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A Point Mutation in the Transmembrane Domain of the Hemagglutinin of Influenza Virus Stabilizes a Hemifusion Intermediate That Can Transit to Fusion

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A hemagglutinin (HA) of influenza virus having a single semiconserved Gly residue within the transmembrane domain mutated to Leu (G520L) was expressed on cells; these cells were bound to red blood cells. By decreasing pH at 23°C rather than 37°C, an intermediate with properties expected of hemifusion just as the membranes are about to transit to full fusion was captured. As evidence: 1) increasing temperature to 37°C at neutral pH allowed fusion to proceed; 2) after achieving the intermediate, the two membranes did not separate from each other after proteolytic cleavage of G520L because cells treated with proteinase K could not fuse upon temperature increase but could fuse upon the addition of chlorpromazine; and 3) at the point of the intermediate, adding exogenous lipids known to promote or inhibit the creation of hemifusion did not significantly alter the lipid dye spread that occurred upon increasing temperature, implying that at the intermediate, contacting membrane leaflets had already merged. A stable intermediate of hemifusion that could transit to fusion was also generated for wild-type HA, but pH had to be reduced at the significantly lower temperature of 4°C. The fusion pores generated by G520L did not enlarge, whereas those induced by wild-type HA did. The finding that a state of transitional hemifusion can be readily obtained via a point mutation without the need for unusually low temperature supports the hypothesis that hemifusion occurs before pore formation.

INTRODUCTION

Membrane fusion is a central event in a wide range of cellular processes. The proteins responsible for fusion have been unambiguously identified for viruses, and in the case of intracellular fusion, candidate proteins have been specified. It is often hypothesized that the underlying mechanism of fusion will be essentially the same in many, if not all, of the varied settings. The finding that many viral fusion proteins and the best candidates for intracellular fusion proteins (the v- and t-SNAREs) share similarities in core structures, namely, assembly into bundles of α-helices (Skehel and Wiley, 1998), provides support for this hypothesis. Fusion caused by hemagglutinin (HA) of influenza virus has been studied more extensively than fusion caused by any other protein. Despite this, the mechanism of fusion is not yet understood, and the key states in a sequence of molecular rearrangements of proteins and membranes have not yet been unambiguously identified. The means used by different domains of fusion proteins to create these states and to induce passage to the next state also remain unclear.

In the leading hypothesis of fusion, it is posited that membranes reach a state of hemifusion that transits to full fusion. Hemifusion is the merger of contacting, outer monolayers while inner monolayers remain distinct (see Figure 9 for the topology of membranes in a state of hemifusion). For HA and mutants of HA, hemifusion has been observed directly as the spread of lipid dye without mixing of aqueous contents. But these observed states have not proceeded on to fusion (Kemble *et al.*, 1994; Melikyan *et al.*, 1995, 1997; Chernomordik *et al.*, 1998; Qiao *et al.*, 1999; Frolov *et al.*, 2000; Markosyan *et al.*, 2000). That is, the states of hemifusion experimentally identified by dye spread have been end states rather than bona fide intermediates of fusion. If states of hemifusion do not permit passage of lipid dye yet can subsequently transit to fusion, they are termed states of "transitional hemifusion." Because lipid dye does not spread at the state of transitional hemifusion, the state must be identified by other means.

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At end-state hemifusion, osmotic swelling of cells (Melikyan *et al.*, 1995) or the addition of chlorpromazine (CPZ) to cells (Melikyan *et al.*, 1997) ruptures hemifusion diaphragms, permitting passage of aqueous dye. Therefore, a means to identify candidates for states of transitional hemifusion is to create states with suboptimal conditions that can be induced to proceed on to fusion and then test whether osmotic swelling and the addition of CPZ to these states induce aqueous dye transfer. Decreasing pH at 4°C followed by reneutralization created one such intermediate: fusion was induced by increasing temperature to 37°C, by adding CPZ, and by osmotic shock (Chernomordik *et al.*, 1998). But because this candidate for transitional hemifusion was formed at an unusually low temperature, it was possible that processes unrelated to fusion could have been responsible for its creation.

We found a means to produce another candidate in which this low-temperature problem was avoided. An HA with a point mutation (G520L) of the semiconserved residue 520 within the TM domain does not yield either end-state hemifusion or fusion under conditions that support full fusion for HA (Melikyan *et al.*, 1999). As we now report, optimizing conditions by increasing temperature at the G520L arrested state allows fusion to proceed. We show that the addition of CPZ or osmotic shock induced the G520L intermediate on to full fusion. By also using HA to create several new states that fulfill the criteria of transitional hemifusion, we were able to compare intermediates achieved by various means and to characterize properties of the intermediates. The facts that G520L is identical to HA except for one residue within the TM domain and that it was much more readily stabilized at transitional hemifusion than was wild-type HA support the hypothesis that the ectodomain induces transitional hemifusion and that the TM domain, in cooperation with the ectodomain, completes fusion by rupturing the hemifusion diaphragm. We present a model that describes the means by which the TM domain may cause the transition from hemifusion to fusion.

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). Japan/305/57 HA and its mutants were expressed in CV-1 cells from a recombinant SV40 vector (Naim and Roth, 1994). The expression levels of wild-type HA and mutants were similar as judged by flow cytometry (Melikyan *et al.*, 1999). The similarity was confirmed for viral stocks used in the present study.

Labeling of Erythrocytes and Quantification of Fusion by Fluorescence Microscopy

Red blood cells (RBCs) were colabeled with the membrane probe octadecylrhodamine B (R18, Molecular Probes, Eugene, OR) and with the aqueous dye carboxyfluorescein (CF; Molecular Probes) as described (Melikyan *et al.*, 1995, 1997) and used for fusion experiments (Melikyan *et al.*, 1999). Briefly, ~39 h after infection with the recombinant SV40 virus, CV-1 cells were lifted from 60-mm culture dishes, transferred into several 35-mm dishes, and cultivated for 3 h in a CO_2 incubator. Cells were then incubated 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 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 and intermediates induced by reducing the pH for a time and at a temperature as indicated in the text. Cells were then returned to a neutral pH solution (PBS supplemented with 20 mM raffinose; referred to as PBS/raffinose). Explicitly, GL23 (i.e., the intermediate obtained with the mutant G520L) was obtained by a 2-min exposure to pH 4.8 at 23°C followed by 10 min in PBS/raffinose (neutral pH) at 23°C. In the case of cells expressing wild-type HA, HA4-23 was created by maintaining pH 4.8 at 4°C for 2 min followed by a 10-min incubation in PBS/raffinose at 4°C and then a 10-min incubation in PBS/raffinose at 23°C. The extent of fusion was quantified 10–15 min later by examining several areas of the dish under a fluorescence 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 increasing temperature or by treating the HA cell–RBC pairs with 0.25–0.5 mM CPZ (Sigma Chemical, St. Louis, MO) in PBS/raffinose for 1 min at 23°C. HA activated by low pH was proteolytically cleaved by treating the HA cell–RBC complexes with the indicated concentration of proteinase K (Sigma) dissolved in PBS/raffinose. The reaction was stopped by washing cells three times with PBS. The intermediates of fusion were also coaxed on to fusion by osmotic swelling of RBCs, achieved by replacing the medium with a 72-mOsm medium at neutral pH for 30s followed by reintroducing the isotonic solution.

