The 1−127 HA2 Construct of Influenza Virus Hemagglutinin Induces Cell−Cell Hemifusion

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ABSTRACT: Conformational changes in the HA2 subunit of influenza hemagglutinin (HA) are coupled to membrane fusion. We investigated the fusogenic activity of the polypeptide FHA2 representing 127 amino-terminal residues of the ectodomain of HA2. While the conformation of FHA2 both at neutral and at low pH is nearly identical to the final low-pH conformation of HA2, FHA2 still induces lipid mixing between liposomes in a low-pH-dependent manner. Here, we found that FHA2 induces lipid mixing between bound cells, indicating that the "spring-loaded" energy is not required for FHA2-mediated membrane merger. Although, unlike HA, FHA2 did not form an expanding fusion pore, both acidic pH and membrane concentrations of FHA2, required for lipid mixing, have been close to those required for HA-mediated fusion. Similar to what is observed for HA, FHA2-induced lipid mixing was reversibly blocked by lysophosphatidylcholine and low temperature, 4 °C. The same genetic modification of the fusion peptide inhibits both HA- and FHA2-fusogenic activities. The kink region of FHA2, critical for FHA2-mediated lipid mixing, was exposed in the low-pH conformation of the whole HA prior to fusion. The ability of FHA2 to mediate lipid mixing very similar to HA-mediated lipid mixing is consistent with the hypothesis that hemifusion requires just a portion of the energy released in the conformational change of HA at acidic pH.

Influenza virus enters its host cell by fusing the viral envelope with an endosome membrane (*1*). This well-characterized fusion reaction involves multistep membrane rearrangements apparently starting with merger of only contacting membrane monolayers, referred to as "hemifusion" (*2*−*5*). In the productive fusion event, local and transient hemifusion rapidly advances to complete fusion: merger of the distal membrane monolayers accompanied by the opening and expansion of an aqueous connection (=fusion pore) between the volumes enclosed by the membranes.

This fusion reaction is mediated by hemagglutinin (HA),[1] a homotrimeric glycoprotein of the viral envelope (*1*, *6*). Each HA monomer is synthesized as a single polypeptide chain (HA0) which is later cleaved by a protease into the fusion-competent form composed of two subunits: HA1 and HA2. The HA1 subunit is responsible for virus binding to sialic acid receptors at the cell surface. The membrane-anchored HA2 subunit and, in particular, its amino-terminal peptide (referred to as "fusion peptide") are essential for fusion. In the native neutral pH conformation, HA1 is thought to confine HA2 in a metastable or "spring-loaded" state (*7*−*11*). Acidification of the endosome triggers a cascade of conformational changes in HA, which brings the fusion peptides and the transmembrane domains of HA2 into a close proximity (*12*) and allows HA2 to reach its final, lowest energy state. Detailed characterization of the structure of HA2 fragments indicates that, in the absence of HA1, HA2 assumes this lowest energy conformation, supporting the spring-loaded state of the native HA (*10*, *13*−*15*). The conformational change of low-pH-activated HA should release the stored energy that can perhaps be used for membrane fusion. However, the mechanism of how this low-pH-triggered refolding of HA is coupled to the membrane rearrangements is not known. It has been proposed that this hypothetical energy released upon refolding is the driving force for membrane fusion (*8*, *9*, *16*−*18*).

It appears that individual domains of HA play specific roles in the fusion process. For example, the lipid-anchored ectodomain of HA is perfectly capable of mediating lipid mixing between the membranes (*2*, *4*) and even of forming small fusion pores (*19*−*21*). In contrast, the specific

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[1] Abbreviations: HA, influenza virus hemagglutinin; FHA2, residues 1−127 of the HA2 polypeptide chain of HA; RBC, red blood cells; LPC, lysophosphatidylcholine; CPZ, chlorpromazine; CELISA, cell surface enzyme-linked immunosorbent assay; CF, 6-carboxyfluorescein.

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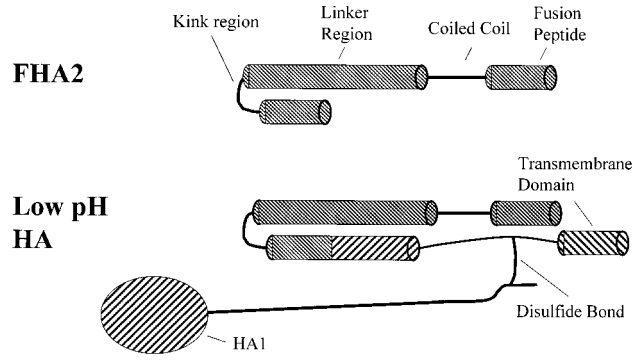

FIGURE 1: Schematic diagram showing the relationship between FHA2 and HA structures.

structures of transmembrane and cytoplasmic domains affect only the stages of fusion downstream of hemifusion (*20*, *22*−*25*).

Several recent studies have focused on the structure and fusogenic activity of smaller fragments of HA2. A trimeric polypeptide, referred to as FHA2, corresponds to the first 127 amino acid residues out of 221 residues of HA2 (Figure 1) and is particularly interesting. FHA2 shows characteristic features of the lowest energy form of the HA2 (*7*, *14*, *15*, *26*, *27*). The fusion peptide of each FHA2 monomer is connected to an extended coiled coil followed by a kink region (aa 105−113) with a flexible loop in the turn of the kink. The remaining half of the HA2 molecule lies antiparallel against the first half (*28*). The only detectable change in the conformation of FHA2 between neutral and acidic pH reflects aggregation of their kink regions (*15*). While this polypeptide represents the "discharged", lowest energy conformation of HA2, and lacks the transmembrane domain, it was found to induce lipid mixing between liposomes in a low-pH-dependent manner (*29*). It remained possible that FHA2 can fuse only protein-free liposome membranes but not cell membranes covered with glycoproteins and, thus, separated by a much larger distance than liposomes. To test whether there is a mechanism of membrane fusion, distinct from the hypothetical spring-loaded mechanism, it was important to find out whether FHA2 is capable of merging biological membranes and, if yes, what the difference is between fusogenic activity of FHA2 and that of full-sized HA.

We report the answers to these questions. While FHA2 did not form expanding fusion pores, it induced lipid mixing between bound cell membranes at acidic pH and at polypeptide surface concentrations similar to those required for HA-mediated fusion. FHA2-induced hemifusion, dependent on the kink−kink and fusion peptide−membrane interactions at acidic pH, shared many important properties with lipid mixing in fusion mediated by full-sized HA.

