Evolution of Intermediates of Influenza Virus Hemagglutinin-Mediated Fusion Revealed by Kinetic Measurements of Pore Formation

Ruben M. Markosyan, Grigory B. Melikyan, and Fredric S. Cohen
Department of Molecular Biophysics and Physiology, Rush Medical College, Chicago, Illinois 60612 USA

ABSTRACT Cells expressing wild-type influenza virus hemagglutinin (HA) or HA with a point mutation within the transmembrane domain (G520L) were bound to red blood cells and exposed to low pH for short times at suboptimal temperatures followed by reneutralization. This produced intermediate states of fusion. The ability of intermediate states to proceed on to fusion when temperature was raised was compared kinetically. In general, for wild-type HA, fusion occurred more quickly by directly lowering pH at 37°C in the bound state than by raising temperature at the intermediate stage. When pH was lowered for 1–2 min, kinetics of fusion upon raising temperature of an intermediate slowed the longer the intermediate was maintained at neutral pH. But for a more sustained (10 min) acidification, kinetics was independent of the time the intermediate was held at neutral pH before triggering fusion by raising temperature. In contrast, generating intermediates in the same way with G520L yielded kinetics of fusion that did not depend on the time intermediates were maintained after reneutralization. For both HA and G520L, the extents of fusion did not depend on the temperature at which pH was lowered, but fusion from the intermediate was extremely sensitive to the temperature to which the cells were raised. The measured kinetics and temperature dependencies suggest that the rate-limiting step of fusion occurs subsequent to formation of any of the intermediates; the conformational change of HA into its final configuration may be the rate-limiting step.

INTRODUCTION

The formation of a fusion pore is a complex and multi-step process in which proteins and lipids undergo a series of rearrangements. If the structure of the fusion protein could be determined at stages that are intermediate to binding and fusion, and the status of the membranes themselves at these stages identified, the mechanism of fusion would become significantly clearer. Experimentally, states that are partway toward fusion have been captured by employing suboptimal conditions. Conditions such as temperature, pH, density of fusion proteins, and lipid composition can be made suboptimal. States captured in this manner are called intermediates. Intermediates have been identified for the fusion proteins of influenza (Chernomordik et al., 1997, 1998; Melikyan et al., 1999, 2000c; Schoch et al., 1992), HIV (Hart et al., 1996; Melikyan et al., 2000b), vesicular stomatitis virus (Pak et al., 1997), rabies (Gaudin, 2000), baculovirus (Chernomordik et al., 1995; Kingsley et al., 1999), and other viruses.

When lipid dye spreads from red blood cells (RBCs) to HA- (or mutant HA)-expressing cells without pore formation, hemifusion has clearly taken place, but it has been found that fusion does not subsequently occur (Chernomordik et al., 1998; Kemble et al., 1994; LeDuc et al., 2000; Leikina and Chernomordik, 2000; Markosyan et al., 2000; Melikyan et al., 1995, 1997a; Qiao et al., 1999). Hemifusion is termed end-state if it does not lead to full fusion. Adding chlorpromazine (CPZ) (Melikyan et al., 1997a) or osmotically swelling cells (Melikyan et al., 1995) induces aqueous dye spread from end-state hemifusion.

Acidic pH is the trigger for HA-mediated fusion. By lowering pH at low temperatures, states have been captured in which lipid dye has not spread, but subsequent raising of temperature causes these intermediates to transit on to full fusion (Chernomordik et al., 1998; Melikyan et al., 2000c). If optimizing conditions leads to fusion, and the addition of CPZ and osmotic swelling leads to aqueous dye mixing, the intermediate is termed transitional hemifusion. It has not yet been rigorously shown that states said to be transitional hemifusion have actually merged contacting lipid leaflets. Thus, although hemifusion is thought to be an intermediate of full fusion, this has not been definitively established.

It may be expected that transitional hemifusion would be kinetically more advanced toward fusion than the bound state before acidification, but this has not yet been experimentally explored. Nor has the kinetic stability of states of transitional hemifusion been investigated. Furthermore, it is not known whether upon relief of the block, fusion proceeds via a route other than one that occurs when optimal conditions are directly used to induce fusion. In this work we devise methods to address these questions and answer them.

By rapidly raising temperature, the kinetics and the temperature dependence for converting different states of transitional hemifusion to fusion were compared with those obtained by triggering fusion directly out of the bound state at a constant and fusion-permissive temperature. Surprisingly, the kinetics was faster when fusion was directly induced from the bound state than from any of the intermediates. The kinetics of some intermediates was dependent on how long the state was maintained, but for other intermediates it was independent. Dependence correlated with dif-

Received for publication 21 July 2000 and in final form 25 October 2000.
Address reprint requests to Dr. Fredric S. Cohen, Department of Molecular Biophysics and Physiology, Rush Medical College, 1653 W. Congress Parkway, Chicago, IL 60612. Tel.: 312-942-6753; Fax: 312-942-8711; E-mail: fcohen@rush.edu.

0006-3495/01/02/812/10 $2.00

ferences in states of transitional hemifusion based on effects of lysophosphatidylcholine (LPC) and proteolytic treatment of HA (Melikyan et al., 2000c). The temperature dependence of fusion from all the intermediate states and from the bound state was also investigated and found to be similarly steep. Our findings show that the captured states are before the major rate-limiting step, and that rate-limiting step is pH independent but strongly temperature dependent.

MATERIALS AND METHODS

Cell growth and expression of HA

CV-1 green monkey kidney cells were grown in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% Cosmic calf serum (HyClone Laboratories, Logan, UT). A/Japan/305/57 HA or a point mutant of HA, G520L, was expressed in CV-1 cells from a recombinant SV40 vector, provided by Dr. M. Roth, as described previously (Naim and Roth, 1994; Melikyan et al., 1999). This vector led to high expression levels, and thus HA density was optimized.

Labeling of erythrocytes and quantification of fusion by fluorescence microscopy

RBCs were co-labeled with the membrane probe octadecylrhodamine B (R18, Molecular Probes, Eugene, OR) and with the aqueous dye carboxyfluorescein (CF, Molecular Probes) and used for fusion experiments (Melikyan et al., 1995, 1999). Briefly, approximately 39 h after infection with the recombinant SV40 virus, CV-1 cells were lifted from a 60-mm culture dish, transferred into several 35-mm dishes, and cultivated for 3 h in a CO_2 incubator. Cells were then treated with 0.1 mg/ml neuraminidase and 0.01 mg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin for 10 min at room temperature. After quenching the trypsin with serum-containing medium and then removing the trypsin, a dilute suspension of labeled RBCs (~0.005%) was added to the dish and allowed to bind to the HA-expressing cells for 10 min. Fusion was triggered by reducing the pH for an indicated time at the indicated temperature. Cells were then returned to a neutral pH solution (PBS supplemented with 20 mM raffinose). The extent of fusion was quantified 10–15 min later by examining several areas of the dish under a fluorescent microscope; the number of cells stained with either membrane or aqueous dye was normalized by the total number of cells with bound RBCs. Intermediates of fusion were revealed by raising temperature or by treating the HA-cell-RBC pairs with 0.5 mM CPZ (Sigma Chemical Co., St. Louis, MO) in PBS/raffinose for 1 min (at the indicated temperature).