Fusion Pore Measurements

For electrophysiological experiments, HA-expressing cells were grown on No. 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 maintained at either ~4 or 23°C. Unless stated otherwise, an HA-expressing cell patched in the whole-cell configuration was voltage clamped and capacitance (i.e., time-resolved admittance) measurements were performed by superimposing a 200-Hz, 50-mV peak-to-peak sine wave voltage on a 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; Melikyan *et al.*, 1999). The specific "phase angle" introduced by the experimental system of pipette and cells was determined by phase tracking (Fidler and Fernandez, 1989). The pH of the solution surrounding the cells was decreased by ejecting a pH 4.8 solution buffered with 20 mM succinate from another micropipette positioned near the HA cell–RBC pair, maintaining 4°C (for HA) or 23°C (for G520L). After 1 min, the microperfusion was stopped and the solution around the patched cell was allowed to reneutralize for at least 30 s. Fusion pores were evoked from intermediate states by rapidly increasing the temperature from 4 or 23°C to 37°C; the steady-state temperature was reached within 2–4 s (Melikyan *et al.*, 2000). Fusion pore conductances were calculated off-line (Ratinov *et al.*, 1998).

RESULTS

The existence of the state of transitional hemifusion has never been demonstrated definitively, but there is a great deal of accumulated indirect evidence that it does occur. Although the behavior of membranes at the point at which transitional hemifusion would take place is not known explicitly, characteristics of such a state have been proposed (Chernomordik *et al.*, 1998): 1) HA trimers can still induce fusion pores. 2) Lipid dye has not yet spread. Lipid dye spread through small fusion pores is extremely limited and difficult to detect experimentally (Tse *et al.*, 1993; Zimmerberg *et al.*, 1994). Because dye movement through the site of transitional hemifusion is expected to be even more con-

strained than that through the small pore, it would not, as a practical matter, be detectable. Moreover, when dye spread has been observed, the hemifusion has been an end state (Chernomordik *et al.*, 1998; Markosyan *et al.*, 2000). 3) The hemifusion diaphragm is capable of rupture. Protocols that promote rupture of an end-state hemifusion diaphragm should also rupture the diaphragm in transitional hemifusion. To keep observable behaviors from being conflated with states of transitional hemifusion, we refer to states that satisfy the above criteria as "CPZ-sensitive" rather than states of transitional hemifusion.

We characterized whether two independent manipulations—one directed against membrane merger, the other against HA—eliminated the ability of the state to proceed to fusion based on related criteria: 1) Does the lipid composition of the contacting leaflets affect the ability of lipid to spread when the suboptimal condition used to capture the intermediate is made optimal? Some lipids, when incorporated into such leaflets, greatly increase or decrease the ability of the membranes to reach hemifusion and therefore fusion. Even if hemifusion has been achieved, some lipids may cause reversion back to two separate membranes (Leikina and Chernomordik, 2000). We tested whether the incorporation of these lipids affects the ability of lipid dye to spread when HA is further activated at the point of the intermediate. 2) Do the HA trimers need to retain their ability to induce fusion to maintain the CPZ-sensitivity of the state? We determined whether proteolytically cleaving HA trimers to the point that they can no longer promote fusion reduced the efficacy of protocols that disrupt hemifusion diaphragms. These two tests are independent but complement each other. The first alters lipid properties and determines whether HA can still induce dye spread. The second degrades HA and determines whether CPZ can still alter membranes to induce aqueous continuity. We refer to the CPZ-sensitive intermediates that could still exhibit dye movement after both of these tests as "secure" and those that no longer could exhibit dye movement as "vulnerable." For the intermediates we studied, if an intermediate was vulnerable to one intervention, it was vulnerable to the other.

Viable Intermediates That Can Proceed to Fusion at 37°C Were Stabilized at 23°C

Japan/305/57 HA or G520L and other mutants were expressed on surfaces of CV-1 cells with a recombinant SV40 vector. Because this method of expression yields extremely high levels of HA (Gething and Sambrook, 1981), HA density in the present study was optimized. These cells were bound to RBCs and briefly exposed to low pH (typically for 2 min) and then reneutralized. For G520L cells, when pH was decreased at 23°C, neither lipid nor aqueous dye transferred (Figure 1, A and B). But increasing the temperature to 37°C (at neutral pH) resulted in lipid and aqueous dye spread (Figure 1, C and D). Thus, an intermediate of fusion was created for G520L by acidifying at 23°C. We denote the activated complexes of G520L at this point as GL23. For the sake of simplicity, we also refer to the conditions used to achieve these complexes as GL23 and to the cells that contained these structures as GL23 cells. When pH was decreased at 4°C followed by reneutralization, increasing the temperature to 23°C gave rise to a similar, but not identical, intermediate referred to as GL4-23.

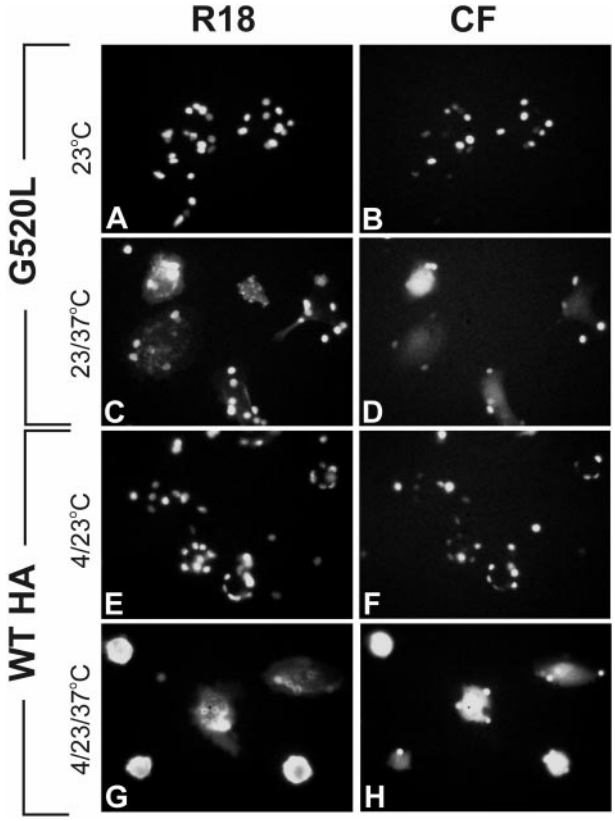

Figure 1. Membrane (octadecylrhodamine B [R18], left column) and aqueous (CF, right column) dye redistribution between HA-expressing cells and double-labeled RBCs. (A and B) G520L cell–RBC complexes were exposed to pH 4.8 for 2 min at 23°C, followed by incubating at neutral pH at 23°C for 10 min to achieve the GL23 intermediate. Neither dye spread. (C and D) Upon increasing temperature to 37°C, both aqueous and lipid dye spread, signifying fusion. (E and F) HA cell–RBC complexes were exposed to pH 4.8 for 2 min at 4°C, followed by reneutralization at 4°C for 10 min. The temperature was then increased to 23°C (i.e., it is HA4-23). Neither lipids nor contents mixed. (G and H) The cells were subsequently incubated at 37°C for 3 min, which induced extensive fusion, as seen by the aqueous and membrane dye transfer.

