete loss of fusion activity. There is also partial loss of lipid mixing activity with liposomes of the G1E mutant in the 90 amino acid fragment of HA2 (37) or in the 185 amino acid fragment (Fig. 2A). Fusogenic activity of the G1E mutant of HA2* detected in DOPC:DOPE:cholesterol (1:1:1) liposomes, even at neutral pH, suggests that for these liposomes protein-mediated fusion is overshadowed by spontaneous lipid mixing between very fusogenic bilayers (39). However, with the less fusogenic liposomes (DOPC:cholesterol 1:1) the G1E mutant of HA2* has very little activity (Fig. 2B). These results indicate that the use of liposomes comprised of very fusogenic lipids can give rise to non-physiological fusion.

There is a significantly greater helix content in the G1E-HA2* mutant protein, compared with HA2* (Table 1). Some factors that would promote more helix in the G1E mutant are the fact that E is a better helix former than G, charge-dipole interactions between the negative charge on E and the helix dipole would stabilize the helix. In addition, there is a bend in the conformation of the fusion peptide when inserted into a membrane, bringing the amino terminus close to the membrane interface (40;41). The negative charge on E at the N-terminus would facilitate forming this kinked structure and hence facilitate achieving the peptide conformation that promotes membrane fusion. Hence the greater helicity that we observe with G1E-HA2* follows expectations. The quantitative extent of increase in helicity is difficult to predict, but our results are in accord with theoretical calculations indicating that the conformational change should not be local, but be more extensive (42).

Our findings argue against the hypothesis that the energy for membrane fusion is provided by the HA2 restructuring from its initial conformation
to the final “spring-unloaded” form of HA2 (21;43-45). Even at neutral pH, HA2* is already in the final low pH form, equivalent to the HA2 subunit of the intact HA that is capable of fusion. Therefore, our results would indicate that at least the early stages of HA-mediated fusion, up to an opening of a small fusion pore, are not driven by the restructuring of an individual HA2 trimer towards its final conformation, but rather by low pH-dependent interactions between this conformation and the membrane and by lateral interactions between adjacent low pH forms of the trimers and/or among the subunits of the trimer (Fig. 6).

Proteins that fuse membranes during enveloped virus entry (46;47), as well as established intracellular and developmental fusogens (48-51), are anchored in the membranes by their transmembrane domains. Moreover, for several viral fusion reactions and for SNARE-dependent intracellular fusion, the sequence of the fusion protein TMD affects its fusogenic activity (52;53). However, our finding that HA2* forms fusion pores argues against the hypothesis that only integral membrane proteins can serve as protein fusogens. Initial findings with the influenza HA protein indicated that when the transmembrane segment was replaced by a GPI-anchor, opening of fusion pores did not occur with membrane merger arrested at the hemifusion stage (54;55). However, subsequent to those studies, several groups showed that lipid-anchored HA does form small, non-expanding fusion pores (56-58) indicating that transmembrane and cytosolic domains of HA are required for fusion pore expansion. Thus, the present study is an independent confirmation of these more recent studies demonstrating that neither the transmembrane nor cytosolic domain is required for an opening of a fusion pore.

This conclusion is also in agreement with reports that transmembrane proteins are not required for fusion in autophagy (59), in nuclear membrane fusion in yeasts (60), in membrane repair (61) and in fusion at the early stages of nuclear envelope assembly in Xenopus oocytes (62;63). The fusogenic activity of the HA2 ectodomain is also consistent with the hypothesis that both fusion and fission of biological membranes are driven by membrane elastic stresses (64;65). Note that many of the curvature generating proteins involved in membrane remodeling lack transmembrane domains (66).

Finding that HA2* does not produce fusion pores large enough to allow passage of 10 kDa fluorescent dextran (Stokes radius of 2.4 nm) and does not produce syncytia substantiates the hypothesis that the transmembrane domain (and perhaps its interactions with the fusion peptide) as well as the cytoplasmic domain of HA, can be especially important for later fusion stages including fusion pore expansion (26;57;67;68).