EXPERIMENTAL PROCEDURES

Polypeptides. FHA2, representing 127 amino-terminal residues of the ectodomain of X31 HA2, and other polypeptides were expressed in *Escherichia coli* BL21(DE3)pACYC and purified to approximately 98% in the following buffer: 0.05−0.5% Triton X-100, 150 mM NaCl, 0.4 mM DTT, 1 mM EDTA, 1 mM PMSF, and 5 mM citrate−phosphate buffer, pH 7, as previously described (*15*, *30*).

Cells. HAb2 cells expressing the Japan strain of HA (*31*), HA300a cells expressing X31 HA (*32*), CV1 cells, and Sf9 cells were grown as described earlier (*33*, *34*). HA300a cells were treated by 5 μg/mL trypsin (Fluka, Buchs, Switzerland) for 10 min at 22 °C to cleave HA0 into its fusion-competent HA1-S-S-HA2 form. In contrast, HAb2 cells were used without trypsin pretreatment to keep HA in a fusion-incompetent form. For fusion experiments we used cells in a confluent monolayer ($\sim 10^5$ cells/dish in 0.4 mL of the medium). Human red blood cells (RBC) were isolated and labeled with the fluorescent membrane dye PKH26 and, in some experiments, loaded with the fluorescent water-soluble dye carboxyfluorescein (CF) as described in ref *34*. Sf9 cells were labeled with *N*-(lissamine Rhodamine B sulfonyl)-diacylphosphatidylethanolamine from egg as described in ref *33*. Labeled cells were then mixed with unlabeled Sf9 cells.

Cell Binding. To test whether FHA2 is capable of mediating fusion of membranes bound by physiologically relevant interactions between HA1 and sialic acid receptors, we studied fusion of HAb2 cells with RBC. RBC binding to HAb2 cells, expressing Japan strain of HA, is mediated by HA1 binding to sialic acid receptors on RBC. HAb2 cells were incubated for 10 min with a 1 mL suspension of RBC (0.05% hematocrit). The cells with 0−2 bound RBC per cell were washed three times with PBS to remove unbound RBC.

To test whether FHA2 fuses cells in the absence of any wild-type HA, we also studied RBC fusion to CV1 cells and fusion between Sf9 cells. RBC were prebound to CV1 cells by mild treatment with paraformaldehyde [4% in PBS (without Ca^{2+} and Mg^{2+}), 10 min at 22 °C]. Neither HA1 nor paraformaldehyde was required to establish contacts between Sf9 cells, which tend to establish physiological contacts and thus rapidly form clusters of bound cells.

Binding of Antibodies. The accessibility of the kink region of intact HA expressed at the surface of HA300a cells was evaluated using LC89 antibody (kindly provided by Drs. John Skehel and Stephen Wharton, MRC, NIMR). The binding site of LC89 includes residue HA2 107 (*13*). HA300a cells with bound RBC were treated by acidic pH and then incubated at neutral pH in the presence of the antibody LC89 to test the effect of this antibody on fusion. For comparison, we also worked with two other antibodies to HA2 [ascitic fluid of monoclonal antibody IIF4 (*13*, *35*) kindly provided by Dr. Frantisek Kostolansky (Institute of Virology, Bratislava) and rabbit polyclonal serum made against the fusion peptide of Japan HA (Covance Laboratories, Inc., Vienna, VA)]. Low-pH-dependent binding of all these antibodies to HA300a cells for the dilutions of the antibodies used in the functional experiments was verified by cell surface enzyme-linked immunosorbent assay as described in ref *36*.

Treatments of the Cells. To treat cells with FHA2 and other polypeptides, bound cells (HAb2−RBC or CV1−RBC or Sf9−Sf9 complexes) attached to the bottom of 35 × 10 mm tissue culture dishes (Nunc, Rochester, NY) were incubated with PBS supplemented with the required concentration of the polypeptides for 5 min prior to low-pH application. Experiments with lauroyllysophosphatidylcholine (LPC, Avanti Polar Lipids, Birmingham, AL) were performed as in ref *34*. PBS titrated by citrate to acidic pH and supplemented with LPC was used to trigger fusion. At the end of the low-pH pulse, cells were washed with LPC-free PBS, and fusion was assayed.

Chlorpromazine (CPZ; Sigma, St. Louis, MO) was prepared as a 0.25 mM solution in PBS. Cells with bound RBC were treated with low-pH medium and then exposed to CPZ-containing solution for 60 s at 22 °C.

In some experiments, the cells with bound FHA2 were treated with thermolysin (Sigma) and then washed twice with complete medium to terminate the enzymatic reaction.

Functional Assays. Fusion was triggered by incubation of the bound cells with PBS titrated by citrate to acidic pH. Acidic solution was replaced by PBS. Lipid mixing between labeled RBC and HAb2 or CV1 cells was assayed by fluorescence microscopy as the ratio of PKH26-redistributed bound RBC to the total number of the bound RBC. Lipid mixing between Sf9 cells was quantified in a similar way as rhodamine lissamine phosphatidylethanolamine transfer from prelabeled to unlabeled Sf9 cells. We also used light microscopy to count Sf9 cells in syncytia (*33*). Final fusion extent (lipid mixing or syncytia formation) was quantified more than 20 min after low-pH application. Longer incubations did not increase the extent of fusion. Each set of experiments for each graph presented here was repeated on at least three occasions with similar results. Presented data were averaged from the same set of experiments.

Evaluation of Polypeptide Binding and Stability of FHA2 Trimers with Western Blotting. Approximately 10^6 cells with bound polypeptides were lifted from the plates with EDTA/EGTA (0.5 mg/mL each) in Ca^{2+},Mg^{2+}-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]. This same buffer was used to equalize the protein concentration between samples. Immediately after lysis, samples were diluted 2-fold with denaturing 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 antisera against HA2 LOOP-36 (*9*) 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.

To analyze the stability of FHA2 trimers in SDS sample buffer, approximately 10^6 CV1 cells were incubated with 1 μM FHA2 peptide for 5 min and then washed twice with PBS. The cells were treated or not treated with pH 4.9 for 10 min and immediately treated with 5 mM bis(sulfosuccinimidyl)suberate (BS^3; Pierce Chemical Co., Rockford, IL) for 10 min at 37 °C. Cell lysate was analyzed by Western blotting as above.