In contrast to G520L cells, for HA-expressing cells bound to RBCs, decreasing pH at 23°C induced fusion (Melikyan *et al.*, 1999). We sought conditions that would create an HA intermediate at 23°C that would proceed to fusion when temperature was increased to 37°C at neutral pH to compare the intermediate generated for G520L with an HA intermediate at the same temperature. We made this intermediate by decreasing pH for the HA cells for 2 min and then reneutralizing, all at 4°C. At this point, as described by others (Chernomordik *et al.*, 1998), lipid dye incorporated into RBCs does not spread and pores do not form. We found that after maintaining 4°C for as little as 30 s (after reneutralization), the temperature could be increased to 23°C without either lipid or contents mixing. The dyes did not spread for as long as 1 h after increasing the temperature to 23°C (Figure 1, E and F). Electrical capacitance measurements verified that pores had not yet formed. Increasing the

temperature to 37°C resulted in both lipid and aqueous dye spread (Figure 1, G and H), demonstrating that the fusion process had proceeded partway before temperature was increased from 23 to 37°C. We denote the intermediate obtained after the cells had been reneutralized at 4°C and the temperature increased to 23°C as HA4-23. These complexes allowed the cells to proceed on to fusion when temperature was increased to 37°C at neutral pH. Except where stated otherwise, we created HA4-23 by maintaining 4°C for 10 min after reneutralization and then for an additional 10 min at 23°C. Complexes with properties similar to HA4-23 also occurred for two cells lines we tested that stably express HA: one expressing Japan/305/57 HA (HAb2 cells) and the other expressing X:31 HA (HA300a cells) (our unpublished observations). The target RBCs had to be bound to the HA-expressing cells during the HA4-23 protocol to form the complexes. Carrying out the HA4-23 protocol on the HA-expressing cells in the absence of RBCs and then binding the RBCs did not yield fusion when temperature was subsequently increased to 37°C.

Increased Temperature Is More Effective Than CPZ in Converting HA4-23 to Full Fusion, but the Reverse Holds for GL23

Although HA4-23 does not proceed on to fusion at 23°C (Figure 2B, first column), increasing the temperature to 37°C induced the same extent of fusion (second column) as when the entire process was carried out at 37°C (last column). In contrast, although fusion did not occur at 23°C for GL23 (Figure 2A, first column), fusion was greater, as monitored by aqueous dye spread, when GL23 was first created and temperature was then increased to 37°C (Figure 2A, second column) than when fusion was triggered directly at 37°C (last column).

Increasing temperature was not the only method of inducing HA4-23 or GL23 to fuse. We could also cause fusion by adding CPZ to the external solution. CPZ was chosen because it destabilizes the hemifusion diaphragm connecting hemifused cells (Melikyan *et al.*, 1997); it is known to induce fusion from another intermediate of a previous study (Chernomordik *et al.*, 1998). When 0.5 mM CPZ was added for brief times (Figure 2, A and B, third column), GL23 or HA4-23 cells fused to RBCs. Increasing the temperature was more effective for HA4-23 cells' ability to fuse than was the addition of CPZ (Figure 2B, second and third columns), whereas the reverse was the case for GL23 (Figure 2A). Thus, although HA4-23 fused more extensively than GL23 when temperature was increased to 37°C, GL23 was the more CPZ-sensitive intermediate. In separate experiments, replacing the external isotonic solution with a hypotonic (72 mOsm/L) solution induced aqueous content mixing, although it also induced cell lysis; in fact, the amount of lysis greatly exceeded content mixing (Melikyan *et al.*, 1995; our unpublished observations).

Because fusion was sensitive to a substitution at position 520, we made and tested several mutations at positions 520 and 521 to determine whether other proteins with mutations in this region exhibited behaviors similar to those of G520L. For each mutant tested, pH was decreased at 23°C exactly as for G520L. Some mutations were not deleterious: for G520S (Melikyan *et al.*, 1999) as well as G520A and G520V, fusion

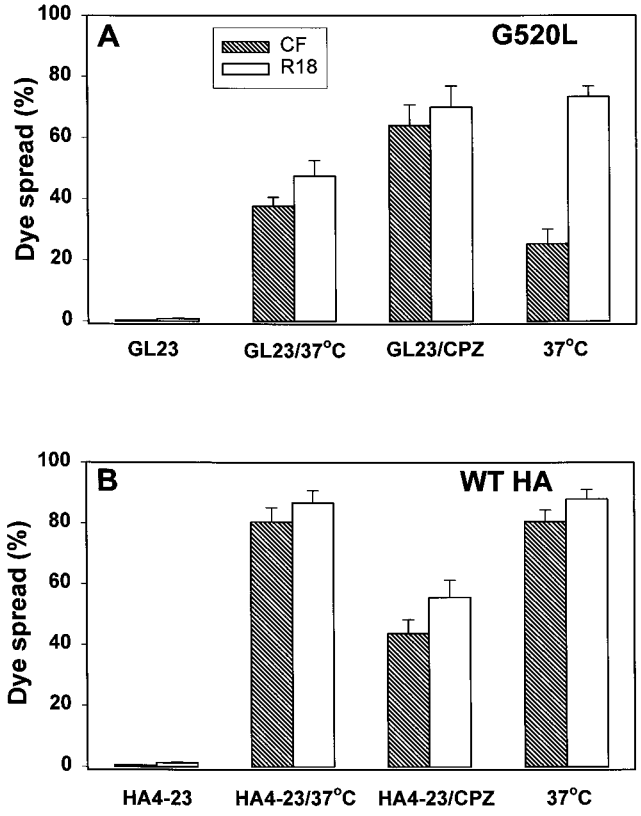

Figure 2. Temperature- and CPZ-induced fusion of GL23 (A) and HA4-23 cells (B). Neither aqueous (striped bars) nor lipid (open bars) dye redistributed when GL23 and HA4-23 were established (first columns in A and B, respectively). Increasing the temperature to 37°C for 3 min (second columns) or exposing cells to 0.5 mM CPZ for 1 min at 23°C (third columns) led to both aqueous and lipid dye spread. The extent of fusion attained by directly triggering fusion at 37°C with a 5-min, pH-4.8 exposure, followed by incubation at neutral pH for 10 min, is represented in the last columns. Bars show the SE of the mean; each experiment was repeated 4–10 times.

was not impaired significantly (our unpublished observations). But for several others, neither lipid nor aqueous dye spread significantly (Figure 3, first column). Increased dye spread occurred upon increasing temperature to 37°C (second column). Dye spread was greatly augmented by the addition of CPZ at 23°C (third column). Thus, a mutation at 521 (i.e., S521A) was as effective in promoting an intermediate as was G520L. Because G520L yielded the lowest lipid and aqueous dye spread when fusion was triggered at 23°C (first column) and yet yielded reasonable fusion upon increasing temperature, we chose G520L for further investigation.

The Fusion Intermediates Were Stable over Time at 23°C

When HA4-23 (Figure 4A, first hatched bar) was held at standard conditions (23°C maintained for 10 min) or after 23°C was maintained for a total of 40 min (first open bar) and then stepped to 37°C, the extent of fusion was the same.