Specific mechanisms by which the complete ectodomain HA2 fuses both model and biological membranes as well as the role of the fusogenic properties of the final conformation of HA2 subunit in the context of fusion mediated by full-sized HA, remain to be established. However our findings suggest that formation of the hairpin conformation of HA2, often referred to as a “post-fusion” conformation, does not signify the end of functionally important HA- and membrane- restructuring but rather represent the beginning of the key fusion stage. Restructuring of protein fusogens into a hairpin-like final structure under fusion conditions is a strikingly conserved mechanistic motif shared by very diverse viruses (47;69). Thus the question whether
these hairpin conformations represent discharged post-fusion forms of the proteins or their functional fusogenic forms, is not limited solely to fusion mediated by HA.
Reference List


FOOTNOTES

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The abbreviations used are: AEBSF, 4-(2-aminoethyl)benzenesulfonfyl fluoride; CD, circular dichroism; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; HA2*, full-length ectodomain of the HA2 subunit of the hemagglutinin protein of influenza virus; IPTG, isopropyl β-D-galactopyranoside; LUV, large unilamellar vesicle; N-Rh-PE, N-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; N-NBD-PE, N-(7-nitro-2,1,3-benoxadiazo]-4-yl) phosphatidylethanolamine; RBC, red blood cell; TMD, transmembrane domain.
**FIGURE LEGENDS**

**Figure 1.** Schematic diagram of three different HA2 constructs of influenza virus hemagglutinin. (A) HA2, corresponding to residues 1-221 of the full-length intact protein of the HA2 subunit containing the transmembrane and short cytoplasmic domains; (B) HA2*, corresponding to residues 1-185 of the full-length ectodomain of the HA2 subunit; (C) HA2(1-127), corresponding to residues 1-127 of the ectodomain of the HA2 subunit.

**Figure 2.** Promotion of lipid mixing in LUVs. Panel A. 0.5 μM of HA2* (curve 1) or G1E-HA2* (curve 2), with 100 μM LUVs of DOPC:DOPE:cholesterol (1:1:1) at pH 5.0, 37 ºC. The mixture at pH 7.4 was acidified to pH 5.0 at time zero. Curve 3 shows the spontaneous rate of lipid mixing in the absence of peptide.

Panel B. Lipid mixing curves as in panel 2A, except that the lipid composition of the LUVs is DOPC:cholesterol (1:1).

Panel C. pH dependence of lipid mixing. Lipid mixing was measured with HA2* at different pHs in 100 μM LUVs composed of DOPC:DOPE:cholesterol (1:1:1) (■) or DOPC:cholesterol (1:1) (□). The % lipid mixing is expressed as the value measured at each pH at 300 s, relative to that measured at pH 7.4.

Panel D. pH dependence of the effects on membrane fusion with 100 μM LUVs composed of DOPC:DOPE:cholesterol (1:1:1) as in panel C but with G1E-HA2*.

**Figure 3.** CD analysis of the proteins. Panel A. Secondary structure. CD spectrum at 25 ºC in 10 mM phosphate buffer, 0.14 M NaF, pH 7.4; (■)13.6 μM HA2* and (□)12.8 μM G1E-HA2*. Solutions contained 0.00033 mg/mL reduced Triton X-100.

Panels B-E. Temperature dependence of the ellipticity at 222 nm. The bottom curve in each graph shows the heating scan and the top curve is the cooling scan. Each data point was accumulated over a period of 20 s, with a point taken every degree at a scan rate of 1.5 ºC/min.

Panel B. 13.6 μM HA2* at pH 7.4 in phosphate buffer.

Panel C. 12.8 μM G1E-HA2*, at pH 7.4 in phosphate buffer.

Panel D. 13.6 μM HA2* at pH 5 in phosphate buffer.

Panel E. HA2(1-127) at pH 7.4 in phosphate buffer.

**Figure 4.** Low pH dependent HA2*-mediated fusion between HA0-cell and bound RBC.

The points in panels A and B represent the final extents of lipid mixing (mean +/- s.e., n=3) assayed more than 20 minutes after low pH application.

Panel A. Concentration dependence. Lipid mixing between PKH26-labeled RBC and HA0-cells after a 2-min application of pH 4.9 medium in the presence of different concentrations of HA2* (filled circles), G1E mutant HA2* (filled squares) or I173E mutant HA2* (open circles).

Panel B. pH dependence. Lipid mixing between PKH26-labeled RBC and HA0-cells after a 5-min application of low pH medium in the presence of 1.5 μM HA2* or I173E mutant HA2* (filled and open circles, respectively).

Panel C. HA2* induces lipid mixing, and opens fusion pores that transfer CF but not large enough to pass 10kD dextran. Fusion between HA0-cells and bound RBC was triggered by a 5-min application of pH 4.9 medium in the presence of 2.5 μM HA2* (Bars 3, 17 by guest on January 1, 2018 http://www.jbc.org/ Downloaded from
In control experiments, we omitted application of either HA2* (1) or low pH medium (2). Fusion was assayed as lipid mixing by redistribution of the probe PKH26 (1, 2, 3), redistribution of CF (4) or 10 kDa dextran (5). Data presented as mean +/- s.e., n=3.

