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Intermediates in influenza induced membrane fusion

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Our results show that the mechanism by which influenza virus fuses with target membranes involves sequential complex changes in the hemagglutinin (HA, the viral fusion protein) and in the contact site between virus and target membrane. To render individual steps amenable to study, we worked at 0°C which decreased the rate of fusion and increased the efficiency. The mechanism of fusion at 0°C and 37°C was similar. The process began with a conformational change in HA which exposed the fusion peptides but did not lead to dissociation of the tops of the ectodomain of the trimer. The change in the protein led to immediate hydrophobic attachment of the virus to the target liposomes. Attachment was followed by a lag period (4–8 min at 0°C, 0.6–2 s at 37°C) during which rearrangements occurred in the site of membrane contact between the virus and liposome. After a further series of changes the final bilayer merger took place. This final fusion event was not pH dependent. At 0°C efficient fusion occurred without dissociation of the top domains of the HA trimer, suggesting that a transient conformation of HA is responsible for fusion at physiological temperatures. The observations lead to a revised model for HA mediated fusion.

Key words: hemagglutinin/influenza/membrane fusion

Introduction

Membrane fusion events are involved in a multitude of vital cellular and physiological functions. Although some progress has been made in identifying proteins which are required for intracellular vesicular transport (Goud *et al.*, 1988; Malhotra *et al.*, 1989), the only proteins demonstrated to play a direct role in membrane fusion are the envelope proteins of animal viruses (for recent reviews see Ohki *et al.*, 1987; Marsh and Helenius, 1989; Stegmann *et al.*, 1989a; Wilschut and Hoekstra, 1990; Sowers, 1988; White, 1990). These proteins mediate fusion between the viral membrane and host cell membranes and thus allow the viral genome to enter the cytosol.

Influenza HA is the best characterized of the viral fusion factors. It is a homotrimeric integral membrane protein composed of three 84 kd subunits; each subunit in turn is composed of two disulfide bonded polypeptides, HA1 and HA2. The trimer forms a spike extending 135 Å from the viral membrane (Wilson *et al.*, 1981). HA mediated fusion takes place in endosomes (see Doms *et al.*, 1989), where it is triggered by a low pH dependent conformational change in HA (Skehel *et al.*, 1982; Doms and Helenius, 1986; White and Wilson, 1987). As a result of this conformational change the so-called 'fusion peptides', or the hydrophobic N-termini of the HA2 polypeptides, are exposed. In the X-ray structure of the neutral pH form, these peptides are located in the subunit interface at a distance of 35 Å from the viral membrane (Wilson *et al.*, 1981). Once exposed, the fusion peptides of bromelain solubilized ectodomains of HA have been shown to insert into the outer leaflet of a bilayer, adopting an α-helical configuration (Harter *et al.*, 1989; Brunner, 1989). Biochemical and morphological studies as well as analysis of fusion mutants have revealed extensive additional low pH induced changes in HA structure, including the dissociation of the top domains of HA1 (reviewed in Doms *et al.*, 1989; Wiley and Skehel, 1987). On the basis of these observations, we have proposed a model for fusion in which the exposure of the fusion peptides and the opening of the top domain are crucial features (see Doms and Helenius, 1987; Doms *et al.*, 1989; Stegmann *et al.*, 1989a).

In this study we analyzed the fusion reaction between influenza virus and liposomes (or erythrocyte ghosts) at reduced temperatures. We found that the reaction was dramatically slowed down at 0°C, and several kinetically and biochemically distinct steps could be detected. Most importantly, we observed that there is a 4–8 min long, low pH dependent lag before the onset of fusion during which a liposome–virus complex undergoes a series of complex changes. Further low pH dependent changes occur after the lag period, but the fusion event itself, i.e. the merger of membranes, proved to be pH independent. Our analysis of the conformational changes in HA indicated that most of the changes previously observed at higher temperatures, including the opening of the top of the trimer, are not only unnecessary but in fact inhibitory to fusion. The results provide the basis for a re-evaluation of our model for HA mediated membrane fusion.

Results

The temperature dependence of fusion

The fusion assay we used takes advantage of the resonance energy transfer between two fluorescent lipids (*N*-NBD-PE and *N*-Rh-PE) when they are both present in the target liposomes (Struck *et al.*, 1981). Fusion of the liposome with a virion causes dilution of the fluorescent lipids, resulting in decreased energy transfer and an increase in fluorescence emission from the energy donor, *N*-NBD-PE (Stegmann *et al.*, 1986). The target membranes used were large unilamellar liposomes prepared by extrusion (Mayer *et al.*, 1986). With a diameter of 0.1–0.2 μm, they were in the same size range as the virus particles. They were composed of natural zwitterionic phospholipids (PC and PE) with added gangliosides. While not required for fusion (White *et al.*,

1982; Stegmann *et al.*, 1989b), the gangliosides serve as receptors for the influenza virus HA so that virus–liposome complexes can form prior to fusion. To allow the formation of virus–liposome complexes, purified virus was incubated with liposomes at 0°C, at neutral pH and at a stoichiometry of one virus particle per liposome. Aliquots of the mixture were then injected into a thermostatted fluorometer cuvette containing buffers at different pH values.

A low pH dependent increase in fluorescence was observed both at 37°C and at 0°C (Figure 1A). To confirm that the increase in fluorescence was caused by fusion and not by other modes of lipid transfer between the membranes, virus alone was incubated at pH 5.0, 37°C and then added to liposomes at pH 5.1. Virus treated in this way remains capable of binding to ganglioside-containing liposomes but the fusion activity is completely abolished (Stegmann *et al.*, 1987). The lack of fluorescence increase at pH 7.4 and the lack of fluorescence increase in this control (Figure 1A) confirmed that the fluorescence increase at 37°C and 0°C was due to virus–liposome fusion.

That virus–liposome fusion was taking place at 0°C, could also be demonstrated directly by negative stain electron microscopy (Figure 1B). At neutral pH, complexes of virus and liposomes were seen (Figure 1B, panel a). Virus particles were readily distinguished from liposomes because of the spikes and their more electron-dense appearance. After incubation at pH 5.1, 0°C for 90 min, fusion products were observed (Figure 1B, panel b). They were larger than the original virus or liposomes. Spikes were present on their surface (arrows in Figure 1B).

At 37°C, fusion started immediately after acidification and was completed within 1–2 min (Figure 1A). At 0°C, there was a period of ~7 min during which no detectable fusion occurred, followed by fusion at a rate 40 times lower than at 37°C. The duration of the lag was highly reproducible between aliquots from the same preparation of virus, but it varied within a range of 4–8 min between virus preparations. The final increase in fluorescence was 12% at 37°C and 20% at 0°C (Figure 1A). These values corresponded to final fusion efficiencies of 24% and 40%, respectively, when calculated and corrected as outlined in Materials and methods. The lower overall efficiency of fusion at 37°C was probably due to the rapid, acid induced loss of viral fusion activity which is observed at 37°C (White *et al.*, 1982; Sato *et al.*, 1983; Stegmann *et al.*, 1987; Junankar and Cherry, 1986). Since such inactivation does not occur at 0°C (Stegmann *et al.*, 1987), virus remains fusion active for a longer period of time at 0°C and consequently more virus is able to fuse.

To obtain a more complete picture of the temperature dependence of fusion, we determined the initial rates of fluorescence increase and the lag times at different temperatures in the 0–37°C range. The rates of fluorescence increase were plotted in Arrhenius plots (Figure 2A), and it was found that the plot of the initial rates was linear (squared regression coefficient 0.98). The linearity indicated that the activation energy of the rate-limiting step in fusion was the same over the entire temperature range and thus suggested that the mechanism of fusion at these temperatures was the same. The lag times could only be measured with confidence in the 0–25°C range. Lag time was found to decrease steeply with increasing temperature (Figure 2B).

