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Lipid-Anchored Influenza Hemagglutinin Promotes Hemifusion, Not Complete Fusion

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Summary

It has been proposed that membrane fusion events such as virus-cell fusion proceed through a hemifusion intermediate, a state where lipids but not contents of the fusing compartments mix. We engineered the influenza hemagglutinin (HA) such that it would be anchored in membranes via a glycosylphosphatidylinositol (GPI) tail. GPI-anchored HA forms a trimer that can bind red blood cells (RBCs) and change conformation under fusion-inducing conditions. Using RBCs labeled with fluorescent lipid or fluorescent soluble content probes, we found that GPI-anchored HA mediated lipid mixing with similar time course and efficiency as wt-HA, yet did not mediate transfer of soluble contents. Hence, GPI-anchored HA appears to initiate, but not complete, a fusion reaction. We interpret our results as evidence for uncoupling a physiological fusion reaction, for trapping a hemifusion intermediate, and for assigning a role to a transmembrane domain in a fusion event.

Introduction

Membrane fusion is essential for an array of cellular functions including synaptic transmission, fertilization, and intracellular membrane traffic. The fusion reactions of enveloped viruses remain the best characterized, owing to the relative ease of identifying, purifying, and manipulating viral membrane fusion proteins. The most thoroughly studied membrane fusion protein is the hemagglutinin (HA) of influenza virus. HA is a homotrimer that performs two functions leading to virus entry: it binds the virus to host cell receptors, and it induces membrane fusion (for reviews, see Stegmann et al., 1989; White, 1992; Wiley and Skehel, 1987).

HA is sufficient to induce membrane fusion (Stegmann et al., 1987; White et al., 1982). Under fusion-inducing conditions (exposure to mildly acidic pH), HA changes conformation. It releases its apolar fusion peptides from the trimer interface (White and Wilson, 1987), and it binds hydrophobically to the target membrane (Harter et al., 1989; Stegmann et al., 1991). The importance of the fusion peptides has been well documented. Mutations within the fusion peptides (Gething et al., 1986; Guy et al., 1992) or ones that prevent fusion peptide exposure (Godley et al., 1992; Kemble et al., 1992) impair or abolish fusion activity. Designed inhibitors (Bodian et al., 1993) that lock the fusion peptides in place prevent fusion.

After the primary conformational change that exposes the fusion peptides (Kemble et al., 1992; White and Wilson, 1987), HA undergoes additional changes (Stegmann et al., 1990) that ultimately lead to the opening and dilation of a fusion pore (Spruce et al., 1991; Spruce et al., 1989). Models have been proposed (Bentz et al., 1990; Ellens et al., 1990; Stegmann et al., 1990; White, 1992) in which the fusion pore is lined with several conformationally altered HA trimers (e.g., Figures 2A and 2B in White, 1992). In view of these models, it is important to dissect which parts of the trimer are crucial for the fusion pore. A possibility suggested by the models is that the transmembrane domain may be critical.

Although HA must be membrane anchored to be fusogenic (Wharton et al., 1986; White et al., 1982), the role of the HA transmembrane domain in the overall fusion process has not been clarified. To address this question, we engineered HA such that it would be anchored in only the outer leaflet of cell membranes via a glycosylphosphatidylinositol (GPI) tail, rather than through both leaflets of the membrane via its normal transmembrane domain. We recently showed (Kemble et al., 1993) that two GPI-anchored HAs that vary in the exact placement of the GPI anchor addition signal resemble wild-type (wt) HA in most important respects. Both form 9S trimers that are highly mobile in the plane of the membrane. When produced in the presence of deoxymannojirimycin (dMM), to prevent terminal oligosaccharide processing, both GPI-anchored HAs bind red blood cells (RBCs). When exposed to low pH, both GPI-anchored HAs change conformation, expose their fusion peptides, and become hydrophobic. Since the ectodomain of GPI-anchored HA can bind target membranes and can undergo the preparatory steps for fusion (Kemble et al., 1993), we asked, in this study, whether GPI-anchored HA is fusion competent.

Results

Membrane fusion involves mixing of both the membrane lipids and the aqueous contents of donor and target compartments. We therefore assessed the ability of cells expressing GPI-anchored HA to fuse with target RBCs prelabeled with either fluorescent lipid or fluorescent soluble content probes.

HA is expressed in fibroblasts as a nonfusogenic precursor (HA0) that can be cleaved by trypsin to generate the fusogenic (HA1–S–S–HA2) form (Lazarowitz and Choppin, 1975; White et al., 1982). One of the nine amino acids added to the ectodomain of GPI-anchored HA, from the GPI anchor additional signal, is a lysine (see Figure 1 in Kemble et al., 1993). To rule out the possibility of cleav-

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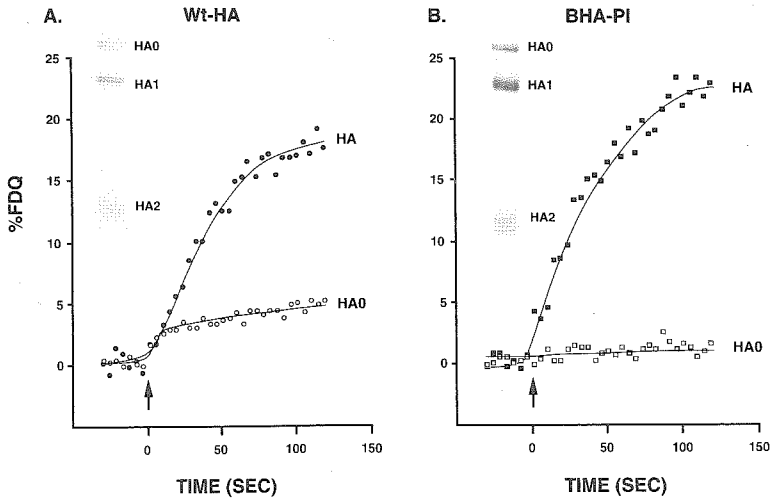