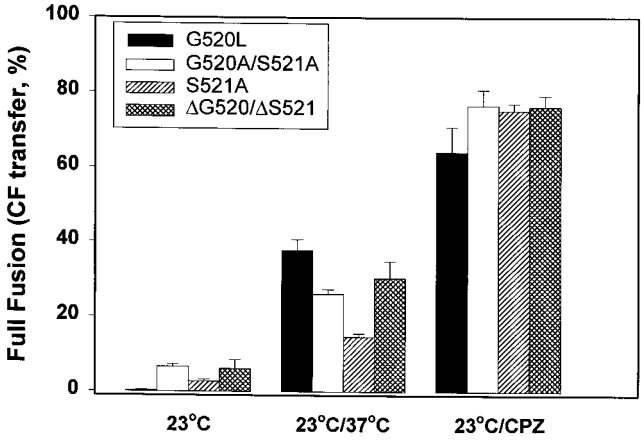

Figure 3. Fusion is sensitive to mutations at residues 520 and 521. G520L (closed bars), the double mutation G520A/S521A (open bars), S521A (hatched bars), and the double deletion ΔG520/ΔS521 (cross-hatched bars) are shown. None yielded appreciable fusion upon acidification at 23°C (first column). All yielded more fusion when the temperature was increased to 37°C at neutral pH (second column) or when 0.5 mM CPZ was added at 23°C for 1 min (third column).

Similarly, the ability of CPZ to induce fusion did not depend on how long HA4-23 was maintained at 23°C (second column). Thus, the ability of HA4-23 to fuse did not vary over time. In contrast, the extent of fusion induced by increasing the temperature to 37°C was less when GL23 was maintained for 40 min (third column, open bar) than for 10 min (hatched bar), although CPZ-sensitive fusion was stable over time. (Similar results were observed for GL4-23.) In other words, the temperature-sensitive G520L protein-mediated fusion decreased as the intermediate was maintained for longer times, but the membrane structure that CPZ acted on to induce fusion did not vary.

The creation of the intermediates depended on pH, as was expected for an HA-mediated process. When either HA4-23 or GL23 was created by decreasing pH to 5.0 (Figure 4B), less fusion resulted upon increasing temperature to 37°C or by adding CPZ than when pH was decreased to 4.8 (Figure 4A). Importantly, CPZ induced fusion more effectively for GL23 than increasing temperature, regardless of whether pH had been decreased to 4.8 or 5.0. The pH dependence for generating intermediates that could be induced on to fusion was steep. For HA4-23, the threshold for decreasing pH was ~5.4, which is typical for Japan/305/57 HA (Morris *et al.*, 1989; Melikyan *et al.*, 1999), and for GL23 it was somewhat lower, about pH 5.2. Maximal fusion upon increasing temperature to 37°C was achieved for both HA4-23 and GL23 when they were created at pH 4.8 or lower.

The Ability of CPZ to Promote Fusion from GL23 Does Not Require Intact Complexes of HA

The finding that G520L slowly lost its ability to induce fusion with time but that CPZ did not lose effectiveness in promoting fusion (Figure 4A) is consistent with characteristic 2 for a secure intermediate in that fusion proteins that can still induce fusion are no longer required to maintain the

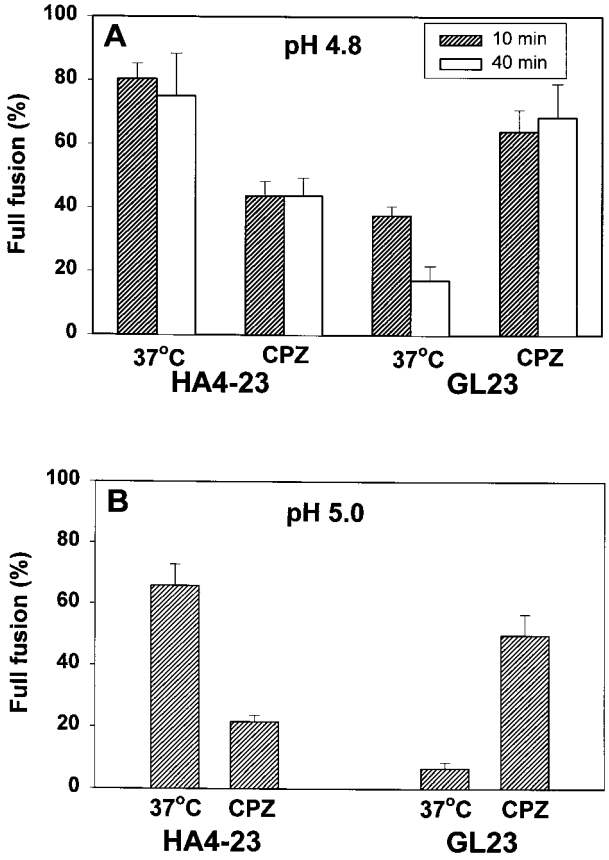

Figure 4. The pH dependence of formation of the GL23 and HA4-23 intermediates and their stability with time. (A) The GL23 and HA4-23 intermediates were generated and their ability to convert to fusion was measured after a 10-min (striped bars) or 40-min (open bars) incubation at 23°C at neutral pH. Fusion from HA4-23 (first and second columns) or GL23 (third and fourth columns) was induced either by increasing temperature to 37°C (first and third columns) or by treating with 0.5 mM CPZ (second and fourth columns). (B) The GL23 and HA4-23 intermediates were generated by decreasing pH to 5.0 (rather than to 4.8), with all other aspects of the protocol performed as usual. The extent of fusion induced by increasing the temperature to 37°C or by adding CPZ after maintaining the intermediates for 10 min was less than when pH had been decreased to pH 4.8.

CPZ-sensitive intermediate once it has been created. We more rigorously tested this criterion by establishing the intermediates and then completely eliminating the ability of HA and G520L to induce fusion. Proteinase K is known to cleave HA after HA has undergone low pH–induced conformation changes, but not before (Doms *et al.*, 1985). Adding proteinase K to HA4-23 cells eliminated the ability of increased temperature or CPZ to promote either lipid (our unpublished observations) or aqueous dye (Figure 5A, second column versus first column) spread. In contrast, proteinase K abolished the ability of increased temperature to promote aqueous dye spread from GL23 (fourth column versus third column, open bar), but CPZ was still effective in promoting aqueous (fourth column, hatched bar) and lipid (our unpublished observations) dye movement. Unlike

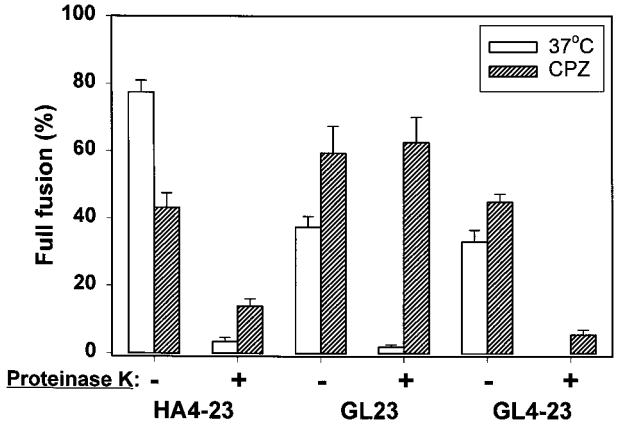

Figure 5. Sensitivity of the intermediates to proteolytic digestion by proteinase K. GL23 or HA4-23 was generated, followed by an additional 10-min incubation at 23°C in the absence (−) or presence (+) of proteinase K (0.01 mg/ml for G520L and 0.05 mg/ml for HA). The reaction was stopped by washing cells three times with PBS/raffinose, fusion was triggered by increasing temperature to 37°C (open bars) or by treating with 0.5 mM CPZ (striped bars), and transfer of CF was measured. Whereas for HA4-23 a proteinase treatment that abolished the ability of increased temperature to induce fusion also eliminated the ability of CPZ to promote fusion, for GL23 the CPZ sensitivity was retained.