'Cold fusion' with erythrocyte ghosts

Before analyzing the fusion event at 0°C in further detail, it was important to determine whether fusion with natural membranes could also occur at reduced temperatures. To do so, we investigated the fusion of influenza virus with erythrocyte ghosts. The fusion assay used in these experiments employed the fluorescent fatty acid octadecylrhodamine (R18), which was incorporated into the viral membrane at a self-quenching concentration (Hoekstra

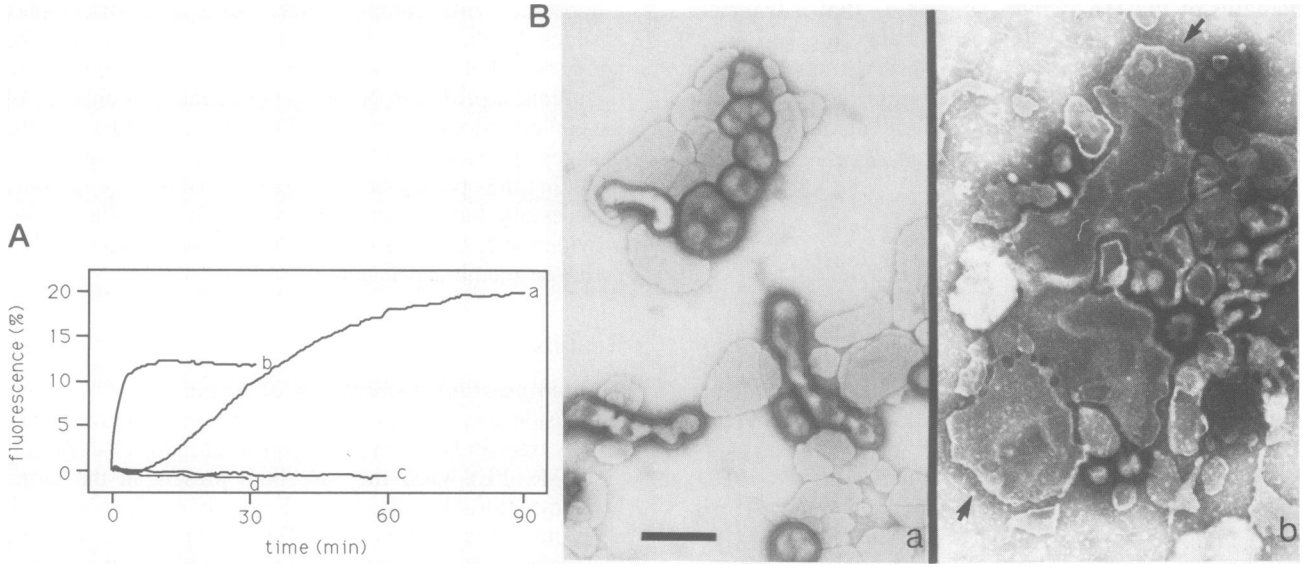

Fig. 1. Fusion of influenza virus, strain X-31, with liposomes. (**A**) Virus and liposomes were prebound in a small volume for 15 min at neutral pH at 0°C. The mixture was then injected into a cuvette at (**a**) pH 5.1, 0°C; (**b**) pH 5.1, 37°C; (**c**) pH 7.4, 0°C. In (**d**), virus was pretreated at pH 5, 37°C for 5 min, neutralized, bound to liposomes at 0°C for 15 min and the mixture was injected into a cuvette at pH 5.1, 0°C. Liposome to virus ratio was 1:1 (5 μM of membrane phospholipid phosphorous each). Liposomes consisted of egg PC: egg PE: bovine brain gangliosides 6:3:1 and trace amounts of *N*-NBD-PE and *N*-Rh-PE (0.6 mol% each). Fluorescence was measured as described in Materials and methods. (**B**) Direct visualization of fusion at 0°C by negative stain electron microscopy. Virus–liposome mixtures were either kept at pH 7.4, 0°C (panel **a**) or incubated at pH 5.1, 0°C for 90 min (panel **b**) after aggregation for 15 min at neutral pH, 0°C. Arrows indicate spikes on fusion product. Bar corresponds to 100 nm.

et al., 1984; Stegmann *et al.*, 1986). The labeled virus was allowed to bind to the ghosts on ice at neutral pH, and aliquots of the mixture were injected into cuvettes at the appropriate pH and temperatures.

We found that fusion occurred efficiently throughout the 0–37°C range. The Arrhenius plot of the rates of fluorescence increase is shown in Figure 2A. Again, the results indicate that the mechanism of fusion is similar at 0°C and 37°C. Overall, the fusion rates were higher than with liposomes, but the lag times were similar. Since the fluorescence increase was faster than with liposomes, lag times could be measured with precision even at elevated temperatures. A lag of 0.6 s could be observed at 37°C. By extrapolation, a similar lag (0.6–2 s) could be calculated for fusion with liposomes at 37°C. Taken together, the results indicated that the mechanism of influenza virus fusion with target membranes is similar at 0 and 37°C. The temperature dependence was similar whether liposomes or erythrocyte ghosts were used as target membranes. Furthermore, the results did not depend on the particular fusion assay used. However, the slope of the line in the Arrhenius plot for virus–liposome fusion was different from that measured with ghosts, indicating that the detailed interactions of virus with ghosts or liposomes might differ.

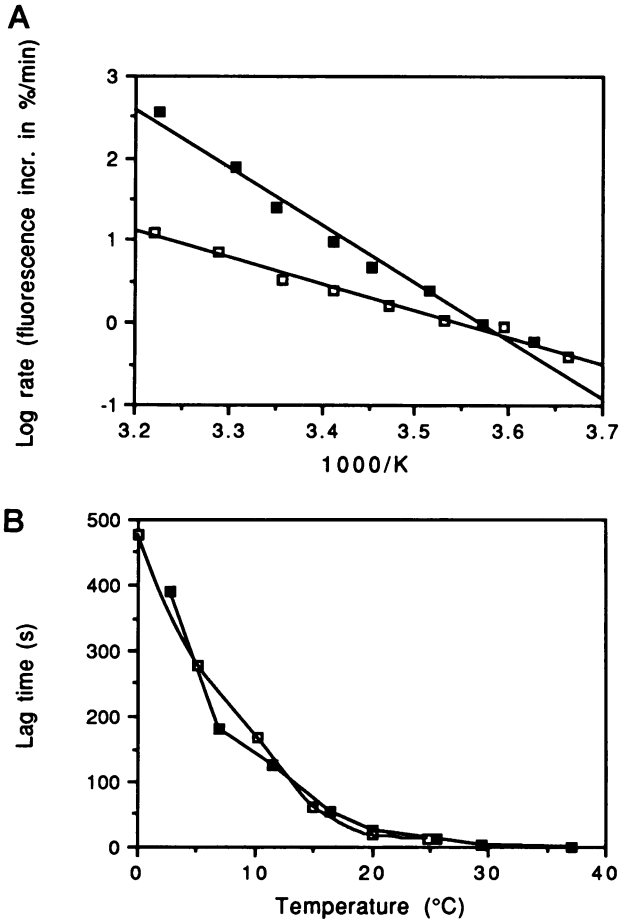

Fig. 2. Temperature dependence of the initial rates of fluorescence increase and the duration of the lag phase. (**A**) Arrhenius plots of the fusion of influenza virus with fluorescently labeled liposomes (open squares; at different temperatures but otherwise as in the legend to Figure 1) or erythrocyte ghosts (closed squares; as described below). The log of the initial rate of fluorescence increase (the slope of the steepest part of the fusion curve, after the lag phase) was plotted against the reciprocal of absolute temperature. Results shown are the averages of duplicates; the same preparation of virus was used in all experiments. Virus concentration 10 μg/ml; ghost concentration 100 μg/ml. Fluorescence was measured as described in Materials and methods. Data were fitted by linear regression, with squared regression coefficients of 0.98 for virus–liposome fusion and 0.99 for virus–ghost fusion. (**B**) Lag times of virus–liposome (open squares) or virus–ghost fusion (closed squares) as described above, at different temperatures.