Figure 1. Lipid Mixing Activity of BHA-PI-Expressing Cells: R18 Fluorescence Dequenching

Cells expressing wt-HA ([A], circles) or BHA-PI ([B], squares) were treated for 5 min at room temperature with neuraminidase and either 5 μg/ml TPCK–trypsin (closed symbols) or 5 μg/ml TLCK–chymotrypsin (open symbols). R18-labeled-RBCs were bound and the complexes analyzed for FDQ as described. Arrows indicate the time of addition of 1 M citric acid to lower the pH to 5.2. Insets: cells expressing either wt-HA or BHA-PI were metabolically labeled with [^{35}S]TransLabel for 16 hr at 37°C and then treated with 5 μg/ml TPCK–trypsin for 5 min at room temperature. The HA was immunoprecipitated from detergent cell lysates, separated by SDS–PAGE (10%), and detected by autoradiography.

ing at this lysine, while trypsin-activating GPI-anchored HAs, we engineered a third GPI-anchored HA in which the lysine from the GPI anchor addition sequence was changed to a serine (see Experimental Procedures). The new construct and the protein expressed from it are referred to as BHA-PI. BHA-PI resembles the previously characterized GPI-anchored HAs (Kemble et al., 1993) in its biochemical properties and in its target cell binding activity (G. W. K., P. Straight, H. Qiao, and J. M. W., unpublished data): BHA-PI forms a trimer that changes conformation at low pH. When grown in the presence of dMM, as described previously (Kemble et al., 1993), cells expressing BHA-PI bind RBCs. Therefore, all experiments presented in this paper employed cells grown in the presence of dMM, to allow RBC binding.

Lipid Mixing Activity of GPI-Anchored HA

Like wt-HA (Figure 1A, inset), BHA-PI is expressed at the surface of Chinese hamster ovary (CHO) cells as an HA0 precursor that can be cleaved by mild trypsinization to yield HA1 and HA2 (Figure 1B, inset). Following trypsin activation, HA-expressing cells were incubated with RBCs labeled with the fluorescent lipid probe octadecylrhodamine (R18). The RBC–cell complexes were then analyzed for R18 fluorescence dequenching, a monitor of lipid mixing, as described previously (Kemble et al., 1992). Following acidification, wt-HA mediated rapid and efficient lipid mixing (Figure 1A). BHA-PI also promoted rapid and efficient lipid mixing (Figure 1B). As seen for wt-HA (Figure 1A), the lipid mixing activity of BHA-PI-expressing cells (Figure 1B) required both mild trypsinization and exposure to low pH. Hence, the lipid mixing activity of BHA-PI has the same requirements as that of wt-HA.

To test whether the lipid mixing activity of BHA-PI-expressing cells was due to GPI-anchored HA, wt-HA- and BHA-PI-expressing cells were treated with phosphatidylinositol-specific phospholipase C (PI-PLC). PI-PLC treatment removes GPI-anchored HAs, as trimers, from the cell surface (Kemble et al., 1993). Wt-HA-expressing cells mediated lipid mixing equally well, whether or not they were pretreated with high concentrations of PI-PLC (Figure 2); wt-HA is not removed from the cell surface by this procedure (Kemble et al., 1993). In contrast, PI-PLC treatment reduced the lipid mixing activity of BHA-PI-expressing cells in a dose-dependent fashion. When pretreated with ≥25 mU/ml PI-PLC, the lipid mixing activity of BHA-PI-expressing cells was abolished (Figure 2). Under these conditions, a fraction of BHA-PI is removed from the cell surface (Figure 3). Therefore, the lipid mixing activity of BHA-PI-expressing cells is due to GPI-anchored HA.

R18 is a single chain lipid analog that incorporates into the outer leaflet of biological membranes; R18-labeled membranes have been used extensively in membrane fusion studies. However, R18 has, on occasion, been subject to nonspecific membrane transfer (Di Simone and Baldeschwieler, 1992; Wunderli-Allenspach and Ott,

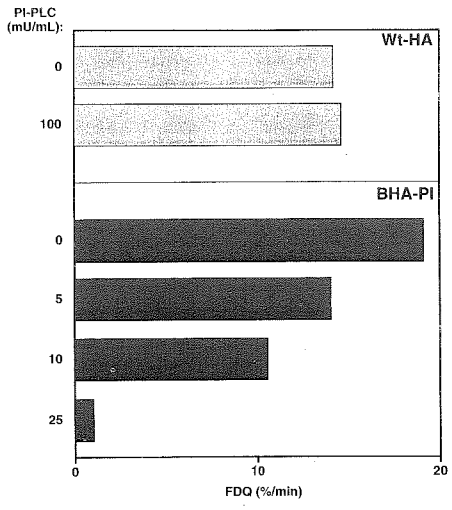


Figure 2. PI-PLC Treatment Abolishes the Lipid Mixing Activity of BHA-PI-Expressing Cells

Cells expressing wt-HA (shaded bars) or BHA-PI (solid bars) were incubated with R18-labeled RBCs, washed, and then treated with the indicated amount of PI-PLC at 37°C for 60 min. This treatment did not significantly reduce RBC binding. RBC–cell complexes were then removed from their dishes and FDQ measured as described in the legend to Figure 1.