GL23, the addition of CPZ did not promote fusion when GL4-23 had been treated with proteinase K (sixth column versus fifth column). We have thus shown that HA in the HA4-23 complex and G520L in the GL23 complex have undergone at least some pH-induced conformational changes. The procession of all of the intermediates to full fusion at 37°C requires the actions of low pH–activated HA. To maintain the CPZ-sensitive sites of HA4-23, at least some of the HAs that make up the complexes must be intact; otherwise, CPZ would cause fusion after the proteinase K treatment. In contrast, all of the G520L within the complex need not be intact to retain CPZ-sensitive fusion once the GL23 complex has formed. These sensitivities to CPZ after proteinase K treatment would be expected if GL23 was a secure state but HA4-23 was a vulnerable state.

GL23 Is a Secure Intermediate but GL4-23 and HA4-23 Are Not

We further tested GL23, GL4-23, and HA4-23 by altering the spontaneous curvature of outer monolayers. If hemifusion has not yet occurred after an intermediate has formed, the addition of lysophosphatidylcholine (LPC), with its positive spontaneous curvature (Chernomordik *et al.*, 1995), would inhibit the merger of outer leaflets and therefore inhibit fusion when temperature is increased. In the same situation, oleic acid (OA), with its negative spontaneous curvature, would promote hemifusion (Chernomordik *et al.*, 1997). What effects these agents would have if hemifusion already occurred and remained stable over time have not yet been investigated.

As a control, LPC was added before GL23 was established, and neither lipid nor aqueous dye spread when temperature was increased to 37°C (Figure 6A, second column), whereas the addition of OA promoted lipid dye transfer (fourth column). Both results were as expected. The addition of LPC after GL23 was created did not inhibit the spread of lipid dye when temperature was increased to 37°C (third column, open bar), but it did prevent the transfer of aqueous dye (hatched bar). Adding OA at the point of GL23 promoted both lipid dye and aqueous dye spread upon increased temperature (fifth column). In fact, the very addi-

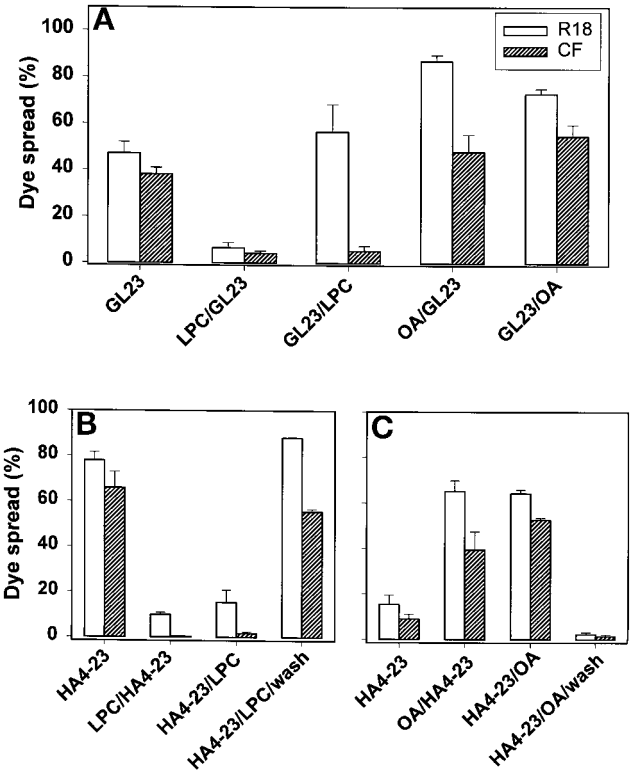

Figure 6. The effect of altering membrane lipid composition on GL23 and HA4-23 cell–RBC fusion. (A) Adding 270 μM lauryl-LPC before establishing GL23 (i.e., LPC was added before decreasing pH) inhibited content and lipid mixing upon increasing temperature to 37°C (second column) compared with control (first column). Adding LPC at the point of GL23 did not inhibit membrane dye redistribution but did suppress aqueous dye spread at 37°C (third column). Adding 50 μM OA before creating GL23 cells promoted transfer of the lipid dye (fourth column). The addition of OA after creating GL23 (fifth column) also induced transfer of lipid dye at 37°C (fifth column). (B) Increasing the temperature of HA4-23 to 37°C yielded a high extent of aqueous (hatched bars) and lipid (open bars) dye transfer (first column). Adding 270 μM lauryl-LPC to the bathing solution either before (second column) or after (third column) HA4-23 was established prevented both lipid and aqueous dye transfer when temperature was subsequently increased to 37°C. The effect of LPC was reversible: washing out LPC (fourth column) restored the ability of HA4-23 to fuse at 37°C. (C) Temperature was increased to 30°C (rather than 37°C) to determine the consequences of adding OA to HA4-23. In the absence of OA, increasing the temperature of HA4-23 elicited minimal dye transfer (first column). Adding 50 μM OA either before (second column) or after creating HA4-23 (third column) promoted fusion. Dye did not transfer when OA was removed before increasing the temperature to 30°C (fourth column).

tion of OA induced some fusion without the need to increase the temperature (our unpublished observations). That is, the addition of OA destabilized the GL23 intermediate toward fusion. These actions of LPC and OA indicate that by the state of GL23, the ability of contacting leaflets to merge or for the hemifusion diaphragm to be maintained is no longer strongly "lipid sensitive," i.e., is not dependent on the specific lipid composition of the target membrane. (The augmentation of fusion by the addition of OA provides support for the interpretation that LPC prevents fusion through its positive spontaneous curvature.) Both the observed sensitivity to CPZ after proteinase K treatment and the results of altering lipid composition indicate that GL23 is a secure intermediate.

Analogous experiments were performed for the GL4-23 (our unpublished observations) and HA4-23 intermediates. LPC was added to the solution bathing bound cells either before (as a control) (Figure 6B, second column) or, in separate experiments, after HA4-23 was established (third column). In both cases, lipid and aqueous dye transfer (first column shows transfer in the absence of LPC) was minimal when temperature was increased to 37°C. That is, LPC blocked fusion. The LPC effect was reversible: when LPC was removed by washing, dye spread at 37°C (fourth column). The addition of OA, either before (Figure 6C, second column) or after (third column) reaching HA4-23, promoted both lipid and aqueous dye transfer upon increasing temperature to 30°C (compare with first column). The effect of OA on fusion was monitored at 30°C rather than the optimal 37°C (at which temperature dye spread is significantly greater) so that augmentation of dye spread could be observed. OA's effect was also reversible (fourth column). The addition of OA to cells at HA4-23 did not promote dye spread when temperature was not increased (in contrast to the situation for GL23). Thus, at the point of assay, the transition of HA4-23 cells to fusion is still lipid-sensitive. However, adding OA before decreasing pH at 4°C resulted in some lipid and aqueous dye spread upon increasing temperature to 23°C. Thus, the ability to create HA4-23 was dependent not only on HA but on the properties of the lipids as well. The behavior of GL4-23 in response to LPC and OA treatments was similar to that of HA4-23 (our unpublished observations). Both the inability of CPZ to promote fusion after proteinase K treatment (Figure 5) and the ability of LPC and OA to affect fusion (Figure 6) indicate that HA4-23 is a vulnerable intermediate. It is possible that at this state contacting leaflets have not merged.