Temperature-jump technique

To quickly step temperature from one that did not permit fusion (usually 4°C) to 37°C, we used an infrared (IR) laser diode emitting at 980 nm (model A001-FC/100, Opto Power Corp., Tucson, AZ) focused on the cells of interest. Water does not absorb at this wavelength, and thus to locally heat the cells we made an experimental chamber with the bottom made of IR-absorbing glass (KG5, Chroma Technology Corp., Brattleboro, VT). The chamber was placed in a temperature-controlled microscope stage (20/20 Technology, Wilmington, NC.). To estimate the temperature in the irradiated portion of the solution, we spread thin layers of various hydrocarbons with known melting temperatures on number 1.5 coverglasses and placed them on the top of the IR-absorbing glass. The laser diode output required to locally heat the coverglass to ~37°C, for example, was adjusted to melt eicosane (melting point 36–38°C, Sigma) but not henecosane (melting point 40–42°C). The melted region was ~200–300 μm in diameter (depending on the temperature of surrounding solution), providing the effective size of the heated spot. The validity of this procedure was verified by monitoring the change in conductivity of the bathing solution loaded into a pipette. Because the solution surrounding the illuminated spot was free to mix, terminating the laser power rapidly lowered the temperature of the spot. As estimated by both hydrocarbon melting and the resistance of the second pipette, a steady-state temperature was established within 2–4 s of turning the laser on, depending on the initial temperature of the buffer. We devised this procedure to induce and locally maintain desired increments and decrements in temperature (Melikyan et al., 2000a), with a time course fast enough for kinetic measurements whenever the time required for fusion was appreciably longer than the rise time of the temperature jump.

Fusion pore measurements

For electrophysiological experiments, HA-expressing cells were cultivated on number 1.5 coverglass for 1 h, treated with trypsin/neuraminidase, and bound to RBCs as described (Melikyan et al., 1999). Pieces of the coverglass were transferred into the experimental chamber, which was usually maintained at ~ 4°C. Unless stated otherwise, an HA-expressing cell patched in the whole-cell configuration was voltage clamped with a 200-Hz, 50-mV peak-to-peak, sine wave voltage superimposed on the holding potential of −40 mV. A software-based lock-in amplifier determined the components of the output current that were in phase and out of phase with the applied sine wave voltage (Ratinov et al., 1998). The T-jump reproducibly and reversibly caused a decrease in the in-phase component and an increase in out-of-phase component of the electrical admittance of the cells in whole-cell patch-clamp mode. This was due to the increase in conductivity of the solution within the patch pipette ($1/R_s$) and the increase in cell membrane capacitance, C_m, both caused by raised temperature. Similar changes were observed when the temperature was increased more slowly through a Peltier device. (The increase of C_m is probably due to the thinning of membranes with temperature, as directly determined by x-ray diffraction for model phospholipid systems (Rand and Pangborn, 1973).) We employed phase tracking (Fidler and Fernandez, 1989) to find the correct phase angle after the altered electrical parameters had stabilized. The pH of the solution surrounding the cells was lowered by ejecting a pH 4.8 solution buffered with 20 mM succinate from another micropipette positioned near the cell-RBC pair, maintaining 4°C or 23°C. After 1 min, the microperfusion was stopped and the solution around the patched cell was allowed to reneutralize for at least 30 s. Fusion between an HA-expressing cell and an RBC was subsequently induced by jumping the temperature to 37°C through IR illumination. In the case that acidification at pH 4.8 was maintained for the relatively long time of 10 min (i.e., HA4*), the intermediate was first established at 4°C and only then was the HA cell patch clamped. Temperature was raised from this point. Fusion pore conductances were calculated off-line (Melikyan et al., 1999).

RESULTS

Stabilization of intermediates

HA-expressing cells were bound to RBC ghosts co-labeled with the aqueous dye carboxyfluorescein (CF) and the membrane dye R18. By lowering pH at a suboptimal temperature for different times before reneutralization, we generated several intermediates. We chose intermediates for which lipid dye had not yet spread, but would proceed on to fusion by raising temperature at neutral pH to 37°C, by adding CPZ and by osmotic swelling (Chernomordik et al., 1998; Melikyan et al., 2000c).

We created intermediate states for wild-type (wt) HA by lowering pH to 4.8 at 4°C for 1 min followed by reneutralization at 4°C for the indicated time. We refer to these intermediates as HA4. We also created an intermediate, denoted HA4*, by maintaining low pH for 10 min (rather than 1 min), followed by reneutralization at 4°C. Fusion did not result upon raising temperature of either HA4 or HA4* to 23°C. We refer to the intermediate of HA4 held at 4°C (after reneutralization) for 5 min, followed by raising and holding temperature at 23°C for an additional 5 min as HA4–23. If the ability of a state to go on to fusion by addition of CPZ is not vulnerable to HA proteolysis and the addition of LPC to outer leaflets does not prevent lipid dye mixing upon raising temperature, the state is said to be secure. If either of the interventions prevents the subsequent dye spread, the state is termed vulnerable. HA4* is secure whereas HA4 and HA4–23 are vulnerable, as demonstrated by both interventions (Melikyan et al., 2000c).

A point mutation within the transmembrane (TM) domain of HA (G520L) yields less fusion, and more stringent conditions are required for appreciable extents of fusion as compared with wt HA. But intermediates can be generated by acidifying at higher temperatures for G520L than is possible for wt HA. For example, lowering pH to 4.8 for 1–2 min at 23°C does not lead to either lipid or aqueous dye spread, whereas it does for wt HA (Melikyan et al., 2000c). Importantly, this lowering of pH gives rise to an intermediate that proceeds on to fusion when temperature is increased to 37°C at neutral pH, although the fusion pores do not enlarge (Melikyan et al., 2000c). Because the abilities of wt HA and G520L to cause fusion were different, but both could generate intermediates, we kinetically compared pore formation from intermediates of G520L and wt HA. In this study, we acidified G520L cells for 1 min and refer to the intermediate state of fusion that has been arrested after reneutralization as GL23. Either raising the temperature of GL23 to 37°C or adding CPZ at 23°C promoted both lipid and aqueous dye transfer. If acidification and reneutralization were performed at 4°C and cells maintained at that temperature, we refer to the state as GL4. (The time the reneutralized cells were maintained is indicated in appropriate figure legends.) Raising the temperature of GL4 (held at neutral pH, 4°C for 1 min) to 23°C yields the intermediate GL4–23. GL23 is a secure intermediate whereas GL4–23 is a vulnerable one. The abbreviations for the intermediates and whether they are secure or vulnerable, as well as the conditions used to obtain the intermediates, are summarized in Table 1.