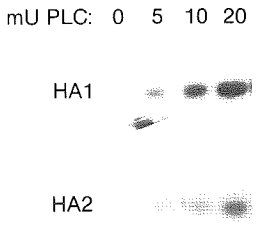


Figure 3. PI-PLC Releases BHA-PI from the Cell Surface

BHA-PI-expressing cells were biotinylated, treated with trypsin, and then incubated with the indicated amount of PI-PLC (mU/ml) at 37°C for 60 min. Supernatants were collected, immunoprecipitated with an anti-HA monoclonal antibody, separated by SDS–PAGE (10%), transferred to nitrocellulose, and probed with strepavidin–horseradish peroxidase as described previously (Kemble et al., 1992; Kemble et al., 1993). The marking between lanes 1 and 2 is due to nonspecific residue.

1990). Although we observed little (Figure 1A; wt-HA) or no (Figure 1B; BHA-PI) evidence for nonspecific R18 fluorescence dequenching, we confirmed the ability of BHA-PI to mediate outer leaflet lipid mixing with a phospholipid probe, rhodamine dipalmitoylphosphatidylethanolamine (Rh-DPPE). This probe is not subject to nonspecific membrane transfer (Nichols and Pagano, 1982). Rh-DPPE-labeled RBCs were bound to cells expressing either wt-HA or BHA-PI. Following a brief exposure to low pH and reculturing in neutral pH medium, the cells were observed by confocal microscopy. Rh-DPPE was transferred from RBCs to wt-HA- (Figure 4A) and to BHA-PI- (Figure 4B) expressing cells. As shown for the R18 fluorescence dequenching activity (Figure 1), the precursor (HA0) forms of wt-HA (Figure 4C) or BHA-PI (Figure 4D) failed to mediate phospholipid transfer. Hence, BHA-PI can promote the transfer of phospholipid probes from the outer leaflet of target membranes to the plasma membrane of BHA-PI-expressing cells.

Content Mixing Activity of GPI-Anchored HA

We employed RBCs preloaded with the small (MW ~500) soluble fluorescent probe, lucifer yellow (Spruce, et al., 1989), to monitor the content mixing activity of BHA-PI. Lucifer yellow–filled RBCs were bound to wt-HA- and BHA-PI-expressing cells. Following exposure to low pH and reculturing in normal medium, the cells were observed by

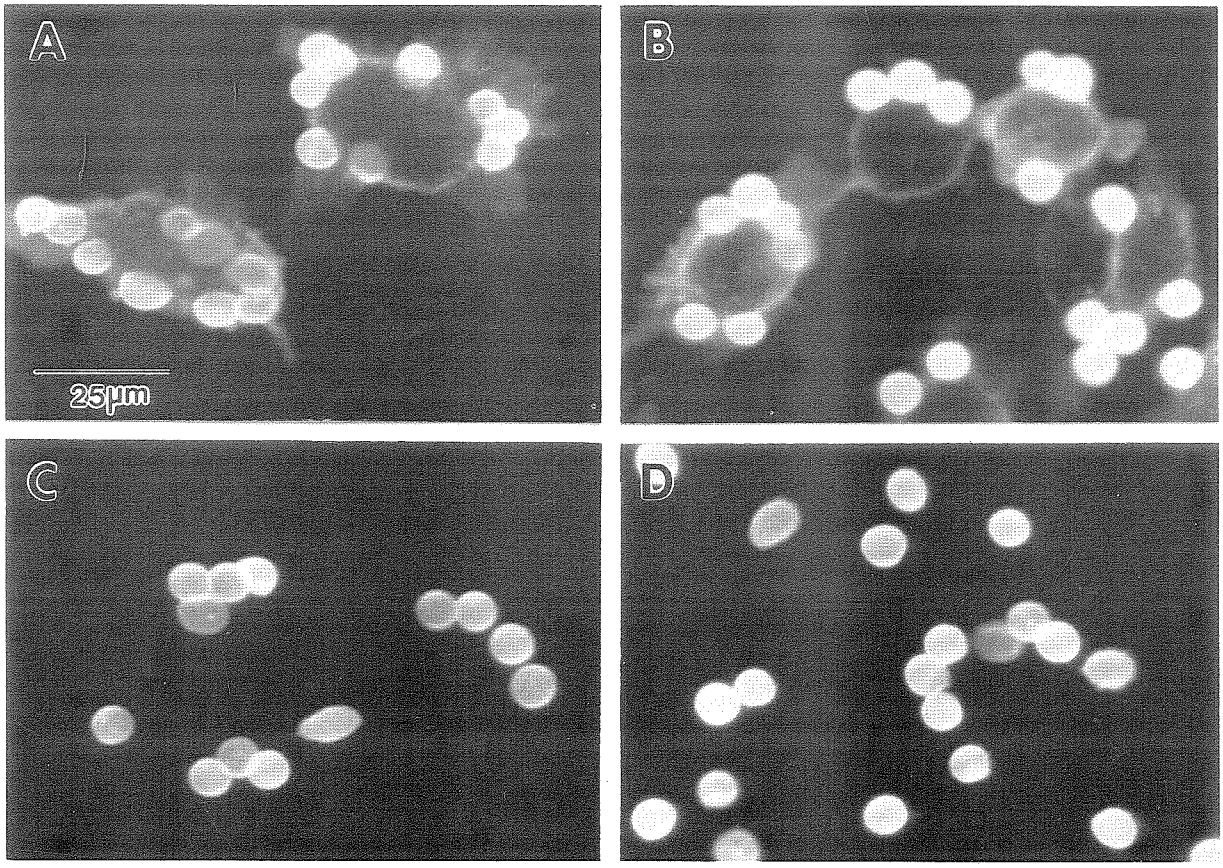


Figure 4. Lipid Mixing Activity of BHA-PI-Expressing Cells: Rh–DPPE Transfer

Cells expressing wt-HA (A and C) or BHA-PI (B and D) were treated with neuraminidase and either 5 μg/ml trypsin (A and B) or 5 μg/ml chymotrypsin (C and D) for 7 min at room temperature. After quenching with trypsin inhibitor, Rh–DPPE-labeled RBCs were bound. After exposure to pH 5.2 medium for 2 min at 37°C, the cells were returned to neutral pH medium at 37°C and then observed and photographed (within 10 min) with a confocal microscope.