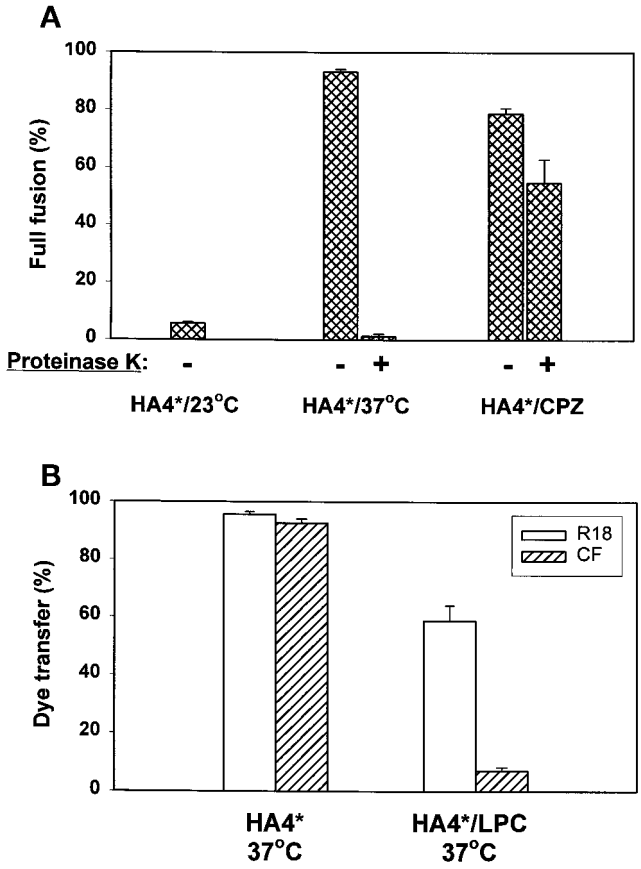

Figure 7. Fusion induced from HA4*. HA4* was generated by decreasing pH to 4.8 for 10 min at 4°C before reneutralization. (A) Little fusion occurred by increasing the temperature to 23°C (first column), but extensive fusion occurred by increasing the temperature to 37°C (first bar, second column). Adding 0.1 mg/ml proteinase K for 10 min at 4°C abolished the ability of fusion to occur when the temperature of HA4* was increased to 37°C (second bar of second column) but only slightly inhibited CPZ-induced aqueous dye transfer (third column). (B) Generating HA4* and then increasing the temperature to 37°C resulted in extensive lipid and aqueous dye transfer (first column). But the addition of LPC severely inhibited aqueous dye transfer (second column, hatched bar) but only moderately prevented lipid dye transfer (open bar).

HA Can Induce a Secure Intermediate State

Having determined that G520L could generate a secure intermediate (GL23), we sought to determine whether the secure state could occur not only for a mutant but for the unaltered HA as well. Therefore, we decreased pH for a longer time than for HA4-23. Decreasing pH for 10 min (rather than the 2 min used to obtain HA4-23) at 4°C, followed by reneutralization at 4°C, did not induce fusion. We refer to this state as HA4*. As was the case for HA4-23, minimal fusion resulted when the temperature of HA4* was increased to 23°C (Figure 7A, first column). (This is in contrast to the situation in which a different cell line expressing the same strain of HA was used: increasing temperature to 23°C yielded dye spread [Chernomordik *et al.*, 1998]. Thus, HA4* may not be precisely the same state as that used in the previous study.) Extensive fusion was observed upon increasing temperature to 37°C (first bar of second column), as reported previously (Chernomordik *et al.*, 1998). Proteinase K treatment abolished the ability of increased temperature to induce fusion from the state of HA4* (second bar of second column). But the addition of CPZ still induced fusion after the proteinase K treatment (second bar of third column). The addition of LPC at the point of HA4* only somewhat reduced the spread of lipid dye (Figure 7B, open bar of second column) but greatly reduced the transfer of aqueous dye (hatched bar of second column) upon increasing temperature compared with the situation without the addition of LPC (first column). These effects of LPC are exactly the same as those that occurred when LPC was added at the point of

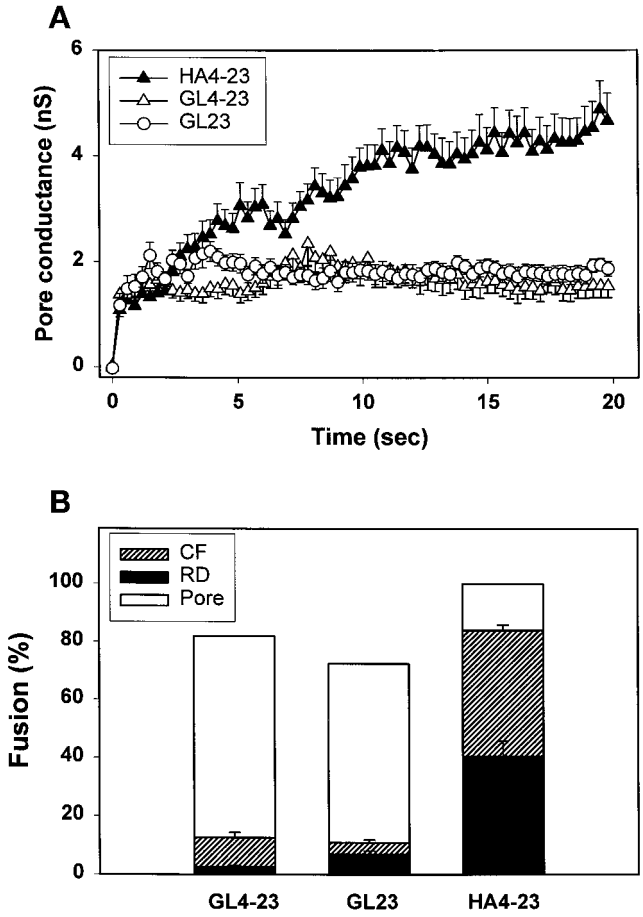

Figure 8. Enlargement of fusion pores induced by HA and G520L. (A) Average conductance of fusion pores induced by a temperature increase to 37°C from GL23 (○, n = 7), GL4-23 (△, n = 5), and HA4-23 (▲, n = 8). Pores induced by G520L had not enlarged within 20 s after formation, whereas HA pores had. Pores were aligned at their opening, and average conductances were calculated at 5-ms intervals (points on the graph were decimated for visual clarity). The error bars show SEM. (B) Pore enlargement was assessed at later times by the ability of a small (CF) and large (rhodamine-tagged dextran [RD]) aqueous marker to transfer between RBCs and HA-expressing cells. The GL23, GL4-23, and HA4-23 intermediates were established, and fusion was then triggered by exposing cells to 37°C for 3 min. As a reference, the probability of pore formation per bound RBCs was determined electrically as described (Melikyan *et al.*, 1999) (open bars). Striped bars show the fraction of HA-expressing cells stained with CF, and closed bars show the fraction of cells that have acquired RD.

GL23. Therefore, by our two tests, HA4* has the properties that define a secure CPZ-sensitive intermediate of fusion.