Intermediates of wt HA exhibit slow kinetics of fusion and can evolve with time

We patch clamped HA-expressing cells with a bound RBC in the whole-cell mode and monitored fusion pores in real time by electrical admittance measurements. This allowed us to compare the kinetics of fusion pore formation from the point of the different intermediates (Fig. 1). The kinetics of fusion obtained by maintaining the temperature at 37°C and applying a low pH solution (i.e., F-37, Fig. 1, *A* and *B*, open squares) was compared with the kinetics when fusion was induced from HA intermediates by increasing the temperature to 37°C at neutral pH. Obviously, temperature should be increased quickly to obtain the true kinetics; any configurations that might form at intermediate temperatures should be avoided. To increase temperature rapidly, we used a temperature jump (T-jump) technique (see Materials and Methods). For F-37, time = 0 was set as the moment pH was lowered whereas for a T-jump experiment, time = 0 was set as the moment temperature was increased. Because pH for an intermediate had already been lowered when temperature was raised, zero time referred to a later event from an intermediate than from the bound state. This, and the fact that the HA of intermediates had already undergone some low-pH-induced conformational changes, would tend to make the kinetics of HA intermediates appear faster than the kinetics of F-37. We established HA4–23 and found that despite the prior acidification, fusion pore formation from HA4–23 after raising temperature (Fig. 1 *A*, filled triangles) was decidedly slower than for F-37 (open squares). If pH was lowered at 4°C for 10 min and then reneutralized, raising temperature to 37°C 10 min later (HA4*, open triangles) led to statistically faster fusion than raising the temperature of HA4–23. Importantly and unexpectedly, even though all the intermediates have attained CPZ sensitivity, they all exhibited slower kinetics of fusion than did F-37.

We tested whether raising the temperature from 4°C to 23°C to create HA4–23 affected the behaviors of the intermediate complexes. That is, are the complexes the same at

TABLE 1 Experimental conditions used to establish HA fusion intermediates

Abbreviations (type of intermediate)	Temperature, time at low pH	→	Temperature, time at neutral pH
HA4 or GL4 (vulnerable)	4°C, 1 min	→	4°C, 10 min
HA4(0.5) or GL4(0.5) (vulnerable)	4°C, 1 min	→	4°C, 0.5 min
HA4-23 or GL4-23 (vulnerable)	4°C, 1 min	→	4°C, 5 min followed by 23°C, 5 min
HA4* (secure)	4°C, 10 min	→	4°C, 10 min
GL23 (secure)	23°C, 1 min	→	23°C, 10 min

Cells were acidified at a temperature for the time shown followed by reneutralization at the temperature for the time indicated before fusion. Fusion was triggered by raising temperature to 37°C at neutral pH.

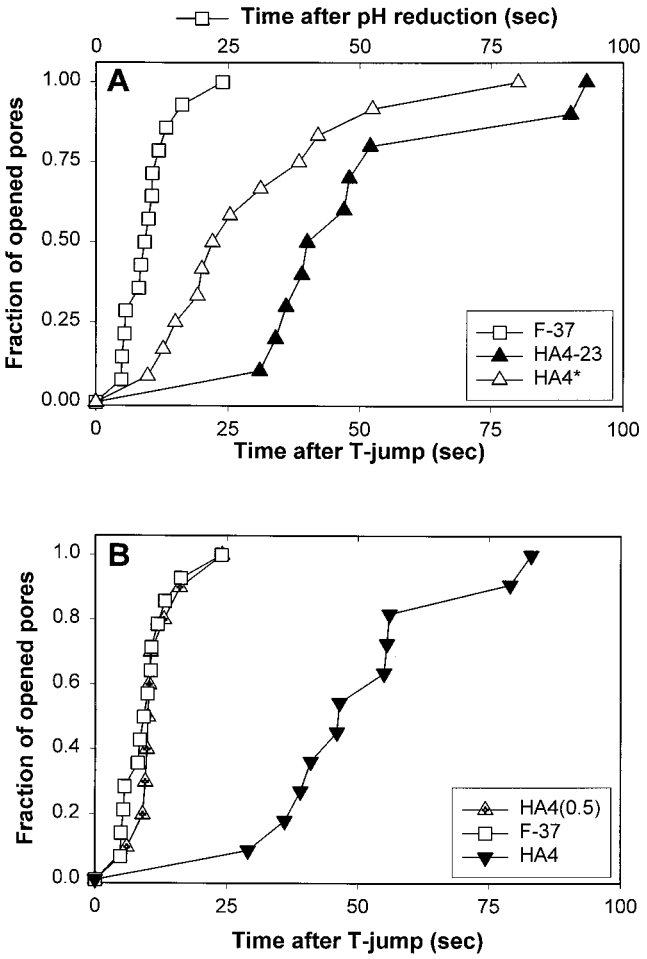

FIGURE 1 Kinetics of fusion pore formation induced by wt HA intermediates by a T-jump to 37°C. (*A*) For F-37 and HA4–23, cells were patch clamped before lowering pH. For HA4* the intermediate was induced, and at this point the cell was patch clamped and fusion was induced by raising temperature. Because F-37 (□) denotes fusion induced by directly lowering pH at 37°C, a T-jump was not employed (lag times shown on the upper axis are times after acidification). HA4–23 (▲) was established by exposing the patch-clamped cells to low pH at 4°C for 1 min and incubating them at neutral pH for 5 min at 4°C and then for 5 min at 23°C. HA4* (△) was created by lowering pH for 10 min at 4°C and then reneutralized for 10 min at 4°C. Fusion induced from HA4* was statistically ($p < 0.05$) faster than for HA4–23. (*B*) HA4(0.5) (*triangles with cross*) was produced by lowering pH at 4°C for 1 min and reneutralizing for 0.5 min before stepping temperature to 37°C. For HA4 (▼), reneutralization was maintained for 10 min before raising temperature.

4°C and after raising temperature to 23°C? The kinetics of fusion pore formation for HA4 (maintaining the reneutralized pH, 4°C for 10 min before stepping temperature to 37°C, Fig. 1 *B*, filled inverted triangles) was virtually identical to that of HA4–23 (Fig. 1 *A*, filled triangles). Thus, it did not matter at which temperature the intermediate was held after reneutralization (so long as it was a temperature too low to induce fusion). Not only were kinetics similar, but so were the extents of fusion: HA4 and HA4–23 proceeded on to roughly the same percentage of contents mixing when temperature was raised to 37°C, and the addition of CPZ induced similar percentages of contents mixing for both states (data not shown). In short, raising the temperature of HA4 to 23°C did not change the behavior of the complexes.

In contrast, the time HA4 was held at neutral pH was consequential. Creating the intermediate and then holding the complexes after reneutralization at 4°C for only 0.5 min before stepping temperature to 37°C (Fig. 1 *B*, triangle with cross) yielded kinetics that was the same as for F-37 (open squares), significantly faster than holding HA4 after reneutralization at 4°C for 10 min (filled inverted triangles). (It is possible that stepping the temperature to 37°C from HA4(0.5) led to somewhat faster fusion than F-37, but this could not be detected because of the few seconds needed to reach 37°C with a T-jump.) Holding cells at 4°C for 3 min after reneutralization yielded fusion kinetics intermediate between those of the 0.5-min and 10-min incubations (Fig. 2 *A*, open triangles). That is, the longer the cells were held at 4°C (after reneutralization) the longer it took to fuse at 37°C. This evolution of complexes can be readily appreciated from the linear increase in the time it took for 50% of the cells to fuse when temperature was raised to 37°C (Fig. 2 *A*). (Presumably, HA4 would eventually stop evolving at neutral pH, but we did not pursue the extremely long time course.) In contrast, HA4* was stable over time. The fusion kinetics was the same when temperature was raised to 37°C after maintaining HA4* at neutral pH for 2 min and 10 min (Fig. 2, filled triangles).