RESULTS

Approaches Applied To Establish Cell−Cell Contacts. To fuse, membranes must be bound to each other. FHA2 on its own did not cause RBC aggregation and did not bind RBC to CV1 cells either at neutral or at low pH (not shown). To establish cell−cell contacts, we used three experimental approaches. In the first approach, HA1−receptor connections were used to provide the contact characteristic for HA-mediated fusion. Labeled RBCs were added to HAb2 cells expressing HA in trypsin-uncleaved and, thus, fusion-incompetent HA0 forms. As expected, low-pH application to these cells with bound RBC caused no lipid or content mixing (see below). In the second approach, nonspecific contacts between RBC and CV1 cells were stabilized by paraformaldehyde cross-linking. In the third approach, we studied fusion of Sf9 cells, which bind to each other to form natural cell clusters. This allowed us to establish cell−cell contacts without using artificial means.

FHA2 Mediates Lipid Mixing between Bound Membranes. Addition of up to 1.5 μM FHA2 to HAb2 cells with bound RBC did not induce measurable lipid mixing while at neutral pH. However, application of acidic pH resulted in fast redistribution of lipid dye from labeled to unlabeled membranes. Figure 2 demonstrates how the extent of lipid mixing depends on pH and FHA2 concentration. Concentrations of FHA2 that induced lipid mixing between the cells also lysed the cells as evidenced by the release of CF from RBC (not shown). Neither lipid mixing nor lysis was observed if low pH was applied to cells prior to addition of FHA2. FHA2 was added to cells in 0.05% Triton X-100. In the control experiments we found neither lipid mixing nor lysis after adding FHA2-free 0.05% Triton X-100, followed by low-pH application. While HA expressed in HAb2 cells was not cleaved by trypsin into its fusion-competent form and, therefore, could not induce fusion on its own, one may hypothesize that uncleaved HA molecules or a small percentage of cleaved HA molecules, present on the membranes even without trypsin application, were somehow involved in FHA2-mediated lipid mixing. However, lipid mixing mediated by FHA2 in a low-pH-dependent manner was also obtained for Sf9 cells and for CV1 cells with bound RBC (see below). Since the properties of FHA2-induced lipid mixing appeared to be identical for all three experimental systems studied, all of the results below will be presented for only one of the systems.

Washing cells with PBS after FHA2 application did not inhibit lipid mixing observed after a subsequent low-pH pulse, indicating that FHA2 irreversibly binds to the cells. Since Triton X-100 is highly water-soluble, washing with PBS removed most of the detergent present. Thus, this result yielded additional evidence that the observed effects of FHA2 were caused by the polypeptide rather than by Triton X-100.

HA mediates fusion when anchored in only one of the two contacting membranes. To test whether FHA2 can also induce lipid mixing between the membranes when added to only one of them, we incubated HAb2 cells with FHA2, washed unbound polypeptide away, added RBC, and then applied low pH (Figure 2B, plot 2). Similar results were obtained for CV1 cells. While it remains possible that some FHA2 had been directly transferred from treated to untreated membrane in the contact region, these results suggest that, to induce lipid mixing, FHA2 could be added to only one of the two membranes.

HA inactivates if low pH is applied in the absence of the target membrane (*37*). Similarly, no lipid mixing was observed in the experiments where HAb2 cells with bound FHA2 (1 μM) were incubated at pH 4.9 for 10 min at 37 °C

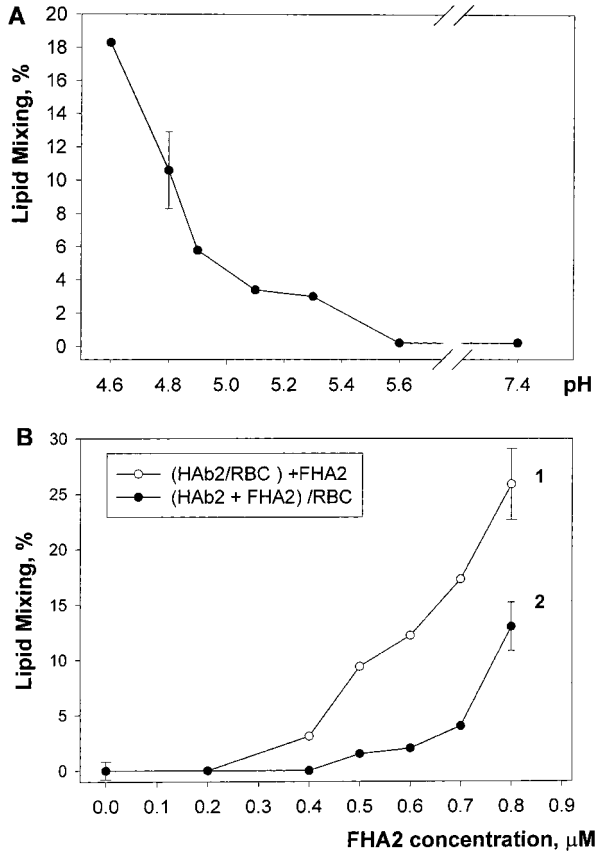

FIGURE 2: Low-pH form of FHA2 mediates lipid mixing. (A) Lipid mixing between PKH26-labeled RBC and HAb2 cells after a 2 min application of low-pH medium in the presence of 0.9 μM FHA2. Note that here and in all other figures HA0 expressed on HAb2 membranes was not cleaved by trypsin into the fusion-competent HA1-S-S-HA2 form. The points represent the final extents of lipid mixing assayed more than 20 min after low-pH application. The representative standard error is shown for pH 4.8, $n = 3$. (B) Dependence of lipid mixing extent on the concentration of FHA2 added either to both contacting membranes (1) or to only one of them (2). (1) FHA2 was added to HAb2 cells with bound RBC at neutral pH 5 min prior to applying pH 4.8 medium for 10 min. (2) FHA2 was added only to HAb2 cells. After washing cells with polypeptide-free medium, we added RBC and 15 min later treated HAb2−RBC pairs with a low-pH pulse. The representative standard errors are shown for 0.8 μM FHA2 and in the absence of the polypeptide, $n = 3$.

prior to addition of RBC (0% vs 16 ± 1.2%, $n = 2$, in the control experiment where a 10 min pH 4.9 pulse followed addition of RBC). Loss of FHA2 ability to mediate lipid mixing upon preincubation at acidic pH was also reported for liposomes (*29*).