HA Pores Enlarge with Time, G520L Pores Do Not

We characterized the growth of a pore formed under a given condition by averaging the conductance of all pores formed under that condition and aligning the times by the moment the pores opened. The pores formed when HA4-23 (Figure 8, closed triangles) was induced on to fusion grew continually during the time in which they were electrically followed. The conductance of the initial pore and its growth were independent of the precise intermediate from which fusion was induced (our unpublished observations). Pores generated by G520L did not depend on the route used to achieve them: GL23 (open circles) and GL4-23 (open triangles) yielded the same pores. In other words, HA yielded similar or the same fusion pores independent of the route taken; the same was true for G520L. In contrast to HA pores, the G520L pores did not enlarge.

It is not practical to electrically follow HA-induced pores for long times because, in addition to its ability to cause fusion, HA will also perturb membranes and cause leaks (Jiricek *et al.*, 1997; Qiao *et al.*, 1999; Bonnafous and Stegmann, 2000). The degree of pore enlargement at later times can be determined by following the movement of a small (CF, $M_r \sim 400$) and a large (rhodamine-tagged dextran [RD], $M_r \sim 40{,}000$) aqueous dye from RBC ghosts into HA- and G520L-expressing cells. We established HA4-23 and then increased the temperature to 37°C and, in separate experiments, either electrically determined the extent of fusion or measured the spread of the aqueous dyes (Figure 8B, third column). Almost the same percentage of cells that fused as determined electrically (open bars) also permitted passage of CF (hatched bars); RD transferred for ~40% of the cells (shaded bars). In contrast, for G520L, only ~15% of the fused cells allowed CF to transfer and almost none allowed RD to spread, regardless of whether fusion was achieved through GL4-23 (first column) or GL23 (second column). In short, HA pores grow over time, but G520L pores do not.

DISCUSSION

Characterizing the State of Transitional Hemifusion

The ability of CPZ and osmotic shock to induce fusion from the point of the intermediates shows that the membranes have either locally come into intimate contact or have actually hemifused without the spread of lipid dye. Although it is not known with certainty whether hemifusion has occurred for any intermediates, the inability of LPC to prevent lipid dye spread at the point of GL23 (Figure 6A) and HA4* (Figure 7B) is consistent with membrane merger having already occurred for these intermediates. Although treating cells at GL23 or HA4* with proteinase K eliminated HA-mediated fusion achieved by increasing the temperature to 37°C, it did not abolish the ability of CPZ to induce fusion (Figures 5 and 7A), indicating that a functional complex of G520L or HA was no longer required to maintain the membrane configuration(s) sensitive to CPZ. If only intimate contact was achieved at these states, the proteolytic cleavage of G520L or HA should cause the membranes to separate further. If, however, a stable state of transitional hemifusion was achieved, it is more likely that the hemifusion diaphragm would be maintained. HA4* and GL23 are thus better candidates than HA4-23 as states with merged outer leaflets. Another previously identified intermediate, termed a frozen intermediate of fusion (Chernomordik *et al.*, 1998), is similar to HA4* but somewhat different in behavior and probably also has merged outer leaflets. If any of these states have merged outer leaflets, it would mean that fusion does proceed through transitional hemifusion and would demonstrate that lipid is not free to move through the initial hemi-

fusion diaphragm. It has been suggested that multiple HA trimers associate with each other at sites of hemifusion to form a "fence" that prevents lipid movement (Chernomordik *et al.*, 1998).

HA4-23 was distinguished from GL23 and HA4*: LPC still inhibited lipid dye spread when added to HA4-23 (criterion 1), and CPZ was unable to promote fusion after proteolytically cleaving HA at the point of HA4-23 (criterion 2). Although we do not know the fraction of HA (or G520L) cleaved at the local sites, it was enough to prevent HA-mediated fusion when temperature was increased. Also, our assays are clearly sufficiently sensitive to distinguish between vulnerable and secure states. It could be argued that because acidification has to be carried out at 4°C to produce a secure state for HA, unintended phenomena caused by the low temperature (such as lipid phase separation [Scheiffele *et al.*, 1997]) could be responsible for the creation of the state. But the fact that the secure state of GL23 was created by acidifying at room temperature indicates that the state is intrinsic to the fusion process rather than an anomaly. The use of G520L thus provides additional support for the hypothesis that transitional hemifusion is a precursor of full fusion.

It is possible that proteinase K and LPC could not gain access to secure sites but were able to reach vulnerable sites and that this was the basis for the observed differences. We have found (our unpublished data) that the kinetics of fusion were independent of how long secure intermediates were maintained before the temperature was increased, whereas the kinetics slowed as vulnerable intermediates were held for longer times. The kinetic measures do not depend on accessibilities and also indicate that secure intermediates were more stable than vulnerable intermediates.

The properties of captured intermediates depend on the precise conditions used to establish them. In the case of HA, increasing the time at low pH (at 4°C) promoted the creation of a secure state rather than a vulnerable state. The fact that precise conditions affect the route toward fusion was perhaps more apparent in the case of G520L. This mutant protein induced more fusion and less end-state hemifusion when the GL23 intermediate was first established than when fusion was triggered directly at 37°C (Figure 2). That is, by creating the GL23 intermediate, the reaction was more effectively routed toward full fusion at the expense of end-state hemifusion. It may be that kinetic factors affect the ability of multiple copies of fusion proteins to assemble into configurations that can support fusion at the site at which a pore will form.

LPC in Outer Leaflets Has Different Effects before and after Transitional Hemifusion

The inhibition of dye spread by LPC and its promotion by OA at the point of assaying HA4-23 has been traditionally taken as evidence that hemifusion has not yet occurred. But it is also possible that HA4-23 had been at hemifusion but had "fallen back" to intimate contact either with time or by the incorporation of LPC. That is, the incorporation of LPC into cells may not only prevent the attainment of hemifusion but also could destabilize the net negative curvature of early connecting structures (Chernomordik *et al.*, 1995) and thereby promote the reversion of hemifusion back to adhesion. Exactly the opposite should be true for the action of OA: LPC and OA are invasive probes, and their very presence may affect the conversions between membrane adhesions and hemifusion. In fact, it has been demonstrated that membranes that have reached end-state hemifusion, as evidenced by dye spread, can revert back to two membranes (Leikina and Chernomordik, 2000).

The incorporation of LPC into outer leaflets at the point of both GL23 (Figure 6A) and HA4* (Figure 7B) inhibited aqueous dye transfer upon increasing temperature. The LPC could prevent the formation of fusion pores (without reducing the lipid mixing of end-state hemifusion) and/or inhibit pore enlargement. It has been shown, with phospholipid bilayers as target membranes (Razinkov *et al.*, 1998), that the degree of asymmetry of lipid composition between outer leaflets and inner leaflets does in fact affect the growth of HA-mediated pores. This study provides the first evidence that the presence of LPC in outer leaflets affects the fusion process even after transitional hemifusion (i.e., GL23 and HA4*) has been achieved.

The TM Domain Strongly Affects Fusion Pore Formation and Growth

It was notable that the HA pores characterized in this study did not flicker at 37°C. It had been thought that flickering is a general feature of HA-induced pores. But the previous electrophysiological studies of fusion pores with RBCs as targets used cells that expressed a lower density of HA (Spruce *et al.*, 1989; Zimmerberg *et al.*, 1994; Markosyan *et al.*, 1999; Qiao *et al.*, 1999) than were used in the present study and/or lower temperatures (Melikyan *et al.*, 1999). It may be that HA-induced pores flicker only when conditions are suboptimal.