The lag phase

The existence of a lag before the onset of fusion suggested that preparatory processes are needed before the actual merger of the two membranes takes place. To determine the molecular basis for the lag, we analyzed the properties of virus–liposome mixtures at 0°C using a variety of approaches.

Binding studies using a centrifugation assay (Stegmann *et al.*, 1987) indicated that attachment of virus to the liposomes could only account for a small fraction of the lag (Figure 3); one third of the virus was already bound to the liposomes prior to acidification, and the rest was bound within the first few seconds at pH 5.1 (half time < 15 s). The length of the lag was, moreover, independent of the total virus and liposome concentration over a 10-fold concentration range at a constant 1:1 virus to liposome ratio. This confirmed that the rate limiting events in the lag were not determined by mass action kinetic processes such as binding of the virus to liposomes. Rather, they involved processes subsequent to the binding, i.e. processes within individual virus–liposome complexes.

As expected, the rate of fluorescence increase after the lag depended on the ratio of virus to liposomes (not shown). The lag time was, however, independent of virus–liposome

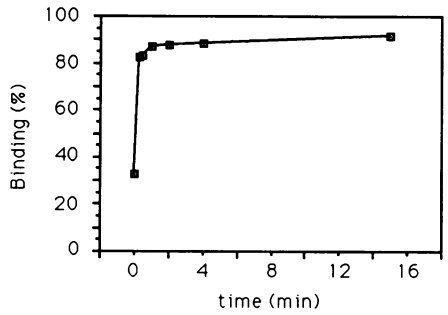

Fig. 3. Binding of ganglioside-containing liposomes to virus. Virus was incubated with liposomes (ratio, concentration and composition as in the legend to Figure 1) at pH 5.1, 0°C for various amounts of time and neutralized. The virus and virus–liposome complexes were then pelleted by centrifugation. Binding was determined as the amount of liposomes in the pellet relative to the total amount of liposomes, by measuring fluorescence in pellet and supernatant.

Table I. Duration of the lag phase preceding virus–liposome fusion at 0°C

Virus:liposome ratio	Lag time (min)
1:2	7.0
1:1	6.9
2:1	6.7
4:1	7.0

Concentration of liposomes was kept constant, while the amount of virus was varied. Liposome:virus ratio based on membrane phospholipid phosphorous; liposomes at 5 μM. Results shown are for one particular preparation of virus; variation between replicates within one preparation was ~0.4 min.

ratio at least in the 4:1−1:2 range (Table I). This confirmed that the duration of the lag was constant and uniform for all virus−liposome complexes. Apparently, every complex had to undergo similar changes during the lag phase, and these changes required a minimum time of 4−8 min.

Two additional observations confirmed that the lag phase was determined by events taking place in virus−liposome complexes and not in the virus alone. First, preincubation of virus alone at pH 5.1 on ice for up to 10 min prior to mixing with liposomes did not change the duration of the subsequent lag. Therefore, the lag started only upon addition of liposomes. Secondly, the lag time varied with the composition of the liposomes. With phosphatidylcholine/phosphatidylethanolamine (PC/PE) liposomes devoid of gangliosides, the lag was 30 min instead of 4−8 min (not shown). We found that this difference was not due to slower binding (see below). Apparently, the effect was specific for gangliosides, as inclusion of cholesterol in liposomal membranes containing either PC and PE or PC, PE and gangliosides had no additional effect on the duration of the lag or on the rate of fusion. The size of the liposomes was not critical; the same lag time was measured using vesicles with diameters in the 0.1−0.8 μm range.

Further experiments indicated that the events which determined the duration of the lag required low pH and were additive. When virus−liposome mixtures at 0°C were neutralized at any time during the lag, no fusion occurred even after 90 min. Upon re-acidification fusion began but only after a further lag (Figure 4). The total lag time, i.e. the sum of the lag times before and after neutralization, always equalled the lag observed in controls that had not been neutralized. The combined lag time did not depend on the amount of time the samples were incubated at neutral pH after neutralization, nor did it matter whether the interruption occurred early or late in the lag phase (Figure 4). These results indicated that the low pH dependent reactions which determine the length of the lag phase (and which were necessary for fusion) progressed steadily and additively during the lag phase.

While the duration of the lag phase was determined by low pH dependent steps, the rate with which fusion proceeded after the lag phase was affected by steps which did not require low pH. This is evident in Figure 4, which shows that fusion in samples that had undergone neutralization during the lag was 1.5−2 times faster upon re-acidification than was fusion in controls which had not been neutralized. Fusion was accelerated to the same extent whether the interruptions took place early or late in the lag period.

Taken together, these results indicated that the lag phase encompasses low pH dependent reactions in individual liposome−virus complexes. The reactions were continuous and additive. They were faster when the liposomes contained gangliosides. They probably represented a series of molecular rearrangements at the sites of virus−liposome contact, and constituted crucial preparatory events in fusion.

pH dependence of fusion at 0°C

To determine whether the lag was the only pH dependent step in fusion several experiments were performed. We first determined the overall pH dependence of virus−liposome fusion at 0°C, the rate of fusion at different pH values (Figure 5A) and the duration of the lag (Figure 5B). The apparent pH threshold for fusion was found to be 5.5 and the pH optimum was 5.1. The threshold pH for fusion was

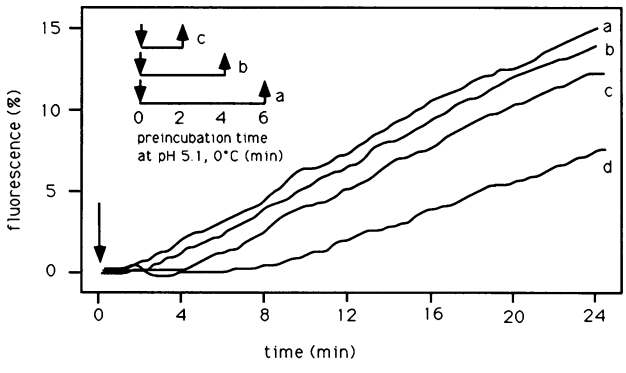

Fig. 4. Fusion of virus with liposomes after a preincubation of virus−liposome complexes at low pH. Virus and liposomes (ratio, composition and final concentration as in Figure 1) were incubated for 15 min in a concentrated suspension at pH 7.4, 0°C, acidified to pH 5.1, 0°C (arrow down in inset) and reneutralized after (**a**) 6 min; (**b**) 4 min; (**c**) 2 min (arrow up in inset) The sample in (**d**) was not acidified and served as a control. The mixtures were then transferred to a cuvette at a final pH of 5.1, 0°C. Tracings began the moment the mixtures were re-acidified (large arrow down).

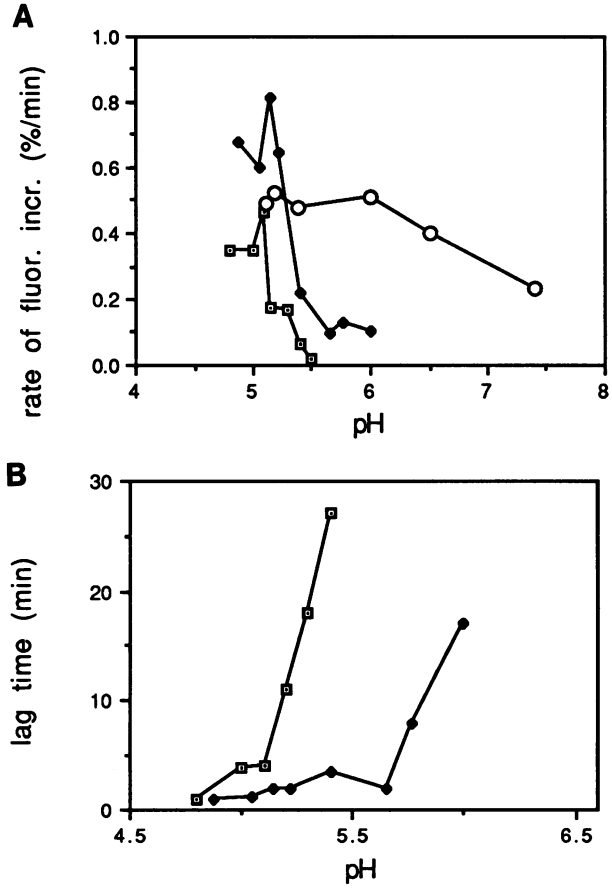

Fig. 5. pH dependence of fusion and lag time at 0°C. (**A**) Rate of fluorescence increase. (**B**) Duration of the lag phase. Open squares: fusion of virus and liposomes at different pH values at 0°C. Open circles: virus and liposomes were allowed to fuse at pH 5.1, 0°C for 25 min, neutralized and re-acidified to the pH indicated at 0°C. Closed diamonds: virus and liposomes were acidified to pH 5.1 for 2 min (half the duration of the lag phase for this preparation of virus), neutralized and then re-acidified to the pH indicated at 0°C. Other conditions as in Figure 1.