Whereas kinetics slowed over time after reneutralization for HA4 (Fig. 2 *A*, open triangles), patch-clamp experiments showed that they did not for GL4 (Fig. 2 *A*, filled circles). Explicitly, holding GL4 at 4°C, neutral pH for only 0.5 min before stepping temperature to 37°C (Fig. 3, GL4(0.5), open circles with cross) gave the same kinetics of fusion as when the intermediate was maintained at neutral pH for 10 min (Fig. 3, GL4, open hexagon). To put this phenomenon in different terms, G520L, unlike HA, never yielded fast fusion. G520L kinetics did not change greatly with the (suboptimal) temperature of acidification; acidifying at 23°C (Fig. 3, GL23, filled circles) led to statistically the same kinetics as did acidifying at 4°C and then reneutralizing at 4°C for 5 min followed by 5 min at 23°C (GL4–23, open circles). In summary, acidification may have been marginally more effective at 23°C than at 4°C, but the intermediates created with G520L did not evolve with time. In fact, all the G520L intermediates yielded similar kinetics (Fig. 3). Thus, the TM domain can affect the evolution of intermediates before raising temperature.

The extent of fusion upon raising temperature is not reduced when HA intermediates are maintained

When 4°C was maintained (after reneutralizing) for only 20 s after creating HA4, the level of fusion upon increasing

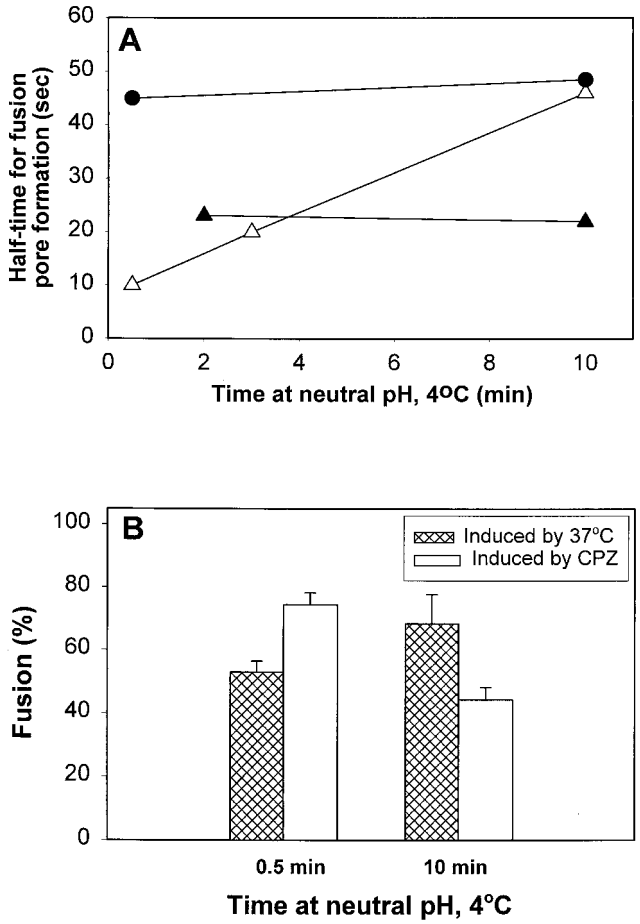

FIGURE 2 The kinetic stability of intermediates with time. (*A*) The time for 50% of the cells to fuse after raising temperature at an intermediate to 37°C are shown for GL4 (●), HA4* (▲), and HA4 (△). HA4 was produced in exactly the same manner as was GL4, but its kinetics slowed (kinetics of GL4 were stable) as the time of maintaining the intermediate was increased after reneutralization to pH 7.2 at 4°C. (*B*) HA4 was maintained for 0.5 min (HA(0.5)) or for 10 min (HA4*). The extent of fusion induced by either raising temperature to 37°C (*cross-hatched bars*) or adding 0.5 mM CPZ (*open bars*) is shown.

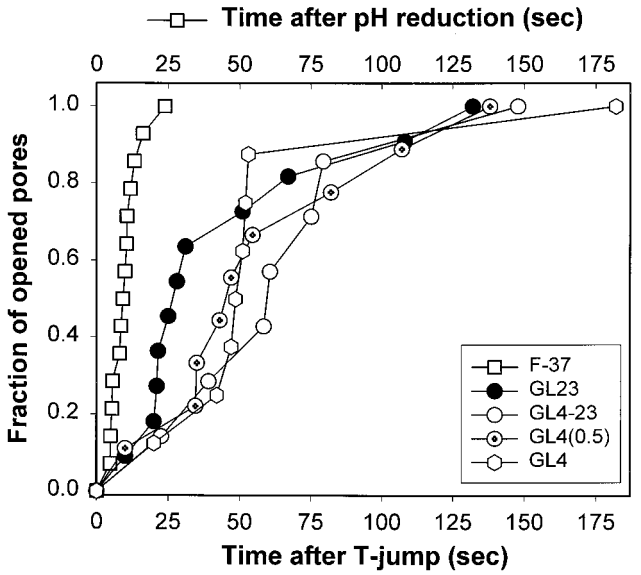

FIGURE 3 Kinetics of fusion for intermediates generated with G520L. Kinetics were obtained by creating an intermediate and electrically measuring fusion pore formation after stepping temperature to 37°C. The kinetics were statistically the same from all intermediates. All the intermediates were created by lowering pH for 1 min followed by reneutralization: GL23 (●), pH lowered at 23°C, followed by reneutralization for 10 min; GL4–23 (○), pH lowered at 4°C, followed by reneutralization at 4°C for 5 min and then at 23°C for 5 min; GL4(0.5) (⊕), pH lowered at 4°C, followed by reneutralization at 4°C for 30 s; GL4 (○), pH lowered at 4°C, followed by reneutralization at 4°C for 10 min. Kinetics of wt HA F-37 (here, time refers to time after acidification) is plotted for comparison.

temperature to 23°C reached ~20% of F-37 (based on dye spread). After maintaining 4°C for 30 s, then raising temperature to 23°C at neutral pH, little fusion occurred. Clearly, the complexes are changing quickly at these very early times. Whereas HA4 maintained for 30 s yielded faster kinetics than HA4 maintained for 10 min when the temperature of each was raised to 37°C (Fig. 2 *A*, open triangles), the extent of fusion for the two HA4 intermediates was statistically the same (Fig. 2 *B*, cross-hatched bars). In contrast, when 4°C was maintained, a greater extent of fusion was observed upon adding CPZ to the 30-s HA4 intermediate (first open bar) than to the 10-min HA4 (second open bar). When cells were held at HA4 for times longer than 10 min, the extents of fusion (caused either by increasing temperature to 37°C or by adding CPZ) did not change further (data not shown). Thus, as HA4 was held at neutral pH for times beyond 30 s, pore formation was slowed and adding CPZ became less effective in promoting fusion. But the extent of fusion upon elevating temperature to 37°C did not depend on how long the intermediate was maintained; it remained the same as for F-37.