We previously showed that GPI-HA could induce fusion pores, but under more stringent conditions than those for HA, and that GPI-HA pores did not enlarge but HA pores did (Markosyan *et al.*, 2000). We have now found that G520L can also generate pores, but under more restricted conditions than HA, and that G520L pores do not enlarge (Figure 8). The GPI-HA and its HA counterpart used in the previous study are H3 subtype (X:31), whereas G520L and HA used in the present study are H2 subtype. The amino acid sequences of TM domains of different subtypes (~14) of HA fall into two classes: Japan/57/305 of the present study is prototypic of one class and X:31 is prototypic of the other class (Tatulian and Tamm, 2000). Independent of class, we envision that the TM domain participates only after transitional hemifusion has been achieved, inducing pore formation and regulating pore growth as well.

HA induces fusion when pH is decreased at 23°C, whereas G520L does not. Thus, although strict amino acid sequence of the TM domain is not required for efficient fusion (Melikyan *et al.*, 1999), a point mutation within the TM domain can cause fusion to become arrested, probably at the point of transitional hemifusion, under conditions that would normally induce fusion. Because a mutation at residue 521 (Figure 3) can also arrest fusion, this local region of the TM domain appears to perform a critical role in inducing fusion to proceed from the point of transitional hemifusion to pore formation. A location within the TM domain of the Env fusion protein of Moloney murine leukemia virus has also been suggested as critical in allowing fusion to proceed from the state of hemifusion (Taylor and Sanders, 1999). It is

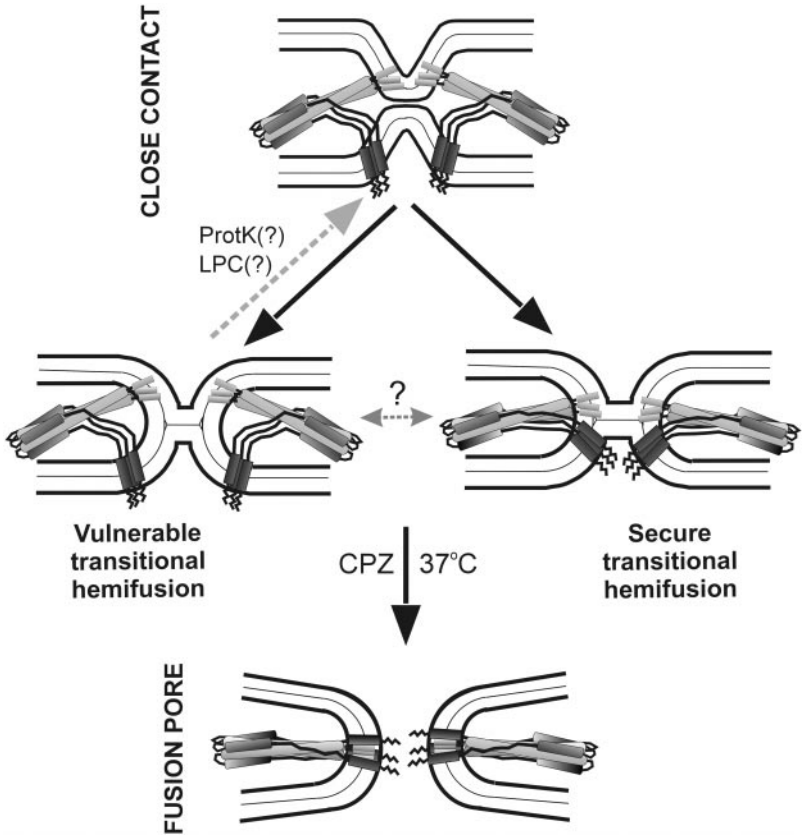

Figure 9. Hypothesized steps in membrane fusion. The addition of LPC hinders, whereas OA promotes, hemifusion. The presence of LPC in outer leaflets or proteolysis of HA may also destabilize and break, through changes in spontaneous curvature, the hemifusion diaphragm of vulnerable hemifusion and thereby reestablish close contact between membranes. It is not known if vulnerable and secure states transit between each other. The conformations of HA and the precise forms of hemifusion diaphragms could be different at vulnerable and secure states of transitional hemifusion. After transitional hemifusion is established, the completion of folding into a six-helix bundle and interactions between residues of the C and N termini cause the TM domain to break the hemifusion diaphragm and intermingle with the fusion peptides. The precise step at which the bundle forms is not yet known.

possible that at the point of transitional hemifusion, the TM domain naturally, without direct aid by the ectodomain, interacts with and destabilizes the hemifusion diaphragm. Particular structural motifs may facilitate this process.

We favor the alternative view, as originally proposed (Melikyan *et al.*, 1995), that conformational changes of the ectodomain (at the state of transitional hemifusion) cause the TM domain to insert into and destabilize the hemifusion diaphragm—the structure that continues to separate aqueous compartments—and that this induces pore formation. The ectodomains of many fusion proteins, including HA, assume a six-helix bundle as a final structure, with N-terminal (i.e., the region adjacent to the fusion peptide) α-helices forming a central triple coiled-coil core surrounded by three C-terminal α-helices that pack antiparallel to the core (Skehel and Wiley, 1998). For some proteins, the C-terminal α-helices are adjacent to the TM domain (Baker *et al.*, 1999), and for these proteins the antiparallel orientation of C- and N-terminal helices necessitates that the fusion peptides and TM domains come into close proximity upon bundle formation. But in the case of HA, a long stretch of amino acids intervenes between the C-terminal α-helices and the TM domains, so it would be conceivable that the TM domain and the fusion peptide do not come into close proximity upon bundle formation. However, the crystallographic structure displays, in addition to the six-helix bundle of HA, bonding between residues adjacent to the TM domain and residues of and immediately adjacent to the structure that terminates (the N cap) the α-helices of the central core (Chen *et al.*, 1999). (These bonds form between amino acids that are largely conserved among the various strains of HA.) As a direct consequence of formation of these bonds, the fusion peptide and TM domain must approach each other (Figure 9). For contact to be made between these two membrane domains, inserted in different membranes, the membranes must merge fully. That is, a pore must form. It is well known that the fusion peptide is critical to fusion and that mutation can abolish fusion and/or transitional hemifusion (Qiao *et al.*, 1999). The TM domains within an HA trimer must separate from each other for the six-helix bundle to form. It may be that the TM domains of G520L do not separate easily and therefore cannot readily insert into the hemifusion diaphragm to meet the fusion peptide, as does HA. This difficulty of separation would account for the finding that a higher temperature is required for G520L to induce fusion.

Temporally, after the fusion peptide inserts into the target membrane, dimples are created (Markosyan *et al.*, 1999; Frolov *et al.*, 2000) that locally allow close contact between membranes and subsequent hemifusion (Figure 9). The state of transitional hemifusion may be able to revert back to separate membranes in close contact; this reversion may be enhanced by proteolysis of HA or by the addition of LPC. If all of the CPZ-sensitive states are indeed hemifusion intermediates, the states identified as vulnerable would revert readily and the secure states would resist reversion. Once transitional hemifusion has been achieved, the free energy released by interactions between the C and N termini of the ectodomain drives the fusion peptide and TM domain mem-

branes together, rupturing the diaphragm and thereby creating the pore.

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