therefore 0.1–0.2 pH units lower at 0°C than at 37°C (Doms *et al.*, 1986). The length of the lag phase increased dramatically with increasing pH. While it was 4–8 min at pH 5.1, it was 30 min at pH 5.3, increasing monotonously with pH (Figure 5B).

Next, we assayed the pH dependence during different stages of the lag. Virus–liposome complexes were acidified to pH 5.1 at 0°C for 2 min (corresponding to half the duration of the lag phase for the virus preparation used), and then neutralized. Aliquots were then re-acidified to different pH values, and fusion was recorded. In agreement with our previous results (Figure 4), we found that fusion took place after a further lag (Figure 5B) and at a faster rate (Figure 5A) than without prior acidification. It was found, however, that the pH threshold for fusion had shifted from pH 5.5 to pH 6. This showed that while the lag was low pH dependent throughout, the overall pH dependence of the fusion reaction was determined by early events in the lag.

These results suggested that the fusion reaction itself might be pH independent. To obtain an estimate for the pH dependence of the final fusion reaction, we neutralized samples which had proceeded beyond the lag phase at pH 5.1 and 0°C and were actively fusing (Figure 6A). We found that, in contrast to our previous results at 37°C where fusion stopped immediately upon neutralization (Stegmann *et al.*, 1986), fusion at 0°C continued for 30 min. The rate was 1/5 to 1/2 of the original rate. Upon re-acidification, the remaining virus fused at a rate faster than the original rate and finally reached the same level as controls. These data indicated that virus can fuse with liposomes at neutral pH once the complex has undergone all the low pH requiring preparatory steps. At any given time after the end of the lag phase only a fraction of the virus has reached this state.

In further studies, it was found that the virus population which did not fuse after neutralization had undergone changes which allowed it to fuse at a higher pH. Virus neutralized during the fusion phase as in Figure 6A, was incubated at different pH values between 5.1 and neutral (Figure 6B). The lower the pH after re-acidification was made, the more virus was able to fuse. The rates of fusion, measured from the steepest part of the curves after re-acidification (as shown in Figure 6B) are plotted in Figure 5A. The pH optimum for fusion was found to be broadened so that considerable fusion occurred at pH values as high as pH 6.

Several conclusions can be drawn from these results, (i) While the events leading to fusion are low pH dependent, the final membrane merger is not. (ii) The step which requires the highest concentration of protons, and hence determines the overall pH dependence of fusion, occurs early in the lag phase. This step is most likely the initial low pH induced conformational change in HA. (iii) All fusion competent virus–liposome complexes undergo a series of low pH dependent changes during the lag phase, and the duration of these changes is constant. (iv) Thereafter, they undergo a further series of changes, occurring at a highly variable rate, including some which are not low pH dependent.

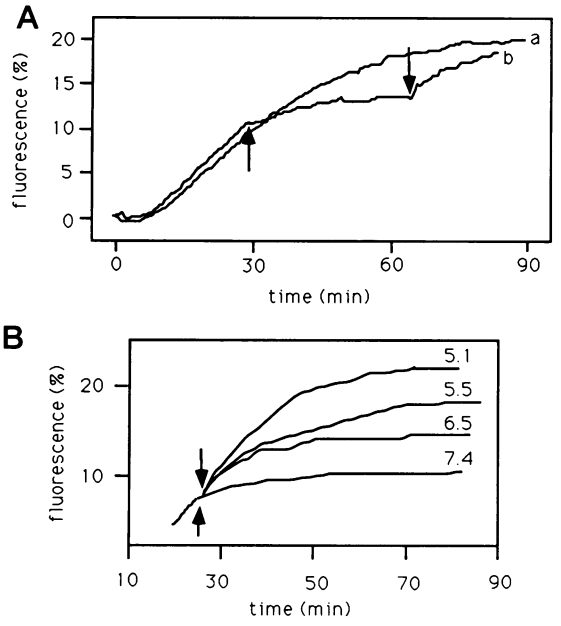

Fig. 6. Effect of neutralization and re-acidification during fusion at 0°C. (**A**) Virus–liposome complexes were neutralized during fusion at pH 5.1, 0°C (**b**, arrow up) and later re-acidified to pH 5.1 (**b**, arrow down). Curve (**a**) is a control fusing at pH 5.1, 0°C throughout. (**B**) Virus–liposome complexes were neutralized after 25 min (arrow up), and re-acidified to the pH indicated shortly thereafter (arrow down). Other conditions as in Figure 1.

Hydrophobic virus–liposome attachment

To analyze what was happening in the contact zone between the membranes, we characterized the mode of binding between virus and liposomes at reduced temperatures. We have previously shown that influenza A virus X-47 rapidly attaches to liposomes, consisting only of the zwitterionic phospholipids PC and PE, in a salt resistant manner at low pH and low temperature (Stegmann *et al.*, 1987). When the experiment was repeated with the strain used in this study, X-31, identical results were obtained. The virus and liposomes were found to associate with each other with a half time of < 15 s at pH 5.1 at 0°C (Figure 7). At neutral pH, no such binding took place. The pH dependence of the

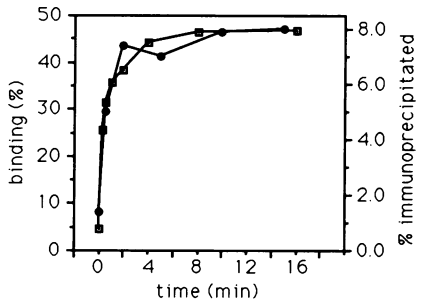

Fig. 7. Binding of zwitterionic liposomes to virus and exposure of the fusion peptide as measured by immunoprecipitation. Open squares: binding. Virus was incubated with liposomes (5 μM of membrane phospholipid phosphorous each; composition of the liposomes: egg PC: egg PE 2:1, and trace amounts of [^{3}H] cholesterol) at pH 5.1, 0°C for various amounts of time and neutralized. The virus and virus–liposome complexes were then pelleted by centrifugation and binding, measured as the amount of liposomes in the pellet relative to the total amount of liposomes, was determined by liquid scintillation counting. Closed circles: amount of HA immunoprecipitated by an antibody against the fusion peptide (see also text). A mixture of trace amounts of ^{35}S-labeled virus and liposomes were prebound for 15 min at neutral pH, subjected to pH 5.1, 0°C (concentrations and ratio as in Figure 1) for the times indicated, neutralized and then immunoprecipitated as in White and Wilson (1987) with antibody against the fusion peptide.

association was virtually identical to that of the overall fusion process. The attachment could not be reversed by neutralization or incubation with 1 M NaCl (not shown). Preincubation of the virus alone at pH 5.1, 0°C followed by neutralization and addition of liposomes at neutral pH also resulted in binding, but at a lower level. This indicated that the change in the virus that resulted in attachment was irreversible.