Raising temperature for short times neither causes fusion nor kinetically advances it

To test whether HA4–23 could be advanced further in the fusion process by increasing the temperature to 37°C for only a short time, we T-jumped HA4–23 to 37°C for 20 s, then let the temperature rapidly fall back to 23°C for 1 min and repeated this process two more times. Fusion did not occur after this series of T-jumps. We T-jumped one final time to 37°C and maintained it. Fusion then did occur but, significantly, it was not faster (Fig. 4 *A*, open diamonds) than after a single sustained T-jump (open inverted triangles); in fact, surprisingly, it was somewhat slower. In other words, short times at 37°C did not advance HA4–23 toward fusion; fusion did not proceed until temperature was sustained at 37°C for ~30 s or more. The sigmoidal kinetics upon raising temperature to 37°C indicates that from the point of HA4–23, fusion is still a multi-step process. Jump-

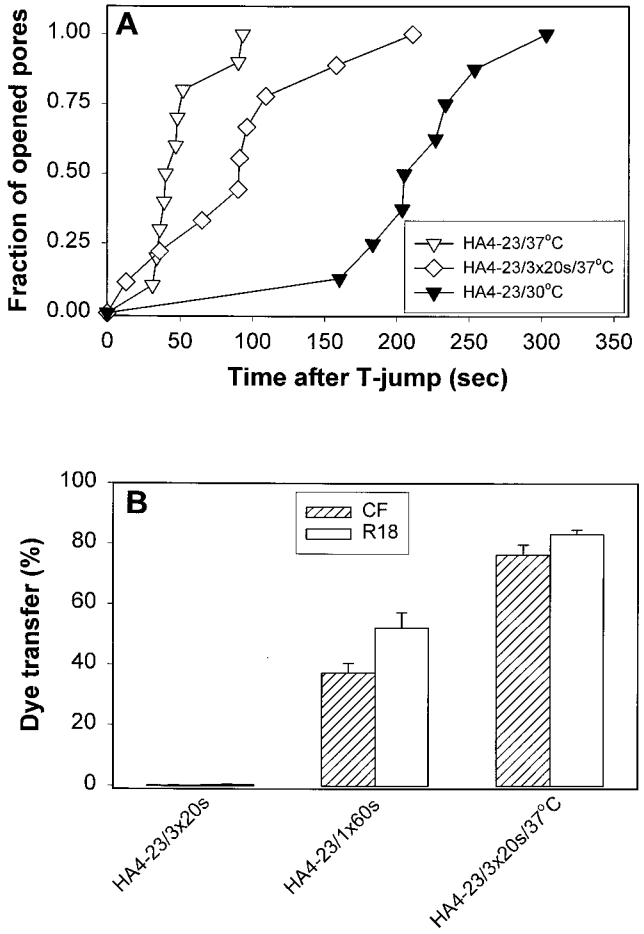

FIGURE 4 Probing HA4–23 by repetitive short exposures to 37°C. (*A*) Normalized fusion pore kinetics. When cells trapped at HA4–23 were exposed to three 37°C pulses (20 s each), with an interval of 1 min at 23°C, fusion pores did not form. A subsequent prolonged exposure to 37°C resulted in fusion pore opening, albeit at a somewhat slower rate (◇) than if the pre-pulses of temperature had not been applied (▽, replotted from Fig. 1 *A* for comparison). Raising temperature to 30°C (▼) led to slower kinetics than raising temperature to 37°C. (*B*) Increasing the temperature of HA4–23 cells to 37°C for 20 s and then returning to 23°C for 1 min and repeating twice more did not lead to either aqueous or lipid dye spread (*first column*). A subsequent incubation at 37°C for 3 min resulted in efficient fusion (*third column*). Exposing cells at HA4–23 to 37°C for an uninterrupted 60 s promoted significant lipid (*open* bar) and content (*hatched bar*) mixing (*second column*).

ing the temperature of HA4–23 to 30°C (closed inverted triangles) yielded significantly slower kinetics of fusion than occurred at 37°C. Thus, the electrical measurements have shown that 1) the kinetics for formation of fusion pores after generating HA4–23 was slower than that of F-37 (Fig. 1) and 2) HA4–23 was not induced to fuse by short temperature increases (Fig. 4 *A*). Both results indicate that large energy barriers intervene between the intermediate configurations and fusion pores.

We used fluorescent dye spread measurements to determine whether short increases in temperature, obtained by transferring cells between water baths at 23°C and 37°C, drove HA4–23 into an end-state hemifusion, one that does not lead to pores. We used the same scheme as for the electrically determined kinetics: three increases in temperature to 37°C, each maintained for 20 s, with a 1-min wait at 23°C between temperature increases. (The absence of pore formation with labeled RBCs using this temperature pulse protocol was verified in all nine experiments in which it was tested, using electrical admittance measurements.) In these experiments, neither lipid nor aqueous dye spread (Fig. 4 *B*, first columns). Thus, neither end-state hemifusion with lipid dye transfer nor fusion pore formation was induced by increases in temperature held for only short times. After the protocol described, a subsequent incubation at 37°C sustained for 3 min evoked lipid and aqueous dye spread for 80% of the cells (Fig. 4 *B*, third column). The same extent of lipid and aqueous dye transfer was observed when the temperature of HA4–23 cells was raised to 37°C for 3 min without any pre-pulses (data not shown). Raising the temperature of cells in HA4–23 to 37°C and maintaining it for 1 min (matching the total time of 60 s at 37°C through three 20-s temperature increases) also led to aqueous dye spread, but the extent was less; dye transferred into ~40% of the HA4–23 cells (Fig. 4 *B*, second column). (Fusion pores were electrically detected in ~80% of the cells after they were held at 37°C for 60 s (Fig. 4 *A*, open inverted triangles), showing that only about one-half of the pores had enlarged sufficiently to allow passage of the small aqueous dye by this time.) Thus, the effects of holding cells at 37°C were not additive in time, and dropping the temperature back to 23°C prevented pores from forming and enlarging; fusion did not proceed from HA4–23 at 23°C even if the cells had previously been at 37°C, and exposures to 37°C that were too short to induce fusion did not irreversibly spur the process on to more advanced intermediates. Similar results were obtained for HA4, except that repetitive short 37°C pulses yielded a small extent of fusion (data not shown); this was the only difference we detected between the behaviors of HA4 and HA4–23. In summary, the inability of transient temperature increases to speed fusion shows that the system has not irreversibly overcome a significant energy barrier during the short temperature increase.