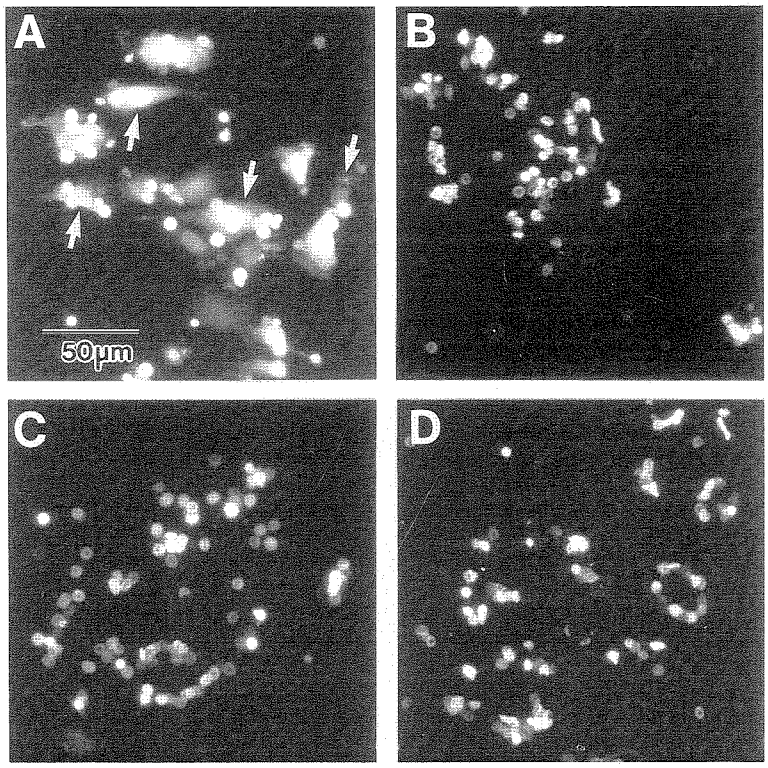


Figure 5. Content Mixing Activity of BHA-PI-Expressing Cells: Lucifer Yellow Transfer

Cells expressing wt-HA (A and C) or BHA-PI (B and D) were treated with neuraminidase and either 5 μg/ml trypsin (A and B) or 5 μg/ml chymotrypsin (C and D). After quenching, they were incubated with lucifer yellow–labeled RBCs. The pH of the media was lowered to pH 5.2 for 1 min at 37°C. Following incubation at 37°C for 30 min in neutral pH media, the cells were mounted and photographed with an IMT2 fluorescence microscope (Olympus Corporation, Lake Success, New York) using a fluorescein filter set. Arrows in (A) mark the location of several wt-HA-expressing cells that have become filled with lucifer yellow.

fluorescence microscopy. Lucifer yellow was transferred to the cytoplasm of wt-HA-expressing cells (Figure 5A). Approximately 90% of the wt-HA-expressing cells with bound RBCs became fluorescent. In striking contrast, lucifer yellow was not transferred to the cytoplasm of any BHA-PI-expressing cells (Figure 5B), even though similar numbers of RBCs bound. The fidelity of the lucifer yellow delivery assay was confirmed by demonstrating the absence of lucifer yellow delivery to the cytoplasm of wt-HA-expressing cells that had not been treated with trypsin to cleave the HA0 precursor (Figure 5C). Hence, even though BHA-PI can promote efficient transfer of outer leaflet lipids, it fails to promote transfer of cytoplasmic contents.

Phenotype of GPI-Anchored HA Is Due to the Lipid Anchor

The construction of BHA-PI added nine amino acids, from the GPI anchor addition signal, to the C-terminus of the BHA-PI ectodomain. To eliminate the possibility that the fusion phenotype of BHA-PI (lipid mixing without content mixing) was due to these nine amino acids, they were inserted into the analogous juxtamembrane region of wt-HA (see diagram in Experimental Procedures). The new construct and the protein expressed from it are referred to as wt-9. COS7 cells were transfected with plasmids encoding wt-HA, wt-9, or BHA-PI. Two days later, RBCs prelabeled with either R18 (Figure 6, left panels) or calcein AM (Figure 6, right panels), another small soluble fluorescent probe, were bound to HA-expressing cells. Cells were exposed to low pH for 2 min, recultured in normal medium, and then observed by confocal microscopy. Wt-9 expressing cells (Figures 6C and 6D) behaved identically to wt-HA-expressing cells (Figures 6A and 6B), exhibiting both R18 (Figure 6C) and calcein (Figure 6D) transfer. In sharp contrast, whereas BHA-PI mediated efficient R18 transfer (Figure 6E), it failed (Figure 6F) to transfer calcein from target RBCs. Calcein was not transferred to BHA-PI-expressing cells even if they were exposed to low pH or recultured in neutral pH medium for longer periods of time (T. D., unpublished data). Since wt-9 mediates transfer of soluble RBC contents, the inability of BHA-PI to mediate content transfer is not due to the nine amino acids appended from the lipid anchor addition signal, but rather to the lipid anchor itself.