The irreversibility of the binding of FHA2 to cells allowed us to estimate the surface density at which FHA2 induced low-pH-dependent lipid mixing. After adding FHA2 to the CV1 cells, we washed unbound polypeptide away and lifted the cells from the bottom of the plate well. Cells with bound polypeptide were transferred to a glass tube to dispose of the polypeptide bound to the plastic of the plate well. We lysed cells, performed Western blotting with antiserum against the loop peptide of HA2 (residues 54−89), and quantified the amount of bound FHA2 by comparing its band with the bands obtained for known amounts of the free polypeptide. To estimate the surface density of FHA2, we calculated the total surface area of the cells from the number of cells and the average surface area per cell of ~ 4000 μm^2 as judged by the capacitance of CV1 cells, ~40 pF (G. Melikyan, personal communication). This estimate suggested that FHA2 induced lipid mixing at a surface density of ~ 900 trimers/μm^2. This surface density is actually lower than that of HA in fusion-competent membranes, which ranges from 1000 to 15 000 trimers/μm^2 for gp4/6 cells and viral particles, respectively (*6, 38*). Using the same quantitative Western blotting approach, we found that the mutant polypeptides G1E, L110C, T111C, and 1−90 (see below) bind to plasma membranes of CV1 cells similarly to FHA2 (900−1400 trimers of the mutant polypeptides/μm^2).

FHA2 Does Not Cause Syncytium Formation. Lipid mixing mediated by wild-type HA is accompanied by content mixing (complete fusion) or involves only outer monolayers of the membranes with no aqueous connection between the cells (unrestricted hemifusion). To distinguish between these two types of fusion reactions, one needs to follow the merger of aqueous contents. While we observed no FHA2- and low-pH-induced transfer of the aqueous probe CF from preloaded RBC into unlabeled cells, low-pH-dependent FHA2-induced release of CF from RBC clearly hinders assaying for complete fusion. An alternative, albeit less sensitive assay for complete fusion involves counting cells in syncytia (*33*). Lipid mixing between Sf9 cells incubated with FHA2 at acidic pH was not accompanied by syncytia formation (Figure 3). One may hypothesize that FHA2 mediates local aqueous connections between cells but somehow blocks the later changes in cell morphology required for syncytia formation. However, the results of the control experiment, in which we found that syncytia formation mediated by baculovirus-infected Sf9 cells (*33*) was not inhibited in the presence of 1 μM FHA2, argued against this hypothesis. Thus the inability of the low-pH form of FHA2 to form syncytia indicates that FHA2 mediates hemifusion rather than complete fusion.

Restricted Hemifusion Phenotype Mediated by FHA2. A recently identified phenotype of HA-mediated membrane interaction, referred to as "restricted hemifusion" is accompanied by neither lipid nor content mixing and apparently corresponds to a situation in which lipid mixing through a site of local hemifusion is restricted by low-pH conformations of HA (*5*). One can detect such restricted hemifusion by its transformation into complete fusion by breaking the hemifusion diaphragms with the amphiphilic agent CPZ, which preferentially incorporates into the inner membrane monolayers (*3, 5*). Adding CPZ to the RBC−HAb2 cell complexes treated by low pH in the presence of FHA2 resulted in a profound increase in the percentage of cells demonstrating lipid mixing and content mixing (Figure 4). Note that in this experiment the concentration of FHA2 was low enough to avoid leakage of CF, and almost no lipid mixing was observed prior to CPZ application. Similar results were obtained for RBC−CV1 cell complexes. No lipid mixing was detected in the control experiments when CPZ was applied to the RBC−HAb2 and RBC−CV1 cell complexes treated with low pH with no FHA2 added or treated with FHA2 but not incubated with low pH. These findings indicate that some of the membrane merger sites yielded by FHA2 did not allow lipid and content mixing prior to CPZ application, suggesting that FHA2 mediates restricted hemifusion as does native HA and lipid-anchored HA ectodomain (*5, 36*). These results indicate that FHA2 induces membrane

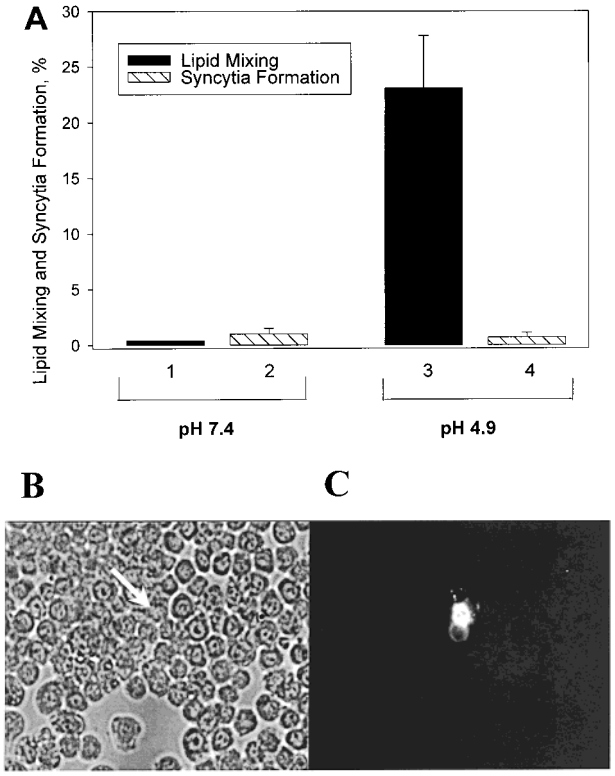

FIGURE 3: FHA2-mediated lipid mixing is not accompanied by syncytia formation. (A) Sf9 cells were treated (bars 3 and 4) or not treated (bars 1 and 2) with a 10 min pulse of pH 4.9 in the presence of 1 μM FHA2. The percentages of cells forming syncytia (bars 2 and 4) and those demonstrating lipid mixing (bars 1 and 3) were evaluated by phase contrast and fluorescence microscopy, respectively. (B, C) Sf9 cells were treated with a 10 min pulse of pH 4.9 in the presence of 1 μM FHA2. Twenty minutes later the same field of view was photographed with phase-contrast (B) and fluorescence microscopy (C). Arrow in (B) points to a cell prelabeled with fluorescent lipid and transferring the dye to one of the adjacent cells.