The transition from an intermediate to fusion is highly temperature dependent

The conformational changes of HA that occurred before establishing an intermediate were clearly pH dependent. We examined the temperature dependence of steps that required low pH by briefly exposing HA cell-RBC pairs to low pH at temperatures between 4°C and 37°C, reneutralizing at the same temperature, and then increasing the temperature to 37°C. The extents of aqueous dye spread were significantly greater for HA (Fig. 5 *A*, filled diamonds) than for G520L

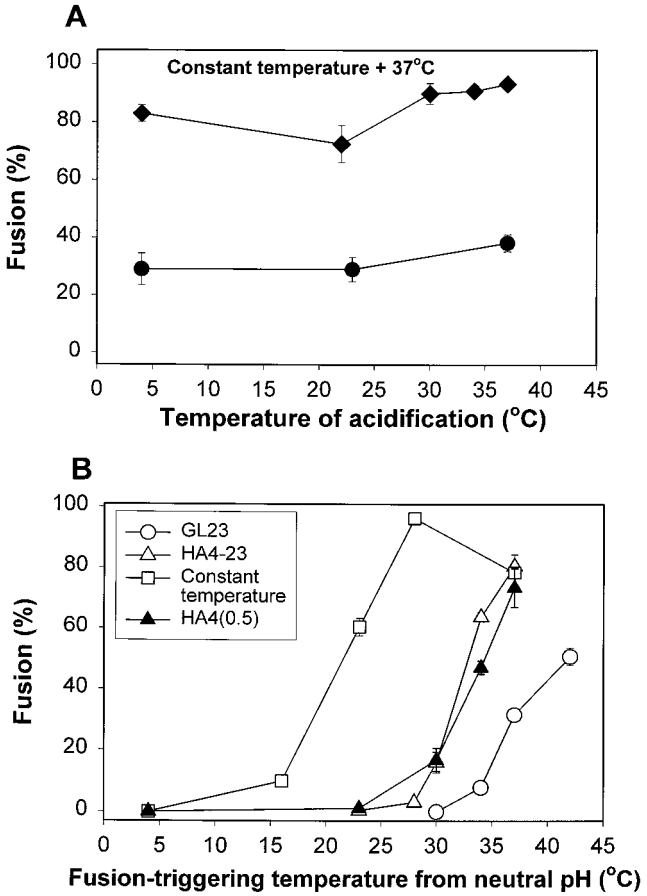

FIGURE 5 The temperature dependence of extent of fusion between HA-expressing cells and RBCs measured by spread of CF. (*A*) The temperature dependence of early steps of fusion was revealed by applying low pH for 2 min for HA4 cells and G520L cells at the indicated temperature, incubating at neutral pH for 10 min at the same temperature, and then inducing fusion by raising temperature to 37°C for 5 min. Fusion was independent of the temperature at which pH was lowered for both wt HA (◆) and G520L (●). (*B*) GL23 and HA4–23 were created by lowering pH for 2 min; HA4(0.5) was created by lowering pH for 1 min. In all cases, the extent of fusion depended strongly on the temperature to which an intermediate was raised. After establishing an intermediate, temperature was raised to values between that of the intermediate and 37°C for 5 min (followed by incubation at 23°C for 5 min before examining the fraction of fused cells). Maintaining temperature throughout with pH 4.8 for 2 min, followed by incubation at neutral pH for 15 min (all at the same temperature), led to a similarly steep temperature dependence (□).

(filled circles). The electrically measured extents of pore formation were, however, similar. This occurs because the G520L pores do not enlarge (Melikyan et al., 2000c). For both HA and G520L, the extents of aqueous dye spread were virtually independent of the temperature at which the cells were acidified (Fig. 5 *A*; the abscissa gives the temperature at which pH was lowered). That is, it does not matter at what temperature acidification occurs. Acidifying at 4°C was as effective in yielding fusion-competent cells (revealed by raising temperature to 37°C) as acidifying at higher temperatures.

In striking contrast, the transition from an intermediate to full fusion exhibited a remarkably steep temperature dependence. For HA4–23, fusion was ~16-fold greater when the temperature was raised to 37°C than to 28°C (Fig. 5 *B*, open triangles show the extent of fusion at the elevated temperature). Raising the temperature of cells at HA4 to varied values led to a steep temperature dependence of fusion that was independent of the time of reneutralization at 4°C (shown for 30 s, filled triangles). By lowering pH and allowing fusion to proceed at the same temperature, we again observed a steep temperature dependence (Fig. 5 *B*, open squares) for the extent of fusion. This curve for temperature dependence was similar to those when any of the intermediates were induced to fuse by raising temperature, except that the curve for constant temperature was shifted approximately 10°C lower. A steep and similar temperature dependence was also observed for GL23 (open circles). The kinetic measures (Fig. 1) suggested that the rate-limiting step for fusion occurs subsequent to the creation of the intermediates. The steep temperature dependence provides an independent verification that the rate-limiting step has not been passed by the point of the intermediate.

The creation of intermediates does not alter the nature of the fusion pore

We quantitatively tested whether fusion pores, as measured electrically, were independent of the intermediate state from which they were generated by averaging the conductance of all pores formed under a given condition, aligning the times by the moment the pores opened. The average conductance of a pore formed when HA4–23 (Fig. 6, filled triangles) was induced on to fusion was statistically the same as when a pore was generated by acidifying directly at 37°C (filled circles, F-37). Similarly, pores formed from GL4 (open

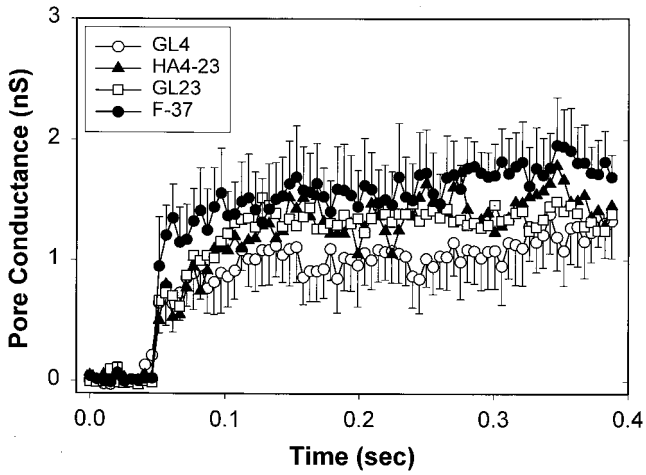

FIGURE 6 Pore conductance at early times after pore formation. Pores were aligned at their opening. Shown are GL4 (○; $n = 5$), GL23 (□; $n = 7$), HA4–23 (▲; $n = 8$), and F-37 (●; $n = 7$).

circles) were the same as pores generated from GL23 (open squares). Thus, although fusion through an intermediate may take a somewhat different route than through lowering pH at 37°C (i.e., F-37), we have shown that the routes converge to a similar or the same fusion pore. Also, because the pores of F-37 were created at low pH, whereas those from the intermediates formed at neutral pH, the conductance of the initial pore is independent of pH. As noted previously, the behavior of pores induced by HA and G520L are not identical: electrically following pores for longer times showed that HA pores enlarge over time, and G520L do not (Melikyan et al., 2000c). This accounts for the greater extent of aqueous dye spread observed for HA than for G520L.