Discussion

The results presented in this paper indicate that GPI-anchored HA promotes rapid and efficient mixing of lipids, but not soluble contents, from target membranes. We therefore conclude that GPI-anchored HA promotes hemifusion, but not complete fusion (Figure 7A). We believe that the hemifusion phenotype is due to the fact that GPI-anchored HA possesses a lipid anchor in place of its normal transmembrane domain rather than because of differences in its ectodomain or lack of a cytoplasmic tail. Support for our contention comes from the following observations. First, when grown in the presence of dMM (as done here), the ectodomain of GPI-anchored HA behaves virtually the same as the wild-type ectodomain; it forms a trimer that can bind RBCs, and, when exposed to low pH (0.2 pH units higher than for wt-HA), its ectodomain changes conformation and exposes its fusion peptides (Kemble et al., 1993). Second, the nine C-terminal amino

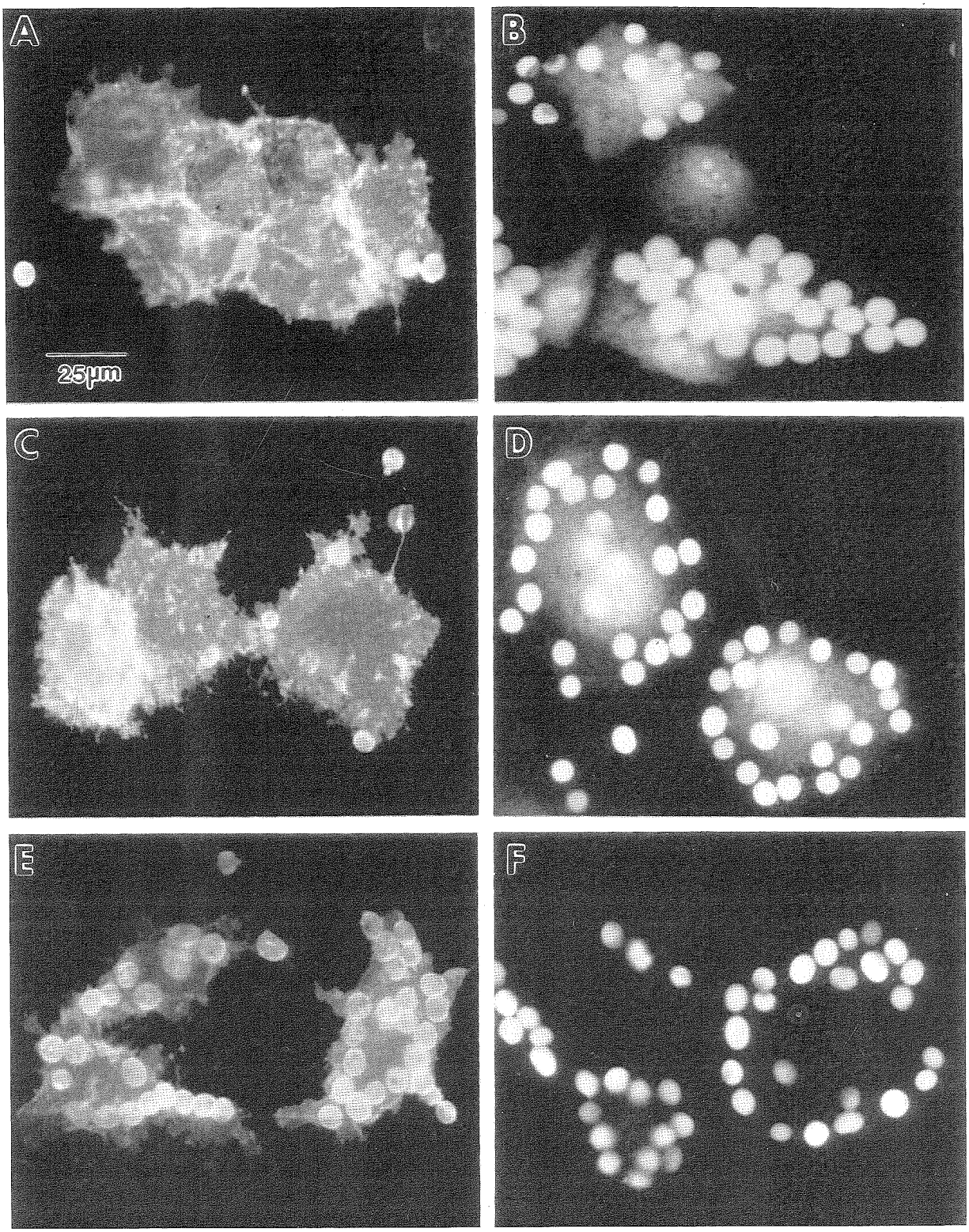


Figure 6. Lipid and Content Mixing Activities of wt-9 and BHA–PI

COS7 cells were transfected with plasmids encoding wt-HA (A and B), wt-9 (C and D), or BHA–PI (E and F). Two days later, when the cells were ~95% confluent, cells were washed, prepared for fusion as described in the legend to Figure 1, and then incubated with either R18-labeled (A, C, and E) or calcein AM–labeled (B, D, and F) RBCs. Binding, fusion, and observation by confocal microscopy were conducted as described in the legend to Figure 4. Photographs of the calcein AM samples were taken within 5–15 min and those of the R18 samples within 30–40 min following the low pH treatment. Nontrypsinized cells, which expressed the HA0 forms of wt-HA, wt-9, and BHA-PI, did not mediate transfer of either R18 or calcein, despite their ability to bind labeled RBCs (not shown).

acids that remain with the ectodomain of GPI-anchored HA, from the lipid anchor addition signal, do not confer the hemifusion phenotype (Figure 6). Third, chimeric HAs containing foreign ectodomain (6–12 amino acids) and/or transmembrane and cytoplasmic tail domain sequences bind to and fuse completely with target cells, as assessed by content mixing assays (Dong et al., 1992; Roth et al., 1986). Fourth, an HA that lacks a cytoplasmic tail mediates efficient cell–cell fusion (Simpson and Lamb, 1992).