We assume that the binding is hydrophobic in nature and that it involves the hydrophobic fusion peptides which are irreversibly exposed under these conditions (see below). This mode of binding occurs during the first few seconds of the lag period, and it provides the starting point for further low pH dependent changes during the lag. It is important to note that this binding is different from the binding of virus to liposomes containing gangliosides at neutral pH (Figure 3). In that case, binding occurs via HA–receptor interactions, is not hydrophobic in nature and does not involve the fusion peptides. When acidified, the ganglioside-bound HA molecules apparently expose their fusion peptides, and attach via additional hydrophobic interactions.

The conformational changes in HA

To determine the conformational changes which occur in HA during fusion at low temperatures, we used conformation specific antibodies which had previously been employed to characterize low pH induced changes in BHA (the bromelain released soluble ectodomain of HA) and HA (White and Wilson, 1987; Copeland *et al.*, 1986; Daniels *et al.*, 1983; Webster *et al.*, 1983; Wilson *et al.*, 1984; Green *et al.*, 1982). The analysis was performed by quantitative immunoprecipitation. In each case the amount of radioactive HA precipitated was counted, and compared with the amount precipitated with conformation independent anti-HA antibodies. Non-specific control antibodies were used as background controls.

First we tested polyclonal antipeptide antibodies to the N-terminus of HA2 (the fusion peptide). Our previous studies at 25°C and 37°C have shown that these antibodies recognize exposed fusion peptides in low pH treated HA and BHA (White and Wilson, 1987). As shown in Figure 7, the amount of viral HA precipitated from virus–liposome mixtures at 0°C reached maximum values <1 min after acidification. Kinetically, the exposure of the antibody binding sites at 0°C coincided with the hydrophobic attachment of the virus to liposomes (Figure 7). The conformational change was not reversible after neutralization, and it was not dependent on the presence of liposomes (not shown). An antiserum to the C-terminal peptide of HA1 (White and Wilson, 1987) was also found to precipitate the HA after acidification of either virus–liposome mixtures or virus alone at 0°C (not shown). The C-terminus of HA1 is located in the stem of the trimer close to the fusion peptide (Wilson *et al.*, 1981).

The antipeptide antibodies thus indicated that viral HA undergoes a rapid, irreversible conformational change upon acidification at 0°C, and this change results in the exposure of the fusion peptides and a region of the C-terminus of HA1, both located in the stem. Experiments with BHA confirmed this conclusion, and furthermore, revealed that the protein becomes proteinase K sensitive with kinetics that coincide with this change (not shown).

Further quantitative immunoprecipitations with a panel of conformation specific monoclonal antibodies are summarized in Table II. The antigen used in this case was purified HA in the presence of non-ionic detergent. On the basis of their reactivity with BHA and detergent solubilized HA before and after acid treatment at room temperature or at 37°C, these antibodies have previously been classified as acid- or

Table II. Low pH induced conformational changes in HA

		Incubation	pH 7.4, 0°C	pH 5.1, 0°C			pH 5.1, 37°C	
			60 min	4 min	60 min	60 min	60 min	60 min
		Precipitation	pH 7.4, 0°C			pH 5.1, 0°C		pH 7.4, 0°C
			I	II	III	IV	V	VI
Antibody		Epitope						
Neutral specific antibodies								
N1	(1)	HA1 192 (B)	89.9	88.5	92.7	nd	nd	7.2
N2	(1)	HA1 192 (B)	101.7	93.0	92.7	nd	nd	5.2
HC-31	(2)	HA1 198	92.6	89.0	94.5	nd	nd	9.2
14-4	(3)	HA1 (B/D)	96.4	84.2	83.9	nd	nd	4.2
Acid specific antibodies								
A1	(1)	HA2	3.9	5.4	20.5	nd	0.5	98.3
A2	(1)	HA1	1.9	1.5	0.8	0	55.5	79.7
H26D08	(4)	HA1 98–106	0.4	0.5	0.5	0	29.0	64.4
88/1	(3)		0.4	4.6	20.0	21.6	10.6	47.0
Polyclonal rabbit antibody			100.8	96.2	103.9	88.5	42.8	77.4

Conformational changes in detergent solubilized, purified HA. The number in brackets denote the references (1) Copeland *et al.* (1986) (2) Daniels *et al.* (1983) (3) Webster *et al.* (1983) (4) Wilson *et al.* (1984). The letters in brackets denote epitopes as defined in Wiley *et al.* (1981). Antibodies N1 and N2 are specific for monomers of HA, A1 and A2 for trimers (Copeland *et al.*, 1986). H26D08 reacts specificially with an epitope in the trimer interface that appears after low pH induced dissociation of the tops of HA (Wilson *et al.*, 1984). The rabbit antibody sees both neutral and acid forms of HA. See also text. Immunoprecipitations performed at pH 5.1 were not completely quantitative; the results shown are for two rounds of immunoprecipitation. Antibody A1 did not recognize its epitope at low pH (column V) and was therefore not used at pH 5.1 (column IV). The nature of the experiment precluded testing of the low pH form with a neutral specific antibody at low pH.

neutral specific (White and Wilson, 1987; Copeland *et al.*, 1986; Daniels *et al.*, 1983; Webster *et al.*, 1983; Wilson *et al.*, 1984; Green *et al.*, 1982). Their conformation specificity was confirmed by the virtually complete reversal of the precipitation patterns before and after low pH treatment at 37°C in the presence of detergent (Table II, columns I and VI). However, after acidification at 0°C, few changes in the antigenic properties of HA were detected by these antibodies. Four neutral specific and two of the acid specific antibodies failed to detect any alterations in purified detergent solubilized HA after acidification and reneutralization at 0°C (Table II, columns II and III). To determine whether the antibodies detected changes during fusion, mixtures of virus, radioactive virus and liposomes (at the same concentrations as in the fusion experiments, cf. Figure 1) were prepared and incubated at 0 or 37°C. Samples were then neutralized, lysed with detergent and immunoprecipitated. The results were similar to those obtained with purified HA (Table II) i.e the same antibodies did not detect changes (not shown). Since most of the conformation dependent binding sites for these antibodies have been mapped to interfaces in the top domain of the HA trimer (binding sites B and D, see Wiley *et al.*, 1981), we conclude that major irreversible changes do not take place in the top domain during fusion at 0°C. Apparently, the tops do not open up.

Of the four acid specific monoclonal antibodies in Table II, only two, 88/1 and A1, detected changes. While the epitope for 88/1 has not been mapped, A1 binds to HA2 and is therefore located in the lower half of the molecule

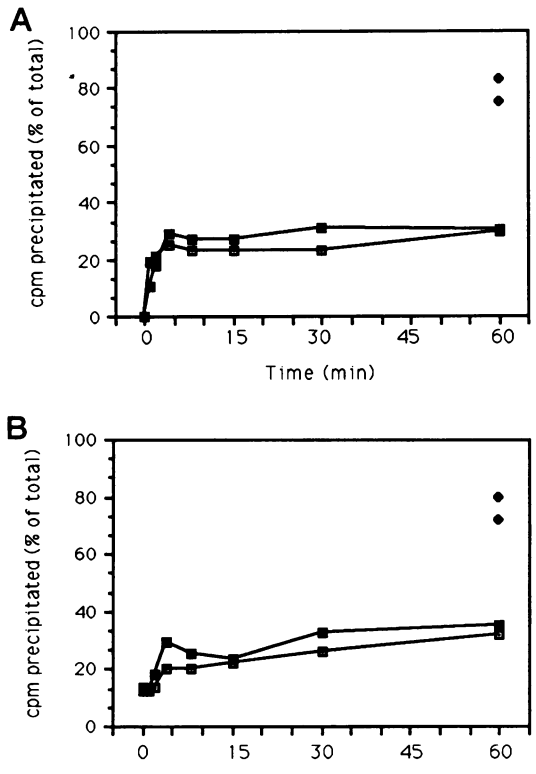

Fig. 8. Kinetics of exposure of the 88/1 and the A1 epitopes. A mixture of ^{35}S-labeled virus, egg-grown virus (open symbols) or ^{35}S-labeled virus, egg-grown virus and liposomes (closed symbols) were incubated at pH 5.1, 0°C (squares) or 37°C (diamonds) for the time indicated, neutralized, detergent-lysed, then immunoprecipitated and analyzed by liquid scintillation counting. **A**: 88/1 antibody (see also Table II) **B**: A1 antibody (see also Table II).