DISCUSSION

In this study, we kinetically characterized intermediates of HA-mediated fusion. Our most surprising observation was that even though the pH-dependent steps required for fusion have been completed by the point of the intermediates, raising temperature at that point at neutral pH induced fusion more slowly than occurs directly from the bound state by lowering pH at 37°C. Furthermore, transient temperature increases before a sustained increase did not kinetically advance fusion. These results indicate that a significant energy barrier is interposed between the intermediates and fusion pores. We have also found that the kinetics of fusion for the two secure intermediates, HA4* and GL23, did not depend on how long the intermediate was maintained. In contrast, fusion slowed with the time the vulnerable intermediates created by wt HA, HA4 and HA4–23, were maintained, but did not for the vulnerable intermediates generated by G520L, GL4 and GL4–23. That is, a point mutation within the TM domain tended to eliminate the kinetic evolution of intermediates.

Potential sites of fusion may be lost with time

HA-expressing cells are in contact with an appreciable fraction of the RBC area in the bound state (Doxsey et al., 1985; Danieli et al., 1996), and when pH is lowered, hundreds of sites where the cells are puckered toward each other are observed by electron microscopy (Frolov et al., 2000). The spatial resolution needed to determine whether these are sites of local hemifusion or merely sites of close contact between membranes has not yet been achieved. Only a small fraction of sites are thought to proceed on to fusion (Frolov et al., 2000; Leikina and Chernomordik, 2000). Our finding that the rate of fusion from HA4(0.5) is the same as for F-37 and faster than the rate for any of the intermediates held for longer times could be caused by the loss, over time, of potential sites of fusion. Fusion is detected if at least one of many potential sites actually fuses. Thus, even if a large fraction of the sites lost their ability to fuse over time, as long as one site fused, the measured extent of fusion would not be affected. Fusion kinetics measured on the single-cell level, on the other hand, would be sensitive to the number of sites because it reports the time course for formation of the first pore. A loss of sites with time would also account for the reduction in CPZ-induced fusion (Fig. 2 *B*). A functional loss of sites could be due to disassembly of HA trimers from each other within a pre-pore complex, inactivation of some HA trimers within a complex, or even reversion of a site of transitional hemifusion back to two separate membranes. Acidifying for 10 min to create HA4* led to invariant kinetics of fusion over the time of maintaining the intermediate (Fig. 2 *A*), which may indicate that these sites are stable over time. Independent of whether sites are lost, secure CPZ-sensitive intermediates resist invasive manipulations (Melikyan et al., 2000c). Just the opposite pertains to vulnerable intermediates. Because kinetics of fusion did not vary over time for secure intermediates but did vary for vulnerable ones, the two classes of CPZ-sensitive intermediates are truly different in some way.

Relationship between captured intermediates and the route of fusion

It might be assumed that intermediates that can be observed experimentally are states that are passed through naturally in the process of physiological fusion. Viewed from the standpoint of energetics, it is likely that an experimental intermediate can be captured because it is at a local minimum. The energy landscape for proteins to form fusion complexes will depend on conditions; suboptimal conditions may deepen energy minima or create minima that do not exist at optimal conditions. In other words, proteins and lipids may reconfigure differently when fusion proceeds optimally than under suboptimal conditions. The captured intermediate may not consist of complexes of fusion proteins and membrane configurations that precisely occur when fusion progresses unimpeded, without experimental manipulations to capture such intermediates. Although the multiple sites of potential fusion are probably heterogeneous for any given intermediate and the intermediate states are likely to be different energetically from those of uninterrupted fusion, the very fact that the intermediates can proceed on to fusion indicates that their molecular configurations are probably not very different. This interpretation is also supported by the finding that the intermediates exhibited the same steep (albeit shifted) temperature dependence of fusion as did lowering pH from the bound state while holding temperature constant (Fig. 5 *B*). Lastly, the initial fusion pore (Fig. 6) and its enlargement (Melikyan et al., 2000c) were independent of the route of fusion.

Protein, rather than lipid, rearrangements cause the steep temperature dependence of fusion

The highly temperature-dependent, rate-limiting steps for fusion could occur as HA undergoes major conformational changes, as lipids reconfigure into a pore, or as the two occur together. Although HA may normally reside in rafts rich in cholesterol and sphingomyelin (Scheiffele et al., 1997), the precise lipid environment for HA does not appear to be critical for its fusion activity. Removing cholesterol from membranes did not affect fusion; mutations of the TM domain or cytoplasmic tail of HA that eliminated the tendency to enter rafts did not reduce fusion; chimeras with the TM domain of HA replaced by those of other proteins did not enter rafts and yet supported fusion at its normal pace and extent (Melikyan et al., 1997b, 1999). Also, it is unlikely that reconfigurations of lipid in a concerted fashion, as occurs in phase transitions, is the dominant cause of the observed rate-limiting step. The transition temperatures at which intermediates proceeded onto fusion were dependent on the precise protocol (Fig. 5 *B*), whereas if lipid rearrangements were responsible this temperature would be a lipid property, independent of the conditions used to activate HA (or G520L). Because the multiple sites generated within an intermediate may be heterogeneous, some of the steep temperature dependence may be due to different sites having different thresholds for fusion. In any case, all the data are consistent with the notion that the rate-limiting step out of an intermediate occurs late in the reaction, when HA undergoes a conformational change that induces lipid rearrangements. This rate-limiting step is strongly temperature, but not pH, dependent.

The evidence of this study indicates that the major rate-limiting step of fusion has not been overcome at the point reached by the intermediates. Kinetics of fusion would be determined by the number of potential sites of fusion and the energy barriers separating each site from pore formation. Because the rate of fusion from HA4–23 was much slower for temperature raised to 30°C than to 37°C (Fig. 4 *A*), the intervening energy barrier appears to be large. Most importantly, the steep temperature dependence of fusion from the point of these intermediates suggests that the HA has not achieved its final conformation at the stage of the arrested intermediates.

Conformational changes of HA between intermediates and pore formation

In the crystallographically identified final structure of HA2 as derived from ectodomains in solution, the residues that comprise the N cap (that terminate each of the N-terminal α-helices and the triple-stranded central coiled-coil) are proximal to the fusion peptide and extensively interact with conserved residues of the C-terminus that are immediately adjacent to the TM domain (Chen et al., 1999). (The crystal structure was obtained for X:31 HA, whereas this study has used Japan 57 HA. But the residues that create the N cap of these two strains are conserved, and the key residues of the C-terminus that they bond to are also conserved; this is generally true for all the subtypes of HA. The role in fusion of these N- and C-terminal regions and their interactions are likely to be the same among the various subtypes of HA.) For these interactions to occur when HA ectodomains are linked to a membrane, the TM domains would have to come toward and perhaps contact the N-terminal fusion peptide. The means by which the TM domains within a trimer could separate from each other and approach the fusion peptides should be sterically limited and difficult to achieve. At the point of the intermediates, the fusion peptides have inserted into the target membrane (Chernomordik et al., 1998). For contact to be made between these two membrane-inserted domains, the membranes must become continuous. The energy released during this transition could be substantial (Chen et al., 1999) and used to generate the disruptions in the local hemifusion diaphragms that culminate in a fusion pore. We propose that the rate-limiting step in the progression from the intermediates to a fusion pore is the formation of the bonds between the N cap region and the C-terminal residues adjacent to the TM domain.