In addition, we consider it unlikely that the inability of GPI-anchored HA to execute a complete fusion reaction is due to its sequestration in membrane microdomains (Brown and Rose, 1992; Edidin, 1992). This conclusion is based on the high and homogeneous lateral diffusion constant of GPI-anchored HA (Kemble, et al., 1993), its high and homogeneous percent mobile fraction (Kemble et al., 1993), and its homogeneous cell surface distribution (Y. Henis, unpublished data).

The major conclusion of this study, that GPI-anchored HA promotes hemifusion, but not complete fusion, has two important implications for the fusion mechanism of the influenza HA in particular and for membrane fusion proteins in general: HA-mediated fusion proceeds via a hemifusion intermediate, and the transmembrane domain is a critical element in the fusion process.

Hemifusion

Hemifusion is an intermediate on the pathway to complete fusion in which the outer, but not the inner, leaflets of two fusing membranes have merged, and there is not yet mixing of their aqueous contents (Figure 7A). Hemifusion has been detected in pure lipid systems (Ellens et al., 1985; Leventis et al., 1986) and during electrically induced fusion of RBCs (Song, et al., 1991). It has also been inferred from ultrastructural studies of biological fusion events (Chandler and Heuser, 1980; Kalderon and Gilula, 1979; Ornberg and Reese, 1981; Palade and Bruns, 1968; Pinto da Silva and Nogueira, 1977). The results presented in this paper provide the first evidence for hemifusion, as defined as lipid mixing in the absence of content mixing, in a well-characterized protein-mediated fusion system. We propose that the hemifusion state that we have detected with GPI-anchored HA (Figure 7B) represents an intermediate along the normal pathway of HA-mediated membrane fusion (Figure 7C).

Viral membrane fusion proteins possess two hydrophobic domains within a single polypeptide chain, a fusion peptide and a transmembrane domain. It has been proposed that the two hydrophobic domains enable the fusion protein to foster a dual membrane interaction, bringing the viral and target membranes into close contact and thereby facilitating their union (for reviews, see Stegmann et al., 1989; White, 1992). Models for HA-mediated fusion suggest that a ring of conformationally-altered HA trimers, with their fusion peptides exposed, surrounds the fusion junction (Bentz et al., 1990; Ellens et al., 1990; Stegmann et al., 1989; White, 1992). In the context of these models, we propose that the primary role of the fusion peptide

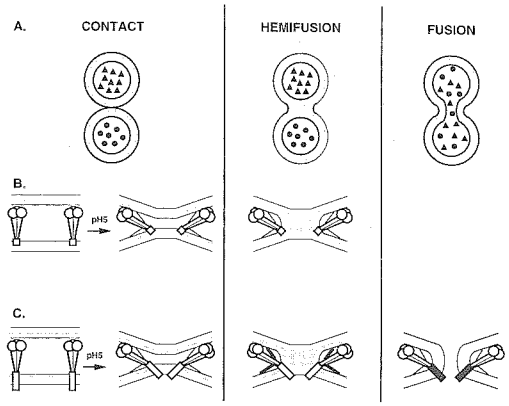


Figure 7. Membrane Fusion via a Hemifusion Intermediate

(A) Hemifusion in model systems. Two membrane-bounded vesicles contact (contact). Next, they form a hemifusion intermediate (hemifusion). In this state, lipids of the outer leaflets (stippled), but not the inner (white), mix. The aqueous contents (triangles and circles) remain separated. Next, the lipids of the inner leaflets mix and complete the fuison process (fusion), resulting in mixing of the aqueous contents. (B) and (C) represent enlargements of the contact areas (from [A], respectively) showing the proposed placement of HA trimers. (B) GPI-anchored HA promotes hemifusion. A ring of GPI-anchored HA trimers promotes contact and hemifusion with target membranes. GPI-anchored HA does not complete the fusion process; no aqueous connection is made between cells expressing GPI-anchored HA and target cells. (C) Model for wt-HA-mediated fusion through a hemifusion intermediate. As in (B), a ring of conformationally-altered HA trimers surrounds a nascent fusion pore in the contact area with the target membrane. The fusion peptide (thickened in hemifusion panel) is postulated to play its primary role in forming the hemifusion intermediate. A transmembrane domain (thickened in fusion panel) is required to complete the fusion process. After opening of the initial narrow fusion pore, the pore dilates (not shown). It will be interesting to determine where along this pathway, including the (unshown) stage of pore dilation, the proposed extended coil–coil conformation of the HA (Carr and Kim, 1993; P. A. Bullough, F. M. Hughson, J. J. Skehel, and D. C. Wiley, personal communication) is involved.

is to facilitate formation of the hemifusion intermediate (Figure 7C). Mutations that lower the hydrophobicity of the fusion peptide abolish the lipid mixing activity of HA (Gething et al., 1986; Guy et al., 1992; T. D., unpublished data). Similarly, preventing fusion peptide exposure abolishes the lipid mixing ability of HA (Godley et al., 1992; Kemble et al., 1992). The isolated HA ectodomain does not promote lipid mixing (Wharton et al., 1986), even though it can expose its fusion peptides (White and Wilson, 1987) and therefore bind, hydrophobically, to target membranes (Doms et al., 1985; Stegmann et al., 1990). Hence, a membrane anchor, be it lipid or protein, is required, in conjunction with an exposed functional fusion peptide, for HA to form a hemifusion intermediate.