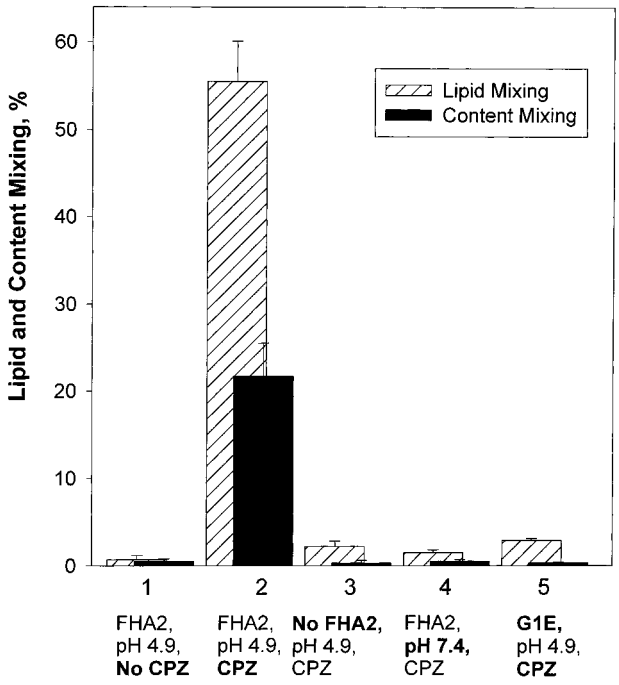

FIGURE 4: FHA2 forms restricted hemifusion intermediates. Fusion of HAb2 cells with bound PKH26- and CF-labeled RBC was triggered at 37 °C by a 2 min application of pH 4.8 in the presence of 0.35 μM FHA2 (bars 2). Immediately after the end of a low-pH pulse cells already at neutral pH were exposed to a 1 min application of 0.25 mM CPZ. The extent of fusion was assayed by fluorescence microscopy at neutral pH 20 min after the end of low-pH application as lipid dye, PKH26 (gray), and aqueous dye, carboxyfluorescein (black), redistribution. Neither lipid nor content mixing was observed in the control experiments where a low-pH pulse applied in the presence of FHA2 was not followed by CPZ (bars 1), or CPZ was applied to the cells treated by low pH in the absence of FHA2 (bars 3) or when FHA2 was replaced with G1E polypeptide (bars 5), or CPZ was applied to the cells treated with FHA2 but not exposed to a low-pH pulse (bars 4). The percentage of RBC−HAb2 cell contacts containing restricted hemifusion intermediates can be estimated as a difference between the extents of lipid mixing with and without CPZ application (bars 2 and 1). Bars are the mean ± SE, $n > 3$.

merger rather than somehow promoting the transfer of lipid probe between distinct membranes and also indicate that this membrane merger is not necessarily accompanied by leakage of aqueous dye.

FHA2-Mediated Lipid Mixing Shares Inhibitors with HA-Mediated Fusion. HA-mediated fusion can be inhibited (1) by cleaving the low-pH conformation of HA with thermolysin or proteinase K (*5*, *6*), (2) by adding lysophosphatidylcholine to alter lipid composition of the membranes to one that is nonpermissive for fusion (*34*), and (3) by lowering the temperature to 4 °C (*5*, *39*, *40*). These same treatments also inhibited FHA2-mediated lipid mixing (Figure 5). As with HA, LPC and 4 °C inhibition of FHA2-induced lipid mixing were reversible. After the end of a low-pH pulse, both removing LPC and raising the temperature at neutral pH, respectively, resulted in lipid mixing, indicating that the actual merger of membranes downstream of LPC- and 4 °C-arrested stages did not require low pH. However, while for HA the LPC arrested and 4 °C arrested intermediates were long-lived structures (*5*, *34*), FHA2-formed LPC-arrested intermediates rapidly disappeared at neutral pH (bar 4 vs bar 3).

Structure of FHA2 and Its Fusogenic Activity. To determine which regions in the FHA2 were important for its fusogenic activity, we studied the activity of some mutant polypeptides. Substitution of glutamic acid for the glycine at position 1 of FHA2 (i.e., in what would be the amino-terminal residue of the HA2 fusion peptide) resulted in complete loss of the lipid mixing activity of FHA2 (Figure 6A, mutant G1E). A single amino acid substitution in the kink region of FHA2 (cysteine for leucine at position 110, mutant L110C) also completely abolished fusogenic activity. Another amino acid substitution in the same region of FHA2 (cysteine for threonine at position 111, mutant T111C) significantly inhibited lipid mixing. Complete deletion of the kink region in the truncated FHA2 polypeptide, corresponding to HA2: 1−90, resulted in the total loss of lipid mixing. Interestingly, this fusion-incompetent peptide was very effective in inducing cell lysis. Not only were the mutant polypeptides G1E and T111C inefficient in inducing lipid mixing, they also did not form a restricted hemifusion phenotype (shown for G1E in Figure 4, bar 5).

As the whole HA ectodomain, FHA2 forms trimers both in aqueous solution and in its membrane-bound form (*14*, *15*). We found that the stability of FHA2 trimers depends on pH (Figure 6B). CV1 cells with bound FHA2 incubated either at neutral or acidic pH were treated or not treated with a cross-linking agent, BS^3, to stabilize the trimers. After