Panel D. **In contrast to HA2(1-127) that mediates only lipid mixing, HA2* also mediates content mixing.** In side-by-side experiment, HA0- cells with bound PKH26-labeled and CF-loaded RBC ghosts were treated with 5 μM of either HA2(1-127) (bars 1, 3) or HA2* (bars 2, 4) for 5-min and then with pH4.9-medium for 5 min at room temperature. Final extents of lipid mixing (bars 1, 2) and content mixing (bars 3, 4) were assayed by fluorescence microscopy and are presented as mean +/- s.e., n>3.

**Figure 5.** Fusion mediated by HA2* reproduces several hallmarks of fusion mediated by full-sized HA. Panel A. **Fusogenic activity of the HA2* is inhibited by mutations known to inhibit fusion mediated by full-sized HA.** Fusion between HA0-cells and bound RBC was triggered by a 2-min application of pH 5.3 medium in the presence of 4 μM HA2* or 15 μM G1E mutant HA2* or 5 μM of I173E mutant HA2*. Fusion was assayed as redistribution of lipid probe PKH26 (gray bars) or the small aqueous probe, CF (black bars). Data presented as mean +/- s.e., n>3.

Panel B. **HA2*-mediated fusion is inhibited at 4°C.** HA0- cells with bound CF-loaded RBC ghosts were treated with 3.2 μM HA2* for 5-min at 37°C. The temperature was lowered to 4°C and pH4.9-medium was applied for 10 min still at 4°C. Then the cold low pH medium was replaced with warm (37°C) medium of pH 7.4 (bar 2) or pH 4.9 (bar 3). Bar 1 - HA0-cells with bound CF-loaded RBC ghosts were first treated with 3.2 μM HA2* for 5-min at 37°C and then incubated at pH 4.9 for 5 min still at 37°C. Final extents of content mixing were assayed by fluorescence microscopy and are presented as mean +/- s.e., n>3.

Panel C. **HA2* fuses membranes when added to only one of them and inactivates if acidified in the absence of target membrane.** (1) HA0-cells with bound CF-loaded RBC ghosts were treated with 3.2 μM HA2* for 5-min at 22°C and then for 5-min with a pH4.9-medium (22°C). (2) HA0-cells were treated with 3.2 μM HA2* for 5-min at 22°C; and washed with HA2*-free medium. Then we applied CF-loaded RBC ghosts, washed the cells to remove the unbound ghosts and, finally, treated HA0-cell/RBC complexes with a 5-min pulse of pH4.9-medium (22°C). (3,4) HA0-cells were treated with 3.2 μM HA2* for 5-min at 37°C; washed with HA2*-free medium and then treated for 5-min with a pH4.9-medium. We then applied CF-loaded RBC ghosts, washed the cells to remove the unbound ghosts and either treated (4) or not treated (3) HA0-cell/RBC complexes with a 5-min pulse of pH4.9-medium. Final extents of content mixing were assayed by fluorescence microscopy and are presented as mean +/- s.e., n≥3.

**Figure 6.** Schematic diagram depicting two mechanisms by which HA restructuring at low pH may be coupled to membrane fusion. HA1 subunits in HA homotrimers are shown as yellow rings. HA2 subunits responsible for HA-mediated fusion are shown as blue shapes. Fusion peptides – amphiphilic amino terminal regions of HA2 exposed only in low pH forms of the protein – are shown as green shapes. For the sake of simplicity one of two fusing membranes is not shown. A, B. Fusion can be driven by the conformational energy released in HA2 restructuring from initial neutral-pH form to the low-pH hairpin form. In this scenario, hairpin conformation represents the “discharged” post-fusion form of HA2. A’,B’,
C’. Low pH-triggered restructuring of HA2 produces hairpin form of the protein and this form mediates low pH-dependent fusion. In this hypothetical scenario the energy for membrane remodeling may be provided by interactions between adjacent HA2 molecules and between HA2 and membranes. Finding that hairpin conformations of HA2 ectodomain form fusion pores in a low pH dependent manner substantiates the pathway A’-B’-C’.
Table 1

Secondary Structure Estimation from CD Measurements

<table>
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<tr>
<th>Protein</th>
<th>% α-helix</th>
<th>% β-structure</th>
<th>% turns</th>
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<td>G1E-HA2*</td>
<td>40</td>
<td>18</td>
<td>19</td>
<td>23</td>
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Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5

A

![Graph showing fusion percentages for w.t.HA2*, G1E, and I173E at different concentrations.]

B

![Graph showing content (CF) mixing for different samples.]

C

![Graph showing content (CF) mixing for different samples.]

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Figure 6.
The final conformation of the complete ectodomain of the HA2 subunit of influenza hemagglutinin can by itself drive Low pH-dependent fusion
Chang Sup Kim, Raquel F. Epand, Eugenia Leikina, Richard M. Epand and Leonid V. Chernomordik

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