(Copeland *et al.*, 1986). With purified HA, the changes recognized by these antibodies were slow and incomplete (Table II). However, when virus−liposome complexes were acidified, faster changes were detected. Even then, the efficiency of precipitation at 0°C remained one third of that seen at 37°C (Figure 8A and B). The increase in precipitation by 88/1 occurred with kinetics identical to the exposure of the fusion peptide, and confirmed that an irreversible conformational change occurred at low pH. Interestingly, we found that the 88/1 antibody, at concentrations of ~5 μg/ml, was the only antibody in the panel of antibodies listed in Table II capable of inhibiting fusion without inhibiting binding of virus to liposomes.

The apparent lack of antigenic conversion at the top of the molecule at 0°C could either mean that the changes previously described at 37°C and room temperature did not occur at the low temperature, or that they occurred but were reversed upon reneutralization prior to immunoprecipitation. To determine whether reversible changes were occurring, immunoprecipitations with four acid specific monoclonal antibodies were performed directly at pH 5.1. First, as a control, HA was treated at pH 5.1, 37°C to induce the complete set of conformational changes, and precipitated at pH 5.1, 0°C. Three of the four acid specific antibodies (A2, 88/1 and H26D08) were able to recognize their epitopes at pH 5.1, albeit with a lower affinity (Table II, column V). Antibody A1 was not able to immunoprecipitate at low pH. When applied to samples that had acidified at 0°C, only one of the three, 88/1, detected its epitope (Table II, column IV). In conclusion, A2 and H26D08 recognized epitopes induced by conformational changes only at elevated temperature, while 88/1's epitope was exposed by conformational changes at high and low temperatures. This demonstrated that the conformational changes at 0°C were much less extensive than at higher temperatures, and not simply reversed by neutralization.

We concluded that fusion at 0°C occurred without the complete opening of the tops of the HA molecule and thus without exposure of the interface of the trimer. A conformational change, which exposed the fusion peptide and modified the antigenic properties of the stem of HA, was, however, required for fusion activity.

Discussion

Two major new insights into the mechanisms of HA mediated membrane fusion resulted from our study. First, we found that fusion is mediated by a conformational intermediate of the low pH treated HA, a form with exposed fusion peptides but without opened top domains. Second, we found evidence for a set of events that occur between attachment of virus to the target membrane and the final fusion event, suggesting complex rearrangements at the site of contact between the membranes.

Conflicting results have been obtained in the past concerning the ability of influenza virus to fuse at low temperatures. While White *et al.* (1982) and Wharton *et al.* (1986) reported that fusion could take place at 0°C, other workers had not seen it (Junankar and Cherry, 1986; Stegmann *et al.*, 1987). The issue is now resolved: not only is fusion possible at 0°C, it is actually more efficient than at 37°C. The negative reports in the literature are explained by the lag time before

the onset of fusion, which prevented the detection of fusion in short recordings. The linear relationship of fusion rate with temperature (when plotted in Arrhenius plots) suggests that the mechanism of fusion is similar at low and high temperatures.

Activation and inactivation of HA

After acidification, the first event that takes place is an irreversible conformational change in HA (Skehel *et al.*, 1982; Doms and Helenius, 1986; White and Wilson, 1987). It is now clear that this primary change, which leads to structural modifications mainly in the stem, is followed by a secondary conformational change at elevated temperatures. As a result of the primary change, the fusion peptides move out of their pockets in the subunit interface. With these peptides exposed, the HA becomes hydrophobic, and the virus binds hydrophobically to target membranes. Also as a result of the primary change, the trypsin cleavage site (between HA1 residues 27 and 28), located in the loop half way up the stem, is exposed (Wharton *et al.*, 1986). During the secondary change the top domains of the trimer dissociate from each other (White and Wilson, 1987). This change results in the opening of the top domains and an additional increase in protease sensitivity such as the trypsin cleavage site HA1 224–225 in the subunit interface in the top domain (Wharton *et al.*, 1986). Studies with the bromelain solubilized ectodomain of HA (BHA) indicate that the secondary change is considerably slower than the primary change (White and Wilson, 1987). The antigenic properties of the top domain are altered at this time, and the interactions between subunits of the trimer are weakened (see Doms and Helenius, 1988).

When we monitored the changes at 0 and 37°C using a panel of conformation specific antibodies (see Table II), binding assays and protease digestion, we found that only the primary change occurs at 0°C. Electron microscopic analysis of vitrified, unstained virus samples confirm this. No detectable changes in overall HA morphology are observed when virus is exposed to low pH at 0°C (Stegmann *et al.*, 1987), while gross conformational dissociation is readily demonstrated by this and other morphological techniques after 37°C incubation (White *et al.*, 1982; Stegmann *et al.*, 1987). Thus, the primary change is sufficient for HA's fusion activity, and HA trimers can mediate fusion without opening of the tops.

The result raises many questions. The most important question, which we will consider later, concerns the mechanism of fusion without trimer dissociation. Others concern the function of the secondary conformational change. What role does it have? How does it affect the fusion activity? In the light of our present data, we conclude that the secondary conformational change is not only unnecessary but most likely inhibitory for fusion. Most strains of influenza A virus rapidly lose fusion activity when incubated at low pH in the absence of target membranes (White *et al.*, 1982; Stegmann *et al.*, 1987; Sato *et al.*, 1983; Junankar and Cherry, 1986).

Concomitantly, the virus loses its ability to interact hydrophobically with the target membrane (Stegmann *et al.*, 1987). At 0°C, the secondary conformational change does not occur (Table II), as we have previously shown (Stegmann *et al.*, 1987) and inactivation does not take place. On the basis of these observations, it seems very likely that the final acid induced conformation of HA observed at physiological temperature is not fusion active. The strong correlation between inactivation and the secondary change argues against the formal possibility that fusion at 0°C is caused by a minor subpopulation of HA, undergoing both primary and secondary changes, and escaping detection by our antibodies. Fusion, therefore, appears to be mediated by a transient form of the protein which has undergone the primary change but not the secondary change. The published kinetics of virus inactivation at low pH indicates that the half time of functional intermediates at 37°C varies between virus strains (Scholtissek, 1985). It is 2–4 min for fowl plague virus (White *et al.*, 1982) and 30 s for X-31. Apparently, the A/Japan strain is not inactivated by low pH (Ellens *et al.*, 1990; Puri *et al.*, 1990).

What biological significance could a built-in deactivation process have? One possibility is that HA needs to be rendered sensitive to proteases after having performed its function in virus entry. After the primary conformational change, the protein is still resistant to a variety of proteases. After the secondary change, it aggregates (Junankar and Cherry, 1986) and becomes quite protease sensitive. This may be important in preventing its recycling from the endosomes to the surface and thus its detection on the cell surface by the immune surveillance of the host. Our studies have shown that the HA of the incoming virus is, indeed, rapidly degraded in lysosomes of CHO cells (Martin and Helenius, 1991).

Events during the lag

The primary conformational change in HA occurs within 15 s after acidification. Regardless of temperature, it is followed instantaneously by salt resistant attachment of the virus to target membranes (Figure 7). The attachment is most likely mediated by the insertion of the exposed fusion peptides of HA into the target bilayer. Recent labeling studies with photo-activatable lipid analogues indicate that the fusion peptides of BHA (isolated HA ectodomains) insert as amphiphilic α-helices into the outer leaflet of liposomal membranes (Brunner, 1989; Harter *et al.*, 1989).