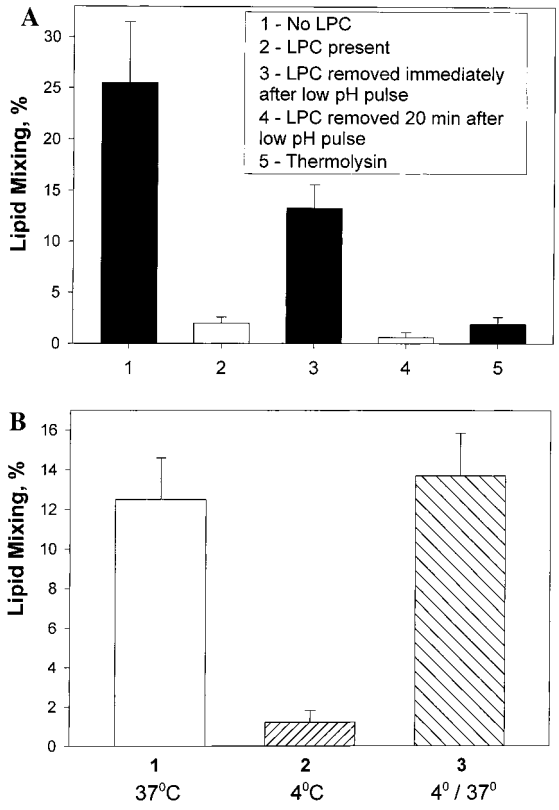

FIGURE 5: FHA2-mediated lipid mixing is inhibited by LPC, thermolysin, and 4 °C. Fusion of HAb2 cells with bound PKH26-labeled RBC was triggered at 37 °C by a 5 min application of pH 4.8 in the presence of 1 μM FHA2. Lipid mixing was assayed by fluorescence microscopy at neutral pH. (A) Inhibition of lipid mixing by LPC and thermolysin. (1) Lipid mixing extent observed in the control experiment with neither LPC nor thermolysin applied. (2−4) After a low-pH pulse was applied in the presence of 170 μM LPC, lipid mixing was assayed either still in the presence of LPC (2) or when LPC was washed out immediately (3) or 20 min (4) after the end of the low-pH pulse. (5) Five minutes after addition of FHA2, HAb2 cells with bound RBC were treated by thermolysin (25 μg/mL) for 5 min at 37 °C. After the end of the thermolysin treatment a low-pH pulse was applied and lipid mixing assayed. (B) 4 °C reversibly blocks FHA2-mediated lipid mixing. Fusion of CV1 cells with bound PKH26-labeled RBC was triggered by a 15 min pulse of pH 4.8 either at 37 °C (1) or at 4 °C (2, 3). In (3) after incubation of the cells for an additional 10 min at 4 °C, the temperature was raised to 37 °C and extent of lipid mixing assayed.

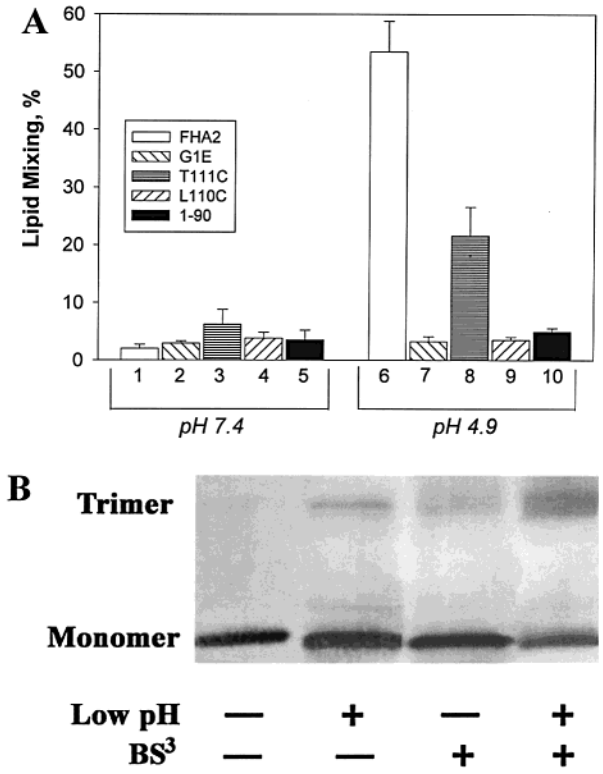

FIGURE 6: Fusogenic activity and stability of the FHA2 trimer depends on its amino acid sequence and pH. (A) Fusogenic activity of FHA2 depends on the sequence of the fusion peptide and kink regions. CV1 cells with bound PKH-labeled RBC were incubated for 5 min with FHA2 (bars 1 and 6) or polypeptides G1E (bars 2 and 7), T111C (bars 3 and 8), L110C (bars 4 and 9), and 1−90 (bars 5 and 10) at a final concentration of 1 μM. Then the cells were treated (bars 6−10) or not treated (bars 1−5) with pH 4.9 for 10 min at 37 °C. The percentage of cell−RBC contacts with lipid mixing was evaluated by fluorescence microscopy. (B) The acidic form of the FHA2 trimer is more stable against dissociation in SDS than the neutral pH form. CV1 cells with bound FHA2 were treated (+) or not treated (−) with pH 4.9, 10 min, and then BS^3 (5 mM, 10 min, 37 °C). The positions of the trimeric and monomeric forms are indicated.

lysing the cells with SDS-containing buffer, we evaluated the amount of trimeric FHA2 by Western blotting. At neutral pH, only BS^3-cross-linked trimers of FHA2 were stable enough to withhold SDS presence. Application of low pH increased the amount of stable FHA2 trimers.

Exposure of the Kink Region in the Low-pH Form of the Whole HA Precedes Fusion. The effects of the kink region on FHA2-mediated lipid mixing are consistent with the reported importance of this region of FHA2 for low-pH-induced aggregation of FHA2 trimers (*15*) and liposome fusion (*29*, *30*). The specific role of the kink region of full-length HA in HA-mediated fusion remains to be understood. To allow interaction between kink regions of HA trimers at acidic pH, similar to that reported for FHA2, the kink region of HA has to be exposed in the low-pH conformation of HA. To probe for the accessibility of the kink region, we used HA300a cells expressing X31 HA and a monoclonal antibody LC89 (*13*). The binding site for this antibody includes residue HA2 107, which is located in the middle of the kink region. Note that in these experiments, in contrast to those where HA was used only to provide binding with receptors, HA was cleaved with trypsin into the HA1-S-S-HA2 form, competent for low-pH-induced conformational change and for fusion. In agreement with earlier studies (*13*), we found that the LC89 antibody binds to the low-pH, but not to the neutral pH, conformation of HA (data not shown). In functional assays, LC89 antibody inhibited HA-mediated lipid mixing if antibody was applied during and after the low-pH pulse (Figure 7). As expected, there was no inhibition if the antibody was applied only before low-pH application. Out of two other tested antibodies known to recognize the low-pH conformation of HA2, antisera to fusion peptide (*41*) and antibody IIF4 (*13*, *35*), only the former one inhibited lipid mixing. While the quantitative comparison between the effects of the different antibodies is impossible because of the differences in their affinities and concentrations, these results indicate that exposure of the binding site for LC89 as well as exposure of the fusion peptide precedes fusion rather than follows it. These results confirm that the kink region of HA is exposed in a fusion-competent HA conformation and can be involved in kink−kink interactions similar to those described for FHA2 (*15*).