We thank Sofya Brener for steadfast technical assistance. Dr. Michael Roth provided the recombinant SV40 stocks used to express HA and G520L. This work was supported by National Institutes of Health grants GM-27367 and GM-54787.

REFERENCES

Chen, J., J. J. Skehel, and D. C. Wiley. 1999. N- and C-terminal residues combine in the fusion-pH influenza hemagglutinin HA(2) subunit to form an N cap that terminates the triple-stranded coiled coil. *Proc. Natl. Acad. Sci. U.S.A.* 96:8967–8972.

Chernomordik, L. V., V. A. Frolov, E. Leikina, P. Bronk, and J. Zimmerberg. 1998. The pathway of membrane fusion catalyzed by influenza hemagglutinin: restriction of lipids, hemifusion, and lipidic fusion pore formation. *J. Cell Biol.* 140:1369–1382.

Chernomordik, L., E. Leikina, and J. Zimmerberg. 1995. Control of baculovirus gp64-induced syncytia formation by membrane lipid composition. *J. Virol.* 69:3049–3058.

Chernomordik, L. V., E. Leikina, V. Frolov, P. Bronk, and J. Zimmerberg. 1997. An early stage of membrane fusion mediated by the low pH conformation of influenza hemagglutinin depends upon membrane lipids. *J. Cell Biol.* 136:81–93.

Danieli, T., S. L. Pelletier, Y. I. Henis, and J. M. White. 1996. Membrane fusion mediated by the influenza virus hemagglutinin requires the concerted action of at least three hemagglutinin trimers. *J. Cell Biol.* 133:559–569.

Doxsey, S. J., J. Sambrook, A. Helenius, and J. White. 1985. An efficient method for introducing macromolecules into living cells. *J. Cell Biol.* 101:19–27.

Fidler, N., and J. M. Fernandez. 1989. Phase tracking: an improved phase detection technique for cell membrane capacitance measurements. *Biophys. J.* 56:1153–1162.

Frolov, V. A., M. Cho, T. S. Reese, and J. Zimmerberg. 2000. Both hemifusion and fusion pores are induced by GPI-linked influenza hemagglutinin. *Traffic.* 1:622–630.

Gaudin, Y. 2000. Rabies virus-induced membrane fusion pathway. *J. Cell Biol.* 150:601–611.

Hart, T. K., A. Truneh, and P. J. Bugelski. 1996. Characterization of CD4-gp120 activation intermediates during human immunodeficiency virus type 1 syncytium formation. *AIDS Res. Hum. Retroviruses.* 12: 1305–1313.

Kemble, G. W., T. Danieli, and J. M. White. 1994. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell.* 76:383–391.

Kingsley, D. H., A. Behbahani, A. Rashtian, G. W. Blissard, and J. Zimmerberg. 1999. A discrete stage of baculovirus GP64-mediated membrane fusion. *Mol. Biol. Cell.* 10:4191–4200.

LeDuc, D. L., Y. K. Shin, R. F. Epand, and R. M. Epand. 2000. Factors determining vesicular lipid mixing induced by shortened constructs of influenza hemagglutinin. *Biochemistry.* 39:2733–2739.

Leikina, E., and L. V. Chernomordik. 2000. Reversible merger of membranes at the early stage of influenza hemagglutinin-mediated fusion. *Mol. Biol. Cell.* 11:2359–2371.

Markosyan, R. M., F. S. Cohen, and G. B. Melikyan. 2000. The lipid-anchored ectodomain of influenza virus hemagglutinin (GPI-HA) is capable of inducing nonenlarging fusion pores. *Mol. Biol. Cell.* 11: 1143–1152.

Melikyan, G. B., S. A. Brener, D. C. Ok, and F. S. Cohen. 1997a. Inner but not outer membrane leaflets control the transition from glycosylphosphatidylinositol-anchored influenza hemagglutinin-induced hemifusion to full fusion. *J. Cell Biol.* 136:995–1005.

Melikyan, G. B., H. Jin, R. A. Lamb, and F. S. Cohen. 1997b. The role of the cytoplasmic tail region of influenza virus hemagglutinin in formation and growth of fusion pores. *Virology.* 235:118–128.

Melikyan, G. B., S. Lin, M. G. Roth, and F. S. Cohen. 1999. Amino acid sequence requirements of the transmembrane and cytoplasmic domains of influenza virus hemagglutinin for viable membrane fusion. *Mol. Biol. Cell.* 10:1821–1836.

Melikyan, G. B., R. M. Markosyan, S. A. Brener, Y. Rozenberg, and F. S. Cohen. 2000a. Role of the cytoplasmic tail of ecotropic Moloney murine leukemia virus Env protein in fusion pore formation. *J. Virol.* 74: 447–455.

Melikyan, G. B., R. M. Markosyan, H. Hemmati, M. K. Delmedico, D. M. Lambert, and F. S. Cohen. 2000b. The transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. *J. Cell Biol.* 151:413–423.

Melikyan, G. B., R. M. Markosyan, M. G. Roth, and F. S. Cohen. 2000c. A point mutation in the transmembrane domain of the hemagglutinin of influenza virus stabilizes a hemifusion intermediate that can transit to fusion. *Mol. Biol. Cell.* 11:3765–3775.

Melikyan, G. B., J. M. White, and F. S. Cohen. 1995. GPI-anchored influenza hemagglutinin induces hemifusion to both red blood cell and planar bilayer membranes. *J. Cell Biol.* 131:679–691.

Naim, H. Y., and M. G. Roth. 1994. SV40 virus expression vectors. *Methods Cell Biol.* 43:113–36.

Pak, C. C., A. Puri, and R. Blumenthal. 1997. Conformational changes and fusion activity of vesicular stomatitis virus glycoprotein: [125I]iodonaphthyl azide photolabeling studies in biological membranes. *Biochemistry.* 36:8890–8896.

Qiao, H., R. T. Armstrong, G. B. Melikyan, F. S. Cohen, and J. M. White. 1999. A specific point mutant at position 1 of the influenza hemagglutinin fusion peptide displays a hemifusion phenotype. *Mol. Biol. Cell.* 10:2759–2769.

Rand, R. P., and W. A. Pangborn. 1973. A structural transition in egg lecithin-cholesterol bilayers at 12°C. *Biochim. Biophys. Acta.* 318: 299–305.

Ratinov, V., I. Plonsky, and J. Zimmerberg. 1998. Fusion pore conductance: experimental approaches and theoretical algorithms. *Biophys. J.* 74:2374–2387.

Scheiffele, P., M. G. Roth, and K. Simons. 1997. Interaction of influenza virus haemagglutinin with sphingolipid-cholesterol membrane domains via its transmembrane domain. *EMBO J.* 16:5501–5508.

Schoch, C., R. Blumenthal, and M. J. Clague. 1992. A long-lived state for influenza virus-erythrocyte complexes committed to fusion at neutral pH. *FEBS Lett.* 311:221–225.