The next detectable event is fusion between the target membrane and the viral membrane. It occurs after a lag period which ranges between 0.6 s and 30 min depending on temperature, pH and lipid composition. At low temperature, where the lag ranges between 4 and 30 min, our results show that the lag involves a series of changes in the virus–target membrane complex. A lag phase has previously been described for fusion of HA-expressing fibroblasts with red cell ghosts (Morris *et al.*, 1989; Sarkar *et al.*, 1989). Using stopped-flow kinetic measurements, a lag was also recently observed for another low pH triggered virus, vesicular stomatitis virus (Clague *et al.*, 1990) suggesting that it may be a general feature in virus fusion. The reason why it has not been routinely observed before is that it is very short at physiological temperature.

Our studies show that the events occurring during the lag are continuous, additive and irreversible. Throughout the lag, the pH must be low, but the pH requirement does not remain constant. The step that requires the lowest pH in the entire fusion reaction, thus determining the overall pH dependence, is probably the primary conformational change in HA. During the lag, the pH required keeps rising. Since the lag time is also determined by the lipid composition, and

since it is initiated only upon addition of target membranes to acidified virus, we can assume that the key reactions take place in the contact sites between the two membranes.

Our antibody probes did not detect changes in HA beyond the initial primary alteration during or after the lag at 0°C. We therefore suspect that the lag is not dependent on major intramolecular changes in HA. To explain the lag, we favor the possibility that it reflects the time needed for lateral movement and reorganization of the HA molecules in the contact zone. Such rearrangement could, for instance, be required for the formation of a multimeric fusion complex. Several independent reports have suggested that HA mediated fusion may require more than one trimeric spike (Doms and Helenius, 1986; Sarkar *et al.*, 1989; Morris *et al.*, 1989; Ellens *et al.*, 1990), and rosette shaped complexes of HA have been observed in acidified virus (Doms and Helenius, 1986).

The fusion phase

The lag is followed by a fusion phase, during which further changes in the virus−liposome interface must be occurring, culminating in the merger of the lipid bilayers. The rate of fusion during this phase depended on temperature, pH and the composition of the target membrane. The main difference with the lag phase was that only some of the changes were low pH dependent; fusion continued for some time even if the sample was neutralized. Thus, while the reactions that lead up to the fusion competent complex are low pH dependent, the final fusion event itself is not.

Given the limited information available on the role of the membrane lipids in the fusion reaction, it is difficult to envisage what exactly occurs during the final fusion step. Freeze fracture studies indicate a local point mechanism (Burger *et al.*, 1988), and the initial formation of small fusion pores has recently been described (Spruce *et al.*, 1989). It is possible that fusion occurs at the center of the HA complex and this is where the initial pore opens up.

A revised model for fusion

Our results provide the basis for a revised fusion model which encompasses five operationally distinct steps, as shown in Figure 9 (B−F). The first is the primary conformational change in HA (Figure 9b). The fusion peptides are exposed without a major, permanent change in the top domain. [Minor alterations throughout the molecule including the tops are likely, however, since the change in the stem is quite dramatic and point mutations in the top domain are known to affect the threshold pH of fusion (reviewed by Wiley and Skehel, 1987)]. The exposure of the fusion peptides leads to hydrophobic attachment of some of the HA molecules to the external bilayer leaflet of the target membrane. Since the ectodomains of HA trimers do not dissociate, it is difficult to envisage how the fusion peptides (which are located close to the viral membrane) can reach the target membrane. To achieve contact the HA trimers may tilt upon contact with the target membrane as shown in Figure 9c. This would bring at least one of the fusion peptides of each trimer into contact with the target membrane and allow insertion. The other fusion peptide(s) of the trimer may interact with the viral membrane as has been proposed previously (Skehel *et al.*, 1982). Attachment of the fusion peptides to the external bilayer leaflet of the target membrane would lead to a stable, salt resistant complex, which is one of the intermediates that we could readily see at low temperature.

Without major additional conformational changes, the HA molecules located in the site of attachment between the

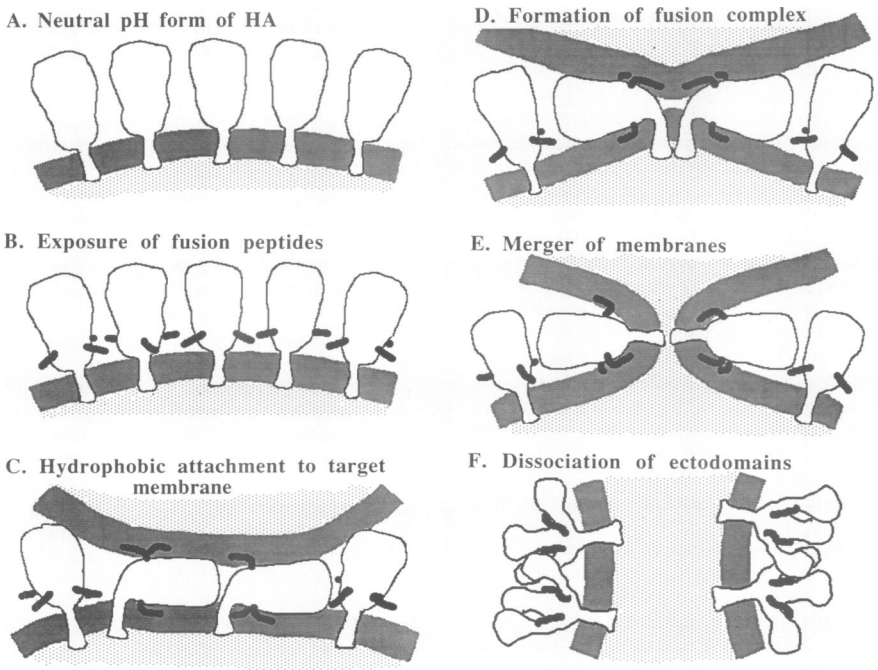

Fig. 9. Schematic model for the conformational changes in HA and the molecular rearrangements in the site of fusion. When combined with the results from previous studies, our results suggest that fusion proceeds in five steps. (**A**) Neutral form of HA. (**B**) The primary conformational change in HA. (**C**) The attachment to the target membrane via the exposed fusion peptides. (**D** The rearrangement of HA trimers in the site of contact between the membranes. (**E**) A period of variable length after which bilayer merger occurs. (**F**) After fusion at 37°C, the HA undergoes secondary conformational changes during which the top domains are dissociated.

membranes may next undergo lateral rearrangement to form a fusion complex. On the basis of electron microscopic data (Doms and Helenius, 1986), we picture it to be a rosette-like structure with three or more HA trimers (Figure 9d). The final fusion reaction may now take place (Figure 9e) resulting in lipid mixing and the opening of a narrow fusion channel. The HA molecules may finally undergo the secondary conformational change (Figure 9f) which—in the cellular context of virus entry—could prepare the HA for rapid degradation.

Materials and methods

Chemicals

N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (*N*-Rh-PE) and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE), egg phosphatidylethanolamine (PE) and egg phosphatidylcholine (PC) from Avanti Polar Lipids (Birmingham, AL) were used without further purification. Cholesterol, bovine brain gangliosides (type III), TPCK-treated trypsin, HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] and sepharose coupled *Ricinus communis* agglutinin were purchased from Sigma (St Louis, MO), octadecyl rhodamine B chloride (R18) from Molecular Probes (Junction City, OR), 2-(4-morpholino)-ethanesulfonic acid (MES) and Tris−HCl from Fisher (Fairlawn, NJ). Goat anti-mouse IgG was purchased from TAGO (Burlingame, CA), scintillation fluid (Opti-fluor) from Packard (Drowners Grove, IL), [1α,2α(*n*)-^{3}H]cholesterol and [^{35}S]methionine (Trans-label) from Amersham (Arlington Heights, IL). Heat-killed *Staphylococcus aureus* (Zysorbin) was obtained from Zymed Laboratories Inc. (San Francisco, CA) and purified by two washes in 20 mM MES, 30 mM Tris, 100 mM NaCl, 0.5% TX-100, pelleting the bacteria at 1500 *g* for 4 min.