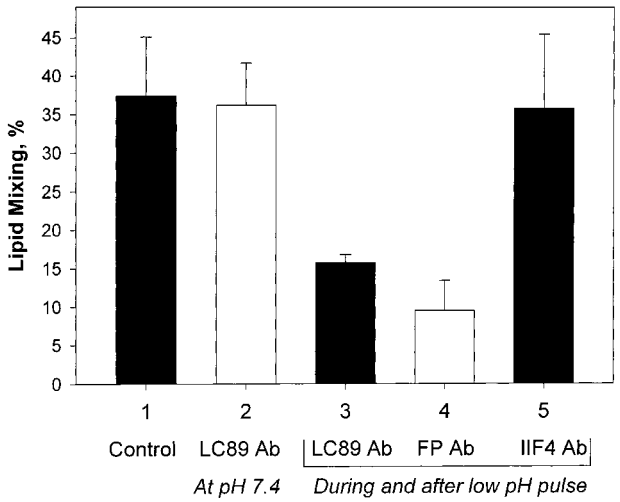

FIGURE 7: Lipid mixing mediated by the whole HA is inhibited by LC89 antibody which binds to the kink region of the acidic pH conformation of HA. Lipid mixing between HA300a cells and PKH26-labeled RBC was triggered by a 2 min pulse of pH 5.1 at 22 °C. The final extents of lipid mixing were assayed by fluorescence microscopy 20 min after the low-pH pulse. (Bar 1) Control experiment, no antibodies applied. (Bars 2 and 3) LC89 antibodies (1:50 dilution) were applied either only at neutral pH before the low-pH pulse (bar 2) or during and after the pulse (bar 3). (Bars 4 and 5) Antisera to fusion peptide (dilution 1:100, bar 4) and IIF4 antibodies (dilution 1:100, bar 5) were applied during and after the pulse. Bars are the mean ± SE, $n > 3$.

DISCUSSION

Our work is aimed at connecting the studies on hemagglutinin-mediated fusion of biological membranes with recent work on the fusogenic properties of different peptide fragments of HA2 in a liposome suspension (*15*, *29*, *30*). A polypeptide's ability to mediate lipid mixing can be dependent on membrane contact and, in particular, on the distance between membranes. One may suggest that membrane merger is easier to achieve for bare, protein-free lipid bilayers than for cell membranes covered with glycoproteins. In fact, the same polypeptide 1−90 induces lipid mixing between liposomes (*30*) but not between cells (Figure 6A), confirming a difference between requirements for merger of protein-free lipid bilayers and those for biological membranes. In contrast to polypeptide 1−90, FHA2 was able to mediate lipid mixing both between liposomes (*29*) and between different cells (Figure 2), including the cells bound by the proper HA1−receptor interactions.

Comparison between Fusogenic Activities of FHA2 and HA. FHA2 represents only about one-fourth of the whole sequence of HA. Thus it is not surprising that fusogenic activity of FHA2 did not include a number of important features observed for native HA. In contrast to HA, FHA2 did not bind cell membranes to each other either at neutral or at low pH. FHA2 was also not capable of forming expanding fusion pores as evidenced by the lack of syncytia formation. This finding is consistent with recent data indicating that fusion pore expansion is critically dependent on the transmembrane domains of HA (*20*−*24*).

Besides these important differences, FHA2-mediated lipid mixing shares many properties with HA-mediated lipid mixing. The fusogenic activity of both HA and FHA2 critically depends on the sequence of the fusion peptide. Mutation in the fusion peptide, known to inhibit HA-mediated fusion (*42*, *43*), resulted in the complete loss of the lipid mixing activity of FHA2 (Figure 6A). A single amino acid substitution in the kink region of FHA2 also significantly inhibited lipid mixing. Complete deletion of the kink region in the truncated FHA2 polypeptide 1−90 resulted in the lack of lipid mixing. For the neutral pH conformation of the whole HA, the kink region is important for stabilization of the fusion peptide at the trimer interface. Substituting glycine for aspartic acid at position 112 of HA2 shifted the pH dependence of fusion to less acidic pH (*44*). Our finding that the kink region of the whole HA upon low-pH application becomes accessible for antibody binding prior to fusion is consistent with the hypothesis that here, as for FHA2, interaction between kink regions plays an important functional role in fusion.

Fusogenic activity of both HA and FHA2 required similar surface concentrations of the polypeptides and was inhibited by the same inhibitors: LPC, 4 °C, and thermolysin. HA-mediated fusion and FHA2-mediated lipid mixing were both triggered at acidic pH. Neither for HA nor for FHA2 does the actual merger of the membranes following low-pH application require continuous application of low pH. To mediate lipid mixing, FHA2 has to be added to only one of the contacting membranes, as in the case of HA. As with HA, FHA2 was inactivated if low pH was applied in the absence of the target membrane (see also ref *29*). At lowered surface concentrations, FHA2 produces the restricted hemifusion phenotype, as does HA. The similarity between FHA2-induced lipid mixing and HA-mediated fusion is consistent with the hypothesis that the FHA2 fragment of HA is required and sufficient to mediate membrane hemifusion, which is hypothesized to occur at the early stage of HA-mediated fusion.

Mechanism of FHA2-Induced Lipid Mixing. Local fusion between membrane monolayers is not the only possible interpretation of the FHA2-mediated lipid mixing observed between liposomes and between cells. Transfer of fluorescent lipid from a labeled membrane to an unlabeled one can proceed without their merger (*45*, *46*) or can be mediated by lysis of the membranes and their subsequent reassembly. Our data suggesting that FHA2 mediates membrane merger into a hemifusion structure argue against both of these interpretations. In addition, lysis and lysis-dependent lipid transfer should be promoted, rather than inhibited, by LPC.

Since FHA2 was reported to cause liposome aggregation (*15*, *29*), one might consider another possible pathway leading to lipid mixing. Strong adhesion of two closed membranes can drive the expansion of the contact area and provide membrane tension. This tension can then break the membranes either in the contact region or in the periphery of the membranes. The adhesion-driven rupture of membranes would lead to leakage and lipid mixing as described for giant phosphatidylserine vesicles in the presence of calcium ions (*47*). FHA2-induced lipid mixing between cells apparently cannot be explained by this mechanism. First of all, cells, in contrast to liposomes, do not aggregate in the presence of FHA2. Even if FHA2 promotes local adhesion between the cells, development of extended contact regions required to develop membrane tension seems rather unlikely. Lipid mixing inhibition by LPC, known to destabilize membranes and promote pore formation, also argues against adhesion-driven rupture/fusion as a possible mechanism of

FHA2-induced lipid mixing.