Our model (Figure 7C) implies that the initial fusion pore contains lipids as well as HA protein. Another model, proposed by Almers and coworkers, suggests that the initial fusion pore is purely proteinaceous. The latter model was based on the observation that an electrical connection is made between HA-expressing cells and RBCs before the detection of lipid flux across the fusion junction (Tse et al., 1993). There are at least two possibilities to explain the apparent differences between our findings and those

of Tse et al. First, it may be extremely difficult to detect the earliest lipid molecules moving through the fusion pore. Second, with wt-HA, lipid motion in the initial fusion pore may be highly constrained (a possibility raised by Tse et al.) by the proteinaceous transmembrane domain, with significant lipid mixing first occurring upon pore dilation; the lipid anchor on GPI-anchored HA may relieve this constraint. Our model (Figure 7C) is in accord with a recent proposal by Fernandez and coworkers for the exocytic fusion pore. In the latter model, a scaffold of proteins surrounds the nascent lipid-containing exocytic fusion pore, and fusion proceeds through a hemifusion intermediate (Monck and Fernandez, 1992). Zimmerberg and colleagues (Curran et al., 1993) have also suggested the presence of lipids in early exocytic fusion pores.

Transient, narrow, and perhaps flickering (i.e., reversible) pores (Alvarez de Toledo et al., 1993; Spruce et al., 1991), which do not dilate and cannot pass detectable levels of small soluble dyes during time intervals sufficient for complete lipid mixing, may form between GPI-HA-expressing cells and RBCs. The formation of small transient pores would not alter our primary conclusion that fusion proceeds through a hemifusion intermediate. Future electrophysiological studies will address this possibility. Regardless of the outcome, since GPI-anchored HA promotes hemifusion, but not complete fusion, as defined by the observation of lipid mixing in the absence of content mixing (Figure 7A), it represents a powerful molecule for further biochemical, biophysical, and ultrastructural dissection of the fusion process.

Role of the Transmembrane Domain

The isolated HA ectodomain does not promote fusion (Wharton et al., 1986; White et al., 1982), although it undergoes the preparatory steps for fusion (Stegmann et al., 1989; White, 1992; Wiley and Skehel, 1987). Our results suggest that whereas a lipid anchor penetrating through only the outer leaflet is sufficient for HA to promote hemifusion, a transmembrane domain is required for HA to promote complete fusion, in other words, to form a stable fusion pore (Figure 7C) that can subsequently dilate. Although future work is necessary to define the requirements for a fusion-competent transmembrane domain, an initial comparison (not shown) of the transmembrane domain sequences of the influenza HA and other viral fusion proteins with those of other glycoproteins reveals no obvious amino acid requirements.

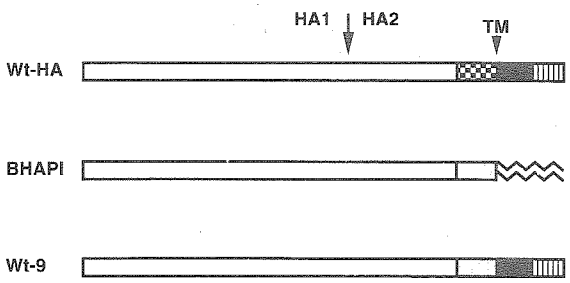
Be it sequence-specific or not, what might the role of a transmembrane domain be in forming a productive fusion pore? The transmembrane domain may stabilize a ring of HA trimers (Bentz et al., 1990; Ellens, et al., 1990; Stegmann et al., 1990; White, 1992) that surrounds the nascent fusion pore (Figure 7C), or it may be a structural element of the pore (Guy et al., 1992; White, 1992), or both. Alternatively, the transmembrane domain may lower the energy barrier to fusion by stabilizing a void in the hemifusion intermediate (Siegel, 1993) or by serving as a bilayer defect (Siegel, 1993).

In conclusion, our results with GPI-anchored HA strongly suggest that HA-mediated fusion proceeds through a hemifusion intermediate and that a lipid anchor (in conjunction with a fusion peptide) suffices for the HA ectodomain to induce hemifusion. Our results also suggest that a transmembrane domain is required to complete the fusion process. Since all known viral membrane fusion proteins (White, 1992) and one candidate cell–cell fusion protein (Blobel et al., 1992) are integral membrane proteins, the requirement for a proteinaceous transmembrane domain is likely generalizable to all proteins that promote fusion events where the ectoplasmic leaflets make initial contact (White, 1992). Moreover, given that cytoplasmically-oriented fusion events, notably regulated exocytosis, also proceed through fusion pores (Almers, 1990; Monck and Fernandez, 1992), and that the machinery involved is conserved through evolution (Schekman, 1992; Wilson et al., 1989) and used for many intracellular fusion events (Bennett et al., 1992; Rothman and Orci, 1992; Sollner et al., 1993), integral membrane proteins may be key players in all membrane fusion reactions.