Virus purified HA, erythrocyte ghosts and liposomes

The X-31 recombinant strain of influenza virus was propagated from a single plaque (C-22; Doms *et al.*, 1986) in the allantoic cavity of embryonated eggs, purified, handled and stored essentially as described before (Stegmann *et al.*, 1985). Viral phospholipid was extracted according to Folch *et al.* (1957) after which phosphate was determined according to Böttcher *et al.* (1961). For the production of radioactive virus and HA, confluent monolayers of MDCK cells were infected with virus at high m.o.i. (10−30) for 1 h at 37°C in Dulbecco's Modified Eagles Minimal Essential Medium (DMEM). The monolayers were then supplemented with DMEM containing 10% fetal calf serum and incubated at 37°C for 3 h. The monolayers were then washed and the medium was replaced with methionine-free DMEM, to which 1 mCi of [^{35}S]methionine was added per 150 cm^2 flask. Virus production was allowed to continue for 6 h at 37°C. TPCK-treated trypsin was than added (5 μg/ml) and the culture was incubated for another 8 h at 37°C. For radioactive virus, the medium was harvested, spun twice at 2000 *g* for 5 min and the supernatant was loaded on a discontinuous sucrose gradient (3 ml 60% sucrose, 20 ml 30% sucrose, in 145 mM NaCl, 2.5 mM HEPES) in an SW 28 rotor and spun at 25 000 r.p.m. for 90 min. The interface between the sucrose layers was collected and used as a source of radioactive virus. For HA, virus infected MDCK monolayers were labeled as described above, trypsin activated as above, and then the cells were collected by centifugation at 2000 *g*, lysed with 0.5% TX-100, 0.5 M KCl, 20 mM MES, 30 mM Tris−HCl pH 7.4, 25 μg/ml soybean trypsin inhibitor and phenylmethylsulfonylfluoride. Nuclei were pelleted at 12 000 *g* for 10 min, the supernatant was loaded onto a 1 ml column containing *R.communis* agglutinin coupled to Sepharose and the column was washed with 40 ml of 145 mM NaCl, 2.5 mM HEPES pH 7.4. HA was eluted off the column with 0.2 M D(+) galactose, 145 mM NaCl, 2.5 mM HEPES pH 7.4 and aliquots were quickly frozen in liquid nitrogen and stored at −70°C.

Large unilamellar liposomes were prepared by repeated low-pressure extrusion of multilamellar liposomes through defined pore polycarbonate filters, 0.2 μm in diameter, according to Mayer *et al.* (1986). Multilamellar liposomes were frozen and thawed three to five times before extrusion. After extrusion, residual multilameller liposomes were removed by centrifugation at 16 000 *g* for 20 min and the radioactivity in pellet and supernatant was determined. Phospholipid phosphate was determined according to Böttcher *et al.* (1961). Erythrocyte ghosts were prepared as in Steck and Kant (1974) with minor modifications (Stegmann *et al.*, 1986).

Fusion and binding experiments

For the resonance energy transfer assay, 0.6 mol% of *N*-NBD-PE and *N*-Rh-PE was incorporated into liposomes (Struck *et al.*, 1981). Measurements were carried out under continuous stirring, in a thermostatted cuvette holder in 2 ml (final volume) of 135 mM NaCl, 15 mM sodium citrate, 10 mM MES, 5 mM HEPES set to various pH values by HCl or NaOH. Temperatures were maintained within 0.1°C and pH within 0.05 pH units. The increase in fluorescence, resulting from dilution of the fluorophores into the viral membrane upon fusion, was recorded continuously at excitation and emission wavelengths of 465 and 530 nm, respectively. A 515 nm long-pass filter was placed between cuvette and emission monochromator (Stegmann *et al.*, 1985). For the R18 assay, virus was labeled with R18 as described earlier (Stegmann *et al.*, 1986) and injected into a cuvette containing erythrocyte ghosts and a buffer as described above. Fluorescence dequenching resulting from probe dilution was monitored continuously at excitation and emission wavelengths of 560 and 590 nm, respectively.

An SLM-8000 fluorometer was used for all measurements. For calibration of the fluorescence scale, the initial residual fluorescence of the liposomes or the labeled virus was set to zero and the fluorescence at infinite probe dilution at 100%. The latter value was obtained after addition of TX-100 (0.5% v/v) to the liposomes and the fluorescence intensity was then corrected for sample dilution and in the case of the resonance energy transfer assay also for the effect of TX-100 on the quantum yield of *N*-NBD-PE (Struck *et al.*, 1981). Initial rates of fluorescence increase were measured as the slope of the fusion curve, immediately after the lag phase. For calculations of the final extent of fusion, it was assumed that viral envelopes and liposomes are of equal size and that, as was found previously (Stegmann *et al.*, 1985, 1989b), at a one to one ratio of virus to liposomes one virus particle fuses with one liposome. For fusion drawings, curves were manually traced from the original stripchart recordings using a digitizer pad (Kurta, Phoenix, AR) and a drawing program (Canvas, Deneba Software, Miami, FL). In either assay, at these concentrations of the fluorescent probes, the fluorescence increases linearly with probe dilution (Hoekstra *et al.*, 1984; Struck *et al.*, 1981). Experiments involving low pH preincubation were performed in a concentrated suspension (100−150 μl). The pH was adjusted by the addition of 1 M HEPES pH 7.8 or 1 M sodium citrate pH 4.0. Temperature was maintained within 0.5°C in these experiments.

For binding experiments, virus was added to liposomes, containing trace amounts of [^{3}H]cholesteryloleate, at a 1:1 (phospholipid phosphate) ratio in buffers at temperatures and pH as described above for fusion experiments. After incubation and if necessary, neutralization by the addition of 1 M HEPES, the mixture was centrifuged at 0°C for 20 min at 16 000 *g*. In the absence of liposomes, ~90% of the virus pellets under these conditions.

Immunoprecipitations

For immunoprecipitations, immune complexes were preformed by incubating 50 μl of a 10% (v/v) slurry of washed, killed *S.aureus* bacteria in 20 mM MES, 30 mM Tris, 100 mM NACl (MNT), pH 7.4 containing 0.1% TX-100 with 5 μl of a 2 mg/ml solution of goat anti-mouse IgG for 1 h at 0°C with continuous agitation. Next, aliquots of mouse monoclonal antibodies from different sources (White and Wilson, 1987; Copeland *et al.*, 1986; Daniels *et al.*, 1983; Webster *et al.*, 1983; Wilson *et al.*, 1984; Green *et al.*, 1982), were added to the mixture and incubated for another hour with continuous agitation. Mixtures were washed with MNT containing 0.5% TX-100, pelleted for 4 min at 1500 *g*, resuspended and added to the purified HA or virus solution, either in MNT (plus 0.1% TX-100 and 0.25% BSA) or in 135 mM NaCl, 15 mM sodium citrate, 10 mM MES, 5 mM HEPES, 0.1% TX, 0.25% BSA pH 7.4 or 5.1) and incubated for 1 h with vigorous agitation. Complexes were then washed three times by pelleting as above and resuspending in 1 ml of buffer as described above, after which pellets were counted in a liquid scintillation counter or loaded on gels. For experiments involving virus, trace amounts of ^{35}S-labeled virus were mixed with egg-grown virus and liposomes prior to acidification, neutralization and the addition of TX-100 to 0.1% final, at concentrations comparable with those used in the fusion experiments.

Electron microscopy

For electron microscopic evaluation of fusion, liposomes and virus were incubated at neutral pH, 0°C for 15 min. For some samples, the pH was then lowered to 5.1, 0°C. Samples were applied to Formvar/carbon coated copper grids which had been freshly glow-discharged, stained with 1% phosphotungsten acid, pH 7.4 and viewed in a Philips 301 electron microscope, operating at 80 kV.

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