As is the case for many amphiphilic peptides mimicking different fragments of viral fusion proteins (*48*−*52*), and for FHA2-induced fusion of liposomes (*29*), lipid mixing in the presence of FHA2 is "leaky" (i.e., accompanied by permeabilization of cell membranes). Release of the aqueous dye CF preloaded into RBC and lipid mixing had similar pH and concentration dependencies. However, the correlation between fusogenic and lytic activity of studied polypeptides was not absolute. While mutant polypeptides L110C and G1E, which did not mediate lipid mixing, did not mediate CF release, the lytic activity of the fusion-incompetent 1−90 peptide was as high as that of FHA2. Note that fusion mediated by wild-type HA is also rather leaky (*43*, *53*, *54*). Also, cell lysis is clearly not a prerequisite for the fusogenic activity of FHA2 as evidenced by detection of a restricted hemifusion phenotype at lowered concentrations of FHA2 in the absence of any detectable CF leakage.

The similarity between fusogenic activities of FHA2 and HA is rather unexpected in the context of the existing models of HA-mediated fusion. Many current hypotheses suggest that the energy for the membrane merger be provided by the conformational change of HA from its initial metastable conformation to the final low-energy structure (*9*, *16*−*18*). Low-pH application is thought to merely trigger this change. The conformation of FHA2 is close to the final low-energy conformation of HA2 already at neutral pH (*14*, *15*), and thus, at least in this case, the energy for hemifusion cannot come from the transition to the rod-shaped α-helical bundle conformation. The notion that protein-mediated hemifusion does not necessarily require such a transition is substantiated by a recent finding that rabies virus G protein, which does not have a coiled coil motif in its structure, fuses membranes through lipidic intermediates apparently similar to those in HA-mediated fusion (*55*).

We still do not know the mechanism by which FHA2 brings together and merges the contacting monolayers of two membranes initially separated, in the case of a biologically relevant HA−receptor connection, by more than 10 nm distance. Since neither the G1E nor 1−90 mutants mediate lipid mixing and since the fusogenic activity of T111C is lowered in comparison with FHA2, both fusion peptide and kink domains of FHA2 are essential for its fusogenic activity. Lowering pH results in the interactions between kink regions of FHA2 both within the same trimer (Figure 6B) and between different trimers (*15*). Low-pH-induced interactions between the kink domains of adjacent FHA2 trimers might tilt these trimers and, thus, alter the fusion peptide's angle of insertion into the membrane to the orientation required to bend and destabilize the membrane monolayers (*56*−*58*). In fact, a larger fragment of HA containing the HA2 subunit and a small portion of HA1 (X31 HA, HA2:1−175 connected with HA1:1−27) undergoes some low-pH-dependent change in orientation, although less profound than those in the whole HA ectodomain (*59*). The same polypeptide also induces low-pH-dependent lipid mixing between liposomes. Interestingly, the whole HA1−HA2 ectodomain (BHA), released from the transmembrane domain by bromelain treatment, has no fusogenic activity (*60*, *61*). It is intriguing that BHA, in contrast to FHA2 and the HA2:1−175 plus HA1:1−27 polypeptide, does not mediate lipid mixing. One may hypothesize that this lack of fusogenic activity of BHA, similarly to its lack of hemolytic activity (*60*, *62*), reflects very fast [faster than for intact HA (*62*) and FHA2 (*29*)] inactivation of this protein at acidic pH and physiological temperature.

FHA2 is clearly only a part of the intact hemagglutinin protein and cannot perform some of the important functions of the HA fusion machine. In particular, FHA2 fails to mediate fusion pore formation and expansion. These stages require more low-pH activated HA molecules than restricted and unrestricted hemifusion (*5*, *36*), demonstrate a more stringent dependence on the HA structure (*23*, *43*), and are apparently the most energy-consuming stages of the fusion process (*63*, *64*). Still, while FHA2 fails to mediate these critical fusion stages, as described above hemifusion mediated by FHA2 shares a number of features with that mediated by intact HA, indicating that membrane hemifusion requires just a portion of the energy released in the conformational change of HA. A fragment of HA with its conformation close to the final HA2 conformation mediates hemifusion by apparently utilizing the energy provided by kink−kink and fusion peptide−membrane interactions at acidic pH.

Further elucidation of FHA2-mediated lipid mixing can result in a radical change in our thinking of how HA merges membranes. Future studies may strengthen the hypothesis that FHA2 is a hemifusion-mediating unit in a greater HA fusion machine containing also the HA domains responsible for progression from hemifusion to complete fusion. It is also possible that FHA2-mediated hemifusion models end-state hemifusion (*36*, *65*, *66*) having a different mechanism from the transient hemifusion in complete fusion. Finally, it remains feasible that FHA2 and HA, two polypeptides with a high degree of homology, merge membranes by entirely different mechanisms. Besides many similarities, we also notice some differences between the lipid mixing activity of FHA2 and HA. For example, HA-induced lipid mixing can be triggered by urea at neutral pH (*8*), although low pH is required for FHA2-induced lipid mixing. Further, the low-pH-induced clustering or aggregation of trimers, similar to the kink-induced clustering for FHA2 (*15*), is not observed for the longer but soluble constructs of HA2 under low-pH conditions (*28*). Aggregation and fusogenic activity of FHA2 can be related to the fact that the central triple-coiled core of HA, normally covered by the polypeptide chains, is exposed in a shorter FHA2 peptide. These exposed nonpolar residues of FHA2 can interact with membranes and adjacent polypeptides in a low-pH-dependent manner. However, if these interactions are indeed instrumental in bringing about fusogenic activity of FHA2, we still need to explain why this activity (i) critically depends on the specific sequence of additional FHA2 domains, the fusion peptide and kink region of FHA2, and (ii) shares a number of functional features with that of full-sized HA. At present we consider it more probable that FHA2 and HA mediate cell hemifusion by similar mechanisms. We hope that better understanding of the connection between the structure and function of the known fusogenic polypeptides will help in identification of the mechanisms by which proteins fuse membranes.

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