Experimental Procedures

Mutagenesis

Plasmid pGPI-BHA (Kemble et al., 1993) was mutagenized with the oligo 5′-CAAAGGTCCGAATTCAGGAAGTGG-3′ to generate pGPI-BHA$_{k/s}$. This procedure substituted a serine for a lysine residue in the nine amino acids from the decay-accelerating factor GPI anchor addition signal that remain with the HA ectodomain (see Figure 1 in Kemble et al., 1993). The nucleotides encoding GPI-BHA$_{k/s}$ were excised from pGPI-BHA$_{k/s}$ by digestion with XbaI and EcoRV and ligated into SmaI- and XbaI-digested pEE14 to generate the plasmid pGSBHAPI$_{k/s}$. The plasmid pSMwt-9 was generated by mutagenizing pSMHA, a plasmid encoding wt-HA, with the oligo 5′-CAACCGGTTT-CAGATCAAAGGTCCGAATTCAGGAAGTGGAACCACTTCATGGAT-CCTGTGGATTTCCTTTGCC-3′. As diagrammed below, this procedure substituted the nine juxtamembrane (lumenal) amino acids of wt-HA with those that remain with BHA-PI from the lipid anchor addition signal. Symbols in the diagram indicate the following: checkerboard, nine C-terminal amino acids of wt-HA ectodomain; black, transmembrane domain of wt-HA; bars, cytoplasmic tail of wt-HA; gray, nine amino acids from GPI anchor addition sequence; zigzag, GPI lipid anchor.



Mutations were confirmed by sequence analysis.

Cells

The plasmid pGSBHAPI$_{k/s}$ was transfected into CHO-K1 cells. A cell line expressing high levels of BHA-PI was selected, by growth of cells in the presence of increasing concentrations of methionine sulfoximine (MSX; Sigma, St. Louis, Missouri), and subcloned as described previously (Kemble et al., 1993). Stable CHO cell lines expressing wt-HA and BHA-PI were grown in glutamine-deficient minimal essential media (Gibco–BRL, Gaithersburg, Maryland) containing 10% supplemented calf serum (SCS, Hyclone, Logan, Utah), 400 μM MSX, and 0.25 mM dMM (Calbiochem). dMM is required to manifest the receptor binding activity of GPI-anchored HAs (Kemble et al., 1993). For tran-

sient expression, COS7 cells, maintained in DME H-16 (University of California, San Francisco, Tissue Culture Facility) with 10% SCS, were transfected with 3 μg of plasmid DNAs encoding wt-HA, wt-9, or BHA-PI.

Membrane Fusion Assays

HA0-expressing cells were treated with 5 μg/ml TPCK–trypsin (Sigma, St. Louis, Missouri) to activate cell surface HA0 to its disulfide-linked HA1 and HA2 subunits, or with 5 μg/ml TLCK–chymotrypsin (Sigma, St. Louis, Missouri) to retain HA0 on the cell surface, in the presence of 0.2 mg/ml neuraminidase for 5 min at room temperature. A solution (50 μg/ml) of soybean trypsin inhibitor (STI; Sigma, St. Louis, Missouri) was then added and the cells washed two times with RPMI. Lipid mixing was measured quantitatively by the R18 fluorescence dequenching (FDQ) assay, essentially as described previously (Kemble et al., 1992). To measure the FDQ of PI-PLC-treated samples, cells were treated with trypsin to activate HA0, and then labeled RBCs were bound. After removing unbound RBCs, the cells were washed two times in PBS (containing 1 g/liter glucose) and then incubated with the indicated amount of PI-PLC (in PBS containing 1 g/liter glucose and 10 μg/ml BSA) for 60 min at 37°C. The RBC–cell complexes were then washed twice with PBS, 0.5 mM EDTA, 0.5 mM EGTA and removed from their dishes. FDQ was measured as described previously (Kemble et al., 1992). The initial rate of the reaction was obtained from the linear portion of the curve after subtraction of the rate of the reaction of chymotrypsin-treated (HA0) control samples.

Lipid mixing was also monitored with Rh-DPPE-labeled RBCs. Since Rh-DPPE cannot be incorporated into RBCs at quenching concentrations, transfer of Rh-DPPE to HA-expressing cells was observed by confocal microscopy. Rh-DPPE (Molecular Probes, Eugene, Oregon) was resuspended at 1 mg/ml in EtOH and added to a 1% suspension of RBCs at a final concentration of 1.5 μg/ml. Following incubation for 30 min at 37°C, RBCs were washed as above. HA0-expressing cells were treated with trypsin (or chymotrypsin) and neuraminidase as described above. Following a recovery period in 10% SCS for 10 min at room temperature, Rh-DPPE-labeled RBCs were added, incubated for 20 min at room temperature, and unbound RBCs were removed. The pH of the medium was lowered to pH 5.2 for 2 min at 37°C and then replaced with 37°C neutral pH medium. Cells were observed and photographed within ~10 min with a confocal microscope (Bio-Rad MRC600, Cambridge, Massachusetts).

Content mixing was assayed with RBCs preloaded with lucifer yellow as described previously (Kemble et al., 1992) or with calcein AM (Molecular Probes, Eugene, Oregon). For calcein labeling, washed RBCs were resuspended to a final concentration of 1% v/v in PBS. To 1 ml of this RBC suspension, calcein AM was added from a 10 mM stock in DMSO to a final concentration of 40 μM. The suspension was immediately vortexed and incubated at 37°C for 30 min. Cells were then washed twice with PBS and resuspended to a final concentration of 0.1% v/v. Binding and fusion to HA-expressing cells were then conducted as described previously (Kemble et al., 1992).

PI-PLC Treatment, Labeling of HA Proteins, and SDS Gel Analysis

Cell surface proteins of BHA-PI-expressing cells were biotinylated with NHS-LC-biotin (Pierce, Rockford, Illinois) as described previously (Kemble et al., 1992). The cells were treated with the indicated amount of PI-PLC for 60 min at 37°C as described above, and the HA was precipitated from the supernatant with an anti-HA monoclonal antibody, separated by SDS–PAGE, transferred to nitrocellulose, and probed with strepavidin–horseradish peroxidase as described previously (Kemble et al., 1992).

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